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Formation of Thiolated Nucleosides Present in tRNA from *Salmonella enterica* serovar Typhimurium Occurs in Two Principally Distinct Pathways

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tRNA from *Salmonella enterica* serovar Typhimurium contains five thiolated nucleosides, 2-thiocytidine (s^2C), 4-thiouridine (s^4U), 5-methylaminomethyl-2-thiouridine (mnm^5s^2U), 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), and *N*-6-(4-hydroxyisopentenyl)-2-methylthioadenosine (ms^2io^6A). The levels of all of them are significantly reduced in cells with a mutated *iscS* gene, which encodes the cysteine desulfurase IscS, a member of the ISC machinery that is responsible for [Fe-S] cluster formation in proteins. A mutant (*iscU52*) was isolated that carried an amino acid substitution (S107T) in the IscU protein, which functions as a major scaffold in the formation of [Fe-S] clusters. In contrast to the *iscS* mutant, the *iscU52* mutant showed reduced levels of only two of the thiolated nucleosides, ms^2io^6A (10-fold) and s^2C (more than 2-fold). Deletions of the *iscU*, *hscA*, or *fdx* genes from the *isc* operon lead to a similar tRNA thiolation pattern to that seen for the *iscU52* mutant. Unexpectedly, deletion of the *iscA* gene, coding for an alternative scaffold protein for the [Fe-S] clusters, showed a novel tRNA thiolation pattern, where the synthesis of only one thiolated nucleoside, ms^2io^6A, was decreased twofold. Based on our results, we suggest two principal distinct routes for thiolation of tRNA: (i) a direct sulfur transfer from IscS to the tRNA modifying enzymes ThiI and MnmA, which form s^4U and the s^2U moiety of (c)mnm^5s^2U, respectively; and (ii) an involvement of [Fe-S] proteins (an unidentified enzyme in the synthesis of s^2C and MiaB in the synthesis of ms^2io^6A) in the transfer of sulfur to the tRNA.

At present more than 80 different modified nucleoside derivatives of the four major nucleosides, adenosine (A), guanosine (G), uridine (U), and cytidine (C), have been characterized from tRNAs from all three domains of life (54). One subgroup of these modifications is the thiolated nucleosides (4, 39), of which 10 have been characterized so far and 5, 2-thiocytidine (s^2C), 4-thiouridine (s^4U), 5-methylaminomethyl-2-thiouridine (mnm^5s^2U), 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), and N-6-(4-hydroxyisopentenyl)-2-methylthioadenosine (ms^2io^6A), are present in tRNA from *Salmonella enterica* serovar Typhimurium (Fig. 1). In *Escherichia coli* the same thiolated nucleosides are present except for ms^2io^6A, which has been replaced by *N*-6-isopentenyl-2-methylthioadenosine (ms^2i^6A).

s^4U, which is present in position 8 of a subpopulation of tRNAs, is the most prevalent thiolated nucleoside in tRNA from *S. enterica* and can act as a sensor for UV radiation, since UV exposure induces the formation of a covalent bond between s^4U8 and a C13 in some tRNAs (17, 63). This structural change results in poor aminoacylation of tRNAs, thereby triggering the stringent response (52). The thio group of mnm^5s^2U34 is part of the recognition element for glutaminyl-tRNA synthetase (36, 60), and it also restricts the ability of the tRNA to read G-ending codons (2, 68). Although lack of the ms^2 group of ms^2io^6A37 does not influence the growth rate (15), it does influence the reading frame maintenance (66) and the speed with which some, but not all, ternary complexes of ms^2io^6A37-containing tRNAs enter the A-site (35). Formation of s^2C32, which is present in only four tRNAs species from *S. enterica*, generates an altered anticodon loop structure (5) that may result in a lower translational efficiency (discussed in reference 9). Although a mutant lacking s^2C32 exhibits wild-type growth, the A-site selection rate for some of the tRNAs normally containing s^2C32 is dependent on this thiolated nucleoside (24a). Thus, all thiolated nucleosides present in tRNA of *S. enterica* influence the activity of the tRNA in several ways and to different degrees.

Iron-sulfur clusters constitute one of the most ancient, ubiquitous, and functionally diverse classes of biological prosthetic groups (6–8, 19, 30). Proteins containing one or more [Fe-S] clusters are commonly called [Fe-S] proteins, and they represent a large class of structurally and functionally diverse proteins that participate in many metabolic processes. The assembly of these [Fe-S] clusters into proteins is facilitated by a set of conserved proteins (IscS, IscU, IscA, HscA, HscB, and ferredoxin [Fdx]), which in many bacteria are encoded by genes organized in a single operon. In *E. coli* these genes constitute an operon of eight genes transcribed in the order *iscR-iscS-iscU-iscA-hscB-hscA-fdx-orf3* (61) (Fig. 2). In front of this operon is a regulator gene, *iscR*, whose product regulates expression of the *isc* operon by sensing the [Fe-S] status of the cell (56). The desulfurase IscS is involved in the assembly of most [Fe-S] clusters in the cell by mobilizing the sulfur from the cysteine. The IscU functions as a scaffold for the [Fe-S] cluster assembly, and it is thought to accept sulfur from IscS and deliver it to the target apoprotein (58, 65). IscA is an alternative scaffold to IscU for IscS-directed [Fe-S] cluster assembly, and it interacts with Fdx, also encoded by the *isc*

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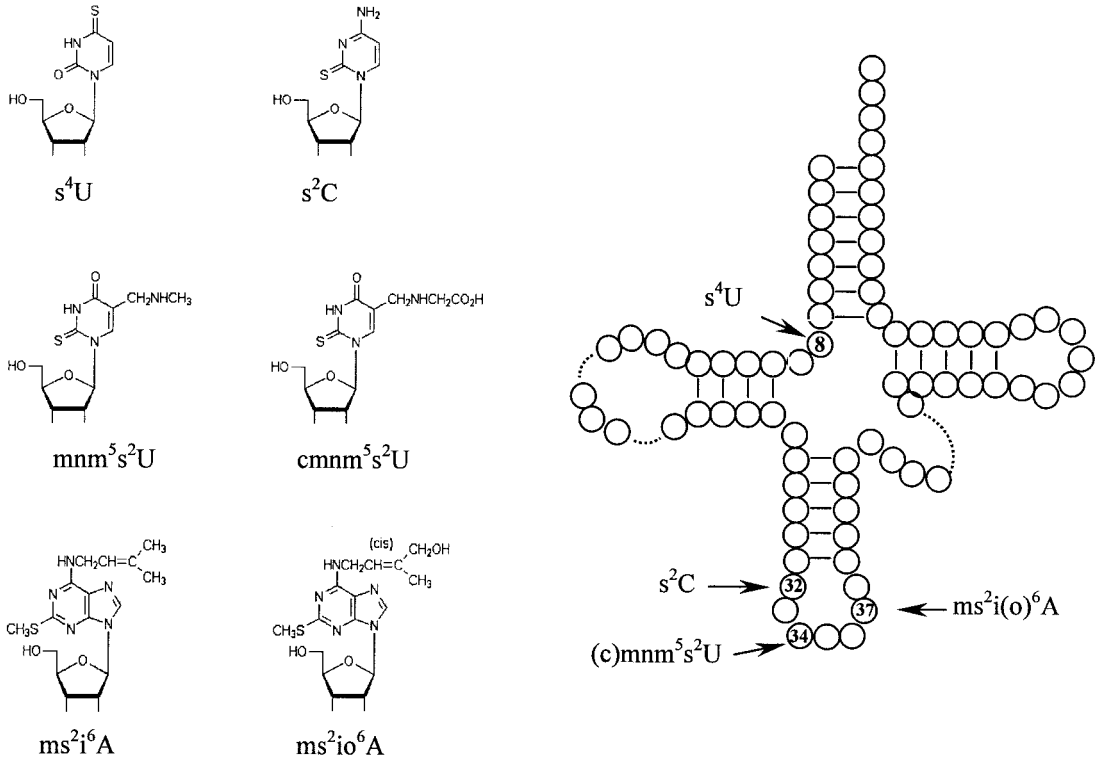

FIG. 1. Structures and positions of thiolated nucleosides in tRNA. Abbreviations: s^2C, 2-thiocytidine; s^4U, 4-thiouridine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; $cmnm^5s^2U$, 5-carboxymethylaminomethyl-2-thiouridine; ms^2io^6A, *N*-6-(4-hydroxyisopentenyl)-2-methylthioadenosine; ms^2i^6A, *N*-6-isopentenyl-2-methylthioadenosine. (c)mnm^5s^2U denotes both mnm^5s^2U and $cmnm^5s^2U34$; $ms^2i(o)^6A$ denotes both ms^2io^6A and ms^2i^6A.

operon (31, 44). The HscA chaperone interacts specifically with IscU by recognizing a specific amino acid sequence (24).

Synthesis of the thiolated nucleosides is a complex and multi-step process (Fig. 3). For many years, our knowledge about the thiolation step was limited to knowing that the sulfur originates from cysteine (3). We know now that IscS is required for the synthesis of s^4U (26, 46) and, further, that IscS is involved in the synthesis of all thiolated nucleosides in tRNA of *S. enterica* (43) and *E. coli* (32). In the synthesis of s^4U, the sulfur is first transferred from cysteine to IscS, thereby forming a persulfide at Cys328 in the active site of IscS. Then the persulfide sulfur from IscS is transferred to a cysteine in ThiI, which in turn transfers the sulfur to a uridine at position 8 of tRNA (26, 41, 46). Alternatively, the sulfur from ThiI may be transferred to another protein, ThiS, which transfers the sulfur to the thiazole moiety in the formation of thiamine (62, 67).

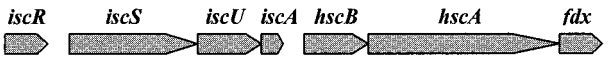

FIG. 2. Organization of the *isc* operon: *iscR* codes for the negative regulator of the operon, *iscS* codes for pyridoxal-phosphate-dependent cysteine desulfurase, *iscU* codes for the scaffold protein for [Fe-S] cluster assembly, *iscA* codes for the alternative scaffold protein for [Fe-S] cluster assembly, *hscB* codes for a J-type molecular cochaperone, *hscA* codes for a Hsp70-type molecular chaperone, and *fdx* codes for [2Fe-2S] Fdx.

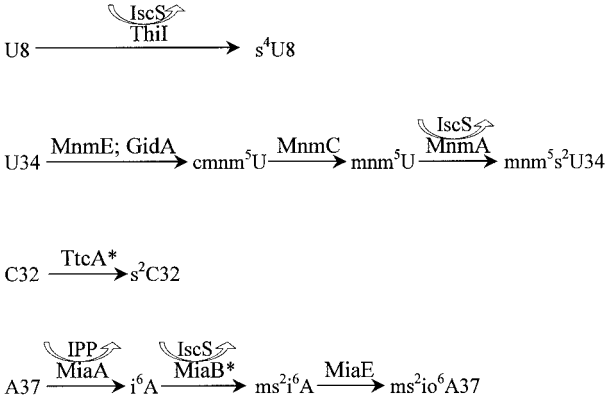

FIG. 3. Biosynthetic pathways of the formation of thiolated nucleosides. U8 is thiolated at position 4 by ThiI, which receives the sulfur directly from IscS (26, 41, 46). U34 undergoes thiolation at position 2 catalyzed by MnmA, which acquires sulfur from IscS (27). The first step in the formation of the mnm^5 side chain is the formation of $cmnm^5$ group by MnmE and GidA. The $cmnm^5$ group is then rearranged in two steps catalyzed by MnmC to synthesize the mnm^5 side chain (23). The thiolation step and the formation of the mnm^5 side chain occur independently, because thiolation may precede or follow the synthesis of the side chain at position 5. In the thiolation of C32 at position 2, the enzyme TtcA is involved (24a). A37 receives isopentenyl group at position 6 from isopentenyl pyrophosphate (IPP) in the reaction catalyzed by MiaA (34, 40, 53). In the later methylthiolation step at position 2 of adenosine, MiaB is involved (15, 16, 49). The MiaE protein is required for the hydroxylation of the i^6 group (48). *, see the text for details.

TABLE 1. *Salmonella* strains used in this study

Strain	Genotype	Source
LT2	Wild type	J. Roth
GT2919	*hisO1242 hisD3749 proL207 zef-2502*::Tn*10d*Tc *miaCl(iscU52)*	50
GT3590	*hisO1242 hisD3749 zef-2516*::Tn*10d*Cm *miaCl(iscU52)*	This work
GT2422	(TT418) *glyA-540*::Tn*10d*Tc	J. Roth
GT6430	*hisO1224 hisC3737 zfh-2525*(STM2545)::Tn*10d*Tc	43
GT6516	*iscU52(miaCl) zee-2526*::Mu*d*SacI	This work
GT4767	*sseA2527*::Tn*10d*Tc	This work
GT4768	*iscU52(miaCl) sseA2527*::Tn*10d*Tc	This work
DM5420	*iscA2*::Mu*d*J	57
GT6594	Δ*iscU53*	This work
GT6593	Δ*iscA54*	This work
GT6582	Δ*hscA51*	This work
GT6645	Δ*fdx51*	This work
GT6595	Δ*iscA54*/piscA1	This work
GT6597	Δ*iscU53*/piscA1	This work

Thus, the syntheses of thiamine and s^4U are metabolically linked. The persulfide sulfur of IscS may also be transferred to another acceptor protein, MnmA, which in turn transfers the sulfur to a uridine in the wobble position of a subset of tRNAs forming the s^2U moiety of mnm^5s^2U (27). The product of the *miaB* gene participates in the methylthiolation of A37 in a subset of tRNAs that read codons starting with U (15, 16). MiaB contains an iron-sulfur complex (49) and is a member of the Radical SAM protein superfamily, which utilizes the combination of a labile iron-sulfur cluster and *S*-adenosylmethionine (SAM) to initiate radical catalysis (10, 18, 59). The synthesis of s^2C is poorly understood; however, it is known that the product encoded by the *ttcA* gene is required for its synthesis (24a). In conclusion, these results suggest that the determinants of thiolation of U at positions 2 and 4 are similar whereas the methylthiolation reaction in the synthesis of ms^2io^6A is different. Interestingly, studies of an *iscS* deletion mutant revealed that the synthesis of s^2C and ms^2io^6A could still occur at lower rates, suggesting the existence of an alternative pathway for the mobilization of sulfur, independent of IscS (32, 43).

This paper addresses the role of IscU, IscA, HscA, and Fdx in the thiolation of tRNA. We show that in contrast to the role of IscS, which is involved in the synthesis of all thiolated nucleosides, IscU, HscA, and Fdx influence only the synthesis of s^2C and ms^2io^6A whereas IscA influences only the synthesis of ms^2io^6A. Based on our results, we suggest that the thiolation of tRNA occurs in two principally distinct ways—one leading to s^4U and (c)mnm^5s^2U formation, and the other leading to s^2C and ms^2io^6A formation.

MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial strains used were derivatives of *S. enterica* (Table 1). Cultures were grown in NAA complex medium (0.8% Difco nutrient broth; Difco Laboratories, Detroit, Mich.) supplemented with the aromatic amino acids, aromatic vitamins, and adenine at concentrations as described previously (12). As the defined rich liquid medium, morpholinepropanesulfonic acid (MOPS) medium, discribed by Neidhart et al. (42), was used. As the rich solid medium, TYS agar (10 g of Trypticase peptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per liter) was used.

Genetic procedures. Transduction with phage P22 HT105/1 (*int-201*) (55) was performed as described previously (12). DNA sequencing was performed on either chromosomal DNA or PCR products as described in the manual for the Applied Biosystems ABI Prism cycle-sequencing BigDye Ready Reaction kit. The deletion mutants used in this study were constructed by first inserting in the gene of interest a PCR fragment coding for antibiotic resistance, which later was eliminated from the chromosome leaving an in-frame "scar" of an 84-nucleotide insertion as described previously (11). The scar in the Δ*iscU53* mutant is inserted between the seventh and the seventh-to-last nucleotide, the scar in the Δ*hscA51* mutant is inserted between the fifth and the fifteenth-to-last nucleotide, the scar in the Δ*iscA54* mutant is inserted between the sixth and the fifth-to-last nucleotide, and the scar in the Δ*fdx51* mutant is inserted between the sixth and the sixth-to-last nucleotide of the respective gene. All mutations were confirmed by DNA sequencing.

Analysis of modified nucleosides in tRNA. Bacterial strains were grown in NAA medium at 37°C to about 4×10^8 to 6×10^8 cells/ml (100 to 150 Klett units). The cells were lysed, and total RNA was prepared (13), dissolved in R200 buffer (10 mM Tris-H_3PO_4 [pH 6.3], 15% ethanol, 200 mM KCl), and applied to a Nucleobond column equilibrated with the same buffer. tRNA was eluted with the same buffer, except that the KCl concentration was raised to 600 mM. The tRNA was precipitated with 2.5 volumes of cold ethanol containing 1% of potassium acetate, washed twice with 70% ethanol, and dried. It was then dissolved in water, and a 100-μg sample was degraded to nucleosides with nuclease P1 followed by treatment with bacterial alkaline phosphatase (22). The resulting hydrolysate was analyzed by high-performance liquid chromatography (HPLC) (21). The chromatograms were scanned at specific wavelengths to optimize the quantification of each of the four thiolated nucleosides. The levels of the various thiolated nucleosides at the specific wavelengths were normalized to that of t^6A at 254 nm. The values for $ms^2i(o)^6A$ represent those for ms^2io^6A and ms^2i^6A taken together; similarly, the values for (c)mnm^5s^2U represent those for mnm^5s^2U and $cmnm^5s^2U$ taken together.

RESULTS

Strain GT2919 has a reduced growth rate and is deficient in both s^2C and ms^2io^6A. Strains with a + 1 frameshift mutation, *hisD3749*, are dependent on added histidine for growth. A defective $tRNA^{Pro}_{GGG}$ (encoded by the *proL* gene) allows $tRNA^{Pro}_{cmo5UGG}$ (encoded by the *proM* gene) to suppress the *hisD3749* mutation, resulting in the His^+ phenotype (51). By using localized mutagenesis in the *proL* region, strain GT2919 (*proL207 hisD3749*) was isolated (50), which, besides being His^+, had a reduced growth rate and a changed tRNA modification pattern. tRNA from strain GT2919 had reduced levels of two modified nucleosides: s^2C and ms^2io^6A (Fig. 4). Note, however, that those two modified nucleosides are not present in $tRNA^{Pro}_{GGG}$. Genetic studies revealed that the *proL207* mutation, which caused the His^+ phenotype, was linked neither to the decreased levels of s^2C and ms^2io^6A nor to the reduced growth rate. A new mutation causing the last two phenotypes

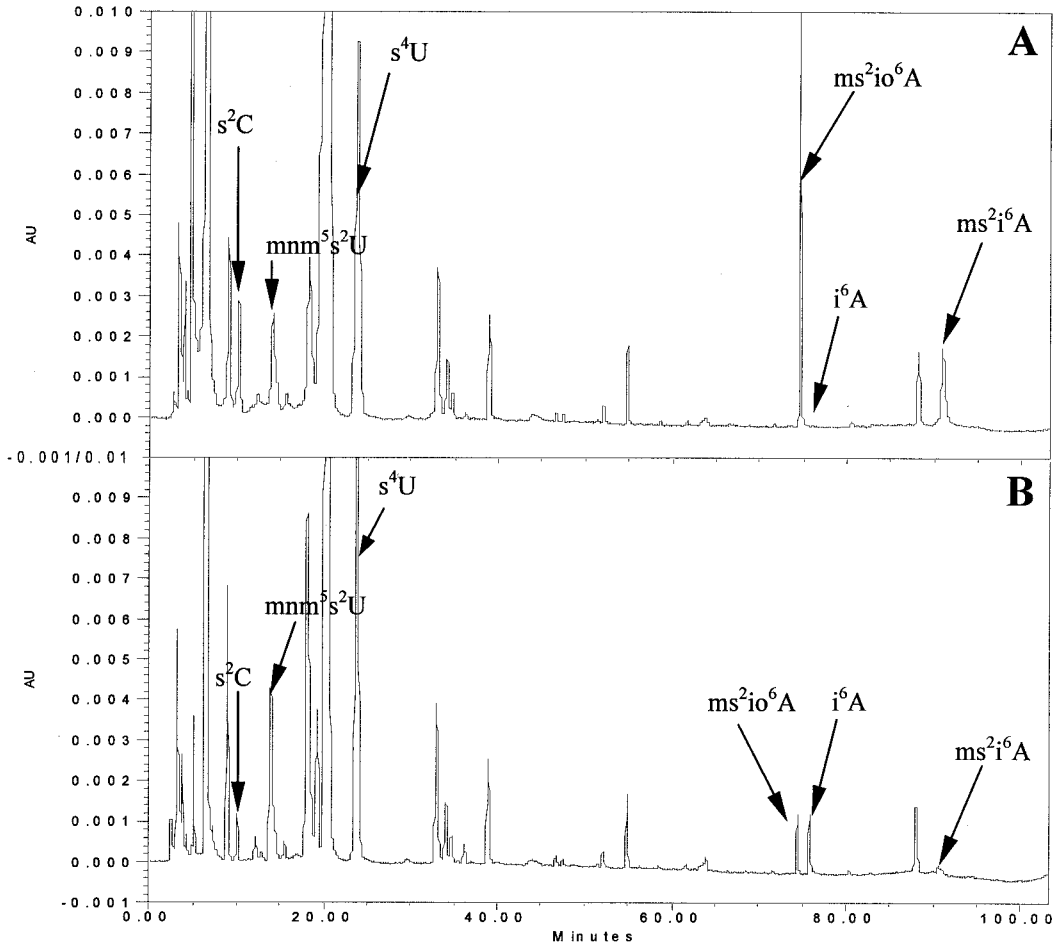

FIG. 4. HPLC chromatograms of tRNA hydrolysates from wild-type (A) and *iscU52* mutant (B) strains. The nucleosides were monitored at 295 nm to maximize the detection of all thiolated nucleosides. mnm^5s^2U, s^4U, ms^2io^6A, and i^6A were identified by comparing UV spectra with published spectra (21); for s^2C, the molecular weight of the protonated form was determined by mass spectrometry (24a). AU, absorbance units.

was temporarily called *miaC1*, since it was the third gene identified to influence the synthesis of ms^2io^6A.

The *miaC1* mutation is located within the *isc* operon. To localize the *miaC1* mutation, a random pool of Tn*10d*Tc insertions in the wild-type strain LT2 was introduced into the slow-growing GT3590 mutant (*miaC1*) and fast-growing colonies on rich medium plates at 30°C were monitored. The Tn*10d*Tc insertion from one of the fast-growing transductants was found to be 28% linked to the *miaC1* mutation, as demonstrated by backcrosses to strain GT3590 (data not shown). The *miaC1* mutation was transferred to the wild-type strain LT2 by P22 transduction. tRNA was prepared from 10 slow-growing and 10 fast-growing transductants for HPLC analysis of their modification patterns. All slow-growing transductants had reduced levels of s^2C and ms^2io^6A in their tRNAs, whereas the fast-growing ones showed the wild-type tRNA modification pattern (Fig. 4; for quantifications, see Fig. 5). One fast- and one slow-growing transductant were saved as congenic strains GT4767 ($miaC^+$) and GT4768 (*miaC1*).

The chromosomal region on each side of the Tn*10d*Tc transposon in strain GT4768 was sequenced with primers specific for the ends of the Tn*10d*Tc transposon. The results showed that Tn*10d*Tc was inserted into the *sseA* gene. The *miaC1* mutation causing slow growth and a deficiency in s^2C and ms^2io^6A in the tRNA was localized to the region between the *sseA* and STM2545 genes by transductional mapping using strains carrying different markers located in the vicinity of the *sseA* gene (*glyA-540*::Tn*10d*Tc, *zee-2526*::Mu*d*SacI, and STM2545::Tn*10d*Tc) (data not shown). The cotransduction frequency (28%) between the *miaC1* mutation and *sseA2527*::Tn*10d*Tc, together with the mapping data, suggested that the mutation was within the *isc* operon.

The *miaC1* mutation is located in the *iscU* gene. To locate the *miaC1* mutation more precisely, we sequenced the entire *isc* operon in strain GT4768. The only mutation that we found was in the *iscU* gene, resulting in a substitution of Thr for Ser at position 107 of the IscU protein. Therefore, we renamed *miaC1* to *iscU52*. A plasmid that contains *iscU* and *iscA* genes complemented the slow growth and modification deficiency of the *iscU52* mutant GT4768 (see the discussion of Fig. 5). These results demonstrate that the slow growth and reduced levels of s^2C and ms^2io^6A in tRNA are caused by the *iscU52* mutation.

To verify the role of IscU in the formation of the two modified nucleosides, s^2C and ms^2io^6A, in tRNA, we deleted the

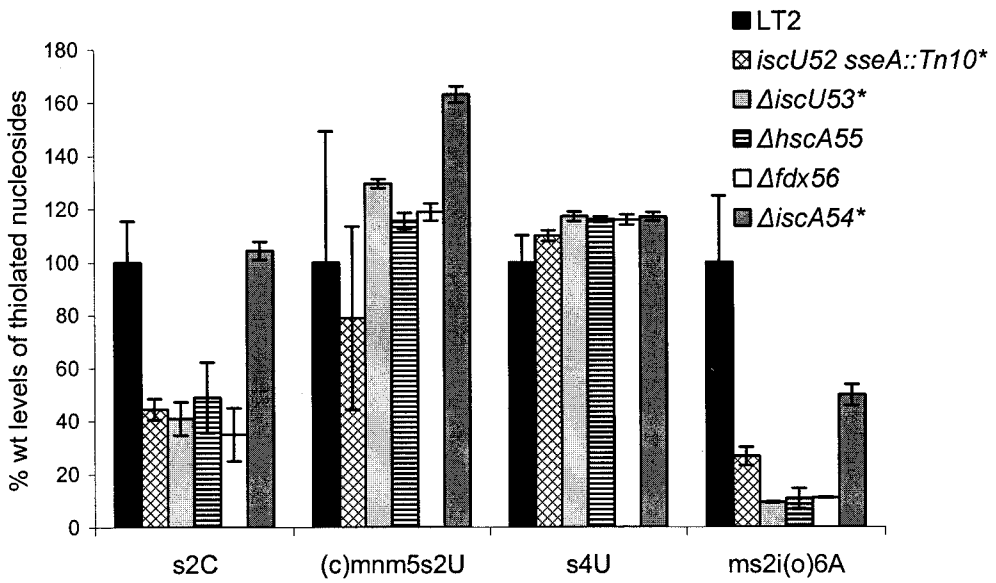

FIG. 5. Mutations in the *iscU*, *hscA*, and *fdx* genes affect the levels of s^2C and ms^2io^6A, whereas mutation in *iscA* reduces only the level of ms^2io^6A. *, The *iscU* and *iscA* mutations were complemented by a plasmid harboring the *iscU* and *iscA* genes in the high-copy-number pGEM vector (data not shown).

iscU gene. The Δ*iscU53* mutation had a similar effect on the modification of tRNA to that of the *iscU52* point mutation: it caused a 60% reduction in the level of s^2C and more than a 10-fold reduction in the level of ms^2io^6A (Fig. 5).

Lack of the HscA chaperone results in s^2C and ms^2io^6A deficiency in tRNA. Three conserved cysteine residues at positions 37, 63, and 106 are all essential for the function of IscU in vivo by providing a scaffold for the sequential assembly of [Fe-S] clusters (1, 28, 58, 69). At residues 99 and 103 of IscU, the motif LPPVK is found, which is required for the interaction with the molecular chaperone HscA (24). Sequence alignment demonstrated that this motif is invariant in all of the IscU homologs identified to date. The amino acid substitution S107T in IscU52 is only 3 amino acids away from this conserved region (Fig. 6) and might prevent the IscU-HscA interaction by altering the structure of the HscA recognition domain. This hypothesis suggests a role of HscA in the modification of tRNA. Therefore, a strain (GT6582) with a nonpolar deletion of the *hscA* gene was constructed. Evidently, tRNA from the Δ*hscA51* mutant showed a similar decrease in the levels of s^2C and ms^2io^6A to that of tRNA from the Δ*iscU53* mutant (Fig. 5), consistent with the view that HscA and IscU interact during the synthesis of these two thiolated nucleosides.

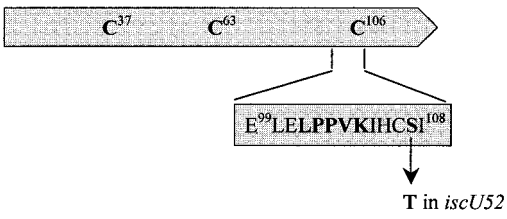

FIG. 6. Schematic presentation of the IscU protein with conserved cysteines indicated. C63 forms a disulfide bridge with C328 of IscS (28). In the expanded region, the HscA binding site, LPPVK (24), and S107, which is altered to T107 by the *iscU52* mutation, are depicted.

IscA influences only the level of ms^2io^6A. Since IscA and IscU have a similar function in [Fe-S] cluster assembly (31, 44), we decided to investigate the involvement of IscA in tRNA thiolation. Strain DM5420 (*iscA2*::Mu*dJ*) contains a MudJ transposon insertion disrupting the *iscA* gene and most probably decreasing the expression of the downstream genes *hscB*, *hscA*, and *fdx* due to polarity effects (57). Analysis of the modification pattern of the tRNA from the strain DM5420 showed that the levels of s^2C and ms^2io^6A were both decreased to levels similar to those reached in the *hscA* mutant (Table 2), suggesting that the effect we observed in DM5420 strain might be caused by the decrease in the synthesis of HscA. To establish which of the two proteins, IscA or HscA, is required for the synthesis of these two thiolated nucleosides, a strain (GT6593) with a nonpolar deletion of the *iscA* gene was constructed. Unexpectedly, analysis of the total tRNA purified from that strain revealed that the presence of IscA is critical only for the synthesis of ms^2io^6A, since its level was decreased twofold (Fig. 5). The levels of the other three thiolated nucleosides were similar (s^2C) or increased [(c)mnm^5s^2U and s^4U] compared to the levels observed in the wild-type strain.

Increased levels of the IscA cannot substitute for the function of the IscU. Since both IscU and IscA can serve as scaffolds for [Fe-S] assembly, we tested whether IscA provided at higher levels could substitute for the activity of IscU. Therefore, a plasmid (piscA1) carrying the $iscA^+$ gene (57) was introduced into the strains GT6594 (Δ*iscU53*) and GT 6593 (Δ*iscA54*). Analysis of the tRNA modification pattern revealed that the low levels of ms^2io^6A in the Δ*iscA54* mutant were restored to wild-type levels when IscA was provided on the plasmid (Table 2). However, tRNA originating from the

TABLE 2. Levels of thiolated nucleosides in tRNA from the different mutants of *iscA*, and *iscU* grown in NAA rich medium

Genotype	Relative level (%) ± SD[a]			
	s^2C (247 nm)	(c)mnm^5s^2U (274 nm)	s^4U (330 nm)	$ms^2i(o)^6A$ (242 nm)
Wild type	100 ± 15.6 (0.59)	100 ± 49.3 (0.38)	100 ± 10.1 (5.28)	100 ± 25.0 (1.10)
iscA::Mu*dJ*	60.6 ± 28.4	123.7 ± 5.9	141.2 ± 0.2	6.3 ± 2.2
Δ*iscA54*	104.5 ± 3.4	163.1 ± 3.1	117.1 ± 1.6	49.8 ± 4.0
Δ*iscU53*	41.0 ± 6.3	129.6 ± 1.7	117.2 ± 1.7	9.3 ± 0.2
Δ*iscA54*/piscA1	112.8	127.4	112.4	110.6
Δ*iscU53*/piscA1	40.5	128.2	116.6	10.4

[a] Results are percentages of the wild-type levels. The numbers in parentheses are the levels of the various thiolated nucleosides at the indicated wavelength relative to the level of t^6A at 254 nm. SD, standard deviation.

Δ*iscU53*/piscA1 strain still had the thiolation pattern characteristic of the Δ*iscU53* mutant. We conclude that IscA cannot substitute for IscU in the synthesis of s^2C or of ms^2io^6A.

Fdx influences the synthesis of two thiolated nucleosides, s^2C and ms^2io^6A. IscA was shown to form a complex with and transfer iron and sulfide to Fdx (Fdx is another member of the *isc* operon) to form [2Fe-2S] holoferredoxin (44). We tested if a lack of Fdx would give a similar phenotype to that resulting from a lack of IscA. A Δ*fdx51* mutant (strain GT6645) was constructed, and its tRNA thiolation pattern was analyzed. In contrast to the Δ*iscA54* tRNA, which was affected only in the levels of ms^2io^6A, the tRNA from the Δ*fdx51* mutant had reduced levels of two thiolated nucleosides, s^2C (35% of the wild-type level remaining) and ms^2io^6A (11% of the wild-type level remaining) (Fig. 5), similar to the reduction observed in the Δ*iscU53* and Δ*hscA51* strains.

Growth characteristics of the mutants defective in the *isc* operon. The *iscU52* mutant forms small colonies on rich-medium agar plates. Therefore, the colony sizes of all the mutants used in this study were determined by measuring the diameters of the colonies grown on rich TYS agar plates at 30°C (the reduction in growth was more pronounced at 30°C than at 37°C) for 24 h. A general reduction in colony sizes to 64 to 77% of the size of the wild-type colonies was observed (Table 3).

The steady-state growth rates in a defined rich medium at 37°C were also reduced in the various mutants compared to that of the wild type (Table 3). In the *iscU52* and Δ*fdx51* mutants, the reduction was 30%, and in the Δ*iscU53*, Δ*iscA54*, and Δ*hscA51* mutants, it was somewhat lower (10 to 20%). We noticed that the *iscU52*, Δ*iscU53*, Δ*hscA51*, and Δ*fdx51* mutants were unable to form dense cultures, since they never grew to to a cell density of more that 2.4 to 2.7 optical density at 420 nm (OD_{420}) units, whereas the wild-type strain reached a cell density of 5.5 to 5.7 OD_{420} units. The Δ*iscA54* mutant had an intermediate final cell density of 3.8 to 4.0 OD_{420} units.

TABLE 3. Growth characteristics of different mutants mutated in the *isc* operon

Genotype	% of the wild-type growth rate in rich medium (growth constant $k[h^{-1}]$ ± SD)[a]	Relative size of the colonies (%)[b]
Wild type	100 (1.54 ± 0.03)	100
iscU+ *sseA*::Tn*10d*Tc	101 (1.55 ± 0.02)	95
iscU52 sseA::Tn*10d*Tc	70 (1.07 ± 0.00)	64
Δ*iscU53*[c]	81 (1.25 ± 0.13)	69
Δ*hscA51*	85 (1.31 ± 0.16)	69
Δ*iscA54*	89 (1.37 ± 0.11)	77
Δ*fdx51*	70 (1.08 ± 0.04)	66

[a] Growth rate in rich MOPS medium (42) is expressed as the specific growth rate constant *k*, which is ln 2/mass doubling time in hours. SD, standard deviation.

[b] The colonies were grown on rich medium plates at 30°C for 24 h. The sizes of the colonies are given relative to the size of the colonies of the wild-type strain. The size of the wild-type colony was 1.05 ± 0.06 mm.

[c] A similar reduced growth rate for an *iscU* deletion mutant in *E. coli* has been reported (64).

DISCUSSION

In this study we showed that different [Fe-S] proteins encoded in the *isc* operon differentially affect the synthesis of the five thiolated nucleosides present in tRNA of *S. enterica*. Whereas mutation in the *iscS* gene reduces the levels of all the thiolated nucleosides in tRNA [s^2C, s^4C, (c)mnm^5s^2U, and ms^2io^6A] (32, 43), mutation in the *iscU*, *hscA*, or *fdx* gene reduced the synthesis of only two of them, s^2C and ms^2io^6A. Mutation in the *iscA* gene reduced the level of only one thiolated nucleoside, ms^2io^6A (Fig. 5).

In the synthesis of s^4U and the s^2U moiety of (c)mnm^5s^2U, the sulfur is delivered from IscS to ThiI and MnmA, respectively; they, in turn, transfer it to tRNA (26, 27, 33, 41, 46). ThiI and MnmA share a weak sequence homology and carry conserved cysteine residues, but neither of them is an [Fe-S] protein. On the other hand, MiaB, which is involved in the synthesis of ms^2io^6A, possesses an oxygen-sensitive [Fe-S] cluster, whose presence is essential for successful methylthiolation of the adenosine of tRNA in vivo (49). Synthesis of s^2C is dependent on the TtcA protein (24a), and its amino acid sequence does not reveal any obvious [Fe-S] cluster motif. However, it contains seven Cys residues, of which four are clustered in two conserved C-X_1-X_2-C motifs that could have the potential for [Fe-S] cluster formation.

The lack of IscU, HscA, or Fdx reduces the activities of [Fe-S] enzymes 5- to 10-fold in *E. coli*, most probably due to the absence of [Fe-S] clusters in these enzymes (64). Assuming that the homologous proteins encoded by the *isc* operon of *S. enterica* have similar effects, we expected that the activity of the [Fe-S] cluster protein MiaB should be reduced in the Δ*iscU53*, Δ*hscA51*, and Δ*fdx51* mutants. Indeed, this was observed, since the level of ms^2io^6A in tRNA was reduced 10-fold compared to the level in the wild type (Fig. 5). The levels of s^2C were reduced two- to threefold, further suggesting that TtcA contains an [Fe-S] cluster of its own or that there are other [Fe-S] cluster-containing proteins upstream or/and downstream of TtcA in the s^2C synthetic pathway. However, the activity of

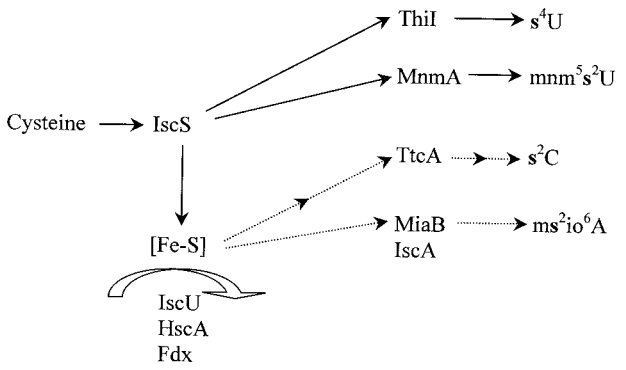

FIG. 7. Working model for sulfur trafficking in tRNA thiolation. Solid lines represent experimentally verified pathways, and dashed lines represent hypothetical productive interactions between the proteins. See the text for further detail.

those unknown [Fe-S] proteins is not absolutely required, since low levels of thiolation are still produced in the various *isc* operon mutants. It is also possible that small amounts of the correct clusters originate from alternative [Fe-S] cluster-forming machinery, such as SufABCDSE.

Based on these results, we suggest that there are two principal distinct routes for the biosynthesis of the thiolated nucleosides (Fig. 7). Following the action of IscS, which affects the formation of all thiolated nucleosides in tRNA, the synthesis diverges into (i) the syntheses of s^4U and (c)mnm^5s^2U, where the sulfur is directly transferred from IscS to the tRNA-modifying enzymes and where apparently no [Fe-S] protein participates, and (ii) the biosynthetic pathways leading to the synthesis of s^2C and ms^2io^6A, which need, besides IscS, other constituents of the ISC machinery since they comprise proteins containing [Fe-S] clusters. Also in support of the presence of two separate pathways is the recent observation that synthesis of s^4U and s^2U is completely dependent on IscS as the sulfur donor, whereas an inefficient synthesis of s^2C and ms^2io^6A occurs in an IscS-independent way (32, 43).

IscU and IscA are both scaffold proteins, presumably with similar functions in the [Fe-S] cluster assembly (1, 44). Since most bacteria seem to have both these scaffold proteins, their function might be not completely overlapping. It is generally thought that IscU is the key player in the assembly process. This view is supported by the fact that deletion of the two IscU homologues in yeast is lethal whereas deletion of both IscA homologs is not (20, 25, 29, 47). We therefore expected that lack of IscA would result in no detectable phenotype if IscU was epistatic to IscA and the target apoproteins were the same for the two scaffold proteins. Alternatively, if these two scaffold proteins had an additive effect, we would expect the lack of IscA to have an effect on the synthesis of s^2C and ms^2io^6A, since lack of IscU reduced the synthesis of both these nucleosides. Surprisingly, only the synthesis of ms^2io^6A was affected by the deletion of the *iscA* gene (Fig. 5). This result could indicate that, for optimal activity, MiaB requires the assistance of the scaffold protein IscA or, more probably, could reflect the possible role of IscA in the restoration of the [Fe-S] cluster in MiaB. MiaB has an oxygen-labile cluster, which is a common feature of proteins belonging to the Radical SAM family. Such clusters are more sensitive to oxidative damage and require more efficient repair. This could explain, why the absence of IscA affected only the synthesis of ms^2io^6A, but not that of s^2C, provided that the protein(s) working in the latter pathway has a more stabile [Fe-S] cluster.

Since IscA and IscU have similar functions in the [Fe-S] cluster assembly, it can be assumed that overproduction of one of them may suppress the lack of the other. However, introduction of a plasmid encoding IscA did not suppress the phenotype of the Δ*iscU53* mutant, since it still had decreased levels of s^2C and ms^2io^6A (Table 2). Hence, IscA cannot substitute for IscU in the assembly of [Fe-S] clusters in MiaB or in the protein(s) participating in the synthesis of s^2C.

Biotin synthase (BioB) is an [Fe-S] enzyme and catalyzes the last step of biotin biosynthesis (38). BioB and MiaB have functional similarity since they both catalyze a C-H to C-S bond conversion and are members of the same family of Radical SAM enzymes (37, 59). Recently, it was shown that [Fe-S] cluster assembly occurred in BioB in vitro when the transient cluster was provided by IscA (45). However, our experiments on the suppression of the Δ*iscU53* mutant by the piscA1 plasmid could not confirm cluster assembly in MiaB by IscA when we tested MiaB for tRNA-modifying activity (Table 2). This could be due to differences in the conditions as we monitored the processes inside the cell, which is difficult to reproduce in the experiments done in vitro, or could be due to the fact that MiaB, in contrast to BioB, needs a cluster provided exclusively by IscU.

While all the mutants analyzed had a decreased synthesis of s^2C and ms^2io^6A, the levels of the other two thiolated nucleosides, s^4U and (c)mnm^5s^2U, were slightly increased (17 to 29% compared to the wild-type levels [Fig. 5]). It is known that the transcriptional repressor of the *isc* operon, IscR, needs a functional [Fe-S] cluster for its activity. In *iscS* and *hscA* mutants (56) as well as in *iscU* and *fdx* mutants (Fig. 5; Table 3), the assembly of [Fe-S] clusters is significantly reduced and therefore IscR loses its repressing abilities, resulting in increased expression of the *isc* operon. Since IscS is directly transferring the sulfur to tRNA-modifying enzymes, the increased levels of s^4U and (c)mnm^5s^2U in tRNA may reflect an increased level of IscS. Such an explanation would require a slight undermodification of the tRNA under the growth conditions used; i.e., some tRNAs would not have a molar content of s^4U and (c)mnm^5s^2U. This may be true, since thiolation of tRNA varies with the growth rate (14). The observed increased levels of s^4U and (c)mnm^5s^2U in various mutants (Fig. 5) suggest that deficiency in any of the [Fe-S] assembly proteins, IscU, IscA, HscA, or Fdx, results in more efficient transfer of sulfur to uridines of tRNA. The very large increase in the level of (c)mnm^5s^2U in the Δ*iscA54* mutant is more difficult to reconcile with such a suggestion, since it would require that about 39% of the possible (c)mnm^5s^2U sites in the tRNA not be thiolated when cells are growing logarithmically in a rich medium.

Observed defects in the growth rate of strains harboring various mutations in the *isc* operon can be due either to a reduction in the activities of some [Fe-S] enzymes critical for cell growth or to the lack of thiolated nucleosides in the tRNA. Since lack of s^2C (24a) and lack of the methylthio group of ms^2io^6A (15) in the tRNA does not influence the growth rate,

we suggest that the reduced growth rate in Δ*iscU53*, Δ*hscA51*, or Δ*fdx51* mutants is caused by the deficiencies of [Fe-S] clusters in a protein(s) critical to obtain a maximal growth rate but not specifically for the synthesis of s^2C and ms^2io^6A.

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