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Unique Organization of *Leptospira interrogans* rRNA Genes

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We cloned *Sau*3AI fragments containing the rRNA genes for *Leptospira interrogans* serovar canicola strain Moulton in the *Bam*HI site of lambda EMBL3 bacteriophage DNA. Physical maps of the fragments were constructed, and the locations of the rRNA genes were determined by Southern blot hybridization and S1 protection. Each fragment of the 23S or the 16S rRNA gene contained at least one copy of the 23S or the 16S sequence. Genomic hybridization showed that there were two genes for the 23S rRNA and the 16S rRNA but only one gene for the 5S rRNA on the chromosome of *L. interrogans*. The results revealed the important fact that each rRNA gene is located far from the other rRNA genes. Our findings, accordingly, also suggest that these rRNA genes are expressed independently in this organism.

Leptospires, aerobic gram-negative bacteria, are thin and spiral shaped and constitute a metabolically and genetically distinct group of eubacteria (10–12). We are interested in this organism because it differs in structure and several genetic and physiological characteristics from other common bacteria.

The number of rRNA genes in bacterial species varies greatly. It has been shown that the *Escherichia coli* chromosome carries seven sets of ribosomal genes (1, 14, 17) and that many more rRNA genes may be carried in *Bacillus subtilis* (2, 15, 21). In contrast, one or two sets of rRNA genes have been reported for *Mycoplasma* (19), *Thermus* (23), *Mycobacterium* (22), and *Halobacterium* (9) species. The organization of the genes for rRNA, however, is preserved in all known eubacterial species. These rRNA genes are arranged in the order 5′-16S-23S-5S-3′, and each rRNA operon is transcribed in that order, with some tRNAs in the transcript. This precursor molecule is processed, and mature rRNAs are produced (17).

On the basis of these reports, we attempted to elucidate the number and structure of the rRNA genes in the genome of this organism. Our earlier work showed that there are at least two 23S rRNA sequences which consist of 2,958 nucleotides in the genome of *Leptospira interrogans* serovar canicola strain Moulton (5, 7). In this report, we provide evidence that there are two genes for 16S rRNA and one gene for 5S rRNA in the leptospiral chromosomal DNA. We also report the important facts that there is no linkage at all among the rRNA genes and that all rRNA cistrons of this organism are far apart on the chromosome.

MATERIALS AND METHODS

Bacterial strains and culture. *L. interrogans* serovar canicola strain Moulton was used throughout this study. The organism was cultivated in the same medium as that used previously (6, 13). *E. coli* K-12 strains HB101, C600, JM109, and NM539 were cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or on LB plates (2% agar with LB broth).

Enzymes and chemicals. Restriction endonucleases were obtained from Takara Syuzo Co. Ltd., Kyoto, Japan; Toyobo Co. Ltd., Osaka, Japan; and New England BioLabs, Inc., Beverly, Mass. Radioactive materials were purchased from Dai-Ichi Kagaku Co. Ltd., Tokyo, Japan. DNA-grade agarose for gel electrophoresis was obtained from Takara Syuzo. All other chemicals were reagent grade.

Preparation of leptospiral DNA and rRNA. Leptospiral cellular DNA was extracted as described previously (7). The DNA was precipitated with ethanol, collected, dried, dissolved in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]), and used for the experiments. Ribosomes of *L. interrogans* were prepared by sucrose gradient centrifugation as described previously (6). rRNAs were extracted by the phenol extraction method (8). The 5S rRNA was electroeluted from 2% agarose gel slices and purified by phenol extraction. Individual species of rRNA were precipitated with 2.5 volumes of 95% ethanol in the presence of 0.15 M ammonium acetate, washed with ethanol, dried, dissolved in TE buffer, and stored at −80°C. DNA digestion with restriction enzymes, agarose gel electrophoresis, electroelution of the DNA fragments from gel slices, and 5′ labeling of rRNA with [γ-^{32}P]ATP (3,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) were performed as described by Maniatis et al. (16). Labeling of the DNA fragment by nick translation was performed with a nick translation kit obtained from Takara Syuzo by using [α-^{32}P]dCTP (3,000 Ci/mmol; Dupont, NEN).

DNA cloning and in vitro packaging. DNA was cloned by the standard method of Maniatis et al. (16) by using lambda EMBL3 bacteriophage or plasmid vectors pBR322 and pUC18. Lambda EMBL3 DNA and in vitro packaging kits, Packagene, were purchased from Promega Biotec, Madison, Wis. Plaques of lambda recombinant phage were formed on LB plates with host bacterium *E. coli* K-12 strain NM539. Transformants were selected on LB plates containing ampicillin at a concentration of 100 μg/ml.

Phage amplification and DNA preparation. Each recombinant phage which contained the rRNA gene was added to host cells and adsorbed to bacterial cells. LB broth (10 ml) was added, and the cultures were incubated at 37°C until the bacterial cells were lysed. Two drops of chloroform was added to the cell lysate, and the mixture was incubated for another 10 min. Bacterial debris was removed by centrifugation at 10,000 × *g* for 30 min, and the cleared lysate was treated with 5 μg each of DNase and RNase per ml at 37°C for 30 min. NaCl (0.5 g) and polyethylene glycol 6000 (1.1 g) were then added to the phage suspension, and the phages were precipitated by allowing the tube to stand at 4°C for a few hours. The phage particles were collected by centrifu-

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gation at 10,000 × *g* for 30 min and suspended in 400 μl of TE buffer. Phages were lysed with sodium dodecyl sulfate, and DNA was extracted as described above. Recombinant plasmid DNA was extracted by the rapid alkaline method (16).

Plaque hybridization and Southern hybridization. Plaques of recombinant phage on LB agar plates were transferred to a colony-plaque screen (82-mm disk; Dupont, NEN). The transferred plaques were denatured by treatment with 0.75 ml of 0.5 M NaOH for 3 min, and this denaturation procedure was repeated with fresh NaOH solution. The disks were then neutralized with 0.75 ml of 1 M Tris hydrochloride buffer (1 M Tris hydrochloride, pH 7.5) twice and dried at room temperature. Electrophoresed DNA fragments in agarose gels were nicked with 0.25 M HCl at room temperature for 20 min and then washed twice in water. The DNA fragments were transferred to the filter by the capillary transfer method in 0.4 M NaOH by using a nylon membrane (7, 20). The disks and filters were washed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) twice and 2× SSC once at 65°C for 30 min. The disks and filters were then rinsed in 0.1× SSC once for 30 min, dried at room temperature, and autoradiographed with Kodak XAR-5 film (Kodak-Nagase Medical Co. Ltd., Osaka, Japan.)

RESULTS AND DISCUSSION

Physical maps of insert DNA containing 23S rRNA gene. The 23S rRNA gene-inserted recombinant lambda EMBL3 phages were obtained by plaque hybridization with 5′-^{32}P-labeled leptospiral 23S rRNA used as a probe. Clones c11 and d1 carrying the 23S rRNA gene were selected for further study. Each recombinant DNA was purified, digested with several restriction enzymes, and electrophoresed in a 1% agarose gel. The patterns of Southern hybridization for clone c11 DNA are shown in Fig. 1A and B. The restriction endonuclease map is shown in Fig. 1C. This physical map was constructed by digestion, partial digestion, and double digestion of end-labeled fragments with each enzyme. The location of the 23S rRNA gene was determined by Southern hybridization and S1 nuclease protection. The physical map for clone d1 was constructed and the location of the 23S rRNA gene was determined by the same procedures as described above for clone c11, and the results are shown in Fig. 1D. The 23S rRNA gene and its flanks were subcloned into pUC18 and sequenced. We have previously reported the nucleotide sequence of the 23S rRNA gene (5).

Southern hybridization patterns and physical maps of lambda recombinant DNA containing 16S rRNA gene. Recombinant phages which hybridized with the ^{32}P-labeled 16S rRNA probe were isolated. Clones M2 and M8 were selected and used for further experiments. Each DNA was digested with several restriction enzymes and electrophoresed. Patterns of Southern hybridization are shown in Fig. 2A (clone M8) and B (clone M2). In the case of digestion with *Bgl*II, a radioactive 4.2-kilobase (kb) fragment of clone M2 appeared, and a 6.5-kb fragment of clone M8 hybridized to the probe. Genomic Southern hybridization with the 16S rRNA probe gave two radioactive bands which corresponded to two similar sizes of the fragments (data not shown). The results are summarized and the physical maps are shown in Fig. 2C (clone M8) and D (clone M2). The 4.2-kb *Bgl*II fragment of clone M2 and the 3.1-kb *Bam*HI-*Sal*I fragment of clone M8 were electroeluted from agarose gel slices, purified by phenol extraction, and subcloned into pUC18 plasmid DNA at the *Bam*HI site and the *Bam*HI-*Sal*I sites, respectively.

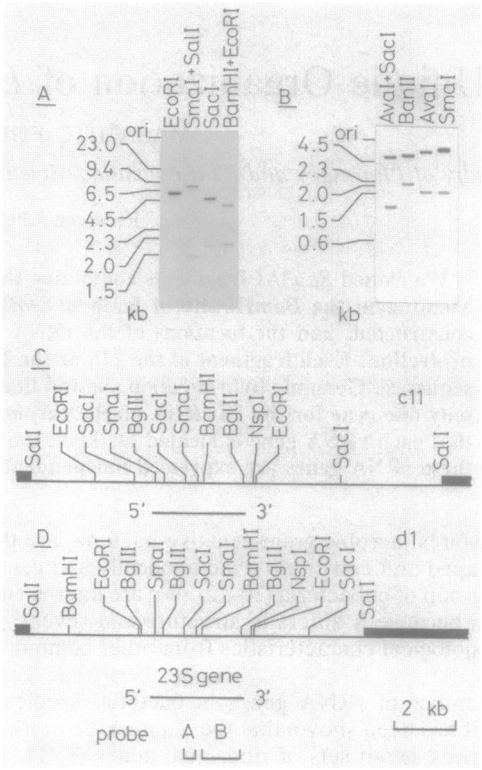

FIG. 1. Patterns of hybridization of a ^{32}P-labeled leptospiral 23S rRNA and physical maps of the insert DNA containing the 23S rRNA gene. DNA was digested with each enzyme indicated in the figure, electrophoresed on 1% (A) and 2% (B) agarose gels, transferred to a nylon membrane (Zeta-probe) by the alkali Southern blotting method, and hybridized to a 23S rRNA probe. Restriction fragment sizes were estimated by using the size marker DNA. rRNA was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and used for the hybridization probes. The other experimental procedures are described in Materials and Methods.

Physical maps of plasmid DNA containing 16S rRNA gene. The DNA fragments which carry the 16S rRNA gene were ligated into pUC18 DNA, and competent cells of *E. coli* K-12 strain HB101 were transformed. Bacterial colonies harboring recombinant plasmid DNA were selected on the ampicillin-containing LB plates. Clone Bg7a which contains the 4.2-kb *Bgl*II fragment and clone BS9 which contains the 3.1-kb *Bam*HI-*Sal*I fragment were selected, cultured, and used for further experiments. Recombinant plasmid DNA was extracted and digested with several restriction enzymes, electrophoresed, and blotted. Patterns of hybridization of clones Bg7a and Bg7b DNA with the probe are shown in Fig. 3A. Clone Bg7b contains the same 4.2-kb *Bgl*II fragment of phage clone M2 but in the opposite direction. The location of the 16S rRNA gene was determined by Southern hybridization. *Ban*II enzyme cleaved DNA inside the 16S rRNA gene and generated a 400-base-pair (bp) fragment. *Acc*I enzyme had a single cleavage site in the 16S rRNA gene (Fig. 3C). The direction of the 16S rRNA gene was determined by using the dideoxy sequencing method for the subcloned 400-bp *Ban*II fragment. Furthermore, it was found by computer analysis that the nucleotide sequence of that fragment is similar to that of *E. coli* (24). Patterns of hybridization of clone BS9, which contains the 3.1-kb *Bam*HI-*Sal*I fragment, which the probe are shown in Fig. 3B. These results also are summarized in Fig. 3D, which shows a pattern similar to that of clone Bg7a.

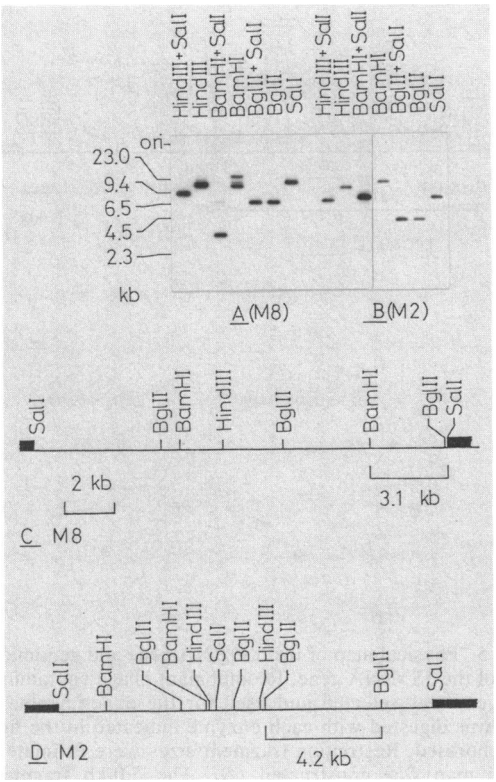

FIG. 2. Hybridization and physical maps of the leptospiral 16S rRNA gene inserted in the recombinant phage EMBL3. DNAs of clones M2 and M8 were extracted and digested with each enzyme indicated in the figure. DNA fragments were electrophoresed in a 1% agarose gel and blotted. The 16S rRNA was labeled with [^{32}P]ATP and used as a hybridization probe. (C and D) Restriction endonuclease cleavage maps. DNA fragments which were subcloned into pUC18 plasmid DNA are indicated in the figure.

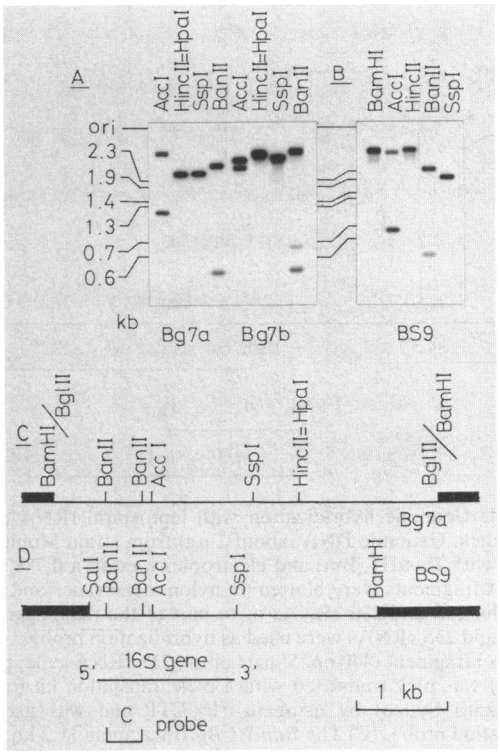

FIG. 3. Patterns of hybridization and restriction maps of recombinant plasmid DNAs. The DNA fragments containing the 16S rRNA gene were subcloned into pUC18 as described in the text. Recombinant plasmid DNAs were digested with the enzymes indicated in the figure, electrophoresed in a 2% agarose gel, blotted, and hybridized. The hybridization probe used was the same as that used for Fig. 2. (C and D) Physical maps. The direction of the 16S rRNA gene was determined by dideoxy sequencing of the subcloned *Ban*II fragment.

Genomic hybridization with an rRNA and the rRNA gene fragments. Leptospiral genomic DNA was digested with *Bam*HI, electrophoresed, blotted, and hybridized with an rRNA or the DNA probe. The DNA probes were labeled with [α-^{32}P]dCTP by nick translation. Figure 4A shows the hybridization pattern of the genomic *Bam*HI digest with 23S and 16S rRNAs. Six radioactive bands appeared. The molecular sizes of these fragments were 24.0, 18.0, 12.5, 12.0, 9.0, and 4.4 kb. Hybridization with DNA probe A (*Sma*I-*Sac*I fragment, 440 bp, 5′ side of *Bam*HI site in the 23S rRNA gene; cf. Fig. 1) showed two bands which correspond to lengths of 24.0 and 4.4 kb (Fig. 4B). The DNA fragment (1.2 kb, DNA probe B, 3′ part of *Bam*HI site in the 23S rRNA gene; cf. Fig. 1) which was generated by *Bam*HI-*Bgl*II digestion of the 23S rRNA gene was hybridized to two leptospiral genomic fragments approximately 12 kb long (Fig. 4C). The *Ban*II fragment (400 bp, DNA probe C, internal part of the 16S rRNA gene; cf. Fig. 3) of clone Bg7a was recovered from a gel slice, labeled, and used as a probe. The hybridization results are shown in Fig. 4D; there were two radioactive bands at 18.0 and 9.0 kb. All of these results are summarized in Fig. 4E. *Bam*HI cleaves the 23S rRNA gene at nucleotide 1587 in mature 23S rRNA and separates it into two fragments (5), but there is no *Bam*HI cleavage site in the 16S rRNA gene. Therefore, the sizes of the *Bam*HI fragments in which entire 23S rRNA genes are included are about 36 and 16 kb, respectively (Fig. 4E). Usually, the rRNA genes in eubacteria are arranged in gene order 16S-23S-5S, the three genes are closely linked, and the sequences corresponding to the three rRNAs are all contained in a maximum size of approximately 5 kb. If the 16S and 5S rRNA genes are adjacent to the 23S rRNA genes in leptospires, both the 16S and 5S rRNA genes must be included within the same 36- and 16-kb *Bam*HI fragments. However, our data demonstrated that the 16S rRNA genes were located on distinct large fragments.

Cloning of the 5S rRNA gene and genomic hybridization. Lambda EMBL3 recombinant phages which contain the 5S rRNA gene were obtained by plaque hybridization. Clone S621 was selected and used for further study. This clone contains the 11-kb leptospiral insert DNA fragment. The 23S rRNA and the 16S rRNA probes were not hybridized to this insert DNA fragment. A detailed restriction map was constructed (Fig. 5A). The 5.0-kb fragment generated by *Bam*HI-*Bgl*II double digestion was subcloned into pBR322 vector DNA. The recombinant pBR322 DNA was digested with several enzymes, electrophoresed, blotted, and hybridized with the leptospiral 5S rRNA probe. The pattern of hybridization is shown in Fig. 5B, and the results clearly show that the 5S rRNA gene is located on the *Eco*RI-*Ssp*I fragment. Since the size of this fragment was 950 bp and indicated rather high radioactivity, it is possible that the 5S rRNA gene was repeated and formed a gene cluster. Using this *Eco*RI-*Ssp*I fragment as a probe (probe D), genomic hybridization was performed to determine the number of 5S

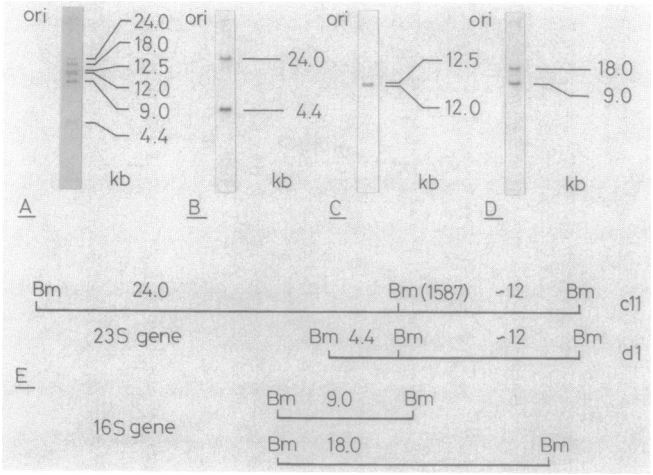

FIG. 4. Genomic hybridization with leptospiral rRNA and the rRNA genes. Genomic DNA (about 1 μg) from strain Moulton was digested with *Bam*HI (Bm) and electrophoresed in a 0.7% agarose gel. DNA fragments were blotted on nylon membranes and hybridized to the 16S and 23S rRNAs or to part of the rRNA genes. (A) The 16S and 23S rRNAs were used as hybridization probes. (B) The *Sma*I-*Sac*I fragment (440 bp, 5′ part of the 23S rRNA gene, probe A in Fig. 1) was nick translated with a nick translation kit (obtained from Takara Syuzo) by using [α-^{32}P]dCTP and was used as a hybridization probe. (C) The *Bam*HI-*Bgl*II fragment (1.2 kb, 3′ part of the 23S rRNA gene, probe B in Fig. 1) was nick translated and used as a hybridization probe. (D) The fragment generated by *Ban*II digestion of the 16S rRNA gene (400 bp, internal part of the 16S rRNA gene, probe C in Fig. 3) was used as a probe. (E) Summary of results.

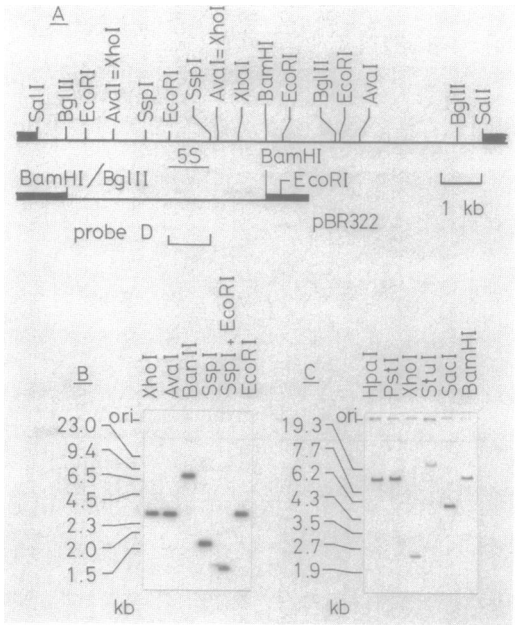

FIG. 5. Physical map of the 5S rRNA gene and genomic hybridization of the 5S rRNA gene. Recombinant phage containing the 5S rRNA gene was selected and used for the experiments. A DNA sample was digested with each enzyme indicated in the figure and electrophoresed. Restriction fragment sizes were estimated, and a physical map was constructed (A). The 5.0-kb fragment from *Bam*HI-*Bgl*II digestion was subcloned into the *Bam*HI site of pBR322 DNA. The recombinant plasmid DNA was digested with each enzyme indicated in the figure, electrophoresed, blotted, and hybridized. (B) Recombinant plasmid pBR322 DNA containing the 5.0-kb fragment digested with each enzyme indicated in the figure and electrophoresed. A 1% agarose gel was used for electrophoresis, and [^{32}P]ATP-labeled 5S rRNA from strain Moulton was used as a probe. (C) Genomic DNA from strain Moulton was digested with each enzyme indicated in the figure, electrophoresed in a 0.7% agarose gel, blotted, and hybridized. The fragment generated by *Eco*RI and *Ssp*I double digestion (950 bp, probe D in Fig. 5A) was nick translated and used as a genomic hybridization probe.

rRNA genes. Leptospiral genomic DNA was digested with each enzyme as indicated in Fig. 5C, electrophoresed, blotted, and hybridized with the 5S rRNA gene. Only one radioactive band appeared in each lane. Genomic hybridization using the 5S rRNA probe showed similar results (data not shown). These results indicate that there is one gene (cluster) for 5S rRNA in the leptospiral genome.

All known eubacterial rRNAs are organized as operons with 16S, 23S, and 5S rRNA genes. *E. coli* has seven cistrons for each rRNA which are present in clusters of tandem 16S, 23S, and 5S rRNA cistrons, and these three RNAs are transcribed consecutively from a single promoter (3, 17). The organization of the rRNA genes for other organisms has also been reported (17, 19, 21–23). The basic structure of rRNA genes in bacteria is as follows: promoter-16S RNA gene-spacer-(tRNA gene)-23S rRNA gene-5S rRNA-(tRNA gene)-terminator.

In contrast, the results of this study indicate that *L. interrogans* has a unique organization of the rRNA genes in its genome. On the leptospiral genome, the three rRNA genes were far apart, a remarkable feature in the organization of the genes. We recently obtained similar results in *Leptospira biflexa*. This unique organization of the rRNA genes thus seems to be the rule in leptospires. The new question raised by our findings is how the transcription of the rRNAs is regulated and coordinated. Experiments to identify the primary transcription products in leptospiral cells and to determine the promoter activity of the 5′ flanks of the rRNA genes are in progress.

Very little is known about the gene organization in leptospires, but some comparative studies using oligonucleotide cataloging of the 16S rRNAs have been reported (4, 18). These phylogenetic studies have demonstrated that the leptospires are phylogenetically a distinct group of eubacteria. The nucleotide sequence determination and elucidation of the organization of the rRNA genes in comparison with those of other organisms are very useful from the evolutionary and taxonomic viewpoints.

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