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Secondary Structure and Phylogeny of *Staphylococcus* and *Micrococcus* 5S rRNAs

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Nucleotide sequences of 5S rRNAs from four bacteria, *Staphylococcus aureus* Smith (diffuse), *Staphylococcus epidermidis* ATCC 14990, *Micrococcus luteus* ATCC 9341 and *Micrococcus luteus* ATCC 4698, were determined. The secondary structural models of *S. aureus* and *S. epidermidis* sequences showed characteristics of the gram-positive bacterial 5S rRNA (116-N type [H. Hori and S. Osawa, Proc. Natl. Acad. Sci. U.S.A. 76:381–385, 1979]). Those of *M. luteus* ATCC 9341 and *M. luteus* ATCC 4698 together with that of *Streptomyces griseus* (A. Simoncsits, Nucleic Acids Res. 8:4111–4124, 1980) showed intermediary characteristics between the gram-positive and gram-negative (120-N type [H. Hori and S. Osawa, 1979]) 5S rRNAs. This and previous studies revealed that there exist at least three major groups of eubacteria having distinct 5S rRNA and belonging to different stems in the 5S rRNA phylogenic tree.

We have previously classified eubacterial 5S rRNAs into two major groups according to their secondary structures, i.e., the eubacterial 120-N type basically having 120 nucleotides and the eubacterial 116-N type basically having 116 nucleotides (5, 11). The classification of eubacteria by the 5S rRNA sequences is generally consistent with that by Gram staining; bacteria having the 120-N type 5S rRNA belong to the gram-negative group (e.g., *Pseudomonas*, *Photobacterium*, *Proteus*, *Yersinia*, *Serratia*, *Aerobacter*, *Escherichia*, *Salmonella*, etc.), and those having the 116-N type 5S rRNA belong to the gram-positive group (e.g., *Bacillus*, *Lactobacillus*, *Streptococcus*, *Mycoplasma*, etc.). We have more recently found that 5S rRNA from *Micrococcus lysodeikticus* (= *M. luteus* [1]) is of the third type of secondary structure that is intermediate between the 120-N type and the 116-N type (6).

To obtain more information on the third type, we have determined the nucleotide sequences of 5S rRNA with the following three bacteria that could have the intermediary type of 5S rRNA: *M. luteus* ATCC 9341 (= *Sarcina lutea*), *Staphylococcus aureus*, and *Staphylococcus epidermidis*, all belonging to the *Micrococcaceae* family (1). At the same time, we have determined the sequence of *M. lysodeikticus* (= *M. luteus* ATCC 4698) to check the reported sequence (6). By comparing these and the known eubacterial 5S rRNA sequences, we have constructed a phylogenic tree to locate the intermediary type of bacteria in the tree.

MATERIALS AND METHODS

Bacteria, medium, and growth conditions. *S. aureus* Smith (diffuse), *S. epidermidis* ATCC 14990, *M. luteus* ATCC 9341 (= *Sarcina lutea*) and *M. luteus* ATCC 4698 (= *M. lysodeikticus*) were cultured in 200 ml of brain heart infusion broth at 37°C with shaking, harvested at the late log phase of growth, washed twice with 0.9% NaCl solution, and then kept at −20°C until use.

Enzymes and biochemicals. Polynucleotide kinase (2.7.1.78) and alkaline phosphatase (3.1.3.1) were obtained from Takara Biomedicals. RNase T_1 (3.1.27.3), RNase T_2 (3.1.27.1) and RNase U_2 (3.1.27.4) were purchased from Sankyo Pharm. Co. T_4 RNA ligase (6.5.1.3), RNase phy M (3.1.27.X), and RNase *Bacillus cereus* (3.1.27.X) were obtained from PL Biochemicals. [γ-^{32}P]ATP (3,000 Ci/mmol) and [5′-^{32}P]pCp (3,000 Ci/mmol) were purchased from Amersham International.

Preparation of 5S rRNA. The 5S rRNA was isolated directly from the cells by the phenol method and purified by DE52 (DEAE) chromatography and polyacrylamide gel electrophoresis as described previously (9). The cells (10 g) were homogenized with quartz sand and mixed with 15 ml of a buffer containing 10 mM Tris-hydrochloride (pH 7.7), 1 mM $MgCl_2$, and 1% sodium dodecyl sulfate; were shaken with an equal volume of 90% phenol for 30 min; and were centrifuged. Ethanol (3 volumes) was then added to the aqueous phase, and the precipitate was dissolved in 50 ml of 20 mM Tris-hydrochloride (pH 7.7)–10 mM $MgCl_2$ containing 0.2 M NaCl. DE52 (2 g) was added and stirred for 1 h at 4°C. The mixture was then filtered and washed with the same solution through a Büchner funnel to remove unabsorbed materials. The cake of DE52 was then washed with 50 ml of 20 mM Tris-hydrochloride (pH 7.7)–10 mM $MgCl_2$ containing 1 M NaCl to elute small RNA species including 5S rRNA. The crude RNA was precipitated with ethanol, dissolved in 15 ml of 10 mM Tris-hydrochloride (pH 7.7)–10 mM $MgCl_2$, and treated with 50 μg of DNase per ml at 37°C for 20 min. The RNA preparation was subjected to electrophoresis on a 12% polyacrylamide gel containing 7 M urea, 0.1 M Tris-borate (pH 8.3), and 1 mM EDTA. The 5S rRNA band was cut out. The RNA was eluted with 0.5 M ammonium acetate–0.1 mM EDTA–0.1% sodium dodecyl sulfate at 37°C and precipitated with ethanol.

5′- and 3′-end analyses of 5S rRNA. The ^{32}P labeling of the 5′ terminus was done with [γ-^{32}P]ATP and polynucleotide kinase after a pretreatment of the 5S rRNA with alkaline phosphatase (3). The ^{32}P labeling of the 3′ terminus was performed with [5′-^{32}P]pCp with RNA ligase (12). The 5′- or 3′-end-labeled 5S rRNAs were digested completely by nuclease P_1 or RNase T_2, chromatographed on a cellulose thin-layer chromatography plate, and autoradiographed (8).

Sequencing of 5S rRNA. The sequencing of 5S rRNA was performed by the enzymatic method of Donis-Keller (3) with 5S [5′-^{32}P]rRNA and by the chemical method of Peattie (12) with 5S [3′-^{32}P]rRNA. For the sequencing of the RNA from the 5′ terminus, 5S [5′-^{32}P]rRNA was digested enzymatically

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in the presence of 5 μg of carrier tRNA by RNase T_1 (0.005 Sankyo unit) in 10 μl of 20 mM sodium citrate–1 mM EDTA–7 M urea buffer (pH 5.0), by RNase U_2 (0.001 Sankyo unit) in 10 μl of 20 mM sodium citrate–1 mM EDTA–7 M urea buffer (pH 3.5), by RNase phy M (0.5 PL unit) in 10 μl of 20 mM sodium citrate–1 mM EDTA–7 M urea buffer (pH 5.0), or by RNase *B. cereus* (0.5 PL unit) in 10 μl of 20 mM sodium citrate–1 mM EDTA buffer (pH 5.0), all at 55°C for 30 min. To analyze the RNA sequence from the 3′ terminus, we modified 5S [3′-^{32}P]rRNA by base-specific chemical reactions, followed by the scission of the RNA strand at the site of the modified base by aniline reaction at pH 4.5. The enzymatically digested or chemically scissed RNA was electrophoresed by using 8, 12, or 20% polyacrylamide gel containing 7 M urea, 50 mM Tris-borate (pH 8.3), and 1 mM EDTA and was autoradiographed.

Secondary structures and sequence alignment of 5S rRNA. The secondary structures of 5S rRNAs were constructed according to the basic method of Tinoco et al. (16) as adopted by us (4, 5). For the alignment of the 5S rRNA sequences, all of the 5S rRNA secondary structures were juxtaposed to obtain the alignment for the helical regions. The best-match alignments of non-base-paired regions were then obtained with minimum gap insertions.

Construction of the phylogenic tree. The evolutionary distance, *Knuc*, and standard error of *Knuc*, σ_k, between two sequences compared were calculated by the following equations from the description by Kimura (7). *Knuc* corresponds to the number of base substitutions per nucleotide site that have occurred in the course of evolution extending over T years.

$$Knuc = -(1/2)\log_e[(1 - 2P - Q)(1 - 2Q)^{1/2}] \quad (1)$$

where P and Q are fractions of nucleotide sites showing transition- and transversion-type differences, respectively.

$$\sigma_k^2 = [(a^2P + b^2Q) - (aP + bQ)^2]/n \quad (2)$$

where n is the number of nucleotide sites to be compared, and a and b are calculated by the following equations:

$$a = 1/(1 - 2P - Q)$$

$$b = [1/(1 - 2P - Q) + 1/(1 - 2Q)]/2$$

One gap (represented by a broken line in alignment in Fig. 2) versus one nucleotide was counted as equal to one transversion-type substitution.

A phylogenic tree was constructed by the weighted pair-group (using arithmetic averages) average-clustering method (4, 15). The *Knuc* values obtained by the method above were used for determination of the branching order and the relative evolutionary distance in the construction of the phylogenic tree (4).

RESULTS

5′- and 3′-end nucleotides of 5S rRNAs. The 5′ ends of *S. aureus* Smith (diffuse), *S. epidermidis* ATCC 14990, *M. luteus* ATCC 9341 (= *Sarcina lutea*), and *M. luteus* ATCC 4698 (= *M. lysodeikticus*) were U, U, U, and G, respectively. The 3′ ends of these four strains were C, C, U, and A, respectively.

Nucleotide sequences and secondary structures of 5S rRNAs. Sequences of ca. 110 nucleotides from the 3′ terminus of all 5S rRNAs were determined by the chemical degradation of [3′-^{32}P]RNA followed by electrophoresis. For the determination of the sequences from the 5′ terminus, the partial digests of the [5′-^{32}P]RNA by RNase T_1, RNase U_2, RNase phy M, and RNase *B. cereus* were analyzed by electrophoresis. This method usually allowed us to sequence 90 to 100 residues from the 5′ terminus. Thus, residues 1 to 10 from the 5′ terminus, which could not be identified by the chemical method, were effectively established in this way. The primary sequences of *S. aureus*, *S. epidermidis*, and *M. luteus* ATCC 9341 and 4698 so obtained are shown in Fig. 1, together with the sequences of *Escherichia coli* (2) and

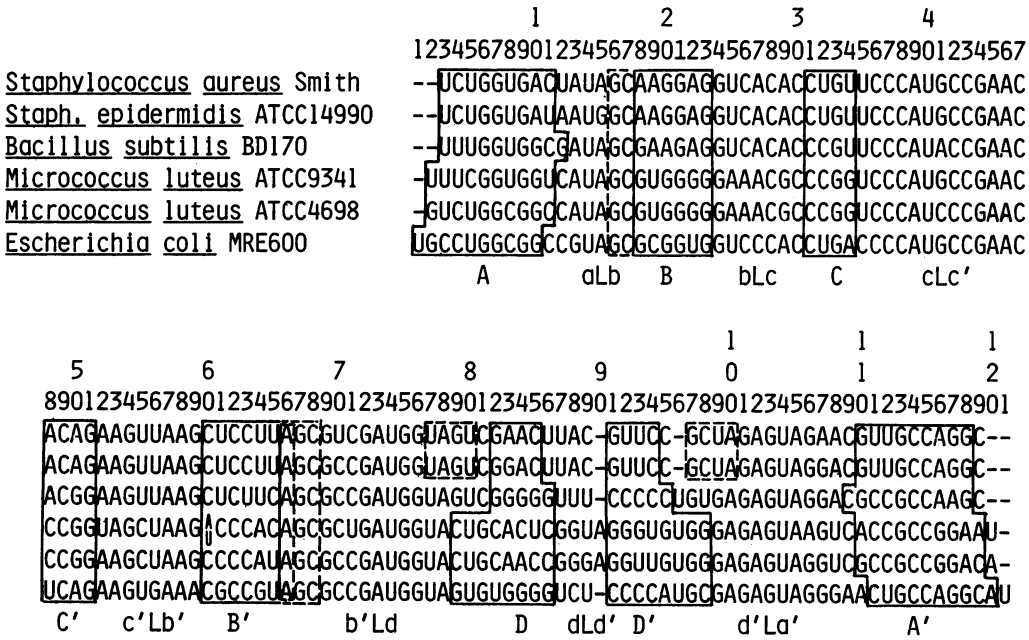

FIG. 1. Sequence alignment of 5S rRNAs from two *Staphylococcus* species, two *Micrococcus* species, *E. coli* (2), and *B. subtilis* (10). The squared-off sequences correspond to the base-paired regions in secondary structures. Broken lines represent the potential base pairs. Those near the B:B′ helix are located according to the findings of Peattie et al. (13). Symbols A, A′, B, B′, etc. in the lowest line designate base-paired regions. Symbols aLb, bLc, etc. designate loop regions (see reference 5).

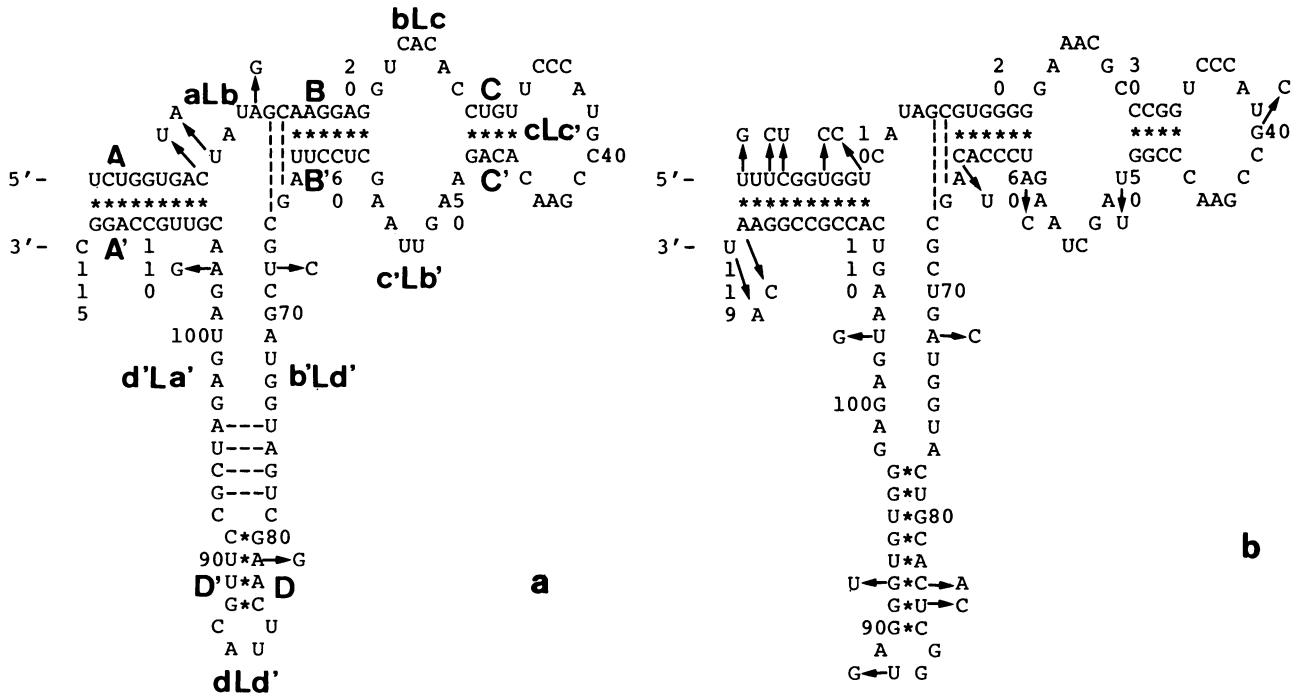

FIG. 2. Secondary structural models of 5S rRNAs. (a) *S. aureus* Smith. Substitutions required to obtain the *S. epidermidis* sequence are indicated by arrows. (b) *M. luteus* ATCC 9341. Substitutions required to obtain the *M. luteus* ATCC 4698 are indicated by arrows. Broken lines indicate potential base pairs. Those near the B:B' helix are located according to the findings of Peattie et al. (13).

Bacillus subtilis (10) for comparison. Both the *S. aureus* and *S. epidermidis* 5S rRNAs are 115 nucleotides long, whereas the two *Micrococcus* 5S rRNAs are composed of 119 nucleotides. The sequence of *M. luteus* ATCC 9341 shows A-U heterogeneity at position 60. The reported sequence of the ATCC 4698 5S rRNA contained some errors. The secondary structures of these RNAs are shown in Fig. 2.

Phylogenic tree. The positions of the two *Staphylococcus* species and the two *Micrococcus* strains together with those of other representative bacteria are shown in a phylogenic tree (Fig. 3) that was constructed from the *Knuc* values (only important ones are shown in Table 1). The two *Micrococcus* species are situated between the typical gram-positive and gram-negative bacteria. An earlier examination of the litera-

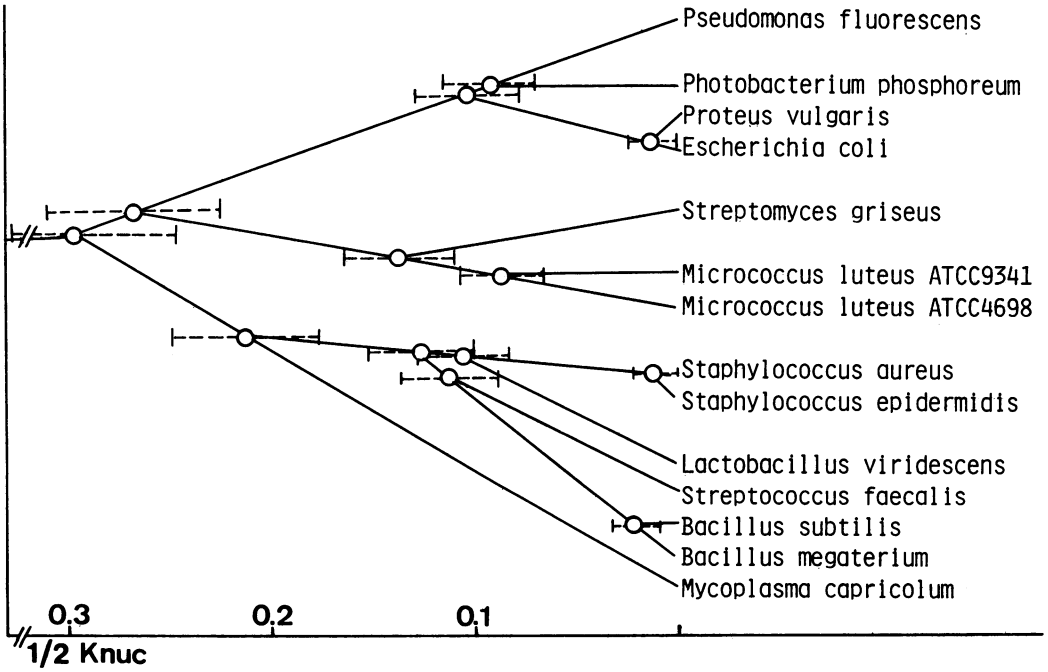

FIG. 3. Phylogenic tree of 5S rRNAs from representative eubacteria. 1/2 *Knuc*, evolutionary distance as calculated from equation 1; |- - -O- - -|, range of standard error as calculated from equation 2.

ture on *Streptomyces* species 5S rRNA sequences (14) reveals that this bacterium also belongs to the same stem as *Micrococcus*. The two *Staphylococcus* species studied here clearly belong to the gram-positive bacterial stem.

DISCUSSION

In a previous paper (5), eubacterial 5S rRNAs have been classified into the 120-N (gram-negative) type and the 116-N (gram-positive) type according to the differences in their secondary structures (see above). The most important difference between the 120-N type and the 116-N type may be seen in two regions: aLb, which connects the base-paired regions A and B, and d'La', which connects D' with A' (Fig. 1 and 2). Both aLb and d'La' of the consensus 120-N type sequence are two bases longer than the 116-N type (compare the *Escherichia* and *Bacillus* sequences in Fig. 1). This difference in length exists because, in the 116-N type, four bases, two from the 3' end and two from the 5' end, are deleted, yet the number of base pairs at the 5':3' (A:A') terminal helix is kept the same with that of the 120-N type at the expense of four bases, two from aLb and two from d'La'. Another difference, although less important, may be found in the length of the D:D' helix. The D or D' of the 120-N type is eight nucleotides long, whereas that of the 116-N type is usually five nucleotides long (6).

The present study has shown that the 5S rRNA sequence of *S. aureus* is very similar to that of *S. epidermidis* (95% identity [Table 1]) and, against expectation, both belong to the 116-N type as seen by the properties of the aLb, d'La', and A:A' regions and others, although pair 10 of the A:A' helix is mismatched (Fig. 1). These sequences are fairly similar to those of *Bacillus* spp. However, the two *Staphylococcus* sequences may contain additional base pairs between positions 77 and 80 and between 100 and 97 at the upper part of the D:D' helix.

The sequences of *M. luteus* ATCC 9341 (= *Sarcina lutea*) and *M. luteus* ATCC 4698 (= *M. lysodeikticus*), both possessing 119 nucleotides, are fairly similar to each other (85% identity [Table 1]). In these two 5S rRNAs, two bases, one from the 5' end and one from the 3' end, are deleted compared with that of the 120-N type. Thus the number of base pairs at the 5':3'-terminal helix is kept the same with the 120-N type at the expense of one base each from regions aLb and d'La'. As already mentioned, in the 116-N type, four bases are deleted from the terminals, and, thus, regions aLb and d'La' are both two bases shorter than those of the 120-N type. In addition, certain nucleotide residues such as U and G at positions 56 and 59, respectively, are unique for the 116-N type, whereas purine, pyrimidine, and G at positions 34, 48, and 81, respectively, are characteristic for the 120-N type. Region D or D' consists of eight bases which can pair with each other as in the case of the 120-N type (usually five in the 116-N type). Essentially the same characteristics as those of the *Micrococcus* 5S rRNA have been found in *Streptomyces griseus* 5S rRNA. The two *Micrococcus* strains and *Streptomyces griseus* have 5S rRNAs of 82% sequence similarity on the average (Table 1). These RNAs have intermediary characteristics between the 120-N type and the 116-N type (Fig. 3) and are situated between the typical gram-negative and the gram-positive groups in the 5S rRNA phylogenic tree (Fig. 3). On the other hand, the two *Staphylococcus* species are phylogenically distant from *Micrococcus* and *Streptomyces* with a 5S rRNA sequence similarity of only 57 to 66% (62% on average [Table 1]) and belong to the gram-positive stem.

TABLE 1. Homology percentage of 5S rRNAs and evolutionary distance calculated from equation 1[a]

Strain no.	Organism	Homology (% and evolutionary distance[a]) with strain no.:						
		1	2	3	4	5	6	7
1.	*S. aureus* Smith		0.05	0.67	0.47	0.70	0.29	0.44
2.	*S. epidermidis* ATCC 14990	95		0.64	0.45	0.67	0.28	0.43
3.	*M. luteus* ATCC 9341	57	58		0.17	0.21	0.51	0.66
4.	*M. luteus* ATCC 4698	66	66	85		0.21	0.45	0.49
5.	*Streptomyces griseus* 45-H	56	57	82	82		0.37	0.65
6.	*B. subtilis* BD170	77	78	63	66	71		0.37
7.	*E. coli* MRE600	67	68	57	57	59	71	

[a] %, Lower left half of table; *Knuc*, upper right half of table.

In conclusion, the present study has shown that there are at least three major groups of eubacteria having the 116-N type, the 120-N type, and the intermediate type of 5S rRNA. These three groups belong to different stems in the phylogenic tree.

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