

## Requirement for IscS in Biosynthesis of All Thionucleosides in *Escherichia coli*

Charles T. Lauhon\*

School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53705

Received 6 June 2002/Accepted 4 September 2002

*Escherichia coli* tRNA contains four naturally occurring nucleosides modified with sulfur. Cysteine is the intracellular sulfur source for each of these modified bases. We previously found that the *iscS* gene, a member of the *nifS* cysteine desulfurase gene family, is required for 4-thiouridine biosynthesis in *E. coli*. Since IscS does not bind tRNA, its role is the mobilization and distribution of sulfur to enzymes that catalyze the sulfur insertion steps. In addition to *iscS*, *E. coli* contains two other *nifS* homologs, *csdA* and *csdB*, each of which has cysteine desulfurase activity and could potentially donate sulfur for thionucleoside biosynthesis. Double *csdA csdB* and *iscS csdA* mutants were prepared or obtained, and all mutants were analyzed for thionucleoside content. It was found that unfractionated tRNA isolated from the *iscS* mutant strain contained <5% of the level of sulfur found in the parent strain. High-pressure liquid chromatography analysis of tRNA nuclease digests from the mutant strain grown in the presence of [<sup>35</sup>S]cysteine showed that only a small fraction of 2-thiocytidine was present, while the other thionucleosides were absent when cells were isolated during log phase. As expected, digests from the *iscS* mutant strain contained 6-*N*-dimethylallyl adenosine (i<sup>6</sup>A) in place of 6-*N*-dimethylallyl-2-methylthioadenosine and 5-methylaminomethyl uridine (mnm<sup>5</sup>U) instead of 5-methylaminomethyl-2-thiouridine. Prolonged growth of the *iscS* and *iscS csdA* mutant strains revealed a gradual increase in levels of 2-thiocytidine and 6-*N*-dimethylallyl-2-methylthioadenosine with extended incubation (>24 h), while the thiouridines remained absent. This may be due to a residual level of Fe-S cluster biosynthesis in *iscS* deletion strains. An overall scheme for thionucleoside biosynthesis in *E. coli* is discussed.

Sulfur is found in the cell not only in essential cofactors, such as biotin and thiamine, but also in macromolecules, such as Fe-S proteins and tRNA (2, 22). The nearly universal source of sulfur for these molecules is L-cysteine. However, the biochemical reactions for the incorporation of the sulfur from cysteine are in many cases poorly understood. It was recently found that the cysteine desulfurase IscS is required for the biosynthesis of 4-thiouridine in tRNA both in vitro (14) and in vivo (17). In addition, *iscS* mutants are unable to synthesize thiazole and nicotinic acid and appear to be deficient in a number of other biosynthetic pathways. There now exists strong evidence from many laboratories that *iscS* is the major sulfur mobilization catalyst required for the biosynthesis of protein Fe-S clusters in both bacteria (32, 33, 40) and yeasts (19).

Base modification in tRNA is found in all organisms and represents the fine-tuning of the many functions of tRNA in protein translation (3, 4). Thionucleosides in particular have been found to influence tRNA aminoacylation (21, 37), codon-anticodon specificity (45), and reading frame maintenance (42) as well as the binding of tRNA to the ribosome (1). There have been several reports linking the biochemistry of tRNA modification to important pathways in primary or secondary metabolism. For example, Lipsett reported that *Escherichia coli* mutants lacking 4-thiouridine in their tRNA were also unable to synthesize thiamine (31). The *thiI* (27, 43) and *iscS* (17) genes, both required for 4-thiouridine and thiazole synthesis, were characterized as a result of these initial studies.

Some tRNA modifications are also thought to act as biochemical sensors for environmental stress. Interestingly, two of these involve sulfur modification. For example, 4-thiouridine has been proposed to act as a sensor for near-UV radiation (39), inducing intramolecular crosslinks into tRNA that result in poor aminoacylation (5) and the stringent response (16). The presence of 6-*N*-dimethylallyl-2-methylthioadenosine has been shown to require soluble iron and is absent in tRNA from bacteria isolated from lethally infected animals, in which iron is scarce (12). It has recently been shown that MiaB, which is implicated in sulfur insertion of 6-*N*-dimethylallyl-2-methylthioadenosine (8) is an Fe-S protein (30).

Four thionucleosides are naturally occurring in *E. coli*. Shown in Fig. 1, these are 4-thiouridine (s<sup>4</sup>U), 2-thiocytidine (s<sup>2</sup>C), 5-methylaminomethyl-2-thiouridine (mnm<sup>5</sup>s<sup>2</sup>U), and 6-*N*-dimethylallyl-2-methylthioadenosine (ms<sup>2</sup>i<sup>6</sup>A). The biosynthesis of 4-thiouridine (Fig. 2A) has been shown to require both the thiamine pathway enzyme ThiI (27) and the cysteine desulfurase IscS (14). The mechanism of sulfur transfer (Fig. 2B) initially involves mobilization of sulfur from L-cysteine by IscS to form a persulfide at Cys328 in the active site (9). This persulfide, or sulfane sulfur, is then transferred to a cysteine on ThiI, which is in turn transferred to the tRNA (15) by a mechanism that likely involves oxidation of ThiI. Evidence for disulfide formation in ThiI during 4-thiouridine synthesis has recently been shown under single-turnover conditions (28). This type of mechanism may also be operating for the synthesis of 2-thiouridine by IscS and MnmA (R. Kambampati and C. T. Lauhon, submitted for publication).

*E. coli* contains three genes, *iscS*, *csdA* (24), and *csdB* (25), that code for cysteine desulfurases. Each gene product has been shown to catalyze cysteine desulfurase activity with vary-

\* Mailing address: School of Pharmacy, University of Wisconsin, 777 Highland Ave., Madison, WI 53705. Phone: (608) 262-3083. Fax: (608) 262-3397. E-mail: clauhon@facstaff.wisc.edu.

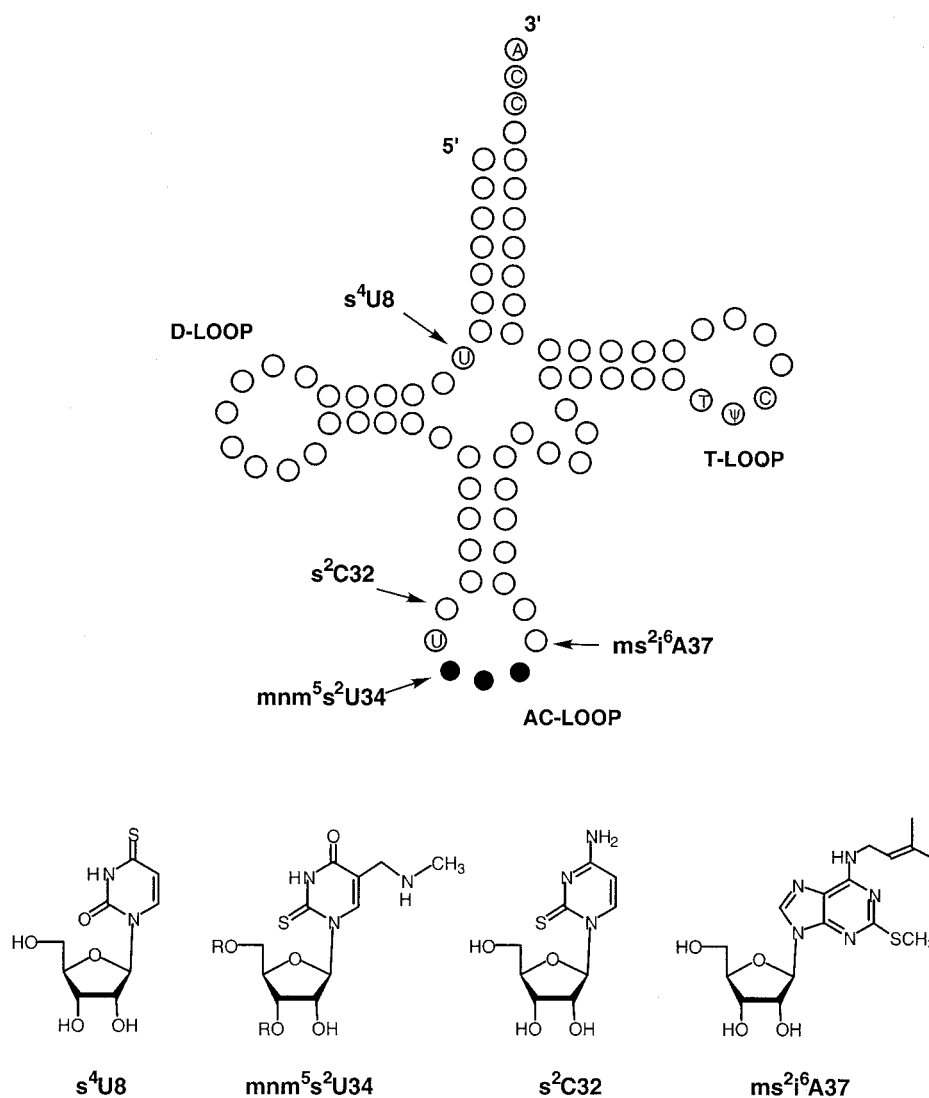


FIG. 1. Structures of the four naturally occurring thionucleosides and their locations in *E. coli* tRNA. Invariant nucleosides are shown in the tRNA secondary structure, and anticodon bases are represented by solid circles. s<sup>4</sup>U8, 4-thiouridine 8; mnm<sup>5</sup>s<sup>2</sup>U34, 5-methylaminomethyl-2-thiouridine 34; s<sup>2</sup>C32, 2-thiocytidine 32; ms<sup>2</sup>i<sup>6</sup>A37, 6-*N*-dimethylallyl-2-methylthioadenosine 37.

ing efficiency in vitro (26). It seemed probable that one or more of these enzymes was required for biosynthesis of the other thionucleosides in *E. coli*. Therefore, deletion mutants of each of the genes were prepared, as well as a double mutant that lacked both *csdA* and *csdB* as well as *iscS* and *csdA*. High-pressure liquid chromatography (HPLC) traces of tRNA nuclease digests from these mutants were then compared. The results showed that the *iscS* gene in *E. coli* is required for the biosynthesis of each of the four thionucleosides and accounts for  $\geq 95\%$  of the sulfur content in *E. coli* tRNA.

#### MATERIALS AND METHODS

**Materials.** 4-Thiouridine, 2-thiocytidine, *E. coli* tRNA<sup>Lys</sup> and tRNA<sup>Phe</sup>, and bacterial alkaline phosphatase were obtained from Sigma. The deletion plasmid pKO3 was generously provided by George M. Church. Nuclease P1 was from Roche Biochemicals. Oligodeoxynucleotides were from Integrated DNA Technologies. The DE3 lysogenization kit used to prepare strain CL100(DE3) was

from Novagen. Strains PK4331 (32) and PK5930 (*ΔiscS ΔcsdA*) were generous gifts of Patricia Kiley, University of Wisconsin.

**General method of gene deletion in *E. coli*.** A combination of the methods of Link et al. (20) and Hamilton et al. (13) was used to perform the in-frame deletion of genes in *E. coli*. The method has previously been described in the deletion of *E. coli iscS* (17). The parent strain for all gene deletions was *E. coli* MC1061. Table 1 shows the primers used for the *csdA* and *csdB* genes.

**Growth media and <sup>35</sup>S labeling of tRNA.** Rich medium was Luria-Bertani (LB). For <sup>35</sup>S labeling, 100-ml cultures containing LB supplemented with 1 mCi of L-[<sup>35</sup>S]cysteine were inoculated with 1 ml of an overnight culture of either the parent strain (MC1061), the *iscS* mutant [CL100(DE3)], or the *iscS* mutant with a plasmid expressing wild-type *E. coli iscS* [CL100(DE3)/pCL010]. The cells were grown overnight, and tRNA was isolated and digested to nucleosides as outlined below.

**Isolation of unfractionated tRNA.** Cells were recovered by centrifugation at  $8,000 \times g$  for 15 min at 4°C. The cell pellet was resuspended in 2 ml of 10 mM Tris (pH 7.5)–1 mM MgCl<sub>2</sub>. An equal volume of equilibrated buffered phenol was added, and the mixture was vortexed for 1 min. After separation of layers by centrifugation ( $13,000 \times g$ , 20 min), the top aqueous layer was transferred to a polypropylene tube, and 0.1 volume of 3 M sodium acetate (pH 5.5) was added,

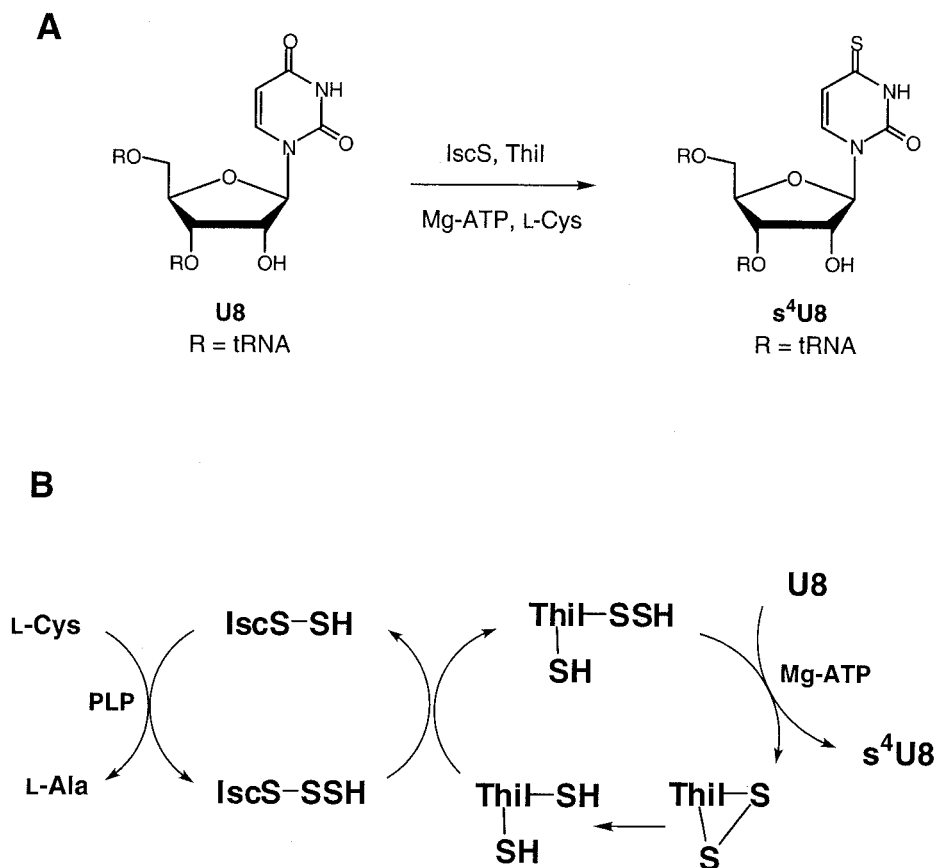


FIG. 2. Biosynthesis of 4-thiouridine in *E. coli*. (A) Factors necessary for conversion of uridine (U8) to 4-thiouridine ( $s^4\text{U8}$ ) in *E. coli*. (B) Currently proposed mechanism for sulfur transfer from IscS to Thil during 4-thiouridine biosynthesis. Thil also utilizes MgATP for activation of the uridine O-4 and requires free thiol for reduction of a disulfide that is formed internally during turnover. IscS-SH, unmodified IscS protein; IscS-SSH, IscS persulfide; PLP, pyridoxal-L-phosphate.

followed by 2.5 volumes of cold ethanol. After storage at  $-20^\circ\text{C}$  for at least 4 h (or overnight), the diffuse precipitate was isolated by centrifugation ( $13,000 \times g$ ,  $4^\circ\text{C}$ , 30 min), washed with 70% aqueous ethanol, dried, and resuspended in 0.5 ml of 10 mM Tris (pH 8) with vortexing. To this solution was added an equal volume of 8 M urea containing 0.01% (wt/vol) bromophenol blue. The resulting viscous solution was heated for 3 min at  $85^\circ\text{C}$  and loaded onto a 10% denaturing polyacrylamide gel.

The band corresponding to tRNA was excised and eluted by crushing and soaking in 0.5 M NaCl overnight. The tRNA was then precipitated with ethanol, resuspended in deionized water, and stored at  $-20^\circ\text{C}$ . Quantitation was done by absorbance at 260 nm. The amounts of tRNA isolated from the parent and mutant strains were within the variation of the isolation procedure when cultures of equal cell density were compared. Amounts ranged from 100 to 200  $\mu\text{g}$  for a 100-ml culture harvested at mid-log phase to double that amount for freshly saturated cultures. Simple "overnight" cultures will yield less tRNA for the *iscS* mutant due to the long time required to reach saturation ( $>24$  h).

**tRNA digestion and nucleoside analysis by HPLC.** A total of 100  $\mu\text{g}$  of gel-purified unfractionated tRNA (concentration determined by  $A_{260}$ ) was treated with 3 U of nuclease P1 in a mixture of 30 mM sodium acetate (pH 5.3) and 20 mM zinc acetate in a total volume of 200  $\mu\text{l}$  at  $37^\circ\text{C}$ . Reaction times could be as little as 4 h but were typically overnight. After reaction with nuclease P1, 20  $\mu\text{l}$  of 1 M Tris (pH 7.5) was added, followed by 3 U of bacterial alkaline phosphatase, and the mixture was incubated at  $37^\circ\text{C}$  for at least 2 h.

For nucleoside analysis, the HPLC method of Gherke et al. (11) was used. Aliquots of the reaction mixture (typically corresponding to 25  $\mu\text{g}$  of tRNA) were loaded directly onto a Supelco  $\text{C}_{18}$  column (catalog no. LC18-S). This column is specially designed for nucleoside separation. For elution of nucleosides that are more polar than adenosine, the following buffer system was chosen: buffer A contained 2.5% methanol in 10 mM ammonium phosphate, and buffer B contained 20% methanol in 10 mM ammonium phosphate, pH 5.3. The

gradient was from 0 to 100% B over 45 min with the five-step program described by Gherke et al. (11) for high-resolution separation. For nucleosides less polar than adenosine (e.g.,  $i^6\text{A}$  and 6-*N*-dimethylallyl-2-methylthioadenosine), the following buffer system was used: buffer A contained 10% acetonitrile in 5 mM ammonium phosphate (pH 5.3), and buffer B contained 35% acetonitrile in 5 mM ammonium phosphate. A 30-min linear gradient from 0 to 100% B was used.

Nucleosides were detected by absorbance at 260 nm, with the additional use of 330 nm for detection of 4-thiouridine. HPLC standards were used for identifying 4-thiouridine, 2-thiocytidine, and  $\text{mnm}^5\text{U}$ , whereas purified *E. coli* tRNAs could provide standards for 5-methylaminomethyl-2-thiouridine ( $\text{tRNA}^{\text{Glu}}$ ) and 6-*N*-dimethylallyl-2-methylthioadenosine ( $\text{tRNA}^{\text{Phe}}$ ). Quantitation (Table 2) was done by dividing the area of peaks from the thionucleosides by that of pseudouridine for 2-thiocytidine,  $\text{mnm}^5\text{U}$ , 5-methylaminomethyl-2-thiouridine, and 4-thiouridine as previously described (6). For 6-*N*-dimethylallyl-2-methylthioadenosine and  $i^6\text{A}$ , buffer A was replaced with 5% acetonitrile to allow separation of  $i^6\text{A}$  and  $\text{m}^2\text{A}$  from adenosine. The area of the 6-*N*-dimethylallyl-2-methylthioadenosine or  $i^6\text{A}$  peak was divided by the area of the  $i^6\text{A}$  peak, the latter of which was proportional to the amount of tRNA in my hands. These relative values served to minimize error due to loading variation.

The assigned thionucleoside peaks were found to be labeled with  $^{35}\text{S}$  when tRNA was analyzed from cells grown in medium supplemented with L- $^{35}\text{S}$  cysteine. In addition, the relative retention times for the nucleosides were very similar to those in the original work of Gherke et al. (11), since the same type of column and buffer system were used in both studies.

**Growth of  $\Delta\text{iscS}$  strain in medium supplemented with sulfide.** Cells of strain CL100(DE3) ( $\Delta\text{iscS}$ ) from 1 ml of overnight culture were added to 100 ml of LB supplemented with sodium sulfide (1 to 10 mM). Cultures were grown to saturation, and unfractionated bulk tRNA was isolated as described above.

TABLE 1. Strains, plasmids, and primers used in this work<sup>a</sup>

Strain, plasmid, or primer	Description or sequence	Source or reference
<b>Strains</b>		
MC1061	F <sup>-</sup> <i>araD139</i> $\Delta$ ( <i>ara leu</i> )7696 $\Delta$ ( <i>lacY74</i> ) <i>galU galK hsdR hsdM<sup>+</sup> strA</i>	BioRad
CL100	<i><math>\Delta</math>iscS</i>	17
CL100(DE3)	DE3 lysogen of CL100	17
CL201	<i><math>\Delta</math>csdB</i>	This work
CL102	<i><math>\Delta</math>csd <math>\Delta</math>csdB</i>	This work
CL103	<i><math>\Delta</math>thiI</i>	This work
CL250	<i><math>\Delta</math>mmmA</i>	— <sup>b</sup>
CL260	<i><math>\Delta</math>miaB</i>	This work
PK4331	MG1655 <i>lacZ</i> $\Delta$ 145 <i><math>\Delta</math>iscS::Kn<sup>r</sup></i>	30
PK5930	MG1655 <i>lacZ</i> $\Delta$ 145 <i><math>\Delta</math>iscS::Kn<sup>r</sup> <math>\Delta</math>csdA::Cm<sup>r</sup></i>	— <sup>c</sup>
<b>Plasmids</b>		
pCL010	Wild-type <i>E. coli</i> <i>iscS</i> in pET21c	14
pCSD	Wild-type <i>E. coli</i> <i>csd</i> in pET21c	This work
pCSDB	Wild-type <i>E. coli</i> <i>csdB</i> in pET21c	This work
pKO3	For deletion of genes in <i>E. coli</i>	20
<b>Primers</b>		
CSD.N	5'-ATC AAG CCG AGG AGT <u>CAT ATG AAC</u> GTT TTT AAT CCC GCG	
CSD.C	5'-CGA ATT GCG GGT <u>GAA TTC</u> TTA ATC CAC CAA TAA TTC CAG	
CsdB.N	5'-GCT GCC AGG AGG TGC <u>CAT ATG</u> ATT TTT TCC GTC GAC AAA	
CsdB.C	5'-AGC CAT AGT GCC <u>GGA TCC</u> TTA TCC CAG CAA ACG GTG AAT	
CSD.No	5'-AAG GAA AAA AGC <u>GGC CGC</u> TAC ATT TAC CCT GTC TGT CCA TAG TGAT T	
CSD.Ni	5'- <b>CAC GCA ATA ACC TTC ACA CTC CAA ATT TAT AAC</b> CAT GGT ACT CCT CGG CTT G	
CSD.Co	5'-CGC ACG CAT <u>GTC GAC</u> GTC TTA TCC TCC GAC CCG GTT CT	
CSD.Ci	5'- <b>GTT ATA AAT TTG GAG TGT GAA GGT TAT TGC GTG</b> TGA CCG CGC GCT GGA ATT A	
CsdB.No	5'-AAG GAA AAA AGC <u>GGC CGC</u> ACG CAA CTC AAT GGC GAA AAC A	
CsdB.Ni	5'- <b>CAC GCA ATA ACC TTC ACA CTC CAA ATT TAT AAC</b> AAT CAT CTT GCA CCT CCT GGC	
CsdB.Co	5'-CGC ACG CAT <u>GTC GAC</u> CAT TGA CCA TCC GGC AAT GTG A	
CsdB.Ci	5'- <b>GTT ATA AAT TTG GAG TGT GAA GGT TAT TGC GTG</b> CAC CGT TTG CTG GGA TAA CAG	

<sup>a</sup> For primers, restriction sites are underlined, and the 33-bp gene replacement tag sequence is shown in bold (see text). Primers labeled No, Co, etc., were used to perform in-frame deletion by the method of Link et al. (20).

<sup>b</sup> R. Kambampati and C. T. Lahun, submitted.

<sup>c</sup> P. J. Kiley, unpublished data.

## RESULTS

**Generation of mutants.** The strains used in this study are shown in Table 1. In addition to *iscS*, *csdA*, and *csdB*, mutations in the other known genes required for thionucleoside biosynthesis were also prepared. These included *thiI*, required for 4-thiouridine modification; *mmmA*, required for introduction of the sulfur at the 2 position of U34; and *miaB*, required for the introduction of sulfur at the 2 position of A37. The modified nucleoside profiles of these mutants were confirmed by HPLC analysis of nuclease digests of unfractionated tRNA isolated from the mutant strains (data not shown). *thiI* (27) and *trmU* (*mmmA*) (7, 36; A. T. Crescenzo, T. Hagervall, J. A.

McCloskey, and D. Soll, unpublished data) mutants of *E. coli* and *thiI* (43) and *miaB* (8) mutants of *Salmonella enterica* serovar Typhimurium have been reported previously. The mutants prepared in this work are isogenic and provide control HPLC traces for the absence of specific thionucleosides. In each mutant, complementation with an expression plasmid containing the wild-type gene restored levels of the modified nucleoside to those found in the parent strain.

**tRNA from  $\Delta$ iscS strain lacks 95% of sulfur in tRNA.** tRNA from the *iscS* mutant was analyzed for the presence of all thionucleosides. When the mutant and parent strains were grown in LB supplemented with L-[<sup>35</sup>S]cysteine and the tRNA

TABLE 2. Modified nucleoside levels for parent strain MC1061, *iscS* mutant CL100(DE3), and CL100(DE3) complemented with plasmid pCL010<sup>a</sup>

Strain	Relative level					
	s <sup>2</sup> C	mmn <sup>5</sup> U	mmn <sup>5</sup> s <sup>2</sup> U	s <sup>4</sup> U	i <sup>6</sup> A	ms <sup>2</sup> i <sup>6</sup> A
MC1061	1.0 (0.2)	(0.015)	1.0 (0.11)	1.0 (1.2)	ND <sup>b</sup>	(0.52)
CL100(DE3)	0.02	(0.15)	ND	ND	(0.65)	ND
CL100(DE3)/pCL010	0.9	(0.01)	0.7	1.0	ND	(0.47)

<sup>a</sup> Levels of 2-thiocytidine (s<sup>2</sup>C), 5-methylaminomethyl-2-thiouridine (mmn<sup>5</sup>s<sup>2</sup>U), and 4-thiouridine (s<sup>4</sup>U) were measured as the area ratio to pseudouridine ( $\Psi$ ) and then normalized relative to the parent strain set at 1.0. Parenthetical values were not normalized. Levels of 6-N-dimethylalyl-2-methylthioadenosine (ms<sup>2</sup>i<sup>6</sup>A) and i<sup>6</sup>A are reported relative to t<sup>6</sup>A and were not normalized to the parent strain. All nucleosides were quantified at 260 nm except 4-thiouridine, which was quantitated at 330 nm.

<sup>b</sup> ND, none detected.

MC1061      CL100  
 (Δ*iscS*)      CL100/pCL010

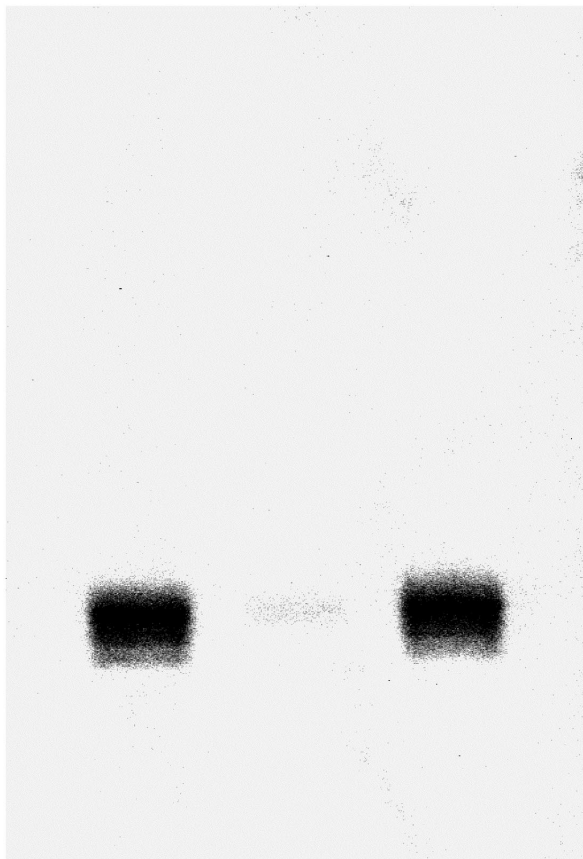


FIG. 3. Sulfur analysis of tRNA isolated from  $\Delta$ *iscS* strain CL100(DE3). PhosphorImager scan of denaturing polyacrylamide gel of total tRNA isolated from parent and *iscS* mutant strain. The strains were grown to saturation in 100 ml of LB containing L-[ $^{35}$ S]cysteine (1 mCi). Equal amounts of tRNA (10  $\mu$ g) from each strain were loaded onto the gel. Left lane shows  $^{35}$ S-labeled tRNA from the parent strain *E. coli* MC1061. Middle lane is tRNA from strain CL100(DE3) ( $\Delta$ *iscS*), and the right lane is tRNA from CL100(DE3) containing plasmid pCL010, which expresses wild-type *iscS*. The specific activities of tRNA from MC1061, CL100(DE3), and CL100(DE3)/pCL010 were 6,720, 330, and 5,789 cpm/ $\mu$ g, respectively.

was isolated, tRNA from the *iscS* mutant had only 2 to 5% of the sulfur level of tRNA of that of the parent strain. Figure 3 shows that expression of *iscS* from a multicopy plasmid in *iscS* mutant CL100(DE3) restored 87% of the sulfur in the tRNA. The reasons for less than complete complementation are not yet clear, but a similar observation has been made with Fe-S enzyme activity in another nonpolar *iscS* mutant (32).

The DE3 lysogen of strain CL100 that was used has significant basal expression of T7 polymerase in the absence of isopropylthiogalactopyranoside (IPTG). This results in overexpression of *iscS* from the T7 promoter-based plasmid pCL010, as measured by cysteine desulfurase activity of soluble cell extracts (not shown). Plasmid pCL010 completely complements both the slow growth rate and auxotrophic requirements of strain CL100(DE3). The presence of IPTG dramatically

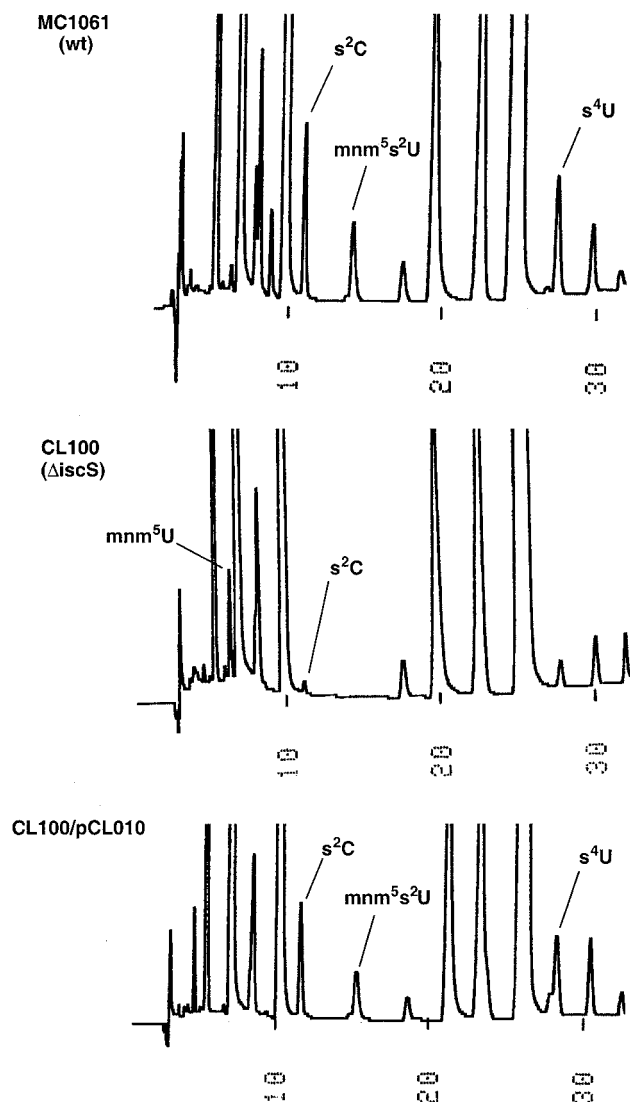


FIG. 4. HPLC profile of pyrimidine region of thionucleosides. (Top) Modified nucleoside HPLC profile of digested tRNA isolated from wild-type (wt) parent strain MC1061, with thionucleosides labeled. (Middle) Same profile from  $\Delta$ *iscS* strain CL100. (Bottom) Profile from strain CL100 with plasmid pCL010 (wild-type *iscS*). The peak near 4-thiouridine in the middle panel is an unidentified compound that was present in all chromatograms but was revealed more clearly in the absence of 4-thiouridine. I independently verified at 330 nm that strain CL100 lacked 4-thiouridine (see Fig. 6) (16).  $s^4U$ , 4-thiouridine;  $s^2C$ , 2-thiocytidine;  $mnm^5s^2U$ , 5-methylaminomethyl-2-thiouridine.

increased *iscS* expression but did not increase the level of thionucleosides by HPLC analysis (not shown).

**Thionucleoside analysis of the  $\Delta$ *iscS* strain.** tRNA digests from the *iscS* strain were examined for the presence of all thionucleosides. Figure 4 shows HPLC traces of the polar nucleoside region of tRNA digests from the parent strain MC1061, the *iscS* strain CL100(DE3), and CL100(DE3) complemented with a plasmid containing *iscS* (pCL010). The thionucleosides 4-thiouridine and 5-methylaminomethyl-2-thiouridine were completely absent in the  $\Delta$ *iscS* strain, and only a small amount of 2-thiocytidine was observed. A proportionate

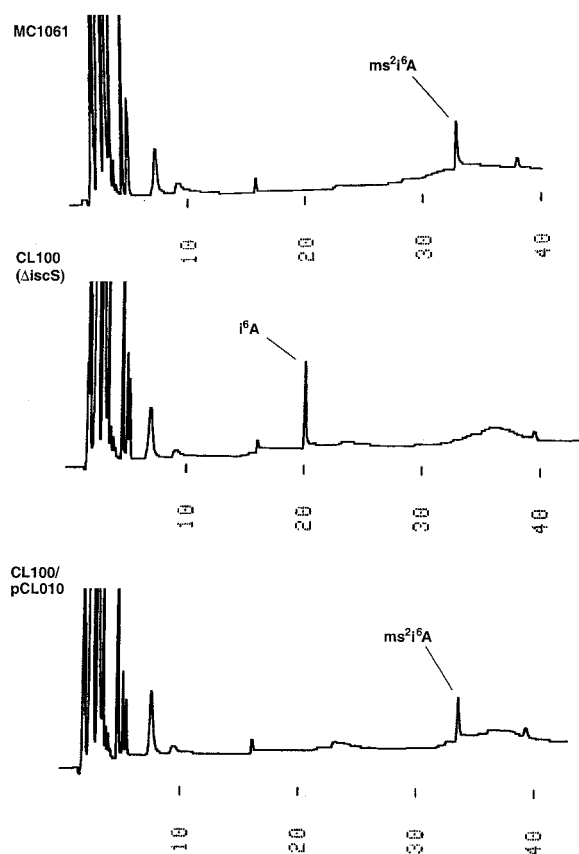


FIG. 5. HPLC profile of modified adenosine region. (Top) Profile of digested tRNA isolated from parent strain MC1061. (Middle) Profile from  $\Delta iscS$  strain CL100. (Bottom) Profile from strain CL100 with pCL010 (wild-type *iscS*). Approximately 25  $\mu$ g of digested tRNA was loaded for each run.  $ms^2i^6A$ , 6-*N*-dimethylallyl-2-methylthioadenosine;  $i^6A$ , 6-*N*-dimethylallyl-adenosine.

amount of  $mnm^5U$  was found in the *iscS* mutant, confirming previous work suggesting that modification of the wobble uridine at the 5 position is independent of thiolation at the 2 position (7, 36; A. T. Crescenzo, T. Hagervall, J. A. McCloskey, and D. Soll, unpublished data).

Figure 5 shows that  $i^6A$  was present in place of 6-*N*-dimethylallyl-2-methylthioadenosine in tRNA from strain CL100, a result that is identical to that with an *E. coli miaB* mutant prepared by me and previously described in *S. enterica* serovar Typhimurium by Esberg et al. (8). This strongly suggests that *miaB* activity is dependent on the presence of *iscS* in vivo. Such dependence is consistent with the observation, from both sequence analysis (35) and in vitro reconstitution (30), that MiaB is an Fe-S protein. The results are shown quantitatively in Table 2. Areas for 2-thiocytidine, 5-methylaminomethyl uridine ( $mnm^5U$ ), 5-methylaminomethyl-2-thiouridine, and 4-thiouridine are given as ratios with pseudouridine as an internal standard, whereas  $t^6A$  was used as the standard for the hydrophobic nucleosides  $i^6A$  and 6-*N*-dimethylallyl-2-methylthioadenosine. Since extinction coefficients vary, relative amounts of the thionucleosides are shown with that of the parent strain set to 1.0 when possible.

#### Increase in 2-thiocytidine and 6-*N*-dimethylallyl-2-methyl-

**thioadenosine levels with growth level.** tRNA was extracted from both the parent and strain CL100 at mid-log phase ( $A_{600} = 0.5$ ), late log phase ( $A_{600} = 1.0$ ), and saturation ( $A_{600} > 1.4$ ) and analyzed. The time required for the *iscS* strain to reach saturation was >24 h. For the parent strain and the mutant strain CL100(DE3) complemented with wild-type *iscS*, final levels of all thionucleosides were reached by early log phase and remained essentially constant. As shown in Fig. 6 for the *iscS* mutant, only a very small fraction (ca. 2%) of 2-thiocytidine was present in the tRNA isolated from mid-log growth. A small increase in 2-thiocytidine levels to about 10% of the normal level was seen at late log phase, with no appearance in 6-*N*-dimethylallyl-2-methylthioadenosine. However, at saturation, 2-thiocytidine levels increased significantly to 70% of the wild-type level. Figure 7 shows that significant amounts of 6-*N*-dimethylallyl-2-methylthioadenosine were also seen at saturation but arose later relative to 2-thiocytidine, as none was detected at late log phase. Quantitation of these results is shown in Table 3.

**Possible supplementary role for *iscS* paralogs in *E. coli*.** Overexpression of the *csdA* and *csdB* genes in the *iscS* mutant did not complement any of the phenotypic characteristics of the *iscS* mutant. In addition, both the *csdA* and *csdB* mutants as well as a *csdA csdB* double mutant showed modified nucleoside profiles that were indistinguishable from that of the parent strain (data not shown). An *E. coli iscS csdA* double mutant (PK5930) was also obtained and analyzed. Thionucleoside levels were found to be identical to those in the isogenic *iscS* mutant PK4331 (32) in all respects, including the increase in 2-thiocytidine and 6-*N*-dimethylallyl-2-methylthioadenosine during stationary phase (Table 3). Thus, *csdA* is not involved in the residual 2-thiocytidine, 6-*N*-dimethylallyl-2-methylthioadenosine synthesis.

It is possible that spontaneous mutations may have caused the increase in these thionucleosides with extended incubation. Strains CL100 and PK4331 were both diluted after extended incubation (40 and 43 h, respectively) and regrown to mid log phase ( $A_{600} = 0.5$ ). Thionucleoside analysis of tRNA showed no increase in 2-thiocytidine or 6-*N*-dimethylallyl-2-methylthioadenosine compared with the initially grown cultures harvested at the same growth level (Table 3). Thus, chromosomal mutations were not the cause of the increase in these thionucleoside levels.

**Attempts to complement the *iscS* phenotype with exogenous sulfide.** Inorganic sulfide can replace IscS/cysteine in 4-thiouridine synthesis in vitro. The  $K_m$  for sulfide is approximately 0.5 mM (C. T. Lauhon, unpublished data). To determine if intracellular sulfide could be the source for residual 2-thiocytidine and/or 6-*N*-dimethylallyl-2-methylthioadenosine synthesis, Strain CL100 was grown in rich medium supplemented with sulfide. Concentrations of 5 mM were tolerated by this strain; higher concentrations led to slow growth and eventual toxicity. HPLC analysis of digested tRNA isolated from strain CL100 grown in medium supplemented with sulfide (Fig. 8) showed a small amount of 4-thiouridine (1 to 2% of that in the parent strain). No increase in the other thionucleosides, including 2-thiocytidine, above those of the mutant grown in LB alone was observed. None of the previously documented slow-growth characteristics or auxotrophic requirements of the *iscS* mutant were complemented by exogenous sulfide up to 10 mM.

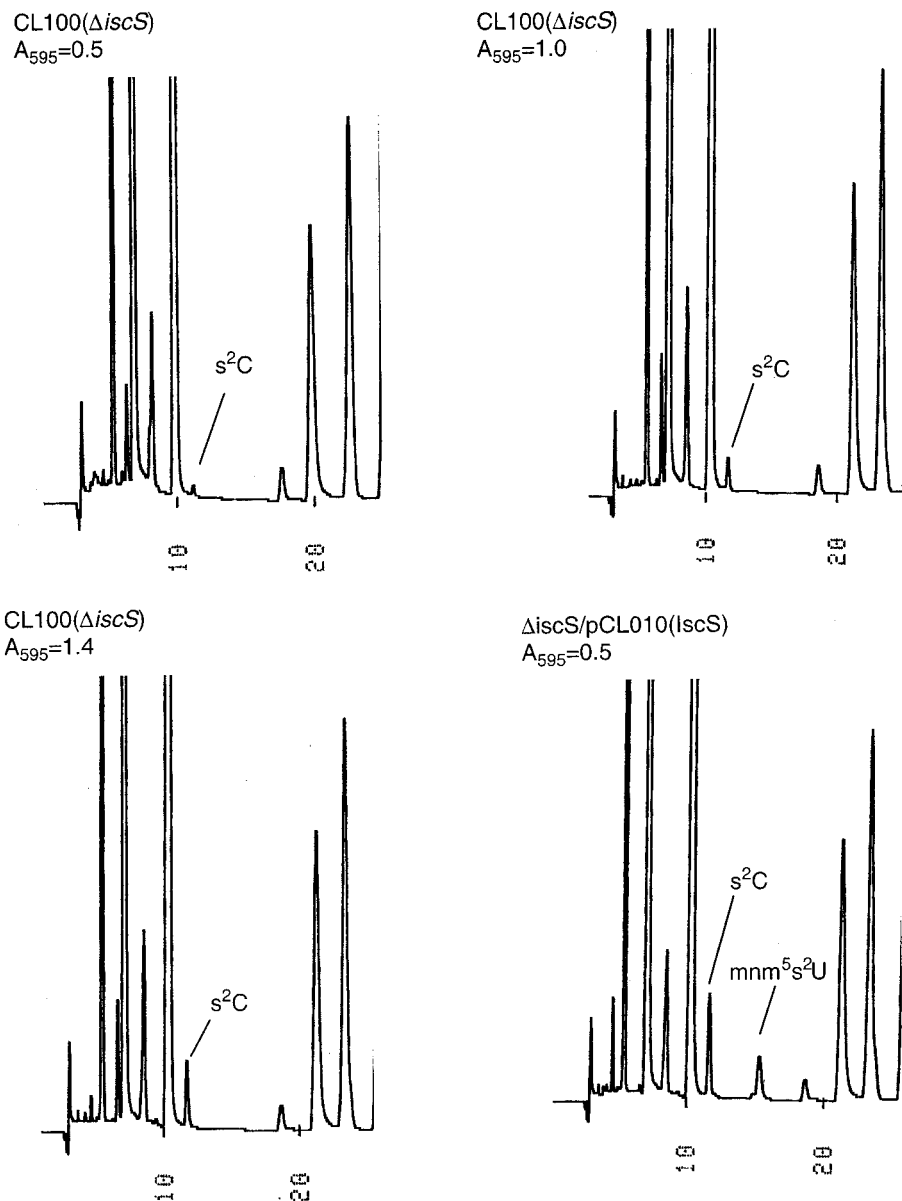


FIG. 6. Increase in 2-thiocytidine with growth (from upper left) of strain CL100 in LB medium. The  $A_{600}$  value is given next to each HPLC trace. The final trace, lower right, is of parent strain MC1061 and shows typical levels of 2-thiocytidine ( $s^2C$ ) and 5-methylaminomethyl-2-thiouridine ( $mnm^5s^2U$ ) at all growth phases.

## DISCUSSION

The present results show that *iscS* is the major cysteine desulfurase required for thionucleoside synthesis in *E. coli*. The *csdA csdB* double mutant showed no variation in thionucleoside levels, and when overexpressed, neither allele can rescue the defects in thionucleoside levels caused by deletion of *iscS*. We (17) and others (32, 33, 40) have established that *iscS* mutants are defective in a wide range of metabolic pathways involving sulfur. These include the biosynthesis of vitamins, such as thiamine and nicotinic acid, as well as the amino acids isoleucine, valine, and methionine. There are undoubtedly other defects as well, since the growth rate of *E. coli iscS* mutants is half that of the parent strains in rich medium.

Many of the metabolic defects are the result of the key role that *iscS* plays in the formation and function of proteins of the *isc* gene cluster that is necessary for Fe-S cluster biosynthesis (46). A direct interaction and persulfide transfer (34, 41) have been shown between *IscS* and the *isc* cluster protein *IscU*. However, this is not the only function of *iscS*. *IscS* is also required for 4-thiouridine synthesis, and recently, Mihara et al. reported that *iscS* is required for both sulfur and selenium insertion into 5-methylaminomethyl-2-thio(seleno)uridine [ $mnm^5s(se)^2U$ ] in *E. coli* (23). Xi et al. (44) showed evidence that *IscS* persulfide is involved in a novel reaction in the biosynthesis of thiazole.

Cysteine desulfurases are also required for sulfur transfer in

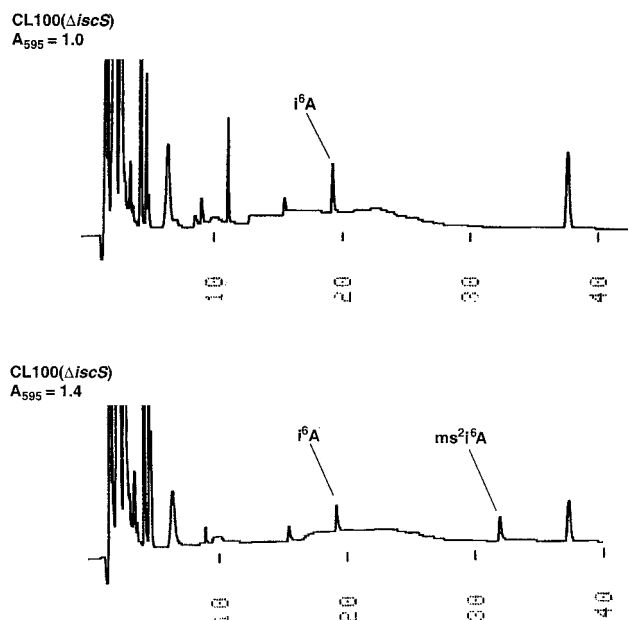


FIG. 7. Increase in 6-*N*-dimethylallyl-2-methylthioadenosine in strain CL100 with extended growth. The top trace is for tRNA isolated at late log phase, while the bottom trace is after extended incubation. There was an additional relative decrease in the amount of 6-*N*-dimethylallyl adenosine ( $i^6A$ ), which is the precursor to 6-*N*-dimethylallyl-2-methylthioadenosine. Parent strain MC1061 or CL100(DE3) with pCL010 showed only 6-*N*-dimethylallyl-2-methylthioadenosine at all growth levels examined (see Fig. 5 for comparison and for abbreviations).

the synthesis of the molybdopterin cofactor (18). These reactions do not involve Fe-S cluster chemistry. The purely “organic” sulfur transfer role of IscS (2) is distinct from that of *iscS* in *isc* cluster-dependent Fe-S biosynthesis and may be more prevalent than is currently known. Thus, IscS likely interacts with a number of other protein sulfur acceptors in addition to IscU and ThiI.

The similar growth dependence of 2-thiocytidine and 6-*N*-dimethylallyl-2-methylthioadenosine levels in the *iscS* mutant differentiates these thionucleosides from 4-thiouridine and 5-methylaminomethyl-2-thiouridine, which are almost certainly Fe-S cluster independent. The increase in levels of 6-*N*-dimethylallyl-2-methylthioadenosine with growth suggests that a residual level of Fe-S cluster biosynthesis exists in the *iscS* mutant. This is supported by the measurement of activities of Fe-S enzymes in *E. coli iscS* mutants (32, 33). A possible candidate for this auxiliary Fe-S synthesis is the *suf* operon. This operon contains the *iscS* paralog *csdB*, also termed *sufS* (29), as well as some homologs of other *isc* genes, and is arguably found in a wider range of organisms. It is clearly involved in Fe-S cluster synthesis, but the extent of its role is unclear.

Takahashi and Tokumoto (38) recently reported that *iscS csdB* (*sufS*) mutants of *E. coli* are not viable, and spontaneous mutations that increase expression of the *suf* operon (which contains *csdB*) can partially complement defects in *isc* operon mutants. To address the possibility of pseudorevertants, two independently isolated *iscS* mutant strains, CL100 and PK4331, with different genetic backgrounds were incubated for

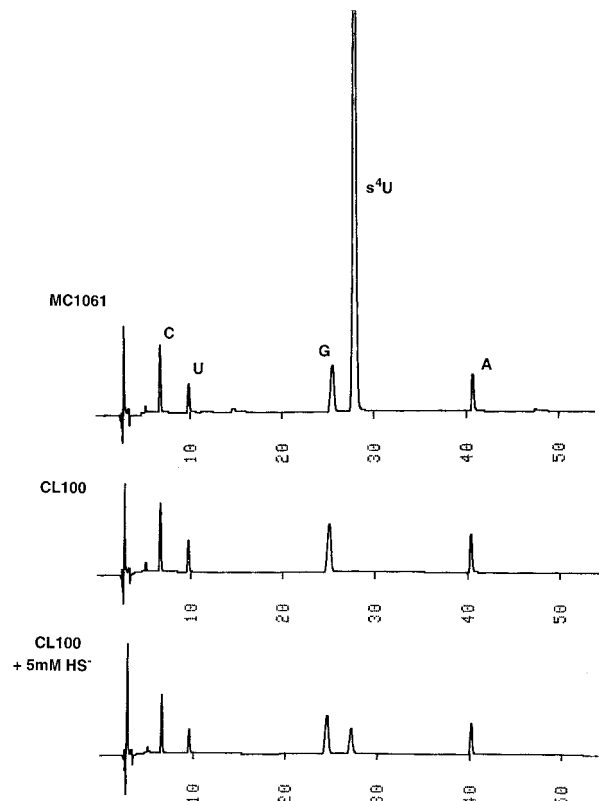


FIG. 8. HPLC profiles of 4-thiouridine ( $s^4U$ ) region at 330 nm of strain CL100 ( $\Delta iscS$ ) grown in rich medium supplemented with sulfide. (Top) Profile from parent strain MC1061, which shows typical levels of 4-thiouridine. (Middle) tRNA nucleoside profile from strain CL100 grown in LB only. (Bottom) Profile from CL100 grown in LB supplemented with 5 mM sodium sulfide ( $HS^-$ ). Residual peaks from the large amounts of C, U, and G are visible for comparison between the three profiles. A similar amount of digested tRNA was loaded for each run shown.

>36 h, diluted 200-fold into fresh medium, and harvested at mid-log phase for tRNA analysis. No differences in thionucleoside levels were observed from the original cultures harvested similarly. This argues against spontaneous mutations and in favor of an auxiliary Fe-S synthesis in the originally isolated

TABLE 3. Levels of modified nucleosides in *iscS* mutant strains relative to the parent strain as a function of growth<sup>a</sup>

Strain	Relative level				
	$s^2C$	$mnm^5s^2U$	$s^4U$	$i^6A$	$ms^2i^6A$
CL100, $A_{600} = 0.5$	0.01	ND <sup>b</sup>	ND	0.6	ND
CL100, $A_{600} = 1.0$	0.4	ND	ND	0.5	ND
CL100, $t = 36$ h	0.7	ND	ND	0.2	0.2
PK4331, $A_{600} = 0.5$	0.01	ND	ND	0.5	ND
PK4331, $t = 43$ h	0.9	ND	ND	0.1	0.1
PK5930, $A_{600} = 0.5$	0.01	ND	ND	0.5	ND
PK5930, $t = 43$ h	0.7	ND	ND	0.1	0.1

<sup>a</sup> 2-Thiocytidine is reported as the area ratio to  $\Psi$  and normalized to the levels in MC1061, which was invariant with growth. Levels of 6-*N*-dimethylallyl adenosine ( $i^6A$ ) and 6-*N*-dimethylallyl-2-methylthioadenosine ( $ms^2i^6A$ ) are reported directly as the area ratio to 6-*N*-threonylcarbamoyl adenosine ( $t^6A$ ). For other abbreviations, see Table 2, footnote a.

<sup>b</sup> ND, none detected.



*iscS* mutant strains. In addition, overexpression of *csdB* alone in strain CL100(DE3) did not affect thionucleoside levels; however, expression of the entire *suf* operon may be necessary to observe an increase in 2-thiocytidine and 6-*N*-dimethylallyl-2-methylthioadenosine levels.

Another possible source of sulfur for residual Fe-S synthesis in an *iscS* mutant is inorganic sulfide. Slow reconstitution of Fe-S enzymes *in vitro* has been demonstrated with soluble iron and sulfide under anaerobic conditions. Flint has reported the reconstitution of dihydroxy acid dehydratase as an assay for isolating the enzymes *O*-acetylserine sulfhydrylase and  $\beta$ -cystathionase in *E. coli*, which can mobilize sulfide from cysteine (10). Free sulfide is thought to be present at a low level in the cytoplasm of *E. coli* but may be sufficient to support very slow synthesis of Fe-S clusters for essential proteins. When strain CL100 was grown in rich medium supplemented with 5 mM sulfide, no difference in growth rate or auxotrophic requirements was observed. Thionucleoside analysis of tRNA showed that levels of 4-thiouridine were increased, which indicates that sulfide levels are likely to be elevated inside the cell. Since 4-thiouridine was observed under these conditions and no increase was observed in the rate of appearance or level of 2-thiocytidine or 6-*N*-dimethylallyl-2-methylthioadenosine, free sulfide is likely not the source for residual production of these thionucleosides.

A working hypothetical scheme for the role of *iscS* in thionucleoside biosynthesis may reflect the general role of *iscS* in sulfur metabolism. This scheme suggests a dual role for IscS as a sulfur source for both the *isc* Fe-S biosynthesis machinery and a more diverse non-Fe-S sulfurtransferase function. The non-*isc* sulfur transfer pathway has been shown definitively only with the 4-thiouridine and thiazole biosynthesis involving ThiI; however, it is likely that others will be found. MnmA, which has many conserved cysteines required for 2-thiouridine activity *in vivo* (C. T. Lauhon, unpublished data) is a likely IscS substrate for 2-thiouridine synthesis.

An unsolved question concerns the specificity of sulfur transfer. If a number of different protein substrates accept sulfane sulfur from IscS, there must be structural determinants for such specificity. Other putative protein substrates are currently being tested to provide a more complete explanation of the extensive role of IscS in sulfur metabolism.

#### ACKNOWLEDGMENTS

I thank Alison Bednar for preparation of the *csdB* mutant. I also thank Glenn Bjork for sharing unpublished results and helpful discussion and Patricia Kiley for strains PK4331 and PK5930.

This work was supported by National Institutes of Health grant GM57002 and a grant from the University of Wisconsin Graduate School.

#### REFERENCES

- Ashraf, S. S., E. Sochacka, R. Cain, R. Guenther, A. Malkiewicz, and P. F. Agris. 1999. Single atom modification (O $\rightarrow$ S) of tRNA confers ribosome binding. *RNA* **5**:188–194.
- Beinert, H. 2000. A tribute to sulfur. *Eur. J. Biochem.* **18**:5657–5664.
- Bjork, G. R. 1995. Genetic dissection of synthesis and function of modified nucleosides in bacterial transfer RNA. *Prog. Nucleic Acid Res. Mol. Biol.* **50**:263–338.
- Bjork, G. R., J. U. Ericson, C. E. Gustafsson, T. G. Hagervall, Y. H. Jonsson, and P. M. Wikstrom. 1987. Transfer RNA modification. *Annu. Rev. Biochem.* **56**:263–287.
- Blondel, M. O., and A. Favre. 1988. tRNAPhe and tRNAPro are the near-ultraviolet molecular targets triggering the growth delay effect. *Biochem. Biophys. Res. Commun.* **150**:979–986.
- Buck, M., M. Connick, and B. N. Ames. 1983. Complete analysis of tRNA-modified nucleosides by high-performance liquid chromatography: the 29 modified nucleosides of *Salmonella typhimurium* and *Escherichia coli* tRNA. *Anal. Biochem.* **129**:1–13.
- Elseviers, D., L. A. Petrullo, and P. Gallaagher. 1984. Novel *E. coli* mutants deficient in biosynthesis of 5-methylaminomethyl-2-thiouridine. *Nucleic Acids Res.* **12**:3521–3534.
- Esberg, B., H. C. E. Leung, H. C. T. Tsui, G. Bjork, and M. E. Winkler. 1999. Identification of the *miab* gene, involved in methylthiolation of isopentenylated A37 derivatives in the tRNA of *Salmonella typhimurium* and *Escherichia coli*. *J. Bacteriol.* **181**:7256–7265.
- Flint, D. H. 1996. *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.* **271**:16068–16074.
- Flint, D. H., J. F. Tuminello, and T. J. Miller. 1996. Studies on the synthesis of the Fe-S cluster of dihydroxy-acid dehydratase in *Escherichia coli* crude extract. Isolation of *O*-acetylserine sulfhydrylases A and B and beta-cystathionase based on their ability to mobilize sulfur from cysteine and to participate in Fe-S cluster synthesis. *J. Biol. Chem.* **271**:16053–16067.
- Gehrke, C. W., K. C. Kuo, R. A. McCune, and K. Gerhardt. 1982. Quantitative enzymatic hydrolysis of tRNAs. Reversed-phase high-performance liquid chromatography of tRNA nucleosides. *J. Chromatogr.* **230**:297–308.
- Griffiths, E., J. Humphreys, A. Leach, and L. Scanlon. 1978. Alterations in the tRNAs of *Escherichia coli* recovered from lethally infected animals. *Infect. Immun.* **22**:312–317.
- Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner. 1989. New method for generating deletions and gene replacements in *Escherichia coli*. *J. Bacteriol.* **171**:4617–4622.
- Kambampati, R., and C. T. Lauhon. 2000. IscS is a sulfurtransferase for the *in vitro* biosynthesis of 4-thiouridine in *Escherichia coli* tRNA. *Biochemistry* **38**:16561–16568.
- Kambampati, R., and C. T. Lauhon. 2000. 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.* **275**:10727–10730.
- Kramer, G. F., J. C. Baker, and B. N. Ames. 1988. Near-UV stress in *Salmonella typhimurium*: 4-thiouridine in tRNA, ppGpp, and ApppGpp as components of an adaptive response. *J. Bacteriol.* **170**:2344–2351.
- Lauhon, C. T., and R. Kambampati. 2000. The *iscS* gene in *Escherichia coli* is required for the biosynthesis of 4-thiouridine, thiamine, and NAD. *J. Biol. Chem.* **275**:20096–21003.
- Leimkuhler, S., and K. V. Rajagopalan. 2001. 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.* **276**:22024–22031.
- Li, J., M. Kogan, S. A. B. Knight, D. Pain, and A. Dancis. 1999. Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution. *J. Biol. Chem.* **274**:33025–33034.
- Link, A. J., D. Phillips, and G. M. Church. 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J. Bacteriol.* **179**:6228–6237.
- Madore, E., C. Florentz, R. Giege, S. Sekine, S. Yokoyama, and J. Lapointe. 1999. Effect of modified nucleotides on *Escherichia coli* tRNA<sup>Glu</sup> structure and on its aminoacylation by glutamyl-tRNA synthetase. Predominant and distinct roles of the mnm<sup>3</sup> and s<sup>2</sup> modifications of U34. *Eur. J. Biochem.* **266**:1128–1135.
- Marquet, A. 2001. Enzymology of carbon-sulfur bond formation. *Curr. Opin. Chem. Biol.* **5**:541–549.
- Mihara, H., S. Kato, G. M. Lacourciere, T. C. Stadtman, R. A. Kennedy, T. Kurihara, U. Tokumoto, Y. Takahashi, and N. Esaki. 2002. The *iscS* gene is essential for the biosynthesis of 2-selenouridine in tRNA and the selenocysteine-containing formate dehydrogenase H. *Proc. Natl. Acad. Sci. USA* **99**:6679–6683.
- Mihara, H., T. Kurihara, T. Yoshimura, K. Soda, and N. Esaki. 1997. Cysteine sulfinate desulfinate, an NIFS-like protein of *Escherichia coli* with selenocysteine lyase and cysteine desulfurase activities. Gene cloning, purification, and characterization of a novel pyridoxal enzyme. *J. Biol. Chem.* **272**:22417–22424.
- Mihara, H., M. Maeda, T. Fujii, T. Kurihara, Y. Hata, and N. Esaki. 1999. 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.* **274**:14768–14772.
- Mihara, H., T. Kurihara, T. Yoshimura, and N. Esaki. 2000. Kinetic and mutational studies of three NifS homologs from *Escherichia coli*: mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. *J. Biochem. (Tokyo)* **127**:559–567.
- Mueller, E. G., C. J. Buck, P. M. Palenchar, L. E. Barnhart, and J. L. Paulson. 1998. Identification of a gene involved in the generation of 4-thiouridine in tRNA. *Nucleic Acids Res.* **26**:2606–2610.
- Mueller, E. G., P. M. Palenchar, and C. J. Buck. 2001. The role of the

- cysteine residues of ThiI in the generation of 4-thiouridine in tRNA. *J. Biol. Chem.* **276**:33588–33595.
29. **Patzer, S. I., and K. Hantke.** 1999. SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in *Escherichia coli*. *J. Bacteriol.* **181**:3307–3309.
  30. **Pierrel, F., G. R. Bjork, M. Fontecave, and M. Atta.** 2002. Enzymatic modification of tRNAs: MiaB is an iron-sulfur protein. *J. Biol. Chem.* **277**:13367–13370.
  31. **Ryals, J., R.-Y. Hsu, M. N. Lipsett, and H. Bremer.** 1982. Isolation of single-site *Escherichia coli* mutants deficient in thiamine and 4-thiouridine syntheses: identification of a *nuvC* mutant. *J. Bacteriol.* **151**:899–904.
  32. **Schwartz, C. J., O. Djaman, J. A. Imlay, and P. J. Kiley.** 2000. The cysteine desulfurase IscS has a major role in in vivo Fe-S cluster formation in *Escherichia coli*. *J. Bacteriol.* **97**:9009–9014.
  33. **Skovran, E., and D. M. Downs.** 2000. Metabolic defects caused by mutations in the *isc* gene cluster in *Salmonella enterica* serovar Typhimurium: implications for thiamine synthesis. *J. Bacteriol.* **182**:3896–3903.
  34. **Smith, A. D., J. N. Agar, K. A. Johnson, J. Frazzton, I. J. Amster, D. R. Dean, and M. K. Johnson.** 2001. Sulfur transfer from IscS to IscU: the first step in iron-sulfur cluster biosynthesis. *J. Am. Chem. Soc.* **123**:11103–11104.
  35. **Sofia, H. J., G. Chen, B. G. Hetzler, J. F. Reyes-Spindola, and N. E. Miller.** 2001. Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization with new analysis and information visualization methods. *Nucleic Acids Res.* **29**:1097–1106.
  36. **Sullivan, M. A., J. F. Cannon, F. H. Webb, and R. Bock.** 1985. Antisuppressor mutation in *Escherichia coli* defective in biosynthesis of 5-methylaminomethyl-2-thiouridine. *J. Bacteriol.* **161**:368–376.
  37. **Sylvers, L. A., K. C. Rogers, M. Shimizu, E. Ohtsuka, and D. Soll.** 1993. A 2-thiouridine derivative in tRNA<sup>Glu</sup> is a positive determinant for aminoacylation by *Escherichia coli* glutamyl-tRNA synthetase. *Biochemistry* **32**:3836–3841.
  38. **Takahashi, Y., and U. Tokumoto.** 2002. A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. *J. Biol. Chem.* **277**:28380–28383.
  39. **Thomas, G., and A. Favre.** 1975. 4-Thiouridine as the target for near-ultraviolet light induced growth delay in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **66**:1454–1461.
  40. **Tokumoto, U., and Y. Takahashi.** 2001. Genetic analysis of the *isc* operon in *Escherichia coli* involved in the biogenesis of cellular iron-sulfur proteins. *J. Biochem. (Tokyo)* **130**:63–71.
  41. **Urbina, H. D., J. J. Silberg, K. G. Hoff, and L. Vickery.** 2001. Transfer of sulfur from IscS to IscU during Fe/S cluster assembly. *J. Biol. Chem.* **276**:44521–44526.
  42. **Urbonavicius, J., Q. Qian, J. M. Durand, T. G. Hagervall, and G. R. Bjork.** 2001. Improvement of reading frame maintenance is a common function for several tRNA modifications. *EMBO J.* **20**:4863–4873.
  43. **Webb, E., K. Class, and D. M. Downs.** 1997. Characterization of *thiI*, a new gene involved in thiazole biosynthesis in *Salmonella typhimurium*. *J. Bacteriol.* **179**:4399–4402.
  44. **Xi, J., Y. Ge, C. Kinsland, F. W. McLafferty, and T. P. Begley.** 2001. Biosynthesis of the thiazole moiety of thiamin in *Escherichia coli*: identification of an acyldisulfide-linked protein-protein conjugate that is functionally analogous to the ubiquitin/E1 complex. *Proc. Natl. Acad. Sci. USA* **98**:8513–8518.
  45. **Yokoyama, S., T. Watanabe, K. Muraio, H. Ishikura, Z. Yamaizumi, S. Nishimura, and T. Miyazawa.** 1985. Molecular mechanism of codon recognition by tRNA species with modified uridine in the first position of the anticodon. *Proc. Natl. Acad. Sci. USA* **82**:4905–4909.
  46. **Zheng, L., V. L. Cash, D. H. Flint, and D. R. Dean.** 1998. Assembly of iron-sulfur clusters. Identification of an *iscSUA-hscBA-fdx* gene cluster from *Azotobacter vinelandii*. *J. Biol. Chem.* **273**:13264–13272.
  47. **Zheng, L., R. H. White, V. L. Cash, R. F. Jack, and D. R. Dean.** 1993. Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. *Proc. Natl. Acad. Sci. USA* **90**:2754–2758.