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The *iscS* Gene in *Escherichia coli* Is Required for the Biosynthesis of 4-Thiouridine, Thiamin, and NAD*

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IscS, a cysteine desulfurase implicated in the repair of Fe-S clusters, was recently shown to act as a sulfurtransferase in the biosynthesis of 4-thiouridine (s^4U) in tRNA (Kambampati, R., and Lauhon, C. T. (1999) *Biochemistry* 38, 16561–16568). In frame deletion of the *iscS* gene in *Escherichia coli* results in a mutant strain that lacks s^4U in its tRNA. Assays of cell-free extracts isolated from the *iscS*$^-$ strain confirm the complete loss of tRNA sulfurtransferase activity. In addition to lacking s^4U, the *iscS*$^-$ strain requires thiamin and nicotinic acid for growth in minimal media. The thiamin requirement can be relieved by the addition of the thiamin precursor 5-hydroxyethyl-4-methylthiazole, indicating that *iscS* is required specifically for thiazole biosynthesis. The growth rate of the *iscS*$^-$ strain is half that of the parent strain in rich medium. When the *iscS*$^-$ strain is switched from rich to minimal medium containing thiamin and nicotinate, growth is preceded by a considerable lag period relative to the parent strain. Addition of isoleucine results in a significant reduction in the duration of this lag phase. To examine the thiazole requirement, we have reconstituted the *in vitro* biosynthesis of ThiS thiocarboxylate, the ultimate sulfur donor in thiazole biosynthesis, and we show that IscS mobilizes sulfur for transfer to the C-terminal carboxylate of ThiS. ThiI, a known factor involved in both thiazole and s^4U synthesis, stimulates this sulfur transfer step by 7-fold. Extracts from the *iscS*$^-$ strain show significantly reduced activity in the *in vitro* synthesis of ThiS thiocarboxylate. Transformation of the *iscS*$^-$ strain with an *iscS* expression plasmid complemented all of the observed phenotypic effects of the deletion mutant. Of the remaining two *nifS*-like genes in *E. coli*, neither can complement loss of *iscS* when each is overexpressed in the *iscS*$^-$ strain. Thus, IscS plays a significant and specific role at the top of a potentially broad sulfur transfer cascade that is required for the biosynthesis of thiamin, NAD, Fe-S clusters, and thionucleosides.

Cysteine has been shown to be the source of sulfur for the biosynthesis of a variety of cofactors such as biotin, lipoic acid, and thiamin (1), as well as metallosulfur clusters in proteins (2) and thionucleotides in tRNA (3). Despite identification of the source, the biochemical pathways for sulfur incorporation into these molecules remain elusive. A major advance in this area was the report by Dean and co-workers (4) that the NifS protein from *Azotobacter vinelandii* is a cysteine desulfurase that is required for the maintenance of the metallosulfur clusters in nitrogenase. A similar enzyme in *Escherichia coli*, termed IscS, has been shown to mobilize sulfur from cysteine for the repair of the [4Fe-4S] cluster in *apo*-dihydroxy-acid dehydratase (5). Zheng *et al.* (6) have reported that many organisms contain *iscS* homologs in a gene cluster thought to be involved in the maintenance of [Fe-S] proteins. Unsuccessful attempts to delete *iscS* in *A. vinelandii* suggest that it is required for viability in that organism (6).

We recently reported that in *E. coli*, IscS also functions as a sulfurtransferase for the *in vitro* biosynthesis of 4-thiouridine in tRNA (8). Lipsett and co-workers (9–11) reported that s^4U[1] was synthesized by two factors, A and C, that were the products of the *nuvA* and *nuvC* genes, respectively. Interestingly, the *nuvC* mutant, which lacked s^4U in its tRNA, also required thiamin for growth in minimal media (12). The *thiI* gene was recently identified as a requirement for both s^4U (13) and thiamin synthesis (16) in *E. coli* and for thiazole biosynthesis in *Salmonella typhimurium* (14).

Begley and co-workers (16) have elegantly shown that in *E. coli* the ultimate sulfur donor in thiamin biosynthesis is a C-terminal thiocarboxylate of the ThiS protein. Studies with a *thiI*$^-$ strain revealed that *thiI* is required for ThiS-COSH synthesis *in vivo* (16). The shared pathway for s^4U and thiamin biosynthesis suggested that ThiI was a sulfurtransferase. However, the factor that initiates the mobilization of sulfur from cysteine for ThiS-COSH synthesis remained unknown (7). Our finding that IscS was able to complete s^4U biosynthesis *in vitro* (outlined in Fig. 1) suggested that it might also function to initiate sulfur transfer in the synthesis of ThiS-COSH. In the present study we have reconstituted ThiS thiocarboxylate formation, and we demonstrate that IscS can indeed provide sulfur *in vitro* for the synthesis of ThiS-COSH.

E. coli contains three genes, including *iscS*, with sequence homology to *nifS*. Thus, it is possible that the *in vitro* activities observed for IscS are not indicative of its role *in vivo*. In order to find evidence for the *in vivo* relevance of *iscS* in s^4U, thiamin, and Fe-S cluster biosynthesis, we deleted the *iscS* gene in *E. coli*.

MATERIALS AND METHODS

Strains and Plasmids—E. coli strain MC1061 was obtained from the *E. coli* Genetic Stock Center. Electrocompetent *E. coli* MC1061 was obtained from Bio-Rad. Plasmid CL010 contains the *E. coli iscS* gene ligated into pET21c as described previously (8).

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[1] The abbreviations used are: s^4U, 4-thiouridine; ThiS-COSH, ThiS thiocarboxylate; S^0, sulfane sulfur; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; HPLC, high pressure liquid chromatography; bp, base pair; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

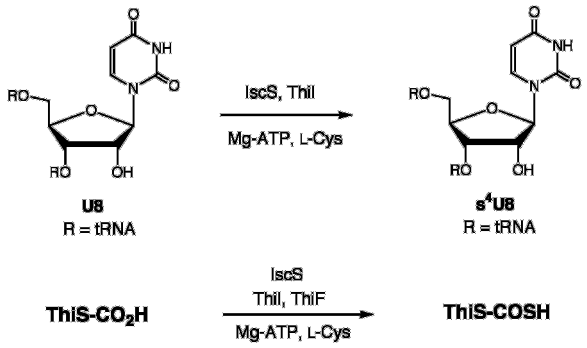

FIG. 1. **Factors required for the biosynthesis of s^4U and ThiS-COSH in *E. coli*.**

Media and Antibiotics—Rich media was LB. LB/suc refers to LB supplemented with 5% (w/v) sucrose. Minimal media were generally M63 supplemented with glycerol (0.2% v/v) and L-leucine (40 μg/ml), in addition to the following nutrients as required. Thiamin, nicotinic acid, THZ, and isoleucine were used at a concentration of 2, 50, 50, 40 μg/ml, respectively. Plasmid pKO3 and its derivatives were propagated in LB containing chloramphenicol (20 μg/ml).

General Methods—Plasmid DNA was isolated by the alkaline lysis method (17). Genomic DNA was isolated using a Qiagen Genomic Tip. Electroporation was performed with a BTX electroporator using a protocol provided by the manufacturer specific for *E. coli* MC1061. DE3 lysogens were prepared using a DE3 lysogenization kit (Novagen). Competent cells of the DE3 lysogens were prepared by the $CaCl_2$ method (18). ThiI was purified from an overproducing strain as described previously (8). Unmodified *E. coli* $tRNA^{Phe}$ was prepared by *in vitro* transcription of linearized plasmid pCF23 (8) and purified by electrophoresis on 10% polyacrylamide, 8 M urea gel using 90 mM Tris borate, 2 mM EDTA as running buffer.

Construction of Deletion Plasmid pID001—Plasmid pID001 contains 1 kb of *E. coli* genomic DNA (500 bp each of the 5′- and 3′-flanking region) surrounding the *iscS* gene. In place of the *iscS* coding sequence, the plasmid contains a 33-bp tag sequence developed by the laboratory of G. M. Church for the generation of deletion strains in *E. coli* (19). The deletion insert was constructed essentially according to the previously reported PCR method (19). Primers (see Table I) were chosen as suggested by computer analysis of the *E. coli* genome, with the exception of the N-terminal inside primer, Ni, which was changed to correspond to the correct start codon, which we and others (5, 8, 21) have identified by N-terminal sequencing of IscS. Two asymmetric PCRs using *E. coli* genomic DNA as a template were performed, one using the No and Ni primers and the other using the Co and Ci primers. The concentration of the outside primers was 0.6 μM, whereas the inside primers were 10-fold lower in order to generate complementary overhangs for the next reaction. After 30 cycles, 1 μl of each reaction was combined in a single new 100-μl PCR containing only the No and Co primers (0.6 μM each) and no additional template DNA. After 15 additional cycles, the desired band at 1.1 kb was purified on a 1% agarose gel followed by extraction with a Qiaquick column (Qiagen). The resulting fragment was digested with *Not*I and *Sal*I and, after gel purification, was ligated into the *Not*I-*Sal*I site of pKO3 and transformed by electroporation into electrocompetent *E. coli* MC1061.

Isolation of $iscS^-$ Strain—A combination of the methods described by Kushner and co-workers (22) and Church and co-workers (19) was used to facilitate isolation of mutant strains that grow very poorly in rich media. After electroporation of plasmid pID001 (Table I) into *E. coli* MC1061, the cells (60 μl) were diluted with 1 ml of SOC medium and allowed to recover with shaking at 30 °C for 1 h. A total of 900 μl of the cells were plated onto 5 LB/cam plates prewarmed to 43 °C, and 10 and 50 μl, respectively, were plated onto two LB/cam plates at 30 °C. Two cointegrate colonies were observed on the 43 °C plates, whereas many colonies appeared on the 30 °C plates. The integration frequency was estimated to be 10^{-5}. The two cointegrate colonies were propagated separately in LB/cam for 3 days, by diluting daily into fresh media. The final saturated cultures were serially diluted (10^{-6}) and plated onto LB/cam plates at 30 °C to isolate resolved cointegrates. Plasmid DNA was isolated from overnight cultures of a master plate of 20 clones, and 4 were found to have the wild type gene on the plasmid, indicating a possible gene replacement event. Two of these colonies were then picked separately into 3 ml of LB, serially diluted (10^{-4}) and plated onto LB/suc plates for plasmid release. A master plate of the LB/suc colonies was replica-plated onto an LB/cam plate to identify colonies that had lost plasmid and were chloramphenicol-sensitive (Cm^S). Several Cm^S colonies were then grown overnight in 3 ml of LB for isolation of genomic DNA. PCR analysis of the genomic DNA was performed using primers that flanked the *iscS* gene.

Measurement of Growth Rates—Growth rates of wild type and $iscS^-$ strains were measured as follows. A single colony of either the wild type or $iscS^-$ strains was grown in 3 ml of LB to saturation. A 200-fold dilution was then made into 100 ml of LB, and cell density was monitored by absorbance at 595 nm. Growth in minimal media was measured in the same manner as above. Growth measurements when switching from rich to minimal media were made by diluting saturated cultures of either the parent or $iscS^-$ strains grown in LB medium into minimal medium containing additives as indicated.

Enzyme Assays—tRNA sulfurtransferase activity for s^4U formation was measured as described previously (8). The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 5 μg of *in vitro* transcribed *E. coli* $tRNA^{Phe}$, 5 μg of ThiI, and 63 μg of cell-free extract from either parent or $iscS^-$ strain.

HPLC Analysis of s^4U in tRNA—tRNA was analyzed for s^4U by digestion with nuclease P1 and bacterial alkaline phosphatase essentially according to Gherke *et al.* (23). Reaction mixtures containing 0.1–0.5 mg/ml tRNA, 30 mM NaOAc, pH 5.3, 0.2 mM $ZnSO_4$, and 3 units of nuclease P1 were incubated at 37 °C for 2 h. To this mixture 1 M Tris-HCl, pH 7.5 (4 μl for a 50-μl reaction), was added followed by 3 units of bacterial alkaline phosphatase and incubated for ≥1 h. Approximately 10 μg of digested tRNA was loaded on a reverse phase HPLC column (Supelco LC-18S) and eluted with a linear gradient (60 min) of 0.5–20% methanol in 10 mM ammonium phosphate, pH 5.3. Peaks were detected at 330 nm, which is specific for the absorbance of s^4U.

Cloning of ThiF and ThiS—Begley and co-workers (16) have reported the overexpression of ThiFS on a single expression plasmid and purification of these proteins as a complex. We cloned ThiF and ThiS separately by PCR amplification from *E. coli* K12 genomic DNA. Primers for ThiF and ThiS are indicated in Table I. Each amplification reaction (100 μl) contained 2.5 units of *Pfu* polymerase (Stratagene), 0.2 mM each of dNTP, 0.5 μM each primer, and 1 μg of genomic DNA. After 30 cycles of amplification, the desired fragment was purified on a 1% agarose gel and extracted using a Qiaquick column (Qiagen). The purified DNA was then digested, gel-purified, and ligated into similarly digested and dephosphorylated pET 21c (Novagen). Ligation mixtures were transformed into NovaBlue cells for the production of supercoiled plasmid for screening by PCR and/or sequencing. Plasmids having the correct insert were transformed into BL21(DE3) cells for protein expression. Cells were grown at 37 °C to an A_{600} of 0.6, and IPTG was added to 0.3 mM. After 3 h, the cells were harvested by centrifugation and lysed with lysozyme (0.3 mg/ml) in lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5% glycerol, 1 mM dithiothreitol, 1 mM PMSF) at 0 °C for 1 h, followed by treatment with DNase I (1 μg/g cells) for 30 min. Centrifugation at 30,000 × *g* for 30 min gave the S-30 supernatant.

Purification of Overexpressed ThiF—ThiF S-30 supernatant (27 mg) was applied to a DEAE-Sephacel column (15-ml bed volume) equilibrated with 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 14 mM 2-mercaptoethanol, 1 mM PMSF, 10% glycerol. The column was washed with 30 ml of equilibration buffer, and the bound protein was eluted with a linear gradient of 50–500 mM KCl in 100 ml of equilibration buffer. Aliquots of the fractions were electrophoresed to determine ThiF peak. DEAE eluate (12.9 mg) was purified by gel filtration on Sephadex G-50 (225 ml bed volume) equilibrated with 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 14 mM 2-mercaptoethanol, 10% glycerol, 1 mM PMSF. Pure ThiF fractions were pooled and stored at −80 °C until use.

Purification of Overexpressed ThiS —ThiS S-30 supernatant (23.4 mg) was applied to a DEAE-Sephacel column and eluted as described above. The DEAE eluate (20.8 mg) was purified by gel filtration on Sephadex G-50 as described above. Pure ThiS fractions were pooled and concentrated using a Centricon (YM-3) centrifugal concentrator (Millipore) and stored at −80 °C.

In Vitro Reconstitution of ThiS Thiocarboxylation—ThiS (176 pmol), ThiI (36 pmol), IscS (22 pmol), and ThiF (24 pmol) were incubated together as indicated in the figure legends. Reactions were carried in 50 μl of 50 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$ 50 mM KCl, 1 mM PMSF, 1 mM dithiothreitol, 5% glycerol, 20 μM pyridoxal phosphate, 10 μM [^{35}S]cysteine (4000 cpm/pmol) either in the presence or absence of 1 mM ATP. Incubations were for 10 min at 37 °C. 0.25 volume of 5× SDS sample buffer minus 2-mercaptoethanol was added to the assay mixtures, heated at 70 °C for 2–5 min, and subjected to electrophoresis on SDS-15% polyacrylamide gels. Proteins were visualized by Coomassie staining followed by phosphorimage analysis (Molecular Dynamics).

TABLE I
Strains, plasmids, and primers used in this work
For primers, restriction sites are underlined, and the 33-bp tag sequence is shown in bold (see "Materials and Methods").

Strains	
MC1061	*F⁻araD139 Δ(ara leu)7696 Δ(lacY74) galU galK hsdr hsdM⁺ strA*
CL100 (iscS⁻)	*F⁻araD139 Δ(ara leu)7696 Δ(lacY74) galU galK hsdr hsdM⁺ ΔiscS strA*
Plasmids	
pKO3	Deletion plasmid, from Link *et al.* (19)
pID001	pKO3 containing iscS deletion construct
pCL010	pET21c derivative containing wt *E. coli* iscS; Kambampati and Lauhon (8)
Primers	
iscS-No	5′-AAGGAAAAAAGCGGCCGCACCGTTGGCTGATATTTCCGA
iscS-Ni	5′-**CACGCAATAACCTTCACACTCCAAATTTATAAC**CACTCAATGCAAGGAATCAGGCT
iscS-Ci	5′-**GTTATAAATTTGGAGTGTGAAGGTTATTGCGTG**TAATCGGTATCGGAATCAGGAGAATTTATAATGG
iscS-Co	5′-CGCACGCATGTCGACACCCTTTACCGCGGTTAGC
ThiF-5′	5′-TTGCAGGAGTTGCATATGAATGACCGTGACTTTATGCGT
ThiF-3′	5′-CACTGCATCGCTAAGCTTTTAAACAGGATCTGCATTGCT
ThiS-5′	5′-CGG TATGCGGAGGAACATATGCAGATCCTGTTTAACGAT
ThiS-3′	5′-TCCGCAATACGTAAGCTTTCAACCCCCTGCAATAACCTG

Mass Spectrometry—The *in vitro* ThiS thiocarboxylation assay mixtures were prepared exactly as above except unlabeled cysteine was used. After incubation, samples were loaded onto a liquid chromatography/mass spectrometry system. HPLC was performed on Vydac C18 column. The eluents were solvent A, 0.5% trifluoroacetic acid in water, and solvent B, 95% CH_3CN in 0.5% trifluoroacetic acid. A linear gradient from 1 to 99% solvent B (flow rate 20 μl/min) was used for elution. The mass spectrometer (PE Sciex API 365) equipped with an ion spray source was used for mass detection. Protein masses were deconvoluted using the Biospec reconstruct program. The error in the determined mass is ±1 per 5000 atomic mass units.

RESULTS

Construction of the iscS⁻ Strain—We used a method for the precise in frame deletion of *E. coli* genes that was originally developed by Kushner and co-workers (22) and recently modified by Church and co-workers (19). The deletion insert was constructed using a two-step PCR method, which replaces the coding sequence of *iscS* with a 33-bp sequence (Table I) that codes for an innocuous peptide (19). This in frame insert is designed to prevent polar effects on downstream genes within the same operon. This concern is applicable to *iscS* since it is known to be located in a multicistronic operon (6). The *iscS* deletion construct was ligated into plasmid pKO3 (19), which contains a temperature-sensitive origin of replication that allows the selection of cells with plasmid integrated into the genome (cointegrates) at the nonpermissible temperature (43 °C) in the presence of chloramphenicol. Growth of isolated cointegrates at the permissible temperature (30 °C) in the presence of antibiotic gives resolved cointegrates, in which the plasmid has been excised from the genome. Plasmid pKO3 also contains the *Bacillus subtilis sacB* gene for levansucrase. This gene is deleterious for growth in *E. coli* in the presence of high sucrose concentrations. Thus, it is possible to select for cells that have both resolved and released the plasmid from their genomic DNA by plating cointegrates on LB/suc plates at 30 °C. Replica plating from the LB/suc plate onto LB/cam plates will identify those colonies that grow in the presence of sucrose, but are chloramphenicol-sensitive, and have thus released the plasmid.

Screening potential deletion mutants by colony PCR can identify those cells that contain the desired deletion in the genomic DNA. However, we have found that deletion strains that grow poorly in LB/suc relative to the parent strain will be selected against at this stage and require the screening of many colonies before the desired mutant is found. This was true for the *iscS⁻* strain, which grows relatively slowly in rich medium. We found it more convenient to resolve the cointegrates using Kushner's original procedure by propagation at 30 °C for several days in LB/cam and then plating and screening the plasmid DNA of individual colonies for the presence of the wild type gene. Such cells should have the desired deletion in their genomic DNA because of the double crossover event. Dilution and plating these candidates onto LB/suc then gives a large proportion (>50%) of the desired deletion mutants free of plasmid DNA. For *iscS⁻* strains, such colonies required an additional 12–16 h to appear on the LB/suc plates. As shown in Fig. 2, we verified the gene deletion by PCR analysis of genomic DNA with primers flanking the *iscS* gene. *Lane 2* shows the expected 2.2-kb PCR product of the parent strain MC1061 genomic DNA, and *lane 3* shows the expected smaller 1.1-kb product for the *iscS* deletion strain (CL100). In addition, complementation with wild type *iscS* (see below) gave a phenotype that was indistinguishable from the parent strain.

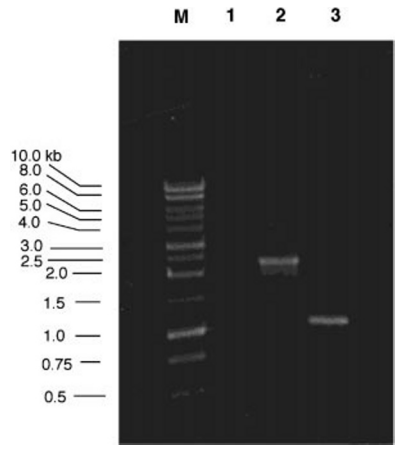

FIG. 2. **PCR analysis of genomic DNA in parent strain MC1061 and *iscS* deletion strain CL100.** *Lane M,* kilobase molecular weight markers; *lane 1,* no template (negative control); *lane 2,* MC1061 DNA with No and Co primers; *lane 3*, *iscS⁻* DNA with No and Co primers.

Growth Analysis of iscS⁻ Strain CL100—Fig. 3*A* shows the growth of the *iscS⁻* and *E. coli* parent strains in LB medium at 37 °C. The *iscS⁻* strain was significantly growth-impaired in rich medium. The doubling time of the i*scS⁻* strain is 55 min, which is 2-fold greater than that of the parent strain (27 min). The *iscS⁻* strain did not grow in M9/glucose or M63/glycerol minimal media supplemented with leucine (parent strain MC1061 is a leucine auxotroph). Because of earlier reports (12–14) on the relationship between s^4U and thiamin biosynthesis and our recent work, we suspected that thiamin was also necessary for growth. However, addition of thiamin to the minimal medium was insufficient for growth. Only after combined addition of nicotinic acid and thiamin was growth observed.

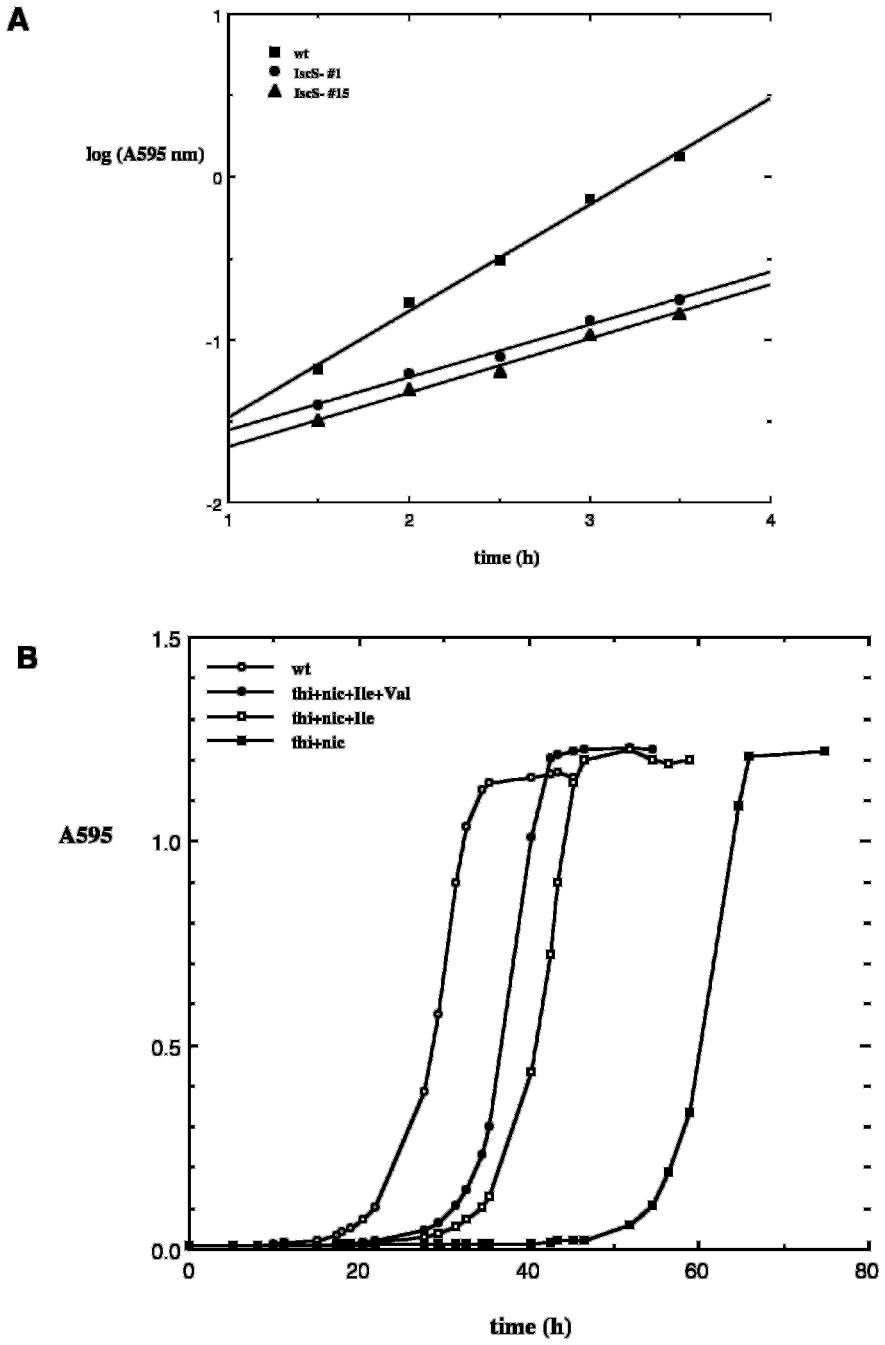

FIG. 3. **Relative growth of parent (MC1061) and *iscS*$^-$ strains.** *A*, growth of MC1061 and two *iscS*$^-$ strains, CL100 and CL115, in LB at 37 °C. *B*, growth of parent and deletion strains after dilution of saturated LB culture into M63/glycerol/leucine with the following additives: ○, MC1061 without additives; ■, CL100 with thi and nic;, □, CL100 with thi, nic, and Ile; ●, CL100 of MC1061 with thi, nic, Ile, and Val.

A significant lag phase relative to the parent strain was observed when the *iscS*$^-$ strain was switched from rich to minimal medium containing thiamin and nicotinate. Flint (5) has reported that IscS can repair the Fe-S clusters of apodihydroxy-acid dehydratase, an enzyme required for the biosynthesis of isoleucine and valine. Therefore, we measured the duration of the lag phase in the presence of these amino acids. Fig. 3*B* shows that the lag time relative to the parent strain is substantially reduced in the presence of isoleucine and is reduced further in the presence of both isoleucine and valine. This effect suggests that an enzyme involved in isoleucine/valine biosynthesis, possibly dihydroxy-acid dehydratase, is defective in the *iscS*$^-$ strain. No other amino acid had any observable effect. Interestingly, the exponential rates of growth are the same for both parent and mutant strains under all minimal medium conditions tested; however, it should be noted that this is a much slower rate of growth than in rich medium.

Complementation of the DE3 Lysogen CL100(DE3)—Transformation of the *iscS*$^-$ DE3 lysogen CL100(D3) with a pET-derived IscS expression plasmid, pCL010 (see Table I), resulted in complementation of all of the observed phenotypic effects of *iscS* deletion. The growth rate of CL100(DE3)/pCL010 was equal to the parent MC1061 in rich medium in the presence or absence of IPTG, indicating that the uninduced expression of *iscS* in the lysogen is significant. SDS gel analysis of cell extracts from the *iscS*$^-$ strain grown in the presence of IPTG shows a large band at the correct relative mass of IscS (data not shown). CL100 was also fully complemented by a derivative of pKO3 that contains the *E. coli iscS* gene with the same flanking 500-bp regions as in the deletion construct.

The iscS Gene Is Required for s^4U Biosynthesis in E. coli—Extracts from the parent and *iscS*$^-$ strains were assayed for tRNA sulfurtransferase activity. The *iscS*$^-$ extract contained significantly reduced activity (2.6% of parent strain extract).

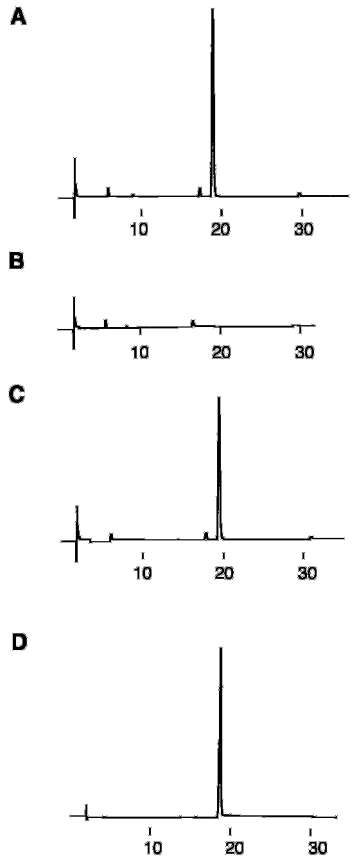

FIG. 4. **HPLC analysis of the tRNA isolated from the *iscS*$^-$ mutant and the *iscS*$^-$ mutant complemented with IscS.** *A–D* represent chromatograms of tRNA digests from *E. coli* parent strain (MC1061), *iscS*$^-$ mutant, *iscS*$^-$ mutant transformed with pCL010 (wild type *iscS*), and parent strain + standard s^4U, respectively.

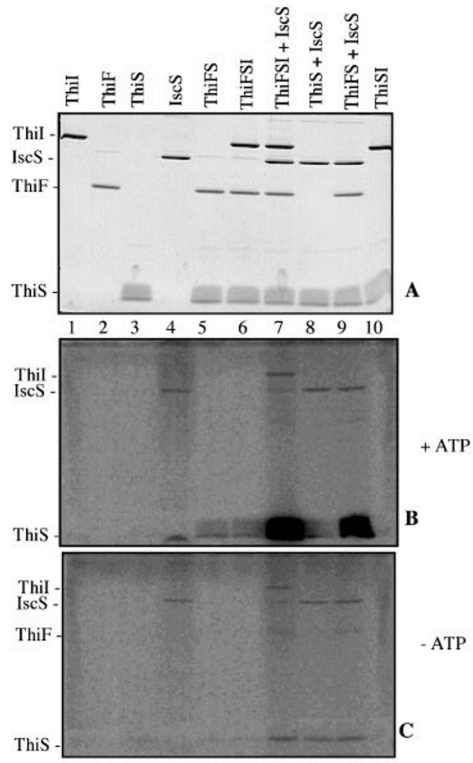

FIG. 5. ***In vitro* thiocarboxylation of ThiS.** Reactions were carried as described under "Materials and Methods." *Lanes 1–4*, ThiI, ThiF, ThiS, and IscS respectively; *lane 5*, ThiFS; *lane 6*, ThiFSI; *lane 7*, ThiFSI + IscS; *lane 8*, ThiS + IscS, *lane 9*, ThiFS + IscS; *lane 10*, ThiSI. *A* is Coomassie-stained gel. *B* and *C* show the phosphorimage scans of reactions performed in the presence and absence of ATP, respectively.

tRNA isolated from the *iscS*$^-$ strain grown in LB was analyzed for the presence of s^4U. The UV-visible spectra of tRNA from *E. coli* MC1061 (parent strain) shows a characteristic peak due to absorbance of s^4U at 330–340 nm, whereas for the CL100 tRNA there is no evidence of a peak in this region. Complementation with pCL010, which contains wild type *iscS*, restores the absorbance in this region of the tRNA spectrum (data not shown). To confirm further the presence or absence of s^4U, unfractionated tRNA isolated from each strain was digested to nucleosides as described (23). HPLC analysis of the tRNA digests (Fig. 4) shows clearly that the *iscS*$^-$ strain is completely devoid of s^4U (Fig. 4*B*). As predicted, tRNA from the complemented strain CL100(DE3)/pCL010 shows a peak for s^4U in the chromatogram that is similar in magnitude to that of the parent strain (Fig. 4*C*). These results show conclusively that *iscS* is required for the biosynthesis of s^4U *in vivo*.

Purification of Overexpressed ThiF and ThiS—Overexpressed proteins were purified as described under "Materials and Methods." ThiS, a small protein of 7.3 kDa, stains as a diffuse band on 15% SDS-polyacrylamide gels (Fig. 5*A, lane 3*). Although we did not determined the nucleotide sequence of plasmids, the molecular masses of purified proteins as determined by mass spectroscopy were essentially identical to their predicted values. Unlike the report by Begley and co-workers (16), we have not detected complex formation between ThiF and ThiS as analyzed by native PAGE (data not shown).

IscS Mobilizes Sulfur from Cysteine for ThiS-COSH Synthesis in Vitro—Begley and co-workers (16) have shown that the ultimate sulfur donor in the biosynthesis of thiazole is ThiS-COSH. These authors reconstituted ThiS-COSH biosynthesis using ThiFSI, Mg-ATP, and [^{35}S]cysteine and an uncharacterized factor in *E. coli* crude extract (24). We attempted to reconstitute the *in vitro* reaction by excluding cell extract and adding purified IscS to the above system. Fig. 5 shows the SDS-PAGE and PhosphorImager analysis of reactions designed to identify the requirements for ThiS-COSH formation *in vitro*. Addition of IscS to ThiFS results in the transfer of ^{35}S from [^{35}S]cysteine to ThiS (Fig. 5*B, lane 9*). In the absence of ATP, this sulfur transfer was reduced by more than 99% (Fig. 5*C, lane 9*). This is in agreement with the report that ThiF requires ATP for adenylation of ThiS (16). Interestingly, the presence of ThiI is not required in our assay, although ThiI is found to stimulate ThiS-COSH production 7-fold (Fig. 5*B, lane 7*). Begley and co-workers also reported that, in contrast to the *in vivo* production of ThiS-COSH, ThiI is not required for the *in vitro* reaction (24), but its addition further enhances its rate. We also observed that both IscS and ThiI are labeled with ^{35}S during the *in vitro* synthesis of ThiS-COSH (Fig. 5*B*, *lanes 4, 7,* and *9*). Absence of label on ThiI alone (Fig. 5*B, lane 1*) indicates that IscS mobilizes ^{35}S from [^{35}S]cysteine and transfers it to ThiI during ThiS-COSH formation (Fig. 5*B, lane 7*). Quantitation of ^{35}S-labeled bands (Fig. 5*B*) revealed that addition of ThiI to the *in vitro* reaction mixture results in the accelerated loss of ^{35}S-label from IscS (*lane 7*) by 3-fold (compare *lane 7* with *lane 9*). This indicates that ThiI stimulates reduction of IscS persulfide. Similarly, we have previously reported (15) that addition of ThiI increases the cysteine desulfurase activity of IscS by 2-fold.

Further evidence that IscS initiates sulfur transfer for ThiS-COSH synthesis is provided by liquid chromatography/mass

[2] T. P. Begley, Xi, J., Kinsland, C., Taylor, S., and Mclafferty, F., unpublished data.

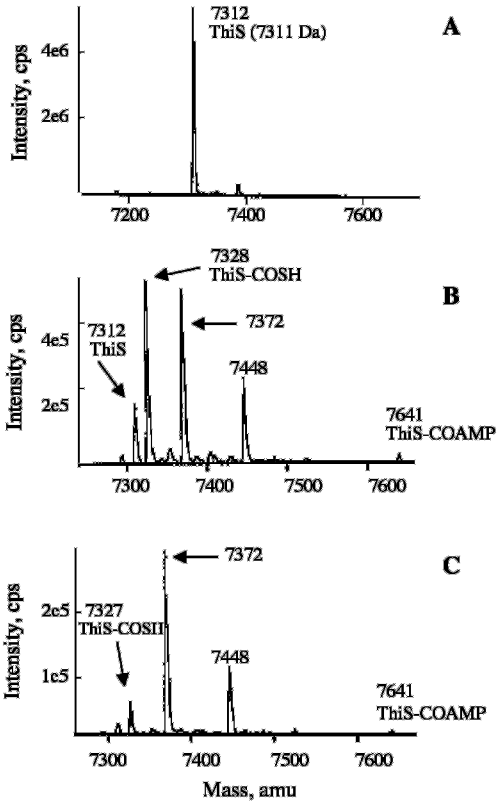

FIG. 6. **Characterization of ThiS-COSH by mass spectrometry.** ThiFSI, IscS, Mg-ATP, and cysteine were incubated together in buffer and analyzed by liquid chromatography/mass spectrometry as described under "Materials and Methods." Only the mass region of ThiS is shown here. *A*, mass spectrum of ThiS alone. *B* and *C* show the mass spectrum of ThiS incubated with ATP and cysteine in the presence and absence of ThiI, respectively.

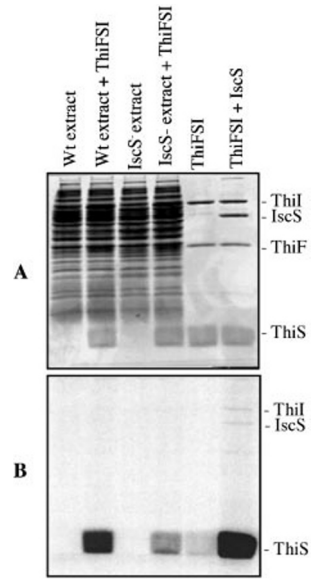

FIG. 7. **Effect of *iscS* deletion on the *in vitro* thiocarboxylation of ThiS.** S-30 supernatant (23.2 μg) from either wild type or *iscS*$^-$ mutant was incubated with purified ThiFSI and [^{35}S]cysteine and subjected to SDS-PAGE (15% polyacrylamide) and PhosphorImager analysis as described under "Materials and Methods." *A* and *B* represent Coomassie-stained gel and its phosphorimage, respectively. *Wt,* wild type.

spectrometry analysis. Fig. 6*A* shows unreacted ThiS with observed molecular mass of 7312 Da, which is within instrument error (±0.02%) of the calculated mass of 7311 Da (16). Fig. 6*B* shows the molecular mass of ThiS after reaction with ThiFI, IscS, and the substrates Mg-ATP and cysteine. A relatively large peak with mass of 7328 Da was observed, which is consistent with the predicted mass for ThiS-COSH. However, additional peaks with mass of 7372 Da and 7448 Da were also observed. Fig. 6*C* shows the same reaction in the absence of ThiI. Again a peak consistent with ThiS-COSH (7327) is observed which is smaller than the ThiS-COSH peak in Fig. 6*B*. Peaks with mass 7372 Da and 7448 Da also decreased relative to the reaction containing ThiI (Fig. 6*B*). Repeating the complete reaction in the absence of cysteine results in the observation of only the peaks with mass 7372 Da and 7448 Da (data not shown). This suggests that these products are formed in competition with ThiS-COSH formation and are not derived by reaction with cysteine. At present, we have not identified these larger mass peaks. However, the observed cysteine-dependent peak with mass 7328 Da (Fig. 6*B*) and the transfer of ^{35}S from [^{35}S]L-cysteine to ThiS shown in the SDS gel analysis (Fig. 5*B*) provide substantial evidence that ThiS-COSH formation is dependent on IscS.

Extracts of the parent and *iscS*$^-$ *E. coli* strains were analyzed for the ability to support ThiS-COSH formation in the presence of all the necessary factors except IscS (Fig. 7). As shown in Fig. 7*B, 2nd lane*, the parent strain extract catalyzed efficient ^{35}S transfer from [^{35}S]cysteine to ThiS. The *iscS*$^-$ strain showed significantly reduced sulfurtransferase activity (Fig. 7*B, 4th lane*). Quantitation of the ^{35}S-labeled ThiS-COSH band formed by the *iscS*$^-$ cell extract gave a value of 16% that of wild type.

DISCUSSION

The cysteine desulfurases of the NifS family are important candidates for sulfur mobilizers and distributors in the cell. IscS, an NifS homolog, was initially isolated from *E. coli* and, like NifS, was shown to convert cysteine into alanine and sulfane sulfur (S^0) in the form of a cysteine persulfide in its active site (5). Since then, *nifS*-like genes have been found in most organisms. Unlike prokaryotes, eukaryotes appear to contain only one *nifS* gene, which in many organisms is highly homologous to *iscS*. For example, the *NFS1* gene of *Saccharomyces cerevisiae* has 56% identity to *E. coli iscS* at the amino acid level, excluding its N-terminal signal sequence. Both the yeast and human forms are directed at least in part to the mitochondria (27, 28), which is the location of many Fe-S cluster-containing enzymes. *NFS1* in yeast is required for viability and was recently shown to be required for the *in vivo* activity of many Fe-S proteins and for the regulation of iron transport (29). Our recent finding that IscS can provide sulfur for s^4U biosynthesis without binding to tRNA (8, 15) prompted us to consider it for a more generalized role in sulfur metabolism. Since it has been previously shown that *thiI* is required for the biosynthesis of both s^4U and thiazole (13, 14, 16), we proposed that *iscS* might also be required for thiazole biosynthesis. By using an in frame gene deletion method, we have inactivated the *iscS* gene within the *orf1-orf2-iscSUA-hscBA-fdx* (or *isc*) operon and confirm that *iscS* is required for both s^4U and thiazole biosynthesis *in vivo*.

To elucidate the biochemical step responsible for the thiamin deficiency, we developed an *in vitro* assay for ThiS-COSH biosynthesis, based on the transfer of ^{35}S from [^{35}S]cysteine to ThiS. The minimal requirements for activity are ThiF, IscS, Mg-ATP, and L-cysteine. Although ThiI is not required in our assay, we find that it stimulates the formation of ThiS-COSH, an effect that must be necessary for sufficient production of ThiS-COSH *in vivo*. We have recently shown evidence for the sequential transfer of S^0 from IscS to ThiI and then to tRNA during the biosynthesis of s^4U (15). Results shown in this paper suggest that a similar pathway is likely in the biosynthesis of ThiS-COSH. However, there are significant differences be-

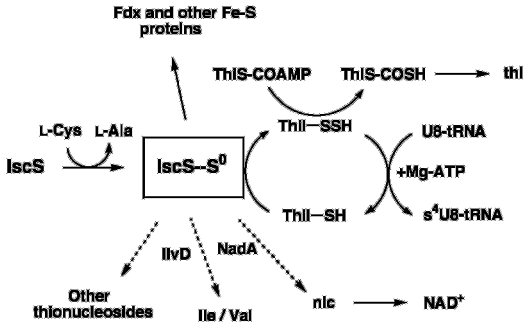

FIG. 8. **Overview of the mobilization and scope of distribution of sulfur by *E. coli* IscS.** The *dashed arrows* indicate interactions that are proposed or for which evidence is found *in vitro*.

tween the two reactions. ThiS-COSH synthesis requires adenylation by ThiF (16), and ThiI appears to act solely as a sulfurtransferase. Alternatively, in s^4U synthesis, ThiI accepts sulfur from IscS and also likely activates the uridine on the tRNA using Mg-ATP and transfers the sulfur to give s^4U. We have found no evidence for the stimulation of s^4U biosynthesis by ThiF nor have we observed transfer of ^{35}S to ThiF during ThiS-COSH synthesis. It is possible that a sulfur-modified ThiF is a metastable intermediate under the assay conditions and is merely not observed; however, the results thus far suggest that ThiI is the ultimate sulfurtransferase for both s^4U and ThiS-COSH synthesis.

For the two pathways (Fig. 8), the initial step is the mobilization of sulfur from L-cysteine by IscS, resulting in the formation of an IscS-derived cysteine persulfide (IscS-SSH). This S^0 is transferred from IscS-SSH to ThiI to give a putative ThiI persulfide (ThiI-SSH). Sulfur is then mobilized from ThiI-SSH and subsequently transferred to the activated uridine or ThiS adenylate (ThiS-COAMP) to give the product. We have found that IscS/cysteine can be replaced by millimolar concentrations of inorganic sulfide (data not shown). This suggests the possibility that nascent sulfide is produced in the ThiI-active site for addition to an enzyme-bound activated intermediate. We have previously shown that the thiol-specific alkylating agent 5-((2-iodoacetamido)ethyl)-1-aminonapthalene sulfonic acid can abolish the tRNA sulfurtransferase activity of ThiI (15). Thus, one or more of the cysteine residues in ThiI is required for s^4U synthesis, which is consistent with the mechanism outlined in Fig. 8. Flint (5) initially proposed that IscS may donate S^0 to a variety of protein acceptors for the synthesis of Fe-S clusters. It now appears that IscS provides S^0 for the incorporation of sulfur into a variety of substrates, in addition to Fe-S clusters.

Although the *iscS*$^-$ strain shows significantly impaired growth relative to the parent strain in rich medium, we have found that the two strains have identical exponential growth rates in minimal medium. This is likely due to the much longer doubling time (180 min) observed for each strain in either M9/glucose or M63/glycerol medium supplemented with leucine. It is likely that the activity of the enzymes affected by the *iscS* deletion is sufficient for the lower growth rate but becomes rate-limiting during faster growth in rich medium. A similar effect was recently observed for an *E. coli tufA*$^-$ strain (20).

The growth delay observed when the *iscS*$^-$ strain was switched from rich to minimal medium was largely alleviated by the addition of isoleucine and, to a lesser extent, valine. From earlier published work (5), we suspect that a partial explanation for the growth delay is a deficiency in the formation of Fe-S clusters in the Ile/Val pathway enzyme dihydroxy-acid dehydratase. We are currently attempting to confirm this experimentally. Since the growth delay is not completely alleviated with Ile/Val, there are likely other pathways affected. A gene expression analysis of the *isc* gene cluster also suggests that *iscS* is involved in the synthesis of a wide variety of Fe-S proteins (39). In addition, it has been recently reported that inactivation of *iscS* affected Fe-S cluster assembly in ferredoxins (36).

The *iscS*$^-$ strain also requires nicotinic acid for growth in minimal medium. This requirement has precedence, since in *B. subtilis*, an *nifS* gene is required for the biosynthesis of NAD (30). The proposed target of the nicotinate deficiency is quinolinate synthetase (NadA), one component of a system that condenses L-aspartate and dihydroxyacetone phosphate to give quinolinate (30). Interestingly, this enzyme and dihydroxy-acid dehydratase are both Fe-S enzymes known to be inactivated by hyperbaric oxygen (31, 32).

E. coli contains three genes with homology to *nifS*. In addition to *iscS*, these are cysteine-sulfinate desulfinase (33) and *csdB* (34). The *csdB* gene is identical to *sufS*, which was found to be required for the stable maintenance of the [2Fe-2S]-containing Fhuf protein in *E. coli* (35). Each of the three proteins has been purified and shown to catalyze the desulfuration of cysteine and the deselenation of selenocysteine with varying efficiency. CsdB shows significant selectivity for selenocysteine, and the recently reported crystal structure (36) reveals fundamental differences in structure compared with that predicted for IscS. Cysteine-sulfinate desulfinase and CsdB (SufS) have been grouped separately from IscS based on amino acid sequence homology (33). We have found no obvious phenotype for deletion of cysteine-sulfinate desulfinase or *csdB*,[3] although it has been reported that *sufS* mutants cannot utilize ferrioxamine B as an iron source (35). In addition, the overexpression of either cysteine-sulfinate desulfinase or CsdB in an *iscS*$^-$ strain, CL100(DE3), does not complement any observed characteristics of the *iscS*$^-$ phenotype.[4] This may not be surprising for CsdB, in light of its unique structure and substrate specificity.

Fig. 8 shows a summary of the proposed role for IscS in the biosynthesis of sulfur-containing metabolites. It is possible that the distribution of sulfur in all of these cases involves the transfer of S^0 to a distinct protein acceptor. In the case of tRNA thionucleoside biosynthesis, additional potential sulfur acceptors have already been identified. These include TrmU, which is required for the biosynthesis of 2-thiouridine derivatives (37), and MiaB, which is required for the synthesis of the 2-methylthio group in ms^2i^6A (38). We are currently investigating the potential role of *iscS* in the biosynthesis of each of these modified bases. Future studies are directed at further elucidation of the scope of *iscS*-initiated sulfur transfer as well as the mechanism of the transfer itself.

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Addendum—After submission of this paper Palenchar *et al.* (26) showed that mutation of Cys-456 of ThiI impairs tRNA sulfurtransferase activity and the synthesis of s^4U *in vivo*. We have also recently learned of two other groups that have isolated *iscS* mutants and report findings that are comparable with our results.[5]

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