JOURNAL OF BACTERIOLOGY, June 1990, p. 3264–3268
0021-9193/90/063264-05$02.00/0

Vol. 172, No. 6

Isolation and Characterization of the 5S rRNA Gene of *Leptospira interrogans*

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Received 2 January 1990/Accepted 23 March 1990

The gene encoding the 5S rRNA for *Leptospira interrogans* serovar canicola strain Moulton was isolated and sequenced. The 5S rRNA gene occurs as a single copy within the genome and encodes a 117-nucleotide-long RNA molecule. The 5S rRNA gene is flanked at both the 5′ and 3′ ends by regions of A + T-rich sequences, and the 5′-flanking region contains a promoter sequence. *L. interrogans* has a unique and remarkable organization of the 5S rRNA gene. The 5S rRNA molecule exhibits a strong similarity to typical eubacterial 5S rRNA in terms of overall secondary structure, while the primary sequence is conserved to a lesser degree. Restriction analysis of the 5S rRNA gene indicated that the DNA sequence including the 5S rRNA gene is highly conserved in the genomes of parasitic leptospires.

The number of rRNA genes varies greatly among eubacterial chromosomes (3, 9, 14, 15). The organization of the genes for rRNA, however, is preserved in almost all eubacterial species studied (1, 2, 19).

We cloned the DNA fragments containing the rRNA genes of *Leptospira interrogans* serovar canicola strain Moulton (7, 8). The location, linkage, and number of these rRNA genes have been previously determined by Southern blot hybridization. We have reported that the leptospiral DNA has two genes for 16S rRNA and two genes for 23S rRNA (8). In contrast to these larger rRNA genes, the leptospiral genome was found to carry a single fragment which hybridizes to the 5S rRNA probe. Since the fragment was 950 base pairs long and it indicated rather high intensity, it seemed that the 5S rRNA gene was part of a gene cluster (8). Furthermore, there is no linkage at all among those rRNA genes. Our results indicated that *L. interrogans* has a unique and remarkable organization of the rRNA genes in its genome. The question raised by these findings is how the transcription of the rRNAs is regulated and coordinated. Determination of the nucleotide sequences, expression experiments, and elucidation of the organization of the *L. interrogans* rRNA genes in comparison with those of other organisms are very important from evolutionary and taxonomic viewpoints.

In this study, we found that the parasitic leptospire *L. interrogans* serovar canicola strain Moulton carries a single gene for 5S rRNA in its genome. We also determined the nucleotide sequence of the 5S rRNA gene and its flanking regions in this strain. The 5S rRNA gene sequence has less than 70% identity with that of *Escherichia coli*, but the predicted secondary structure (17, 28) showed close similarity to those of other eubacterial forms. Primer extension experiments revealed that the 5S rRNA gene of strain Moulton has its own promoter for transcription.

MATERIALS AND METHODS

Bacterial strains. The leptospiral strains (Table 1) were provided by Yasutake Yanagihara. The leptospires were cultivated in the same bovine serum albumin-Tween 80 medium as that used previously (6, 13) and were harvested by centrifugation.

Preparation of DNA. Total cellular DNA was extracted by the method described in previous papers (7, 8). The hybridization probes were also prepared as described previously (6–8). The 17-mer primer (5′GCGAACCACATAGTACC3′) for primer extension experiments was purchased from Takara Shuzo Co. Ltd., Kyoto, Japan. DNAs were cloned by the standard method of Maniatis et al. (16) by using plasmid vector pUC18.

Southern hybridization. DNA fragments in agarose gels were transferred and hybridized to the probes. Experimental conditions were as described in our previous paper (8) and as described by Southern (23).

RNA purification. Exponential-phase leptospiral cells (400 ml) were collected by centrifugation and suspended in 10 ml of 0.1 M sodium acetate solution (pH 5.2) containing 0.1 M EDTA and frozen at −80°C. The cells were thawed at 65°C and lysed by the addition of 0.7 ml of a 10% sodium dodecyl sulfate solution. The cell suspension was incubated for 2 min at 65°C and then extracted twice with phenol at 65°C. The RNA in the aqueous phase was precipitated with ethanol, and the pellet was washed with ethanol and dried. RNA was dissolved in 1 ml of Tris-EDTA buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]).

Primer extension. RNA from *L. interrogans* serovar canicola strain Moulton (about 50 μg) was mixed with 10 pmol of oligonucleotide primer (17-mer [Fig. 1]) in 35 μl of 0.1 M Tris hydrochloride (pH 8.5)–0.14 M KCl–10 mM $MgCl_2$. The 5′ end of the primer was labeled by means of an end-labeling kit (Takara Shuzo) and [γ-^{32}P]ATP (6,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.). The solution was heated at 65°C for 10 min and cooled to room temperature for 30 min. Dithiothreitol (5 ml, 0.1 M) and the four deoxyribonucleotide triphosphates (1 μl each) were added to the solution (final concentration, 2 mM). Rous-associated virus-2 reverse transcriptase (20 U; Takara Shuzo) was added, and the mixture was incubated at 42°C for 1 h. The reaction was stopped by heating at 75°C for 10 min and then cooled to 37°C. RNA was digested at 37°C for 30 min by the addition of DNase-free RNase at a final concentration of 2 μg/ml. Ammonium acetate (8 M, 0.05 volume) was added, and the reaction products were precipitated by the addition of 3 volumes of ethanol. The mixture was stored at −80°C for 1 h and centrifuged for 10 min. The precipitate was washed twice in 70% ethanol, dried, and dissolved in loading buffer

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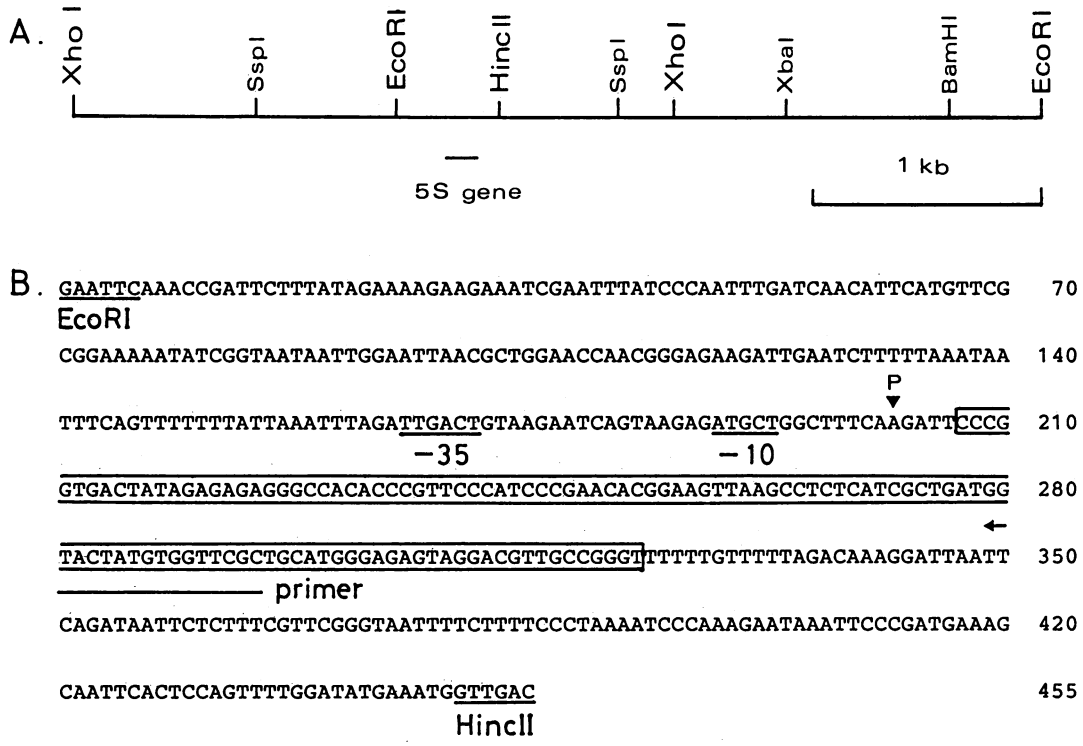

FIG. 1. Physical map (A) and nucleotide sequence (B) of the 5S rRNA gene and its flanking regions for *L. interrogans* serovar canicola strain Moulton. The 2.8-kb *Eco*RI fragment was subcloned into the pUC18 *Eco*RI site, and the 2.8-kb *Xho*I fragment containing the 5S rRNA gene was subcloned into the pUC18 *Sal*I site. Deletions were made by exonuclease III digestion followed by mung bean nuclease digestion. All sequences for both strands were determined by dideoxynucleotide terminating sequencing, as described by Sanger et al. (22). The mature 5S rRNA sequences are boxed. Putative promoter sites and the region complementary to the synthetic oligonucleotide primer are underlined. P, Transcription initiation site for strain Moulton. These data have been submitted to EMBL/GenBank/DDBJ and have been assigned accession number D90074.

(98% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 2 mM EDTA).

Dideoxy sequencing. Ordered deletion mutants were generated by digestion with exonuclease III and sequenced by the dideoxy-chain termination method (22) by using a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]dCTP (1,200 Ci/mmol; Amersham Japan Ltd., Tokyo, Japan). Computer program GENE GIII, prepared by Hideyasu Hirano, Department of Biochemistry, University of Occupational and Environmental Health Japan, Kitakyusyu, Japan, was used for handling the DNA sequences and constructing the secondary structure.

RESULTS AND DISCUSSION

Nucleotide sequence and expression of 5S rRNA gene. *L. interrogans* serovar canicola strain Moulton possesses only one DNA fragment that hybridizes to the 5S rRNA probe (8). A lambda EMBL3 gene bank of strain Moulton was constructed, and recombinant bacteriophages containing the 5S rRNA gene were selected by plaque hybridization (8, 16). One of these clones was selected, and physical maps were constructed. A detailed restriction map for part of this DNA fragment is shown in Fig. 1A. The fragments which were generated by *Eco*RI or *Xho*I digestion were subcloned into *Eco*RI or *Sal*I sites, respectively, in the pUC18 vector DNA. The location of the 5S rRNA gene was determined by Southern blot hybridization with 5′-end-labeled 5S rRNA (Fig. 1A). Deletion mutants were made, and the nucleotide sequence of the *L. interrogans* 5S rRNA gene and its flanking regions were then determined by using the universal primer and Sequenase. The resulting nucleotide sequence contained transcription information for a single copy of the 5S rRNA gene (Fig. 1B). Limitation of the 5S rRNA gene to a single copy in the genome is a constant feature among the parasitic leptospires (Table 1). In contrast, there are two copies of the genes encoding 5S rRNA from saprophytic leptospires (e.g., *Leptospira biflexa*), and there are also two copies of the 16S and the 23S rRNA genes in all of these leptospiral strains. (7, 8; M. Fukunaga, T. Masuzawa, N. Okuzako, I. Mifuchi, and Y. Yanagihara, submitted for publication). Why the 5S rRNA gene occurs as a single copy in several parasitic leptospires is not known.

The nucleotide sequence of the strain Moulton DNA encompassing the 5S rRNA gene is shown in Fig. 1B. The bases encoding the 5S rRNA gene begin at position 207 and continue to position 323. The 3′ terminus of the gene was assigned according to the homologous sequence of *E. coli* 5S rRNA (28). In contrast to the G+C content of the coding region (57%), the regions flanking both the 5′ and the 3′ ends are rather A+T rich (more than 70%). A promoterlike signal for transcription is present adjacent to and upstream from the coding region. Figure 2 shows the primer extension analysis of the gene. To identify possible precursor rRNA transcripts, a 17-mer synthetic primer was made (marked on the sequence in Fig. 1B) and used for primer extension experiments. The primer extension reaction products were run alongside of the sequence analysis with the same primer in order to identify the exact location of the 5′-end bands.

TABLE 1. Sizes of restriction endonuclease-digested genomic DNA fragments hybridized with the 5S gene probe

Species	Serogroup	Strain	Size of DNA fragment(s) (kb) digested by:						
			*Eco*RI	*Hin*cII	*Ssp*I	*Xho*I	*Eco*RI + *Ssp*I	*Eco*RI + *Xho*I	*Eco*RI + *Hin*cII
L. interrogans	Autumnalis	Akiyami A	2.8	4.2	1.6	2.8	0.9	1.2	0.5
		Congo 21-543	2.7	3.4	1.1	>20	0.4	2.7	ND[a]
	Javanica	Veldrat Batavia 46	1.5	7.8	1.5	>20	0.9	1.3	1.3
	Canicola	Moulton	2.8	4.2	1.6	2.8	0.9	1.2	0.5
	LIGP[b]		2.8	4.2	1.6	2.8	0.9	1.2	0.5
L. biflexa	Semaranga	PatocI	4.3	8.5	3.0	>20	1.4	4.3	2.6
			1.4	6.4	1.5	>20		1.4	1.4
	?	Urawa	4.8	8.2	2.0	12.0	1.4	4.5	4.0
			2.7	6.9	1.3	7.5		2.6	2.7

[a] ND, No fragment detected.
[b] LIGP, Strains Akiyami C (serogroup Australis), Hebdomadis (serogroup Hebdomadis), RGA, Ictero No. I (serogroup Icterohaemorrhagiae), Pomona (serogroup Pomona), Salinem (serogroup Pyrogenes), Hardjo (serogroup Sejiroe).

The major transcript (+6) apparently represents the start site of mature 5S rRNA, and the faint band (+1) corresponds to the primary transcripts of 5S rRNA. No other transcripts extending further upstream could be detected, by these experiments even with a 10-times-prolonged exposure. A typical −35 sequence, resembling the *E. coli* promoter consensus sequence (21), and a less-stringent −10 sequence are found very near to and upstream from the primary transcription start site (Fig. 1A). This revealed that the invaluable 5S gene of *L. interrogans* has its own promoter for transcription.

The DNA fragment including this promoter region (from the *Eco*RI cleavage site to position 262) was prepared and ligated into plasmid pKK232-8 (Pharmacia, Uppsala, Sweden), which contains a promoterless chloramphenicol acetyltransferase gene. The recombinant plasmid was introduced into *E. coli* but failed to express the chloramphenicol acetyltransferase gene. The results may indicate that *E. coli* does not utilize this sequence for transcription initiation. The nucleotide sequences that preceded the 16S and the 23S rRNA genes in strain Moulton contained tandem promoter sequences. These tandem promoter sequences were recognized in *E. coli*, and the chloramphenicol acetyltransferase gene was expressed (unpublished results). These findings suggest that another (main?) promoter exists further upstream from the *Eco*RI site of the sequence. The 5S rRNA of strain Moulton is 117 nucleotides long and is strikingly similar to 5S rRNA from *E. coli* (17), whereas the primary structure is conserved to a lesser degree. For instance, rRNA identity with the rRNA of *E. coli* is 66%. By analogy, sequence similarities between strain Moulton and other eubacteria, such as *Pseudomonas aeruginosa*, are also around 66%. 5S rRNA can be folded according to the secondary-structure model (17, 28). In spite of many nucleotide substitutions (both transitions and transversions), the secondary structure agrees with a model based on comparative analysis of the eubacterial sequences (data not shown). Some bands that appeared in the primer extension experiments (+12, +16 through +22; Fig. 2) also support this model because these bands resulted from detachment of the reverse transcriptase when it encountered the secondary structure on the RNA. Therefore, many shorter primer extension products appearing in the gel are located in a stem-loop structure of the mature 5S rRNA.

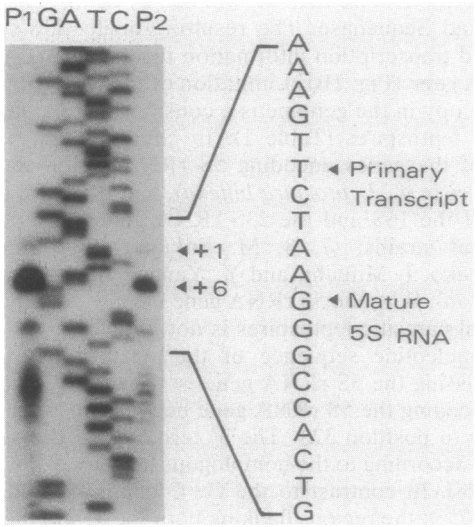

FIG. 2. Dideoxy sequence analysis and primer extension experiments with 5′-end-labeled oligonucleotide primer. For primer extension, the end-labeled synthetic primer (indicated in Fig. 1) was hybridized to RNA from *L. interrogans* and extended by reverse transcriptase. The products of primer extension experiments were electrophoresed in 8% polyacrylamide–7 M urea gels. The dideoxy sequencing ladder was made with a Sequenase kit by using the same primer and [α-^{32}P]dCTP (Dupont, NEN). Lane P1, Primer extension products; lane P2, same primer extension products in 1/5 the volume; lanes G, A, T, and C, sequence ladder.

Comparison of restriction patterns in *Leptospira* strains. The DNA fragment including the entire sequence of the 5S rRNA gene from strain Moulton (*Dra*I-*Hin*cII, nucleotide numbers 135 to 452 [Fig. 1B]) was electroeluted from the gel slices, radiolabeled, and used as a probe against restriction endonuclease digests of genomic DNA from strains of *L. interrogans* and *L. biflexa*. All of the hybridization experiments with strains of *L. interrogans* yielded a single radioactive band, and the patterns of hybridization for these strains were quite alike. Therefore, the genomic DNA digests of these parasitic strains were electrophoresed simultaneously in the same gels to compare their exact molecular sizes. Probing of *Eco*RI-*Xho*I, *Eco*RI-*Ssp*I, and *Ssp*I digests with the DNA probe is shown in Fig. 3 as an example. The molecular sizes of the hybridization fragments, in kilobases, were calculated by using *Hin*dIII-*Kpn*I digests of lambda phage DNA and *Hin*cII digests of ϕX174 DNA as molecular size markers. The five strains produced a single radioactive

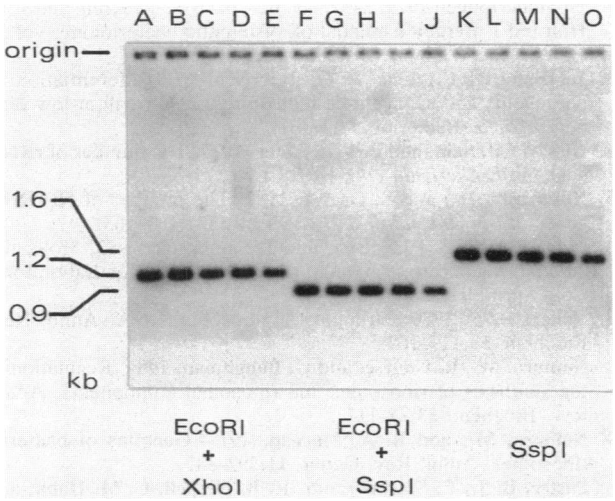

FIG. 3. Patterns of hybridization of the 5S rRNA gene probe to restriction endonuclease-digested leptospiral genomic DNA. Genomic DNA of five *L. interrogans* strains was digested with *Eco*RI-*Xho*I (lanes A through E), *Eco*RI-*Ssp*I (lanes F through J), and *Ssp*I (lanes K through O) and electrophoresed in 1% agarose gels. The 5S gene fragment of strain Moulton (a 321-base-pair *Dra*I-*Hin*cII fragment including the entire 5S rRNA gene) was labeled with [^{32}P]dCTP by using a nick translation kit, as described in a previous paper (7), and used as a hybridization probe. The filters were washed in 6× SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) twice and in 2× SSC once at 65°C (each washing, 30 min). Then the filters were rinsed in 0.1× SSC once for 30 min, dried at room temperature, and autoradiographed with XAR-5 film (Kodak Nagase Medical Co. Ltd., Osaka, Japan). The strains used were Akiyami C (lanes A, F, and K), Moulton (lanes B, G, and L), Pomona (lanes C, H, and M), Salinem (lanes D, I, and N), and Hardjo (lanes E, J, and O).

band in each restriction endonuclease digestion (Fig. 3). Molecular sizes were 1.2 kilobases (kb) for *Eco*RI-*Xho*I digestion, 0.9 kb for *Eco*RI-*Ssp*I digestion, and 1.6 kb for *Ssp*I digestion. Eleven strains with typical serologically diverse serovars were tested (Table 1). Hybridization of the probe to *Ssp*I digests and to *Hin*cII digests of genomic DNA from nonparasitic leptospiral strains are shown in Fig. 4 as an example. Probing the restriction digests with the 5S rRNA gene probe yielded two bands of equal intensity in almost all digests. The sizes of the fragments, however, varied widely, in contrast to those of parasitic strains.

The sizes of the fragment in almost all parasitic strains (9 of the 11 strains) were identical in each restriction endonuclease digestion. Only strains Congo 21-543 and Veldrat Batavia 46 revealed genomic DNA fragments of different sizes. These results clearly confirm that the 5S rRNA gene in parasitic leptospires occurs as a single copy. They also indicate that the sequences of the 5S rRNA gene and its flanking regions are well conserved in these strains. We determined the number of 5S rRNA genes in two saprophytic leptospiral strains (Table 1). The results strongly suggest that there are two genes for 5S rRNA in these strains. In the genomic hybridization analysis, we found that the patterns of hybridization with the 5S rRNA probe are quite alike. These results, therefore, indicate that the sequences flanking the 5S rRNA gene are well conserved in parasitic leptospires. We have also shown that nonparasitic leptospires have two 5S rRNA genes and that the sequences flanking their 5S rRNA genes are conserved to a lesser degree.

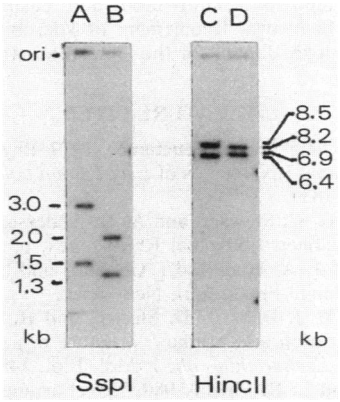

FIG. 4. Patterns of hybridization of the 5S rRNA gene probe to restriction endonuclease-digested leptospiral genomic DNA. Genomic DNA of each leptospiral strain was digested with *SSp*I (lanes A and B) and *Hin*cII (lanes C and D) and electrophoresed in 1% agarose gels. Electrophoresed DNA fragments in agarose gels were nicked, transferred to filters, and hybridized to the probe as described in the text and in the legend for Fig. 3. The strains used were *L. biflexa* PatocI (lanes A and C) and Urawa (lanes B and D).

Our recent work on the organization of the rRNA genes in the genera *Leptospira* and *Leptonema* showed that there is no linkage at all among the genes for 16S, 23S, and 5S rRNAs (M. Fukunaga et al., submitted). In the eubacterial species examined so far, the three rRNA genes are closely linked, with a gene order of 5′ 16S-23S-5S 3′. Recently, some exceptions to this rule have been reported. In *Thermus thermophilus* (25), 16S and 23S rRNA genes are separated; in *Mycoplasma gallisepticum* (4), the 16S gene is separated from the other genes; and in the other *Mycoplasma* strain, *M. hypopneumoniae* (24), the 5S rRNA gene is separated from the other genes. However, there is no reported example like that of the *Leptospira* genome, in which each rRNA gene is located far from the other rRNA genes. The spirochetes, including the genera *Leptospira* and *Leptonema*, are one of the relatively ancient evolutionary branches of the eubacteria (5, 20, 27). The results reported here, therefore, reconfirm the phylogenetic situation of leptospires in the evolution of the eubacteria.

rRNAs are most useful as molecular chronometers because of their functional constancy and are now the most used molecular chronometers (18, 26). In fact, as we showed in this study, the secondary structure of 5S rRNA exhibits a strong similarity to that of the eubacterial model, while the primary sequence is not well conserved. Conservation of this secondary structure across such a wide phylogenetic gap implies that this molecule has a constant function. Leptospires are now divided into several groups on the bases of their biochemical and serological characteristics (10–12), but they are the descendants of a common progenitor which later diverged into the parasitic and saprophytic leptospires. The determination of the number of 5S rRNA genes and the comparison of restriction patterns of the regions flanking the gene clearly divided leptospires into two groups. Thus, analysis of the genetic organization, as we demonstrated here, would be useful in taxonomic and phylogenetic studies of leptospires.

ACKNOWLEDGMENTS

We thank Yasutake Yanagihara for his encouragement and valuable discussion; Noriko Okuzako and Toshiaki Kanata for their

assistance with leptospiral cultivation; and Yasutake Yanagihara and Toshiyuki Masuzawa, Department of Microbiology, Shizuoka University, Shizuoka, Japan, for the leptospiral strains.

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