JOURNAL OF BACTERIOLOGY, Dec. 1980, p. 991-998
0021-9193/80/12-0991/08$02.00/0

Vol. 144, No. 3

Methylation Patterns of Mycoplasma Transfer and Ribosomal Ribonucleic Acid

CHUEN-CHIN HSUCHEN† AND DONALD T. DUBIN*

Department of Microbiology, College of Medicine and Dentistry of New Jersey-Rutgers Medical School, Piscataway, New Jersey 08854

The methylation patterns of transfer and ribosomal ribonucleic acid (RNA) from two mycoplasmas, *Mycoplasma capricolum* and *Acholeplasma laidlawii*, have been examined. The transfer RNA from the two mycoplasmas resembled that of other procaryotes in degree of methylation and general diversity of methylated nucleotides, and bore particular resemblance to *Bacillus subtilis* transfer RNA. The only unusual feature was the absence of m^5U from *M. capricolum* transfer RNA. The methylation patterns of the mycoplasma 16S RNAs were also typically procaryotic, retaining the methylated residues previously shown to be highly conserved among eubacterial 16S RNAs. The mycoplasma 23S RNA methylation patterns were, on the other hand, quite unusual. *M. capricolum* 23S RNA contained only four methylated residues in stoichiometric amounts, all of which were ribose methylated. *A. laidlawii* 23S RNA contained the same ribose-methylated residues, plus in addition approximately six m^5U residues. These findings are discussed in relation to the phylogenetic status of mycoplasma, as well as the possible role of RNA methylation.

Mycoplasmas, or, more precisely, members of the class *Mollicutes*, are generally considered to constitute a group of organisms separate from other procaryotes such as the eubacteria. In addition to the prime taxonomic characteristic defining the group, the absence of a cell wall, mycoplasmas occupy an extreme evolutionary position with regard to their small cell and genome sizes (see references 20, 32, and 43).

We have examined the methylation patterns of rRNA and tRNA from two mycoplasmas, with two aims in mind. In view of the relative evolutionary preservation of such patterns (e.g., references 12, 23, 25, and 30) we hoped they might provide new insights on the phylogenetic status of mycoplasmas, and in view of the limited quantity of genetic information in mycoplasmal genomes we hoped to obtain clues as to the possible relative dispensability of the various methylated residues of more conventional RNA. We examined one species from each of the two main mycoplasma families, *Mycoplasma capricolum* and *Acholeplasma laidlawii*. The results for tRNA and 16S rRNA indicated that mycoplasmas are evolutionarily closely related to eubacteria, in particular to *Bacillus*. The results for 23S RNA, on the other hand, indicated a special status for mycoplasma. Especially for *M. capricolum*, this special status represents a striking simplification of the procaryotic 23S RNA pattern at least as typified by that of *Escherichia coli*.

† Present address: Department of Biochemical Sciences, Princeton University, Princeton, NJ 08544.

MATERIALS AND METHODS

M. capricolum (formerly *Mycoplasma* sp. Kid) and *A. laidlawii* strains were obtained from the American Type Culture Collection, Rockville, Md. (ATCC numbers 27343 and 14192, respectively). Separate samples were obtained for each of three experiments. Cultures were grown in shaking flasks at 37°C in 50 ml of medium consisting of 2% tryptose, 0.5% NaCl, 0.5% glucose, 1% PPLO serum (Difco), 0.5% Tris, and 100 U of penicillin G per ml, brought to pH 8.2 (33). Labeled precursors were added to cultures at initial cell densities of approximately 4×10^7 colony-forming units per ml. For one experiment, we added 8 mCi of [*methyl*-^{3}H]methionine (14 Ci/mmol) and $^{32}P_i$ (1 mCi); for the other two we added 10 mCi of [*methyl*-^{3}H]methionine (10.5 Ci/mmol) and 5 μCi of [2-^{14}C]methionine (58 Ci/mmol). After 2 h, the cells were harvested by centrifugation (10,000 rpm, 10 min at 4°C) and washed twice in 10 mM Tris-hydrochloride (pH 7.4) containing 0.9% NaCl. RNA was extracted by holding the cells for 20 min at 37°C in 2 ml of 10 mM sodium acetate, pH 5.1, containing 1% sodium dodecyl sulfate, 8 mM EDTA, 25 μg of polyvinyl sulfate per ml, 1% diethylpyrocarbonate, and 5 mg of bentonite per ml. The extract was shaken four times with phenol, and the RNA was precipitated from the aqueous phase with ethanol. Yields of ^{3}H ranged from 40,000 to 60,000 cpm, yields of ^{14}C were approximately 15,000 cpm, and the yield of ^{32}P was approximately 50,000 cpm. RNA was separated into 4S, 16S, and 23S fractions by velocity sedimentation in sucrose gradients (11). Our procedures for analyzing RNA have been described. Briefly, sam-

ples were digested with dilute acid (1 N HCl; 9), alkali (0.5 N KOH; 9), or T2 RNase (17) and fractionated using DEAE column chromatography (16), paper electrophoresis (9), and a variety of paper and thin-layer procedures, in some cases after secondary degradation. Details relevant to particular analyses are presented below.

RESULTS

On fractionation by density gradient sedimentation, RNA from both mycoplasma species yielded discrete peaks of ^3H and of ^{14}C or ^{32}P, corresponding to 4S, 16S, and 23S RNA (data not shown). The ratios of ^3H to general label across these peaks are summarized in Table 1. These ratios, appropriately corrected, were used to estimate degrees of methylation (see below).

Analysis of methylated nucleotides was modeled after our earlier studies on animal cell tRNA (9). Samples were hydrolyzed with dilute acid, and these hydrolysates were subjected to fractionation by electrophoresis at pH 3.5. To characterize any ribose-methylated residues present, samples were also hydrolyzed with alkali or T2 RNase to release such residues as $O^{2'}$-methyl-dinucleotides, and these latter were separated from mononucleotides by DEAE column chromatography.

The results for ribose-methylated residues are summarized in Table 2. The 4S RNA from both mycoplasmas had essentially no ribose-methylated residues, differing in this regard from an *E. coli* preparation examined for comparison. The 16S RNA yielded dinucleotide peaks accounting for roughly 20% of the total ^3H. These peaks were characterized by chromatographic analysis of ribosides released by phosphatase and of bases released by acid (17). In the case of alkaline hydrolysates, 75 to 80% of the label was found to be in the $O^{2'}$-ribose-methylated dinucleotide m^4CmpCp (where m^4Cm is $N^4,O^{2'}$-dimethylcytidine), and the rest was found in the partially alkali-resistant dinucleotide of N^6,N^6-dimethyladenosine, $m_2^6Ap \cdot m_2^6Ap$ (see reference 29). Similar results were obtained for *E. coli* 16S RNA. These results suggest that some (at least) of the m_2^6A's of mycoplasmal 16S RNA are vicinal; they also explain the slightly smaller dinucleotide fraction in T2, as opposed to alkaline, digests of 16S RNA.

The results for 23S RNA were the most striking: about two-thirds of the ^3H of *M. capricolum* 23S RNA and one-third of the ^3H of *A. laidlawii* 23S RNA appeared in the dinucleotide fraction, in sharp contrast to the results for *E. coli*. These dinucleotide fractions were recovered and subjected to paper electrophoresis, as illustrated for *M. capricolum* in Fig. 1. Three discrete peaks were displayed, the identities of which were ascertained essentially as in an earlier study (16). Peak 1 (30% of the ^3H) was found to be CmpGp; peak 2 (also 30%) was GmpGp; and peak 3 (40%) was GmpUp. The results for *A. laidlawii* were similar. Each of these dinucleotides was also subjected to acid hydrolysis, followed by electrophoresis at pH 3.5; CmpGp yielded a peak in the Cp region (presumably Cmp-ribose), whereas the other two yielded primarily peaks just beyond Up. Analysis of *methyl*-^3H, ^{32}P-labeled samples from hamster cell 28S RNA, which is relatively rich in ribose-methylated nucleotides (16), has shown that this latter peak is methyl-

TABLE 1. *Ratios of ^3H from [methyl-^3H]methionine to general RNA*

Strain	RNA fraction	Ratio[a]
M. capricolum	4S	3.09 ± 0.15
	16S	1
	23S	0.28 ± 0.02
A. laidlawii	4S	2.83 ± 0.32
	16S	1
	23S	0.54 ± 0.02

[a] Ratios represent the averages across density gradient peaks of ^3H (from [*methyl*-^3H]methionine) to ^{14}C or ^{32}P (from [2-^{14}C]uridine or $^{32}P_i$), normalized to that of 16S RNA, for each of the three experiments described in the text; we indicate also standard deviations. In view of the undefined nature of the growth medium and the slight leakage of ^3H into purine rings (see the text) we did not attempt to convert counts per minute to micromole equivalents. The actual counts per minute ratios of 16S RNA were approximately 3 for ^3H:^{14}C and 0.2 for ^3H:^{32}P.

TABLE 2. *DEAE column chromatography of T2 and alkaline digests of mycoplasma RNA*

Strain	Hydrolysis	Label from [*methyl*-^3H]- or [*methyl*-^{14}C]methionine (% of total)[a]					
		4S RNA		16S RNA		23S RNA	
		Mono	Di	Mono	Di	Mono	Di
M. capricolum	T2			82	18	42	58
	KOH	97	<3	79	21	35	65
A. laidlawii	T2			85	15	68	32
	KOH	96	<4	74	26	67	33
E. coli	T2			85	15		
	KOH	86	14	82	18	85	15

[a] The mycoplasma results were obtained from *methyl*-^3H-labeled samples as described in the text, and the *E. coli* results were obtained from *methyl*-^{14}C-labeled samples prepared as described in reference 14. The mononucleotide fraction (Mono) is defined as the peak eluting in the region of mononucleotide marker from an RNase A digest of unlabeled RNA, and the dinucleotide fraction (Di) was that eluting in the region of the dinucleotide marker (corresponding to net charges of approximately −2 and −3, respectively) (9, 16).

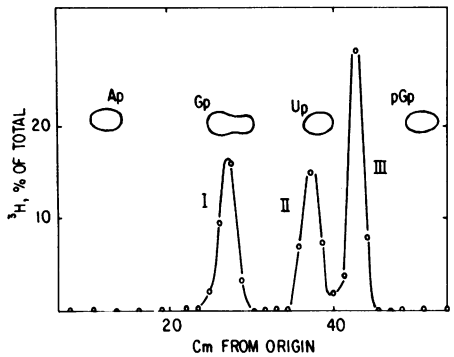

FIG. 1. *Paper electrophoresis of the dinucleotide fraction from M. capricolum methyl-^{3}H-labeled 23S RNA. The dinucleotide peak from a DEAE column eluate was desalted, a portion was subjected to paper electrophoresis at pH 3.5 (3,000 V, 4 h), and segments of the electropherogram were assayed for ^{3}H. The ovals represent 260-nm-absorbing markers. Only the region between Ap and pGp is shown; there was essentially no ^{3}H elsewhere.*

ribose-phosphate (Baer and Dubin, unpublished data).

Representative electrophoretic patterns from acid hydrolysates of intact *M. capricolum* RNA are illustrated in Fig. 2. We processed ^{3}H,^{32}P-labeled mycoplasma RNA together with *methyl-*^{14}C-labeled *E. coli* RNA as an added internal standard. Including m^2Ade (which occurred only in *E. coli*), there were nine main electrophoretic fractions. Each was characterized by (i) comparison of mobility with 260-nm-absorbing markers; (ii) comparison of mobility and ^{3}H content with those expected for acid degradation products or ribose-methylated residues; and (iii) elution from parallel preparative electropherograms followed by paper or thin-layer chromatography (in the case of pyrimidine nucleotides, after further degradation to ribosides or bases). Samples of *A. laidlawii* RNA were processed in parallel, and the findings are summarized in Table 3. We note first that there was a small degree of "leakage" of ^{3}H into purine rings, as evidenced by counts in adenine. Subsequent data have been corrected for this, and we shall hereafter refer to "methyl label" as opposed to "^{3}H." The mycoplasmal 4S patterns of Fig. 2 can be interpreted without reference to degradation products of ribose-methylated residues, since there is none (see Table 2). The only ribose-methylated species in the 16S RNA pattern was m^4CmpCp; being acid resistant, this dinucleotide appears as such, and accounts for all the ^{3}H of peak 7 (m_2^6Ap·m_2^6Ap is acid sensitive and yields only m_2^6Ade). The acid degradation products of the 23S ribose-methylated residues more markedly influence interpretations of acid hydrolysate patterns. We believe CmpGp contributed all (in the case of *M. capricolum*) or approximately one-half (*A. laidlawii*) of the counts in peak 6 as Cmp-ribose and that GmpGp and GmpUp contributed all of the counts in peak 9 as methyl-ribose-phosphate. Other less well defined acid degradation products of ribose-methylated residues probably contributed to the small amounts of heterodisperse label running between Cp and Up (Fig. 2C).

We note, finally, that there was no discrete peak corresponding to m^5Up (peak 8) in the *M. capricolum* 4S pattern (Fig. 2A) and a relatively small one in the *S. laidlawii* pattern (not shown). In view of the fact that in some bacteria the methyl group of this residue arises from folate rather than methionine (2, 10, 36), we checked the m^5U content of RNA samples using [^{14}C]uridine as a label. Alkaline hydrolysates were subjected to electrophoresis at pH 3.5, and the ^{14}C corresponding to Up (which would include any m^5Up and ψp present) was fractionated chromatographically (38). Again no m^5Up was found in *M. capricolum* samples (<0.01% of the Up), whereas a modest amount (1.1% of the Up) occurred in *A. laidlawii* 4S RNA. A by-product of these analyses was the determination of 4S RNA ψp contents, which amounted to 6.1 to 6.2% of the Up for both 4S RNA samples. This leads to values of about two ψ per 100 nucleotides, only slightly lower than for *E. coli* 4S RNA (14). Uridine-labeled RNA also provided a check on one of the more surprising of the findings from methyl-labeled samples, the preponderance of m^5U in *A. laidlawii* 23S RNA. In this case 0.93% of the ^{14}C in the Up peak was found to be m^5Up. Taking into account the base ratio and chain length of 23S RNA, this amounts to $0.93 \times 24 \times 2{,}904 \times 10^{-4}$ or 6.5 mol per molecule, a value similar to that based on methyl label (see below).

Degrees of methylation and nucleotide composition. The degrees of methylation of the several mycoplasmal RNA fractions were estimated as follows. (i) We assumed that mycoplasmal 16S RNA resembles 16S RNA from other sources (see Discussion) in containing one m^4Cm and two m_2^6A residues. Thus the methyl label corresponding to these residues was taken to correspond to six methyl groups (two in m^4Cm, two in each m_2^6A) per molecule. This led to average values of 11.5 and 12.5 methyl groups per molecule for *M. capricolum* and *A. laidlawii* 16S RNA, respectively. The chain length of mycoplasma 16S RNA was taken to be 1,510, by comparison (34) with *E. coli* (1,541; reference 6). This yields values of 0.76 and 0.83 methyl group per 100 nucleotides for *M. capricolum* and *A. laidlawii*, respectively. (ii) Comparisons of ra-

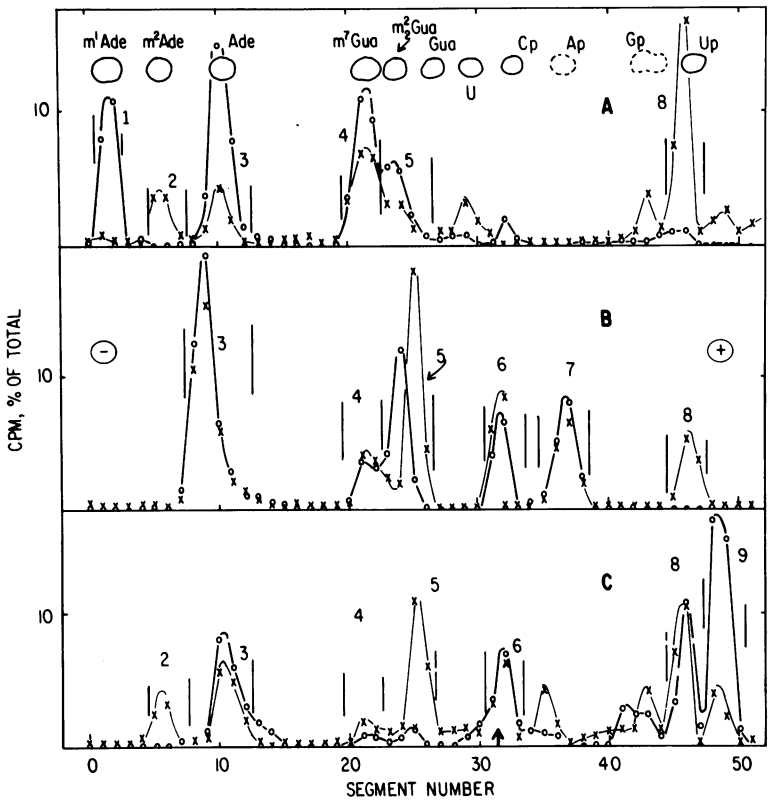

FIG. 2. *Electrophoretic analysis of acid hydrolysates of methyl-labeled M. capricolum and E. coli RNA. Methyl-^3H, ^{32}P-labeled mycoplasma RNA samples were mixed with methyl-^{14}C-labeled E. coli RNA and subjected to hydrolysis with HCl followed by paper electrophoresis as described in the text (3,000 V, 3 h). The arrow at segment 31 indicates the origin. Each sample contained about 1,000 cpm in ^3H, ^{14}C, and (not plotted) ^{32}P; to facilitate comparisons, counts are plotted as percentages of totals recovered. UV-absorbing markers added to samples are indicated by solid-line ovals (see footnote to Table 3 for abbreviations); these ran similarly for all samples. Markers run in parallel are indicated by dashed ovals. No ^3H ran beyond peaks 1 or 9. Strips were cut into 2-cm segments (no. 0 to 5 and 40 to 50) or 1-cm segments (no. 6 to 39) for assaying in a scintillation counter.* (○) *^3H;* (×) *^{14}C. (A) 4S RNA; (B) 16S RNA; (C) 23S RNA.*

tios of ^3H to ^{32}P or ^{14}C (Table 1), corrected for ^3H in adenine rings, yielded values of 0.199 and 0.443 methyl group per 100 for *M. capricolum* and *A. laidlawii* 23S RNA, respectively, taking chain lengths to be 2,904 (5, 34). (iii) We corrected the ^3H:^{14}C and ^3H:^{32}P ratios of 4S RNA (Table 1) for dilution of methyl label by 5S RNA (8% of the 4S peak for *M. capricolum*, 7% for *A. laidlawii*). Comparison with 16S RNA ratios then yielded values of 2.49 and 2.58 methyl groups per 100 nucleotides for *M. capricolum* and *A. laidlawii* tRNA, respectively.

In Table 4 we have put together all our mycoplasma data and added similarly obtained data for *E. coli*. Results are expressed as residues per molecule, or (for tRNA) per "average" molecule. Note that the sum of identified methylated residues does not quite add up to the values for total methyl groups; this reflects the presence of small amounts of unidentified ^3H running between discrete peaks. If this ^3H is excluded and the other results are rounded off to the nearest integers, *E. coli* 16S RNA would contain 13 methyl groups (a value in agreement with that which we obtained by other methods [17]), *M. capricolum* 16S RNA would contain 10, and *A. laidlawii* 16S RNA would contain 13. Comparable values for 23S RNA would be 4 (*M. capricolum*), 12 (*A. laidlawii*), and 13 (*E. coli*).

The mycoplasmal 23S RNA data yielded a number of fractional values. In particular, the four base-methylated residues detected in *M. capricolum* 23S RNA, and three of those detected in *A. laidlawii* 23S RNA, fell in the range of 0.2 to 0.5 residues per molecule. Whether this reflects incomplete methylation at specific 23S

TABLE 3. *Distribution of ^3H from [methyl-^3H]-methionine among electrophoretic fractions of acid hydrolysates of mycoplasma RNA*[a]

Peak	Methylated residue[b]	% Total ^3H recovered from:					
		M. capricolum			*A. laidlawii*		
		4S	16S	23S	4S	16S	23S
1	m^1Ade	19			13		
2	m^2Ade						
3	Ade		2.3	12		3.0	4.6
	m^6Ade	31	2.0	6.0	16		3.6
	m_2^6Ade		36	4.0		31	2.3
4	m^7Gua	29	8.6		29	11	
5	m^1Gua	11		6	19		4
	m^2Gua	1.6			2.6	22	
	m_2^2Gua		20				
6	m^5Cp	2.0	12		2.6		~10
	Cmp-ribose			17			~10
7	m^4CmpCp		16			15	
8	Total	4[c]	2[c]	16[c]	15	13	46
	m^3Up			8		9	
	m^5Up				15	4	46
9	m-ribose-p			36			18

[a] Results are expressed as percent of total ^3H recovered from runs such as those illustrated in Fig. 2. The values represent averages of three experiments, the ranges being within 10% of the average. Strips were divided into regions generally corresponding to discrete peaks of ^3H or ^{14}C, as indicated by the bars (Fig. 2). Identifications were confirmed, and for peaks containing more than one compound, their relative amounts were determined on samples eluted from parallel runs, as follows: Peaks 1, 2, and 3, paper chromatography in system 4 (11, 26) (*n*-butanol–water, 86:14, in NH_3 atmosphere); peaks 4 and 5, paper chromatography in system 1 (16, 26) (isopropanol–concentrated HCl, 68:17.6 in water); peaks 6 and 7, phosphatase digestion followed by thin-layer chromatography in isopropanol–water–concentrated NH_4OH–*n*-butanol, 3:2:2:1 (17); peak 8, paper chromatography in isopropanol–concentrated NH_4OH–water, 70:29:1 (28, 38), or by perchloric acid hydrolysis followed by thin-layer chromatography in system A (17, 35) (*n*-butanol–isobutyric acid–water–concentrated NH_4OH, 30:15:10:1); peak 9 was presumptively identified as described in the text.

[b] Abbreviations of methylated residues are as follows: m^1Ade, 1-methyladenine; m^2Ade, 2-methyladenine; m^6Ade, N^6-methyladenine; m_2^6Ade, N^6,N^6-dimethyladenine; m^7Gua, N^7-methylguanine; m^1Gua, 1-methylguanine; m^2Gua, N^2-methylguanine; m_2^2Gua, N^2,N^2-dimethylguanine; m^5Cp, 5-methylcytidylate; Cmp-ribose, $O^{2'}$-methyl,$O^{3'}$-(ribose 5-phosphate)-cytidine (a presumed acid degradation product of CmpGp; see text); m^4Cm, $N^4,O^{2'}$-dimethylcytidine; m^3Up, 3-methyluridylate; m^5Up, 5-methyluridylate; m-ribose-p, ($O^{2'}$-methyl)ribose 5-phosphate (a presumed degradation product of RmpRp, where R is a purine riboside; see text).

[c] In these cases none, or only a portion of, the counts in peak 8 ran with m^3Up, m^5Up, or their derivatives on subsequent analysis. The remainder was not identified.

RNA loci, or nonspecific or artifactual effects or both, remains to be determined.

DISCUSSION

Transfer RNA. It has been implied that mycoplasma tRNA is substantially less heavily modified and has a lesser variety of modified residues than does typical procaryotic tRNA (see, e.g., references 24, 31, and 32). The present study indicates that this does not apply at least to ψ and the methylated residues. The levels of these latter were similar to those we determined in parallel analyses of *E. coli* tRNA; furthermore, except for the dearth of ribose methylation and (especially with *M. capricolum*) of m^5U, the variety of methylated residues of the mycoplasma tRNA also resembled that of *E. coli* tRNA. Our mycoplasmal data are actually closer to those reported for *Bacillus subtilis* tRNA by Chia et al. (8), in that these authors found relatively large amounts of m^1A and no m^2A (but see also references 1 and 40).

The most unusual features of our tRNA results is the absence of m^5U in *M. capricolum* tRNA. However, even in this regard *M. capricolum* is not unique. Mixed tRNA preparations from several other procaryotes (*Mycobacterium smegmatis* [39], *Thermus thermophilus* [41], *Methanobacter vanniellii* [4], and *Micrococcus luteum* [10]) appear to contain unmodified U or odd variants of m^5U in the "universal" loop IV sequence that usually contains the m^5U residue, and animal mitochondrial tRNA lacks this sequence entirely (38). Clearly, the m^5U that is so plentiful in tRNA from *Enterobacteriaceae* and *Bacillus* is not essential for the function of tRNA.

Three earlier studies have been published on the modification status of mycoplasma tRNA (18, 21, 22). Perhaps most relevant to this work is the paper of Hayashi et al. (21), which reported semiquantitative findings on *M. capricolum* and *A. laidlawii* that are largely in accord with our findings. A notable discrepancy between our results and those of two of the previous studies (21, 22) is the failure to detect m^1G in the latter. However, our results for *E. coli* resemble those from other recent relatively rig-

TABLE 4. *Modified residues of mycoplasma RNA*

Residue	No. of residues per molecule[a]								
	4S tRNA			16S RNA			23S RNA		
	M. capricolum	*A. laidlawii*	*E. coli*[b]	*M. capricolum*	*A. laidlawii*	*E. coli*	*M. capricolum*	*A. laidlawii*	*E. coli*
m^1A	0.38	0.27							
m^2A			0.23						
m^6A	0.62	0.33	0.11	0.02			0.35	0.49	2.43
$m_2{}^6A$				2.22	2.00	1.90	0.23	0.31	
m^7G	0.58	0.60	0.65	1.01	1.42	0.98			0.83
m^1G	0.22	0.39	0.08				0.35	0.54	0.79
m^2G	0.03	0.05			2.83	3.12			2.38
$m_2{}^2G$				1.18					
m^5C	0.04	0.05		1.41		2.18		1.35	1.29
m^4Cm				0.95	0.97	1.09			
m^3U					1.16	0.97	0.47		
m^5U		0.31	0.78		0.53			6.25	3.01
CmpC			0.03						0.68
CmpG							1.23	1.35	
GmpG			0.13				1.23	1.35	0.85
GmpU							1.63	1.82	
UmpG									0.97
Total methyl	1.99	2.06	2.40	11.5	12.5	14.4	5.78	12.9	16.2

[a] For 4S tRNA we refer to an "average" molecule of chain length 80. For 16S RNA we have used a value of 1,510 nucleotides for mycoplasma and 1,541 for *E. coli*, and for 23S RNA, we used a value of 2,904 nucleotides (6, 34). Values were calculated from the data of Tables 1 to 3 as described in the text. Abbreviations follow the same system as in Table 3 except that for simplicity we have used symbols for ribosides, or (in the case of ribose methylation) for nucleotidylribosides, throughout.

[b] *E. coli* tRNA was also found to contain substantial amounts of CmpU and UmpU, bringing its total methylated ribose to 0.34 residues per molecule.

orous studies using differing analytical procedures (e.g., reference 8), and in addition the sequence established for *M. capricolum* $tRNA^{phe}$ (24) indeed contains an m^1G residue. We thus believe that our results are reasonably accurate and can be used as the basis for the following inferences. (i) *Mycoplasma* and *Acholeplasma* tRNA methylation patterns resemble each other more closely than they resemble tRNA patterns reported for other procaryotes. (ii) They resemble *Bacillus* tRNA more closely than that of *E. coli*; this is in agreement with other inferences on the phylogenetic status and evolutionary origin of mycoplasma (e.g., reference 43). (iii) The mycoplasma patterns are clearly procaryotic rather than eucaryotic, although there are some provocative similarities to animal mitochondrial tRNA (9): the overall level of methylation, the absence of T and of ribose methylation, and the prominence of m^1A.

23S RNA. Our most novel findings were those for the 23S RNAs. The methylation pattern for *M. capricolum* 23S RNA superficially resembles that of eucaryotic cytoplasmic rRNA in the relative prominence of ribose- versus base-methylated residues. However, the main difference between *M. capricolum* and *E. coli* 23S RNAs is simply that the former lacks, or is at least markedly deficient in, the base-methylated residues that dominate the *E. coli* pattern. The absolute numbers of ribose-methylated residues in *M. capricolum* 23S RNA, either per molecule (approximately 4) or per 100 nucleotides (0.141), are much closer to the respective values for *E. coli* 23S RNA (3, 0.10) than to those for 28S RNA (e.g., for hamster cells, approximately 70 per molecule, 1.3 per 100 nucleotides [our unpublished data]). Why base-methylated moieties might be more "dispensable" than ribose-methylated ones is an interesting evolutionary question.

Aside from the present work and some earlier studies on *E. coli* and *Staphylococcus aureus* (14, 19, 20), there is little information available on 23S RNA modification patterns. An earlier analysis of *M. hominis* 23S RNA (22) yielded a pattern of base-methylated residues qualitatively resembling one obtained by the same authors in parallel analyses of *E. coli*; however, the authors' results for *E. coli* were aberrant, and in

addition the "post-labeling" technique employed would not have registered ribose-methylated residues. Interestingly, we observed a pattern resembling that of the *M. capricolum* 23S RNA in the large ribosomal subunit ("17S") RNA of hamster mitochondria. This RNA is also very poorly methylated, containing three methyl groups per molecule (approximately 0.18/100 nucleotides) as the ribose-methylated nucleotides GmpG and UmpGmpU (16). We propose that the simple patterns of the large subunit rRNA from both sources represent parallel evolution; that the parasitic life-styles of mitochondria and of mycoplasma have obviated the requirement for whatever subtle contributions to ribosome function the base-methylated residues provide to more independent forms.

Although *Acholeplasma* 23S RNA is as heavily methylated as that of *E. coli*, its methylation pattern is simpler by virtue of the extraordinarily large number (six) of m^5U's. This feature is, to our knowledge, unprecedented. However, again the resemblance to procaryotic RNA is closer than to eucaryotic; *E. coli* 23S RNA contains three m^5U's (present studies), whereas 28S RNAs contain none (our published data; see also reference 7). The relative conservation of ribose-methylated residues in *A. laidlawii* 23S RNA is, again, noteworthy.

16S RNA. There are T_1 oligonucleotide catalogs available on 16S RNA from many bacterial species, and indeed it is largely these data that have suggested the usefulness of rRNA as an evolutionary probe, the general conservation of rRNA sequences within phylogenetic groups, and the special conservation of modified oligomers that frequently spans major groups (see reference 42). A recent review (3) summarizes current knowledge on this latter, highly conserved set of oligonucleotides. All or almost all eubacteria contain m^4Cm (in m^4CmCCG), vicinal m_2^6A's (in one of two relatively conserved oligomers), and m^7G (in CCm^7GCG). Seven of the 11 to 14 methyl groups in our mycoplasma and *E. coli* 16S RNA patterns can be accounted for by this set: hence the similarity among the three methylated nucleotide patterns. These findings are in accord with oligonucleotide catalog data, published by Woese et al. (43) during preparation of this manuscript, on 16S RNA from a group of mycoplasma. The modest differences we found between *A. laidlawii* and *M. capricolum* involve poorly conserved residues, and are in the same direction as implied by the findings of Woese et al. (43). These authors detected an oligonucleotide containing a modified G and one containing a modified U in *A. laidlawii* that were absent from *M. capricolum*, whereas we detected three mG's in *A. laidlawii* versus one in *M. capricolum*, and one or two mU's in *A. laidlawii* versus none in *M. capricolum*. They also detected a modified C-containing oligonucleotide in *M. capricolum* that was absent from *A. laidlawii*; this may account for our *M. capricolum* m^5C (see Table 4). As there are no reliable surveys of the poorly conserved methylated residues of 16S RNA, we can infer from our data only that *Mycoplasma* and *Acholeplasma* 16S RNAs are typically eubacterial (3).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM-14957 from the National Institute of General Medical Sciences. C.-C.H. also received support from Public Health Service Institutional National Research Service Award no. CA-09069 from the National Institutes of Health and MSRP Award no. 27-9699.

We thank Cheryl Winters for her expert technical assistance.

LITERATURE CITED

1. **Arnold, H. H., and H. Kersten.** 1973. The occurrence of ribothymidine, 1-methyladenosine, methylated guanosines and the corresponding methyltransferases in *E. coli* and *B. subtilis*. FEBS Lett. **36**:34–38.
2. **Arnold, H. H., W. Schmidt, and H. Kersten.** 1965. Occurrence and biosynthesis of ribothymidine in tRNA's of *B. subtilis*. FEBS Lett. **52**:62–65.
3. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
4. **Best, A. N.** 1978. Composition and characterization of tRNA from *Methanococcus vannielii*. J. Bacteriol. **133**: 240–250.
5. **Brosius, J., T. J. Dull, and H. F. Noller.** 1980. Complete nucleotide sequence of a 23S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:201–204.
6. **Carbon, P., C. Ehresmann, B. Ehresmann, and J.-P. Ebel.** 1979. The complete nucleotide sequence of the ribosomal 16S RNA from *Escherichia coli*. Eur. J. Biochem. **100**:399–410.
7. **Cecchini, J.-P., and R. Miassod.** 1979. Studies on the methylation of cytoplasmic ribosomal RNA from cultured higher plant cells. Eur. J. Biochem. **98**:203–214.
8. **Chia, S. S., H. P. Morris, K. Randerath, and E. Randerath.** 1976. Base composition studies on mitochondrial 4S RNA from rat liver and morris hepatomas 5123D and 7777. Biochim. Biophys. Acta **425**:49–62.
9. **Davenport, L. W., R. H. Taylor, and D. T. Dubin.** 1976. Comparison of human and hamster mitochondrial transfer RNA: physical properties and methylation status. Biochim. Biophys. Acta **447**:285–293.
10. **Delk, A. S., J. M. Romeo, D. P. Nagle, and J. C. Rabinowitz.** 1976. Biosynthesis of ribothymidine in the transfer RNA of *Streptococcus faecilis* and *Bacillus subtilis*. J. Biol. Chem. **251**:7949–7656.
11. **Dubin, D. T.** 1974. Methylated nucleotide content of mitochondrial ribosomal RNA from hamster cells. J. Mol. Biol. **84**:257–273.
12. **Dubin, D. T., R. J. Baer, L. W. Davenport, R. H. Taylor, and K. D. Timko.** 1979. Post-transcriptional modification of mitochondrial ribosomal RNA from animal cells, p. 389–398. *In* E. Usdin, R. J. Borchardt, and C. R. Creveling (ed.), Transmethylation. Elsevier/North Holland, New York.
13. **Dubin, D. T., and D. A. Friend.** 1972. Comparison of

cytoplasmic and mitochondrial 4S RNA from cultured hamster cells: physical and metabolic properties. J. Mol. Biol. **71**:163–175.

14. **Dubin, D. T., and A. Günalp.** 1967. Minor nucleotide composition of ribosomal precursor, and ribosomal ribonucleic acid in *Escherichia coli*. Biochim. Biophys. Acta **134**:106–123.
15. **Dubin, D. T., and R. H. Taylor.** 1975. The methylation state of poly A-containing messenger RNA from cultured hamster cells. Nucleic Acids Res. **2**:1653–1668.
16. **Dubin, D. T., and R. H. Taylor.** 1978. Modification of mitochondrial ribosomal RNA from hamster cells: the presence of GmG and late-methylated UmGmU in the large subunit (17S) RNA. J. Mol. Biol. **121**:523–540.
17. **Dubin, D. T., R. H. Taylor, and L. W. Davenport.** 1978. Methylation status of 13S ribosomal RNA from hamster mitochondria: the presence of a novel riboside, N^4-methylcytidine, Nucleic Acids Res. **5**:4385–4397.
18. **Feldmann, H., and H. Falter.** 1971. Transfer ribonucleic acid from *Mycoplasma laidlawii A*. Eur. J. Biochem. **18**:573–581.
19. **Fellner, P.** 1974. Structure of the 16S and 23S ribosomal RNA's, p. 169–191. *In* M. Nomura, A. Tissieres, and P. Lengyel (ed.), Ribosomes. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
20. **Freundt, E. A.** 1974. The mycoplasmas, p. 929–952. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
21. **Hayashi, H., H. Fisher, and D. Söll.** 1969. Transfer ribonucleic acid from mycoplasma. Biochemistry **8**: 3680–3686.
22. **Johnson, J. D., and J. Horowitz.** 1971. Characterization of ribosomes and RNAs from *Mycoplasma hominis*. Biochim. Biophys. Acta **247**:262–279.
23. **Khan, M. S. N., M. Salim, and B. E. H. Maden.** 1978. Extensive homologies between the methylated nucleotide sequences in several vertebrate ribosomal ribonucleic acids. Biochem. J. **169**:531–542.
24. **Kimball, M. E., S. Szeto, and D. Söll.** 1974. The nucleotide sequence of phenylalanine tRNA from mycoplasma sp. (Kid). Nucleic Acids Res. **1**:1721–1732.
25. **Klagsbrun, M.** 1973. An evolutionary study of the methylation of transfer and ribosomal ribonucleic acid in prokaryotic and eukaryotic organisms. J. Biol. Chem. **248**:2612–2620.
26. **Lai, C. J., and B. Weisblum.** 1971. Altered methylation of ribosomal RNA in an erythromycin-resistant strain of *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. **68**:856–860.
27. **Littlefield, J. W., and D. B. Dunn.** 1958. The occurrence and distribution of thymine and three methylated adenine bases in ribonucleic acids from several sources. Biochem. J. **70**:642–651.
28. **Markham, R., and J. D. Smith.** 1952. The structure of ribonucleic acids. 1. Cyclic nucleotides produced by ribonuclease and by alkaline hydrolysis. Biochem. J. **52**:552–558.
29. **Nichols, J. L., and B. G. Lane.** 1966. N^4-Methyl-2′-*O*-methyl cytidine and other methyl-substituted nucleoside constituents of *Escherichia coli* ribosomal and soluble RNA. Biochim. Biophys. Acta **119**:649–651.
30. **Randerath, K., and E. Randerath.** 1973. Chemical characterization of unlabeled RNA and RNA derivatives by isotope derivative methods. Methods Cancer Res. **9**: 3–69.
31. **Razin, S.** 1973. Physiology of mycoplasmas. Adv. Microb. Physiol. **10**:1–80.
32. **Razin, S.** 1978. The mycoplasmas. Microbiol. Rev. **42**: 414–470.
33. **Razin, S., H. J. Morowitz, and T. M. Terry.** 1965. Membrane subunits of *Mycoplasma laidlawii* and their assembly to membrane-like structures. Proc. Natl. Acad. Sci. U.S.A. **54**:219–225.
34. **Reff, M. E., E. J. Stanbridge, and E. K. Schneidr.** 1977. Phylogenetic relationships between mycoplasmas and other procaryotes based upon the electrophoretic behavior of their ribosomal ribonucleic acids. Int. J. Syst. Bacteriol. **27**:185–193.
35. **Roggs, H., R. Brambilla, G. Keith, and M. Staehelin.** 1976. An improved method for the separation and quantitation of the modified nucleosides of transfer RNA. Nucleic Acids Res. **3**:285–295.
36. **Schmidt, W., H.-H. Arnold, and H. Kersten.** 1977. Tetrahydrofolate-dependent biosynthesis of ribothymidine in transfer ribonucleic acids of gram-positive bacteria. J. Bacteriol. **129**:15–21.
37. **Sprinzl, M., F. Grueter, A. Spelzhaus, and D. H. Gauss.** 1980. Compilation of tRNA sequences. Nucleic Acids Res. **8**:r1–r22.
38. **Taylor, R. H., F. Varrichio, and D. T. Dubin.** 1980. Hamster mitochondrial transfer RNA lacks T and the "universal" GUUCG sequence. Biochim Biophys. Acta **607**:521–526.
39. **Vani, B. R., T. Ramakrishnan, Y. Taya, S. Noguchi, Z. Yamaizumi, and S. Nishimura.** 1979. Occurrence of 1-methyladenosine and absence of ribothymidine in transfer ribonucleic acid of *Mycobacterium smegmatis*. J. Bacteriol. **137**:1084–1087.
40. **Vold, B.** 1976. Modified nucleosides of *Bacillus subtilis* transfer ribonucleic acids. J. Bacteriol. **127**:258–267.
41. **Watanabe, K., T. Oshima, M. Saneyoshi, and S. Nishimura.** 1974. Replacement of ribothymidine by 5-methyl-2-thiouridine in sequence GTψC in tRNA of an extreme thermophile. FEBS Lett. **43**:59–63.
42. **Woese, C. R., G. E. Fox, L. B. Zablen, T. Uchida, L. Bonen, K. Pechman, B. J. Lewis, and D. Stahl.** 1975. Conservation of primary structure in 16S ribosomal RNA. Nature (London) **253**:83–86.
43. **Woese, C. R., J. Maniloff, and L. B. Zablen.** 1980. Phylogenetic analysis of the mycoplasmas. Proc. Natl. Acad. Sci. U.S.A. **77**:494–498.