Biochemical and genetic characterization of mercaptopyruvate sulfurtransferase and paralogous putative sulfurtransferases of *Escherichia coli*

Promjit Jutabha

Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Biochemistry

Timothy J. Larson, Chair
Jiann-Shin Chen
Dennis R. Dean
David L. Popham
Ann M. Stevens

June 11, 2001
Blacksburg, VA

Keywords: Mercaptopyruvate sulfurtransferase, rhodanese, sulfurtransferase ortholog, *Escherichia coli*

Biochemical and genetic characterization of mercaptopyruvate sulfurtransferase and paralogous putative sulfurtransferases of *Escherichia coli*

Promjit Jutabha

(ABSTRACT)

Sulfurtransferases, including mercaptopyruvate sulfurtransferase and rhodanese, are widely distributed in living organisms. Mercaptopyruvate sulfurtransferase and rhodanese catalyze the transfer of sulfur from mercaptopyruvate and thiosulfate, respectively, to sulfur acceptors such as thiols or cyanide. There is evidence to suggest that rhodanese can mobilize sulfur from thiosulfate for *in vitro* formation of iron-sulfur clusters. Additionally, primary sequence analysis reveals that MoeB from some organisms, as well as ThiI of *Escherichia coli*, contain a C-terminal sulfurtransferase domain. MoeB is required for molybdopterin biosynthesis, whereas ThiI is necessary for biosynthesis of thiamin and 4-thiouridine in transfer ribonucleic acid. These observations led to the hypothesis that sulfurtransferases might be involved in sulfur transfer for biosynthesis of some sulfur-containing cofactors (e.g., biotin, lipoic acid, thiamin and molybdopterin). Results of a BLAST search revealed that *E. coli* has at least eight potential sulfurtransferases, besides ThiI. Previously, a *glpE*-encoded rhodanese of *E. coli* was characterized in our laboratory. In this dissertation, a mercaptopyruvate sulfurtransferase and corresponding gene (*sseA*) of *E. coli* were identified. In addition, the possibility that mercaptopyruvate sulfurtransferase could participate or work in concert with a cysteine desulfurase, IscS, in the biosynthesis of cofactors was examined.

Cloning of the *sseA* gene and biochemical characterization of the corresponding protein were used to show that SseA is a mercaptopyruvate sulfurtransferase of *E. coli*. A strain with a chromosomal insertion mutation in *sseA* was constructed in order to characterize the physiological function of mercaptopyruvate sulfurtransferase. However, the lack of SseA did not result in a discernable phenotypic change. Redundancy of

sulfurtransferases in *E. coli* may prevent the appearance of a phenotypic change due to the loss of a single sulfurtransferase. Subsequently, other paralogous genes for putative sulfurtransferases, including *ynjE* and *yceA*, were cloned. Strains with individual deletions of the chromosomal *ynjE* and *yceA* genes were also constructed. Finally, strains with multiple deficiency in potential sulfurtransferase genes, including *sseA*, *ynjE* and *glpE*, as well as *iscS*, were created. However, no phenotype associated with combinations of *sseA*, *glpE* and/or *ynjE* deficiency was identified. Therefore, the physiological functions of mercaptopyruvate sulfurtransferase and related sulfurtransferases remain unknown.

Acknowledgments

I would like to express my sincere gratitude to Dr. Timothy J. Larson, my advisor, for his generosity, patience and guidance. He provided valuable suggestions and encouragement throughout my research, which allowed me to gain more experience during work in his laboratory.

I am truly grateful to my committee members, Dr. Jiann-Shin Chen, Dr. Dennis R. Dean, Dr. David L. Popham, and Dr. Ann M. Stevens. Their suggestions and concern led me to the completion of the Doctorate. Thanks are also extended to Dr. Thomas W. Keenan for being chairman in my preliminary examination. In addition, I appreciate Dr. John L. Hess, the Department Head, who supported my travel to the ASBMB conference 2000 at Boston, Massachusetts.

I am thankful to other faculty members of Department of Biochemistry who brightened for me the knowledge in biochemistry and molecular biology. These include Dr. Peter J. Kennelly, Dr. David R. Bevan, Dr. Walter G. Niehaus Jr., Dr. Robert H. White, Dr. William E. Newton, Dr. Eugene M. Gregory, Dr. Malcolm Potts, Dr. Thomas O. Sitz, Dr. Shirley Luckhart and Dr. Glenda E. Gillaspy. Thanks to Dr. Brian Storrie who gave me an opportunity to rotate in his interesting laboratory for a semester.

My appreciation also goes to Janet L. Donahue and her lovely husband, Joel, for their kindness and unforgettable assistance. Besides biochemistry, I gained knowledge about language and American's culture from their family. Additionally, I thank previous lab specialist, Ali Bhattacharya, for training in general molecular techniques.

I especially thank graduate students in Larson's laboratory, Farzana Ahmed, Bekir Col and Hui Cheng, for their friendship, assistance and useful opinion. Activities that we did together were very cheerful and impressive. Thanks to graduate students in Department of Biochemistry, especially, Kanit Vichitphan and Hong Li for their help and friendship. In addition, I would like to thank Dr. Somchai Santiwatanakul and Capt. Eakasit Weeratumanoon, who provided assistance when I stayed in Blacksburg.

Appreciation is extended to the Royal Thai Government for a scholarship to study at Virginia Tech. Moreover, I would like to thank to the Department of Pathology, Faculty of Medicine, Srinakarinwirot University for allowing me to pursue my Ph.D.

Additional thanks go to my special friend, Lt. Col. Dr. Thongchai Chutivut, who heartened and supported me all along.

I am deeply indebted to my parents and my two sisters for their unconditional love and everlasting support throughout my education. Finally, my grandmothers and grandfathers are unforgettable to thank for their love and care from my earliest memory, although three of them have no opportunity to see my success.

Table of Contents

Acknowledgments iv
Table of Contents v
Figures vii
Tables ix
Abbreviations x
Chapter 1 Introduction 1
1.1. Sulfur: an essential component of biological compounds 1
1.2. Assembly of iron-sulfur clusters and the role of cysteine desulfurase 2
1.3. Sulfur-containing cofactors 6
1.3.1 Biotin: Functions and biosynthesis 6
1.3.2 Lipoic acid: Functions and biosynthesis 9
1.3.3 Thiamin: Functions and biosynthesis 11
1.3.4 Molybdopterin: Functions and biosynthesis 13
1.4. Thionucleosides in tRNA 15
1.5. Sulfurtransferases and proposed physiological roles 18
1.5.1 Rhodaneses: catalytic reaction, distribution 18
1.5.2 MST: catalytic reaction, distribution 20
1.5.3 Thiosulfate: thiol sulfurtransferase: catalytic reaction, distribution 22
1.5.4 Proposed functions of sulfurtransferases 23
1.6: Statement of Hypothesis 24
1.7. References 29
Chapter 2 Identification of a gene encoding mercaptopyruvate sulfurtransferase of *Escherichia coli* 42
2.1: Abstract 42
2.2: Introduction 42
2.3: Experimental Procedures 45
2.4: Results 57
2.5: Discussion 64

2.6: References 81
Chapter 3 Construction of *Escherichia coli* strains deficient in multiple sulfurtransferase genes 85
3.1: Abstract 85
3.2: Introduction 85
3.3: Experimental Procedures 87
3.4: Results and discussion 102
3.5: Conclusions and possible future work 110
3.6: References 120
Curriculum Vitae 123

Figures

Chapter 1

Figure 1.1 The carbon-sulfur bond forming reactions in the biosynthesis of biotin and lipoic acid. 25
Figure 1.2 Biosynthesis of thiamin and molybdopterin. 26
Figure 1.3 Proposed pathways and potential roles of IscS and sulfurtransferases. 27
Figure 1.4 Structures with the abbreviations of some commonly found thionucleosides in tRNA. 28

Chapter 2

Figure 2.1 Schematic for disruption of *sseA*. 70
Figure 2.2 SDS-PAGE illustrating the overexpression and purification of SseA-His_6. ... 71
Figure 2.3 SDS-PAGE illustrating the overexpression and purification of SseA. 72
Figure 2.4 Native molecular weight of MST. 73
Figure 2.5 Dependence of MST activity on pH. 74
Figure 2.6 Mass spectrometric analysis of the precipitate obtained from the incubation of SseA with 3-mercaptopyruvate. 75
Figure 2.7 Dependence of MST activity on substrate concentration. 76
Figure 2.8 Substrate inhibition by mercaptopuvate and cyanide. 77
Figure 2.9 Southern analysis for verification of *sseA*::Sp^r in the genome. 78
Figure 2.10 Growth of the *sseA*::Sp^r strain and with the wild type on mercaptopyruvate versus sulfate. 79
Figure 2.11 The predicted amino acid sequence of SseA. 80

Chapter 3

Figure 3.1 Sequence alignment of the conserved regions of the predicted sulfurtransferases of *Escherichia coli*. 112
Figure 3.2 The pFRT-K and pKO3 vectors used in the gene replacement experiments. 113
Figure 3.3 Disruption of *ynjE*. 114
Figure 3.4 Construction of *ynjE*-deletion in the genome. 115

Figure 3.5 Verification of the *ynjE*-disrupted strains by PCR, using primers YNJE-F and YNJE-RXH. 116
Figure 3.6 Introduction of Δ*yceA*::Km^r into the genome..... 117
Figure 3.7 SDS-PAGE analysis of YnjE-His$_6$. 118
Figure 3.8 Comparison of amino acid sequences around the active site cysteine region of *Escherichia coli* IscS with *Azotobacter vinelandii* IscS and NifS. 119

Tables

Chaper 2

Table 2.1 *E. coli* strains and plasmids 47
Table 2.2 Oligonucleotides used in this study 50
Table 2.3 MST activities in crude extracts of wild type and *sseA*::Sp^r strains. 62

Chapter 3

Table 3.1 Strains of *E. coli* used in this study 98
Table 3.2 Plasmids used in this study 100
Table 3.3 Oligonucleotides used in this study 101
Table 3.4 MST and rhodanese activities of the control and *ynjE* overexpression strains. 103
Table 3.5 MST and rhodanese activity of the wild type and of strains with putative sulfurtransferase gene(s) deletion. 105

Abbreviations

ACP	acyl-carrier protein
AMP	adenosine monophosphate
ARNR	anaerobic ribonucleotide reductase
ATP	adenosine triphosphate
bp	base pair
C-DES	cysteine desulfurylase
Cm	chloramphenicol
CMP	cytidine monophosphate
CSD	cysteine-sulfinate desulfinase
DNA	deoxyribonucleic acid
DNP	dinitrophenylhydrazine
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
GMP	guanosine monophosphate
GSH	glutathione
IMP	inosine monophosphate
IPTG	isopropylthiogalactoside
kDa	kilodaltons
kb	kilobase pair
Km	kanamycin
K_m	Michaelis constant
LAM	lysine 2,3-aminomutase
MGD	molybdopterin guanine dinucleotide
MoCo	molybdenum cofactor
MPT	molybdopterin
ms^2i^6A	2-methylthio-N^6-isopentenyladenosine
MST	mercaptopyruvate sulfurtransferase
NAD	nicotinamide adenine dinucleotide

NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PFL AE	pyruvate formate-lyase activating enzyme
PLP	pyridoxal-5-phosphate
PMSF	phenylmethylsulfonyl fluoride
Sp	spectinomycin
s^2U	2-thiouridine
s^4U	4-thiouridine
SAM	S-adenosyl-L-methionine
SDS	sodium dodecyl sulfate
Tc	tetracycline
TPP	thiamin pyrophosphate
Tris-HCl	[Tris (hydroxymethyl) aminomethane] hydrochloride
tRNA	transfer ribonucleic acid
UBA	ubiquitin activation enzyme

Chapter 1
Introduction

1.1. Sulfur: an essential component of biological compounds

Sulfur is a versatile nonmetallic element essential for life. It is required for the biosynthesis of vital cofactors (e.g. biotin, lipoic acid, molybdopterin and thiamin), metal-sulfur clusters (e.g. Fe-S) of proteins and thionucleosides (e.g. 4-thiouridine) of transfer ribonucleic acids (tRNA). Regarding the form of sulfur in biological systems, sulfate (SO_4^{2-}), the most oxidized form of sulfur, is of limited use to higher organisms, being used only for sulfation and detoxification reactions (1). On the other hand, the versatile chemistry of the lower oxidation states of sulfur is vital in nature for anabolic reactions. Sulfenate (SO^-) is probably at the upper limit of oxidation compatible with its use, as it can be recovered via the reaction: RSOH + cysSH → RSScys + H_2O (1). In fact, sulfenate has been identified in proteins as a catalytically essential redox center or as an intermediate, for example in NADH peroxidase and NADH oxidase (2). Sulfane sulfur, which has an apparent oxidation state of zero (6 electrons), is of interest here because it is the mobilized form of reactive sulfur that is used for metal cluster formation and initiation of radical reactions (1). The term sulfane designates sulfur atoms that are bonded covalently only to other sulfur atom (3). It is often incorrectly called sulfur zero, S^0. However, zero-valent sulfur does not exist because sulfur has the tendency to self-associate and is not found as a single atom. Sulfane compounds involved in biochemical systems include elemental sulfur (S_8), persulfides (R-S-S^-), polysulfides (R-S_n-R) where n ≥3, polythionates ($^-SO_3$-S_n-SO_3^-), thiosulfate ($S_2O_3^{2-}$) and alkylthiosulfonate [$RS(O)_2S^-$] (4, 5). Sulfanes in small molecules are fairly labile, readily oxidizing in air, reducing with thiols and decomposing slowly in dilute acid to release free sulfur (3), while sulfanes on proteins can be protected and transported to sites of biosynthesis (1). Elemental sulfur is not soluble and may not be accessible to microorganisms. However, sulfur or sulfides are rapidly incorporated into polysulfide chains, or into rings of S_8, or exchange to similar compounds (1). The most characteristic reaction of sulfane sulfur is

its reaction at pH 8.5-10, with cyanide ion (CN^-) to form thiocyanate (3, 5). Other compounds that contain cyanolyzable sulfur, i.e. reactive sulfur atoms with properties similar to the sulfane mentioned above, are disulfides with a double-bonded carbon adjacent to the sulfur-bonded carbon such as in alkenyl disulfides (R-S-S-CH_2-CH=CH-R), or in disulfides of 3-mercaptopyruvate (R-S-S-CH_2-CO-CO_2H). The sulfur of reduced mercaptans is not cyanolyzable (4). This may be important in some reactions to be discussed later.

Sulfane sulfur is the precursor of the sulfur that becomes incorporated into biological compounds. Two main categories of sulfur incorporation can be differentiated here: those involving reactions with metal to form metal-sulfur clusters (inorganic) and those involving formations of carbon-bound sulfur (organic). Only one example of the inorganic system will be discussed here, the iron-sulfur (Fe-S) cluster, although there are many vital reactions in microorganisms that rely on other metals bound to sulfur, including Ni-S, Mo-S and Cu-S clusters. At present, the pathways of Fe-S cluster synthesis and disassembly are not completely established. However, the key proteins participating in providing the sulfur in a reactive form for Fe-S cluster formation have been recently discovered and this will be discussed in part B. The organic system includes the C-S bond forming reactions. The discussion will be limited here to the biosynthesis of four types of sulfur-containing cofactors (part C) and two types of thionucleosides of transfer RNA (part D). Likewise, the complete mechanisms for the incorporation of the sulfur atom into thionucleosides and cofactors remain to be elucidated. The main hypothesis of the research in our laboratory is that some aspects of sulfur delivery for the biosynthesis of some of the S-containing compounds listed above require the activity of a group of enzymes named sulfurtransferases. Background information regarding sulfurtransferases will be covered in part E.

1.2. Assembly of iron-sulfur clusters and the role of cysteine desulfurase

Iron-sulfur (Fe-S) clusters contained in Fe-S proteins are widely distributed in the three domains of living organisms. These proteins display a broad range of functions,

including electron transfer, catalysis in redox and nonredox reactions, and regulation of gene transcription and translation (6-8). Protein containing Fe-S clusters are important for the regulation of de novo synthesis of proteins and also for the repair of certain proteins that are damaged during oxidative stress. An example is that of the transcription factor FNR of *E. coli*, which governs the transition from anaerobic to aerobic metabolism, with alternation between the holo $[4Fe\text{-}4S]^{2+}$ form, the inactive $[2Fe\text{-}2S]^{2+}$ form and the apo-form, by means of its oxygen-sensitive Fe-S cluster (9). In order to maintain the functions of the Fe-S proteins, recycling (disassembly and reassembly or repair) of the clusters must occur continuously *in vivo*. However, the biological mechanism for the assembly and disassembly of Fe-S clusters is not completely known. Research has shown that cysteine sulfur is the origin of the sulfide sulfur of iron-sulfur proteins in aerobically grown *E. coli* (10). Studies on *nif* mutants of *Azotobacter vinelandii* identified *nifS* as an essential gene for the activity of nitrogenase, an enzyme required for nitrogen fixation (11). The characterization of NifS by Dean and coworkers (12, 13) lent insight into the process of Fe-S cluster formation for nitrogenase. The NifS protein is a pyridoxal phosphate (PLP)-dependent cysteine desulfurase, and was found to catalyze the *in vitro* reconstitution of Fe-S clusters of several proteins, including the nitrogenase iron protein component (13).

$$\text{L-cysteine} \rightarrow \text{alanine} + \text{sulfur}_{\text{elemental}} \quad \text{(catalyzed by NifS or IscS or Nfs1)}$$

NifS is the key enzyme in providing the sulfur in a reactive form. The enzyme is the carrier for sulfur, which is present in a sulfane structure attached to an active site cysteinyl residue of NifS in the form of RSSH (protein-bound persulfide). In fact, there are enzymes that have long been known to be potential sulfane carriers: rhodanese (14, 15) and mercaptopyruvate sulfurtransferase (16), which will be discussed later in part E. NifU and IscU, proteins involved in building the iron assembly for the formation of Fe-S centers, have also been identified (17). However, only proteins participating in sulfur mobilization will be focused on here.

Cysteine desulfurases homologous to NifS have been discovered in non-nitrogen-fixing bacteria, eukaryotes and archaea (18, 19). These enzymes might have a general role in Fe-S cluster formation. NifS-like proteins have been purified from *A. vinelandii* (13) and *E. coli* (20). These proteins were named IscS to indicate a role in iron-sulfur cluster assembly and to distinguish them from the one encoded by the *nif* clusters of nitrogen-fixing bacteria (18). In addition, Nfs1p and m-Nfs1 were identified in the yeast *Saccharomyces cerevisiae* (21) and mouse (22), where they appear to play a role in Fe-S cluster assembly in mitochondria.

An alternative to the IscS or NifS proteins that should be mentioned here is cysteine desulfhydrase. The enzyme has been found in *Salmonella typhimurium* (23) and *Synechocystis* (24). It catalyzes the formation of cysteine hydrogen disulfide (or persulfide, CysSSH), pyruvate and ammonia from cyst(e)ine (1). Therefore, cysteine desulfhydrase may also provide sulfane sulfur for Fe-S center synthesis.

$$\text{L-cysteine} + H_2O \rightarrow \text{pyruvate} + NH_3 + H_2S$$

(catalyzed by cysteine desulfhydrase)

The *iscS* gene in *A. vinelandii* and the *Nfs1*gene in yeast seem to be essential for the viability of those organisms, since attempts to delete the genes were unsuccessful (18). In contrast, disruption of *iscS* in *E. coli* did not preclude its survival. Thus, studies of the function of IscS *in vivo* were performed (25, 26). The *E. coli* genome contains three genes, including *iscS*, with sequence homology to *nifS*. The remaining two NifS homologues are cysteine-sulfinate desulfinase (CSD) and CsdB, which also have cysteine desulfurase activity although cysteine sulfinate and selenocysteine are the preferred substrates, respectively (27). Using the *iscS* deletion strain, Kiley and coworkers (25) observed decreased specific activities for proteins containing [4Fe-4S] clusters, including membrane-bound proteins (NADH dehydrogenase I and succinate dehydrogenase) and soluble proteins (aconitase B, 6-phosphogluconate dehydratase, glutamate synthase, fumarase A, and FNR). Furthermore, studies using FNR* mutants provided evidence indicating that the decrease in enzyme activity is directly caused by a defect in Fe-S

cluster synthesis. FNR is normally active only under anaerobic conditions, because its Fe-S cluster is O_2-labile. However, the FNR* variants have an altered requirement for the Fe-S cluster and remain active under aerobic growth conditions. It was found that the deletion of *iscS* has no effect on the aerobic activity of FNR*. This agrees with the fact that the FNR* activity is independent of the Fe-S cluster under aerobic conditions. In contrast, the *iscS* deletion decreased the activity of FNR* under anaerobic conditions (where the activity depends on the Fe-S cluster), as is found in the wild type FNR (25). These results indicate that IscS plays a direct role in providing the sulfur for construction of Fe-S clusters *in vivo*. Another experiment that implicates IscS in the *in vivo* process of Fe-S cluster assembly was done by examination of the co-overexpression of the ORF1-ORF2-*iscS*-*iscU*-*iscA*-*hscB*-*hscA*-*fdx*-ORF3 gene cluster along with ferredoxins (Fd) encoded by compatible plasmids (28). A dramatic increase in holoFd (protein-bound Fe-S clusters) production was observed with increased dosage of the *isc* gene cluster. These results indicate that the proteins encoded by the *iscS* gene cluster are involved in the synthesis of Fe-S clusters.

Deletion of *iscS* has a general effect on the growth rate of *E. coli*. Δ*iscS* strains grow at half the rate of the wild type even in rich medium (25, 26). The addition of isoleucine and valine reduces the lag time of the Δ*iscS* strain growing on minimal medium, which suggests that an enzyme involved in isoleucine/valine biosynthesis, possibly the Fe-S cluster enzyme dihydroxy-acid dehydratase, is defective in the absence of IscS (26). Furthermore, the lack of IscS causes an auxotrophy for thiamin and nicotinic acid under aerobic condition, whereas only nicotinic acid was required under anaerobic conditions (25, 26). The requirement for nicotinic acid is most likely explained by a defective Fe-S cluster in one of the biosynthesis enzymes. A previous study in *Bacillus subtilis* demonstrated that *nadA* is required for nicotinamide adenine dinucleotide (NAD) biosynthesis (29). NAD is synthesized from quinolinic acid. The Fe-S cluster enzyme NadA is a putative quinolinate synthetase, one component of the system for quinolinate production from L-aspartate and dihydroxyacetone phosphate. A defect in NadA led to an auxotrophy for nicotinic acid. The requirement for thiamin is quite complex and will be explained later in part C. The effects of Δ*iscS* suggest that IscS plays a significant

broad role as a potential sulfurtransferase. IscS appears to be required for the *in vivo* formation of Fe-S clusters and the biosynthesis of a variety of sulfur-containing compounds, including thiamin and 4-thiouridine of tRNA (26, 30). The requirement of IscS for synthesis of the last compound will be discussed in part D.

1.3. Sulfur-containing cofactors

Cofactors are small organic molecules or metallic cations that possess special chemical reactivities or structural properties that are essential for enzyme-catalyzed reactions. The cofactor usually binds tightly to a special site on the enzyme, and is sometimes referred to as a prosthetic group, which makes an inactive apoenzyme become an intact holoenzyme. Eight sulfur-containing cofactors have been identified in living organisms, including coenzyme A, S-adenosyl methionine (SAM), thiamin pyrophosphate (TPP), biotin, molybdopterin, lipoic acid, coenzyme M and coenzyme B [*N*-7(-mercaptoheptanoyl) threonine phosphate] (31-34). The C-S bond formation chemistry in the biosynthesis of coenzyme A and SAM is relatively simple (35) and will not be covered here. Additionally, little is known about the formation of coenzyme M and coenzyme B, cofactors that are involved in the final steps of methane formation, and will not be covered here. The enzymology of C-S bond forming reactions and genetics involved in the biosynthesis of biotin, lipoic acid, thiamin, and molybdopterin will be discussed in this chapter.

1.3.1 Biotin: Functions and biosynthesis

Biotin (vitamin H) is a water-soluble vitamin that can be produced by bacteria, plants and a few fungi (36). Biotin plays a very important physiological role as the prosthetic group of several carboxylases involved in central metabolism, including gluconeogenesis and fatty acid biosynthesis, and also in secondary metabolism (36). Biotin functions in ATP-dependent carboxylation reactions involving bicarbonate as the carboxylating agent. No severe biotin deficiency has been observed in humans, except in cases of some genetic diseases (37). However, cases of biotin deficiency related to diet were observed in animals (38), so biotin is added to animal food. Additionally, it is used in fermentation

broth and in cosmetics. Therefore, production of biotin by fermentation is a subject of interest. This requires identification and characterization of the *bio* genes and the gene products in a variety of microorganisms and plants (36). The discussion in this part will include mainly work done in *E. coli*.

The biotin biosynthetic pathway is not completely characterized, especially the steps leading up to synthesis of pimeloyl-CoA and the final step where a sulfur atom is inserted between the saturated C6 and C9 carbons of dethiobiotin to form biotin (Figure 1.1a). The last step is of interest here. Identification of the source of sulfur for biotin synthesis has been a very challenging problem. There is evidence that cysteine acts as the metabolic origin of the sulfur atom of biotin in *E. coli* (39-41), but this does not mean that cysteine is the immediate sulfur donor. Early studies by genetic complementation identified the *bioB* gene product from *E. coli* as an essential component of biotin synthase (EC 2.8.1.6) that catalyzes biotin formation (42). Biotin synthase (BioB) is a dimeric protein of 38 kDa monomers that contains two $[2Fe\text{-}2S]^{2+}$ clusters per BioB dimer, when it is purified under aerobic conditions (43), and contains two $[4Fe\text{-}4S]^{2+}$ clusters per dimer under strict anaerobiosis (44, 45). Sanyal *et al.* (46), working with an *in vitro* assay with well-defined mixtures, found that biotin was generated by a mixture of purified BioB along with flavodoxin, flavodoxin reductase, NADPH, S-adenosyl-L-methionine (SAM), cysteine and dithiothreitol. However, only about 1 mole of biotin per mole of BioB was made under these conditions (no turnover). They also found that the sulfur of [^{35}S] cysteine or [^{35}S] SAM was not incorporated into biotin and suggested that the Fe-S center could be involved. Later work, using isotopically labeled sulfide ($^{34}S^{2-}$) in the Fe-S cluster of BioB, demonstrated that the sulfur of biotin is actually derived from the Fe-S cluster of the synthase itself and explained that biotin synthase functions as a substrate rather than as a catalyst (47, 48). The participation of the Fe-S cluster explains why no turnover was observed *in vitro*.

The exact mechanism of sulfur insertion by biotin synthase is still to be unraveled. BioB and the sequences of other biotin synthases from 14 sources contain highly conserved motifs, namely, a Cys triad CxxxCxxC, an isolated C and a cluster YNHNL that belong to the active site (36). The Cys triad motif, which is probably liganded to the iron-sulfur

center, is also found in pyruvate formate-lyase activating enzyme (PFL AE), anaerobic (type III) ribonucleotide reductase (ARNR), lysine 2,3-aminomutase (LAM) and lipoic acid synthase (LipA). PFL AE and ARNR have been shown to use SAM and reduced flavodoxin to catalyze the formation of an intermediate glycyl radical on the protein backbone, whereas LAM appears to use SAM and pyridoxal-5'-phosphate to catalyze direct formation of substrate radical (49, 50). The requirement for SAM, NADPH and the flavodoxin system in BioB-catalyzed biotin formation led to the suggestion that BioB is also an Fe-S enzyme with a radical-based mechanism. It was proposed that two molecules of SAM are used to synthesize one molecule of biotin, i.e. one for dethiobiotin to form the intermediate, and a second for the intermediate to form biotin (51). Begley *et al.* (31) proposed a detailed pathway for the mechanism of reductive cleavage of SAM by the reduced Fe-S cluster of BioB and sulfur transfer from the Fe-S cluster (immediate sulfur donor) to the dethiobiotin radical, producing biotin and an incomplete Fe-S cluster. However, many aspects of the very complex mechanism of biotin synthase have to be elucidated. For example, it is not clear how SAM is reductively cleaved to deoxyadenosyl radical and methionine through the reduced Fe-S cluster despite unfavorable redox potentials. In addition, the enzyme(s) involved in the reassembly of the Fe-S cluster (in order to participate in another round of catalysis) remain to be determined. Recently, *in vitro* experiments to reconstitute *E. coli* apo-biotin synthase with sulfide-producing systems have been carried out. NifS protein from *A. vinelandii* and cysteine desulfurylase (C-DES) from *Synechocystis*, as well as rhodanese from bovine liver, were able to mobilize the sulfur, respectively, from cysteine and thiosulfate for the formation of a [2Fe-2S] cluster in the apoprotein of *E. coli* biotin synthase (52). However, this study showed that although the apoenzyme could be reconverted to holoform with cysteine and NifS, addition of these two components to the complete *in vitro* system had no significant effect on the turnover of the enzyme (52). Other unidentified factors may be involved *in vivo* besides NifS-like enzymes.

1.3.2 Lipoic acid: Functions and biosynthesis

Lipoic acid (6,8-dithioctanoic acid or 1,2-dithiolane-3-pantanoic acid) is a widely distributed protein-bound cofactor among plants, animals and microorganisms (36). It is essential for the activity of a variety of enzyme complexes that catalyze oxidative decarboxylations. *E. coli* cells express three such lipoate-dependent multienzyme complexes, including the well-studied pyruvate and α-ketoglutarate dehydrogenases, which are the enzymes involved in the glycolytic pathway and the tricarboxylic acid cycle, as well as the recently-identified glycine cleavage enzyme, glycine decarboxylase (53). Lipoic acid is attached by an amide linkage via its carboxylic acid group to the ε-amino group of a specific lysyl residue within a subunit of these enzymes. The redox-active disulfide bond of the lipoated protein functions as a covalently-bound carrier of reaction intermediates between successive active sites within these large multisubunit enzyme complexes.

Early studies established that octanoic acid is the fatty acid precursor to lipoic acid synthesized by *E. coli* (54, 55). The pathway of lipoic acid biosynthesis consists of two steps in which two sulfur atoms are inserted into the carbon chain at C6 and C8 of octanoic acid (Figure 1.1b). Although the sulfur atom is known to originate from cysteine, the mechanism for sulfur incorporation is poorly understood. The basic mechanism for C-S bond formation is thought to be very similar to the one established for biotin biosynthesis. However, the situation is slightly different since two sulfur atoms have to be inserted during lipoic acid synthesis whereas a single sulfur atom is inserted between two carbon atoms in biotin synthesis. Stable isotope studies showed that 8-mercaptooctanoic acid is a likely intermediate, indicating that insertion of sulfur occurs first predominantly at C8 of octanoic acid and then at C6 of 8-mercaptooctanoic acid (54). There is evidence to indicate that in *E. coli*, lipoate synthase, the *lipA* gene product, is involved in the insertion of at least the first sulfur into the octanoic acid backbone (56). The *lipA* mutants could grow in the presence of 8-mercaptooctanoic or to a lesser extent with 6-mercaptooctanoic acid, but not with octanoic acid (56). Biochemical data that 6-

mercaptooctanoate was found to be incorporated into lipoic acid to a significant degree, also agree with a preference for sulfur insertion first at C8, but that insertion first at C6 occurs occasionally (55).

Regarding lipoic acid biosynthesis in eukaryotes, the *LIP5* gene from *Saccharomyces cerevisiae* (57) and the *LIP1* gene from *Arabidopsis* (58) have been isolated. The protein sequences deduced from these genes share 43% and 44% identical residues with LipA of *E. coli*. The regions of these two proteins that are different from the LipA contain mitochondrial targeting signals (83 and 36-amino acid extensions at the N-termini).

LipA and lipoate synthase from other organisms (14 sources) display some amino acid sequence similarity (36). They contain two conserved Cys motifs: CEExxCPNxxC and CxxxCxxC. The latter Cys motif is probably liganded to the Fe-S cluster, and is strictly conserved in both lipoate and biotin synthases. In recent studies (32, 44), it was demonstrated that the purified lipoate synthase from *E. coli* contains Fe-S clusters although there is some discrepancy about the number and form of Fe-S clusters per monomer. By analogy with BioB, it is probable that the sulfur source for lipoic acid synthesis is also the Fe-S cluster of LipA. *E. coli* requires the *lipB* gene product to transfer the lipoate that is synthesized by LipA to apoproteins, forming lipoylated proteins (59). LipB utilizes lipoyl-acyl-carrier-protein (ACP) as a source of lipoyl groups, and can use the octanoyl groups from octanoyl-ACP when lipoyl-ACP is limiting (60). Miller *et al* (61) found that lipoylated pyruvate dehydrogenase complex (PDC) was formed *in vitro* when reduced LipA was incubated with octanoyl-ACP, LipB, apo-PDC, and SAM. This result provides information that LipA utilizes SAM for radical-based chemistry in C-S bond formation (61). However, there is still no evidence for the requirement for the flavodoxin system, which is needed in biotin synthase catalysis. Further characterization of the mechanism to regenerate an active Fe-S cluster is also required.

1.3.3 Thiamin: Functions and biosynthesis

Thiamin or vitamin B_1 is an essential vitamin in the human diet; deficiency can cause beriberi. Thiamin is the precursor of the coenzyme thiamin pyrophosphate (TPP), which is important for carbohydrate metabolism. Specifically, TPP is a cofactor of enzymes such as α-ketoacid decarboxylase, α-ketoacid dehydrogenase, transketolase and acetolactate synthase. The mechanistic role of thiamin in all these reactions is to stabilize the acyl carbanion. Nevertheless, the biosynthetic pathway for thiamin biosynthesis is still not completely understood (62).

The thiamin molecule is comprised of two parts: the pyrimidine moiety (4-amino-5-hydroxymethyl-2-methylpyrimidine) and the thiazole moiety (5-(2-hydroxyethyl)-4-methylthiazole). Prokaryotes and eukaryotes utilize different pathways for thiamin biosynthesis, especially for construction of the pyrimidine unit (62). The pathway is very complicated and not yet entirely known. Thiamin biosynthesis in *E. coli* is emphasized here. In *E. coli*, the precursors of the thiazole moiety are tyrosine, cysteine and deoxy-D-xylulose-5-phosphate, which provide the nitrogen, the sulfur and the carbon atoms, respectively. The coupling of thiazole with pyrimidine pyrophosphate, followed by a final phosphorylation (catalyzed by thiamin phosphate synthase and thiamin phosphate kinase, respectively), gives TPP (62). Genes involved in thiamin biosynthesis that have been identified in *E. coli* comprise three operons and four single-gene loci. Each of the *thi* operons is transcriptionally regulated by TPP, while the single genes are not (63).

The biosynthesis of the thiazole moiety involves at least 6 gene products, including ThiF, ThiS, ThiG, ThiH, ThiI and ThiJ (63). Cysteine is the original source of sulfur. The immediate sulfur source in thiazole assembly was demonstrated to be ThiS-thiocarboxylate (ThiS-COSH), a small protein that is post-translationally modified at its carboxy terminal glycine, via an ATP-dependent reaction (64). Studies with a *thiI*-deletion strain revealed that *thiI* is required for ThiS-COSH synthesis *in vivo* (64). In a recent study, the solution NMR structure of ThiS showed that it possesses structural

homology (with 14% sequence identity, including the same C-terminal Gly-Gly motif) to ubiquitin, an abundant protein in eukaryotes involved in targeting proteins for degradation (65). In the ubiquitination process of eukaryotes, the C-terminal glycine of ubiquitin is covalently attached to lysine side chains of target proteins through sequential reactions of ubiquitin activation enzyme E1 (UBA1), conjugation enzyme E2 and protein ligase enzyme E3. The key features are the initial formation of acyl adenylate at the C-terminus of ubiquitin and then the thioester bond formation between the ubiquitin C-terminus and the active site Cys of UBA1.

Ubiquitin-COOH → Ubiquitin-CO-AMP → Ubiquitin-CO-S-E1

An evolutionary link between the ubiquitin system and the pathway of sulfur-carrier protein synthesis in prokaryotes has been suggested by the identification of an ubiquitin-related modifier protein (Urm1) in yeast that has sequence similarlity with prokaryotic ThiS and MoaD (a molybdopterin enzyme) (66). In *E. coli*, ThiF (homologous in sequence to part of UBA1) catalyzes the initial formation of an acyl adenylate intermediate at the C-terminus of ThiS (ThiS-COAMP). Subsequently, ThiS-COAMP is converted to ThiS-COSH by the actions of ThiF, ThiI and ThiJ (31). Figure 1.2a shows the formation of ThiS-thiocarboxylate for thiazole assembly. While ThiJ is the limiting factor in ThiS-COSH biosynthesis (31), ThiI appears to act solely as a sulfurtransferase. A carboxyl terminal domain of ThiI displays sequence similarity to rhodanese and also is suggested to be the ultimate sulfur source for ThiS-COSH synthesis (67). The factor that initiates sulfur mobilization from cysteine for ThiS-COSH formation is IscS, a cysteine desulfurase involved in Fe-S cluster synthesis. Indeed, evidence for sulfur transfer has been obtained from the *in vitro* reconstituted ThiS-COSH formation and the studies in an Δ*iscS* strain (26). Figure 1.3 shows a proposed pathway of sulfur mobilization from IscS-derived cysteine persulfide (IscS-SSH) to ThiI, forming ThiI persulfide (ThiI-SSH). Sulfur is then transferred from ThiI-SSH to ThiS-COAMP.

1.3.4 Molybdopterin: Functions and biosynthesis

Molybdopterin (MPT), or one of its dinucleotide forms, e.g. molybdopterin guanine dinucleotide (MGD), is a component of the molybdenum cofactor (MoCo). MoCo is required for all molybdoenzymes (68), except nitrogenase (69), in all organisms. MoCo-containing enzymes usually catalyze the net transfer of an oxygen atom, ultimately derived from or incorporated into water, to or from their substrates in a two-electron redox reaction. Four families of MoCo-containing enzymes have been identified, on the basis of sequence alignments and spectroscopic properties (70). In humans, MoCo-containing enzymes play important roles in sulfite detoxification and the metabolism of xenobiotics. MoCo biosynthesis, an evolutionarily-conserved pathway present in prokaryotes, eukaryotes and archaea, has been studied most extensively in *E. coli* and will be discussed here.

The molybdenum-containing enzymes of wild-type *E. coli* include nitrate reductase, formate dehydrogenases N and H, biotin-D-sulfoxide reductase, dimethyl-sulfoxide reductase and trimethylamine *N*-oxide reductase (71). Since molybdoenzyme-independent anaerobic respiratory pathways are present, none of the above enzymes is essential for growth of *E. coli*. Therefore, the absence of these enzymes is nonlethal and allows isolation of mutants. Most information on the biosynthesis of MoCo derives from studies of nitrate reductase-deficient mutants of *E. coli* which are resistant to chlorate under anaerobic conditions. In wild-type cells, chlorate is reduced to the highly toxic chlorite ion by nitrate reductase and is thus lethal. Large numbers of chlorate-resistant mutant strains were isolated and some were found to carry defects in MoCo biosynthesis (72). The corresponding genes were subsequently identified. These mutants were called *chl* mutants, and are now renamed *mo* mutants to more accurately reflect the individual genes in MoCo biosynthesis. MoCo is synthesized in *E. coli* as follows:

Guanosine-X → Precursor Z → MPT → MPT-Mo cofactor → MGD-Mo cofactor

The MPT biosynthetic pathway has three stages in common in all organisms utilizing molybdoenzymes. Initially, a guanosine nucleotide is converted into sulfur-free

precursor Z (by functions of MoaA, B and C in the case of *E. coli*). The second step involves the conversion of precursor Z to MPT (Figure 1.2b). This includes a multi-step sulfur transfer mechanism that appears to be similar to the biosynthetic pathway leading to the formation of thiamin. In the final step, molybdenum is incorporated into MPT to form MoCo. In most bacteria, however, additional modification of MoCo occurs with the attachment of GMP, AMP, IMP (inosine monophosphate) or CMP to the phosphate group of MPT, forming the dinucleotide variants. In the case of *E. coli*, the dinucleotide variant is molybdopterin guanine dinucleotide (MGD). The conversion of precursor Z into MPT will be focused on here.

MPT synthase catalyzes the insertion of the dithiolene moiety into precursor Z, converting it to MPT. In *E. coli*, MPT synthase is a heterotetramer composed of two large MoaE subunits (16.8 kDa) and two small MoaD subunits (8.5 kDa). The C-terminal sequence of the small subunit of MPT synthase (MoaD) is Gly-Gly, the same as that of ubiquitin, yeast Urm1 and ThiS. This similarity suggests that thiocarboxylate is formed at the C-terminal glycine residue of MoaD in MPT synthase. Actually, the structural homology to ubiquitin and the insertion of the C-terminus of each MoaD subunit into the large MoaE subunit (to form the active site) have been recently demonstrated from the high-resolution crystal structure of MPT synthase (73). Additionally, MPT synthase in its inactive form has a smaller molecular mass because it lacks the thiocarboxylate at the C-terminal glycine of MoaD (74). MoeB is most likely the MPT synthase sulfurylase, involved in recharging MPT synthase with a sulfur atom after each cycle of catalysis (74). This assertion is based on evidence from an *E. coli moeB* mutant in which precursor Z accumulated and the inactive desulphoMPT synthase complex was isolated. Interestingly, most MPT synthase sulfurylase enzymes from organisms other than enteric bacteria contain a sulfurtransferase domain at their C-termini. These include *Mycobacterium tuberculosis* MoeB, as well as fungal, plant and mammalian MoeB orthologs (PSI BLAST search). In *E. coli*, the MoeB protein, which lacks a C-terminal sulfurtransferase domain, has a nucleotide-binding motif near the N-terminus and two CxxC motifs in the sequence. MoeB has significant sequence similarity to UBA1, the ubiquitin-activating enzyme E1, and ThiF, a thiamin pathway

enzyme that is required for ThiS adenylation. The ubiquitin-dependent protein degradation process includes ATP-dependent protein-linked thioester formation. The sequence similarities to ThiF and UBA1 suggest that MoeB catalyzes the adenylation of the C-terminal glycine of MoaD (33). The presence of a sulfurtransferase domain fused to MoeB of many organisms suggests its possible role in mobilization of sulfur for MPT biosynthesis. The CxxC motifs of MoeB may first serve as sulfur acceptors from a sulfurtransferase, and then as a sulfur donor to generate the thiocarboxylate of MoaD (active MPT synthase). Finally, the sulfur is transferred from MoaD thiocarboxylate into precursor Z to form the dithiolene group of MPT. Recently, Leimkuhler and Rajagopalan (75) demonstrated that L-cysteine could serve as the sulfur source for MPT formation *in vitro* with the requirement of a persulfide-containing sulfurtransferase such as IscS, CSD or CsdB (CSD had the highest activity). However, IscS is not required for the *in vivo* sulfuration of MPT synthase, since an extract of the *iscS*-deletion strain was able to convert externally added precursor Z to MPT. Conclusive demonstration of which other persulfide-containing sulfurtransferase is the physiological sulfur donor for MPT synthesis in the cell is still lacking.

MoCo factor deficiency in humans leads to a combined deficiency of the molybdoenzymes sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase. This rare disease results in serious neurological symptoms including attenuated growth of the brain and untreatable seizures. It is an autosomal recessive trait and usually causes death in early childhood. The molecular basis of MoCo deficiency has been recently identified (76, 77). Two genes found to be involved in MoCo biosynthesis are *MOCS1*, which encodes two enzymes functioning in precursor Z formation (homologous to MoaA and MoaC of *E. coli*), and *MOCS2*, which encodes the small and large subunits of MPT synthase (homologous to MoaD and MoaE in *E. coli*).

1.4. Thionucleosides in tRNA

Many different modified nucleosides are found in transfer RNA. Most of the modified nucleosides are synthesized after transcription of the tRNA has been completed. These

modified nucleosides improve the efficiency of tRNA in translation, can increase or decrease translational fidelity, and make the tRNA less codon-context-sensitive (78-80). Thionucleosides have been found in a wide variety of species although the number and type of sulfur-containing modifications vary between organisms. Figure 1.4 shows structures of some commonly found thionucleosides. 2-Thiopyrimidines, 4-thiouridine and a variety of adenosine derivatives containing a methylthio group at the C^2 position have been found, whereas there is no report of a naturally-occurring thioguanosine modification. Each type of thiolation results from the activity of different enzyme(s) (81). Cysteine was identified as the origin of the sulfur atom in thionucleosides about 30 years ago by using [^{35}S] cysteine labeling (82). However, the exact molecular species involved in transfer of the sulfur to the nucleosides in tRNA remains unknown.

The biosynthesis of 4-thiouridine (s^4U) in *E. coli* has been extensively studied. Initial studies showed that two distinct protein factors are involved in the biosynthesis of s^4U, factors A and C, which are the products of *nuvA* and *nuvC*, respectively (82-84). Furthermore, factor C requires pyridoxal phosphate (PLP) as a cofactor (82). In addition, cysteine and Mg-ATP are necessary for s^4U formation. Surprisingly, *nuvC* mutants are deficient in both s^4U and the thiazole moiety of thiamin. Addition of thiazole to the growth medium satisfies the thiamin requirement but does not restore s^4U in tRNA (84). In two independent studies, the *thiI* gene was isolated and shown to be required for both the biosynthesis of s^4U and thiazole in *E. coli* (67, 85) and thiazole biosynthesis in *S. typhimurium* (86). However, *thiI* may not correspond to the *nuvC* locus because ThiI protein does not bind PLP (87). The shared pathway for s^4U and thiamin biosynthesis suggested that ThiI is a sulfurtransferase. In fact, the C-terminal domain of ThiI displays amino acid sequence similarity to a sulfurtransferase domain and is required for biosynthesis of s^4U (67). Nevertheless, the steps of sulfur mobilization from cysteine to tRNA are not known in detail. Kambampati and Lauhon (30) attempted to find the s^4U sulfurtransferase by using wild-type extracts of *E. coli* to complement the *in vitro* assay for s^4U biosynthesis. The assay consisted of unmodified tRNA as substrate and included purified ThiI for reconstitution of tRNA thiolation. This led to the identification a 45-kDa protein containing PLP as a cofactor. The 45-kDa protein turned out to be IscS, the

protein participating in the biosynthesis of Fe-S clusters, mentioned earlier (part B). IscS can catalyze the transfer of sulfur from cysteine for the *in vitro* generation of s^4U in tRNA (tRNA sulfurtransferase activity) (30). Thus the formation of s^4U requires ThiI and IscS as well as Mg-ATP, and L-cysteine as the sulfur donor. IscS requires PLP for activity analogous to factor C. However, the location of *iscS* (57.3 min) in the *E. coli* genome is not the same as that reported for *nuvC* (42-46 min). It is unclear whether *iscS* and *thiI* can be assigned as *nuvA* or *nuvC*.

IscS catalyzes the conversion of cysteine to alanine and sulfane sulfur in its active site (in the form of cysteine persulfide). Evidence for sulfur mobilization to ThiI and tRNA has been reported recently (88). Irreversible inhibition that resulted from the treatment of ThiI with 5-((2-iodoacetamido)ethyl)-1-aminonapthalene sulfonic acid suggests the presence of a reactive cysteine that is required for binding and/or catalysis by ThiI (88). In addition, gel mobility shift studies and protease protection assays showed that ThiI, but not IscS, binds to unmodified *E. coli* $tRNA^{(Phe)}$. These results suggest that ThiI is a recipient of the sulfane sulfur generated by IscS and catalyzes the ultimate sulfur transfer step in the *in vitro* synthesis of s^4U of tRNA (88). Figure 1.3 shows a proposed pathway of sulfur mobilization from persulfide-containing IscS to ThiI, forming ThiI persulfide (ThiI-SSH). Sulfur is then transferred to the uridine of tRNA, forming s^4U. A recent study (26) of an *iscS*-deletion strain demonstrated that IscS plays a significant role in sulfur mobilization for the biosynthesis of thiamin, NAD and s^4U in tRNA *in vivo*.

Biosynthesis of 2-thiouridine (s^2U) in *E. coli* requires an additional protein sulfur-acceptor in addition to IscS. This protein, TrmU, is analogous to ThiI, and is the product of the *mnmA* gene (formerly named *trmU* or *asuE*) (89, 90). However, reconstitution reactions *in vitro* using TrmU, IscS, cysteine, Mg-ATP and tRNA substrates did not result in synthesis of 2-thiouridine (Lauhon, personal communication). Since TrmU does not have a rhodanese homology domain as found in ThiI, other sulfurtransferases may be required in this reaction.

The biosynthesis of 2-methylthio derivatives of adenosine involves a multi-step process. Normally, thiolation occurs first, followed by SAM-dependent methylation. In the case of 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A), the isopentenyl group is added before the synthesis of the 2-methylthio group. In *E. coli*, the tRNA prenyltransferase encoded by *miaA* gene was identified to catalyze the formation of i^6A. i^6A is further methylthiolated to form ms^2i^6A by the recently-identified gene product, MiaB (91) and another enzyme, MiaC. Since iron has been implicated in the methylthiolation of i^6A (92) and MiaB protein contains a conserved cysteine motif (iron-binding site), MiaB is proposed to be a thiotransferase (91). In *S. typhimurium*, ms^2i^6A can be further modified to the hydroxylated form, 2- methylthio-N^6-hydroxyisopentenyladenosine (ms^2io^6A) by the activity of the *miaE* gene product (91, 93). Defects in translation, which confer broadly pleiotropic phenotypes, have been identified in *miaA* mutants (93) and in *miaB* mutants (94). The mechanism for the sulfur transfer reaction is probably different from that of 4-thiouridine synthesis. In this case, sulfur is added directly to an unsubstituted carbon atom of the purine ring, while in the case of s^2U or s^4U, the sulfur replaces the oxygen atom present at C2 or C4. Further study is necessary for a complete understanding of the sulfur transfer mechanism.

1.5. Sulfurtransferases and proposed physiological roles

Sulfurtransferases comprise a group of enzymes widely distributed in nature: they are found in animals, plants, bacteria (95) and archaea (BLAST search). Sulfurtransferases catalyze the transfer of a sulfane sulfur atom from a donor molecule to a thiophilic acceptor substrate, such as thiols, cyanide, sulfite and sulfinates.

1.5.1 Rhodaneses: catalytic reaction, distribution

Rhodanese, which is defined by its *in vitro* activity as a thiosulfate: cyanide sulfurtransferase (EC 2.8.1.1), is the best-characterized sulfurtransferase and catalyzes the following reaction:

$$SSO_3^{2-} + CN^- \rightarrow SO_3^{2-} + SCN^-$$

In the reaction, the sulfane sulfur atom of thiosulfate is transferred to the cysteine residue of the enzyme to form a persulfide intermediate that is subsequently attacked by cyanide ion to produce thiocyanate. The enzyme has rather wide substrate specificity with respect to sulfur donor and sulfur acceptor. Sulfur compounds other than thiosulfate, for instance, thiosulfonates or persulfides, may function as sulfur donors. Sulfite, sulfinates or various dithiol compounds, e.g., dihydrolipoate and thioredoxin, may substitute for cyanide as sulfur acceptors (96, 97).

Rhodanese was first identified in animals, then in plants and bacteria (97, 98). In animals, rhodanese is present in large quantities in liver and kidney tissues, where it is localized in the matrix of mitochondria as well as in the mitochondrial membrane (99). The enzyme has been purified to homogeneity from various animal tissues, including rat liver (100), bovine liver and kidney. Moreover, recombinant rhodaneses from various animal tissues (101, 102) were overexpressed in *E. coli* and have been characterized. Bovine liver rhodanese was isolated and crystallized (103, 104) and its primary and three-dimensional structures were determined (105-108). The enzyme, a protein of 33 kDa, is comprised of two very similar structural domains. The structural similarity of the two domains has been considered as the prototype of divergent evolution from a common ancestral protein (generated by gene duplication) (107). Two rhodanese cDNAs from *Arabidopsis thaliana* (*At*RDH1 and *At*RDH2) were isolated and characterized. *At*RDH1 and *At*RDH2 cDNAs were found to encode proteins from mitochondria (379 amino acids) and cytosol (318 amino acids), respectively (109).

Rhodanese is also widely distributed in bacteria; for example, it is present in gram-negative *A. vinelandii* (110), *Acinetobacter calcoaceticus lwoffi* (111), *Pseudomonas spp.* (112), *E. coli* (98) and the gram-positive *Bacillus subtilis* (113). In *A. vinelandii*, the gene *rhdA* coding for rhodanese has been identified and cloned (114). The amino acid sequence of RhdA, including the conservation of the active-site Cys residue, displays about 22% identity to bovine liver rhodanese. The crystal structure of RhdA was determined (110). The overall protein fold of RhdA has similarity to that of bovine

rhodanese. In addition, it was found that the active-site loop of RhdA displays structural similarity to the active-site loop of the similarly-folded catalytic domain of dual specificity phosphatase Cdc25, suggesting a common evolutionary origin of both enzyme families (110). The enzyme from *E. coli* has been isolated and characterized. It has a molecular weight of about 14,000 (98). Recently, Ray and coworkers (115) defined the gene, *glpE*, and characterized the corresponding 12-kilodalton rhodanese from *E. coli*. The amino acid sequence identity between GlpE and the active-site domain of mammalian rhodaneses is limited (approximately 17%), indicating the variation in amino acid sequences present in the sulfurtransferases. Kinetic analysis revealed that catalysis by GlpE, similar to other characterized rhodaneses, occurs via a double-displacement mechanism requiring an active site cysteine (115). Thioredoxin 1, with an apparent K_m of 34 μM, may serve as a physiological sulfur acceptor substrate for GlpE (115). GlpE has been crystallized recently (116).

1.5.2 MST: catalytic reaction, distribution

3-Mercaptopyruvate: cyanide sulfurtransferase (MST, EC 2.8.1.2) which is evolutionarily-related to rhodanese (102), prefers mercaptopyruvate to thiosulfate as a sulfur donor:

$$HSCH_2COCOO^- + CN^- \rightarrow CH_3COCOO^- + SCN^-$$

The enzyme appears to be specific for 3-mercaptopyruvate although it reacts slowly with 3,3'-dimercaptodipyruvate [*bis*(2-carboxy-2-oxoethyl) disulfide] and ethyl mercaptopyruvate. On the other hand, other compounds, including 3-mercaptolactate, 3,3'-dimercaptodilactate [*bis*(2-carboxy-2-hydroxyethyl) disulfide], 2-mercaptoethanol, 2-mercaptoethylamine, L-cysteine, L-alanine 3-sulfinate, L-cystine disulfoxide, 2-iminothiazolidine carboxylic acid, DL-homocysteine, DL-cystathionine or sulfide ions cannot be utilized by the enzyme (117). As with rhodanese, MST also has a broad range of sulfur acceptor substrates, e.g. cyanide, 2-mercaptoethanol, sulfite and sulfinates.

MST was discovered in rat liver more than 40 years ago (118, 119), but compared with rhodanese, the enzyme has received little attention. The rat liver enzyme was purified to homogeneity (102) and a recombinant form of the enzyme, which was overexpressed in *E. coli*, was characterized (120). Nagahara's group (102) showed that rat MST has 66 percent identity in amino acid sequence to rhodanese. It was possible to convert the sulfur donor specificity of MST to rhodanese and vice versa, by site-directed mutagenesis at the active sites of each enzyme. Two arginine residues, at positions 187 and 196 of rat MST, are critical in determining substrate specificity for mercaptopyruvate, whereas in rhodanese, arginine residues at 185 and 247 and also lysine residue at 248 determine substrate specificity for thiosulfate (120).

Bovine kidney MST (33 kilodalton) was purified to homogeneity, and its kinetic properties were studied (121, 122). In contrast to rhodanese, it was found that the reaction catalyzed by MST is of the sequential type mechanism (121, 122).

Recently, two cDNAs from *A. thaliana*, *At*MST1 and *At*MST2, were isolated and characterized (123, 124). The corresponding proteins, MST1 (378 amino acids) and MST2 (365 amino acids), prefer mercaptopyruvate to thiosulfate as substrate. The K_m values for mercaptopyruvate and cyanide of both enzymes are almost identical (3.7, 3.8 mM for mercaptopyruvate and 5 mM for CN^-). An *Arabidopsis* mutant with a T-DNA insertion in the *At*MST1, designated as *mst1-1*, was shown to have morphology and growth similar to the wild type control. Furthermore, the *mst1-1*mutation had no effect on the activities of Fe-S enzymes, including aconitase and NADH dehydrogenase, which indicated that MST1 is not directly involved in Fe-S cluster assembly (125). However, the presence of MST2 may compensate for the loss of MST1 function in the *mst1-1* mutant. Potential roles of plant MST in senescence- and stress-associated processes have been discussed (123).

MST from *E. coli* was first isolated and characterized by Vachek and Wood about 30 years ago (126). However, the gene responsible for this enzyme was not mapped nor was the sequence for the protein determined. It was reported that the purified enzyme has a

molecular weight of about 23,000 and contains 0.5 mole of copper and 1 mole of zinc per mole of enzyme, but no iron. The optimum pH for enzyme activity was reported to be between 9.3 and 9.6. Pyruvate, cysteine and cystine at 0.1 M and glutathione at 1 mM inhibited MST activity (126).

1.5.3 Thiosulfate: thiol sulfurtransferase: catalytic reaction, distribution

Thiosulfate: thiol sulfurtransferases (or thiosulfate reductases, thiol-dependent) are widespread in nature (127). These enzymes use electrons from thiols, which *in vivo* probably use electrons from glutathione (GSH), to reduce the sulfane sulfur atoms of inorganic thiosulfate and organic thiosulfonate anions to the sulfide level. Sulfide production from these thiol-dependent reductases was thought to be used in the synthesis of Fe-S proteins (95).

$$SSO_3^{2-} + GSH \rightarrow SO_3^{2-} + GSSH$$

$$(GSSH + GSH \rightarrow H_2S + GSSG) \text{ ------ spontaneous reaction}$$

In mammals, thiosulfate reductases are found in liver, kidney, heart, brain, intestine and testis. The subcellular distribution is similar to that of MST (95). Because the enzyme is very unstable, it is difficult to purify. Only the yeast enzyme has been purified to homogeneity and characterized. The enzyme is a monomeric globular protein with an apparent molecular weight of 17,000. Donor substrates are $S_2O_3^{2-}$ and $RS(O_2)S^-$. Acceptor substrates for the enzyme include GSH, cysteine and homocysteine (127). Kinetic analysis with benzene thiosulfonate and GSH as substrates indicated that the formal mechanism of the catalytic reaction is rapid equilibrium-ordered with GSH as the leading substrate (128). At pH 8.1 and 37 °C, the K_m for benzene thiosulfonate is 0.24 mM. The first reaction written above is enzyme-catalyzed, whereas the reaction of the persulfide product with excess thiol substrate occurs spontaneously, releasing the inorganic sulfide. Despite the fact that thiosulfate reductase contains one cysteine residue, its sulfhydryl group is not directly involved in the catalytic mechanism (95). In fact, the enzyme uses the sulfhydryl group of the acceptor substrate for cleavage of the

sulfur-sulfur bond of the donor substrate. The overall reaction is the reductive dismutation of thiosulfonate by two molecules of GSH. Furthermore, unlike rhodanese and MST, this enzyme will not transfer sulfur to cyanide. Therefore, the presence of a thiol substrate is required for persulfide production (95). However, when cyanide is present, sulfur from persulfide can transfer to cyanide and form thiocyanate in a nonenzymic reaction (95).

The production of hydrogen sulfide from thiosulfate via thiosulfate reductase has also been studied in *S. typhimurium*. DNA sequence analysis indicates that thiosulfate reductase contains 3 subunits, which are encoded by the *phsABC* (production of hydrogen sulfide) operon at 41.5 min (129, 130). Expression of the *phs* operon is dependent on anaerobiosis and the presence of reduced sulfur. PhsA (82.7 kDa), PhsB (21.3 kDa) and PhsC (28.5 kDa) have significant homology with the catalytic, electron transfer (Fe-S cluster) and hydrophobic (intrinsic membrane) subunits, respectively, of other anaerobic molybdoprotein oxidoreductases, including dimethyl sulfoxide reductase, nitrate reductase and formate dehydrogenase of *E. coli*, and polysulfide reductase (PsrABC) of *Wolinella succinogenes*. A role for thiosulfate reduction in anaerobic energy conservation has been suggested (129).

1.5.4 Proposed functions of sulfurtransferases

The physiological roles of rhodanese and related sulfurtransferases have not yet been established. In mammals, the enzymes are thought to be involved in a defense against cyanide toxicity since cyanide is metabolized to less toxic thiocyanate via transsulfuration. Thiocyanate is excreted mainly in urine (131). It is possible that herbivorous animals need the enzyme to defend against various cyanides derived from plants. The results of kinetic studies, however, suggest that cyanide is not a natural substrate of rhodanese and MST, since the K_m values for cyanide are typically in the millimolar range (102). The ubiquity of sulfurtransferases suggests that they have additional physiological functions. Some studies have implicated rhodanese in the formation of iron-sulfur centers. For example, rhodanese could generate sulfur for *in*

vitro reconstitution of the iron-sulfur clusters of iron sulfur proteins (succinate dehydrogenase, NADH dehydrogenase, spinach- and clostridial-ferredoxin, and nitrogenase of *Klebsiella pneumoniae*) (14, 132-136). However, the results obtained from *in vitro* reconstitution experiments do not reveal how the Fe-S clusters are being formed in the cell (137). One study proposed a role for rhodanese in the modulation of mitochondrial respiratory activity (99). In addition, Westley and coworkers showed a rhodanese isoform that functions as a thioredoxin oxidase and suggested a role in detoxification of oxygen free radicals in mitochondria (138).

To obtain a better understanding of the physiological roles of sulfurtransferases, the *E. coli* system was used as a model here. Both genetic and biochemical approaches were used to characterize the individual genes and the corresponding sulfurtransferases predicted to be present in *E. coli*. Chapter 2 of this dissertation describes work designed to identify the gene (*sseA*) and enzyme relationships for mercaptopyruvate sulfurtransferase(s) of *E. coli*. Moreover, it was shown that the disruption of a single gene (*sseA*::Sp^r) encoding MST does not confer a phynotype upon the mutant, which may be due to the presence of redundant sulfurtransferases in *E. coli*. The genome sequence of *E. coli* is predicted to encode seven proteins that display amino acid sequence similarity to SseA. Chapter 3 describes genetic techniques designed for construction of strains multiply-deficient in sulfurtransferase genes (including *sseA*, *glpE*, *iscS* and other *sseA*-paralogous genes) in an attempt to find a phenotype associated with sulfurtransferase deficiency.

1.6: Statement of Hypothesis

The main hypothesis of the research is that sulfurtransferases are required for sulfur delivery for the formation of Fe-S clusters, or biosynthesis of some sulfur-containing cofactors, including thiamin, molybdopterin, biotin and lipoic acid, or for synthesis of thionucleosides in tRNA.

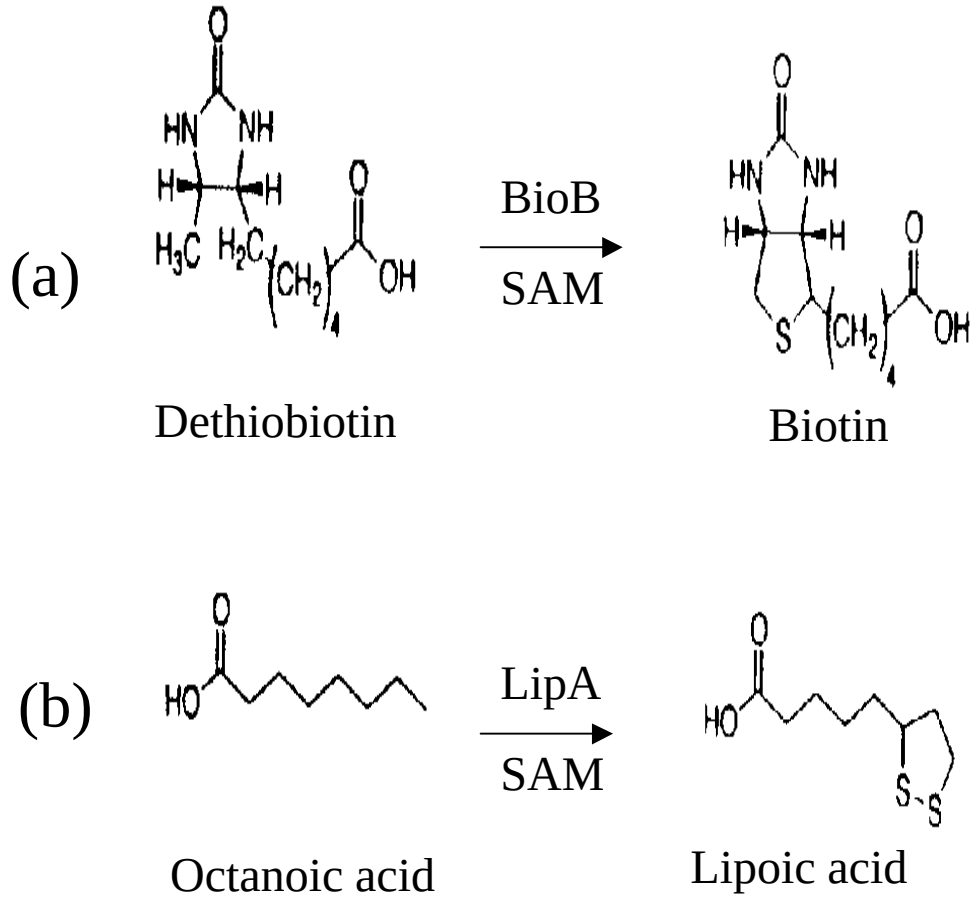


Figure 1.1 The carbon-sulfur bond forming reactions in the biosynthesis of biotin and lipoic acid.

(a) Biotin synthase (BioB) catalyzes a sulfur insertion into the saturated carbon at positions C6 and C9 of dethiobiotin to form biotin. (b) Lipoate synthase (LipA) catalyzes the two-sulfur atom insertion into the C6 and C8 of octanoic acid, forming lipoic acid. S-adenosyl-methionine (SAM) is required for both reactions.

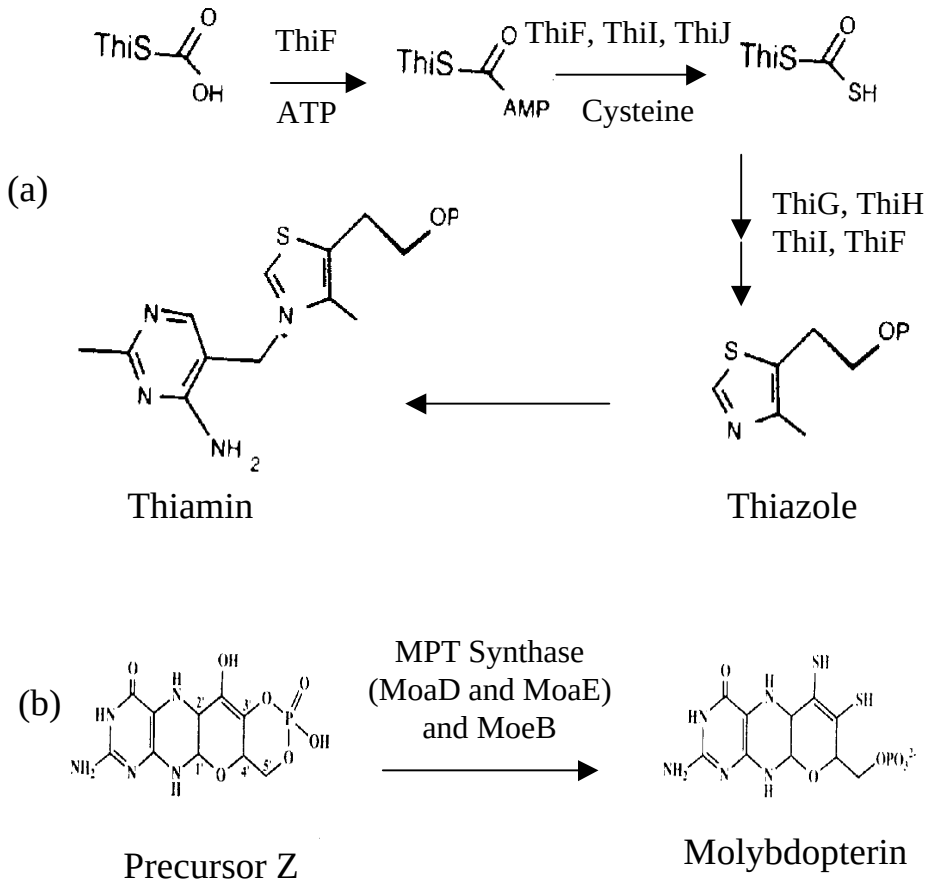


Figure 1.2 Biosynthesis of thiamin and molybdopterin.

(a) The formation of ThiS-thiocarboxylate. ThiF first converts ThiS to a C-terminal acyl adenylate (ThiS-COAMP). ThiS-COAMP is then converted to the thiocarboxylate form of ThiS (ThiS-COSH), the immediate sulfur donor for thiazole assembly, by ThiF, ThiI, ThiJ and cysteine. Thiazole is then incorporated into thiamin. (b) The conversion of precursor Z into molybdopterin. MPT synthase, which is composed of 2 subunits (MoaD and MoaE), and MoeB catalyze the incorporation of the dithiolene moiety into precursor Z.

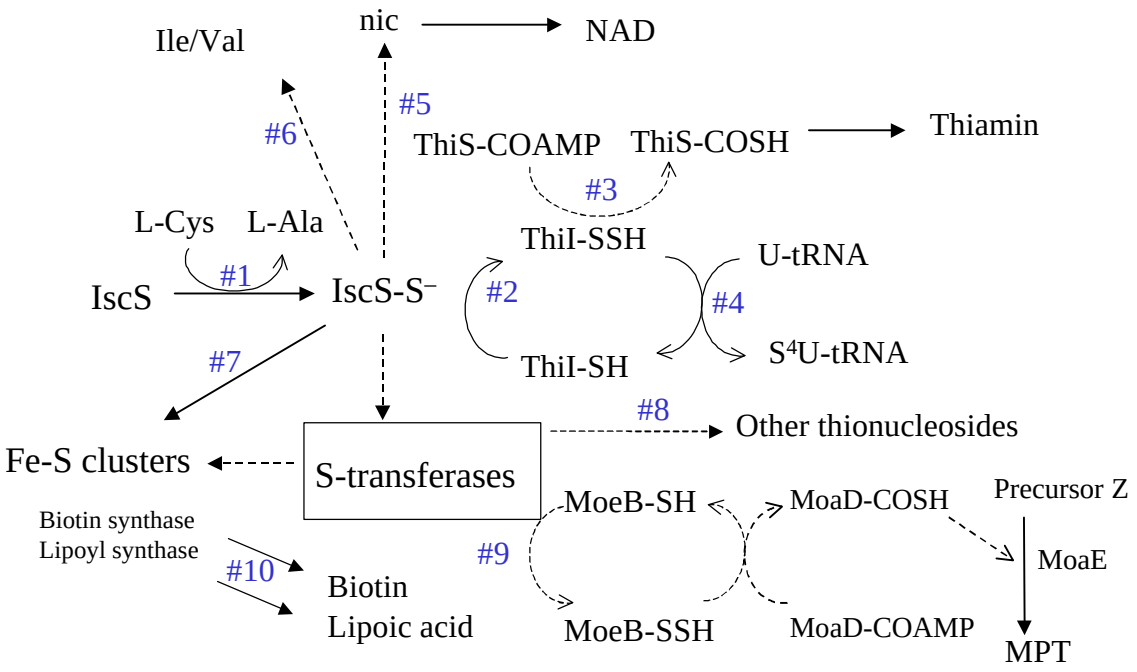
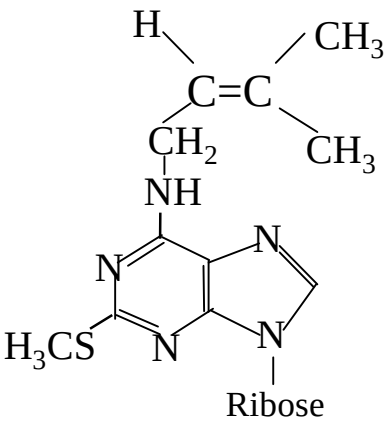
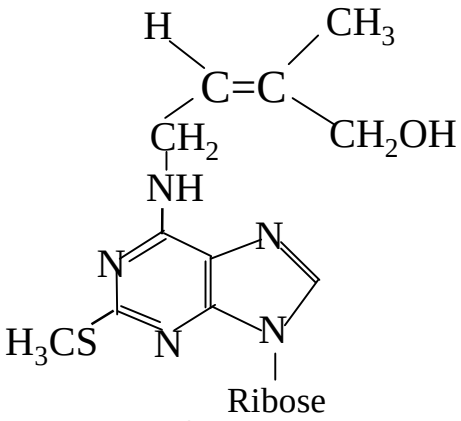


Figure 1.3 Proposed pathways and potential roles of IscS and sulfurtransferases.

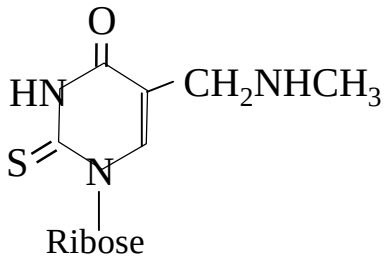
IscS catalyzes the conversion of L-cysteine to L-alanine by incorporating the sulfur into its active site as the cysteine persulfide form (IscS-S) (reaction #1). Sulfur can be mobilized from IscS-S to ThiI, forming ThiI persulfide (ThiI-SSH) (reaction #2). Sulfur is then transferred from ThiI-SSH to ThiS-COAMP (reaction #3), forming ThiS-COSH, which is the immediate sulfur donor for thiamin synthesis. In 4-thiouridine (S^4U) biosynthesis, sulfur from ThiI-SSH is transferred to uridine of tRNA (reaction #4). IscS is also need for provision of iron-sulfur (Fe-S) clusters for enzymes of the biosynthetic pathway for nicotinic acid (nic, reaction #5) and isoluecine/valine (Ile/Val) (reaction #6), as well as for other Fe-S clusters (reaction #7). Additional sulfurtransferases may be required for other thionucleoside biosyntheses (reaction #8) as well as for the sulfur transfer for molybdopterin (MPT) biosynthesis (reaction #9). The sulfur atom of biotin and lipoic acid are derived from the Fe-S clusters of biotin synthase and lipoyl synthase, respectively (reaction #10).



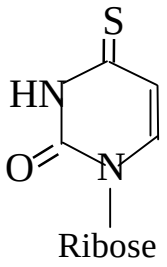
ms^{2}i^{6}A



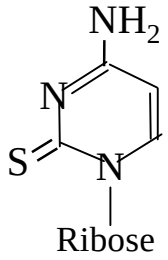
ms^{2}io^{6}A



mnm^{5}s^{2}U



s^{4}U



s^{2}C

Figure 1.4 Structures with the abbreviations of some commonly found thionucleosides in tRNA.

ms^{2}i^{6}A, 2-methylthio-N^6-isopentenyladenosine; ms^{2}io^{6}A, 2-methylthio-N^6-hydroxyisopentenyladenosine; mnm^{5}s^{2}U, 5-methyl-N-methyl-2-thiouridine; s^{4}U, 4-thiouridine; s^{2}C, 2-thiocytosine.

1.7. References

1. Beinert H. A tribute to sulfur. Eur J Biochem 2000;267:5657-64.
2. Claiborne A, Yeh JI, Mallett TC, et al. Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. Biochemistry 1999;38:15407-16.
3. Wood JL. Sulfane sulfur. Methods Enzymol 1987;143:25-9.
4. Toohey JI. Sulphane sulphur in biological systems: a possible regulatory role. Biochem J 1989;264:625-32.
5. Westley AM, Westley J. Biological sulfane sulfur. Anal Biochem 1991;195:63-7.
6. Beinert H, Holm RH, Munck E. Iron-sulfur clusters: nature's modular, multipurpose structures. Science 1997;277:653-9.
7. Beinert H. Iron-sulfur proteins: ancient structures, still full of surprises. J Biol Inorg Chem 2000;5:2-15.
8. Johnson MK. Iron-sulfur proteins: new roles for old clusters. Curr Opin Chem Biol 1998;2:173-81.
9. Kiley PJ, Beinert H. Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. FEMS Microbiol Rev 1998;22:341-52.
10. White RH. Origin of the labile sulfide in the iron-sulfur proteins of *Escherichia coli*. Biochem Biophys Res Commun 1983;112:66-72.
11. Dean DR, Bolin JT, Zheng L. Nitrogenase metalloclusters: structures, organization, and synthesis. J Bacteriol 1993;175:6737-44.
12. Zheng L, White RH, Cash VL, Dean DR. Mechanism for the desulfurization of L-cysteine catalyzed by the *nifS* gene product. Biochemistry 1994;33:4714-20.
13. Zheng L, Dean DR. Catalytic formation of a nitrogenase iron-sulfur cluster. J Biol Chem 1994;269:18723-18726.
14. Bonomi F, Pagani S, Cerletti P, Cannella C. Rhodanese-mediated sulfur transfer to succinate dehydrogenase. Eur J Biochem 1977;72:17-24.
15. Westley J. Rhodanese. Adv Enzymol 1973;39:327-368.

16. Taniguchi T, Kimura T. Role of 3-mercaptopyruvate sulfurtransferase in the formation of the iron-sulfur chromophore of adrenal ferredoxin. Biochim Biophys Acta 1974;364:284-95.
17. Yuvaniyama P, Agar JN, Cash VL, Johnson MK, Dean DR. NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. Proc Natl Acad Sci U S A 2000;97:599-604.
18. Zheng L, Cash VL, Flint DH, Dean DR. Assembly of iron-sulfur clusters. Identification of an *iscSUA-hscBA-fdx* gene cluster from *Azotobacter vinelandii*. J Biol Chem 1998;273:13264-72.
19. Altschul SF, Madden TL, Schaffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389-402.
20. Flint DH. *Escherichia coli* contains a protein that is homologous in function and N-terminal sequence to the protein encoded by the *nifS* gene of *Azotobacter vinelandii* and that can participate in the synthesis of the Fe-S cluster of dihydroxy-acid dehydratase. J Biol Chem 1996;271:16068-16074.
21. Li J, Kogan M, Knight SA, Pain D, Dancis A. Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution. J Biol Chem 1999;274:33025-34.
22. Nakai Y, Yoshihara Y, Hayashi H, Kagamiyama H. cDNA cloning and characterization of mouse *nifS*-like protein, m-Nfs1: mitochondrial localization of eukaryotic NifS-like proteins. FEBS Lett 1998;433:143-8.
23. Kredich NM, Foote LJ, Keenan BS. The stoichiometry and kinetics of the inducible cysteine desulfhydrase from *Salmonella typhimurium*. J Biol Chem 1973;248:6187-96.
24. Jaschkowitz K, Seidler A. Role of a NifS-like protein from the cyanobacterium *Synechocystis* PCC 6803 in the maturation of FeS proteins. Biochemistry 2000;39:3416-23.
25. Schwartz CJ, Djaman O, Imlay JA, Kiley PJ. The cysteine desulfurase, IscS, has a major role in *in vivo* Fe-S cluster formation in *Escherichia coli*. Proc Natl Acad Sci U S A 2000;97:9009-14.

26. Lauhon CT, Kambampati R. The *iscS* gene in *Escherichia coli* is required for the biosynthesis of 4- thiouridine, thiamin, and NAD. J Biol Chem 2000;275:20096-103.

27. Mihara H, Maeda M, Fujii T, Kurihara T, Hata Y, Esaki N. A *nifS*-like gene, *csdB*, encodes an *Escherichia coli* counterpart of mammalian selenocysteine lyase. Gene cloning, purification, characterization and preliminary x-ray crystallographic studies. J Biol Chem 1999;274:14768-72.

28. Nakamura M, Saeki K, Takahashi Y. Hyperproduction of recombinant ferredoxins in *Escherichia coli* by coexpression of the ORF1-*ORF2-iscS-iscU-iscA-hscB-hs cA-fdx*-ORF3 gene cluster. J Biochem (Tokyo) 1999;126:10-18.

29. Sun D, Setlow P. Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis nadB* gene and a *nifS*-like gene, both of which are essential for NAD biosynthesis. J Bacteriol 1993;175:1423-32.

30. Kambampati R, Lauhon CT. IscS is a sulfurtransferase for the *in vitro* biosynthesis of 4- thiouridine in *Escherichia coli* tRNA. Biochemistry 1999;38:16561-8.

31. Begley TP, Xi J, Kinsland C, Taylor S, McLafferty F. The enzymology of sulfur activation during thiamin and biotin biosynthesis. Curr Opin Chem Biol 1999;3:623-9.

32. Busby RW, Schelvis JPM, Yu DS, Babcock GT, Marietta MA. Lipoic acid biosynthesis: LipA is an iron-sulfur protein. J Am Chem Soc 1999;121:4706-4707.

33. Rajagopalan KV. Biosynthesis and processing of the molybdenum cofactors. Biochem Soc Trans 1997;25:757-761.

34. Howell DM, Harich K, Xu H, White RH. Alpha-keto acid chain elongation reactions involved in the biosynthesis of coenzyme B (7-mercaptoheptanoyl threonine phosphate) in methanogenic Archaea. Biochemistry 1998;37:10108-17.

35. Jackowski S. Biosynthesis of pantothenic acid and coenzymeA. In: Neidhardt FC, Curtiss R, Ingrahan JL, et al., eds. *Escherichia coli* and *Salmonella*: cellular and molecular biology. 2nd ed. Washington, D.C.: ASM Press, 1996:687-694.

36. Marquet A, Bui BT, Florentin D. Biosynthesis of biotin and lipoic acid. Vitam Horm 2001;61:51-101.
37. Bonjour JP. Biotin in human nutrition. Ann N Y Acad Sci 1985;447:97-104.
38. Whitehead CC. Assessment of biotin deficiency in animals. Ann N Y Acad Sci 1985;447:86-96.
39. Birch OM, Fuhrmann M, Shaw NM. Biotin synthase from *Escherichia coli*, an investigation of the low molecular weight and protein components required for activity in vitro. J Biol Chem 1995;270:19158-65.
40. DeMoll E, Shive W. The origin of sulfur in biotin. Biochem Biophys Res Commun 1983;110:243-9.
41. DeMoll E, White RH, Shive W. Determination of the metabolic origins of the sulfur and 3'-nitrogen atoms in biotin of *Escherichia coli* by mass spectrometry. Biochemistry 1984;23:558-62.
42. Cleary PP, Campbell A. Deletion and complementation analysis of biotin gene cluster of *Escherichia coli*. J Bacteriol 1972;112:830-9.
43. Sanyal I, Cohen G, Flint DH. Biotin synthase: purification, characterization as a [2Fe-2S]cluster protein, and in vitro activity of the *Escherichia coli bioB* gene product. Biochemistry 1994;33:3625-31.
44. Ollagnier-De Choudens S, Sanakis Y, Hewitson KS, et al. Iron-sulfur center of biotin synthase and lipoate synthase. Biochemistry 2000;39:4165-73.
45. Ugulava NB, Gibney BR, Jarrett JT. Iron-sulfur cluster interconversions in biotin synthase: dissociation and reassociation of iron during conversion of [2Fe-2S] to [4Fe-4S] clusters. Biochemistry 2000;39:5206-14.
46. Sanyal I, Gibson KJ, Flint DH. *Escherichia coli* biotin synthase: an investigation into the factors required for its activity and its sulfur donor. Arch Biochem Biophys 1996;326:48-56.
47. Bui BT, Florentin D, Fournier F, Ploux O, Mejean A, Marquet A. Biotin synthase mechanism: on the origin of sulphur. FEBS Lett 1998;440:226-30.
48. Gibson KJ, Pelletier DA, Turner IM, Sr. Transfer of sulfur to biotin from biotin synthase (BioB protein). Biochem Biophys Res Commun 1999;254:632-5.

49. Kulzer R, Pils T, Kappl R, Huttermann J, Knappe J. Reconstitution and characterization of the polynuclear iron-sulfur cluster in pyruvate formate-lyase-activating enzyme. Molecular properties of the holoenzyme form. J Biol Chem 1998;273:4897-903.

50. Lieder KW, Booker S, Ruzicka FJ, Beinert H, Reed GH, Frey PA. S-Adenosylmethionine-dependent reduction of lysine 2,3-aminomutase and observation of the catalytically functional iron-sulfur centers by electron paramagnetic resonance. Biochemistry 1998;37:2578-85.

51. Shaw NM, Birch OM, Tinschert A, Venetz V, Dietrich R, Savoy LA. Biotin synthase from *Escherichia coli*: isolation of an enzyme-generated intermediate and stoichiometry of S-adenosylmethionine use. Biochem J 1998;330:1079-85.

52. Bui BT, Escalettes F, Chottard G, Florentin D, Marquet A. Enzyme-mediated sulfide production for the reconstitution of [2Fe-2S] clusters into apo-biotin synthase of *Escherichia coli*. Sulfide transfer from cysteine to biotin. Eur J Biochem 2000;267:2688-94.

53. Vanden Boom TJ, Reed KE, Cronan JE. Lipoic acid metabolism in *Escherichia coli*: isolation of null mutants defective in lipoic acid biosynthesis, molecular cloning and characterization of the *E. coli lip* locus, and identification of the lipoylated protein of the glycine cleavage system. J Bacteriol 1991;173:6411-20.

54. White RH. Stable isotope studies on the biosynthesis of lipoic acid in *Escherichia coli*. Biochemistry 1980;19:15-9.

55. White RH. Biosynthesis of lipoic acid: Extent of incorporation of deuterated hydroxy- and thiooctanoic acids into lipoic acid. J Am Chem Soc 1980;102:6605-7.

56. Reed KE, Cronan JE. Lipoic acid metabolism in *Escherichia coli*: sequencing and functional characterization of the *lipA* and *lipB* genes. J Bacteriol 1993;175:1325-36.

57. Sulo P, Martin NC. Isolation and characterization of *LIP5*. A lipoate biosynthetic locus of *Saccharomyces cerevisiae*. J Biol Chem 1993;268:17634-9.

58. Yasuno R, Wada H. Biosynthesis of lipoic acid in *Arabidopsis*: cloning and characterization of the cDNA for lipoic acid synthase. Plant Physiol 1998;118:935-43.

59. Morris TW, Reed KE, Cronan JE, Jr. Lipoic acid metabolism in *Escherichia coli*: the *lplA* and *lipB* genes define redundant pathways for ligation of lipoyl groups to apoprotein. J Bacteriol 1995;177:1-10.

60. Jordan SW, Cronan JE, Jr. A new metabolic link. The acyl carrier protein of lipid synthesis donates lipoic acid to the pyruvate dehydrogenase complex in *Escherichia coli* and mitochondria. J Biol Chem 1997;272:17903-6.

61. Miller JR, Busby RW, Jordan SW, et al. *Escherichia coli* LipA is a lipoyl synthase: *In vitro* biosynthesis of lipoylated pyruvate dehydrogenase complex from octanoyl-acyl carrier protein. Biochemistry 2000;39:15166-78.

62. White RL, Spenser ID. Biosynthesis of thiamin. In: Neidhardt FC, Curtiss R, Ingrahan JL, et al., eds. *Escherichia coli* and *Salmonella*: cellular and molecular biology. 2nd ed. Washington, D.C.: ASM Press, 1996:680-686.

63. Begley TP, Downs DM, Ealick SE, et al. Thiamin biosynthesis in prokaryotes. Arch Microbiol 1999;171:293-300.

64. Taylor SV, Kelleher NL, Kinsland C, et al. Thiamin biosynthesis in *Escherichia coli*. Identification of ThiS thiocarboxylate as the immediate sulfur donor in the thiazole formation. J Biol Chem 1998;273:16555-60.

65. Wang C, Xi J, Begley TP, Nicholson LK. Solution structure of ThiS and implications for the evolutionary roots of ubiquitin. Nat Struct Biol 2001;8:47-51.

66. Furukawa K, Mizushima N, Noda T, Ohsumi Y. A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. J Biol Chem 2000;275:7462-5.

67. Palenchar PM, Buck CJ, Cheng H, Larson TJ, Mueller EG. Evidence that ThiI, an enzyme shared between thiamin and 4-thiouridine biosynthesis, may be a sulfurtransferase that proceeds through a persulfide intermediate. J Biol Chem 2000;275:8283-6.

68. Rajagopalan KV, Johnson JL. The pterin molybdenum cofactors. J Biol Chem 1992;267:10199-202.

69. Ludden PW, Shah VK, Roberts GP, et al. Biosynthesis of the iron-molybdenum cofactor of nitrogenase. In: Stiefel EI, Coucouvanis D, Newton WE, eds. Molybdenum enzymes, cofactors, and model systems. Washington DC: Am Chem Soc, 1993:196-215.
70. Kisker C, Schindelin H, Rees DC. Molybdenum-cofactor-containing enzymes: structure and mechanism. Annu Rev Biochem 1997;66:233-67.
71. Rajagopalan KV. Biosynthesis of the molybdenum cofactor. In: Neidhardt FC, Curtiss R, Ingrahan JL, et al., eds. *Escherichia coli* and *Salmonella*: cellular and molecular biology. 2nd ed. Washington, D.C.: ASM Press, 1996:674-9.
72. Johnson ME, Rajagopalan KV. Involvement of chlA, E, M, and N loci in *Escherichia coli* molybdopterin biosynthesis. J Bacteriol 1987;169:117-25.
73. Rudolph MJ, Wuebbens MM, Rajagopalan KV, Schindelin H. Crystal structure of molybdopterin synthase and its evolutionary relationship to ubiquitin activation. Nat Struct Biol 2001;8:42-6.
74. Pitterle DM, Johnson JL, Rajagopalan KV. *In vitro* synthesis of molybdopterin from precursor Z using purified converting factor. Role of protein-bound sulfur in formation of the dithiolene. J Biol Chem 1993;268:13506-9.
75. Leimkuhler S, Rajagopalan KV. A sulfurtransferase is required in the transfer of cysteine sulfur in the *in vitro* synthesis of molybdopterin from precursor Z in *Escherichia coli*. J Biol Chem 2001;276:22024-31.
76. Reiss J, Dorche C, Stallmeyer B, Mendel RR, Cohen N, Zabot MT. Human molybdopterin synthase gene: genomic structure and mutations in molybdenum cofactor deficiency type B. Am J Hum Genet 1999;64:706-11.
77. Reiss J. Genetics of molybdenum cofactor deficiency. Hum Genet 2000;106:157-63.
78. Hagervall TG, Ericson JU, Esberg KB, Li JN, Bjork GR. Role of tRNA modification in translational fidelity. Biochim Biophys Acta 1990;1050:263-6.
79. Persson BC, Esberg B, Olafsson O, Bjork GR. Synthesis and function of isopentenyl adenosine derivatives in tRNA. Biochimie 1994;76:1152-60.

80. Li J, Esberg B, Curran JF, Bjork GR. Three modified nucleosides present in the anticodon stem and loop influence the *in vivo* aa-tRNA selection in a tRNA-dependent manner. J Mol Biol 1997;271:209-21.

81. Ajitkumar P, Cherayil JD. Thionucleosides in transfer ribonucleic acid: diversity, structure, biosynthesis, and function. Microbiol Rev 1988;52:103-13.

82. Abrell JW, Kaufman EE, Lipsett MN. The biosynthesis of 4-thiouridylate. Separation and purification of two enzymes in the transfer ribonucleic acid-sulfurtransferase system. J Biol Chem 1971;246:294-301.

83. Lipsett MN. Enzymes producing 4-thiouridine in *Escherichia coli* tRNA: approximate chromosomal locations of the genes and enzyme activities in a 4-thiouridine-deficient mutant. J Bacteriol 1978;135:993-7.

84. Ryals J, Hsu RY, Lipsett MN, Bremer H. Isolation of single-site *Escherichia coli* mutants deficient in thiamine and 4-thiouridine syntheses: identification of a *nuvC* mutant. J Bacteriol 1982;151:899-904.

85. Mueller EG, Palenchar PM. Using genomic information to investigate the function of ThiI, an enzyme shared between thiamin and 4-thiouridine biosynthesis. Protein Sci 1999;8:2424-7.

86. Webb E, Claas K, Downs DM. Characterization of *thiI*, a new gene involved in thiazole biosynthesis in *Salmonella typhimurium*. J Bacteriol 1997;179:4399-402.

87. Mueller EG, Buck CJ, Palenchar PM, Barnhart LE, Paulson JL. Identification of a gene involved in the generation of 4-thiouridine in tRNA. Nucleic Acids Res 1998;26:2606-10.

88. Kambampati R, Lauhon CT. Evidence for the transfer of sulfane sulfur from IscS to ThiI during the *in vitro* biosynthesis of 4-thiouridine in *Escherichia coli* tRNA. J Biol Chem 2000;275:10727-30.

89. Sullivan MA, Cannon JF, Webb FH, Bock RM. Antisuppressor mutation in *Escherichia coli* defective in biosynthesis of 5-methylaminomethyl-2-thiouridine. J Bacteriol 1985;161:368-76.

90. Hagervall TG, Pomerantz SC, McCloskey JA. Reduced misreading of asparagine codons by *Escherichia coli* $tRNA^{Lys}$ with hypomodified derivatives of 5-

methylaminomethyl-2-thiouridine in the wobble position. J Mol Biol 1998;284:33-42.

91. Esberg B, Leung HC, Tsui HC, Bjork GR, Winkler ME. Identification of the *miaB* gene, involved in methylthiolation of isopentenylated A37 derivatives in the tRNA of *Salmonella typhimurium* and *Escherichia coli*. J Bacteriol 1999;181:7256-65.

92. Buck M, Griffiths E. Iron mediated methylthiolation of tRNA as a regulator of operon expression in *Escherichia coli*. Nucleic Acids Res 1982;10:2609-24.

93. Esberg B, Bjork GR. The methylthio group (ms^2) of N^6-(4-hydroxyisopentenyl)-2- methylthioadenosine (ms^2io^6A) present next to the anticodon contributes to the decoding efficiency of the tRNA. J Bacteriol 1995;177:1967-75.

94. Zhao J, Leung HC, Winkler ME. The *miaA* mutator phenotype of *Escherichia coli* K-12 requires recombination functions. J Bacteriol 2001;183:1796-1800.

95. Westley J, Adler H, Westley L, Nishida C. The sulfurtransferases. Fundam Appl Toxicol 1983;3:377-82.

96. Villarejo M, Westley J. J Biol Chem 1963;238:4016-4020.

97. Westley J. Thiosulfate: cyanide sulfurtransferase (rhodanese). Methods Enzymol 1981;77:285-91.

98. Alexander K, Volini M. Properties of an *Escherichia coli* rhodanese. J Biol Chem 1987;262:6595-604.

99. Ogata K, Volini M. Mitochondrial rhodanese: membrane-bound and complexed activity. J Biol Chem 1990;265:8087-93.

100. Wasylewski Z, Basztura B, Koj A. Comparison of some physicochemical properties of rat and beef liver rhodanese. Bull Acad Pol Sci [Biol] 1979;27:807-14.

101. Dooley TP, Nair SK, Garcia REt, Courtney BC. Mouse rhodanese gene (*Tst*): cDNA cloning, sequencing, and recombinant protein expression. Biochem Biophys Res Commun 1995;216:1101-9.

102. Nagahara N, Okazaki T, Nishino T. Cytosolic mercaptopyruvate sulfurtransferase is evolutionarily related to mitochondrial rhodanese. Striking similarity in active site amino acid sequence and the increase in the mercaptopyruvate

sulfurtransferase activity of rhodanese by site-directed mutagenesis. J Biol Chem 1995;270:16230-5.

103. Sorbo BH. Crystalline rhodanese. Purification and physicochemical examination. Acta Chem Scand 1953;7:1137-1145.

104. Horowitz P, DeToma F. Improved preparation of bovine liver rhodanese. J Biol Chem 1970;245:984-5.

105. Ploegman JH, Drent G, Kalk KH, Hol WG. Structure of bovine liver rhodanese. I. Structure determination at 2.5 A resolution and a comparison of the conformation and sequence of its two domains. J Mol Biol 1978;123:557-94.

106. Ploegman JH, Drent G, Kalk KH, Hol WG. The structure of bovine liver rhodanese. II. The active site in the sulfur-substituted and the sulfur-free enzyme. J Mol Biol 1979;127:149-62.

107. Ploegman JH, Drent G, Kalk KH, et al. The covalent and tertiary structure of bovine liver rhodanese. Nature 1978;273:124-9.

108. Russell J, Weng L, Keim PS, Heinrikson RL. The covalent structure of bovine liver rhodanese. Isolation and partial structural analysis of cyanogen bromide fragments and the complete sequence of the enzyme. J Biol Chem 1978;253:8102-8.

109. Hatzfeld Y, Saito K. Evidence for the existence of rhodanese (thiosulfate:cyanide sulfurtransferase) in plants: preliminary characterization of two rhodanese cDNAs from *Arabidopsis thaliana*. FEBS Lett 2000;470:147-50.

110. Bordo D, Deriu D, Colnaghi R, Carpen A, Pagani S, Bolognesi M. The crystal structure of a sulfurtransferase from *Azotobacter vinelandii* highlights the evolutionary relationship between the rhodanese and phosphatase enzyme families. J Mol Biol 2000;298:691-704.

111. Aird BA, Heinrikson RL, Westley J. Isolation and characterization of a prokaryotic sulfurtransferase. J Biol Chem 1987;262:17327-35.

112. Lanyi B. Rhodanese activity: a simple and reliable taxonomic tool for gram-negative bacteria. J Med Microbiol 1982;15:263-6.

113. Villarejo M, Westley J. Sulfur metabolism of *Bacillus subtilis*. Biochim Biophys Acta 1966;117:209-16.

114. Colnaghi R, Pagani S, Kennedy C, Drummond M. Cloning, sequence analysis and overexpression of the rhodanese gene of *Azotobacter vinelandii*. Eur J Biochem 1996;236:240-8.
115. Ray WK, Zeng G, Potters MB, Mansuri AM, Larson TJ. Characterization of a 12-kilodalton rhodanese encoded by *glpE* of *Escherichia coli* and its interaction with thioredoxin. J Bacteriol 2000;182:2277-84.
116. Bordo D, Larson TJ, Donahue JL, Spallarossa A, Bolognesi M. Crystals of GlpE, a 12 kDa sulfurtransferase from *Escherichia coli*, display 1.06 A resolution diffraction: a preliminary report. Acta Crystallogr D Biol Crystallogr 2000;56:1691-3.
117. Roy AB, Trudinger PA. Rhodanese and 3-mercaptopyruvate sulphurtransferase. The biochemistry of inorganic compounds of sulphur. London: Cambridge university press, 1970:190-206.
118. Meister A. Conversion of the α-keto analog of cysteine to pyruvate and sulfur. Fed Proc 1953;12:245.
119. Wood JL, Fiedler H. β-mercaptopyruvate, a substrate for rhodanese. J Biol Chem 1953;205:231-234.
120. Nagahara N, Nishino T. Role of amino acid residues in the active site of rat liver mercaptopyruvate sulfurtransferase. CDNA cloning, overexpression, and site-directed mutagenesis. J Biol Chem 1996;271:27395-401.
121. Jarabak R, Westley J. Steady-state kinetics of 3-mercaptopyruvate sulfurtransferase from bovine kidney. Arch Biochem Biophys 1978;185:458-65.
122. Jarabak R, Westley J. 3-Mercaptopyruvate sulfurtransferase: rapid equilibrium-ordered mechanism with cyanide as the acceptor substrate. Biochemistry 1980;19:900-4.
123. Papenbrock J, Schmidt A. Characterization of two sulfurtransferase isozymes from *Arabidopsis thaliana*. Eur J Biochem 2000;267:5571-9.
124. Papenbrock J, Schmidt A. Characterization of a sulfurtransferase from *Arabidopsis thaliana*. Eur J Biochem 2000;267:145-54.

125. Nakamura T, Yamaguchi Y, Sano H. Plant mercaptopyruvate sulfurtransferases molecular cloning, subcellular localization and enzymatic activities. Eur J Biochem 2000;267:5621-5630.
126. Vachek H, Wood JL. Purification and properties of mercaptopyruvate sulfur transferase of *Escherichia coli*. Biochim Biophys Acta 1972;258:133-46.
127. Chauncey TR, Uhteg LC, Westley J. Thiosulfate reductase. Methods Enzymol 1987;143:350-4.
128. Chauncey TR, Westley J. The catalytic mechanism of yeast thiosulfate reductase. J Biol Chem 1983;258:15037-45.
129. Heinzinger NK, Fujimoto SY, Clark MA, Moreno MS, Barrett EL. Sequence analysis of the *phs* operon in *Salmonella typhimurium* and the contribution of thiosulfate reduction to anaerobic energy metabolism. J Bacteriol 1995;177:2813-20.
130. Alami N, Hallenbeck PC. Cloning and characterization of a gene cluster, *phsBCDEF*, necessary for the production of hydrogen sulfide from thiosulfate by *Salmonella typhimurium*. Gene 1995;156:53-7.
131. Nagahara N, Ito T, Minami M. Mercaptopyruvate sulfurtransferase as a defense against cyanide toxication: molecular properties and mode of detoxification. Histol Histopathol 1999;14:1277-86.
132. Bonomi F, Pagani S, Cerletti P. Insertion of sulfide into ferredoxins catalyzed by rhodanese. FEBS Lett 1977;84:149-52.
133. Bonomi F, Pagani S, Kurtz DM, Jr. Enzymic synthesis of the 4Fe-4S clusters of *Clostridium pasteurianum* ferredoxin. Eur J Biochem 1985;148:67-73.
134. Pagani S, Bonomi F, Cerletti P. Enzymic synthesis of the iron-sulfur cluster of spinach ferredoxin. Eur J Biochem 1984;142:361-6.
135. Pagani S, Eldridge M, Eady RR. Nitrogenase of *Klebsiella pneumoniae*. Rhodanese-catalysed restoration of activity of the inactive 2Fe species of the Fe protein. Biochem J 1987;244:485-8.
136. Pagani S, Galante YM. Interaction of rhodanese with mitochondrial NADH dehydrogenase. Biochim Biophys Acta 1983;742:278-84.

137. Sandberg W, Graves MC, Rabinowitz JC. Role for rhodanese in Fe-S formation is doubful. TIBS 1987;12:56-57.

138. Nandi DL, Horowitz PM, Westley J. Rhodanese as a thioredoxin oxidase. Int J Biochem Cell Biol 2000;32:465-73.

Chapter 2
Identification of a gene encoding mercaptopyruvate sulfurtransferase of *Escherichia coli*

2.1: Abstract

3-Mercaptopyruvate sulfurtransferase (EC 2.8.1.2) catalyzes the cleavage of a C-S bond and transfer of the sulfur atom from 3-mercaptopyruvate to thiophiles. In this work, the gene and the corresponding mercaptopyruvate sulfurtransferase from *Escherichia coli* were defined. *sseA*, previously identified as a gene that enhances serine-sensitivity when present in multicopy, in fact, encodes mercaptopyruvate sulfurtransferase. A SseA-His_6 variant was purified and characterized. The apparent molecular weight of the protein and the pH optimum for mercaptopyruvate sulfurtransferase activity disagreed with a previously-characterized mercaptopyruvate sulfurtransferase from *E. coli*. A chromosomal insertion mutation in *sseA* was constructed. Characterization of a strain harboring this mutation demonstrated that SseA is the only protein possessing mercaptopyruvate:mercaptoethanol sulfurtransferase activity in *E. coli*. However, no discernable phenotype of the *sseA*::Sp^r mutant was found. Therefore, the physiological function of mercaptopyruvate sulfurtransferase is unknown.

2.2: Introduction

Mercaptopyruvate sulfurtransferase (EC 2.8.1.2, MST) catalyzes the cleavage of a carbon-sulfur bond and transfer of the sulfur atom from 3-mercaptopyruvate to any of a variety of thiophiles, including thiols, cyanide, sulfite and sulfinates.

$$HSCH_2COCOO^- + CN^- \rightarrow SCN^- + CH_3COCOO^- \text{ or}$$
$$HSCH_2COCOO^- + RSH \rightarrow RSSH + CH_3COCOO^-$$

MST was first discovered in rat liver over 40 years ago (1, 2). This enzyme is widely distributed in nature, being found in eukaryotes and prokaryotes (3-5). The enzyme is located in the cytosol (mainly) and the mitochondria of eukaryotic cells (3). MST has received little attention when compared with rhodanese. Rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) is the best characterized enzyme in the family of sulfurtransferases. This enzyme catalyzes the transfer of sulfur from thiosulfate to cyanide by way of a double-displacement mechanism.

$$SSO_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}$$

Rhodaneses are found ubiquitously in organisms from all three domains of life (6). The enzyme is located mainly in the mitochondria of eukaryotes (3, 7).

Rat liver MST was purified to homogeneity (8) and a recombinant form of the enzyme, which was overexpressed in *E. coli*, was characterized (9). The amino acid sequence of rat MST displays 66% identity to that of rat liver rhodanese, indicating that the genes for these enzymes have evolved from a common ancestor (8). Bovine kidney MST was purified to homogeneity, and its kinetic properties were studied (10). The enzyme was found to catalyze the reaction by a sequential formal mechanism (10). However, the nature of the acceptor substrate determines the subclass of sequential mechanism. When the acceptor is cyanide ion, the mechanism is rapid equilibrium-ordered; when it is 2-mercaptoethanol, the mechanism appears to be a random type (11). Recently, two cDNAs from *Arabidopsis thaliana*, *At*MST1 and *At*MST2, were isolated and characterized (5, 12, 13). The corresponding proteins, MST1 (378 amino acids) and MST2 (365 amino acids), are located in the mitochondria and the cytoplasm of *A. thaliana*, respectively.

The physiological roles of rhodanese and related sulfurtransferases are still in question. In mammals, rhodanese and MST appear to be involved in a defense against cyanide toxication since cyanide is metabolized to less toxic thiocyanate via transsulfuration (14). But the low affinity for cyanide of these enzymes (8) suggests that cyanide detoxification

is unlikely to be the predominant role of these ubiquitous enzymes. Other roles in the formation of iron-sulfur clusters (15-18) and in sulfur metabolism (19) have been proposed.

MST from *E. coli* was first isolated and characterized in 1972 (20). However, the gene responsible for this enzyme was not mapped nor was the sequence for the protein determined. Previously, Ray and coworkers found that *glpE,* a gene in the glycerol-3-phosphate regulon of *E. coli,* encodes a 12-kilodalton rhodanese (21). A PSI-BLAST search (22) using GlpE as the query revealed that there are at least seven other proteins in *E. coli* that show amino acid sequence similarity to GlpE. One of these proteins is the so-called SseA protein. Indeed, the *sseA* gene was first identified by the Tsuchiya group (23) as a gene that enhances serine-sensitivity when present in muticopy. Addition of serine to *E. coli* cells growing on lactate (or other carbon sources) causes growth inhibition, since serine inhibits the function of homoserine dehydrogenase I, which is need for threonine-isoleucine biosynthesis (24). They also reported that the protein encoded by the *sseA* gene was similar in sequence to bovine rhodanese and reported a twofold increase in rhodanese activity in crude extract of cells harboring a plasmid carrying *sseA.*

The amino acid sequence and size of SseA are more similar to rat MST than to rhodanese. Therefore, we considered the possibility that *sseA* may encode MST rather than rhodanese. In this study, we cloned the *sseA* gene, purified the protein and demonstrated that SseA, in fact, catalyzes MST activity. This is the first time that the gene for a mercaptopyruvate sulfurtransferase of bacterial origin has been identified. The apparent molecular mass and some catalytic properties of the enzyme were determined and compared to those of the MST previously described by Vachek and Wood (20). Finally, a chromosomal insertion mutation in *sseA* was constructed to test the effect of SseA deficiency on MST activity as well as on growth phenotype. The results revealed that SseA is the only protein possessing mercaptopyruvate:mercaptoethanol sulfurtransferase activity in *E. coli*.

2.3: Experimental Procedures

Materials

Unless listed below, the reagents used were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical Company (St. Louis, MO). Restriction endonucleases were purchased from several sources, including New England Biolabs (Beverly, MA) and Promega (Madison, WI) and used with the buffers supplied by the manufacturers. Vent (exo^-) DNA polymerase, T4 polynucletide kinase, T4 DNA ligase and deoxyribonucleotides for PCR were acquired from New England Biolabs and used with buffers from the supplier. Shrimp alkaline phosphatase, with the appropriate buffer, and isopropylthio-β-D-galactopyranoside (IPTG) were obtained from United States Biochemical (Cleveland, OH). Difco Laboratories (Detroit, MI) was the source of yeast extract, tryptone and bacto agar. Ammonium thiosulfate was purchased from Aldrich (Milwaukee, WI). Ferric nitrate and the sodium salt of mercaptopyruvic acid were purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Oligonucleotides for PCR were synthesized by DNAgency (Malvern, PA).

Bacterial strains and plasmids

All bacterial strains are derivatives of *E. coli* K-12, except for BL21(DE3),which is an *E. coli* B derivative. Strains and plasmids used in this study are listed in Table 2.1.

Growth media and conditions

Cultures were routinely grown in Luria-Bertani broth (LB) (25) at 37°C. Antibiotics were included at 100 μg/ml for ampicillin, 50 μg/ml for kanamycin, 30 μg/ml for chloramphenicol, 10 μg/ml for tetracycline and 25 μg/ml for spectinomycin when required. M9 salts or AB minimal media (25) were used in some experiments and supplemented with one or more of the following: 0.2% glucose or 0.4% glycerol, 2 μg/ml thiamin, 0.01 M $MgSO_4$ and 0.005 M $CaCl_2$ where appropriate. However, to study

growth on limiting sulfur, $MgSO_4$ (or Na_2SO_3 or cysteine) at a concentration of 65 μM, instead of 0.01 M, was added to the minimal medium. In this case, 0.01 M $MgCl_2$ was added as the Mg^{2+} source. Cells were grown overnight in this medium, then diluted into fresh medium before the optical density at 600 nm was monitored.

To determine whether 3-mercaptopyruvate can serve as the sulfur source, or both sulfur and carbon sources, concentrations of 65 μM and 1 mM were used, respectively.
Colonies were picked from the plates of glucose minimal medium containing 0.01 M $MgSO_4$ and inoculated into liquid medium (M9) with limiting sulfur (65 μM $MgSO_4$). Cells cultured at 37 °C overnight were diluted into fresh medium with limiting sulfur (same as overnight) and grown at 37 °C from an OD_{600} of 0.04 until an OD_{600} of about 0.3. The cells were subjected to centrifugation and washed twice in M9 salts without any sulfur or carbon source. Then cells were diluted into 1) fresh M9 glucose containing 65 μM 3-mercaptopyruvate without any other sulfur source, or 2) fresh M9 containing 1 mM 3-mercaptopyruvate without any other carbon or sulfur source. The OD_{600} of the cultures was monitored. M9 glucose without sulfur addition was used as the control medium to determine the extent of sulfur contamination in M9 salts.

To test the involvement of the *sseA* gene in MPT biosynthesis, LB medium containing 20 mM $NaClO_3$ was used as previously described (36). Cultures were grown at 37 °C under anaerobic conditions. Strain S1247 (*bioA24*, *moaA29*, *zbh-283*::Tn*10*) was used as the positive control for the chlorate-resistant phenotype (29). Anaerobic conditions were obtained in a closed container containing a GasPak Plus™ anaerobic generator from Becton Dickinson (Sparks, MO).

Table 2.1 *E. coli* strains and plasmids

Strains	Genotype	Description or Reference
BL21(DE3)	*hsdS gal* (λcI*ts857 ind-1 S*am7 *nin-5 lacUV5-T7* gene *1*)	(26)
DH5αZ1	(ϕ80*d lacZ*Δ*M15*) *endA1 recA1 hsdR17 supE44 thi-1 gyrA relA* Δ(*lac ZYA-argF*)*U169* (λ*att lacI*^{q} *tetR* Sp^{r})	(27)
CAG18470	*purC80*::Tn*10*	(28)
S1247	*bioA24*, *moaA29*, *zbh-283*::Tn*10*	(29)
MG1655	Wild type isolate (F^{-} *rph-1* λ^{-})	(30)
TL524	MG1655 Δ(*lac ZYA-argF*)*U169*	Tc^{s} derivative of TL504 (31)
PJ1	TL524 *sseA*::Sp^{r}	This work
Plasmids		
pT7-7	ColE1 origin Ap^{r} T7 promoter	(32)
pZE2 P_{N25}	ColE1 origin Km^{r} P_{N25}-*luc*	(27)
pGZ117	$(His)_6$ linker cloned into *Sal*I/*Hin*dIII of pT7-7	(33)
pHP45Ω	Sm^{r}/Sp^{r} in pHP45	(34)
pKO3	M13 origin, *repA*^{ts}, *cat*, *sacB*	(35)
pAG101	*sseA* cloned into *Nde*I/*Sal*I sites of pGZ117	This work
pJYA2	*sseA* in *Eco*RI/*Xba*I of pZE2 P_{N25}-*luc*	This work
pPJA2	Sp^{r} cloned into *Eco*RV site of *sseA* gene of pJYA2	This work
pPJ13	*sseA*::Sp^{r} cloned into *Sal*I/*Xba*I sites of pKO3	This work

General microbiological and molecular biological techniques

Polymerase chain reaction (PCR)

Approximately 0.5 μg of chromosomal DNA was used as a template for DNA amplification. A PCR kit from New England Biolabs and a GeneAmp PCR 9600 thermocycler (Perkin Elmer Cetus, Boston, MA) were used following the manufacturer's instructions. Primers used in these reactions are listed in Table 2.2. PCR fragments were purified from an agarose gel as described below.

Agarose gel electrophoresis

DNA was analyzed by electrophoresis on 0.8% to 1.2% agarose gels in 0.05 M Tris, 0.05 M boric acid, 0.01 M EDTA (TBE) buffer containing 0.5 μg/ml ethidium bromide, as described by Sambrook et al. (37).

Extraction of DNA fragments

DNA was extracted from the agarose gel with the GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ) following the instructions provided by the manufacturer.

Dephosphorylation of linear DNA

In some cases, linear DNA was dephosphorylated before use in cloning procedures, according to the manufacturer's instructions. Two-tenths unit of shrimp alkaline phosphatase (USB) was used per 1.0 picomole of DNA termini.

Fill-in reaction

When required, products of restriction enzyme digests with 5' protruding ends were filled in to generate blunt ends. This was done using 1-3 units of T4 DNA polymerase (NEB) in the recommended buffer supplemented with 100 μM of each dNTP. The reaction was incubated for 20 minutes at 12 °C.

Ligation reaction

For ligation reactions, approximately 0.1 pmol of digested vector and 0.4 pmol of insert were usually used. T4 DNA ligase (NEB) was used as suggested by the manufacturer's instructions. The reactions were usually carried out at 16 °C overnight (12-16 hours) and the enzyme was heat inactivated at 65 °C for 10 minutes.

Isolation of DNA

Plasmid DNA was isolated using the materials in the Wizard Minipreps kit (Promega) following the instructions provided by the manufacturer.

Transformation of *E. coli* with plasmid DNA

Competent *E. coli* cells were prepared by using a standard procedure involving treatment with ice-cold solutions of $CaCl_2$ and $MgCl_2$ as described by Sambrook et al. (37). About 100 μl of competent cells and 0.1 pmol of DNA were used for each transformation. The mixture of cells and DNA was incubated on ice for 30 minutes and then at 42°C for 2 minutes to allow plasmid entry into cells. The culture was then grown in 1 ml LB for 1 hour at 37°C and plated on LB agar containing the appropriate antibiotic.

Maintenance of strains

Stationary cultures of *E. coli* grown in LB broth were stored frozen at –70°C after the addition of 15% (v/v) glycerol.

Cloning of *sseA*

A former undergraduate student, Yo Jin Yoon, performed this work. The *sseA* gene was amplified from chromosomal DNA of strain MG1655 using the primer pair SSEA1 and SSEA2. The PCR product was cloned into *Eco*RI/*Xba*I-cut pZE2 P_{N25}, forming pJYA2. The expected nucleotide sequence of *sseA* in pJYA2 was confirmed by DNA sequencing with the dideoxynucleotide chain termination sequencing method first described by Sanger et al. (38). Sequencing was done using the DNA sequencing kit from Amersham Life Sciences by the method described in the manufacturer's instructions. The reactions were analyzed by electrophoresis on 6% polyacrylamide gels with 7 M urea in TBE buffer.

Table 2.2 Oligonucleotides used in this study

Name	Oligonucleotide sequences [a]	Restriction sites
SSEA1	5'-TTCAgaaTTCATCCCTGCCACAATGGC-3'	*Eco*RI
SSEA2	5'-ACCTtCTaGAAAGGCCGCGAACCAGACTAG-3'	*Xba*I
SSEA3	5'-gcttattaacaTATGCGTGAGAATTTACG-3'	*Nde*I
SSEA4	5'-CACTaagTCgaCCGGTAAATCTGC-3'	*Sal*I

[a] The sequence mismatches are lowercase and the restriction sites are underlined.

Construction of a plasmid for overproduction of SseA-His_6

An undergraduate student, Ashley Garth, initially did this work. To facilitate overexpression and purification of SseA, a pT7-7 derivative containing *sseA* fused to a cassette encoding a hexahistidine affinity tag was constructed. The *sseA* coding sequence was amplified using chromosomal DNA from strain MG1655 as the template and primers SSEA3 and SSEA4. Primer SSEA4 binds to the sequence just upstream of the last four codons of *sseA* and contains a *Sal*I site that allows in-frame fusion of *sseA* to the hexahistidine linker sequence of pGZ117 (33). Four native amino acids (EPVK) at the C-terminal end of SseA are replaced by a thrombin cleavage site and the hexahistidine tag (VDLVPRGS$(H)_6$). Following PCR, the *Nde*I/*Sal*I fragment was inserted into the same sites of pGZ117 to create pAG101.

Overexpression of SseA

Plasmids pJYA2 and pAG101 were introduced into strain DH5αZ1 and BL21(DE3), respectively, by transformation and used for overexpression of the SseA proteins. Overnight cultures were diluted 1:50 into LB medium containing the appropriate antibiotic and cultured further at 37 °C. At an optical density at 600 nm of about 0.4, expression in BL21(DE3)(pAG101) was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), whereas in DH5αZ1(pJYA2), expression was induced by the addition of 0.1 μg of tetracycline per ml. Cultures were grown for 2 hours at 37 °C after induction and then harvested by centrifugation. For the MST activity assay, cells from a 5-ml culture were resuspended in 0.5 ml 25 mM Tris-acetate (pH 8.6) and disrupted by sonication. After centrifugation, the supernatant fraction was assayed for MST activity. For protein purification, cells from a 200-ml culture were washed once in 100 ml 25 mM Tris-acetate (pH 7.8) and subjected to centrifugation. Cell pellets were stored at −70 °C until purification.

Purification of SseA-His_6

Cells from a 200 ml culture was resuspended in 8 ml of 25 mM Tris-acetate (pH 7.8), 150 mM NaCl, 1 mM DTT, 1mM PMSF, and disrupted by two passages through a French pressure cell at 16000 psi. Cell lysate was clarified by centrifugation at 8,000 X g for 15 min at 4°C yielding a cell-free extract, which was subjected to ultracentrifugation at 100,000 X g for 90 min at 4°C. The resulting supernatant was immediately loaded onto a 1 x 2 cm column of Ni^{2+}-chelating Sepharose (Pharmacia Biotech) previously equilibrated with 25 mM Tris-acetate (pH 7.8), 150 mM NaCl. The column was washed with 20 ml of 25 mM Tris-acetate (pH 8.0), 50 mM NaCl, 10 mM imidazole and the protein was eluted with a 60-ml gradient of 50 to 200 mM imidazole in the same buffer. The absorbance at 280 nm was monitored during the purification. Peak fractions were assayed for MST activity. The purest fractions (>95%), as judged by SDS-PAGE analysis, were pooled and stored at 4°C.

Purification of SseA

The purification of SseA was done so that its properties could be compared with the SseA variant with the C-terminal hexahistidine tag. Janet L. Donahue performed this work. SseA protein was purified from DH5αZ1 harboring pJYA2. The expression of SseA was described above. After harvesting, the cell pellet was stored at –70 °C. To release SseA from the cells, the frozen cells were thawed on ice, resuspended in 1/50 the original volume of buffer A (50 mM Tris-HCl (pH 7.2), 3 mM Na_2EDTA), and incubated on ice for 30 min. Cells were collected by centrifugation, and the supernatant fraction was saved. Incubation in buffer A and centrifugation was repeated twice more, and the three supernatant fractions were combined as the crude extract. Any debris was removed from the crude extract by centrifugation at 10,000 X g for 20 min. The majority of MST activity was precipitated by addition of $(NH_4)_2SO_4$ between 0.2 g ml^{-1} and 0.3 g ml^{-1}. The $(NH_4)_2SO_4$ pellet was dissolved in 2 ml buffer A and loaded onto a QHR15 anion exchange column (Waters) equilibrated at room temperature with buffer A. The flow rate was maintained at 1.5 ml min^{-1}. The column was washed with buffer A until no more

protein came off the column (about 45 ml of buffer A used). Then the column was developed with a 45-ml gradient from 0 to 105 mM NaCl in buffer A, followed by a 112-ml gradient of 105 mM to 255 mM NaCl in buffer A. There was only one major peak of protein that eluted from the column at about 220 mM NaCl. Unsatisfactorily, we found that most of the MST activity appeared in the flow-through fraction (unbound to the column). The MST activity eluted during the salt gradient contained less than 3% of that found in the flow-through. Therefore, to purify more MST, the flow-through fraction was reapplied to the anion exchange column and elution procedures were repeated twice. Similarly, most of MST activity appeared in the flow-through fraction. However, the peak fractions containing MST activity that were more than 90% homogenous as judged by SDS-PAGE were pooled and precipitated with 0.5 g of $(NH_4)_2SO_4$ ml^{-1}, redissolved in buffer A, and then dialyzed against 20 mM Tris-HCl (pH7.5), 0.5 M NaCl. The purified protein was stored at 4 °C for further characterization.

Determination of protein concentration

The method of protein determination first described by Bradford (39) was performed, using reagents purchased from Pierce Chemical Company (Rockford, IL) and the manufacturer's microassay method. A standard curve using 0 to 3 μg of bovine serum albumin was routinely made.

Estimation of subunit and native molecular masses

(i) Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE).

Proteins were electrophorosed on 12% polyacrylamide gels as described by Laemmli (40). A modified sample loading buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.02% bromophenol blue, and 1.5 M 2-mercaptoethanol was used. Protein was visualized with Fast Stain from Zoion Biotech, Inc. (Shrewsbury, MA).

(ii) Gel filtration chromatography.

This work was performed, with the assistance of Janet Donahue, to determine the apparent molecular mass of MST under nondenaturing conditions using a Waters Protein Pak Glass 300SW column in 20 mM Tris-HCl (pH 7.5)-50 mM NaCl. Void volume and total column volume were determined by using blue dextran and vitamin B_{12}, respectively. The protein standards included ribonuclease (17 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), and bovine serum albumin (66 and 132 kDa). Elution volumes for protein were determined by monitoring the optical density at 280 nm.

Assay of MST activity

Two assay methods were used to measure mercaptopyruvate sulfurtransferase activity for this study.

(i) The first assay utilized 3-mercaptopyruvate as the sulfur donor and cyanide as sulfur acceptor. The assay is based on a spectrophotometric method for detection of thiocyanate, one of the reaction products. The 0.5-ml assay mixture consisted of 100 mM Tris-acetate buffer (pH 8.6), 50 mM sodium mercaptopyruvate, 50 mM potassium cyanide, and cell-free extract or purified enzyme. The reaction was initiated by the addition of enzyme and terminated, after a 3- to10- min incubation at 25 °C, by the addition of 0.25 ml of 15% formaldehyde. Color was developed by the addition of 0.75 ml of 6.7% (w/v) ferric nitrate solution. A reaction mixture containing all reagents except the cell extract or SseA was used as the reagent blank. Thiocyanate (complexed with iron) was quantitated by absorbance at 460 nm. One unit of enzyme is defined as the amount that catalyzes the production of 1 μmole of thiocyanate per min. This assay is a modification of the assay used for rhodanese (7), with substitution of mercaptopyruvate for thiosulfate. This method is simpler to perform and was used for screening extracts for expression levels and during enzyme purification.

(ii) The second assay used 3-mercaptopyruvate as the sulfur donor and 2-mercaptoethanol as the sulfur acceptor. The production of pyruvate was measured after

reaction with 2,4-dinitrophenylhydrazine (DNP). This method is modified from the assay previously described (4). Cell-free extract or enzyme was added to a 0.4-ml assay mixture containing 100 mM Tris-HCl, pH 8.3, 15 mM sodium mercaptopyruvate, and 14 mM 2-mercaptoethanol. For preparation of a reagent blank, the cell extract or enzyme was omitted. Incubation was carried out at 30 °C for 3 minutes (unless indicated otherwise). Addition of 0.2 ml of 0.5 M $CdCl_2$ stopped the reaction. After 10 min, the sample was cleared by centrifugation and 0.2 ml of the supernatant was mixed with 0.2 ml of 0.1% DNP solution in 2 M HCl. After 5 min, 1 ml of 1.5 M NaOH was added. The precipitate was removed by centrifugation, and the absorbance at 435 nm was measured. This assay is more sensitive and specific for pyruvate, and it was used for the kinetic studies.

Disruption of the chromosomal *sseA* gene

To disrupt the *sseA* gene, a plasmid containing *sseA*::Sp^r was constructed (Figure 2.1) and then integrated into the chromosome using a method previously described (35). A 2-kb spectinomycin resistance cassette (Sp^r) from pHP45Ω (34) was blunt-end ligated into the unique *Eco*RV site (inside *sseA*) of dephosphorylated pJYA2 (3.7 kb) to create pPJA2 (5.7 kb) (Figure 2.1a). pPJA2 was then digested with *Xba*I at the 3' end of *sseA* and filled in to generate a blunt end. The DNA was subsequently cleaved at a unique *Xho*I site upstream of *sseA* (Figure 2.1b). The 3.1-kb *Xho*I-*Xba*I fragment of pPJA2, containing *sseA*::Sp^r, was then ligated into the *Sal*I/*Sma*I sites of pKO3 to create pPJ13. pKO3 (Figure 2.1d) is a gene replacement vector containing genes conferring resistance to chloramphenicol (*cat*), sensitivity to sucrose (*sacB*), and a temperature-sensitive origin of replication ($repA^{ts}$) (35). pPJ13 (pKO3 carrying *sseA*::Sp^r) was introduced by transformation into a wild-type strain (TL524) by selection for chloramphenicol resistance at 30 °C (Figure 2.1e). Transformants were purified once at 30 °C and then streaked on LB chloramphenicol at 43 °C. At the non-permissive temperature for plasmid replication, cells maintain chloramphenicol resistance only if pPJ13 integrates into the chromosome by homologous recombination at the *sseA* locus (Figure 2.1f). A single colony growing at 43 °C was diluted into LB medium and plated on LB

spectinomycin containing 5% sucrose at 30 °C. Sucrose-tolerant colonies appear if the integrated vector has been excised from the chromosome by a second recombination event (Figure 2.1g). Colonies from sucrose plates were replica plated to LB chloramphenicol at 30 °C to test for loss of the integrated pPJ13 vector (Figure 2.1h). Colonies that were sucrose-tolerant, spectinomycin-resistant and chloramphenicol-sensitive were purified. The strain was designated PJ1.

The integration site was verified genetically by showing that *sseA*::Sp^r was cotransducible with *purC80*::Tn*10*. The transduction was performed essentially as described by Silhavy et al. (41). Strain CAG18470 (*purC80*::Tn*10*) served as the P1 donor. LB tetracycline and LB tetracycline/spectinomycin served as the medium for selection and scoring, respectively.

Southern analysis of DNA of strain PJ1, containing the insertional mutation, compared with wild type DNA (strain TL524), was also performed to confirm the integration of the Sp^r gene in the *sseA* gene. Chromosomal DNA of both strains was digested with restriction enzymes (*Eco*RI and *Eco*RI/HindIII) and electrophoresed through a 1% agarose gel. DNA was transferred to the Hybond^{TM}-N+ nylon membrane (Amersham Biotech, Piscataway, NJ) and fixed by baking at 80 °C for 2 hours. Subsequently, the chromosomal DNA restriction fragments immobilized on the nylon membrane were detected using a direct nucleic acid labelling and detection system (ECL^{TM}, Pharmacia) as described by the manufacturer's instructions. The *Eco*RI-*Xba*I (1.1 kb) DNA fragment from pJYA2, containing the entire *sseA* gene (1 kb), was used as probe. The probe DNA was labeled with enzyme horseradish peroxidase, which was achieved by completely denaturing the probe (about 150 ng of DNA) before the addition of the peroxidase. Subsequent addition of a glutaraldehyde solution resulted in covalent cross-linking of the enzyme to the nucleic acid. Hybridization was performed at 42 °C for 18 hours in a solution that included 6M urea and 0.5 M NaCl to control the stringency of hybridization. After hybridization, the membrane was washed twice in a primary wash buffer (0.4 % SDS, 0.1x SSC) at 55 °C for 10 min, and then washed twice in a secondary wash buffer (0.2x SSC) at room temperature for 5 min, to remove unlabeled probe. The detection

system includes hydrogen peroxide (the substrate for peroxidase) and luminol (a chemiluminescence-producing substance). Coupling of the reduction of hydrogen peroxide by the enzyme to the oxidation of luminol produces blue light that was detected on X-ray film.

2.4: Results

SseA is a mercaptopyruvate sulfurtransferase

Sulfurtransferase activities were determined in cell-free extracts after induction of SseA. We found that overexpression of SseA resulted in about a one hundred-fold increase in MST activity. The MST specific activity in extracts containing overexpressed SseA was about 20 units mg^{-1}, while those of the vector controls was about 0.2 unit mg^{-1}. We also found a two- to three-fold increase in rhodanese activity associated with overexpression of SseA (data not shown). The high ratio of MST to rhodanese activity indicated that *sseA* encodes MST.

Purification of SseA-His_6

Expression of SseA with a C-terminal hexahistidine affinity tag from a T7 promoter in pAG101 produced an abundant protein in the cleared lysate (Figure 2.2, lane 2). Immobilized-metal affinity chromatography was very efficient for purification of SseA-His_6. After most of the loosely (nonspecifically) bound proteins were washed from the Ni^{2+}-chelating column with buffer containing 10 mM imidazole, two prominent protein peaks were eluted with a gradient of 50-200 mM imidazole. The second peak exhibited MST activity. SseA-His_6 in the pooled fractions was more than 95% homogeneous, as visualized by protein staining of an SDS-gel (Figure 2.2, lane 1). From a 200-ml culture, a total of 3.2 mg of protein was purified 11-fold to a MST specific activity of 300 U mg^{-1}. The purified enzyme was tested for stability under a variety of storage conditions. We found that freezing (–20 °C and –70 °C in the presence or absence of 15% (vol/vol)

glycerol) destroyed MST activity. However, loss of activity was minimized when the purified protein was stored in solution containing 0.5 M NaCl at 4 °C for up to 6 weeks.

Purification of SseA

The repeated treatment of cells with Tris-HCl buffer containing Na_2 EDTA provided an effective primary fractionation of proteins from strain DH5αZ1(pJYA2). Most of the overexpressed protein, exhibiting the size of SseA on an SDS-gel (Figure 2.3, lane 1), was present in the crude extract but not in the cleared lysate that was obtained from sonication of the remaining cells. The purification step using anion exchange chromatography described in the "Experimental procedures" was not efficient since the protein did not bind well to the column. Dialysis of the $(NH_4)_2SO_4$ fraction against low ionic strength solution before loading onto the column, using a different type of column, or adjusting the pH of the buffer may be required to obtain better binding. However, we obtained enough purified SseA for characterization. A total of 0.9 mg of protein, from a 200 ml-culture, was purified to a MST specific activity of about 200 U mg^{-1}. Although the yield of purified MST was low, after the final purification step, we found that the protein was more than 95% homogeneous as visualized by protein staining of an SDS-gel (Figure 2.3, lanes 3 and 4).

Molecular mass of SseA

As determined by SDS-PAGE, the apparent molecular masses of SseA and SseA-His_6 were 33 kDa (Figure 2.3) and 34 kDa (Figure 2.2), respectively, which are near the expected sizes based on the deduced amino acid sequences (30.8 and 31.9 kDa, respectively). Gel filtration chromatography was used to determine the apparent size of SseA under nondenaturing conditions. SseA and SseA-His_6 eluted at positions corresponding to proteins of 50 kDa and 45 kDa, respectively (Figure 2.4). We cannot conclude from the results whether SseA is a monomer or a dimer under these conditions. Strangely, as determined by gel filtration chromatography, the size of SseA-His_6 seemed

to be smaller than that of SseA. One explanation is that the His_6-tag has an influence on the conformation of the protein such that it elutes more slowly.

Optimum pH for enzyme activity

To maintain the pH of reactions between pH 7.3 to pH 10.7 during the assay, two buffer systems were used (0.1 M Tris-HCl, pH 7.3-9.3, and 0.1 M glycine-NaOH, pH 9.2-10.7). Both purified proteins (SseA and SseA-His_6) were analyzed for MST activity in these ranges of pH. The assay at each pH had its individual blank reaction (mixture without the enzyme). The results obtained from both enzymes were similar, so only results for SseA are shown here. By using the thiocyanate detection assay, it was found that the higher the pH (pH 7 up to pH 10.7 was tested), the higher the enzyme activity (Figure 2.5 a). When measuring pyruvate production, pH 8.3 was the optimum pH for MST activity (Figure 2.5b).

Catalytic properties of SseA

In the MST assay, addition of the enzyme into the reaction mixture is the last step for initiation of reaction. If mercaptopyruvate was mixed with the enzyme first, we noticed a characteristic yellow color, which precipitated upon longer incubation (>1 hour). However, this yellow color disappeared upon addition of cyanide or mercaptoethanol. We confirmed that the yellow precipitate was elemental sulfur by mass spectrometry as shown in Figure 2.6. The results agree with the report of the thiocyanate detection assay by Jarabak and Westley (11). They suggested that the production of thiocyanate is a nonenzymic step. It is likely that MST catalyzes the removal of sulfur from mercaptopyruvate, with rapid released of pyruvate, while the primary sulfur product is not discharged from the enzyme as thiocyanate, but as elemental sulfur. The sulfur product will be partitioned between non-enzymatic reactions to form thiocyanate or unreactive colloidal sulfur. Hence, there is a discrepancy in apparent enzyme activity when measuring pyruvate versus thiocyanate formation (11).

The well-characterized bovine kidney MST utilizes a single-displacement (sequential) mechanism (10, 11). Kinetic data from activity measurements of purified MST at various concentrations of 3-mercaptopyruvate, at fixed concentrations of 2-mercaptoethanol, were fit to this type of mechanism. Both SseA-His_6 and SseA had the same kinetic pattern. Results for SseA are shown in Fig. 2.7. The double reciprocal plots for 3-mercaptopyruvate form a linear, intersecting pattern (Fig. 2.7), indicating the sequential mechanism. The apparent K_m for mercaptopyruvate was about 5 mM when mercaptoethanol was fixed at 21 mM.

Despite the similarity of MST to rhodanese, substitution of thiosulfate for 3-mercaptopyruvate in the standard thiocyanate assay resulted in less than 1% of the rate of thiocyanate formation. We also found that 2-mercaptoethanol, cysteine and sodium sulfite (Na_2SO_3) were unable to substitute for 3-mercaptopyruvate as the sulfur donor. Previous studies (42) indicated that the alpha-keto group of 3-mercaptopyruvate is necessary for its utilization as a substrate for MST. In addition, it was reported that alpha-keto acids including alpha-ketobutyrate, alpha-ketoglutarate and pyruvate could inhibit MST activity in a concentration-dependent manner (43). Not surprisingly, several previously-characterized MSTs, including the bovine kidney enzyme and the MST of *E. coli*, are inhibited by pyruvate, a product of the reaction (10, 20). In addition to pyruvate inhibition, we observed substrate inhibition by cyanide and mercaptopyruvate when they were present at high concentration (greater than 25 mM, Figure 2.8). Inhibition may be explained by the earlier finding that mercaptopyruvate itself can serve as a sulfur acceptor when present at high concentrations, resulting in formation of mercaptopyruvate persulfide (11). The possibility of mercaptopyruvate cyanohydrin participation in the reaction was also considered to complicate the kinetic data (11). Mercaptopyruvate cyanohydrin is formed by an uncatalyzed reaction of cyanide ion with mercaptopyruvate. The cyanohydrin might be a competing sulfur-acceptor substrate in the system (11).

Disruption of the chromosomal *sseA* gene

To address the function of SseA in *E. coli*, a gene disruption was created by insertion of a gene encoding Sp^r (including its transcriptional and translational termination sequences) into the *sseA*-coding region (Figure 2.1). The genotype of the *sseA*::Sp^r strain was verified by Southern analysis, using a probe that specifically hybridizes to the *sseA* region. Chromosomal DNA of the wild type and the *sseA*::Sp^r strains were digested with *Eco*RI (Figure 2.9, lanes 1 and 3) as well as *Eco*RI and *Hin*dIII (Figure 2.9, lanes 2 and 4). *Eco*RI cuts twice, at positions about 1.7 kb upstream and 0.2 kb downstream of the *sseA* sequence. *Hin*dIII cuts at each end of the Sp^r gene, but there is no native *Hin*dIII site in the *sseA* region (Figure 2.9, upper panel). The patterns of Southern analysis obtained from the wild type DNA (Figure 2.9, lanes 1 and 2) and the *sseA*::Sp^r DNA (Figure 2.9, lanes 3 and 4) were different. In the case of digestion with *Eco*RI, only one band appeared at 2.9 kb for the wild type and at 4.9 kb for the *sseA*::Sp^r strains, as expected. In the case of digestion with *Eco*RI and *Hin*dIII, a 2.9-kb band and a 2.0-kb band appeared for the wild type and the *sseA*::Sp^r DNAs, respectively. These agree with the expected sizes. In the case of the *sseA*::Sp^r strain, we expected to see one more band at 0.9 kb. However, it is possible that the small fragment of DNA might not be transferred from the agarose gel or bind to the nylon membrane.

Genetic mapping by P1 transduction using a donor strain with a Tn*10* (*purC80*, 55.9 min) linked to *sseA* (54.8 min) also showed that the Sp^r marker was at the expected location in the *E. coli* chromosome. The observed co-transduction frequency between *purC80*::Tn*10* and *sseA*::Sp^r was 5% (expected 5%). Ten spectinomycin-sensitive transductants were obtained from a total of 194 tetracycline-resistant transductants tested.

Comparison of MST activities in wild type and *sseA*::Sp^r strains

The disparity in molecular weight between SseA (33,000) and previously-purified MST (23,800) (20) suggests that there may be more than one MST in *E. coli*. To find out if this may be true, the *sseA*-disrupted strain was assayed for MST activity and compared with the activity found for the wild type. Both assay methods were used. The results showed that the *sseA*-disrupted strain had no detectable MST activity when using the pyruvate detection assay (Table 2.3). We therefore conclude that SseA is the only or the predominant protein possessing mercaptopyruvate:mercaptoethanol sulfurtransferase activity in *E. coli*. An explanation for the activity detectable by using the thiocyanate assay is that crude extracts contain nonspecific sources of sulfur, including cystinyl and cysteine persulfide residues of proteins that can react with CN^-. However, assay mixtures without an addition of mercaptopyruvate had no SCN^- formation. These results suggest that unknown sulfurtransferases present in crude extracts may catalyze thiocyanate production from mercaptopyruvate.

Table 2.3 MST activities in crude extracts of wild type and *sseA*::Sp^r strains.

Strains	MST Specific Activity (U/mg) [a]	
	SCN^- assay	Pyruvate assay [b]
TL524 (wild type)	0.38	0.82
PJ1 (*sseA*::Sp^r)	0.24	ND [c]

[a] Determined using supernatant fractions of sonicated cells. The average values from duplicate assays are shown.

[b] Used 2-mercaptoethanol as the S acceptor

[c] No detectable activity (less than ~ 0.005 U/mg)

Characterization of the *sseA*::Sp^r mutant

To study the function of MST in *E. coli*, the growth of the *sseA*::Sp^r mutant and the wild-type were compared on both rich and minimal media. On LB medium, the *sseA*::Sp^r mutant (PJ1) grew at a rate similar to the wild-type parental strain (TL524). Moreover, the *sseA*::Sp^r mutant grew on glucose or glycerol minimal medium (M9 or AB salts) without supplements. Similar doubling times in liquid medium (M9 or LB) were observed for both the mutant and the parent strains. Growth rates on various sulfur sources, including sulfate, sulfite and cysteine, or on limiting sulfur (SO_4^{2-} or cysteine) were determined. The absence of MST had no apparent effect on the growth pattern under all conditions we investigated.

If MST catalyzes *in vivo* the production of sulfide and pyruvate from 3-mercaptopyruvate, the *sseA*::Sp^r mutant strain may be unable to utilize 3-mercaptopyruvate as sole source of sulfur or carbon (assuming mercaptopyruvate is transported into the cell). To find out if this is the case, the wild type (TL524) and the *sseA* mutant (PJ1) were grown overnight in M9 medium with limiting sulfur (65 μM $MgSO_4$) to adjust the physiological state. The cells were diluted into fresh medium of the same composition, grown to log phase, washed and transferred to different types of media with various sulfur and carbon sources (Fig 2.10). We found that in glucose medium with limiting sulfur (65 μM $MgSO_4$), both strains (TL524 and PJ1) grew to a maximum OD_{600} of about 1.5 (Fig 2.10 a). There was no significant difference in growth pattern between PJ1 and TL524 when 3-mercaptopyruvate was the sulfur source. Two possible explanations are 1) other sulfurtransferases compensate for the loss of MST in strain PJ1 or 2) mercaptopyruvate is chemically unstable and breaks down to form a utilizable source of sulfur. In the experiment where 1 mM mercaptopyruvate was provided as sole source of C and S, both strains grew equally poorly in this medium. Control experiments need to be performed to find out whether the concentration of mercaptopyruvate provided (break down to pyruvate + S) was too low for the cells to grow or whether the cells can take up pyruvate. The controls should include growing the cells in minimal salts containing 1 mM pyruvate as the sole source of C and with or

without 65 μM SO_4^{2-}. In sulfur-free medium (containing 0.2% glucose), both strains also grew about one doubling (probably utilizing either the sulfur that accumulated inside the cell from the previous generation, or the small amount of sulfur that contaminated the medium) and then stopped growing (when the sulfur was exhausted).

An experiment was carried out to study possible involvement of MST in the biosynthesis of molybdopterin (MPT). This was done because the immediate sulfur donor for the MPT synthase sulfurylase is unknown. Additionally, the MPT synthase sulfurylase from some organisms contain a C-terminal sulfurtransferase domain. MPT is required for the function of molybdenum-containing enzymes, including nitrate reductase. Mutants defective in nitrate reductase are chlorate resistant during anaerobic growth. Therefore, the chlorate phenotype of strains PJ1 (*sseA*::Sp^r), TL524 (wild-type) and S1247 (*moaA29*, a chlorate-resistant mutant) were compared under anaerobic conditions. We found that the wild type and *sseA*::Sp^r mutant were chlorate-sensitive, whereas S1247 was chlorate resistant. LB medium was used as the control for anaerobic growth; all three strains grew well. The results indicate that MST is not directly involved in the MPT biosynthetic pathway.

To determine whether MST is required for the biosynthesis of s^2U and s^4U of tRNA, strain PJ1 was sent to Dr. C. Lauhon at the University of Wisconsin for the assay. He found that strains deficient in MST (*sseA*::Sp^r) had wild type levels of s^2U and s^4U in tRNA (personal communication).

2.5: Discussion

Comparison of SseA with a previously characterized MST of *E. coli*

MST previously purified by Vachek and Wood (20) had an apparent molecular weight of 23,800 as determined by sedimentation equilibrium ultracentrifugation. The enzyme dissociated into two active fragments of about 12 kDa in solutions of low ionic strength (20). In contrast, we found that the apparent size of MST as determined by SDS-PAGE

was much larger (33 kDa). This discrepancy in size, along with the existence of multiple sulfurtransferase paralogs in *E. coli*, suggested there may be more than one MST in *E. coli*. The lack of detectable MST activity in the *sseA*-disrupted strain revealed that *sseA* is the only or the predominant gene encoding MST. However, conflicting results were obtained depending on the assay method used. Sulfurtransferases, including MST, have rather wide specificity for sulfur acceptor substrates and the actual physiological acceptor is not yet known. Based on our data from *in vitro* activity assays, we proved that SseA is the only or the predominant 3-mercaptopyruvate:mercaptoethanol sulfurtransferase in *E. coli*. It remains possible that other proteins of *E. coli* may be 3-mercaptopyruvate:cyanide sulfurtransferases. Identification of *in vivo* sulfur acceptor substrates is necessary to assign specific function(s).

Link et al. (44) previously analyzed the apparent molecular masses on SDS-PAGE and N-terminal amino acid sequences of 381 proteins encoded by the *E. coli* genome. They found that the observed values of some proteins deviate from the expected values as deduced from the DNA sequence. The deduced amino acid sequence of SseA is shown in Figure 2.11. Link et al. (44) found SseA in the cold osmotic shock fraction, suggesting SseA may be secreted to the periplasm. If secreted, an N-terminal signal sequence may be encoded by the *sseA* gene. The N-terminal amino acid sequence of SseA is STTWF as determined by Edman sequencing (44), which indicates that the actual translation start site is the fourth in-frame AUG codon, located 53 codons downstream of the first possible in-frame AUG. A good ribosome-binding site is found just upstream of this fourth in-frame AUG codon. Taken together, the results lead us to believe that the actual SseA protein contains a total of 281 amino acids (30.8 kDa), and that the upstream 53 amino acids do not constitute a signal sequence for secretion. Since the protein is acidic (the theoretical pIs for SseA (30.8 kDa) and SseA-His_6 (31.9 kDa) are 4.56 and 4.83, respectively), it may migrate more slowly on SDS gels (45). Our results show a slightly larger apparent size (SseA, 33 kDa and SseA- His_6, 34 kDa). Compared to the sizes of MSTs in other organisms, e.g. 32.8 kDa for rat liver MST (296 amino acids) (8) and 33 kDa for bovine kidney MST (4), it is reasonable that SseA contains 281 amino acids

(30.8 kDa). *Arabidopsis* MST1, however, is larger in size (378 amino acids) because it contains a 57 amino acids targeting-sequence at its N-terminus (12).

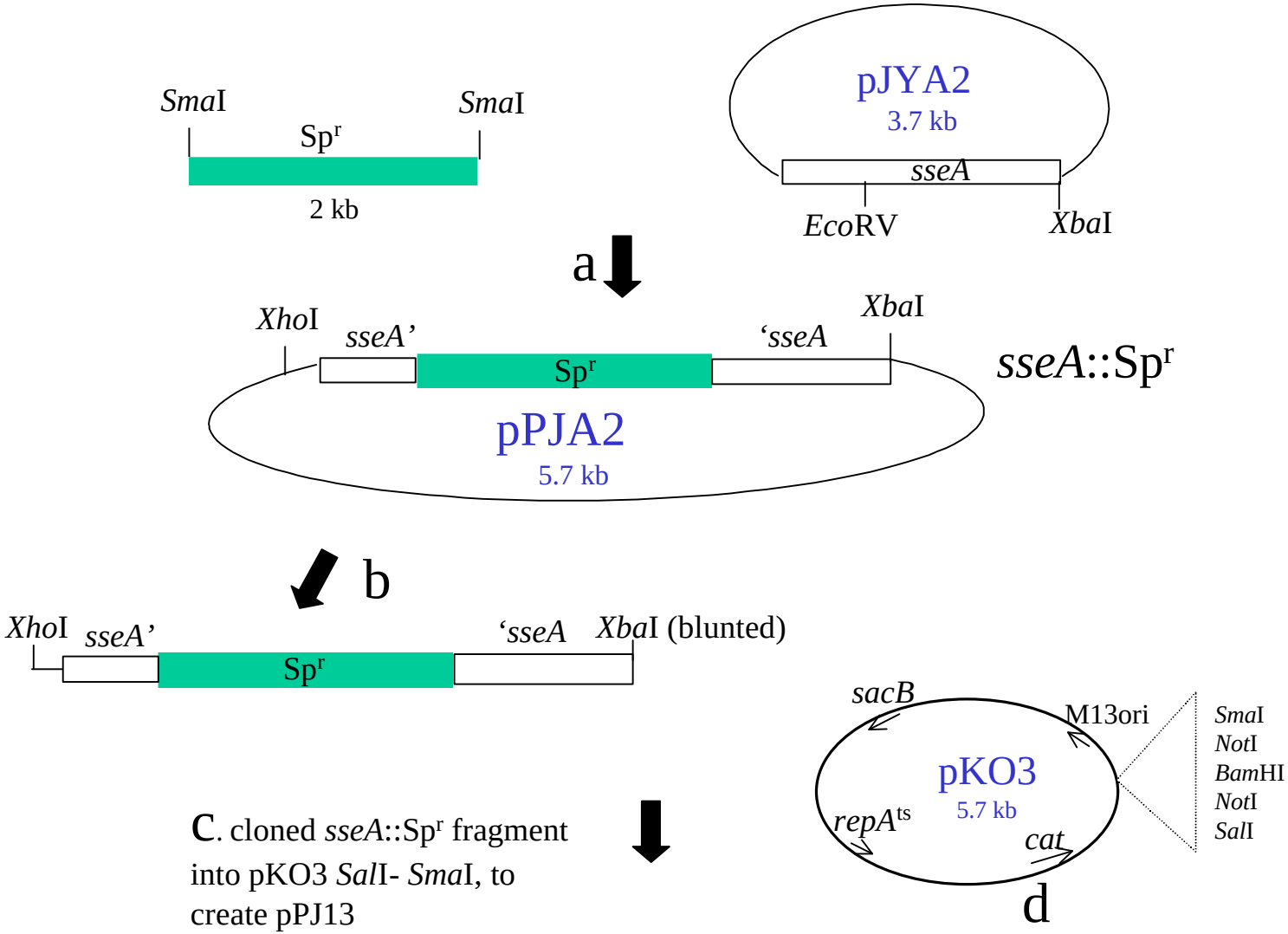
The MST purified by Vachek and Wood showed that pH values between 9.3-9.6 were optimum for enzyme activity by the thiocyanate detection assay (20). In contrast, we found that the more basic the pH, the greater the thiocyanate detected. One explanation is that the ratio of CN^- to HCN is determined by the pH (increases at higher pH). Thus, the rate of SCN^- production is higher at higher pH.

Physiological role of MST.

The physiological role of MST is still in question. Cyanide detoxification has been proposed as a possible role for sulfurtransferases. The enzymes are located prominently in liver and kidney tissues of mammals, which are the important organs where detoxification processes occur. The distribution of mammalian rhodanese in mitochondria and MST in both mitochondria and the cytoplasm led to the proposal that MST provides a first defense against cyanide as it enters the cytoplasm and then assists rhodanese with detoxification of any cyanide that enters mitochondria (14). However, the low affinity for cyanide of rhodanese from mammals as well as from *E. coli* makes this role seem unlikely. A role in cysteine metabolism cannot be excluded (19). Another function of rhodanese and related sulfurtransferases may be to provide sulfur to generate iron-sulfur centers (16-18, 46). In addition, a possible role of these enzymes in sulfur mobilization for biosynthesis of some S-containing cofactors cannot be disregarded, since some proteins such as ThiI and MoeB, participating in thiamin and MPT biosynthesis, contain a sulfurtransferase domain fused to their C-termini (47).

A *sseA*::Sp^r mutant strain was created to address the *in vivo* function of MST. Although the mutant was devoid of MST activity, it had no other discernible phenotype distinct from the wild-type strain, including growth using various sulfur sources or with limiting sulfur. Moreover, the completely prototrophic phenotype on minimal medium lacking supplements, chlorate-sensitivity during anaerobic growth, and the normal composition

of thionucleosides of the *sseA*::Sp^r mutant indicate that SseA may not be involved in the biosynthesis of thiamin, biotin, lipoic acid, MPT and thionucleosides. Nevertheless, we are aware that the presence of redundant sulfurtransferase enzymes in *E. coli* may prevent the detection of the potential phenotype for individual sulfurtransferase. Construction of strains with multiple deficiencies in genes encoding potential sulfurtransferases may be necessary in order to reveal a phenotype. Therefore, the physiological function of MST remains to be elucidated.


SmaI
Spr
SmaI
2 kb
pJYA2
3.7 kb
sseA
EcoRV
XbaI
a
XhoI
sseA'
'sseA
XbaI
Spr
pPJA2
5.7 kb
sseA::Spr
b
XhoI
sseA'
'sseA
XbaI (blunted)
Spr
C. cloned sseA::Spr fragment into pKO3 SalI- SmaI, to create pPJ13
sacB
M13ori
pKO3
5.7 kb
repAts
cat
SmaI
NotI
BamHI
NotI
SalI
d

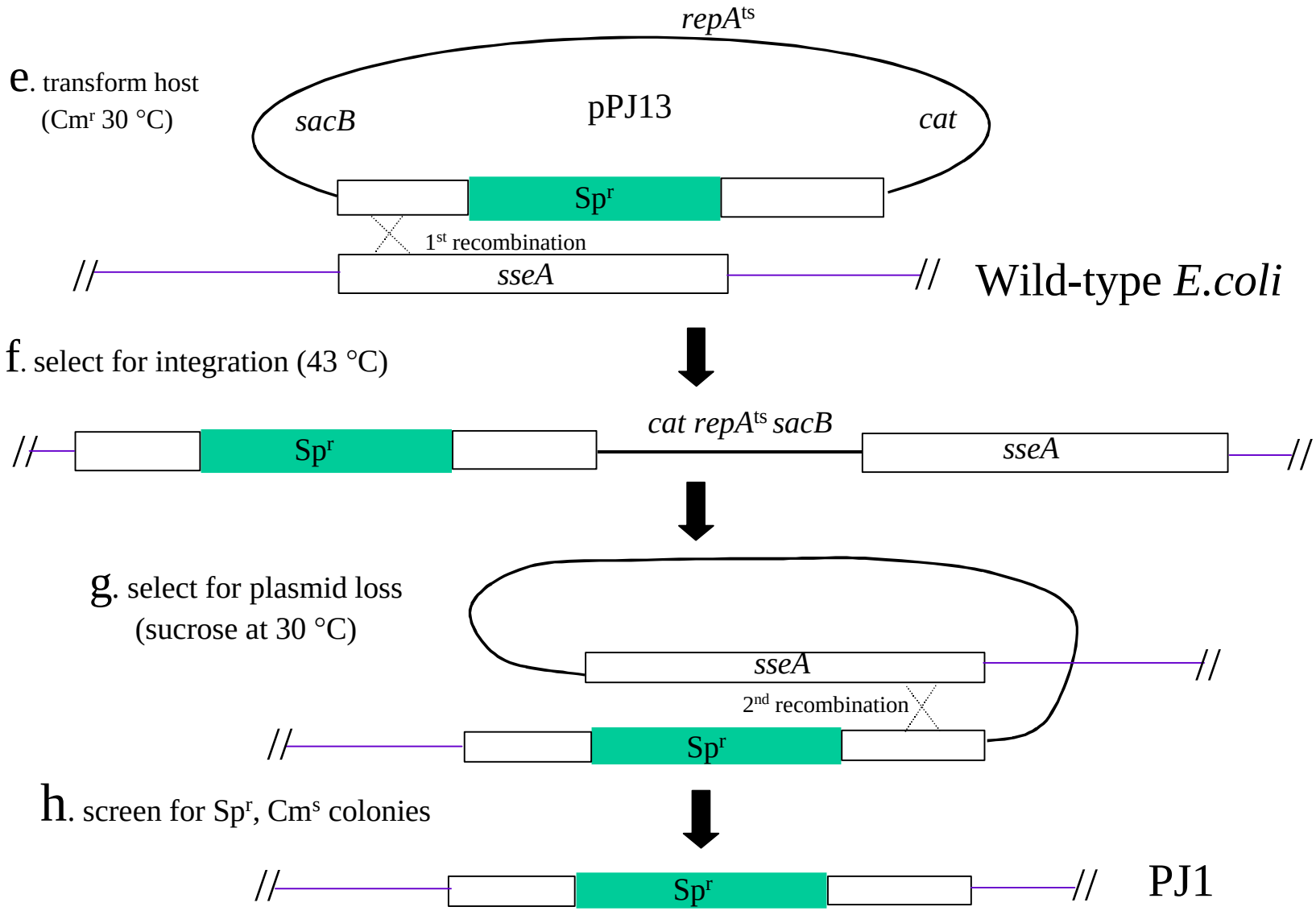

e. transform host
(Cmr 30 °C)
repAts
pPJ13
sacB
cat
Spr
1st recombination
sseA
Wild-type E.coli
f. select for integration (43 °C)
cat repAts sacB
Spr
sseA
g. select for plasmid loss
(sucrose at 30 °C)
sseA
2nd recombination
Spr
h. screen for Spr, Cms colonies
Spr
PJ1

Figure 2.1 Schematic for disruption of *sseA*.

Strain PJ1 was created by insertion of a Sp^r cassette into the *Eco*RV site of *sseA*, using the pKO3 (containing *repA*^{ts}, *sacB*, *cat*) plasmid/ chromosomal integration selection system (see Experimental Procedures). To identify the strains that contained the chromosomal insertion, techniques used included replica plating to medium containing spectinomycin, and Southern analysis.

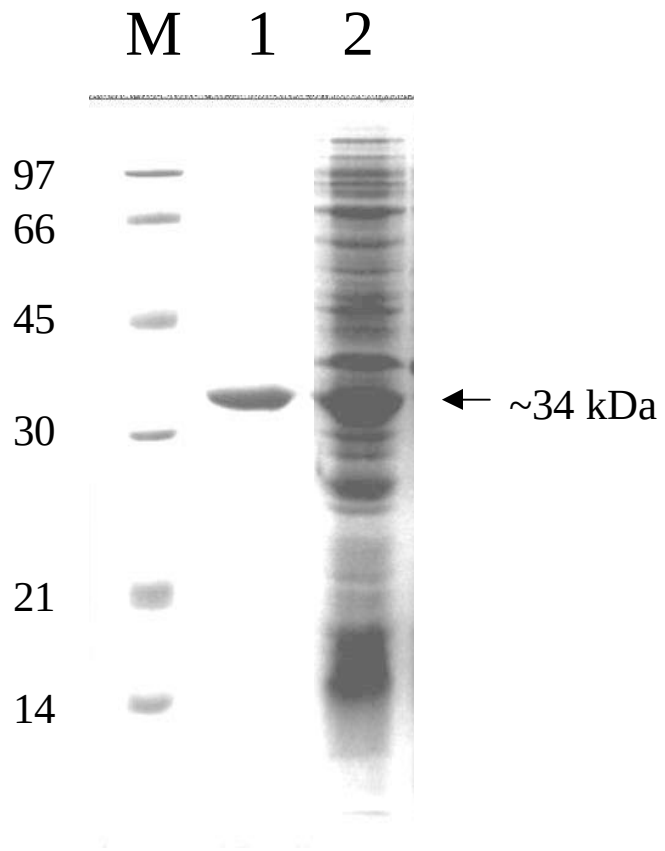


Figure 2.2 SDS-PAGE illustrating the overexpression and purification of SseA-His_6.

Lane M, molecular mass markers, with sizes indicated (kDa) at the left. Lane 1, purified SseA-His_6 (1 μg). Lane 2, total cellular protein of BL21(DE3)(pAG101) (10 μg).

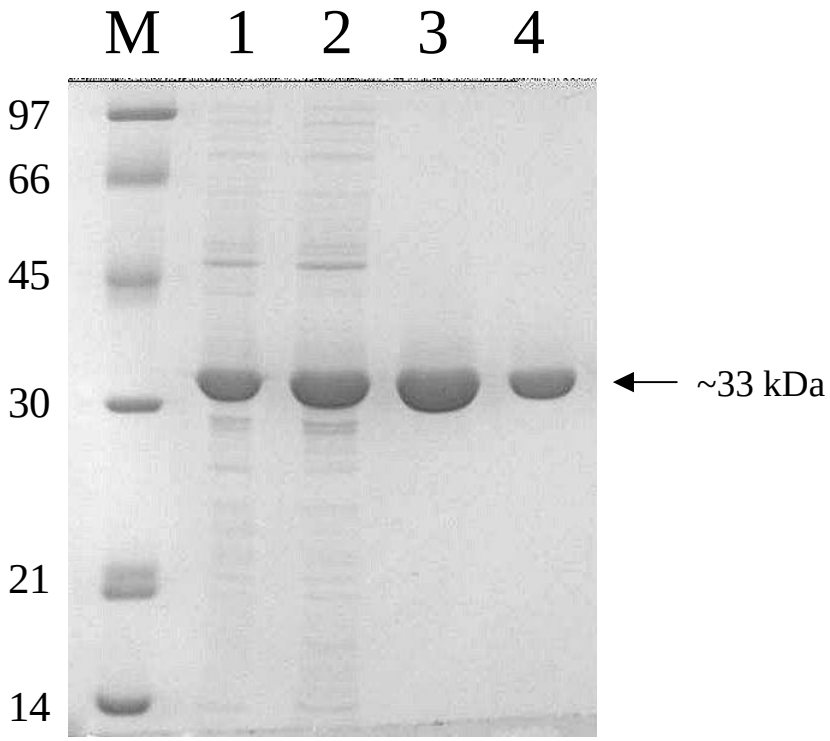


Figure 2.3 SDS-PAGE illustrating the overexpression and purification of SseA.

Lane M, molecular mass markers, with sizes indicated (kDa) at the left. Lane 1, crude extract of strain DH5αZ1(pJYA2) (5 μg). Lane 2, protein fraction after precipitation by ammonium sulfate (5 μg). Lanes 3 and 4, SseA after final purification (5 and 2 μg, respectively).

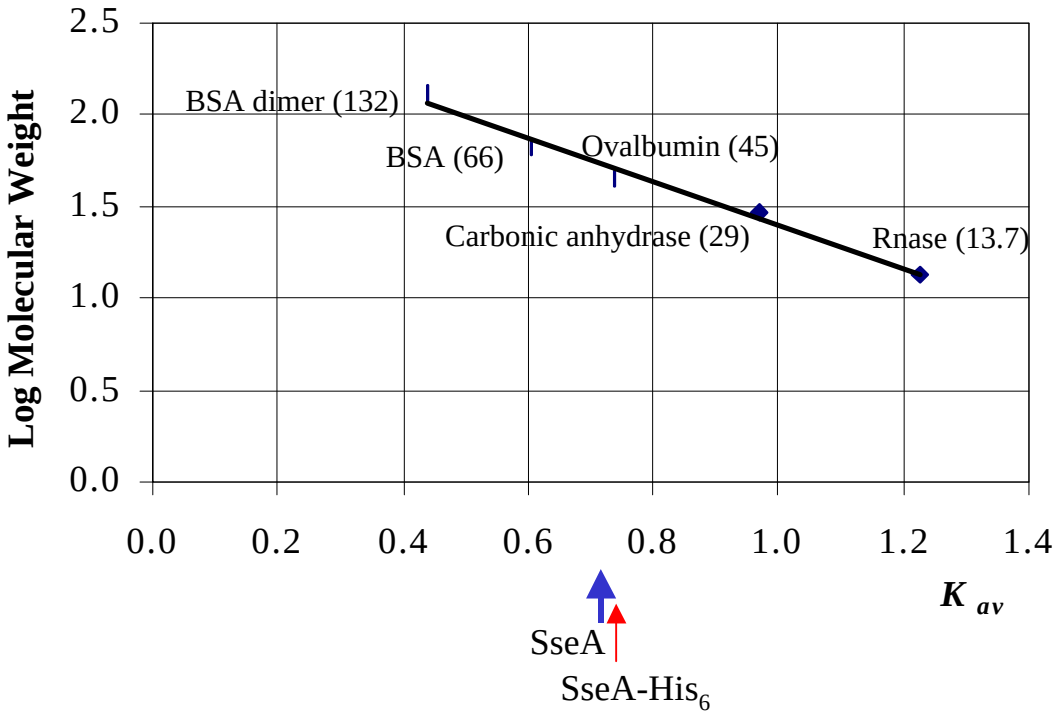


Figure 2.4 Native molecular weight of MST.

The logs of molecular weights of protein standards are plotted versus the K_{av} for the protein. Proteins and their sizes in kDa (parentheses) are shown next to the plotted data points. K_{av} is defined as $(V_e-V_v)/ (V_t-V_v)$, where V_e is the elution volume of the protein, V_v is the void volume of the column and V_t is the total volume of the column. For this column, V_v was 5.4 ml and V_t was 13.4 ml. The arrows indicate the K_{av} determined for SseA (0.74, thick blue arrow) and SseA-His_6 (0.77, thin red arrow).

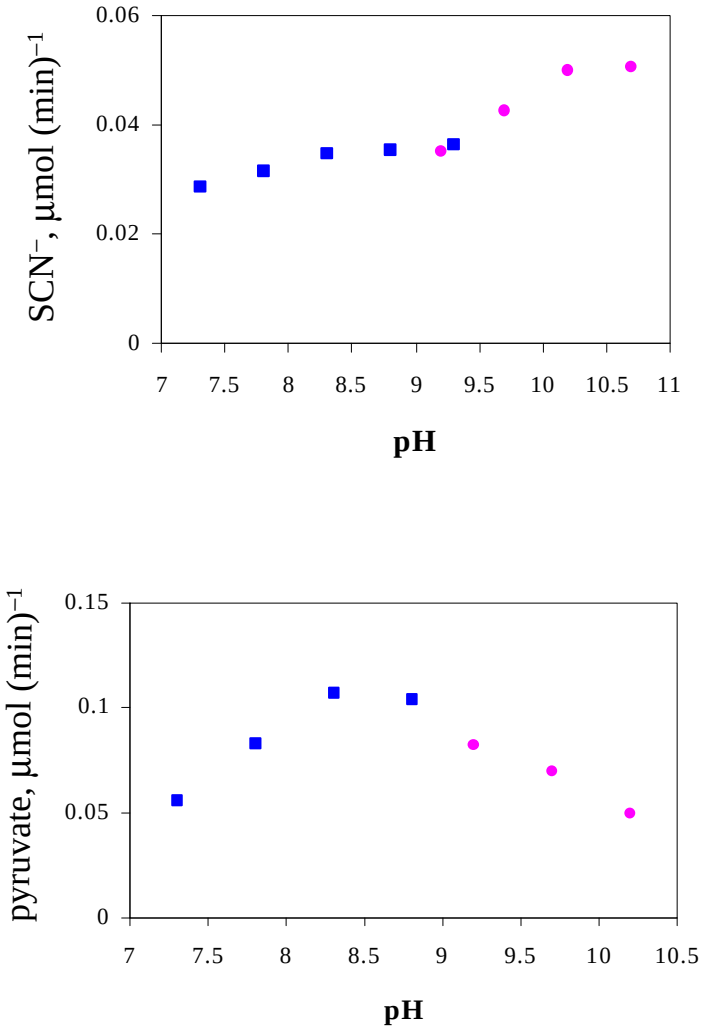


Figure 2.5 Dependence of MST activity on pH.

The reaction mixtures contained 30 ng of purified enzyme (SseA) and buffers as designated in the graph: 100 mM Tris-HCl (■), 100 mM glycine-NaOH (●). The average values of duplicate assays from two-independent experiments were plotted. (A) Assay of SCN^- formation in the presence of 50 mM 3-mercaptopyruvate and 50 mM KCN with an incubation time of 10 min. (B) Assay of pyruvate formation in the presence of 15 mM 3-mercaptopyruvate and 14 mM 2-mercaptoethanol with an incubation time of 3 min. Assays were performed as described under "Experimental Procedures". The results obtained from SseA-His_6 were similar to those from SseA.

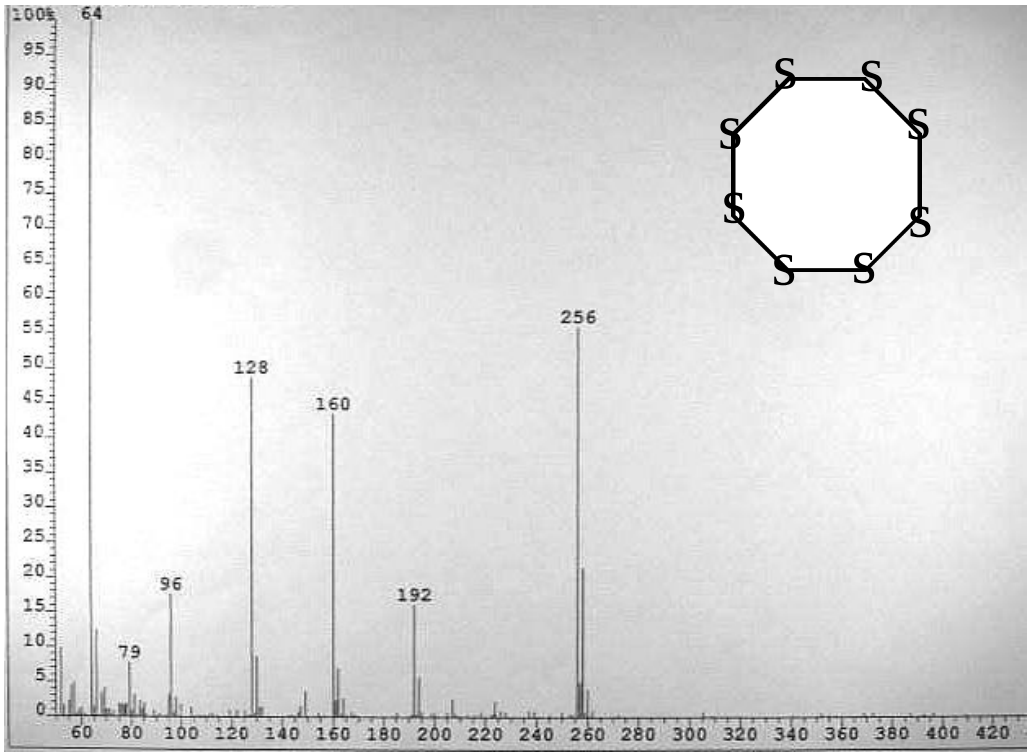


Figure 2.6 Mass spectrometric analysis of the precipitate obtained from the incubation of SseA with 3-mercaptopyruvate.

The mass spectrum revealed the production of elemental sulfur.

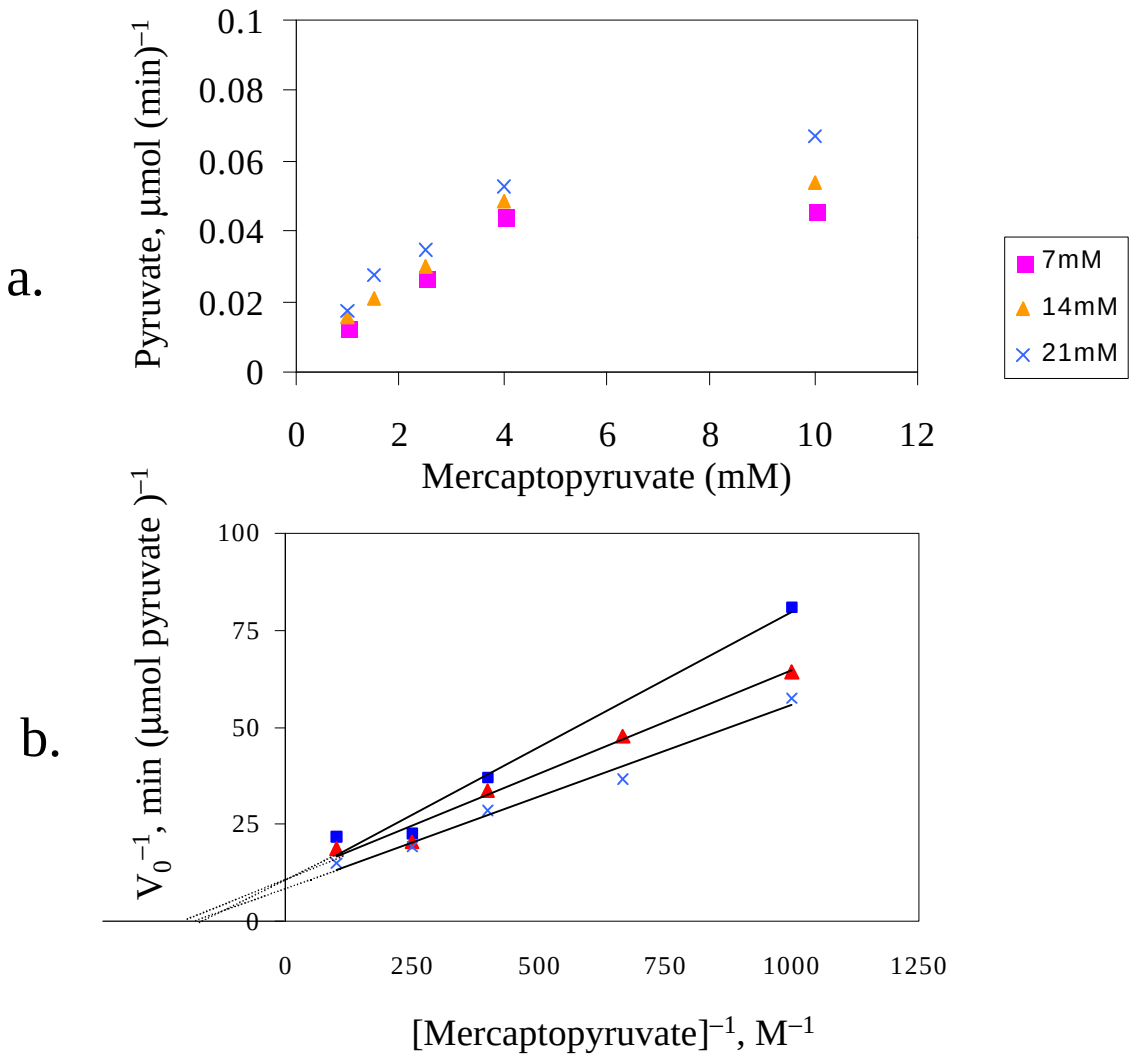


Figure 2.7 Dependence of MST activity on substrate concentration.

(a) Initial velocity data were obtained by measuring pyruvate formation. The reaction mixtures contained 30 ng of purified enzyme (SseA) and determinations were carried out as described under "Experimental Procedures". The incubation times were 3 min. (b) Double-recipical plot of pyruvate formation versus 3-mercaptopyruvate concentration at 7 mM (■), 14 mM (▲), and 21 mM (X) 2-mercaptoethanol. The results obtained from SseA-His_6 were similar to those from SseA.

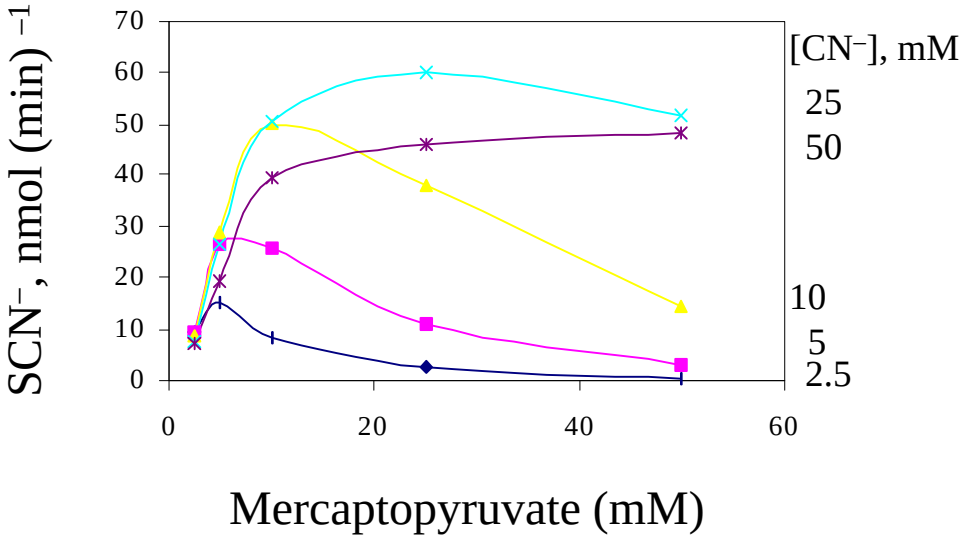


Figure 2.8 Substrate inhibition by mercaptopuvate and cyanide.

Initial velocity data were obtained by measuring SCN^- formation. The reaction mixtures contained 40 ng of purified enzyme (SseA-His_6) and determinations were carried out as described under "Experimental Procedures". The incubation times were 5 min. Concentrations of CN^- are indicated on the right. The experiment using SseA was also performed and the results were similar to those from SseA-His_6.

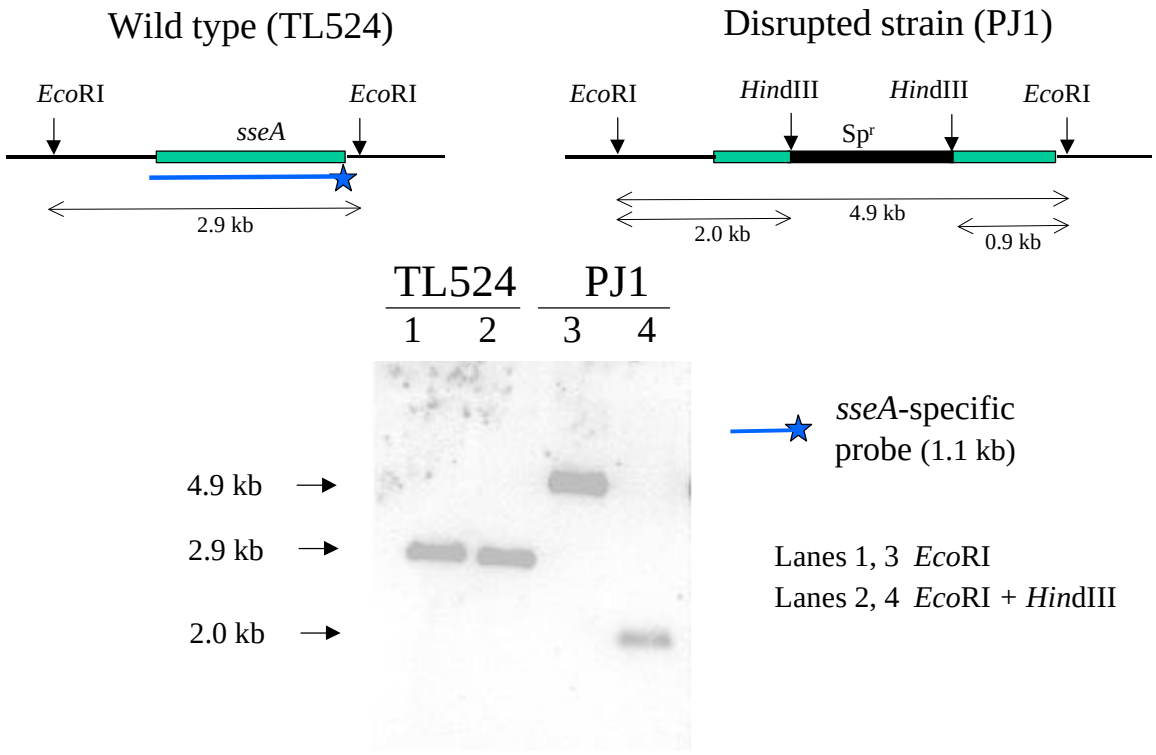


Figure 2.9 Southern analysis for verification of *sseA*::Sp^r in the genome.

Upper panel: Chromosomal DNA of TL524 containing *sseA*$^+$ (left), and PJ1 with *sseA*::Sp^r (right), are depicted with *Eco*RI and *Hin*dIII restriction sites. Lower lines show expected sizes of DNA fragments after appropriate restriction digestion. A probe (1.1 kb) corresponding to the entire *sseA* gene is depicted as a blue line with star. Bottom panel: Southern analysis of *E. coli* genomic DNA from strains indicated at the top (TL524, lanes 1 and 2, PJ1, lanes 3 and 4). Chromosomal DNA was digested with *Eco*RI (lanes 1 and 3) or with *Eco*RI and *Hin*dIII (lanes 2 and 4). DNA specific to the *sseA* gene was labeled with peroxidase and used for hybridization to the digested DNA. Substrate of peroxidase (H_2O_2) and enhancer (luminol) were added for detection. X-ray film was exposed for 5 minutes. λ DNA digested with *Hin*dIII was used as standard. The arrows indicate the sizes of restriction fragments derived from chromosomal DNA.

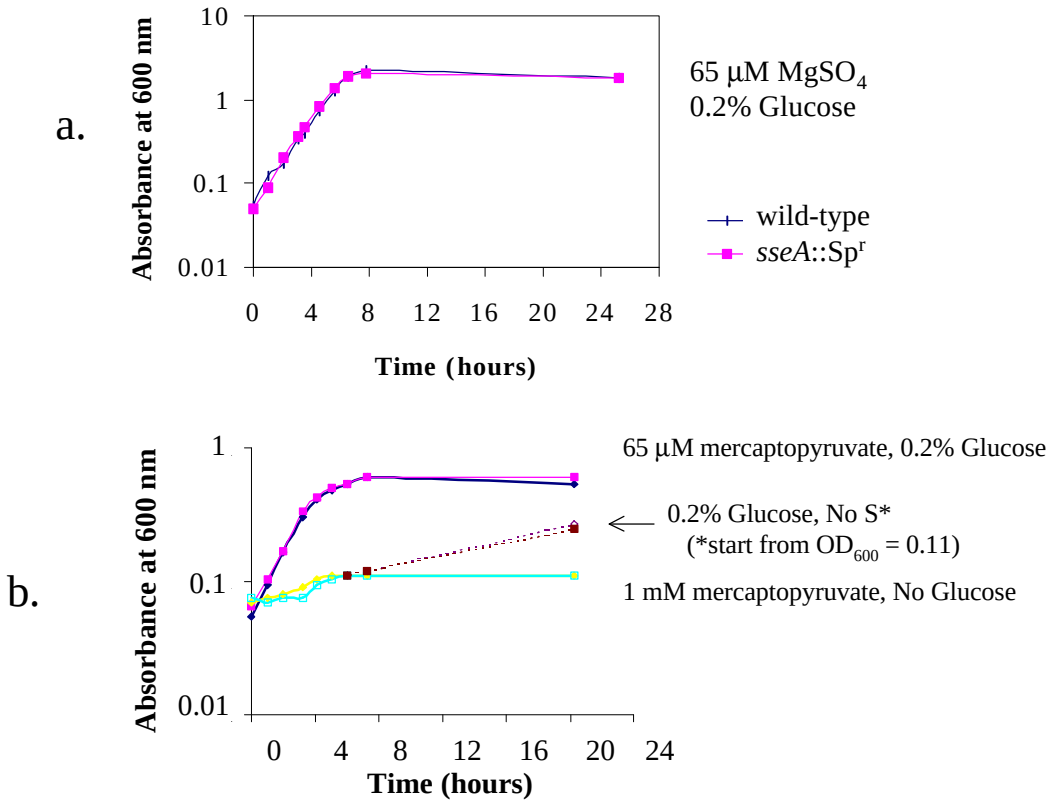


Figure 2.10 Growth of the *sseA*::Sp^r strain and with the wild type on mercaptopyruvate versus sulfate.

(a) Cells were grown at 37 °C in minimal medium containing 0.2% glucose with limiting sulfur (65 µM $MgSO_4$). (b) Cells were grown at 37 °C in minimal medium, containing 0.2% glucose without sulfur (dashed line), or containing 0.2% glucose and 65 µM 3-mercaptopyruvate (solid symbols), or containing 1 mM 3-mercaptopyruvate without glucose (open symbols). Diamonds (♦) and squares (■) indicate plots of the wild type (TL524) and the *sseA*::Sp^r strain (PJ1), respectively.

```
  1 MRENLRYVIQYHAHTIRILVRIVAQTAKSASFACRFSLNIRQFSLTPAKM
 51 EMPMSTTWFVGADWLAEHIDDPEIQIIDARMASPGQEDRNVAQEYLNGHI
101 PGAVFFDIEALSDHTSPLPHMLPRPETFAVAMRELGVNQDKHLIVYDEGN
151 LFSAPRAWWMLRTFGVEKVSILGGGLAGWQRDDLLLEEGAVELPEGEFNA
201 AFNPEAVVKVTDVLLASHENTAQIIDARPAARFNAEVDEPRPGLRRGHIP
251 GALNVPWTELVREGELKTTDELDAIFFGRGVSYDKPIIVSCGSGVTAAVV
301 LLALATLDVPNVKLYDGAWSEWGARADLPVEPVK
```

Figure 2.11 The predicted amino acid sequence of SseA.

The protein sequence deduced from the DNA sequence consists of 334 amino acids. The experimentally determined N-terminal amino acid sequence is **STTW,** as indicated in bold letters, yielding a protein of 281 amino acids.

2.6: References

1. Meister A. Conversion of the α-keto analog of cysteine to pyruvate and sulfur. Fed Proc 1953;12:245.
2. Wood JL, Fiedler H. β-mercaptopyruvate, a substrate for rhodanese. J Biol Chem 1953;205:231-234.
3. Westley J, Adler H, Westley L, Nishida C. The sulfurtransferases. Fundam Appl Toxicol 1983;3:377-82.
4. Jarabak R. 3-Mercaptopyruvate sulfurtransferase. Methods Enzymol 1981;77:291-7.
5. Nakamura T, Yamaguchi Y, Sano H. Plant mercaptopyruvate sulfurtransferases molecular cloning, subcellular localization and enzymatic activities. Eur J Biochem 2000;267:5621-5630.
6. Tatusov RL, Galperin MY, Natale DA, Koonin EV. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res 2000;28:33-6.
7. Westley J. Thiosulfate: cyanide sulfurtransferase (rhodanese). Methods Enzymol 1981;77:285-91.
8. Nagahara N, Okazaki T, Nishino T. Cytosolic mercaptopyruvate sulfurtransferase is evolutionarily related to mitochondrial rhodanese. Striking similarity in active site amino acid sequence and the increase in the mercaptopyruvate sulfurtransferase activity of rhodanese by site-directed mutagenesis. J Biol Chem 1995;270:16230-5.
9. Nagahara N, Nishino T. Role of amino acid residues in the active site of rat liver mercaptopyruvate sulfurtransferase. CDNA cloning, overexpression, and site-directed mutagenesis. J Biol Chem 1996;271:27395-401.
10. Jarabak R, Westley J. Steady-state kinetics of 3-mercaptopyruvate sulfurtransferase from bovine kidney. Arch Biochem Biophys 1978;185:458-65.
11. Jarabak R, Westley J. 3-Mercaptopyruvate sulfurtransferase: rapid equilibrium-ordered mechanism with cyanide as the acceptor substrate. Biochemistry 1980;19:900-4.

12. Papenbrock J, Schmidt A. Characterization of two sulfurtransferase isozymes from *Arabidopsis thaliana*. Eur J Biochem 2000;267:5571-9.
13. Papenbrock J, Schmidt A. Characterization of a sulfurtransferase from *Arabidopsis thaliana*. Eur J Biochem 2000;267:145-54.
14. Nagahara N, Ito T, Minami M. Mercaptopyruvate sulfurtransferase as a defense against cyanide toxication: molecular properties and mode of detoxification. Histol Histopathol 1999;14:1277-86.
15. Pagani S, Eldridge M, Eady RR. Nitrogenase of *Klebsiella pneumoniae*. Rhodanese-catalysed restoration of activity of the inactive 2Fe species of the Fe protein. Biochem J 1987;244:485-8.
16. Pagani S, Bonomi F, Cerletti P. Enzymic synthesis of the iron-sulfur cluster of spinach ferredoxin. Eur J Biochem 1984;142:361-6.
17. Bonomi F, Pagani S, Cerletti P. Insertion of sulfide into ferredoxins catalyzed by rhodanese. FEBS Lett 1977;84:149-52.
18. Bonomi F, Pagani S, Cerletti P, Cannella C. Rhodanese-Mediated sulfur transfer to succinate dehydrogenase. Eur J Biochem 1977;72:17-24.
19. Donadio S, Shafiee A, Hutchinson CR. Disruption of a rhodaneselike gene results in cysteine auxotrophy in *Saccharopolyspora erythraea*. J Bacteriol 1990;172:350-60.
20. Vachek H, Wood JL. Purification and properties of mercaptopyruvate sulfur transferase of *Escherichia coli*. Biochim Biophys Acta 1972;258:133-46.
21. Ray WK, Zeng G, Potters MB, Mansuri AM, Larson TJ. Characterization of a 12-kilodalton rhodanese encoded by *glpE* of *Escherichia coli* and its interaction with thioredoxin. J Bacteriol 2000;182:2277-84.
22. Altschul SF, Madden TL, Schaffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389-402.
23. Hama H, Kayahara T, Ogawa W, Tsuda M, Tsuchiya T. Enhancement of serine-sensitivity by a gene encoding rhodanese-like protein in *Escherichia coli*. J Biochem (Tokyo) 1994;115:1135-40.

24. Hama H, Sumita Y, Kakutani Y, Tsuda M, Tsuchiya T. Target of serine inhibition in *Escherichia coli*. Biochem Biophys Res Commun 1990;168:1211-6.
25. Miller JH. A short course in bacterial genetics. A laboratory manual and handout for *Escherichia coli* and related bacteria. New York: Cold Spring Harbor Laboratory, 1992.
26. Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 1986;189:113-30.
27. Lutz R, Bujard H. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. Nucleic Acids Res 1997;25:1203-10.
28. Nichols BP, Shafiq O, Meiners V. Sequence analysis of Tn*10* insertion sites in a collection of *Escherichia coli* strains used for genetic mapping and strain construction. J Bacteriol 1998;180:6408-11.
29. del Campillo-Campbell A, Campbell A. Molybdenum cofactor requirement for biotin sulfoxide reduction in *Escherichia coli*. J Bacteriol 1982;149:469-78.
30. Blattner FR, Plunkett G, Bloch CA, et al. The complete genome sequence of *Escherichia coli* K-12. Science 1997;277:1453-74.
31. Podkovyrov SM, Larson TJ. Identification of promoter and stringent regulation of transcription of the *fabH, fabD* and *fabG* genes encoding fatty acid biosynthetic enzymes of *Escherichia coli*. Nucleic Acids Res 1996;24:1747-52.
32. Tabor S, Richardson CC. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc Natl Acad Sci U S A 1985;82:1074-8.
33. Zeng G, Larson TJ. Use of engineered thrombin cleavage site for determination of translational reading frames. Biotechniques 1996;21:822-4.
34. Prentki P, Krisch HM. *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene 1984;29:303-13.
35. Link AJ, Phillips D, Church GM. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J Bacteriol 1997;179:6228-37.

36. Johnson ME, Rajagopalan KV. Involvement of *chlA*, *E*, *M*, and *N* loci in *Escherichia coli* molybdopterin biosynthesis. J Bacteriol 1987;169:117-25.
37. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory, 1989.
38. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 1977;74:5463-7.
39. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
40. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
41. Silhavy TJ, Berman ML, Enquist LW. Experiments with gene fusions. New York: Cold Spring Harbor Laboratory, 1984.
42. Porter DW, Baskin SI. Specificity studies of 3-Mercaptopyruvate sulfurtransferase. J Biochem Toxicol 1995;10:287-92.
43. Porter DW, Baskin SI. The effect of three alpha-keto acids on 3-mercaptopyruvate sulfurtransferase activity. J Biochem Toxicol 1996;11:45-50.
44. Link AJ, Robison K, Church GM. Comparing the predicted and observed properties of proteins encoded in the genome of *Escherichia coli* K-12. Electrophoresis 1997;18:1259-313.
45. Garfin DE. One-dimensional gel electrophoresis. In: Deutscher MP, ed. Methods in enzymology. Guide to protein purification. SanDiego: Academic Press, Inc, 1990:425-441.
46. Pagani S, Galante YM. Interaction of rhodanese with mitochondrial NADH dehydrogenase. Biochim Biophys Acta 1983;742:278-84.
47. Palenchar PM, Buck CJ, Cheng H, Larson TJ, Mueller EG. Evidence that ThiI, an enzyme shared between thiamin and 4-thiouridine biosynthesis, may be a sulfurtransferase that proceeds through a persulfide intermediate. J Biol Chem 2000;275:8283-6.

Chapter 3

Construction of *Escherichia coli* strains deficient in multiple sulfurtransferase genes

3.1: Abstract

The genome of *Escherichia coli* is predicted to contain eight genes that encode sulfurtransferase-like proteins. The studies in our laboratory demonstrated that insertion or deletion mutation of *sseA* and *glpE* that encode MST and rhodanese, respectively, did not abolish all sulfurtransferase activity. Moreover, no discernable phenotype was observed from the *sseA*::Sp^r and Δ*glpEGR*::Km^r mutants. In the present study, other predicted sulfurtransferase genes, including *ynjE* and *yceA*, were cloned. Characterization of the corresponding proteins demonstrated that YnjE did not possess either MST or rhodanese activity. Strains deficient in multiple sulfurtransferase genes, including *sseA*, *ynjE* and *glpE*, and a gene encoding cysteine desulfurase, *iscS*, were constructed. A chlorate-resistant phenotype was observed in the IscS-deficient strains, which suggests an involvement of IscS in the pathway for biosynthesis of nitrate reductase. However, no phenotype associated with sulfurtransferase deficiency has been observed.

3.2: Introduction

Sulfurtransferase enzymes catalyze the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors, such as cyanide, thiols, dithiols, sulfite and sulfinates. The well-characterized sulfurtransferases are thiosulfate:cyanide sulfurtransferase (rhodanese, EC 2.8.1.1) and 3-mercaptopyruvate:cyanide sulfurtransferase (MST, EC 2.8.1.2), which catalyze, *in vitro*, the transfer of sulfane sulfur from thiosulfate (reaction A) and 3-mercaptopyruvate (reaction B), respectively, to cyanide.

$$SSO_3^{2-} + CN^- \rightarrow SO_3^{2-} + SCN^- \text{…….. (reaction A)}$$

$HSCH_2COCOO^- + CN^- \rightarrow CH_3\ COCOO^- + SCN^-$…….. (reaction B)

Two distinct rhodaneses (1) and MST (2) were detected in *E. coli* many years ago. However, the genes and the sequences for these enzymes have only recently been identified in our laboratory. Ray and coworkers (3) characterized a rhodanese encoded by *glpE*. However, the deletion of *glpE* gene (Δ*glpEGR*) did not significantly decrease the total rhodanese activity measured in cell extracts, which indicates the presence of other proteins possessing rhodanese activity in *E. coli*. My work described in chapter 2 identified an MST of *E. coli* that is encoded by the *sseA* gene. Two different assays were used in that study to detect MST activity in extracts of the *sseA*::Sp^{r}mutant: an assay for the detection of pyruvate and an assay for thiocyanate formation. 2-Mercaptoethanol and cyanide are used as the sulfur acceptor substrates in the pyruvate and thiocyanate assays, respectively. Disruption of *sseA* decreased 3-mercaptopyruvate:2-mercaptoethanol sulfurtransferase activity to non-detectable level. However, 60% of 3-mercaptopyruvate:cyanide sulfurtransferase activity remained in the cell extract of the *sseA*::Sp^r mutant. When cyanide was the sulfur acceptor, it was not completely clear whether thiocyanate was produced from unknown sulfurtransferase proteins that may be present in the extract. Rhodanese displays amino acid sequence similarity to MST, and it is known that rhodanese exhibits weak MST activity and vice versa (4). GlpE is able to use 3-mercaptopyruvate as a sulfur donor, but at less than 1% of the rate when compared to the use of thiosulfate as a substrate (3). At least eight genes in the *E. coli* genome are predicted to encode sulfurtransferase-like proteins, including SseA (Figure 3.1). Besides GlpE, other proteins homologous to SseA in the extract may catalyze thiocyanate formation from mercaptopyruvate and cyanide.

Previous studies showed that the *sseA*::Sp^r and the Δ*glpEGR* deletion mutants did not exhibit discernable phenotypic changes. Due to the redundancy of the sulfurtransferases in *E. coli*, single mutations may not necessarily confer a phenotypic change. In the present work, we tried to identify whether other genes of *E. coli* encode an active MST as well as whether disruption of these genes results in a phenotypic change. We started by cloning the *ynjE* and *yceA* genes, since their amino acid sequences are most similar to the

SseA protein. We found that YnjE does not possess MST or rhodanese activity. The expression of YceA, however, could not be demonstrated by SDS-gel analysis. A method generally used in our laboratory to inactivate a chromosomal gene, which facilitates subsequent construction of strains with multiple gene disruption, was developed. This system was used to construct a strain with a *ynjE*-deletion in the genome. Furthermore, a deletion of *yceA* in the chromosome was also made, following several attempts. We then constructed strains deficient in multiple sulfurtransferase genes (*sseA, ynjE* and *glpE*) to find out if a phenotype related to sulfurtransferase deficiency could be identified.

The *iscS* gene of *E. coli*, which encodes a cysteine desulfurase, is involved in mobilization of sulfur from cysteine for formation of Fe-S clusters (5), and biosynthesis of thiamin and 4-thiouridine (6). *iscS* deletion mutants have thiamin and nicotinic acid auxotrophies. Since the sulfur transfer mechanism for biosynthesis of Fe-S clusters and sulfur-containing cofactors, including thiamin, molybdopterin, biotin and lipoic acid are not completely known, we hypothesize that some of the sulfurtransferases may cooperate with IscS or participate in some aspects of the sulfur transfer. Here, we created the strains deficient in *sseA*, or *glpE*, or *ynjE* along with Δ*iscS* to find out whether the double mutants have additional defects, besides thiamin and nicotinic acid deficiencies.

3.3: Experimental Procedures

Materials

Unless listed below, the reagents used were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical Company (St. Louis, MO). Restriction endonucleases were purchased from several sources, including New England Biolabs (Beverly, MA) and Promega (Madison, WI) and used with the buffers supplied by the manufacturers. *Taq* DNA polymerase was obtained from Promega. T4 polynucletide kinase, T4 DNA ligase and deoxyribonucleotides for PCR were acquired from New England Biolabs and used with buffers from the supplier. Shrimp alkaline phosphatase, with the appropriate buffer,

and isopropylthio-β-D-galactopyranoside (IPTG) were obtained from United States Biochemical (Cleveland, OH). Difco Laboratories (Detroit, MI) was the source of yeast extract, tryptone and bacto agar. Ammonium thiosulfate was purchased from Aldrich (Milwaukee, WI). Ferric nitrate and the sodium salt of mercaptopyruvic acid were purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Oligonucleotides for PCR were synthesized by DNAgency (Malvern, PA).

Bacterial strains and plasmids

E. coli strains and plasmids used in this study are listed in Tables 3.1 and 3.2, respectively.

Media and growth conditions

E. coli cells were grown aerobically at 37 °C in Luria-Bertani broth (LB) (7). Cells containing plasmids were grown in the presence of antibiotic as appropriate. Antibiotics were included at 100 μg/ml for ampicillin (Ap), 50 μg/ml for kanamycin (Km), 30 μg/ml for chloramphenicol (Cm), 10 μg/ml for tetracycline (Tc) and 25 μg/ml for spectinomycin (Sp) when required. M9 salts or AB minimal media (7) were used in some experiments and supplemented with one or more of the following: 0.2% glucose or 0.4% glycerol, 2 μg/ml thiamin, 0.01 M $MgSO_4$ and 0.005 M $CaCl_2$ where appropriate. In some cases, 50 μg/ml nicotinic acid, 40 μg/ml L-leucine, 40 μg/ml isoleucine, 5 mM adenosine, and 0.3 mM guanosine were supplemented as required.

LB-chlorate medium (containing 20 mM $NaClO_3$) was prepared as previously described (8). Cultures were grown at 37 °C under anaerobic conditions using a kit (GasPak Plus™) from Becton Dickinson (Sparks, MO). Strain S1247 (*bioA24*, *moaA29*, *zbh-283*::Tn*10*) was used as the positive control for the chlorate-resistant phenotype (9). Anaerobic growth at 37 °C on LB plates (without chlorate) was also performed as a control.

General molecular biological and biochemical techniques

Polymerase chain reactions (PCR) were done using the reagents from New England Biolabs, *Taq* DNA polymerase from Promega, and a GeneAmp PCR System 9600 thermocycler (Perkin Elmer Cetus, Boston, MA). Approximately 0.5 μg of chromosomal DNA or a suspension of an isolated colony in water heated at 95 °C for 30 minutes was used as a template. PCR primers used are listed in Table 3.3. DNA was extracted from agarose gel through use of the DNA gel band and purification kit (Amersham Pharmacia Biotech, Piscastaway, NJ) following the instructions provided by the manufacturer. Ligation reactions were performed as suggested by New England Biolabs using their T4 DNA ligase. Plasmid DNA was isolated by the QIAprep® Minipreps kit from QIAGEN (Valencia, CA) following the instructions provided by the manufacturer. Analysis of DNA by gel electrophoresis with 0.8 to 1.2 % agarose was performed using TBE buffer (0.05 M Tris, 0.05 M boric acid, 0.01 M Na_2 EDTA), containing 0.5 μg/ml ethidium bromide, as described by Sambrook et al.(10). Competent cells were prepared by the $CaCl_2$ method and the transformation of cells with plasmid DNA was performed essentially as described by Sambrook et al. (10). P1 transductions were performed basically as described by Silhavy et al. (11). Protein concentrations were determined using the method first described by Bradford (12) with bovine serum albumin as the standard. SDS-PAGE was performed as described by Laemmli (13) with 12% polyacrylamide gels.

Cloning *ynjE*, *ynjE*-His$_6$ tagged and *yceA*

In order to determine whether the *ynjE* and *yceA* genes encode an active MST, expression vectors containing these genes under control of promoters P_{T7} and P_{N25} were constructed. Farzana Ahmed, another graduate student in our laboratory, cloned *ynjE* and *yceA*, while I cloned *ynjE*-His$_6$. Chromosomal DNA of the *E. coli* strain MG1655 was used as the template for the polymerase chain reaction. The oligonucleotide primers used for *ynjE* amplification were YNJE-F and YNJE-R. The PCR product was cloned into the same restriction sites of pZE2 P_{N25}, creating pFA82. This plasmid was used to transform strain DH5αZ1 for expression of the protein.

Primers YNJE-F2 and YNJE-R2 were used for PCR amplification and cloning of *ynjE* into the *Eco*RI and *Bam*HI of pT7-7. Chromosomal DNA of *E. coli* strain MG1655 was used as the template. The resulting plasmid was designated pFA11, which was used to transform strain BL21(DE3) for protein expression. The primer YNJE-F2 was designed to have an *Eco*RI site (instead of *Nde*I site, which is located next to the ribosome binding site of pT7-7) because there is a native *Nde*I site in the coding sequence of *ynjE*. This cloning caused the overexpressed protein from pFA11 to contain five extra amino acids (MARIP) at its N-terminus.

To facilitate the purification of YnjE, a pT7-7 derivative containing *ynjE* with a C-terminal hexahistidine sequence was constructed. The *ynjE* coding sequence was amplified by PCR using chromosomal DNA from strain MG1655 as the template. Primers YNJE-F2 and YNJE-RXh were used. Since pGZ117 (14) does not have an *Eco*RI site, a new vector (pPJ14) was created to contain a hexahistidine linker sequence for cloning the PCR fragment. pPJ14 is a pT7-7 derivative that contains a part of *glpR* gene (211 bp) insertion at its *Eco*RI/*Sal*I sites and a hexahistidine linker sequence (44 bp) between its *Sal*I/*Hin*dIII sites. The *Eco*RI-*Hin*dIII fragment of pPJ14 was derived from pLK100. Removal of the *glpR* sequence from pPJ14 was accomplished by digestion with *Eco*RI and *Sal*I. The PCR fragment was then inserted into the *Eco*RI/*Sal*I digested pPJ14. During cloning, the stop codon of *ynjE* was changed to an *Xho*I site that allows

in-frame ligation to the hexahistidine linker sequence from *Sal*I to *Hin*dIII of pPJ14. The resulting plasmid was designated pPJ15. This plasmid was introduced into strain BL21(DE3) by transformation for overexpression of YnjE-His_6.

The DNA fragment containing *yceA* was PCR amplified using oligonucleotide primers YCEA-F and YCEA-R. Chromosomal DNA of *E. coli* strain MG1655 was used as the template. The *Eco*RI and *Xba*I digested *yceA* fragment was cloned into the same sites of pZE2 P_{N25} to create pFA81.

Primers YCEA-F2 and YCEA-R2, containing *Nde*I and *Sal*I sites, respectively, were used to amplify *yceA* for insertion into pT7-7 cut with the same enzymes. The plasmid was named pFA13. During design of the latter primer for construction of pFA13, the stop codon of *yceA* was mistakenly changed within the *Sal*I site. Overexpression of pFA13 resulted in larger-sized YceA protein, presumably due to a C-terminal extension.

Induction of YnjE, YceA and YnjE-His_6 expression

Overexpression of YnjE, YnjE-His_6, or YceA was carried out in BL21(DE3) carrying pFA11, pPJ15, or pFA13, respectively. Cultures were induced in mid-log phase by the addition of 0.5 mM IPTG into a 5-ml culture. In DH5αZ1 cells carrying pFA82, or pFA81, overexpression was induced similarly by the addition of 0.1 μg of tetracycline per ml. Cultures were grown for 2 hours after induction and then harvested by centrifugation. Cells were resuspended in 0.5 ml of 25 mM Tris-acetate (pH8.6) and disrupted by sonication. After centrifugation, the supernatant fraction was collected and used for assay of MST activity.

Assay of MST activity

The assay was performed using both methods described in chapter 2.

Purification of YnjE-His_6

After harvesting a 5 ml culture of BL21(DE3)(pPJ15), cells were resuspended in 0.5 ml of 25 mM Tris-HCl buffer, pH8.0, and disrupted by sonication. A portion (0.2 ml) of the supernatant fraction (~ 0.1 mg protein) was used for YnjE-his_6 purification using Ni^{2+}-chelating Sepharose (Pharmacia) by a centrifugation protocol as described in the manufacturer's instructions. The starting buffer contained 20 mM $NaHPO_4$, pH 7.4, 0.5 M NaCl and 10 mM imidazole. The extract was loaded to the 50% slurry of Ni^{2+}-chelating Sepharose (40 μl), mixed for 20 min for binding and then centrifuged at 500 X g for 3 min to sediment the Sepharose gel. The supernatant was decanted and saved for SDS-PAGE analysis. For washing, a 5-gel volume of the starting buffer was added, mixed for 5 min and then centrifuged as above. The washing steps were repeated two more times. Elution of the YnjE-His_6 was done by the addition of 2-gel volume of 500 mM imidazole in the same buffer, mixed for 5 min and centrifuged as above. The elution steps were repeated two more times. The eluates were saved for SDS-PAGE analysis. YnjE-His_6 was eluted in the first 0.5 M imidazole elution. It was partially purified (see results).

Construction of the knockout vector (pFRT-K) containing the Km-resistance gene flanked by FRT sites

In order to facilitate the construction of strains with multiple-gene disruptions, vector pFRT-K was constructed. The vector is derived from pBluescript-KS^+ (Stratagene®) and contains a 1.5 kb kanamycin-resistance (Km^r) gene flanked by *FRT* sites (Figure 3.2). *FRT* sites contain directly repeated sequences that are the targets for Flp (the recombinase of yeast) recognition (15). The vector was also designed to contain appropriate multiple cloning sites on both sides of the cassette. 5'- and 3'-flanking regions of the desired gene are cloned into the multiple cloning sites on respective sides of the Km^r *FRT* cassette for construction of the gene deletion/Km^r insertion mutation. The antibiotic resistance gene, Km^r, facilitates the selection of clones harboring the deletion/insertion gene or subsequent movement of the mutated gene into any desired genetic background by P1

transduction. After the mutated gene is introduced into the genome, the Km^r gene between the *FRT* sites can be excised, if desired, by yeast Flp recombinase (15). This is accomplished by transformation of the mutated strain with pCP20, a plasmid that contains the gene encoding Flp recombinase. Elimination of the antibiotic resistance gene makes it easy to subsequently move another Km^r-disrupted gene into the genome by P1 transduction. In this way, mutants containing multiple-gene disruption can be constructed.

Janet L. Donahue did the cloning. The Km^r *FRT* cassette (*Eco*RI-*Hin*dIII fragment) derived from pCP15 (15) was cloned into the same sites of pBS-KS^+ (Stratagene®) to create pFRT-K. The pFRT-K vector contains the Km^r gene flanked by *FRT* sites and multiple cloning sites. The gene for ampicillin-resistance (*bla*) is derived from pBS-KS^+.

Disruption of the chromosomal *ynjE* gene

In order to address the function of the *ynjE* gene *in vivo*, a strain containing a deletion within the *ynjE* locus was constructed. First, a plasmid was constructed of cloned *E. coli* genomic DNA in which 0.3 kb of the 5' and 0.7 kb of the 3' region of the *ynjE* gene were ligated into pFRT-K such that these regions flanked the Km^r *FRT* cassette. A total of 1234 nucleotides of *ynjE* (1323 bp total) were deleted. Secondly, the mutated region, containing the *ynjE* flanking regions and the Km^r *FRT* cassette insertion, was cut and then inserted into a gene replacement vector, pKO3 (Figure 3.2b). Finally, the Δ*ynjE*::Km^r *FRT* was introduced into the wild-type chromosome by a process that includes two steps of homologous recombination. Figure 3.3 depicts the strategy used for disruption of *ynjE*. Details of the construction follow.

The N-terminal flanking region of *ynjE* was amplified by PCR using chromosomal DNA of strain MG1655 as template (Figure 3.3A). The N-terminal outside primer was YNJENo and the N-terminal inside primer was YNJENi. The ynjENo primer has no restriction site because the genomic sequence at about 30 bp downstream of the YNJENo binding site contains a native *Bam*HI site. The C-terminal flanking region of *ynjE* was

amplified by PCR using chromosomal DNA of strain MG1655 as template. The C-terminal inside primer was YNJECi and C-terminal outside primer was YNJECo. The PCR product of the N-terminal flanking region was digested with *Bam*HI and *Eco*RI (330 bp), and then ligated into the same sites of pFRT-K, creating pPJ16. Subsequently, the *Hin*dIII-*Xho*I cut C-terminal flanking region (700 bp) was inserted into the same sites of pPJ16 to create pPJ20 (Figure 3.3B). The 2.53 kb *Bam*HI-*Xho*I fragment of pPJ20, containing the Δ*ynjE*::Km^r *FRT*, was then ligated to pKO3 vector (containing *cat*, *sacB* and *repA*^{ts} genes) (16) at its *Bam*HI and *Sal*I sites, creating pPJ24 (Figure 3.4A). The pPJ24 was used to transform a wild-type *E. coli* strain (TL524). At the non-permissive temperature (43 °C), cells maintain Cm resistance only if pPJ24 integrates into the chromosome by homologous recombination between the cloned fragment and chromosomal *ynjE* (Figure 3.4B). A Cm-resistant colony was diluted into LB medium and plated on LB 5% sucrose-kanamycin plates at 30 °C. At this stage, the integrated vector was allowed to excise from the chromosome by the second recombination event (Figure 3.4C). Screening for loss of the excised vector was done by replica plating colonies from LB-sucrose-Km plates to LB Cm plates at 30 °C (Figure 3.4D). Cells that were sucrose-tolerant, Km^r and Cm^s were purified. Gene replacement on the chromosome was verified by bacteriophage P1 mediated co-transduction of Δ*ynjE*::Km^r with nearby Tn*10* markers (*zdj-276*::Tn*10* and *zea-225*::Tn*10*), as well as PCR with primers flanking the *ynjE* gene (YNJE-F and YNJE-RXh) (Figure 3.5). The resulting strain was designated PJ2.

Elimination of antibiotic resistance (Km^r) gene from *ynjE*::Km^r of chromosome

pCP20 is an Ap^r and Cm^r plasmid that has a temperature-sensitive origin of replication and thermal induction of yeast Flp recombinase synthesis (15). Excision of the Km^r gene from *ynjE*::Km^r was done by transformation of PJ2 with pCP20. Ampicillin-resistant transformants were selected at 30 °C. Some were colony-purified once nonselectively at 43 °C and tested for loss of all antibiotic resistances. The majority lost the *FRT*-flanked Km^r gene and the FLP helper plasmid simultaneously. Four colonies that were both Km- and Ap-sensitive were purified. PCR using primers flanking the *ynjE* gene (YNJE-F and

YNJE-RXh) verified that the cells have the Δ*ynjE* replacement on the chromosome (Figure 3.5). The newly created strain was named PJ202.

Disruption of the chromosomal *yceA* gene

A plasmid was constructed of cloned *E. coli* genomic DNA in which 0.4 kb each of the 5'- and 3'-flanking regions of the *yceA* gene were ligated on either side of the Km^r *FRT* cassette (Figure 3.6). A total of 975 nucleotides of *yceA* (1053 bp total) were deleted. The N-terminal flanking region of *yceA* was PCR amplified using primers YCEANo and YCEANi. The C-terminal flanking region of *yceA* was PCR amplified using primers YCEACi and YCEACo. After cleavage with *Bam*HI and *Eco*RI, the N-terminal flanking fragment of *yceA* (442 bp) was cloned into the same sites of pFRT-K to create pPJ18. The *Hin*dIII-*Sal*I digested C-terminal flanking fragment of *yceA* (432 bp) was then ligated into the same sites of pPJ18 to create pPJ23. The *Bam*HI-*Sal*I fragment (2.37 kb) from pPJ23, consisting of Δ*yceA*::Km^r *FRT*, was cloned into the same restriction sites of pKO3 to make pPJ26. The pPJ26 plasmid was used to transform the wild type strain. The selection was done using the same protocol as that used for disruption of *ynjE* as described above. Gene replacement on the chromosome was assessed by PCR with primers flanking the *yceA* gene (YCEA-F and YCEA-R). To determine whether the disrupted gene was replaced at the *yceA* locus on the chromosome, attempted P1-mediated co-transduction of Δ*yceA*::Km^r with Tn*10* markers from strains CAG 18466 and CAG 12078 was performed. However, after several trials, creation of the Δ*yceA* strain by this method was unsuccessful. It is possible that *yceA* may be needed for survival of the cell under the growth conditions used.

Therefore, another approach was performed by using a strain containing the wild type *yceA* gene in *trans* as the host strain for introducing the disrupted gene. Janet Donahue created pZE14-C11 (cloned *Eco*RI-*Xba*I fragment from pFA81 into pZE14), carrying an ampicillin resistance gene and wild type *yceA* gene under the control of $P_{lac/ara}$. pZE14-C11 was introduced intoTL524 cells by transformation. The transformant was used as the host strain for construction of the *yceA*-deletion strain as previously described.

Attempts to obtain the disrupted strain failed again. An antibiotic-resistant clone that was finally obtained contains a disrupted *yceA* gene that was mislocated on the chromosome, along with the wild type *yceA* gene. To find out if *yceA* in pZE14-C11 can be expressed, a culture of DH5αZ1 harboring pZE14-C11 was induced by the addition of 0.5 mM IPTG and 0.05% arabinose, and checked for YceA protein expression by SDS-PAGE analysis. No protein band corresponding to YceA was overexpressed relative to the vector control.

A plasmid (pZS34-C12) was constructed that carries a wild type *yceA* gene (from an independent PCR) under control of the $P_{lac/ara}$ promoter in a low-copy-number vector. An alternative technique for replacement of the mutated gene into the genome as described by Murphy (17) was used. By this technique, bacteriophage λ recombinase functions to generate an *E. coli* strain whose chromosome recombines with linear DNA fragments (Figure 3.6). This work was performed by Janet Donahue. To prove whether the *yceA* gene is essential, two host strains for introducing the disrupted gene were used side by side, including BW25113(pKD46) and BW25113(pKD46) carrying pZS34-C12 (providing $yceA^+$ in *trans*). pKD46 is a λred helper plasmid, carrying genes encoding λred recombinase (*g b exo*) (18). The linear *Bam*HI-*Sal*I DNA fragment consisting of Δ*yceA*::Km^r *FRT* (from pPJ23) was introduced by transformation into strains BW25113(pKD46) and BW25113(pKD46)(pZS34-C12). Selection of Km^r colonies was performed at 30 °C because we suspected from my failures that the growth of *yceA*-deleted cells might be affected by growth at 37 °C. The Km^r colonies obtained from both host strains were counted, and some of them were purified. After 2 days of incubation, only one small Km^r colony was obtained from BW25113(pKD46) whereas 80 small and 15 tiny colonies were obtained from the host strain carrying $yceA^+$ in *trans*. A small colony from each plate was purified. The presence of the Δ*yceA*::Km^r gene replacement in the chromosome was verified by PCR using primers YCEA-F and YCEA-R. The strains obtained from BW25113 and BW25113(pZS34-C12) were named JLD18801 and 18802, respectively.

P1 transductions were performed to transfer the Δ*yceA*::Km^r gene from JLD18801 and 18802 (BW25113 derivatives) into wild type cells, including strains TL524 and MG1655. The resulting transductants (Km^r) were purified and named JLD19401 and JLD19402 for TL524 derivatives and JLD19403 and 1 JLD9404 for MG1655 derivatives. P1-mediated transductional mapping using the nearby Tn*10* markers (from strains CAG18466 and CAG12078) as well as PCR using primers YCEA-F2 and YCEA-R2, flanking the *yceA* gene, were performed and verified the expected gene replacement at the *yceA* chromosomal locus.

Construction of strains with multiple sulfurtransferase gene disruptions

Using phage P1-mediated transduction (11), mutant strains deficient in multiple sulfurtransferase genes as listed in Table 3.1 were generated. For phenotypic comparisons of mutant strains in this study, all mutations were transferred to the wild-type recipient background strain TL524. P1 lysates were generally prepared from the previously-created single-gene disrupted strains that contain antibiotic marker insertions, including *sseA*::Sp^r, Δ*ynjE*::Km^r and Δ*glpEGR*::Km^r. The donor of Δ*glpEGR*::Km^r is strain NZ41, a derivative of strain MC4100 constructed in our laboratory by Ningyue Zhao. Transfer of Δ*glpEGR*::Km^r into strain TL524 background by P1 transduction resulted in strain PJ14. The construction of a disruption affecting only the *glpE* gene was performed later by Jennifer Brichetti, an undergraduate in our laboratory.

In order to transfer a disrupted gene lacking an antibiotic resistance insertion or a nearby marker, addition to the strain of a co-transducible selectable marker was required. This was done in the case of Δ*iscS* in strain CL100, obtained from C. T. Lauhon (19). Transfer of other mutated genes into CL100 is another choice, but the parent of CL100 (MC1061) is a different genetic background than our standard wild-type strain, TL524. Therefore, CL100 and its derivatives could not be used directly for phenotypic comparison with the TL524 derivatives. Selectable markers of strains CAG18470 (*purC80*::Tn*10*) and PJ1 (*sseA*::Sp^r) are expected to be co-transducible with the *iscS* gene. Therefore, CAG18470 and PJ1 were used as P1 donors for recipient strain CL100.

The selected tetracycline- or spectinomycin-resistant colonies were screened for Δ*iscS* by replica plating on minimal medium lacking nicotinic acid, isoleucine and valine. Leucine was supplemented into the replica plate since the parent strain of CL100 (MC1061) is a leucine auxotroph. Additionally, adenine and guanine were supplemented into the replica plate of the tetracycline-resistant colonies because the insertion in *purC* causes purine auxotrophy. Δ*iscS* transductants with *purC80*::Tn*10* and with *sseA*::Sp^r were named PJ19 and PJ20, respectively. Δ*iscS* was subsequently transferred into strain TL524 by co-transduction with *sseA*::Sp^r or *purC80*::Tn*10*, creating strains PJ22 and PJ23, respectively.

Table 3.1 Strains of *E. coli* used in this study

Strains	Genotype	Description or Reference
MG1655	Wild type isolate (F$^-$ *rph-1* λ$^-$)	(20)
TL524	MG1655 Δ(*lac ZYA-argF*)*U169*	Tc^s derivative of TL504 (21)
MC1061	F$^-$ *araD139* Δ(*ara leu*)*7696* Δ(*lacY74*) *galU galK hsdR hsdM*$^+$ *strA*	*E. coli* Genetic Stock Center
CL100	MC1061 Δ*iscS*	(19)
S1247	*bioA24*, *moaA29*, *zbh-283*::Tn*10*	(9)
BL21(DE3)	*hsdS gal* (λcI*ts857 ind-1 S*am7 *nin-5 lacUV5-T7* gene *1*)	(22)
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	(18)
CAG12078	*zce-726*::Tn*10*	(23)
CAG18464	*zdj-276*::Tn*10*	(23)
CAG18465	*zea-225*::Tn*10*	(23)
CAG18466	*zcc-282*::Tn*10*	(23)
CAG18470	*purC80*::Tn*10*	(23)
DH5αZ1	(ϕ80*d lacZ*Δ*M15*) *endA1 recA1 hsdR17 supE44 thi-1 gyrA relA* Δ(*lacZYA-argF*)*U169* (λ*att lacI*^{q} *tetR* Sp^r)	(24)

NZ41	MC4100 *recB21 recC22 sbcB15* Δ*glpEGR::*Km^r	Ningyue Zhao (25)
JLD18801	BW25113 Δ*yceA*::Km^r *FRT*	This work
JLD18802	BW25113 Δ*yceA*::Km^r *FRT* (pZS34-C14)	This work
JLD19401	TL524 Δ*yceA*::Km^r *FRT*	P1(JLD18801) x TL524
JLD19402	TL524 Δ*yceA*::Km^r *FRT*	P1(JLD18802) x TL524
JLD19403	MG1655 Δ*yceA*::Km^r *FRT*	P1(JLD18801) x MG1655
JLD19404	MG1655 Δ*yceA*::Km^r *FRT*	P1(JLD18802) x MG1655
JLD20501	TL524 Δ*yceA FRT*	JLD19401, Km^r cassette removal
PJ1	TL524 *sseA*::Sp^r	This work
PJ2	TL524 Δ*ynjE*::Km^r *FRT*	This work
PJ202	TL524 Δ*ynjE FRT*	PJ2, Km^r cassette removal
PJ6	TL524 Δ*ynjE sseA*::Sp^r	P1(PJ1) x PJ202
PJ8	TL524 Δ*ynjE* Δ*glpEGR*::Km^r	P1(NZ41) x PJ202
PJ12	TL524 *sseA*::Sp^r Δ*glpEGR*::Km^r	P1(NZ41) x PJ1
PJ13	TL524 *sseA*::Sp^r Δ*ynjE* Δ*glpEGR*::Km^r	P1(NZ41) x PJ6
PJ14	TL524 Δ*glpEGR*::Km^r	P1(NZ41) x TL524
PJ17	CL100 Δ*glpEGR*::Km^r	P1(NZ41) x CL100
PJ18	CL100 Δ*ynjE*::Km^r *FRT*	P1(PJ2) x CL100
PJ19	CL100 *purC80*::Tn*10*	P1(CAG18470) x CL100
PJ20	CL100 *sseA*::Sp^r	P1(PJ1) x CL100
PJ22	TL524 *sseA*::Sp^r Δ*iscS*	P1(PJ20) x TL524
PJ23	TL524 Δ*iscS purC80*::Tn*10*	P1(PJ19) x TL524

Table 3.2 Plasmids used in this study

Plasmids	Description	Source or Reference
pBS-KS+	ColE1 origin Ap^r	Stratagene®
pCP15	FRT-flanked Km^r cassette in a pUC19 derivative	(15)
pCP20	FLP^+ Ap^r Cm^r *repA*^{ts}	(15)
pKD46	oriR101 Ap^r *repA*^{ts} *araC*-P_{araB} λRed (*g b exo*)	(18)
pKO3	M13 origin, *repA*^{ts}, *cat*, *sacB*	(16)
pT7-7	ColE1 origin Ap^r T7 promoter	(26)
pGZ117	hexahistidine linker sequence in *Sal*I/*Hin*dIII of pT7-7	(14)
pZE2 P_{N25}	ColE1 origin Km^r P_{N25}-luc	(24)
pZE14	ColE1 origin Ap^r $P_{lac/ara}$	(24)
pZS34	pSC101 origin Cm^r $P_{lac/ara}$	(24)
pDB551	*nifS* of *A. vinelandii* in pT7-7	D.R. Dean
pDB943	*iscS* of *A. vinelandii* in pT7-7	D.R. Dean
pFA11	*ynjE* in *Eco*RI/*Bam*HI of pT7-7	This work
pFA13	*yceA* in *Nde*I/*Sal*I of pT7-7	This work
pFA81	*ynjE* in *Eco*RI/*Xba*I of pZE2 P_{N25}	This work
pFA82	*yceA* in *Eco*RI/*Xba*I of pZE2 P_{N25}	This work
pFRT-K	FRT-flanked Km^r cassette in *Eco*RI/*Hin*dIII of pBS-KS+	This work
pLK100	*glpR* in *Nde*I/*Sal*I of pGZ117	This work
pPJ14	*Eco*RI-*Hin*dIII fragment of pLK100 in pT7-7	This work
pPJ15	*ynjE* in *Eco*RI/*Sal*I of pPJ14	This work
pPJ16	5'- flanking region of *ynjE* in *Bam*HI/*Eco*RI of pFRT-K	This work
pPJ18	5'- flanking region of *yceA* in *Bam*HI/*Eco*RI of pFRT-K	This work
pPJ20	3'- flanking region of *ynjE* in *Hin*dIII/*Xho*I of pPJ16	This work
pPJ23	3'- flanking region of *yceA* in *Hin*dIII/*Sal*I of pPJ18	This work
pPJ24	5'- and 3'- flanking region of *ynjE* with FRT-flanked Km^r cassette insertion in *Bam*HI/*Sal*I of pKO3	This work

pPJ26	5'- and 3'- flanking of *yceA* with FRT-flanked Km^r cassette insertion in *Bam*HI/*Sal*I of pKO3	This work
pZE14-C11	*yceA* in *Eco*RI/*Xba*I of pZE14	This work
pZS34-C12	*yceA* in *Nde*I/*Xba*I of pZS34	This work

Table 3.3 Oligonucleotides used in this study

Name	Sequences [a]	Restriction site
YCEACi	5'-CTTTAATAAGctTCGTGGACGTCTG-3'	*Hin*dIII
YCEACo	5'-CAGCGTCGAcACTAATCACGCC-3'	*Sal*I
YCEA-F	5'-ATCCgaAttCGAGGCGGTAGATAACCGG-3'	*Eco*RI
YCEA-F2	5'-CtTACcatATGCCAGTGTTACACAAC-3'	*Nde*I
YCEANo	5'-GAAGCACgGaTCCCAGGTTGCGATG-3'	*Bam*HI
YCEANi	5'-GTCGTTGGAAtTcCGGTTGTGTAACAC-3'	*Eco*RI
YCEA-R	5'-TGGAtctaGaACAGCAGAAGTAAGCGTG-3'	*Xba*I
YCEA-R2	5'-GCAATGgTcgacATTCTGTTGGATCAG-3'	*Sal*I
YNJECi	5'-TATGACGGaaGCtTGTACGAATGGA-3'	*Hin*dIII
YNJECo	5'-CTGTGCTcGAgCGCCATCTTCATC-3'	*Xho*I
YNJE-F	5'-ATAGgaaTtcgaTGGATATGGCGCAGTG-3'	*Eco*RI
YNJE-F2	5'-CTCAgAAtTcCAATGTCGGGAT-3'	*Eco*RI
YNJENo	5'-CCGTGAATGATGCACTTGAGCGT-3'	
YNJENi	5'-GTTTCATCGgAAtTCCCGACATTGTAG-3'	*Eco*RI
YNJE-R	5'-TTGTtCTaGATGACGACCTTTACGCGG-3'	*Xba*I
YNJE-R2	5'ACGTGgAtccTTATTTGCTACTG-3'	*Bam*HI
YNJE-RXh	5'-TGCAATGCTcGagGCTACTGTCCGG-3'	*Xho*I

[a] The sequences mismatches are lower case and the restriction sites used are underlined.

3.4: Results and discussion

The protein encoded by *ynjE* does not possess MST activity.

Disparity in the MST activity between assays using 2-mercaptoethanol and cyanide as the sulfur acceptor in the *sseA*::Sp^r mutant suggested that there may be more than one type of MST in *E. coli*. Among eight paralogs, the primary sequences in the region of the active site cysteine of proteins encoded by *ynjE* and *yceA* are most similar to that of the SseA protein (Figure 3.1). In order to characterize potential sulfurtransferases encoded by *ynjE*, the gene was cloned into expression vectors, including pZE2 P_{N25} and pT7-7, where expression of the proteins is under the control of P_{N25} and P_{T7}, respectively. Addition of either tetracycline to cultures of DH5αZ1 harboring derivatives of pZE2 P_{N25}, or IPTG to BL21(DE3) harboring derivatives of pT7-7 resulted in induction of YnjE expression. Three different expression vectors for *ynjE* were constructed, including pFA82, pFA11 and pPJ15. SDS-PAGE analysis of whole cell proteins from DH5αZ1 harboring pFA82, as well as BL21(DE3) harboring pFA11, showed an overexpressed protein band of about 48 kDa, which is the expected size of YnjE based on amino acid sequence (data not shown). In contrast, the fractionated proteins from BL21(DE3) harboring pPJ15 showed an overexpressed band at about 50 kDa (Figure 3.7, lane 2). To make sure that the 50 kDa protein seen on this gel was the His_6-tagged protein, purification of YnjE-His_6 protein from BL21(DE3)(pPJ15) extract was performed using Ni^{2+}-chelating Sepharose (Pharmacia) by a small scale centrifugation protocol as described in the manufacturer's instructions. After the binding and washing steps, the addition of 500 mM imidazole-containing buffer yielded partially purified YnjE-His_6. The most abundant protein has an apparent molecular mass of 50 kDa as shown in Figure 3.7 (lane 3), which agrees with the expected size of YnjE-His_6 (50.8 kDa). Nevertheless, MST activity of the supernatant fraction obtained from the strains carrying these expression vectors was not significantly different from that of the vector controls (Table 3.4). The results indicated that YnjE does not possess MST activity under these conditions. An assay of rhodanese activity was also performed. Results shown in Table 3.4 exclude the possibility that

YnjE is a rhodanese. However, the partially purified YnjE-His_6 protein was not assayed for MST and rhodanese activities.

Table 3.4 MST and rhodanese activities of the control and *ynjE* overexpression strains.

Strains	Sulfurtransferase activity [a] (units/ mg protein)	
	MST [b]	Rhodanese
BL21(DE3) (pT7-7)	0.22	0.08
BL21(DE3) (pFA11)	0.27	0.08
BL21(DE3) (pPJ15)	0.22	0.07

[a] Enzyme activity was measured in the supernatant fractions from sonicated cells.

[b] Assayed for thiocyanate production

Disruption of the chromosomal *ynjE* gene of *E. coli*

In vitro analysis suggested that YnjE has neither MST nor rhodanese activity. In order to find the gene functions *in vivo*, strain PJ2 (Δ*ynjE*::Km^r) was constructed. Genetic mapping using P1-mediated co-transduction with Tn*10* markers closely linked to *ynjE*, including *zdj-276*::Tn*10* (located ~3 kb counterclockwise relative to *ynjE*) and *zea-225*::Tn*10* (located ~32 kb clockwise relative to *ynjE*), provided evidence that the Km^r marker was at the expected location in the *E. coli* chromosome. The observed co-transduction frequencies of Δ*ynjE*::Km^r with *zdj-276*::Tn*10* and *zea-225*::Tn*10* were about 85% and 19%, respectively (expected values are 86% and 29%, respectively). Result of PCR with primers flanking the *ynjE* gene (YNJE-F and YNJE-RXh) confirmed that strain PJ2 has the Δ*ynjE*::Km^r *FRT* replacement on the chromosome. Figure 3.5 shows that a 1.7 kb-PCR product was obtained from strain PJ2 (lane 3), as the expected size, whereas the 1.4 kb-product was obtained from the wild type (lanes 4 and 5).

Strain PJ202 was derived from PJ2 by the expression of Flp recombinase, which eliminated the Km^r gene between the FRT sites. This step was very useful for construction of strains with multiple gene disruptions. After removal of the Km^r gene, strain PJ202 was subsequently used as the recipient for moving another Km^r-disrupted gene, such as Δ*glpEGR*::Km^r, into the chromosome.

To determine if deletion of *ynjE* has an effect on MST or rhodanese activity, an extract from PJ202 was assayed for MST activity. It was found that there was no significant difference in MST activity relative to the wild-type control [0.85 vs. 0.82 units mg^{-1} protein, pyruvate detection assay (mutant vs. wild type)]. The MST activities determined using cyanide as sulfur acceptor, as well as rhodanese activities, for wild type and several mutants are shown in Table 3.5. The results show that deletion of *ynjE* has no influence on mercaptopyruvate:cyanide sulfurtransferase activity. It would appear, however, that one or more of the rhodaneses (GlpE, PspE, or YgaP) contributes to this activity.

Table 3.5 MST and rhodanese activity of the wild type and of strains with putative sulfurtransferase gene(s) deletion.

Strain (genotype)	Sulfurtransferase activity [a] (units/ mg protein)	
	MST [b]	Rhodanese [c]
TL524 (wild type)	0.13	0.20
PJ1 (*sseA*::Sp^r)	0.10	0.20
PJ202 (Δ*ynjE FRT*)	0.12	0.21
PJ6 (*sseA*::Sp^r Δ*ynjE FRT*)	0.10	0.21
JLD20501 (Δ*yceA FRT*)	0.12	0.21
JLD19901 (*sseA*::Sp^r Δ*ynjE FRT* Δ*ygaP FRT* Δ*glpE FRT* Δ*pspE FRT*) [d]	0.03	0.01
JLD20502 (*sseA*::Sp^r Δ*ynjE FRT* Δ*ygaP FRT* Δ*glpE FRT* Δ*pspE FRT* Δ*yceA FRT*) [d]	0.03	0.01

[a] Enzyme activity was measured in the supernatant fractions from sonicated cells. The average values of duplicate assays are shown.

[b] Sodium mercaptopyruvate used was not fresh.

[c] The reactions were initiated by the addition of crude extracts.

[d] This strain was made by Janet L. Donahue

Cloning and attempted expression of *yceA*

Two different vectors, pFA81 and pFA13, for *yceA* expression under control of P_{N25} and P_{T7}, respectively, were constructed. There was no significant increase in either MST or rhodanese activity from these strains compared to the vector control. While YceA expression from the P_{N25} system could not be demonstrated on SDS-PAGE gel, there was an apparent protein band of approximately 50 kDa from the pT7 system, which was larger than the expected size of YceA (39.8 kDa). This larger protein was first suspected to result from an error of reverse primer design. Because the primer (YCEA-R2) was designed to have two-nucleotide changes from TT to AC at the 5' end, the stop codon of *yceA* (TAA) became a cysteine codon (TGT) instead. Therefore, translation further into

the pT7-7 DNA sequence must have occurred. To find out how many amino acids were added to the C-terminus of YceA, the pT7-7 sequence beyond the *Sal*I site was then carefully analyzed. It was found that only ten more amino acids (CRPAAQAYHA) could be translated through the same reading frame before termination would occur at two consecutive stop codons. The calculated size of YceA plus extra 10 amino acids is 40.9 kDa (not 50 kDa). However, there was no overexpressed protein band around this position when compared to the vector control. The apparent protein band at about 50 kDa actually contained at least two bands close together, which may cause misinterpretation in the first place. Apparently, YceA overexpression did not occur from either vector relative to the control as assessed by SDS-PAGE analysis. In fact, *yceA* was also cloned into other expression vectors, such as pZE14-C11 and pZS34-C12. Similarly, no YceA expression could be demonstrated. SDS-PAGE analysis may not be sensitive enough to detect a small amount of overexpressed protein, if it was present. Therefore, we could not ascertain if YceA has MST or rhodanese activity. Nevertheless, we stepped forward to an *in vivo* system for disruption of the chromosomal *yceA* gene.

Disruption of the chromosomal *yceA* gene of *E. coli*

Failure to disrupt chromosomal *yceA* as described in "Experimental Procedures" suggested that the *yceA* gene is essential in *E. coli*. The *yceA* gene is located next to the *htrB* gene, which is transcribed divergently from *yceA*. A part of the *htrB* promoter also overlaps with the promoter region of *yceA*. It is possible that deletion of the *yceA* gene might effect *htrB* expression. The *htrB* gene was identified as a gene that was required for growth in rich media at temperatures above 32 °C, but not at lower temperature (27). Taken together with the previous failure to disrupt *yceA*, we considered the possibility that Δ*yceA* may confer a temperature-sensitive phenotype. Therefore, Janet Donahue carried out the selection for the Km^r *yceA* disruption at 30 °C. The transformation of strain BW25113, harboring the λ Red recombinase, with linear Δ*yceA*::Km^r *FRT* DNA resulted in recombination with the homologous *yceA* region on the chromosome (see methods). The total number of Km^r transformants obtained from BW25113 was about 1% of that obtained from BW25113 with a $yceA^+$covering plasmid (pZS34-C12). The

greater number of transformants from BW25113 (pZS34-C12) than from BW25113 suggests that *yceA*$^+$ confers an advantage for survival. PCR using primers YCEA-F and YCEA-R1, as well as P1 transductional mapping, verified that both of the newly constructed strains, JLD18801 and JLD18802, which were made from BW25113 and BW25113 (pZS34-C12), respectively, were correct.

Strains JLD18801and JLD18802 were used as the donors for P1 transduction to transfer Δ*yceA*::Km^r into the wild type TL524 and MG1655 recipients. Although the recipient strains did not carry a *yceA*$^+$covering plasmid, kanamycin-resistant transductants were obtained at 30 °C. The resulting strains (JLD19401 to JLD19104) grew normally at 30 °C, however, a phenotype of two-populations (normal and tiny colonies) was found at 37 °C when growing on kanamycin-containing medium. The normal-sized colonies are probably suppressor mutants of temperature-sensitive cells that allow growth at 37 °C. Subculture of a tiny colony yielded the two-population pattern at 37 °C. The results suggest a requirement for *yceA*$^+$ for optimal growth at temperatures above 30 °C.

Strain JLD20501 was derived from JLD19401 by removal of the Km^r gene. An extract from this strain was used to assay for MST and rhodanese activity. It was found that there was no significant difference in either MST or rhodanese activity relative to the wild type control (Table 3.5).

Phenotypic characterization of *sseA*, *ynjE* and *glpEGR* mutants

To test whether a deficiency in sulfurtransferase(s) has any effect on biosynthesis of cofactors, e.g. thiamin, biotin and lipoic acid, growth phenotypes on various media of eight strains were compared. The eight strains included the wild type (TL524) and seven disrupted strains: three single mutants, PJ1 (*sseA*::Sp^r), PJ202 (Δ*ynjE*) and PJ14 (Δ*glpEGR*::Km^r); three double mutants (PJ6, PJ8, PJ12); and a triple mutant (PJ13). Phenotypic testing started by using minimal glucose or glycerol medium without any cofactor supplements. If a sulfurtransferase is needed for biosynthesis of one or more of the mentioned cofactors, some of the disrupted strains are expected to be auxotrophic. In

minimal glucose or glycerol medium (AB without thiamin or any supplement), no significant differences in growth pattern between the wild type and strains lacking all possible combinations of *sseA*, *ynjE* and *glpEGR* were observed. This was also true of growth in minimal medium with a limiting sulfur source (65 μM $MgSO_4$ or cysteine, instead of 10 mM).

In order to determine whether the deletion of these sulfurtransferase genes has an effect on molybdopterin (MPT) biosynthesis, the mutants were tested for chlorate-resistance under anaerobic conditions. The rational is that molybdoenzymes, including nitrate reductase, require MPT for activity. Nitrate reductase, which normally reduces NO_3^- to NO_2^-, can also convert chlorate to chlorite, which is lethal to wild type cells. Therefore, mutants that are defective in MPT synthesis are chlorate-resistant. The chlorate phenotype of the knockout strains was compared to those of the wild type and a chlorate-resistant control (S1247, *moaA29*). All of the strains with single- and multiple-disrupted sulfurtransfurase genes were chlorate-sensitive, similar to the wild type. The results indicate no direct involvement of these sulfurtransferases in biosynthesis of MPT, as well as other cofactors mentioned above. Another possibility is that one or more of the other sulfurtransferase paralogs compensate for the loss of SseA, YnjE, and/or GlpE.

Interestingly, the Δ*iscS* strains (CL100, PJ17, PJ18, PJ19 and PJ20, as well as PJ22 and PJ23) were chlorate-resistant, which suggests a deficiency in nitrate reductase. Possibly, Fe-S clusters required for active nitrate reductase are not assembled properly in the Δ*iscS* mutant. Recent work (28) demonstrated that NifS-like cysteine desulfurases, including CSD, CsdB and IscS, were capable of mobilizing sulfur from cysteine to precursor Z for MPT synthesis *in vitro*, with CSD showing the highest activity. Nevertheless, it was found that IscS was not required for *in vivo* conversion of precursor Z to MPT (28). *In vitro* experiments showed that the extract of strain CL100 could convert externally added precursor Z to MPT. It is possible that other persulfide-containing sulfurtransferases, such as CSD and/or rhodanese, may serve this function *in vivo*.

Mutants deficient in IscS and sulfurtransferases

To test whether a sulfurtransferase has function in some aspects of sulfur mobilization associated with a cysteine desulfurase (IscS), Δ*iscS* mutants containing in addition a sulfurtransferase gene disruption were constructed and tested for growth phenotypes. Strain CL100 (Δ*iscS*) requires thiamin and nicotinic acid for growth on minimal medium, and addition of isoleucine and valine promotes growth. CL100 also has a Δ*leu* on its chromosome, so leucine is required in the medium. When *sseA*::Sp^r, Δ*glpEGR*::Km^r, or Δ*ynjE*::Km^r was introduced into the chromosome of CL100 (making strains PJ17, PJ18 and PJ20), no additional supplement was required for growth of these derivative strains, which indicated that there is no phenotype associated with mutation of these genes. Possibility for the lack of an additional phenotype is that other *sseA*- or *glpE*- paralogous gene products play a role redundant with that of the missing sulfurtransferase.

Complementation of Δ*iscS* in *E. coli* by NifS and IscS of *A. vinelandii*

A complementation test was performed in order to solve the problem of preparing P1 lysates from strain CL100 derivatives. Unexpectedly, upon repeated attempts, P1 lysates made from CL100 derivatives (PJ19 and PJ20) were found to have abnormally low titers (less than 10^8 phage/ ml compared with $>10^{10}$ for *iscS*$^+$ strains). Although an increased amount of P1 lysate of strain PJ19 was added to the TL524 recipient, only 13 colonies of Tc^r-transductants were obtained. None of the Tc^r-transductants (*purC80*::Tn*10*) were Δ*iscS* because the expected co-transduction frequency between *purC80*::Tn*10* and Δ*iscS* is ~7%. An increase in P1 titer was obtained by introducing in *trans* pDB943 (carrying the *iscS*$^+$ gene of *A. vinelandii*) into PJ19 prior to preparation of the P1 lysate. Transduction of TL524 using this lysate yielded one colony out of 84 Tc^r-colonies with the Δ*iscS* phenotype on minimal medium. This raises an interesting question: is there any relationship between phage P1 propagation and defective host proteins caused by Δ*iscS*? Further experiments should be performed to find out whether the production of other

bacteriophages, e.g. T4 and λ, is influenced by lack of IscS in the host strain. The results may provide a better understanding of host factors required for phage propagation.

Interestingly, the *iscS* and *nifS* genes from *A. vinelandii* carried by plasmids pDB943 and pDB551, respectively, were able to complement the nicotinic acid, isoleucine and valine auxotrophies of strains CL100, PJ17, PJ18 and PJ20 (thiamin complementation was not tested). The capability of *A. vinelandii* IscS and NifS to replace the functions of IscS of *E. coli* may be due to the fact that the sequences of these proteins are highly conserved, especially around the active site cysteine (Figure 3.8). Although *iscS* and *nifS* were cloned into pT7-7 and therefore should not be expressed (CL100 derivatives have no T7 RNA polymerase), expression of *iscS* and *nifS* is apparently high enough to allow complementation.

3.5: Conclusions and possible future work

In sum, the physiological functions of sulfurtransferases in *E. coli* remain unknown. Although SseA and GlpE have defined *in vitro* functions as MST and rhodanese, respectively, their functions *in vivo* cannot yet be addressed because of the redundancy of the sulfurtransferase genes in *E. coli*. The results presented here are a progressive step in that we started construction of strains deficient in multiple sulfurtransferases. Our hypothesis was that sulfurtransferases are involved in biosynthesis of some sulfur-containing cofactors. However, to prove our hypothesis by using *E. coli* as the model organism, all eight gene disruptions need to be completed. My work could not demonstrate a requirement for SseA or the other two paralogous proteins (GlpE and YnjE) in the biosynthesis of thiamin, MPT, biotin, or lipoic acid. In fact, besides the *sseA*, *glpE* and *ynjE* disruptions, two other genes disruptions, including *ygaP* and *pspE*, were successfully constructed in our laboratory. Recently, we created a strain with all five genes disrupted (quintuple mutant). Moreover, a strain with sextuple mutant (including Δ*yceA*) was also created. A dramatic decreased in either MST or rhodanese activity was found in the quintuple and the sextuple mutant (Table 3.5). Nevertheless, no discernable phenotypic change has been demonstrated yet. To continue the loss-of-

function approach, we need to continue introduction into the sextuple mutant the disruption in *ybbB* and *yibN*, the remaining sulfurtansferase paralogs. In fact, a student in our laboratory cloned the *ybbB* gene into an expression vector. However, there was no increase in the MST or rhodanese activity in the extract from the YbbB-overexpressing strain. The putative active site of YbbB contains the sequence CCxRGxxRS (Fig. 3.1), suggesting this protein may have protein tyrosine phosphatase (PTP) activity. The consensus sequence for PTP active site is HCxxGxxR(S/T) (29). Farzana Ahmed assayed for PTP activity. However, PTP activity was not increased in the extract of the YbbB-overexpressing strain relative to the vector control. Nevertheless, disruption of the *ybbB* gene should be continued.

The YibN protein is less likely to have MST or rhodanese activity, since its sequence contains an aspartate residue instead of cysteine at the predicted active site (Fig. 3.1). A *yibN*-deletion strain has been constructed in Lauhon's laboratory (personal communication), so it is available for further work. Defining the function of sulfurtransferases will be of broad biological relevance, since the enzymes are present in multiple forms in all three domains of life.

Since SseA and GlpE have *in vitro* MST and rhodanese activity, respectively, a further biochemical phenotype should be investigated. One of our hypotheses is that sulfurtransferases function downstream of IscS, which catalyzes the mobilization sulfur from cysteine to its active site. To find out whether cysteine-derived sulfur from IscS can be mobilized to SseA, a coupled IscS-sulfurtransferase reaction should be performed. The assay mixture will contain cysteine, IscS, SseA and CN^-. Control reactions should include the mixture without cysteine, and/ or IscS and/ or SseA. If sulfur is transferred from cysteine to IscS, then from IscS to SseA, and then from SseA to CN^-, SCN^- will be detected. If this is the case, the result will suggest that an analogous sulfur transfer process may occur in the biological system.

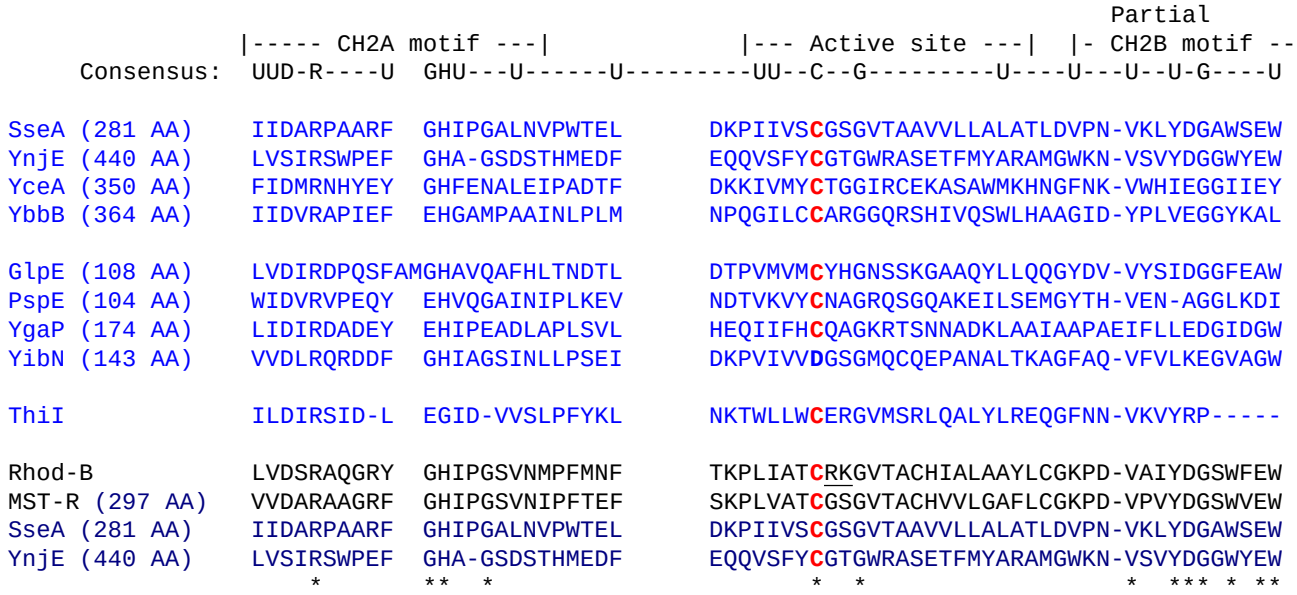


Figure 3.1 Sequence alignment of the conserved regions of the predicted sulfurtransferases of *Escherichia coli*.

The active site and two conserved structural motifs, designated CH2A and CH2B, are labeled. A consensus is indicated. U, uncharged residue: D, aspartic acid: G, glycine: H, histidine: R, arginine. SseA and YnjE of *E. coli* are compared to those representative sulfurtransferases [rat liver mercaptopyruvate sulfurtransferase (MST-R) and bovine rhodanese (Rhod-B)]. The asterisks highlight positions that are identical in four sequences. The active site cysteine (**C**) is shown in bold. The two basic residues important for binding of thiosulfate (R and K) in rhodanese are underlined. These residues are replaced by G and S, respectively, in MST. The sequence of *E. coli* ThiI (a protein required for s^4U and thiamin biosynthesis) that contains a sulfurtransferase domain is also shown.

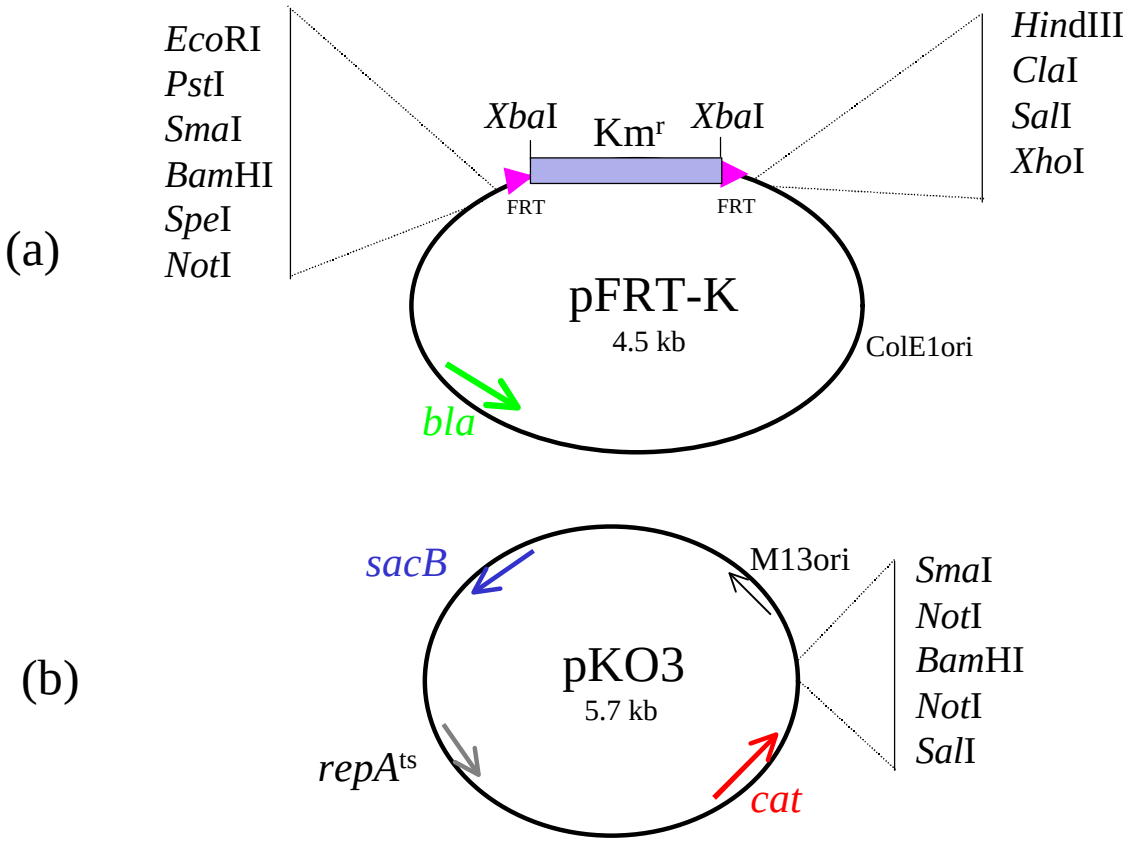


Figure 3.2 The (a) pFRT-K and (b) pKO3 vectors used in the gene replacement experiments.

The cloning region is enlarged and the restriction sites are shown. Arrows in the circular plasmid indicate the directions of transcription. Arrowheads in the pFRT-K vector represent Flp recombination target (FRT) sites.

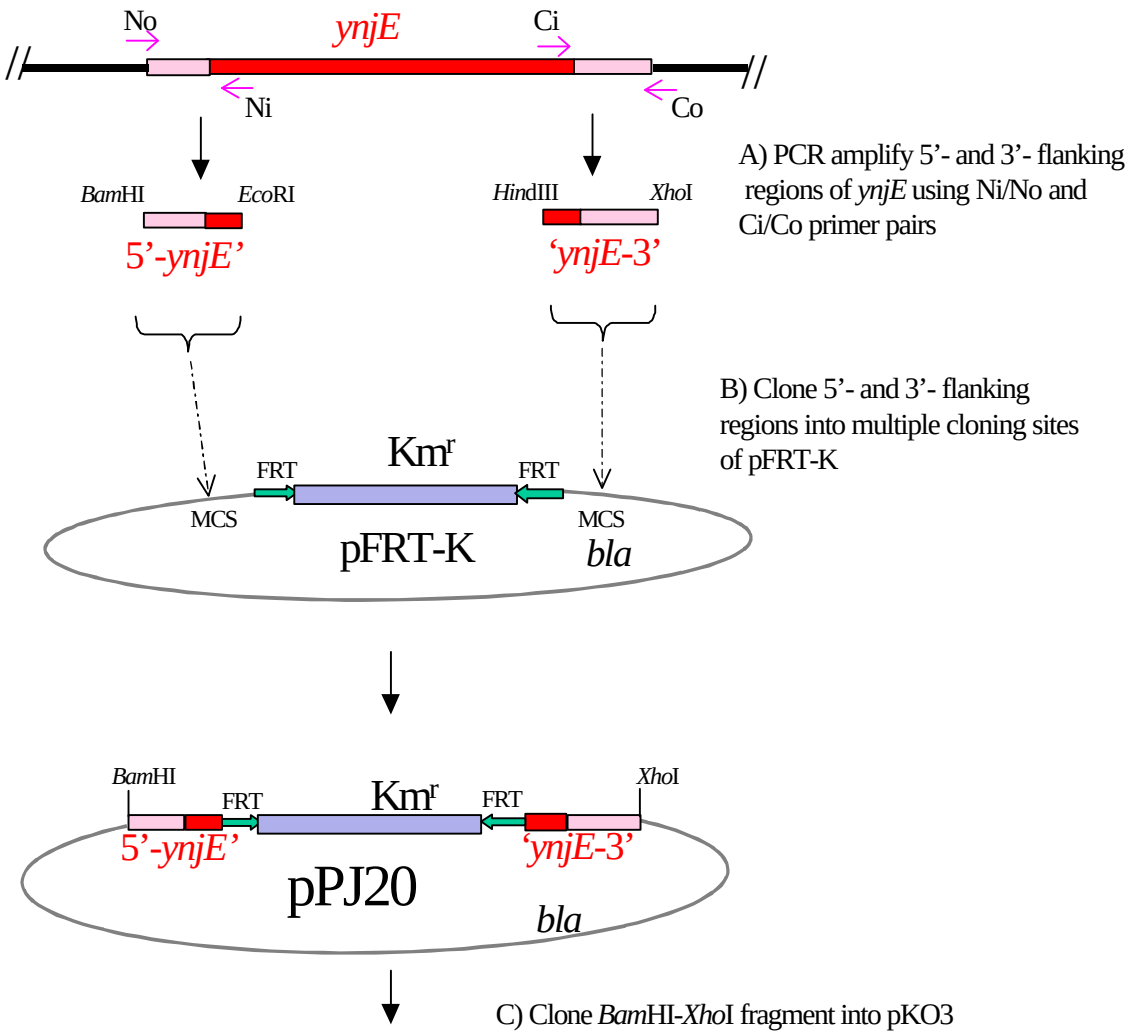


Figure 3.3 Disruption of *ynjE*.

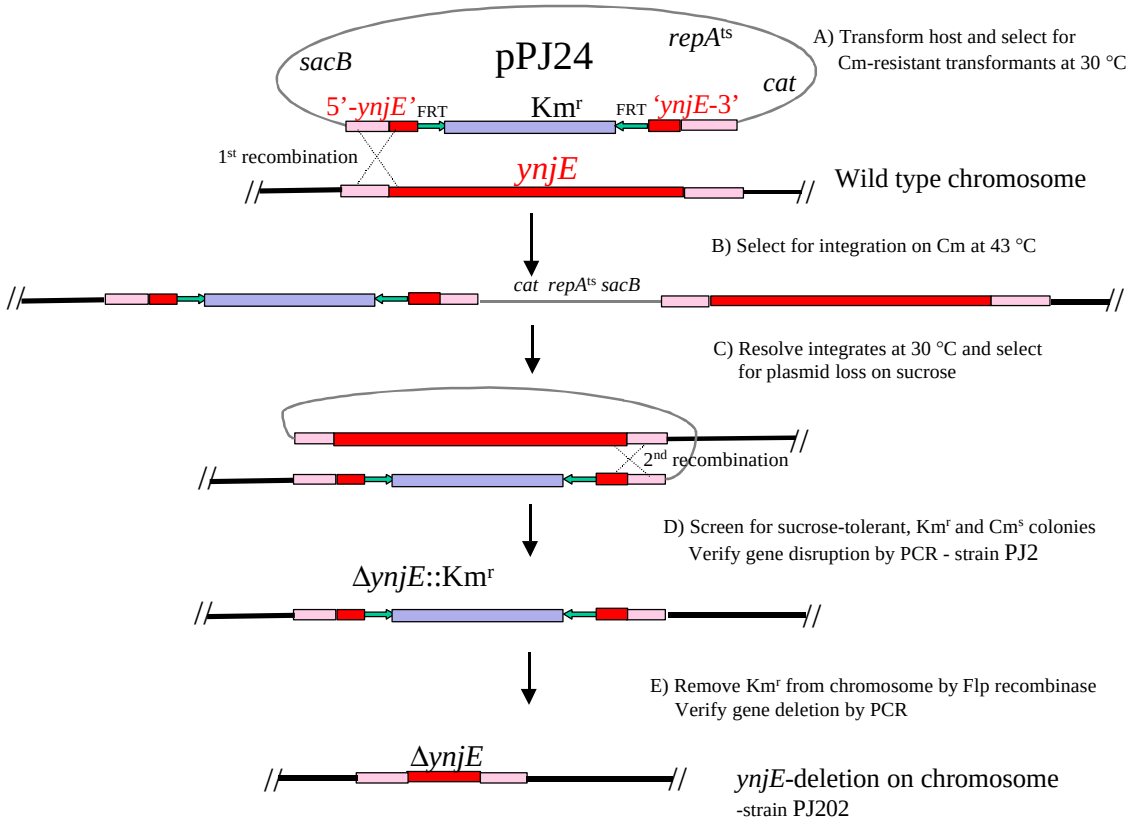


Figure 3.4 Construction of *ynjE*-deletion in the genome.

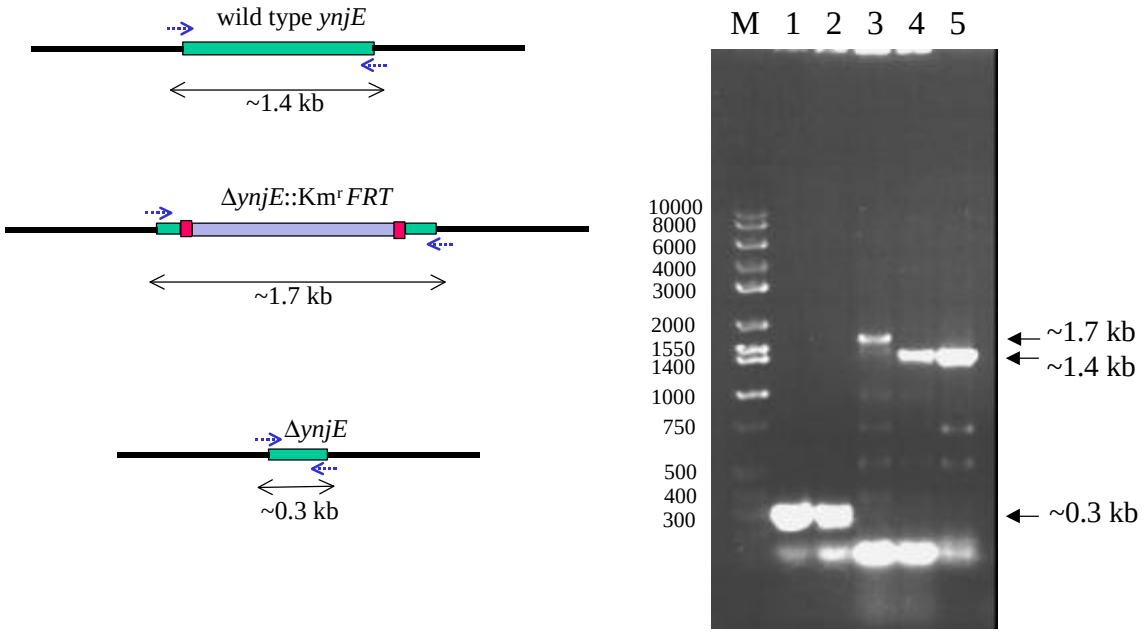


Figure 3.5 Verification of the *ynjE*-disrupted strains by PCR, using primers YNJE-F and YNJE-RXH.

Left panel: schematic of the templates used for PCR. Top, middle, and bottom indicate DNA from the wild type, the Δ*ynjE*::Km^r *FRT* and the Δ*ynjE* strains, respectively. Dashed arrows on each template indicate the primers. Two-headed arrows under the template indicate the expected size in kilobase (kb) of PCR products. Right panel: agarose gel electrophoresis of PCR products obtained from different DNA templates. Lanes 1 and 2, the Δ*ynjE* colonies. Lane 3, the Δ*ynjE*::Km^r *FRT* colony. Lanes 4 and 5, the wild type colony and chromosomal DNA, respectively. Lane M, DNA markers with the size in base pairs labeled on the left. Arrows on the right indicate the expected sizes of PCR products.

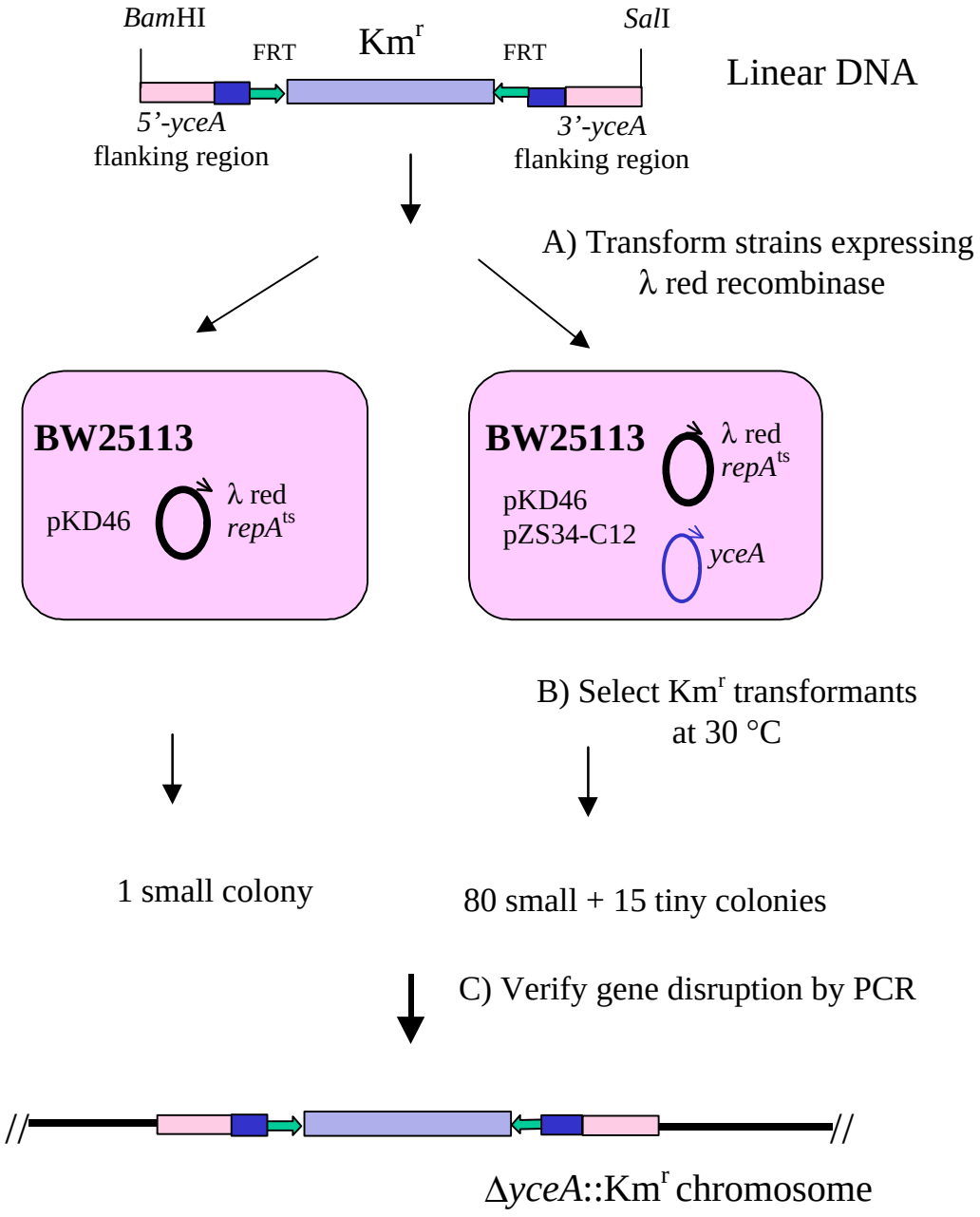


Figure 3.6 Introduction of Δ*yceA*::Km^r into the genome.

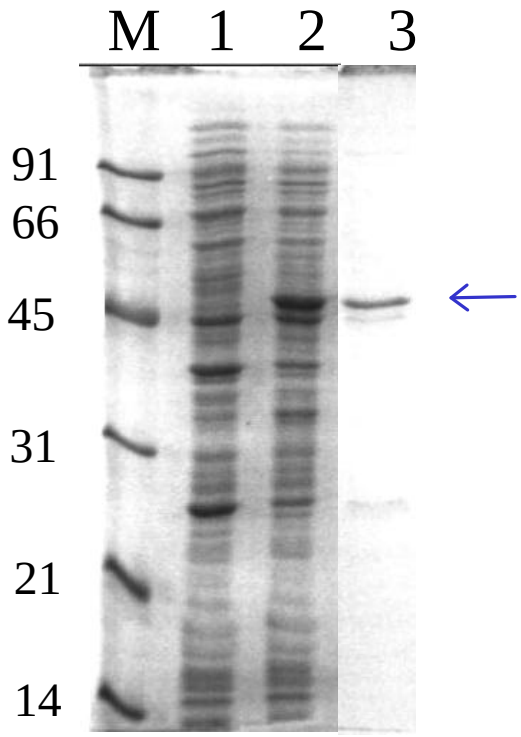


Figure 3.7 SDS-PAGE analysis of YnjE-His_6.

Lane M, the molecular mass markers; lane 1, BL21(DE3)(pT7-7) cells treated with 0.5 mM IPTG (10 μg); lane 2, BL21(DE3)(pPJ15) cells treated with 0.5 mM IPTG (10 μg); lane 3, partially purified YnjE-His_6. The arrow shows the position of an abundant protein with apparent molecular mass of 50 kDa.

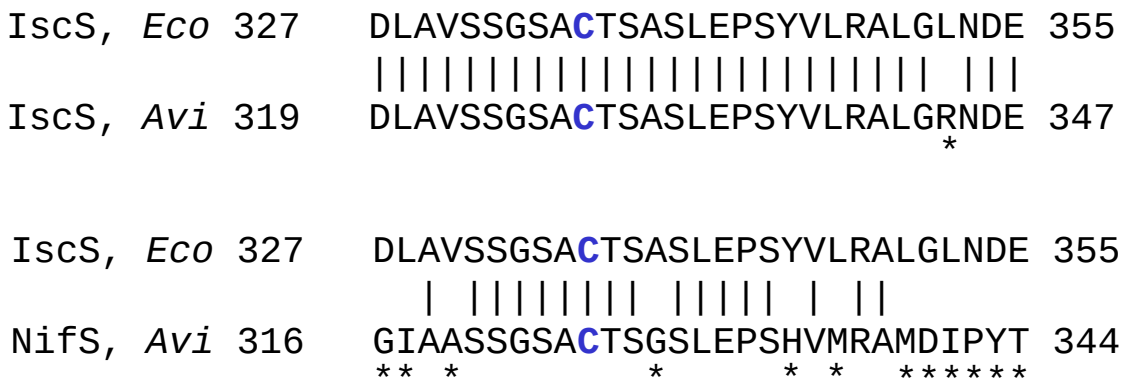


Figure 3.8 Comparison of amino acid sequences around the active site cysteine region of *Escherichia coli* (*Eco*) IscS with *Azotobacter vinelandii* (*Avi*) IscS and NifS.

Asterisk (*) under the *Avi* sequence and vertical line (|) between the two sequences highlight the positions that are different and identical, respectively. The active site cysteine (**C**) is shown in bold.

3.6: References

1. Alexander K, Volini M. Properties of an *Escherichia coli* rhodanese. J Biol Chem 1987;262:6595-604.
2. Vachek H, Wood JL. Purification and properties of mercaptopyruvate sulfur transferase of *Escherichia coli*. Biochim Biophys Acta 1972;258:133-46.
3. Ray WK, Zeng G, Potters MB, Mansuri AM, Larson TJ. Characterization of a 12-kilodalton rhodanese encoded by *glpE* of *Escherichia coli* and its interaction with thioredoxin. J Bacteriol 2000;182:2277-84.
4. Nagahara N, Okazaki T, Nishino T. Cytosolic mercaptopyruvate sulfurtransferase is evolutionarily related to mitochondrial rhodanese. Striking similarity in active site amino acid sequence and the increase in the mercaptopyruvate sulfurtransferase activity of rhodanese by site-directed mutagenesis. J Biol Chem 1995;270:16230-5.
5. Schwartz CJ, Djaman O, Imlay JA, Kiley PJ. The cysteine desulfurase, IscS, has a major role in *in vivo* Fe-S cluster formation in *Escherichia coli*. Proc Natl Acad Sci U S A 2000;97:9009-14.
6. Kambampati R, Lauhon CT. IscS is a sulfurtransferase for the *in vitro* biosynthesis of 4- thiouridine in *Escherichia coli* tRNA. Biochemistry 1999;38:16561-8.
7. Miller JH. A short course in bacterial genetics. A laboratory manual and handout for *Escherichia coli* and related bacteria. New York: Cold Spring Harbor Laboratory, 1992.
8. Johnson ME, Rajagopalan KV. Involvement of *chlA*, *E*, *M*, and *N* loci in *Escherichia coli* molybdopterin biosynthesis. J Bacteriol 1987;169:117-25.
9. del Campillo-Campbell A, Campbell A. Molybdenum cofactor requirement for biotin sulfoxide reduction in *Escherichia coli*. J Bacteriol 1982;149:469-78.
10. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory, 1989.

11. Silhavy TJ, Berman ML, Enquist LW. Experiments with gene fusions. New York: Cold Spring Harbor Laboratory, 1984.
12. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
14. Zeng G, Larson TJ. Use of engineered thrombin cleavage site for determination of translational reading frames. Biotechniques 1996;21:822-4.
15. Cherepanov PP, Wackernagel W. Gene disruption in *Escherichia coli*: Tc^r and Km^r cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 1995;158:9-14.
16. Link AJ, Phillips D, Church GM. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J Bacteriol 1997;179:6228-37.
17. Murphy KC. Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. J Bacteriol 1998;180:2063-71.
18. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 2000;97:6640-5.
19. Lauhon CT, Kambampati R. The *iscS* gene in *Escherichia coli* is required for the biosynthesis of 4- thiouridine, thiamin, and NAD. J Biol Chem 2000;275:20096-103.
20. Blattner FR, Plunkett G, Bloch CA, et al. The complete genome sequence of *Escherichia coli* K-12. Science 1997;277:1453-74.
21. Podkovyrov SM, Larson TJ. Identification of promoter and stringent regulation of transcription of the *fabH*, *fabD* and *fabG* genes encoding fatty acid biosynthetic enzymes of *Escherichia coli*. Nucleic Acids Res 1996;24:1747-52.
22. Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 1986;189:113-30.

23. Nichols BP, Shafiq O, Meiners V. Sequence analysis of Tn*10* insertion sites in a collection of *Escherichia coli* strains used for genetic mapping and strain construction. J Bacteriol 1998;180:6408-11.
24. Lutz R, Bujard H. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. Nucleic Acids Res 1997;25:1203-10.
25. Yu F, Jen Y, Takeuchi E, et al. Alpha-glucan phosphorylase from *Escherichia coli*. Cloning of the gene, and purification and characterization of the protein. J Biol Chem 1988;263:13706-11.
26. Tabor S, Richardson CC. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc Natl Acad Sci U S A 1985;82:1074-8.
27. Karow M, Georgopoulos C. Isolation and characterization of the *Escherichia coli msbB* gene, a multicopy suppressor of null mutations in the high-temperature requirement gene *htrB*. J Bacteriol 1992;174:702-10.
28. Leimkuhler S, Rajagopalan KV. A sulfurtransferase is required in the transfer of cysteine sulfur in the *in vitro* synthesis of molybdopterin from precursor Z in *Escherichia coli*. J Biol Chem 2001;4:4.
29. Peters GH, Frimurer TM, Olsen OH. Electrostatic evaluation of the signature motif (H/V)CX_5R(S/T) in protein-tyrosine phosphatases. Biochemistry 1998;37:5383-93.

Curriculum Vitae

Promjit Jutabha

Department of Pathology, Faculty of Medicine, Srinakharinwirot University,

Vajira Hospital, 681 Samsen Road, Dusit, Bangkok 10300, Thailand.

E-mail: promjit@psm.swu.ac.th or promjit@vt.edu

Education

Doctor of Philosophy (Biochemistry), 2001.
Department of Biochemistry, Virginia Polytechnic and State University, Blacksburg, VA.
Master of Science (Clinical Pathology), 1994.
Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.
Bachelor of Science (Medical Technology), First Class Honors, 1989.
Faculty of Medical Technology, Mahidol University, Bangkok, Thailand.

Honors

Bruce M. Anderson Award, 1997.
For outstanding first year graduate student in Department of Biochemistry.

Professional Experience

Lecturer in Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand, 1994-1996.
Guest Lecturer in Department of Pathology, Bangkok College of Medicine, Thailand, 1995-1996.
Medical Technologist, Suanpai Clinic, Bangkok, Thailand, 1990-1996.

Professional Membership

The Medical Technologist Association of Thailand.

Presentation at Professional Meeting

Jutabha P, Yoon YJ, Larson TL. Mercaptopyruvate Sulfurtransferase of *Escherichia coli*. Poster presented at the American Society for Biochemistry and Molecular Biology Meeting, Boston, MA, June 2000.