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Escherichia coli Mutants with Defects in the Biosynthesis of 5-Methylaminomethyl-2-Thio-uridine or 1-Methylguanosine in Their tRNA

GLENN R. BJÖRK* AND KERSTIN KJELLIN-STRÅBY
Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden

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Two tRNA methyltransferase mutants, isolated as described in the accompanying paper (G. R. Björk and K. Kjellin-Stråby, J. Bacteriol. **133**:499-507, 1978), are biochemically and genetically characterized. tRNA from mutant IB13 lacks 5-methylaminomethyl-2-thio-uridine in vivo due to a permanently nonfunctional methyltransferase. Thus tRNA from this mutant is a specific substrate for the corresponding tRNA methyltransferase in vitro. In spite of this defect in tRNA, such a mutant is viable. Mutant IB11 is conditionally defective in the biosynthesis of 1-methylguanosine in tRNA due to a temperature-sensitive tRNA(1-methylguanosine)methyltransferase. In mutant cells grown at a high temperature, the level of 1-methylguanosine in bulk tRNA is 20% of that of the wild type, demonstrating that in this mutant an 80% deficiency of 1-methylguanosine in tRNA is not lethal. Genetically these two distinct lesions, *trmC2*, causing 5-methylaminomethyl-2-thio-uridine deficiency, and *trmD1*, giving a temperature-sensitive tRNA(1-methylguanosine)methyltransferase, are both located between 50 and 61 min on the *Escherichia coli* chromosome.

Only a few mutants with specific defects in tRNA modification are known. In the preceding paper (5), we presented preliminary information on two new mutants, IB11 and IB13, that seemed to be methyl deficient in their tRNA since the in vitro methylated RNA was soluble in 2M LiCl. We now show that mutant IB11 harbors a temperature-sensitive tRNA(1-methylguanosine)methyltransferase and that mutant IB13 permanently lacks mam^5s^2U in its tRNA.

MATERIALS AND METHODS

Abbreviations. Abbreviations used in this paper are those recommended by the Commission on Biochemical Nomenclature (CBN-1970): Tris, tris(hydroxymethyl)aminomethane; U, uridine; m^5U, 5-methyluridine (ribothymidine); Um, 2'-0-methyluridine; mam^5s^2U, 5-methylaminomethyl-2-thio-uridine; C, cytidine; m^5C, 5-methylcytidine; Cm, 2'-0-methylcytidine; m^1I, 1-methylinosine; G, guanosine; m^1G, 1-methylguanosine; m^2G, 2-methylguanosine; m^7G, 7-methylguanosine; A, adenosine; m^2A, 1-methyladenosine; m^6A, 6-methyladenosine; Am, 2'-0-methyladenosine; and Gm, 2'-0-methylguanosine. In addition, the tRNA methyltransferase producing 1-methylguanosine is called tRNA(1-methylguanosine)methyltransferase (EC 2.1.1.31), and *trm* and Trm^- designate the genotype and phenotype, respectively, of tRNA methylation-defective mutants.

Bacterial strains, media, and growth conditions. The *Escherichia coli* K-12 strains used are listed in Table 1. Growth conditions and media (salt medium E, rich medium LB, and others) are as described in the accompanying paper (5). In the genetic experiments, the following medium was used: medium E, glucose (0.2%), all amino acids (25 μg/ml), adenine (30 μg/ml), guanine (30 μg/ml), uracil (30 μg/ml); vitamins thiamine (1 μg/ml), riboflavine (1 μg/ml), biotine (2.5 ng/ml), nicotinic acid (1 μg/ml), pantothenic acid (2 μg/ml), inositol (5 μg/ml), paraaminobenzoic acid (0.5 μg/ml), pyridoxine (1 μg/ml), and folic acid (0.4 μg/ml); and streptomycin (100 μg/ml).

In vivo labeling. Cells were grown in LB medium containing either L-[*methyl*-^{14}C]methionine (parental strain; 5 μCi/ml of a 56-mCi/mmol solution) or L-[*methyl*-^{3}H]methionine (mutant strain, 50 μCi/ml of a 11-Ci/mmol solution). The final specific activity of L-methionine in the medium is not known since unlabeled L-methionine is present in the rich medium used. Cells were grown at different temperatures from 4×10^7 to 6×10^8 cells per ml, harvested, washed once in 10 mM Tris-hydrochloride (pH 8.0)-10 mM magnesium acetate, and total RNA was prepared as described in the accompanying paper. The aqueous phase after the phenol extraction was directly applied to a Sephadex G200 column, operated with 0.05 M triethyl-ammonium acetate buffer (pH 5.2) (9). The fractions containing rRNA (both 23S and 16S RNA) and those containing tRNA and 5S RNA were pooled. [*methyl*-^{14}C]tRNA (3×10^4 to 5×10^4 dpm) from a wild-type strain grown at 28, 37, or 43.5°C was mixed with [*methyl*-^{3}H]tRNA (3×10^5 to 5×10^5 dpm) from mutant strains grown at the same respective temperatures. The mixed tRNA was lyophilized and transferred to a siliconized test tube and degraded to nucleosides (5). The hydrolysate was subjected to two-

TABLE 1. *E. coli K-12 strains*

Strain	Sex	Relevant genetic markers[a]	Reference
PK191	Hfr	Origin is shown in Fig. 7	(13)
KL16	Hfr	Origin is shown in Fig. 7	(13)
GB6[b]	Hfr	Origin as in KL16	This paper
KL96	Hfr	Origin is shown in Fig. 7	(13)
KL983	Hfr	Origin is shown in Fig. 7	(13)
GB4	F⁻	*metB his relA fdp rpsL ampA1*	(5)
GB5	F⁻	*metB his relA pyrB valS*(Ts) *rpsL ampA1*	(5)
GM19	F⁻	*trmC1*	(14)
IB11	F⁻	*trmD1 ilvA aroC asp*[c]	(5)
IB1102	F⁻	*trmD1 relA ilvA aroC asp metB his rpsL ampA1*	This paper[d]
IB13	F⁻	*trmC2*,[c] some additional uncharacterized auxotrophic markers	(5)
IB1302	F⁻	*trmC2 metB his relA rpsL ampA1*, some additional uncharacterized auxotrophic markers	This paper[e]

[a] The genetic abbreviations are according to Bachmann et al. (2).
[b] GB6 is a spontaneous nalidixic acid (100 μg/ml)-resistant mutant of KL16.
[c] In addition, this strain contains the markers of strain GB5.
[d] P1(G11a1) × IB11, selection for PyrB⁺, and scoring for *valS*⁺.
[e] P1(G11a1) × IB13, selection for PyrB⁺, and scoring for *valS*⁺.

dimensional thin-layer chromatography (20), and radioactivity of ^3H and ^{14}C was determined as described in the accompanying paper (5). The average of all ratios obtained from one analysis was set to 1.0, and then all normalized ratios were calculated. Thus all experiments can be compared, and when the level of a nucleoside is as in the wild type, the normalized ratio should be 1.0.

Preparation of tRNA. The tRNA preparations used for experiments on methyl group incorporation into tRNA in vitro were prepared by the method of Avital and Elson (1). The cells were grown in LB-MHU medium (5) and washed with 0.9% NaCl. The method involves phenol extraction in the presence of chloroform, stripping the amino acids (1 h at 37°C in 0.5 M Tris, pH 9.7), and fractionation of rRNA and tRNA with 2 M LiCl in 0.1 M potassium acetate (pH 5.0). The tRNA was dialyzed for 22 h against 6 liters of 0.1 mM magnesium acetate. Such tRNA preparations are reported to have a very small amount of high-molecular-weight contaminants (1), and this has been confirmed by Sephadex G100 molecular sieve chromatography, in which only a very small amount of rRNA was detected.

Methylation of tRNA in vitro. The procedure was essentially as described in reference 6.

Conjugation procedures. The conjugation procedure of Miller was used (15). Donor and recipient strains were mated at a ratio of 1:20 for indicated times at 37 or 30°C. Portions of the culture were blended for 1 min and directly plated in streptomycin-containing soft agar overlays on selective plates. If conjugation was performed for a long time, as with strain IB1302, proper dilution was made before plating. Different prototropic recombinants were selected and purified twice before scoring for the Trm⁻ phenotype. Nonselected markers were found to segregate, as expected, from their locations on the *E. coli* chromosome.

Test for TrmC phenotype. Single cell recombinants from crosses with IB1302 were inoculated into 2 ml of LB medium containing 100 μg of streptomycin per ml and incubated overnight at 30°C. The next day, cells were grown at 37°C in 12.5 ml of LB medium containing [^3H]uracil (0.25 μCi/ml of a 25-Ci/mmol solution) from 4×10^7 to 4×10^8 cells per ml. At time of harvest, samples were taken to test markers and bacterial purity. ^3H-labeled total RNA was prepared, and half of the amount was methylated in vitro in a total volume of 0.2 ml using *S*-[*methyl*-^{14}C]adenosyl-L-methionine as methyl donor as described (5). A partially purified enzyme was used and, therefore, the in vitro methylation reaction was terminated by adding 3 ml of 0.01 M $HClO_4$ containing 0.5 M $La(NO_3)_3$. Precipitated RNA was collected on a glass fiber filter and washed twice with 5 ml of 5% trichloroacetic acid and 20 ml of acetone. The $^{14}C/^3H$ ratios for *trmC*⁺ and *trmC2* recombinants were 0.06 ± 0.04 and 0.4 ± 0.1, respectively. Three independent analyses were run with control strains GB4 and IB1302. Recombinants with $^{14}C/^3H$ ratios higher than twice the $^{14}C/^3H$ ratios obtained with the control strain were judged as TrmC⁻.

Test for TrmD phenotype. Recombinants from crosses with strain IB1102 were grown and tested as described for TrmC recombinants, but at a temperature of 43.5°C. ^3H-labeled total RNA was prepared, and half the amount was methylated in vitro in a total volume of 0.2 ml. After in vitro methylation, which was catalyzed with a crude enzyme extract as described in the accompanying paper (5), carrier RNA (rRNA, 500 μg, and tRNA, 100 μg) was added, and the in vitro methylated RNA was reisolated by phenol extraction. After precipitation with ethanol, tRNA was extracted from the pellet by 2 M LiCl in 0.05 M ammonium acetate, pH 5.0. tRNA was precipitated with trichloroacetic acid (10% final concentration), collected on a glass fiber filter, and washed as described above. When strain IB11 was the recipient, the purified recombinants were grown overnight at 30°C in 0.5 ml of LB medium containing [^3H]uracil. Duplicates of each purified recombinant were grown.

The next day the culture was diluted with 10 ml of the same medium and incubated for 4 to 6 h at 43.5°C. After addition of carrier cells (10^7 cells), the culture was harvested and cells were washed once with 10 mM Tris-hydrochloride (pH 8.0) containing 0.01 M magnesium acetate. The ^{3}H-labeled RNA was methylated in vitro as described above for IB1102 recombinants.

The $^{14}C/^3H$ ratios for *trmD*$^+$ and *trmD1* were 0.027 ± 0.002 and 0.11 ± 0.03, respectively. Three independent analyses were run with control strains GB4 and GB5 and mutants IB11 and IB1102. Recombinants with $^{14}C/^3H$ ratios higher than twice the $^{14}C/^3H$ ratios obtained with the control strains were judged as Trm$^-$. The *trmD*$^+$ recombinants were tested at least twice to ascertain that the genotype was correct.

RESULTS

Methyl group acceptor in IB11 and IB13 is tRNA. When total RNA from mutants IB11 and IB13 was methylated in vitro, the label is soluble in 2 M LiCl. This could result from either tRNA being the methyl group acceptor or from an extensive degradation of in vitro labeled rRNA resulting in small components that are LiCl extractable. To discriminate between these two possibilities, strains IB11 and IB13 and the parental strain GB5 were grown at 28°C in rich medium (LB-MHU) supplemented with [^{3}H]uracil. At 2 × 10^8 cells per ml, the cultures were shifted to 44°C and incubated for 4 h. Total RNA was extracted and methylated with a wild-type enzyme and *S-[methyl-*^{14}*C]*-adenosyl-L-methionine as methyl donor. The ^{14}C- and ^{3}H-labeled total RNAs were then subjected to polyacrylamide gel electrophoresis (Fig. 1). In no case did the rRNA region contain any ^{14}C-labeled methyl groups. As expected, the tRNA from the parental strain was already fully methylated in vivo and did not incorporate any additional methyl groups in vitro (Fig. 1a). In both mutants, the methyl group acceptor in vitro was tRNA. Figure 1 also shows that methyl-deficient tRNA chains from IB13 have about the same size distribution as does bulk tRNA while in IB11; in addition, larger tRNA chains were methylated as judged from $^{14}C/^3H$ ratios.

Extent of methyl deficiency corresponds to a lack of one methyl group in 3 to 5% of the tRNA chains. Mutants IB11 and IB13 carry the *valS*(Ts) allele, which hampers studies on the extent of methyl deficiency at different temperatures. This allele was, therefore, replaced by the corresponding wild-type allele by P1 transduction (Table 1). Resulting strains, IB1102 and IB1302, are thus *valS*$^+$ and able to grow also at 43.5°C. tRNA isolated from cells grown at different temperatures were tested for plateau levels of methyl group incorporation.

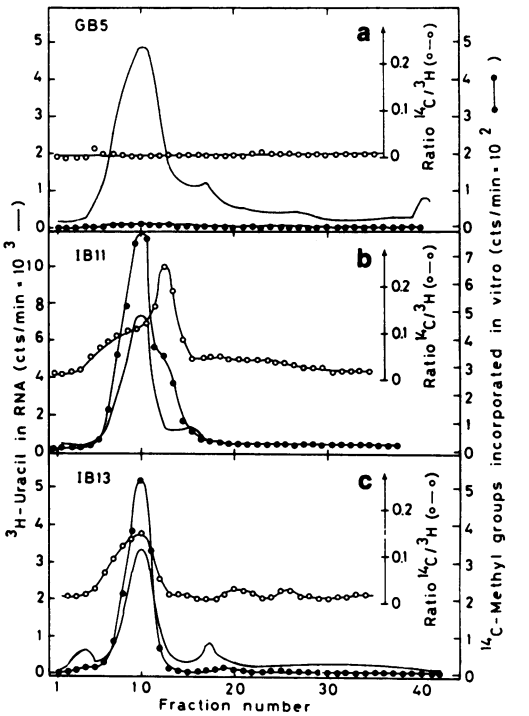

FIG. 1. *Electrophoresis of ^{3}H-labeled total RNA from parental strain GB5 (a), mutant strains IB11 (b), and IB13 (c). RNA from cells incubated at 44°C was methylated in vitro using S-[methyl-^{14}C]adenosyl-L-methionine as methyl donor. The ^{3}H- and ^{14}C-labeled RNA was reisolated and applied on a 3% polyacrylamide gel containing 0.5% agarose (17). The gel tube was 19 cm long, and the electrophoresis was performed at 220 V, 2 mA/tube, for 250 min. The gel was sliced in 2-mm sections. The RNA in each slice was hydrolyzed in 0.5 ml of 0.5 M NaOH overnight at 37°C. After neutralization with 0.5 ml of 0.5 M acetic acid, the radioactivity was determined with a scintillation counter. tRNA migrates around fraction 10, 5S rRNA around fraction 17, and rRNA around fraction 67. Fractions 42 to 72 are not shown.*

Table 2 shows that tRNA from strain IB1102 accepted methyl groups in vitro only when tRNA was isolated from cells grown at 43.5°C. The extent was about 10 times higher than that of the control tRNA, corresponding to one methyl group incorporated into 5% of the chains. In IB1302 tRNA, the methyl deficiency was independent of growth temperature, and 3 to 4% of the tRNA chains are always able to accept one methyl group each.

Product made in vitro is m^{1}G in IB11 tRNA and mam^{5}s^{2}U in IB13 tRNA. tRNA was prepared from strain IB1102 grown at 43.5°C and from strain IB1302 grown at different temperatures. After methylation in vitro using extract from wild-type cells as the enzyme

source, the *methyl*-^{14}C-labeled tRNA was reisolated with phenol extraction and ethanol precipitations. tRNA from IB1102 was enzymatically degraded (5), and the hydrolysate was subjected to two-dimensional thin-layer chromatography. The positions of radiaoctive compounds were determined by radioautography. The chromatogram shown in Fig. 2 demonstrates that the only product made was m^{1}G. Radioactive IB1102 tRNA was hydrolyzed also with 1 M HCl to purine bases and pyrimidine nucleotides (7). Two-dimensional thin-layer chromatography of this hydrolysate verified that 1-methylguanine was the only methylated product formed.

tRNA from strain IB1302 grown at different temperatures was methylated in vitro and degraded to nucleosides. When analyzed, three different products were always obtained (Fig. 3): compound 13, now identified as mam^{5}s^{2}U, and compounds 14 and 15 (65, 20, and 10% of the recovered radioactivity [9,000 cpm], respectively). Since strain GM19 (14) is known to be defective in the biosynthesis of mam^{5}s^{5}U, it could be used for comparison. Hydrolysate from in vitro methylated tRNA from GM19 gave the same three products as obtained with tRNA from our mutant. Synthetically made mam^{5}s^{2}U was incubated, degraded, and chromatographed exactly as mutant tRNA. Compound 13 was observed as a UV-absorbing spot, but no UV-absorbing material was observed at the positions of compounds 14 and 15. These two compounds might still be degradation products of mam^{5}s^{2}U, but their identification must await further experimentation. RNase T2 degradation of in vitro methylated tRNA from IB1302 or GM19 gave one predominating compound (5-methylaminomethyl-2-thio-uridylic acid) and several minor spots when the nucleotide mixture was analyzed by two-dimensional thin-layer chromatography by the method of Nishimura (16). Thus, our mutant IB1302 and strain GM19 both seem to be defective in their biosynthesis of this highly modified nucleoside.

TABLE 2. *Extent of methylation in vitro into tRNA from wild-type and mutant cells grown at different temperatures*[a]

Growth temp (°C)	Moles of CH_3 per mole of tRNA		
	GB5	IB1102	IB1302
30	0.002	0.004	0.04
37	ND[b]	0.006	0.03
43.5	0.004	0.05	0.07

[a] Experimental details are given in reference 6.
[b] ND, Not determined.

Mutant IB11 lacks m^{1}G, and mutant IB13 lacks mam^{5}s^{2}U in vivo. Results from in vitro experiments (Fig. 2 and 3) suggest that in vivo the tRNA of the mutants should have a deficiency of m^{1}G in one of the strains and of mam^{5}s^{2}U in the other. This tentative conclusion was verified by a direct measurement of the in vivo level of these nucleosides at different temperatures. Since all methylated nucleosides found in tRNA were not available to us as markers, we did the experiment in the following way. The wild-type strain, GB4, was grown in the presence of L-[*methyl*-^{14}C]methionine while the mutants were grown in L-[*methyl*-^{3}H]methionine. Total RNA was extracted, rRNA and tRNA were separated on a Sephadex G200 column, and ^{14}C-labeled tRNA from wild-type cells was mixed with ^{3}H-labeled tRNA from one mutant at a time. The mixed tRNA was concentrated, digested to nucleosides, and separated on two-dimensional thin-layer chromatography. The ^{14}C-labeled nucleosides from wild-type

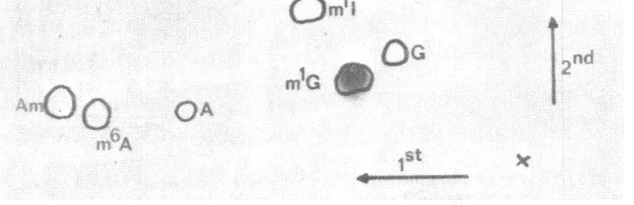

FIG. 2. *Two-dimensional thin-layer chromatography of hydrolysate from tRNA isolated from strain IB11 after incubation at 44°C. tRNA, methylated in vitro with enzyme extract from parent strain GB5, was hydrolyzed to nucleosides. The hydrolysate was subjected to chromatography as described in the text. Solid lines indicate areas in which UV markers were found. A total of 91% of recovered counts (1,963 cpm) were in m^{1}G.*

tRNA were visualized by autoradiography as shown in Fig. 4. Added synthetic markers localized by UV are indicated by full lines, while compounds for which no markers were available are indicated by dashed lines. Each area on the chromatogram that coincides with a spot on the autoradiogram was cut out for determining the *methyl*-^{3}H/*methyl*-^{14}C ratios.

The results of such analyses for the two mutants IB1102 and IB1302 grown at different temperatures are shown in Table 3. Data are given as normalized ratios, allowing direct comparisons between the different experiments. Thus at 43.5°C, IB1102 tRNA had a m^1G level that was only 20% of the normal level (0.12 mol/mol of tRNA), whereas at 28°C the level was normal. Compound 5 was the only other spot for which a significant difference was found. Table 3 shows that the only compound affected in mutant IB1302 is mam^5s^2U (compound 13 in Fig. 4). At 28°C the level of this nucleoside is only 10% of the level found in wild-type tRNA, which agrees

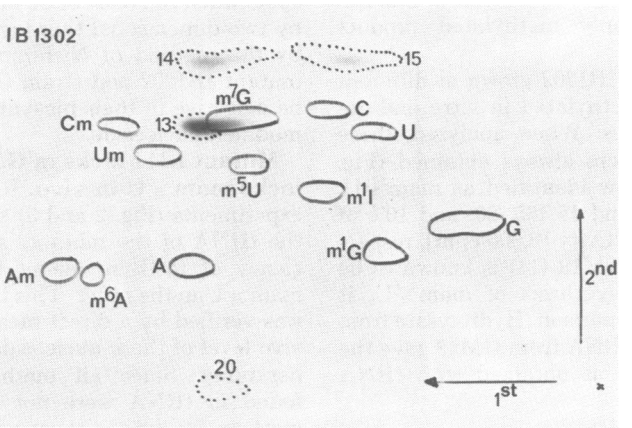

FIG. 3. *Two-dimensional thin-layer chromatography of hydrolysate from tRNA originating from strain IB1302 grown at 43.5°C. Before digestion to nucleosides, tRNA was methylated in vitro using enzyme fraction PII, which is an enzyme extract purified through a two-phase system, diethylaminoethyl-cellulose and phosphocellulose chromatography. Dashed areas indicate locations of radiaoctive spots visualized by autoradiography. The same results were obtained with tRNA from cells grown at 28°C.*

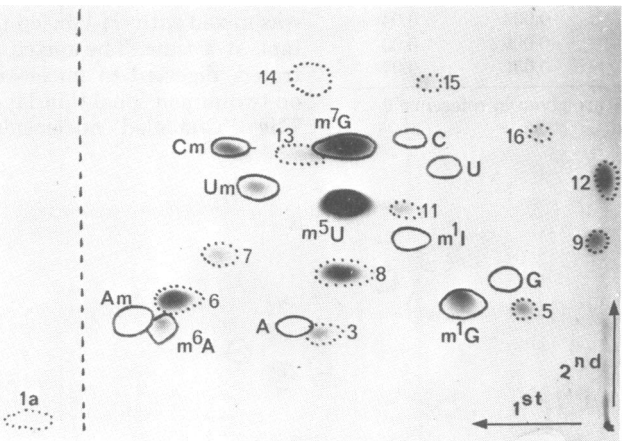

FIG. 4. *Two-dimensional thin-layer chromatography of hydrolysate of tRNA from IB1102 grown at 28°C and labeled in vivo with* L-*[methyl-*^{14}*C]methionine. After chromatography in the first dimension, the chromatogram was cut (dashed line) and subjected to chromatography in the second dimension. Thus, compound 1a was only run in the first dimension. By using authentic markers, compound 13 was identified as* mam^5s^2U *and compound 8 as Gm. Compound 6 is probably* m^2A *since the level of this compound agrees with the level found when a comparable sample is analyzed as a free base (2-methyladenine) (6). Other dashed areas denote the position of unidentified methylated nucleosides.*

TABLE 3. *Normalized ^3H-CH_3/^{14}C-CH_3 ratios of in vivo labeled tRNA from mutant (^3H) and wild type (^{14}C)*

Compound[a]	Moles of CH_3 per mole of tRNA in wild type[b] (37°C)	Normalized ratios					
		IB1302			IB1102		
		28°C	37°C	43.5°C	28°C	37°C	43.5°C
1a	0.06	0.84	1.14	1.23	0.90	1.14	
m^6A	0.06	0.87	0.69	1.17	0.97	0.64	1.17
m^2A (6)[c]	0.20	0.77	0.85	0.82	0.81	0.83	0.87
3	0.05	1.01	1.06	0.95	1.09	0.93	0.96
m^1G	0.12	1.10	0.88	0.97	1.00	0.75	**0.23**[d]
5	0.03	0.91	1.04	0.86	0.90	0.77	**0.62**[d]
7	0.04	1.06	1.12	0.85	1.05	0.84	0.98
Gm (8)	0.14	2.02[e]	1.87[e]	1.88[e]	1.55[e]	1.57[e]	1.88[e]
9	0.12	0.10[f]	0.07[f]	0.13[f]	0.13[f]	0.29[f]	0.08[f]
12	0.06	1.03	1.35	1.08	0.26	1.27	1.42
m^5U	1.00	0.97	1.01	0.88	1.02	1.01	0.91
Um	0.07	0.97	0.66	0.97	1.15	0.68	0.81
Cm	0.06	1.05	1.08	1.00	1.08	0.97	1.00
mam^5s^2U (13)	0.04	**0.09**[d]	**0.74**[d]	**0.53**[d]	0.96	0.91	1.05
m^7G	0.53	0.99	0.93	0.93	1.10	0.95	1.06

[a] Compounds are defined in Fig. 4. Included in these analyses are those compounds which constitute in one scintillation vial more than 1% of recovered ^{14}C counts per minute. On each chromatogram, 4×10^3 to 8×10^3 ^{14}C cmp were recovered. Compound 11 is not listed in the table since it was not well separated from m^5U in some analyses.

[b] This was calculated as counts per minute/counts per minute in m^5U for three independent experiments with strain GB4. Standard error of the mean was ≤0.01 for each compound except for compound 1a (0.02) and m^7G (0.04).

[c] Numbers in parentheses refer to spot numbers in Fig. 4.

[d] Values in boldface are considered to be significantly different from the corresponding wild-type value (here 1.0) since they differ by more than one standard deviation. The methodological error is ±5 − 15% for all compounds except for compounds 9 (±24%) and 12 (±48%).

[e] This high ratio depends on the unexpected low level (0.07 to 0.14) of Gm found in the wild-type strain GB4. In *S. typhimurium*, the level of Gm is 0.13 to 0.20 mol/mol of tRNA, which we assume to be the normal level even in *E. coli* GB5, the parental strain. This strain is temperature sensitive and cannot be used as a reference strain at temperatures above 32°C.

[f] This low ratio depends on isotopic effect, since it was obtained by mixing ^3H-CH_3-labeled tRNA with ^{14}C-CH_3-labeled tRNA, both from wild-type cells.

with in vitro data (cf. Table 2). mam^5s^2U and m^7G migrate close to each other, and m^7G counts can easily contaminate the mam^5s^2U area considerably (Fig. 4), especially since m^7G in wild-type tRNA is at least 10 times as abundant as is mam^5s^2U. We therefore believe that contamination with m^7G has affected the ratios for mam^5s^2U at 37 and 43.5°C (Table 3). In summary, mutant IB1302 had, at all temperatures, less than 10% of the normal level of mam^5s^2U in vivo in its tRNA. Mutant IB1102 had no methyl deficiency at low temperatures, but had only 20% of the normal m^1G level in its tRNA at high temperatures.

Mutant IB11 harbors a temperature-sensitive tRNA(1-methylguanosine)methyltransferase. To establish whether the lack of m^1G in vivo is due to a defective tRNA(1-methylguanosine)transferase, the following experiments were performed. We used enzyme extract made from strain IB11 to methylate generally methyl-deficient tRNA from methionine-starved *E. coli* W6 at low and high temperatures. At 25°C, many methylated compounds, including m^1G, were made (ratio, $m^1G/m^5U = 0.15$). This pattern was similar to that obtained with extract from the parental strain ($m^1G/m^5U = 0.35$) (data not shown). When the methylation reaction was performed at 37°C, the only dramatic change was in the amount of m^1G made ($m^1G/m^5U = 0.01$). Thus, in vitro tRNA(1-methylguanosine)methyltransferase was the only methylating enzyme in strain IB11 that was affected by temperature. Results presented in Tables 2 and 3 support the finding that this enzyme from IB11 is temperature sensitive. If so, the half-life for tRNA(1-methylguanosine) methyltransferase from mutant and wild-type cells should be different. This is clearly shown in Fig. 6, in which the half-life at 35°C of the

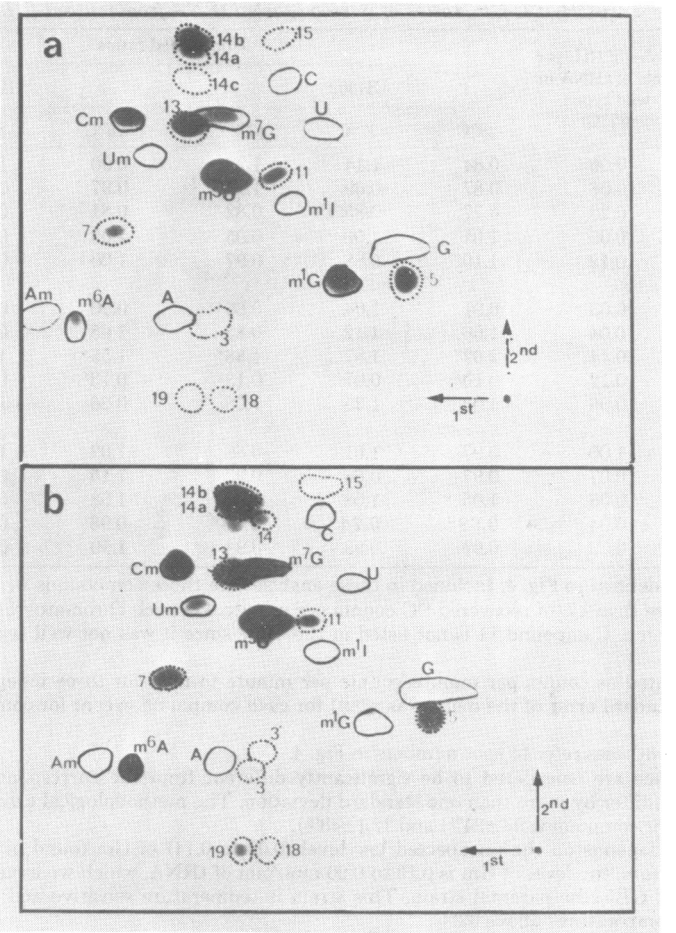

FIG. 5. *Two-dimensional thin-layer chromatography of hydrolysate of generally methyl-deficient tRNA methylated in vitro using enzyme extract from mutant strain IB11 grown at 28°C. Incubation temperature was 25°C (a) and 37°C (b). In vitro methylated tRNA was degraded to nucleosides and separated by thin-layer chromatography. (a) m^5U, 5,900 cpm; m^1G/m^5U, 0.15, (b) m^5U, 15,000 cpm; m^1G/m^5U, 0.01.*

mutant enzyme ($T_{1/2}$ = 9 min) was 10 times shorter than that for the parental enzyme ($T_{1/2}$ = 90 min).

Preliminary mapping of the tRNA methyl-deficient character in mutants IB1102 and IB1302. Since, at present, the Trm$^-$ phenotype cannot be selected, the identification and mappings of this character must be laborious. This was particularly true for the temperature-sensitive phenotype of the mutation in IB1102. It was therefore necessary to select for other phenotypes, and among such recombinants determine the linkage to the Trm$^-$ phenotype. This was done by extracting [^{3}H]RNA from each recombinant and testing for the ability to accept methyl groups in vitro. Since crude enzyme was used, it was necessary to reisolate the in vitro methylated total RNA by phenol extraction, precipitate the RNA with ethanol, extract tRNA with 2M LiCl, then collect the tRNA on a filter, and determine the [*methyl*-^{14}C]RNA/[*methyl*-^{3}H]RNA ratios. Since this tedious procedure had to be used, only about 10 recombinants of each kind were screened for their Trm$^-$ phenotype. The difference in the $^{14}C/^3H$ ratio for the Trm$^+$ and Trm$^-$ phenotypes was about five times. Trm$^-$ phenotype was easily detected as a high $^{14}C/^3H$ ratio. However, if the methylation reaction for some reason did not function properly, theoretically we could get a false Trm$^+$ phenotype. Clones giving a Trm$^+$ phenotype were therefore tested at least twice. The results of the different crosses are shown in Table 4, and the origins of the different Hfr strains are depicted in Fig. 7.

When strain IB1302 (His$^-$, Trm$^-$) was mated

with Hfr KL96 (His$^+$, Trm$^+$) and His$^+$ recombinants were selected for, none was Trm$^+$. However, by mating IB1302 with either KL16 (His$^+$, Trm$^+$) or PK191 (His$^+$, Trm$^+$) and selecting for His$^+$ recombinants, Trm$^+$ recombinants were found. Thus, the Trm$^-$ phenotype in IB1302 is located between the injection points of KL96 and KL16, i.e., between 46 and 16 min. The mutation *trmC1*, present in strain GM19, is located between the injection points of KL983 (51 min) and KL16 (14). Thus, the product made in vitro and the map position seem to be the same in our mutant and in strain GM19.

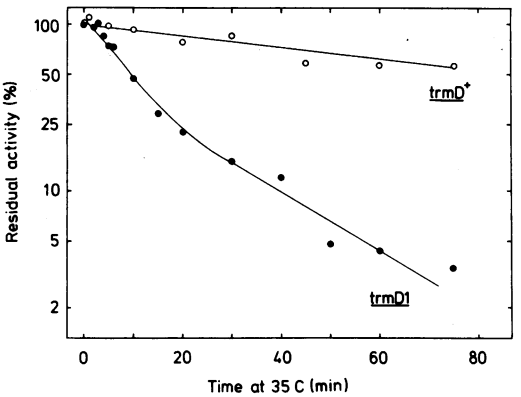

FIG. 6. *Heat inactivation of tRNA(1-methylguanosine)methyltransferase from mutant strain IB11 (trmD1) and wild-type strain (trmD$^+$). Ribosome-free crude enzyme extracts were preincubated at 35°C for the indicated times in the presence of 0.14 M Tris-hydrochloride (pH 8.0), 1.4 mM dithiothreitol, 0.14 mM EDTA, 14 mM $MgSO_4$, and 29 mM NH_4Cl. Thereafter, samples from the preincubation mixture were used as methylating enzymes (10 μg of protein per ml) for transfer of methyl groups at 25°C from S-[methyl-^{14}C]adenosyl-L-methionine (45 nmol/ml, 56 mCi/mmol) to tRNA (1.5 mg/ml) prepared from IB11 cells grown at 44°C. Conditions for in vitro methylation are as described in the text of the accompanying paper (5); 100% residual activity corresponds to 1,060 (trmD$^+$) and 1,030 (trmD1) cpm/30 min.*

The mutation *trmD1* that causes tRNA(1-methylguanosine)methyltransferase to become temperature sensitive was tentatively localized in the area between the injection points of KL16 and PK191 (cf. Fig. 7). Since the strain IB1102 also had acquired different auxotrophic characters, we did an interrupted mating experiment with IB1102 and PK191. Among His$^+$ recombinants isolated at 14 min (data not shown) and *aroC*$^+$ recombinants isolated as late as 20 min after mixing the two strains, none was Trm$^-$ (Table 4). When selecting for a late marker (*metB*$^+$, 87 min), *trmD*$^+$ recombinants were obtained with PK191 (data not shown). Thus, the *trmD* gene should be located clockwise of *aroC*$^+$ (50 min). Using donor strain GB6, which injects counterclockwise at 61 min, *trmD*$^+$ recombinants were obtained among selected *aroC*$^+$ recombinants. Thus, the *trmD* gene must be located between the injection point of KL16 and *aroC*. This is supported by crosses at 30°C with the temperature-sensitive recipient IB11 (Table 4). Considering the kinetics at 37°C of *aroC*$^+$ entry and the lack of Trm$^+$ recombinants as late as 20 min after mixing with strain PK191, the *trmD* gene should be located between the injection point of KL16 (61 min) and min 56 to 58.

DISCUSSION

This paper describes two mutants with defects in their methylation of tRNA. Mutant IB13 is permanently deficient in the synthesis of mam^{5}s^{2}U. This conclusion is based on the following facts. Irrespective of growth temperature, tRNA from IB13 is able to accept methyl groups in vitro (Table 2, Fig. 3), and consistently these tRNA preparations all lack mam^{5}s^{2}U in vivo (Table 3). The other mutant, IB11, contains a mutated tRNA(1-methylguanosine)methyltransferase, which upon raising the temperature becomes inactivated. At 43.5°C, newly synthe-

TABLE 4. *Location of trmC and trmD genes on the E. coli K-12 chromosome*

Donor	Recipient	Time (min)	Selected marker[a]	Unselected marker	Fraction Trm$^+$	Linkage (%)
PK191	IB1102	20	*aroC*$^+$	*trmD*$^+$	0/8	0
GB6	IB1102	40	*aroC*$^+$	*trmD*$^+$	2/15	13
PK191	IB11	40[b]	*aroC*$^+$	*trmD*$^+$	0/12	0
GB6	IB11	160[b]	*aroC*$^+$	*trmD*$^+$	4/10	40
PK191	IB1302	75	*his*$^+$	*trmC*$^+$	10/10	100
KL96	IB1302	75	*his*$^+$	*trmC*$^+$	0/10	0
KL16	IB1302	75	*his*$^+$	*trmC*$^+$	10/10	100

[a] In the interrupted mating experiments performed, *his*$^+$ recombinants appeared 6 min after mixing the strains, and *aro*$^+$ appeared after 16 min. Thus, the likely aromatic gene mutated in IB11 is *aroC*.

[b] These matings were performed at 30°C.

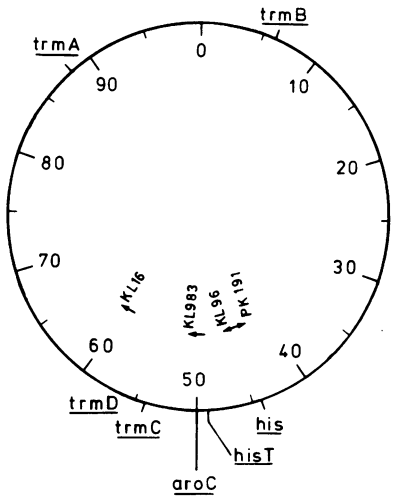

FIG. 7. *Location of genes involved in tRNA maturation (after Bachmann et al. [2]). The origins of indicated Hfr strains used are shown as well as the pertinent genetic markers used in crosses described in the text.*

sized tRNA lacks m^1G in vivo (Table 3) and thus constitutes a specific substrate for m^1G formation in vitro (Fig. 2). At low temperatures, mutant enzyme extract contains as many different methylating activities as a wild-type extract. At high temperatures, all activities except for m^1G formation were present (Fig. 5). This temperature sensitivity of the tRNA(1-methylguanosine)methyltransferase became evident also in the shorter half-life of the mutant enzyme when incubated at high temperature as compared with that of the wild-type enzyme (Fig. 6).

Only a few mutants affected in tRNA modification have been characterized earlier. Among eucaryotic organisms, a yeast mutant is known that lacks N^2-dimethylguanosine (18). Among bacteria, some *Salmonella* and *E. coli* strains have been isolated that are defective in pseudouridine modification. This mutation has an impact on the regulation of amino acid biosynthesis (10, 11, 12, 21). Aberrant translational suppression in the *supK* mutants of *S. typhimurium* is due to an unidentified defect in tRNA methylation (19). The first description of a bacterial mutant with a specific defect in methyltransferase activity was *trmA* of *E. coli*. This mutant lacks m^5U in tRNA as a consequence of a mutation in the tRNA(5-methyluridine)-methyltransferase gene (3, 4). This defect gives the cells inferior survival properties compared to those of wild-type cells (6). Later, Marinus et al. (14) characterized *E. coli* mutants with defects in the biosynthesis of m^7G (*trmB*) and of mam^5s^2U (*trmC*).

One of our strains, IB13, seems to be mutated in the *trmC* gene. tRNA from both *trmC* mutants, i.e., IB13 and Marinus' mutant GM19, gave the same products in vitro and both mutations mapped in the same area. We therefore denote our mutation *trmC2*. In our hands the extent of tRNA methylation from IB13 and GM19 is about 0.04 mol of CH_3 per mol of tRNA. Marinus et al. (14) report a level of 0.01 mol of CH_3 per mol of tRNA. We believe that this low level of methyl-group acceptance is due to differences in technique and is not biologically significant. When IB13 or GM19 were methylated in vitro, three different products were obtained, but preferentially mam^5s^2U. With strain GM19, Marinus et al. (14) found only this modified nucleoside. The three products we found were formed when both crude extract and a more purified enzyme fraction (PII, cf. Fig. 2) catalyzed the in vitro reaction. The two minor components might be either intermediates in the synthesis of the final product mam^5s^2U or degradation products of this modified nucleoside. Our attempts to demonstrate that the two minor compounds could be degradation products have so far given only negative results. Although we cannot exclude that the two minor components result from additional mutations, that identical results were obtained with two independent mutants (IB13 and GM19) indicate a relationship between the two minor compounds and mam^5s^2U.

Strain IB11 is the first mutant isolated with a defect in the biosynthesis of m^1G in tRNA. Furthermore, the mutation that has occurred renders the tRNA(1-methylguanosine)methyltransferase temperature sensitive, which is of technical advantage, and also proves that our new screening method (5) works as predicted. Of the *E. coli* tRNA chains hitherto sequenced, only $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ contain a modified guanosine (8), probably m^1G. These tRNA's contain 87 nucleosides and are among the largest of all the different tRNA species in the cell. When IB11 was methylated in vitro, we found that methylation also occurred on larger tRNA chains (cf. Fig. 1), as expected, since $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ should have sites for m^1G formation. Figure 1 also shows that other tRNA chains besides $tRNA_1^{Leu}$ may normally contain m^1G. Our mutant will thus allow us to determine which tRNA chains in *E. coli* contain m^1G. IB11 tRNA isolated from cells grown at high temperatures is a specific substrate for m^1G formation in vitro, and consistently this tRNA is deficient in this nucleoside in vivo. However, in vivo there also appears a deficiency in an unknown nucleoside, called compound 5. At 37°C the decrease

in compound 5 is of the same magnitude as for m^1G, whereas at 43.5°C the decrease is more pronounced for m^1G than for compound 5. Our enzyme extracts from wild-type cells contained activity for formation of compound 5 (cf. Fig. 5), but no (i.e., <2% of m^1G) such compound was formed with tRNA from IB1102 as substrate. A closer examination of these contradictory results might clarify whether biosyntheses of compound 5 and m^1G are interrelated, or whether two independent mutations cause the concominant decrease in the in vivo levels of m^1G and compound 5 at higher temperatures.

Mutations like those described in this paper could result in either inactive enzymes, presence of enzyme inhibitors, or changes in the methyl donor level in the cell. Enzyme extract from the mutant IB1302 lacks almost all activity for the production of mam^5s^2U in vitro, and, furthermore, the extract contains no inhibitor to this activity as tested in mixing experiments (unpublished data). The temperature sensitivity of the tRNA(1-methylguanosine)methyltransferase in vitro strongly indicates that the enzyme itself is structurally changed. Thus, in both strains IB13 and IB11, we believe that the mutations are located in the structural genes for the respective enzymes. Aside from genes *trmC* and *trmD* mentioned here, other genes (*trmA, trmB,* and *hisT*) involved in tRNA modifications have been investigated with respect to map positions (cf. Fig. 7). Thus, with the new mutants in tRNA modification now available, a picture is emerging that genes involved in tRNA biosynthesis are scattered all over the *E. coli* chromosome.

Deficiency of mam^5s^2U or m^1G in tRNA is not lethal to the cell in that genetic background in which they were isolated. Although the mutants have a reduced growth rate compared to that of the parental strain, it is premature to evaluate this as being due to a deficiency in tRNA methylation, since the mutants probably contain additional mutations. A transfer of the *trm* genes to a known genetic background must be performed before decisive experiments can be made to elucidate the consequences of these mutations for the cell.

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