JOURNAL OF BACTERIOLOGY, Feb. 2004, p. 750–757 Vol. 186, No. 3
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.3.750–757.2004

The Conserved Cys-X_1-X_2-Cys Motif Present in the TtcA Protein Is Required for the Thiolation of Cytidine in Position 32 of tRNA from *Salmonella enterica* serovar Typhimurium

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Received 30 June 2003/Accepted 22 October 2003

The modified nucleoside 2-thiocytidine (s^2C) has so far been found in tRNA from organisms belonging to the phylogenetic domains *Archaea* and *Bacteria*. In the bacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, s^2C is present in position 32 of only four tRNA species—$tRNA^{Arg}_{ICG}$, $tRNA^{Arg}_{CCG}$, $tRNA^{Arg}_{mnm^5UCU}$, and $tRNA^{Ser}_{GCU}$. An in-frame deletion of an *S. enterica* gene (designated *ttcA*, for "two-thio-cytidine") was constructed, and such a mutant has no detectable s^2C in its tRNA. The TtcA protein family is characterized by the existence of both a PP-loop and a Cys-X_1-X_2-Cys motif in the central region of the protein but can be divided into two distinct groups based on the presence and location of additional Cys-X_1-X_2-Cys motifs in terminal regions of the sequence. Mutant analysis showed that both cysteines in this central conserved Cys-X_1-X_2-Cys motif are required for the formation of s^2C. The Δ*ttcA1* mutant grows at the same rate as the congenic wild-type strain, and no growth disadvantage caused by the lack of s^2C was observed in a mixed-population experiment. Lack of s^2C32 did not reduce the selection rate at the ribosomal aminoacyl-tRNA site (A-site) for Arg-$tRNA^{Arg}_{ICG}$ at any of its cognate CGN codons, whereas A-site selection at AGG by Arg-$tRNA^{Arg}_{mnm^5UCU}$ was dependent on the presence of s^2C32. The presence of s^2C32 in peptidyl-$tRNA^{Arg}_{CCU}$ or in peptidyl-$tRNA^{Arg}_{mnm^5UCU}$ interfered with decoding in the A-site. The presence of s^2C32 in $tRNA^{Arg}_{ICG}$ decreased the rate of translation of the CGA codon but not that of the CGU codon.

The source of sulfur for the synthesis of the thiolated nucleosides found in tRNA from *Escherichia coli* or *Salmonella enterica* serovar Typhimurium is cysteine (1). About 30 different modified nucleosides are present in tRNA from *E. coli* or *S. enterica*, and of these, five [2-thiocytidine (s^2C), 4-thiouridine (s^4U), 5-methylaminomethyl-2-thiouridine (mnm^5s^2U34), 5-carboxymehylaminomethyl-2-thiouridine ($cmnm^5s^2U34$), and 2-methylthio-N^6-(*cis*-4-hydroxy-isopentenyl)adenosine (ms^2io^6A, in *S. enterica*) or 2-methylthio-N^6-isopentenyl-adenosine (ms^2i^6A, in *E. coli*)] are thiolated. Although the biochemical source of sulfur is known, the mechanism by which sulfur is incorporated into these various molecules has been an enigma for many years. However, recently we and others showed that the IscS cysteine desulfurase, which is not an RNA binding protein, is involved in the synthesis of all five thiolated nucleosides present in tRNA of *S. enterica* and *E. coli* (29, 37). It is likely that the synthesis of each thiolated nucleoside requires at least one enzyme that recognizes a specific subset of tRNAs. One characterized example of this is the enzyme that catalyzes the formation of s^4U8 at position 8 of tRNA (26, 38). The sulfur of cysteine is transferred first to IscS, thereby forming an IscS-SSH persulfid, which in turn transfers a sulfane sulfur to the protein ThiI, forming a ThiI-SSH persulfid. In the presence of ATP-Mg, this protein transfers the sulfur to the particular tRNA substrates, thereby forming s^4U8. A mutation in the *mnmA* (*asuE*, *trmU*) gene inhibits the thiolation step in the formation of mnm^5s^2U34 (37, 53). Similar to the synthesis of s^4U, the protein MnmA obtains a persulfid from IscS and, in turn, transfers the sulfur to a subset of tRNAs, thereby forming the s^2U moiety of mnm^5s^2U34 (27). A defective *miaB* gene results in a lack of the ms^2 moiety of ms^2io^6A37 in *S. enterica* (22), and the gene encodes an iron-sulfur protein (39) belonging to the recently identified Radical SAM superfamily (50). The *thiI*, *mnmA*, and *miaB* genes are specific for the syntheses of s^4U, mnm^5s^2U, and ms^2io^6A in tRNA, respectively. Apart from *iscS*, no gene has been identified that influences the synthesis of s^2C. This nucleoside is found in tRNA from organisms belonging to the phylogenetic domains *Archaea* and *Bacteria* and so far not in tRNA from organisms belonging to the domain *Eucarya* (2, 32). It is present at position 32 of only four tRNA species, $tRNA^{Arg}_{ICG}$, $tRNA^{Arg}_{CCG}$, $tRNA^{Arg}_{mnm^5UCU}$, and $tRNA^{Ser}_{GCU}$ in *E. coli* and *S. enterica* (52). As a first step to elucidate the mechanism of thiolation of C32 in tRNA, we have identified a gene, *ttcA* (for "two-thiocytidine"), whose product is required for the formation of s^2C32. Interestingly, there exist genes that are very similar to the *ttcA* gene in organisms from all three domains, including eukaryotic organisms, in whose tRNAs no s^2C has so far been found.

MATERIALS AND METHODS

Bacteria, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. Cultures were grown in either Luria-Bertani (LB) broth by the method of Bertani (6) or 0.8% nutrient broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl, adenine, tryptophan, tyrosine, phenylalanine, *p*-hydroxybensoate, dihydroxybensoate, and *p*-aminobenzoate at concentrations given in reference 17. As rich solid medium, TYS agar plates (10 g of Trypticase peptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per liter) were used. Minimal agar plates contained medium E (55) and appropriate metabolites and carbon sources as described previously (17). As defined liquid medium, morpholinepropanesulfonic acid (MOPS) medium (36) supplemented

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TABLE 1. *E. coli* and *Salmonella* strains and plasmids used in this study

Strain or plasmid	Genotype	Origin
E. coli		
GRB105	*strL aroE tsaA1 trmG1 ttcA1*	Laboratory collection
S. enterica		
LT2	Wild type	J. Roth
GT5969	Congenic wild type to GT5970	This work
GT5970	Δ*ttcA1*	This work
GT6443	*ttcA2* (C122A, C125A)	This work
GT6635	*ttcA3* (C122S) *zdf-2528*::Kan^r	This work
GT6636	*ttcA4* (C125S) *zdf-2528*::Kan^r	This work
Plasmids and phages		
DD301	λ, Charon 30 containing *Bam*HI fragment from *E. coli*	M. Rydéen-Aulin
pGJ8	pUC19 containing 3.5-kb *Bam*HI-*Bam*HI fragment from DD301	This work
pGJ13	*ttcA* gene from *E. coli* cloned into vector pCL1921	This work
pTH34	+1 frameshifting site CCC-AGA-A inserted in the beginning of *lacZ* as described previously (25)	Tord Hagervall
pTH35	As for pTH34, but the +1 frameshifting site was CCC-AGG-A	Tord Hagervall

with the relevant carbon source at 0.4% was used. Rich-MOPS medium was described by Neidhardt et al. (35). Antibiotics were used at concentrations of 50 μg/ml for carbenicillin, 12.5 μg/ml for chloramphenicol, 50 μg/ml for kanamycin, and 100 μg/ml for spectinomycin in *E. coli* and 800 μg/ml for spectinomycin in *S. enterica*.

Genetic techniques. The Hfr mapping procedure was that of Singer et al. (48), and phage P1 transduction was done by the method of Miller (33). Transduction with phage P22 HT105/I (*int201*) (47) was done as described previously (17).

tRNA preparation, analysis of modified nucleosides, and mass spectrometry analysis. Bacterial strains were grown in LB 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 (20). tRNA was separated from rRNA by LiCl extraction (4). The tRNA was precipitated with 2.5 volumes of cold ethanol twice, washed 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 alkaline phosphatase (24). The resulting hydrolysate was analyzed by high-performance liquid chromatography (HPLC) by the method of Gehrke and Kuo (23) on a Supelcosil LC_{18} column (Supelco) with a Waters HPLC system. For mass spectrometry analysis, the nucleosides were eluted using the gradient of Pomerantz and McCloskey (40). The outlet from the UV detector was directly interfaced to a VG platform mass spectrometer equipped with an electrospray ionization source (Fison Instruments, Altrincham, United Kingdom). The UV chromatograms were recorded continuously and the mass spectra were recorded every 1.0 s during the 60-min chromatography. The procedure and interpretation of data for qualitative liquid chromatography-mass spectrometry analysis of nucleosides in RNA hydrolysate by a soft ionization technique have been described previously (40).

DNA sequencing and bioinformatics. DNA sequencing of PCR fragments purified by the PCR Kleen spin kit (Bio-Rad) or of plasmid DNA purified by the Nucleospin kit (Clontech) was performed in an ABI Prism 377 DNA sequencer. DyeEnamic ETerminator cycle-sequencing premix (Amersham-Biotech) was used for sequencing. The sequences were analyzed using the University of Wisconsin Genetics Computer Group programs for sequences from *S. enterica* serovar Typhimurium (http://genome.wustl.edu) and the BLAST server at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Multiple sequence alignments were conducted using global progressive algorithms (MAP program, available at http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Phylogenetic analysis was carried out, and neighbor-joining trees were constructed using the PHYLIP software package with the Fitch method (on-line server at the Pasteur Institute: http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html).

In vivo labeling with $^{35}SO_4^{2-}$. Cells were grown in 15 ml of MOPS minimal medium containing 0.081 mM K_2SO_4 supplemented with 0.4% glucose. Radioactive sulfate (final concentration, 150 μCi/ml) was added to the culture, and the cells were grown to 150 Klett units. tRNA was prepared as described above. The labeled tRNA was digested to nucleosides, and the hydrolysate was subjected to HPLC analysis. The radioactivity of the eluate was monitored with a 500TR flow scintillation analyzer (Packard) coupled to the UV detector of the HPLC system.

Construction of mutants. An in-frame deletion in the *ttcA* gene was constructed by the method of Link et al. (31). Flanking primers A (5′-CGC GTC GAC CGC AGG GAT GGT TCG CAT TG-3′) and D (5′-CGC GAG CTC GCT CCA CGG CGA TCT GGA AC-3′) complementary to sequences located about 600 bp outside the gene were used together with primers B (5′-CCC ATC CAC TAA ACT TAA ACA GTT ACG GCG CAG GCG CTT TTG-3′) and C (5′-TGT TTA AGTT TAG TGG ATG GGC CGG AAG AAG ATG ACA CCG CC-3′), which were complementary for DNA 36 bp internal from the beginning and 54 bp internal from the end of the *ttcA* gene, respectively. Primers B and C contained an overlapping region at their 5′ ends (underlined bases), with an additional 21 bases coding for an amino acid sequence not present in bacteria. PCR fragments generated by primer pairs A-B and C-D were purified from an agarose gel and mixed in a 1:1 ratio. A new PCR was performed with the mixture using primers A and D at an annealing temperature of 50°C. A fragment of 1,234 bp was purified and cloned into the pGEM(T/A) vector (Promega). The construct was sequenced, and the entire fragment was cloned into the *Sal*I-*Sph*I site of the vector pDM4 (34). Using this suicide vector, which harbors the *sacB* gene that makes the bacteria unable to grow on 5% sucrose, the constructed in-frame deletion between codons 25 and 292 was transferred to the chromosome.

The two cysteines at positions 122 and 125 in the TtcA protein were altered by the Quikchange protocol (Stratagene). The wild-type allele of the *ttcA* gene from *S. enterica* was amplified by PCR using primers s^2C-st1 (5′-CGT CGC CGT ACG TCA AGC AG-3′) and s^2C-st2 (5′-GTA GCG ATG TTG AAT GCG CG-3′), which were complementary to sequences 120 bp 3′ and 140 bp 5′ of the *ttcA* gene, respectively, and cloned into the pGEM(T/A) vector. Primers cysmutL (5′-CCG ACG CAA ACG CGA CGC CAG CGA CGC GGT GGT TTT TCC-3′) and cysmutR (5′-GGA AAA ACC ACC GCG TCG CTG GCG TCG CGT TTG CGT CGG-3′) were used to change the cysteine codons (TGC) into alanine codons (GCG; underlined). The *Sal*I-*Sph*I fragment was cut out and cloned into the suicide vector pDM4. This mutated fragment was later transferred to the chromosome as described previously (34).

Single-amino-acid changes of Cys 122 or Cys 125 in the TtcA protein were made as follows. First, a kanamycin resistance cassette was inserted 9 nucleotides downstream of the *ttcA* gene by using linear transformation (16). The chromosome of the resulting strain was then used as a template for PCR, where one of the primers bound within the Km^r cassette and the other primer bound to a site where the mutation was going to be introduced. This second primer was 60 nucleotides long and contained 41 nucleotides complementary to the sequence upstream the mutated codon, AGC, and 16 nucleotides homologous to the downstream sequence (designed to place a Ser instead of the Cys present in the wild type). The obtained PCR fragment of 2.2 kb was purified and used in linear transformation using electroporation of the wild-type *S. enterica*. Km^r transformants were selected for and screened for the mutated gene. Individual transformants were used to generate PCR fragments over the region of *ttcA*, where the mutation was constructed. Because the mutagenic change in sequence also introduced a *Sac*I (C125S) or an *Alu*I (C122S) restriction site, we used restriction analysis of the PCR fragments to differentiate between the wild-type and mutated *ttcA* forms present in the transformants.

Determination of A-site selection and P-site frameshifting activity. The rate of aminoacyl-tRNA (aa-tRNA) selection to the A-site and the P-site frameshifting ability were determined as described previously (11, 13, 54).

Determination of sensitivity to various amino acid analogs. Strains GT5969 (*ttcA*$^+$) and GT5970 (Δ*ttcA1*) were tested for the sensitivity to different amino acid analogs as described previously (10, 21). Strains were grown overnight in LB rich medium at 37°C. A sample (0.1 ml) was mixed with 2 ml of 0.5% agar in 0.9% NaCl and poured onto an agar plate containing medium E (55) and 0.2% sodium citrate. Paper disks (6 mm in diameter) were placed on the surface of the plates, and a certain amount of each analog as specified by Ericsson and Björk (21) was applied to the respective disk. The plates were scored after incubation at 37°C for 24 h. The following analogs were tested: DL-aspartic acid β-hydroxamate (Asn), 1,2,4-triazole (Cys), L-glutamic acid γ-hydrazide (Gln), DL-methi-

onine hydroxmate (Gln), azaserine (Gln), L-methionine-DL-sulfoximine (Gln), 1,2, 4-DL-triazole-3-alanine (His), *S*-2-aminoethyl-L-cysteine (Lys), β-(2-thienyl)-DL-alanine (Phe), *m*-fluoro-DL-phenylalanine (Phe), thioproline (Pro), L-thiazolidine-4-carboxlicacid (Pro), DL-5-fluorotryptophan (Trp); 5-methyl-DL-tryptophan (Trp), 3-aminotyrosine (Tyr), β-DL-hydroxynorvalin (Tyr), *m*-fluoro-DL-tyrosine (Tyr), fluoroacetic acid (acetate), 3,4-dehydro-DL-proline, and 3-nitrotyrosine. All analogs were obtained from Sigma Chemical Co., St. Louis, Mo.

Biolog MicroPlate assay. ES MicroPlates were purchased from Biolog Corp. and used as recommended by the manufacturer. Log-phase cells (grown in LB broth at 37°C) were washed and then diluted in 0.9% NaCl to an optical density at 590 nm of 0.35. Diluted cells (150 μl) were added to each well, and the plates were incubated at 37°C without shaking. The optical density at 620 nm was measured by a Titertek Multiscan MCC/340 reader after 6 and 16 h of incubation.

Analysis of sulfur metabolism. To investigate the sulfur metabolism in the deletion mutant, strains GT5969 and GT5970 were grown in MOPS minimal medium supplemented with different sulfur compounds as a sole sulfur sources. The carbon source in the experiment was succinate, and the cells were grown at 37°C. The following sulfur sources were used: *S*-methylcysteine, methionine sulfone, *N*-acetyl-L-cysteine, and isethionic acid. All chemicals were purchased from Sigma Chemical Co.

RESULTS

Isolation and characterization of *ttcA1* mutants of *E. coli* and *S. enterica*. *E. coli* GRB105 lacks cmo^5U in its tRNA as a result of the *aroE* mutation (7) and is also deficient in m^6t^6A and in m^2A (43). Moreover, tRNA from this strain is also deficient in a compound with migration properties similar to those of a compound suggested to be s^2C on the basis of the published retention time (23). The mutation causing the lack of this unknown compound was named *ttcA1*, and conventional mapping placed it in the area of 28 to 33 min on the chromosomal map of *E. coli*. DNA was prepared from an ordered set of lambda transducing phages covering this region of the chromosome and subcloned. One of the obtained plasmids, pGJ8, complemented the *ttcA1* mutation. Cloning of single open reading frames generated plasmid pGJ13, which contained an open reading frame, annotated as *ydaO* (b1344), and which complemented the *ttcA1* mutation. We conclude that the annotated *ydaO* gene in *E. coli* is the *ttcA* gene.

We have for many years studied the formation of another thiolated nucleoside, ms^2io^6A37, in *S. enterica*. Moreover, we have isolated a mutant of *S. enterica* which is distinct from the *ttcA1* mutant of *E. coli* and is defective in the synthesis of both s^2C and ms^2io^6A (29a). To investigate the relationship between these two mutations, we decided to make an in-frame deletion in the *ttcA* (STM1654) gene of *S. enterica* and use this deletion mutant to study the physiological consequences of s^2C32 deficiency for *S. enterica*. tRNA modification patterns from the two resulting congenic strains, GT5969 ($ttcA^+$) and GT5970 (Δ*ttcA1*), differing only in the allelic state of the *ttcA* gene, were determined by HPLC analyses. Only one compound was lacking in the tRNA from the mutant with respect to the tRNA from the congenic wild-type strain (Fig. 1). After introducing the plasmid pGJ13, which contains the *E. coli* $ttcA^+$ gene, into the Δ*ttcA1* mutant, the same compound reappeared in the tRNA (data not shown). We identified this compound as s^2C by the following criteria. (i) The migration in the chromatography system developed by Gehrke and Kuo (23) was similar to that observed for s^2C in their analysis. (ii) The UV spectrum is the same as that published for s^2C (23). (iii) By mass spectrometry, the molecular weight of the unknown compound was the same as that of the protonated form of s^2C (data not shown). (iv) The ^{35}S-labeled nucleoside migrating as s^2C is present in wild-type tRNA but absent in tRNA from the mutant (data not shown). Hence, we conclude that the *ttcA* gene product is required for the thiolation of C32 to form s^2C32 in tRNA.

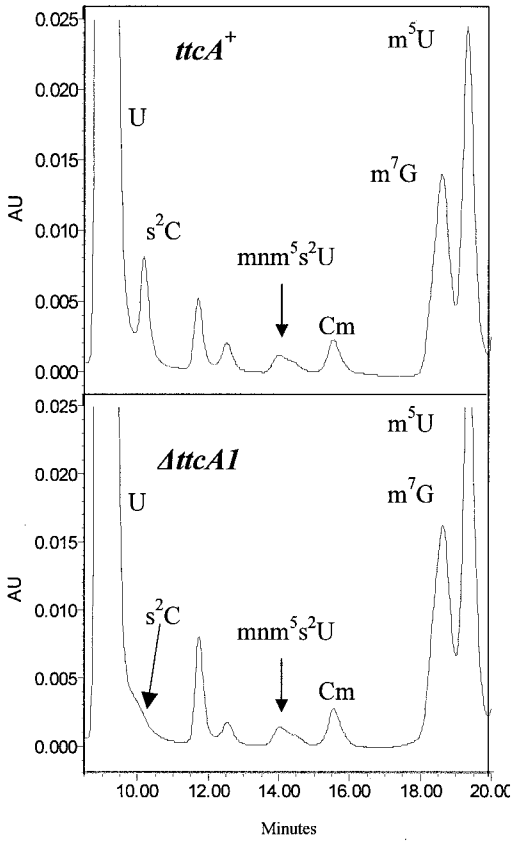

FIG. 1. HPLC chromatograms of tRNA hydrolysates from wild-type $ttcA^+$ (top) and the Δ*ttcA1* mutant (bottom). The nucleosides were monitored at 254 nm. The positions of U, s^2C, mnm^5s^2U, Cm, m^7G, and m^5U are indicated. The arrow in the bottom panel denotes the position where s^2C32 should migrate if present. Only part of the chromatogram (5- to 25-min retention time) is shown.

TtcA is part of a broadly distributed protein family. Sequence analysis of TtcA reveals that it contains several motifs that are conserved in similar proteins from a wide variety of organisms. These elements are located in the central domain of the protein and include the SGGKDS motif (positions 47 to 52) characteristic of ATPases in the PP-loop superfamily as well as the C-X_1-X_2-C motif (positions 122 to 125), which is characteristic of proteins in the thioredoxin superfamily. Alignment of over 125 similar proteins from available sequence databases revealed that these proteins cluster into two major groups based both on elements in the intervening sequence of the central domain and on conserved motifs in the N and C termini (Fig. 2). The presence and juxtaposition of the PP-loop and C-X_1-X_2-C motifs (designated T1 in Fig. 2) is the unifying attribute for this family of proteins. The subgroup of this family that includes TtcA from *S. enterica* contains one additional C-X_1-X_2-C motif in the C terminus of the protein. The other major subgroup, which includes almost all family members

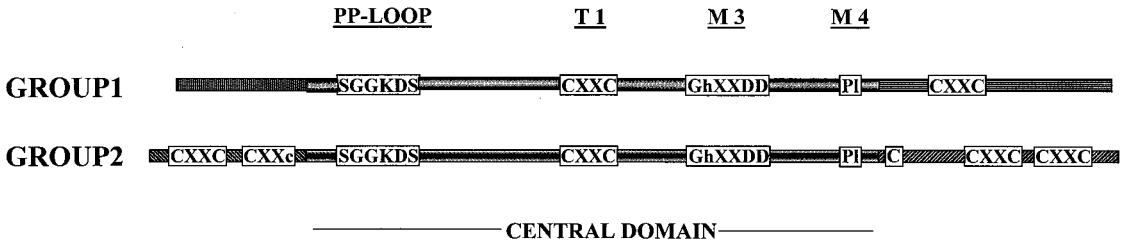

FIG. 2. Domain structure of TtcA family proteins. There are two major subgroups of proteins closely related to TtcA, as exemplified by the known 74 bacterial members and 1 eukaryotic member of group 1 and by the 26 archeael, 6 bacterial, and 16 eukaryotic members of group 2. The presence of the four indicated motifs and their juxtaposition within the central domain is the unifying feature of the TtcA family as a whole. T1 is the only thioredoxin-like motif whose position is conserved in all family members. Sequences intervening between these motifs and the terminal domains of these proteins partition into two major groups, as shown. Lowercase letters represent residues that are not entirely conserved.

from eukaryotic and archaeal species, contains two additional C-X_1-X_2-C motifs at the N terminus and five additional conserved cysteines at the C terminus of the protein, four of which are spaced as C-X_1-X_2-C motifs.

TtcA family members are present in all major bacterial taxa (where genomic data are available), except for the actinobacteria and cyanobacteria, but are present only sporadically in eubacteria outside of the β and γ proteobacteria (Fig. 3). In *Firmicutes*, homologs are found primarily in the clostridia, many of which contain several homologs in group 1. Horizontal transfer seems to account for the presence of this gene in many organisms as a phylogenetic tree representing the relationship between these proteins does not correspond in many places to a standard tree based on rRNA sequences (data not shown). Many archaeal species possess two distinct genes of the TtcA family, both in group 2. Family members from *Aquifex*, *Thermotoga*, and other "archaeon-like" eubacteria partition with the partial group of archaeal homologs (Archaea I in Fig. 3). All archaeal species represented in cluster Archaea I are also present in cluster Archaea II, which partitions more closely to the eukaryotic family members. There also exists one example of a eukaryotic group 1 homolog from *Giardia lamblia*.

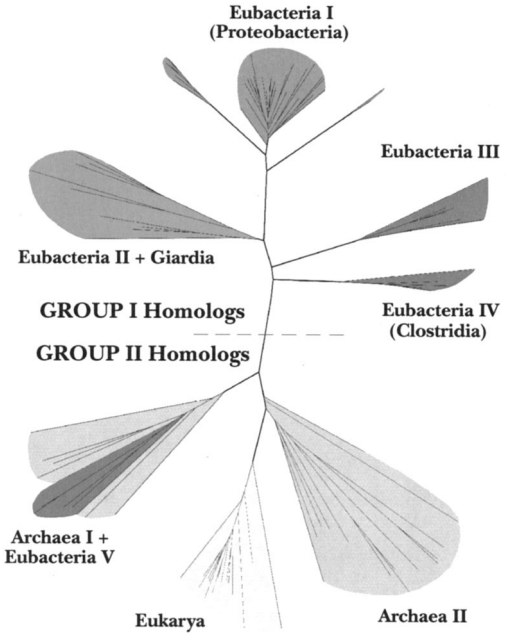

FIG. 3. Phylogenetic tree depicting the relationship between the various groups of TtcA family proteins. Group I homologs in the top half of the diagram cluster into several branches which constitute different eubacterial lineages, and the second of these also includes a representative from the *Eucarya* (*Giardia lamblia*). Group II homologs in the bottom half of the tree cluster into three distinct groups. The cluster labeled Archaea I + Eubacteria V includes TtcA homologs which are more similar to the group I proteins than to their counterpart archaeal homologs in the branch labeled Archaea II. The eubacterial representatives in this cluster are also predominantly from deeply branching "archaeon-like" bacteria.

The two cysteines at positions 122 and 125 in the central C-X_1-X_2-C motif of TtcA are both required for the formation of s^2C. Since the -CX_1X_2C- sequence is conserved in the TtcA protein, it was of interest to investigate its influence on the formation of s^2C in tRNA. We therefore replaced these two Cys residues with Ala in strain GT6443 [*ttcA2* (C122A; C125A)]. Next, we exchanged Cys122 for Ser122 [strain GT6635, *ttcA3* (C122S)] and Cys125 for Ser125 [strain GT6636, *ttcA4* (C125S)]. tRNA from these three strains was analyzed by HPLC, and all three strains lacked s^2C (data not shown). Hence, both cysteines in the conserved T1 motif C-X_1-X_2-C are essential for the activity of TtcA in the formation of s^2C in tRNA.

Lack of s^2C does not affect the steady-state growth rate, nor does it show any growth disadvantage in a mixed-population experiment. Several mutants lacking a modified nucleoside in the tRNA grow more slowly than their wild-type counterparts do (8). We therefore investigated whether the s^2C deficiency influences growth rate in various media. Steady-state growth cultures of the congenic strains GT5969 (*ttcA*$^+$) and GT5970 (Δ*ttcA1*) were grown in MOPS-glucose, MOPS-acetate, and Rich-MOPS media at 37°C. We did not observe any difference in growth rates between the wild type and the Δ*ttcA1* mutant in the three tested media (data not shown).

Next, we did a mixed-population experiment, which compares not only the growth in logarithmic phase but also the ability to sustain and to recover from stationary phase. The Δ*ttcA1* mutant and the wild type were mixed in a ratio of about 1:1 in LB medium. The culture was inoculated and grown at 37°C overnight into stationary phase and diluted 200-fold each day. On day 7 the bacteria were plated onto TYS plates. The colonies were screened for the mutant versus wild-type genotype by PCR. A total of 61 clones were tested from the samples

taken on day 1, just after mixing the strains, and 99 clones were tested from the samples taken on day 7. The ratio between Δ*ttcA1* and *ttcA*$^+$ cells was the same on day 1 as it was on day 7 following about 340 doublings in mass (day 1, 60.6% of the clones were Δ*ttcA1*; day 7, 61.6% were Δ*ttcA1*; the *t* test showed no statistical difference). Hence, the *ttcA1* mutation did not influence the ability of the mutant to compete with the wild type in a growth competition experiment in the rich medium LB broth.

Lack of s^{2}C does not affect the growth response to 32 amino acid analogs nor oxidation of various compounds. It was shown previously that mutants deficient in ψ38, ψ39, and ψ40 (10), ms^{2}io^{6}A37 (21, 22), or m^{1}G37 (30) show an altered response to various amino acid analogs, suggesting an altered expression of the corresponding biosynthetic enzymes. We therefore tested 20 analogs, but the responses of the wild type and of the Δ*ttcA1* mutant were the same for all tested analogs.

Some tRNA modification mutants oxidize certain compounds differently from their congenic wild-type strains, suggesting a changed metabolism (22, 30). Therefore, we tested the ability of the Δ*ttcA1* mutant to oxidize 95 compounds. Indicator plates (Biolog ES MicroPlates) that contained different test compounds and the dye 2,3,5-triphenyl tetrazolium chloride were used (9). If the compound is oxidized, the redox dye is reduced and gives a purple color. However, the Δ*ttcA1* mutant responded similarly to the wild-type control to all compounds.

Sulfur metabolism of the Δ*ttcA1* mutant. Although one phenotype induced by the deletion of the *ttcA* gene was the deficiency in thiolation of C32 in tRNA, this phenotype might be a secondary consequence of a defect in sulfur metabolism prior to the sulfur transfer reaction leading to the formation of s^{2}C. We therefore tested the ability of the Δ*ttcA1* mutant to reduce thiosulfate or sulfite to H_2S. Compared to the wild type, no difference in H_2S production was noticed for the Δ*ttcA1* mutant (data not shown).

The two strains GT5969 (*ttcA*$^+$) and GT 5970 (Δ*ttcA1*) were grown overnight in MOPS-succinate minimal medium with 0.081 mM sulfate as the sole sulfur source. The cultures were diluted 20-fold into a different minimal medium containing 0.552 mM *S*-methylcysteine, methionine sulfone, *N*-acetyl-L-cysteine, or isethionic acid instead of sulfate as the sole sulfur source. The wild-type strain and the mutant strain grew equally well on the different sulfur sources.

The presence of s^{2}C32 in tRNA$^{Arg}_{mnm^5UCU}$ increases the rate of selection at A-site located AGG codons. The synthesis of release factor 2 (RF2) requires a frameshift to avoid a stop codon in the zero frame. The frequency of frameshifting, which occurs in the P-site, is dependent on the rate of selection of the ternary complex (aa-tRNA/EF-Tu/GTP) at the codon in the ribosomal aa-tRNA site (A-site). This RF2-programmed frameshifting site has been placed upstream of the *lacZ* gene in such a way that the synthesis of β-galactosidase requires a shift into the +1 frame (13). At this RF2-programmed frameshifting site, frameshifting competes with the in-frame decoding at the A-site, which makes the frameshift-dependent β-galactosidase activity inversely proportional to the rate of aa-tRNA selection at the A-site. The tRNA$^{Arg}_{ICG}$, which contains s^{2}C32, decodes CGU, CGC, and CGA. Plasmids with these codons as A-site codons in the programmed frameshifting site were introduced into the congenic strains GT5969 (*ttcA*$^+$) and GT5970 (Δ*ttcA1*). However, in all three cases, lack of s^{2}C32 did not influence the rate of selection of Arg-tRNA$^{Arg}_{ICG}$ (Data not shown).

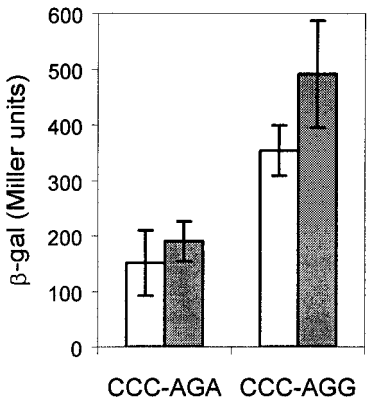

FIG. 4. The rate of A-site selection at AGA and AGG codons in wild-type (open bars) and Δ*ttcA1* mutant (gray bars) strains was determined by the efficiency of programmed +1 frameshifting at the *prfB* gene, which replaces the regulatory UGA codon by the indicated codons (13). The +1 frameshifting by tRNA$^{Pro}_{cmo^5UGG}$ decoding CCC in the P-site is influenced by the rate of Arg-tRNA selection at the AGA or AGG codon at the A-site. The difference observed at the CCC-AGG site is significant based on a *t* test ($P = 0.023$).

The rare AGA codon is decoded by tRNA$^{Arg}_{mnm^5UCU}$, whereas either tRNA$^{Arg}_{mnm^5UCU}$ or tRNA$^{Arg}_{CCU}$ decodes AGG. The rate of selection of these tRNAs at AGA or AGG codons was determined by using a nonprogrammed +1 frameshifting site. We have previously shown that at a site CCC-N, in which the CCC codon is in the zero frame and the CC-N codon is in the +1 frame, the frameshifting event occurs by tRNA$^{Pro}_{cmo^5UGG}$ slippage at the CCC codon in the P-site (41, 42). The frequency of this slippage is inversely dependent on the rate of selection of the ternary complex at the A-site. Plasmids containing CCC AGA or CCC AGG as frameshifting sites were introduced into congenic strains differing only in the allelic state of the *ttcA* gene. As expected, in the wild-type strain the Arg-tRNA selection at AGG was less efficient (higher β-galactosidase) than at the AGA codon (Fig. 4). Interestingly, an s^{2}C32-dependent enhancement of about 30% was observed for the selection at the AGG codon. We conclude that the presence of s^{2}C32 in tRNA$^{Arg}_{mnm^5UCU}$ increases the Arg-tRNA selection at the AGG codon.

The presence of s^{2}C32 in tRNA$^{Arg}_{mnm^5UCU}$ interferes with the decoding in the A-site. The s^{2}C32 modification is located on the 5′ side of the anticodon loop of the tRNA. When the tRNA is residing in the P-site, its 5′ side is facing the A-site tRNA. In an assay monitoring how changes in the P-site tRNA influence the aa-tRNA selection at the A-site, a nucleoside change in position 32 had a strong influence (49). Therefore, it was of interest to investigate whether the thiolation of C32 also influenced the ability of tRNA to read the A-site codon. To do this we used an assay system that monitors the influence of the P-site tRNA on readthrough/termination (level of suppression) at the UAG codon in the A-site. The assay measures the interference in vivo by the P-site-located peptidyl-tRNA with the decoding activity in the A-site. Only at one site, AGG-

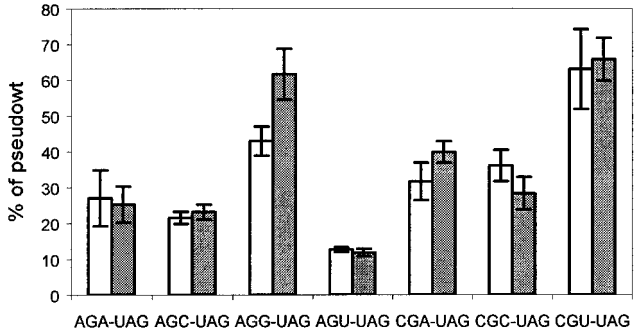

FIG. 5. Influence of the s^2C32-containing peptidyl-tRNA on *supF30*-mediated suppression of UAG. Various plasmids containing P-site codon(s) CGA CGC, CGU, AGA, AGC, AGG, or AGU were introduced into wild-type (open bars) and Δ*ttcA1* mutant (gray bars) strains, and readthrough was monitored by measuring β-galactosidase activity. A significant difference between the wild type and the mutant was observed only in the case of AGG as the P-site codon ($P < 0.0003$).

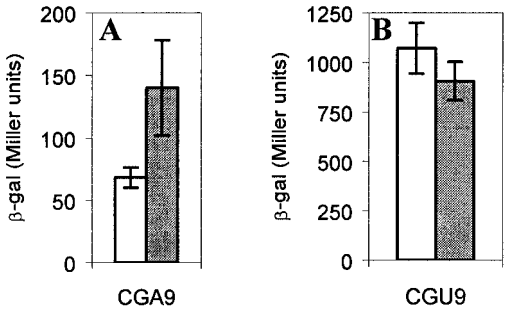

FIG. 6. Efficiency of tRNA in translating nine consecutive CGA codons (pJC1113) (A) or nine consecutive CGU codons (pJC1119) (B) (12). β-Galactosidase activity (β-gal) was measured in wild-type (open bar) and Δ*ttcA1* mutant (gray bar) strains. Standard error is indicated by the error bars. A *t* test indicated that the difference in the wild type and in the Δ*ttcA1* mutant was significant in panel A ($P = 0.0003$)

UAG, of the seven sites tested did we observe a difference between the mutant and the wild type (Fig. 5). In that site, at the time when the suppressor tRNA reads the UAG codon, the P-site AGG codon is decoded either by $tRNA^{Arg}_{mnm^5UCU}$ or by $tRNA^{Arg}_{CCU}$. A 40% increase in the readthrough was observed in the mutant, suggesting that the presence of s^2C32 in the P-site tRNA decreased the decoding efficiency of the UAG suppressor tRNA or increased the ability of RF1 to decode UAG.

s^2C32 in $tRNA^{Arg}_{ICG}$ decreases the rate of translation of the CGA codon but not of the CGU codon. One way to increase a small difference in the efficiency of reading a codon is to measure the rate of migration of the ribosome over a repeated sequence of the same codon. Curran (12) has constructed two plasmids (pCGA9 and pCGU9), which have nine consecutive CGA or CGU codons, respectively. These consecutive codons are translationally coupled to the *lacZ* mRNA in such a way that the synthesis of β-galactosidase requires that the ribosomes translate the upstream cistron with the consecutive codons, terminate, and then reinitiate at the AUG codon of *lacZ* (12). The frequency of reinitiating is dependent on the number of ribosomes terminating at the end of the upstream cistron. In wild-type cells the CGU codons were translated more rapidly than the CGA codons (Fig. 6), in agreement with earlier results (12). Whereas there was no difference between the wild type and the Δ*ttcA1* mutant in reading the CGU codons, a twofold increase was observed in the mutant in reading the CGA codons. Surprisingly, the lack of s^2C32 in $tRNA^{Arg}_{ICG}$ increased the rate of decoding of the CGA codons, revealing that the presence of this modified nucleoside may have a negative modulating effect on the activity of $tRNA^{Arg}_{ICG}$.

DISCUSSION

The IscS protein is pivotal in the synthesis of the five thiolated nucleosides present in tRNA of *S. enterica*. Whereas the synthesis of s^4U and the s^2U moiety of (c)mnm^5s^2U has recently been elucidated (26, 27, 38), the syntheses of ms^2io^6A and s^2C following the participation of IscS are much less understood. Studies of the Δ*iscS* mutant revealed that formation of ms^2io^6A and s^2C can proceed, albeit with low efficiency, in an IscS-independent way (29, 37). Thiolation of both of those nucleosides requires the presence of IscU, which is a scaffold for [Fe-S] cluster assembly (29a). This suggests that [Fe-S] proteins are involved in the thiolation of s^2C32 and ms^2io^6A37. MiaB, which catalyzes the last step in the synthesis of ms^2io^6A, is a Radical SAM enzyme containing an [Fe-S] cluster (39). Biochemical characterization of the TtcA protein, which contains several conserved cysteines, will reveal whether TtcA is the [Fe-S] protein involved in the synthesis of s^2C32. Still, we conclude that the TtcA peptide appears to act in a manner distinct from the ThiI and MnmA enzymes but may share the requirement of [Fe-S] cluster assembly proteins with MiaB.

Proteins closely related to TtcA are found in a wide variety of organisms in all three domains, but s^2C32 has not been identified in many of these (e.g., *Saccharomyces cerevisiae* contains a TtcA family member, but no s^2C is known to exist in its tRNA). There are only limited data concerning the taxonomic distribution of s^2C32 itself, but it is consistent to speculate that group 1 family members, as defined here (Fig. 2), play a role in mediating reactions that ultimately lead to the production of s^2C32 in certain tRNAs and to assume that group 2 family members from the *Eucarya* and the *Archaea* II group (Fig. 3) are present in species where enzymatic interactions resulting from a similar type of reaction do not lead to the formation of s^2C32 in tRNA. The central domains of all these proteins contain enough similar features to suggest that they are all performing reactions of a similar nature, perhaps to generate a sulfur intermediate that is involved not only in the synthesis of s^2C32 in some species but also in another part of general sulfur metabolism. In species where a TtcA family member does in fact mediate interactions that lead to the formation of s^2C32 in tRNA, it is likely that the enzyme associates directly with a tRNA substrate because of the high degree of similarity to other PP-loop proteins known to interact with tRNA (e.g TilS [51]).

It is generally assumed that the tRNA anticodon loop comprises 7 nucleotides. However, since a bifurcated hydrogen bond contact is present between the first base (position 32) and the last base (position 38) in the anticodon loop (44, 56), such a loop comprises 5 instead of 7 nucleotides. Although this contact is with only one hydrogen bond between the residues at

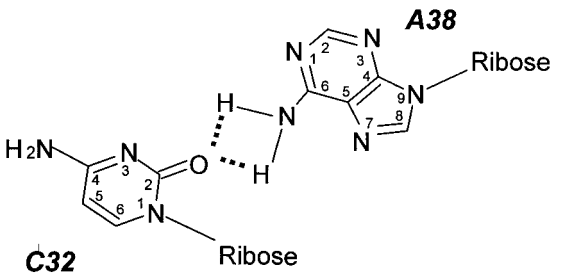

FIG. 7. Base pairing through a bifurcated hydrogen bond between the first (C32) and the last (A38) nucleotides in the anticodon loop. A dashed line indicates a hydrogen bond. This diagram was modified from reference 3.

positions 32 and 38 (Fig. 7), it is surprisingly strong, since it is not disrupted even if the tRNA is severely unfolded by its interaction with its cognate aa-tRNA synthetase (14, 15, 45, 46). All four s^2C32-containing tRNAs have an A38, and thus an S2(s^2C32)—N6(A38) bifurcated hydrogen bond is present (3). However, the N-H—S hydrogen bond may be weaker than the corresponding N-H—O hydrogen bond (18). If so, a change from an S2(s^2C32)—N6(A38) hydrogen bond to an O2(C32)—N6(A38) hydrogen bond as a result of a mutation in the *ttcA* gene should strengthen the interaction between these bases. On the other hand, a sulfur atom stabilizes stacking interactions by its greater polarizability than oxygen as well as its contribution to dispersion forces. Since the base at position 32 is partially stacked between bases at positions 31 and 33, the change from s^2C32 to C32 in the *ttcA* mutant should destabilize the interaction between positions 32 and 38. The final influence on the stability of the anticodon loop by these two opposing forces is not clear. In either case, the structure of the anticodon of *E. coli* $tRNA^{Arg}_{ICG}$ free in solution is influenced by s^2C32, since the nuclease digestion pattern is dependent on the presence of this modification (5). We have shown that selection of Arg-$tRNA^{Arg}_{mnm^5UCU}$ to the AGG codon (Fig. 4), peptidyl-$tRNA^{Arg}$ interference with A-site decoding (Fig. 5), and the rate of decoding of CGA by $tRNA^{Arg}_{ICG}$ (Fig. 6) are dependent on s^2C32. These results also suggest that this postulated s^2C32-A38 interaction is important to keep the anticodon loop in its proper 5-base-loop structure.

Arginylation of $tRNA^{Arg}_{ICG}$ in vitro, formation of a ternary complex, and binding of Arg-$tRNA^{Arg}_{ICG}$ to the four CGN triplets were not dependent on s^2C32 (28). We also did not observe any dependence on s^2C32 for the Arg-$tRNA^{Arg}_{ICG}$ selection at the A-site-located CGN codons (data not shown). However, the rate at which $tRNA^{Arg}_{ICG}$ read nine contiguous CGA codons was higher for s^2C32-depleted $tRNA^{Arg}_{ICG}$ than for wild-type s^2C32-containing $tRNA^{Arg}_{ICG}$ (Fig. 6). This may seem inconsistent, since the wild-type form of the tRNA is apparently less efficient that the mutant form (Fig. 6). However, the translation apparatus is optimized and reflects a trade-off between speed and cost of accuracy (19). Therefore, the role of s^2C32 in this optimization appears to be as an enhancement of fidelity. We observed the s^2C32 dependence of translation rate only when $tRNA^{Arg}_{ICG}$ decoded the CGA codon, not when it decoded the CGU codon (Fig. 6). The latter codon is more efficiently decoded than the CGA codon due to the poor interaction of the I34 wobble nucleoside with the A-ending codon (Fig. 6) (12). Thus, the impact of s^2C32 on the function of the tRNA appears to depend on which codon is read. Alternatively, the impact of s^2C32 abolition may be more easily observed when the interaction is inefficient. Similarly, we observed an s^2C32 dependence in the decoding of AGG but not of AGA. The AGA codon is read by $tRNA^{Arg}_{mnm^5UCU}$, whereas the AGG codon is read efficiently by $tRNA^{Arg}_{CCU}$ and less well by $tRNA^{Arg}_{mnm^5UCU}$. We observed an increased readthrough at the A-site-located UAG codon when $tRNA^{Arg}_{CCU}$ or $tRNA^{Arg}_{mnm^5UCU}$ was present in the P-site and interacting with AGG, but not when $tRNA^{Arg}_{mnm^5UCU}$ was present in the P-site and interacting with AGA (Fig. 5). Since it is not likely that the tRNA in the P-site directly interacts with a tRNA in the A-site, this interference may be caused by a direct interaction between the P-site tRNA and RF1 decoding the stop codon in the A-site. Alternatively, the P-site-located tRNA may induce a conformational change of the A-site that influences the decoding by either RF1 or a tRNA causing readthrough. Considering these findings together, although the lack of s^2C32 does not influence the physiology of the bacterium as monitored by growth rate, several specific steps in the translational decoding process were affected, suggesting that the function of s^2C32 could be to modulate the flexibility of the anticodon loop, thereby optimizing the decoding process.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Cancer Foundation (Project 680) and Swedish Science Research Council (Project BU-2930).

We thank Kerstin Jacobsson for her skilful analysis of modified nucleosides by HPLC, and we thank Tord Hagervall for critical reading of the manuscript. We also thank Tord Hagervall for the generous gift of plasmids pTH34 and pTH35.

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