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Systematic Difference in the Methylation of Ribosomal Ribonucleic Acid from Gram-Positive and Gram-Negative Bacteria

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A survey of gram-positive and gram-negative organisms was performed to compare the distribution of N^6-methylated adenine. It was found that (i) all the gram-positive strains tested, *Staphylococcus aureus*, *Sarcina lutea*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus megaterium*, contain neither N^6-monomethyl adenine (m^6A) nor N^6-dimethyladenine (m_2^6A) in 23*S* ribosomal ribonucleic acid (rRNA). In the case of *S. aureus* and *Streptococcus pyogenes*, strains which are clinically resistant to erythromycin contain m_2^6A. (ii) The gram-negative strains *Salmonella typhimurium*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* all contain m^6A but not m_2^6A in 23*S* rRNA. These observations suggest the existence of at least one systematic structural difference between the ribosomes of the two classes of bacteria. Because of the demonstrated relationship between N^6-dimethylation of adenine in 23*S* rRNA and clinical resistance to macrolide, lincosamide, and streptogramin B-type antibiotics in staphylococci and streptococci, the observed systematic differences found in rRNA methylation combined with greater cellular permeability may be related to the relatively greater efficacy of macrolide, lincosamide, and streptogramin B-type antibiotics in treating infections caused by gram-positive organisms.

A casual relation between erythromycin resistance and the presence of N^6, N^6-dimethyladenine (m_2^6A) in 23*S* ribosomal ribonucleic acid (rRNA) of *Staphylococcus aureus* has been shown by ribosome reconstitution studies (8). Inducible and constitutive modes of expression of erythromycin resistance associated with adenine methylation have been described (7). In inducible strains, subinhibitory concentrations of erythromycin (10^{-8} to 10^{-7} M) induce both 23*S* rRNA adenine methylation and a characteristic pattern of resistance to three different classes of antibiotics which inhibit 50*S* subunit function: the macrolides, lincosamides, and streptogramin-B type (MLS) antibiotics (11). In the constitutively resistant strains both the resistant phenotype and methylation are present continuously and do not require the presence of erythromycin to be expressed. A tetranucleotide AAAG, shown previously to contain all the m_2^6A present in 23*S* rRNA, was isolated from both the induced and constitutively resistant cells (6).

Additional studies (C. J. Lai, Ph.D. thesis, Univ. of Wisconsin, Madison, 1972) revealed the absence of any form of N^6-methylated adenine in the 23*S* rRNA of *Streptococcus pyogenes* and the presence of this base in a (nonisogenic) erythromycin-resistant strain of clinical origin. Moreover, the erythromycin-resistant strain also displayed the MLS phenotype. The MLS phenotype in streptococcal isolates of clinical origin has also been reported by other investigators (3-5, 9).

In view of the well-known suceptibility of gram-positive organisms to erythromycin, it occurred to us that other gram-positive organisms might also lack N^6-methylated adenine. We therefore undertook a survey in which 10 different bacterial strains (five gram positive and five gram negative) were tested for the presence of N^6-methylated adenine in 23*S* rRNA.

Cells were grown in a medium supplemented with [*methyl*-^{14}C]methionine and 23*S* rRNA was prepared. Purines obtained from 23*S* rRNA by treatment with HCl were separated by column chromatography and methylated adenines were further analyzed by paper chromatography. The results are shown in Fig. 1. We note the absence of N^6-methylated adenine from all the gram-positive strains tested

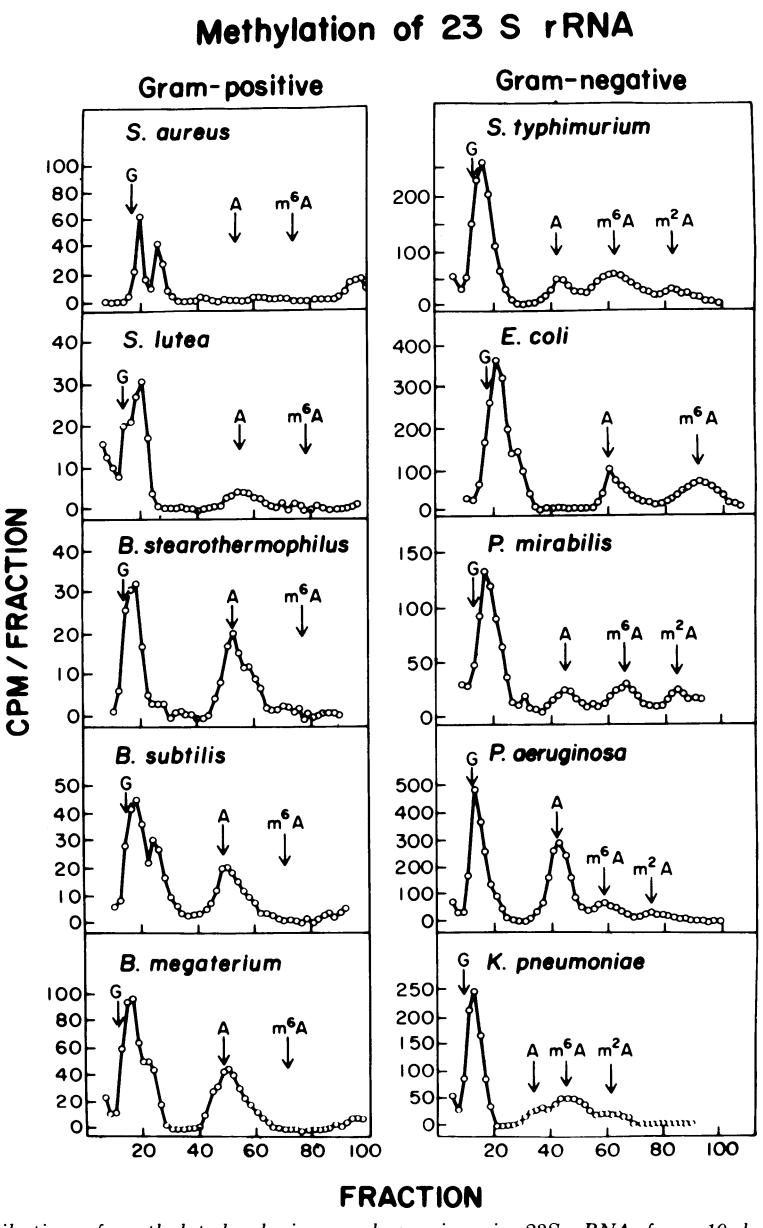

FIG. 1. *Distribution of methylated adenines and guanines in 23S rRNA from 10 bacterial strains. Methyl-^{3}H-labeled 23S rRNA was prepared from each bacterial strain after growth in [methyl-^{3}H]methionine. Purified 23S rRNA was degraded to free purines by treatment with 1 N HCl for 45 min in a boiling water bath. The hydrolysate was analyzed by chromatography on Dowex 50 × 12. For each fraction, the optical density at 260 nm was determined to locate guanine, adenine, m^{6}A, and m^{2}A added as markers. In this system, m^{6}A and m$_2$^{6}A have the same mobility. A sample from each fraction was dried in a vial and radioactivity was determined in a liquid scintillation counter. S. aureus 1206$^+$ and its properties have been described previously (11). The other bacterial strains used in this study and their respective sources are: Sarcina lutea and Bacillus stearothermophilus 1503, E. McCoy; Bacillus subtilis 23, H. Kubinski; Bacillus megaterium B6, H. Halvorson; Escherichia coli 3000, Salmonella typhimurium, Proteus mirabilis Pm 8, and Pseudomonas aeruginosa Ps 49, J. Davies; Klebsiella pneumoniae MSA 1, W. Brill. Bacterial cells were grown in enriched broth medium which contained (in grams per liter): yeast extract, 5; bactopeptone, 5; glucose, 2; and K_2HPO_4. 1. Cells were labeled with [methyl-^{14}C]methionine (0.2 μCi/ml, 50 μCi/μmol) for 1 or 2 h (depending on the rate of growth) in the presence of added adenine and guanine, 10^{-4} M each.*

whereas N^6-methylated adenine was found to be present in all the gram-negative strains. (Further analysis of the methylated adenine by paper chromatography shows it to be indistinguishable from N^6-monomethyl adenine [m^6A].) In several of the rRNA samples tested, significant levels of labeling of adenine itself appears to be present. This cannot account for the appearance of label in the methylated adenine fraction since there are approximately 1,000 adenine residues in 23S rRNA, whereas m_2^6A residues number 2 or 3 at most. Thus, the specific activity of m_2^6A is several-hundredfold higher than the specific activity of adenine.

Our tentative extrapolation from this limited survey is that the absence of N^6-methylated adenine in 23S rRNA may be a general property of other (if not all) gram-positive bacteria, whereas the presence of N^6-methylated adenine in 23S rRNA may be a property of other (if not all) gram-negative bacteria. In addition to the wild-type gram-positive and gram-negative bacteria are the clinical isolates of certain gram-positive bacteria such as *S. aureus* and *S. pyogenes* which, by virtue of acquiring a specific plasmid or other form of extrachromosomal genetic information, also acquire the enzymatic capacity for N^6-dimethylation of adenine and concomitant resistance to MLS antibiotics.

A preliminary investigation of *methyl*-labeled oligoribonucleotides obtained from a pancreatic ribonuclease digest of 23S rRNA has indicated that the N^6-dimethylated adenine in *S. aureus* 23S rRNA occurs as part of a heptanucleotide sequence and that the sequence AAAG previously described is embedded within this heptanucleotide.

The feasibility of reconstituting the 23S rRNA from *S. aureus* with the ribosomal proteins of *Bacillus stearothermophilus* to form particles active in protein synthesis (8) implies that at least the specificity and therefore, presumably, nucleotide sequences of the ribosomal-protein-binding sites in these two diverse gram-positive organisms have been conserved to a significant degree. It therefore should not be surprising to find that other features of rRNA structure and function have also been conserved in the staphylococci and streptococci. The uniform clinical sensitivity of all gram-positive organisms to MLS antibiotics, particularly erythromycin, is well known and provides further indirect support for the notion that 23S rRNA methylation plays a direct role in determining the MLS phenotype at least by virtue of its absence in clinically sensitive isolates.

An additional feature of 23S rRNA adenine methylation to emerge from these studies is the presence of m^6A in all the gram-negative organisms tested, suggesting that this may be a general property of gram-negative organisms. It should be stressed that our analysis revealed only the presence of m^6A and failed to disclose the presence of any m_2^6A. The clinical unresponsiveness of gram-negative organisms to MLS antibiotics suggests a possible functional significance of this finding, namely that m^6A residues in 23S rRNA of gram-negative bacteria contribute, in part, to this phenotype. From studies of Taubeneck (10), it appears that cellular impermeability might also play a role. In these studies, the minimal inhibitory concentration of erythromycin for intact cells of *Proteus mirabilis* was 1,000 μg/ml, whereas for a line of stable L-forms the minimal inhibitory concentration was 1 μg/ml. However, marked differences between the sensitivities of 50S subunits from gram-positive and gram-negative bacteria can be readily demonstrated if one uses lincomycin instead of erythromycin. Thus, in our previous studies (1), 10^{-6} M lincomycin produced 50% inhibition of polyphenylalanine synthesis in a cell-free *B. stearothermophilus* system, whereas a cell-free *Escherichia coli* system required in excess of 10^{-4} M. In addition, lincomycin formed much more stable complexes with *B. stearothermophilus* 50S subunits than with *E. coli* 50S subunits (2).

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