Reduced intracellular SAM can increase the expression of *met* gene under the SAM-MetJ mechanism in *Escherichia coli*

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ABSTRACT

Reduced intracellular SAM can increase the expression of *met* gene under the SAM-MetJ mechanism in *Escherichia coli*

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S-adenosylmethionine(SAM) is synthesized from methionine by SAM synthetase. In *E. coli* this enzyme is encoded by *metK*. Since *metK* is an essential gene and SAM cannot cross bacterial cell membranes, it is difficult to study the the role of SAM directly in vivo. We introduced the SAM transporter gene from *Rickettsia* (kindly provided by Dr. D. O.Wood) to study the effect of exogenous supply of SAM on expression of genes of methionine biosynthesis in *E .coli* strains carrying a deletion of *metK*. Such strains could not make their own SAM but could transport SAM provided in the medium. The effect of growth with SAM, and SAM starvation on expression of the genes encoding most of enzymes in methionine biosynthesis, *metA, metB, metC, metE, metF, metK, metR*, was studied by means of reporter gene constructs using their promoters fused to *lacZ*. The Beta-Galactosidase coded by *lacZ* will reflect the *met* genes' expression in the SAM including medium or non-SAM medium. By using the above system, the effect of SAM on regulating *met* genes was studied. The result showed that in rich medium depleting the SAM supply of the Δ*metK* strain dramatically induced *metB* and *metR* (both 19.2 fold). The other genes were little affected. During growth in minimal medium with methionine and glucose, depleting the SAM pool had a much greater effect, inducing all the genes tested 10 fold to 100 fold. Moreover, there is no siganificant increase for all met genes in *metJ* deletion strain. These results support the in vitro model of MetJ-SAM repressor

complex binding met gene promoters to block their transcription. Furthmore, result of studying a metJ deficient mutant MNR6 implies that the truncated MetJ in MNR6 retains a considerable function. All these studies give one step further to understand the mechanism of regulating *met* gene expression by MetJ-SAM complex in vivo.

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Furthermore, I show my thankfulness to Dr.Wood who provided us the SAM transporter gene in *Rickettsia* and Rodrigo Reyes, my colleage, who cloned the above SAM transporter gene into pLTET vector.

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1 Introduction

1.1 The functions of S-adenosylmethionine (SAM)

S-adenosylmethionine (SAM) is a very versatile metabolite. It serves as donor of methyl groups in many reactions, reflecting the favorable energetics over the use of other one carbon unit carriers such as tetrahydrofolic acid (Greene, 1996). SAM is the methyl donor for DNA, RNA and proteins. *The Escherichia coli* (*E.coli*) K-12 genome encodes three DNA methyltransferases, which can transfer methyl group from SAM, Dam, Dcm and *EcoK* I ((M.G, 1996). Modification of nucleosides in the ribosomal RNA (rRNA) and transfer RNA (tRNA) plays an important role in the maturation of these molecules, with more than 95 different chemical modifications reported to exist in various organisms (Limbach et al., 1994; Rozenski et al., 1999). Methylation is a common modification in proteins, occuring at different amino acid residues in various organisms: lysine, arginine, proline, histidine, alanine, methionine, glutamic acid, asparagine and glutamine. In bacteria, all of these modifications occur except the methylation of arginine (Kim, 1980). For example, some ribosomal proteins and translation factors in *E.coli* have been found methylated (Lhoest and Colson, 1981; Young and Bernlohr, 1991).

In *E. coli*, SAM can work as corepressor for MetJ and together to inhibit *met* gene expressions, which are genes in *E.coli* that encode enzymes in methionine biosynthesis. This conclusion was confirmed by many in vitro experiments. Recently, Dr. Hughes's research group showed the reduction in the SAM pool induced the *met*

regulon (LaMonte and Hughes, 2006). This function will be elucidated in the following part.

In *E. coli* SAM also is involved in the synthesis of polyamines spermidine by being the aminopropyl group. SAM is first decarboxylated to S-adenosyl-methiioninamine catalyzed by SAM decarboxylase (enzyme coded by *speD*). Then, spermindine synthase (enzyme coded by *speE*) catalyzes transfer of the aminopropyl moiety to putrescine and yields spermine (Roje, 2006). Moreover, SAM has been recognized as an important precusor in reactions that requires the formation of organic radicals (Banerjee, 2003).

The product of *metK*, SAM, is involved in many metabolic pathways, and plays a variety of biological roles. SAM is best known as the primary methyl group donor in all organisms. This includes methylation of many cellular constituents, including DNA, RNA, proteins, and small molecules (Miller et al., 2003). However, it also acts as a precursor of the polyamines, biotin and lipoate, and is involved in DNA repair and in signal transduction system involved in the *E. coli* adaptation response during chemotaxis (Levit M.N., 1998).

1.2 Biosynthesises of methionine and SAM

The biosynthesis of methionine and S-adenosylmethionine can start with homoserine, which is the common precursor for some amino acids of the aspartate family, isoleucine, threonine and methionine (Rodionov DA, 2004). The enzymes involved are MetA, MetB, MetC, MetE, MetH and MetK (Figure 1). The functions of

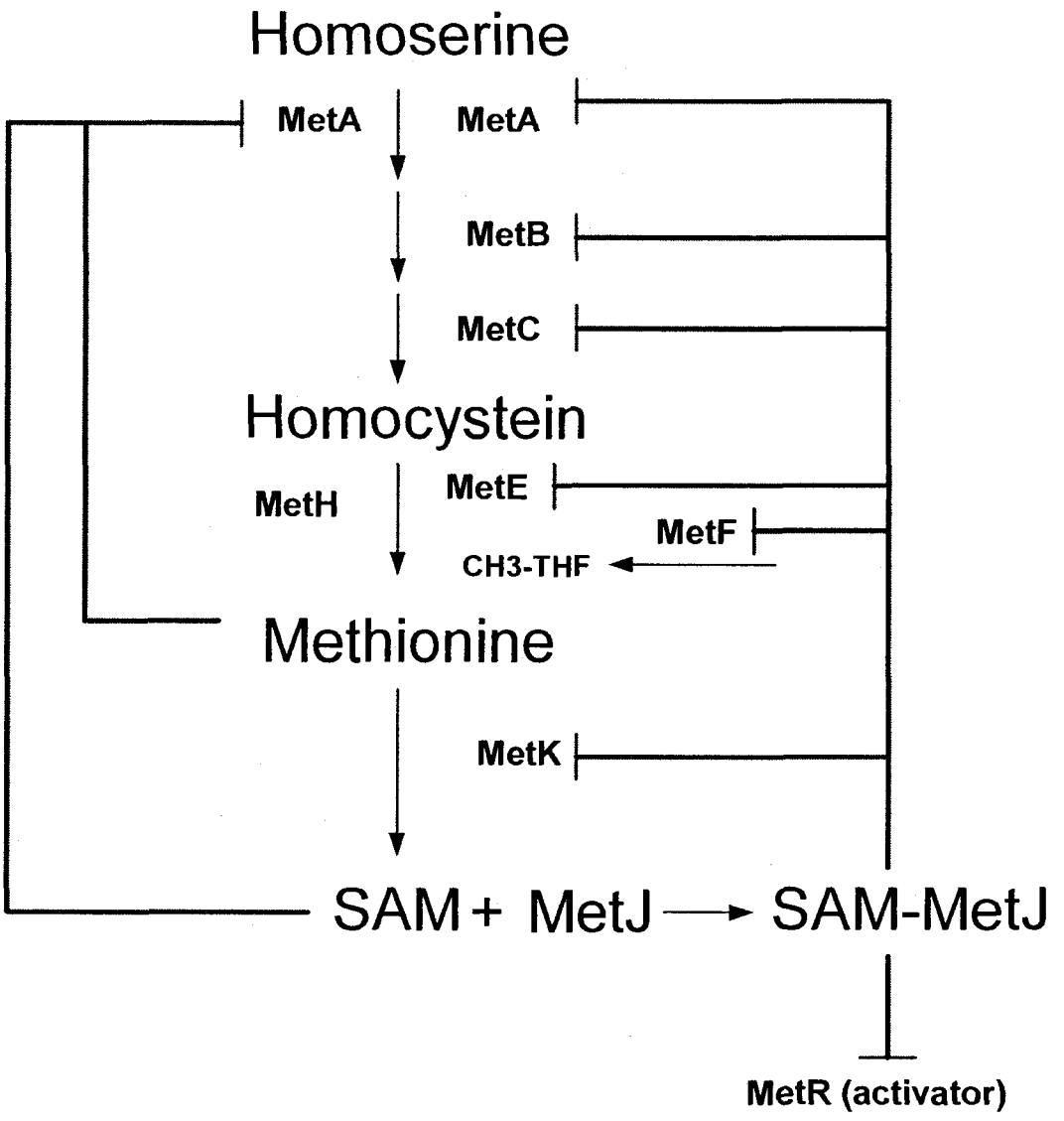


Figure 1. Regulation of the synthesis of methionine and SAM.
MetA, B, C, E, H, F, K, R are enzymes involved or related methionine biosynthesis.
The blunt end represents the inhibition.

the biosynthetic enzymes can be summarized as follows.

The *metA* gene codes for homoserine O-succinyltransferase (EC2.3.1.46, 90.79minute), which catalyzes the first reaction on the pathway to homocysteine and methionine. It catalyzes the o-succinylation of homoserine, using succinylCoA and producing o-succinylhomoserine.The *metB* gene codes for cystathionine γ-synthase(EC 4.2.99.9, 88.94minute), which catalyzes the displacement of the succinyl moiety of O-succinyl-L-homoserine by L-cysteine to yield L-cystathionine and succinate.

The *metC* gene codes for cystathionine β-lyase (cystathionase)(EC 4.4.1.8, 67.91minute) which catalyzes the straightforward β elimination that converts L-cystathionine to L-homocysteine, pyruvate, and ammonia. Two different enzymes can catalyze the terminal step in methionine biosynthesis, MetE and MetH. *MetE* and *metH* both code for homocysteine-methyltetrahydrofolate methyltransferases, which introduce the methyl group to homocysteine. These enzymes differ in their catalytic mechanism. MetE is cobalamin-independent homocysteine transmethylase (EC 2.1.1.14, 86.45minute), which is produced during cell growth in minimal media and shows an absolute specificity for methyltetrafolic acid modified with polyglutamate. MetH is a cobalamin-dependent enzyme (EC 2.1.1.13, 90.99minute) and is active only in media supplemented with vitamin B_{12}(Greene, 1996). MetH prefers to accept methyl group by 5-methyl THF instead of a methyltetrafolic acid modified with polyglutamate. However, MetE prefers to accept methyl group coming from a methyltetrafolic acid modified with polyglutamate. This is thought to be consistent

with the assumption that the MetH protein is primarily active during cell growth in the intestinal tract, where anaerobic conditions prevail and cobalamin is available (Greene, 1996).

The *metK* gene codes for the enzyme SAM synthetase (EC 2.5.1.6, 66.49minute) which catalyzes the condensation of methionine with the adenosyl group coming from ATP. This reaction is unusual in that the ATP is cleaved at the 5' carbon forming a tripolyphosphate enzyme-bound molecule which is released later as pyrophosphate and phosphate. SAM synthetase is a highly conserved enzyme, being present in all organisms examined to date. The SAM synthetases of as distant organisms as *E. coli* and humans share 57 % sequence identity (Newman et al., 1998). *MetK* is an essential gene in *E. coli* (Wei and Newman, 2002). MetR and MetJ can regulate transcription of *metK*, positively and negatively respectively. *MetK* can also be regulated negatively by the leucine responsive protein (Lrp) (Newman et al., 1998). For some Lrp-regulated genes, the presence of leucine in the medium modulates greatly the action of Lrp (Newman and Lin, 1995). In the *metK* case, leucine promotes the transcription of the gene probably by releasing Lrp from the promoter. The strain MetK84 has a point mutant in the promoter region of the *metK* gene. This decreases transcription causing an unusually low level of SAM synthetase, such that the strain needs leucine to induce the enzyme further (Wei and Newman, 2002).

E. coli is not able to transport SAM from the medium (Holloway et al., 1970). This has made the study of the effect of variations in intracellular SAM concentration very difficult (Posnick and Samson, 1999). Indeed, there was no description of a protein

capable of transporting SAM in bacteria until 2003, when the product of the gene RP076 from the alpha proteobacteria *Rickettsia prowazekii* was described in the study of Dr. Woods' group (Tucker A.M., 2003; Driskell et al., 2005). They transferred this transporter to *E. coli* and showed that it can function in this strain. They were kind enough to provide us with a plasmid containing SAM transporter gene from *Rickettsia prowazekii.* Thus, by designing two primers of this SAM transporter gene and using clone PCR product method, Rodrigo Reyes in our lab was able to make our pSAM which contains this SAM transporter gene in pLtet01, a low copy number expression vector with a p15A origin of replication and carrying a Pltet promoter with a convenient multiple cloning site (Lutz and Bujard, 1997). Reyes also optimized the function of the transporter in *E. coli*(Reyes, 2005). Therefore, I can use this plasmid to study the cellular functions of SAM by investigating the effects of changing the intracellular SAM concentration in *E. coli.*

1.3 Control of the *met* regulon

The pathway of methionine and SAM biosynthesis is controlled by both regulation of gene expression and feedback inhibition. The enzyme MetA is subject to feedback inhibition, and the products of the *metJ* and *metR* genes are also involved in regulation of *met* gene expression. As indicated in Figure 1, both methionine and *S*-adenosylmethionine can inhibit the function of the first enzyme of methionine biosynthesis, MetA, a phenomenon known as feedback inhibition. The feedback inhibition occurs at early stage of biosynthsis of methionine and SAM. Methionine

and SAM exert appreciable inhibitory effect alone when the concentration of methionine and SAM is high. At a low concentration level of methionine and SAM, feedback inhibition for *metA* only happens when these two metabolites combine to work (Lee et al., 1966).

Besides the negative regulation due to the feedback inhibition, the amount of methionine biosythesis enzymes made can also be regulated by the level of transcription. It has been showed that mutations in *metJ* and *metK* increased the level of the methionine biosynthetic enzymes (Lawrence et al., 1968). Purified MetJ protein was shown to be a DNA-binding protein which protected a region from cleavage by DNase I in the promoter between *metB* and *metJ* (Kirby et al., 1986). Consistent with this is the finding of Shoeman that pure MetJ protein inhibited in vitro transcription of the *metB, metF, metJ,* and *metL* genes and that *S*-adenosylmethionine enhanced that inhibition (Shoeman et al., 1985). Belfaiza noticed the presence of repeated octameric sequences at the 5′ ends of four *met* genes (including the region of the *metB-metJ* footprint) and suggested that they are the binding sites for MetJ repressor (Belfaiza et al., 1986). Results of subsequent studies supported this conclusion and octameric repeats have been found adjacent to most of the genes of the *met* regulon (Phillips et al., 1989). The consensus sequence for these repeats, 5′-AGACGTCT-3′, is called the "MET Box". SimonE.V.Phillips' data supported the hypothesis that the *E. coli met* repressor, MetJ with its complex with the corepressor SAM, can bind to synthetic and natural *met* operators (Phillips et al., 1989). Because of cooperative interactions between the bound repressor molecules, the number of adjacent octamers is an

important parameter in determining the affinity of the MetJ protein for a given operator. Highly repressible transcription units (e.g., *metB* and *metF*) have more MET Box repeats than a poorly repressible one (*metC*) (Holloway et al., 1970; Greene et al., 1973).

Operator consensus sequences for MetJ-SAM repression
[refered from (Phillips et al., 1989)]

Consensus	AGACGTCT	AGACGTCT	AGACGTCT	AGACGTCT	AGACGTCT
*met*A	AGctaTCT	gGAtGTCT	AaACGTaT	AagCGTaT	
	62.5%	75%	75%	62.5%	
*met*B	AtACGcaa	AGAaGTtT	AGAtGTCc	AGAtGTaT	tGACGTCc
	50%	75%	75%	75%	75%
*met*C	AGACaTCc	AGACGTaT			
	75%	87.5%			
*met*E	gGAtGaaT	AaACtTgc	cGcCtTCc		
	50%	50%	50%		
*met*F	cttCaTCT	ttACaTCT	gGACGTCT	GaACGgaT	AGAtGTgc
	50%	62.5%	87.5%	62.5%	62.5%
*met*R	AGgatTtT	AGcCGTCc	AGAtGTtT	AcACaTCc	
	50%	75%	75%	62.5%	

In addition to the repression by MetJ, several *met* genes are also subject to regulation by a second protein, MetR (the product of *metR* gene). It was reported that the native MetR protein from *E. coli* has a molecular mass of 68 kDa and it functions as a dimer (Maxon et al., 1990). Moreover, a leucine zipper motif functions in the dimerization (Neidhardt, 1996). The MetR protein is a member of homologous bacterial activator proteins known as the LysR family (Henikoff et al., 1990). It binds to DNA near the promoters of *metE* and *metH* and stimulates transcription (Maxon et al., 1989; Marconi et al., 1991). In addition, MetR increases the expression of *metA* and *metF* genes, and represses its own transcription (Maxon et al., 1989). It also can counter the MetJ repression of *metF* (Cowan et al., 1993). Moreover, DNase I footprints of MetR binding and the effects of several mutations on the response to MetR protein have identified TGAANN(T/A)NNTTCA as the consensus MetR target sequence (Greene, 1996). The *metR* mutants grow rapidly if methionine is fed, while they grow very slowly on medium supplemented with vitamin B_{12}. The product of the *metR* gene is required for an appropriate level of *metE* expression, and it stimulates the expression of *metH* (Urbanowski ML, 1987).

In summary, on the one hand, the SAM-MetJ complex, working as repressor, has a high affinity to sequences known as *met* boxes AGACGTCT, which are found at the upstream of the *metA, B, C, E, F* and *R* genes. Recently, the *met* regulon has been extended tentatively to genes not apparently related to methionine metabolism (Marincs et al., 2006). On the other hand, MetR is an activator for *met* regulon. It has a remarkable effect on the stimulation of expression of the two *met* genes (*metE* and

metH), while it has smaller effects on other genes, which are either inhibitory or stimulatory.

1.4 Motivation and Objectives

Although many data in vitro proved that SAM can regulate *met* regulon, the quantitative details of SAM regulating *met* gene expression in vivo are little understood, particularly because SAM until recently could not be provided from outside of the cell. The recent discovery of the *Rickettsia* SAM transporter (Driskell et al., 2005) allows the study of the effects of adding varying amounts of SAM to our Δ*metK* strain. With *Rickettsia* SAM transporter gene, Δ*metK* strain can grow normally in rich medium and it can grow normally in minimal medium when methionine is provided. If SAM represses methionine biosynthesis, addition of SAM should be inhibitory unless methionine is also provided. Reyes found this to be true and added methionine to SAM dependent strain cultures grown in minimal medium (Reyes, 2005). If the methionine requirement of SAM dependent strain reflects the inhibition of methionine biosynthesis by SAM, it should be possible to isolate deregulated mutants by screening methionine non-requiring strains.

The availability of an *E. coli* strain expressing a SAM transporter allows us to monitor the effect of varying the availability of exogenous SAM on *met* gene expression in vivo. The hypothesis of this work is that reducing intracellular SAM can increase the *met* gene expression under the SAM-MetJ mechanism. According to this hypothesis, the following questions will be focused: Is MetJ-SAM mechanism

effective in vivo? If so, what are the quantitative data on reducing SAM regulating *met* gene expression? Can SAM dependent mutants be isolated, which do not require methionine to grow in minimal medium? If so, what is their nature?

2 Materials and Methods

2.1 Bacterial strains and plasmids

All bacterial strains used in this study were derivatives of *E.coli* K-12 and listed in Plasmids used are also listed in .

In all cases, the cells used to inoculate liquid cultures came from colonies raised overnight on agar plates inoculated with transformants stored in 25%(v/v)glycerol at -80□ and streaked for purification.

2.2 Media and growth conditions

2.2.1 Luria-Bertani medium (LB)

Per liter contained:

1) Bacto-peptone 10 g;
2) Yeast extract 5 g;
3) NaCl 5 g;

PH adjusted to 7.0. The media was sterilized by autoclaving.

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

Table 1. Strains and plasmids were used

Strain name	Genotype	phenotypes	Source
Cu1008	*E. coli* K-12 $^{\Delta}$*ilvA*	Need isoleucine and valine	L.S.Wiliams
Cu/pSAM	*E. coli* K-12 $^{\Delta}$*ilvA* pLtet-SAM transporter	Need isoleucine and valine; Cm^r	L.S.Wiliams and Dr.Wood
SAD16/pSAM	*E. coli* K-12 $^{\Delta}$*ilvA* $^{\Delta}$*metK* pLtet-SAM transporter	Need isoleucine, valine and SAM in all medium; need methionine in minimal medium; Cm^r	This work
SAD16$^{\Delta}$*metJ*/pSAM	SAD16$^{\Delta}$*metJ*::kan SAM requiring and relative growth rate is low	The same as SAD16 and Kan^r grow very slowly in minimal medium	This work
MNR6/pSAM	SAD16 *metJ*::Tn10 pLtet-SAM transporter	The same as SAD16 except it does not need methionine in minimal medium and it contains Kan^r	This work
SM10(*λpir*)	*thi-1 thr leu ton*A *lac*Y *sup*E *rec*A::RP4-2-Tc::Mu,	Kan^r	Kenneth N.Timmis
XL1-blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacIqZΔM15 Tn10 (Tetr)]	blue/white color screening	Stratagene
JWK 3909-1	*E. coli* K-12 W3110 Δ*metJ*	Kan^r	Dr.Mori Janpan
Plasmid name			
pL*tetsam* (pSAM)	pLtet01 carrying the *sam* gene from Rickettsia prowazekii,	Cm^r	Rodrigo Reyes
pmetA	pMC1871 carrying the *metA* promoter and operator fused in frame to a promoterless *lacZ* gene	tet^r	This study
pmetB	pMC1871 carrying the *metB* promoter and operator fused in frame to	tet^r	This study

	a promoterless *lacZ* gene		
pmetC	pMC1871 carrying the *metC* promoter and operator fused in frame to a promoterless *lacZ* gene	tet^r	This study
pmetE	pMC1871 carrying the *metE* promoter and operator fused in frame to a promoterless *lacZ* gene	tet^r	This study
pmetF	pMC1871 carrying the *metF* promoter and operator fused in frame to a promoterless *lacZ* gene	tet^r	This study
pmetK	pMC1871 carrying the *metK* promoter and operator fused in frame to a promoterless *lacZ* gene	tet^r	This study
pmetR	pMC1871 carrying the *metR* promoter and operator fused in frame to a promoterless *lacZ* gene	tet^r	This study
pZ1	Lac promoter and operator fused in frame with *lacZ*	Cm^r	N/A
pLOF	Tn10 based delivery plasmid with Kan^r	Amp^r Kan^r	Marta Herrero(Herrero et al., 1990)
pMC1871		color blue/white tet^r Amp^r	Pharmacia

2.2.2 Minimal medium (NIV)

Per liter contained:

1) $(NH4)_2SO_4$ 2 g ;
2) K_2HPO_4 15 g ;
3) KH_2PO_4 5.25 g ;
4) L-isoleucine 0.5 g ;
5) L-valine 0.5 g.

PH adjusted to 7.0. The media was sterilized by autoclaving.

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

2.2.3 Carbon source for NIV minimal medium

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

2.2.4 R-top agar

1) Bacto-tryptone 10g,
2) Yeast extract 1g,
3) NaCl 8g ,
4) Bacto-agar 8g,

were dissolved in 1 liter distilled water. Sterile $CaCl_2$ (1M) and Glucose (20%, W/V)

were added to the medium to the final concentration of 2mM and 0.1% separately after autoclaving.

2.2.5 SOC medium for electro-transformation

1) Bacto-tryptone 20g,
2) Yeast extract 5g,
3) NaCl 0.58 g,
4) KCl 0.185 g,
5) $MgCl_2$ 2.03 g
6) Glucose 3.6 g

were dissolved in 1 liter distilled water. And this media was sterilized by autoclaving

2.2.6 Preparation of SAM Stock solution

The nutritional supplement of SAM was obtained from a commercial pharmaceutical brand "FoodScience of Vermont" as 200 mg tablets of SAM-e. The tablets were ground and dissolved in 1 mM HCl to a final concentration around 100 mM. The mixture was centrifuged at 4000 rpm for 5 minutes to pellet the insoluble material and the supernatant was filtered through a 0.4 m pore size filter and stored as aliquots at –20 °C. An estimate of the real concentration of SAM was obtained by measuring the absorbance at 260 nm (= 15, 400 $M^{-1}cm^{-1}$). SAM as p-toluenesulfonate salt was purchased from SIGMA. It was diluted in 1 mM HCl to a final concentration of 100 mM, sterilized by filtration and stored as aliquots at –86 °C.

2.2.7 Antibiotics and Supplements

Final concentrations used for antibiotics were: 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, 40 μg/ml kanamycin and tetracycline at 20 μg/ml. The supplement of methionine is used in the minimal medium as 40 μg/ml.

2.2.8 Growth of SAM-requiring strains

Strains containing the deletion of *metK* and pSAM were grown in LB with 200 μM SAM or in NIV with 200 μM SAM and 40 μg/ml methionine.

For β-galactosidase assays, cultures were grown overnight with shaking at 37□ in 5ml liquid rich and minimal medium with 200μM SAMe, with the appropriate antibiotics to select strains, transporter plasmid and reporter plasmid. After chill the cultures on ice and subculture to the same medium the next day and grew the cells until they reach the log phase (3-4 hours). Collect some cells and then wash the left cells with LB or minimal medium to get rid of SAM. Subculture to the same medium without SAM and starve SAM for 4 hours. Then measure Beta-galactosidase in presence or absence of SAM in different strains immediately.

2.3 Solutions:

2.3.1 SM buffer

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

1) NaCl 5.8g;

2) MgSO4·7H2O 2g;

3) 1M Tris·Cl (pH 7.5) 50ml;

4) 2% gelatin solution 5ml.

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

2.3.2 Beta-Galactosidase assay solution

Z buffer: per 50 ml contains 0.80g $Na_2HPO_4.7H_2O$ (0.06M) ; 0.28g $NaH_2PO_4.H_2O$ (0.04M); 0.5 ml 1M KCl (0.01M); 0.05 ml 1M $MgSO_4$ (0.001M); 0.135 ml beta-mercaptoethanol (BME) (0.05M); bring to approximately 40 ml with H_2O, dissolve all the salts; adjust the pH to 7.0, store at 4 C.

O-nitrophenyl-D-galactopyraniside (ONPG): 4 mg/ml prepared in 0.1M sodium phosphate PH 7.5.

1 M Na_2CO_3: 10.6 g Na_2CO_3 was dissolved in 100 ml double distilled water.

2.3.3 Southern analysis solution

Denaturing solution includes:

1) 20 g of NaOH,

2) 58.4 g NaCl

were dissolved in 1 liter of ultrapure water.

Neutralizing solution includes:

1) 78.8 g Tris base,

2) 87.6 g NaCl

in 800 mls of ultrapure water. Adjust the pH to 7.4. Add ultrapure water to 1 liter.

20X SSC includes:

1) 175.3 g NaCl,

2) 88.2 g of trisodium citrate (citric acid);

add ultrapure water to 1 liter.

2.4 Enzymes and related buffers

Taq polymerase, pfu DNA polymerase, T4 ligase, Calf Intestine Alkaline Phosphatase (CIAP) and restriction enzymes were purchased from MBI fermentas (Montreal, Canada). Kits for plasmid extraction QIAprep Spin Miniprep Kit and GenElute Plasmid Miniprep Kit were purchased from QIAGEN (Montreal, Canada) or SIGMA (USA) respectively. DNA mini Kit was obtained from QIAGEN (Montreal, Canada). QIAquick PCR Purifcation Kit was obtained from QIAGEN (Montreal, Canada).

2.5 P1 phage transduciton

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

2.5.1 P1 phage lysate preparation

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

2.5.2 P1 phage-mediated transduction

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

2.6 Plasmid isolation and restriction enzyme digestion

2.6.1 Plasmid isolation

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

2.6.2 Restriction enzyme digestion

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

2.6.3 DNA gel electrophoresis analysis

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

2.7 Electro-transformation

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

2.8 Plasmid and Constructions

I constructed seven plasmids: pmetA, pmetB, pmetC, pmetE, pmetF, pmetK and pmetR, which contain the different natural *met* gene promoter and operator sequences fused in frame to a promoterless *lacZ* gene. The vector pMC1871 contains the promoterless *lacZ* gene and PCR products containing different *met* promoters were cloned into the *Sma* I site (Figure 2).

The strain XL1-blue list in strain table was used as host for plasmids. Transformants were selected on LB Tet X-Gal plates, and screened for blue colonies. The blue colour indicates that the cells are expressing beta gal, suggesting the recombinant plasmids have an active promoter.

The primers for PCR were designed to amplify the promoter sequence for different *met* genes. The sequence of primers used and their locations are listed in Table 2. The PCR reaction was carried in a volume of 50 µL and the mixture contained 1µM concentration of each primer, 50 ng of template *E. coli* Cu 1008 genomic DNA, Reaction buffer [75mM Tris-HCl (pH8.8), 20 mM $(NH_4)_2$ SO_4, 0.1% Tween 20], 2 mM $MgCl_2$, 300 µM dNTPs, 1 U of *pfu* DNA Polymerase (Fermentas, USA). The reaction was performed using the following program:

94 °C 5 min

35 cycles 94 °C 30 s

55 °C 30 s

72 °C 2 min

72 °C 10 min

After amplification, the products were analyzed on a 1% agarose gel and DNA was purified using QIAquick PCR Purifcation Kit (QIAGEN Montreal, Canada). The vector pMC1871 was cut by *Smal* 1, and the band was extracted from the gel using QIAquick® Gel Extraction Kit from Qiagen. The blunt ended PCR products were cloned into the using T4 DNA ligase.

XL1-Blue competent cells were transformed by electorporation and selected in LB tetracycline X-Gal plate. Blue colonies were selected and the plasmids were extracted and checked using restriction enzyme. Finally the positive plasmids were sent to sequencing to determine if the appropriate promoter regions were cloned.

2.8.1 Δ*metK* Δ*metJ* double deletion strain (SAD16 Δ*metJ*/pSAM) construction

P1 phage was prepared in the deletion strains JWK 3909-1(*metJ*:: kan^r), kindly provided by Dr.Mori. P1 was then used to infect SAD16/pSAM and transductants were selected on LB with kanamycin and SAM. The Kan^R colonies were checked with PCR using the *metJ* primers which located upstream and downstream of *metJ* gene. Colonies with the *metJ* deletion will give a 1883 bp product whereas a colony with a wild type *metJ* gene will give *a* 909 bp product.

2.8.2 Isolation of methionine non-requiring derivatives of SAD16/pSAM

From the previous study, we know that *E. coli* can use externally provided SAM if it has a transporter (Reyes, 2005). However in minimal medium, addition of SAM is not sufficient to allow growth of the *metK* mutant (Reyes, 2005). Methionine must also be provided. We hypothesized that this is because SAM can inhibit methionine biosynthesis. Therefore, we tried to select mutants that can grow much faster than *metK* mutant alone without methionine in the minimal medium using mini-Tn10 based transposon insertion (Herrero et al., 1990).

Grow *metK* mutant strain SAD16/pSAM on the LB SAM Cm plates with different concentrations of Nalidixic acid. SAD16, which can grow under the Nalidixic acid 5 ng/ml after overnight on the above selecting plates, was selected as the recipient strain. The mini-TN10 donor strain SM10(λpir)/pLOF was cultured in LB Kan 50 μg/ml Amp100μg/ml. Then mix with recipient using a ratio of 1:4.

Centrifuge down the mixture and put all cells on the surface of LB plates with

50μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 8 hours at 37□. Then the cells were suspended in 10mM MgSO4 and the appropriate dilutions were plated on selective medium (NIV Glu, SAM100μM, Nal 5μg/ml, Kan 50μg/ml). We selected four mutants which can grow much faster than original *metK* mutant SAD16 on this minimal medium lacking methionine. We called these mutants as methionine nonrequiring mutants (MNR).

After recombinant plasmids were obtained, all pmet plasmids as well as a control plasmid pZ1 were transformed into four strains, namely, wild type (Cu/pSAM), Δ*metK* strain (SAD16/pSAM), Δ*metK* Δ*metJ* double deletion strain (SAD16Δ*metJ*/pSAM) and methionine-non requiring mutant (MNR6/pSAM) by electro-transformation on LB SAM chloramphenicol, tetracycline selected medium. All these strains include the SAM transporter gene carried on plasmid pLtet01. All genetic phenotypes for each strain were verified by PCR using corresponding primers.

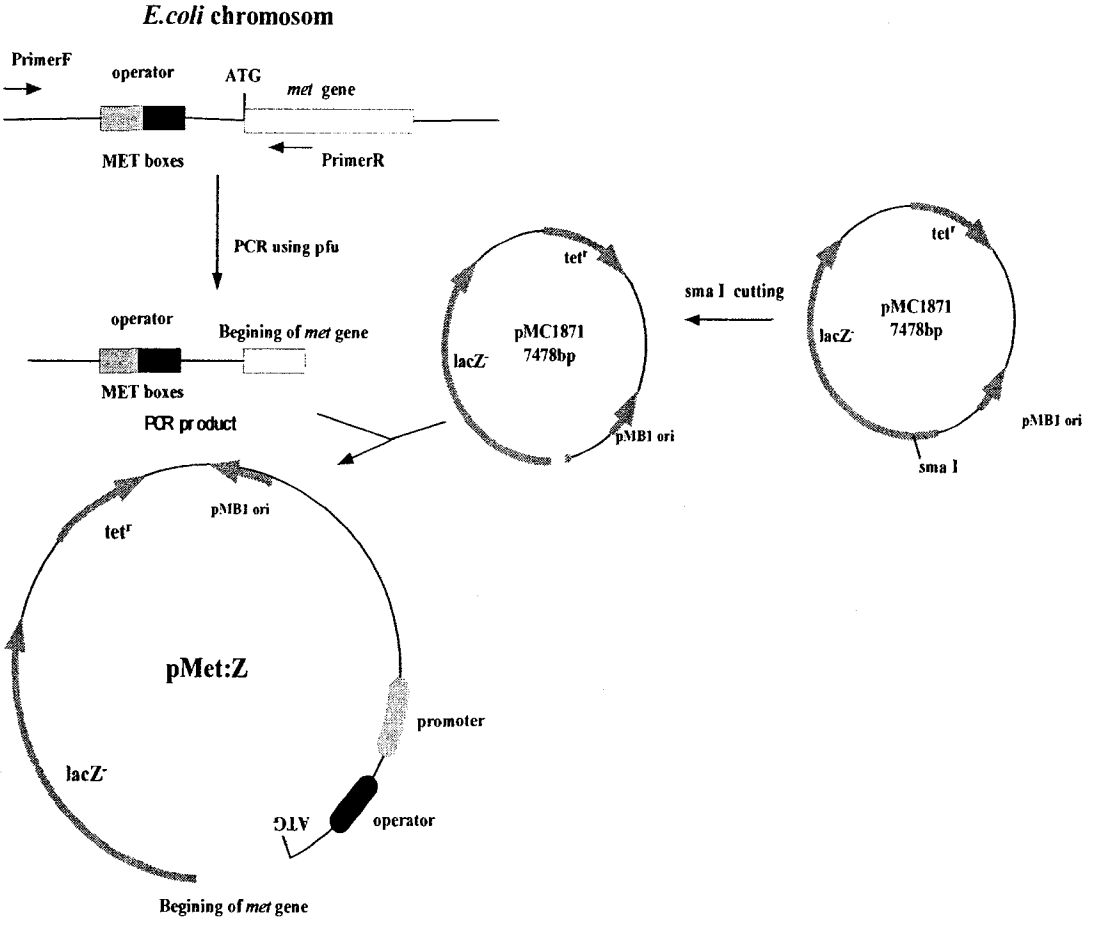


Figure 2. The construction of pmet.

The operater regions of *metA, metB, metC, metE, metF, metK and metR* were amplified with two primers. One locates upstream of operater region, another locates inside of *met* gene. These PCR products were fused in frame with a promoterless *lacZ* in the vector pMC1871 at *Sma* I site.

2.9 Plasmid and strain verification

All the plasmids and strains constructed has been verified by using PCRs method with appropriate primers (Table 4.) The template of these PCRs was prepared by putting *E. coli* cells into PCR reaction tubes with autoclaved toothpicks. The cell density in PCR tubes becomes just visible in pure water. Freeze them at -70°C for 5 min before they were put PCR reagents and put them into PCR thermal cycler.

Table 4.

2.10 Beta-Galactosidase assay

Grow cells overnight. Subculture and allow them to reach OD_{600}=0.5 and record the number. In triplicate, take 100 µl of the cell culture and place it in glass tubes. Then add 900 µl of Z buffer and 1 drop of prewarmed 0.1% SDS and 2 drops of chloroform were added to each tube and vortex for 10 seconds. Incubate at 28°C for 5min. Add 0.2 ml of ONPG and vortex at 23°C (room temperature). Then record time in minuites of development of yellow color and stop reaction by adding 0.5ml of 1M Na_2CO_3 and vortex. Read absorbance at 420 and 550 nm and record the number. Calculate units of Beta-galactosidase activity using following equation:

Units=1000x[OD_{420}-(1.75xOD_{550})/(time in min)x(volume used of cell culture)xOD_{600}]

reference for method (Miller, 1972).

2.11 Southern Blotting

Southern hybridization was carried out as described previously (Sambrook and Russell, 2001) with minor modifications. Total genomic DNA from MNR mutants isolated using Qiagen genomic DNA extracting Kit and around 100 µg DNA were digested overnight with restriction enzyme *Hind*III. Digest was precipitated by a standard ethanol precipitation and DNA was diluted in 30 µl TE buffer. 100 µg of the digest was separated by standard agarose gel electrophoresis using a 1% agarose gel and transferred overnight to a Hybond N+ nylon membrane by capillary blotting using alkaline transfer according the instructions provided by the manufacturer (Amersham Biosciences).

The DNA probe was generated by digesting pLOF with *Mlu*I (Figure 5). The digest was separated by agarose gel electrophoresis, and a 1500 bp fragment containing the mini-TN10kan was purified using Quiagen QuiaexII gel extraction kit. This fragment (100 ng) was denatured in boiling waterbath for 2 min and immediately transferred on ice. 1X Klenow buffer (supplied by MBI Fermentas), 0.5 mM dNTP (dAGT), 1 µl DTT (100 mM), 80uCi of [α^{32}P] dCTP (3000 Ci/mmole), 1 µl Klenow fragment and water were added up to 25 µl and the mixture was incubated at room temperature for 1 hour. Removal of unincorporated dNTPs was done by passing the reaction through a Sephadex G50 column (Sambrook and Russell, 2001).

Combination of 50 ng of the labeled probe and hybridization solution brought the final volume to 2.5 ml and the sample was denatured for 5 minutes in boiling water bath. Hybridization was carried out in glass roller bottles as described by (Sambrook

and Russell, 2001). Washing steps were carried out as described previously (Sambrook and Russell, 2001). The hybridization signal was detected by autoradiography.

2.12 Mass spectrometry for checking SAM degradation

SAMe from the tablets was put into 5 ml double distilled water (PH=7.0) and final concentration is 100μM. The solution was divided into two parts, one part was sent to pass mass spectrometry immediately and another part was incubated at 37 °C for 20 hours, then pass mass spectrometry. The sample were analyzed with the Q-TOF 2^{TM} (Micromass, USA) mass spectrometer, using electrospray ionization (ESI) (Nano spray) and tandem mass spectrometry (MS/MS).

2.13 Sample preparation for Beta-Galactosidase assay

To study the effect of SAM on *met* gene expression, I constructed seven plasmids: pmetA, pmetB, pmetC, pmetE, pmetF, pmetK and pmetR, which contained the different natural *met* gene promoter and operator sequences fused in frame to a promoterless *lacZ* gene. Then I transformed them together with a control plasmid pZ1 into four strains, namely, wild type (Cu/pSAM), Δ*metK* strain (SAD16/pSAM), Δ*metK* Δ*metJ* double deletion strain (SAD16 Δ*metJ*/pSAM) and methionine-non requiring mutant (MNR6/pSAM). The pSAM is the plasmid with SAM transporter gene carried on vector pLtet01.

To study the effects of SAM starvation in rich medium, I grew these four kinds of strains in LB medium overnight with 200 μM SAM and proper antibiotic (25 μg/ml

chloramphenicol, 20 μg/ml tetracycline for Cu/pSAM/pmet, SAD16/pSAM/pmet and additional 40 μg/ml kanamycin for SAD16 Δ*metJ*/pSAM/pmet and MNR6/pSAM/pmet). In the next day, I chilled the cultures on ice for a least 5 min, and subcultured them in the same medium with 1/200 dilution for 3-4 hours (OD600 is around 0.5-0.7). These resulting log phase cells were chilled, and divided into two parts, starved and non-starved samples. Starved samples were prepared by washing with cold LB medium incubated in LB medium without SAM with proper antibiotics for 3 hours (Cu/pSAM/pmet) or 4 hours (Δ*metK* strains including SAD16/pSAM/pmet, SAD16 Δ*metJ*/pSAM/pmet and MNR6/pSAM/pmet). Non-starved samples were prepared by subcuturing overnight culture to the same LB medium with SAM and incubated with their starved ones for the same period of time. The β-galactosidase values of both non-starved and starved samples were assayed at the same time. For each strain, three parallel samples for each *met* gene were measured under the same condition. This whole set experiment was done once to three times according to different strains (Cu/pSAM/pmet, twice; SAD16/pSAM/pmet, three times; SAD16 Δ*metJ*/pSAM/pmet, once; MNR6/pSAM, twice). The average data of Beta-Galactosidase of each strain under the same condition was used to represent the expression of each *met* gene in rich medium.

The similar method was used to study these *met* gene expression in minimal medium (NIV). The only difference is to put 40 μg /ml methionine in the the medium NIV to grow strains. Only two kinds of strains (Cu/pSAM/pmet and SAD16/pSAM/pmet) were used to study in minimal medium since the other two

strains (MNR6/pSAM/pmet and SAD16 Δ*metJ* /pSAM/pmet) grew too slow in minimal medium to give enough amount of cell to measure Beta-Galactosidase. The whole process is described as follows.

I grew these strains (Cu/pSAM/pmet and SAD16/pSAM/pmet) in NIV overnight with 200 μM SAM, 40 μg /ml methionine and 25 μg/ml chloramphenicol, 20 μg/ml tetracycline. In the next day, I chilled the cultures on ice for a least 5 mins, then subcultured them in the same medium with 1/100 dilution for 3-4 hours (OD600 is around 0.5-0.7). The resulting log phase cells were chilled, and divided into two parts, starving and non-starved samples. Starving samples were prepared by washing with cold NIV+ 40 μg /ml methionine medium, then incubated in the same medium without SAM with proper antibiotics for 3 hours (Cu/pSAM/pmet) or 4 hours (SAD16/pSAM). The non-starved samples were prepared by subcuturing overnight culture to the same NIV, methionine medium with SAM and incubated with their starved ones for the same period of time. Then the β-galactosidase values of both non-starved and starved samples were assayed immediately. For each strain, three parallel samples for each *met* gene were measured for Beta-Galactosidase under the same condition. The set of experiments were repeated three times for Cu/pSAM/pmet and twice for SAD16/pSAM/pmet. The average data of Beta-Galactosidase of each strain under the same conditions was used to present the expression of each *met* gene in minimal medium.

I have to methion that, in order to achieve similar cell density before measuring Beta-Galactosidase, "starve" SAM for Cu/pSAM for 3 hours and starve *metK*

deficient strain for 4 hour were used. Actually, although we did not put SAM in the medium, Cu/pSAM did not starve SAM since it can make SAM itself and it has a relatively high growth rate. However, for *metK* deficient strains, since they can not make SAM and when they grow in the medium without SAM, they had low growth rate and will use up SAM after 4 hours.

3 Results

3.1 Determing the optimal SAM working concentration by Mass spectrum

SAM is an unstable molecule and it can be easily broken due to heat or alkaline environment. Since the most strains used in this study are SAM dependent strains and they have to grow in the medium provided with SAM. The effect of SAM concentration of the medium on the growth of SAM dependent cell is large. On one hand, there should be enough SAM to support cell growth and division. On the other hand, too much SAM cannot be put into the medium, since it would result in a very acidic medium, which would hinder the cell growth. Therefer, the level of SAM concentration needs to be studied. I used Mass sprectrum to determine whether 100μM SAM is enough to support overnight culture. The result was shown in Figure 3. The ratio of m/z for SAM is 399, and another compound (m/z 298) is selected to be used as a reference compand since it is the second highest peak, which is more accurate to measure and it is a relative stable compound. The middle figure showed non-incubated SAM under the ESI. From it, we can see that the ratio of peak

399/peak 298 is around 2. The top figure showed the result for non-incubated SAM under the MS/MS. The ratio of peak 399/peak 298 is reduced to 1/5. It is obvious that under this high energy, SAM can be easily broken down (m/z for SAM is 399). The bottom figure showed incubated SAM under the ESI. From it, we can see that the ratio of peak 399/298 is around 1. Compared with non-incubated SAM, which ratio of peak 399/298 is around 2, SAM of the incubated sample degrades around 50% after incubating 37°C for 20 hours. It means that in the normal SAM dependent strain growth condition, after overnight, SAM concentration in the medium should be around 50 μM if 100 μM SAM was used. Reyes in our lab before showed that 50 μM SAM is not enough to support chromosome DNA methylation(Reyes, 2005). In order to support all cell growth under the normal condition, I use 200 μM SAM as the working concentration. It should be noted that methionine can not be produced during the SAM degradation, neither from MS/MS nor from incubating for 20 hours in pure wather (methionine m/z is 150)(Figure 3). Thus SAM degradation can not contribute methionine which is required by SAM deficient strain when it grows in minimal medium.

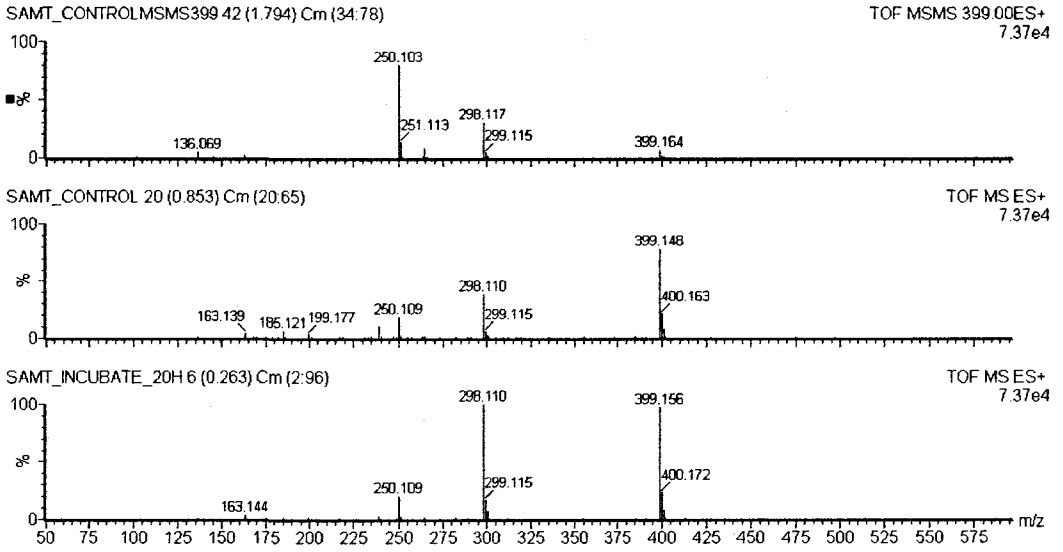


Figure 3. Mass spec result for testing SAM degradation.
SAM: m/z is 399; the top figure is the result for non-incubated SAM under the MS/MS; the middle figure shows non-incubated SAM under the ESI; the bottom figure shows incubated SAM under the ESI

3.2 Construction and verification of plasmids and strains

3.2.1 Construction and verification of reporter plasmids

In order to study the effect of extracellular SAM on the expresson of *met* genes, I made plasmids in which *met* promoters fused with the reporter gene lacZ gene. I designed primers to amplify the *metA, metB, metC, metE, metF, metK and metR* promoter region in frame from wildtype *E. coli* genomic DNA using on line software primer3 http://frodo.wi.mit.edu/. Then I amplify the responsible bands using error correcting enzyme *pfu*. One designed primer locates upstream of CDS (coding sequence) and another locates inside of the CDS. Table 4 shows the primers sequence and the expected band size for PCR product. From figure 4, all expected bands are seen on the DNA gel picture.

After I successfully cloned these seven *met* promoters into pMC1871, the PCR technique was used to verify these plasmids. Since I cloned the PCR product of promoter region of *met* genes, we should make sure that there is no any mutation at the promoter region, which is caused by PCR amplification. Then, I sent all seven plasmids to Genome Quebec Innovation Center for sequencing the insert using the primer from the vector. The results were compared with Collibri *E. coli* database using EBI tools: Clustalw2 http://www.ebi.ac.uk/Tools/clustalw2/index.html. The alignment results for all constructed plasmids showed that the inserted sequences are 100% identical to the reference sequences. From that I am sure that all MET boxes as

well as promoter regions of all met gene cloned into pMC1871 are intact.

Table 2. *Met* gene primer designation and localization

[one primer locates upstream of operater(MET boxes), another primer locates inside of *met* gene]

Gene name	up primer to ATG	Down primer to ATG	*Met* boxs to ATG
metA	655bp	299bp	19 bp
metB	661bp	225bp	71 bp
metC	584bp	231bp	34bp
metE	663bp	228bp	71bp
metF	515bp	256bp	81bp
metK	612bp	231bp	No met boxes
metR	683bp	256bp	95bp

Table 3. Primers used to amply *met* genes and expected size

Met gene	Forward primer	Reverse primer	Expected bands
metA	ACCATTGGTGGGTACTAAACC	ACAGTAGAAATTATTCAG ATG CTC	934bp
metB	CAA TAT GAC GTA GGC CTG AT	AGC ACC ACC TTC CAG TTC	880 bp
metC	GGT ACG TTC TTT AAT ACC TTC G	CGC TTG TTG TAA GGA GAA AT	833 bp
metE	TTA CCA GAT CCA GCT CTC C	ATG ATC GTA CCA GGC AAA AT	877 bp
metF	GTG AAG ATC ATC CGT TGG	AAT GCC TTT AAT AAT GCT GTG	754 bp
metR	CTC GTC CAC TTC GTC CAG	GCT AAT TTG CGG CAG TAC CT	922 bp
metK	GCG AAA CCA CGA GAA AAC TC	ACG GGT GAT CTC TTC GAT GT	841 bp

Table 4. Primers used for checking strains

primer	Sequence(5'-3')	Amplified genes	Expected bands (wild type)	Expected bands (deletion mutant)
SAMF	TTGGTACCTAAT GAATGATGCATT AAA AAC	SAM transporter gene	830 bp	N/A
SAMR	TTGGATCCTTAC TGCGATTCATGT TTG C			
metKF	GATAAATACGCAGAATGCCG	*metK* gene	1800 bp	1000 bp
metKR	GCTGTTATCTGCAATTAAGAC			
metJF	CAGGCACCAGAGTAAACATT	*metJ* gene	909 bp	1883 bp
metJR	CATACCGGTGTGGCTATAAT			
KanF	ATGAGCCATATTCAACGGGA	Kanamycin Resistant gene	820 bp	N/A
KanR	TTAGAAAAACTCATCGAGCA			

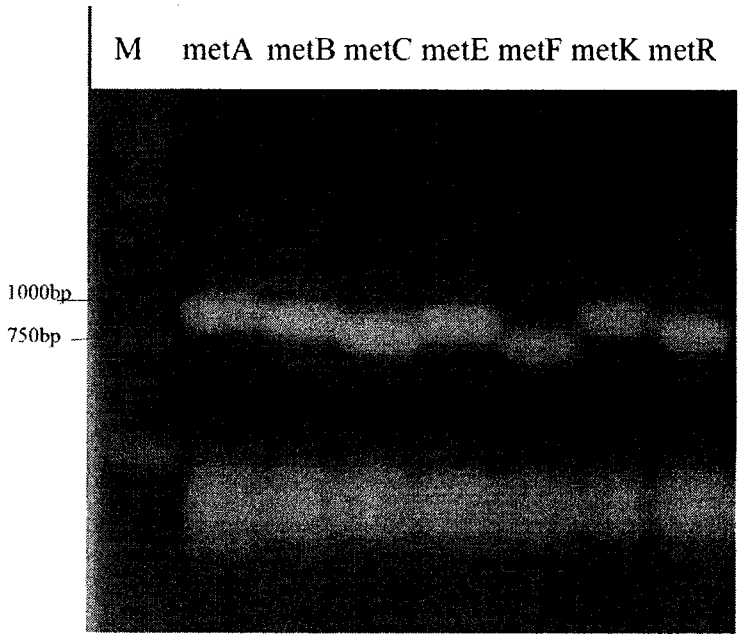


Figure 4. PCR products for *met* gene protomers

M: 1kb DNA ladder; the expected bands for *met* genes were showed in Table 3 and all bands from this result match all expected bands. The expect band for *metA* is 934bp, *metB* is 880 bp, *metC* is 833 bp, *metE* is 877 bp, *metF* is 754 bp, *metK* is 922 bp, *metR* is 841 bp.

3.2.2 MNR6 construction and verification

In order to find out why SAM dependent strain can not grow in minimal medium without methonine, we need study strains, which are SAM dependent and does not need methionine to grow in the minimal medium. I used miniTn10Kan insertion to generate this mutant. The details were showed in the chapter 2 (method part). Prior to finding out which genes were disrupted by Tn10, we should first know how many Tn10 insertions exist on the chromosome of these mutants. To this aim, southern blot technology was used. It was reported that minTn10kan used in this study has a high propobility of causing only one insertion in the chromosome (Herrero et al., 1990). Thus, I chose to use one insertion strategy to design southern blot to test MNRs strains. *Hind*III is selected since there is only one site inside miniTn10Kan. Firstly, I cut genomic DNA of MNRs using *Hind*III Completely. At the same time, the probe, Tn10-transferring sequence, is gotten by *Mlu*I cutting from the pLOF. Then I hybridize the probe with the *Hind*III treated MNRs genomic DNA. If there is only one insertion, two hybridization bands would be seen. The diagram of the probe is shown in Figure 6.

The procedure is showed in method part and the result showed in Figure 7. From this result, we can see that all MNR mutants have two bands and from the strategy of southern blot for MNR mutants (Figure 6), we conclude that there is only one Tn10 insertion on the chromosome for all MNRs.

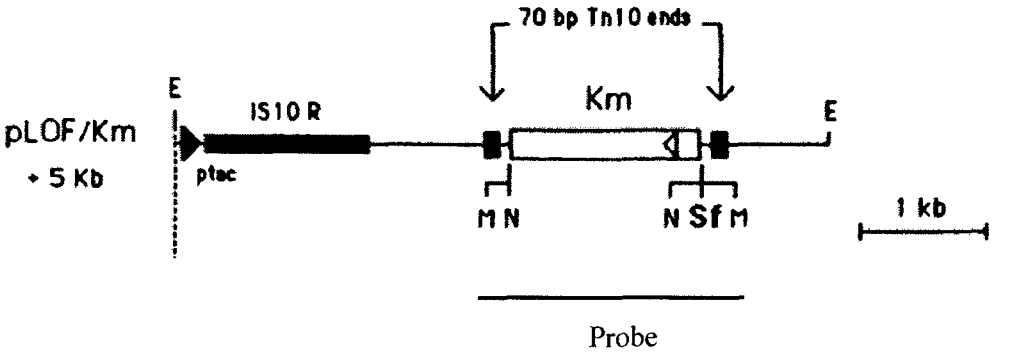
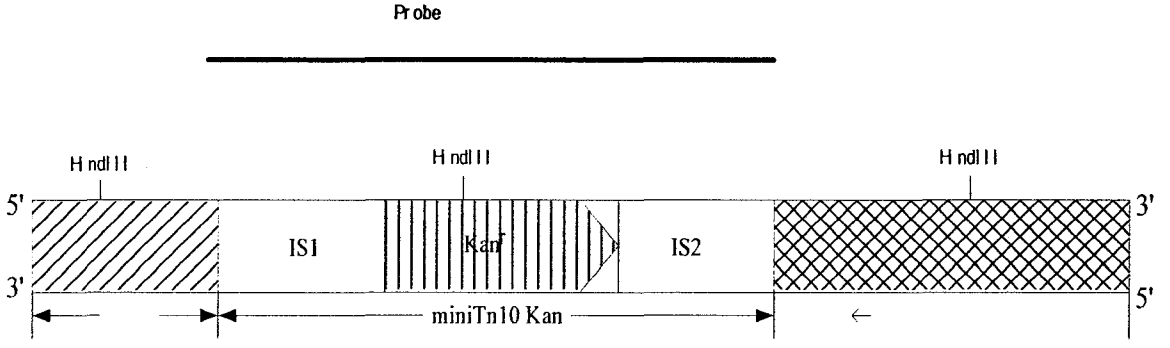


Figure 5. The probe of southern blot.
The Tn10 transfer sequence coming from the pLOF which is gotten by *Mlu*I digestion was selected to be the probe of southern blot. M, N, Sf represent the restriction enzyme *Mlu*I, *Not*I, *Sfi*I respectively.



Chromosome of MNR mutants

Figure 6. The one insertion strategy of southern blot for MNR mutants.
If MNR mutant has only one Tn10 insertion, two bands coming from the *Hind*III digested products can partially hybridize to the radio labeled probe. So there should be two bands on the film. In the other words, two bands on the southern film can prove that this is only one Tn10 insertion in the MNR mutant if the same method were used.

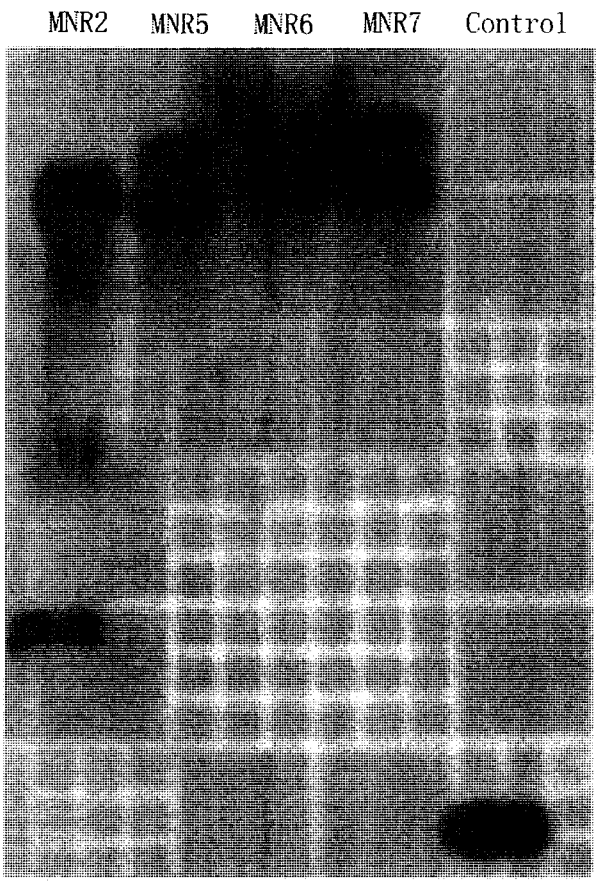


Figure 7. Southern blot result for MNR mutants.
Control samlple is the probe hybridizes itself.

3.2.3 Verification of *metJ* mutant among MNRs

All MNR mutants cannot make SAM since they contain *metK* deletion and SAM has to be provided in any medium for their growth. However, unlike SAD16/pSAM, they do not need methionine to grow in minimal medium although extracellular SAM can inhibit methionine biosynthesis. Based on the knowledge that SAM can inhibit methionine biosynthesis by binding the *met* regulon repressor MetJ as the corepressor, there is a chance that *metJ* mutant could grow without methionine in minimal medium. In order to verify whether there is *metJ* mutant among the MNR mutants, I used *metJ* primers (metJF and metJR), which located upstream and downstream of *metJ* gene to amply all MNR mutants. The result showed there is a bigger band for MNR6 than wildtype *metJ* and the band size that equals wildtype *metJ* gene plus Tn10-transferring sequence was gotten. So there is a great chance that *metJ* gene in MNR6 is disrupted by miniTn10Kan since PCR product was bigger than wild type *metJ* gene 909 bp and the difference band size was matched to miniTn10Kan. The DNA gel result was shown in Figure 9. In order to know which position was disrupted in *metJ* gene and whether this insertion mutantion is caused by miniTn10kan, PCR product for MNR6 was sequenced using metJF primer. The sequencing result showed that the 7th to last codon of metJ gene from MNR6 is disrupted. The sequence after 7^{th} to last codon came from the transposon miniTn10kan. The sequencing result is shown in Figure 8. From the result of Southern blot, we know that there is only one miniTn10kan insertion in MNR6. Thus, by combining all results, I can conclude that

MNR6 phenotype which it does not need methionine to grow in minimal medium because *metJ* gene is not functional.

Figure 8

GATTGAGCAAATCCCAAATAGCCGTTAAAATTATATGCATTATCACGCCG
ACAGGTGCATTACACGATGTCACGGTAACGCCTGTACGGTAAACTATGCG
GGTTTACGGTCAGTACCCACATCAACTGTGTGGTCTGGTCTCAATTTATT
GACGAAGAGGATTAAGTATCTC***ATGGCTGAATGGAGCGGCGAATATATCA***
GCCCATACGCTGAGCACGGCAAGAAGAGTGAACAAGTCAAAAAGATTACG
GTTTCCATTCCTCTTAAGGTGTTAAAAATCCTCACCGATGAACGCACGCG
TCGTCAGGTGAACAACCTGCGTCACGCTACCAACAGCGAGCTGCTGTGCG
AACGTTTCTGCATGCCTTTACCGGGCAACCTTTGCCGGATGATGCCGATC
TGCGTAAAGAGCGCAACGGCGAAATCCCGGAAGCGGCAAAAGAGATCATG
CGTGAGATGGGGATTAACCCCATAAGCGCTAACTTAAGGGTTGTGGTATT
ACGCCTGATATGATTTAACGTGCCGATGAATTACTCTCACGATAACTGGT
CAGCCATTCTGGTCCATATTGGTAAGCCCGAAGAACTGGATACTTCGGTC

Figure 8. Sequencing result for PCR product of *metJ* gene of MNR6.
The italic and bold sequence are completely matched with *metJ* gene and underline sequence comes from the transposon.

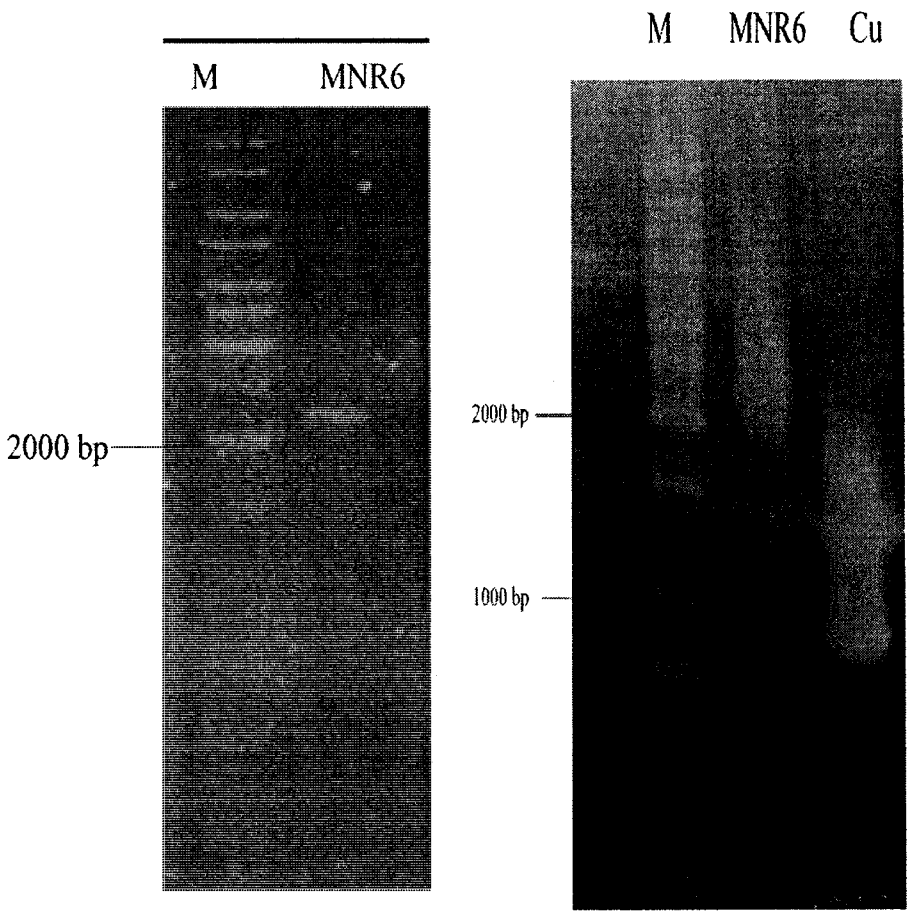


Figure 9. Approval *metJ* gene is disrupted in MNR6 mutant by PCR.
Using metJF and metJR primers, the expected band of wildtype metJ gene is 909 bp and the expected band for *metJ*::Tn10 is 2400 bp.

3.2.4 Construction and verification of strain SAD16 Δ*metJ*/pSAM

We know that SAM can bind MetJ and the complex works as the inhibitor to repress *met* genes expresson. In order to study the *met* gene expression influenced by this mechanism, we need a strain as a negative control which cannot make both SAM and MetJ. Our SAD16/pSAM is the SAM dependent strain and we need to construct *metJ* deletion strain derived from SAD16/pSAM. The P1 transduction technology was used to construct this strain. P1 phage from JWK 3909-1(*metJ*:: kan) was used to infect SAD16/pSAM and transductants were selected on LB with kanamycin, SAM. The tranductant colonies were checked with PCR using the *metJ* primers (metJF and metJR). The positive colonies will amplify 1883 bp and the negative clone will amplify wild type *metJ* 909 bp. The PCR product gel picture approving the SAD16Δ*metJ/pSAM* is shown in Figure 10.

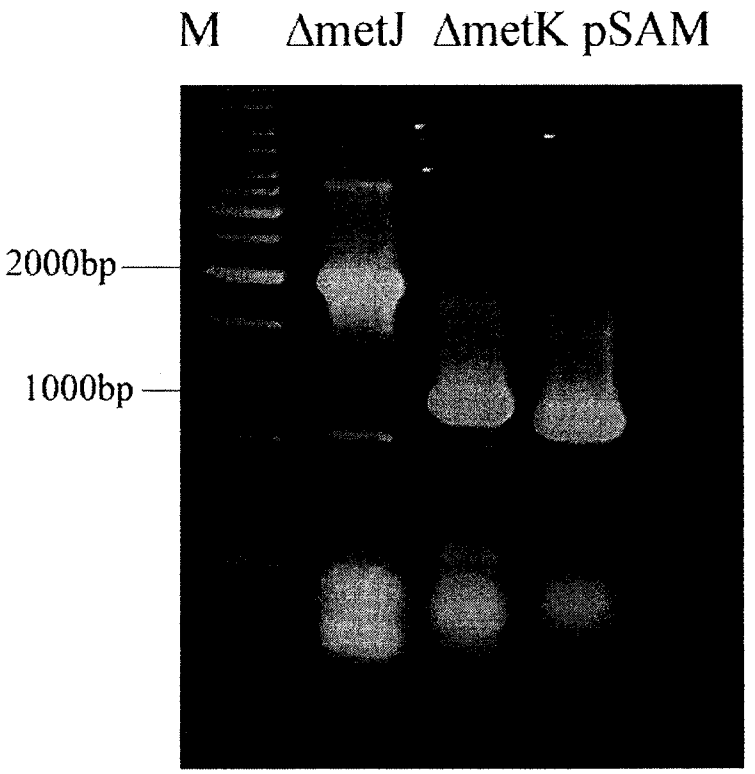


Figure 10. The strain SAD16 Δ*metJ* verification.
M: 1 kb DNA ladder. Using metJF and metJR primers, the expected band for Δ*metJ* is 1883 bp(the wildtype is 909 bp). Using metKF and metKR primers, the expected band for Δ*metK* is 1000 bp(the wildtype is 1800 bp). Using SAMF and SAMR primers, the expected band for SAM transporter encoded gene is 830 bp.

3.2.5 Construction and verification strain Cu /pSAM/pmet, SAD16/pSAM/pmet, MNR6/pSAM/pmet, SAD16Δ*metJ*/pSAM/pmet

In order to study the *met* genes expression, I transformed seven pmet plasmids that contain different *met* promoter fused to *lacZ* gene into 4 different strains. They are wildtype Cu pSAM, which can make SAM itself; SAD16/pSAM, which cannot make SAM and must use SAM in the medium to support its growth; MNR6/pSAM and SAD16Δ*metJ*/pSAM, both strains cannot make both SAM and MetJ. The difference between MNR6/pSAM and SAD16Δ*metJ*/pSAM is that MNR6/pSAM is the *metJ* insertion strain and SAD16Δ*metJ*/pSAM is the total deletion *metJ* strain. All tranformants were verificated by PCR using proper primers. The primer sequences were shown in Table 3 and Table 4. The SAM-transporter gene for all strains was verified by amplifying 830 bp band using primers SAMF and SAMR. Deletion of *metK* gene in the strain SAD16/pSAM and MNR6/pSAM was verified by amplifying 1000 bp instead of 1800 bp using MetKF and MetKR primers. The miniTn10 insertion in MNR6 was verified by amplifying 820 bp kanamycin resistant gene using KanF and KanR primers. Deletion of *metJ* gene in SAD16 Δ*metJ* was verified by using metJF and metJR primers and the 1883 bp band was amplified. As for the verification of pmet, the used primers and expected amplified bands are shown in Table 3. The all expected bands of strain verification were obtained.

3.3 Result for Beta-Galactosidase in rich medium

To study the effects of SAM starvation, I constructed seven *met* plasmids which contained the different natural *met* gene promoter and operator sequences fused in frame to a promoterless *lacZ* gene. Then I transformed them and a control plasmid pZ1 into four strains, wild type (Cu/pSAM), Δ*metK* strain (SAD16/pSAM), Δ*metK* Δ*metJ* double deletion strain (SAD16 Δ*metJ*/pSAM) and methionine-non requiring mutant (MNR6/pSAM). The details of sample preparation and measuring Beta-Galacdosidase have been shown in chapter 2 (method section). The measured Beta-Galactosidase coming from the *lacZ* gene should reflect the expression of *met* gene in one particular strain. Through Beta-Gal activity data in the same strain, we can compare the *met* gene expression in vivo between treated SAM and starved SAM conditions. All the data coming from rich medium are shown in Table 5 .

I chose Cu/pSAM/pmet as negative control, which contains the wild type *metK* gene and can make intracellular SAM itself. Theoretically, the changed extracellular SAM should have nearly no effect on regulating *met* gene expressions in this strain. The result showed that, for the same *met* gene, the Beta-Gal units is relatively smaller in the strain Cu/pSAM/pmet than in other *metK* deficient strains under both conditions with and without SAM in rich medium. Moreover, the no SAM /SAM ratio data showed that all *met* gene expression has no siganificant increase when starving extracellular SAM in rich medium. Although both *metB* and *metR* increased 3.5 times after "starving" SAM, which is much smaller than in SAD16pSAMpmet strain (both increased nearly 20 times after starving SAM). Therefore, we can conclude that

reducing exogenous SAM concentration can not significantly increase *met* gene's expression in Cu/pSAM/pmet.

The *metK* defective strain SAD16/pSAM cannot make SAM itself and it needs to get extracellular SAM to support its growth. In addition, this strain contains the wildtype MetJ protein. According to the previous in vitro work, *met* genes can be inhibited by the MetJ-SAM mechanism, the expression of *met* gene in SAD16/pSAM/pmet should be inhibited if SAM is present in the medium. The details how to grow SAD16/pSAM and how to measure Beta-Galactosidase have been shown in chapter 2(method part). From the result, we can see that SAM depletion leads to a nearly 20 times expression of *metB* and *metR*. Moreover, it is surprised to observe that no dramatically influences were seen in other *met* genes.

MNR6/pSAM is a *metK* and *metJ* defective strain. Theoretically, the expression of *met* gene cannot be influenced by the MetJ-SAM mechanism. The growth condition of MNR6/pSAM was exactly the same as the one of SAD16/pSAM except that 40μg/ml kanamycin was put in the medium. Kanamycin resistant came from the miniTn10kan insertion. The details of culture growth were shown in method part. The results of *met* gene expression in vivo in MNR6/pSAM in rich medium (Table 5) showed that, for all the *met* genes that we tested, depletion of SAM cannot upregulate their gene expression, except *metE*, which has a little increase (2.2 times). Some genes expression became lower, which is probably caused by the influence of SAM starvation (the explaination is shown in the discussion chapter). The relative higher Beta-Gal data of all *met* genes in MNR6/pSAM/pmet than in SAD16/pSAM/pmet

with SAM was gotten, except *metE*. This can explain that *met* gene expression is inhibited when both MetJ and SAM exist and no inhibition when only SAM exists. The data also implied that except the MetJ-SAM mechanism, there is perhaps another SAM-involving mechanism of regulating *metE* gene.

As MNR6/pSAM/pmet, SAD16Δ*metJ*/pSAM/pmet is also a *metK* and *metJ* defective strain. The only difference is that SAD16Δ*metJ*/pSAM/pmet is the total *metJ* deletion and MNR6/pSAM/pmet is *metJ* deficient strain, which is disrupted by Tn10 at the last 7^{th} codon. The growth condition of SAD16Δ*metJ*/pSAM/pmet was exactly the same as the one of MNR6/pSAM/pmet. The kanamycine resistance of SAD16Δ*metJ*/pSAM/pmet comes from the deletion of *metJ* gene instead of coming from the miniTn10 insertion. The details of culture growth and Beta-Galactosidase measurement were shown in the method part. The result showed thatm, for all the *met* genes, there is no significant increase of Beta-Gal units after depletion of SAM (Table 5). It reconfirms that MetJ-SAM mechanism is not functional in both MetJ and SAM deficient strain. In addition, the Beta-Gal units for SAD16Δ*metJ*/pSAM/pmet strain with SAM is more than 10 times higher than SAD16 /pSAM/pmet strain with SAM for most tested *met* genes, except *metK*, which is two fold. Furthermore, we can see that for both SAM and no SAM conditions, SAD16Δ*metJ*/pSAM/pmet has higher Beta-Gal unit than MNR6/pSAM/pmet, although they have similar genetic background. The possible explaination is that the truncated MetJ protein in MNR6/pSAM/pmet has part of MetJ function, such as binding to MET boxes on the operator region of *met* genes. This binding somehow influences the *met* gene

expression. Moreover, SAM is not involved in this truncated MetJ binding process.

In summary, from the data in the rich medium, we can conclude that MetJ-SAM mechanism is functional in vivo. In addition, *metB* and *metR* are most sensitive to be regulated by this mechanism in LB.

3.4 Result for Beta-Galactosidase in minimal medium

Since SAD16Δ*metJ*/pSAM/pmet and MNR6/pSAM/pmet grow very slow in minimal medium and they cannot provide enough cells to measure Beta-Galactosidase, I only studied the effect of SAM in minimla medium NIV for strain Cu/pSAM/pmet and SAD16/pSAM/pmet. Since SAD16/pSAM/pmet needs both SAM and methionine in minimal medium, in order to keep the same growth condition, I put 40 μg/ml methionine in minimal medium for Cu/pSAM/pmet. The details of growing strains and Beta-Galactosidase measurement were shown in method part. The result of minimal medium is shown in Table 6.

From the result of Cu/pSAM/pmet in minimal medium, depletion exogenous SAM cannot increase *met* genes expression significantly (The no SAM/SAM ratio ranges from 0.5 to 2.8). Cu/pSAM/pmet contains the wild type *metK* gene and can make intracellular SAM itself. Theoretically, the changed extracellular SAM should have almost no effect on regulating *met* gene expressions in this strain. The Beta-Gal data here imply that above theory can be applied in Cu/pSAM/pmet in minimal medium.

Table 5. Effect of SAM starvation in rich medium (LB)

Medium A (LB glucose 200uM SAM with antibiotic)
Medium B(LB gluscose no SAM with antibiotic)

Beta-Gal units												
Strain	Cu/ pSAM/pmet			SAD16/pSAM /pmet			SAD16Δ*metJ*/pSAM/pmet			MNR6/pSAM/pmet		
Medium / plasmids	A	B	B/A	A	B	B/A	A	B	B/A	A	B	B/A
pmetA	38	79	2.1	162	852	5.2	11095	11925	1.1	1128	553	0.5
pmetB	8	28	3.5	22	429	19.2	537	676	1.3	594	268	0.5
pmetC	220	311	1.4	463	951	2.1	10390	8147	0.8	2317	1918	0.8
pmetE	51	53	1.1	1667	2499	1.5	13218	12403	0.9	1527	3308	2.2
pmetF	125	78	0.6	1610	3585	2.2	18440	23617	1.3	5363	5486	1.0
pmetK	2463	6253	2.5	6927	7405	1.1	11693	18515	1.6	16284	11741	0.7
pmetR	29	101	3.5	356	6849	19.2	18362	12945	0.7	2987	3108	1.0
pZ1	366	435	1.2	201	125	0.6	1769	838	0.5	589	312	0.5

For the strain SAD16/pSAM/pmet in minimal medium, the result showed that except *metK*, the depletion of exogenous SAM can dramatically increase nearly all tested *met* genes' expression (from nearly 10 times to 100 times, according to different *met* gene tested). This result strongly supports the MetJ-SAM inhibition mechanism for *met* genes. Also this result shows that unlike other *met* genes, *metK* gene has relatively little affected by exogenous SAM.

Table 6. Effect of SAM starvation in minimal medium (NIV)

Medium C (NIV Glu 40 µg /ml methionine 200uM SAM with antibiotic)
Medium D (NIV Glu 40 µg/ml methionine with antibiotic)

Beta-Gal units						
Strain	Cu/pSAM/pmet			SAD16/pSAM/pmet		
Medium / plasmids	C	D	D/C	C	D	D/C
pmetA	703	353	0.5	391	3857	9.9
pmetB	81	201	2.5	16	697	44.2
pmetC	1651	1795	1.1	407	10564	26.0
pmetE	1292	588	0.5	86	8695	100.8
pmetF	2042	3627	1.8	248	11881	48.0
pmetK	3853	6889	1.8	3749	15534	4.1
pmetR	984	2758	2.8	478	8889	18.6
pZ1	1463	3113	2.1	1512	1205	0.8

4 Discussion

The present work deals with the study of the physiological roles of SAM on regulating methionine biosythesis by using a SAM transporter. The hypothesis of this work is that SAM deficiency can increase the expression of genes encoding enzymes of Met biosynthesis (*met* genes) under the SAM-MetJ mechanism.

Previous researchers have achieved reduced intracellular SAM level by regulating its synthesis or degradation indirectly. Wei reduced the internal pool with a mutation in the *metK* gene (Wei and Newman, 2002) and Hughes used a plasmid expressing the SAM hydrolase gene (Hughes, 2006). The role of SAM has not been shown directly in vivo since exogenously provided SAM cannot cross the *E. coli* membrane and *metK* is an essential gene (Wei and Newman, 2002) . Recently, LaMonte and Hughes circumvented this problem with the use of T3 SAM hydrolase carried on a relatively high copy vector to lower endogenous SAM levels (LaMonte and Hughes, 2006). They showed that the ensuing reduction in the cellular SAM pool induced the *met* regulon. When SAM-hydrolase is used, SAM pools cannot be completely depleted since the synthetase is still present. In fact, sudden changes in the intracellular concentration are not possible since this system requires a change in the concentration of the protein due to a gradual decrease in SAM. Thus, they could not study the effects of higher than normal concentrations of SAM. In addition, they studied some *met* genes from *E. coli* and some from *Salmonella typhimurium.*

The following sections are devoted to the discussion of my own data and

comparability with Dr. Hughes group's data on the effect of SAM on methionine biosynthesis.

4.1 Regulation of intracellular levels of SAM

I used SAM transporter gene to alter the intracellular SAM concentration, which was obtained from a screen of a cosmid bank of *Rickettsia prowazekii* (Tucker A.M., 2003), and cloned into p_L*tet01* vector by Reyes(Reyes, 2005). The SAM transporter used in this work shows clear advantages over other systems in the rapid regulation of intracellular concentrations of SAM, being only regulated by the concentration of SAM in the medium and the amount of the transporter in the cell.

SAM is chemically unstable and it can degrade even at -20°C. In order to examine the stability of SAM, I tested 100 μM SAM in double distilled water immediately and incubated at 37°C for 20 hours in mass spectrometry. From the results, I can roughly estimat that about 50% SAM degraded after incubating 37°C for 20 hours in pure water. Since it is reported that 50 μM extracellular SAM is not enough to support chromosome DNA methylation of SAM deficient strain (Reyes, 2005), I chose to use 200 μM SAM instead of 100 μM to grow *metK* deficient strain to make sure cells can get enough SAM through their growth. Moreover, methionine cannot be produced after SAM degradation (methionine m/z is 150)(Figure 3). Thus, SAM degradation cannot contribute methionine which is required by SAM deficient strain when it grows in minimal medium.

4.2 The effect of SAM on *met* gene expression in LB broth medium

In order to study the effect of SAM on expression of *met* genes, seven plasmids which contain *met* genes promoter fused with *lacZ* gene in four different strains were used. All the strains contain SAM transporter. They are wild type strain Cu/pSAM/pmet which contains the wildtype *metK* gene, *metK* deletion strain SAD16/pSAM/pmet, *metK metJ* double deletion strain SAD16Δ*metJ*/pSAM/pmet and one of methionine-non requiring mutants, MNR6/pSAM/pmet, which is *metK* deletion strain as well as its *metJ* gene is disrupted at the last 7^{th} codon.

In the wild type strain Cu/pSAM/pmet, which contains the wild type SAM pool, the result shows that reducing exogenous SAM concentration cannot increase *met* gene's expression siganificantly. The experimental result of wild type is consistent with the expected result, which can be explained theorically. Since it contains *metK* gene, it has physiological SAM pool inside cells. This intracellular SAM pool has a big capacity. In Cu/pSAM/pmet even with SAM transporter, the exogenous SAM is hard to influence intracellular SAM pool. Dr. Hughes group cannot study the effect of extracullar SAM on $metK^{+}$ strains since instead of using depletion extracullar SAM to deplete intracellular SAM pool, they used T3 SAM hydrolase to deplete SAM pool.

In the *metK* defective strain, the result shows that SAM depletion leads to a nearly 20 times increase of expression of *metB* and *metR*. We are surprised to observe that no dramatically influences were seen in other *met* genes. We got the similar result for *metB* and *metR* (both increase 17 times, after decrease SAM pool) in *metK* mutant with Dr. Hughes group's data published at 2006 (Table 7). However, their data also

showed that *metE* and *metF* derepressed after decreasing SAM pool in *metK*::Tn5 strains. This may be explained that they used *met* promoter from *S. typhimurium* instead of from *E. coli*. The difference may be caused by the difference of promoter sequence between *E. coli* and *S.typhimurium*. This also can be explained by different *metK* defective strain. Dr.Hughes used *metK*::Tn5 strains, which can make enough SAM to support cell growth since their *metK* mutant can grow in rich medium without SAM. However, our Δ*metK* strain cannot make SAM at all and it must get exogenous SAM to support its growth. The fact that we starved SAM by depletion SAM in the medium actually can cause its death. When we starved SAM in the medium, the intracellular SAM pool became lower and lower as Δ*metK* strain continued growing and dividing within 4 hours, around 3 generations, proved the plating on SAM plate(Reyes, 2005). When SAM pool became low enough, many SAM-involving metabolisms in the cells stopped, for example, DNA stop replicating since it need SAM to initate and ribosome stops making proteins due to the fact that some residues need to be methylated before start to work. Resulted in many reasons, the cells die of SAM starvation. So the depletion of SAM can cause Δ*metK* strain grow slow and the mechanism of gene expression also is influenced and is not efficient as in normal SAM pool. So if we counted the effect of SAM starvation, actually *metB* and *metR* gene expression should be more than 20 times after depletion SAM. And *metE* and *metF* expression should be higher than they seem to be. Moreover, we have to mention that when we compare the Beta-Gal units between Δ*metK* strain(SAD16/pSAM/pmet) with SAM and wild type strain (Cu/pSAM/pmet) without

SAM in LB, we found that except *metK*, all other *met* genes got much higher units in Δ*metK* strain than in wild type (Table 5). This represented that even with enough SAM supply for both strains, nearly all *met* genes were more repressed in wildtype strain than Δ*metK* strain, except *metK* gene. This may be explained that no *met* box locates the promoter of the *metK* gene. Thus the expression of *metK* has little been effected by SAM-MetJ mechanism. Moreover, this comparation showed that there is more concentration of intracellular SAM in wild type strain than that in *metK* defective strain. Therefore, we can conclude that in rich medium, SAM transporter cannot work efficient enough to reach the same SAM pool as in the wild type.

The previous research showed that cells containing *metJ* allele can produce constitutive expression of most genes in the *met* regulon and excess production and even export of methionine and its metabolites (Usuda and Kurahashi, 2005). To prove whether MetJ as an aporepressor binds corepressor SAM to become repressor complex, which can block *met* genes transcription. We removed the corepressor and prevented formation of the complex to see whether *met* genes can be upregulated. Thus we constructed *ΔmetK ΔmetJ* double strain (SAD16Δ*metJ*/pSAM/pmet) to see whether in vivo data can support the model of a MetJ-SAM repressor complex. The results showed that for all the *met* genes that we tested, depletion of SAM cannot upregulate. Moreover, the Beta-Gal units for *ΔmetK ΔmetJ* strain (SAD16Δ*metJ*/pSAM/pmet) in the medium with SAM is more than 8 times higher than *ΔmetK* strain (SAD16/pSAM/pmet) in the same medium for most tested *met* genes, except *metK*, which is nearly two fold (Table 5). This result is consistent with

that of Dr.Hughes' group. Dr.Hughes' group also got more than 10 times expression of *metB, E, F, R* of the *metJ* defective strain than that of those in wild type strain when there is no T3 SAM hydrolase (

Table 8). It confirms that there is very similar influence of MetJ deficiency on both *met* promoter in *E.coli* and in the *S.typhimurium.*

We know that *ΔmetK* strain needs exogenous supply of SAM in every medium. Nonetheless, addition of SAM is not sufficient to allow growth of the *metK* mutant in minimal medium (Reyes, 2005). Methionine must also be provided, consistent with the idea that methionine is not a repressor, and SAM inhibits both methionine and SAM methionine biosynthesis. By using MiniTn10Kan, we selected the mutant that can grow only with SAM in minimal medium. We called them MNR mutant. MNR6 is one of them.

By sequencing result, we found that the last 7^{th} codon is disrupted in MNR6mutant (

Figure 8). It means that this mutant is MetJ mutant. We tested *met* genes expression in MNR6 mutant. We found that in this mutant, as SAD16Δ*metJ*/pSAM/pmet, depletion of SAM cannot increase *met* genes expression in MNR6/pSAM/pmet. Some *met* genes expression became lower, which is caused by the influence of SAM starvation. And nearly for all the *met* genes, we got relatively high data for MNR6/pSAM/pmet in the LB with SAM compared to *ΔmetK* strain (SAD16/pSAM/pmet) in the same medium. This also confirms that MetJ is the important molecule for repressor of *met*

genes. Moreover, the data of MNR6/pSAM/pmet in the LB with SAM is much lower than that of *ΔmetK ΔmetJ* double deletion strain (SAD16Δ*metJ*/pSAM/pmet) for all the *met* genes except *metB and metK* in the same medium (Table 5). All these data showed that MetJ in MNR6 may work at part of function such as binding to MET boxes on the promoter region of *met* genes. This binding somehow influences the *met* gene expression. In addition, SAM is not involved in this truncated MetJ binding process. From the 2.2 Å resolution of wildtype MetJ crystal structure, one can conclude that MetJ dimers bind to two MET box site of DNA sequence (He et al., 1996). However, other recent data suggested that there is a structural basis for the differences observed in the regulatory effectiveness of MetJ for the various genes of the *met* regulon(Augustus et al., 2006). Moreover, SAM as the co-repressor, can lie on the opposite side of the protein from the DNA-binding motif (Saint-Girons et al., 1986; Rafferty et al., 1989). From the concerved domain analysis (Figure 11), although 3D structure showed that SAM binding sites and DNA binding sites locate at the opposite direction, their primary sequence location is very near. But none of them locate at C terminal end of MetJ protein. From above, it seems that MNR6 MetJ mutant has intact corepressor binding sites and DNA binding sites. However, the data I showed in this thesis suggested that disrupted C terminal of MetJ will definitely influence its SAM binding and its repressor function. The possible explanation is that the disruption of C terminal of MetJ will influence the conformation of corepressor binding site. And this can lead to SAM can not bind MetJ correctly anymore and MetJ loses its function for repressor.

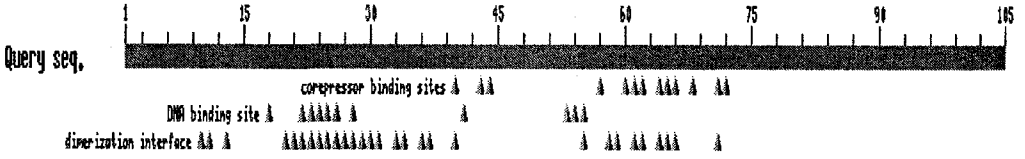


Figure 11 Concerved domain analysis for MetJ

The ruler represents the amino acid sequence of MetJ (105 amino acids).
Corepressor binding sites:40,43..44,57,60..62,64..66,68,71..72;
DNA binding sites: 18, 22..26,28,41,53..55;
dimerization inferface sites: 10..11,13,20..31,33..34,36..37,40,55,58..59,61..62, 64..66,71

Table 7. Data comparation between this study and Dr.Hughes's study for *metK* deficient strain in rich medium

[the data of Dr.Hughes' study was refered from the published paper (LaMonte and Hughes, 2006)]

	This study	Dr.Hughes's study
Medium	LB	YT
strain	SAD16/pSAM/pmet (*ΔmetK*)	*metK*:: Tn5 cells
Met gene-*lacZ* fusion	plasmid	chromosome
Origin of *met* promoter in *met-lacZ* fusion	*E.coli*	*S.typhimurium*
plasmid / ratio	No SAM/SAM	pHBBR2/pBR322[a]
metA	5.2	N/A
metB	19.2	16.8
metC	2.1	N/A
metE	1.5	13.4
metF	2.2	14.8
metK	1.1	N/A
metR	19.2	16.7

a : pHBBR2: T_3 SAM hydrolase expression vector, whose backbone vector is pBR322

pBR322: Backbone of expression vector, no T_3 SAM hydrolase encoded gene. This is the negative control.

Table 8. The result comparation between this study and Dr.Hughes's study for *metJ* deficient strain in rich medium

[the data of Dr.Hughes' study was calucated according to the published data (LaMonte and Hughes, 2006)]

	This study	Dr.Hughes's study
Medium	LB+200μMSAM	YT
strain	SAD16 Δ*metJ* /pSAM/pmet SAD16/pSAM/pmet	*metJ*:: Tn5/pBR322[b] BW545[a]/pBR322
Met gene-*lacZ* fusion	plasmid	chromosome
Origin of met promoter in *met-lacZ* fusion	*E.coli*	*S.typhimurium*
plasmid / ratio	SAD16 Δ*metJ* / SAD16	*metJ*:: Tn5/ BW545
metA	68	N/A
metB	24	16.7
metC	22	N/A
metE	8	10
metF	11	16
metK	1.7	N/A
metR	52	13.4

a : BW545 is the wildtype strain which is used to make *metJ*:: Tn5

b: pBR322: Backbone of expression vector, no T_3 SAM hydrolase encoded gene. This is the negative control.

4.2 The effect of SAM on *met* gene expression in minimal medium

When we grew *ΔmetK* strain (SAD16/pSAM) in minimal medium, we found that we have to put SAM as well as methionine to support cell growth. Thus, when we checked *met* gene expression in minimal medium, we always put 40 μg/ml methionine in the medium for both wild type (Cu/pSAM/pmet) and *ΔmetK* cell growth. The data showed that in wild type strain, depletion exogenous SAM cannot increase *met* genes expression. However, when we observe the data of *ΔmetK* strains(SAD16/pSAM/pmet) in the minimal medium, depletion of exogenous SAM can dramatically increase nearly all tested *met* genes' expression (from 10 times to 100 times, according to different *met* gene tested). The data from minimal medium strongly supports SAM working as corepressor for regulating *met* genes expression. Also, we noticed that in minimal medium, nearly all *met* genes had higher expression in wild type strain (Cu/pSAM/pmet) in the minimal medium without SAM than in *ΔmetK* strains (SAD16/pSAM/pmet) in the minimal medium with SAM. It means that SAM pool in *ΔmetK* strains in minimal medium is higher than SAM pool in the wild type. The above result is opposite to that in LB, in which SAM pool in the *ΔmetK* strains is much less compared to wild type strain. Moreover, the above result can be used to explain why we have to put methionine in the minimal medium to grow *ΔmetK* strains. The reason is that the intracellular SAM pool in *ΔmetK* strains is much higher than wild type, and thus higher SAM pool can inhibit more methionine biosynthesis. Therefore, we have to put methionine in the medium.

4.3 Comparison the effect of SAM on *met* gene expression in rich and minimal medium

Other questions arose when we compared the data from rich medium and minimal medium (Table 9). From the result of no SAM/SAM ratio, we can find that *met* genes expressions are more highly regulated by SAM in minimal medium than in rich medium. It indicates that there is another regulator for methionine biosynthesis in rich medium. It can also be seen that in wild type strain, except *metK*, all the other *met* genes enzyme activity is much higher in minimal medium without SAM than in LB without SAM. This result represents that even for wild type strain, intracellular SAM pool can change in different media. In this dissertation, intracellular SAM pool in LB is much higher than that in minimal medium in wildtype strain. This phenomenon may be explained by using the mechanism of leucine responsive protein (Lrp), which can control the expression of *metK* gene, since *metK* gene is induced by leucine and is repressed by Lrp (Newman et al., 1998). In rich medium, which contains lots of leucine, Lrp binds to leucine to derepress *metK* gene expression. However, in minimal medium, there is no any leucine there, so Lrp can bind the promoter region of *metK* and repress its expression. When we compared the data of *ΔmetK* strain in rich medium with SAM and minimal medium with SAM, the result is opposite to that in wild type strain without SAM in both media. For *ΔmetK* strain, nearly all the *met* genes have higher expression in LB with SAM than in minimal medium with SAM. This trend implies that SAM pool is higher in minimal medium than in LB. It can be explained by the different efficiency of SAM transporter in different medium.

Table 9. The comparation of effect of SAM in rich and minimal medium for Cu/pSAM/pmet and SAD16/pSAM/pmet

Medium A (LB glucose 200uM SAM with antibiotic)
Medium B(LB gluscose no SAM with antibiotic)
Medium C (NIV Glu 40 μg /ml methionine 200uM SAM with antibiotic)
Medium D (NIV Glu 40 μg /ml methionine with antibiotic)

Beta-Gal units																
Strain	**Cu/pSAM/pmet**								**SAD16/pSAM/pmet**							
Medium / plasmid	A	C	C/A	B	D	D/B	B/A	D/C	A	C	C/A	B	D	D/B	B/A	D/C
pmetA	38	703	18.5	79	353	4.5	2.1	0.5	162	391	2.4	852	3857	4.5	5.2	9.9
pmetB	8	81	10.1	28	201	7.2	3.5	2.5	22	16	0.7	429	697	1.6	19.2	44.2
pmetC	220	1651	7.5	311	1795	5.8	1.4	1.1	463	407	0.9	951	10564	11.1	2.1	26
pmetE	51	1292	25.3	53	588	11.1	1.1	0.5	1667	86	0.1	2499	8695	3.5	1.5	100.8
pmetF	125	2042	16.3	78	3627	46.5	0.6	1.8	1610	248	0.2	3585	11881	3.3	2.2	48
pmetK	2463	3853	1.6	6253	6889	1.1	2.5	1.8	6927	3749	0.5	7405	15534	2.1	1.1	4.1
pmetR	29	984	33.9	101	2758	27.3	3.5	2.8	356	478	1.3	6849	8889	1.3	19.2	18.6
pZ1	366	1463	4.0	435	3113	7.2	1.2	2.1	201	1512	7.5	125	1205	9.6	0.6	0.8

4.4 Conclusions

From the above, I can reach the following conclusions according to the data I got.

1. In general, all results support the in vitro model of MetJ-SAM repressor complex binding *met* gene promoters to block their transcription.
2. All *met* genes expressions are more highly regulated by SAM in minimal medium than in rich medium. It indicates that there is another regulator for methionine biosynthesis in rich medium.
3. *MetK* gene is surprisingly highly expressed and relatively little affected by exogenous SAM.
4. In MNR6, *met* genes expressions are highly derepressed, which, however, can not reach the level in Δ*metJ* strain. It suggests that the truncated MetJ in MNR6 retains the considerable function.

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