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A study of the *metK* gene encoding S-adenosylmethionine synthetase in *Escherichia coli* K-12

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A Thesis

In

The Department

Of

Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science at

Concordia University

Montreal, Quebec, Canada

August 2001

ABSTRACT

A Study Of The *metK* Gene Encoding S-Adenosylmethionine Synthetase in *Escherichia coli* K-12

Yuhong Wei

The *metK* gene of *Escherichia coli* encodes S-adenosylmethionine synthetase, which catalyzes the reaction between L-methionine and ATP to form S-adenosylmethionine (SAM), the universal methyl group donor in the cell. SAM synthetase is generally assumed to be essential in *E.coli*, but this has not been proven. I demonstrated here that the *metK* gene could be deleted in *E.coli* by gene replacement only in the presence of a rescue plasmid carrying a functional copy of *metK*. Growth of cells containing the *metK* deletion was absolutely dependent on the expression of *metK* from the rescue plasmid. This clearly proved that *metK* is an essential gene in *E.coli* K-12. Studies of limited *metK* expression indicated that a reduction of SAM concentration decreased the methylation of genomic DNA and caused a cell division defect in glucose minimal medium.

One commonly used *metK* mutant "*metK84*" had a markedly reduced SAM synthetase level and exhibited a complex phenotype under certain growth conditions. Through DNA sequencing analysis and primer extension analysis, I showed that this mutant carried an A→G transition in the −10 region of the *metK* promoter. This mutation greatly reduced transcription from the *metK* promoter, which resulted in a reduction of SAM synthetase activity and deficiency in intracellular SAM.

In this work, the initiation site for *metK* transcription was determined by primer extension. The *metK* promoter was characterized as an *E.coli* σ^{70} promoter. The −10 region

sequence was TAAAAT, located at 146-151bp upstream of the *metK* ATG codon; and the –35 region sequence was TGGAAA. The spacing between these two regions is 18bp.

Acknowledgements

I would like to thank all those who helped, supported and encouraged me during my two years study.

First of all, I wish to express my deep appreciation to my supervisor Dr. E.B. Newman for her scientific guidance, financial support and continuous encouragement. Without these, I would not be able to start and finish my Master degree study.

I sincerely thank Dr. R. Storms and Dr. P. Gulick for being my committee members and providing helpful advice and suggestions.

I would also like to thank Dr. P. Albert, Dr. W. Zerges, Dr. L. Giraldeau and Dr. P. Joyce for their help and counsel. I am indebted to Dr. Z. Shao, Dr. R. Lin and Dr. W. Shen for their invaluable advices and great help, which allow me to overcome the obstacles and make progress in the course of my project.

I am grateful to J. Lan, Y. Zhen, P. Pitsikas, K. Dorion, N. Brodeur, J. Rokas and A. Spurmanis for their helpful suggestions, many assistances and wonderful friendship.

Finally, I want to thank all my family members for their understanding, encouragement and endless love, which support me finishing through this work.

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Introduction

1. SAM, SAM synthetase and the *metK* gene

S-adenosylmethionine (SAM) is a natural metabolite formed from the amino acid methionine. SAM is of great importance in many organisms because it takes part in a large variety of biological reactions. It is the ubiquitous intracellular methyl group donor in a wide variety of biosynthetic reactions; the aminopropyl donor for the biosynthesis of polyamine spermidine; and the corepressor of the methionine biosynthetic pathway in *Escherichia coli*.

The enzyme named S-adenosylmethionine synthetase (SAM synthetase) catalyzes a reaction between L-methionine and ATP to produce SAM. The synthetic reaction consists of two unusual sequential steps, SAM formation and the subsequent hydrolysis of triphosphate to PP_i and P_i, before SAM is released from the enzyme (Cantoni, 1953). SAM synthetase is an exceptionally well-conserved enzyme through evolution. The sequence of *E. coli* SAM synthetase has been found to be 55.7% identical and 70% similar to the human enzyme (Newman *et al.*, 1998), and the polar active site residues are all conserved in the more than 30 reported SAM synthetase sequences (Taylor and Markham, 2000). In *E. coli*, SAM synthetase is a tetramer with a molecular mass of 108kDa.

In *E. coli*, *metK* is the structural gene encoding SAM synthetase (Greene *et al.*, 1973). *metK* is mapped at min 66.4 on the *E. coli* genome and is transcribed clockwise. With the completion of the *Escherichia coli* K-12 genome sequence, the DNA sequence of *metK*

gene was determined. The open reading frame extends 1155 bp and codes for 384 amino acids.

2. Three major roles of SAM in *E. coli* cell metabolism

To better understand the importance of SAM in metabolism, more detailed explanations about the key roles of SAM will be discussed, including the major pathways of the synthesis and degradation of SAM.

2.1. SAM as a major source of methyl groups

After SAM is formed, it functions as an activated methyl group donor. As shown in Fig.1, the methyl group is transferred from SAM to a wide variety of methyl acceptors in reactions catalyzed by the SAM-dependent methyltransferases, producing components with far-reaching metabolic effects. Once SAM loses its methyl group, it breaks down to form homocysteine, which can then be remethylated to methionine by the reactions catalyzed by methionine synthetases.

SAM is the methyl donor for much of metabolism. SAM-dependent methylation reactions play significant roles in controlling and regulating a variety of cellular functions. Methylation of DNA or RNA has regulatory effects on DNA replication, gene expression, DNA repair, restriction and modification and RNA translation. Methylation of protein residues regulates amino acid biosynthesis and bacterial chemotaxis (Hughes *et al.*, 1987).

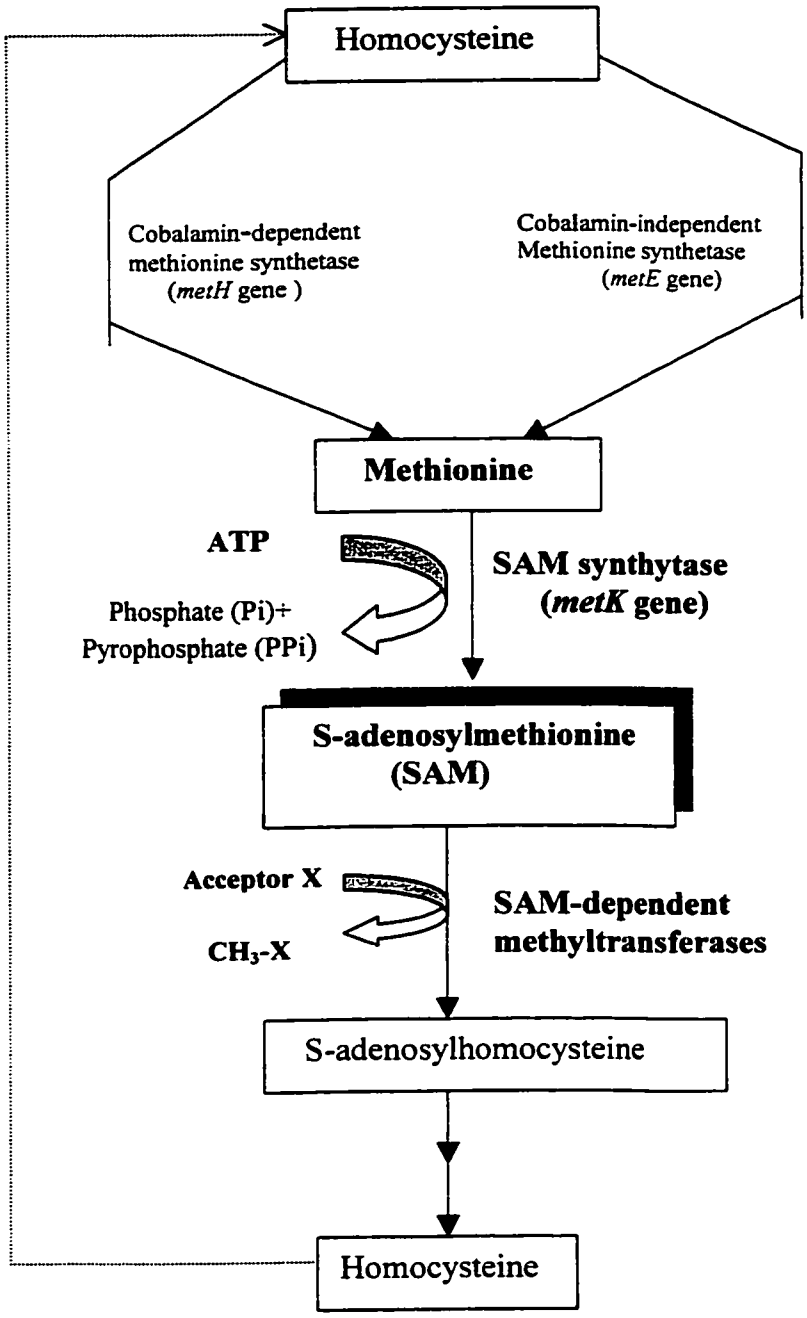


Figure 1. The pathway of SAM biosynthesis and SAM as a major methyl group donor in the remethylation cycle.

In *E. coli*, many methylation reactions are known. Table 1 includes the 43 methyltransferases of *E. coli* that are listed in the SWISS-PROT database, most using SAM as the methyl group donor. DNA adenine methyltransferase and DNA cytosine methyltransferase are the most thoroughly studied of these enzymes.

DNA adenine methyltransferase (Dam) modifies almost all of the GATC sites in *E. coli* DNA and generates 6-meAde. The state of methylation can be determined by restriction enzyme digestion, in which *Mbo*I or *Dpn*II cut only unmethylated GATC, while *Dpn*I cuts only methylated GATC, and *Sau*3AI cuts regardless of methylation status. Dam has been suggested to have two SAM-binding sites: the catalytic site and the other site that increases specific DNA-binding due to allosteric change. Study of *dam* mutants indicates that *dam* methylation has multiple functions in the cell. In DNA mismatch repair, *dam* methylation signals the strand to be modified, so that the repair system removes replication errors (base mismatch, deletion or insertion) only in the newly synthesized unmethylated DNA strand. In initiation of chromosome replication, DNA methylation takes part in two ways. On one hand, it helps the initiation of chromosome replication to occur more efficiently through generating fully methylated oriC DNA; on the other hand, it modulates the GATC sites within the oriC region and the GATC sites at the promoter of the dnaA gene, which encodes the DnaA protein that is required for the initiation of chromosome replication, so as to ensure that initiation at oriC happens only once per generation (Marinus, 1996). Dam methylation is also involved in the regulation of genes such as those of the pap operon. Pyelonephritis–associated pilus (Pap) expression is regulated by the phase variation mechanism in which

Table 1. Methyltransferases in *E. coli*

Category	Name of Methyltransferase	Encoding gene	Used methyl donor
DNA Mtase	DNA cytosine methytransferase	dcm	SAM
	DNA adenine methylase	dam	SAM
	Modification methylase Eco57I	eco57IM	SAM
	Modification methylase EcoRI	ecoRIM	SAM
	Modification methylase EcoRII	ecoRIIM	SAM
	Modification methylase EcoRV	ecoRVM	SAM
	Modification Methylase Eco47II	eco47IIM	SAM
	6-O-methylguanine-DNA-methyltransferase	ogt	NC
	Type IIS restriction enzyme Eco57I	eco57IR	NC
	EcoP15I methyltransferase	ecoP15I	SAM
rRNA MTase	rRNA adenine N-6-methyltransferase	ermBC	SAM
	SAM--6-N',N'-adenosyl(rRNA) dimethyltransferase	ksgA	SAM
	rRNA(Guanine-N1-)-methyltransferase	rrmA	SAM
	rRNA (Uridine-2'-O)-methyltransferase	ftsJ or rrmJ	SAM
	rRNA(Guanine-N2-)-methyltransferase	rsmC	SAM
	Putative ribosomal RNA small subunit methyltransferase D	ygjO	SAM
	Hypothetical RNA methyltransferase	ybjF	NC
	Hypothetical RNA methyltransferase	ygcA	NC
tRNA MTase	tRNA (Uracil-5-)-methyltransfease	trmA	SAM
	tRNA(Guanine-N1)-methyltransferase	trmD	SAM
	tRNA (Guanosine-2'-O-)methyltransferase	trmH or spoU	SAM
	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	trmU	SAM

rRNA/tRNA Mtase	Hypothetical tRNA/rRNA methyltransferase	lasT	NC
	Hypothetical tRNA/rRNA methyltransferase	yfiF	NC
	Hypothetical tRNA/rRNA methyltransferase	yibK	NC
	Hypothetica tRNA/rRNA methyltransferase	yjfH	NC
Protein Mtase	Chemotaxis protein methyltransferase	cheR or cheX	SAM
	Protein-L-Isoaspartate O-methyltransferase	pcm	SAM
	Ribosomal protein L11 methyltransferase	prmA	NC
	ADA regulatory protein	ada	NC
Other MTase	Cobalamin-independent methionine synthetase	metE	5'-methyl-tetrahydropteroyl-triglutamate
	Cobalamin-dependent methionine synthetase	metH	5'-methyl-tetrahdrofolate
	Aminomethyltransferase	gcvT	5', 10'-methelene-tetrahydrofolate
	Serine hydroxymethyltransferase	glyA	5', 10'-methelene-tetrahydrofolate
	Uroporphyrin-III C-methyltransferase	cysG	SAM
	Trans-aconitate methyltransferase	tam	NC
	Putative uroporphyrin-III C-methyltransferase	hemX	SAM
	Type 4 prepilin-like proteins leader peptide processing enzyme	hofD/hopD	NC
	SAM:2-demethylmenaquinone methyltransferase	menG	SAM
	Homocysteine S-methyltransferase	mmuM	SAM
	3-methyl-2-oxobutanoate-hydroxymethyltransferase	panB	5,10-methylene-tetrahydrofolate
	Ubiquinone/Menaquinone-biosynthesis methyltransferase	ubiE	NC
	3-Demethylubiquinone-9 3-methyltransferase	ubiG	SAM

Abbreviations: Mtase: methyltransferase. NC: not clear

cells either express pili (on) or not (off) (Van de Woude *et al.*, 1993). Through the competition between Dam and transcription regulators Lrp and PapI, the methylation status of two important GATC sites ($GATC_{1028}$ and $GATC_{1130}$) is regulated and the expression of the pap operon transits between on or off phases.

DNA cytosine methyltransferase (Dcm) methylates sites CCA/TGG in the *E. coli* DNA and yields 5-meCyt. The status of methylation at *dcm* sites can be monitored by digestion with *Eco*RII, which cleaves only the unmethylated sequence, and *Mva*I which cleaved both methylated and unmethylated sequences. Dcm also uses SAM as the methyl group donor in the methylation reactions, but its SAM binding sites have not been demonstrated. Study of the *dcm* mutants did not show obvious phenotypes but Dcm is thought to be involved in very-short-patch (VSP) repair and protection from restriction endonucleases (Marinus, 1996).

2.2. SAM as the precursor of polyamine biosynthsis

Another important role of SAM is as the precursor in the biosynthesis of the polyamine spermidine. As shown in Fig.2, SAM undergoes decarboxylation catalyzed by SAM decarboxylase, the product of the *speD* gene in *E. coli*, thus generating the propylamine donor-decarboxylated SAM. This derivative of SAM reacted with another polyamine putrescine, and thus generates spermidine. Polyamines (putrescine, spermidine and spermine) are present in almost all living organisms and can modulate the functions of RNA, DNA, nucleotide triphosphates, proteins and other acidic substances. In *E.coli*, spermidine has been shown not to be essential for cell growth but is necessary for optimal growth (Tabor and Tabor, 1985; Xie *et al.*, 1993).

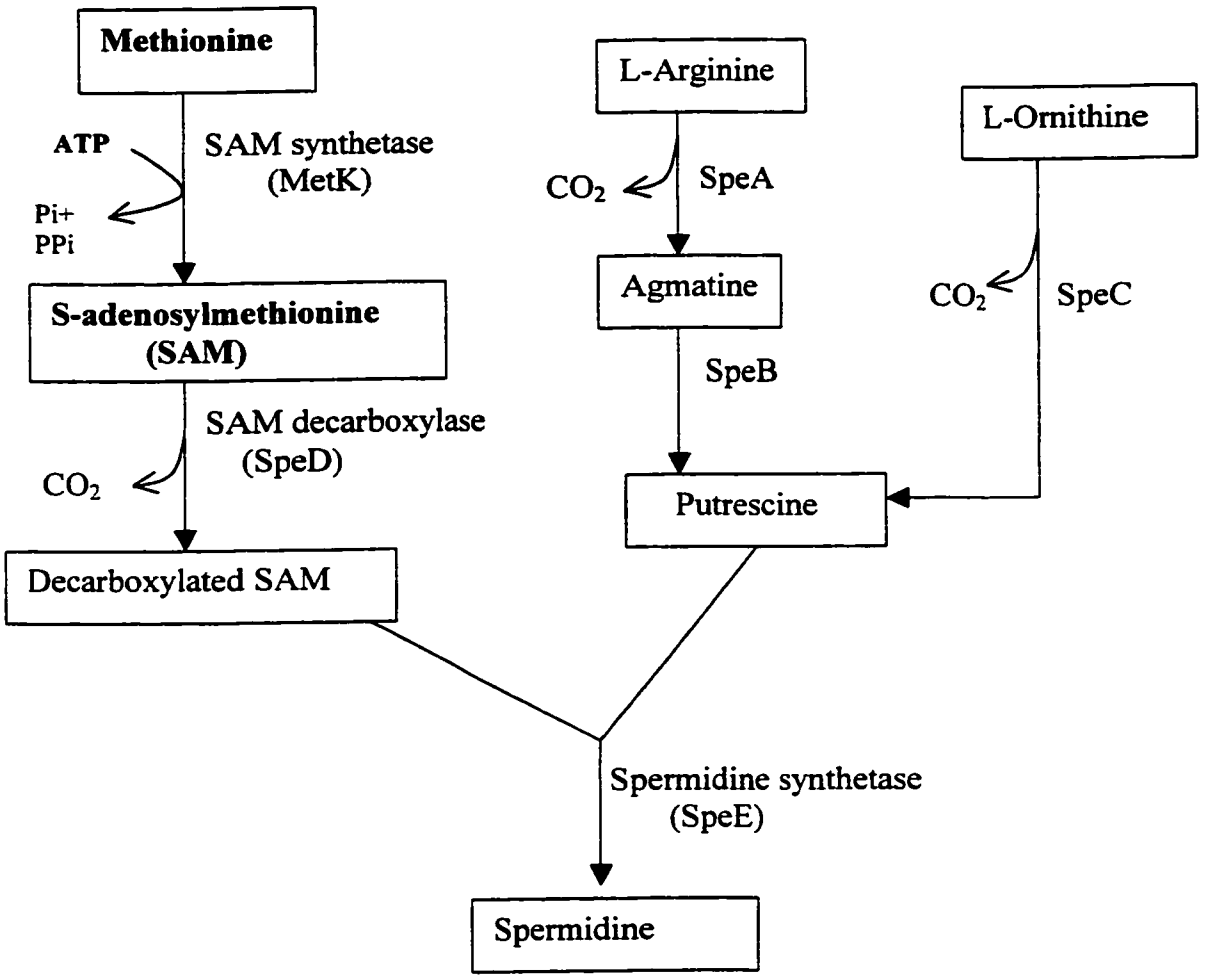


Figure 2. SAM as propylamine donor in the biosynthesis of spermidine.

SpeA: arginine decarboxylase; SpeB: arginine ureohydrolase; SpeC: ornithine decarboxylase; SpeD: S-adenosylmethionine decarboxylase; SpeE: spermidine synthetase.

2.3. SAM as the corepressor of the methionine biosynthetic regulon

SAM is synthesized from methionine and actually is the bioactive form of methionine in the cell. After participation in the methyl donor reactions, SAM is then recycled back to methionine. Therefore, between SAM and methionine there exists a complicated relationship. To further understand this relationship, the discussion will begin first with the methionine biosynthetic pathway.

2.3.1. The methionine biosynthetic pathway and the *met* regulon

The methionine biosynthetic pathyway is highly branched with both divergent and convergent segments (Greene *et al.*, 1973). As illustrated in Fig.3, methionine is derived from another amino acid, aspartate. The first three biosynthetic reactions from aspartate to homoserine are shared with several other metabolites, such as lysine and threonine. From homoserine, methionine starts its own synthetic pathways. A group of methionine biosynthetic enzymes, whose structural genes are scattered on the chromosome to form the met regulon, catalyze the conversion of homoserine to methionine (Greene, 1996).

The first reaction is O-succinylation of homoserine catalyzed by the product of the *met*A gene. This is the only step that is subject to feedback inhibition by methionine and/or SAM. Then, the sulfur atom (-S) is incorporated through the addition of cysteine to form cystathionine. The subsequent step is catalyzed by cystathionine-β-lyase that releases ammonia and pyruvate and generates homocysteine. At last methionine is formed by adding the methyl group to the sulfur of homocysteine.

This last reaction lies at the point of convergence of two major biosynthetic pathways, for homocysteine and for methyl group transfer via tetrahydrofolate derivatives. The

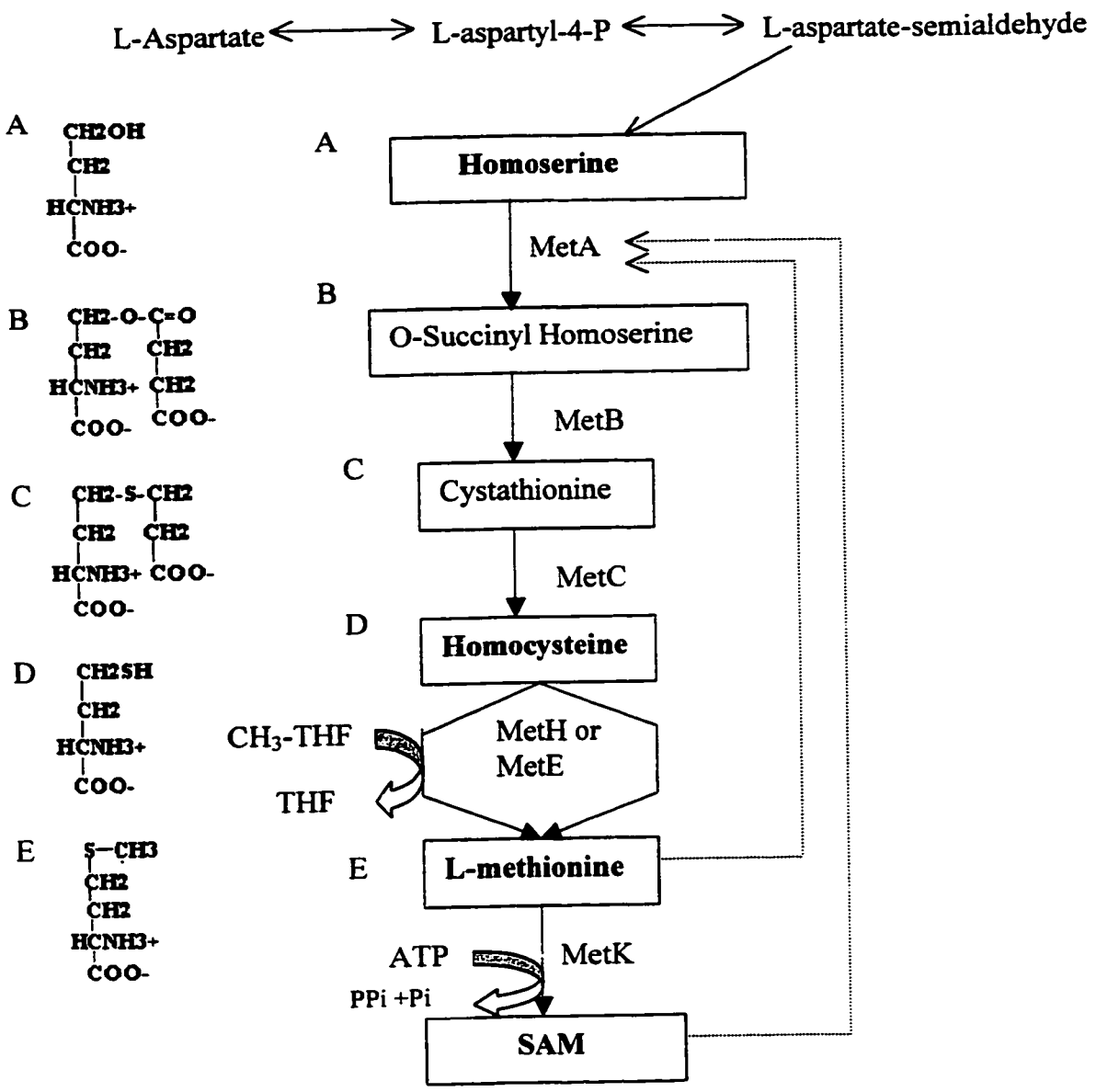


Figure 3. The methionine biosynthetic pathway in *E. coli*

MetA: Homoserine transsuccinylase; MetB: Cystathionine synthetase; MetC: Cystathione-β-lyase; MetE: cobalamin-independent methionine synthetase; MetH: cobalamin-dependent methionine synthetase; MetK: S-adenosylmethionine synthetase; CH_3-THF: N5-methyltetrahydrofolate; THF: tetrahydrofolate.

⟵ : Feedback inhibition

methyl group comes from the reduction of N5, N10-methylene-tetrahydrofolate to N5-methyltetrahydrofolate, which then reacts with homocysteine to biosynthesize methionine. In *E. coli*, transfer of methyl groups can be catalyzed by two enzymes. They are the cobalamin-dependent methionine synthetase, the product of the *metH* gene, and the cobalamin-independent methionine synthetase, the product of the *metE* gene.

The product of the *metH* gene is similar to the enzyme of mammals, which depends on cobalamin as cofactor and also requires SAM. It accepts both the monoglutamate and triglutamate forms of methyltetrahydrofolate as methyl donors. In contrast, the product of the *metE* gene accepts only the triglutamate form of methyltetrahydrofolate as a methyl donor. It does not require SAM or cobalamin for activity. Instead, adding methionine or cobalamine to the growth medium can repress MetE enzyme activity (Shoeman *et al.*, 1985).

2.3.2. SAM as the cofactor in the negative control of the met regulon

In addition to its role in inhibition of homoserine transsuccinylase (product of the *metA* gene), SAM is thought to function at the transcription level in regulation as a corepressor in the negative control of the methionine biosynthetic enzymes (Greene, 1996).

The idea first came from the observations that *metJ* or *metK* mutants showed derepression in methionine biosynthesis and had elevated levels of methionine biosynthetic enzymes such as cystathionine synthetase (the *metB* gene product) or cystathionase(the *metC* gene product).Thus, it was hypothesized that the *metJ* gene codes for an aporepressor and the *metK* gene codes for SAM synthetase to produce SAM as a

corepressor functioning in the repression of methionine biosynthesis (Greene *et al.*, 1973). This hypothesis was supported by later studies. *In vitro* transcription assays showed that pure MetJ protein inhibited the transciption of the *metB, metJ* and *metL* genes and SAM enhanced repression (Shoeman *et al.*, 1985). Study of the 5'ends of met genes (including *metA, metBJ, metC, metER and metF*) found that they all had a tandemly repeated consensus sequence "AGACGTCT", which is called a Met box and is supposed to be the MetJ protein binding site. Finally, the three-dimensional structure of the MetJ protein and its complex confirmed the involvement of SAM in MetJ repression of the met regulon (Greene, 1996).

3. Is the *metK* gene encoding SAM synthetase essential in *E. coli*?

The *metK* gene encodes SAM synthetase, which then combines L-methionine and ATP to form SAM. Since SAM functions as a central metabolite, SAM synthetase and its encoding gene might be expected to be essential.

3.1. SAM synthetase has been proven to be essential in yeast

In yeast, mutations causing loss of SAM synthetase result in cell death, unless exogenous SAM has been provided in the medium to support growth (Cherest *et al.*, 1978). Therefore, the essentiality of SAM synthetase and its encoding genes are clearly demonstrated.

3.2. SAM synthetase or *metK* gene is generally assumed to be essential in *E. coli*

The same demonstration has not been established in *E. coli*, because the *E. coli* cell does not take up SAM from medium and the standard techniques for permeabilizing *E. coli* to SAM cannot be used with living cells (Holloway *et al.*, 1970; Posnick and Samson, 1999). Studies of the functions of SAM, SAM synthetase and/or its structural gene *metK* have to depend on *metK* mutants. Most *metK* mutants were isolated by their phenotype of resistance to methionine analogues. This resistance is due to the derepression of methionine biosynthesis resulting from reduced SAM synthetase activity and reduced intracellular SAM levels. Even though some of mutants may have markedly reduced SAM synthetase activity, all such mutants still have enough residual enzyme activity to grow, and their intracellular SAM levels are not severely changed (Greene *et al.*, 1973; Posnick and Samson, 1999). Attempts to screen for null mutants of *metK* or temperature-sensitive, conditional lethal mutants were not successful (Hafner *et al.*, 1977; Hobson and Smith, 1973). Indeed, no non-conditional, non-leaky *metK* mutant of *E. coli* has been isolated (Cheng and Blumenthal, 1999). Considering these facts, and the great importance of SAM in cell metabolism, SAM synthetase and its structural gene *metK* are generally assumed to be essential in *E. coli*.

3.3. Hypothesis of duplicated genes encoding SAM synthetase in *E. coli*

Dr. Markham and his colleagues constructed certain temperature-sensitive *metK* mutants, and reported that one of the mutants showed extremely low intracellular SAM levels and could not grow in minimal medium at the nonpermissive temperature, but did grow normally in rich medium (LB). They thus concluded that the functional *metK* gene

is essential in minimal medium, while in rich medium (LB) SAM synthetase is encoded by a second gene, *metX* (Satishchandran *et al.*, 1990). However, those mutants later proved to be genetically unstable and could not be well characterized (Greene, 1996). With the whole *E. coli* genome sequenced, the position tentatively mapped for the *metX* gene has been found to be occupied by the *galP* gene. A blast search did not reveal any *metK* homolog in the *E. coli* genome (Newman *et al.*, 1998). Therefore, the hypothesis of duplicated genes (*metK* and *metX*) coding SAM synthetase in *E. coli* is not supported by present data. Therefore, till now it has not been demonstrated whether or not the *metK* gene or its product, SAM synthetase, is essential in *Escherichia coli* K-12, and if it is, for what?

4. Using gene replacement for demonstrating gene essentiality

4.1. Classical gene replacement methods

A bacterial gene essential for growth is always a potential target for finding new antibiotics. To demonstrate the essentiality of a gene for the growth of a certain bacterium, gene replacement is one of the widely used methods (Brown and Fournies, 1984; Satishchandran *et al.*, 1990; Chang *et al.*, 1989). It is based on the principle of homologous recombination. An alteration of the target gene is first engineered on a plasmid or a linear DNA, the *in vitro*-altered sequence is then used to replace the chromosomal wildtype sequence using strains with specific genetic backgrounds such as *polA*, *recBC sbcB*, *recD*, *strR*, or F' (Gutterson and Koshland, 1983; Link *et al.*, 1997). In a first recombination event, the mutant allele is integrated into the chromosome; in a second recombination event; the chromosomal wildtype allele is exchanged with the

mutant allele, thus resulting in gene replacement on the chromosome. Using this replacement method, the essentiality of the target gene can be examined directly or indirectly.

4.2. A new method for gene replacement using the pKO3 vector

In order to let allele replacement be performed directly in the wildtype strains, Dr. Church and his colleagues refined the original method for gene replacement in wildtype *E. coli* (Hamilton *et al.*, 1985) and developed a new gene replacement vector pKO3 (Link *et al.*, 1997).

Vector pKO3 (Fig.4) has three major characteristics: (1) A temperature sensitive pSC101 origin of replication (*repA*(ts)) (2) the *cat* gene coding chloramphenicol resistance so as to positively select chromosomal integrants at a non-permissive temperature; and (3) the *sacB* gene for counterselection of loss of vector in 5% sucrose medium.

The mechanism of using pKO3 for gene replacement also relies on double recombination. At the non-permissive temperature, pKO3 integrates into the chromosome by homologous recombination, thus creating a tandem duplication of the wildtype and mutant alleles with plasmid sequence between. When shifted back to the permissive temperature, the plasmid *ori* in the chromosome is detrimental, so that the integrated plasmid resolves from the chromosome, leaving either the wildtype or the mutant allele on the chromosome. When the cells are plated on media containing 5% sucrose, the expression of *sacB* gene is lethal to *E. coli*; only cells that have lost the *sac*B gene can

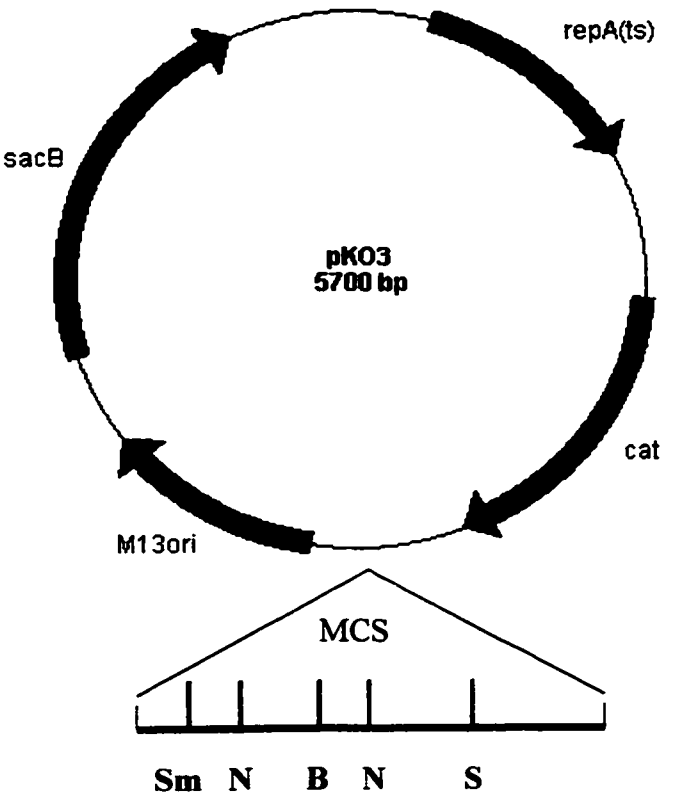


Figure 4. Plasmid map of gene replacement vector pKO3.

Abbreviations: MCS: multiple cloning site; Sm: *Sma*I; N: *Not*I ; B: *Bam*HI; S: *Sal*I.

repA(ts): pSC101 origin of replication (temperature-sensitive)

cat: the *cat* gene encoding chloramphenicol (Cm)-resistance

sacB : the *sacB* gene encoding levansucrase

survive. This makes it easy to select for the loss of plasmid pKO3. Finally either PCR or Southern blotting is used to screen for the expected gene replacement.

Using vector pKO3 for gene replacement has been shown to work efficiently and simply and is thus suitable for examining gene essentiality (Link *et al.*, 1997; Brown *et al.*, 1995). Therefore, in this thesis work, the pKO3 vector and its protocol have been used for gene replacement.

5. New revelations from the study of the *metK84* mutant

5.1. Characteristics of the *metK84* mutant

The short name, *metK84* mutant, is commonly used to describe *metK* mutant strain MEW402 (Table 2), because it has the *metK*84 mutation on the chromosome. The *metK84* mutation was originally named from one *metK* mutant of *E. coli*, RG62 (Greene *et al.*, 1973), which showed elevated levels of methionine biosynthetic enzymes and an extremely low level of SAM synthetase. This *metK84* mutation was then transduced into strain MEW1 (wildtype *E. coli* background) and characterized by phenotypes of leucine-requirement and GGME (a methionine analogue) resistance. One such isolate was picked, named MEW402 (*metK84* mutant) and used for further study. This newly constructed unsuppressed *metK*84 mutant showed a complex phenotype under certain growth conditions. It grew normally in minimal medium with a high concentration of leucine (50 μg/ml), but had a deficiency in cell division at lower concentrations of leucine and showed long filaments, some as long as 50 times the regular *E. coli* cell length (2μm). However, this filamentation could be suppressed by complementation with a plasmid-

carrying a functional *metK* gene or by generation of Lrp (Leucine-Responsive Regulatory Protein) deficiency (Newman *et al.*, 1998; Budman, 1998).

In association with all these observations, two new revelations have been proposed. They are reviewed in section 5.2. and section 5.3.

5.2. SAM might play a role in cell division

5.2.1. Cell division and cell division genes in *E. coli*

Cell division in gram-negative bacteria such as *Escherichia coli* is also referred to as septation. It is a process whereby after an *E. coli* cell elongates to a certain point for subdivision, a number of essential proteins participate in the assembly of a ring at a selected site so as to form a septum, such that the parental cell is divided into two daughter cells. Totally nine proteins have been identified as being required for cell division, FtsZ, A, I, Q, L, K, N, W and ZipA. A defect in cell division results in filamentous morphology, due to defects in DNA segregation (Par) or in septation (Fts). Cells exhibiting the Fts phenotype are filamentous with regularly distributed nucleoides, which suggests that DNA segregation has no observable defect and the *fts* genes are specifically essential for formation of the septum (Lutkenhaus and Mukherjee, 1996).

The most studied cell division *fts* genes are described as follow:

(1) *ftsZ* gene: the *ftsZ* gene is the most well-studied *fts* gene and encodes the FtsZ protein. Dr.Lutkenhaus once described the role of the FtsZ protein as "The first visible event in prokaryotic cell division is the assembly of the soluble, tubulin-like FtsZ GTPase into a membrane-associated cytokinetic ring that defines the division plane in bacterial and archaeal cells"(Lutkenhaus, 1998). This clearly illustrates the character and the function

of FtsZ in cell division and its importance. Like tubulin in eukaryotes, FtsZ acts as a GTPase, and polymerizes into long tubules in a GTP-dependent manner (Lutkenhaus, 1993; Ma *et al.*, 1996). Together with the help from MinC (division inhibitor) and MinE proteins (topological specificity protein), FtsZ condenses into a ring at the future division site to initiate cell division. Other cell division proteins then attach in a hierarchical order to assemble the septum. It has been noted that excess FtsZ and loss of FtsZ both lead to filamentous phenotype (Lutkenhaus and Mukherjee, 1985).

(2) *ftsA* gene: the *fts*A gene encodes the FtsA protein, which fuctions as an ATPase. The ratio of FtsZ to FtsA is important for cell division to proceed (Dai and Lutkenhaus, 1992). Overexpression and alteration of FtsA both result in inhibition in cell division and cause indented filaments. It is possible that the specific interactions between FtsA and FtsZ and/or other division proteins are required for cell division (Wang and Gayda, 1990).

(3) *ftsI* gene: the *ftsI* gene located close to the 5'end of the 2-min cluster(position for *fts* genes on *E. coli* chromosome) and is transcribed using a promoter far upstream. The product of the *ftsI* gene is the penicillin-binding protein 3 (PBP 3). PBP3 is specifically needed for peptidoglycan biosynthesis and is an essential cell division component (Lutkenhaus and Mukherjee, 1996).

(4) *ftsQ and ftsL* genes: both genes encode transmembrane proteins based on primary sequence analysis, while the biochemical activities of their products are not known (Lutkenhaus and Mukherjee, 1996).

5.2.2. The role of SAM in cell division

The *metK*84 mutant showed filamentous morphology when grown in minimal medium with a low concentration of leucine. Fluorescence microcopy examination showed nucleoids were segregated uniformly along the filaments, which indicated that DNA segregation was regular (Newman *et al.*, 1998). Thus, the filamentous phenotype should result from a defect in septation (Fts). Tested in the same conditions as above, SAM synthetase activity had been found to be markedly decreased. From this evidence, it has been proposed that SAM might influence the methylation of certain steps or specific components in cell division and that lack of SAM results in a cell division defect in *E. coli* (Newman *et al.*, 1998). However, it is still not clear how SAM is involved in cell division, and further investigations are needed.

5.3. The Leucine/Lrp regulon regulates *metK* gene expression

5.3.1. The Leucine/Lrp regulon

The leucine/Lrp regulon, is regulated by Lrp, with leucine as cofactor. Lrp, the leucine responsive regulatory protein, is a global regulator encoded by the *lrp* gene. It regulates the transcription of a number of genes by activating the expression of some genes such as *serA*, *ilvIH*, *gcv*, *gltD*, *ompF*, *etc.* while repressing the expression of some others like *sdaA*, *ompC* and *lysU*, *etc.* (Newman *et al.*, 1995; 1996).

Lrp as a DNA binding protein has three binding domains: the DNA binding domain, the leucine binding domain and the transcription activation binding domain (Willins *et al.*, 1991). The possible binding consensus sequence is: YAGHAWARRWTDCTR (Y=C/T, H=not G, W=A/T, D=not C, R=A/G) (Cui *et al.*, 1996). Nevertheless, this

sequence is not so specific and may be found in many places in the genome (Newman *et al.*, 1996). When Lrp binds to the upstream region of the regulated gene, it could bend DNA to help RNA polymerase bind and activate the transcription. On the other hand, it also could prevent or interfere with the binding of RNA polymerase, thus repressing transcription (Lin *et al.*, 1992).

Leucine as the cofactor, binds to Lrp and causes an allosteric change. This conformation change does not prevent Lrp from binding to its target DNA, but alters the effect of Lrp on transcription (Newman *et al.*, 1995).

5.3.2. Lrp represses *metK* expression

The promoter region and the transcription regulation of the *metK* gene have not yet been studied. Therefore, the regulatory mechanism of *metK* gene expression and the proteins and factors involved are still unknown. Study of the properties of the *metK*84 mutant found that the slow growth and filamentation of the *metK*84 mutant strain grown in minimal medium could be avoided by the presence of a high concentration of leucine or by the loss of Lrp. SAM synthetase activity examination also gave consistent results in which the enzyme activity is markedly decreased in minimal medium, but increased several fold in an *lrp metK*84 double mutant background or by adding high concentrations of leucine. Therefore, it is suggested that Lrp represses *metK* expression while leucine antagonizes Lrp to release the repression, analogous to the regulation of the *sdaA* gene (Newman *et al.*, 1998).

However, the mutation in *metK*84 has not yet been identified. The position of the mutation (*metK* coding region or upstream) and the type of mutation are still riddles. The

involvement of the leucine/Lrp regulon in the regulation of *metK* expression needs further characterization from the molecular levels.

6. The purpose of this study

From the above introduction, we know SAM is very important in *E. coli* metabolism. But on the other hand, many things related to SAM, SAM synthetase and the *metK* gene are still not clear. The purpose of this thesis work is to answer two basic questions: (1) Is the *metK* gene encoding SAM synthetase essential to *Escherichia coli* K-12? (2) What is the *metK*84 mutation and why does it result in such a complex phenotype?

In this thesis, I present data to demonstrate that the *metK* gene and/or its product SAM synthetase are absolutely required for *E. coli* cell growth, whether in rich medium (LB) or in minimal medium. I sequenced and identified the *metK*84 mutation and determined the transcription initation site of the *metK* promoter.

Materials and Methods

1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Tables 2 and 3. Here all the bacterial strains used were derivatives of *E. coli* K-12.

2. Media and growth conditions

2.1. Luria-Bertani medium (LB)

1% bacto-peptone, 0.5% yeast extract, 0.5% NaCl.

For solid medium, 2% bacto-agar is added.

2.2. Glucose minimal medium

0.54% K_2HPO_4, 1.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.02% $MgSO_4$,0.001% $CaCl_2$,pH7.0

2% bacto-agar was added for solid medium.

D-glucose was added at final concentration of 0.2% after autoclaving. 50 μg/ml

L-isoleucine and L-Valine were added for strain MEW1 and its derivatives to compensate the deletion of the *ilvA* gene.

2.3. SOC medium

2%Bactotryptone, 0.5%yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$,

10 mM $MgSO_4$, 20 mM glucose

Table 2. Bacterial strains used in this study

Strains	Relevant Genotype	Reference/Source
CU1008	*E. coli* K-12 *ilvA*	L.S. Williams
MEW1	CU1008 Δ*lac*	Newman *et al.*, 1985
CA	MEW1 Δ*ara*	Cheng, C.F. 1997
MEW 402	MEW1 *metK*84, leucine-requiring	Newman *et al.*, 1998
MEW522	CA Δ*metK* pBAD22 *metK*	This study
MEW523	CA Δ*metK recA*::Tn10 pBAD22 *metK*	This study
MEW646	CA Δ*metK* pLtet-K	This study
MEW647	CA Δ*metK tetR* pLtet-K	This study
MEW648	CA Δ*metK tetR recA*::Kn pLtet-K	This study
DH5αZ1	DH5α *tetR lacI* Sp^r	Lutz et *al.*, 1997
BW26355	Δ*recA*635::Kan, Δ(*araD-araB*)567	Datsenko and
	Δ*lacZ*4787(::rrnB-4),	Wanner, 2000
RecA::Tn10	*ara*, Δ(*gpt-lac*)5, *thi*, *recA*::Tn10	C.G. Cupples

Table 3. Plasmids used in this study

Plasmids	Description	Reference/Source
pZAPA-TA	pBlueScript SK+ derivative	L.Des. Groseillers
pZAP-K31	pZAPA-TA containing 3.1-kb *metK* (1-kb upstream+1.15-kb *metK*+1-kb downstream)	This study
pZAP-K25	pZAPA-TA containing 2.5-kb deleted *metK*(1-kb upstream+ 472 bp *metK*+1-kb downstream)	This study
pZAP-K18	pZAPA-TA containing 1.8-kb *metK*84 fragment (700 bp *metK*84 upstream +1.15-kb *metK*)	This study
pKO3	*cam-sac*B pSC101(ts) replicon	Link *et al.*, 1997
pKO3-DK	pKO3 carrying the 2.5-kb deleted *metK* insert from pZAP-K25	This study
pZE21	PLtetO-1 promoter, ColE1 replicon, MCS-1, Kn^R	Lutz and Bujard, 1997
pZA31-luc	PLtetO-1 promoter, p15A replicon, *luc* gene, Cm^R	Lutz and Bujard, 1997
pLtet01	PLtetO-1 promoter, p15A replicon, MCS-1, Cm^R	This study
pLtet-K	pLtet01 containing 1155 bp wildtype *metK* gene, Cm^R	This study
pLtetK::*lacZ*	pLtet01 containing *metK*::*lacZ* fusion	This study
pBAD22	A derivative of pBR322, carrying P_{BAD} promoter	Guzman *et al.*, 1995
pBAD22*metK*	pBAD22 containing 1155 bp *metK*, Amp^R	Newman *et al.*, 1998
pMC1871	pBR322 with a truncated *lacZ* gene	R.K. Storms

pRS415	*lac* operon fusion vector, Amp^R	Simons *et al.*, 1987
pRS-K	pRS415 containing *metK*::*lacZ* operon fusion	This study
pRS-K84	pRS415 containing *metK*84::*lacZ* operon fusion	This study

2.4. Other additions to the media

Antibiotics were used at the following concentrations: Ampicillin (Amp) 200 μg/ml; Tetracycline (Tet) 20 μg/ml; Kanamycin (Kn) 50 μg/ml; Spectinomycin (Spec) 50 μg/ml; Chloramphenicol (Cm) 25 μg/ml.

3. Buffers and solutions

MC buffer: 0.1M $MgSO_4$, 0.5 M $CaCl_2$

1X TBE buffer: 0.09 M Tris-borate, 0.002 M EDTA (pH8.0)

Z buffer: 1.61%$Na_2HPO_4.H_2O$, 0.53% $NaH_2PO_4.H_2O$, 0.075%KCL, 0.0264% $MgSO_4.7H_2O$, 0.27% (v/v) β-mercaptoethanol, pH7.0

X-gal solution: 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside in N-N – dimethyl-formamide

ONPG solution: 4 mg/ml o-nitrophenyl-β-D-galactoside in 0.1M phosphate buffer, pH7.0

4. Chemicals

Restriction enzymes, Taq polymerase, Pfu polymerase and DNA modifying enzymes were purchased from MBI fermentas, New England Biolab, Promega or Stratagene. [γ-^{32}P]ATP was purchased from Amersham. Oligonucleotides used in this study were purchased from Biocorp Montreal (Table 4).

Table 4. Oligonucleotides used in this study

Oligonucleotide	Sequence (5' to 3')	Use
metK-U1	TGG CCC GGG TCG TTA ACG TC	PCR; Sequencing
metK-D2	TAG GTC GAC GCG TTC GGC AT	PCR
metK-In1	TTA CGT CCG AGT CCG TCT CT	PCR; Sequencing
metK-In2	TAC TTC AGA CCG GCA GCA TC	PCR
metK-Del1	ACG GGT GAT CTC TTC	PCR; Sequencing
metK-Del2	CAG GTT TCC TAC GCA	PCR; Sequencing
metK-UPI	AGC AGT GAA TGC TGA CGC TCC AAC	Sequencing
metK-UPII	GCT GGA AGT GGC AAC ACG AA	Sequencing
metK-UPIII	AGC AGA GAT GCA GAG TGC GGG GA	Sequencing
metK-Mid	CGG CTC ACC AGG GTC TGA TGT TT	Sequencing
metK-In3	GCA GCA CGT TAT GTC GCG AA	Sequencing
MetK-In5	CCC GCT GAA TGG CTG ACT TCT	Sequencing
metK-UPB	ATG CCC TTC AGA GAC GGA CTC GGA	Sequencing
metK-In6	CTG AGT GGA AAG CAC GAC AGC A	Sequencing
metK-KP505	GCA GGA ATT CAA AGC AGA GAT G	PCR
metK-In7	GAC GGA CTC GGA CGT AAA AAG GT	Primer Extension

5. β-galactosidase assay

Cells were grown to the mid-log phase in the test medium.β-Galactosidase activity was assayed in whole cells according to the method described by Miller and expressed in his units (Miller, 1972).

6. Transformation and Transduction

Electro-transformation was done by using Gene Pulser(Bio-Rad) according to the instruction manual provided by the manufacturer.

P1 phage-mediated transduction was performed according to the method described by Miller (Miller, 1972).

7. DNA Manipulation

7.1. DNA isolation and purification

Plasmid or Chromosomal DNA isolation, PCR product purification and Gel extraction were performed by using kits supplied from QIAGEN and following the protocols.

7.2. Molecular biology methods

Recombinant DNA methods used protocols recommended by the suppliers of the enzymes and other standard procedures described by Maniatis (Sambrook *et al.*, 1989).

8. DNA Sequencing

DNA Sequencing was performed as described with the Beckman CEQ2000 Dye Terminator Cycle Sequencing Kit. Primers used for sequencing were included in Table 4. Sequencing reaction products were sent to Concordia University Genomic Centre for analysis.

9. Gene replacement

To construct a chromosomal *metK* deletion mutant, the gene replacement method was performed by using the pKO3 vector provided by Dr.Church (Harvard Univ.) and following their protocols (Link, *et al.*, 1997) with slight modifications. In brief, the deleted *metK* mutant allele in the pKO3 vector was transformed into an *E. coli* wildtype strain CA (MEW1 Δ*ara*) and allowed to recover for1h-1h 30min at 30^{0}C. The cells were then plated on prewarmed LB-Cm plates and incubated at 30^{0}C. Colonies were picked into LB broth, serially diluted and plated on the same kind of plates but incubated at 43^{0}C to select for integrants. From the 43^{0}C plate, several integrants were picked, resuspended in 1ml of LB broth separately, serially diluted and immediately plated on LB-5% sucrose plates and shifted back to 30^{0}C. The 5% sucrose plates were then replica plated to chloramphenicol containing plates at 30^{0}C to screen for sucrose-resistant and chloramphenicol sensitive colonies. The gene replacement event was confirmed by PCR using primers flanking the *metK* ORF.

10. Primer Extension

10.1. Isolation of total cellular RNA

For preparation of total RNA, wildtype *E. coli* strain MEW1 carrying plasmid pZAP-K31or pZAP-K18 was used. Overnight cultures in LB or in glucose minimal medium were subcultured in the same medium to $O.D_{600}$ around 0.5. Total cellular RNA was isolated from 10^9 cells with the QIAGEN RNeasy Mini Kit, following the instructions provided by the manufacturer.

10.2. Primer labelling

The gene specific primer, *metK* In7 (5'-GAC GGA CTC GGA CGT AAA AAG GT-3') complementary to an internal region within *metK* (+33 to +11bp) was used for RNA primer extension. The primer was synthesized and 5'-end labelled with Cy5 fluorescent dye by ACGT company (Toronto). The obtained oligo was resupended into 200 µl of DEPC-treated water and was diluted to a final concentration of 10 µM.

10.3. RNA primer extension

One µl of Cy5-labelled primer In7 (10 µM) was mixed with 5 µg of total RNA and the volume was adjusted to 10 µl with DEPC-treated water. RNA and primer were incubated at 65^0C for 5 min and chilled on ice. Then 10 µl cDNA synthesis mix was added, which contained 4 µl 5× cDNA synthesis buffer (250 mM Tris acetate, 375 mM potassium acetate, 40 mM magnesium acetate, stabilizer), 1 µl 0.1M DTT, 2 µl 10 mM dNTP mix, DEPC-treated H_2O and 1 unit ThermoScript Reverse Transcriptase (LIFE TECHNOLOGIES, CA). The reaction mixture was incubated at 53^0C for 60 min for

primer extension. The reaction was stopped by adding 20 µl stop solution [95%(v/v) formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue]. Eight µl of the reaction sample was electrophoresed on a packed sequencing gel (ReproGel, Amersham Pharmacia), and detected by automated "A.L.F" DNA Sequencer (Amersham Pharmacia). A sequencing reaction with the same primer was run in the adjacent lane.

11. Plasmid constructions

11.1. Construction of a *metK* deletion on plasmid pZAP-K25

To construct a chromosomal *metK* deletion mutant, the deletion of *metK* was first constructed on a plasmid using the inverse PCR technique. The strategy for this construction is shown on Figure 5.

For this construction, I used two gene specific primers Del 1 and Del 2 (See Table 4) to anneal to the internal regions of *metK* on plasmid template pZAP-K31 (See Table 3). These two primers went outward in the PCR reaction to amplify the two ends of the *metK* sequence, and all the remaining sequence of pZAP-K31, thus generating a linear plasmid, missing the central 683 bp of the *metK* coding region. This linear DNA was then self-ligated and formed plasmid pZAP-K25.

The above construction was performed as follows. In the first step, PCR was done in a 40 µl reaction mixture containing 50 ng template pZAP-K31, 0.25 µM of each of the two primers, 200 µM each of dNTPs, Cloned Pfu reaction buffer [200 mM Tris-HCl(pH 8.8), 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH4)_2SO_4$, 1% Triton X-100, 1 mg/ml nuclease-free BSA] and 2.5 U cloned Pfu polymerase (Stratagene,USA). The reaction

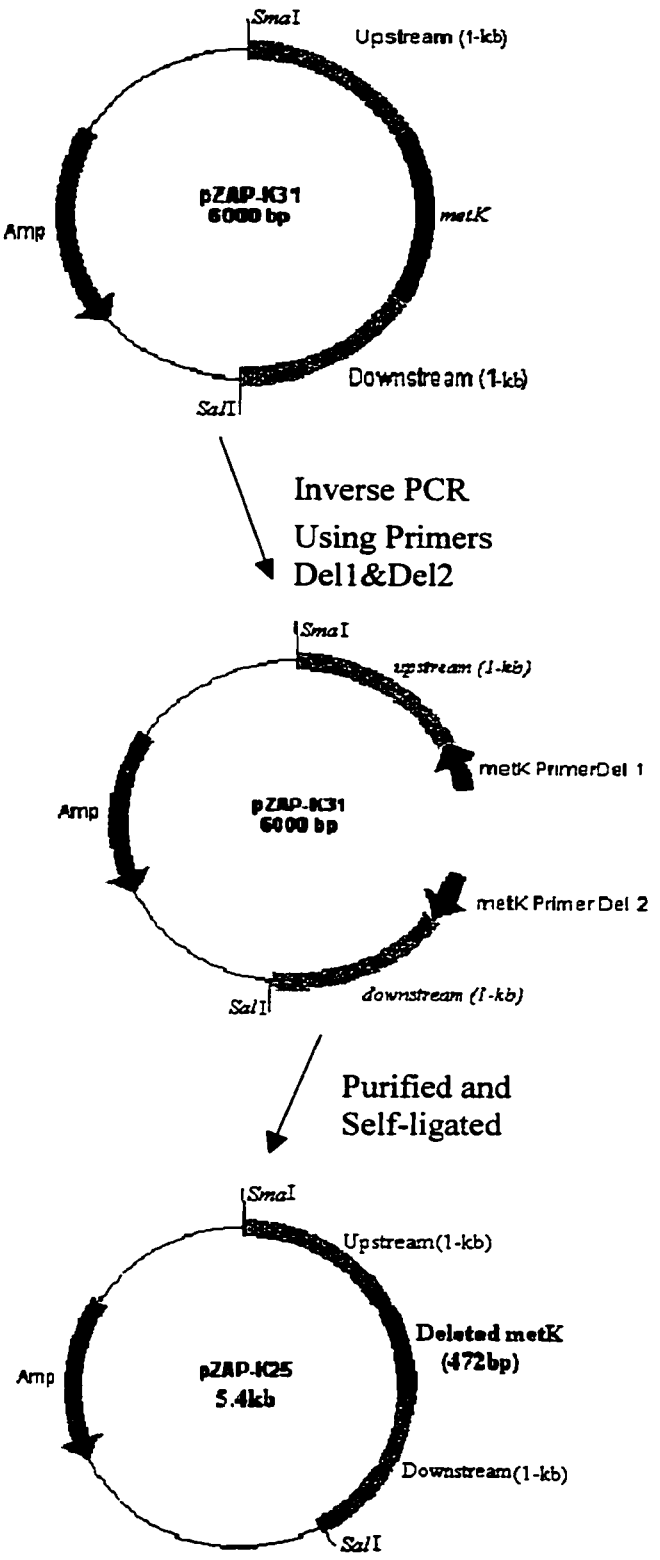


Figure 5. Construction of the deleted-*metK* carrying plasmid pZAP-K25.

mixture was then run on the DNA thermocycler (Interscience) using the thermal cycle file as:

	95^0C 5 min to denature DNA
28 cycles	95^0C 1 min
	43^0C 30 sec
	72^0C 10 min
2 cycles	95^0C 1 min
	43 ^{0}C 30 sec
	72^0C 12 min

The PCR product was analyzed on a 1% agarose gel to confirm that the amplification was successful. The well-amplified sample was further purified with PCR purification Kit (QIAGEN) to remove the template and unincorporated primers. In the second step, purified PCR sample was self-ligated by adding T4 DNA ligase. The ligated product was then electroporated into *E. coli* strain CA (MEW1 Δ*ara*) and plated on the LB ampicillin plate for selection. Plasmid from one of the ampicillin-resistant colonies was confirmed to be right clone by appropriate enzyme digestions. This plasmid was named pZAP-K25.

11.2. Construction of plasmid pKO3-DK

A 2.5-kb *Sma*I-to-*Sal*I fragment, which contained the deleted *metK* sequence and its intact upstream and downstream regions, was isolated from pZAP-K25 and inserted into the *Sma*I and *Sal*I sites of pKO3 vector to yield plamid pKO3-DK, which later was used in the gene replacement experiment to replace the chromosomal wildtype *metK* sequence with the deleted *metK* sequence.

11.3. Construction of a highly inducible and tightly controlled expression vector pLtet01

In order to tightly control plasmid gene expression, I took advantage of the new Bacterial Expression Vector System designed by Lutz *et al.* (1997) and used it to generate a new vector having the PLtet promoter, chloramphenicol resistance and p15A origin of replication.

To do this, two vectors pZE21-MCS-1 and pZA31-luc were first double digested with restriction enzymes *Aat*II and *Avr*II. The 371 bp fragment from pZE21-MCS-1 and the 1716 bp fragment from pZA31-luc were isolated by gel extraction and ligated together. The ligation product was transformed into wildtype strain MEW1 and selected on LB-Cm plate. Plasmid isolated from one Cm^r transformant showed the right size after enzyme digestion, this plasmid was named pLtet01. Figure 6 illustrates the construction of this plasmid.

11.4. Construction of *metK*-carrying plasmid pLtet-K

A 1155 bp *Nco*I -to-*Hind*III fragment carrying the whole structural *metK* gene was isolated from plasmid pBAD22*metK*, and the restriction sites filled in with Klenow enzyme and dNTPs, prior to insertion into the *Sma*I site of plasmid PLtet01, thus producing the new *metK* carrying plasmid pLtet-K, which had the *metK* gene under the control of PLtet promoter (Figure 7). Restriction enzyme digestion was performed to make sure that this recombinant plasmid had the right size and was ligated in the right direction.

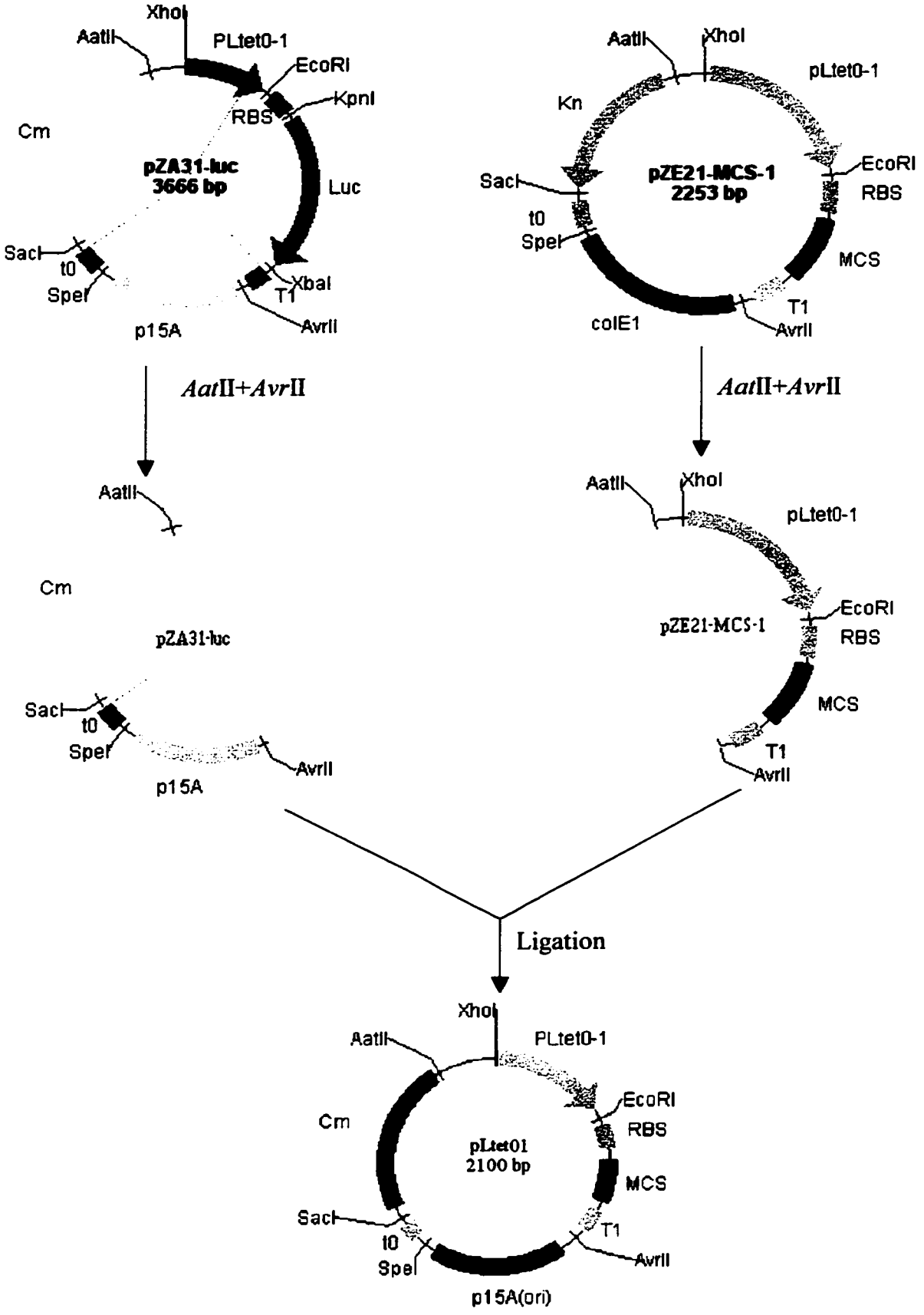


Figure 6. Construction of plasmid pLtet01.

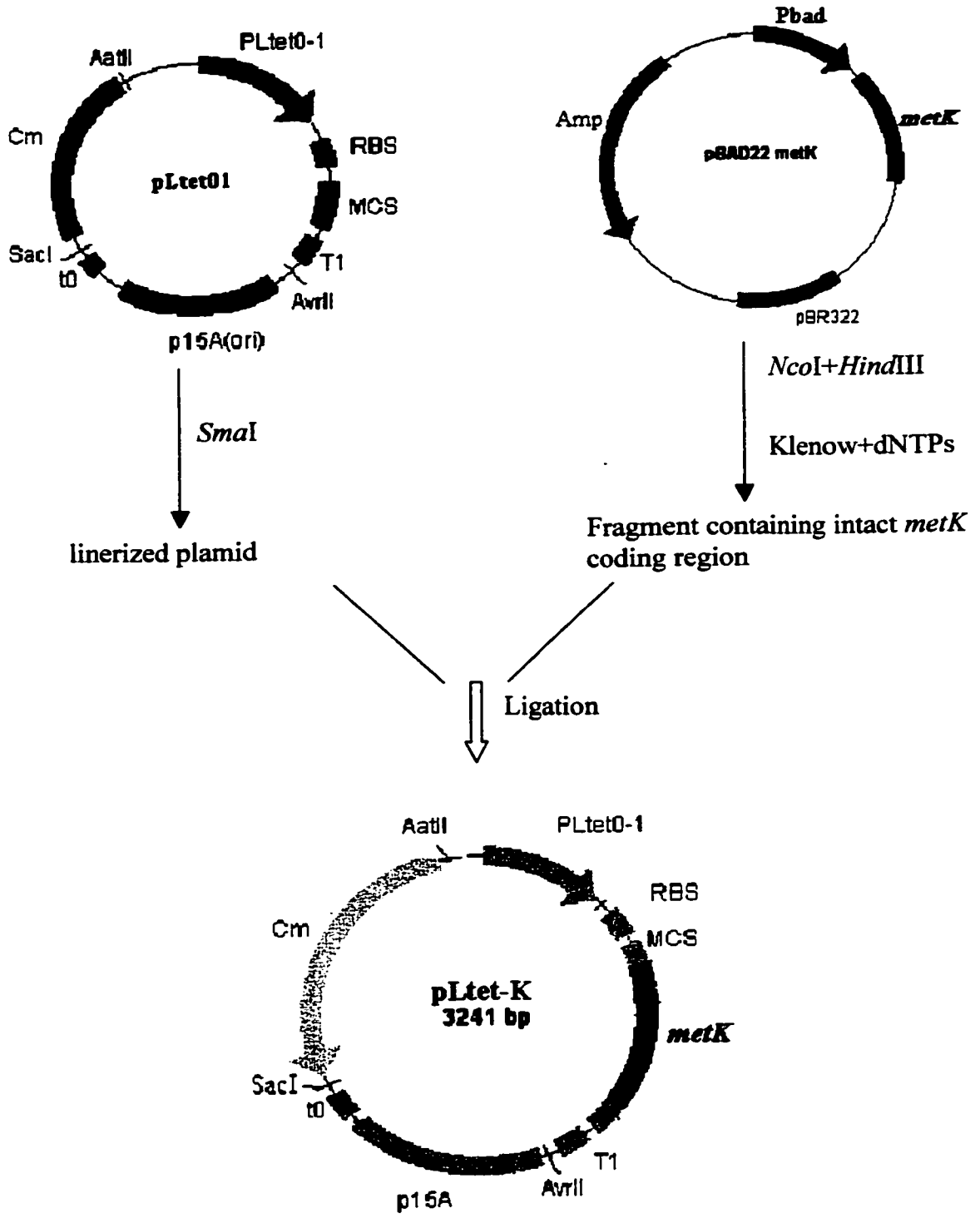


Figure 7. Construction of plasmid pLtet-K.

11.5. Construction of pLtetK::*lacZ* fusion

In order to measure the level of plasmid *metK* gene induction at different concentrations of inducer aTc, a *lacZ* reporter gene was fused in frame to the *metK* coding region on plasmid pLtet-K, generating plasmid pLtetK::*lacZ* (Figure 8).

A 3-kb *Bam*HI fragment containing the promoterless *lacZ* gene from plasmid pMC1871 was isolated, and subcloned into the *Bam*HI site of a 2.1-kb dephosphorylated pLtet-K fragment. The ligated mixture was transformed into strain DH5αZ1 and plated onto LB plate containing chloramphenicol, X-gal (40 μg/ml) and aTc 50 ng/ml. After digestion with restriction enzyme *Mlu*I, plasmids from several dark blue colonies were confirmed to have the right sizes and the right orientations.

11.6. Construction of plasmid pZAP-K18 carrying the *metK*84 promoter

First, genomic DNA was isolated from the *metK*84 mutant strain. Two oligonucleotides *metK*-UPI (5'-AGCAGTGAATGCTGACGCTCCAAC-3') and *metK*-In2 (5'-TACTTCAGACCGGCAGCATC-3'), complementary to the 701 bp- 678 bp upstream of the *metK* ATG start codon and 1154 bp-1135 bp within the *metK* gene were selected as primers to amply this DNA using Taq polymerase. The 1.8-kb PCR amplified fragment containing the 700 bp upstream region and the whole *metK* gene were then purified and inserted into the *Xcm*I site of plasmid pZAPA-TA. The ligation mixture was transformed into strain XL1-blue and selected on LB ampicillin plates containing X-gal and IPTG. Plasmids from white colonies were isolated and examined by restriction enzyme digestion. The verified recombinant plasmid was named pZAP-K18.

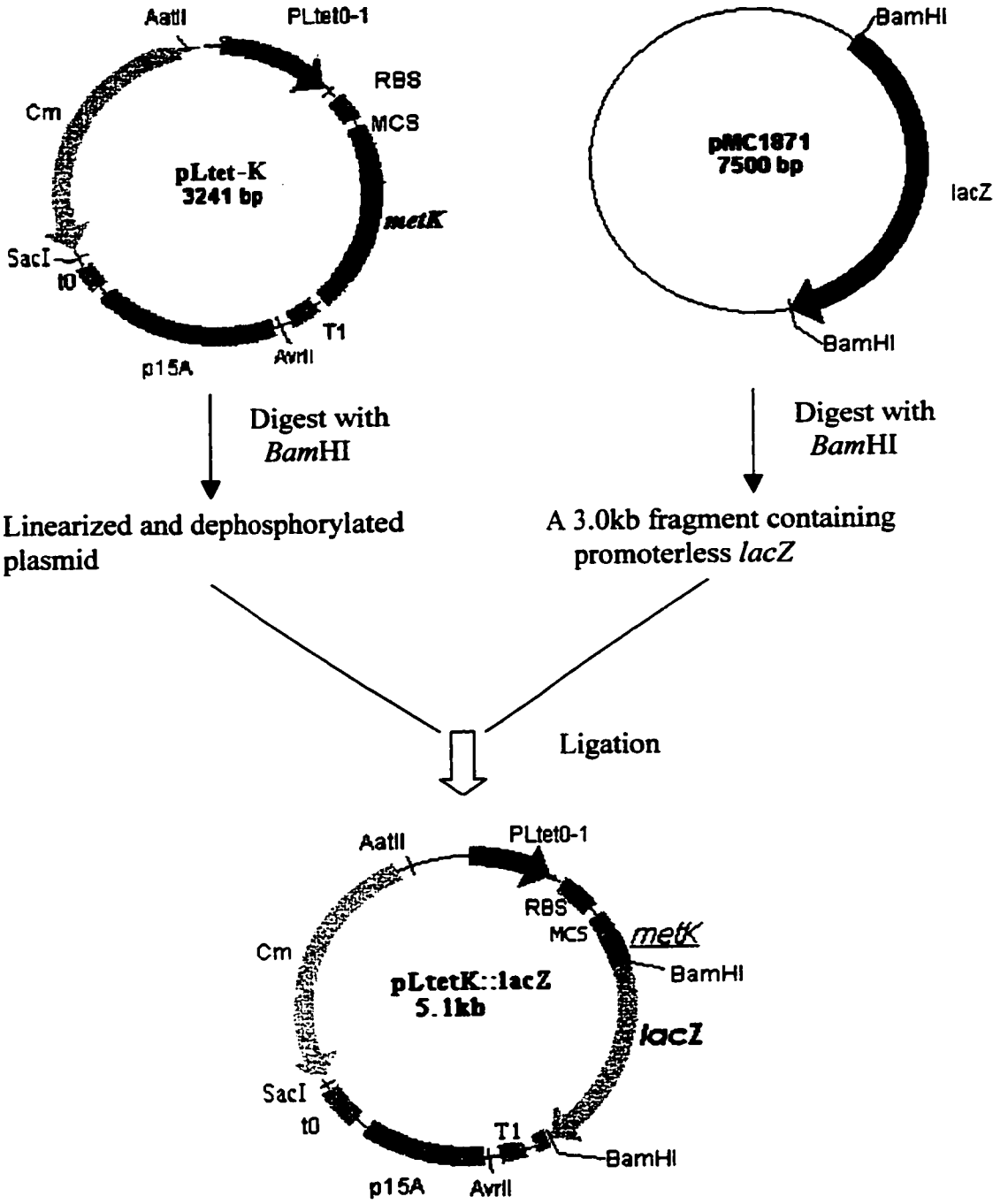


Figure 8. Construction of *lacZ* fusion plasmid pLtetK::*lacZ*.

11.7. Construction of *metK::lacZ* and *metK*84*::lacZ* operon fusion plasmids

To create operon fusions, a DNA fragment covering –504 bp to +45 bp from the *metK* ATG start codon was amplified by PCR. Primer *metK*-KP505, 5'-GCAGGAATTCAAA GCAGAGATG-3', corresponded to the region from –504 bp to –483 bp upstream of *metK* ATG and included the *Eco*RI restriction site. Primer *metK*-UPB, 5'-ATGCCCTTC AGAGACGGACTCGGA-3', corresponded to +45 bp - +22 bp of the *metK* coding region.

The amplified fragment was digested with *Eco*RI, purified and cloned into the operon fusion plasmid pRS415 at *Eco*RI to *Sma*I sites. The ligation mixture was transformed into wildtype strain MEW1 and plated on LB plate with X-gal and ampicillin. Plasmids from blue colonies were isolated and checked by restriction enzyme digestion.

For *metK*::lacZ fusion, plasmid pZAP-K31 containing the wildtype *metK* upstream region was used as template in PCR amplification, and the resulting clone was named pRS-K. Also, plasmid pZAP-K18 carrying the *metK*84 mutant upstream (-10 region mutation) was used as template for construction of *metK84::lacZ* fusion, and the resulting fusion clone was named pRS-K84. Figure 9 illustrated the construction of these two operon fusions.

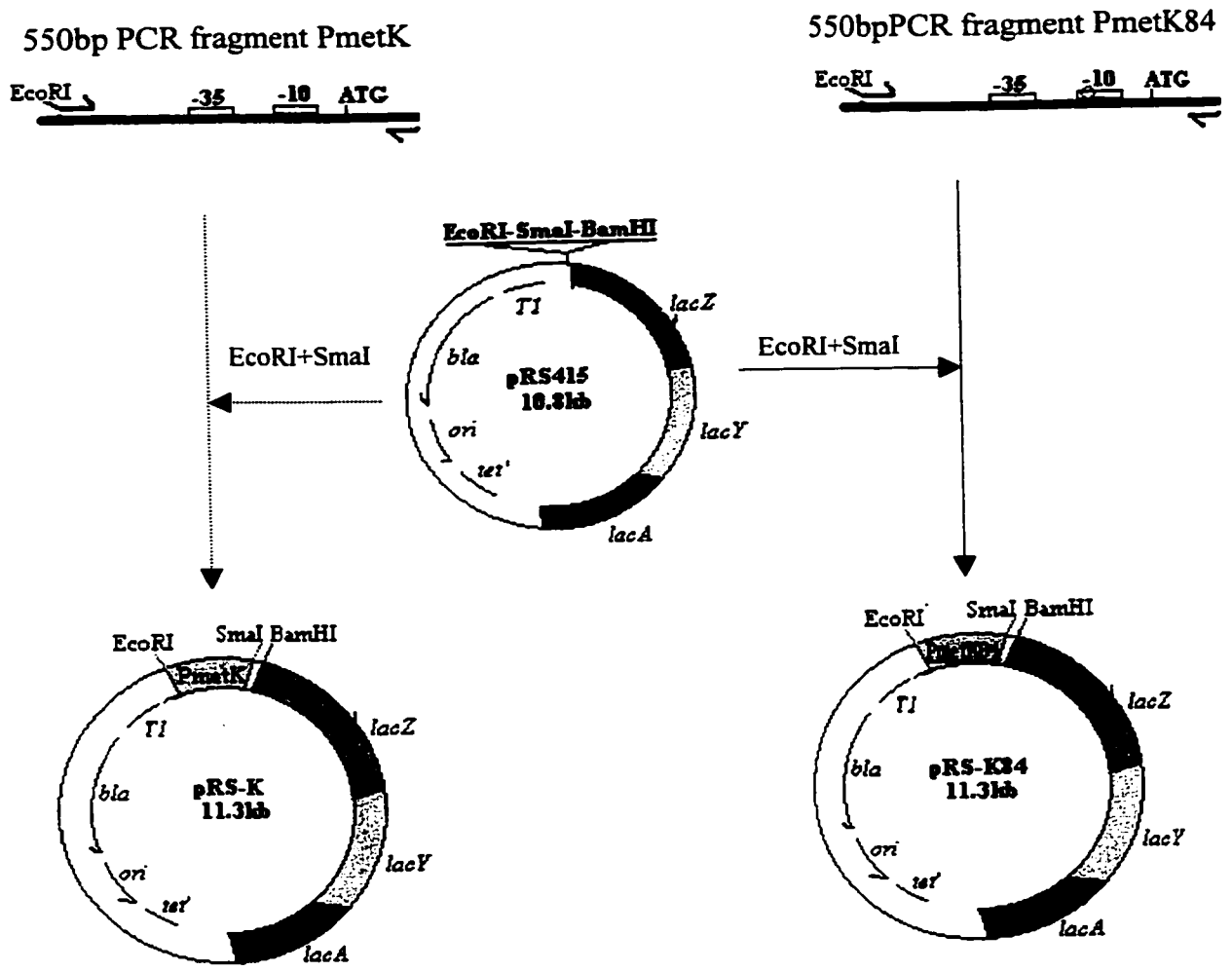


Figure 9. Construction of lac operon fusion plasmid pRS-K and pRS-K84

PmetK: Fragment including 504 bp **wildtype** *met*K upstream and 45 bp *met*K coding region

PmetK84: Fragment including 504 bp**mutated** *met*K upstream (from mutant *met*K84) and 45 bp *met*K coding region

-10 region point mutation is labeled as a circle (o).

Results

In this study, I focused on studying the role of *metK* gene in *E. coli* K-12. The results are reported in two major sections. In part A, I report experiments using the gene replacement method to construct a chromosomal *metK* deletion mutant, complementing with a plasmid carrying the functional *metK* under the anhydrotetracycline-induced PLtet promoter, and demonstrating that the *metK* gene, which encodes S-adenosylmethionine synthetase, is an essential gene in *Escherichia coli* K-12. In part B, I report the sequencing of the *metK* gene from the *metK*84 mutant (which has a complex phenotype presumed to relate to low S-adenosylmethionine concentration) and the identification of the *metK* promoter via primer extension.

Part A. The *metK* gene, encoding S-adenosylmethionine synthetase, is an essential gene in *Escherichia coli* K-12

The *metK* gene of *E. coli* encodes SAM synthetase, which catalyzes the reaction between methionine and ATP to form SAM. SAM is chemically unstable and is thought not to be taken up by *E. coli* cell. Therefore, though SAM synthetase is generally assumed to be essential in *E. coli*, this has not been demonstrated until now. However, no single SAM-dependent methylation has been shown to be indispensable in *E. coli*. I, therefore, set out to test whether or not the *metK* gene is essential to *E. coli* cell growth.

1. Construction of a chromosomal deletion of *metK* and a plasmid containing *metK* under anhydrotetracycline control

1.1. Generation of a *metK* deletion allele on a plasmid

A 683 bp, out-of-frame deletion of the *metK* gene was first engineered on plasmid pZAP-K25 (See Figure 5). A deletion of 60% of the *metK* coding region was generated and the reading frame was completely disrupted. Therefore, it was reasonable to think that the remaining 472 bp could not produce a functional SAM synthetase.

1.2. Replacement of the intact chromosomal *metK* with the deleted *metK*

1.2.1. pKO3 gene replacement vector

I used the newly developed pKO3 gene replacement vector (Link *et al.*, 1997; http://www.arep.med.harvard.edu/) to replace the intact chromosomal *metK* with the deleted *metK* construct described above. A 2476 bp *Sma*I to *Sal*I fragment, which contained the 472 bp deleted *metK*, as well as 952 bp upstream and 1052 bp downstream of *metK*, was cut from plasmid pZAP-K25 and subcloned into the same sites of vector pKO3, thus generating plasmid pKO3-DK, which was used for producing a chromosomal deletion following the pKO3 gene replacement protocols (Link *et al.*, 1997).

To verify that the deleted *metK* had the desired sequence, I sequenced the plasmid pKO3-DK in both 5' to 3' and 3' to 5' directions using 4 primers In1, Del2, In2 and Del1. After comparing the sequence data with the *metK* sequence of the *E. coli* genome, it was confirmed that the sequence of deleted *metK* was as expected, i.e., missing the central 683 bp. Figure 10 illustrates the sequence of the deleted *metK* gene.

atg gca aaa cac ctt ttt acg tcc gag tcc gtc tct gaa ggg cat cct gac aaa att gct

gac caa att tct gat gcc gtt tta gac gcg atc ctc gag cag gat ccg aaa gca cgc gtt

gct tgc gaa acc tac gta aaa acc ggc atg gtt tta gtt ggc ggc gaa atc acc acc agc

gcc tgg gta gac atc gaa gag atc acc cgt aac acc gtt cgc gaa att ggc tat gtg cat
tcc gac atg ggc ttt gac gct aac tcc tgt gcg gtt ctg agc gct atc ggc aaa cag tct
cct gac atc aac cag ggc gtt gac cgt gcc gat ccg ctg gaa cag ggc gcg ggt gac cag
ggt ctg atg ttt ggc tac gca act aat gaa acc gac gtg ctg atg cca gca cct atc acc
tat gca cac cgt ctg gta cag cgt cag gct gaa gtg cgt aaa aac ggc act ctg ccg tgg
ctg cgc ccg gac gcg aaa agc cag gtg act ttt cag tat gac gac ggc aaa atc gtt ggt
atc gat gct gtc gtg ctt tcc act cag cac tct gaa gag atc gac cag aaa tcg ctg ctg
gaa gcg gta atg gaa gag atc atc aag cca att ctg ccc gct gaa tgg ctg act tct gcc
acc aaa ttc ttc atc aac ccg acc ggt cgt ttc gtt atc ggt ggc cca atg ggt gac tgc
ggt ctg act ggt cgt aaa att atc gtt gat acc tac ggc ggc atg gcg cgt cac ggt ggc
ggt gca ttc tct ggt aaa gat cca tca aaa gtg gac cgt tcc gca gcc tac gca gca cgt
tat gtc gcg aaa aac atc gtt gct gct ggc ctg gcc gat cgt tgt gaa att cag **gtt tcc**

tac gca atc ggc gtg gc**t ga**a ccg acc tcc atc atg gta gaa act ttc ggt act gag aaa

gtg cct tct gaa caa ctg acc ctg ctg gta cgt gag ttc ttc gac ctg cgc cca tac ggt

ctg att cag atg ctg gat ctg ctg cac ccg atc tac aaa gaa acc gca gca tac ggt cac

ttt ggt cgt gaa cat ttc ccg tgg gaa aaa acc gac aaa gcg cag ctg ctg cgc gat gct

gcc ggt ctg aag **taa**

Figure 10. Nucleotide Sequence of the deleted *metK* gene.

The nucleotide sequence of the 472 bp deleted *metK* gene is shown in black letters. The deleted 683 bp nucleotides within the *metK* coding region are shown in light letters. The translation start codon (**atg**) and the stop codon (**taa**) of wildtype *metK* sequence are marked in boldface. A newly encountered stop codon (**tga**) is marked in boldface and underlined.

1.2.2. First attempt at gene replacement in the wildtype strain background

In the first attempt to delete *metK*, wildtype *E. coli* strain CA (MEW1 Δ*ara*) was transformed with pKO3-DK, plated on LB-chloramphenicol and incubated overnight at the permissive temperature (30°C). Several colonies were picked and serially diluted in LB liquid. An estimated 10,000 cells were then spread on prewarmed LB-chloramphenicol plates and incubated at the non-permissive temperature (43°C) to select for cointegrants. The integration frequency was found to be 10^{-2} to 10^{-3}. Integrate colonies (6) from the 43°C plate were picked, suspended in LB liquid, serially diluted and plated on LB plates with 5% sucrose at 30°C overnight to allow plasmid resolution so as to result in the replacement of chromosomal *metK* with deleted *metK* and the loss of plasmid pKO3. After replication on LB-5%sucrose-Cm plates, all the sucrose resistant colonies were chloramphenicol- sensitive, which indicated that the plasmid had been lost as expected. However, when I screened these sucrose-resistant chloramphenicol-sensitive colonies for the desired gene replacement by using colony PCR and primers U1 and D2, which hybridized to the genomic DNA sequence flanking the *metK* gene, none showed a *metK* deletion. All the amplification products gave the same size as the wildtype *metK* gene (3200 bp including 1-kb upstream, 1-kb downstream and 1.15-kb of *metK* coding region). This indicated that the attempt to replace the chromosomal intact *metK* with deleted *metK* did not succeed. I tried the same experiments several times, but the results were the same. I could not substitute the chromosomal *metK* gene with the *metK* deletion. This led me to speculate that the *metK* gene may be essential for *E. coli* cells.

1.2.3. Construction of a chromosomal deletion of *metK* in the presence of a plasmid containing *metK* under arabinose control

In order to further test the possibility that *metK* is essential, I repeated the above gene replacement experiments but with a rescue plasmid pBAD22*metK*, in which a wildtype *metK* structural gene was carried under the control of the arabinose-induced P_{bad} promoter.

After the pKO3-DK was integrated into the chromosome at the non-permissive temperature (43°C), I picked a few cointegrants and tested by colony PCR to confirm that both the deleted *metK* allele and wildtype *metK* allele were on the chromosome. I then made competent cells from the confirmed cointegrants, transformed them with plasmid pBAD22*metK* and plated on LB-chloramphenicol-ampicillin at 43°C overnight, thus generating CA pKO3-DK/pBAD22*metK*. Colonies of CA pKO3-DK/pBAD22*metK* were then serially diluted and plated onto LB plates containing 5% sucrose, 500 μg/ml arabinose and ampicillin and incubated at 30°C. In these conditions, vector pKO3 should resolve from the chromosome and get lost in the presence of 5% sucrose, and 500 μg/ml arabinose should fully induce plasmid *metK* expression from pBAD22*metK*. All the sucrose-resistant colonies were replicated and tested for choloramphenicol sensitivity to see if the plasmid pKO3 was present. As in the previous experiments, the sucrose-resistant isolates were all chloramphenicol-sensitive. I randomly picked 19 colonies and screened by colony PCR with the same pair of primers U1& D2 for gene replacement. Eight colonies showed 2500-bp size bands, which indicated that the intact chromosomal *metK* had been successfully replaced by the deleted *metK* (1-kb upstream+ 472-bp deleted *metK*+ 1-kb downstream); nine colonies showed 3200-bp size bands, which still

conserved the wildtype *metK* allele on the chromosome; two colonies had both 2500 bp and 3200 bp bands, presumably carrying both deleted and intact *metK* alleles on the chromosome (Fig.11).

1.2.4. Attempt to cure plasmid pBAD22*metK*

In order to investigate the null phenotype of the *metK* deletion, I tried to cure the cells of plasmid pBAD22*metK*. I grew the *metK* deletion mutant MEW522 (Δ*metK* pBAD22*metK*) in LB broth without antibiotic for 16 hours, serially diluted and spread on LB agar plates and incubated overnight at 37°C. Plates containing around 200 colonies at 10^{-6} dilution were then replicated to LB-ampicillin plates. No ampicillin sensitive isolates were obtained, which indicated that plasmid pBAD22*metK* was not lost. Experiments were perfomed more than 3 times with variations in incubation time and screening more cells. However, I still could not cure the plasmid pBAD22*metK* from *metK* deletion mutants.

1.2.5. Plasmid *metK* expression was enough for cell growth even in the repressed state

Since the plasmid pBAD22*metK* could not be cured from the *metK* deletion strain MEW522, I set out to study the effect of the *metK* gene on cell growth through controlling the plasmid *metK* expression. Vector pBAD22*metK* is a derivative of pBAD22, one of a series of pBAD expression vectors (Guzman *et al.*, 1995). With a promoterless *metK* coding sequence cloned under the arabinose –induced P_{bad} promoter, the plasmid *metK* gene expression could be controlled by varying the concentrations of

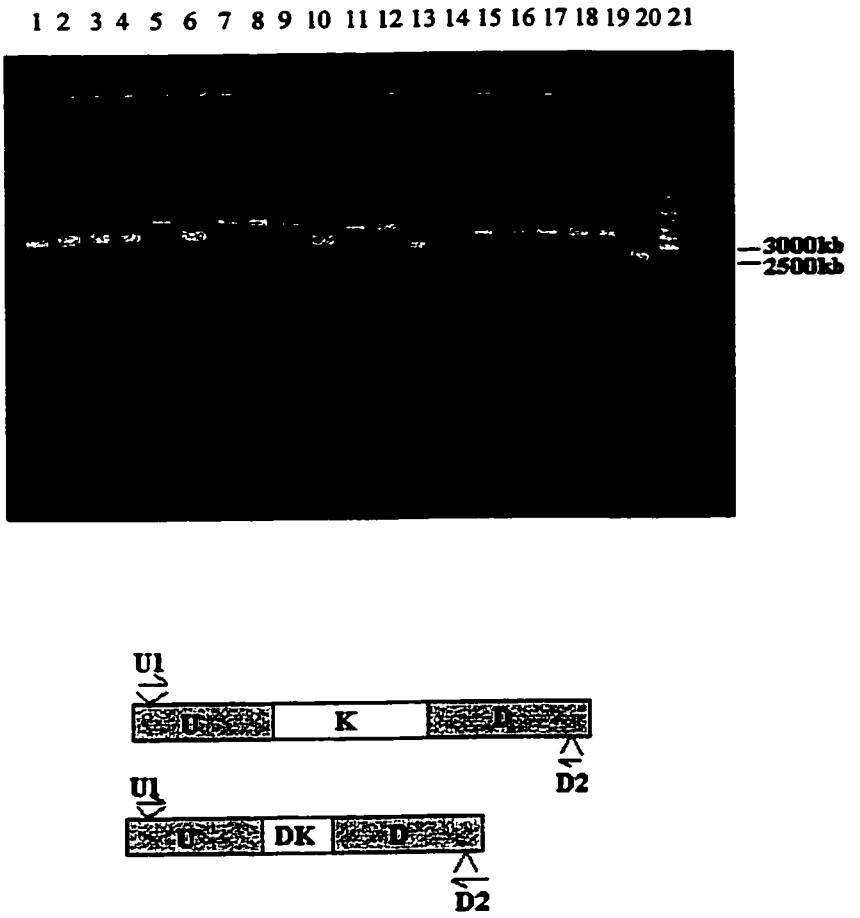


Figure 11. Verification of gene replacement.

PCR amplification from 19 sucrose-resistant but chloramphenicol-sensitive colonies with primer metK-U1 (5'-TGGCCCGGGTCGTTAACGTC-3') and primer metK-D2 (5'-TAGGTCGACGCGTTCGGCAT-3') are shown. Eight colonies show a 2500 bp amplification fragment, indicating the deleted *metK* gene has replaced the wildtype *metK* gene (lanes 1,2,3,4,6,10,13,20). Nine colonies gave only 3.2-kb amplification product, indicating that they still have the wildtype *metK* sequence on the chromosome (lanes 5,7,8,9,11,12,15,17,18). Two colonies show bands of both sizes, suggesting that they have both the deleted *metK* and the intact *metK* genes on the chromosome (lanes 16,19). Lane 14 is a negative control without DNA template. Lane 21 is a 1kb DNA ladder.

the inducer, arabinose. To prevent recombination from the plasmid *metK* to the chromosome, P1-phage mediated transduction was used to introduce a *recA* $^{-}$ allele into strain MEW522. The new transductant (Δ*metK recA*::Tn10 pBAD22*metK*) named MEW523 was used to study the effect of the *metK* deletion on growth. When the inducer, arabinose, was given at 500 μg/ml, the concentration at which plasmid *metK* expression should be fully induced, cells grew as well as wildtype *E. coli*. However, even when inducer arabinose was not provided, mutant cells still could grow although they grew slowly and the colonies appeared tiny. This suggested that the pBAD-driven *metK* gene expression could not be shut off completely and that the background of expression in the repressed state was high enough to complement the null mutation.

1.3. Tightly controlled plasmid *metK* expression

1.3.1. A new *metK*-containing rescue plasmid pLtet-K

To tightly control plasmid *metK* gene expression and reduce the background of *metK* expression in the repressed state, I used a new prokaryotic expression vector system from Dr.Bujard's lab (Lutz *et al.*, 1997) to construct the vector pLtet01 (Fig.6). This vector had the following characteristics:

(1). PLtet0-1 promoter: a novel hybrid regulatory unit that contained the elements of the phage lambda PL promoter and the operator of the Tn10 tetracycline resistance operon. PLtet0-1 was tightly repressed by the specific TetR repressor and showed very low background expression; however it could be highly induced in response to

anhydrotetracycline (aTc), an ideal inducer, which is the analogue of tetracycline but exhibits no antibiotic activity at the concentrations used.

(2). p15A replication origin: low copy number helps to reduce background expression

(3). *cat*^{r} gene: chloramphenicol resistance for selection

The new vector was much better for the purposes of this work because of its tight control and the ease of induction. Another very great advantage is that it could be used in glucose minimal medium because its expression is not sensitive to catabolite repression. I, therefore, excised *metK* from pBAD22*metK* and subcloned into pLtet01, thus generating a new *metK*-containing rescue plasmid pLtet-K (Fig.7).

1.3.2. The replacement of pBAD22*metK* by pLtet-K

Plasmid pBAD22*metK* carries the ColE1 origin of replication and ampicillin resistance gene (β-lactamase), whereas plasmid pLtet-K uses p15A origin of replication and carries chloramphenicol resistance. Therefore the two plasmids can coexist in the cell and can be individually selected. I transformed plasmid pLtet-K into *metK* deletion strain MEW522 (CA Δ*metK* pBAD22*metK*). Plasmids from 4 purified transformants were extracted and subjected to endonuclease *Bam*HI digestion, which confirmed both of the plasmids were present. The confirmed transformants (CA Δ*metK* pBAD22*metK*/pLtet-K) were inoculated into LB-Chloramphenicol cultures, grown overnight at 37°C, serially diluted and plated onto LB-Chloramphenicol plates to lose plasmid pBAD22*metK*. A number of isolates were replicated onto LB-chloramphenicol-ampicillin plates to screen for cells that would be Cam^{r} Amp^{s}, indicating the loss of the pBAD plasmid. I randomly picked 6 colonies, extracted plasmids and digested with the same restriction enzyme

*Bam*HI, the results showed that the plasmid pBAD22*metK* had been lost (See Fig.12). This new strain (CA Δ*metK* pLtet-K) was named MEW646 and kept for future study.

As control, I also performed the identical experiments using control vector pLtet01 (which did not carry the *metK* gene); all the isolates were $Cm^R$and Amp^R. I could not replace pBAD22*metK* with pLtet01 in *metK* deletion strain MEW522 and so conclude that the *metK* function is necessary as expected.

1.3.3. Transferring the TetR repressor

To tightly control plasmid *metK* expression from the PLtet-driven promoter, so that the expression can be shut off as efficiently as possible in the repressed state, the regulatory protein TetR repressor is required (Lutz and Bujard., 1997). The gene encoding this repressor molecule, *tetR*, was transferred from strain DH5α Z1 (DH5α *tetR* *lacR* $spec^R$) to the *metK* deletion strain MEW646(CA Δ*metK* pLtet-K) by P1-phage transduction, and selected by spectinomycin resistance. This generated strain MEW647 (CA Δ*metK* *tetR* pLtet-K).

1.4. Generating a RecA$^-$ derivative to prevent recombination

Since I made only a partial deletion of *metK*, albeit a long one and out-of-frame, plasmid *metK* gene on the rescue vector pLtet-K could recombine into the chromosome. To avoid this possibility, I therefore made the *metK* deletion strain MEW647 *recA*$^-$ by P1-phage mediated transduction from strain BW26355 (*recA*::Kn), thus constructing strain MEW648 (CA Δ*metK* *tetR* *recA*$^-$ pLtet-K).

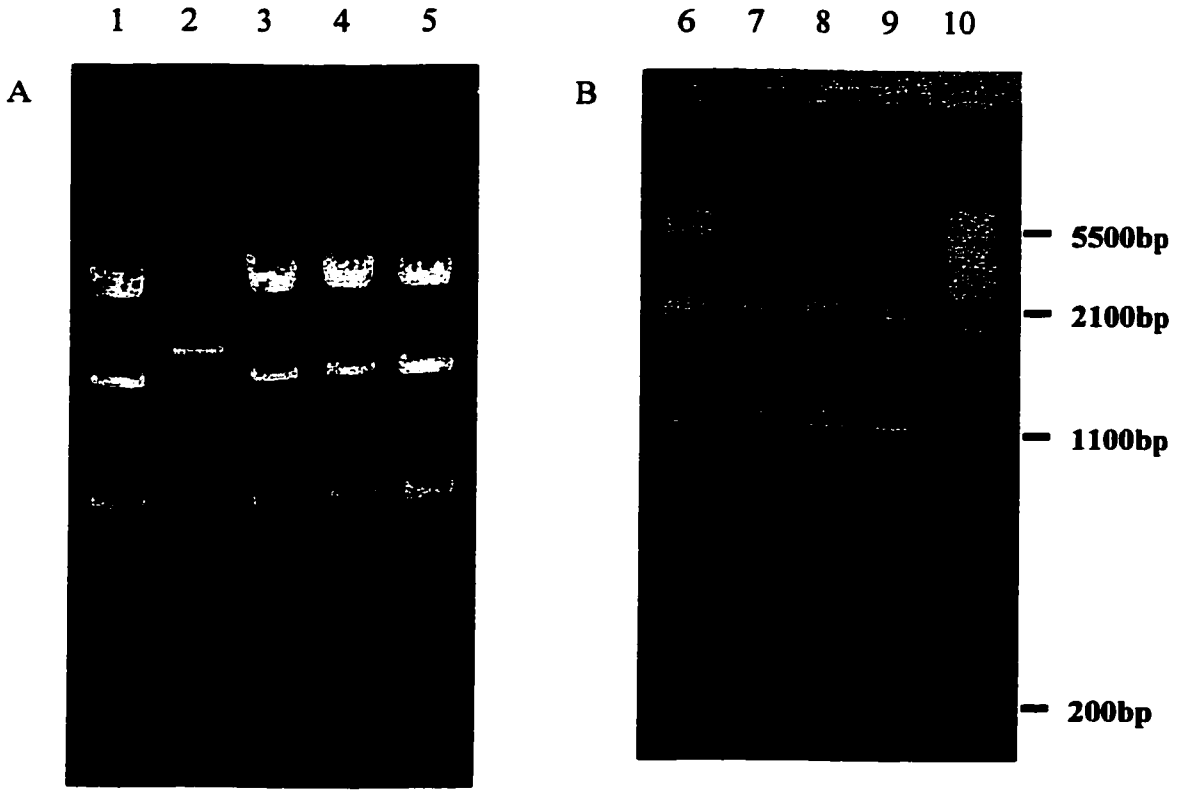


Figure 12. The replacement of pBAD22*metK* by pLtet-K.

(A). *Bam*HI digestion of plasmids isolated from 4 transformants (CA Δ*metK* pBAD22*metK*/pLtet-K). Two plasmids pBAD22*metK* (5500 bp and 200 bp bands) and pLtet-K (2100 bp and 1100 bp bands) coexist in each sample [lanes1& 3-5].
(B). *Bam*HI digestion of plasmids isolated from 3 Cam^RAmp^S colonies, in which the plasmid pBAD22*metK* has been lost (no 5500 bp and 200 bp bands) [lanes 7-9].
In the control, *Bam*HI digestion of plasmid isolated from a Cam^RAmp^R colony indicates that both plasmids still existed in the cell [lane 6]. Lanes 2 and 10 show 1kb DNA ladders.

The Cam^RAmp^S or the Cam^RAmp^R colonies were isolated by growing the confirmed transformants (CA Δ*metK* pBAD22*metK*/pLtet-K) in LB chloramphenicol culture, plating on LB chloramphenicol plates and screening on LB-ampicillin-chloramphenicol plates.

1.5. Summary of three types of *metK*-deletion mutants

After a series of construction steps, I finally constructed three types of *metK* deletion mutants. They all carried the deletion of *metK* on the chromosome and a rescue plasmid carrying a functional *metK* gene under the control of the anhydrotetracycline inducible PLtet promoter but had different alleles of *tetR* and *recA* (See Table 5). Figure13 shows a diagram of the construction of these strains.

Table 5. Three Types of isogenic metK deletion mutant strains

Strain	Genotype		Chracteristics
	Chromosome	Plasmid	
MEW646	Δ*met*K	pLtet-K	-No TetR repressor -RecA$^+$ Strain, functional *metK* gene can recombine from plasmid pLtetK to chromosome.
MEW647	Δ*met*K *tetR*$^-$	pLtet-K	- *tetR* gene on the chromosome; Constitutive synthesis of repressor keeps PLtet promoter repressed; -RecA$^+$ Strain, recombination possible.
MEW648	Δ*met*K *tetR*$^+$ *recA*$^-$	pLtet-K	- *tetR* gene on the chromosome. Constitutive synthesis of repressor; -*recA*$^-$ allele prevents most recombination.

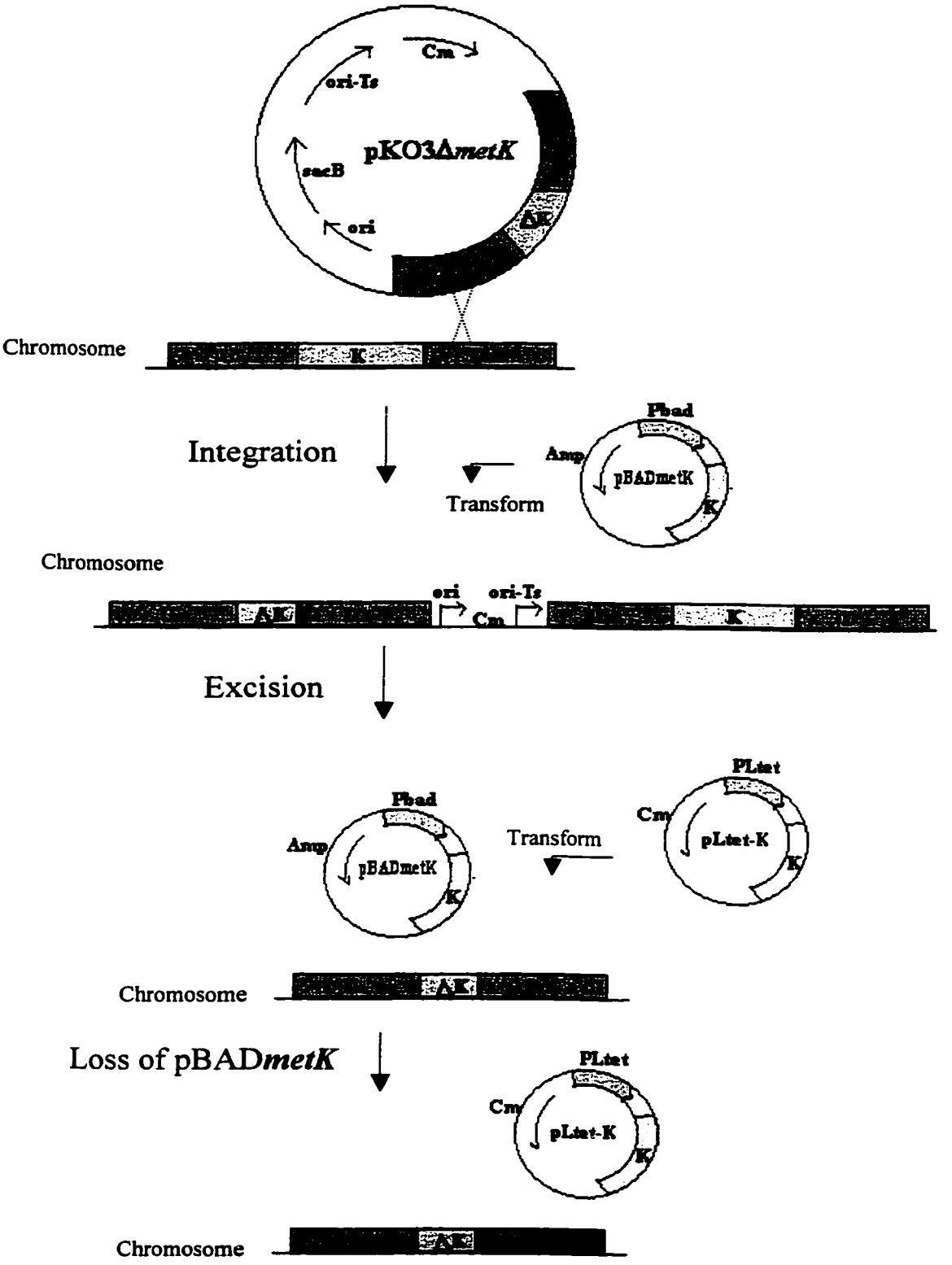


Figure 13. Strategy for the construction of a chromosomal deletion of *metK*.
Symbols: K: *metK*; ΔK: Δ*metK*; U: upstream; D: downstream

2. Strain MEW648 proves that *metK* gene is essential to *E. coli* K-12

To determine whether *metK* gene is essential to the *E. coli* cell, as it is in yeast, the growth characteristics of these three deleted *metK* strains: MEW646 (Δ*metK* pLtet-K), MEW647 (Δ*metK tetR* pLtet-K) and MEW648 (Δ*metK tetR recA*⁻ pLtet-K) were examined.

2.1. *metK* expression is required even in rich medium (LB)

I first streaked the deletion strains: MEW646, MEW647 and MEW648 on chloramphenicol-containing LB agar plates in the absence of inducer aTc or in the presence of aTc 50 ng/ml, a relatively high concentration to inactivate the TetR repressor and fully turn on plasmid *metK* expression. The results of overnight incubation at 37°C are shown in Fig.14:

Strain MEW646 (Δ*met*K pLtetK) grew equally well on both plates, no matter with or without inducer aTc. Since this strain was a TetR repressor-free strain, the PLtet promoter activity was always identical to the activity in the fully induced state (Lutz *et al.*, 1997). Therefore, enough *metK* was expressed from the plasmid to permit growth even though the inducer was not provided.

With TetR regulatory proteins constantly present, strain MEW647 (Δ*metK tetR* pLtetK) showed an inducer-dependent phenotype: with aTc, full growth; without aTc, no growth (Fig.14 MEW647 #11). However, since the strain had a functional *recA* gene, recombination from plasmid *metK* into the chromosome could happen and this obscured results. For example, colony #10 grew well in the presence of aTc, but also showed considerable growth when inducer was not provided.

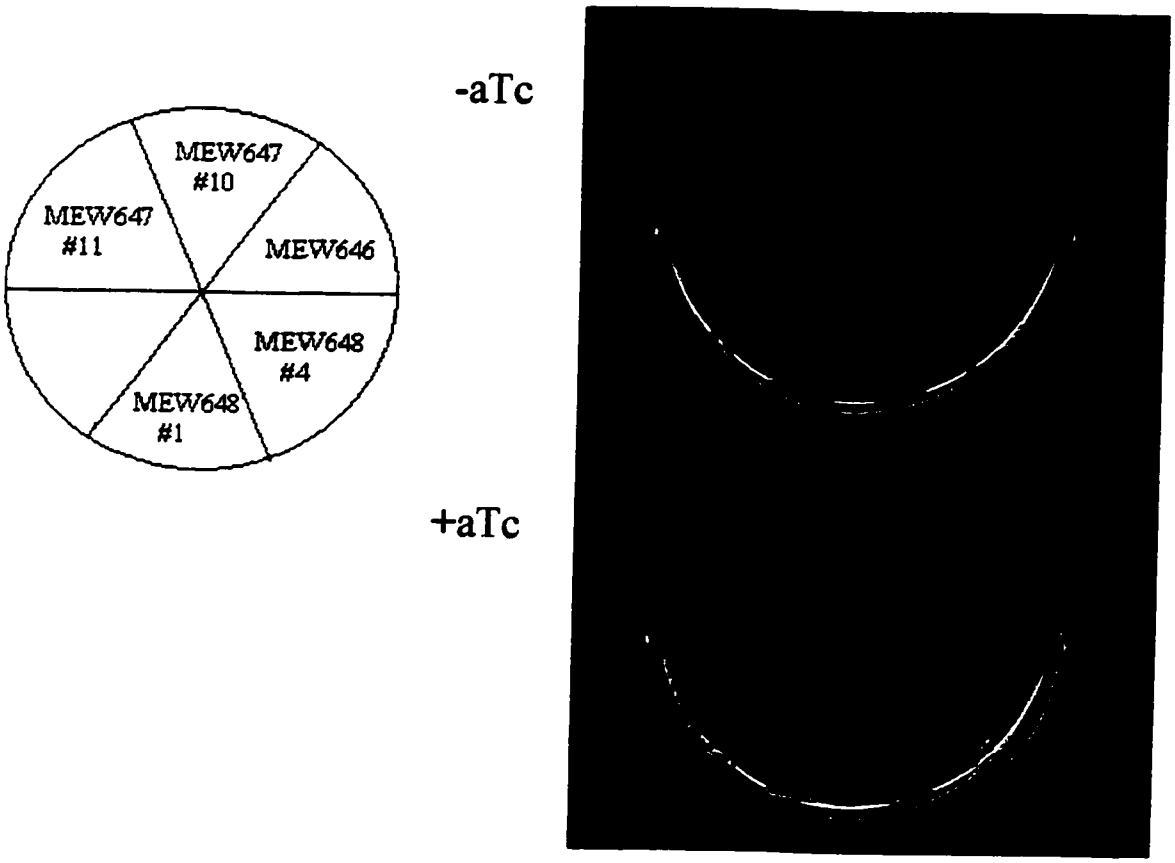


Figure 14. Demonstration of *metK* as an essential gene in LB.
Three types of isogenic *metK* deletion mutant stains: MEW646 ((Δ*metK* pLtet-K), MEW647 (Δ*metK tetR* pLtet-K), and MEW648 (Δ*metK tetR recA*⁻ pLtet-K) were streaked onto LB plates containing 25 μg/ml chloramphenicol with or without inducer anhydrotetracycline(aTc) 50 ng/ml as shown above. Strain MEW648 grew only in the presence of aTc.

Strain MEW648 (Δ*metK tetR recA*- pLtetK), with both *tetR*$^{+}$ and *recA*$^{-}$ backgrounds, showed absolute dependence on inducer aTc for cell growth. In the presence of aTc, plasmid *metK* expression was fully induced and cells grew normally (Fig.14, +aTc plate); but, in the absence of aTc, plasmid *metK* expression was almost completely shut off by the TetR repressor acting at the PLtet promoter, these cells were unable to grow, even with a longer incubation time (Fig.14 –aTc plate). This result clearly demonstrated that the ability of MEW648 to grow on LB medium plates corresponded well to the expression of *metK* induced by aTc.

Having established the aTc-dependent growth of mutant MEW648 on solid media, I also tested its growth in LB broth. Fig.15 shows the growth of mutant strain MEW648 in LB containing chloramphenicol without aTc, and that of mutant strain MEW646 in LB containing chloramphenicol. This graph indicates that strain MEW646, with TetR repressor free and plasmid *metK* gene always fully induced, exhibited the same growth pattern as that of wildtype *E. coli*. In contrast, mutant strain MEW648 was almost lack of growth without aTc, presumably because in the presence of the TetR repressor plasmid *metK* gene expression was repressed to an extremely low level. When inducer aTc was subsequently added to the culture of MEW648 at a final concentration of 50 ng/ml, cells were stimulated to grow and reached OD_{600} 1.3 within 5 hours. The growth rate was similar to that of strain MEW646. This clearly demonstrated that expression of the *metK* gene is absolutely required for the growth of *E. coli* cells in LB.

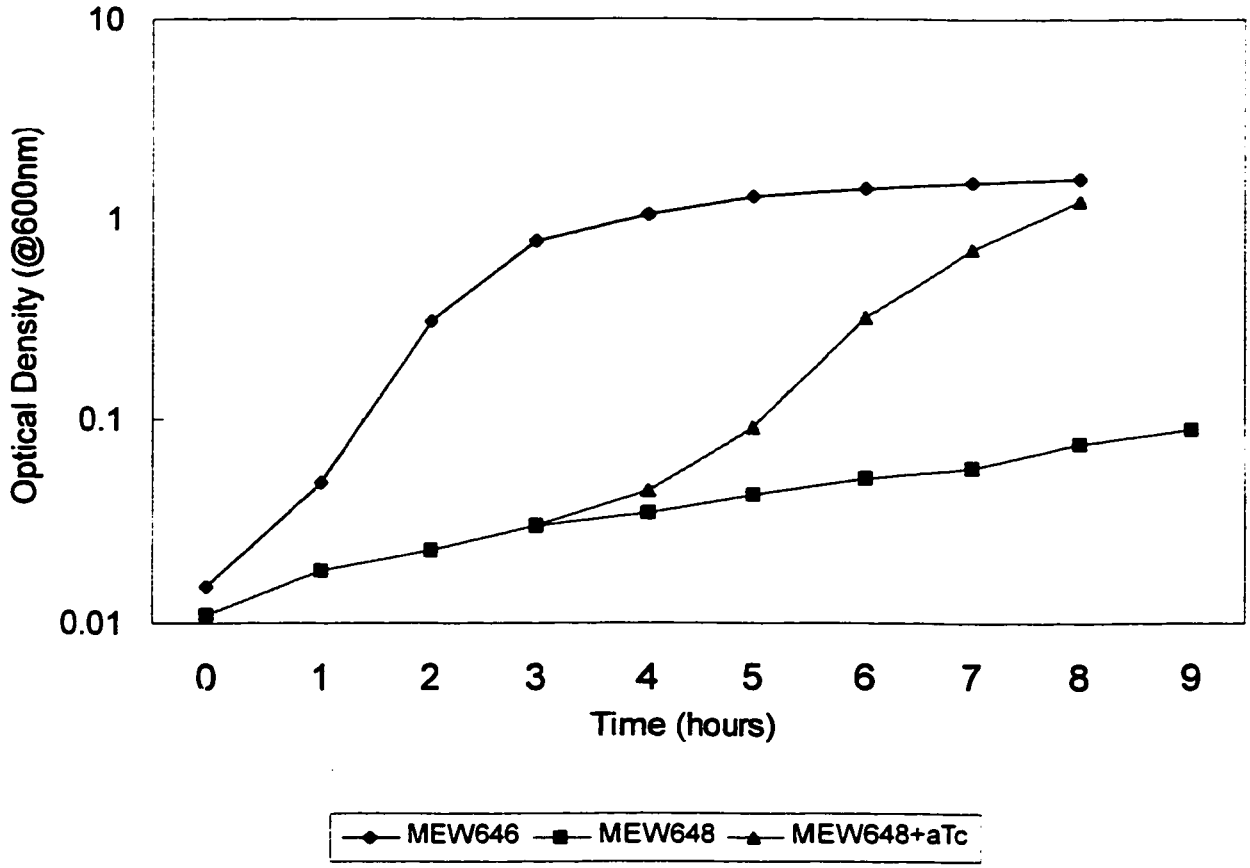


Figure 15. Growth of *metK* deletion strains MEW646 and MEW648 in the absence of anhydrotetracycline. The growth curves shown are for 100 ml shaking cultures of strain MEW646 (◆) or strain MEW648 (■) in LB with chloramphenicol but without inducer anhydrotetracycline(aTc). For strain MEW648, after 3 hours incubation, 20ml of the culture is transferred to another flask and aTc is added to a final concentration of 50ng/ml (▲) and the optical density is followed.

Prior to this experiment, 5 ml LB containing 25 μg/ml Chlramphenicol and 50 ng/ml aTc was inoculated with cells from a single colony of MEW646 or MEW648 (from LB-chloramphenicol-aTc50 ng/ml plates). And 1 ml of well-grown overnight cultures was centrifuged, washed 3 times and resuspended into 1 ml LB liquid. Subsequently 0.6 ml of the resuspension was used as inoculum for the 100 ml LB culture.

2.2. *metK* Expression and exogenous methionine are both needed for growth in glucose minimal media

The preceding experiment shows that SAM is required for growth in LB. It is also necessary, but not sufficient, for growth in glucose minimal medium.

In glucose minimal medium, mutant MEW648 was unable to grow whether inducer aTc was provided or not. Addition of 1% casamino acids to the glucose minimal medium allowed the same inducer-dependent growth as LB. Therefore, it could be inferred that some other amino acid(s) was(were) also needed to enable cell growth even with enough *metK* gene expression. Through adding methionine 50 μg/ml or leucine 100 μg/ml to glucose minimal medium individually and testing for growth in the presence or absence of aTc, I found methionine could support cell growth when inducer aTc was also present to turn on the plasmid *metK* gene expression, but leucine could not. This result indicated that the growth of *metK* deletion strain MEW648 in minimal media not only depended on the induction of plasmid *metK* gene expression, but also required methionine.

3. Effects of limited *metK* expression on cell growth, cell division and DNA methylation

By turning on and off plasmid *metK* expression in *metK* deletion strain MEW648, I had shown that the *metK* gene was essential to *E. coli*. To further explore the roles of *metK* gene expression in cell physiological and biochemical processes, I was interested in studying the effects of various levels of *metK* gene expression. Since the plasmid pLtetK had been shown to be well-regulated by inducer aTc, it was possible for me to vary plasmid *metK* expression in response to the level of aTc.

3.1. Plasmid *metK* expression in response to different concentrations of aTc

3.1.1. Construction of pLtetK::*lacZ* fusion

In order to quantitate the level of plasmid *metK* induction corresponding to different concentration of aTc added to the media, I constructed pLtetK::*lacZ* fusion (Fig.8). A promoterless *lacZ* gene missing its translation initiation site was fused in frame to the *metK*-coding region, 34 codons downstream of the *metK* ATG on plasmid pLtetK. In this construct, as the *metK*::lacZ fusion was under the control of the PLtet promoter and translated from the *metK* translation start codon, I could estimate the extent of plasmid *metK* induction simply by measuring β-galactosidase activity.

3.1.2. Induction of pLtetK::*lacZ* in response to aTc

The fusion plasmid pLtetK::*lacZ* was transformed into *metK*$^+$*tetR*$^+$ strain DH5αZ1, and β-galactosidase activitities were measured in LB culture or in glucose minimal media containing chloramphenicol 25μg/ml and various concentrations of aTc. The Results are shown in Table 6 and Figure 16.

In LB culture, without inducer aTc or with 1ng/ml aTc, plasmid *metK* expression from PLtet promoter was efficiently shut off. The β-galactosidase activities were insignificant, less than 5 Miller Units. With 5 ng/ml and 10 ng/ml of aTcs slight induction (about 1.5-to 5-fold) was apparent. When the concentration of aTc increased to 20 ng/ml, the induction of pLtetK::*lacZ* increased substantially, with the β-gal units elevated to 20 fold higher than the basal level. At 30 ng/ml of aTc maximum induction was seen with no further effect on adding up to 100 ng/ml.

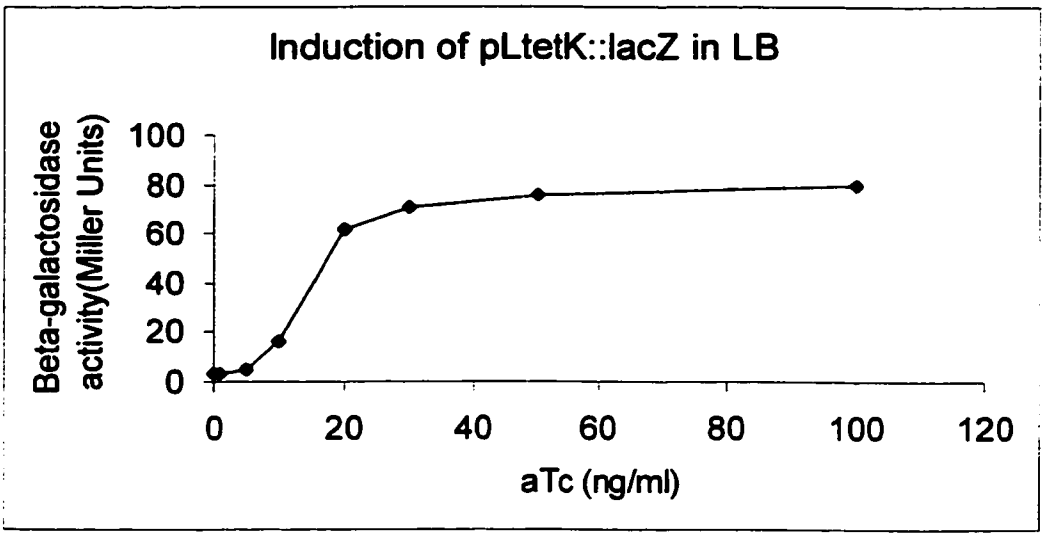
Table 6. Induction of pLtetK::*lacZ* fusion in response to aTc concentrations.

aTc(ng/ml)	β-galactosidase activity (Miller Units)	
	LB	Glu MM+1%CAA
0	<5	<5
1	<5	<5
5	5	5
10	15	10
20	60	80
25	ND[a]	115
30	70	135
50	75	145
100	80	160

β-galactosidase activities produced from *lacZ* fused into the *metK* coding region on plasmid pLtet-K were measured in strain DH5αZ1($metK^{+}tetR^{+}$) transformed with plasmid pLtetK::*lacZ* . Cultures were grown in LB or Glucose minimal medium with 1% casamino acids (CAA). Each contained 25 μg/ml chloramphenicol and various concentrations of aTc from 0 to 100 ng/ml. The results given are the average of the data from three determinations and the β-galactosidase units are expressed to the nearest 5 units.

a: not determined

A.



B.

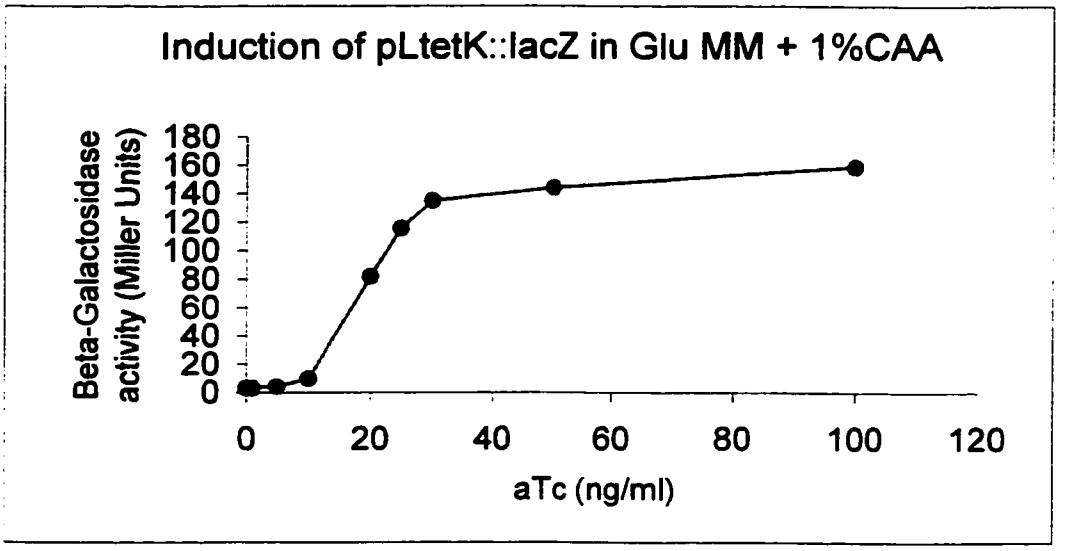


Figure 16. Inductions of the pLtetK::*lacZ* fusion in response to various concentrations of aTc in two different growth conditions. These are graphs of the data presented in Table 6. The β-galactosidase activities were measured from pLtetK::*lacZ* fusion in strain DH5αZ1 transformed with this plasmid. Abbreviations: Glu MM: glucose minimal medium; CAA: casamino acids; aTc: anhydrotetracycline

In glucose minimal medium, 1% casamino acids were added since DH5αZ1 could not grow. The correlation between induction of *lacZ* expression and varied concentrations of aTc was similar to that in LB, but at all levels tested above 20 ng/ml, the induction was about 2 fold higher than that in LB; and the induction could be regulated within a 50-fold range.

Based on all of the above results, I was able to estimate the regulatory range of plasmid *metK* expression, which allowed me to precisely control plasmid *metK* expression and investigate the influences of limited *metK* expression.

3.2. Effects of limited *metK* expression on cell growth and cell division

Previous study of the *metK* mutant (*metK*84) in our lab found that this mutant produced a markedly reduced level of SAM synthetase; it grew poorly on glucose minimal medium plates and exhibited filamentation when starved for leucine. This suggested that lack of SAM resulted in a defect in cell division (Newman *et al.*, 1998). In the *metK* deletion strain, the level of SAM synthetase was solely determined by plasmid *metK* expression. By regulating plasmid *metK* expression, it was possible to vary the intracellular SAM pool to a certain extent. If SAM influenced cell division as suggested, reducing plasmid *metK* expression would be expected to interfere with cell division.

To examine this, I streaked the *metK* deletion strain MEW648 onto glucose minimal medium plates, containing 50 μg/ml methionine, chloramphenicol and various concentrations of aTc (0, 5,10, 20, 30, 50, 100 ng/ml), and tested the relationship between plasmid *metK* expression and cell division.

It was observed that without inducer aTc, mutant cells did not grow even after 3 days. With 5 ng/ml of aTc, cells could grow a bit, but slowly and poorly; no single colony was visible even in 2 days. Examined under the microscope, the cells were present as bunches of long filaments, 20-25 times of the normal *E.coli* cell length and few short single cells were seen (Fig.17 aTc 5ng/ml). Adding 10 ng/ml of aTc helped cells to grow better. Small single colonies were visible as were medium-sized or short filaments (Fig.17 aTc 10 ng/ml). Using 20 ng/ml of inducer aTc allowed cells to grow normally on the plate; the majority of the cells seen were normal *E. coli* cells, though a few short or medium length filaments could also be seen (Fig.17 aTc 20 ng/ml). With still higher concentrations of aTc (30,50 and 100 ng/ml), cells grew normally and gave good single colony isolations after 18 hours incubation. They all appeared as short single *E. coli* rods and no filaments were observed (data not shown).

This result indicated that cell growth and cell division correlated with *metK* gene expression. Lower *metK* expression, which resulted in reducing the intracellular SAM level, slowed cell growth and caused a defect in cell divison.

The effects of varied *metK* gene expression on cell growth and cell division were also tested in LB. Overnight cultures of strain MEW648 in LB containing chloramphenicol and 50 ng/ml of aTc were washed, resuspended and serially diluted. Cells (10^4) were then plated onto LB chloramphenicol plates with aTc added at varied concentrations: 0, 5,10, 20, 50, 100 ng/ml and grown at 37^0C overnight. The results confirmed that without inducer added to turn on plasmid *metK* expression, *E. coli* cells were unable to grow. With the concentrations of inducer aTc ranging from 5 to 100 ng/ml, the number of

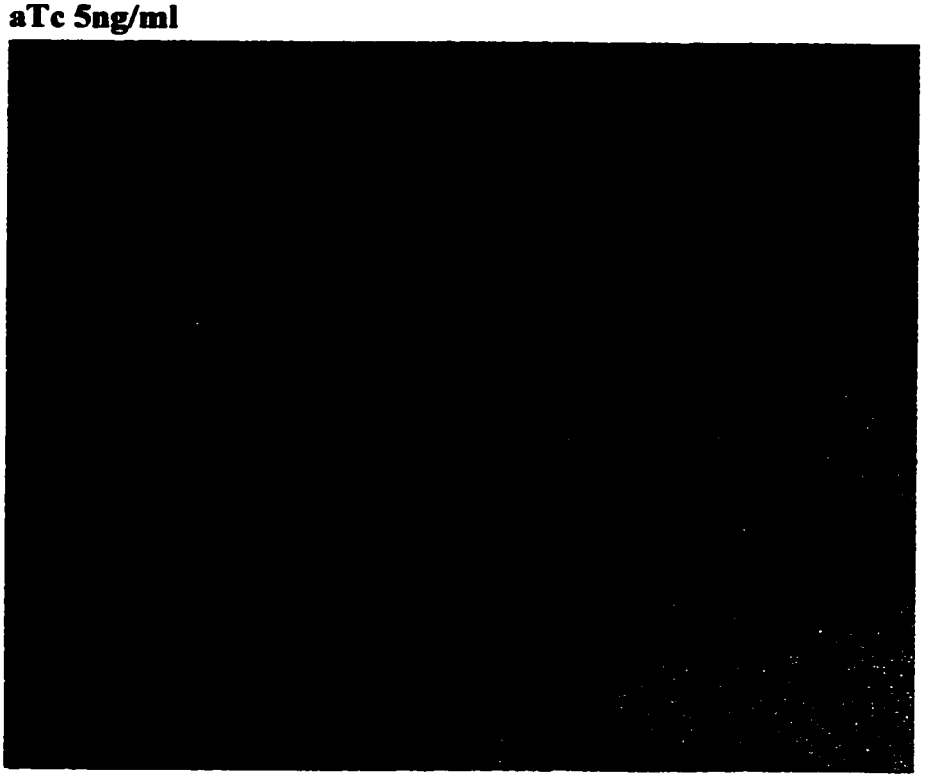
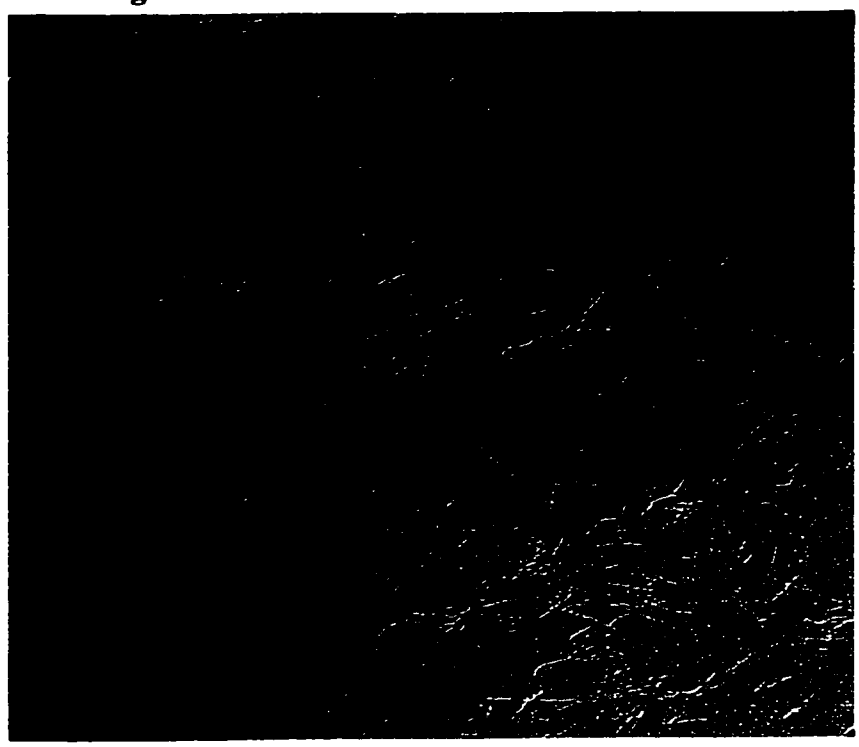


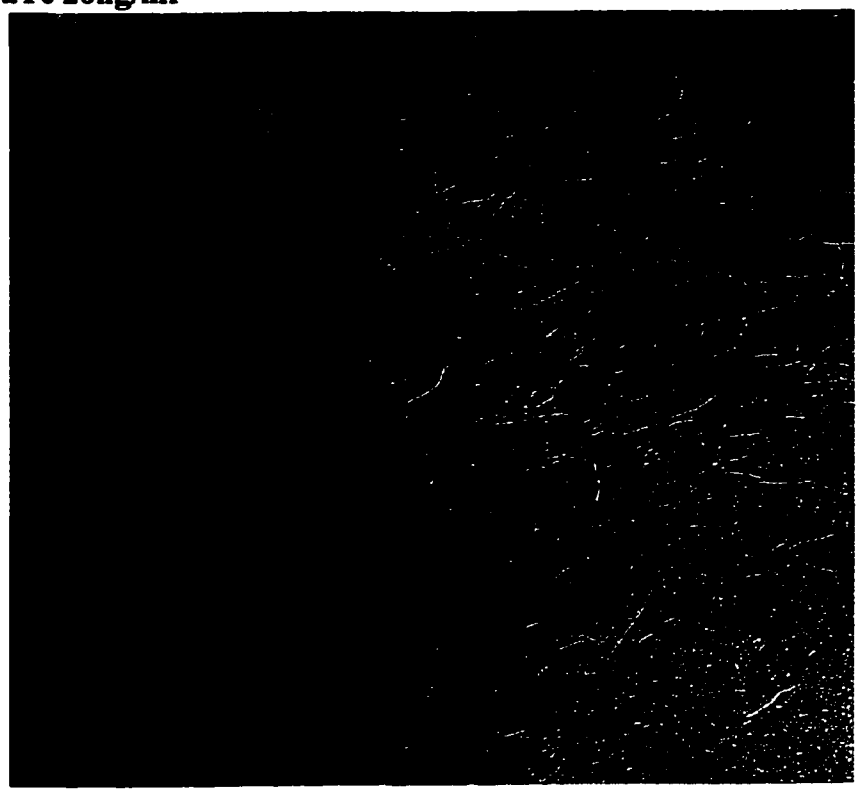
Figure 17. Effects of limited *metK* expression on cell division.

Photomicrographs of *metK* deletion mutant cells grown under limited *metK* expression conditions. The *metK* deletion mutant MEW648 was streaked onto glucose minimal medium plates containing methionine 50 μg/ml, chloramphenicol 25 μg/ml and various concentrations of aTc, and incubated at 37°C for two days. Cells were immobilized on object slides and photographed on a Zeiss Axiophot microscrope with DIC optics. The total magnification is 400X and the filter is 3200/5500 K conversion filter. Images are captured using Kodak MDS 120 Microscopy Documentation System and converted from KDC to TIF.

aTc 10ng/ml



aTc 20ng/ml



colonies varied little but the size of each colony increased proportionally, from needle-like, tiny, small to regular size (data not shown). Examining the colonies under the microscope, I did not observe filaments. Even at 5 ng/ml of aTc, only a few short filaments were seen.

3.3. Effects of limited *metK* expression on genomic DNA methylation

E. coli DNA is normally methylated at GATC sequences by Dam methyltransferase, and methylated at CCA/TGG sites by Dcm methyltransferase. Both DNA methyltransferases transfer the methyl group from S-adenosylmethionine to their specific residues in double stranded DNA. It had been shown that induction of bacteriophage T3 SAM hydrolase (T3SH) decreased genomic DNA methylation by Dam methyltransferase (Posnick and Samson, 1999) and also reduced Dcm methylation of genomic DNA (MacIntyre *et al.*, 2001). I, therefore, wished to test how DNA methylation would be affected when I varied plasmid *metK* induction to modulate the intracellular SAM pool level.

To test this, the *metK* deletion strain MEW648 was grown at 37^0C overnight in LB chloramphenicol broth with various concentrations of aTc (10, 20, 50, 100 ng/ml), chromosomal DNA was isolated and analysed for Dam and Dcm methylation.

To examine Dam methylation, chromosomal DNA was digested with restriction enzymes that cut at GATC sequences in a methylation-dependent fashion. Results are shown in Fig.18. Chromosomal DNA isolated from cells grown at a low concentration of aTc (10 ng/ml) was extensively digested by enzyme *Mbo*I, which cuts only unmethylated

methylated GATC (lane 7). In contrast, chromosomal DNA from cultures with 20, 50 or 100 ng/ml of aTc, where plasmid *metK* expression was greatly induced, was insensitive to *Mbo*I digestion (lanes 3-5), but was cleaved extensively by *Dpn*I (lanes 8-10).

To examine Dcm methylation, chromosomal DNA was subjected to *Mva*I digestion or *Eco*RII digestion. *Mva*I cuts DNA at CCA/TGG sites irrespective of Dcm methylation while *Eco*RII cleaves only unmethylated Dcm sites. It was observed that the entire chromosomal DNA was equally cut by *Mva*I (Fig.19 lanes 2-5), whereas only the chromosomal DNA extracted at the lower level of aTc (10 ng/ml) could be cleaved by *Eco*RII (Fig.19 lane 7).

Together, these results indicated that at low *metK* expression (aTc 10ng/ml), the chromosomal DNA was in its hypomethylated status, with a majority of the GATC sites unmethylated. With an increase of *metK* expression, the majority of the GATC sites on the chromosomal DNA were restored to the methylated state. Therefore, it could be concluded that the extent to which DNA was methylated depended on the extent to which *metK* was expressed. Lower *metK* expression decreased the Dam or Dcm methylation of chromosomal DNA.

4. Attempts to screen for SAM-using mutants

With the *metK* deletion strain, plasmid pLtet-K could not be cured, which indicated that *metK* gene expression was critical to the *E. coli* cell. Although I could well control plasmid *metK* expression by varying the concentration of inducer in the medium, the modulation of intracellular SAM level was still indirect. The intent of this experiment was to search for a SAM-using mutant, which could use SAM provided from outside, so

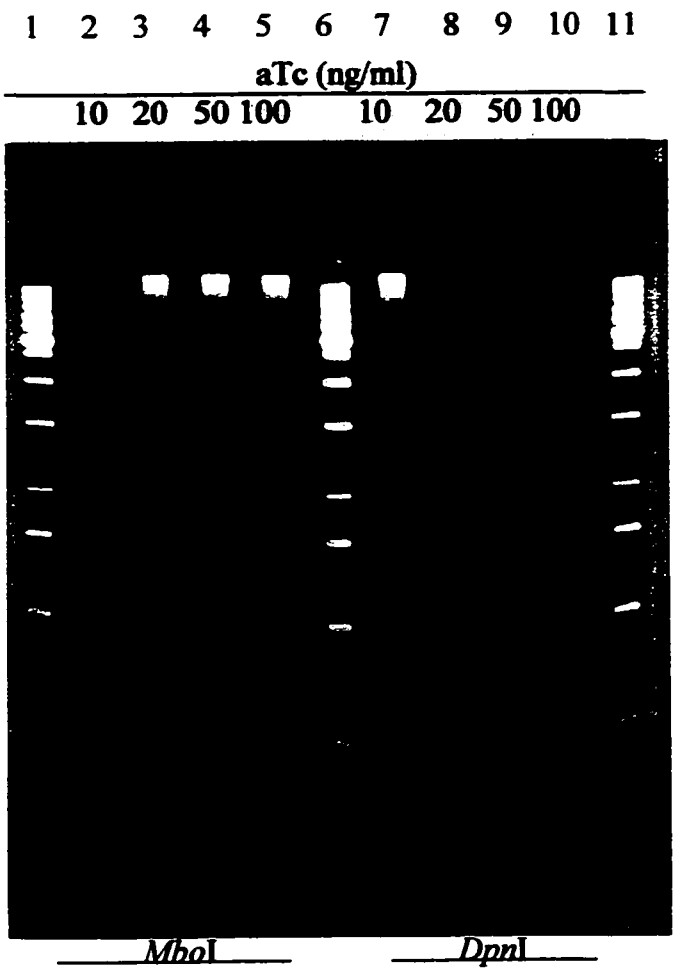


Figure 18. Effects of varied plasmid *metK* expression on Dam methylation.
The *metK* deletion strain MEW648 (Δ*metK tetR recA*⁻ pLtetK) was grown overnight in LB containing 25 μg/ml chloramphenicol and different concentrations of aTc. Genomic DNA was isolated and digested with restriction enzymes *Mbo*I or *Dpn*I.

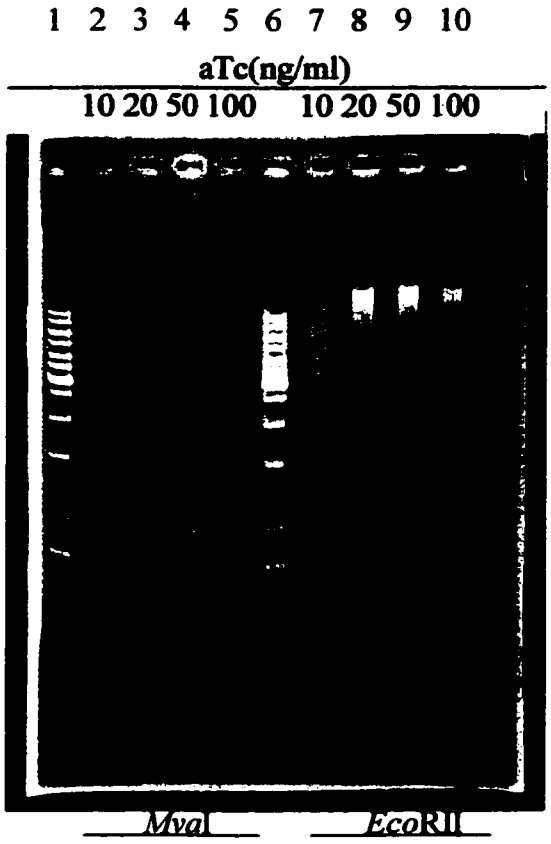


Figure 19. Effect of varied plasmid *metK* expression on Dcm methylation.

The *metK* deletion strain MEW648 (Δ*metK tetR recA*⁻ pLtetK) was grown overnight in LB containing 25 μg/ml chloramphenicol and different concentrations of aTc. Genomic DNA were isolated and digested with restriction enzymes *Mva*I or *Eco*RII.

that we could study the SAM-dependent effects (cell division, DNA methylation *etc.*) directly.

To establish this, SAM was first purified from commercial SAMe tablets through a series of chemical precipitation steps. Purified SAM was then added to LB agar plates at final concentration of 40 μg/ml.

Well-grown, single colonies of strain MEW648 (Δ*metK tetR recA*⁻ pLtet-K) from LB-Cm-aTc 50 ng/ml plates were individually resuspended into LB liquid, plated on LB SAM 40 μg/ml plates, and incubated at 37^0C until colonies could be seen (usually 4-5 days). I isolated 24 such colonies. These were further tested on five kinds of selective plates (Glu MM, Glu MM+Met, LB, LB-SAM 40μg/ml and LB-Cm-aTc50ng/ml), 14 of them could grow on all plates; 8 could grow on LB plates but not on Glu MM; and the other 2 did not grow. Based upon this result, I concluded that none of the colonies was a real SAM-using mutant as I wanted, i.e., one which could grow on SAM plate or SAM plate and aTc plate, but should not grow on all of the other plates where no SAM and inducer aTc had been provided. The attempt to isolate a SAM-using mutant did not succeed.

PART B. Characterization of the *metK*84 mutation

*metK*84 (CU1008 Δlac *metK*84) is an unsupressed, leucine-requiring and GGME-resistant *metK* mutant strain. It has markedly reduced SAM synthetase activity and exhibits a complex phenotype when grown in glucose minimal medium (Newman *et al.*, 1998; Budman, 1998). Which includes: (1) growth at a normal rate only with supplementation with high concentrations of leucine; (2) cells grown at lower concentrations of leucine are hindered in cell division and produce long filaments; (3) This filamentation can be suppressed by a plasmid-carried *metK* gene or by Lrp deficiency. Based on these results, the *metK*84 mutation has been suggested to be either in the regulatory region or in the coding region of the *metK* gene. In order to define where the *metK84* mutation is and what kind of mutation it is, I started by sequencing this *metK*84 mutant.

1. Sequencing and identifying the mutation of *metK*84 mutant

1.1. Using the real *metK*84 mutant for sequencing

As the *metK*84 mutant strain accumulates suppressor mutations easily in the lrp+ background, it was important to assure that the right strain was used for sequencing. Therefore, mutant strain *metK*84 was always streaked for use from the –80°C glycerol stock vial, tested for GGME-resistance and leucine-starved filamentation to confirm it was the correct, unsuppressed *metK*84.

1.2. Using PCR-amplified template DNA for sequencing

To sequence, I first isolated genomic DNA of *metK*84 from glucose minimal medium culture supplemented with a high concentration of leucine, amplified it with Taq polymerase and two gene specific primers U1 and D2, which hybridized with the upstream and downstream regions of *metK*. The 3.2-kb PCR-amplified fragment was used as template for DNA sequencing.

1.3. Identification of the mutation of *metK*84 mutant

The above 3.2-kb PCR fragment was sequenced from the 5' to 3' direction with 8 nested gene specific primers (Table 4) using a primer-walking strategy. To avoid the random mistakes generated by Taq polymerase, DNA sequencing was performed at least twice with three different PCR amplifications. Totally 2241 nucleotides were sequenced, which covered the whole *metK* coding region, 897 bp upstream and 189 bp downstream of the *metK* gene. After comparing the data with the *metK* sequence of the *E. coli* genome (http:://genolist.pasteur.fr/Colibri/genome.cgi), I noted one distinct difference, which is a single basepair change from "A" to "G", located 150 basepairs upstream of the ATG translation start codon of *metK* gene. This change appeared consistently in all the DNA sequencings. However, there was also a questionable part (+370 bp--+373 bp) within the *metK* coding sequence, which sometimes was the same as the standard sequence, and sometimes varied by one base. Except for the above two areas, no other differences were found. To double-check those two specific regions, I designed two new primers and sequenced from the 3' to 5' direction. Finally, this confirmed the "A" to "G" single basepair change in the upstream region as true, and showed that the questionable part in

the coding region was still the same as the standard sequence. Therefore, I could conclude that the mutation in the *metK*84 mutant was an A→G transition mutation at the 150^{th} position upstream of *metK* ATG translation start codon.

2. Characterization of the *metK*84 mutation

Though just a single base pair change in the upstream region, the *metK*84 mutant strain had shown remarkably decreased SAM synthetase activity. Therefore, it was rational to infer that this point mutation must be located in a very important regulatory region of *metK*. Little work had been done on the molecular mechanism of *metK* gene regulation, but a computer homology search suggested the postulated –10 and –35 sequences, TAAAAT at –151 bp to -146 bp and TGGAAA at –175 bp to –170 bp upstream of the *metK* ATG (Greene, 1996). If this were true, then the point mutation found in *metK*84 would be located in the –10 region. To investigate whether this hypothesis was true and to initiate study of the *metK* promoter, I set out to map the transcriptional start site of the *metK* gene.

2.1. Mapping of 5'-end transcription initiation sites of wildtype *metK* promoter and *metK*84 promoter

To identify the transcription start site, primer extension analysis was performed using multicopy plasmid pZAP-K31 or pZAP-K18 (Table 3). The pZAP-K31 clone had the wildtype *metK* promoter, the whole *metK* gene and its downstream region, while the pZAP-K18 clone carried the *metK*84-mutated promoter and the intact *metK* gene. Host strain MEW1 containing *metK* clone and MEW1 containing *metK*84 clone were grown in

glucose minimal medium and LB medium, respectively. RNAs from different growth conditions were extracted, except that MEW1 with *metK* clone could not grow in glucose minimal medium due to the toxicity of overexpression of *metK*. For the primer extension reaction, a 5'-end Cy5 labelled gene specific primer In7 was used to hybridize nucleotides +33 to +11 within the *metK* gene, and extended by reverse transcriptase. The extension products were sent to Dr. Z. Shao for polyacrylamide gel electrophoresis and computer program analysis, in parallel with the products of sequencing reactions run with the same In7 primer and clone pZAP-K31 or pZAP-K18 as template.

The extension product from wildtype *metK* promoter (MEW1 pZAP-K31) in LB culture revealed a single putative start point (Fig. 20 B). This indicated the 5'-end of the *metK* mRNA was thus located at 140 bp upstream of the *metK* ATG start codon. Proceeding from this transcription initiation site, positioned as +1 in Fig. 20 A, I found a promoter-like sequence that conformed relatively well to the consensus for *E. coli* σ^{70} RNA polymerase. In this sequence, an AT-rich sequence (TAAAAT) located from –11 to –6 relative to the transcription initiation site (+1T) matched the consensus *E. coli* –10 hexamer (TATAAT) at five of six nucleotides, which suggested it functioned as the –10 region for *metK* promoter. At 18 bp upstream of this –10 region, a hexamer TGGAAA matched the consensus *E. coli* –35 hexamer (TTGACA) at four of six positions, which was assigned as the –35 region of the *metK* promoter.

As the region from 151 bp to 146 bp upstream of the *metK* ATG codon had been defined as the –10 region of the *metK* promoter, the *metK*84 mutation identified above was actually a –10 region point mutation of *metK*, in which the adenine at position 2 of the –10 hexamer (TAAAAT) had been converted to guanine. This change must have

Figure 20. Promoter sequence and primer extension analysis of *metK* gene.

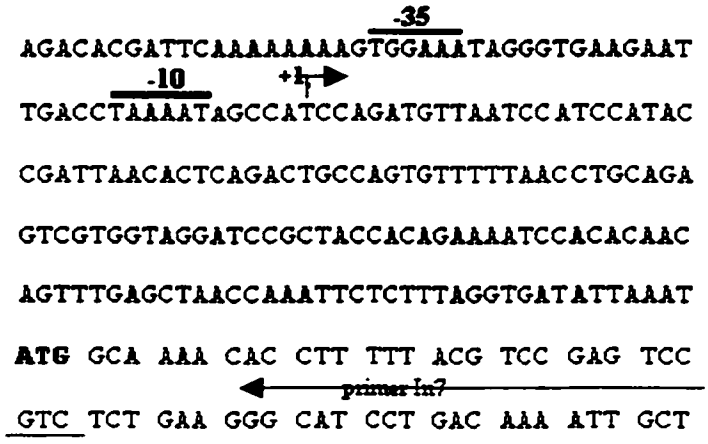
(A) The sequence containing the wildtype *metK* promoter is presented and numbered relative to the 5'end of the *metK* gene. The –10 and –35 consensus sequences for RNA polymerase binding were indicated by black bars. The 5'end of the RNA transcript is denoted by the arrowhead labelled as +1. The translation start codon is shown in boldface.

(B) The primer –extended product using total RNA isolated from strain MEW1/pZAP-K31 (carrying wildtype *metK* upstream) grown in LB ampicillin culture was electrophoresed with sequencing reaction products generated by using the same primer and plasmid templates pZAP-K31 or pZAP-K18. A portion of the deduced nucleotide sequence is shown.

Panel I is the sequence from plasmid template pZAP-K18, which carried the *metK84* mutated upstream, with a point mutation in the –10 region. Panel II is the sequence from plasmid template pZAP-K31, which carried the wildtype *metK* upstream. The difference between the two sequences is marked by a rectangle.

Panel III showed the primer extension product of wildtype *metK*. The transcription start site is labelled by a circle.

A)



B)

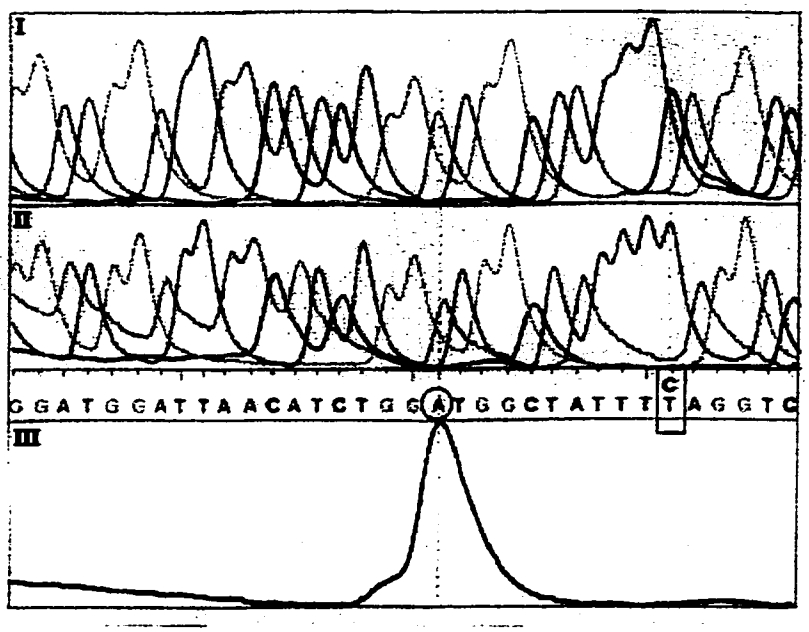


Figure 20. Promoter sequence and primer extension analysis of the *metK* gene.

reduced the transcription activity of the *metK* promoter, thus, resulting in the marked reduction of SAM synthetase activity.

No transcripts from the *metK*84 mutant promoter (MEW1 pZAP-K18) had been detected, no matter whether the cells were grown in LB or in glucose minimal medium. I repeated the primer extension analyses with different primers and/or increasing the amount of total cellular RNA, but have not found any detectable extension products yet. These results might reflect the fact that the level of transcription from the *metK*84 mutant promoter decreased so much, so as not to be detectable under the present experimental conditions.

2.2. The expression and regulation of the *metK*84 promoter

I had demonstrated that the *metK*84 mutation was actually a point mutation at the –10 region of the *metK* promoter. When the promoter –10 region was altered, it was very possible that the transcription of *metK* would be affected. However, study of the *metK*84 mutant also showed that high concentrations of leucine or Lrp deficiency could help to increase the SAM synthetase activity in glucose minimal medium, which suggested that Lrp and /or leucine might be involved in the regulation of *metK* gene expression. In order to investigate the effect of this mutation on transcription of the *metK* gene and the influence of leucine/Lrp on this mutant promoter, I tried the following experiments.

2.2.1. Construction of *metK*::*lacZ* transcription fusions carrying the wildtype *metK* promoter or the *metK*84 mutant promoter

In order to test the effect of the *metK*84 mutation on the transcription activity of *metK*, I constructed two transcription fusions between the *metK* promoter and *E. coli lacZ* or between the *metK84* promoter and *lacZ*. A DNA fragment containing the 504 bp upstream and the first 45 bp of the *metK* coding region was generated by PCR and ligated into the operon fusion plasmid pRS415, which carried a promoterless *lac* operon. The pZAP-K31 clone, carrying the wildtype *metK* upstream region and the pZAP-K18 carrying the mutant *metK*84 upstream region, were used for PCR to obtain the two promoters and each was fused to *lacZ*.

2.2.2. Evaluations of *metK::lacZ* and *metK84::lacZ* fusions

To evaluate the level of expression from wildtype *metK* and *metK*84, β-galactosidase activities expressed from the *metK*::lacZ fusion and from the *metK*84::lacZ fusion were measured in different host strain backgrounds and in various growth conditions. The results are illustrated in Table 7.

In glucose minimal medium, the β-galactosidase activity expressed from the *metK*84 mutant promoter was less than 10% of the activity from wildtype *metK* promoter in wildtype host strain background (Row 2). This suggested that the A→G transition mutation in the −10 region markedly reduced the *metK* transcription activity. It was consistent with results of SAM synthetase activity, which had been found remarkably decreased in *metK*84 mutant.

Adding 100 μg/ml of leucine helped to increase the β-galactosidase activity expressed from the *metK*84 promoter to a relatively small extent (about 2.5 fold). While leucine had even less effect on the wildtype *metK* promoter. When Lrp was absent (in lrp^- strain), the *lacZ* expression from the *metK*84 promoter increased to 6 fold higher than that seen in the presence of Lrp. These results reflected that Lrp repressed the *metK*84 promoter, and that a high concentration of leucine could release this repression at a certain point.

In LB culture, the β-galactosidase activity expressed from the mutant promoter decreased 3.3 fold in comparison with the activity expressed from wildtype *metK* promoter (Row 1).

Unfortunately, DNA sequencing of *metK*84::*lacZ* fusion found a second point mutation introduced by one of the several PCR amplification steps. It was positioned at

Table 7. Expressions of *metK*::*lacZ* and *metK*84::*lacZ* operon fusions

	β-Galactosidase Activity (Miller Units)		
	lrp$^+$ (MEW1)		lrp$^-$ (CT4A)
Growth Conditions	*metK*::*lacZ*	*metK*84::*lacZ*	*metK84*::*lacZ*
LB	11795	3560	ND[a]
Glu MM	17125	1435	8520
GluMM+Leu100	20080	3285	ND[a]

β-galactosidase activities produced from the *metK*::*lacZ* operon fusions were assayed in lrp$^+$ host strain transformed with multicopy plasmid pRS-K. And β-galactosidase specific activities expressed from the *metK84*::*lacZ* operon fusion were measured in lrp$^+$ and lrp$^-$ host strains transformed with multicopy plasmid pRS-K84. For measurements, the cultures were grown in different growth conditions as mentioned aboved. β-galactosidase units (Miller Units) were the average of three or more independent experiments and are expressed to the nearest 5 units.

Abbreviation: Glu MM: glucose minimal medium; Leu100:L-leucine 100 μg/ml.

a: not determined.

52 bp upstream of the *metK* ATG. However, searching through the region around this point did not show any important binding sites. Therefore, I thought the above β-galactosidase results should mainly reflect the influence resulted from the mutation at –10 region of the *metK* promoter.

Discussion

The work in this thesis is directed to an understanding of the roles of S-adenosyl-methionine (SAM) in *Escherichia coli* K-12. It has been devoted to answering two basic questions: (1) Is the *metK* gene, which encodes SAM synthetase, an essential gene in *E. coli* K-12? (2) Where is the mutation in the *metK*84 mutant?

I used gene replacement to demonstrate here that expression of the *metK* gene was absolutely required for the growth of *E. coli* K-12, no matter whether in rich medium (LB) or in minimal medium. I also found that the *metK*84 mutation is a point mutation in the –10 region of the *metK* promoter. In this discussion, I will first review the evidence for concluding that *metK* gene expression is essential for the growth of *E. coli*. Secondly, I will suggest an explanation for the fact that the *metK* deletion strain requires methionine. Thirdly, I will discuss the role of SAM in *E. coli* cell division. Finally, I will discuss the characterization of the *metK84* mutation and the possible role of the Leucine/Lrp regulon in the regulation of *metK* expression.

1. The *metK* Gene, which encodes S-adenosylmethionine synthetase, is an essential gene in *Escherichia coli* K-12.

1.1. Indirect and direct proofs for concluding *metK* to be an essential gene in *E. coli*

To conclude that the *metK* gene is an essential gene in *E. coli* K-12, several indirect proofs could be found by reviewing the process of construction of the chromosomal *metK* deletion mutants.

Using wildtype *E. coli* strain (MEW1) as the starting strain, I attempted but failed to substitute the chromosomal wildtype *metK* gene with the deleted and inactive version of *metK*. However, the same substitution was accomplished when a rescue plasmid (pBAD22*metK*) carrying a functional copy of *metK* gene under the arabinose-induced promoter was provided and plasmid *metK* expression was turned on in the presence of arabinose. This rescue plasmid (pBAD22*metK*) could not be cured from the *metK* deletion mutant. It could only be replaced by another type of rescue plasmid (carrying a different origin of replication and different antibiotic resistance gene) that also held a functional copy of the *metK* gene (pLtetK). All of these facts imply that *metK* gene expression is essential to *E. coli* K-12.

Direct proofs came from a series of growth ability tests of the *metK* deletion mutant strains. In strain MEW648 (Δ*metK tetR recA*- pLtetK), the chromosomal *metK* gene is non- functional due to a large deletion, a functional *metK* gene is provided on a plasmid under the control of the PLtet promoter, the *tetR* repressor gene prevents expression of that gene in the absence of inducer and recombination onto the chromosome is prevented by the *recA* mutation. Growth of that strain was shown to be absolutely dependent on the presence of inducer aTc to turn on plasmid *metK* expression, both in rich and minimal media. This was also observed in an isogenic strain MEW647 (Δ*metK tetR* pLtetK) that had the functional RecA system. Because recombination was possible, it was frequently overgrown by strains with a functional chromosomal *metK* gene. Strain MEW646 appeared to be independent of the inducer aTc. However, this was due to expression of the plasmid *metK* gene in the absence of TetR repressor. All of these results clearly demonstrate that *metK* gene expression is essential to *E. coli* cell growth.

The pair of primers Del1 and Del2 were designed to generate an in frame deletion of *metK*, in which 681 bp of the internal region of *metK* was supposed to be deleted. But later sequencing found this deletion was 683 bp, due to the 3'-5'exonuclease activity of Pfu polymerase which was used for amplification in the construction of the *metK* deletion (Fig.5). Thus the deletion of *metK* became an out-of-frame deletion. It was good that the deleted *metK* allele totally lost its function but it also could cause a polar effect that might affect the upstream (*speA*) or downstream (*galP*) gene expression. However altered *speA* or *galP* expression cannot be causing the growth problems seen here, because the rescue plasmid contained only the functional copy of the *metK* gene, and expression from the rescue plasmid restored the growth of the *metK* deletion mutants. This in turn reflected that the *metK* gene was the real essential gene for the growth of *E. coli* cell.

Based on all of these direct and indirect proofs, I conclude that the *metK* gene of *E. coli* K-12 is essential for its growth.

1.2. The commonality of essential genes

Many genes have been determined to be essential genes by using gene replacement methods. Brown *et al.* (1995) used the same gene replacement vector pKO3 to test the essentiality of the *murA*(*murZ*) gene in *E. coli*. They could not delete the gene in the wildtype background but succeeded in deletion by using a wildtype strain carrying a plasmid on which a functional copy of the *murA*(*murZ*) gene was cloned under the control of the P_{bad} promoter. As in my study, growth of the mutants depended on adding the inducer arabinose to turn on plasmid gene expression. They, therefore, concluded that the gene was essential in *E. coli* (Brown *et al.*, 1995). In gram-positive bacteria, a similar

example could be found. Wada and Watanabe (1998) used gene replacement to study the essentiality of *pbpA* gene, which codes penicillin-binding protein 1 in *Staphylococcus aureus*. They found the *pbpA* gene could only be disrupted in the presence of a plasmid carrying the functional *pbpA* gene, and observed the retention of this rescue plasmid in the chromosomal p*bpA* disruptant. Based on these observations, they concluded that the *pbpA* was essential for the growth of *S.aureus* (Wada and Watanabe, 1998). Pan *et al.* (2001) demonstrated that the *glf* gene, coding UDP-galactopyranose mutase in *Mycobacterium* could only be knocked out by allelic replacement in the presence of appropriate rescue plasmids and the loss of rescue plasmids correlated with the loss of the ability of the bacterium to grow. They thus concluded the enzyme was necessary for the growth of Mycobacteria (Pan *et al.*, 2001). All of these samples are based on the principle that if a gene is essential for the growth of the bacterium, it could not be disrupted or inactivated, unless a rescue plasmid carrying a functional copy of that gene is provided, and the growth ability of the bacterium then relies on the expression from the rescue plasmid.

1.3. *metK* Is the only gene coding for SAM synthetase in *E. coli*

When the inducer aTc was not present and plasmid *metK* expression was almost shut off, the *metK* deletion mutant MEW648 (Δ*metK tetR recA*- pLtetK) was unable to grow in both rich medium (LB) and in minimal medium supplemented with methionine. This clearly illustrated that *metK* was the only gene in *E. coli* that codes for SAM synthetase, as well as being an essential gene. It excluded the hypothesis that a second gene encoding SAM synthetase is expressed in complex medium (Satishchandran *et al.*, 1990) and is

consistent with the Blast search result in which no *metK* homologue had been found on the *E. coli* genome (Newman *et al.*, 1998).

2. Methionine auxotrophy of deletion mutant MEW648

In glucose minimal medium, the *metK* deletion mutant MEW648 required both inducer aTc and exogenous methionine for growth. The inducer aTc was needed to turn on plasmid *metK* expression, because *metK* gene expression is essential for *E. coli* growth, while the reason for the methionine requirement is not so obvious. This requirement might be attributed to overproduction of SAM synthetase. When inducer aTc was added at 50 ng/ml, *metK* expression from plasmid pLtetK was fully induced. pLtetK is a relatively low copy plasmid, its 15-20 copies result in a much higher level of *metK* coding DNA than is seen in the wildtype cell. Therefore, SAM synthetase was probably overexpressed. This high level of SAM synthetase might pull the reaction between methionine and ATP to synthesize SAM, which resulted in depletion of the methionine pool and an increased level of SAM in the cell. The increased SAM could also repress methionine biosynthesis since SAM is the corepressor of the *met* regulon. The depletion of the methionine pool and the prevention of methionine biosynthesis finally led to methionine auxotrophy. Adding exogenous methionine not only compensated for this deficiency, but probably also could repress *metK* expression to a certain extent (Holloway *et al.*, 1970), thus restoring a balance between methionine and SAM. Methionine requirement caused by overproduction of SAM has also been seen in other reports (Yocum *et al.*, 1996; Posnick and Samson, 1999; Budman, 1998).

It was also noted that the isogenic mutant MEW646 (Δ*metK* pLtetK), in which plasmid *metK* expression was always fully induced due to TetR repressor free, did not exhibit a methionine requirement for growth on glucose minimal medium with or without aTc 50 ng/ml (data not shown). It was presumably because recombination from the plasmid gene into the chromosome obscured this requirement.

3. SAM and cell division

3.1. SAM plays a role in cell division

To see if SAM plays a role in cell division, I studied the effects of varied *metK* expression on cell division using the *metK* deletion mutant MEW648. In MEW648, SAM synthetase activity was solely determined by plasmid *metK* expression, therefore, variations in plasmid *metK* expression could be expected to result in different intracellular SAM concentrations. By adding different concentrations of inducer aTc to the medium, plasmid *metK* expression could be easily tuned between low and high levels.

A good correlation between plasmid *metK* expression and cell division was found in cells grown on glucose minimal medium with methionine 50 μg/ml. It was observed that at low *metK* expression and low SAM level, cells of mutant MEW648 grew badly and exhibited extensive filamentous morphology, which indicated deficiency in cell division; while with the increase of *metK* expression and SAM level, filamentation gradually disappeared and cells were restored to the short, single *E. coli* rod morphology. This indicates that cells divided normally. This result was consistent with other observations that expression of T3 bacteriophage SAM hydrolase in *E. coli* cells reduced SAM level and caused elongated and occasionally filamentous cells (Hughes *et al.*, 1987; Posnick

and Samson, 1999) and that SAM synthetase- deficient *metK84* mutants underwent filamentation in leucine-starved conditions (Newman *et al.*, 1998). Therefore, it can be deduced that SAM plays a direct or indirect role in cell division.

Under complex medium (LB) growth condition, no clear filamentous morphology had been found at low *metK* expression. Any amount of *metK* that allowed growth resulted in the formation of short filaments. Posnick and Samson (1999) reported that *E. coli* cells expressing T3 SAM hydrolase were more elongated at saturation than control cells, but the elongate cells illustrated in the picture were 4- cell length short filaments. This reflected that some component(s) of LB might compensate for the effect of SAM deficiency at low *metK* expression levels and prevent filamentation, even though *metK* expression was absolutely required for *E. coli* growth in LB.

3.2. SAM-dependent methylation might affect cell divison

The primary role of SAM is to act as the methyl group donor in most of the methylation reactions. It had been confirmed that reduced SAM level decreased Dam methylation of *E. coli* genomic DNA as well as Dcm methylation (This work Fig.18 & Fig.19; Posnick and Samson, 1999; MacIntyre *et al.*, 2001). Therefore, it is possible that reduced SAM level causes a deficiency in methylation, which finally affects cell division. To date, the targets of methylation required for cell division have not been determined. But some hypotheses about how SAM-dependent methylation affects cell division have been proposed. Decreased DNA methylation caused by reduced SAM could induce the SOS regulon, thus bringing about SOS-mediated division inhibition (Hughes *et al.*, 1987). As part of the SOS response, transcription of the *sulA* gene would be induced and

the level of SulA protein elevated. SulA protein had been found to target the most important cell division protein FtsZ, inhibiting its polymerization, thus blocking FtsZ from localizing to the division site, and thereby causing division inhibition (Trusca *et al.*, 1998). Dr. Newman has postulated the existence of a methylase, which uses SAM as methyl donor and catalyzes the methylation reactions of cell division targets, so as to allow cell division to proceed (Dr. Newman, personal communication). One possible candidate was thought to be the product of the *ftsJ* gene. FtsJ protein has been shown to have the SAM-binding motif and was thought to be a methyltransferase involved in cell division. The other candidate is the product of the *mraW* gene, located in the upstream region of the *E. coli* 2 min cluster, where most of the cell division genes are included. MraW protein had been found to catalyze SAM-dependent methylation of 3 unidentified proteins (Ayala *et al.*, 1999) and also had been considered as a possible division methylase. It is possible that lack of SAM results in loss of methylation by these or other methylases, so that the cell division targets could not be properly methylated, which then inhibits cell division.

Cell division is a complicated process. A number of proteins take part in and act together so as to let the cell divide properly. At present, the methylation status of these proteins is still unclear, but it is possible that any or all of them may require methylation to function properly in cell division.

At present, the question about how SAM affects cell division still could not be answered; more work will be needed to further understand this mechanism.

4. Characterization of the *metK84* mutation

4.1. Using DNA sequencing analysis to identify the *metK*84 mutation

Based on the biological behaviours of the *metK84* mutant, it had been suggested that the *metK84* mutation could possibly be in the regulatory region or within the structural region of the *metK* gene (Greene *et al.*, 1973). Therefore, a 3.2-kb PCR amplified DNA fragment, containing the whole *metK* coding region, and 1-kb upstream and 1-kb downstream regions should be long enough to include the *metK84* mutation. Direct PCR amplification from the genomic DNA of the *metK84* mutant circumvented the complexity in construction of the clones and the need to grow individual cultures of each clone to obtain enough DNA for sequencing. However, it also introduced the risk of producing random mutations that would interfere with the mutation analysis. To avoid this disadvantage, each region was sequenced at least twice from independent PCR amplifications.

As the PCR-amplified template DNA was less than 5-kb, and the sequences of the *metK* gene and its flanking areas were known, I chose the Primer Walking strategy to analyze the mutation. Primer Walking has several advantages. No subcloning is required, and the location and direction of each sequencing run is known. After repetitive primer walking, and running a second sequencing from the opposite direction to verify the questionable parts, I finally confirmed a single base pair difference from the standard *E. coli* genome sequence, which is an A→G transition mutation located at the 150th position upstream of the *metK* ATG translation start codon. This point mutation seems to be the reason for the complex phenotype of the *metK84* mutant.

4.2. Characterization of the *metK84* mutation

4.2.1. The transcription initiation site of *metK*

To identify the transcription initiation site of the *metK* gene, primer extension analysis was performed in this work using a wildtype *E. coli* strain (MEW1) with plasmid PZAP-K31 carrying the wildtype *metK* upstream region. For cells grown in LB medium, a single transcription start site was observed, located 140 nucleotides upstream of the *metK* ATG start codon. This predominant transcription initiation nucleotide was recognized as +1T, in which a less preferred TTP nucleotide was used instead of the typical ATP or GTP nucleotide. This choice in the *metK* promoter might help to impose a severe restriction on the transcription of *metK* gene.

In a *serA* promoter study, the *serA* gene has two promoters P_1 and P_2. Transcription from P_2 is blocked by Lrp (Leucine-responsive regulatory protein), so is seen in the *lrp* mutants. However some transcription from P_2 is also seen in LB where the intracellular Lrp concentration is low. Could *metK* also have two or more promoters? In this case, I did the primer extension only in LB and saw only one primer extension product. It seems that only one is used to transcribe *metK* in LB, but it does not exclude that other promoters might be used in other growth conditions.

4.2.2. The *metK* promoter

Results presented in this work for the first time characterized the *metK* promoter. The sequence of the *metK* promoter deciphered by primer extension resembles that of the *E. coli* σ^{70} promoter. Its promoter elements such as the –10 hexamer (TAAAAT), the –35 hexamer (TGGAAA) and the spacing between these two regions (18bp) all showed high

homology to the consensus sequences of the *E. coli* σ^{70} promoters, in which the canonical –10 and –35 hexamers are TATAAT and TTGACA, and the inter-region spacing is 17±1bp (Harley and Reynolds, 1987). The highly conserved T_6, T_1 and A_2 in the –10 sequence and the highly conserved G_3 in the –35 sequence are all maintained in the *metK* promoter.

4.2.3. *metK84* mutation is a *metK* promoter point mutation

With the identification of the *metK* promoter, the –10 region has been positioned at 146 to 151 bp upstream from the *metK* ATG. Sequencing analysis confirmed the *metK84* mutation was an A→G transition mutation at 150bp position upstream of the *metK* ATG start codon, therefore, it is concluded that the *metK84* mutation is a point mutation in the –10 region of the *metK* promoter.

5. The effect of an A→G transition in the –10 region on the transcription activity of the *metK* gene

To examine if this A to G single basepair change within the –10 region is in fact sufficient to reduce the transcription activity of the *metK* promoter, I compared the β-galactosidase activities expressed from two transcription vectors in which one carries a *metK*::*lacZ* fusion and the other carries a *metK84*::*lacZ* fusion. *lacZ* expression from the *metK84* promoter was 12- fold lower than that from the wildtype *metK* promoter in glucose minimal medium, and 3.3 fold reduced in LB (Table 7). This would be totally convincing that the *metK*84 mutation reduces transcription. Therefore, it was a great disappointment to find a second point mutation introduced by PCR amplification at the

52 bp upstream of the *metK* ATG codon. Examination around that position did not find important binding sites such as a ribosome binding site, an Lrp binding site or a Crp binding site. Therefore, the obviously reduced level of β-galactosidase activity from the *metK84* promoter should be mainly ascribed to the point mutation at the –10 region, although I could not rule out the possible influence of the second point mutation. It is most likely then that the transition mutation from A→G at position 2 in the –10 hexamer ($T_1A_2A_3A_4A_5T_6$) markedly reduced the transcription activity of the *metK* promoter.

Other experimental observations also supported this point. In primer extension experiments, using RNAs isolated from the wildtype *E. coli* host strain transformed with the *metK84* clone (pZAP-K18) did not detect any transcripts, although the copy number of *metK84* clone was the same as the *metK* clone for which I did detect transcript and all the experiments were performed with the same conditions. Increasing the amount of total cellular RNA to 5-6 fold higher still did not allow transcripts to be detected. All of this suggests that the mRNA level transcribed from the *metK*84 clone was very low, and that the *metK*84 mutation greatly reduce the transcription level.

The same effect had also been reported in the *E. coli fis* P promoter study. The –10 consensus sequence of *E. coli fis* P promoter is $T_1A_2A_3T_4A_5T_6$. Changing the A at position 2 to either G or T severely reduced *fis* P transcription. The A→G mutation caused 21- and 111-fold decreases in *fis* P transcription in *fis* $^+$ and *fis* cells, respectively; and the A→T mutation caused 14- and 47-fold reductions (Walker *et al.*, 1999).

In comparison of the σ^{70} promoter -10 consensus sequences of different bacteria, it could be found that nucleotides at three positions are highly conserved within this short AT-rich region, they are: T_6, A_2, and T_1 (Wosten *et al.*, 1998). This high conservation

suggests that they are crucial to this AT-rich region. The mutation of A_2 to G not only disrupted the high conservation but also reduced the high A+T content, and all these would be expected to affect the recognition specificity of RNA polymerase complex (Patek *et al.*, 1996), resulting in severe reduction of the transcription activity of the *metK* promoter.

6. Some considerations about the regulation of the *metK* promoter

The β-galactosidase activities measured from the *metK*84::*lacZ* fusion in glucose minimal medium showed a great difference in the *lrp*$^+$ background from those in the *lrp*$^-$ background, indeed the removal of Lrp (lrp-) resulted in a 6-fold increase in *lacZ* expression. This suggested that Lrp repressed the transcription of the *metK*84 promoter. Adding a high concentration of leucine (100 μg/ml) also increased the transcription level of the *metK84* promoter, although to a relatively small extent (2.5-fold). The effect of leucine on the regulation of the *metK*84 transcription supported the idea that Lrp plays a negative role on the *metK*84 promoter. In the leucine/Lrp regulon, leucine acts as the cofactor of the regulatory protein Lrp and affects the regulation of Lrp on the expression of a number of genes. Based on the observation that Lrp repressed the *metK84* promoter, it could be implied that Lrp might be involved in the transcription regulation of the *metK* gene and represses its expression, analogous to its action on the *sdaA* promoter.

The repressive effect of Lrp on *metK* expression could be either a direct action or an indirect action. Through searching the *metK* promoter, 12 Lrp binding sites had been observed around the –10 and –35 regions, with most of them in the template strand. It provides the possibility for Lrp to function directly on the promoter structure, but could

not define it, because studies showed that the consensus sequences of the Lrp binding site are not specific enough (Newman *et al.*, 1995; 1996). To fundamentally understand how Lrp regulates the *metK* promoter, many further and deeper studies are needed. For example, DNA footprinting is needed to confirm that Lrp really binds to the *metK* promoter; the effects of Lrp on the transcription of the wildtype *metK* promoter have to be studied to reinforce that Lrp represses *metK* expression; gel retardation assays can be performed to investigate binding affinities of Lrp on wildtype or mutant *metK* promoters.

Searching around the *metK* promoter, a CRP binding site was found 60 bp upstream of the *metK* transcription initiation site (+1T), 3 bp away from the –35 hexamer (TGGAAA). Based upon this structure, it is also possible that Crp might be involved in the regulation of *metK* expression.

Summary

S-adenosylmethionine (SAM) is a central metabolite in *E. coli* and other cells. It has numerous roles in metabolism, mainly as a methyl group donor. SAM is synthesized from L-methionine and ATP by SAM synthetase, the product of the *metK* gene in *E. coli*. SAM synthetase and its structural gene *metK* have been thought to be essential for *E. coli*. However, before this work this had not been tested.

In order to test whether the SAM synthetase and/or *metK* gene are essential, I took advantage of the gene replacement vector pKO3 and tried to replace the chromosomal wildtype *metK* with a deleted *metK* gene. I could not produce this gene replacement using wildtype *E.coli* as starting strain, but succeeded in constructing a chromosomal deletion of *metK* in the presence of a plasmid carrying a functional *metK* under the anhydrotetracycline (aTc)-induced PLtet Promoter. I showed that the growth ability of *metK*-deletion mutant cells corresponded well to the expression of *metK* induced by aTc, both in rich medium (LB) and in minimal medium with methionine 50 μg/ml. This clearly demonstrated that the *metK* gene, coding SAM synthetase, is an essential gene in *E. coli* K-12. It also indicated that the *metK* gene was the only gene that encodes SAM synthetase in *E. coli*.

It was noted that the deletion mutant cells required methionine when grown in glucose minimal medium. This presumably resulted from the overproduction of SAM.

By adding different concentrations of inducer aTc into the media, I varied the level of plasmid *metK* expression of deletion mutant cells, and studied the effect of limited *metK* expression on cell growth, cell division and genomic DNA methylation. It was observed that reduced *metK* expression slowed cell growth and caused a defect in cell division and

producing filamentous morphology during incubation on glucose minimal media with methionine 50 μg/ml. This suggests that SAM plays a role in cell division. I also showed that lower *metK* expression decreased the Dam methylation and Dcm methylation of chromosomal DNA.

In the second part of this project, I made a study of the *metK84* mutant. The *metK84* mutant had a remarkably low level of SAM synthetase activity and showed a complex phenotype under certain growth conditions. I sequenced the *metK* gene and its flanking regions in this *metK84* mutant, and found out it carried an A→G transition mutation at the 150 bp upstream of the *metK* ATG start codon. Further primer extension analysis identified that this point mutation was located within the −10 region of the *metK* promoter, changing the highly conserved A_2 of the −10 hexamer (T<u>A</u>AAAT) to G. By comparing the β-galactosidase activities expressed from *metK::lacZ* and *metK84::lacZ* operon fusions, I showed that this point mutation within the −10 region markedly reduced the transcription activity of *metK*, with a 12-fold reduction in glucose minimal medium and a 3-fold reduction in LB. Results also reflected that the global regulator Lrp repressed this mutant promoter, while adding high concentrations of leucine could release this repression to a certain extent.

In this work, I characterized the *metK* promoter for the first time. Primer extension analysis determined the transcription start point of *metK* as +1T, located at the 140 bp upstream of the *metK* ATG start codon. The −10 hexamer is TAAAAT, from position −11 to −6, relative to the transcription start site (+1T); and the −35 hexamer is TGGAAA, positioned from −35 to −30. The consensus sequence of the *metK* promoter showed high

homology to that of an *E. coli* σ^{70} promoter, of which the canonical –10 and –35 consensus sequences are TATAAT and TTGACA.

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