JOURNAL OF BACTERIOLOGY, Jan. 1977, p. 15-21

Vol. 129, No. 1
Printed in U.S.A.

Tetrahydrofolate-Dependent Biosynthesis of Ribothymidine in Transfer Ribonucleic Acids of Gram-Positive Bacteria

W. SCHMIDT, H.-H. ARNOLD, AND H. KERSTEN*

Physiologisch-Chemisches Institut der Universität Erlangen-Nürnberg, 8520 Erlangen, Wasserturmstrasse 5, Germany

Received for publication 27 September 1976

Trimethoprim, an inhibitor that prevents tetrahydrofolate-dependent transmethylation reactions in bacteria, was used in a comparative study to discriminate between two possible biosynthetic pathways, either the *S*-adenosylmethionine or the tetrahydrofolate-dependent formation of ribothymidine (rT) in transfer ribonucleic acids (tRNA's) of several strains of gram-positive and gram-negative microorganisms. rT-deficient tRNA's accumulate in trimethoprim-treated gram-positive *Streptococcus faecium, Staphylococcus aureus, Corynebacterium bovis, Arthrobacter albidus,* and all examined *Bacillaceae,* except *Bacillus stearothermophilus.* The rT-deficient tRNA's accept the methyl moiety from *S*-adenosylmethionine in vitro, with extracts from *Escherichia coli* (wild type) as a source of methylating enzymes; 90% of the incorporated methyl groups are present in rT. Trimethoprim does not inhibit the biosynthesis of rT in tRNA of gram-negative *Enterobacteriaceae, Rhizobium lupini,* and *Pseudomonadaceae,* suggesting that the rT-specific tRNA methyltransferases of these gram-negative strains use *S*-adenosylmethionine as coenzyme.

The methyl groups in prokaryotic transfer ribonucleic acids (tRNA's) are mainly derived from methionine and introduced in tRNA precursor molecules by specific *S*-adenosylmethionine (SAM)-dependent tRNA methyltransferases (9). We have recently observed that in *Bacillus subtilis* the methyl group of ribothymidine (rT), i.e., 5-methyluracil (m^5U), is derived from serine (4) and transferred during maturation to tRNA by a tetrahydrofolate-dependent enzyme (10). A tetrahydrofolate-dependent pathway in the biosynthesis of rT in tRNA was also shown to be present in two other gram-positive microorganisms, e.g., *Streptococcus faecalis* (7) and *Micrococcus lysodeikticus* (16). These observations raise the question as to whether the rT-specific tRNA methyltransferases of gram-positive microorganisms are generally tetrahydrofolate dependent. The answer to this question is of particular interest, first with respect to the evolution of tRNA methylating enzymes and second with respect to tRNA modification in different taxa.

In the tetrahydrofolate transmethylation assay described previously (2), rT-deficient tRNA is methylated to an extent of 1 to 4% of the expected value. This low degree of rT formation in vitro is probably caused by the fact that the tetrahydrofolate-dependent transmethylation, unlike the SAM-dependent methyl group transfer, involves more than one enzymatic step. The assay system is thus not suitable to measure the activity of the tetrahydrofolate-dependent tRNA methyltransferase in crude extracts. A rapid indirect procedure was therefore developed to discriminate between an SAM or tetrahydrofolate-dependent pathway in the biosynthesis of rT in tRNA of a variety of gram-positive and gram-negative microorganisms.

This procedure is based on the observation that rT-deficient tRNA accumulates in *B. subtilis* in the presence of trimethoprim provided that the growth medium is supplemented with tetrahydrofolate-dependent metabolites: deoxythymidine, purines, methionine, and glycine (3). Heterologous methyltransferase from wild-type *Escherichia coli* is able to effect in vitro transfer of methyl groups from SAM to rT-deficient tRNA; 80 to 90% of the incorporated methyl groups are present in rT. Extracts from a mutant of *E. coli* (Trm^-) lacking the rT-specific enzyme transfer only trace amounts of methyl groups to this tRNA. The differential ability of extracts from wild-type and mutant (Trm) strains of *E. coli* to transfer methyl groups to tRNA can be used as an index of rT deficiency. tRNA's were isolated from a variety of trimethoprim-treated gram-positive and gram-negative microorganisms. rT-deficient tRNA's have been found to accumulate in the

presence of trimethoprim in gram-positive bacteria, except *B. stearothermophilus*, but not in gram-negative microorganisms.

(The results of this publication were presented at the Frühjahrstagung der Gesellschaft für Biologische Chemie München, 1976 [reference 17].)

Chemicals. Chemicals were from the following sources: [^{14}C]SAM (55 mCi/mmol), Radiochemical Centre, Amersham; ribonuclease T_2, Calbiochem; X-ray films for autoradiography, Osray T4, Agfa; all other reagents, solvents, and cellulose thin-layer plates, Merck AG. Trimethoprim [2,4-diamino-5'-(3,4,5-trimethoxybenzyl)pyrimidine] was a generous gift of Hoffmann-La Roche.

Media. The following media were used: medium A, 10 g of peptone (Difco), 5 g of yeast extract (Difco), 5 g of glucose, and 5 g of NaCl per liter, pH 7.2; medium B, the glucose-salt medium described in a previous communication (2); medium C, 5 g of peptone, 5 g of meat extract (Oxoid), and 5 g of NaCl per liter, pH 7.0; medium D, described by Björk et al. (5); medium E, 8 g of nutrient broth (Difco) per liter.

Bacterial strains. Bacterial strains were from the following sources: *B. circulans, Pseudomonas aeruginosa, Serratia marcescens,* and *Xanthomonas pruni,* A. Rösch, Erlangen; *S. faecium,* W. Fischer, Erlangen; *B. stearothermophilus,* H. Schweiger, Berlin; *E. coli* IB5, G. R. Björk, Umeå.

All other bacterial strains (summarized in Table 2) were obtained from Deutsche Sammlung für Mikroorganismen, Munich.

Bacterial growth. An overnight culture of each strain was diluted with fresh medium and grown to an optical density of A_{578} = 0.2. Trimethoprim was then added at concentrations from 10 to 100 μg/ml of medium (see Table 2). The optical density of each culture was measured for an additional 30 min. During this time growth had stopped in those strains cultivated in the minimal medium B. In strains cultivated in complex media (A, C, E) growth was inhibited to about 30 to 40%. After treatment with the inhibitor for 30 min the tetrahydrofolate-dependent metabolites adenosine, guanosine, and deoxythymidine (100 μg/ml each) and methionine and glycine (20 μg/ml each) were added. The bacteria resumed growth immediately at a rate almost identical to that of untreated controls. The bacteria were harvested at an optical density of A_{578} = 1.1 to 1.2. The bacterial strains, media and extent of growth inhibition that can be achieved by treatment with trimethoprim are shown in Table 2 (see below).

For the preparation of enzyme extracts, *E. coli* MRE 600 and *E. coli* IB5 (Trm$^-$) were grown in complex medium C and harvested at A_{578} = 0.8.

For the preparation of tRNA, *E. coli* IB5 was grown in salt-medium D to the stationary phase.

Preparation of tRNA and enzyme extracts. Bulk tRNA was isolated from bacteria harvested from 500-ml cultures. The isolation procedure and criteria of purity were the same as described previously (2). One A_{260} unit of tRNA (dissolved in water) was taken as 1.8 nmol.

Enzyme extracts (S-100) from *E. coli* MRE 600 and *E. coli* IB5 were prepared according to Traub et al. (18). The protein content was estimated by the method of Lowry et al. (12) and was about 10 to 15 mg/ml of S-100 extract.

In vitro methylation of tRNA's with *E. coli* enzymes. The methyl group accepting capacity of tRNA preparations was determined by a slight modification of the method of Kuchino et al. (11). The 180-μl reaction volume contained one A_{260} unit of tRNA, 19 nmol (0.2 μCi) of [*methyl*-^{14}C]SAM, 5 mmol of triethanolamine (pH 8.0), 0.1 μmol of β-mercaptoethanol, 50 mmol of $MgCl_2$, and 180 μg of protein (S-100). Incubation was at 37°C for 2 h. Samples (50 μl) were taken at 30, 60, and 120 min, and the trichloroacetic acid-insoluble radioactivity was measured by the technique of Mans and Novelli (13). For preparative purposes the reaction assay was increased threefold. The specific activity of [*methyl*-^{14}C]SAM was 57 nmol (4.15 μCi). Methylated tRNA was recovered by chromatography on diethylaminoethyl-cellulose (DE 32, Whatman) (20) and twice dialyzed against water.

Hydrolysis of labeled tRNA and analysis of the methylated compounds. tRNA was either hydrolyzed with 72% perchloric acid at 100°C for 1 h or digested with ribonuclease T_2 in 0.05 M sodium acetate, pH 4.5, for 4 h. Free bases were separated after desalting over charcoal (2) on cellulose thin-layer plates with solvent A (first dimension) and solvent B (second dimension). The 3' nucleotides were separated with solvent C (first dimension) and solvent D (second dimension). The solvents were as follows: solvent A, methanol-HCl-water (65:17:18); solvent B, *n*-butanol-water-acetic acid (4:1:1); solvent C, isobutyric acid-0.5 N ammonia (5:3); solvent D, isopropanol-water-HCl (70:15:15).

Autoradiography was performed on X-ray films for several days. Radioactive areas were

scraped out and the radioactivity was determined in a dioxane-scintillation mixture.

RESULTS

Measurement of rT deficiency in tRNA of *B. subtilis*. tRNA from untreated and from trimethoprim-treated *B. subtilis* and rT-deficient tRNA from the *E. coli* Trm⁻ mutant were methylated in vitro by employing [*methyl*-^{14}C]SAM as methyl donor and extracts prepared from wild-type or mutant Trm⁻ strains of *E. coli*.

The amount of methyl groups accepted by bulk tRNA from untreated *B. subtilis* proved to be especially low and is the same with enzyme extracts from the wild-type and mutant strains (Table 1); the sole methylated product is 7-methyl-guanine (Fig. 1a). The extent of methylation is about 10-fold greater when tRNA from trimethoprim-treated *B. subtilis* is used as the methyl group acceptor. The additional methyl groups were found to be present exclusively in rT (Table 1 and Fig. 1b). Under the conditions used, an average of 0.5 mol of rT residue is formed per mol of tRNA. An rT-deficient control tRNA from the *E. coli* mutant Trm⁻ accepts 0.8 mol of methyl groups per mol of tRNA in this in vitro assay (Table 1); 94% of the methylated product is rT as described previously (4a). The results indicate that rT deficiency in tRNA can be detected by comparing the amount of methyl groups transferred from SAM to the tRNA in vitro with extracts from the wild-type and mutant strains.

TABLE 1. *Methyl group acceptance capacity of tRNA from untreated and trimethoprim-treated B. subtilis upon methylation with [methyl-^{14}C]SAM and extracts from E. coli wild type or E. coli Trm⁻ mutant as the source of tRNA methyltransferases*

Source of tRNA[a]	mol of CH_3/mol of tRNA	
	Wild-type extract	Mutant extract
B. subtilis untreated	0.06	0.06
B. subtilis treated	0.45	0.06
E. coli Trm⁻	0.80	0.00

[a] The growth conditions for *B. subtilis* are described in Table 2.

Growth of bacterial strains in the presence of trimethoprim. Trimethoprim inhibits the growth of bacteria by blocking the enzyme dihydro-folate reductase. As a consequence, the tetrahydrofolate-dependent metabolites deoxythymidine, purines, methionine, and glycine are not formed. Moreover the tetrahydrofolate-dependent formylation of the initiator $tRNA_f^{Met}$ is blocked. As has been shown for *B. subtilis* the cells grow even though $tRNA_f^{Met}$ is not formylated, when the culture medium is supplemented with deoxythymidine, purines, methionine, and glycine (H. H. Arnold, W. Schmidt, and H. Kersten, Abstr. 10th FEBS. Meet., Paris, 1975, no. 388). Whether other bacterial strains are able to grow in the presence of trimethoprim under these conditions was tested for several gram-positive and gram-negative bacterial strains.

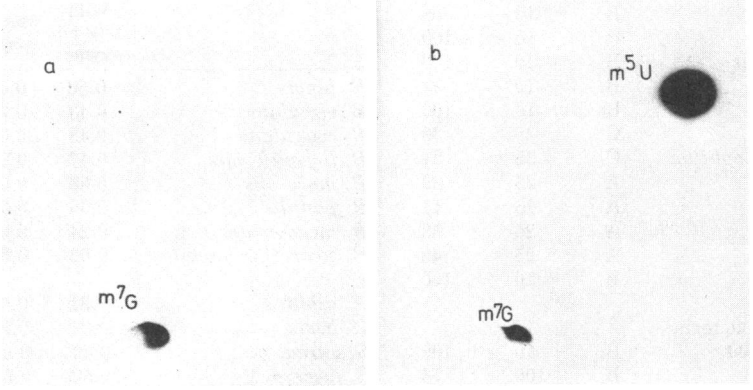

FIG. 1. *Autoradiography of methyl-^{14}C-labeled bases of tRNA of B. subtilis, untreated (a) and trimethoprim treated (b). The tRNA's were methylated in vitro with [methyl-^{14}C]SAM and E. coli extracts. The in vitro methylated tRNA was recovered by chromatography on diethylaminoethyl-cellulose and hydrolyzed to free bases with perchloric acid at 100°C for 2 h. The hydrolysate was desalted over charcoal and chromatographed on cellulose thin-layer plates. First dimension (solvent A), from left to right; second dimension (solvent B), from bottom to top. Radioactive spots were detected by autoradiography. The tRNA's from untreated and trimethoprim-treated B. subtilis do not accept methyl groups in this assay with homologous extracts.*

Overnight cultures of the bacterial strains were diluted into fresh media and grown to a cell density corresponding to an optical density of A_{578} = 0.2. Trimethoprim was added to each culture at concentrations from 5 to 100 μg/ml. The growth inhibitory effect was followed by measuring the optical density at 578 nm for a further 30 min. Upon subsequent addition of the supplements the bacteria were grown to an optical density of 1.1 to 1.2.

As Table 2 shows, all bacterial strains that can be cultivated in minimal medium (B) are totally inhibited by trimethoprim. All strains that had to cultivated in enriched media (A, C, E) are inhibited by trimethoprim at concentrations of 25 μg/ml of medium to an extent of about 30 to 40%. Increasing the concentration of trimethoprim had no further growth inhibitory effect on these strains. The effect of trimethoprim was found to be dependent on the composition of the growth medium and on the permeability of the bacteria. In the enriched media apparently the tetrahydrofolate-dependent metabolites are in excess and a 100% inhibition cannot be achieved. The permeability barrier is evident when comparing the effect of trimethoprim on *E. coli* MRE 600 and *P. aeroginosa* (see Table 2).

Methyl group acceptance capacity of tRNA from trimethoprim-treated gram-positive and gram-negative bacteria. tRNA's were isolated from trimethoprim-treated strains and tested with respect to their methyl group acceptance capacity in vitro with [*methyl*-^{14}C]SAM as the donor of methyl groups and enzymes from *E. coli* wild type and *E. coli* Trm$^-$ (Table 3). In tRNA populations from trimethoprim-treated *Bacillaceae, Streptococcus faecium, Staphylococcus aureus, Arthrobacter albidus,* and *Corynebacterium bovis,* on an average, each second tRNA molecule accepted one methyl group with enzyme extracts from *E. coli* wild type. With extracts from the mutant, lacking the rT-specific tRNA methyltransferase, the extent of methylation was at least one order of magnitude lower. No difference in methyl group acceptance capacity was found for *B. stearothermophilus* tRNA from trimethoprim-treated cells upon in vitro methylation with the wild-type or mutant extracts.

From the difference in methyl group acceptance of the tRNA from trimethoprim-treated gram-positive bacteria with either extract, we

TABLE 2. *Growth inhibitory concentrations of trimethoprim for several gram-positive and gram-negative bacterial strains grown in different media*

Strain	Medium[a]	Concn of trimethoprim	% Inhibition of growth rate
Gram-positive bacteria			
B. brevis	B	10	86
B. circulans	B	10	100
B. licheniformis	B	10	100
B. macerans	B	10	92
B. megaterium	B	10	100
B. pumilis	C	25	39
B. stearothermophilus	C	25	31
A. albidus	A	25	29
C. bovis	A	25	44
S. aureus	A	25	38
S. faecium	A	25	42
B. subtilis	B	10	100
Gram-negative bacteria			
E. coli MRE 600	B	10	100
P. aeruginosa	B	100	73
S. typhimurium	B	10	93
S. marcescens	B	10	100
X. pruni	B	10	100
R. lupini	E	25	72

[a] A, C, E = complex media; B = minimal medium. The composition of the media are described in the text.

TABLE 3. *Methyl group acceptance capacity of tRNA's from trimethoprim-treated gram-positive bacteria upon methylation in vitro with [methyl-^{14}C]SAM and extracts from E. coli wild type or E. coli Trm$^-$*

Strain	mol of CH_3/mol of tRNA[a]		mol of rT/mol of tRNA calculated
	Wild-type enzyme	Mutant enzyme	
B. brevis	0.50	0.04	0.46
B. circulans	0.43	0.06	0.37
B. coagulans	0.48	0.04	0.44
B. licheniformis	0.47	0.03	0.44
B. macerans	0.58	0.04	0.54
B. pumilis	0.50	0.06	0.44
B. megaterium	0.24	0.05	0.19
B. stearothermophilus	0.02	0.02	
A. albidus	0.21	0.06	0.15
C. bovis	0.27	0.03	0.24
S. aureus	0.40	0.07	0.33
S. faecium	0.50	0.07	0.43

[a] The difference between the values obtained with enzymes from the wild type and enzymes from the mutant represents methylation of uridine to rT. One mole of the methyl group incorporated into 1 mol of tRNA corresponds to 29,000 cpm. Blanks (without tRNA) were substracted. Further details are described in the text and in Table 2.

have calculated that about 80 to 90% of the methyl groups accepted with the wild-type enzyme are used to form rT. Whether this calculation is correct was tested for tRNA's from six different gram-positive strains treated with trimethoprim. The tRNA's were methylated in vitro with extracts from *E. coli* wild type and digested by ribonuclease T_2, and the labeled 3' nucleotides were analyzed (Table 4). About 80 to 90% of the incorporated radioactivity was found in ribothymidylic acid, indicating that the tRNA's from trimethoprim-treated gram-positive microorganisms, except the tRNA from *B. stearothermophilus*, are rT deficient.

In control experiments tRNA's were isolated from different families as representatives of the gram-positive strains. The bacteria were grown under identical conditions but without added trimethoprim. The isolated tRNA's do not accept extra methyl groups from SAM with enzymes from wild type *E. coli* (Table 5). This shows that the tRNA's from different families of gram-positive bacteria are fully modified with respect to rT.

From the fact that trimethoprim inhibits the formation of rT in several strains of gram-positive bacteria, we conclude that the rT-specific tRNA methyltransferases in these strains are tetrahydrofolate dependent.

To determine whether a tetrahydrofolate-dependent enzyme might also occur in gram-negative bacteria, we have treated several gram-negative strains with trimethoprim at growth inhibitory concentrations. The isolated tRNA's did not exhibit any differences in methyl group acceptance with wild-type or mutant enzymes (Table 6).

TABLE 4. *Formation of rT in tRNA's from trimethoprim-treated gram-positive bacteria upon methylation in vitro*[a]

Source of tRNA	Total cpm	cpm in rT	% Recovered as rT
A. albidus	16,980	13,131	77.0
B. brevis	17,460	15,150	86.8
B. coagulans	25,890	23,004	88.6
B. subtilis	21640	19,101	89.0
C. bovis	16,031	14,772	92.1
S. aureus	17,369	14,676	84.5

[a] The tRNA was methylated in vitro with enzymes (S-100) from *E. coli* wild type and [*methyl*-^{14}C]SAM. The tRNA was enzymatically digested to 3' nucleotides. The nucleotides were resolved by two-dimensional thin-layer chromatography, and the radioactivity of each compound was measured as described in the text. The remaining 10 to 20% of the label refers to 7-methylguanine, 1-methylguanine, and ribose methylations. These products are also formed with tRNA from untreated gram-positive bacteria as substrate.

DISCUSSION

This paper describes that trimethoprim, an inhibitor of the dihydrofolate reductase, can be used as a tool to discriminate between a tetrahydrofolate- or SAM-dependent pathway in the biosynthesis of rT in tRNA of bacteria. In the case of a tetrahydrofolate-dependent pathway the inhibitory effect of the antibiotic leads to a tRNA that is deficient in rT. The deficiency of rT can be detected by the formation of rT in tRNA in vitro in a SAM-dependent assay with *E. coli* extracts. With the homologous rT lacking control tRNA from the *E. coli* Trm mutant as substrate, 0.8 mol of m^5U are formed in vitro per 1 mol of tRNA. With rT-deficient tRNA's from trimethoprim-treated gram-positive strains, less than 0.8 mol of rT are always formed per 1 mol of tRNA. This can be explained by the following reasons. (i) The tRNA from trimethoprim-treated cells is heteroge-

TABLE 5. *Methyl group acceptance capacity of tRNA's from untreated gram-positive bacteria upon methylation in vitro with [methyl-^{14}C]SAM and extracts from E. coli wild type and E. coli Trm$^-$ mutant*

Strain	mol of CH_3/mol of tRNA	
	Wild-type enzyme	Mutant enzyme
A. albidus	0.06	0.06
B. brevis	0.05	0.04
B. coagulans	0.05	0.05
B. licheniformis	0.04	0.04
B. subtilis	0.06	0.06
C. bovis	0.04	0.04
S. aureus	0.07	0.07

TABLE 6. *Methyl group acceptance capacity of tRNA's from trimethoprim-treated gram-negative bacteria upon methylation in vitro with [methyl-^{14}C]SAM and enzymes from E. coli wild type and E. coli Trm$^-$ mutant*

Strain	mol of methyl group/mol of tRNA	
	Wild-type enzyme	Mutant enzyme
E. coli MRE 600	>0.01	>0.01
P. aeruginosa	0.02	0.02
R. lupini	0.03	0.03
S. typhimurium	0.04	0.04
S. marcescens	0.03	0.02
X. pruni	>0.01	>0.01

nous with respect to the rT content because completely modified tRNA is already present before trimethoprim is added. (ii) tRNA's are methylated in vivo at the precursor level. It is therefore possible that the assay conditions for the methylation of heterologous mature tRNA are not optimal for all tRNA's tested. (iii) Trimethoprim inhibits the dihydrofolate reductase by a competitive mechanism. Small amounts of the tetrahydrofolate coenzyme might thus be present in trimethoprim-treated cells.

The results clearly show that 11 out of 12 tRNA populations which were isolated from trimethoprim-treated gram-positive strains are rT deficient. Control experiments with seven selected gram-positive bacteria reveal that in their tRNA's rT is present. Altogether these results support the conclusion that the biosynthesis of rT in most of gram-positive bacteria is tetrahydrofolate dependent.

B. stearothermophilus is one exception. The tRNA from treated *B. stearothermophilus* is not deficient in rT. This fact is consistent with the observation that methionine serves as the donor of the methyl group of rT in this strain (1).

In the tetrahydrofolate-dependent biosynthesis of rT in tRNA of *B. subtilis*, formate or serine are used as precursors (4). Labeling experiments with [^{14}C]formate reveal that in *S. aureus* and *C. bovis*, which were tested as representatives of gram-positive bacteria, the methyl moiety of rT is derived from formate (data not shown).

In six different gram-negative bacterial strains that were treated with trimethoprim, a deficiency of rT in their tRNA was not detected. This means that rT is formed via SAM.

The findings show that different pathways have been evolved in the biosynthesis of rT in tRNA of different taxa, e.g., gram-positive and gram-negative bacteria. Not only the biosynthetic pathways but also the patterns of modified nucleosides in tRNA of gram-positive and gram-negative strains show characteristic variations. The patterns are almost identical in different species of the same taxon (W. Schmidt, unpublished data).

rT occurs in the GTψC loop of tRNA's and might be involved in the binding of tRNA to ribosomes via a complementary sequence in 5S ribosomal RNA (8, 14). The precise role of rT is not yet known, but it has been shown that the presence of rT in a tRNA molecule is an evolutionary advantage: the *E. coli* Trm mutants are less viable than the wild-type strains that have rT in their tRNA (6). As has been discussed by other workers (19), the presence of rT in the GTψC sequence increases the fidelity of translation. This could explain why rT-specific tRNA methyltransferases are essential and occur early during the evolution of prokaryotic tRNA's.

It is striking that the m^{5}U-specific tRNA methyltransferases from several gram-positive bacteria use a tetrahydrofolate derivative, like the thymidylate synthetase (15) which catalyzes the transfer of the methyl group from deoxyuridylate to deoxythymidylate. In contrast to the formation of rT in tRNA, the methylation of deoxyuridylate occurs at the nucleotide level. One can speculate that both enzymes might be related. Whether such a relationship exists can be clarified only after the isolation and characterization of the tetrahydrofolate-dependent rT-specific tRNA methyltransferase.

ACKNOWLEDGMENTS

We are indebted to the Deutsche Forschungsgemeinschaft and the Fond der Chemischen Industrie for support of this work.

ADDENDUM IN PROOF

In a previous communication we reported that considerably less rT is present in tRNA from *M. lysodeikticus* than in tRNA from *B. subtilis* (17). J. Rabinowitz (personal communication) investigated a strain of *M. lysodeikticus* lacking rT in tRNA. The question as to whether various gram-positive micrococci lack rT-specific tRNA methyltransferase or have low levels of this enzyme is currently being investigated.

LITERATURE CITED

1. **Agris, P. F., H. Koh, and D. Söll.** 1973. The effect of growth temperatures on the in vivo ribose methylation of Bacillus stearothermophilus tRNA. Arch. Biochem. Biophys. 145:277–282.
2. **Arnold, H. H., and H. Kersten.** 1973. The occurrence of ribothymidine, 1-methyladenosine, methylated guanosine and the corresponding methyltransferases in E. coli and B. subtilis. FEBS Lett. 36:34–38.
3. **Arnold, H. H., and H. Kersten.** 1975. Inhibition of the tetrahydrofolate-dependent biosynthesis of ribothymidine in tRNAs of B. subtilis and M. lysodeikticus by trimethoprim. FEBS Lett. 53:258–261.
4. **Arnold, H. H., W. Schmidt, and H. Kersten.** 1975. Occurrence and biosynthesis of ribothymidine in tRNAs of B. subtilis. FEBS Lett. 52:62–65.

4a. **Arnold, H. H., W. Schmidt, R. Raettig, L. Sandig, H. Domdey, and H. Kersten.** 1976. S-adenosylmethionine and tetrahydrofolate-dependent methylation of tRNA in B. subtilis: incomplete methylation caused by trimethoprim, pactamycin or chloramphenicol. Arch. Biochem. Biophys. 176:12–20.

5. **Björk, G. R., and L. A. Isaksson.** 1970. Isolation of mutants of E. coli lacking 5-methyluracil in tRNA or 1-methylguanine in rRNA. J. Mol. Biol. 51:83–100.
6. **Björk, G. R., and F. C. Neidhardt.** 1975. Physiological and biochemical studies on the function of 5-methyluridine in transfer ribonucleic acid of *Escherichia coli*. J. Bacteriol. 124:99–111.
7. **Delk, A. S., and J. C. Rabinowitz.** 1975. Biosynthesis of

ribosylthymine in the transfer RNA of streptococcus faecalis: a folate dependent methylation not involving S-adenosylmethionine. Proc. Natl. Acad. Sci. U.S.A. 72:528–530.

8. **Erdmann, V. A., M. Sprinzl, and O. Pongs.** 1973. The involvement of 5S RNA in the binding of tRNA to ribosomes. Biochem. Biophys. Res. Commun. 54:942–948.
9. **Kerr, S. J., and E. Borek.** 1973. Enzymic methylation of natural polynucleotides, p. 167–195. *In* P. D. Boyer (ed.), The enzymes, vol. 9. Academic Press Inc., New York.
10. **Kersten, H., L. Sandig, and H. H. Arnold.** 1975. Tetrahydrofolate-dependent 5-methyluracil-tRNA transferase activity in B. subtilis. FEBS Lett. 55:57–60.
11. **Kuchino, Y., and S. Nishimura.** 1970. Nucleotide sequence specificities of guanylate residue-specific tRNA methylases from rat liver. Biochem. Biophys. Res. Commun. 40:306–313.
12. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
13. **Mans, R. J., and G. D. Novelli.** 1961. Measurements of the incorporation of radioactive amino acids into protein by a filter-paper disk method. Arch. Biochem. Biophys. 94:48–53.
14. **Ofengand, J., and C. Henes.** 1969. The function of pseudouridylic acid in transfer ribonucleic acid. J. Biol. Chem. 244:6241–6253.
15. **Rader, J. I., and F. M. Huennekens.** 1973. Folate coenzyme-mediated transfer of one-carbon groups, p. 197–223. *In* P. D. Boyer (ed.), The enzymes, vol. 9. Academic Press Inc., New York.
16. **Schmidt, W., H. H. Arnold, and H. Kersten.** 1975. Biosynthetic pathway of ribothymidine in B. subtilis and M. lysodeikticus involving different coenzymes for transfer RNA and ribosomal RNA. Nucleic Acids Res. 2:1043–1052.
17. **Schmidt, W., H. H. Arnold, and H. Kersten.** 1976. Ribothymidin-Biosynthese in Transfer-RNA von Bakterien. Hoppe Seyler's Z. Physiol. Chem. 357:303.
18. **Traub, P., S. Mizushima, C. V. Lowry, and M. Nomura.** 1971. Reconstitution of ribosomes from subribosomal components, p. 391–407. *In* K. Moldave and L. Grossman (ed.), Methods in enzymology, vol. 20. Academic Press Inc., New York.
19. **Yang, S., E. R. Reinitz, and M. L. Gefter.** 1973. Role of modifications in tyrosine transfer RNA. II. Ribothymidylate-deficient tRNA. Arch. Biochem. Biophys. 157:55–62.
20. **Yang, W. K., and G. D. Novelli.** 1968. Isoaccepting tRNAs in mouse plasma cell tumors that synthesize different myeloma protein. Biochem. Biophys. Res. Commun. 31:534–539.