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# Deoxyribonucleic Acid Methylation in Mycobacteria

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Deoxyribonucleic acid modification in six strains of mycobacteria was investigated. The presence of 5-methylcytosine in the virulent strain *Mycobacterium tuberculosis* $H_{37}R_v$ and its absence in the avirulent strain *M. tuberculosis* $H_{37}R_a$ and other saprophytic, fast-growing mycobacteria appear to be the salient features. However, deoxyribonucleic acid from *M. smegmatis* SN2 lysogenized with the temperate phage I3 showed the presence of 5-methylcytosine. All of the strains had $N^6$-methyladenine.

The guanine plus cytosine (GC) content of DNA in mycobacteria ranges from 66.3% for *M. tuberculosis* $H_{37}R_v$ to 71.4% for *M. phlei* (1), and the molecular weights of DNA range from $3.0 \times 10^9$ for *M. tuberculosis* (wild type) to $5.55 \times 10^9$ for *M. smegmatis* 108 (1). Recently, Norgard and Imaeda (7) reported two types of DNA molecules (differing in GC content) in mycobacteria as determined by careful fractionation. Here, we report a comparative account of DNA modification in various strains of mycobacteria. Although $N^6$-methyladenine ($m^6A$) was present in the DNAs of all species tested, no cytosine modification could be detected in the DNAs of strains other than *M. tuberculosis* $H_{37}R_v$ and *M. smegmatis* SN2 lysogenized with temperate mycobacteriophage I3 (12).

The bacterial strains used in the present study are listed in Table 1. They were grown in synthetic minimal medium (15). Polysaccharide-free DNA was isolated (10), digested with RNase A (50 μg/ml) for 18 to 24 h, and re-extracted. For the preparation of $^{32}P$-labeled DNA, the cells were grown in low-phosphate medium containing 150 μCi of $^{32}P_i$ per ml. The amount of unlabeled phosphate present was 0.01% of that mentioned previously (15). For [8-$^{14}$C]adenine labeling, the medium contained 10 μCi of [8-$^{14}$C]adenine per ml. In [$^{14}$C]methyl-labeling experiments, the growth medium contained 10 μCi L-[*methyl*-$^{14}$C]methionine per ml, 10 mM sodium formate, and 20 μM concentrations each of unlabeled adenine, guanine, cytosine, thymine, and uracil.

DNA preparations were completely hydrolyzed to the base level with 95% formic acid at 175°C for 30 min in nitrogen-filled, sealed ampoules, or with 70% perchloric acid for 1 h at 100°C. The bases were analyzed by high-performance liquid chromatography, using a Bondapack C-18 (Waters Associates) reverse-phase column. Samples (1 to 2 μl) were injected through a UK6 injection port and eluted with 4.0% methanol containing 0.2% acetic acid. Flow rate was adjusted to 0.7 ml/min. The bases were monitored by their absorbance at 254 nm. The elution patterns of DNA bases from the slow-growing pathogenic *M. tuberculosis* $H_{37}R_v$ and its avirulent variant *M. tuberculosis* $H_{37}R_a$ are shown in Fig. 1. In addition to the four standard bases, an extra base (peak 2, Fig 1a) was seen for *M. tuberculosis* $H_{37}R_v$. This peak was identified as 5-methylcytosine ($m^5C$) based on its retention time, UV spectrum, and co-chromatography results with the standard $m^5C$. There was no detectable presence of $m^5C$ in *M. tuberculosis* $H_{37}R_a$ (Fig. 1b). By injecting higher concentrations of DNA digests (at which the peaks corresponding to normal bases exceeded the limit of the recorder several times), we also observed peaks corresponding to $m^6A$ in DNA digests of both virulent and avirulent strains (peak 6, Fig. 1c and d).

Elution patterns from fast-growing mycobacteria, namely, *Mycobacterium* sp. "lacticola" (a laboratory isolate), *M. phlei*, and *M. smegmatis* SN2, are shown in Fig. 2a, b, and c. There was complete absence of $m^5C$, whereas $m^6A$ was present in all of these strains. The quantitation of $m^6A$ in all of the strains was carried out by paper chromatography (4) of [8-$^{14}$C]adenine-labeled DNA digests. Since $m^5C$ has been found in the DNA of mycobacteriophage I3 (S. S. Karnik and K. P. Gopinathan, unpublished data), the presence of $m^5C$ was tested in *M. smegmatis* SN2 cells lysogenized with phage I3. Figure 2d shows that an *M. smegmatis* SN2 lysogen harboring the phage I3 genome contained $m^5C$. To confirm that there was absolutely no $m^5C$ in the nonlysogenized *M. smegmatis* SN2 DNA and that it was entirely because of the lysogenization by phage I3, high-voltage

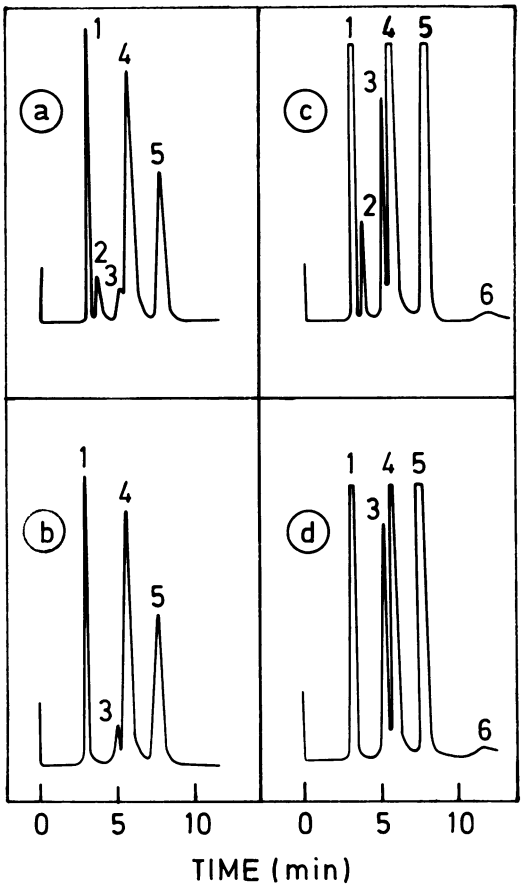


FIG. 1. *Elution pattern of DNA bases in high-performance liquid chromatography. a and c, M. tuberculosis $H_{37}R_v$; b and d, M. tuberculosis $H_{37}R_a$. Peaks 1, 3, 4, and 5 represent cytosine, guanine, adenine, and thymine, respectively. Peak 2 represents $m^5C$, and peak 6 represents $m^6A$. In a and b, 50-μg samples of DNA digests were injected. In c and d, three times larger samples were injected.*

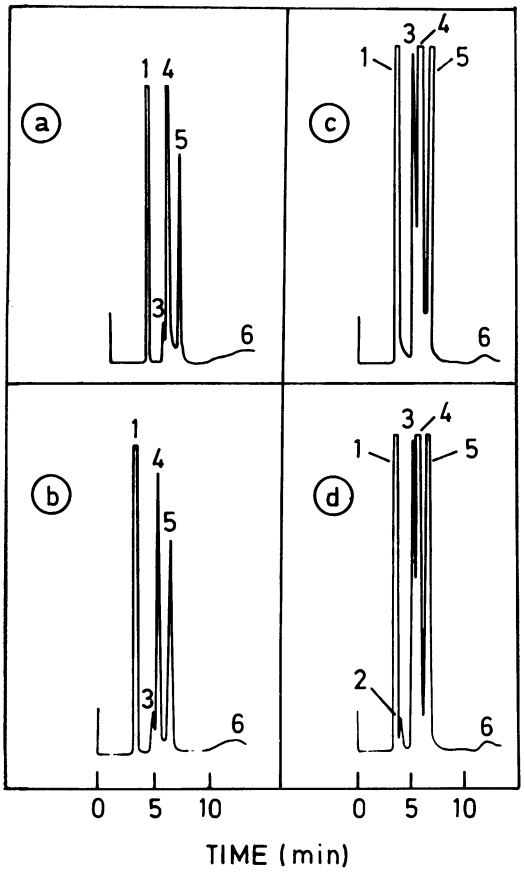


FIG. 2. *DNA base composition of saprophytic mycobacteria. The elution patterns of DNA bases on high-performance liquid chromatography are shown. a, M. phlei; b, M. lacticola; c, M. smegmatis; d, M. smegmatis lysogenized with phage I3. Numbers of different peaks correspond to those in Fig. 1. All of the experiments were repeated with severalfold higher concentration of DNA digests, but the qualitative patterns remained same.*

paper electrophoresis (data not shown) and two-dimensional thin-layer chromatography of [$^{32}$P]DNA digests of very high specific activity (70,000 cpm/μg of DNA) were carried out. The [$^{32}$P]DNA was digested by the combined actions of DNase I and snake venom phosphodiesterase (11). The solvent systems used for the two-dimensional thin-layer chromatography were those which resolve $m^5C$ very distinctly from the normal four nucleotides (8). As shown in Fig. 3, no $m^5C$ could be detected in the DNase I and snake venom phosphodiesterase digest of [$^{32}$P]DNA from *M. smegmatis* SN2. The $^{32}$P-labeling study also revealed that the GC content of *M. smegmatis* SN2 DNA was 67%, which is less than that reported for other strains of *M. smegmatis* (1).

From the comparative study of DNA methylation in the six different strains of mycobacteria used here, the following features have emerged.

(i) The virulent strain of *M. tuberculosis* contained $m^5C$, the absence of which was conspicuous in the avirulent strain, *M. tuberculosis* $H_{37}R_a$. Although $m^5C$ was first discovered in the DNA of *M. tuberculosis* (5), Wyatt (14) could not confirm its presence. However, no mention has been made as to whether the *M. tuberculosis* strain used was virulent. Also, the techniques used here to detect the modified bases are more sensitive. No comparative studies of modified bases have been done on the DNAs of virulent and avirulent strains. The question is whether the expression of $m^5C$ is connected to the virulence of *M. tuberculosis* in any way. Most of the earlier attempts to show any metabolic differ-

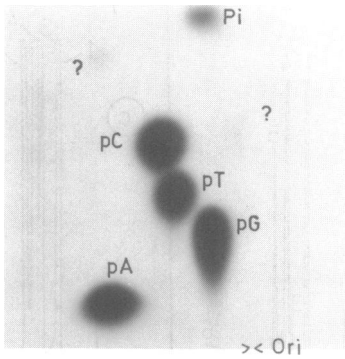


FIG. 3. *Nucleotide analysis by thin-layer chromatography. Autoradiogram of $^{32}P$-labeled nucleotides from DNase I and snake venom phosphodiesterase digests of M. smegmatis DNA, chromatographed on a thin-layer cellulose plate (10 by 10 cm). Chromatography in the first dimension (right to left) was in isobutyric acid–$H_2O$–$NH_4OH$ (66:20:1 [vol/vol], and chromatography in the second dimension (bottom to top) was in isopropanol–1 M sodium acetate–saturated $(NH_4)_2SO_4$ (2:18:80 [vol/vol]). About 400,000 cpm was spotted. Autoradiograms were developed after 48 h of exposure to X-ray films. No spot corresponding to $m^5C$ was seen. Ori, origin.*

TABLE 1. *Content of methylated bases in the DNAs of different mycobacteria*[a]

| Strain | mol % $m^6A$[b] | mol % $m^5C$[c] | No. of determinations |
|---|---|---|---|
| *M. tuberculosis* $H_{37}R_a$ | 0.5 | <0.01 | 6 |
| *M. tuberculosis* $H_{37}R_v$ | 0.45 | 0.46 | 6 |
| *M. smegmatis* SN2 | 3.0 | <0.01 | 12 |
| *M. smegmatis* SN2 lysogenized with phage I3 | 3.0 | 0.04 | 5 |
| *Mycobacterium* sp. "lacticola" | 0.35 | <0.01 | 5 |
| *M. phlei* | 0.55 | <0.01 | 3 |

[a] The *M. tuberculosis* strains were grown as stationary cultures in synthetic minimal medium (15) for 14 days at 37°C. The other strains were grown until late-log phase as shake cultures in medium containing 0.2% Tween 80. The values shown are the means of the analysis of several independent DNA preparations as indicated for each strain. The variations were within ±10% of the mean.

[b] Based on [$^{14}C$]adenine and L-[*methyl*-$^{14}C$]methionine labeling and separation of bases by paper chromatography.

[c] Based on high-performance liquid chromatography.

ences between the virulent and avirulent strains have only shown some quantitative variations in the metabolic pathways (see Ramakrishnan et al. [9]). So far, no definite biochemical difference to which the virulence of *M. tuberculosis* can be totally attributed has been documented. The possibility that the virulence of *M. tuberculosis* is a consequence of its lysogenic state exists by analogy to the virulence of other related bacteria (2, 6). If so, for final proof, one should be able to "cure" the virulent strain and show the associated loss of virulence. Experiments are under way to look for the presence of a plasmid in *M. tuberculosis* $H_{37}R_v$ which is absent in the avirulent strain.

(ii) The relatively fast-growing mycobacteria showed complete lack of $m^5C$. All of these strains contained $m^6A$. Vanyushin et al. earlier reported the presence of 0.3 mol% each of $m^5C$ and $m^6A$ in *M. luteum* (13). The $m^6A$ content of *M. smegmatis* SN2 DNA observed during the present investigation was considerably higher (3 mol%) than that reported previously for mycobacteria, specifically, for *M. tuberculosis* bovine strain (3).

(iii) The GC content of *M. smegmatis* SN2 DNA reported here (67%), as monitored by direct analysis of $^{32}P$-labeled nucleotides, was lower than the GC content reported for other strains of *M. smegmatis* (1).

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## ADDENDUM

After acceptance of this manuscript, we detected a plasmid with a molecular weight of about $1.2 \times 10^6$ in *M. tuberculosis* $H_{37}R_v$ by using transmission electron microscopy. No such plasmid could be detected in *M. tuberculosis* $H_{37}R_a$. A plasmid was detected previously in *M. tuberculosis* $H_{37}R_v$, using other techniques (J. T. Crawford and J. H. Bates, Infect. Immun. **24**:979–981, 1979). It will be of interest to know the role of this plasmid in the virulence of *M. tuberculosis*.

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