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General Screening Procedure for RNA Modificationless Mutants: Isolation of *Escherichia coli* Strains with Specific Defects in RNA Methylation

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A general method for the isolation of mutants of *Escherichia coli* that are defective in RNA modification is described. The method is based on the fact that RNA with specific undermodifications accumulates under nonpermissive growth conditions and that such a defect can be detected by remodification either in vivo at permissive conditions or in vitro. The method provides a means by which to study mutations affecting essential modification reactions. The usefulness of the method was demonstrated by the isolation of two rRNA and two tRNA methylation defective mutants. Both rRNA mutants accept methyl groups into their 23S rRNA in vitro. Analyses of in vitro methylated 23S rRNA from one of the mutants revealed the presence of several methylated nucleosides, of which 6-methyladenosine was the most abundant (40% of recovered radioactivity). In 23S rRNA from the other mutant, the only product formed in vitro was 5-methylcytidine. The tRNA mutants are characterized in the accompanying paper.

RNA from most cells, procaryotic as well as eucaryotic, contains a large number of modified nucleosides. Hitherto at least 50 different modified nucleosides have been identified (29). All modification reactions seem to occur on the polynucleotide level, although this has been shown experimentally only for the methylated, thiolated, and isopentenylated nucleosides and pseudouridine (11, 20).

So far, little has been revealed about the function of these nucleosides. It is known that a hypermodified adenosine in $tRNA^{Tyr}$ is important for binding tRNA to the ribosome and that the presence of ψ in the anticodon stem in $tRNA^{His}$ is necessary for proper regulation of the histidine operon (19, 33). Almost all tRNA species contain m^5U in the common m^5U-ψ-C-G sequence. Results from our laboratory have shown that the presence of m^5U in this sequence seems to give the cell a survival advantage, but the precise function of m^5U is not yet known (8). Baumstark et al. (3) claim that m^5U is important in initiation of protein synthesis, but we have been unable so far to confirm these results. Finally, methylated nucleosides may be important in both the aminoacylation reactions and in proper codon recognition of the tRNA (6, 10, 30, 31, 36).

One of the most effective ways to study the function of a modified nucleoside, and thus of tRNA, is to isolate mutants that are defective in the biosynthesis of such a nucleoside. Since their function is unknown, no selection procedure for such mutants is available. Therefore Björk and Isaksson (5) devised a screening procedure for isolating *Escherichia coli* mutants with aberrant RNA methylation. Mutants that were defective in rRNA (Rrm^-), as well as in tRNA (Trm^-) methylation, were found. The latter type was mutated in the *trmA* locus and was shown to be unable to synthesize m^5U in tRNA. This is the most abundant methylated nucleoside in tRNA, and the sensitivity of the screening method, as it was used by us, only allowed detection of mutations affecting the formation of this nucleoside. A similar screening procedure was used by Marinus and Morris (27) in their isolation of DNA and tRNA methyltransferase mutants.

Our earlier screening method for isolating mutants that specifically lack one modified nucleoside in RNA relied on the following conditions for its success: (i) a modification defect in vivo could be identified by remodification in vitro, and (ii) the physiological consequences of such a mutation could not have a serious impact on the cell, since the cells were required to grow (5). Thus, mutations affecting biologically necessary modification reactions could never be explored. Therefore, the aim of our present project was to devise a general screening method that allows the isolation of mutants containing lethal,

as well as nonlethal, defects in RNA modification.

A condition that allows the accumulation of RNA in the cell even when protein synthesis is inhibited, i.e., relaxed conditions, should permit the isolation of the desired types of modificationless mutants, among them those in which the mutation causes lethal effects to the cells. In this case mutants may be identified by remodification either in vitro, as previously noted, or in vivo if the mutation is reversible under permissive conditions. Remodification in vitro also reveals, in addition, such mutants in which the modifying enzyme is permanently nonfunctional even at permissive temperatures. Our new screening method is based on growth under relaxed conditions and is designed to work for all types of RNA modificationless mutants. Its usefulness is demonstrated by the successful isolation of mutants defective in the methylation of rRNA and tRNA.

(A preliminary report of part of this investigation was presented at the Tenth International Congress of Biochemistry, Hamburg, West Germany, 1976, abstr. p. 33, Brönners Druckerei, Breidenstein, Frankfurt, W. Germany.)

MATERIALS AND METHODS

Abbreviations. Abbreviations used in this paper are those recommended by the Commission on Biochemical Nomenclature (CBN-1970): EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; U, uridine; m^5U, 5-methyluridine (ribothymidine); Um, 2'-0-methyluridine; ψ, pseudouridine; 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^6A, 6-methyladenosine; Am, 2'-0-methyladenosine; compound 5, depicted in Fig. 4 in the accompanying paper (7); DNase, deoxyribonuclease; RNase, ribonuclease; R_c, relative migration of a component to that of cytidine.

Bacterial strains, media, and growth conditions. The *E. coli* K-12 strains used are listed in Table 1. Medium E, described by Vogel and Bonner (38), was supplemented with 0.2% glucose, 1 μg of thiamine per ml, and 50 μg of L-epimer of the required amino acids or bases per ml. The complete medium used was LB, described by Bertani (4), supplemented with medium E and 0.2% glucose. When the parental strain GB5 and its derivatives were grown in this medium, it was supplemented with L-histidine, L-methionine, and uracil, all at a concentration of 50 μg/ml (LB-MHU). The LB medium was solidified with 1.5% agar (LA plates).

The bacteria were cultivated on a rotary shaker, and growth was recorded by optical density readings by using a Klett Summerson colorimeter with filter W66. In the LB medium, 100 Klett units corresponded to about 4×10^8 cells per ml.

Construction of the parent strain GB5. Strain GB2 (*thr leu arg his relA rpsL*), a spontaneous streptomycin-resistant mutant of strain CP79, was crossed

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

Strain	Sex	Relevant genetic markers[a]	Reference
G11	HfrC	*metB ilv relA1*	35
G11a1	HfrC	*metB ilv relA1 ampA1*	13
CP79	F⁻	*thr leu arg his relA*	14
W6	F⁺	*met relA1*	26
GB2	F⁻	*thr leu arg his relA rpsL*	Derivative of CP79, this paper
GB3	F⁻	*metB his relA rpsL*	Derivative of CP79, this paper
GB4	F⁻	*metB his relA fdp rpsL ampA1*	Derivative of CP79, this paper
GB5	F⁻	*metB his relA pyrB valS*(Ts) *rpsL ampA1*	Derivative of CP79, this paper
NP910212	F⁻	*pyrB valS*(Ts) *trp*	F. C. Neidhardt (cf. 37)
KLF18/132	F′	*pro thy* episome covering *fdp* and *ampA* region	*E. coli* Genetic Stock Center, Yale University, New Haven, Conn. CGSC 4259
JC411-6	F⁻	*fdp*	17
JC411-6a	F⁻	*ampA1 fdp*	Ampicillin-resistant transductant using G11a1 as donor
LMUR-5-226	F⁻	*lys ura trmA5 rel*	8
IB11	F⁻	*trmD1 aroC ilvA asp*[b, c]	Nitrosoguanidine treatment of GB5, this paper
IB13	F⁻	*trmC2*, some additional uncharacterized auxotrophic markers[b]	Nitrosoguanidine treatment of GB5, this paper
IB14	F⁻	Rrm⁻[b]	Nitrosoguanidine treatment of GB5, this paper
IB15	F⁻	Rrm⁻[b]	Nitrosoguanidine treatment of GB5, this paper

[a] The genetic abbreviations are according to Bachmann et al. (2).
[b] In addition, these strains contain the markers of strain GB5.
[c] Shown genetically to be *aroC* and by feeding tests to be *ilvA*.

with the HfrC strain G11 (*metB ilv relA1*). Recombinants, prototrophic for threonine and leucine, were selected on plates supplemented with methionine, leucine, and valine. Out of 400 such recombinants, one was *metB, his, relA,* and *rpsL*. This strain, GB3, was then simultaneously crossed with two other strains JC411-6a (*fdp ampA1*) and KLF18/132 (*pro thy rpsL*). Ampicillin-resistant recombinants were selected on plates containing methionine, histidine, ampicillin (6 μg/ml), and streptomycin (100 μg/ml). These recombinants were screened for the Fdp phenotype by testing for inability to use glycerol or succinate as the carbon source. One such recombinant, which had received the *fdp* allele is GB4. *pyrB* and *valS*(Ts) were introduced into this strain by transduction with phage Plv grown on strain NP910212 [*pyrB valS*(Ts) *fdp*$^+$]. Strain GB4 (*metB his relA pyrB valS*$^+$ *fdp*) was used as recipient, and *fdp*$^+$ transductants were selected. These transductants were screened for *pyrB* and *valS*(Ts). One transductant, which had received both these alleles, is strain GB5 [F$^-$ *metB his pyrB valS*(Ts) *relA ampA1 rpsL*] used as parent strain in the mutation experiment described below.

Mutagenesis. Log-phase culture of strain GB5 was grown at 30°C and treated with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (400 μg/ml) for 30 min as described by Miller (28).

Isolation of Trm$^-$ mutants. Single colonies were picked up and placed into 0.5 ml of LB-MHU medium containing streptomycin (100 μg/ml) and grown for 2 days at 28°C in microculture containers with 25 wells (9; G. Bertani, personal communication). After incubation, drops were transferred to LA plates containing streptomycin and [^{3}H]uracil (0.5 μCi/μg, 50 μg/ml) using a replicator consisting of 25 steel needles. Radioactive uracil was included to assure that all cells at harvest contained RNA with the same specific activity. These plates, each containing 25 different clones, were incubated for 2 to 3 days at 28°C. A sample from each clone was grown in a test tube in 0.5 ml of LB-MHU medium supplemented with [^{3}H]uracil with a specific activity of 0.5 μCi/μg. After overnight growth at 28°C, the culture was diluted with 10 ml of the same growth medium, but prewarmed to 44°C. The cell density after this dilution was below 2×10^8 cells per ml. The cells were incubated at 44°C for 4 to 6 h, during which time relaxed RNA was synthesized. The cultures were combined in groups of three, and the cells were harvested and washed once in 5 ml of 10 mM Tris-hydrochloride (pH 8.0), containing 10 mM magnesium acetate. Total RNA was prepared and methylated in vitro by using enzyme extract from strain LMUR-5-266 with *S*-[*methyl*-^{14}C]adenosyl-L-methionine as methyl donor as described below. If a combined set of three clones gave evidence of a Trm$^-$ mutant, it was restreaked and tested once more for ability to accept methyl groups in vitro as described in footnote *a* of Table 3.

Preparation of methylating enzyme extract. Cells (5 g) were suspended in 10 ml of 10 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM dithiothreitol, 1 mM EDTA, and 10% glycerol (Buffer A). The cells were disintegrated in a French pressure cell (18) and centrifuged to remove cell debris. The supernatant was made 0.6 M in ammonium sulfate and 10 mM in magnesium acetate and centrifuged for 1 h at 192,000 to 275,000 × *g* in a Spinco L65B ultracentrifuge to remove the ribosomes. The supernatant was dialyzed against buffer A, made 50% in glycol, and stored at −20°C. Such an extract retained its original methylating activities for several months.

Preparation of total RNA. Cells were suspended in 1 ml of 10 mM Tris-hydrochloride buffer (pH 8.0) containing 10 mM magnesium acetate, lysozyme (200 μg), and DNase (25 μg). The suspension was frozen and thawed three times and then incubated for 10 min at 37°C. A 1-ml amount of buffer-saturated phenol and 0.2 ml of 1% sodium dodecyl sulfate were added, and the mixture was shaken at 30°C for 20 min. After low-speed centrifugation, the aqueous phase was withdrawn, and total RNA was precipitated by adding 2 ml of cold (−20°C) ethanol and 0.1 ml of 20% potassium acetate. The RNA was precipitated overnight at −20°C, washed once in 2 ml of 67% ethanol, dried, and dissolved in 0.2 ml of water.

Methylation of total RNA in vitro. A 0.2-ml amount of incubation mixture was added to the solution (0.2 ml) containing total RNA, giving a final concentration of: 0.1 M Tris-hydrochloride (pH 8.0), 1 mM dithiothreitol, 0.1 mM EDTA, 10 mM $MgSO_4$, 20 mM NH_4Cl, 38.5 μM *S*-[*methyl*-^{14}C]adenosyl-L-methionine (26 μCi/μmol), and enzyme extract from strain LMUR-5-266 (*trmA5*), with a final protein concentration of 250 μg/ml. After 2 to 3 h of incubation at 37°C, the reaction was stopped by adding 1 ml of 10 mM Tris-hydrochloride (pH 8.0) containing 10 mM magnesium acetate, 1.5 mg of carrier RNA, and 1 ml of buffer-saturated phenol. The mixture was shaken for 20 min at 30°C, and total RNA was precipitated from the aqueous phase by making it 10% in trichloroacetic acid. The RNA was collected on a Whatman GF/C glass fiber filter and washed with 10 ml of ice-cold 5% trichloroacetic acid and 20 ml of acetone. The filters were dried for 15 min at 110°C, and the radioactivity was determined in a scintillation counter. In other in vitro methylation experiments, the reaction was stopped as above, but carrier RNA was omitted. Total RNA was then prepared as described above.

Sucrose gradient centrifugation. In vitro methylated total RNA from GB5, IB14, and IB15 was layered on a 5 to 20% sucrose gradient (13 ml) in 0.1 M Tris-hydrochloride, 120 mM KCl, and 1 mM $MgCl_2$ (pH 8.0) and centrifuged for 24.5 h at 24,500 rpm using a Spinco SW40 rotor (22). Optical density and radioactivity were measured in the 25 fractions obtained. To obtain more material for identification of the product made in vitro, a larger sucrose gradient (58 ml) was made using the Spinco 25.2 rotor for 26 h at 25,000 rpm. 23S rRNA was pooled, dialyzed against distilled water, lyophilized, and digested to nucleosides as described below.

Enzymatic digestion of tRNA to nucleosides: chromatographic methods. After lyophilization, the RNA was transferred to siliconized glass tubes and digested essentially as described by Rogg et al. (32) in 0.05 ml of 0.08 M ammonium formate (pH 7.6), 0.5 mM $MgCl_2$ containing 25 μg of snake venom phosphodiesterase, 25 μg of pancreatic RNase A, and 25 μg of alkaline phosphatase. The procedure of Rogg et al. was modified by decreasing the incubation vol-

ume, increasing the molarity of the buffer, and increasing the incubation time to 26 to 40 h at 37°C. Usually 0.01 ml of hydrolysate and marker nucleosides were applied to thin-layer cellulose-coated aluminium foil, which were developed first in solvent I: *N*-butanol–isobutyric acid–concentrated ammonium hydroxide–water (75:27.5:2.5:25, vol/vol) and then in solvent II: saturated ammonium sulfate–0.1 M sodium acetate (pH 6.0)–isopropanol (79:19:2, vol/vol) as described by Rogg et al. (32). Radioactive material was visualized by autoradiography. To determine the amount of radioactivity, the thin-layer chromatogram was sprayed with lacquer, and the spots were cut out and placed in scintillation vials. A 1-ml amount of water was added, and 10 ml of scintillation cocktail containing Triton X-100 was added. The vials were shaken for 2 s to remove the cellulose layer from the aluminum foil. Care was taken not to place too large an area of the chromatogram in one scintillation vial because of the high salt content in solvent II. Moreover, the external standard ratio was always measured to monitor possible changes in counting efficiency. If the area of the chromatogram was smaller than the cross area of a scintillation vial, the counting efficiency was constant for both ^3H and ^{14}C.

Other methods. Protein was measured by the method of Lowry et al. (25), using bovine serum albumin as the standard.

Materials. [^{3}H]uracil (28,000 mCi/mmol) and *S*-[*methyl*-^{14}C]adenosyl-L-methionine (56 mCi/mmol) were obtained from Amersham, England. Lysozyme, DNase (1× crystallized), RNase T2, alkaline phosphatase from *E. coli*, snake venom phosphodiesterase from *Bothrops atrox*, and RNase A from bovine pancreas were purchased from Sigma Chemical Co., St. Louis, Mo. Cellulose-coated aluminum foil was obtained from E. Merck, Darmstadt, Germany. All methylated nucleosides used as markers were purchased from Cyclo Chemical Corporation, Los Angeles, Calif.

RESULTS

Necessary conditions for the screening procedure. The rationale for the screening method is presented in Fig. 1. An *E. coli* strain containing a temperature-sensitive mutation [*valS*(Ts)] in combination with a *relA* allele produces RNA at elevated temperatures, while protein synthesis is inhibited (12). Mutagenic treatment of such a strain resulting in a defective modifying enzyme causes the RNA that accumulates under nonpermissive conditions to be undermodified. A strain with the desired combination of genetic markers was not available, and the construction of such a strain, GB5 [*valS*(Ts) *relA pyrB*] is described in Materials and Methods. The reason for including the *pyrB* allele into the parent strain was to allow later replacement of the *valS*(Ts) allele with its wild-type allele by utilizing the high cotransduction frequency between *pyrB* and *valS* genes (37).

In strain GB5, a temperature shift to 44°C stops protein synthesis, but RNA production is

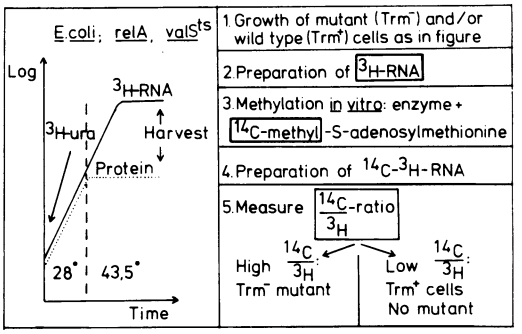

FIG. 1. *Principle of the screening method. An E. coli strain containing a temperature-sensitive valyl-tRNA ligase [valS(Ts)] in combination with a defect relA allele accumulates RNA at high temperature, while protein synthesis is inhibited. To relate the degree of methylation (^{14}C) to the amount of RNA accumulated, RNA was labeled in vivo with [^3H] uracil.*

supposed to continue. If the cell density was high at the time of temperature shift, the synthesis of relaxed RNA was almost abolished. If the cell density was 2×10^8 cells per ml or lower at the time of temperature shift, net RNA synthesis continued for about 2 h. Thereafter, the amount of total RNA decreased, but that of tRNA increased. This preferential accumulation of tRNA continued for at least 6 h. Thus, in screening for tRNA modificationless mutants, the temperature should be shifted to 44°C at about 2×10^8 cells per ml and kept at a high temperature for about 6 h to allow maximal accumulation of tRNA. Accumulation, as well as extraction, of RNA is somewhat irreproducible and, therefore, RNA was labeled in vivo with [^{3}H]uracil, so that the amount of RNA present in the later in vitro methylation reaction was known.

A further prerequisite for a screening method based on remodification in vitro is a functional enzyme extract. Our aim was to isolate tRNA methylation defective mutants and at the same time avoid previously isolated mutants (*trmA*). Therefore, we used a ribosome-free crude extract from a *trmA* strain that lacks tRNA(m^5U) methyltransferase activity completely. The extract contained at least four different methylating activities as shown by thin-layer chromatography of hydrolysates from generally methyl-deficient tRNA, methylated in vitro (Table 2). Lack of other methylating activities was not due to nonoptimal pH and ionic conditions. This extract was rather stable during storage and contained as many different activities as was found in more purified extracts from this strain. Thus we used this extract for screening mutants presented

TABLE 2. *Products formed by the enzyme extract*[a]

Compound formed	cpm	Recovered counts (%)
5	46	4.4
m^1G	323	31
Cm	49	4.6
mam^5s^2U[b]	384	37

[a] Enzyme extract was prepared from strain LMUR-5-266 (*trmA5*) as described in the text. Enzyme activities were measured with generally methyl-deficient tRNA as substrate and *S*-[*methyl*-^{14}C]adenosyl-L-methionine as methyl donor. Reaction volume was 0.5 ml, and no carrier RNA was added. After the incorporation of methyl groups, the tRNA was reisolated by phenol extraction and enzymatically digested to nucleosides. The hydrolysate was then subjected to two-dimensional thin-layer chromatography (32). This procedure separates the 21 different compounds, among them the unknown methyl-labeled compound 5, depicted in Fig. 4 of the accompanying paper (7). All compounds not indicated in the table were present in less than 40 cpm (4%) of recovered radioactivity.

[b] mam^5s^2U, 5-Methylaminomethyl-2-thio-uridine.

here in spite of the lack of desirable activities for production of m^7G and m^2A.

The screening procedure was tested by using methionine-starved strain W6 (*relA1, met*) as a model for methyltransferase-negative mutants. Methionine-starved cultures of strain W6 were mixed with different amounts of strain GB5, which had been incubated at 44°C for 6 h. Total RNA from such mixtures of cells was methylated in vitro under different conditions. We found that a high concentration of RNA was inhibitory to the methylation reaction. Using a reaction volume of 0.4 ml, the methylation in vitro could be performed with total RNA from three combined clones, i.e., from about 10^{10} cells, without any inhibition.

Mutagenesis of the parent strain. Logarithmically growing cells of strain GB5 were treated with nitrosoguanidine. The mutagenized culture was grown at 25°C for 6.5 h to allow phenotypic expression. Parameters chosen to monitor the efficiency of the mutagenization were survival (4 to 5%), auxotrophy (20%), and frequency of rifampin-resistant mutants (10^{-3}, as compared to 10^{-8} for the untreated control). Thus, a mutant allele of a gene might be found with a frequency of at least 10^{-3}. Since, later, no selection was to be used, a high frequency of mutants was a prerequisite for our screening experiment.

Screening for RNA methylation defective mutants. Samples from the mutagenized culture were plated on rich medium (LA). Individual colonies were later grown in rich (LB-MHU) medium and then transferred to LA plates. These plates (storage plates) were saved throughout the entire screening experiment. Samples from each clone were then grown in liquid medium and incubated at a high temperature. Cultures were combined in groups of three, the cells were harvested, and total RNA was prepared. After RNA methylation in vitro, total RNA was reisolated by phenol extraction. This step lowered the ^{14}C incorporation into control RNA by 90%, probably due to separation of RNA from methyl-labeled proteins.

A total of 300 clones were screened as described above. In these experiments, where relaxed RNA was produced in test-tube cultures, the [^{14}C]methyl/[^{3}H]uracil ratio, i.e., methyl groups/RNA, was 0.07 ± 0.002 for the parent strain. Whenever this ratio was higher than 0.1 and the ^{14}C counts were significantly increased for a combination of three mutated clones, these three clones were individually tested in flasks.

Single cell colonies from potential mutants were retested as described in footnote *a* of Table 3. During these conditions the parental strain had a ^{14}C/^{3}H ratio of 0.024. The methyl group incorporation and ^{14}C/^{3}H ratio for six potential mutants are listed in Table 3. The values obtained with different mutants cannot be quantitatively compared. During relaxed RNA synthesis, there is a preferential degradation of rRNA, and it is quite possible that this degradation is not the same in the different mutants. However, both the amount of methyl groups incorporated and the ^{14}C/^{3}H ratio are higher in all the mutants than in the parent strain, and for one mutant (IB13) the ratio was increased 10 times.

TABLE 3. *Methyl group incorporation in vitro into RNA originating from parent strain and different mutants of E. coli*[a]

Strain	$^{14}CH_3$ (cpm)	$^{14}CH_3$/[^{3}H]uracil
GB5[b]	467	0.024 (±0.004)
IB11	2,509	0.153
IB13	2,379	0.255
IB14	1,196	0.063
IB15	1,063	0.039
NG174	642	0.039
NG180	794	0.095

[a] Single colonies were picked into 2 ml of LB-MHU-[^{3}H]uracil medium and grown at 28°C. Part of this cell suspension was grown overnight in 15 to 20 ml of the same medium. Cells were then diluted to about 2 × 10^8 cells per ml with the same medium (12 ml) prewarmed to 44°C and incubated at this temperature for 4 to 5 h, to allow production of relaxed RNA. Cells were harvested and washed, and total RNA was prepared. After methylation in vitro using *S*-[*methyl*-^{14}C]adenosyl-L-methionine as the methyl donor, total RNA was reisolated by phenol extraction, and radioactivity was determined.

[b] Average of five determinations.

Mutants are defective in rRNA or tRNA. Our first step in characterizing four of the mutants was to determine the type of RNA that accepted the methyl groups in vitro and whether this ability was temperature dependent. Table 4 shows that, of the total RNA originating from IB11, only tRNA from cells incubated at 43.5°C accepted methyl groups in vitro. tRNA from IB13 accepted methyl groups when the cells were grown at 28°C as well as at 43.5°C. Mutants IB14 and IB15 are defective in rRNA methylation.

Characterization of rRNA mutants. To determine whether 23S or 16S rRNA was the methyl group acceptor, total RNA from IB14 and IB15 cells grown at 28°C was extracted, methylated in vitro, and centrifugated in a 5 to 20% sucrose gradient. In both mutants, 23S rRNA was the methyl group acceptor (Table 5). The two rRNA species were well separated, and the small amount of radioactive methyl groups also found in 16S rRNA region originates from tailing of the degraded radioactive 23S rRNA. To determine the product(s) made in vitro, the methyl-^{14}C-labeled 23S rRNA was degraded to nucleosides and analyzed by the method of Rogg et al. (32). Figure 2 shows that when 23S rRNA originated from strain IB14, more than 75% of recovered radioactivity (2,735 cpm) was found in one spot. The radioactivity in this spot comigrates with m^{5}C in this two-dimensional system and also in the following solvents: isopropanol–1% ammonium sulfate, 2:1, vol/vol (R_f = 0.59; R_C = 1.05); *N*-butanol–water, 86:14, vol/vol NH_3(gas) (R_f = 0.08, R_C = 1.27); and isopropanol–concentrated hydrochloric acid–water, 171:41, water to 250 vol/vol (R_f = 0.44; R_C = 1.12). However, 23S rRNA from strain IB15 is not a specific substrate for the formation of a single methylated nucleoside,

TABLE 4. *Methyl group incorporation in vitro into rRNA and tRNA*[a]

Strain	[*methyl*-^{14}C] RNA/[*uracil*-^{3}H]RNA ratio			
	Cells grown at 28°C		Cells incubated at 43.5°C	
	rRNA	tRNA	rRNA	tRNA
GB5	0.02	0.01	0.04	0.02
IB11	0.04	0.03	0.07	**0.37**
IB13	0.05	**0.06**	0.06	**0.08**
IB14	**0.13**	0.03	**0.22**	0.04
IB15	**0.09**	0.01	0.04	0.01

[a] Experiments were performed as described in footnote *a* of Table 3, but in vitro methylated total RNA was precipitated by ethanol, and rRNA and tRNA was separated by extraction with 2 M LiCl in 0.05 M NH_4^+-CH_3COOH, pH 5.0 (1). Values in boldface are considered significantly different from those of wild type.

TABLE 5. *Methyl group acceptor activity of 23S rRNA and 16S rRNA*[a]

Strain	Moles of CH_3 per mole of RNA	
	23S rRNA	16S rRNA
GB5	0.01	0.004
IB14	0.26	0.04
IB15	0.13	0.04

[a] Pooled lyophilized 23S rRNA and 16S rRNA were incubated in a total volume of 0.5 ml with wild-type enzyme and *methyl*-^{14}C donor (26 or 58 mCi/mmol) as described in the text. No carrier RNA was added. Incubation time was 60 min. Moles of CH_3 per mole of RNA was calculated from the amount of radioactivity found in 23S rRNA and 16S rRNA, assuming a molecular weight of these RNA species of 1.1×10^6 and 0.56×10^6, respectively (23).

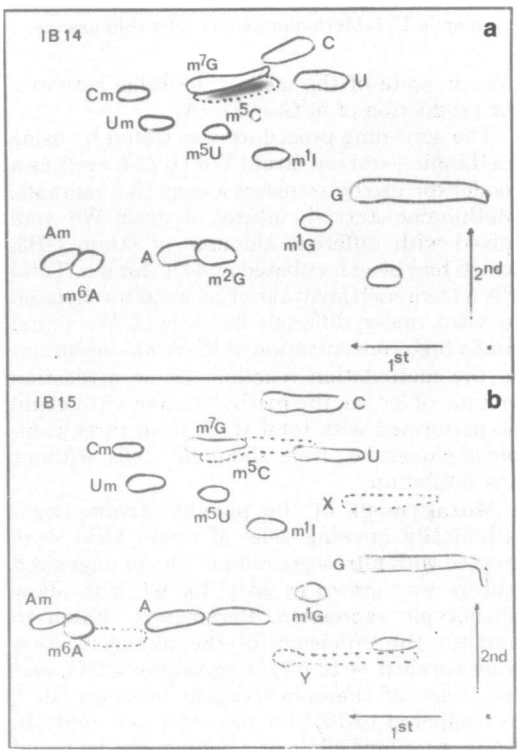

FIG. 2. *Two-dimensional thin-layer chromatography of hydrolysates of 23S rRNA from strain IB14 (a) and strain IB15 (b). The RNA was methylated in vitro with S-[methyl-^{14}C]adenosyl-L-methionine (50 mCi/mmol) using enzyme extract from the strain LMUR-5-266 before complete digestion to nucleosides. Dashed areas indicate location of radioactive materials visualized by radioautography, and solid lines indicate location of markers detected by UV light. Compounds X and Y are unknown methylated nucleosides.*

since radioactivity was found in m^6A (38% of recovered radioactivity, 1,855 cpm), m^5C (16%), unknown compound X (11%), and unknown compound Y (8%). Thus, strain IB14 seems to be defective in the biosynthesis of m^5C in 23S rRNA, whereas 23S rRNA from strain IB15 accepts methyl group in vitro into at least four different methylated compounds.

DISCUSSION

This paper describes a general screening procedure designed to isolate mutants defective in any RNA modifying enzyme producing lethal, as well as nonlethal, effects in cells. The usefulness of this method has been demonstrated by the successful isolation of several mutants defective in methylation of rRNA and tRNA. The tRNA mutants are characterized in the accompanying paper (7). The two rRNA mutants were shown to accept a methyl group only into the 23S rRNA. One single product, m^5C, was formed in vitro when 23S rRNA originated from mutant strain IB14, whereas more unspecific methylation was observed with the other mutants, IB15, in which m^6A was the most abundant product formed.

The earlier method devised by Björk and Isaksson (5) to screen for RNA methylation mutants required that cells be able to grow, and thus mutants deficient in an essential methylation reaction could never be isolated. This limitation is circumvented in our new method, since it is based on the accumulation of methyl-deficient RNA under a physiological condition during which cell growth is not required. The basic design of the method thus allows the isolation of all possible types of modificationless mutants, provided the mutation can be detected by an in vivo or in vitro assay. The efficiency of the in vitro method is dependent on the presence and activity of different modifying enzymes in the crude extract used. One way to selectively screen for one type of modificationless mutants is to purify the desired modifying activity. Another way to achieve some selectivity is to prepare enzyme extract from mutants that are known to lack some specific modification activity. In our screening for new methylation-defective mutants, we used the latter method and thus avoided reisolation of the already known *trmA* mutants. In theory, it should be possible to isolate mutants in any RNA modification, such as methylation, thiolation, or isopentenylation in tRNA or rRNA, by this method, provided an in vitro assay is functional.

In applying this method, we found mutants that were defective in rRNA methylation. Our enzyme extract used for in vitro assays contained the corresponding activities, although we had not checked extracts for rRNA methyltransferase activities before we started the screening. We knew that our extract could preferentially catalyze the formation of 5-methylaminomethyl-2-thio-uridine and m^1G in generally methyl-deficient tRNA. Consequently the corresponding tRNA mutants (see accompanying paper, 7) were isolated among the 300 clones tested. The strain we used as the enzyme source was later shown to be inferior to other strains in our collection concerning the number of different methylating activities. Thus, since the set and degree of different methylating activities in a crude enzyme extract is strain dependent, it is important to know the quality and property of the enzyme extract used to evaluate the possible success for screening certain types of modificationless mutants. The apparent frequency of mutants found was seemingly high. However, a later screening of 600 clones gave no mutant of the kind described here. Therefore, the frequency of mutants was as expected. One of the tRNA mutants (IB13) found lacks a methylated nucleoside normally present in 4% of the tRNA chains (7). We, therefore, estimate that our method is sensitive enough to detect a loss of one modified nucleoside normally present in 2% of the tRNA chains (cf. Table 3).

Few mutants in rRNA modification have so far been isolated. The first such mutant to be characterized was a mutant completely lacking m^1G in its 23S rRNA (5). These authors also isolated three mutants, the rRNA of which could be methylated in vitro with the formation of m^2G, although no deficiency of this nucleoside was found in vivo (5). In *Staphylococcus aureus*, resistance or induced resistance toward erythromycin results in the formation of 6-dimethyladenosine in 23S rRNA, a nucleoside that is absent in sensitive strains (24). Aberrant methylation in the 16S rRNA has been found in kasugamycin-resistant mutants that lack two 6-dimethyladenosine near the 3′ terminal of the 16S rRNA and also lack the corresponding methyltransferase activity (21).

Thus, IB14 and IB15 isolated by us are new rRNA methylation-defective mutants. Although we have not checked the in vivo level of m^5C in IB14 23S rRNA, it is likely that this level is decreased in view of the methylation specificity in vitro (5). On the other hand, it might be that error in processing or assembly of the ribosomal particles leads to aberrant methylation in vitro. The unspecific methylation in vitro with rRNA from IB15 suggest such an error instead of a specific defect in one methyltransferase, e.g., rRNA(6-methyladenosine)methyltransferase

(34). However, further studies of these mutants are necessary to clarify the biochemical nature of these mutations.

The two rRNA species in *E. coli* each contain 1 mol of m^5C per mol of RNA (15, 16). Since only 23S rRNA and not 16S rRNA from IB14 is a methyl-group acceptor, another enzyme is probably involved in the biosynthesis of m^5C in 16S rRNA. The rRNA(6-dimethyladenosine)methyltransferase (21) does not use naked 16S rRNA as substrate. Analysis in vivo of IB14 will reveal whether the level of m^5C differs in the 23S rRNA and 16S rRNA and thus unravel part of the complexity of rRNA maturation. Following genetical analysis and transfer of the mutated modification genes to a known genetic background, these mutations might help to elucidate the biological function(s) of these methylated nucleosides in rRNA.

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