Isolation and Partial Characterization of Three *Escherichia coli* Mutants with Altered Transfer Ribonucleic Acid Methylases

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Seven transfer ribonucleic acid (tRNA) methylase mutants were isolated from *Escherichia coli* K-12 by examining the ability of RNA prepared from clones of unselected mutagenized cells to accept methyl groups from S-adenosylmethionine catalyzed by crude enzymes from wild-type cells. Five of the mutants had an altered uracil-tRNA methylase; consequently their tRNA's lacked ribothymidine. One mutant had tRNA deficient in 7-methylguanosine, and one mutant contained tRNA lacking 2-thio-5-methylaminomethyluridine. The genetic loci of the three tRNA methylase mutants were distributed over the *E. coli* genome. The mutant strain deficient in 7-methylguanosine biosynthesis showed a reduced efficiency in the suppression of amber mutations carried by T4 or λ phages.

Transfer ribonucleic acid (tRNA) from many organisms contains a great variety of modified nucleotides (12, 23, 27). Most abundant among them are the methylated nucleotides. In all known cases they are formed in a post-transcriptional modification of macromolecular tRNA or precursor tRNA by tRNA-specific methylases that use S-adenosylmethionine (SAM) as methyl donor (16). To date only a few tRNA methylases have been studied in detail partly due to the lability of these enzymes and partly due to the lack of defined RNA substrates (e.g., 32).

Bacterial mutants defective in certain tRNA methylases would be very desirable. Not only would they facilitate enzymatic studies of these enzymes, but they might also reveal the biological role of a specific methylated base in tRNA. So far only two tRNA methylase mutants are known. One is a mutant yeast strain in which the tRNA lacks 2-dimethylguanosine (25). The other is an *Escherichia coli* mutant unable to form ribothymidine in tRNA (4). Despite the specific undermethylation in their tRNA's, both strains grow normally (5, 25, 34). However, this gross observation does not rule out subtle changes in their metabolism or its regulation.

Björk and Isaksson (4) isolated the *E. coli* tRNA methylase mutant by in vitro screening of mutagenized colonies for methyl-deficient RNA. Using a similar, more sensitive proce-

dure, we had earlier identified a number of deoxyribonucleic acid (DNA) methylase mutants (20). During that work we also discovered a number of RNA methylase mutants. In this paper we describe the isolation of three different tRNA methylase mutants, each defective in the formation of one methylated nucleoside, namely, 7-methylguanosine, 2-thio-5-methylaminomethyluridine, and ribothymidine.

MATERIAL AND METHODS

Bacterial strains and bacteriophages. The bacterial strains used in this study are described in Table 1. T4 amber phages B17, N65 (7) and E605, H39X (8) were obtained from S. Champe and J. Karam, and λ gam-210 (35) was obtained from A. Skalka.

Media and reagents. Complete medium consisted of 20 g of brain heart infusion (Difco) per liter plus 10 μ g of thymine per ml solidified when required with 1.6% agar. M9 (1) was the minimal medium used and was supplemented with the required growth factors (2). Streptomycin was added at a final concentration of 200 μ g/ml.

[methyl-¹⁴C]SAM (50 mCi/mmol), [methyl-³H]SAM (379 mCi/mmol), [³H]phenylalanine (5.1 Ci/mmol), and [³H]glutamic acid (20 Ci/mmol) were obtained commercially. Ribonuclease (RNase) T2 (Sankyo Co.) and bacterial alkaline phosphatase were obtained from Calbiochem and Worthington Biochemical Corp., respectively. A sample of 2-thio-5-methylaminomethyluridine 3'-phosphate was a generous gift of S. Nishimura.

Mutagenesis. Log-phase cultures of strain GM1 were treated with N-methyl-N-nitro-N-nitrosoguanidine as described by Adelberg et al. (3).

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TABLE 1. *Description of bacterial strains*

Strain	Sex	Relevant genotype	Source
GM1	F ⁻	<i>trm</i> ⁺	Marinus and Morris (20)
GM18	F ⁻	<i>trmB1</i> ^a	NG ^b treatment of GM1
GM19	F ⁻	<i>trmC2</i> ^a	NG treatment of GM1
GM20-24	F ⁻	<i>trmA11-trmA15</i> ^a	NG treatment of GM1
Hfr 3000	Hfr	<i>thi-1 rel-1</i> λ ⁻	CGSC ^c
KL16	Hfr	<i>thi-1 rel-1</i> λ ⁻	CGSC
KL154	Hfr	<i>purF1 mtl-2 xyl-7 gal-6 lacY1</i> or <i>Z4</i>	CGSC
KL983	Hfr	<i>xyl-7 lacY1 mglP1</i>	CGSC
PK191	Hfr	<i>thi-1 Δ(proB-lac)_{X111} sup-56</i>	CGSC
C6	Hfr	<i>cysG49 metB1 rel-1</i> (λ)	Harris et al. (13)
W3679	F ⁻	<i>argC24 galT23</i> λ ⁻	CGSC
KL356	F ⁻	<i>trmA14 galT23</i> λ ⁻	P1 (GM23) × W3679 → Arg ⁺

^a In addition, these strains contain the following mutations: *thr-1 leu-6 proA2 his-4 metB1 lacY1 galK2 ara-14 xyl-5 mtl-1 str-31 tsx-33 thi-1 thyA12 drm-6 supE44 rel*⁺.

^b NG, Nitrosoguanidine.

^c CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

Isolation of mutants. Single colonies, obtained after mutagenesis, were inoculated in 2 ml of complete medium and grown at 30 C overnight. The next day, the cultures were pooled in groups of 10 and an equal volume of fresh medium was added (40 ml total). After growth had commenced at 30 C, the temperature was shifted to 42 C. The cultures were incubated for 1 h and the cells were harvested. Total nucleic acids were prepared essentially by the method of Marmur (21) except that the RNase and subsequent steps were omitted. Methyl group incorporation into RNA (and also into DNA; see ref. 20) was determined as described below. If methyl acceptor activity was found, the 10 colonies were retested in groups of five and then individually.

Preparation of methylating enzymes (used for screening mutagenized colonies). Late log-phase cultures of strain GM1 grown in complete medium at 30 C were harvested and washed twice with 0.01 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.8)-0.001 M ethylenediaminetetraacetic acid (EDTA). The cells were ground with twice their wet weight of alumina and extracted with 5 volumes of 0.04 M Tris (pH 7.8)-0.001 M EDTA-0.001 M dithiothreitol-10% (vol/vol) glycerol. The crude extract was clarified by 2 centrifugations at 12,000 × *g* for 10 min, dispensed into tubes, and stored frozen at -60 C. Such frozen extracts are stable for at least 6 months.

Assay of methyl group incorporation into RNA. The reaction mixture (0.25 ml) contained, per milliliter: 40 μmol of Tris (pH 7.8), 1 μmol of EDTA, 10 μmol of MgSO₄, 20 μmol of NH₄Cl, 6.3 nmol of [*methyl*-³H]SAM, substrate RNA, and 0.25 to 1 mg of methylase enzyme preparation. The reaction time

was 30 min at 37 C. The reaction was stopped by the addition of 0.2 ml of 25% sodium lauryl sulfate and water to a final volume of 2 ml, followed by heating at 60 C for 10 min. Sodium perchlorate was added to a final concentration of 1 M and the mixture was shaken with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was removed after centrifugation and cooled in ice, and the nucleic acids were precipitated with 0.5 M perchloric acid and washed three times with cold 0.5 M perchloric acid. The precipitate was suspended in 0.5 M NaOH and heated at 60 C for 10 min to hydrolyze RNA. After cooling, the solution was precipitated with 0.5 M perchloric acid and centrifuged. Portions of the supernatant fluid (0.2 ml) were dissolved in scintillation medium consisting of 4 g of Omnifluor (New England Nuclear Corp), 667 ml of toluene, and 330 ml of absolute ethanol.

Genetic procedures. The Hfr strains, conjugation and transduction procedures, and methods for the selection of recombinants or transductants have been described in detail elsewhere (19). Ten recombinants or transductants from each cross were scored for *trm* by measuring the methyl group incorporation into RNA as described above.

Plating of bacteriophages. The plating efficiencies were determined as described by Adams (1).

Preparation of tRNA methylases. tRNA methylases (used for characterization of mutants) from strain GM1 were prepared by a procedure that was essentially the method B used by Schaefer et al. (26), except that the ammonium sulfate fractionation step was omitted. Fractions containing the tRNA methylase activities that had been eluted from a diethylaminoethyl-cellulose column by 0.2 M NaCl were designated pool I, whereas those eluted by 0.3 M NaCl (predominantly uracil-tRNA methylase, see reference 26) were called pool II.

Preparation of tRNA and aminoacyl-tRNA synthetases. The preparation of unfractionated tRNA from strains GM1, GM18, GM19, GM23, C6, and from *Mycoplasma* sp. (Kid) (14) and the preparation of aminoacyl-tRNA synthetases from strain GM1 were performed as reported previously (30).

Preparation of rRNA. Ribosomal RNA (rRNA) from strains GM1, GM18, GM19, and GM23 was prepared by the procedure of Nichols and Lane (22) followed by gel filtration on Sephadex G-200.

Assay of methyl acceptance of mutant tRNA or rRNA. Each assay mixture (0.2 ml) contained, per milliliter: 50 μmol of Tris (pH 8.0), 5 μmol of magnesium chloride, 6 μmol of 2-mercaptoethanol, 2.5 μCi of [*methyl*-¹⁴C]SAM, 1.0 to 7.5 absorbancy units at 260 nm of tRNA or rRNA, and 1.25 mg of pool I methylase or 0.75 mg of pool II methylase. The reaction was allowed to proceed at 37 C. Samples were removed at various time intervals and assayed for acid-precipitable radioactivity by the filter paper technique.

Preparation of [*methyl*-¹⁴C]tRNA. Large-scale preparation of [*methyl*-¹⁴C]tRNA for nucleotide analysis was performed by scaling up the methyl acceptance assay. [*methyl*-¹⁴C]tRNA was recovered by phenol extraction of the reaction mixture and then concentrated by alcohol precipitation and extensively

dialyzed against glass-distilled water. Double-labeled [*methyl*- ^{14}C]-[^3H]Glu-tRNA was prepared by aminoacylation (14) of [*methyl*- ^{14}C]tRNA with [^3H]glutamic acid.

Enzymatic digestion of tRNA. [*methyl*- ^{14}C]tRNA (approximately 30 absorbancy units at 260 nm) was digested with RNase T2 (25 units) in 0.1 ml of 0.02 M ammonium acetate (pH 4.5) at 37°C for 4 or 24 h (for m⁷G). The digest was separated by two-dimensional chromatography as described below. Nucleotides were dephosphorylated with bacterial alkaline phosphatase (10 μg) in 10 μl of 10 mM Tris (pH 8.0) for 3 h at 37°C.

Chromatography and electrophoresis. Two-dimensional chromatography in the descending fashion was conducted at room temperature with Whatman no. 1 or 3MM paper. The solvent systems (vol/vol) were: isobutyric acid/0.5 M ammonium hydroxide (5:3) (solvent I); and 2-propanol/concentrated HCl/water (68:17.6:14.4) (solvent II). Cellulose plates (20 by 20 cm) were used for thin-layer chromatography and electrophoresis. The solvent systems for thin-layer chromatography were (vol/vol): solvents I and II; 2-propanol/water/ammonium hydroxide (7:2:1) (solvent III); and 1-butanol/acetic acid/water (5:3:2) (solvent IV).

Thin-layer electrophoresis was conducted in 20 mM potassium phosphate (pH 7.0) at 400 V for 1 h.

Column chromatography. Affinity column chromatography on polyacrylamide-bound yeast tRNA^{Phe} was performed according to Grosjean et al. (10). Unfractionated [*methyl*- ^{14}C]-[^3H]Glu-tRNA from strain GM19 was loaded very slowly (2.7 ml/h) at 0°C onto the column (1 by 1 cm; resin has 5 mg of tRNA attached), which had been previously equilibrated with buffer A (10 mM sodium acetate [pH 5.4]/10 mM magnesium sulfate/1 M sodium chloride). After washing the column with 20 ml of the same buffer, elution of Glu-tRNA was effected at 30°C by buffer B (10 mM sodium acetate [pH 5.4]/1 M sodium chloride/10 mM EDTA) at a flow rate of 10 ml/h. Fractions of 0.5 ml were collected and a sample from each fraction was measured for ^3H and ^{14}C radioactivity. The material in fractions 25 to 33 (see Fig. 3A) was pooled and used for RPC-5 chromatography.

Reversed-phase chromatography (RPC-5) was performed according to Kelmers and Heatherly (15). The pooled fractions of [*methyl*- ^{14}C]-[^3H]Glu-tRNA (see Fig. 3A) were applied to an RPC-5 column (0.6 by 30 cm) that had been previously equilibrated with 0.4 M NaCl/10 mM MgCl₂/10 mM sodium acetate (pH 4.5)/2 mM 2-mercaptoethanol. Elution was carried out at 22°C with a linear gradient (100 ml) of NaCl (0.45 to 0.8 M). One-milliliter fractions were collected every 2 min.

RESULTS

Isolation of mutants. When RNA and a preparation of RNA-methylating enzymes from the same bacterial strain are incubated with SAM, no transfer of methyl groups is observed, since the RNA is already fully methylated in vivo. However, if methyl-deficient RNA (obtained by starving of a *rel*⁻ methionine auxo-

troph for its required amino acid) is used, methyl group transfer does occur (9). Similarly, mutant strains lacking a specific methylated base in RNA will accept methyl groups when incubated with SAM and methylating enzymes from wild-type cells (4, 25). To produce mutants, strain GM1 was mutagenized with nitrosoguanidine. Fifteen hundred nitrosoguanidine-treated survivors were taken at random and screened for RNA methyl acceptor activity with a crude RNA methylase preparation from strain GM1 and [*methyl*- ^3H]SAM as methyl donor. Seven strains were detected that showed methyl incorporation ranging from 1,164 to 5,256 counts/min into the RNA (isolated from 40-ml cultures of each strain). When RNA from the wild strain was tested as a control, it also accepted some methyl groups (an average of 635 counts/min). Therefore, under normal growth conditions RNA is slightly undermethylated. This has also been observed by Björk and Isaksson (4). After treatment with RNase A, all the radioactivity in the methylated mutant RNA was rendered acid soluble. No methyl group incorporation into DNA was detected.

A preliminary characterization showed that strains GM20, GM21, GM22, GM23, and GM24 all had the same lesion. Strain GM23 was chosen for further study.

Preliminary mapping of the tRNA methylase-deficient character. Mapping of the tRNA methylase genes presents certain difficulties. In *trm*⁺ × *trm*⁻ crosses, it is not possible to select for *Trm*⁺ recombinants, since no phenotypic trait has yet been discovered that allows for the direct selection of such recombinants. To map the *trm* genes, an indirect method was used that involved the selection of recombinants prototrophic for a particular genetic marker (e.g., His⁺, Met⁺, etc.) and subsequent analysis of the tRNA from the recombinant strains for the presence of the methylated base. This is accomplished by extracting bulk RNA from each recombinant and assaying methyl group incorporation into tRNA catalyzed by crude extract from strain GM1. In view of the tedious procedure, only 10 recombinants from each cross were analyzed, since the aim of the preliminary genetic analysis was to determine whether the *trm* genes were clustered in a small part of the chromosome or scattered around the genetic map. The results are given in Table 2; the points of origin of the various Hfr strains are shown in Fig. 1.

When strain GM18 was mated with Hfr PK191 and Thy⁺ recombinants were selected, none of them were *trm*⁺ (Table 2). The *trm* mutation, therefore, does not lie between the origin of Hfr PK191 and *thyA*. When strain

TABLE 2. Location of *trm* mutations on the *E. coli* K-12 genetic map

Cross ^a	Selected marker	Unselected marker	Linkage (%)
AB259 × GM18	Thr ⁺ Leu ⁺	<i>trmB1</i> ⁺	50
	ProA ⁺	<i>trmB1</i> ⁺	80
	His ⁺	<i>trmB1</i> ⁺	30
PK191 × GM18	ThyA ⁺	<i>trmB1</i> ⁺	0
KL154 × GM18	Thr ⁺ Leu ⁺	<i>trmB1</i> ⁺	40
PK191 × GM19	ThyA ⁺	<i>trmC1</i> ⁺	50
KL16 × GM19	His ⁺	<i>trmC1</i> ⁺	30
KL983 × GM19	His ⁺	<i>trmC1</i> ⁺	0
AB259 × GM19	His ⁺	<i>trmC1</i> ⁺	0
KL154 × GM19	Thr ⁺ Leu ⁺	<i>trmC1</i> ⁺	0
KL154 × GM23	Thr ⁺ Leu ⁺	<i>trmA14</i> ⁺	50
KL154 × GM23	MetB ⁺	<i>trmA14</i> ⁺	80
AB259 × GM23	His ⁺	<i>trmA14</i> ⁺	0
PK191 × GM23	ThyA ⁺	<i>trmA14</i> ⁺	0

^a Donor and recipient cells were mated at a ratio of 1:20 for 60 min at 37 C. after which the cultures were blended for 1 min, diluted, and plated in soft agar overlays on selective media. Ten recombinants from each cross were scored for *trm* as described in the text.

FIG. 1. Genetic map of *E. coli* K-12 (after Taylor and Trotter [33]) showing pertinent genetic markers on the outside of the circle and the points of origin of various Hfr strains (17) on the inside of the circle.

GM18 was mated with Hfr KL154 (Thr⁺ Leu⁺ selection) or Hfr AB259 (His⁺ selection), some of the selected recombinants were *trm*⁺, which means that the *trm* mutation must lie between the origins of Hfr AB259 and Hfr PK191. To ascertain the position of the *trm* gene more precisely, Hfr AB259 was mated with strain GM18 and selection was made for Thr⁺ Leu⁺, ProA⁺, and His⁺ recombinants. The data in Table 2 show that 80% of the ProA⁺ recombinants were also *trm*⁺, indicating that the *trm*

mutation in strain GM18 is closely linked to *proA*.

In contrast, when Hfr PK191 was mated with strain GM19, 50% of the Thy⁺ recombinants were also *trm*⁺. No *trm*⁺ recombinants were obtained in matings with Hfr KL154 (Thr⁺ Leu⁺ selection), KL983 (His⁺ selection), or AB259 (His⁺ selection). These results indicate that the *trm* mutation in strain GM19 probably lies between the origin of PK191 and *thyA*. This was confirmed by showing that 30% of the His⁺ recombinants, isolated from a cross between Hfr KL16 and strain GM19, were *trm*⁺. Since no *trm*⁺ recombinants were obtained with KL983 as donor strain, the *trm* mutation in strain GM19 must lie between the points of origin of strains KL16 and KL983.

No *trm*⁺ recombinants were recovered in crosses involving strain GM23 and Hfr AB259 (His⁺ selection) or Hfr PK191 (Thy⁺ selection). When KL154 was mated with strain GM23, however, 80% of the MetB⁺ and 50% of the Thr⁺ Leu⁺ recombinants were *trm*⁺. Björk and Neidhardt (5) have shown that the *trmA* gene, which specifies a uracil-tRNA methylase, can be co-transduced by P1 with *argH*. A P1 lysate grown on strain GM23 was used to select Arg⁺ transductants from strain W3679, which is *argC*⁻. Among 10 transductants analyzed for the methylase mutation, 8 were found to be Trm⁻ (80% cotransduction). Thus, the *trm* mutation in strain GM23 can be cotransduced at a high frequency with *argC* and appears to be in the *trmA* gene described by Björk and Neidhardt (5).

The above preliminary genetic analyses are necessarily crude since only 10 recombinants from each cross were examined. In spite of this, it would appear that the *trm* mutations in strains GM18, GM19, and GM23 are not clustered in a small region of the chromosome but are scattered around the genetic map.

Phenotypic characterization. All the *trm* strains are able to grow in broth at 30 and 42 C although they display a wide variety of growth rates. Table 3 summarizes the doubling times of the mutant strains and of strain GM1 when grown in LB broth (18).

Strain GM18 grows slowly in broth and requires 4 to 5 days of incubation at 33 C to form colonies on minimal agar. Growth is enhanced by the addition of 1% Casamino Acids to the minimal medium. Attempts to isolate a derivative of strain GM18 that grows faster in minimal medium were unsuccessful. The slow growth of this strain may be due to the *trm* mutation or the result of extra mutations induced by the mutagen, nitrosoguanidine, which is known to

produce several mutations per chromosome (11). However, all recombinant strains with Trm⁻ character from crosses of strain GM18 with the various Hfr strains (see above) showed slow growth.

The *trmA* strains are quite heterogeneous in their characteristics. Only strain GM23 will grow in minimal medium. However, all are able to grow if Casamino Acids are added to a final concentration of 1%. It is probable that the failure to grow in minimal medium is due to other additional auxotrophic mutations induced by nitrosoguanidine rather than the *trmA* mutation, since strain GM23 and the *trmA* mutant described by Björk and Isaksson (4) show no additional auxotrophy.

Since the *trm* mutants all contain the *supE44* amber suppressor, the efficiency of plating of various T4 and λ amber mutants on the tRNA methylation-defective strains has also been determined. The efficiency of plating of the phages on strains GM19 and GM23 was the same as in the wild-type strain GM1 (data not shown). Strain GM18, however, gave a lower efficiency of plating for some phages (Table 4). The low efficiency of plating may be due to either the loss of 7-methylguanosine in tRNA or to an extraneous mutation induced by nitrosoguanidine (11).

Mutants have altered tRNA methylase activities. Since the majority of methylated nucleosides in RNA are found in tRNA and to a lesser extent in rRNA, we prepared both tRNA

and rRNA from strains GM1, GM18, GM19, and GM23. All RNAs were methylated with [*methyl*-¹⁴C]SAM by using unfractionated tRNA methylases from wild type (GM1). Although there was some methyl group incorporation into rRNA from the *trm* mutant strains (Table 5), the level of incorporation was negligible and comparable with that of wild-type rRNA. However, there was good methyl acceptance by the tRNA of the mutants (Table 5). We therefore conclude that the mutations in strains GM18, GM19, and GM23 affect tRNA methylases.

Methyl acceptances of the tRNA preparations from the three mutant strains varied from the very low, but in reproducible levels in strains GM18 and GM19 (one methyl group accepted per every 50 and 100 molecules of tRNA, respectively), to the much higher value in strain GM23 (one methyl group accepted per every 1.3 molecules of tRNA) (Table 5). Furthermore, the effectiveness of the different mutant tRNA's to serve as substrate for the two enzyme fractions (pools I and II) varied; tRNA from strains GM18 and GM19 were not methylated by pool II enzymes, whereas tRNA from strain GM23 was an excellent substrate. As controls, tRNA from *Mycoplasma* sp. (Kid), which has a low content of methylated components and is devoid of ribothymidine (14), and undermethylated tRNA prepared from *E. coli* C6 (13) were also included as controls. The results suggest that GM23 tRNA is deficient in ribothymidine.

Identification of in vitro methylated components. To identify the products of in vitro methylation, the mutant [*methyl*-¹⁴C]tRNA's were first degraded to the nucleotide level with RNase T2. The hydrolysis products were then resolved by two-dimensional chromatography using solvents I and II. Radioactive regions of the chromatogram were located by autoradiography (Fig. 2). The radioactive materials were recovered for further characterization.

The two-dimensional chromatogram of the nucleotide mixture obtained by RNase T2 digestion of the [*methyl*-¹⁴C]tRNA from strain GM18 yielded two radioactive spots after autoradiography (Fig. 2A). The radioactive nucleotide, which had a slower mobility in solvent II,

TABLE 3. Doubling time of mutant and wild-type strains

Strain ^a	Genotype	Doubling time (min)
GM1	<i>trm</i> ⁺	40
GM18	<i>trmB1</i>	75
GM19	<i>trmC1</i>	40
GM20	<i>trmA11</i>	45
GM21	<i>trmA12</i>	48
GM22	<i>trmA13</i>	48
GM23	<i>trmA14</i>	40
GM24	<i>trmA15</i>	40
KL356	<i>trmA14</i>	40

^a The strains were grown in LB broth (18) with forced aeration at 37 C.

TABLE 4. Efficiency of plating of T4 and λ amber mutants

Strain	Phage				
	T4amB17	T4amE605	T4amH39X	T4amN65	λ gam-210
GM1 (<i>supE44 trm</i> ⁺)	1.0	1.0	1.0	1.0	1.0
GM18 (<i>supE44 trmB1</i>)	1.0	5×10^{-3}	8×10^{-3}	1.0	$< 10^{-3}$

TABLE 5. Methyl acceptance of mutant tRNA and rRNA^a

Enzyme fraction	tRNA						rRNA			
	GM1	GM18	GM19	GM23	<i>Mycoplasma</i>	C6	GM1	GM18	GM19	GM23
S-100 ^b	4 (0.002)	28 (0.017)	15 (0.009)	1200 (0.72)	1805 (1.09)	1035 (0.62)	3	2	1	3
Pool I	6 (0.004)	35 (0.021)	18 (0.011)	151 ^c (0.091)	931 ^c (0.56)	1062 (0.64)				
Pool II	3 (0.002)	3 (0.002)	2 (0.001)	1178 (0.71)	1660 (1.0)	946 (0.57)				

^a Methyl acceptance is expressed as picomoles of [*methyl*-¹⁴C] group accepted per absorbancy unit at 260 nm of tRNA or rRNA after 180 min of incubation at 37 C. The numbers in parentheses represent methyl acceptance values alternatively expressed as moles of [*methyl*-¹⁴C] group per mole of tRNA. All acceptance values are "plateau" values; i.e., there was no further increase in methyl incorporation with time.

^b A 100,000 × *g* supernatant of a cell extract of GM1. It was not subsequently fractionated on a diethylaminoethyl-cellulose column to give pool I and pool II methylases.

^c Not plateau values.

FIG. 2. Two-dimensional thin-layer chromatography of RNase T2 hydrolysates of [*methyl*-¹⁴C]tRNAs. Open circles represent the four major mononucleotides. Hatched areas designate location of the radioactive spots (visualized by radioautography). (A) tRNA from GM18; (B) tRNA from GM19; (C) tRNA from GM23.

was eluted and co-chromatographed in two solvent systems with an authentic marker of 7-methylguanosine 3'-phosphate. Complete co-incidence of radioactivity and ultraviolet light-absorbing material was seen (Table 6). A similar coincidence was seen after enzymatic de-phosphorylation and co-chromatography of the resulting *methyl*-¹⁴C-labeled nucleoside with an authentic marker of 7-methylguanosine (Table 6). The radioactive compound that had a faster mobility in solvent II was redigested with RNase T2 (24 h). As a result, 70 to 80% of the radioactivity became associated with 7-methylguanosine 3'-phosphate. Thus it appears that under the reaction conditions used, the RNA was only incompletely degraded to 7-methylguanosine 3'-phosphate. Thus we conclude that strain GM18 is a mutant defective in the synthesis of 7-methylguanosine in tRNA.

As seen from the results of two-dimensional thin-layer chromatography (Fig. 2B), only one methylated nucleotide was released by RNase T2 digestion of [*methyl*-¹⁴C]tRNA isolated

from strain GM19. The relative mobilities of this methylated compound in these two solvent systems suggest that it might be 2-thio-5-methylaminomethyluridine 3'-phosphate. This modified nucleotide occurs in several *E. coli* tRNA species, among them tRNA^{Glu} (24). Therefore, we decided to isolate tRNA^{Glu} from strain GM19 [*methyl*-¹⁴C]tRNA and ascertain that this tRNA carries a radioactive methyl group. Utilizing the principle of complementary anticodon:anticodon interaction between two tRNA molecules, tRNA^{Glu} can be readily purified by affinity chromatography on polyacrylamide-bound yeast tRNA^{Phe} (10). For easier analysis, this experiment was performed with tRNA aminoacylated with radioactive glutamate. When [*methyl*-¹⁴C]-[³H]glutamyl-tRNA from strain GM19 was passed over the tRNA^{Phe} affinity column (Fig. 3A), only 7% of the ultraviolet light-absorbing material was retarded, but it contained most of the tRNA^{Glu} (indicated by ³H radioactivity) and the bulk of the methylated tRNA (indicated by the ¹⁴C radioactivity).

TABLE 6. R_f values of authentic and biosynthesized nucleotides and nucleosides^a

Strain	Compound ^b	R_f				Electrophoresis at pH 7.0 (cm from origin)
		Solvent I	Solvent II	Solvent III	Solvent IV	
GM18	¹⁴ C-labeled nucleotide	0.65	0.49			(+) 2.2
	m ⁷ Gp marker	0.65	0.49			(+) 2.2
	¹⁴ C-labeled nucleoside	0.64	0.26			(-) 1.1
	m ⁷ G marker	0.64	0.26			(-) 1.1
GM19	¹⁴ C-labeled nucleotide	0.59	0.47			
	Sp marker	0.59	0.47			
	¹⁴ C-labeled nucleoside	0.71	0.25			
	S marker	0.71	0.25			
GM23	¹⁴ C-labeled nucleotide	0.43	0.76			
	rTp marker	0.43	0.76			
	¹⁴ C-labeled nucleoside			0.56	0.52	
	rT marker			0.56	0.52	

^a Thin-layer chromatography and thin-layer electrophoresis were performed as described in Materials and Methods. Unlabeled nucleotides and nucleosides were detected by their ultraviolet absorbance at 260 nm. Radioactive compounds were localized by autoradiography.

^b Sp, 2-thio-5-methylaminomethyluridine 3'-phosphate; S, 2-thio-5-methylaminomethyluridine; rTp, ribothymidine 3'-phosphate; rT, ribothymidine.

From the amount of radioactivity (corrected for double-label settings), one can calculate that there are 0.5 to 0.8 [¹⁴C]methyl groups per mol of [³H]glutamyl-tRNA. To eliminate any doubts about the nonidentity of the ³H- and methyl-¹⁴C-labeled material, this enriched fraction of glutamyl-tRNA was subjected to reversed-phase chromatography (Fig. 3B). The ¹⁴C and ³H radioactivities were eluted together. This strongly supports the idea that the [³H]Glu-tRNA became methylated in vitro. To characterize the methylated nucleotide, the tRNA^{Glu} was recovered from RPC-5 column fractions, discharged of its ³H amino acid, and then digested with RNase T2. The resulting radioactive methylated nucleotide co-chromatographed in the standard two-dimensional system with the authentic marker (Table 6). This was confirmed by co-chromatography at the nucleoside level (Table 6). We conclude that the methyl deficiency of GM19 tRNA, and in particular of tRNA^{Glu}, lies in the undermethylation of the 2-thio-5-methylaminomethyl uridine moiety.

The RNase T2 digest of [methyl-¹⁴C]tRNA from strain GM23 showed only one radioactive spot on the autoradiogram (Fig. 2C). This radioactive compound was at the position where ribothymidine 3'-phosphate is normally found (Table 6). After elution from the chromatogram, this radioactive material, together with authentic ribothymidine, was dephosphorylated and then subjected to thin-layer

chromatography in solvents III and IV. In both cases, the radioactive material co-chromatographed with the unlabeled ribothymidine. Thus, we conclude that strain GM23 is a mutant defective in the synthesis of ribothymidine in tRNA.

DISCUSSION

Although the in vitro screening method used in this work is undoubtedly tedious, the successful isolation of three tRNA methylase mutants shows that it is sensitive enough to detect even mutants that are deficient in only one methyl group per 100 tRNA molecules (e.g., GM19). Therefore further screening, especially of conditional mutants, may lead to the isolation of additional tRNA methylase mutants.

From the initial mapping data of the *trm* loci, it is clear that the genes for tRNA methylases are scattered over the *E. coli* chromosome. This situation is also found with the genes for tRNA (28, 29) and for aminoacyl-tRNA synthetases (31).

The fact that the tRNA in two of the three mutant strains is very little undermodified merits further discussion. In the case of 2-thio-5-methylaminomethyluridine, probably 10% of the total tRNA species harbor this compound in the anticodon (23). 7-Methylguanosine is always located in the middle of the very short extra loop in perhaps 20% of the tRNA species. However, the tRNA isolated from mutant cells (GM18 and GM19) grown

FIG. 3. (A) Affinity chromatography of [*methyl-¹⁴C]-[³H]glutamyl-tRNA from GM19 on a column of polyacrylamide-bound yeast tRNA^{Phe}. At fraction 23 (indicated by arrow), the column was eluted by buffer B. For details, see Materials and Methods. (B) Reversed-phase (RPC-5) column chromatography of [*methyl-¹⁴C]-[³H]glutamyl-tRNA from GM19. For details, see Materials and Methods.**

under normal conditions is found to be undermodified only to 10% of the expected level. This may suggest that there are several tRNA methylases in *E. coli*, all responsible for the formation of the same methylated nucleoside but specific for different tRNA species. Within such a family of methylases only one would be altered, leaving a particular tRNA species undermodified. To date such a case has not been found among tRNA-modifying enzymes. On the contrary, it is known, for instance, that there is only one enzyme in *E. coli* for the formation of isopentenyladenosine in tRNA (27). Pseudouridine biosynthesis in tRNA is catalyzed by at least two enzymes (6). However, even in this case there is only one activity responsible for each site-specific base modification. Another possibility seems at least equally likely: if

different tRNA's have different affinities for the tRNA methylases and if the enzymes are only partially active (as in our case), they might modify first those tRNA's for which they have the highest affinity and not modify those for which they have the lowest affinity. We know, for instance, that the 7-methylguanosine deficiency does not occur in tRNA^{Phe}, since this tRNA had been purified from [*methyl-¹⁴C]-tRNA of GM18 by affinity column chromatography (10) on a tRNA^{Glu} column, and it was not *methyl-¹⁴C labeled (data not shown). Only further genetic characterization of the undermodified species will clarify this situation.**

It is most important, of course, to find out whether there are any physiological effects caused by the genetic lesion. The finding that

strain GM18 is incapable of allowing the *supE* suppressor to function efficiently with certain T4 or λ phage *amber* mutants, although the wild-type GM1 strain does so, is very exciting and might point the way to further investigations on the involvement of tRNA and the role of methylated bases in the expression of the suppression phenomenon. However, it cannot yet be ruled out that this effect is the consequence of a second mutation that may have been introduced into GM18 by the nitrosoguanidine mutagenesis (11).

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