JOURNAL OF BACTERIOLOGY, Dec. 1993, p. 7776–7785 Vol. 175, No. 24
0021-9193/93/247776-10$02.00/0
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Isolation of the Gene (*miaE*) Encoding the Hydroxylase Involved in the Synthesis of 2-Methylthio-*cis*-Ribozeatin in tRNA of *Salmonella typhimurium* and Characterization of Mutants

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Received 6 July 1993/Accepted 6 October 1993

The modified nucleoside 2-methylthio-*N*-6-isopentenyl adenosine (ms^2i^6A) is present at position 37 (3′ of the anticodon) of tRNAs that read codons beginning with U except $tRNA^{Ser}_{I,V}$ in *Escherichia coli*. *Salmonella typhimurium* 2-methylthio-*cis*-ribozeatin (ms^2io^6A) is found in tRNA, probably in the corresponding species that have ms^2i^6A in *E. coli*. The gene (*miaE*) for the tRNA(ms^2io^6A)hydroxylase of *S. typhimurium* was isolated by complementation in *E. coli*. The *miaE* gene was localized close to the *argI* gene at min 99 of the *S. typhimurium* chromosomal map. Its DNA sequence and transcription pattern together with complementation studies revealed that the *miaE* gene is the second gene of a dicistronic operon. Southern blot analysis showed that the *miaE* gene is absent in *E. coli*, a finding consistent with the absence of the hydroxylated derivative of ms^2i^6A in this species. Mutants of *S. typhimurium* which have MudJ inserted in the *miaE* gene and which, consequently, are blocked in the ms^2i^6A hydroxylation reaction were isolated. Unexpectedly, such mutants cannot utilize the citric acid cycle intermediates malate, fumarate, and succinate as carbon sources.

The modified nucleoside 2-methylthio-*N*-6-isopentenyl adenosine (ms^2i^6A) is present at position 37 (next to the anticodon on the 3′ side) of tRNAs that read codons beginning with U, except $tRNA^{Ser}_{I,V}$, in *Escherichia coli* (32, 58). In *Salmonella typhimurium*, 2-methylthio-*cis*-ribozeatin (ms^2io^6A) is found in tRNA, probably in the corresponding species that have ms^2i^6A in *E. coli* (13, 39). The modified nucleoside ms^2io^6A is also present in the tRNAs of *Klebsiella pneumoniae*, *Serratia marcescens* (39), *Pseudomonas aeruginosa* (61), *Citrobacter freundii*, and *Enterobacter cloacae* (9) and in the plant pathogens *Agrobacterium tumefaciens*, *Rhizobium leguminosarum*, and *Corynebacterium fascians* (16) as well as in plant tRNA (34). However, it is not present in either of the facultative anaerobes *E. coli* (13) and *Proteus vulgaris* nor in the strict aerobe *Clostridium prefringens*, all three of which instead have ms^2i^6A in their tRNAs (9). In addition, the ms^2io^6A nucleoside and other i^6A derivatives are able to function as cytokinins in plants (for a review, see reference 56).

The biosynthesis of ms^2io^6A in *S. typhimurium* requires at least four enzymatic activities, and the corresponding genetic loci are designated *miaA*, *-B*, *-C*, and *-E* (Fig. 1). Mutants that are affected in the *miaA* gene of *S. typhimurium* (22), in the *miaA* gene (*trpX*) of *E. coli* [encoding the tRNA (i^6A37) isopentenyltransferase (14, 17, 72)], and in the *miaB* gene of *S. typhimurium* [probably encoding the tRNA (s^2i^6A37)synthetase (24)] have been isolated. A mutation in a locus (*miaD*) suggested to be involved in demodification of the ms^2i^6A nucleotide in *E. coli* has also been characterized (18). Interestingly, the absence of this modified nucleoside has several dramatic effects on gene regulation and cell physiology. A *miaA* mutant is affected in growth rate, attenuation regulation, and translational step time and has increased uptake and synthesis of aromatic amino acids (11, 21, 22, 30, 72, 74). These effects are most likely caused by a decreased efficiency and an increased codon context sensitivity of those tRNAs that normally contain ms^2i^6A37 or ms^2io^6A37 (5, 22, 48, 67). The i^6A level has been suggested to signal the availability of Δ2-isopentenyl pyrophosphate needed for the synthesis of, e.g., menaquinone and ubiquinone (9). The level of synthesis of the 2-methylthio group of ms^2io^6A is sensitive to the presence or absence of sulfur or iron and may function as a signal device for the availability of these compounds (9, 12). Furthermore, deficiency in the methylthiolation of i^6A to form ms^2i^6A37, or in the preceding isopentenylation step, leads to an increased spontaneous mutation frequency (GC-to-TA transversions) in *E. coli* (18). The last step, the hydroxylation of ms^2i^6A, in the pathway to form ms^2io^6A is dependent on the presence of molecular oxygen. The tRNA(ms^2io^6A37)hydroxylase is present under anaerobic conditions, but the hydroxylation reaction does not occur in the absence of oxygen (9), and it was suggested that ms^2io^6A may function as a regulator for aerobiosis, signalling the availability of oxygen.

To further study the synthesis and function of the hydroxyl group of ms^2io^6A, we cloned the *miaE* gene encoding the tRNA(ms^2io^6A37)hydroxylase from *S. typhimurium* by complementation of an *E. coli* strain naturally deficient in ms^2i^6A hydroxylation. Gene organization and complementation studies showed that the *miaE* gene is the second gene of a dicistronic operon encoding a 30,822-Da polypeptide. Mutants of *S. typhimurium* deficient in the tRNA(ms^2io^6A37) hydroxylase activity were obtained by insertion mutagenesis with transposon MudJ. The gene organization of the *miaE* operon is described below. Unexpectedly, *miaE*::MudJ mutants lacking the hydroxyl group of ms^2io^6A are unable to use some citric acid cycle intermediates as carbon sources.

MATERIALS AND METHODS

Strains, media, and enzymes. Strains and plasmids used are listed in Table 1. Rich liquid media were either LB of Bertani (2) or Difco nutrient broth (0.8%) (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl, adenine, tryptophan, tyrosine, phenylalanine, *p*-hydroxybenzoate, dihydroxybenzoate, and *p*-aminobenzoate. As rich solid medium, TYS-agar (10 g of Trypticase peptone, 5 g of yeast extract, 5 g of

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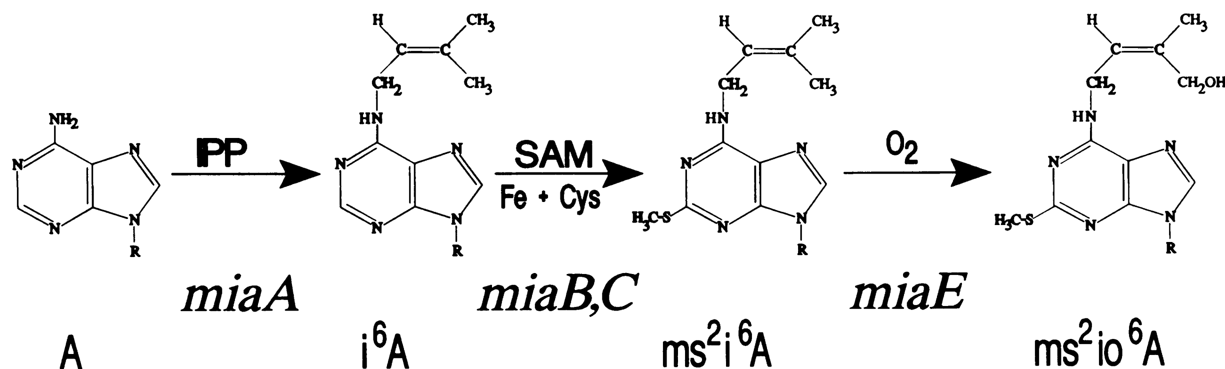

FIG. 1. Suggested biosynthetic pathway for ms^{2}io^{6}A in *S. typhimurium*. Known cofactors and substrates are indicated. The gene designations correspond to either identified genetic loci or postulated, but not yet identified, functions. IPP, Δ2-isopentenyl pyrophosphate (dimethylallyl diphosphate); SAM, *S*-adenosylmethionine.

NaCl, 15 g of agar per liter) was used. As defined liquid medium, MOPS (morpholinepropanesulfonic acid) medium (47) supplemented with 0.2% of the relevant carbon source was used. Plates were of E medium (68) with no citrate added, supplemented with agar (1.5%) and 0.2 to 0.4% of the relevant carbon source. Antibiotics kanamycin, carbenicillin, and spectinomycin were used in concentrations of 50 μg/ml. For maintenance of the pCL1921 plasmid derivatives in *S. typhimurium*, 400 μg of spectinomycin per ml was used. Enzymes were purchased from New England Biolabs (Beverly, Mass.) and Boehringer (Mannheim, Germany).

Isolation of *miaE*$^+$ plasmids. The *S. typhimurium* gene bank used in this study was kindly provided by Tord Hagervall, Umeå, Sweden. It consists of 20,000 clones harboring a partial *Sau*3A digest of chromosomal DNA from strain GT344 (*hisO1242 hisD6404*) inserted into the *Bam*HI site of the vector pLG339 (60). The gene bank was introduced into *E. coli* MM294, clones were individually grown and then pooled five by five, total RNA was prepared and digested to nucleosides, and the presence of ms^{2}io^{6}A was monitored by separation of the nucleosides by high-performance liquid chromatography (HPLC) (see below). One positive clone, harboring plas-

TABLE 1. Strains and plasmids

Strain	Description	Source or reference
S. typhimurium		
LT2	Wild type	John Roth[a]
TT10288	*hisD9953*::MudJ *hisA9944*::MudA	37
TT147	*argI1883*::Tn*10*	John Roth
GT2944	*miaE2506*::MudJ	This study
GT2947	*miaE2507*::MudJ	This study
GT3098	*miaE2506*::MudJ	This study
GT3099	*miaE2507*::MudJ	This study
TT10286	*hisD9953*::MudJ	37
E. coli		
MC1061	Δ(*ara-leu*) *araD139* Δ*lacX74 galU galK strA hsr*	15
MM294	*supE44 hsdR endA1 pro thi*	44
DH5α	F$^-$ f80d*lac*ZDM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* D(*lacZYA-argF*)U169	Bethesda Research Laboratories
Plasmids		
pAI1	Cb^r *argI*$^+$	49
pBR322	Ap^r Tc^r	4
pCL1921	Sp^r	41
pLG339	Tc^r Km^r	60
pUC19	Ap^r	71
pUST104	pLG339 derivative, Km^r *miaE*$^+$	This study
pUST107	15.5-kb chromosomal DNA fragment from pUST104 in pBR322, *miaE*$^+$	This study
pUST108	8-kb chromosomal DNA fragment from pUST107 in pUC19, *miaE*$^+$	This study
pUST114	pUC19 derivative, ORF15.6	This study
pUST115	*Nru*I cutback of pUST108, *miaE*$^+$	This study
pUST116	pUC19 derivative, *miaE*$^+$ ORF15.6	This study
pUST117	As pUST116 but with an internal in-frame deletion of ORF15.6	This study
pUST118	Same chromosomal insert as in pUST115 but inserted into pCL1921	This study
pUST119	Same chromosomal insert as in pUST116 but inserted into pCL1921	This study
pUST120	Same chromosomal insert as in pUST117 but inserted into pCL1921	This study
pUST121	Same chromosomal insert as in pUST114 but inserted into pCL1921	This study

[a] University of Utah.

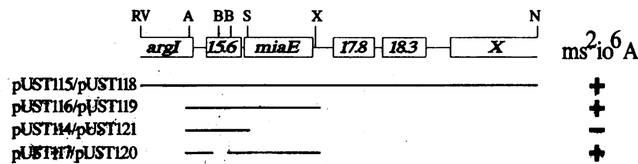

FIG. 2. The sequenced *miaE* region with putative ORFs and relevant restriction enzyme cleavage sites. RV, *Eco*RV; A, *Acc*I; B, *Bst*BI; S, *Sna*BI; X, *Bst*XI; N, *Nru*I. The regions of chromosomal DNA inserted into the plasmids are indicated. Plasmids pUST114 through pUST117 are high-copy-number pUC19 derivatives, and plasmids pUST118 through pUST121 are low-copy-number pLG339 derivatives. + and −, ability and lack of ability, respectively, to complement a strain deficient in tRNA(ms^2io^6A37)hydroxylase activity.

mid pUST104 with a chromosomal insert of 29 kb, was isolated. Plasmid pUST104 was digested with restriction enzyme *Hin*dIII, and the fragments were ligated to *Hin*dIII-digested pBR322 (4). A positive subclone (carrying plasmid pUST107, a 15.5-kb insert) was identified as described above. Plasmid pUST107 was digested with *Eco*RV and ligated to *Eco*RV-digested plasmid pUC19 (71). *E. coli* DH5α was transformed, and clones that were white on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were screened for the presence of ms^2io^6A. One MiaE$^+$ plasmid derivative was isolated and designated pUST108. Chromosomal inserts of other plasmids used in this study are shown in Fig. 2.

Analysis of tRNA nucleoside composition by HPLC. Total RNA was prepared essentially by the method of Isaksson and Phillips (38). tRNA was prepared as described by Buck et al. (10). RNA samples were digested to nucleosides according to the method of Gehrke et al. (28) by nuclease P1 (Boehringer) and bacterial alkaline phosphatase (Sigma) treatment. After centrifugation, appropriate amounts (usually 100 μg of total RNA or 50 μg of tRNA) were applied on a Supelcosil LC-18S column on a Waters HPLC system. The gradient used was a modification of that of Gehrke and Kuo (27): 0 to 12 min, 100% buffer A [2.5% methanol, 0.01 M $(NH_4)H_2PO_4$ (pH 5.3)]; at 20 min, 90% buffer A–10% buffer B [20% methanol, 0.01 M $(NH_4)H_2PO_4$ (pH 5.1)]; 25 min, 75% buffer A–25% buffer B; 32 min, 40% buffer A–60% buffer B; 36 min, 38% buffer A–62% buffer B; 45 min, 100% buffer B; 100 to 120 min, 100% buffer C [35% acetonitrile, 0.01 M $(NH_4)H_2PO_4$ (pH 4.9)]. The gradient change in condition between these points was linear. Flow rate and temperature were 1 ml/min and 26°C, respectively. The spectra of ms^2io^6A and ms^2i^6A both have absorption maxima at 242 and 283 nm (Fig. 3). Therefore, we routinely monitored the eluate at 242 nm to optimize detection of ms^2io^6A or ms^2i^6A.

Oligonucleotides, radioisotopes, and autoradiography. Oligonucleotides were synthesized on a Milligen Cyclone Plus DNA synthesizer (Millipore AB). Radioisotopes were purchased from Amersham. Autoradiography was performed by exposure to Amersham MP X-ray film.

DNA sequencing and computer analyses. Plasmid pUST115 was sequenced by using the T7 polymerase kit and the T7 7-deazaguanosine kit (Pharmacia). Sequences were assembled and characterized by using the GENEUS sequence analysis program (35). The obtained sequences were compared with those in the EMBL sequence data base by using the Wisconsin Genetics Computer Group package (20).

S1 mapping, primer extension analyses, and Southern and Northern blot hybridizations. RNA from *S. typhimurium* LT2 with and without plasmid pUST116 was prepared by the method of Sambrook et al. (54). S1 mapping and primer extension experiments were done essentially by the method of Sambrook et al. (54). The primer extension of the *argI* transcript was made with primer arg3F (5′-CAGTTTGGCG GCAAG-3′; positions 565 to 580 in the sequence submitted to the EMBL data base). The polymerase chain reaction fragment used for S1 nuclease protection of the *miaE* transcript was amplified from *S. typhimurium* LT2 cells with the help of primers arg3F and arg5B (5′-CTCTTCCACTTCCACGCTC-3′; positions 1029 to 1012 in the submitted sequence). Southern and Northern (RNA) blot hybridizations were performed by using Hybond-N filters and as recommended by Amersham, with the following exception. The RNA was separated on a denaturing glyoxal agarose gel (62). Radioactive probes for hybridizations were made by oligolabelling (25) of polymerase chain reaction products covering the DNA region of interest.

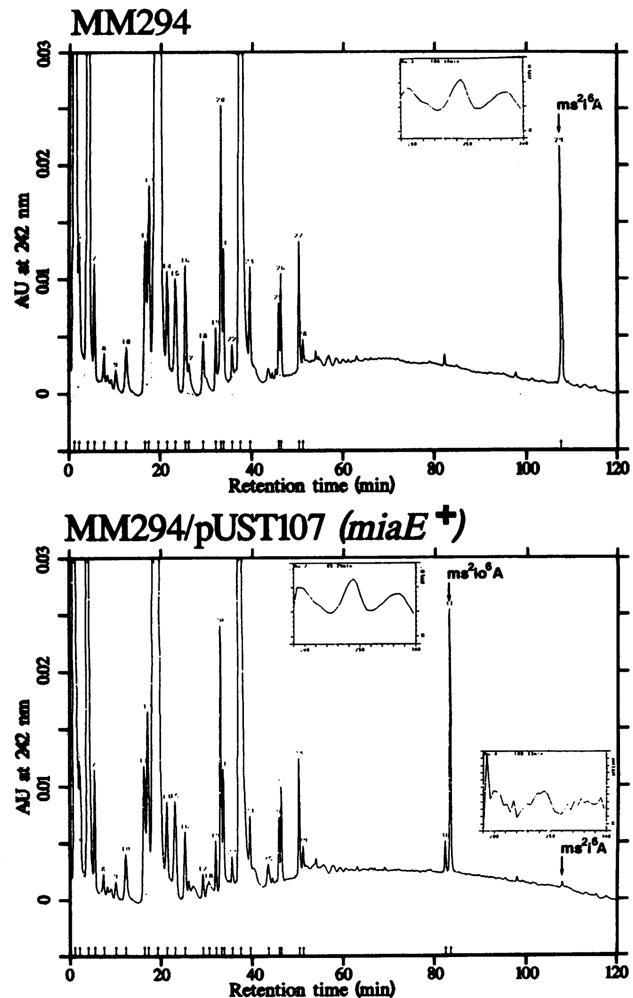

FIG. 3. HPLC chromatograms of RNA hydrolysates from the screening of subclones of pUST104. The spectra obtained from the ms^2io^6A and ms^2i^6A peaks are shown. The only nucleoside with a retention time similar to that of ms^2io^6A is i^6A, which when present elutes after ms^2io^6A and has a spectrum (λ_{max} = 210 and 270 nm) distinct from those of ms^2io^6A and ms^2i^6A (λ_{max} = 242 and 283 nm for both). AU, absorption units.

Nucleotide sequence accession number. The complete se-

TABLE 2. Relative amounts of ms^2io^6A and ms^2i^6A[a]

Strain	ms^2io^6A/Ψ	ms^2i^6A/Ψ
S. typhimurium LT2	0.29	0.15
E. coli MM294		0.30
MM294/pUST104 (*miaE*$^+$)	0.0013	0.34
MM294/pUST107 (*miaE*$^+$)	0.34	
MM294/pUST108 (*miaE*$^+$)	0.35	
MM294/pUST115 (*miaE*$^+$)	0.34	
GT3098		0.30
GT3099		0.33
GT3099/pUST114 (*miaE* mutant)		0.33
GT3099/pUST115 (*miaE*$^+$)	0.34	
GT3099/pUST116 (*miaE*$^+$)	0.34	
GT3099/pUST117 (*miaE* mutant)		0.35

[a] The data are ratios of absorption units of ms^2i^6A or ms^2io^6A per absorption unit of pseudouridine (Ψ) at 242 nm.

quence has been submitted to the EMBL data base and been assigned accession number X73368.

RESULTS

Isolation of the gene (*miaE*) for the tRNA(ms^2io^6A37) hydroxylase. Since *E. coli* contains the unhydroxylated ms^2i^6A in its tRNA, we used the *E. coli* K-12 strain MM294 as a naturally occurring ms^2io^6A-deficient strain to isolate the *miaE* gene from *S. typhimurium* by complementation. This *E. coli* strain was transformed with an *S. typhimurium* gene library in the low-copy-number vector pLG339. Individual clones were grown separately and pooled five by five before total RNA was prepared. The RNA samples were digested into nucleosides, and the nucleoside composition was analyzed by HPLC. Among 480 clones screened for the presence of ms^2io^6A, one positive clone was found. The plasmid of the *miaE*$^+$ clone resulting in ms^2io^6A synthesis in *E. coli* was designated pUST104. Plasmid pUST104 was found to carry an approximately 29-kb large chromosomal insert by restriction enzyme analysis. To possibly increase the activity of the cloned *miaE* gene, the *miaE* gene was subcloned onto the multicopy plasmid pBR322. A MiaE$^+$ plasmid, pUST107, was identified by HPLC analysis. This plasmid had a chromosomal insert of 15.5 kb. The activity of the tRNA(ms^2io^6A)hydroxylase from plasmid pUST107 increased compared with that from plasmid pUST104 (Table 2), but this increase greatly exceeded that which would be expected from the difference in copy number (approximately fivefold). We estimated the increase of hydroxylation to be about 250-fold, using other modified nucleosides as internal standards. We do not know the reason for the unexpected increase in activity. Isolation of an 8-kb fragment from pUST107 ligated with the vector pUC18 gave plasmid pUST108. The tRNA(ms^2io^6A37)hydroxylase activity from this clone seems comparable to that of plasmid pUST107. An *Nru*I cutback of plasmid pUST108 generated plasmid pUST115 with a 4.5-kb chromosomal insert. This MiaE$^+$ clone was chosen for sequencing. Derivatives of plasmid pUST115 on both high-copy-number vector pUC19 and low-copy-number vector pCL1921 used to identify the *miaE* gene are shown in Fig. 2.

The *miaE* gene is the second gene of a dicistronic operon. The chromosomal insert of plasmid pUST115 comprising 4,531 bp was sequenced on both strands, and open reading frames (ORFs) were tentatively assigned as shown in Fig. 4. The sequence from one end of the insert of plasmid pUST115 was found to be similar to that of the *argI* and *argF* genes of *E. coli*. Both genes encode the ornithine carbamoyltransferase (1, 45).

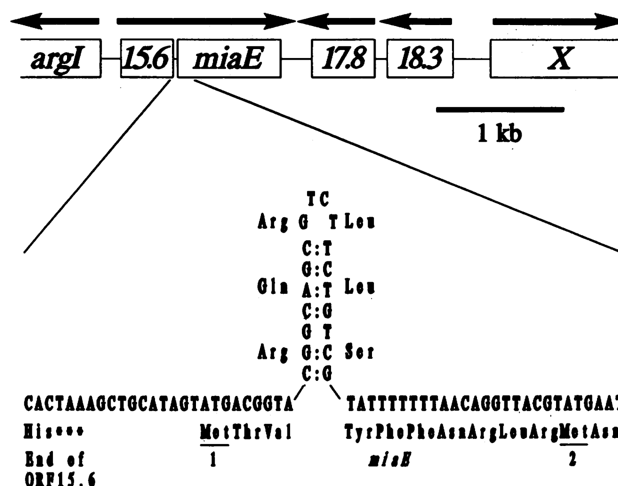

FIG. 4. The translation initiation region of *miaE* and the postulated attenuator terminating most of the ORF15.6 transcription. Arrows, direction of transcription.

In *S. typhimurium*, however, there is only one gene for this enzyme, the *argI* gene (55), which is located at min 99, localizing the *miaE* gene to this minute. A total of four complete and two partial ORFs were identified (Table 3). One of the partial ORFs corresponds to the N-terminal part of the *argI* gene. Two ORFs, upstream of the *argI* and transcribed in a direction opposite to that for *argI*, form a dicistronic operon. Complementation of the ms^2io^6A deficiency in *E. coli* with several plasmids revealed that the second gene in this operon encodes the gene necessary for tRNA(ms^2io^6A37)hydroxylase activity (Fig. 2). The first gene of the *miaE* operon codes for a 15.6-kDa protein that has an estimated pI of 3.5 and consists of 34% aspartate and glutamate residues.

All putative proteins encoded by the sequenced DNA were compared with those in the EMBL sequence data base. Similarities were found only for *argI* and ORF18.3, which showed similarity to a number of acetyltransferases, the protein acetylating the N-terminal alanine of protein S18 (*rimI*) and *N*-acetylglutamate synthase from *E. coli* (*argA*), streptothricin acetyltransferase of *Streptomyces lavendulae* (*sta*), and an ORF downstream of the *trpGDC* operon in *Azospirillum brasilense*. The sequence of *miaE* reveals no similarities to that of *miaA* of *E. coli* or *S. typhimurium*. The hydrophobic amino acids of the *miaE* gene product (~50%) are evenly distributed, and no putative membrane-spanning regions could be found.

The *miaE* gene is absent in *E. coli*. Hybridization of probes specific for genes ORF15.6, *miaE*, and ORF17.8 to *E. coli* MC1061 chromosomal DNA revealed that the *miaE* and ORF17.8 genes are absent in *E. coli*. Strain MC1061 has the

TABLE 3. Properties of the known and putative genes in the *miaE* region

Gene	Function	Number of codons	Mol wt of protein	Estimated pI
argI	Ornithine carbamoyltransferase	>218		
ORF15.6	Unknown	138	15,622	3.5
miaE	tRNA(ms^2io^6A37)hydroxylase	270	30,822	7.9
ORF17.8	Unknown	162	17,755	7.0
ORF18.3	Acetyltransferase?	167	18,256	5.3
ORFX	Unknown	>269		

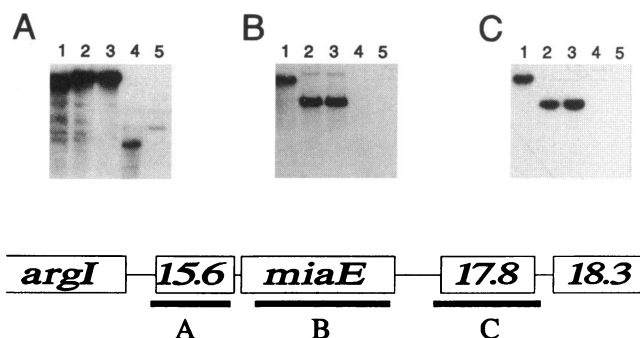

FIG. 5. Southern blot hybridization. Chromosomal DNAs from *S. typhimurium* LT2 (lane 1), GT2944 (*miaE2506*::MudJ) (lane 2), GT2947 (*miaE2507*::MudJ) (lane 3), and *E. coli* MC1061 (lanes 4 and 5). The DNAs in lanes 1 to 4 were digested with *Nru*I; that in lane 5 was digested with *Hinc*II. Panels A to C correspond to hybridizations with probes covering the individual genes as indicated at the bottom.

wild-type alleles of both *pyrBI* and *argI*, which are located counterclockwise and clockwise to *miaE*, respectively, and is not known to have any deletions in this area of the chromosome. The probe specific for ORF15.6 gave a hybridization signal demonstrating that at least part of this gene is present in *E. coli* (Fig. 5), in accordance with the sequence obtained by Piette et al. (49) for *argI* and the first 19 codons of the ORF15.6 gene of *E. coli*. We sequenced the region upstream of the *argI* gene of *E. coli* on plasmid pAI1 (49), which was derived from λd192A originating from *E. coli* K-12 strain RW420 (40). The amino acid sequence of ORF15.6 is highly conserved between the two species, but immediately after the ORF15.6 stop codon the sequence shows no similarity at all to the *S. typhimurium* sequence. Thus, the absence of ms^2io^6A in *E. coli* is due to a deletion, which starts immediately downstream of the ORF15.6 gene and extends at least beyond the *miaE* and ORF17.8 genes.

Transcriptional organization of the *miaE* operon. The promoter region between the *argI* and the *miaE* operon is highly conserved between *S. typhimurium* and *E. coli*. The transcriptional start point for the *S. typhimurium argI* gene is 38 nucleotides upstream of the *argI* translational start. The transcriptional start point for the *miaE* operon is 30 nucleotides upstream of the AUG start codon of ORF15.6, as demonstrated by S1 mapping and primer extension. The experimentally determined transcriptional start points and the putative −10 and −35 promoter sequences are shown in Fig. 6. In Northern blot experiments (Fig. 6) with a probe covering the ORF15.6 gene, two transcripts were detected. With the probe specific for the *miaE* gene, only the longer of these two transcripts was detected. The shorter transcript (approximately 500 nucleotides) is large enough to cover the first cistron and would end close to the translational start of the *miaE* gene, at the putative ρ-independent transcriptional attenuator shown in Fig. 4. The longer transcript (approximately 1,200 nucleotides) is large enough to cover both the ORF15.6 and the *miaE* genes. The shorter transcript is the most abundant and represents approximately 90 to 95% of the transcription from the *miaE* operon.

Isolation of a *miaE*::MudJ insertion mutant. MudJ (conferring kanamycin resistance) transposon insertions around *argI* were made in order to get an insertion mutant of the *miaE* gene (37). In short, P22 phage stock was grown on strain TT10288 (*hisD9953*::MudJ *hisD9944*::MudA) and used to make MudJ insertions into LT2. The MudJ random insertions were pooled and used as the donor in a transduction with strain TT147 (*argI*::Tn*10*) as the recipient. Arg^+ colonies were selected and screened for kanamycin resistance. Total RNA was prepared from 35 Arg^+ Km^r clones, and the level of ms^2i^6A or ms^2io^6A was analyzed by HPLC. Two clones were found to have ms^2i^6A instead of ms^2io^6A in their tRNAs (Fig. 7). These clones were designated GT2944 (*miaE2506*::MudJ) and GT2947 (*miaE2507*::MudJ). That the MudJ insertions were in the *miaE* gene was confirmed by Southern hybridization (Fig. 5). Chromosomal DNAs from the wild-type strain LT2 and from the two insertion mutants (GT2944 and GT2947) were digested with *Nru*I, which cuts within the MudJ element. Hybridization with probes covering either ORF15.6, *miaE*, or ORF17.8 (Fig. 5) showed that ORF15.6 and ORF17.8 are located on each side of the insertion, since the ORF15.6 probe hybridizes to an *Nru*I fragment in the mutant that is larger than the *Nru*I fragment covering the region in the wild type and the ORF17.8 probe hybridizes to a fragment which in the mutant is smaller than the *Nru*I fragment of the wild type. The *miaE*-specific probe hybridizes to both of these fragments and, thus, covers the MudJ insertion (i.e., the MudJ insertion is in the *miaE* gene). Since the *miaE*-specific probe hybridizes only weakly to the longer *Nru*I fragment compared with the hybridization to the shorter fragment, this suggests that the MudJ insertion is close to the end of the probe in the beginning of the *miaE* gene.

The tRNA(ms^2io^6A37)hydroxylase-deficient phenotype was correlated to the *miaE*::MudJ insertions by transduction of the *miaE*::MudJ insertions into *S. typhimurium* LT2, which generated strains GT3098 (*miaE2506*::MudJ) and GT3099 (*miaE2507*::MudJ), which were used for physiological studies.

***miaE*::MudJ mutants show normal growth characteristics after being shifted from anaerobic to aerobic conditions.** Since it has been suggested that the hydroxyl group of ms^2io^6A may function to signal the availability of oxygen for *S. typhimurium* shifted from an anaerobic to an aerobic atmosphere (9), the *miaE2507*::MudJ mutant was tested for its ability to grow anaerobically and to adapt when shifted from anaerobic to aerobic conditions. No differences in growth rate between the *miaE*::MudJ mutants and the wild type in MOPS-glucose media at 37°C, under either anaerobic or aerobic conditions, were detected (data not shown). In addition, the lag phase was not prolonged in the mutant, compared with the wild type, when shifted from anaerobic to aerobic conditions. Therefore, the ms^2io^6A hydroxylation has no effect on the ability of *S. typhimurium* to adapt to aerobic growth under the conditions tested.

It has been reported that a mutant (*miaA*) deficient in the tRNA(i^6A37)isopentenyltransferase in *S. typhimurium* shows increased sensitivity to hydroperoxide compounds (3). It was noted that *E. coli* is as sensitive as an *S. typhimurium miaA* mutant. Since the difference between wild-type *S. typhimurium* and an *S. typhimurium miaA* mutant is the presence of ms^2io^6A in the former and the difference between wild-type *S. typhimurium* and wild-type *E. coli* is the absence of the hydroxyl group of ms^2io^6A in the latter, the sensitivities of our *miaE*::MudJ mutants towards the compounds *tert*-butyl hydroperoxide, cumene hydroperoxide, and hydrogen peroxide were tested. There was no difference in sensitivity between wild type and *miaE* mutant cells. However, we were unable to reproduce the results of Blum and Ames (3) with the *S. typhimurium miaA* mutant, which in our experiments showed no increased sensitivity towards hydroperoxide compounds. Care was taken to ensure that the different strains were all in the same growth phase when tested, since hydroperoxide sensitivity decreases when bacteria enter stationary phase.

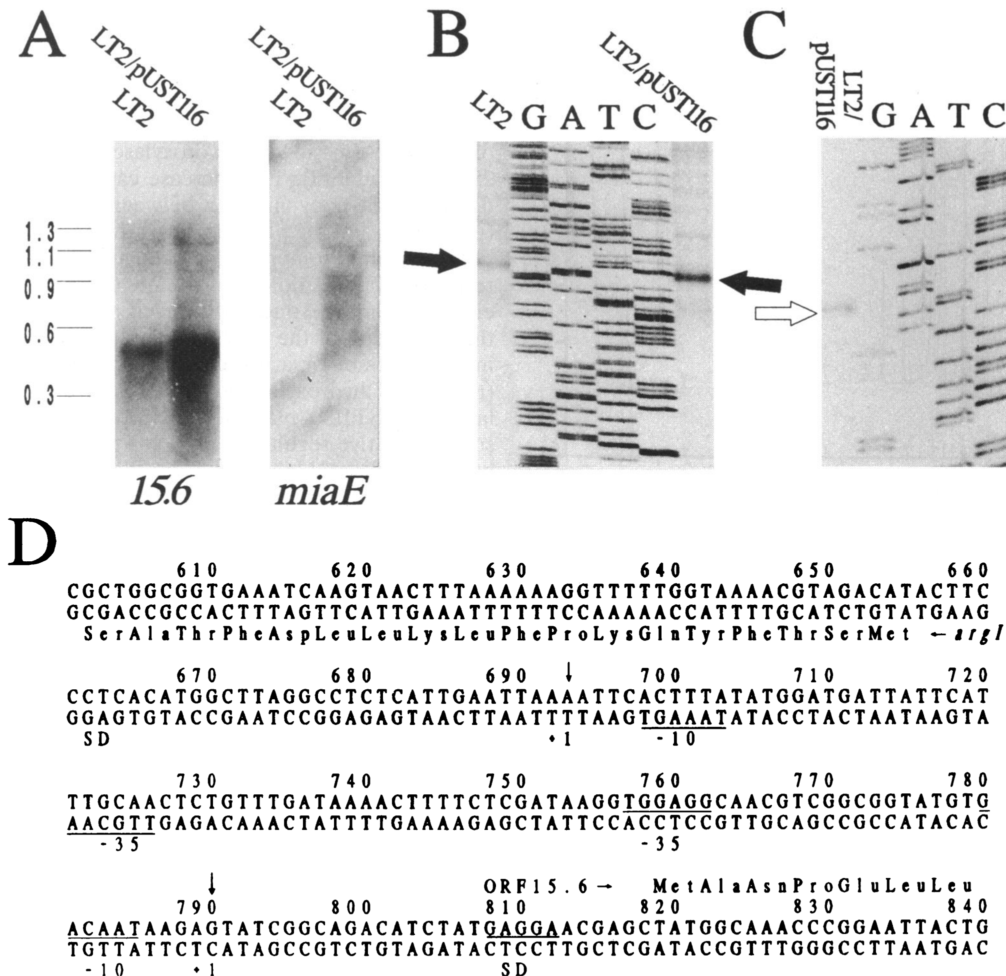

FIG. 6. (A) Northern blot hybridization with probes covering ORF15.6 or *miaE* (A and B, respectively, in Fig. 5). Numbers refer to standard fragments in kilonucleotides. (B) S1 nuclease protection of the *miaE* operon transcript. The sequence ladder is M13mp18 read with the Pharmacia universal M13 primer as supplied with the T7 sequencing kit. The length of the major protected product is 238 nucleotides. (C) Primer extension of the *argI* transcript. The sequence ladder is M13mp8 read with the Pharmacia universal M13 primer as supplied with the T7 sequencing kit. The length of the extension product is 126 nucleotides. (D) Promoter region of the *argI* and *miaE* operons. Promoter and Shine-Dalgarno (SD) sequences are underlined. Arrows, determined transcriptional start points.

The hydroxyl group of ms^2io^6A does not influence the activity of the $tRNA^{Tyr}$ amber suppressor *supF30*. Other mutations in the biosynthesis of ms^2io^6A (*miaA* and *miaB*) influence the translational efficiency of a tRNA (24, 33). Therefore, it was of interest to investigate what influence the hydroxyl group of ms^2io^6A may have on the efficiency of the tRNA. The system of Ericson and Björk (23) in which a *hisD-lacZ* fusion is present on the chromosome, where the *hisD* part contains an amber stop codon followed on its 3′ side by either an A or a C, was used to test whether lack of ms^2i^6A hydroxylation had any effect. The level of read-through of this amber codon measures the efficiency of the amber suppressing tRNA. By comparing the efficiency of read-through of the amber codon followed by a C or an A, an estimate of the influence of the nucleoside 3′ of the amber codon can be made. When we measured the efficiency of the *supF* nonsense suppressor tRNA that has the nucleoside ms^2io^6A (*miaE*$^+$) at position 37 next to its anticodon or the unhydroxylated form ms^2i^6A (*miaE*), no difference in either efficiency or codon context sensitivity was detected (Table 4).

The *miaE*::MudJ mutant is unable to grow on the citric acid cycle intermediates succinate, fumarate, and malate. The ability of the *miaE*::MudJ mutant to grow on minimal plates supplemented with different carbon sources was tested. The *miaE*::MudJ mutant grew as well as wild-type *S. typhimurium* LT2 cells on most carbon sources but significantly more slowly on citrate and not at all on the citric acid cycle intermediates succinate, fumarate, and malate. The wild-type cells continued to grow after a short lag period when cells grown in liquid glucose minimal medium were shifted to acetate, citrate, malate, or succinate minimal medium. In contrast, mutant cells immediately stopped growing when shifted to a medium containing either malate, fumarate, or succinate (data not shown). When shifted to citrate minimal medium, the *miaE* mutant resumed growth after a lag period, but at a lower growth rate than the wild type. If glucose was added to the mutant cells 3 h after they were shifted to MOPS malate or MOPS succinate, the cells immediately started to grow at a rate typical for growth in MOPS glucose medium. Thus, *miaE*::MudJ mutants show impaired growth on the citric acid cycle intermediate citrate and inability to grow on succinate, fumarate, and malate.

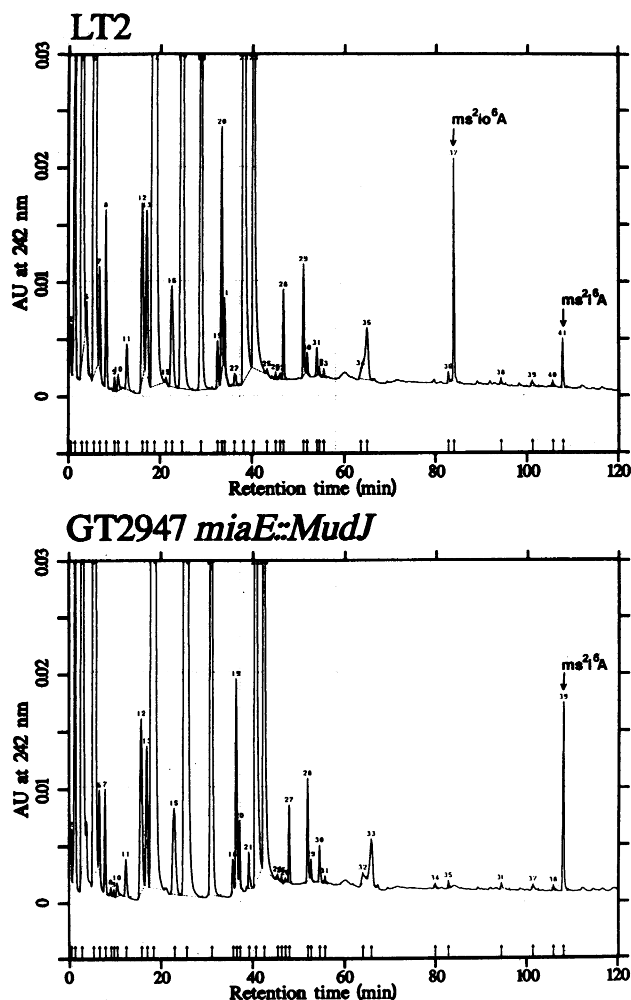

FIG. 7. HPLC chromatograms from the analysis of *miaE*::MudJ insertions. Cells were grown overnight at 37°C in LB medium; tRNA was prepared as described in Materials and Methods.

DISCUSSION

The modified nucleoside ms^2io^6A is synthesized on the tRNA from A (adenosine) in at least four steps (Fig. 1). The last step performed by the tRNA(ms^2io^6A37)hydroxylase takes place in most strains in the family *Enterobacteriaceae* so far examined but not in *E. coli*, which has the unhydroxylated derivative ms^2i^6A in its tRNA (13, 39). Knowing this, we cloned the gene for the tRNA(ms^2io^6A37)hydroxylase (*miaE*) from *S. typhimurium* using *E. coli* as a naturally occurring hydroxylase-deficient strain. Cells harboring the $MiaE^+$ plasmid originally isolated gave a very low hydroxylation activity. Upon subcloning of the *miaE* gene from this low-copy-number derivative (pLG339) to plasmid pBR322, the apparent activity of the tRNA(ms^2io^6A37)hydroxylase increased approximately 250-fold (Table 2). This increase cannot be explained by the difference in copy number between the pLG339 derivative (pUST104) and the pBR322 derivative (pUST107) (copy numbers equivalent to approximately 6 and 25 copies per genome, respectively). We do not yet know the reason for this difference, but it may be due to the effects of plasmid supercoiling on the expression of the *miaE* gene. Alternatively, a gene encoding a repressor or a protein causing inhibition of the tRNA (ms^2io^6A37)hydroxylase activity may have been present on the larger (pUST104) plasmid and lost during subcloning. If so, such a putative regulatory gene for the *miaE* expression must be located several kilobases from the *miaE* gene itself, since it is not present on plasmid pUST108, which has at least 6 kb of chromosomal DNA on each side of *miaE*.

TABLE 4. Suppressor efficiency of *supF30* in wild type and *miaE* strains

Strain	% Read-through[a]		*hisD6404-C1*/ *hisD6404*
	hisD6404 (UAG-C)	*hisD6404-C1* (UAG-A)	
Wild type	32	52	1.6
miaA[b]	2.3	12	5.2
miaE	33	55	1.7

[a] Defined as the percentage of the β-galactosidase activity in a control strain that has the same MudK insertion but no amber stop codon in the *hisD* gene.
[b] Values from reference 23.

Sequencing of plasmid pUST115 revealed that two-thirds of the *S. typhimurium argI* gene is present on this plasmid. The *argI* gene is located at min 99 of the *Salmonella* chromosome (55). Complementation of *miaE*::MudJ mutants showed that the *miaE* gene is the second gene of a dicistronic operon just upstream of *argI*, transcribed in the opposite direction (Fig. 5). The first gene of this operon, ORF15.6, codes for a protein with a calculated molecular size of 15,622 Da and an estimated pI of 3.5 due to the high content of aspartate and glutamate residues (34%). There are two possible translational start points for the *miaE* gene product (Fig. 4). The first AUG start codon is located 11 bp downstream of the stop codon of ORF15.6, and the second AUG is located 49 bp downstream of the ORF15.6 stop codon and downstream of the terminator-like structure indicated in Fig. 4. The calculated molecular size of the tRNA(ms^2io^6A37)hydroxylase would be 30,822 or 28,513 Da if the first or second AUG is used, respectively. Neither AUG seems to be preceded by a good Shine-Dalgarno sequence. The *rnd* gene is the structural gene for RNase D. The *rnd* operon is preceded by a stem-loop structure similar to a ρ-independent terminator. This sequence has been shown to be essential for efficient translation of the *rnd* gene (73). It is tempting to speculate that the terminator structure in the *miaE* operon may function in a similar way, allowing translation to start from the second AUG, although we have no evidence for this.

The *miaE* gene is not very similar to the *miaA* gene encoding the tRNA(i^6A37)isopentenyltransferase. It has been suggested that the tRNA(i^6A37)isopentenyltransferase requires nucleotides 37 to 39 of the tRNA to be adenosines and a 5-bp anticodon stem for substrate recognition (32, 64). Maybe the absence of similarity between the *miaA* and *miaE* genes indicates that the tRNA(ms^2io^6A37)hydroxylase recognizes only the base it modifies and not the tRNA as such. Since tRNA from a *miaB* mutant, which lacks the ms^2 group of ms^2io^6A, has the unhydroxylated derivative i^6A in its tRNA, the tRNA(ms^2io^6A37)hydroxylase requires the presence of the 2-methylthio group for its activity (24).

Transcription of the *miaE* operon gives rise to two different transcripts, both initiated 30 nucleotides upstream of the ATG of the ORF15.6 gene (Fig. 6). The shorter, major transcript (approximately 90 to 95% of total transcription in plasmid-free wild-type cells) is long enough to cover only the first gene, ORF15.6, in agreement with termination at the terminator-like structure shown in Fig. 4. The size of the longer, minor transcript (5 to 10%) suggests that it covers both the ORF15.6

and the *miaE* genes. Thus, the *miaE* gene seems to be the second gene of a dicistronic operon. We cannot exclude the possibility that there is an additional promoter just upstream of the *miaE* gene, but in the Northern hybridization experiment with a *miaE*-specific probe we could see hybridization only to the long transcript covering also ORF15.6. Moreover, we cannot detect any promoter-like sequence upstream of *miaE*.

In *E. coli*, at least part of the ORF15.6 gene is present, whereas the *miaE* and the ORF17.8 genes, transcribed in the opposite direction downstream of *miaE*, are absent from *E. coli*. The absence of the *miaE* gene explains why *E. coli* has ms^2i^6A instead of ms^2io^6A in its tRNA. None of the *E. coli* strains (W3110, K-12 Hfr3000, C-1, B, 15, or W) tested was found to have ms^2io^6A in its tRNA (13). The *miaE* gene may encode either the tRNA(ms^2io^6A37)hydroxylase or a cofactor necessary for the hydroxylation reaction. If the latter is true, *E. coli*, which lacks the *miaE* gene but gets fully hydroxylated tRNA when the *S. typhimurium miaE* gene is introduced, would have the tRNA(ms^2io^6A37)hydroxylase but lack the cofactor needed for its activity. Since it is unlikely that the *miaE* gene encodes a cofactor needed for only one reaction and that *E. coli* would have kept the hydroxylating enzyme through evolution despite the absence of the necessary cofactor, it seems more plausible that the *miaE* gene encodes the tRNA(ms^2io^6A37)hydroxylase.

Buck and Ames (9) suggested that the hydroxylation of ms^2i^6A to form ms^2io^6A in the tRNA of *S. typhimurium* may be a signal for adaptation to aerobic growth, since molecular oxygen was required for the hydroxylation to take place. They showed that the hydroxylating enzyme was present also during anaerobic conditions, although no hydroxylation occurred, and that the tRNA was rapidly hydroxylated as the cells were shifted from anaerobic to aerobic conditions. Although these features only partly fulfill the requirements (rapid synthesis and rapid turnover [59, 63]) of an alarmone, the hydroxylation of ms^2i^6A still may function as a cellular device to sense the oxygen tension as suggested by Buck and Ames (9). However, we could not detect any prolonged growth lag for the mutant, compared with the wild type, when it was shifted from anaerobic to aerobic growth conditions. This means that if hydroxylation is needed to give *S. typhimurium* a signal to adapt to aerobic growth, this effect is too small to be seen in such an oxygen shift experiment.

It is known that the ms^2io^6A modification is important for efficient translation. If the adenosine at position 37 is totally unmodified (5, 22, 48, 67) or lacks the methylthio group (24, 33), the efficiency of the tRNA decreases and its sensitivity to codon context increases. However, no effect on translation efficiency or codon context sensitivity of a *supF* amber suppressor tRNA lacking only the hydroxyl group of ms^2io^6A was observed (Table 4). However, this does not exclude the possibility that there is another tRNA species or another codon context that is dependent on the presence of the ms^2io^6A hydroxyl group for efficient translation. Therefore, the observed phenotypes (see below) of the *miaE*::MudJ mutant may still be mediated through effects on translation.

The *miaE*::MudJ mutants were found to be unable to grow on the citric acid cycle intermediates succinate, fumarate, and malate and grew more slowly than the wild type on citrate. This phenotype was shown to be 100% linked to the *miaE*::MudJ insertion (200 Km^r transductants were also Suc^-) and could be complemented by introduction of $miaE^+$ plasmids. The reason for the growth deficiency of the *miaE*::MudJ mutants may be a translational effect on either the expression of the enzymes needed for growth on citric acid cycle intermediates or a regulator of central metabolism. Alternatively, a fully hydroxylated tRNA is needed for an enzymatic reaction to take place. In plants, algae, and some bacteria, the first step (glutamate→glutamate-1-semialdehyde) in the synthesis of δ-aminolevulonic acid requires that the glutamate be coupled to a $tRNA^{Glu}$ (36, 69). Another alternative would be that the tRNA(ms^2io^6A37)hydroxylase acts on substrates other than the isopentenyl group of ms^2io^6A, maybe modifying a protein needed for growth on citric acid cycle intermediates. Although *E. coli* lacks the hydroxyl group of ms^2io^6A, it is still able to grow on malate, fumarate, and succinate and is obviously not dependent on the hydroxylation of ms^2i^6A for this ability.

Though the tRNA(ms^2io^6A37)hydroxylase is a fairly small enzyme (30 kDa), we cannot exclude the possibility that the protein has two unrelated enzymatic activities. If so, it is intriguing that an enzyme would act both on tRNA and in a reaction of the central metabolism. We are currently investigating why the *miaE*::MudJ mutants are unable to grow on succinate, fumarate, and malate.

The tRNA modification level is often influenced by cellular metabolism; e.g., starvation for methionine causes deficiency in methylation tRNA (42). Cellular metabolism is also influenced by the tRNA modification level. Mutations in *hisT* and *miaA*, which cause deficiencies in pseudouridine (38, 39, 40) and ms^2io^6A37, respectively, influence the synthesis (7, 8, 19, 50, 66) and degradation (29) of many amino acids through tRNA-mediated attenuation. However, several regulatory features of such modification-deficient strains are not easily reconciled with regulation through attenuation (3, 6, 8, 46, 52, 57, 65). The *nuvC* gene in *E. coli* encodes an enzyme that is needed both for 4-thiouridine synthesis in tRNA and for thiamine biosynthesis (53). One case of cellular metabolism and tRNA modification mutually influencing each other has been established. The synthesis of the methylthio group of ms^2io^6A is dependent on the presence of iron (Fig. 1) (31, 51, 70), and bacteria starved for iron have i^6A in their tRNAs. Lack of the ms^2 modification of ms^2io^6A decreases the efficiency of the corresponding tRNAs (24, 26, 33) and causes increased synthesis of aromatic amino acids and the iron-sequestering chelator enterobactin (9, 12, 43). Further studies will reveal whether the hydroxyl group of ms^2io^6A is another example of a tRNA modification affecting cellular metabolism—in this case, the dicarboxylic acid metabolism of *S. typhimurium*.

ACKNOWLEDGMENTS

Gunilla Jäger, Kristina Nilsson, and Kerstin Jacobsson are acknowledged for excellent technical assistance. We thank F. van Vliet for plasmid pAI1. We are grateful to Tord Hagervall and Mikael Wikström for critical reading of the manuscript.

This work was supported by the Swedish Cancer Society (project 680) and the Swedish Natural Science Research Council (project B-BU 2930).

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