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Biosynthetic pathway of ribothymidine in B. subtilis and M. lysodeikticus involving different coenzymes for transfer RNA and ribosomal RNA

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ABSTRACT

Ribothymidine (m^5U) in tRNAs of M. lysodeikticus is not derived from methionine. The results indicate that as in tRNAs of B. subtilis a tetrahydrofolate derivative is involved in the formation of m^5U, whereas methionine serves as precursor in the biosynthesis of m^7G, m^1A and m^6A.

Ribothymidine also occurs in 23S rRNA of B. subtilis and M. lysodeikticus. Approximately 2-3 moles of m^5U residues were found per mole of 23S rRNA. In contrast to m^5U residues present in tRNAs of B. subtilis and M. lysodeikticus, ribothymidine in 23S rRNA of these organisms and of E. coli is synthesized via S-adenosylmethionine. m^6A and m^1G, present in E. coli rRNAs, were not detected in rRNAs of (methyl-^{14}C) methionine labeled B. subtilis and M. lysodeikticus.

INTRODUCTION

Transfer ribonucleic acids of B. subtilis contain approximately one ribothymidine (m^5U) residue per molecule (1-3). However methionine, which normally serves as precursor in tRNA methylation, is not used in this transmethylation reaction (2-4). The methyl groups are donated by formate or serine (3) and are transferred via a tetrahydrofolate derivative as coenzyme (5). We now describe an analogous biosynthetic pathway for ribothymidine in tRNAs of M. lysodeikticus. The aim of the present work was further to clarify, whether ribothymidine occurs as minor component in ribosomal RNAs of B. subtilis and of M. lysodeikticus and whether S-adenosylmethionine or a tetrahydrofolate derivative is involved in the formation of m^5U of rRNA. The 23S rRNAs of B. subtilis and of M. lysodeikticus contain ribothymidine in considerable amounts. Both organisms use a tetrahydrofolate derivative for the biosynthesis of m^5U in tRNA but for 23S rRNA S-adenosylmethionine (SAM) serves as coenzyme in the biosynthesis of m^5U.

MATERIALS AND METHODS

Chemicals

The chemicals were from the following sources: (methyl-^{14}C) methionine (specific activity 56 mCi/mmole) from Radiochemical Center Amersham; methylated nucleosides from Cyclochemical Corporation, Los Angeles, California, USA (free bases were obtained by hydrolysis in $HClO_4$). Resines Voltalef 300 LD-PL micro from Lehmann and Voss, Hamburg; Adogen 464 from Lilachim N.V. Brussels, Belgium; DNase I (Code DC L 115) from Worthington, Freehold, USA. All other chemicals were from sources as described previously (3,4).

Conditions of growth and labeling

E. coli K 12 rel^- met^- was grown in a minimal medium (6) with (methyl-^{14}C) methionine (100 uC = 33 µmoles/100 ml culture).

B. subtilis met^- $indol^-$ was cultured in a glucose-salt medium (4) with (methyl-^{14}C) methionine (100 µC = 7 nmoles/100 ml) and indol (25 µg/ml). *M. lysodeikticus* was grown in the same medium without indol.

The cultures were grown up to the stationary phase, harvested by centrifugation and frozen at -20°C.

Isolation of tRNAs and rRNAs

tRNAs were isolated purified and characterized as described previously (3). For the isolation of rRNA about 0.5 g (wet weight) cells were ground with a two-fold amount of Alcoa at 0°C and extracted with 1.5 ml buffer (0.01M Mg-acetate; 6 mM mercaptoethanol; 0.06M KCl; 0.01M Tris-HCl, pH = 7.6; 100 U/ml DNase I). After centrifugation at 12 000 g for 15 min the supernatant was diluted with the same buffer, but free of Mg^{2+}, to a final concentrations of Mg^{2+} of 2.5 x 10^{-3}M. The extract was treated for 30 min at 0°C with an equal volume of phenol. After centrifugation the upper phase was removed and mixed with 3 volumes of ethanol containing 2 % K-acetate. The RNA was precipitated at -20°C overnight, centrifuged and finally dissolved in 1-2 ml water. About 10-20 A_{260} units of total RNA were applied to an RPC-5 column and eluted with 150 ml 0.01M Mg-acetate, 0.05M Tris-HCl buffer pH 7.3 contain-

ing a linear gradient of NaCl from 0.4M to 1.2M (7). Usually fractions of about 2.5 ml were collected in which UV-absorption and radioactivity were measured. Those fractions containing 16S or 23S rRNA were pooled, dialyzed twice against distilled water and evaporated to dryness. The residues were hydrolyzed to the bases as described previously (4).

Base analysis

The methylated bases were analyzed according to Kahle et al. (8). Analysis of the ^{3}H-postlabeled nucleoside derivatives of tRNAs from unlabeled cells was performed as described by Randerath (9).

RESULTS AND DISCUSSION

Minor components in tRNA

The analysis of tRNA from M. lysodeikticus by ^{3}H-postlabeling reveals that ribothymidine is present and comprises 0.65 % of the total nucleosides (Table I). From this analysis it was calculated that on average one $m^{5}U$-residue occurs per two tRNA molecules. This amount is considerably lower than that found in tRNAs from E. coli or B. subtilis in which $m^{5}U$ constitutes one residue per molecule (1-3). The $m^{5}U$ residue of tRNA isolated from M. lysodeikticus cells, which were grown in the presence of (methyl-^{14}C) methionine, are not labeled. This result clearly shows that ribothymidine in tRNAs of M. lysodeikticus is not derived from methionine. This SAM-independent transmethylation reaction in tRNA maturation was also found in B. subtilis (3, 5) and S. faecalis (9), two other grampositive microorganisms. However a common biosynthetic pathway of ribothymidine in tRNAs of grampositive microorganisms does not exist, since in B. stearothermophilus the $m^{5}U$-tRNA methyltransferase is SAM-dependent (11).

The other methylated bases occurring in tRNAs of M. lysodeikticus at relative high frequency are $m^{7}G$, $m^{1}A$ and $m^{6}A$. The pattern of the methylated bases of tRNA from M. lysodeikticus exhibits great similarities with that of tRNA from B. subtilis (3,12), especially with respect to the methylation of adenine residues at position 1. This modification has not been observed sofar in other procaryotic tRNAs. Moreover two as yet unidentified minor components X and P are common for

Table I. Analysis of M. lysodeikticus tRNA by ^{3}H-postlabeling or methyl-^{14}C labeling

compounds	percentage of total radioactivity recovered		
	boro (^{3}H) hydride incorporation		(methyl-^{14}C) methionine incorporation
	total bases	methyl. bases	methyl. bases
m^5U	0.65	34.0	0
G + I	26.20		
C	31.83		
A	18.20		
U	19.73		
Ψ	1.23		
hU	0.77		
m^7G	0.57 (a)	29.8	36.0
m^1G	0.17	8.9	9.7
m^2G	0.09	4.7	5.2
m^1A	0.27 (b)	14.1	21.4
m^2A	—	2.0	5.0
m^6A	0.16	8.4	11.0
m_2^6A	x		1.0
mC	x		1.0
X	xx		7.8
P	xx		2.9

(a) corrected for recovery of 66 %
(b) m^1A is partially converted to m^6A
x not detectable; xx position unkown

tRNAs of both organisms. With the exception of m^5U all other methylated bases of M. lysodeikticus tRNAs are derived from methionine via S-adenosylmethionine as coenzyme.

Minor components in ribosomal RNA

Ribosomal RNAs of E. coli contain ribothymidine, which can be labeled during growth by (methyl-^{14}C) methionine (13-15). In the following experiments we have compared E. coli, B. subtilis and M.lysodeikticus with respect to the occurrence and biosynthetic pathway of m^5U in ribosomal RNAs.

Ribosomal RNAs were isolated from cells grown with (methyl-^{14}C) methionine and separated on RPC-5 columns which allow sufficient resolution of 16S and 23S rRNA. The extent of methylation of both types of ribosomal RNAs was calculated by using extinction coefficients and molecular weights as described by Kurland (16). Based on this calculation we have found that E. coli 16S rRNA contains approximately 12 methyl groups per molecule. This value agrees well with the content of methyl groups of 16S rRNA estimated from sequence analysis (17). In 16S rRNA of B. subtilis and M. lysodeikticus only 7-9 labeled methyl groups were found per molecule. The 23S rRNA of E. coli contained 17, 23S rRNAs of the grampositive organisms only 8 labeled methyl groups per molecule. Whether additional methyl groups which are not derived from methionine might be present in ribosomal RNAs of grampositive or gramnegative organisms is under current investigation.

The individual labeled methylated bases of ribosomal RNAs were identified by two dimensional thin layer chromatography. The methylation of ribose moieties was found to be less than 10 % of the total methylated bases and is not considered in these results. The patterns of the (methyl-^{14}C) labeled bases of 16 S and 23S rRNAs from E. coli and B. subtilis show variations especially for 23S rRNA (Fig. 1). The patterns of methylated bases from M. lysodeikticus rRNAs are identical with those of B. subtilis and were therefore ommitted. Quantitative data are summarized in Table II and III.

The distribution of the methylated bases of 16S rRNA from the three organisms differs only slightly (Table II). In the position of m^5U trace amounts of radioactivity were found. The identity of this labeled compound with m^5U is not yet established. Recently in 16S rRNA of E. coli a methylated U was detected similar to, but not identical with m^5U (15).

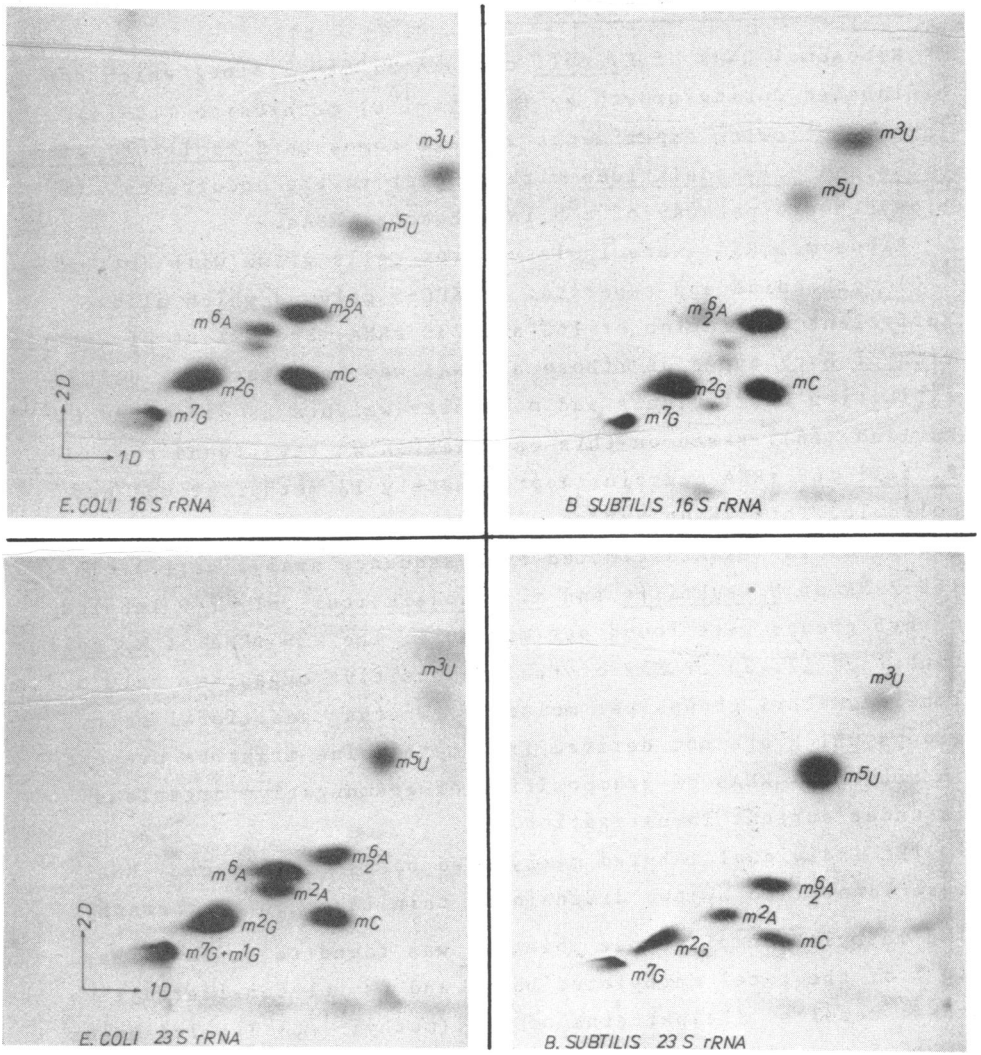

Fig. 1. Autoradiography of the patterns of methyl-^{14}C labeled bases of 16S and 23S ribosomal RNA from E. coli and B. subtilis. Solvent for the first dimension: Methanol/12N HCl/water (65:17:18), for the second dimension: n-butanol/acetic acid/water (4:1:1). For resolution and quantitative estimation of 7-methylguanine 1-methylguanine another solvent system was used in the second dimension (4).

In 16SrRNA of all three organisms mainly m^2G, m_2^6A, and methylated cytosines were found, which represent 85 % of the total methylated bases derived from methionine. Trace amounts of m^6A

Table II. Relative distribution of methylated bases in 16S rRNA of E. coli, B. subtilis and M. lysodeikticus derived from methionine

compounds	methyl-^{14}C incorporation as percentage of total radioactivity recovered		
	E. coli	B. subt.	M. lys.
m^5U	4.6$^+$	2.5$^+$	
m^1G	—	—	—
m^7G	7.3	10.8	13.6
m^2G	26.3	21.2	23.2
m^2A	—	—	—
m^6A	4.8	—	—
m_2^6A	23.1	33.9	35.1
m C	26.6	21.2	20.1
m^3U	4.8	7.2	8.0

$^+$whether the radioactive spot found in the position of m^5U is identical with m^5U is not yet clarified (see text).

observed in hydrolysates of E. coli 16S rRNA probably results from a contamination of the preparation with 23S rRNA which contains this base in relative high amounts (Table III).

Striking variations were observed in the relative distribution of labeled methylated bases of 23S rRNA of the three organisms (Table III). m^5U represents in 23S rRNA of E. coli 8 %, in 23S rRNA of B. subtilis 39 % and in 23S rRNA of M. lsyodeikticus 28 % of the total labeled bases. The relative high radioactivity of m^5U in 23S rRNA of the grampositive organisms can be explained by the absence of labeled m^6A, m^1G and methylated cytosine residues. A calculation of the absolute amount of m^5U per 23S rRNA molecules revealed for E. coli 2, B. subtilis 3 and M. lysodeikticus 2-3 residues. The value obtained for 23S rRNA of E. coli agrees well with that described by Fellner (15).

In a previous work we have shown, that extracts of B. sub-

Table III. Relative distribution of methylated bases in 23S rRNA of E. coli, B. subtilis and M. lysodeikticus derived from methionine

	methyl-^{14}C incorporation as percentage of total radioactivity recovered		
compounds	E. coli	B. subt.	M. lys.
m^5U	8.3	38.7	28.1
m^1G	8.0	—	—
m^7G	9.0	16.7	25.9
m^2G	21.6	19.2	23.4
m^2A	8.1	5.4	12.3
m^6A	17.1	—	—
m_2^6A	7.5	9.8	5.5
m C	18.4	6.0	3.1
$m^3\Psi$	—	1.8	—

tilis contain a specific tetrahydrofolate - dependent 5-methyluracil-tRNA transferase. The enzyme mediates the transfer of one-carbon groups from formaldehyde via tetrahydrofolate to m^5U-deficient tRNA from E. coli IB5 Trm^-. The product of the transmethylation reaction was identified as 5-methyluracil (18). The results presented previously (5) and in this paper indicate, that an analogous enyzme exists in M. lysodeikticus which is involved in the biosynthesis of ribothymidine of M. lysodeikticus tRNA. Furthermore the results of this paper conclusively show that ribothymidine in 23S rRNA of B. subtilis and M.lysodeikticus is formed with S-adenosylmethionine as coenzyme. SAM serves as coenzyme in several other transmethylation reactions of rRNA in both organisms. The total absence of methyl-^{14}C labeled m^6A and m^1G in 23S rRNA either mean that these modification do not occur in 23S rRNA of the two grampositive organisms or are synthesized via another biosynthetic pathway.

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