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Methylated bases in mycoplasmal DNA

Aharon Razin* and Shmuel Razin**

*Department of Cellular Biochemistry, and **Department of Biomembranes and Ultrastructure, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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ABSTRACT

The DNAs of four *Mycoplasma* and one *Acholeplasma* species were found to contain methylated bases. All of the five species contained 6-methyladenine (m^6Ade), the methylated base characteristic of prokaryotic DNA. The extent of methylation of adenine residues in the mycoplasmal DNA ranged from 0.2% in *Mycoplasma capricolum* to about 2% in *Mycoplasma arginini* and *Mycoplasma hyorhinis* with intermediate methylation values for *Mycoplasma orale* and *Acholeplasma laidlawii* DNAs. About 5.8% of the cytosine residues in *M. hyorhinis* DNA were methylated also. Analysis of cell culture DNA for the presence of m^6Ade as a means for detection of contamination by mycoplasmas, and the phylogenetic implications of the finding of methylated bases in mycoplasmal DNAs are discussed.

INTRODUCTION

Methylated bases occur in the DNA of essentially all organisms examined so far (1). The DNA can be methylated either at adenine residues (N^6-methyladenine, m^6Ade) and at cytosine residues (5-methylcytosine, m^5Cyt). DNA of prokaryotes contains m^6Ade, m^5Cyt, or both, whereas the eukaryotes methylate their DNA exclusively at cytosine residues (2). In a very few exceptions, m^6Ade was reported to exist in DNA isolated from eukaryotic cells (3,4). The biological significance of DNA methylation is still obscure. The only biological process in which DNA methylation is knownat the present to play a major role is the restriction-modification phenomenon in bacteria (2).

The mycoplasmas form a very large and prevalent group of prokaryotes with several unique properties. They are the smallest self-reproducing organisms and, in addition, lack cell walls, a property which distinguishes them from all other prokaryotes. The genome size of many mycoplasmas (included in the genera *Mycoplasma* and *Ureaplasma*) is about 5×10^8 daltons, the smallest genome recorded for any self-reproducing prokaryote. The *Acholeplasma*, *Spiroplasma*, and *Thermoplasma* species have a genome size of about 1×10^9 daltons, about the size of the smallest genome of wall-covered bacteria.

Another interesting property of the mycoplasma DNA is its low guanine plus cytosine (G + C) content, the lowest value being close to 25% in some species (5,6).

Although data revealing the scarcity of modified bases (including m^6Ade and m^5Cyt) in transfer RNAs of some mycoplasmas have been reported (6,7), no information is available on methylated bases in mycoplasmal DNA. The recent development of sensitive and specific techniques for detection and quantitation of the minor bases prompted us to study DNA methylation in mycoplasmas. The test organisms were selected to represent mycoplasmas with different genome size and G + C content. Another consideration was to include species of mycoplasmas known to contaminate cell cultures. Eukaryotic cell cultures are now routinely used in studies on DNA methylation. Cell cultures are most frequently contaminated by mycoplasmas, an infection which in many cases goes undetectable (8). It seemed, therefore, of importance to elucidate the DNA methylation patterns of the species which are the most common contaminants of cell cultures: *M. orale*, *M. arginini*, *M. hyorhinis* and *A. laidlawii* (8,9).

MATERIALS AND METHODS

Organisms and growth conditions. *A. laidlawii* (oral strain), *M. capricolum* (ATCC 27343), *M. arginini* (G-230, N.I.H. collection) and *M. orale* (ATCC 23714) were grown in a modified Edward medium (10) supplemented with 3% horse serum. For growth of the more fastidious *M. hyorhinis* (ATCC 17981) the medium was enriched by the addition of 10% (v/v) fresh yeast extract and by raising the serum content to 10% (v/v). The cultures were incubated aerobically, except for *M. orale* which was incubated under anaerobic conditions. *A. laidlawii*, *M. capricolum* and *M. arginini* were harvested after 20-24 h, and *M. orale* and *M. hyorhinis* after 3 days of incubation at 37°C. The organisms were collected by centrifugation at 12,000 x g for 30 min in the cold, and were washed once in cold 0.25 M NaCl containing 0.1 M EDTA. The pellets of washed cells were kept at -20°C until used for preparation of DNA.

In vivo labeling of methyl groups in DNA. *A. laidlawii* and *M. capricolum* were inoculated into one-liter volumes of a methionine-deficient medium, consisting of the salt solution of Razin and Cohen (11), 0.27% (w/v) of an amino acid mixture devoid of methionine (12), 0.7% Difco yeast extract, 0.5% (w/v) glucose, 2% (v/v) horse serum, and 1000 units/ml of penicillin G. When cultures reached the early logarithmic phase of growth, after 8-10 h of incubation at 37°C, 2.5 mCi of [^{3}H methyl]methionine (40-80 Ci/mmole, Amersham, England) were added per liter of culture, and incubation was continued

for another 16 h. *In vivo* labeling of *M. hyorhinis* DNA was carried out in the modified Edward medium described above. The labeled methionine was added to a one-day old culture, and the organisms were harvested after an additional incubation period of two days at 37°C. Collection of the organisms by centrifugation and their washing were as described above.

DNA preparation and hydrolysis. The washed mycoplasma cells suspended in 0.1 M EDTA were lysed by the addition of sodium sarcosinate (Sigma Co.) to a final concentration of 1% (w/v). The resulting clear lysate was digested by 50 µg/ml of Protease K (Merck Co.) for 2 h at 37°C. RNase A (50 µg/ml) was added and incubation was continued for another 30 min. A solution of 5 M $NaClO_4$ (0.1 vol) followed by one vol of chloroform:isoamyl alcohol (24:1, v/v) were added to the lysate and the resulting mixture was shaken for 30 min. The two phases were separated by centrifugation (2,000 rpm, 10 min) and the aqueous upper phase was collected and supplemented with two vol of ethanol. DNA was spooled on a glass rod, dried under vacuum, and dissolved in 0.5 M NaCl. RNase treatment (50 µg/ml) was repeated at this high salt concentration. One vol of 10% polyethylene glycol was added and the DNA spooled. After repeating this treatment the polyethylene glycol was removed by several subsequent extractions with chloroform. The pure DNA obtained was precipitated by two vol of ethanol, dissolved in H_2O and dried in ampoules. Each ampoule received 200 µl of 88% formic acid, ampoules were sealed and hydrolysis was performed at 180°C for 1 h. Formic acid was removed by evaporation under a stream of N_2. The dried samples were dissolved in 0.1 N HCl and used for high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC).

Base composition analysis by high-performance liquid chromatography (HPLC). The bases obtained by the formic acid hydrolysis of the DNA as described above were separated on a Partisil SCX 10/25 (Whatman Co.) column. The samples were applied via an injection port (Rheodyn 7120) and eluted with 0.02 M NH_4COOH, pH 2.4. The column was run at ambient temperature at a pressure of 550 p.s.i. by use of a Simplex Milton Roy Mini Pump at flow rate ~40 ml/h. The bases were detected by their absorbance at 280 nm measured with a UA5 absorbance monitor (ISCO) with a 19 µl flow cell.

Two-dimensional chromatography of free bases. [^{3}H methyl]labeled DNA prepared and hydrolyzed as described above was supplemented with 2.5 µg of each m^5Cyt and m^6Ade and applied to Eastman-Kodak 6064 Cellulose thin layer 20 x 20 cm sheets. The samples were chromatographed in butanol:H_2O (86:14, v/v) under ammonia atmosphere as first dimension, and isopropanol:HCl:H_2O (170:41:

39, v/v) as the second dimension. The bases were visualized as blue spots under a mineralight UV lamp.

RESULTS

Preliminary analysis of the DNA hydrolysates of the *Mycoplasma* and *Acholeplasma* species by HPLC revealed the presence of m^6Ade in all species, and in addition m^5Cyt in *M. hyorhinis*. In order to verify unambiguously the presence of these minor bases in the DNA preparations, cultures of *M. capricolum*, *M. hyorhinis* and *A. laidlawii* were grown in media supplemented with [^{3}H methyl]methionine. The DNA of the organisms was extracted, hydrolyzed and the resulting free bases were separated by two-dimensional TLC. Table 1 shows that in *M. capricolum* and *A. laidlawii* DNA the only minor base labeled was m^6Ade, whereas in *M. hyorhinis* DNA both m^6Ade and m^5Cyt were labeled.

It is very difficult to quantitate minor bases on the basis of labeling experiments. The radioactivity values in the TLC spots of the minor bases were generally low and, as can be seen in Table 1, some of the radioactivity was also associated with spots other than those of the methylated bases, particularly the thymine spot (12,14). We decided, therefore, to apply HPLC for the quantitative analysis of the minor bases. About 5-10 µg of unlabeled DNA sufficed for each assay. The elution patterns of the major and minor bases of the DNA from the five different mycoplasmas is shown in Fig. 1. The results of the quantitative analysis of the major and minor bases of the DNA of the various mycoplasmas, calculated from the peak areas and the molar absorption coefficients of the bases at 280 nm, are presented in Table 2. The data in Table 2 reveal that the DNA of all five mycoplasmas contains m^6Ade. The extent of methylation varied from 0.2% of all adenine residues in

Source of DNA	[^{3}H methyl] radioactivity incorporated (cts/min)					
	Ade	Cyt	Gua	Thy	m^5Cyt	m^6Ade
M. capricolum	26	0	0	38	0	137
M. hyorhinis	0	6	0	30	193	146
A. laidlawii	0	0	0	0	0	360

Table 1. Distribution of radioactivity in bases derived from DNA labeled *in vivo* with [^{3}H methyl]methionine. Labeled DNA was hydrolyzed and the free bases were chromatographed as described in Materials and Methods. The spots observed by uv were scraped off and counted in toluene scintillation liquid. Ade, adenine; Cyt, cytosine; Gua, guanine; Thy, thymine; m^5Cyt, 5-methylcytosine; m^6Ade, N^6-methyladenine.

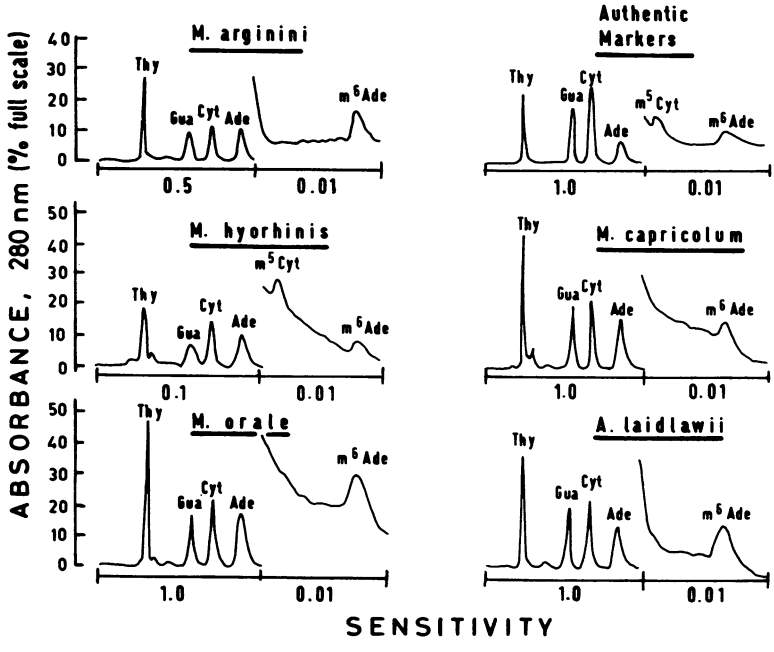

Fig. 1. Separation by HPLC of free bases in DNA hydrolyzates of various species of mycoplasmas. Samples (5-20 µl) were injected and chromatographed as described in Materials and Methods. Analysis time was 20 min for each sample. Note change in the sensitivity scale applied before the elution of the methylated bases. Abbreviations are as in legend to Table 1.

Source of DNA	Molar fraction (%)					
	Thy	Ade	Gua	Cyt	m^5Cyt	m^6Ade
M. capricolum	38.2	36.7	12.6	12.5	N.D.	0.075
M. orale	37.8	36.2	13.0	12.6	N.D.	0.32
M. arginini	35.9	36.1	14.2	13.1	N.D.	0.75
M. hyorhinis	35.4	36.1	12.8	14.2	0.8	0.75
A. laidlawii	33.4	32.7	17.2	16.7	N.D.	0.16

Table 2. Base composition of the DNA from the various mycoplasmas. Bases were separated by HPLC as described in Materials and Methods and recorded in Fig. 2. Peak areas were cut and weighed (21) and the molar fraction of each base was calculated according to the weight of the peak area and the molar absorbance coefficient of the base. Molar fractions given in the table are average values of 3-6 analyses of different DNA preparations. N.D. = not detected. Abbreviations are as in legend to Table 1.

M. capricolum DNA to about 2% in *M. arginini* and *M. hyorhinis* DNA. In addition, about 5.8% of the cytosine residues in *M. hyorhinis* DNA were methylated.

When free bases obtained by formic acid hydrolysis of [^{3}H methyl]-labeled DNA from *A. laidlawii* were separated by HPLC and collected, all the label was found in the m^6Ade fraction (data not shown). This result serves as an additional proof for the identification of the minor peak as mycoplasmal m^6Ade, since it co-chromatographed with authentic m^6Ade.

DISCUSSION

Our results show that the DNA of mycoplasmas contains methylated bases, like the DNA of other organisms. This finding supports the ubiquitous nature of methylated bases, and the notion that they play an important role(s) in the biology of cells (2). The methylated base found in the DNA of all five species tested by us is m^6Ade. In this respect the mycoplasmal DNA resembles DNAs of other prokaryotes and differs from the eukaryotic DNA, which is almost exclusively methylated in cytosine residues (2). The extent of methylation of the adenine residues in the mycoplasmal DNA also resembles that reported for other prokaryotes (15), with the exception of *M. capricolum*, where only 0.2% of the adenine residues are methylated. This value is much lower than that found in other bacteria, and is close to the extremely low methylation value of the bacteriophage $\phi X174$ DNA, where only 0.1% of the cytosine residues are methylated (12).

The different degrees of methylation of adenine noted among the mycoplasmas tested, and the finding of both m^6Ade and m^5Cyt in *M. hyorhinis*, can be taken as another example to illustrate the wide phylogenetic diversity among mycoplasmas (16,17). Analysis of the methylated sequences in the mycoplasma DNA by restriction enzymes is now underway in our laboratory.

As a by-product of our study, the base composition of the DNA of the five species tested was determined directly by the highly sensitive and reproducible HPLC technique. The values obtained (Table 2) are in good agreement with the reported values derived mostly from indirect methods, such as isopycnic centrifugation, or thermal denaturation curves of DNA (5).

Since eukaryotic DNA does not contain m^6Ade, the detection of this methylated base in cell cultures may serve as an indication for contamination by mycoplasmas. The methods employed by us can detect m^6Ade in DNA in which no more than 0.05% of the adenine residues are methylated (see value for *M. capricolum* in Table 2). Obviously, the number of mycoplasmas in contaminated cell cultures varies greatly. Nevertheless, in most cases the number of

viable mycoplasmas in contaminated cell cultures was found to range between 10^5 to 10^8 organisms/ml (9), so that a ratio of 1000 mycoplasmas per eukaryotic cell may be considered feasible and, in fact, was experimentally observed (8). The genome size of mycoplasmas is 5×10^8-1×10^9 daltons (5,6) while that of a diploid mouse or human cell is about $3\text{-}3.6 \times 10^{12}$ daltons (18). Hence the ratio of eukaryotic DNA to mycoplasmal DNA in a cell culture contaminated with approximately 1000 mycoplasmas per cell is between 3:1 to 6:1; i.e. about 15% to 30% of the DNA extracted from the contaminated culture is mycoplasmal DNA. This percentage of mycoplasmal DNA is more than enough for detection of the mycoplasmal m^6Ade.

Analysis of cell culture DNA for m^6Ade may prove particularly useful for the detection of contamination by *M. hyorhinis*. In over half of the cell cultures contaminated by *M. hyorhinis*, the mycoplasmas resist cultivation in cell-free media, making the diagnosis of contamination by this mycoplasma a most difficult task (19). The finding of both m^6Ade and m^5Cyt in *M. hyorhinis* may also have important implications for those working on methylation of eukaryotic DNA in cell cultures. The presence of mycoplasmal m^5Cyt in cell cultures, indistinguishable from the eukaryotic m^5Cyt, may lead to erroneous estimations of the degree of methylation of this base in the eukaryotic cells grown *in vitro*.

The observation of methylated bases in mycoplasmal DNA raises the question whether some of these modifications are associated with restriction-modification systems. It is of great interest and practical importance to search for new restriction enzymes in this group of organisms. One restriction enzyme (ThaI) was already isolated from *Thermoplasma acidophilum* (20). Undoubtedly more restriction enzymes will be found in these small organisms in the future.

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