Studies on a strain of *Escherichia coli* K-12 dependent on an exogenous supply of S-adenosylmethionine

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ABSTRACT

Studies on a strain of *Escherichia coli* K-12 dependent on an exogenous supply of S-adenosylmethionine

Rodrigo Reyes, M.Sc.

S-adenosylmethionine (SAM) is a universal metabolite formed from methionine and ATP. SAM has a highly reactive sulfonium group and is used as methyl donor in a large number of methylations. The *metK* gene, coding for the enzyme which synthesizes SAM, SAM synthetase, is an essential gene in *E. coli*. However the study of the importance of SAM has been hindered by the inability of the cells to incorporate this compound directly from the medium.

A plasmid carrying the SAM transporter from the intracellular parasitic bacterium *Rickettsia prowazekii* was used to construct an *E. coli* strain lacking a functional copy of *metK*. The resulting strain requires a supply of exogenous SAM for growth, demonstrating that SAM is the only essential product of SAM synthetase. The use of the transporter in strain MetK84 confirmed that the filamenting phenotype observed in the strain is explained by its low intracellular concentration of SAM. Studies on the relation of SAM concentration and production of biomass in a strain carrying the SAM transporter showed a linear relation at low SAM concentrations, but suggested that SAM is wasted at high concentrations. Finally, in an attempt to understand the relation between SAM and cell division, the mass of two proteins related to cell division was studied. The

very preliminary results on the characterization of the proteins suggest that FtsZ does not undergo any post-translational modifications.

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Introduction

The work in this thesis concerns the production and use of S-adenosylmethionine (SAM) by *E. coli*. In this introduction I will discuss the nature and role of SAM, including a description of its synthesis, how its production is regulated and the effect of the concentration SAM on the synthesis of methionine. I will also write about the different ways in which the cells use SAM. This will be followed by a brief summary on previous works in which the intracellular concentration of S-adenosylmethionine was externally modified. Finally I will write about cell division and its relation with S-adenosylmethionine.

1. **On S-adenosylmethionine and its functions**

1.1 SAM and SAM synthetase

S-Adenosylmethionine (SAM) is a sulfonium compound involved in many biochemical processes. SAM is thought to be an essential metabolite, and its deficiency has been related in humans to liver diseases (Mato *et al.*, 2002), affective disorders and cancer (Lu, 2000). SAM is synthesized by SAM synthetase (EC 2.5.1.6) through the condensation of methionine with the adenosyl group coming from ATP. This reaction is unusual in that the ATP is cleaved at the 5' carbon forming a tripolyphosphate enzyme-bound molecule which is released later as pyrophosphate and phosphate.

SAM-synthetase is a very well conserved enzyme present in all organisms. The SAM-synthetases of as distant organisms as *E. coli* and humans share 57 % sequence identity (Newman *et al.*, 1998). In *E. coli* SAM-synthetase is composed of 383 amino acids and is encoded by the essential gene *metK* (Wei and Newman, 2002). The peptides

coded by *metK* form dimers, each of them form a tight complex where the two subunits contribute to the active site; two of these dimers come together to form a functional enzyme (a tetramer). The active site is suggested to be divided into two coordinated subsites: the first is responsible for the binding of methionine and ATP and performing the synthetic reaction; the second binds the tripolyphosphate and hydrolyses it (Takusagawa *et al.*, 1996).

1.2 Regulation of SAM synthesis

Transcription of *metK* is regulated negatively by the leucine responsive protein (Lrp) (Newman *et al.,* 1998) and the protein MetJ (Liu *et al.,* 2001). Lrp is a global regulator which acts negatively or positively, depending on the gene and contains a DNA binding helix-turn-helix motif. For some Lrp-regulated genes, the presence of leucine in the medium modulates greatly the action of Lrp (Newman *et al*, 1996). This is the case of *metK* where leucine promotes the transcription of the gene probably by releasing Lrp from the promoter.

MetJ is a homodimeric DNA binding protein of the family β-ribbon-helix-helix which is involved in the negative regulation of five genes of the metabolism of methionine (see figure 1) (Philips *et al.,* 1989). The regulation of MetJ over *metK* has been previously removed resulting in the overproduction of SAM synthetase in *metJ* mutants (Markham *et al.,* 1980) and a putative binding sequence of MetJ in the promoter of *metK* has been reported (Liu *et al.*, 2001), although no detailed description of this regulation is available. The affinity of MetJ for the operator DNA is increased over 100-

fold by the binding of SAM (Philips *et al.,* 1989), indicating that the concentration of SAM acts a signal in the feedback regulation of methionine and SAM.

The SAM concentration is also regulated by the inhibition of SAM synthetase. SAM itself acts a competitive inhibitor against ATP and a noncompetitive inhibitor against methionine. The coproducts pyrophosphate and phosphate also act as noncompetitive inhibitors against ATP and methionine (Markham *et al.,* 1980).

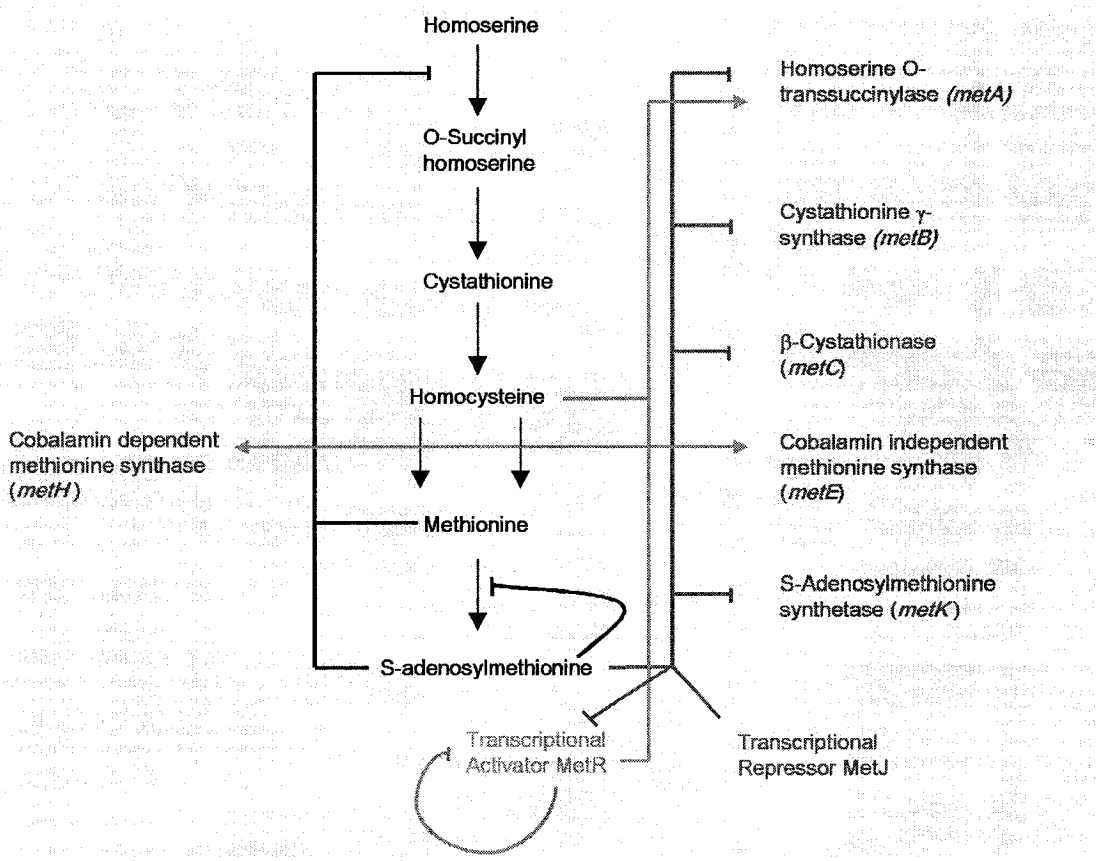


Figure 1, regulation of the synthesis of methionine and SAM

1.3 SAM and methionine metabolism

As described before SAM acts as corepressor along with MetJ to negatively regulate the expression of five genes related to the metabolism of methionine. Of these genes four

code for enzymes involved in methionine biosynthesis (*metA, metB, metC* and *metF*), while the gene *metR* codes for a transcriptional regulator (Greene, 1996). MetR regulates positively the transcription of *metE*, *metH*, *metA* and *metF*, and is also known to repress its own transcription (figure 1). The expression mediated by MetR is increased in the presence of homocysteine (the substrate in the last step of methionine biosynthesis) at least in the case of *metE* (Urbanowski and Stauffer, 1989). Since SAM can be converted into homocysteine after donating its methyl group, the regulation of the transcription of methionine by SAM can be direct through its association with MetJ or indirect through its conversion to homocysteine and interaction with MetR.

SAM can also act in special cases as a precursor in the biosynthesis of methionine. It has been reported that SAM can serve as methyl donor to homocysteine, resulting in the formation of methionine (Tanbichler *et al.*, 1999). On the other hand, SAM is converted into methylthioadenosine (MTA), a byproduct in the synthesis of spermidine. Studies in *Klebsiella pneumoniae* have shown that MTA is in turn converted by a multi-step process into α-keto-4-methylthiobutyrate, the direct ketoacid precursor of methionine, which can be converted into methionine by the action of a tyrosine amino-transferase (Sekowska *et al.*, 2000).

2. **Functions of SAM**

SAM is a very reactive molecule. It can be hydrolyzed in the presence of water at any of the three different chemical groups attached to the sulfur atom. This reactivity converts SAM into a very versatile molecule, reflected in the number of processes in which it is involved. SAM serves as a donor of a variety of groups: methyls to a number of

molecules, aminopropyl groups in the synthesis of spermidine, ribosyl groups in the modification of tRNA, amino groups in the synthesis of biotin. SAM even acts as a free radical initiator in radical enzymatic reactions.

2.1 SAM as methyl donor

SAM is used as a methyl donor for many reactions (Table I), reflecting the favorable energetics over the use of other methyl donors such as tetrahydrofolic acid. Although the substrates and enzymes change, the basic methyltransferase (MTase) reaction is as follows:

$$\text{SAM} + \text{Molecule} \xrightarrow{\text{MTase}} \text{S-Adenosylhomocysteine} + \text{Methylated Molecule}$$

2.1.1 Methylation of DNA

E. coli K-12 DNA contains two modified bases: 6-methyl-adenine (6-meAde) and 5-methyl-cytosine (5-meCyt). Three DNA methyltransferases have been shown to be involved in these methylations: Dam, Dcm and EcoKI.

Dam (DNA adenine methyltransferase) modifies GATC sequences, forming over 99% of the 6-meAde (Marinus, 1996). On average there is one GATC sequence every 214 nucleotides, with the result that a methylated chromosome has over 40,000 adenines modified. The 6-meAde modification is essential in the recognition of the parental strand of DNA (always methylated) during mismatch repair. This modification is also related to the initiation of chromosome replication, since the origin of replication *oriC* is not active when its sequence is hemimethylated, SeqA protein binds to *oriC* when it is

hemimethylated (Lobner-Olesen *et al.*, 2005), and therefore it ensures that replication occurs only once per generation. Finally, Dam methylation has been related to regulation of transcription (Marinus, 1996).

Dcm (DNA cytosine methyltransferase) recognizes the sequence CC(A/T)GG and methylates the second cytosine in it. This is the only enzyme responsible for cytosine methylation, and since there is on average one CC(A/T)GG site every 351 base pairs, there are more than 25,000 methylated cytosines in the fully methylated chromosome (Marinus, 1996). Dcm methylation has no apparent function other than to protect DNA from restriction endonucleases.

The enzyme EcoK is composed of three subunits. The subunits M and S are essential for methylating DNA at the second adenine of the sequence AAC(N_6)GTCG, while the subunit R is essential for restriction but is not able to cut when the 6-meAde is present (Redaschi and Bickle, 1996). This system probably helps to prevent foreign DNA from invading the cell.

2.1.2 Methylation of RNA

Modification of nucleosides in the ribosomal RNA (rRNA) and transfer RNA (tRNA) plays an important role in the maturation of these molecules, with more than 95 different chemical modifications reported to exist in various organisms (Limbach *et al.*, 1994; Rozenski *et al.*, 1999). The modifications (methylations and others) of nucleosides take place after the RNAs are synthesized. Only queuosine is modified prior to incorporation (Bjork, 1996). For most of the modifications the enzymes are site specific, so different enzymes probably catalyze the same modification at different positions.

Table I, Reported methyltransferases in *Escherichia coli*

Name	Substrate	Related Function	Methyl donor
Dam	DNA	Mismatch repair and other	SAM
Dcm	DNA	Not defined	SAM
EcoKI M	DNA	Restriction digestion	SAM
EcoKI S	DNA	Restriction digestion	SAM
YhdJ	DNA	Hypothetical	ND
CheR	MA Chemot Prot	Chemotaxis	SAM
HemK	Prot RF1 & RF2	Translation	SAM
Pcm	Protein L-β aspartate	Degradation of proteins	SAM
PrmA	Rib prot L11	Protein synthesis	SAM
PrmB	Rib prot L3	Protein synthesis	SAM
YabC		Hypothetical	ND
KsgA	16S rRNAm6(2)A1518-A1519	Protein synthesis	SAM
RsmC	16 S rRNA m2G1207	Protein synthesis	SAM
RlmB	rRNA Gm2270	Protein synthesis	SAM
RrmA	23S rRNA m1G745	Protein synthesis	SAM
RrmJ	23S rRNA Um2552	Protein synthesis	SAM
RumA	23S rRNA m5U1939	Protein synthesis	SAM
RumB	23S m(5)U747	Protein synthesis	SAM
SpoU	tRNA Gm18	Protein synthesis	SAM
TrmA	tRNA m5U54	Protein synthesis	SAM
TrmD	tRNA m1G	Protein synthesis	SAM
TrmU	tRNA mnm(5)s(2)U34	Protein synthesis	SAM
YggH	tRNA m7G46	Protein synthesis	SAM
LasT	tRNA/rRNA	Hypothetical	ND
SmtA	rRNA	Hypothetical	ND
YfiF	tRNA/rRNA	Hypothetical	ND
YgdE	RNA	Hypothetical	ND
YibK	tRNA/rRNA	Hypothetical	ND
BioC	Pimelic Acid	Biotin synthesis	SAM
Cfa	Phospholipids	Lipid cyclopropane mod	SAM
CysG	Uroporphyrinogen III	Sirohaem and cobalamine synt	SAM
DhhB	Demthylubiquinone	Ubiquinone synthesis	SAM
GidB	Sterol or Lipid	Possibly DNA replication	SAM
HemX	Uroporphyrin III	Sirohaem and cobalamine synt	SAM
MmuM	Homocysteine	Methionine metabolism	SAM
Tam	Trans aconitate	Unknown	
TehB	Tellurite	Tellurite resistance	SAM
UbiE	DDMQH2	Ubiquinone synthesis	SAM

YafE	Not known	Hypothetical	ND
YafS	Not known	Hypothetical	ND
YbaZ	Not known	Hypothetical	ND
YbiN	Not known	Hypothetical	ND
YcbY	Not known	Hypothetical	ND
YccW	Not known	Hypothetical	ND
YecO	Not known	Hypothetical	ND
YecP	Not known	Hypothetical	ND
YfiC	Not known	Hypothetical	ND
YgfZ	Not known	Hypothetical	ND
YhhF	Not known	Hypothetical	ND
YhiQ	Not known	Hypothetical	ND
YjhP	Not known	Hypothetical	ND
YmfD	Not known	Hypothetical	ND
YnbC	Not known	Hypothetical	ND
YraL	Not known	Hypothetical	ND

In *E. coli* both rRNA and tRNA are methylated at various sites. In the case of the 16S rRNA there are ten methylated nucleosides. All of these involve methylation of the base, but in one case the ribose is also methylated (Bjork, 1996). As occurs for the methylation of other RNA molecules, all of the enzymes responsible have not yet been described (Table I). The methylation of the 16S rRNA is a late event, not occurring just after transcription, but during the maturation of the molecule.

23S rRNA is methylated at 14 sites, three of these at the 2' hydroxyl of the ribose and the other eleven at different positions in the bases. In contrast to the 16s rRNA, the modification of the 23s is an early event (Bjork, 1996).

For both 16S and 23S rRNA, 94% of the modifications are situated at important functional regions, including the peptidyl transferase centre, A, P and E sites, the polypeptide exit tunnel and sites of subunit-subunit interaction (Decatur and Fournier, 2002). This suggests that methylation of rRNA plays a role in the function of ribosomes. Studies of unmodified 16s rRNA *in vitro* showed an imperfect assembly of the 30s subunit and the resulting ribosomes were 40-70% as active as the modified ribosomes (Cunningham *et al.*, 1991). The unmodified 23S rRNA is not able to assemble a functional 50S subunit *in vitro* at all, unless the region comprising nucleosides 2445-2523 is modified (Green and Noller, 1996).

The *in vitro* studies are not easy to extrapolate to the situation inside the cells, since many factors, functions and regulations are excluded. Studies *in vivo* have showed in some cases defects in translation or growth in strains containing mutations at certain methyltransferases or strains that do not contain specific modified sites (Jemiolo *et al.*, 1991; Tan *et al.*, 2002; Liu *et al.*, 2004), although for most of the rRNA

methyltransferases studied so far there is not a significant change in growth. The function of the methylations in the ribosome is still not clear.

E. coli contains 31 different modified nucleosides in the different tRNA species (Bjork, 1996), of which 8 are methylations and 9 others are complex modifications which require methylation during their synthesis; e.g. 2-methylthio-N^6-methyladenosine (ms^2iA37) or 5-methylaminomethyluridine (mnm^5U). A summary of the described tRNA methyltransferases is presented in Table I. All tRNA species contain a methylation at the position U54, and the positions 34 and 37 are frequently modified, and modification in these sites is very diverse, including 7 and 5 methylated nucleosides respectively (Bjork, 1996).

Interestingly, the nucleotide 34 is in the wobble position, and the 37 is just adjacent to the anticodon. Specific nucleoside modifications are seen at the same position in the same tRNA subpopulations in organisms of all 3 kingdoms. For instance, 1-methylguanosine at position 37 (m^1G37) is present in tRNAs for leucine, proline and one of the arginine in all three kingdoms. All this suggests that the modifications play a role in the function of tRNA (Bjork *et al.*, 2001).

Some of the nucleoside modifications in tRNA like m^1G37 and ms^2iA37 have been shown to prevent +1 frameshifting errors (Urbonavicius *et al.*, 2001), and the absence of methylation in m^1G37 produces severe growth defects (Bjork *et al.*, 2001). Nucleoside modifications have also been shown to affect metabolism, like changing the ability to use carbon sources as in ms^2iA37 or the ability to grow without thiamine, although the reasons are not very clear (Bjork *et al.*, 1999). As in the methylation of rRNA, the

identification of all the enzymes responsible for the methylations will help us to gain insight to their role in the function of tRNA.

2.1.3. Methylation of Proteins

Methylation is a common modification in proteins, occurring at different amino acid residues in various organisms: lysine, arginine, proline, histidine, alanine, methionine, glutamic acid, asparagine and glutamine. In bacteria all of these modifications occur except the methylation of arginine (Paik and Kim, 1980).

In *E. coli* only fourteen methylated proteins have been identified (Table II) and for eight of them the enzymes responsible for the modification are known. The methylated proteins can be arranged in two groups: the first are proteins related to the chemotaxis and the second are those related to translation.

The role of methylation in chemotaxis has been studied extensively. *E. coli* monitors the chemical composition of its medium via five different transmembrane chemoreceptors (Tar, Tsr, Tgr, Tap and Aer). After binding to an attractant or a repellent, one protein associated to the receptor (CheA) changes its own phosphorylation state and this in turn gives a signal to the flagella several steps later. The methyltransferase CheR modifies all of the chemoreceptors at specific glutamate and glutamine residues, and this methylation modulates the level of autophosphorylation of CheA. The ability of protein methylation to direct a rapid response in this system is even greater thanks to the presence of the protein CheB, which removes the methylated residue (Levit *et al.,* 1998).

Table II_ Reported methylated proteins in *Escherichia coli.*

Protein	Function	Methyltransferase
MCP-I	Chemoreception	CheR
MCP-II	Chemoreception	CheR
MCP-III	Chemoreception	CheR
MCP-IV	Chemoreception	CheR
S11	30s ribosomal protein (A1)	ND
L3	50s ribosomal protein (Q150)	YfcB
L11	50s ribosomal protein (9 times)	PrmA
L12 (L8)	50s ribosomal protein (K81)	ND
L16	50s ribosomal protein	ND
L33	50s ribosomal protein (A1)	ND
IF-3	Initiation factor	ND
EF-Tu	Elongation factor	ND
RF-1	Release factor	HemK
RF-2	Release factor	HemK

The function of protein methylation associated with translation is less understood. There are five methylated ribosomal proteins. The effect of unmodified proteins in the cell has been studied only for L3 and L11, and a phenotype was only observed for the former. Absence of L3 methylation led to cold sensitivity (Lhoest and Colson, 1981). The translation factors IF-3, EF-Tu, RF1 and RF2 are also methylated. The elongation factor Tu is methylated at a lysine residue in response to starvation for carbon or nitrogen sources (Young and Bernlohr, 1991). Finally, both RF1 and RF2 are methylated by HemK constitutively, and the lack of this methylation affects translation and consequently growth (Heurge-Hamard *et al.*, 2002).

2.1.4. Methylation of small molecules

SAM participates in the biosynthesis of some molecules as methyl donor. A summary of the enzymes which use SAM in this way is presented in table I. Among the molecules synthesized are the vitamin biotin, the electron carriers ubiquinone (used in aerobic respiration) and menoquinone (used in anaerobic respiration) and the coenzyme cobalamin. SAM dependent methylation is also involved in the formation of cyclopropane fatty acids, produced particularly during stationary phase and related to stress resistance.

2.2 SAM in the synthesis of spermidine

E. coli synthesizes a large amount of polyamines. The two most represented are putrescine and spermidine for which a typical cell has been calculated to contain 5.6 and 1.1 million molecules respectively (Neidhardt and Umbarger, 1996). Putrescine is

synthesized by the decarboxylation of L-ornithine or the deamination of agmantine. Spermidine synthesis requires two steps (figure 2): in the first SAM is decarboxylated (enzyme coded by *speD*), and in the second step the aminopropyl group of the decarboxylated SAM is transferred to putrescine by the enzyme spermidine synthase (gene *speE*) forming spermidine (Glansdorff, 1996).

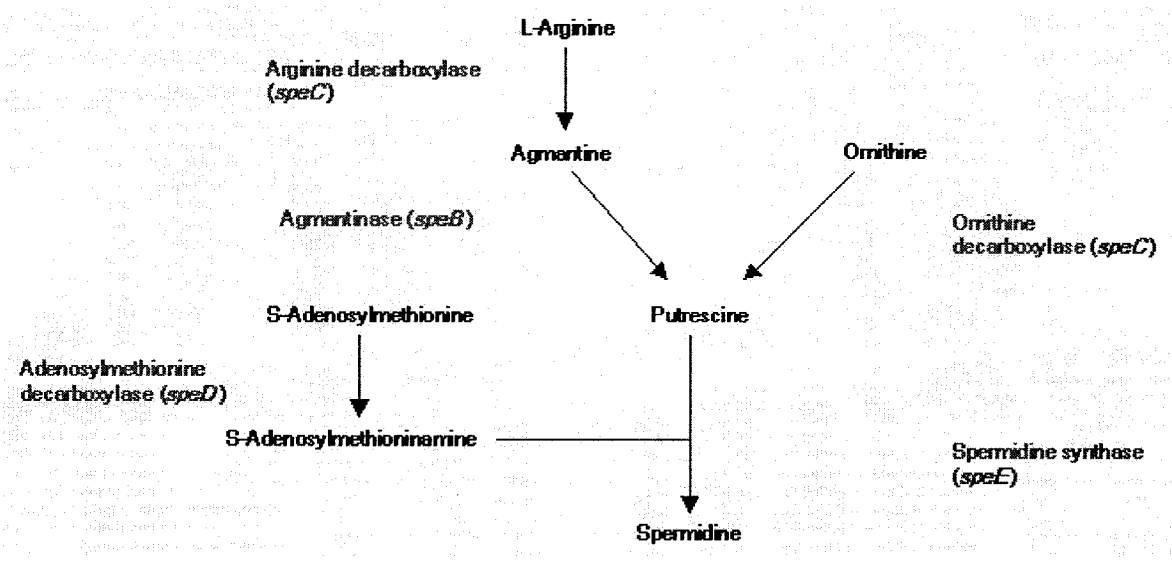


Figure 2, Synthesis of spermidine

Polyamines are believed to be the most abundant polycation molecules after Mg^{2+} and they have been related to a number of cellular processes. Most of the polyamine molecules are bound to macromolecules and especially to RNA, in the case of spermidine 90% (Igarashi and Kashiwagi, 2000). Therefore, is not unexpected that they are believed to play a fundamental role in protein synthesis. In this sense, polyamines have been shown to increase the efficiency and fidelity of protein synthesis, stimulate the assembly

of 30S subunits and the formation of specific aminoacyl-tRNAs, among other processes (Igarashi and Kashiwagi, 2000).

Nevertheless, spermidine is not essential for viability (nor is putrescine). Mutant strains that do not produce any spermidine can still grow. Such a mutant strain has been reported to grow at 55% growth rate of the original strain and produce instead aminopropyl cadaverine (Igarashi *et al.*, 1986). This deficiency in growth can be repaired by the addition of any of various polyamines in the medium (Tholl *et al.*, 1998).

2.3 Other functions of SAM

2.3.1 SAM as donor of other chemical groups

Apart from serving as a methyl or aminopropyl donor SAM is also used, although with less frequency, as donor of amino or ribosyl groups. Indeed, the only known reaction where SAM acts as an amino group donor is in the antepenultimate step of biotin biosynthesis (enzyme coded by *bioA*). SAM acts as a ribosyl group donor for the synthesis of queuosine a modified nucleoside present in position 34 of certain species of tRNA. This reaction is catalyzed by the enzyme SAM-tRNA ribosyltransferase-isomerase (gene *queA*) (Fontecave *et al.*, 2004).

2.3.2 SAM in radical enzymatic reactions

In recent years SAM has been recognized as an important precursor in reactions that require the formation of organic radicals. Some reactions that are chemically challenging, like the interchange of a hydrogen atom for a variable group on vicinal carbons (catalyzed by carbon skeleton isomerases), require the use of radical chemistry (Banerjee,

2003). Organic radicals can be generated with molecular oxygen in a reaction that requires iron, manganese or copper centers. Alternatively they can be generated in an oxygen independent manner using either adenosylcobalamin (AdoCbl) or SAM (Tarret, 2003).

The reactions requiring AdoCbl have been studied extensively and AdoCbl was thought to be the only organic molecule capable of generating radicals via a transient formation of an adenosyl radical. Later it was discovered that SAM was able to act in a similar way. Moreover, it was found that SAM-dependent reactions were not limited to atom rearrangements but they also included insertions of sulfur into C-H bonds and introduction of glycyl radicals into polypeptides necessary for a number of reactions (Frey and Magnusson, 2003). The interest for SAM-dependent radical reactions has greatly increased after the discovery by sequence analysis of a superfamily of enzymes which includes more than 600 members in various organisms (Sofia *et al.*, 2001).

SAM-dependent radical enzymes utilize a Fe-S cluster to facilitate the cleavage of SAM. The first step in all these enzymes is the reduction of the [4-Fe-4S] center (figure 3). In the case of *E. coli* this is achieved using a reduced flavodoxin as an electron donor. The reduced [4-Fe-4S] center is then believed to transfer an electron to the sulfonium group of SAM, resulting in the homolytic cleavage to methionine and the highly reactive 5'-deoxyadenosyl radical. Finally the 5'-deoxyadenosyl radical abstracts a hydrogen from a properly positioned carbon, which can either be an organic molecule or alternatively a protein, having as consequence an active enzyme (Gunhild *et al.*, 2004).

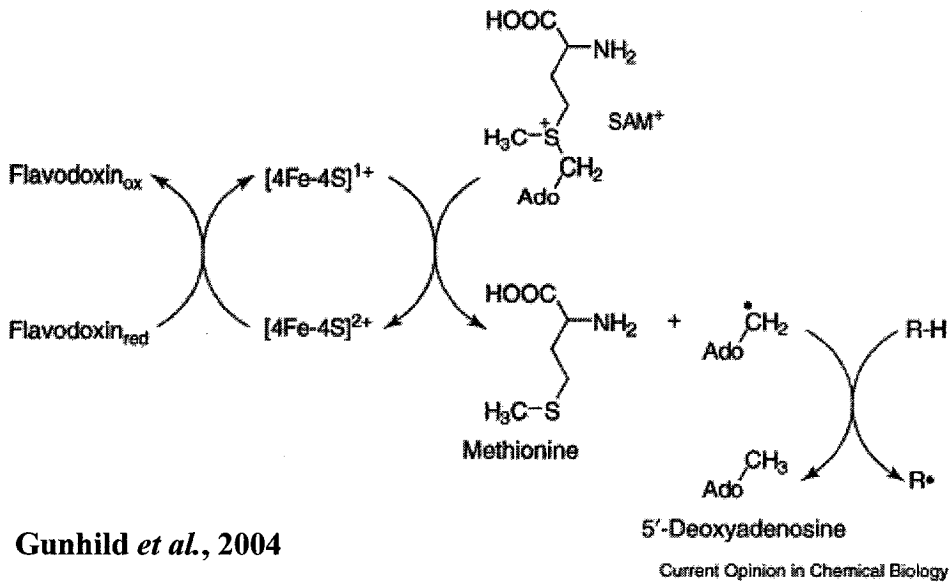


Figure 3, Generation of radicals from SAM

The processes in *E. coli* in which the SAM-dependent radical enzymes are involved include biotin biosynthesis (gene *bioB*), anaerobic heme biosynthesis (*hemN*), anaerobic reduction of ribonucleotides (*nrdG*), lipoic acid biosynthesis (*lipA*), thiamine biosynthesis (*thiH*) and tRNA methyltholation (*miaB*), although a wider vision of the importance of the role of SAM in this reactions will be obtained as the study of this superfamily proceeds.

3. **Manipulation of intracellular SAM concentration**

The study of the effect of various intracellular SAM concentrations has been obstructed by the lack of a system able to transport SAM from the medium. Previous studies used abnormally low or high intracellular SAM concentrations to study their effects. The strategies used to externally change the concentration included the use of a

mutation in the promoter of the *metK* gene which does not allow growth in minimal medium unless leucine is provided (Newman *et al.*, 1998). Another study made use of an enzyme of the T3 phage which is able to hydrolyze SAM. The gene coding for this enzyme was cloned in a plasmid and expressed from there inside the cells to reduce SAM levels (Hughes *et al*, 1987; Posnick and Samson, 1999). Finally the SAM synthetase from rat has been overproduced in *E. coli* to partially avoid the feedback repression and increase SAM concentration (Posnick and Samson, 1999).

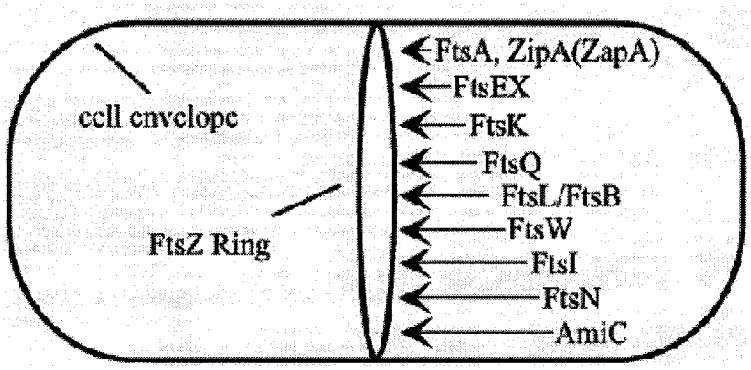
4. **SAM and cell division**

4.1 Cell division in *E. coli*

Cell division begins with the polymerization of the tubulin-like protein FtsZ at the midcell, resulting in the formation of a ring around this zone of the cell (Z ring). To this protein others are subsequently joined in a stepwise process in a defined order. These proteins are FtsA, ZipA, FtsEX, FtsK, FtsQ, FtsL/FtsB, FtsW, FtsI, FtsN and AmiC (figure 4). The process ends with the constriction of the zone where the ring was formed and the separation into two new cells (Weiss, 2004).

FtsZ seems to function as a scaffold for the formation of the septum and to act in the contraction of the ring during the process of invagination (Romberg and Levin, 2003). The other cell division proteins have been functionally grouped into: those that serve to modulate the assembly state of FtsZ (FtsA, ZipA); those that connect FtsZ to the membrane (FtsA, ZipA); those that coordinate division with chromosome segregation (FtsK); those that synthesize cell wall (FtsI, FtsW); and those that hydrolyze the cell wall to separate the daughter cells (AmiC) (Weiss, 2004). An activity has only been

demonstrated for two of these proteins, FtsK, which acts as a DNA translocase and promotes recombination (Aussel *et al.*, 2002; Pease *et al.*, 2005), and FtsI, which has transpeptidase activity and crosslinks chains of peptidoglycan in the cell wall (Pisabarro *et al.*, 1986).



Weiss, 2004

Figure 4, Stepwise incorporation of cell division proteins into the septum

The formation of the Z ring is the only step at which cell division is known to be regulated. There are two mechanisms which are thought to participate in the timing of FtsZ polymerization: nucleoid occlusion and the Min system. The first one, nucleoid occlusion, refers to the observation that the Z ring does not form if a section of the nucleoid is present at the midcell. The second one, the Min system, consists of two inhibiting proteins (MinC and MinD) which prevent FtsZ polymerization and which are thought to oscillate from pole to pole, and a third protein MinE which is a positive regulator of polymerization (Weiss, 2004).

Other proteins that stop cell division at particular physiological circumstances also act through the inhibition of FtsZ polymerization. This is the case of SulA which is a component of the SOS response (Mukherjee *et al.*, 1998).

4.2 *metK84* mutation and cell division

Defects in any of the essential components for cell division results in filamentous morphology. Indeed, screening for this filamentous morphology in conditional lethal mutants led to the identification of the genes involved in cell division (Lutkenhaus and Mukherjee, 1996). The importance of the phenotype to identify the components of the cell division machinery is reflected in their name, fts, standing for filamenting temperature sensitive.

It is interesting therefore that a mutant strain that produces low levels of the enzyme SAM synthetase shows a filamenting phenotype. Previous studies in this lab showed that the mutant strain MetK84 when grown in minimal medium with low concentration of leucine (*metK* inducer) showed extensive filamentation, but this did not occur when the concentration of leucine was high, supposedly because of an increase in the production of SAM synthetase and therefore SAM (Newman *et al.*, 1998). A more recent study in this same lab demonstrated not only that indeed not all of the Fts proteins were being incorporated into the ring in *metK* mutants, but furthermore it suggested that the problem may arise at the point of incorporation of FtsK (Wang, 2004). The study of the MetK84 strain has therefore suggested two important things: firstly that SAM plays a role in the regulation of cell division and secondly that this regulation is unique since the point where it is exerted is not at the polymerization of FtsZ but at the incorporation of FtsK.

Materials and Methods

1. Bacterial strains and plasmid

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

The bacterial strains and plasmids are listed in the Table III and IV.

2. Media and growth conditions

2.1 Luria-Bertani medium (LB)

Per liter contained:

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

pH adjusted to 7.0

The media was sterilized by autoclaving.

For plates, a final concentration of 2% v/w of bacto-agar was added to the LB liquid before autoclaving it.

2.2 Minimal medium (NIV)

Per liter contained:

$(NH4)_2SO_4$ 2 g K_2HPO_4 15 g KH_2PO_4 5.25 g.

L-isoleucine 0.5 g L-valine 0.5 g

pH adjusted to 7.0

The media was sterilized by autoclaving.

Table III_ Strains used in this study

Strain	Relevant Genotype	Source
CU1008	*E. coli* K-12 *ilvA*	L.S. Williams
MEW1	CU1008 Δ*lac*	Newman *et al.*, 1985
CuA	MEW1 Δ*ara*	Cheng *et al.*, 1997
MEW402	MEW1 *metK*84, leucine requiring	Newman *et al.*, 1998
MEW648	CA Δ*metK tetR* sp^r *recA*::kan^r pLtet-K	Wei and Newman, 2002
SAD1	MEW648 pSAD1	This study
SAD16	CA Δ*metK tetR* Sp^r pSAM	This study
BW25113	$LacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* Δ*araBA-*D_{AH33} Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner, 2000
JWK2880_1	BW25113 Δ*serA*::kan^r	H. Mori
JWK3350_2	BW25113 Δ*dam*::kan^r	H. Mori
JWK1944_2	BW25113 Δ*dcm*::kan^r	H. Mori
JWK0116_1	BW25113 Δ*speD*::kan^r	H. Mori
SAD20	SAD16 Δ*dam*::kan^r	This study
SAD37	SAD16 Δ*dcm*::kan^r	This study
SAD39	SAD16 Δ*speD*::kan^r	This study

Table IV_ Plasmids used in this study

Plasmid	Description	Source
pLtet01	PLtetO-1 promoter, p15A replicon, MCS-1, Cm^r	Wei and Newman, 2002
pLtet-K	pLtet01 containing wildtype *metK* gene, Cm^r	Wei and Newman, 2002
pSAM	pLtet01 containing *sam* gene from Rickettsia prowazekii, Cm^r	This study
pSMART	Blunt end high copy cloning vector, Amp^r	Lucigen
pMW1402	Derivative of pSMART containing *sam* coding sequence flanked by 56 and 32 bp upstream and downstream respectively	Tucker *et al.*, 2003
pSAD1	Derivative of pMW1402 containing a mutation which allows cells to use SAM more efficiently, Amp^r	This study
pCA24N	T5-lac promoter, ColE1 replicon, *lacI*, N-terminal His tag, NotI removable C-terminal GFP, Amp^r	
pftsZ-his	pCA24N derivative without GFP carrying *ftsZ* gene	H. Mori
pftsA-his	pCA24N derivative without GFP carrying *ftsA* gene	H. Mori

For plates, 2X NIV is prepared and sterilized. Separately 4% v/w of bacto-agar in water is prepared and sterilized. After autoclaving equal volumes of both the 2X NIV and the 4% bacto-agar are mixed.

2.3 Carbon source for NIV minimal medium

The carbon source, D-glucose, was sterilized separately by filtration and then added to the NIV. The final concentration was 0.2% (w/v).

2.4 R-top agar

Bacto-tryptone	10g	Yeast extract	1g
NaCl	8g	Bacto-agar	8g

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

2.5 SOC medium for electro-transformation

Per liter contained:

Bacto-tryptone	20g	Yeast extract	5g	NaCl	0.58 g
KCl	0.185 g	$MgCl_2$	2.03 g	Glucose	3.6 g

2.6 L-leucine concentration for leucine-requiring strain, m*etK84* (MEW402)

100μg/ml leucine (final concentration) in NIV medium for normal growth

5μg/ml leucine (final concentration) in NIV medium for filament formation

2.7 Preparation of S-Adenosyl Methionine (SAM) Stock

SAM-e, the nutritional supplement of SAM was obtained from a commercial pharmaceutical brand as 200 mg tablets. The tablets were ground and dissolved in 1 mM HCl to a final concentration around 100 mM. The mixture was centrifuged at 4000 rpm for 5 minutes 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. An estimate of the real concentration of SAM was obtained by measuring the absorbance at 260 nm (ε= 15, 400 $M^{-1}cm^{-1}$).

SAM as p-toluenesulfonate salt was purchased from SIGMA. It was diluted in 1 mM HCl to a final concentration of 100 mM, sterilized by filtration and stored as aliquots at –86 °C.

2.8 Antibiotics and Supplements

Final concentrations used for antibiotics were: 200 μg/ml ampicillin, 25 μg/ml chloramphenicol and 30 μg/ml kanamycin. The supplements and concentrations used were: methionine 60 μg/ml, serine 15μg/ml and spermidine 10 μM.

2.9 Anhydrotetracycline (ATC) as inducer of *tet* promoter in pLtet

Strains containing *metK* or *sam* (coding for S-Adenosyl Methionine transporter from *Rickettsia prowazekii*; synonym RP076) inserted in pLtet were grown in the presence of 100 ng/ml of the gratuitous inducer ATC to maintain the expression of the relevant genes.

2.10 S-Adenosylmethionine (SAM) for SAM-requiring strains

Strains containing the deletion of *metK* and pSAM were grown in LB with 100 μM SAM in the presence of the inducer of the transport gene.

1. Buffers and solutions

1.1 SM buffer

SM buffer is used for storage and dilution of bacteriophage λ stocks. Per liter contained:

NaCl	5.8g	MgSO4·7H2O	2g
1M Tris·Cl (pH 7.5)	50ml	2% gelatin solution	5ml

Sterilize the buffer by autoclaving for 20 minutes at 151b/sq on liquid cycle and store at room temperature.

1.2 Phosphate-buffered saline (PBS) buffer

Per liter contained:

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

Adjust the pH to 7.4 with HCl. Sterilize the buffer by autoclaving for 20 minutes at 151b/sq on liquid cycle and store at room temperature.

1.3 Binding Buffer (His-tag affinity chromatography)

Four times concentrated stock contained per liter:

80 mM Tris (pH 7.4)

600 mM NaCl

20 mM Imidazole

The Buffer was prepared using double distiled water and the pH adjusted to 7.4. The solution was diluted five times with the same water before using it.

In case denaturing conditions were used, urea was added to the buffer to a working concentration of 8 M.

1.4 Washing Buffer (His-tag affinity chromatography)

80 mM Tris (pH 7.4)

600 mM NaCl

180 mM Imidazole

Buffer prepared as in section 3.7

1.5 Elution Buffer (His-tag affinity chromatography)

80 mM Tris (pH 7.4)

600 mM NaCl

2 M Imidazole

Buffer prepared as in section 3.7

1.6 Stripping Buffer (His-tag affinity chromatography)

20 mM Tris (pH 7.4)

500 mM NaCl

100 mM EDTA

1.7 Charging Solution (His-tag affinity chromatography)

50 mM $NiSO_4$

2. Others

TAQ polymerase, T4 ligase, Calf Intestine Alkaline Phosphatase (CIAP) and restriction enzymes were purchased from MBI fermentas (Montreal, Canada), except for Bsp143I, which was obtained from New England Biolabs (USA).

Kits for plasmid extraction QIAprep Spin Miniprep Kit and GenElute Plasmid Miniprep Kit were purchased from QIAGEN (Montreal, Canada) or SIGMA (USA) respectively. DNA mini Kit was obtained from QIAGEN (Montreal, Canada). QIAquick PCR Purifcation Kit was obtained from QIAGEN (Montreal, Canada).

Concentration and desalting of proteins was done using Centricom columns (Millipore, USA) YM-10 for volumes up to 2 mls, and Microcon columns (Millipore, USA) YM-10 or Vivaspin concentrator (VivaScience, Germany) 5.000 MWCO PES for volumes up to 0.5 ml.

His Tag Affinity Chromatography was performed using His-Bind® Resin (Novagen, Germany).

3. P1 phage transduction

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

3.1 P1 phage lysate preparation

A single colony of donor strain was incubated in LB+Ca^{2+} overnight at 37°C without shaking. 0.1ml P1 phage (10^{5-6} phage/ml) was then added to 1ml of overnight culture and incubated at 37°C for 15-30 minutes to let the phage infect the donor strain. Then 3ml LB+Ca^{2+} and 3ml melted R-top agar are added, and the whole mixture is poured onto a fresh LB plate. After incubating the plate without inversion for 8 hours, the phage lysate was collected and stored at 4°C with the presence of the chloroform.

3.2 P1 phage-mediated transduction

The recipient strain was harvested and resuspended in 1/10 volume of LB+ Ca^{2+} when it reached late log phase (OD600=0.7-1.0). 0.1ml of the resuspended culture was mixed with a dilution of P1 phage lysate containing the desired gene. The mixture was incubated at 37 °C for 15 minutes, 1ml of SM phage buffer was added and cells were resuspended in 1ml of LB+ glucose. After incubation at 37°C for 1 hour, 0.2ml aliquot was plated in the appropriate selection plates.

4. Plasmid isolation and restriction enzyme digestion

4.1 Plasmid isolation

Plasmid was isolated using QIAprep Spin Miniprep Kit (QIAGEN) or GenElute Plasmid Miniprep Kit (SIGMA) following manufacturer's instructions.

4.2 Restriction enzyme digestion

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

4.3 DNA gel electrophoresis analysis

DNA agarose gel electrophoresis analysis was performed as described elsewhere (Sambrook *et al.*, 1989). The final concentration was 1.0% (W/V) agarose.

5. Electro-transformation

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

6. Plasmid Constructions

6.1 Construction of plasmid pSAM carrying the SAM Transporter gene

To construct pSAM the gene coding for the SAM Transporter (RP076) was first amplified by PCR from the original plasmid pMW1402 in which the gene was cloned (Tucker, A.M., *et al.*, 2003). The strategy used is shown in figure 5.

The primers used SAM-F (5'- TTG GTA CCT AAT GAA TGA TGC ATT AAA AAC – 3') and SAM-R (5'- TTG GAT CCT TAC TGC GAT TCA TGT TTG C– 3') were design to amplify almost only the coding sequence of the gene and contained a KpnI and BamHI site respectively. The reaction was carried in a volume of 50 µl and the mixture contained 1µM concentration of each primer, 50 ng of template pMW1402, Reaction

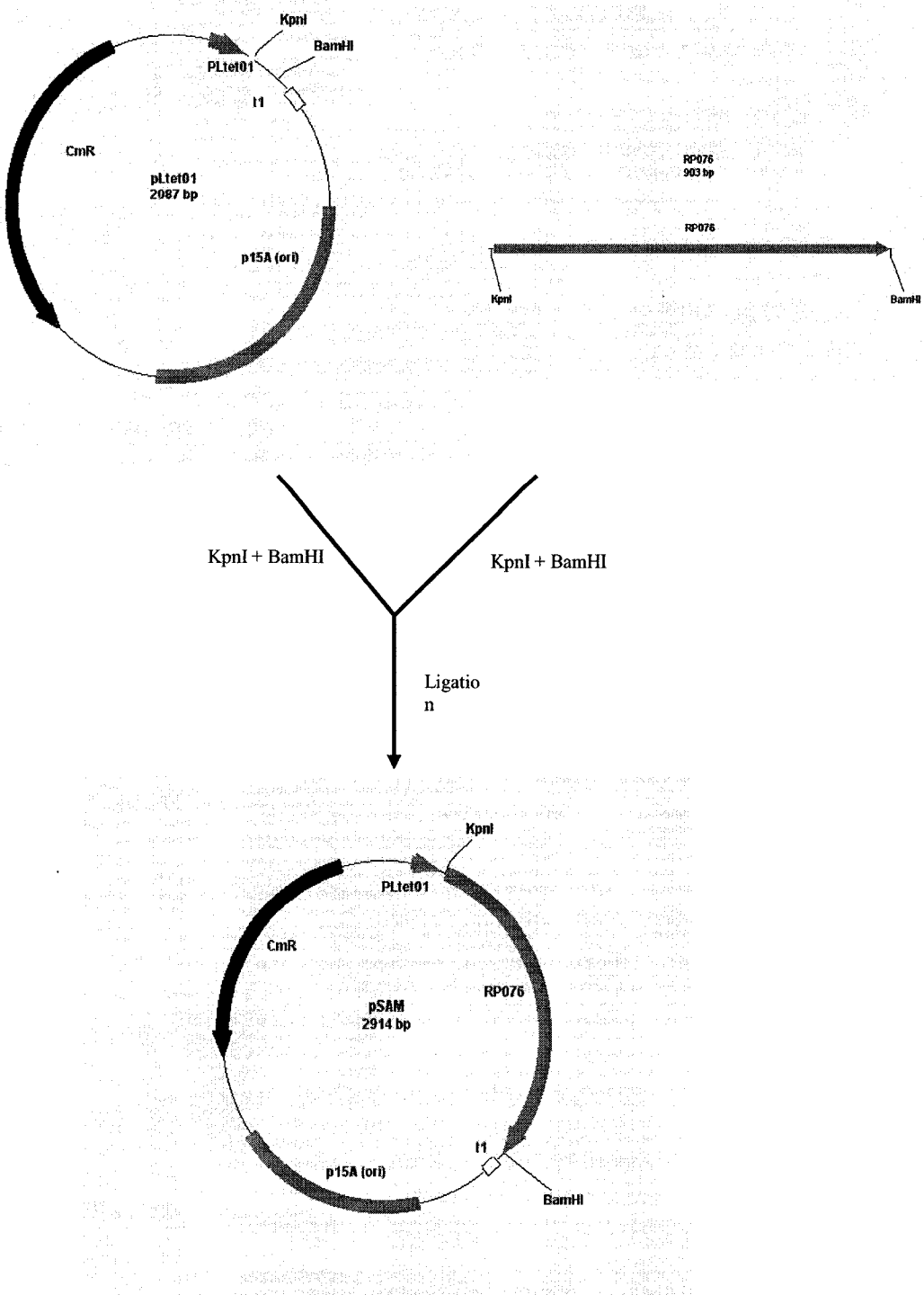


Figure 5, Construction of pSAM

buffer [75mM Tris-HCl (pH8.8), 20 mM $(NH_4)_2$ SO_4, 0.1% Tween 20], 2 mM $MgCl_2$, 300 μM dNTPs, 1 U of *Taq* DNA Polymerase (Fermentas, USA). The reaction was performed using the following program:

94 °C 4 min

30 cycles 94 °C 30 s

55 °C 30 s

72 °C 1 min

72 °C 6 min

After amplification the 902 bp product was analyzed on a 1% agarose gel and DNA was purified using QIAquick PCR Purifcation Kit (QIAGEN Montreal, Canada). The fragment was then digested sequentially with BamHI and KpnI and inserted into pLtet01 plasmid using T4 DNA ligase. DH5α competent cells were transformed by electroporation and selected on LB chloramphenicol plates. Finally some colonies were picked and their plasmid extracted and digested with the appropriate restriction enzymes to confirm its structure. The plasmid was named pSAM.

7. Construction of strains

9.1 SAD16 (CuR Δ*metK* pSAM)

SAD16 was constructed in three steps. First the deletion of the gene *serA* containing a marker of kanamycin resistance was transduced to CuR from the strain JWK2880_1 using P1 phage. The gene *serA* was used as a marker since its position in the chromosome of *E. coli* is less than one minute away from *metK*, so there was around 30% probability of co-transduction between these two genes. This strain was then transformed

with pSAM. Finally the deletion of the *metK* gene was transduced from the strain MEW648 by P1 and the co-transductants for *serA*+, including those which also carried Δ*metK*, were selected in minimal medium without serine but containing SAM. After three passes of selection through the same medium, the transductants were screened for kanamycin sensitivity and SAM dependence.

9.2 Construction of deletion strains for *dam*, *dcm* and *speD* genes

P1 phage was prepared in the deletion strains JWK3350_2(*dam*::kan^r), JWK1944_2 (*dcm*::kan^r) or JWK0116_1 (*speD*::kan^r) kindly provided by Dr. Mori. P1 was then used to infect SAD16 and the transductants were selected on LB with chloramphenicol, ATC, SAM and kanamycin. To test if the deletion was successfully transferred an assay to determine the methylation pattern in DNA was used for Δ*dam* and Δ*dcm*. In the case of a PCR using primers flanking the gene Δ*speD* was used to verify the correct transduction of the deletion.

9.3 Construction of Cu derivative strains containing the plasmids pftsZ-his and pftsA-his

The strains containing the plasmid pftsZ-his (JW0093) and pftsA-his (JW0092) were kindly provided by Dr. Mori. The plasmids were extracted from them and analyzed by digestion with the restriction enzyme SfiI, whose sites are flanking the genes inserted. The plasmids were transformed into Cu competent cells by electroporation and selected in LB with chloramphenicol.

10. Growth and viability experiments in the SAM dependent strains

10.1 Growth curves

Cells were grown overnight in rich medium containing 100 μM SAM and required supplements and antibiotics. Cells were then subcultured in fresh medium with the same supplements and grown for some hours until the culture reached an OD at 600 nm between 0.5-1. At that point the cultures were chilled in ice water and centrifuged for 5 minutes at 4000 rpm in cold conditions (4 °C). The pellets were washed twice with cold LB without any supplement and resuspended in the same medium to remove SAM from the medium. The OD of the cell suspension was determined and a dilution of it in 20 ml of fresh medium with different concentrations of SAM was made, beginning with an OD600 near 0.05. The cultures were incubated at 37 °C and changes in density were followed using a Klett-Sumerson colorimeter with a green filter (OD500-580).

10.2 Viability experiments

Cells were treated as in the growth curve experiments, except that after the washes they were resuspended in medium containing no SAM. The cell suspension was incubated at 37 °C and changes in viability were studied by plating dilutions of the culture on LB with SAM and ATC in quadruplicate at different times after SAM was removed from the medium. The number of colonies was determined after one day of incubation at 37 °C and corrected by the dilution to obtain the colony forming units (cfu) per ml of culture.

10.3 Yield experiments

Cells were grown in LB containing SAM, ATC (when required) and antibiotics. A subculture was done in the same medium and the cultures grown for some hours until they had turbidity in OD600 between 0.5- 1. Then a 1/1000 dilution was made in flasks containing LB medium with the supplements needed and different concentrations of SAM. For each concentration of SAM two flasks were used per strain in each single experiment. The cells were grown overnight and the optical density was measured at hourly intervals until no significant change was seen within 2 average doubling times. The volume of the cultures was corrected for the loss of volume due to evaporation. Then 3 ml of each culture were transferred to three eppendorf tubes (1 ml in each tube), the cells pelleted at 5,000 rpm for 5 minutes and washed once with PBS to remove the remnants of the medium culture and stored as pellet until used.

To determine the protein concentration the pellets were resuspended in 1 ml of 5% trichloracetic acid and stored on ice for 10 minutes after which the proteins precipitated. The tubes were centrifuged at 13,000 rpm for 2 minutes and the pellet was kept and resuspended by vortexing in 100 μl of 1 N NaOH. Finally 0.9 ml of distilled water were added to the mixture and an aliquot of it was used to quantify protein concentration using Biorad's Protein Assay system as specified by the manufacturer.

11. Determination of the DNA methylation

11.1 Dam methylation assay

Chromosomal DNA was extracted from overnight cultures using QIAGEN's DNA mini kit following manufacturer's instructions. DNA was quantified by determining the

absorbance at 260 nm. The same amount of DNA was digested with Bsp143I (cuts both methylated and not methylated DNA), MboI (cuts only not methylated DNA), DpnI (cuts only methylated DNA) or not digested at all. The results were analyzed in a 1% agarose gel.

11.2 Dcm methylation assay

Chromosomal DNA was extracted from overnight cultures using QIAGEN's DNA mini kit following manufacturer's instructions. DNA was quantified by determining the absorbance at 260 nm. The same amount of DNA was digested with EcoRII (cuts only not methylated DNA), BstNI (cuts methylated and not methylated DNA) or not digested at all. The results were analyzed in a 1% agarose gel.

12. Protein purification

Cells containing the plasmids pftsZ-his or pftsA-his were grown at 37 °C overnight in rich medium containing chloramphenicol and of glucose 1% to repress the basal expression from the plasmids. The cells were subcultured in the same medium and incubated until they reached an OD600 between 0.5- 1. A dilution of the culture was made in rich medium without glucose and the cultures were incubated until they reached again an OD600 near 0.5, at which point they were induced with 1 mM IPTG. The cultures were incubated for 3 to 4 hours after the induction, they were then centrifuged at 4,000 rpm for 20 minutes at 4 °C, washed with PBS once and the pellets resuspended in binding buffer and stored at -86 °C until they were used. Before purification cells were disrupted by sonication and cellular debris was removed by centrifugation at 30,000 x g

for 15 minutes at 4 °C. The supernatant was transferred to a new tube and used for protein purification.

A column containing new His-Bind resin was first washed using two volumes of double distilled water and then charged with the same volume of nickel sulfate solution. The column was washed with water again and then equilibrated with two volumes of binding buffer. The sample was then loaded into the column at a flow rate of 0.4 ml/min. The column was washed with four volumes of binding buffer and with eight volumes of washing buffer at the same flow rate, and finally the protein was recovered using elution buffer. The amount of protein obtained was determined by the Biorad's protein assay system and the level of purity of the sample was analyzed using an SDS-PAGE gel.

13. Protein analysis by mass spectrometry

13.1 Analysis of the proteins using ESI-TQ mass spectrometer

13.1.1 Preparation of samples

The purified protein was first concentrated using centricom filters, the sample was diluted ten times in 10 mM ammonium bicarbonate and concentrated again. The same process was repeated two times more and then done two times in 1 mM ammonium bicarbonate and two times in double distilled water. The protein concentration of the treated sample was determined and a 1:1 dilution of it was made in a solution containing 50% acetonitrile and 0.1% trifluoracetic acid in double distilled water.

13.1.2 Analysis of samples

The samples were analyzed with the Q-TOF 2^{TM} (Micromass, USA) mass spectrometer, using electrospray ionization (ESI) (Nano spray).

Results

The present study explored two aspects of the physiological role of S-adenosylmethionine (SAM) in *E. coli* K12. Most of the experiments, found in Part A, deal with the use of a SAM transporter from *Rickettsia prowazekii* to rescue the mutation in strain MetK84 and characterize a strain with a deletion of *metK*, and thus totally devoid of SAM synthetase. The second section, Part B, reports an investigation of the possible methylation of the first two proteins of the cell division.

PART A_ Studies on the role of SAM in *E. coli* using a SAM transporter

1. Construction and characterization of a strain which requires an exogenous supply of SAM

1.1 Selection of a variant efficient in the use of SAM

Escherichia coli is not able to transport SAM from the medium (Holloway *et al*, 1970) and this has hindered the studies on the function of this molecule. Indeed, there was no described protein capable of transporting SAM in bacteria until 2003, when the product of the gene RP076 (*sam*) from the alpha proteobacteria *Rickettsia prowazekii* was described (Tucker *et al.*, 2003). This study showed that the transporter when it was expressed in *E. coli*, permitted the cell to incorporate radioactive SAM from the medium.

Given the potential of the SAM transporter to facilitate the investigation of the role of SAM, I decided to study the activity of the transporter in *E. coli* and the effect of supplying SAM from the medium This was possible because the plasmid pMW1402, which expresses constitutively the gene coding for the SAM transporter, was kindly

supplied by Dr Wood. To test if the protein was produced and functional, I started with a strain (MEW648) containing a deletion of *metK* and a rescuing plasmid which transcribes the *metK* gene only in the presence of the inducer (ATC). MEW648 was previously used in this lab to demonstrate that the *metK* gene is essential (Wei and Newman, 2002) by showing that cells can only grow when ATC is present. A MEW648 derivative containing the SAM transporter should be able to grow in two ways: either first when the inducer is present resulting in the production of SAM synthetase or second when SAM is added to the medium and taken up by the transporter. The transporter would allow growth when SAM is supplemented, assuming that the transport is efficient enough to supply enough SAM for the basic functions of the cell, and that the production of SAM is the only essential role of *metK*.

To construct the appropriate strain the plasmid pMW1402 carrying the gene for the SAM transporter was introduced into strain MEW648 (Δ*metK* pLtet-K) and transformants were selected on rich medium plates containing antibiotics and 100 μM SAM but no ATC. The resulting colonies were very heterogeneous in size and were only visible after two days of growth at 37 °C. The ability of these cells to form colonies when SAM is provided externally demonstrates for the first time that the synthesis of SAM is the only essential function of *metK*.

The slow formation of colonies suggested that SAM was not being introduced or used efficiently in the cells. Therefore to facilitate subsequent studies, I decided to select a variant in which the growth was improved. The heterogeneity in colony morphology noted above in cells selected for growth using SAM was probably caused by mutations, which could be on the plasmid pMW1402 and therefore modify the amount of SAM

introduced into the cell or alternatively they could be in the chromosome changing the way in which SAM was used. An increase in growth rate due to a mutation on the plasmid would most likely result from an increase in expression or function of the transporter. This plasmid mutation could then be transferred to any cell conferring the same efficiency. I therefore looked for a more efficient mutant plasmid. To do this, I extracted plasmid from several variants able to form big colonies visible after one day of growth, introduced the plasmids into strain MEW648 and examined the colonies developed in rich medium supplemented with SAM. One of the plasmid variants conferred the ability to form big colonies on all of its transformants when SAM was supplied. The plasmid was given the name pSAD1 (SAM-Dependent1). The strain containing it (Cu Δ*metK* pSAD1) was named SAD1. This strain SAD1 clearly contains a mutant plasmid which allows the cells to incorporate SAM more efficiently from the medium. Whether this results from a mutation in the transporter gene *sam* itself or from a mutation elsewhere in the plasmid possibly affecting expression of the gene was not determined but it did not matter for the purposes of this study.

1.2 Characterization of growth of the SAM dependent strain SAD1

Until the present study, the effect of variations in the intracellular concentration of SAM in *E. coli* could be studied in a few, somewhat indirect ways. These include the use of a plasmid carrying the gene for SAM synthetase with a promoter regulatable by an external inducer (Posnick and Samson, 1999), or of strains containing mutations in the *metK* gene which alter the expression or functions of SAM synthetase (Newman *et al.*, 1998), or by the introduction of a regulatable copy of the gene coding SAM-hydrolase

from the phage T3 (Hughes *et al.*, 1987; Posnick and Samson, 1999). Each of these strategies has its own problems mainly because the concentration of SAM in each case is dependent on the activity of an enzyme and therefore cannot be regulated very closely.

The SAD1 strain allows a much more direct analysis. It can obtain SAM in either of two ways: by inducing *metK* expression with ATC and thus synthesizing its own SAM, or by using the transporter to incorporate to incorporate SAM from the medium. A comparison of these two methods of growth is seen in figure 6. When SAM synthetase was not induced, SAD1 cells depended on an external source of SAM. SAD1 cells grown overnight in LB with 100 μM SAM had a doubling time of 58 minutes reaching a turbidity of 355 klett units (KU) at the stationary phase when subcultured in medium containing 100 μM SAM.

When subcultured in a medium without SAM, the culture did not increase in density. Thus, when using external SAM for growth, these cells were absolutely dependent on an external supply and did not accumulate enough SAM to grow for an appreciable time without it. In contrast, when the cells were cultured in medium containing ATC they continued growing for some hours after the inducer was removed reaching a turbidity of 110 KU, presumably because the SAM synthetase which has been induced could still function. This suggests that intracellular SAM synthetase is quite stable once made, though there might be some inducer remaining after the cells were washed and subcultured. On the other hand, when cells were grown supplying the SAM exogenously

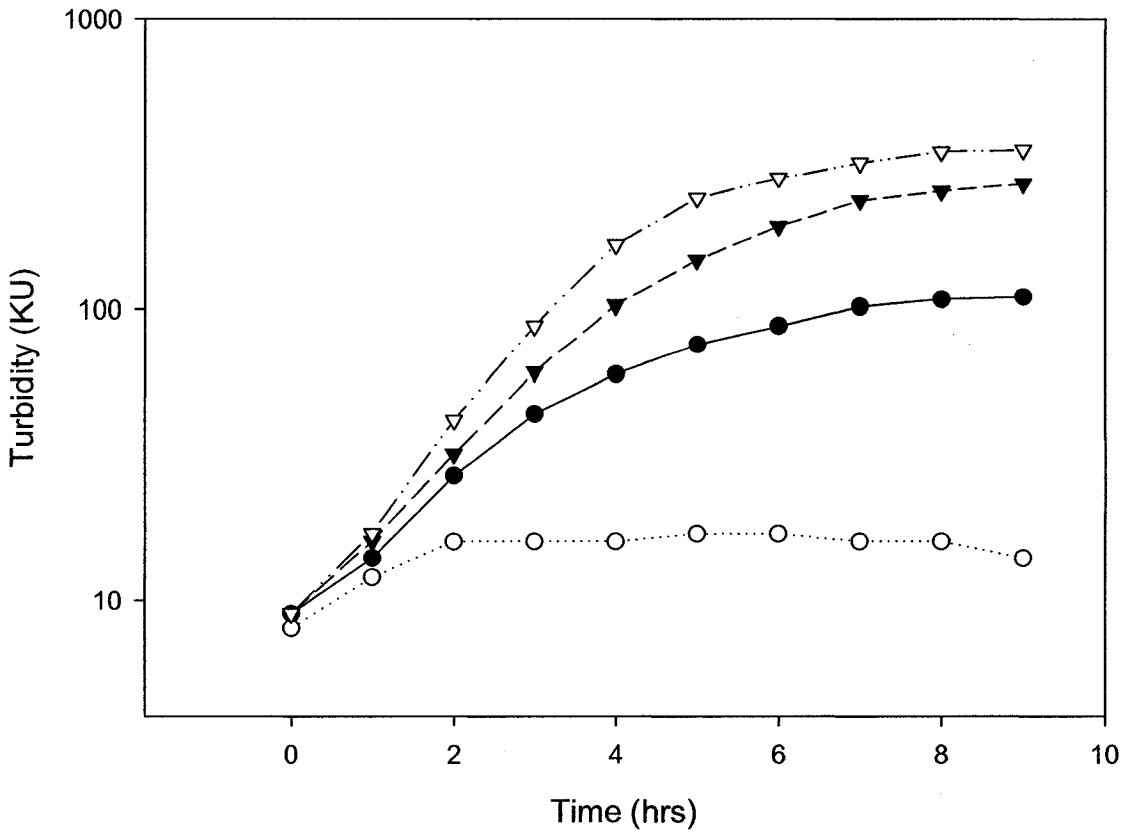


Figure 6, SAD1 grown in presence of SAM or ATC_ SAD1 was either grown ovenight in LB containing 100 ng/ml ATC, washed and resuspended in LB without the inducer (●), or it was grown overnight in LB containing 100 μM SAM and resuspended in LB (○), LB with 100 ng/ml ATC (▼) or LB with 100 μM SAM (▽).

the increase in turbidity immediately stopped, suggesting that the intracellular pool of SAM was not sufficient to maintain growth.

The effect of SAM concentration in the medium on the growth of SAD1 was studied further. When cells were incubated with an appropriate range of SAM concentrations and allowed to grow until the turbidity was constant for a time equivalent to two generations, the final turbidity depended directly on the amount of SAM provided. Thus cultures with 5, 10, 50 or 100 μM SAM reached a turbidity of 101, 133, 358 or 450 KUs respectively (figure 7). Increasing the SAM concentration above 100 μM, did not increase the final turbidity much. The cultures reached only about 515 KU when given 5 or 10 times more SAM, scarcely more than the 450 seen with 100 μM. Indeed, a concentration of 5 mM was shown to be somewhat toxic and decreased the growth rate markedly (figure 7). As shown in the previous experiment, the strain did not grow when no SAM was added.

SAM is involved in many reactions. SAM is the direct methyl donor for a large variety of methylations (Chiang *et al.*, 1996). SAM is also known to act as donor of other chemical groups (Fontecave, 2004) and as regulator of methionine biosynthesis (Greene, 1996). We wanted to determine how the growth of the SAM dependent strain is affected by an external supply of SAM-derived compounds, like spermidine and methionine, both of which can be incorporated from the medium. Supplying them in the medium should reduce the requirement for exogenous SAM. Spermidine has been related to a number of processes in the cells, where it acts through binding of RNA, DNA, nucleotide triphosphates and other acidic substances (reviewed in Igarashi and Kashiwagi, 2000). Spermidine is synthesized from SAM by two successive reactions, the first decarboxylating SAM and the second transferring the aminopropyl group to

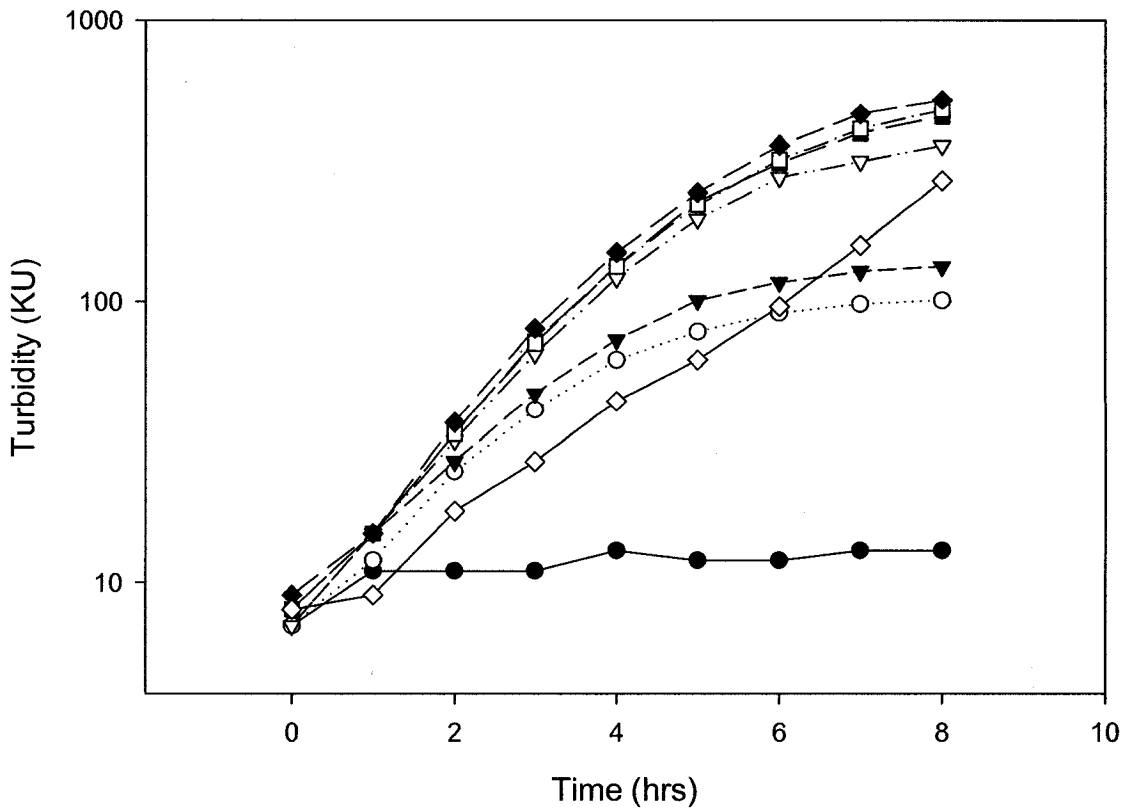


Figure7, Growth curve for SAD1 at different SAM concentrations_ SAD1 was precultured in LB containing 100 μM SAM, washed and grown in LB with no SAM (●), 5 μM (○), 10 μM (▼), 50 μM (▽), 100 μM (■), 500 μM (□), 1 mM (◆), 5 mM (◇).

putrescine. The activity of the first enzyme is negatively regulated by spermidine itself. Similarly it is known that an excess of SAM represses the synthesis of methionine (Greene, 1996). Indeed, although methionine usually obtains the methyl group in the last step of its synthesis from methyltetrahydrofolate, SAM itself can also be used as methyl donor of homocysteine to generate methionine but this activity is inhibited by high concentrations of methionine (Tanbichler *et al*, 1999).

To investigate the effect of methionine and spermidine on the requirement for SAM, SAD1 was grown with a low concentration of SAM (5 μM), chosen because changes in yield are most closely proportional to changes in the SAM provided or the intracellular SAM pool in this range of SAM concentrations, and either spermidine or methionine were added to the medium. The supplementation of the growth medium with spermidine or methionine should change the yield of the cultures, resulting from an increase in the intracellular SAM pool, if the amount of SAM involved in their synthesis or metabolism required a significant proportion of the total SAM available to the cell. However no change in growth rate or yield of the strain was observed when spermidine at 50 μM was supplied in the medium (figure 8). Neither was there a change when methionine at 30 μM was used in the medium or even when both supplements were added together. The results suggest that the synthesis of these compounds was not a considerable drain on the intracellular SAM pool, at least in the conditions tested, or that the concentrations of the supplements were not high enough to prevent their synthesis from SAM. That is, the growth of SAD1 at suboptimal concentrations of SAM was not improved by the supplementation of both spermidine or methionine. Other compounds might be studied in the same way.

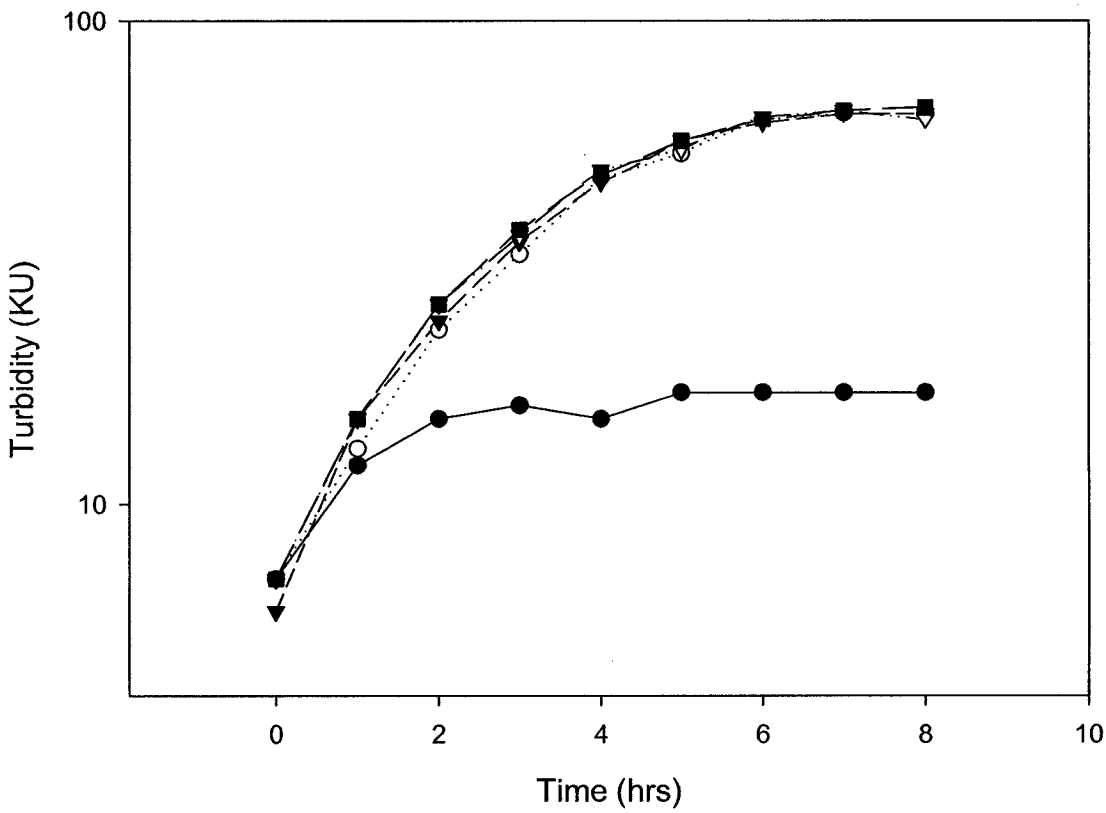


Figure 8, Effect of spermidine and methionine on SAD1 growth _ SAD1 was grown in LB with 100 μM SAM, washed and resuspended in LB without SAM (●), or with 5 μM SAM (○), 5 μM SAM and methionine 30 μgml (▼), 5 μM SAM and spermidine 50 μgml or with 5 μM SAM (▽), with the same concentrations of methionine and spermidine (■)

1.3 Viability of SAD1 during SAM starvation

The strain *metK*84 has a point mutation at the -10 region of the promoter and produces less SAM-synthetase than the wild type strain (Wei and Newman, 2002). Since the global regulatory leucine responsive protein (Lrp) negatively controls the expression of *metK*, the transcription of this gene in the MetK84 mutant cells is dependent on the presence of high leucine concentrations in the medium in order to remove Lrp from the *metK* promoter. The growth of *metK*84 cells in minimal medium with low concentrations of leucine (5 μg/ml) results in extensive filamentation (Newman *et. al.*, 1998). A study in which the gene of T3 SAM Hydrolase was introduced in the cells showed that when the hydrolase was induced, the intracellular SAM levels dropped and the cells became elongated (Posnick. and Samson, 1999). Both studies suggested that low levels of intracellular SAM affect cell division, resulting in the formation of elongated cells. For this reason it was surprising not to see filaments in the cultures when SAM was removed from the cultures of SAD1.

Not only do the cells not filament, but also they do not increase significantly in mass as judged by the fact that the culture turbidity does not increase in the absence of SAM (figure 6). Unexpectedly, the cultures also decreased in viability. The intriguing possibility that death was caused by a process related to SAM starvation and independent of cell division lead us to study further the timing and the extent of death.

The first experiments performed were done using logarithmic phase cultures of SAD1 and starved for SAM. Aliquots of these cultures were chilled on ice, diluted and plated. The results showed that a considerable proportion (more than 80 percent) of the original population died before one hour after starvation.

These experiments clearly demonstrated that the cultures starved of SAM could not form colonies after dilution and plating. To see if the cells were actually dead prior to dilution, I starved the cells for various lengths of time, and then added SAM and followed the turbidity of the culture (figure 9) When SAM was restored after 1 hour, turbidity could be seen to increase. If as many cells had died as was indicated by the determination of colony forming units, 80% of the cells would have been dead. The remaining 20% might divide- but the increase in turbidity would be much less than the increase actually seen (figure 9). Readdition of SAM actually allowed most of the cells to grow, and I was able to calculate a growth rate for the period from one to three hours after refeeding. For cells starved for 0,1,2 and 3 hours, the growth rate was 45, 57, 64 and 122 minutes. Thus after 2 hours, for instance, the cells were slowed down somewhat. Even if this was due to death of some of the cells, at least half the culture must still be alive, a much different result from that seen by determining the viable count.

Thus one of the major differences between these two experiments is the transient change in temperature during plating. To see whether exposure to cold temperatures of cells starved for SAM increased the rate of death, I diluted starved cultures in LB tubes preincubated at 37°C, and plated on prewarmed LBagar plates, comparing this to diluting in and plating on cold medium (4 °C). As shown in figure 10 the number of viable colonies dropped after the onset of SAM starvation even when the cells were maintained at 37 °C, but death was markedly lessened. 80% of the cells were still alive after three hours of starvation. The decrease in viability was more dramatic if the cells were spread on 4 °C plates even when the plates were immediately placed in a 37 °C incubator, in which cases only around 30 % of the cells survived after one hour starvation. While it is

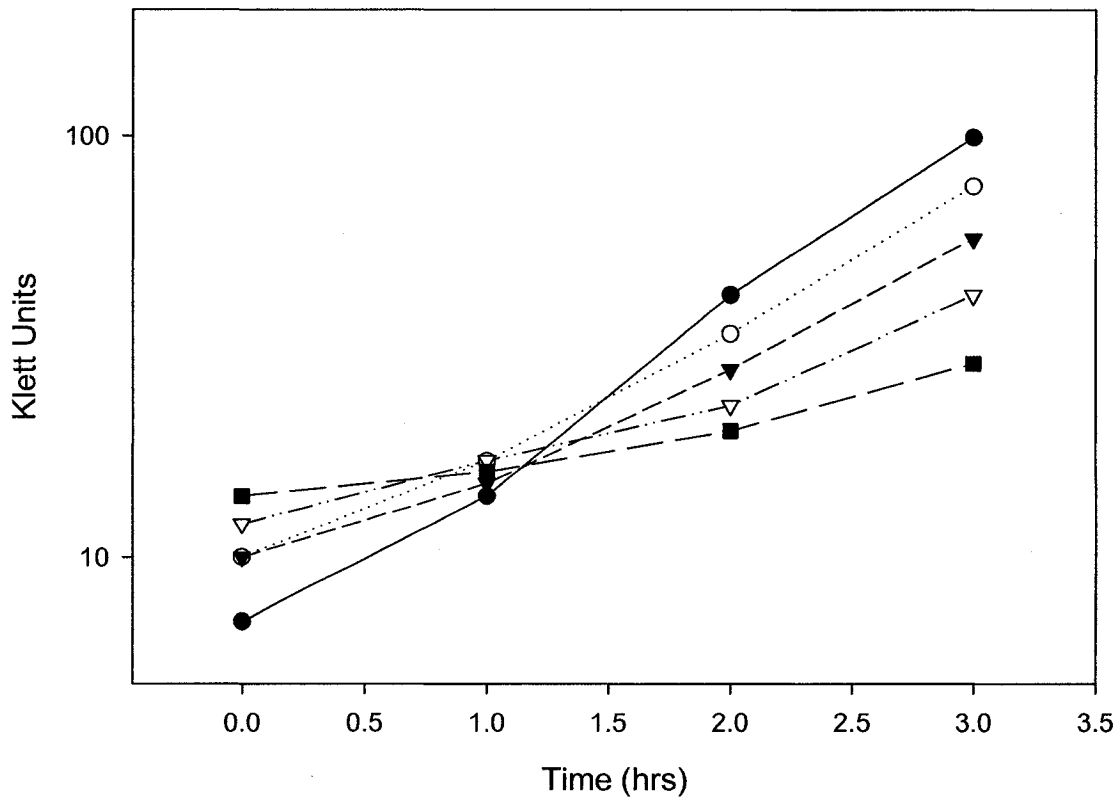


Figure 9, Effect of SAM starvation on SAD1 growth rate SAD1 cells were grown in LB containing 100 μM SAM and either subcultured in medium with the same concentration of SAM (●) or in LB with no SAM at all and added after 1 (○), 2 (▼), 3 (▽) or 4 hrs (■) of incubation. The graph show only the the behavior of the strain from the point where SAM was added to three hours later after that.

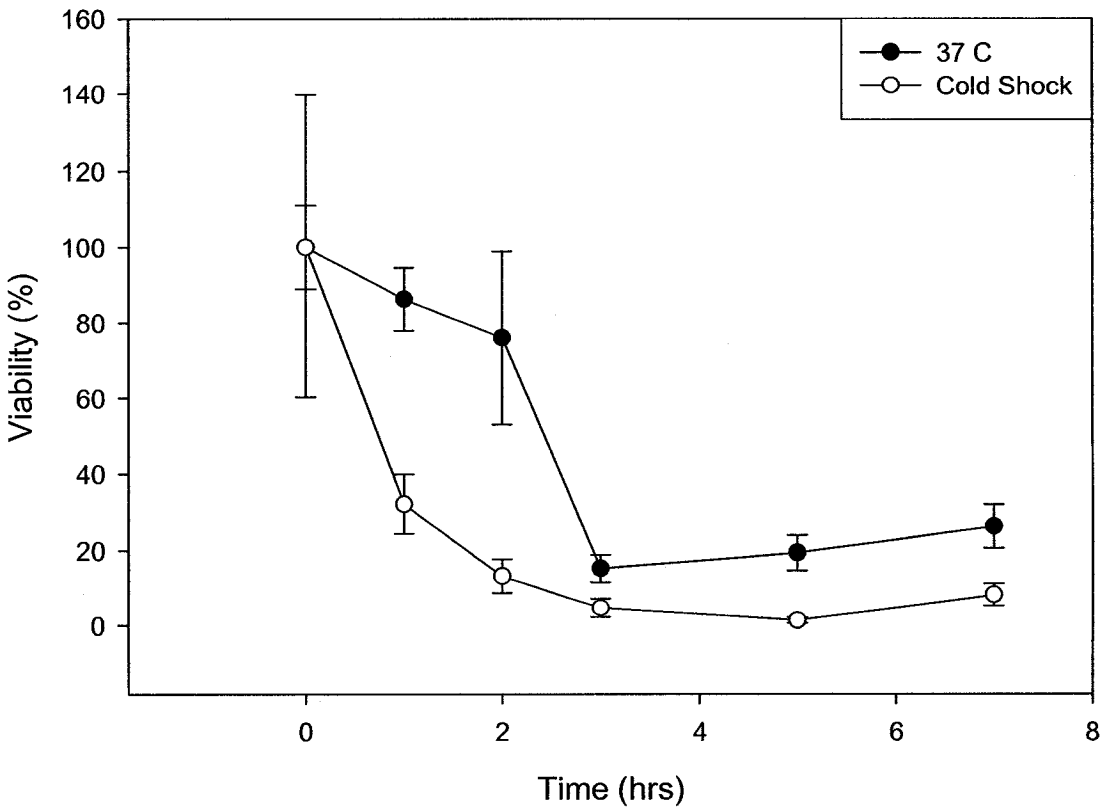


Figure 10, SAM starvation and death SAD1 cells were grown in LB with 100 μM, washed and subcultured in LB with no SAM. Aliquots of the culture were serially diluted and plated on warm (●) or cold (○) LB agar.

clear that SAM starvation leads to death in SAD1, further studies will have to determine if this is caused by the inability of the cells to perform an essential SAM dependent process(es) or if the cells die because of a more unspecific response to stress (Hazan, 2004). The effect of starvation is increased in cold conditions, which may or may not be the same process as causes death at 37 °C.

2. Attempt to complement strain MetK84 by *sam* gene in pSAD1 and SAM

MetK84 forms filaments in minimal medium with low concentrations of leucine because the rate at which *metK* is transcribed drops enough to affect an activity related to cell division (Wei and Newman, 2002). The easiest way to explain this is by assuming that the cell will have fewer copies of SAM-synthetase and therefore a lower concentration of SAM. However it is also possible that either the *metK* gene or the protein for which it codes, or indeed both, is essential for a secondary unknown function. The following experiment demonstrates that SAM is the only essential product of *metK*. If the formation of filaments is caused by suboptimal levels of SAM inside the cells, the presence of the SAM transporter and SAM in the medium should allow the *metK*84 cells to divide normally even without the presence of leucine and SAM synthetase.

To test this hypothesis, the plasmid pSAD1 was introduced into *metK84* cells which were grown in minimal medium with different concentrations of leucine (*metK* inducer) and SAM. Unexpectedly, the presence of the plasmid resulted in a much increased requirement of leucine for growth. As shown in figure 11c the cells containing the plasmid did not divide normally even when a concentration of 200 μg/ml of leucine was used whereas the *metK* mutant normally requires only 100 ug/ml. Even the vector without

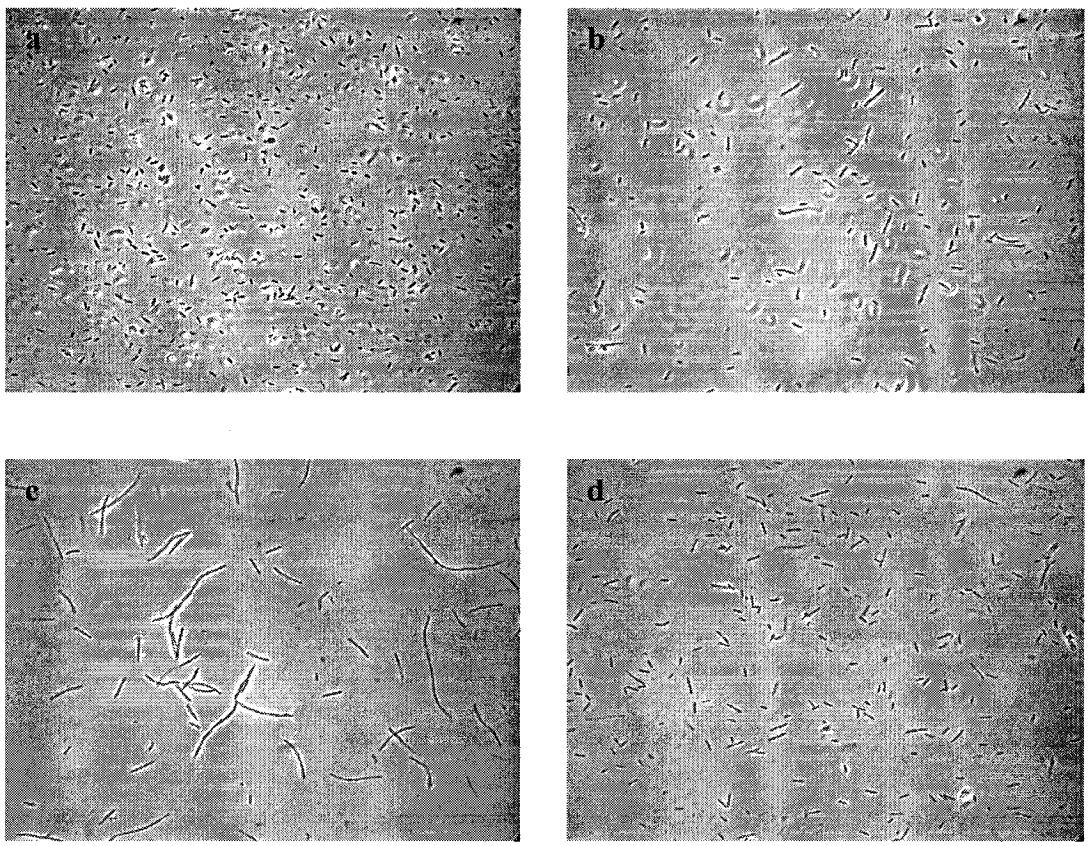


Figure 11, Supplementing SAM to MetK84 pMW1402_ MetK84 cells grown in minimal medium with 200 μg/ml of leucine with no plasmid (a), empty vector (b), pMW1402 (c) and the same with 1 mM SAM. The vector by itself affects the way MetK grows. The cells are even more affected when the gene is in the plasmid, but a difference can be observed when SAM is present.

the transporter cloned on it interfered with growth, suggesting that the problem was associated with plasmid replication.

Whereas the cells could not be grown with normal concentrations of SAM, they did grow with 1mM SAM if leucine was also present in the medium (figure 11d). This is in some sense equivalent to the conditions in LB with added SAM. LB of course contains a great deal of leucine. It is only in these experiments which were done in minimal medium that the problems caused by the plasmid became evident. These are probably caused by its high copy number, which may also increase the need for SAM to methylate plasmid DNA, and inefficient transcription of the *sam* gene from the plasmid. In summary, the presence of pSAD1 in *metK*84 cells makes them more prone to filamentation. High concentrations of SAM and leucine restored normal growth of the *metK84* strain. However the use of the transporter gene as carried on this vector did not allow us to clearly demonstrate that SAM alone can revert the phenotype since leucine had always to be added in addition.

3. Construction of pSAM

To overcome the limitations imposed by the original plasmid in which *sam* was cloned, the *sam* gene was cloned in pLtet01. This plasmid is a low copy number expression vector with a p15A origin of replication (Lutz and Bujard, 1997). This carries a Pltet promoter with a convenient multiple cloning site, and therefore the cloned gene is transcribed when induced by tetracycline or analogues. This system was previously demonstrated to be sensitive enough to allow physiological studies of the

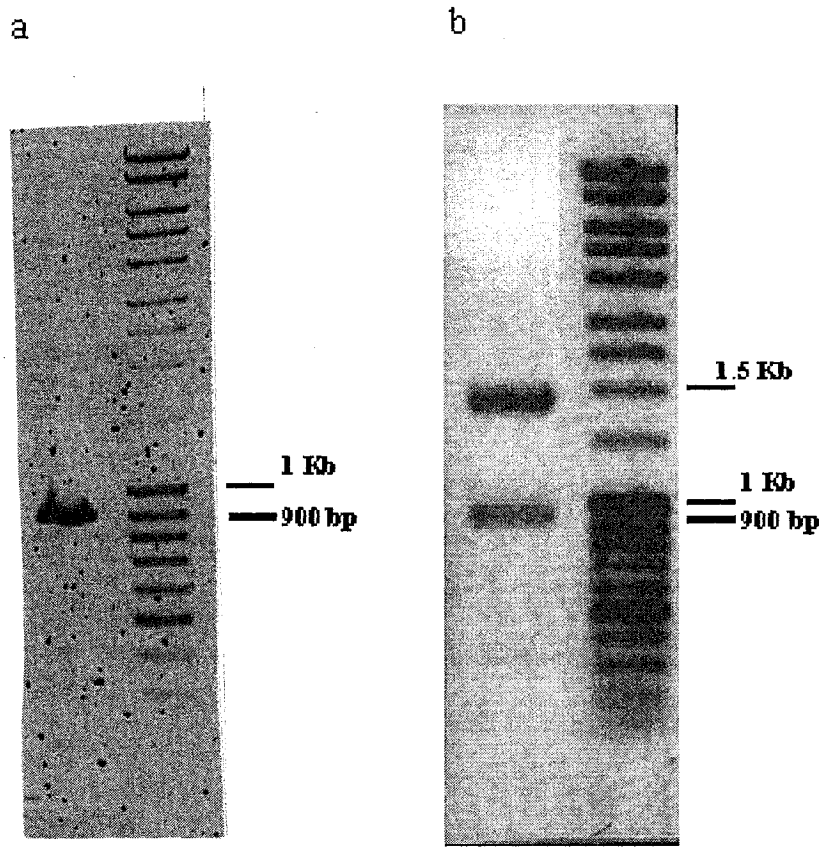


Figure 12, construction of pSAM_ SAM transporter coding sequence was amplified from pMW1402 by PCR (a), and later inserted into KpnI – BamHI restriction sites of the expression vector pLtet01. The new plasmid was called pSAM.

metK gene (Wei, 2001), and has been afterwards used extensively for this purpose in this lab.

To clone the SAM transporter gene, the coding sequence was amplified from pMW1402 by PCR using a 5' primer which contained a KpnI restriction site and a 3' primer with a BamHI to facilitate cloning into pLtet01. As was expected the PCR gave a product of 903 bp (figure 12b) which was then digested first with KpnI and then with BamHI and inserted into pLtet01, giving a plasmid of 2914 bp. To analyze the plasmid construction, it was digested with BamHI-XhoI, the size of the fragments expected were of 994 and 1920 bp, which as shown in figure 12b corresponds to the results obtained for one of the clones. The plasmid containing the gene *sam* regulated by a tet promoter was named pSAM and used for subsequent studies.

4. Complementation of MetK84 by pSAM

I first used the pltet plasmid to study the relation between SAM and filamentation in the *metK84* mutant. pSAM did indeed give significantly clearer results than pSAD1. The *metK84* cells did filament when grown with a low concentration of leucine (5 μg/ml) without inducer (figures 13a), but they showed a normal phenotype when grown in presence of higher concentrations (200 μg/ml)(figure 13b), showing that the plasmid by itself did not affect the growth. Furthermore, induction of the gene with 100 ng/ml ATC did not cause the cells to filament, reinforcing the idea that the SAM transporter is not toxic. Indeed strain *metK84* pSAM behaved as the parental strain *metK84* when SAM was not added to the medium.

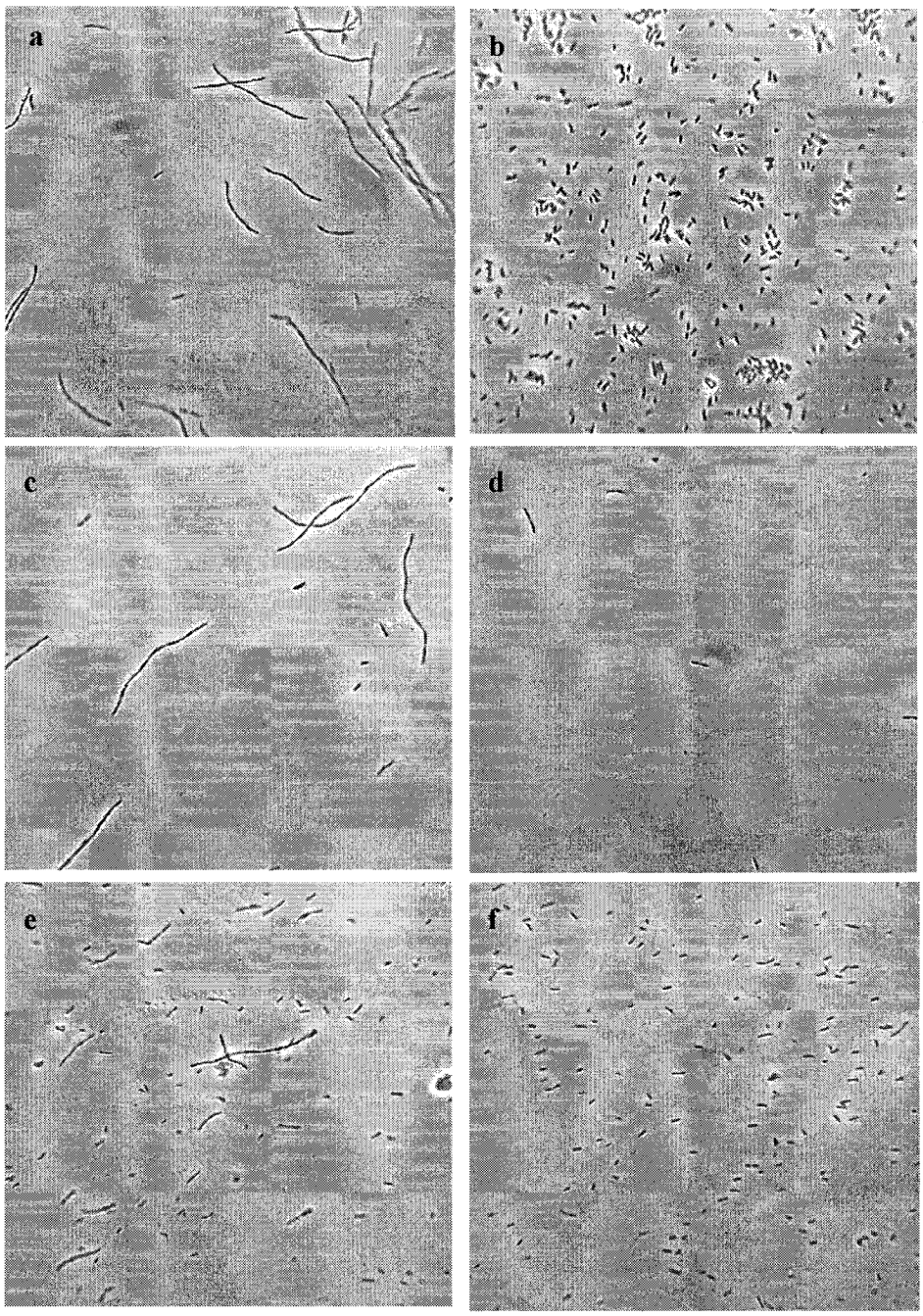


Figure 13, Supplementing SAM to MetK84 pSAM_ Cells were grown in minimal medium supplemented with leucine at 5 (a) or 100 μg/ml (b); 60 μg/ml methionine (c); SAM 100 μM (d); SAM 100 μM and methionine 60 μg/ml with ATC 1 (e) or 100 ng/ml (f).

SAM could prevent filamentation of *metK84* pSAM, but only if methionine and ATC were also added (figure 13f). In the absence of methionine and ATC, the cultures provided with 100 μM SAM reached a very low density (figure 13d). When SAM, methionine and low concentrations of ATC (1 ng/ml) were present, the cells were able to grow but showed some filamentation (figure 13e). Cells only exhibited a normal morphology when ATC was present at a high concentration (100 ng/ml) in medium containing SAM and methionine (figure 13f).

This demonstrates that the plasmid pSAM produces a functional protein when the inducer ATC is present, and is thus subject to regulation by ATC. It also showed that the SAM transporter carried on this plasmid (pltet) is not toxic to the cells as it seemed to be when carried on the original plasmid pSAD1, since single cells could be observed at high concentrations of leucine even when the gene was induced by ATC. Methionine was shown to be an important factor when growing cells by providing exogenously SAM. Finally, the results showed that the formation of filaments observed in MetK84 in low concentrations of leucine can be prevented just by introducing SAM into the cells, demonstrating that the filamentation of MetK84 is caused by a suboptimal intracellular concentration of SAM. Filamentation thus is due to a deficiency in a SAM-dependent process related to cell division. It is now clear that *metK* gene and the SAM-synthetase protein itself do not have any participation in cell division other than the production of SAM in the SAM-dependent process related to cell division.

5. Construction and characterization of Δ*metK* pSAM strain SAD16

5.1 Construction of SAD16

The use of SAD1 permitted us to demonstrate that cells can grow when SAM is provided exogenously. Nevertheless neither the growth nor the morphology of the cells was similar to the wild type strain. The studies on strain MetK84 using the SAM transporter in pSAD1 showed that the production of the protein from this plasmid was not as efficient as when pSAM was used, probably due to low levels of transcription of *sam* gene from the original plasmid. However one would expect that provision of the transporter transcribed from the plasmid pSAM would allow cells of a *metK* deletion strain to grow in a way more similar to the wild type.

Strain SAD1 contained a mutant copy of the *recA* gene, originally introduced to prevent recombination between the wild type copy of *metK* in pLtet-K and the deletion in the chromosome. This made it impossible to transduce any genes into it. Having a wild type *recA* gene with a deletion in *metK* would allow us to introduce further mutations in specific genes to study different aspects of SAM utilization in the cell. Since a plasmid containing *metK* is not needed if the SAM transporter is present and SAM is provided, the mutation in *recA-* would not longer be necessary. For these reasons, I decided to construct a strain containing the newly cloned pSAM and a deletion of *metK* which would allow us to introduce SAM more efficiently into the cells and transduce alleles from other strains.

In order to construct a Δ*metK* pSAM strain, I began by transducing a *serA*::kan^r gene replacement into strain Cu pSAM forming strain Cu *serA* pSAM. Since the *serA* gene is only 0.6 minutes away from *metK* in the chromosome, about 34% of *serA*+ transductants

will acquire the donor *metK* allele. Thus using MEW648 (Δ*metK* pLtet-K) as a donor, transductants of strain Cu *serA* pSAM were therefore selected for the autotrophy for serine, growing them in minimal medium containing SAM, methionine and ATC to permit growth of both SAM dependent and SAM independent cells. Then the transductants containing the deletion of *metK* were identified by their inability to grow in minimal medium without SAM. Some transductants with the desired phenotype were picked and purified further by selecting them in minimal medium with SAM, methionine and ATC. The strains constructed in this way were found to produce a phage which appeared without any discernable pattern, either coming from the strain used to generate the *serA*::kan^r or present always in the parental strain and only activated in conditions when SAM was supplied from the medium. To continue with the characterization of the roles of SAM we decided to isolate a variant strain resistant to infection by this phage. One such variant was selected and named SAD16. The strain SAD16 contains the SAM transporter gene in pSAM plasmid and no *metK* gene either in the chromosome or in plasmid. The absence of *metK* gene has since been confirmed by PCR.

5.2 Characterization of growth of SAD16 with exogenously provided SAM

Growth of SAD16 showed a direct relation between the concentration of SAM and the yield of the cultures (figure 14). This relation was linear only at low concentrations of SAM. The yield for cultures grown with 2.5, 5 and 10 μM was of 181, 228 and 312 KU in the experiment shown (figure 14), but 50 or 100 μM gave yields of only 376 and 413 KU. The growth of strain SAD16 on LB supplemented with SAM was markedly faster than that of SAD1, the apparent doubling times being 40 and 60 minutes respectively.

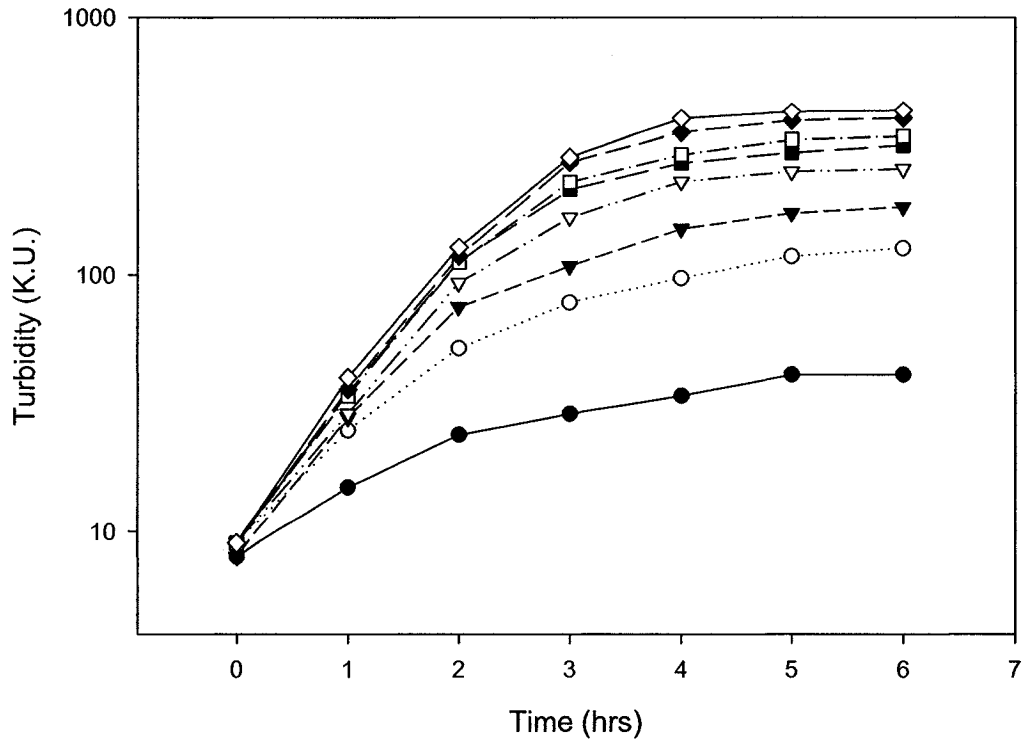


Figure 14, Response of SAD16 to different concentrations of SAM_ SAD16 was grown in LB medium containing 100 μM SAM, washed and resuspended in LB with no SAM (●), 2.5 (○), 5 (▼), 10 (▽), 15 (■), 20 (□), 50 (◆) or 100 μM (◇).

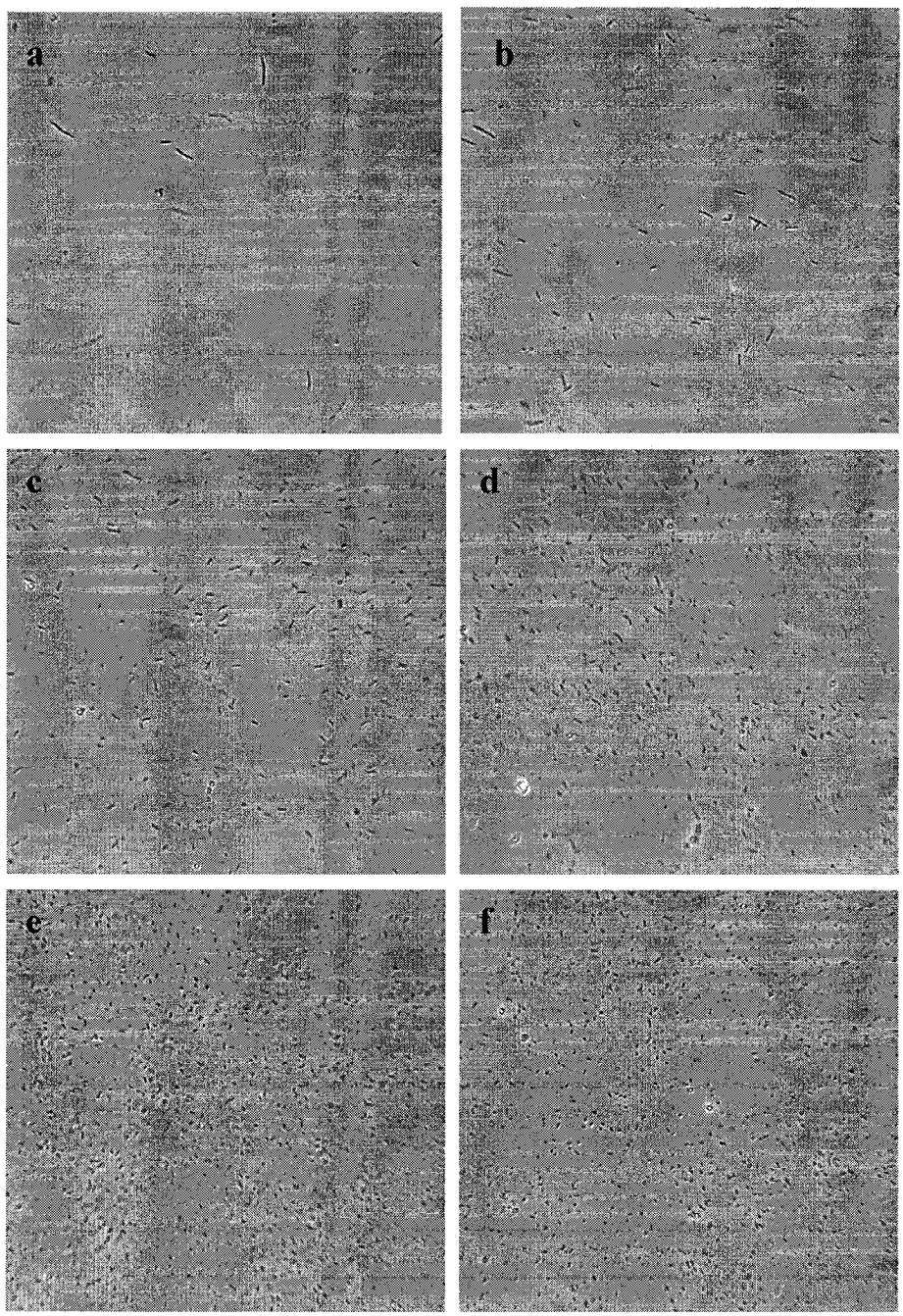


Figure 15, Morphology of SAD16 cells at different SAM concentrations_ SAD16 was grown in rich medium supplemented with no SAM (a), 2.5 (b), 10 (c), 20 (d), 50 (e) or 100 μM SAM (f). The size of the cells was visibly reduced as higher concentrations of SAM were used.

Contrary to what was observed before for strain SAD1, the increase in turbidity of a SAD16 culture did not stop immediately after SAM was removed from the medium, but increased approximately 4-fold. This extended period of growth shows that SAD16 cells are able to store or use SAM more efficiently than SAD1. It also suggests that not much SAM is needed to accomplish the basic necessities of the cells require for growth.

While the density of cultures increased as the SAM concentration in LB cultures increased, the cells themselves became gradually smaller. At high SAM concentrations the cells seem to behave as the wild type both in growth rate and morphology. On the other hand, a lack of SAM led to the formation of long cells (figure 15) but filaments were not observed in LB.

SAD16 cells did not show the same dramatic decrease in viability on SAM starvation as SAD1 cells did. Whereas SAD1 showed a great loss of viability after 3 hours of starvation, SAD16 showed no change after 6 hours. Indeed death was only visible after 1 day. This may suggest that it is not the lack of SAM in the cell but the change of a cellular condition related to it that leads the cells to die.

5.3 Yield and SAM Sparing

SAM is involved in many reactions. To find out how much SAM is needed for its major products, I expected to use strain SAD16 for classic sparing experiments. That is, I would determine the yield of cell material from the cultures of wild-type and mutant cells grown at different concentrations of SAM and determine the increase in yield consequent on the cell's being able to avoid using SAM for particular purposes. This methodology

has been successfully used earlier in this laboratory to study the role of L-serine deaminase (Ramotar and Newman, 1986).

As a first step to study sparing, the yield of SAD16 when grown at different SAM concentrations was determined. To have an estimate of the total cell mass produced, total protein was extracted from cultures grown in LB containing different SAM concentrations at the stationary phase. The results show a non-linear relation between SAM concentration and cell yield (figure 16), similar to that observed in the growth curves when measuring turbidity, suggesting that SAM is more efficiently used at lower concentrations. Since changes in SAM concentration are more or less linearly related to yield at low SAM concentrations, I decided to use 2.5 and 5 μM SAM for subsequent experiments.

The products of *dam* and *dcm* are SAM-dependent DNA methyltransferases which recognize and methylate specifically the adenine and cytosine at the sequences GATC or CC(A/T)GG respectively. The gene *speD* codes for the enzyme SAM-decarboxylase, which catalyzes one of the steps for spermidine formation. Since some reports suggest that mutants containing no spermidine show a decrease in growth (Tholl *et. al.*, 1998) I decided to grow the *speD* mutant in presence of 10 μM spermidine. All these three enzymes are thought to use a considerable amount of SAM.

I obtained SAD16 derivatives containing deletion mutations in the genes *dam*, *dcm* and *sped* by transduction from strains in which the respective genes have being replaced by a kanamycin resistance cassette (Datsenko and Wanner, 2000), these been kindly provided by Dr. H. Mori of the Nara Institute. The mutations were transduced into SAD16 using the kanamycin resistance marker and then their presence was confirmed by

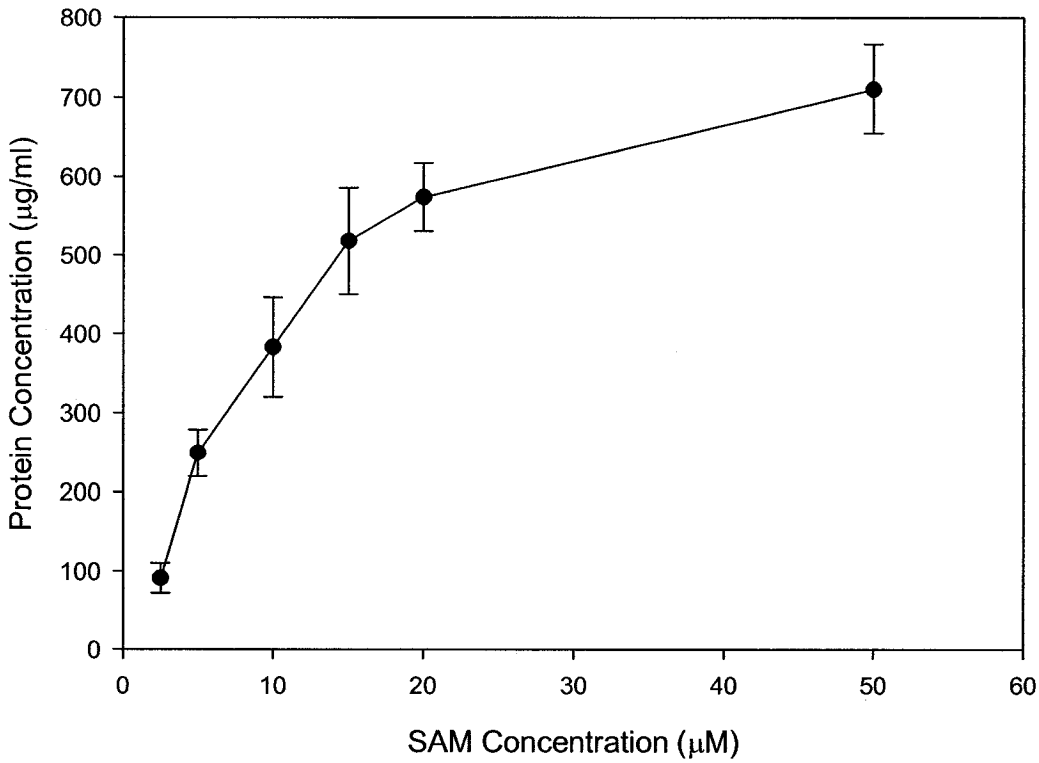


Figure 16, Protein Yield per SAM concentration_ SAD16 cells were cultured in LB with SAM 100 µM and resuspended in LB containing different concentrations of SAM. Protein concentration was measured in the stationary phase for each of these cultures Each point represents the average of twelve independent measurements.

the determination of DNA methylation patterns for *dam* and *dcm* mutations or by PCR in the case of Δ*speD*.

Unexpectedly a change in yield was only observed as a result of the *dam* deletion and this change was greater at 2.5 than at 5 μM SAM in the medium (figure 17). The *dam* deletion strain showed an increase in yield of around 30 % over the parental strain when the medium was supplemented with 2.5 μM SAM, but this increase was reduced to less than 10 % when a concentration of 5 μM was used instead. No big change was observed for the deletion strains of *dcm* and *speD*. The result is surprising in the sense that we were expecting to see a proportional change in yield for both concentrations and did not find it. It is also interesting that the yield of the *dcm* mutant did not change as much as that of the *dam* mutant, since the two enzymes have similar activity. Could the behavior of the strains be just reflecting the extent of activity of the enzymes in those conditions? How active are the enzymes with that much of SAM? We did answer those questions for Dam and Dcm since the degree of DNA methylation can be studied by digestion with restriction enzymes sensitive to this modification. Figure 18 shows the digestion of chromosomal DNA with MboI which recognizes and cuts unmethylated GATC sites and DpnI which cuts the same sequence only when methylated. Methylated DAM sites in the DNA of parental strain SAD16 were almost undetectable in cells grown with less than 20 μM SAM. DNA methylation by Dcm was also very poor at low concentrations of SAM as observed when the methylation sensitive enzyme EcoRII was used (figure 19). However at higher SAM concentrations, DNA was in fact methylated. We therefore compared the yield of cells of the parent and mutant cells using higher concentrations of SAM (between 10 and 80 μM), but did not find any difference between the strains.

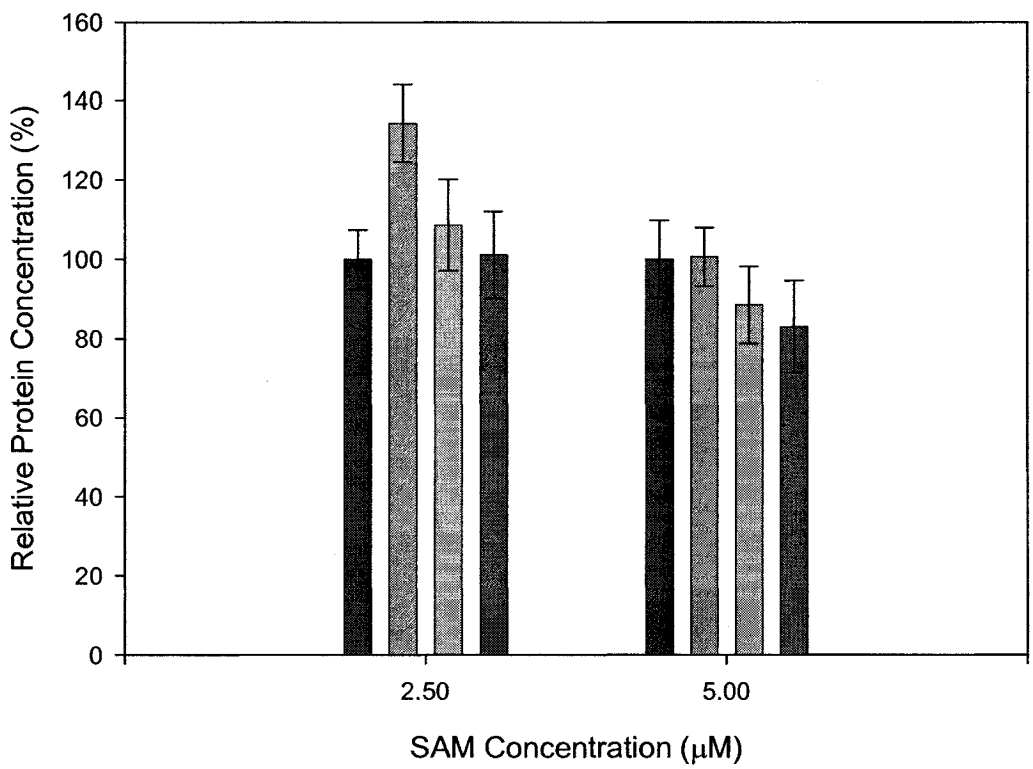


Figure 17, SAM sparing in *dam*, *dcm* and *speD* deletion strains_ SAD16 cells (■) or derivatives carrying deletions for *dam* (▒), *dcm* (▒), or *speD* (■) were grown in LB containing 100 μM SAM, washed and resuspended in medium with either 2.5 or 5 μM SAM. The cultures were incubated until the optical density did not change for two generations and the protein concentration was determined. Each point is the average of twelve independent measurements.

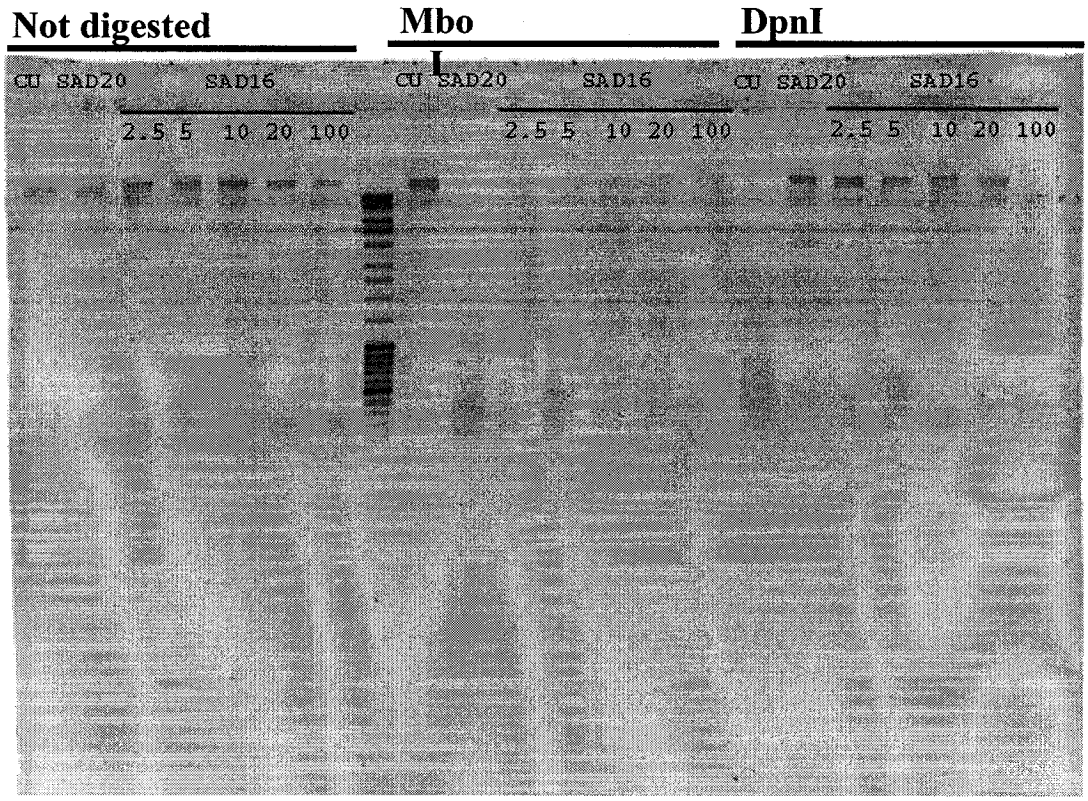


Figure 18, Effect of SAM on DAM methylation_ SAD16 cells were grown overnight in LB with 2.5, 5, 10, 20 or 100 μM SAM respectively and DNA extracted. DNA was also extracted from strain CU, used as positive control and SAD20 (SAD16 Δ*dam*) as negative control for methylation. The DNA was loaded directly into a 1 % agarose gel (lanes 1-7), digested with methylation sensitve restriction enzyme MboI (lanes 9-15) or methylation dependent restriction enzyme DpnI (lanes 16-22).

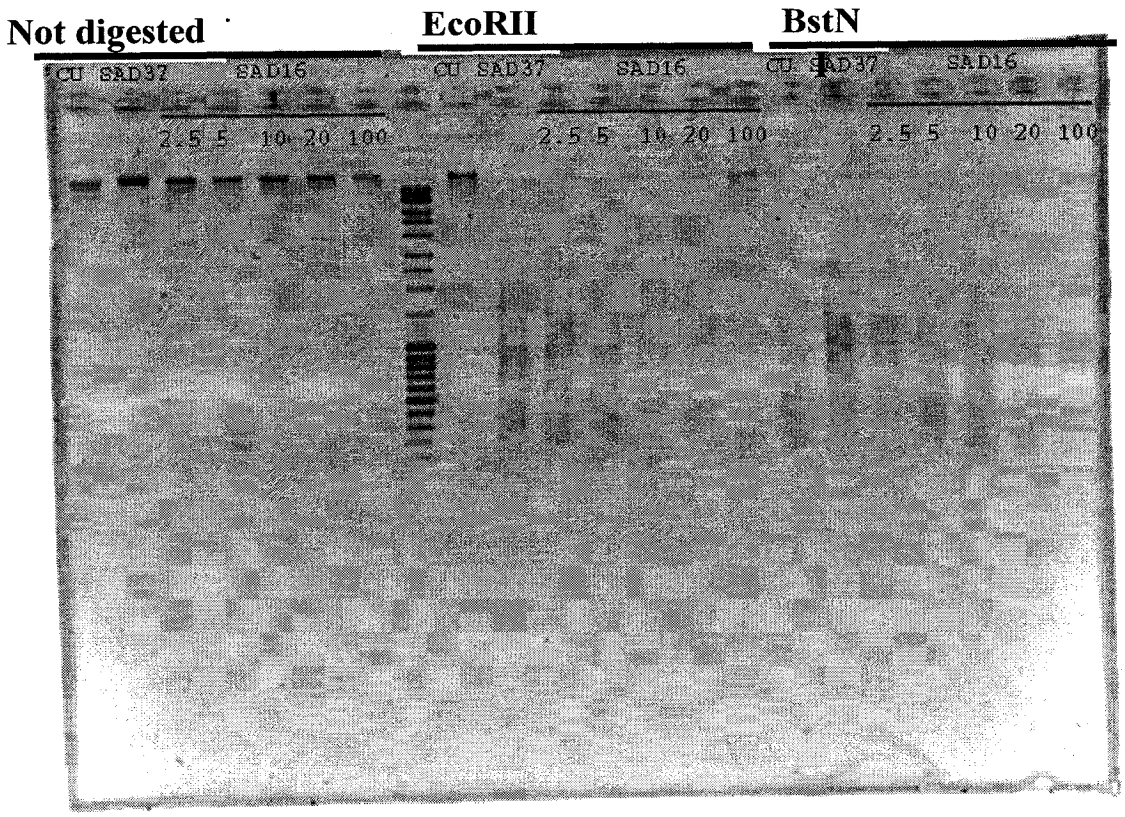


Figure 19, Effect of SAM on DCM methylation_ SAD16 cells were grown overnight in LB with 2.5, 5, 10, 20 or 100 μM SAM respectively and DNA extracted. DNA was also extracted from strain CU, used as positive control and SAD37 (SAD16 Δ*dcm*) as negative control for methylation. The DNA was loaded directly into a 1 % agarose gel (lanes 1-7), digested with methylation sensitve restriction enzyme MvaI (lanes 9-15) or methylation insensitive restriction enzyme BstNI (lanes 16-22).

However these experiments were done in the concentration range where the response to SAM is not linear.

All the above suggest that at low SAM concentrations the cultures are more sensitive to the changes of this compound, which makes them ideal to look for changes caused by sparing, but at the same time the activity of the SAM dependent enzymes drop. At higher concentrations the changes are much smaller but the activity of the enzymes is higher. Further studies should try to solve this limitation of the system.

PART B_ Analysis of the early cell division proteins by mass spectrometry

1. Study of FtsZ and FtsA

1.1 Purification of proteins

Cell division in *E. coli* is mediated by a complex of proteins (the septal complex) assembled at the middle of the cell. FtsZ is the first protein to localize at the middle of the cell where it polymerizes to form a ring, the Z-ring, around the periphery of the cell. The rest of the cell division proteins then associate with this ring sequentially in a defined order (reviewed in Weiss, 2004). Defects in the function or incorporation of the cell division proteins into the ring result in filamentation. Previous work in this laboratory studied the localization of cell division proteins in strain MetK84 grown in minimal medium with low lecucine, conditions in which the cells filament (Wang, 2004). This study used cell division proteins fused to the green fluorescent protein (GFP) to determine their position inside the cells and showed that the cells were able to incorporate the early division proteins FtsZ, FtsA and ZipA into the septal ring but not the later ones.

Formation of filaments in MetK84 strain can be suppressed if SAM is provided and transported from the medium. To explain the relation between SAM and cell division, the most parsimonious hypothesis is that methylation of one of the cell division proteins affects the formation of the septal complex in two possible ways, first it can change the efficiency of incorporation of the same protein, or second it can change the efficiency of incorporation of one of the proteins which gets into the ring after it. Since the problems of incorporation occur prior to ftsQ, we considered that the most possible targets for methylation are FtsK and those proteins before it (FtsZ, FtsA, ZipA, FtsX and FtsE).

To study the modifications present in the cell division proteins, I decided to overproduce the proteins just mentioned, purify them and analyze them by mass spectrometry looking specifically for changes in the mass corresponding to methylation. Because of time limitation, the experiments were attempted with FtsZ and FtsA.

To purify the proteins FtsZ and FtsA, we introduced into strain Cu a plasmid, also a gift of Dr. Mori (Nara Institute of Science and Technology, Japan), which contained either one gene or the other fused to a sequence coding for His-tag at the 5' and regulated by a *lac* promoter. The proteins were overexpressed and then analysed in an SDS-PAGE gel, showing a marked expression band for FtsZ and a less intense band for FtsA both following the expected migration pattern, around 42 KDa for FtsZ and 46 KDa for FtsA (Figure 18a). The cells were sonicated, the cellular debris removed by centrifugation, the supernatant passed through a column charged with nickel for His-tag binding and the bound proteins eluted with high concentrations of imidazole (500 mM). Figure 18b shows the extent of purification for both proteins. The yield was always higher in the case of FtsZ , around 10 mg of protein per 200 ml of culture. The amount of protein recovered for FtsA increased when the purification was carried out in denaturing conditions, possibly because this protein binds to the membrane (Sanchez *et al.*, 1994). But the yields obtained even in this way were only less than 1 mg of protein for 400 ml culture of cells. The amount of protein obtained was nevertheless enough to proceed with the analysis.

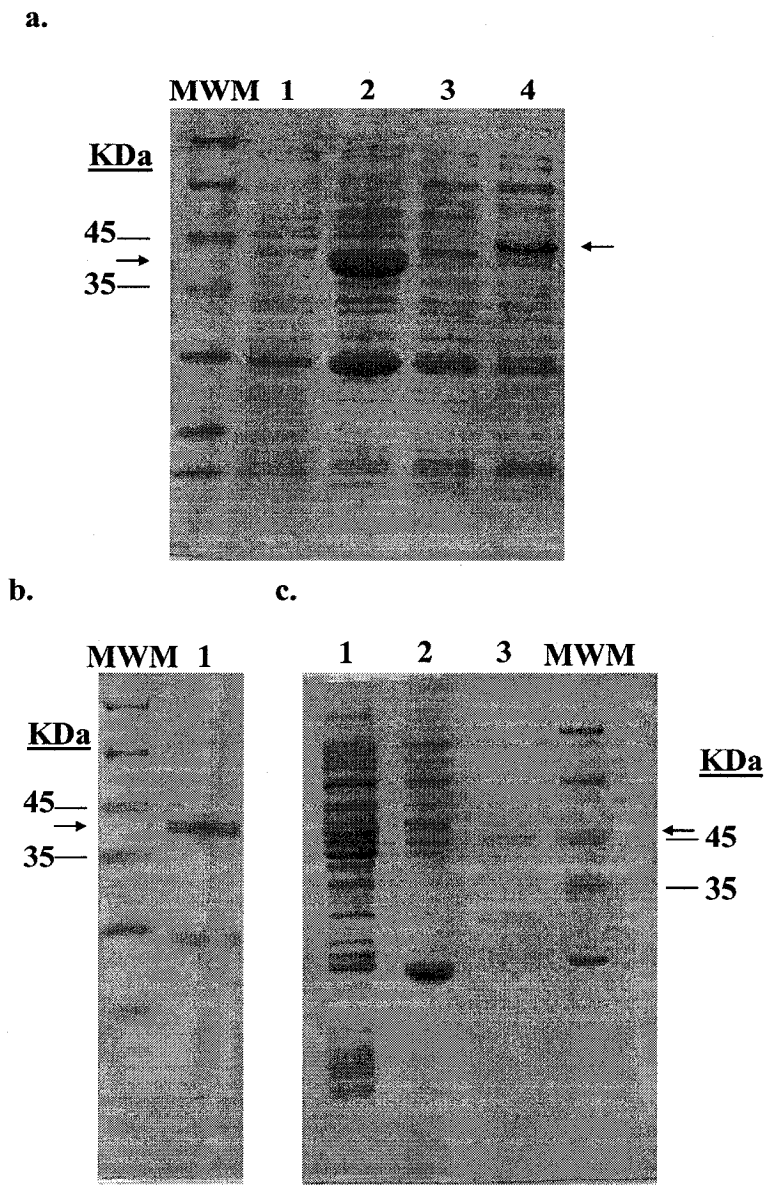


Figure 20, Overexpression and purification of FtsZ and FtsA_ Figure A shows the separation by an SDS-PAGE of the total protein extract from cells containing the plasmid pftsZ-his (lanes 1-2) or pftsA-his (lanes 3-4) before (1 and 3) and after induction (2 and 4) with 1 mM IPTG. Figure B shows the end result of the purification of FtsZ (lane 1). Figure C shows the separation of protein mixtures collected during the purification of FtsA after binding (lane1), washing (lane2) or the eluted protein after the purification (lane 3).

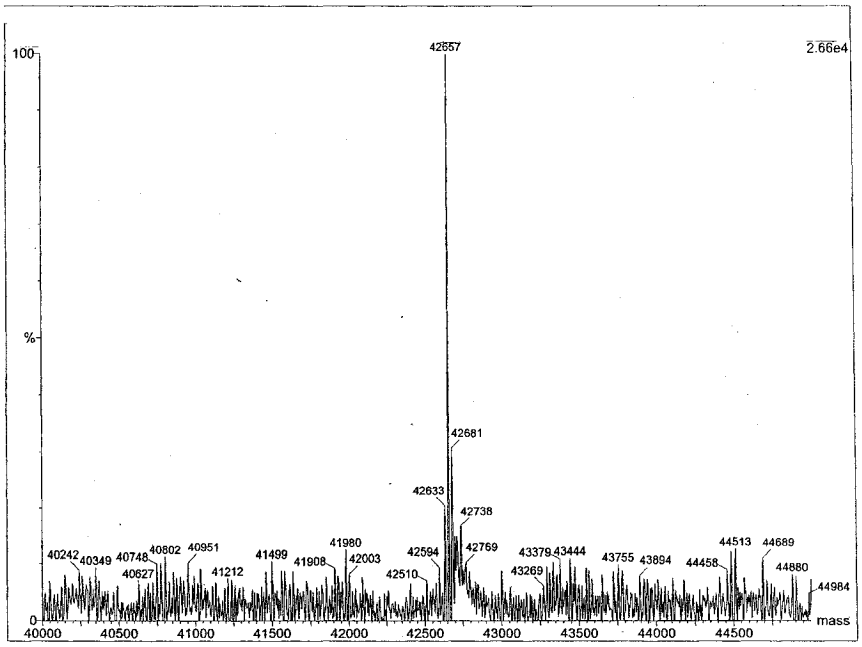
1.2 Preliminary mass determination for FtsZ and FtsA

The study of methylation of proteins in *E. coli* has not been extensive. Due to the inability of *E. coli* to incorporate SAM from the medium, the transfer of the methyl group could not previously be followed by radioactivity. The change in mass and charge that are used to study other protein modifications like phosphorylation cannot be exploited to detect methylated proteins, because this change is very small. Mass spectrometry has become an essential technique for protein characterization, and has been successfully used for the study of post-translational modifications, including methylation (Heurgue-Hamard *et al.*, 2002; Arnold and Reilly, 1999). We decided to use mass spectrometry to determine if the proteins studied were methylated.

We wanted to look for a modification that could be in any region of the protein. Many studies analyze proteins after trypsin digestion, determining the mass of the resulting peptides and recognizing which peptide and therefore the region of the protein modified, but this has the disadvantage that not all these peptides generated can be detected. Since the aim was to determine methylation in the proteins without caring about the position of it, we decided to analyze the whole protein.

The purified FtsZ and FtsA proteins were desalted and prepared for the analysis. The proteins were subjected to electrospray ionization (ESI) and analyzed using a triple quadropole (TQ) mass spectrometer. The results for FtsZ (figure 19a) showed a peak representing a peptide of 42.657 KDa which corresponds to the predicted mass for FtsZ fused to the his-tag produced from the plasmid used as calculated according to its sequence. Methylation of the protein could be represented by a peak 14 Da higher or a multiple of 14 in the case of more than one methylation. No peaks were

a.



b.

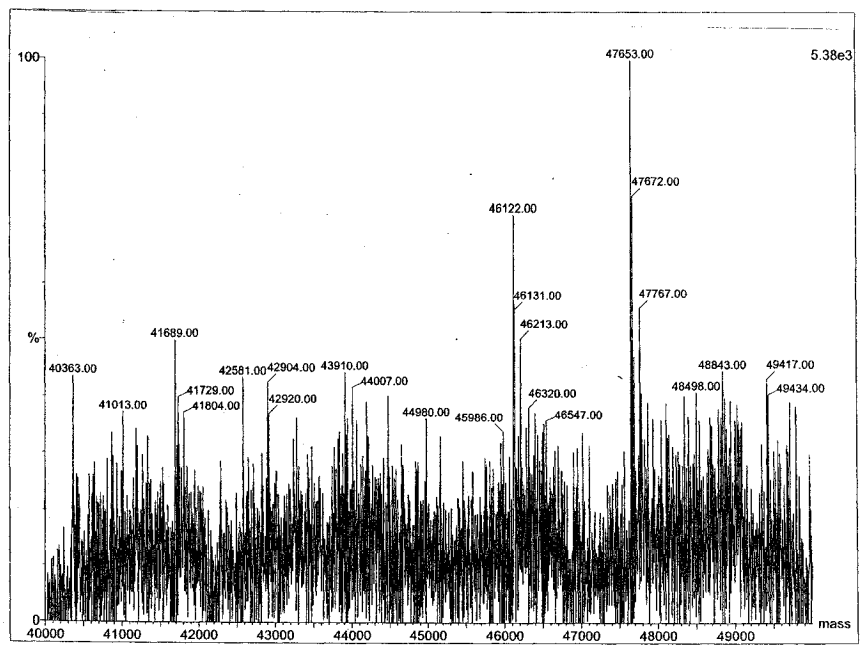


Figure 21, Mass determination for FtsZ-His and FtsA-His proteins_ FtsZ-His (a) and FtsA-His (b) proteins overproduced in Cu strain and purified by his-tag affinity purification were analyzed in a triple quadropole mass spectrophotometer.

observed which could correspond to the modified protein. In the case of FtsA (figure 19b), the study showed a peak of 47.651 Kda, which corresponded to what we had calculated but there was a considerable amount of background noise and therefore is difficult to draw a conclusion on whether there is or there is not a modified peptide. The results suggest that there is no methylation in FtsZ. Indeed there was no evidence for any other modification either. Nevertheless the results are preliminary. They are limited by the level of contamination of the samples with other proteins, which was higher for FtsA that can mask peptides present only in low concentration and therefore lower the sensitivity of detection. Further studies using these methods would have to deal with this problem and try to optimize the detection.

Discussion

The present work dealt with two different subjects. The first one was the use of a SAM transporter to study the physiological roles of SAM, and the second was an attempt to determine the mass of cell division proteins FtsZ and FtsA looking for post-translational modifications in them.

The results showed that the SAM transporter is efficient enough as to provide SAM from the medium to a strain containing a deletion of the *metK* gene. They showed that SAM is the only essential product of the *metK* gene and confirmed previous suppositions that low levels of SAM can explain the filamenting phenotype of strain MetK84. In a strain containing the SAM transporter, there was a direct relation between SAM concentration and mass production at low external SAM concentration. However SAM was less efficiently used at high concentrations. Finally, the very preliminary results on the characterization of the proteins FtsZ and FtsA suggest that FtsZ does not undergo any post-translational modifications, and indicate the methods to be used for the further studies which would have to be done for both these proteins, and any others.

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

1. Transportation of SAM into the cells

1.1 Previous attempts to transport SAM

It is interesting that *E. coli* has no system to transport SAM, though other organisms do. This may reflect the scarcity in nature of this molecule due to its chemical instability, and to the fact that organisms rarely excrete, or even maintain high internal pools, of

compounds directly activated with ATP. Nonetheless there are transporters for the two molecular groups of which SAM is constituted. *E. coli* transports methionine by a high affinity system (*metD*) and at least one low affinity system (Greene, 1996). Adenosine is transported along with all other nucleosides using proteins coded by genes *nupG* and *tsx*, and is also transported along with pyrimidine nucleosides by the protein coded by *nupC* (Zalkin and Nygaard, 1996).

Early attempts to transport SAM into *E. coli* from the medium demonstrated that very little or none was introduced into cells (Holloway *et al*, 1970) and similar results have been reported for other bacteria (Dartois, 1997). The introduction of SAM into *E. coli* was only achieved by toluene mediated permeabilization of membranes (Paoni and Koshland, 1979), a semi-disruptive technique which improved the detection of methylated proteins related with chemotaxis, but which by no means maintained the normal physiological state of the cells. Uptake of radioactive SAM was demonstrated in *E. coli* carrying a SAM transporter cloned from *Rickettsia prowazekii* (Tucker *et al*, 2003). In this study we have shown that using the SAM transporter from that clone, that *E. coli* can introduce SAM efficiently enough to allow a mutant unable to synthesize SAM to grow in a similar way to the wild type.

1.2 Regulation of intracellular levels of SAM

Previous studies have achieved alterations in the intracellular levels of SAM indirectly, by the regulation of its synthesis or degradation. The strategies have reduced the internal pool with a mutation in the *metK* gene (Wei and Newman, 2002) or with a plasmid expressing the SAM hydrolase gene (Hughes *et al*, 1987; Posnick and Samson,

1999) or increased it with expression vectors carrying SAM synthetase (Posnick LM and Samson, 1999).

The ability of these systems to regulate intracellular SAM concentration have limitations imposed by their indirect action in the SAM pool: when SAM synthetase is used to increase SAM concentration, the system is subject to feedback regulation of the enzyme (having SAM, pyrophosphate and orthophosphate as inhibitors); when SAM-hydrolase is used, SAM pools cannot completely deplete because the synthetase has always to be present. In any case, sudden changes in the intracellular concentration are not possible since these systems produce a sudden change in the concentration of the protein but a more gradual decrease or increase in SAM.

The SAM transporter used in this work shows clear advantages over other systems in that it results in the rapid regulation of intracellular concentrations of SAM, which depend only on the concentration of SAM in the medium and the amount of the transporter in the cell.

1.3 Possible applications for SAM transportation

The impermeability of *E. coli* to SAM has hindered the study of the role of SAM in the physiology of cells. Using the SAM transporter will help, among other things, to determine the processes in which SAM is used in different situations. It will also allow the study of the role of SAM in the regulation of biosynthesis of SAM itself, methionine and other sulfur-containing molecules in the cells. Finally it can be used to introduce labeled SAM into the cells with different purposes, among them the study of protein methylation.

Protein methylation has been classically studied providing radioactive methionine while inhibiting protein synthesis, thus assuring that only the methyl group of methionine can be incorporated. The deficiencies of this method are reflected in the scarcity of information about methylation in proteins. The SAM transporter allows radioactive SAM to be introduced by cells carrying it, directly labeling methylatable proteins, and therefore inhibition of protein synthesis is unnecessary. Alternatively, techniques using mass spectrometry which recognize changes in mass due to methylation by non-radioactive isotope have been recently developed (Wan *et al.*, 2004; Ong *et al.,* 2004).

In order to use labeled SAM in the study of protein methylation future work should focus on the construction of a strain where reactions, other than methylation, that result in the movement of methyl groups from SAM into proteins do not take place.

2. Essentiality of SAM

2.1 Growth of *E. coli* is dependent on the presence of SAM

Previous work in our lab demonstrated that *metK* is an essential gene (Wei, 2002), and although the only known function for this gene is the production of SAM, the possibility of it having another essential role could not be ruled out. In this work it was demonstrated that the lethal phenotype of a deletion in *metK* can be suppressed by the expression of the SAM transporter gene when SAM is present in the medium. This showed that the synthesis of SAM is the only essential function of *metK*, and therefore showed conclusively that SAM is an essential molecule in the cells.

2.2 Why is SAM essential?

SAM is a very versatile metabolite. It serves as donor of methyl groups in many reactions, but also is used as source of amino, aminopropyl, amino-carboxypropyl, oxyadenosyl radical and ribosyl groups (Fontecave *et al*, 2004; Sekowska *et al*, 2000). SAM participates in the synthesis of biotin, quinones, deoxyribunocleotides and spermidine and the modification of macromolecules modulating processes as replication, transcription and translation. Given the number of reactions in which SAM is involved, it is interesting that no single essential SAM-dependent enzyme has been described in *E. coli,* though this may only be because the correct enzyme has not been tested.

If we assume that there is no essential SAM-dependent reaction in the cell, it is possible that what makes the cells inoperable in the absence of SAM is the sum of small defects in the processes in which SAM participates. As an alternative to this general breakdown of metabolism, it should also be considered the effect that the absence of SAM would have in one of the variety of general processes in which it is involved, particularly protein synthesis, because of the large number of SAM dependent reactions associated with it. For the sake of clarity I will only discuss the second possibility, although the explanation should no doubt be expanded to include a general effect of methylation.

E. coli has 23 nucleotide methylations in functionally important regions of ribosomal RNA (Decatur and Fournier, 2002), some widely conserved through different organisms (Sirum-Connolly *et al.*, 1995). The description of the methyltransferases associated is far from finished, and therefore the importance of individual modifications have not been studied for all of the sites. Nevertheless it is interesting that none of those studied is essential for survival and some of them show no visible effect when missing, although

the lack of others causes defects in translation and growth (Jemiolo *et al.*, 1991; Tan *et al.,* 2002). To the methylation of rRNA we should add that at least 5 ribosomal proteins become methylated (Arnold and Reilly, 1999). For two of these protein methylations the enzymes responsible have been identified, one of them producing growth defects when absent (Lhoest and Colson, 1981). A number of methylations and other SAM dependent modifications are also present in tRNA and translation factors, each of them with specific functions. The absence of some of these is associated with growth defects. Finally we should consider also the effect of spermidine, which along other polyamines, is supposed to bind preferentially to RNA and modify ribosomal function (Igarashi and Kashiwagi, 2000). Furthermore, mutants unable to synthesize spermidine show a reduction in growth rate (Tholl *et al.*, 1998) though the deficiency in spermidine can be compensated by other compounds. Although there is no single essential SAM dependent activity related to protein synthesis, the widespread association of SAM with this process suggests that SAM is important for efficient protein synthesis. An acute defect in translation would inevitably lead to a complete halt in growth. Could the absence of SAM produce such an impact in translation when all these small defects are summed? Can *E. coli* live without these activities? Hitherto no effort has been made to determine if cells can survive multiple defects in the methylation of the translation machinery, but a study like that would help to clarify the importance of this modification in protein synthesis and growth.

2.3 SAM and death

Early in the study we observed a fast loss in viability when SAM was removed from the medium, leading to death of around 80 % of the population after three hours. This

rapid decrease in viability contrasted with the slow death reported when cells are starved for amino acids, carbon or phosphate sources, where no significant change is observed after days of incubation (Hecker *et al.*, 1986; Moreau *et al.*, 2001). The experiments using a more efficient plasmid (pSAM) to transcribe the SAM transporter gene did not showed the same outcome, and only a reduction in viability after one day of starvation. Other than the plasmid they carried, a difference between strains SAD1 (Δ*metK* pLtet-*metK* pSAD1) and SAD16 (Δ*metK* pSAM) was that the former had a mutation in the *recA* gene. Cells containing a mutation in *dam* require functional copies of *recA* for survival (Marinus, 2000). Since the chromosomal DNA is undermethylated at low concentrations of SAM, the cells may have a phenotype similar to that of *dam* mutants. So one possibility to explain the difference in viability between the two strains is because of the presence of *recA* mutation. It might also be true that the pSAM transporter also allows accumulation of a larger pool of SAM and thus delays killing.

As mentioned before, fast changes in viability are rarely observed, but similar effects as response to stressful situations has been observed previously to be mediated by the toxin-antitoxin (TA) systems (reviewed in Gerdes *et al.*, 2005). The TA systems were first described in plasmids as a way of plasmid maintenance (plasmid addiction systems). The toxin and antitoxin are both proteins that have no effect when present in the cell at the same time, but when the antitoxin, usually metabolically unstable, is degraded as response to a cellular signal, the action of the inactivated toxin leads to killing or stasis. In *E. coli* there are five reported TA systems encoded in the chromosome (Gerdes *et al.*, 2005), supposed to respond to starvation or stressful situations (Hazan *et al.*, 2004). One of these systems composed of *mazE* (antitoxin) and *mazF* (toxin) genes mediates

thymineless death, in which viability is reduced to less than 10 % after two hours when cells are starved for thymine (Sat *et al.*, 2003). Due to the involvement of the TA systems in different stressful situations, further studies should determine their participation in the death of starved cells.

In the experiments with SAD1 (Δ*metK* pLtet-*metK* pSAD1) we also observed that the viability at starvation decreased when cells were exposed to cold temperatures. It is interesting to note that mutants carrying a defective PrmB methyltransferase, the enzyme which methylates the ribosomal protein L3, exhibit cold sensitivity (Lhoest J and Colson C, 1981), although the phenotype was reported at 22 and not 4 °C. Another possible relation between SAM and cold sensitivity can be inferred from a study of protein-protein interactions in *E. coli* (Butland *et al.*, 2005), where a putative methyltransferase (gene *ycbY*) interacts with proteins related to cold response (coded by *cspA*, *cspC, cspD* and *cspE*). These proteins, in contrast with most of other proteins, are synthesized more actively after a temperature downshift and are believed to act in the adaptation of the ribosome to cold temperatures (Ermolenko and Makhatadze, 2002). No methylation has been reported for the cold shock proteins but the association with the *ycbY* gene product suggests this possibility. Whether PrmA or YcbY have anything to do with the sensitivity to cold observed in SAM starved cells will have to be determined.

3. Cell division and intracellular SAM concentration

The strain MetK84 has been previously shown to form filaments when low levels of leucine, the *metK* inducer, are present in the medium (Newman *et al.*, 1998). Because filamentation is associated with defects in cell division, a relation between the levels of

SAM-synthetase and cell division were previously postulated and demonstrated in this lab (Wei and Newman, 2002). In this work we have demonstrated that this defect in cell division is caused by a suboptimal concentration of SAM and that the phenotype is suppressed when SAM is provided exogenously into the cell, ruling out any other possible participation of the SAM synthetase or the *metK* gene.

Furthermore, the study showed that cells did not form filaments when SAM was completely removed from the medium (figure 15a). In order to become a filament a cell has to be metabolically active, so it is able to increase in mass, but it should be incapable of dividing. The fact that cells did not continue growing during starvation shows that suboptimal concentrations of SAM results in two independent situations: inability of the cell either to divide or to increase in mass. Furthermore, defects in cell division seem to be observed when an abnormally low concentration of SAM is present in the cells, but an even lower concentration seems to be related to a total halt in growth.

Considering all the above, one possible scenario to understand the relation between SAM, division and growth considers two enzymes or group of enzymes, one related to cell division and the other to cell growth. The SAM dependent enzyme related to cell division is less efficient in the use of SAM and more sensitive to changes in concentration of this metabolite than the other essential enzyme, whatever it may be. Cells having a suboptimal SAM concentration within a certain range would have problems with cell division, if the concentration is increased single cells will be formed (since the cell division related enzyme would be able to carry out the reactions needed in order to divide), but if the concentration is decreased even more cells will stop growing

entirely because the SAM-dependent enzyme(s) related to growth would not be able to function anymore.

This models predict that filamentation requires a low SAM concentration to be maintained during time, which is what happens when the strain MetK84 is grown in minimal medium or when a plasmid containing *metK* in a strain containing a deletion of *metK* is induced at low levels. We should be able to observe filaments using the SAM transporter only when it is expressed at low levels and a high concentration of SAM is provided in the medium. When strain SAD16 (Δ*metK* pSAM) was grown with varying SAM concentrations in LB, no filaments were observed because a low enough concentration was only maintained at much for a couple of generations, so the cells had only a limited increase in size.

4. SAM consumption

4.1 Efficiency of the utilization of SAM

In this study we analyzed the relation between SAM concentration and mass incorporation and showed that there is a direct relation between them, but that this relation is not linear. A non-linear relation suggests that SAM is inefficiently used at high concentrations. There are two possible explanations imaginable, both using the consideration that the lower concentrations used in our experiments were near the K_T (4.7 μM) described for the transporter in *E. coli* (Tucker *et al.*, 2003), and therefore a change in SAM concentration in the medium would affect the velocity of transportation.

The first possibility is an active regulation of the intracellular levels of SAM. At low concentrations SAM is transported slower than at high concentrations, introducing SAM

fast would lead to accumulation of it in the cells and, as we have suggested before (figure 7) high intracellular concentrations of SAM seems to be toxic. Cells may degrade SAM to avoid these toxic effects and to maintain the concentration of this metabolite within a certain range.

The second possibility is a passive regulation. At low concentrations some SAM dependent enzymes have slower velocity of reaction due to a suboptimal concentration of SAM. This does not happen when SAM is present at high concentrations, at which the same enzymes show normal kinetics in the reactions they catalyze, and therefore more SAM is used. Further studies should be done to distinguish between these two possibilities.

5. Cell division and protein methylation

Two recent studies have given more importance to the study of modifications of the cell division proteins. In the first work the authors constructed a chimeric ftsQ gene, which produces an FtsQ protein able to interact directly with FtsZ (Goehring *et al.*, 2005). Using this system they showed that the presence of FtsQ in the septal ring is enough to allow the localization of downstream proteins (FtsB, FtsL, FtsW and FtsI) and even upstream protein FtsK when the early protein FtsA is not present. The work reinforces the previous observation that FtsQ, FtsB and FtsL forms a complex independently of the formation of the septal complex (Buddelmeijer and Beckwith, 2004), and furthermore adds to this complex FtsW, FtsI and FtsK proteins.

The second study used culture conditions which permitted a constant age distribution and relative frequency of cells in a certain age class (despite of an overall increase of the

population) to determine the time in which different cell division proteins are incorporated into the septum (Aarsman *et al.*, 2005). They observed a temporal separation of 16 minutes in cells with a doubling time of 85 minutes, between the formation of the Z-ring and the localization of FtsQ and downstream proteins. In summary both articles suggest a physical and temporal separation between the early (FtsZ, FtsA and ZipA) and late (from FtsK downstream) division proteins, which also insinuate the possibility of an independency of regulation.

In a previous study in this lab it was demonstrated that the cell division defects encountered by strain MetK84 result from a deficiency in the incorporation of late cell division proteins. Since filamentation in MetK84 is caused by a suboptimal concentration of SAM and since this metabolite is used as methyl donor for protein methylation, it seemed worthwhile to study the post-translational modifications of cell division proteins. Until now no systematic search for post-translational modifications has been done for the cell division proteins. In this study the first two proteins, FtsZ and FtsA, were purified and analyzed by mass spectrometry. The results are preliminary and should be confirmed, but they suggest that at least for FtsZ no modification is encountered. Further studies should be done with FtsA and other proteins to determine if they undergo post-translational modifications.

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