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The Methylthio Group (ms^2) of N^6-(4-Hydroxyisopentenyl)-2-Methylthioadenosine (ms^2io^6A) Present Next to the Anticodon Contributes to the Decoding Efficiency of the tRNA†

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A *Salmonella typhimurium* LT2 mutant which harbors a mutation (*miaB2508*::Tn*10d*Cm) that results in a reduction in the activities of the amber suppressors *supF30* ($tRNA^{Tyr}_{CUA}$), *supD10* ($tRNA^{Ser}_{CUA}$), and *supJ60* ($tRNA^{Leu}_{CUA}$) was isolated. The mutant was deficient in the methylthio group (ms^2) of N^6-(4-hydroxyisopentenyl)-2-methylthioadenosine (ms^2io^6A), a modified nucleoside that is normally present next to the anticodon (position 37) in tRNAs that read codons that start with uridine. Consequently, the mutant had i^6A37 instead of ms^2io^6A37 in its tRNA. Only small amounts of io^6A37 was found. We suggest that the synthesis of ms^2io^6A occurs in the following order: A-37→i^6A37→ms^2i^6A37→ms^2io^6A37. The mutation *miaB2508*::Tn*10d*Cm was 60% linked to the *nag* gene (min 15) and 40% linked to the *fur* gene and is located counterclockwise from both of these genes. The growth rates of the mutant in four growth media did not significantly deviate from those of a wild-type strain. The polypeptide chain elongation rate was also unaffected in the mutant. However, the *miaB2508*::Tn*10d*Cm mutation rendered the cell more resistant or sensitive, compared with a wild-type cell, to several amino acid analogs, suggesting that this mutation influences the regulation of several amino acid biosynthetic operons. The efficiencies of the aforementioned amber suppressors were decreased to as low as 16%, depending on the suppressor and the codon context monitored, demonstrating that the ms^2 group of ms^2io^6A contributes to the decoding efficiency of tRNA. However, the major impact of the ms^2io^6 modification in the decoding process comes from the io^6 group alone or from the combination of the ms^2 and io^6 groups, not from the ms^2 group alone.

Modified nucleosides are present in tRNA from organisms of all three domains, *Archaea*, *Bacteria*, and *Eucarya* (68). Seventy-nine modified bases have been identified, and they are present at different positions of the tRNA molecule (45). One position that is often modified is position 37, the base immediately 3′ of the anticodon. Depending on the identity of the tRNA, different modifications are found in this position (for reviews, see references 5 and 6). In *Escherichia coli*, tRNAs that read codons that start with uridine (except $tRNA^{Ser}_{I,V}$) (38) have the hydrophobic residue 2-methylthio-N^6-(isopentenyl) adenosine (ms^2i^6A) present at position 37. In *Salmonella typhimurium* and several other gram-negative organisms, the 4-hydroxy derivative ms^2io^6A is most likely present in the corresponding tRNAs (17, 56). Two of the genes involved in synthesis of ms^2io^6A, the *miaA* and *miaE* genes, have been identified. The *miaA* gene is the structural gene (18, 19) for tRNA(i^6A)synthase (2). Since a mutation in this gene results in an unmodified A at position 37, the formation of i^6A37 may be the first step in the synthesis of ms^2io^6A. The fact that 2-methylthioadenosine (ms^2A) either is not found or is present only in small amounts in a *miaA* mutant (27, 65) implies that the ms^2-forming enzyme(s) requires the presence of the isopentenyl group or the MiaA peptide. It has also been suggested that the hydroxylation enzyme, which is encoded by the *miaE* gene (55), requires the ms^2 group to act (13). If so, a mutation which inhibited formation of the ms^2 modification would result in the presence of N^6-isopentenyladenosine (i^6A), not N^6-(4-hydroxyisopentenyl)adenosine (io^6A). This paper describes the isolation and characterization of a mutant that is blocked in formation of the ms^2 modification. Characterization of this mutant supports the stepwise formation of ms^2io^6A that is depicted in Fig. 1.

Although all known tRNA species contain modified nucleosides and a considerable part of the bacterial genome (about 1%) is devoted to modification of tRNA, no modified nucleoside in tRNA has yet been shown to be essential. However, the *trmA* gene that encodes tRNA(m^5U54)methyltransferase is essential, although the 5-methyluridine (m^5U) in tRNA is not (57). Loss of a modification, however, can have slight or rather profound effects on cell physiology. The *miaA1* mutation of *S. typhimurium* is an example of the latter. This mutation, which results in an unmodified A at position 37, induces strong pleiotropic effects on cell physiology (27). The growth and polypeptide chain elongation rates in vivo are reduced, and several amino acid biosynthetic operons show altered expression in a *miaA1* strain (23, 27, 48). Amber suppressors normally contain $ms^2i(o)^6A37$, but in a *miaA1* mutant, these suppressor tRNAs contain an unmodified A-37. [Since the hydroxylation reaction does not occur in *E. coli*, the abbreviation $ms^2i(o)^6A$ is used hereafter to refer to data for both *E. coli* and *S. typhimurium*.] By using amber suppressors, the presence of $ms^2i(o)^6A37$ was shown to dramatically increase the efficiency of tRNA in vivo (11). Since ms^2i^6A has no influence on the aminoacylation reaction (15, 32, 67), these results imply that $ms^2i(o)^6A$ is important in anticodon-codon interaction (11, 28, 63). This has also been established in vitro (22, 32, 63, 67). In model experiments in which dissociation of tRNA dimers with complementary anticodons was monitored, it was shown that this modification stabilizes anticodon-anticodon interaction by increased stacking (63). Such experiments have also shown that the ms^2 group is the major stabilizing factor (39). Thus, the effects

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† This paper is dedicated to Helga and Walter Kersten on the occasion of their retirement from active research.

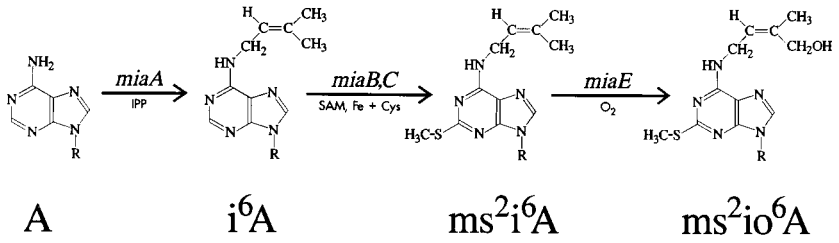

FIG. 1. Biosynthesis of ms^2io^6A in *S. typhimurium*. The *miaA* gene encodes tRNA(i^6A37)synthase (2). We postulate that the conversion of i^6A to ms^2i^6A is catalyzed by at least two enzymes (a tRNA sulfurtransferase and a tRNA methyltransferase); therefore, two genes, *miaB* and *miaC*, are designated to represent this step. The last gene, *miaE*, is thought to encode tRNA(ms^2io^6A37)hydroxylase (55). IPP, Δ^2-isopentenyl-pyrophosphate; SAM, *S*-adenosyl-L-methionine; R, the ribose moiety.

observed in the aforementioned in vivo and in vitro experiments with tRNA from a *miaA* mutant were expected to be primarily due to lack of the ms^2 group. This paper shows that this is not the case. Instead, the major contribution of the ms^2io^6A modification on the decoding efficiency of tRNA is exerted by the io^6 group.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used were all derivatives of *S. typhimurium* LT2 (Table 1). F′ plasmids that carried *proAB*$^+$ and a *lacI-lacZ* gene fusion with nonsense mutations in different positions in the *lacI* part and also conferred resistance to kanamycin were obtained from Jeffrey H. Miller, University of California, Los Angeles (50). Cells were grown either in medium E (64) supplemented with 0.2% glucose, histidine, and leucine or in MOPS (morpholinepropanesulfonic acid) medium (53). Rich MOPS medium was that described by Neidhardt et al. (52). To determine the ability of cells to oxidize different carbon sources, cells were grown in a complex medium called NAA. NAA consists of Difco nutrient broth (0.8%) (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl, adenine, tryptophan, tyrosine, phenylalanine, *p*-hydroxybenzoate, 2,3-dihydrobenzoate, and *p*-aminobenzoate. All supplements were provided at the concentrations recommended by Davis et al. (21).

Genetic procedures. Transductions with phage P22 HT105/I (*int-201*) (61) were performed as described elsewhere (21). F′ plasmids were introduced into appropriate strains by conjugal transfer, which was achieved by mixing F′-carrying donor cells with recipient cells directly on selective agar plates. Preparation of Mu*d*-P22 lysates was carried out according to the method of Benson and Goldman (3), and the method of selection for tetracycline sensitivity was that of Bochner et al. (8) as modified by Maloy and Nunn (46). Bochner plates were prepared as described by Benson and Goldman (3).

Analysis of contents of modified nucleosides in tRNA. Strains GT1825 and GT1975 were grown in rich MOPS medium at 37°C. Cells were harvested at a density of approximately 4×10^8 cells per ml, and tRNA was prepared by lithium chloride fractionation and DEAE-cellulose chromatography (14). tRNA was digested with nuclease P1 and alkaline phosphatase (34), and the hydrolysate was analyzed by high-performance liquid chromatography (HPLC) according to the method of Gehrke and Kuo (33).

Determination of growth and polypeptide chain elongation rates. Growth rates at 37°C were determined as described by Björk and Neidhardt (7). The procedure involved pregrowth for several generations to ensure that cells were in balanced growth. The increase in mass was monitored at 420 nm. Growth rates were determined in four MOPS media with different carbon sources (Table 2). Polypeptide chain elongation rates were determined as described by Ericson and Björk (27) and Schleif et al. (60).

Determination of sensitivities to different amino acid analogs. The sensitivities of strain GT2176 (*miaB2508*::Tn*10d*Cm) to different amino acid analogs were compared with those of strain GT522 (wild type). The procedure was performed essentially as described by Ericson and Björk (27) and Cortese et al. (20). Conditions with and without shift were employed. The conditions without shift consisted of growth of bacteria in NCE medium (21)–0.2% glycerol–5 mM $(NH_4)_2SO_4$, and then cells were plated on corresponding plates. Shiftdown conditions were achieved by pregrowth in the rich medium NAA with subsequent plating on plates that contained medium E and 0.2% citrate. The amino acid analogs were purchased from Sigma Chemical Co., St. Louis, Mo., with the exception of 5,5,5-trifluoroleucine, which came from Serva Biochemica GmbH & Co., Heidelberg, Germany. Azatyrosine was a gift from Susumu Nishimura, Tsukuba, Japan. In addition to the amino acid analogs listed in Table 3, there were other analogs with which no differences in response between mutant and wild-type strains were obtained. These analogs were L-methionine-DL-sulfoximine, 2-thiazolyl-DL-alanine, 1,2,4-DL-triazole-3-alanine, 3-amino-1,2,4-triazole, β-chloro-L-alanine, 4-aza-DL-leucine, L-ethionine, L-norleucine, *m*-fluoro-DL-phenylalanine, *p*-fluoro-DL-phenylalanine, DL-β-hydroxynorvaline, DL-7-aza-tryptophan, 5-methyl-DL-tryptophan, *m*-fluoro-DL-tyrosine, and azatyrosine. With 10 of the analogs tested, only small differences were observed. Since the significance of these differences is unsure, only the amino acid analogs with which considerable differences were observed are listed in Table 3. Small differences between mutant and wild-type strains were observed under conditions without shift when DL-aspartic-β-hydroxamate, DL-methionine-hydroxamate, L-2-acetidinecarbonate, and 3-aminotyrosine were used. The addition of analogs α-methyl-DL-methionine, β-(2-thienyl)-DL-alanine, DL-β-3-thienyl-alanine, thioproline, and DL-5-fluorotryptophan resulted in small differences when shift was employed. Only 3-nitrotyrosine gave small differences under both conditions.

TABLE 1. *S. typhimurium* strains used in this study

Strain	Genotype	Source or reference
LT2	Prototrophic	John R. Roth (University of Utah, Salt Lake City)
GT522	Prototrophic	27
GT523	*miaA1*	27
GT530	*pro-662*::Tn*10*/F′ *lac*$^+$ *proAB*$^+$	27
GT1696	*hisO1242 hisD2504*::Mu*d*K *leuA414*	28
GT1825	*hisO1242 hisD6404*(Am) *leuA414*(Am) *supF30 pro-688*::Tn*5*/F′ *del-14 lacIam117 proAB*$^+$	This work
GT1975	*hisO1242 hisD6404*(Am) *leuA414*(Am) *supF30 pro-688*::Tn*5 miaB2508*::Tn*10d*Cm/F′ *del-14 lacIam117 proAB*$^+$	This work
GT2176	*miaB2508*::Tn*10d*Cm	This work
GT2901	*pro-662*::Tn*10 miaB2508*::Tn*10d*Cm/F′ *lac*$^+$ *proAB*$^+$	This work
GT3147	*miaB2508*::Tn*10d*Cm *nag-1 zbf-99*::Tn*10d*Tc *fur-1 iroA-lacZ*	This work
TT4427	*hisO1242 hisD6404*(Am) *leuA414*(Am) *zeb-618*::Tn*10 zej-636*::Tn*5*	John R. Roth
TT15232	*purE2154*::Mu*d*Q *leuA414*(Am) *fels2* r$^-$	3
TT15237	*cobD498*::Mu*d*P *leuA414*(Am) *fels2* r$^-$	3
TT15240	*putA1019*::Mu*d*P *leuA414*(Am) *fels2* r$^-$	3
TT15629	*nadA219*::Mu*d*Q *leuA414*(Am) *fels2* r$^-$	3

TABLE 2. Comparison of specific growth and polypeptide synthesis rates in *miaB*$^+$ and *miaB2508*::Tn*10d*Cm cells

Medium	Specific growth rate (k)[a]		Polypeptide chain growth rate (aa/s)[b]	
	miaB$^+$	*miaB*	*miaB*$^+$	*miaB*
MOPS-acetate	0.37 (±0.02)	0.36 (±0.02)	ND	ND
MOPS-glycerol	0.77 (±0.01)	0.76 (±0.01)	16.7 (±1.0)	17.1 (±2.5)
MOPS-glucose	0.88 (±0.02)	0.87 (±0.02)	ND	ND
Rich MOPS	1.68 (±0.08)	1.66 (±0.00)	ND	ND

[a] The strains used were GT522 (*miaB*$^+$) and GT2176 (*miaB2508*::Tn*10d*Cm). Specific growth rate, k = ln 2/generation time (in hours). Data are averages of four determinations, with ranges in parentheses.

[b] The strains used were GT530 (*miaB*$^+$ F′ *lac*$^+$) and GT2901 (*miaB2508*::Tn*10d*Cm F′ *lac*$^+$). The rate of polypeptide elongation was determined by measuring the time required for production of the first β-galactosidase molecule as described by Ericson and Björk (27) and Schleif et al. (60). Data (in amino acids [aa] per second) are averages of 7 to 10 determinations, with in parentheses. ND, not determined.

Biolog MicroPlate assay. ES MicroPlates were purchased from Biolog Corp. and used according to the manufacturer's instructions. Log-phase cells (grown in NAA 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 plates were incubated at 37°C without shaking. Plates were read in a Biolog MicroStation Reader immediately after inoculation and after 6 and 17 h of incubation.

Measurements of β-galactosidase activity. Cells were grown at 37°C for determination of β-galactosidase levels. In order to maintain the F′ plasmids used, these strains were grown in media selective for the F′ plasmid, whereas Mu*d*K-fusion-containing strains were grown in rich MOPS medium. β-Galactosidase activity served as a measure of suppression efficiency and was determined as described by Miller (49) and Putnam and Koch (58). Each value represents the average of three experiments, in which two independent clones of each strain were assayed. For each culture, four enzymatic determinations at two enzyme concentrations were performed. For F′-carrying strains, this activity is presented as the percentage of activity in a strain that is phenotypically Lac$^+$ since the *lacI-lacZ* fusion in that construct does not carry a nonsense mutation. Values for the *hisD2504*::Mu*d*K strains were normalized to that for strain GT1696, which contains the same *hisD2504*::Mu*d*K fusion but lacks the nonsense codon in the *hisD* gene.

RESULTS

Isolation of a mutant with reduced efficiency of *supF30*-mediated suppression. The *miaA1* mutation, which results in an unmodified A-37, acts as an antisuppressor toward *supF30* (27). Since the major effect of the ms^{2}io^{6}A37 modification is thought to reside in the ms^2 part of the modification (39), mutations in other genes involved in biosynthesis of ms^{2}io^{6}A may also reduce the decoding efficiency of *supF30* suppressor tRNA. In order to screen for antisuppressors of *supF30* (amber suppressor tRNA$^{Tyr}_{CUA}$), a strain, GT1825, that harbors amber codons in the *hisD* and *leu* genes and an F′ plasmid was constructed. This F′ carries *lacI* fused to *lacZ* with in-frame amber codons in the *lacI* part. The *supF30* allele makes the strain phenotypically His$^+$, Leu$^+$, and Lac$^+$. Into this strain, a pool of random insertions of the defective minitransposon Tn*10d*Cm (26) was introduced. Cells that contained the transposon were selected and screened for *lacZ* expression by monitoring the level of blue color on plates that contained histidine, leucine, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Of about 12,000 chloramphenicol-resistant (Cm^r) clones, two that were light blue, His$^{(+)}$, and Leu$^+$ were found, compared with the phenotype of the parent, which was blue, His$^+$, and Leu$^+$; apparently, the insertion reduced suppression of amber mutations in the *lacI* (light blue) and *hisD* [His$^{(+)}$] genes but not the one in the *leuA* gene (Leu$^+$; see below also). By using the isolated mutants as donors and strain TT4427 [*hisD6404*(Am) *leuA414*(Am)] as the recipient, we demonstrated that in one of the mutants (strain GT1975) the *supF30* allele was still present. In the other mutant, Tn*10d*Cm was located very close to the *supF30* allele and it was not further analyzed. Strain GT1975, which harbored the potential antisuppressor mutation *zxx-2508*::Tn*10d*Cm, was tested further. The antisuppressor phenotype was found to be 100% linked (202 of 202) to the Cm^r phenotype; Tn*10d*Cm was not located on the F′ *lacI-lacZ* plasmid nor was it cotransducible with either *supF30*, *miaA1*, or *hisT1509*. (The last two mutations are known antisuppressors of *supF30* [4].) Taken together, these results indicate that the transposon insertion *zxx-2508*::Tn*10d*Cm gives rise to an external antisuppressor of *supF30* and that this mutation is different from the *miaA1* and *hisT1509* mutations.

Mutant strain GT1975 has i^{6}A37 instead of ms^{2}io^{6}A37 in its tRNA. In order to examine the composition of modified nucleosides in the tRNA of mutant strain GT1975 and its wild-type parent, GT1825, bulk tRNA was prepared, degraded to nucleosides, and analyzed by HPLC according to the method of Gehrke and Kuo (33). Figure 2A shows that strain GT1825 (wild type) contained ms^{2}io^{6}A and its nonhydroxylated derivative, ms^{2}i^{6}A. The content of ms^{2}io^{6}A in tRNA is dependent on oxygen tension and therefore varies according to the growth phase at which cells are harvested (13). However, the mutant (Fig. 2B) lacked both of these nucleosides. Moreover, the mutant strain contained a new compound that mi-

TABLE 3. Sensitivity of *miaB*$^+$ and *miaB2508*::Tn*10d*Cm strains to amino acid analogs[a]

Analog	Amino acid	Amt (μg)[b]	Zone of inhibition (diameter [mm])[c]							
			Shiftdown				No shift			
			miaAB$^+$	*miaB*	*miaA*	*miaB* relative to *miaB*$^+$	*miaAB*$^+$	*miaB*	*miaA*	*miaB* relative to *miaB*$^+$
1,2,4-Triazole	Cys	800	22 C	34 C	26 C	S	15 C	23 C	14 C	S
Azaserine	Gln	50	48 C	57 C	50 C	S	47 C	50 C	47 C	ND
L-Glutamate-γ-hydrazide	Gln	50, 500	24 T	R	24 T	R	21 C, 37 VT	13 C, 23 VT	19 C, 36 VT	R
5,5,5-Trifluoro-DL-leucine	Leu	740	43 C	58 C	52 C	S	20 C	51 C	26 C	S
S-2-Aminoethylcysteine	Lys	100, 500	9 T	29 C	10 T	S	24 C	26 C	45 VT	ND
3,4-Dehydro-DL-proline	Pro	50	32 C	45 C	38 C	S	35 C	41 C	40 C	S
DL-Serinehydroxamate	Ser	1,000	20 T	27 C	22 T	S	20 C	30 C	16 C	S
Fluoroacetate	Acetate	50, 500	24 VT	21 C	17 VT	S	R	16 T	R	S

[a] Additional analogs to which the mutant did not show altered responses or for which only subtle differences were observed are listed in Materials and Methods.

[b] When two values are listed, the first is the amount used under shiftdown conditions, while the second is the amount used when no shift occurred.

[c] The indicated amount of each analog was placed on a 6-mm-diameter paper disc. R, resistant (no zone was seen); C, clear; T, turbid; VT, very turbid; S, sensitive; ND, no difference.

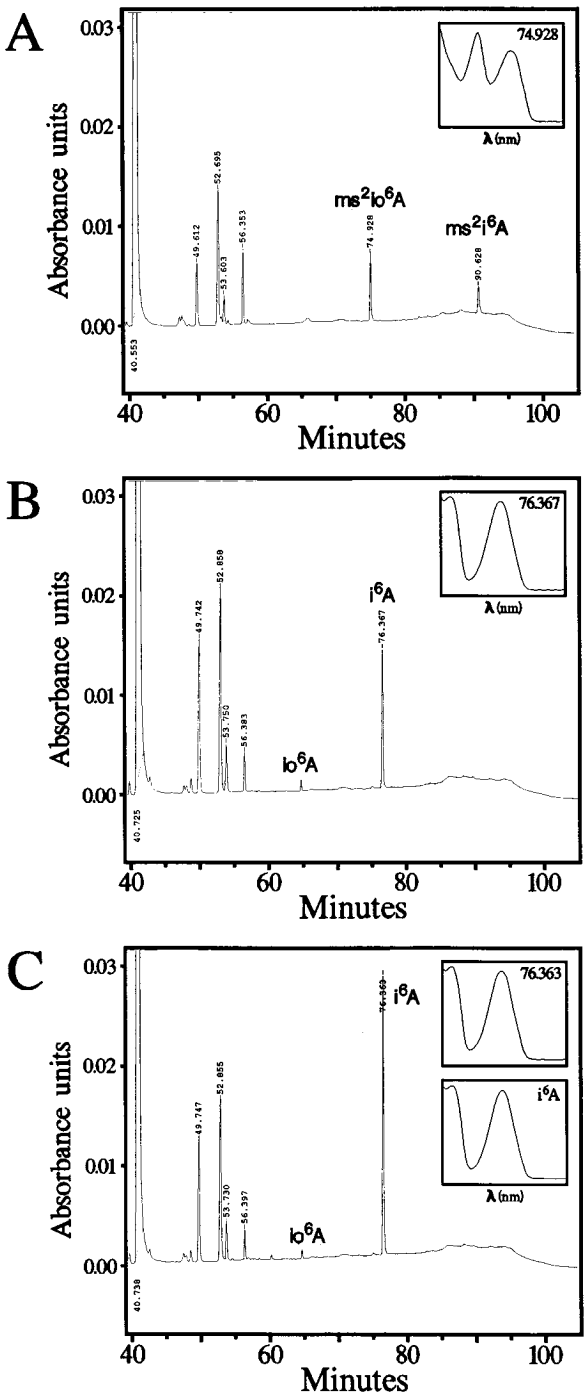

FIG. 2. HPLC profiles (A_{254}) of nucleosides from tRNAs extracted from *S. typhimurium* GT1825 (*miaB*$^+$) (A) and GT1975 (*miaB2508*::Tn*10d*Cm) (B) grown in rich MOPS medium at 37°C. (C) Chromatogram of the tRNA hydrolysate from strain GT1975 to which free i^{6}A was added as a marker. Inserted in each chromatogram is the spectrum from 200 to 350 nm of each relevant compound shown. (A) The compounds that migrated at 74.9 and 90.6 min were ms^{2}io^{6}A and ms^{2}i^{6}A, respectively. (B) The compound that migrated at 76.4 min had a different spectrum from that in panel A but was similar to the compound that migrated at 76.4 min in panel C, which corresponded to added i^{6}A. (The spectrum from analysis of pure i^{6}A is shown for comparison.)

grated with a retention time of about 76 min. This compound had a spectrum which was identical to that of i^{6}A and distinct from that of ms^{2}io^{6}A (Fig. 2). No other differences between the mutant and wild-type strains were observed. Five independent transductants were tested, and they all showed this feature, convincingly showing that lack of the ms^2 group was linked to the Tn*10d*Cm insertion. The addition of i^{6}A to a tRNA preparation from strain GT1975 (Fig. 2C) showed that i^{6}A comigrated with the compound that accumulated in the mutant. We conclude that tRNA from the mutant strain contains i^{6}A instead of ms^{2}io^{6}A. Thus, the mutant has a block in synthesis of the ms^2 group. Note also that io^{6}A, which migrates with a retention time of about 64 min (13), was present only in small amounts in the mutant; only 5% of the accumulated i^{6}A was in hydroxylated form. Moreover, ms^{2}i^{6}A is present in wild-type cells at low oxygen tension, strongly suggesting that ms^{2}i^{6}A, not i^{6}A, is the normal substrate for the hydroxylating enzyme. Taken together, these results suggest that the methylthiolation reaction precedes the hydroxylation reaction (or occurs in conjunction with it) and thus that it is the second step in synthesis of ms^{2}io^{6}A. We suggest that the insertion is in the *miaB* gene; therefore, the mutation is designated *miaB2508*::Tn*10d*Cm.

TABLE 4. Transductional mapping of the *miaB2508*::Tn*10d*Cm mutation[a]

Phenotype				No. of colonies	%
miaB2508::Tn*10d*Cm	*zbf-99*::Tn*10d*Tc	*nag*	*fur*		
D	D	D	D	189	59
D	R	D	D	1	0.3
R	D	D	D	36	11
R	R	D	D	23	7.2
D	D	D	R	57	18
D	R	D	R	0	0
R	D	D	R	9	2.8
R	R	D	R	6	1.9

[a] Transductions were carried out with strain GT3147 (*miaB2508*::Tn*10d*Cm *nag-1 zbf-99*::Tn*10d*Tc *fur-1 iroA-lacZ*) as the recipient, and the transducing phage stock was grown on strain LT2. Nag$^+$ transductants were selected, and donor (D) and recipient (R) phenotypes were scored. The *fur-1* phenotype was scored as Lac$^+$ since strain GT3147 is *iroA-lacZ* and the *iroA* gene is derepressed in a *fur-1* background. The *iroA* mutation is not linked to *miaB2508*::Tn*10d*Cm (data not shown) and is located at min 57 (30) on the chromosome. A total of 321 transductants were scored.

The *miaB2508*::Tn*10d*Cm mutation is located in the vicinity of *nag* at min 15 on the *S. typhimurium* chromosome. To elucidate the position of the *miaB2508*::Tn*10d*Cm mutation on the *S. typhimurium* chromosome, we used the collection of Tn*10d*Tc insertions described by Kukral et al. (43). Only one of these transposon insertions, *zxx-3210*::Tn*10d*Tc, showed linkage to the *miaB2508*::Tn*10d*Cm mutation. Although the location of the *zxx-3210*::Tn*10d*Tc insertion on the chromosomal map of *S. typhimurium* was unknown, it still could be used to aid in the mapping of *miaB2508*::Tn*10d*Cm. Since the Mu*d*P and Mu*d*Q mapping system described by Benson and Goldman (3) requires a tetracycline resistance (Tc^r) marker, the closely linked *zxx-3210*::Tn*10d*Tc was mapped by selection for tetracycline-sensitive clones. By using the collection of Mu*d*-P22 lysates, Tc^s clones were obtained with the *zxx-3210*::Tn*10d*Tc strain when lysates of strains TT15232, TT15237, TT15629, and TT15240 were used as donors. This means that the *zxx-3210*::Tn*10d*Tc insertion, and consequently the linked *miaB2508*::Tn*10d*Cm insertion also, is located between min 13 and 17 on the chromosomal map of *S. typhimurium*. Cotransductions of *miaB2508*::Tn*10d*Cm with different markers in this area showed that it was 60% linked to the *nag* gene and 40% linked to the *fur* gene at min 15 on the chromosome. The relative locations of these markers were determined in a four-

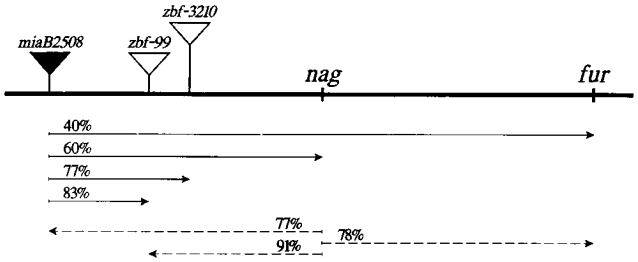

FIG. 3. Gene organization of the min 15 region of the *S. typhimurium* chromosome. Solid arrows represent cotransduction data, with each arrow beginning at the selected marker. Broken arrows denote the cotransduction frequencies obtained from a four-factor cross (Table 4) in which the relative locations of these alleles were established. Filled triangle, Tn*10d*Cm; unfilled triangles, Tn*10d*Tc.

factor cross (Table 4; Fig. 3), with strain LT2 as the donor and strain GT3147 (*miaB2508*::Tn*10d*Cm *nag-1 zbf-99*::Tn*10d*Tc *fur-1 iroA-lacZ*) as the recipient. Nag$^+$ colonies were selected, and the resulting 321 colonies were scored for Cm^r, Tc^r, and Lac$^+$ (Lac$^+$ represents the *fur* phenotype [Table 4]; see text also). Table 4 shows that the *miaB* gene is not located on the same side of *nag* as the *fur* gene, since the *fur* and *miaB* genes were 77.5 and 76.9% linked to the *nag* gene, respectively, but were only 59% (190 of 321) linked to each other. Furthermore, *zbf-99*::Tn*10d*Tc is located between the *miaB* and *nag* genes; among the 247 *miaB*$^+$ (D) and *nag*$^+$ (D) transductants, only 1 retained the recipient Tc^s phenotype, whereas 24 of the 249 Fur$^+$ Nag$^+$ transductants retained the Tc^s phenotype. These results show that the *miaB* and *fur* genes are on opposite sides of the *nag* gene.

Lack of the ms^2 part of the ms^{2}io^{6}A modification has no effect on growth and polypeptide chain elongation rates. Strains GT522 (*miaB*$^+$) and GT2176 (*miaB2508*::Tn*10d*Cm), a transductant of GT522, were grown under steady-state conditions in different media at 37°C (Table 2). No differences in the growth rates of mutant and wild-type strains were observed. The polypeptide chain elongation rate was determined as the rate at which β-galactosidase was synthesized. Thus, the rate of migration of a ribosome along the *lacZ* message was unaffected by introduction of the *miaB2508*::Tn*10d*Cm mutation.

The *miaB2508*::Tn*10d*Cm mutation alters cell metabolism. A difference in response toward analogs of an amino acid indicates that some aspect of the metabolism of the corresponding amino acid in the mutant is altered, compared with that in a wild-type cell. The amino acid analogs which gave substantially different responses in the *miaB2508*::Tn*10d*Cm mutant are listed in Table 3. How the mutant was affected by 1,2,4-triazole (Cys), azaserine (Gln), DL-serinehydroxamate (Ser), *S*-2-aminoethylcysteine (Lys), 3,4-dehydro-DL-proline (Pro), and 5,5,5-trifluoro-DL-leucine (Leu) is noteworthy. The observed effects may have been due to a gene located downstream of the insertion. This issue cannot be resolved until gene organization in the area around the transposon has been established. However, the responses of the *miaB* mutant to the last two analogs were similar to those of a *miaA* mutant, suggesting that the observed effects in these two cases may be caused by aberrant translation by undermodified tRNA.

The ability of the *miaB2508*::Tn*10d*Cm strain to oxidize different compounds was also investigated. Indicator plates (Biolog ES MicroPlates) that contained 2,3,5-triphenyltetrazolium chloride and different test substrates were used (9). If the compound in a well is oxidized, the redox dye is reduced, giving a purple color. Ninety-five substrates were tested, and two of them gave responses in the mutant strain substantially different from those in the wild-type strain. The wild-type strain was almost completely incapable of oxidizing glycyl-L-aspartic acid, whereas after 6 h of incubation, the *miaB* mutant had increased A_{590} by 0.5 unit. On the other hand, the wild-type strain was able to oxidize formic acid, which was not achieved by the *miaB* mutant. These results further support the notion that the *miaB2508*::Tn*10d*Cm mutation influences cell metabolism.

Lack of the ms^2 part of the ms^{2}io^{6}A modification strongly reduces the decoding efficiencies of amber suppressor tRNAs. In order to examine if the *miaB2508*::Tn*10d*Cm mutation had any effect on the efficiencies of certain suppressor tRNAs to read amber codons, we combined the *miaB* mutation with the following suppressor tRNAs that normally contain ms^{2}io^{6}A at position 37: *supD10*, *supF30*, and *supJ60* ($tRNA^{Ser}_{CUA}$, $tRNA^{Tyr}_{CUA}$, and $tRNA^{Leu}_{CUA}$, respectively). To monitor suppression levels, we used an F′ plasmid that carried an in-frame fusion between *lacI* and *lacZ* that produced hybrid β-galactosidase activity (12, 51). In this F′ plasmid, in-frame amber codons have been introduced at different positions in the *lacI* part (50). Therefore, translation is terminated and little or no hybrid protein is produced unless these amber codons are read by suppressor tRNAs. Thus, β-galactosidase activity is a direct measurement of the efficiencies of suppressor tRNAs to read these amber codons. The level of readthrough was related to the β-galactosidase level in a strain with an identical genetic background that harbored an F′ plasmid that carried a *lacI-lacZ* fusion

TABLE 5. Suppression of UAG in various codon contexts by modified and undermodified suppressor tRNAs

F′ plasmid	UAG position[a]	mRNA sequence	*supD10*			*supF30*			*supJ60*		
			β-Galactosidase activity[b]		*miaB*/*miaB*$^+$ ratio[c]	β-Galactosidase activity		*miaB*/*miaB*$^+$ ratio	β-Galactosidase activity		*miaB*/*miaB*$^+$ ratio
			miaB$^+$	*miaB*		*miaB*$^+$	*miaB*		*miaB*$^+$	*miaB*	
112	84	AUU-UAG-UCU	9.9	4.8	0.48	15	5.9	0.39	13	8.1	0.62
117	117	GCG-UAG-CGC	4.9	1.5	0.31	8.8	1.4	0.16	10	4.4	0.44
121	181	CAG-UAG-AUC	22	15	0.68	40	24	0.60	34	22	0.65
122	189	CCA-UAG-AGU	23	13	0.57	39	21	0.54	36	23	0.64
A24	220	GAC-UAG-AGU	18	9.5	0.53	34	17	0.50	29	24	0.83
128	228	CAA-UAG-ACC	22	14	0.64	48	23	0.48	33	26	0.79

[a] Position of the UAG codon in the *lacI* part of the *lacI-lacZ* gene fusion.
[b] β-Galactosidase activity is expressed as a percentage of the activity in a strain that carried the same *lacI-lacZ* fusion without a nonsense codon. The standard error (calculated as described by Ipsen and Feigl [40]) was ±15% or less, except for the standard errors of the values for *supD10* (*miaB* at position 117), *supJ60* (*miaB*$^+$ at positions 181 and 189), and *supJ60* (*miaB* at position 220), which were between 20 and 25%.
[c] Ratio of the β-galactosidase activity of the *miaB* strain to that of the *miaB*$^+$ strain.

TABLE 6. Efficiency of suppression of UAG in two codon contexts by various suppressor tRNAs with different degrees of modification

Suppressor	β-Galactosidase activity[a]				*hisD6404-C1*/*hisD6404* ratio[b]		
	hisD6404 (UAG-C)		*hisD6404-C1* (UAG-A)				
	miaB$^+$	*miaB*	*miaB*$^+$	*miaB*	*miaAB*$^+$	*miaB*	*miaA*[c]
supD10	36	15	50	26	1.4 (1.8)	1.7 (2.0)	5.7 (6.1)
supF30	50	17	71	38	1.4 (2.4)	2.2 (3.0)	5.2 (5.8)
supJ60	51	21	55	30	1.1 (1.2)	1.4 (1.6)	5.3 (7.9)

[a] β-Galactosidase activity is expressed as a percentage of the activity obtained with strain GT1696, which is Lac$^+$. The standard error is ±14% or less (calculated as described by Ipsen and Feigl [40]).

[b] Data are the ratios of suppression in either *miaAB*$^+$, *miaB2508*::Tn*10d*Cm, or *miaA1* cells in the indicated contexts. Since suppression efficiency is defined as a probability and thus cannot exceed 1.0, Smith and Yarus (62) derived an equation which allows quantitative evaluation of tRNA activity from in vivo measurements of suppression efficiency. We compared two contexts (UAG-A and UAG-C) in strains with no other differences. Therefore, the aminoacyl-tRNA concentrations in these two strains are the same. The equation used is as follows: $K_1/K_2 = [E_2(1 - E_1)]/[E_1(1 - E_2)]$, where E_1 and E_2 are the suppression values in the contexts of UAG-A and UAG-C, respectively. Smaller K values mean more active tRNAs; accordingly, a smaller ratio means that context 1 (UAG-A) is more active than context 2 (UAG-C). To conform to the ratio of uncorrected suppression, we present parenthetically each $(K_1/K_2)^{-1}$ ratio to show how much more efficiently UAG-A is translated.

[c] Data from studies by Ericson and Björk (28) have been included for comparison.

without a nonsense codon in the *lacI* part. Table 5 shows that the presence of the *miaB2508*::Tn*10d*Cm allele does indeed reduce (as low as 16%) the decoding efficiencies of these suppressor tRNAs. Furthermore, suppression was more sensitive in certain codon contexts (e.g., position 117 in *lacI*) than in others. This suggests that tRNA that lacks ms^2 is more codon context sensitive than is fully modified tRNA. However, the reductions in suppressor tRNA activity were not as great as those observed for the *miaA1* mutant (11) which contains a totally unmodified A-37 in its tRNA.

miaB2508::Tn*10d*Cm-induced codon context sensitivity was more pronounced at amber codons followed by a pyrimidine (positions 84 and 117), in a fashion similar to that observed for the *miaA1* mutant (11). However, since the amber codons are located at different positions in *lacI* mRNA, no firm conclusions about the influence of the nucleotide on the 3′ side of the codon can be made. To establish whether the ms^2 group senses the identity of the nucleotide 3′ of the amber codon, we utilized the system constructed by Ericson and Björk (28) which takes advantage of a codon context mutation isolated by Bossi and Roth (10), in which a C 3′ of a UAG codon in *hisD* has been changed to an A. Translational fusions between the *hisD* alleles (*hisD6404* [UAG-C] and *hisD6404-C1* [UAG-A]) and the *lacZ* gene of Mu*d*K were used to monitor the decoding efficiencies of the three aforementioned suppressor tRNAs. We compared the effects of the ms^2 group by using different bacterial constructs that differed in the allelic state of the *miaB* gene. Table 6 shows that the decreases in the decoding efficiencies of these suppressors were about the same as those observed with the *lacI-lacZ* system described above. In addition, the decoding efficiencies of the *miaB*$^+$ and *miaB2508*::Tn*10d*Cm strains increased 1.4- and 2-fold, respectively, when the C 3′ of the amber codon was changed to an A. However, for a *miaA1* mutant, the efficiency increased five- to sixfold (28). This demonstrates that lack of the ms^2 group makes tRNA only slightly more sensitive to the identity of the 3′ nucleotide, whereas total lack of the ms^{2}io^{6}A modification increases codon context sensitivity dramatically. Thus, the property of the ms^{2}io^{6}A modification that makes tRNA less sensitive to the base 3′ of the codon seems to reside primarily in the isopentenyl group.

DISCUSSION

Studies with mutants defective in biosynthesis of ms^{2}i(o)^{6}A (25, 27, 55), together with precursor analyses of methionine-, sulfur (1)-, and iron (16, 59)-starved cells, have provided information on the synthesis of this modified nucleoside. In this paper, we have presented the isolation and characterization of a novel mutant that affects the formation of ms^{2}io^{6}A in *S. typhimurium* tRNA. The previously postulated biosynthetic pathway for ms^{2}io^{6}A (Fig. 1) was confirmed by the composition of modified nucleosides in this mutant, since as shown in Fig. 2B, tRNA of this mutant contained i^{6}A and no ms^{2}i^{6}A or ms^{2}io^{6}A was found. These results suggest that the mutant has a block in formation of the ms^2 group of ms^{2}io^{6}A; therefore, we have designated it the *miaB2508*::Tn*10d*Cm mutation. Only small amounts of io^{6}A were present. From that observation, we conclude that the hydroxylation enzyme, which is encoded by the *miaE* gene (55) and operates only under aerobic conditions (13), depends on the presence of the ms^2 group to work efficiently. Even overproduction of the MiaE peptide does not convert more than 30% of the accumulated i^{6}A in a *miaB* mutant to io^{6}A (54). The methylthiolation reaction is believed to be the second step in this biosynthesis, and the enzyme(s) that performs this seems to require the presence of the isopentenyl group since very little or no ms^{2}A is found in a *miaA* mutant (27). The methyl group originates from *S*-adenosyl-L-methionine, while the sulfur atom comes from cysteine (31). This reaction may be performed by one enzyme with two catalytic activities or by two separate enzymes. Whatever the situation is, we suggest that our mutant is impaired in the methylthiolation step of biosynthesis of ms^{2}io^{6}A. However, we cannot be sure that the mutation is actually in the structural gene. It is still possible that the transposon insertion affects some other component of the cell, which in turn influences the expression or activity of the methylthiolating enzyme(s). This reaction is dependent on iron, and starvation of this compound also leads to the accumulation of i^{6}A in tRNA (16, 59, 66). Then perturbations in the availability of iron might cause a phenotype such as that expressed by the *miaB2508*::Tn*10d*Cm mutant.

However, we have five lines of evidence that argue against iron metabolism being affected. (i) The *miaB2508*::Tn*10d*Cm mutation is located at min 15 on the *S. typhimurium* chromosome and does not coincide with any known iron-related gene. The iron regulator gene *fur* is located nearby (29), but it is well separated from the insertion point of Tn*10d*Cm (Fig. 3). If an iron-related gene is affected, it must be one that has not been previously characterized. (ii) The *miaB2508*::Tn*10d*Cm mutant was grown under conditions in which iron was available (MOPS-glucose minimal medium contains ferrous iron [53]). It has been suggested that this form of iron can be taken up by cells in a passive manner (47). (iii) Limitation of iron in a way which inhibits formation of the ms^2 group leads to reduced growth rates for cells (36). This is in great contrast to what was found for the *miaB2508*::Tn*10d*Cm mutant, which grew as well as the wild-type strain. (iv) Formation of the modified nucleoside queuosine (Q) is blocked if *E. coli* cells are starved for iron (42). If the ms^2 deficiency in the *miaB2508*::Tn*10d*Cm mutant is the result of a depleted iron pool, no Q would form in these cells. However, the Q modification was identified in

the tRNA of the *miaB2508*::Tn*10d*Cm mutant (data not shown). (v) The *iroA* gene, which is regulated by the iron-regulatory protein Fur, was not derepressed in the *miaB2508*::Tn*10d*Cm strain (data not shown). If the *miaB* mutation reduced the levels of iron in cells, this would have been sensed by the Fur protein and expression of the *iroA* gene would have been increased as a result. This was not the case. Taken together, these findings make it unlikely that the transposon insertion influences intracellular concentrations of iron.

tRNA of the *miaB2508*::Tn*10d*Cm mutant is undermodified with respect to the ms^{2}io^{6}A modification. This feature makes the mutant suitable for studies of the importance of the methylthio group in the function of tRNA. Our results show that a tRNA which contains i^{6}A instead of ms^{2}io^{6}A at position 37 is less efficient in translation. Since tRNAs that are specific for tyrosine or phenylalanine that contains unmodified A-37, i^{6}A37, or fully modified ms^{2}i^{6}A37 show the same kinetics in the aminoacylation reaction (15, 32, 67), the reduced efficiency of suppression is due to inefficient anticodon-codon interaction. The decoding efficiency of tRNA is reduced to between 83 and 16% (Tables 5 and 6), depending on the codon context and suppressor tRNA used. If tRNA has an unmodified A-37, as in a *miaA1* strain, the decoding efficiency is affected much more (efficiency decreased to between 1 and 40%) (11, 28). These results suggest that although both ms^2 and io^6 groups contribute to the decoding efficiency of tRNA, the major impact originates from the io^6 group or the combination of io^6 and ms^2 groups. This conclusion is contradictory to the results obtained by Houssier and Grosjean (39) in studies of the stability of tRNA dimers with complementary anticodons. In these in vitro experiments, the major stabilizing effect of the ms^{2}i^{6}A modification was due to the ms^2 group. However, the dissociation of complementary tRNAs was measured in solution in the absence of factors such as EF-Tu and GTP, which form complexes with aminoacyl-tRNA when presented to the A site of the ribosome. It has indeed been shown by Janiak et al. (41) that the conformation of aminoacyl-tRNA changes as the ternary complex is formed. This may explain the discrepancies found between data from in vitro model experiments and our in vivo data. Moreover, Gefter and Russell (32) showed that in an in vitro translation system, the ability of i^{6}A-containing suppressor tRNA^{Tyr} from *E. coli* to support protein synthesis is reduced to 50%, compared with that of ms^{2}i^{6}A-containing tRNA, while the decoding efficiency of completely unmodified tRNA (with respect to ms^{2}i^{6}A) is reduced to 10%. In addition, tRNA^{Phe} that lacked the ms^2 group because of iron starvation showed decreased ability (by 20 to 30%) to support protein synthesis in an in vitro system (16). These results are in full quantitative agreement with our data; taken together, a picture that shows that both the isopentenyl and methylthio groups contribute to the decoding efficiency of tRNA in translation emerges. However, quantitatively, the effect exerted by the io^6 group is much more pronounced than that of the ms^2 group.

Lack of the ms^2 group in three amber suppressor tRNAs typically reduced the suppression efficiency two- to threefold (Tables 5 and 6). Therefore, one would expect only small effects on the polypeptide elongation rate (measured as the time required for the first β-galactosidase molecule to appear). Assuming an average twofold reduction in efficiency for reading the 15.8% of codons in *lacZ* decoded by tRNAs with ms^{2}io^{6}A37, one would expect a reduction from 16.8 to 15.4 amino acids per s. Such a small difference (7%) in the translational elongation rate was not observed (Table 2); it would most likely have been overlooked with the method employed. Furthermore, the impact of the ms^2 group may also be tRNA dependent, since we know that m^{1}G37 affects cognate interaction in a tRNA-dependent manner (44). Thus, our observations of two- to threefold reductions in the activities of these three suppressor tRNAs are consistent with the apparent lack of reduction in the time required for synthesis of β-galactosidase. Note also that there was no difference in the growth rates of the *miaB* mutant and the wild type, which suggests that the polypeptide elongation rate was not severely affected.

On the other hand, this does not necessarily mean that lack of the methylthio group of ms^{2}io^{6}A has no impact on cell physiology. For instance, the *miaB2508*::Tn*10d*Cm mutant is more sensitive toward several amino acid analogs (Table 3) and metabolizes some carbon sources differently from the wild type. It is possible that inefficient translation caused by the *miaB2508*::Tn*10d*Cm mutation in some specific circumstances and contexts influences expression of some genes and operons. However, the gene organization around the transposon insertion is unknown (since the mutation has not yet been cloned); therefore, it cannot be ruled out that some or all of the effects seen are derived from a gene that is located downstream of the insertion point. This might be the case for the effects of azaserine, *S*-2-aminoethylcysteine, and DL-serinehydroxamate on the *miaB* mutant, since the *miaA1* strain responded in the same way as the wild-type strain to these analogs. However, it should be noted that the insertion of Tn*10d*Cm does not cause polar effects in some cases (26). Increased sensitivity toward 5,5,5-trifluoroleucine and 3,4-dehydro-DL-proline is, however, likely to be due to undermodification of tRNA since the *miaA1* strain also exhibits increased sensitivity.

Not only might ms^{2}io^{6}A optimize tRNA for its function in translation, but also it is quite possible that cells use the degree of modification as a regulatory device. For instance, expression of the *trp* and *tna* operons is changed in a *miaA* strain of *E. coli* (35, 69). In these cases, inefficient decoding of critical tryptophan codons in leader mRNA leads to relieved attenuation and increased expression. Lack of the ms^2 group also influences expression of the *trp* operon, but the increase is only two- to threefold, compared with the eightfold increase in a *miaA* strain (16). This is consistent with our data for translational efficiency and shows that the level of this modification can influence the regulation of specific operons in a differential manner. The fact that the *miaB2508*::Tn*10d*Cm mutant showed altered responses toward several amino acid analogs (Table 3) indicates that the metabolism of these amino acids is perturbed in some way. Since formation of the ms^2 group of ms^{2}io^{6}A37 is sensitive to iron concentrations, the level of the ms^2 group in tRNA may be part of a regulatory system which senses the availability of iron (13, 15). Furthermore, since bacterial growth in body fluids also results in lack of the ms^2 group, such an induced adaptation of tRNA may be a required character in the pathogenicity of bacteria (37). In fact, another modified nucleoside, Q, whose synthesis is sensitive to iron concentrations influences the pathogenicity of a bacterium (24). Those results together with the results presented here are consistent with the hypothesis that the levels of modified nucleosides in tRNA contribute to physiological adaptations of microorganisms to changing environments. In the case of formation of the ms^2 group, either the activity of the thiolating enzyme may be sensitive to iron concentrations or expression of the *miaB* gene may be sensitive to the availability of iron. The results presented in this paper are the first step in an investigation to reveal the mechanism by which regulation of the synthesis of the ms^2 group occurs.

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