Cloning, characterization and taxonomic significance of genes for the 5S ribosomal RNA of *Leptonema illini* strain 3055

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The genes encoding the 5S ribosomal RNA (rRNA) for *Leptonema illini* strain 3055 were isolated and sequenced. The 5S RNA molecule encoded was 117 nucleotides long. The genome of strain 3055 contained two genes for 5S rRNA that were located close together. The nucleotide sequences of the *Leptonema illini* genes exhibited less similarity to the rRNA gene of *Leptospira interrogans* strain Moulton and also to those of typical eubacterial genes than did the rRNA genes of other leptospires. However, the overall secondary structure of the 5S rRNA encoded exhibited a strong similarity to that of typical eubacterial 5S rRNA. Southern hybridization of the 5S rRNA gene probe with the genomic DNA of strain 965, which is currently classified as *Leptospira biflexa*, showed the latter to have close similarity to that of strain 3055. The physical map of strain 965 was quite similar to that of strain 3055 and was greatly different from that of any other strains of *L. biflexa*. In the organization of 5S rRNA genes, strain 965 is sufficiently different from other members of the genus *Leptospira* to be regarded as a member of the genus *Leptonema*.

Introduction

We have been studying the molecular biology of leptospires with special emphasis on ribosomal RNA (rRNA) genes. Our interest in rRNA genes was aroused because the organization of rRNA genes for leptospires is unique (Fukunaga & Mifuchi, 1989b). Furthermore, the number of 5S rRNA genes depends on parasitism (Fukunaga *et al.*, 1990a, b). In strains of non-parasitic leptospires, there are two genes for 5S rRNA, whereas only one gene for 5S rRNA is carried in parasitic leptospires. Thus, understanding the nature of leptospiral rRNA gene organization might be of great importance. Determination of nucleotide sequences, expression experiments and elucidation of the organization of the rRNA genes in comparison with those of other organisms are very important from the evolutionary and taxonomic viewpoints. Here we report the organization and nucleotide sequences for the 5S rRNA genes of the leptospire *Leptonema illini* strain 3055, and compare them with *Leptospira biflexa* strain 965.

Methods

Bacterial strains and media. The bacterial strains were kindly provided by Drs Y. Yanagihara, University of Shizuoka, Shizuoka, Japan, and R. C. Johnson, University of Minnesota, Minneapolis, Minn., USA. The leptospires were cultivated in the same BSA/Tween 80 medium as used previously (Fukunaga & Mifuchi, 1988) and harvested by centrifugation. *Escherichia coli* strains HB101, Y1090 and JM109 were grown in Luria broth either in the liquid form or with 2% (w/v) agar added (Miller, 1972). L-agar plates supplemented with 100 µg ampicillin ml⁻¹ were used to isolate *E. coli* transformants.

Preparation of DNA, and genomic- and plaque-hybridization. Total cellular DNA was extracted by the method we described in a previous paper (Fukunaga & Mifuchi, 1989a). The hybridization probes were also prepared as described previously (Fukunaga & Mifuchi, 1989b). DNAs were cloned by the standard method of Maniatis *et al.* (1982) by using plasmid vector pUC18 or bacteriophage λ gt11 arm DNA. DNA fragments in agarose gels or the phage plaques on the plates were transferred and hybridized to the probes. Experimental conditions were as described by us in previous papers (Fukunaga & Mifuchi, 1989a, b) and by Southern (1975).

Dideoxy sequencing. Ordered deletion mutants were generated by digestion with exonuclease III and nucleotide sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977) by using a Sequenase kit (United States Biochemical Corp.) and deoxycytosine 5'-[α-³²S]thiotriphosphate ([α-³²S]dCTP) [1200 Ci mmol⁻¹; (44.4 TBq mmol⁻¹); Amersham Japan]. The computer program GENE III, written by Hideyasu Hirano, University of Occupational and Environmental Health, Kitakyushu, Japan, was used for handling the DNA sequences and construction of the secondary structure.

The nucleotide sequence data reported in this paper have been submitted to DDBJ, EMBL and GenBank and have been assigned the accession numbers D90253 and D90254.

Primer extension. The 17-mer primer (5'-GCGAACTCTGCAG-TACC-3') for the primer extension experiment was purchased from Takara Shuzo Co., Japan. Total cellular RNA of *Leptonema illini* strain 3055 was extracted by the method we described in a previous paper (Fukunaga *et al.*, 1990a) from exponential or stationary cultures of the bacterium. Experimental conditions were as described previously by (Fukunaga *et al.*, 1990a).

Results

Cloning of *Leptonema illini* genes encoding 5S rRNA

The 5S rRNA genes in *Leptonema illini* strain 3055 are located on a 4.6 kb *EcoRI* fragment (Fukunaga *et al.*, 1990b). Therefore, a λ gt11 gene bank of strain 3055 was constructed, and recombinant bacteriophages containing the 5S rRNA gene were selected by plaque hybridization. One of these clones was selected, and physical maps were constructed. A restriction map for the *EcoRI* fragment is shown in Fig. 1. The 2.8 kb fragment which was generated by *Bgl*II digestion was subcloned into the *Bam*HI site in the pUC18 vector DNA. A detailed restriction map was then constructed and the location of the 5S rRNA genes (indicated in Fig. 1) was determined by Southern blot hybridization. Ordered deletion mutants were made by exonuclease III digestion, and the nucleotide sequences of the 5S rRNA gene and its flanking regions were determined by using a universal primer.

The resulting nucleotide sequences of the 5S rRNA genes for strain 3055 are shown in Fig. 2. The 5'-terminus of the gene was determined by primer extension experiments with a dideoxynucleotide sequencing ladder using the same synthetic primer, and the 3'-terminus was assigned according to the homologous sequence of *E. coli* (Wolters & Erdmann, 1988). The DNA sequences of the 5S rRNA A and B genes were identical to each other until position -54, and then they diverged. The sequences surrounding the rRNA gene included A+T-

rich sequences and a sequence resembling a bacterial promoter was found immediately upstream from the 5'-terminus of the 5S gene. Results of our primer extension experiments suggested that the 5'-terminus of the 5S rRNA is localized (nucleotide number +1). However, no primary transcript was identified when the primer was annealed to RNA isolated from both stationary and exponential phase cultures of *Leptonema illini* (Fig. 3). No transcripts extending further upstream from the mature 5'-terminus could be detected in our experiments, even when the exposure was prolonged 10 times. The coding region of the 5S rRNA gene was 117 bases long and its G+C content was 56 mol%. *Leptonema illini* strain 3055 has a genomic DNA with a G+C content of 53 mol%, which is considerably higher than that of *Leptospira* (Johnson, 1981; Johnson & Faine, 1984). Secondary structure models for other eubacteria have been proposed (Noller, 1984; Wolters & Erdmann, 1988), and there are close similarities in the secondary structure in spite of their many nucleotide substitutions.

Gene organization in *Leptospira biflexa* strain 965 in comparison with that in *Leptonema illini* strain 3055

Hybridization analysis according to Southern (1975) was used to construct the genomic organization of the 5S rRNA gene. The DNA fragment including the entire 5S rRNA gene was radiolabelled and used as a probe against restriction digests of genomic DNA from those strains (Fukunaga *et al.*, 1990a). The DNA was digested by several enzymes in combination with *Eco*T14I, *Hpa*I or *Eoc*RV. The results of one such experiment are shown in Fig. 4. Hybridization of the 5S probe to double digests of chromosomal DNA from the two strains indicated that fragments with identical sizes had been produced, except in the case of *Cla*I + *Eco*T14I digests (lanes I and J). Detailed restriction maps for part of the chromosomal DNA of both strains including the 5S rRNA genes are

Fig. 1. Physical map of the DNA insert containing the 5S rRNA genes for *Leptonema illini* strain 3055. Recombinant phage DNA or plasmid DNA was digested with each restriction enzyme, electrophoresed on agarose gels, transferred to a nylon membrane (Zeta probe membrane) by the alkaline Southern blotting method (Fukunaga & Mifuchi, 1989a), and hybridized to a 5S rRNA gene probe. Restriction fragment sizes were estimated by using size marker DNA. Bold lines represent λ gt11 and pUC18 vectors; thin lines represent the *Leptonema illini* DNA inserts (4.6 kb *EcoRI* fragment in λ gt11, 2.8 kb *Bgl*II fragment in pUC18). Ec, *EcoRI*; ER, *EcoRV*; Bg, *Bgl*II; Bm, *Bam*HI; Sm, *Sma*I; Hc, *Hinc*II; ET, *Eco*T14I; Pt, *Pst*I; Hp, *Hpa*I; Cl, *Cla*I; Sc, *Sac*I.

acgcattcttt catattcatt gcccggttc taaagAGGCC Ttcgtatacgt -201
 c.agtg.caa gc.tgaa.ac .aagaCAGCT Gggtttcttg gggtt.ca..t
PvuII

Eco105I
 gcaactgttt tcgTACGTAA aggttgcccg cgtccatccg gagaaaagca -151
 t.gg.gt.g. aaca.aactc taag.c.ggc gcg..cc.aa tctc.cccgt

BglI
 gcacGCCgcc gtGGCatttt tctctttttc cggattttcc cattttctat -101
 ..gg.tg..g c.ttgt.aa. c.gaggagat t.a.gCCGCG Gc..c.ggtg
SacII(SsrII)

tttttctatt ttttctatatt tttcaatttt tcccatTTTT tctatgccct -51
 cCCGCGGcag ggc.t.c.aa ..gag.a... ..gca....
SacII(SsrII)

tgatataaat tgtgtttgac cggaagaacc gctcaccata catggttctt -1

-35 -10

BheI(NarI)
 GCTGGCGCCA ATAGAGAGGG TGATACACCT GTTCCCATCC CGAACACAGA +50
 ...GGCGCC.

PstI
 AGTTAAGCCC C'FCATCGCCG ATGGTACTGC AGAGTTCGCT CTGTGGGAGA +100
 CTGC AG.....
← Primer

GCAGGACGGT GCCACACagc actcatacag caatcaaaag accctcgctt +150
a.cg.ta ga.t.a.aaa

Eco52I(XmaIII)
 cggctgggtt tttttatgcc ctgacaCGGC CGccctgacc gccctcgaac +200
 aactcctt.a cgggag.ttt tcttttttat aa..tgctgg ttg..tcc.t

ggagaatgct atcgacatct gctcagcagg ttcgaaagcg gggcacgaac +250
 .a.ccgac.c gg.at.c.tc .a.acagcat c.tcgctctt tccgttatta

cgaattgtaa aaaaagttct ccttatatttg gttttttccg tcagacggat +300
 tc.t.gtctt cgcct.gatc gg..cacaaa .acag...a. cttc.actt.

Fig. 2. Nucleotide sequences of the *Leptonema illini* strain 3055 5S rRNA gene and its flanking regions. The 2.8 kb *Bgl*/II fragment was subcloned into the pUC18 *Bam*HI site and ordered deletion fragments were made by exonuclease III digestion followed by mung bean nuclease digestion. The sequences of both strands were determined by dideoxynucleotide terminating sequencing as described by Sanger *et al.* (1977). The mature 5S rRNA sequences are indicated in capital letters (+1 to +117). Restriction sites are also indicated in capital letters. The upper line of each pair shows the sequence for the 5S rRNA gene B and the lower line for gene A. Dots indicate nucleotides identical with those of gene B. The synthetic primer used for primer extension experiments and promoter-like sequences (-35, -10) are also indicated.

Fig. 3. Primer extension experiments with 5'-end-labelled oligonucleotide primer. For primer extension, the end-labelled synthetic primer (indicated in Fig. 2) was hybridized to RNA prepared from *Leptonema illini* strain 3055 and extended by reverse transcriptase. The products of primer extension experiments were electrophoresed in 8% (w/v) polyacrylamide/7 M-urea gels. Gels were electrophoresed at 1200 V for 3 h and then autoradiographed with Kodak XAR-5 film at -80°C for 1 h. The mature 5'-terminus of the 5S rRNA is indicated; some shorter primer extension products resulted from detachment of the reverse transcriptase when it encountered the secondary structure of the rRNA. Lane P_E, primer extension products using RNA prepared from an exponential cell culture; lane P_S, using RNA extracted from stationary phase cells.

Fig. 4. Southern blot analysis of chromosomal DNAs from different bacterial sources. Genomic DNA was digested with two enzymes and electrophoresed through a 2% (w/v) agarose gel, denatured and transferred to a nylon membrane. Hybridization was done as described previously (Fukunaga & Mifuchi, 1989a) using a 5S rRNA gene probe. *Leptospira biflexa* strain 965 genomic DNA (lanes A, C, E, G and I) and *Leptonema illini* strain 3055 genomic DNA (lanes B, D, F, H and J) were digested with *Eco*T14I + *Sac*I (lanes A and B); + *Pst*I (lanes C and D); + *Hpa*I (lanes E and F); + *Hinc*II (lanes G and H); + *Cla*I (lanes I and J). Size markers are indicated in kb.

Fig. 5. Comparison of physical maps of the 5S rRNA gene cluster in *Leptonema illini* strain 3055 and *Leptospira biflexa* strain 965 chromosomal DNAs. Genomic DNA was digested, electrophoresed and hybridized to a 5S rRNA gene probe. These physical maps were constructed by single, double or partial digestion with restriction enzymes. Arrowheads (B and A) indicate the location of the genes and also indicate the direction of transcription (left-to-right). The different *Cla*I restriction sites are indicated (*).

shown in Fig. 5. The *Pst*I cleavage site was present inside the 5S rRNA gene for *Leptonema illini* strain 3055 as shown in Fig. 2. The 5S rRNA genes in *Leptospira biflexa* strain 965 were also cleaved by *Pst*I at almost the middle of the gene. Cleavage sites for all the restriction enzymes except *Cla*I that were used in our experiments were thus identical in the two strains.

Discussion

Previously, we have reported the patterns of hybridization of the 5S rRNA probe to restricted chromosomal DNA for some saprophytic leptospires (Fukunaga *et al.*, 1990a, b). There were some differences between the *Leptonema illini* strain and the strains of *Leptospira biflexa*. The genomic hybridization of the 5S probe to the strains of *Leptospira biflexa* yielded two radioactive bands in all restriction digests. In contrast, some enzyme digestions of the DNA from *Leptonema illini* yielded only one radioactive band. These results suggest that the 5S rRNA genes in the *Leptonema illini* strain are close to each other and that the genes in the *Leptospira biflexa* strains are located separately on the genome.

To study the relatedness of *Leptonema illini* to other parasitic and saprophytic leptospires, we examined the organization and nucleotide sequences of the 5S rRNA genes. Our results showed that the 5S rRNA genes in *Leptonema illini* strain 3055 are close together on the genome. The secondary structure predicted from the sequence is strikingly similar to that of other leptospires, whereas the primary sequences are conserved to a lesser

degree [27 nucleotides substituted in comparison with the *Leptospira interrogans* strain Moulton (Fukunaga *et al.*, 1990a) and 31 bases substituted in comparison with the *Leptospira biflexa* strain Patoc I, (the DDBJ/EMBL/GenBank accession number for the 5S rRNA gene of strain Patoc I is D90255)]. In spite of many nucleotide substitutions, the secondary structure agrees with a model based upon the comparative analysis of eubacterial sequences.

The pathogenic species *Leptospira interrogans* possesses only one DNA fragment which hybridizes to the 5S rRNA probe and the nucleotide sequence contains the transcription information for a single copy of the 5S rRNA gene (Fukunaga *et al.*, 1990a). Limitation of the 5S rRNA gene to a single copy in the genome is a constant feature among parasitic leptospires (Fukunaga *et al.*, 1990a, b). Furthermore, the sequences flanking the 5S gene are well conserved in many parasitic strains. In contrast, there are two copies of the genes encoding the 5S rRNA in saprophytic leptospires (i.e. *Leptospira biflexa* and *Leptonema illini*). The larger rRNA genes (such as 16S and 23S) in all of these leptospiral strains also occur as two copies (Fukunaga & Mifuchi, 1989a; Fukunaga *et al.*, 1990b). Why the 5S rRNA gene occurs as a single copy in parasitic leptospires is not known.

The physical mapping of the 5S rRNA genes in *Leptospira biflexa* strain 965 indicates that the 5S gene organization in strains 965 and 3055 is highly homogeneous, and this feature distinguishes these strains from the other leptospires. Strain 965 is, however, currently placed in the genus *Leptospira*, and strain 3055 in the genus *Leptonema*. Analysis of the physical maps of these strains revealed close similarity of 5S rRNA gene organization, suggesting that the two strains are very much alike in their genomic DNA, but genetic analysis indicates that strain 965 is sufficiently different to be regarded as a member of the genus *Leptonema*. Our results show that the 5S rRNA gene can be used for taxonomic investigations or to identify and classify related leptospires. *Leptospira* is currently divided into serogroups and serovars based on their antigenic composition (Johnson & Faine, 1984). Restriction endonuclease analysis of leptospiral genomic DNA is very accurate and reliable procedure for classifying leptospires (Marshall *et al.*, 1981, 1984; Thiermann *et al.*, 1985, 1986). Recently, Southern hybridization analysis using an rRNA probe was reported (Perolat *et al.*, 1990). rRNA genes are very useful as molecular chronometers because they are highly conserved (Fox *et al.*, 1980; Paster *et al.*, 1984; Woese, 1987) and therefore suitable for the study of bacterial classification. Thus, analysis of the genetic organization, especially of 5S rRNA genes as we demonstrated here, should be useful in differentiating leptospires.

We have found that the 5S rRNA gene of *Leptospira interrogans* has its own promoter for its transcription and the primary transcript has been detected (Fukunaga *et al.*, 1990a). Primer extension experiments using the synthetic primer located the 5'-terminus of the gene but failed to identify the primary transcripts in *Leptonema illini* strain 3055, even in RNA prepared from an exponential cell culture. The sequence resembling a promoter was found immediately upstream from the 5'-terminus of the gene. These results may indicate that the 5S rRNA of *Leptonema illini* strain 3055 is transcribed as a mature form. They also indicate that *Leptonema illini* may have its own transcription system different from that of the genus *Leptospira*.

In conclusion, our experiments show that *Leptonema illini* has two genes for 5S rRNA which are located close together on the genome. The nucleotide sequences of the genes are less conserved than those of the other leptospires and also less than typical eubacteria such as *E. coli*. The secondary structure of the 5S rRNA, however, shows close similarity to that of a typical secondary model (Noller, 1984). Finally, the unique 5S rRNA gene organization of *Leptonema illini* differentiates this organism from other leptospires and analysis of the 5S rRNA gene would be very useful in the taxonomic study of leptospires.

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