The 23S/5S ribosomal RNA genes (*rrl/rrf*) are separate from the 16S ribosomal RNA gene (*rrs*) in *Borrelia burgdorferi*, the aetiological agent of Lyme disease

MASAHITO FUKUNAGA,^{1*} YASUTAKE YANAGIHARA² and MASAKO SOHNAKA¹

¹Faculty of Pharmacy and Pharmaceutical Sciences, University of Fukuyama, Fukuyama, Hiroshima 729-02, Japan

²Department of Microbiology, School of Pharmaceutical Sciences, University of Shizuoka, Yada, Shizuoka 422, Japan

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DNA fragments containing the rRNA genes for *Borrelia burgdorferi* strain B31 were cloned in bacteriophage λ EMBL3. A restriction map of the fragments was constructed and the organization of the rRNA genes was determined by Southern hybridization. One genomic DNA fragment contained a single copy of the *rrs* sequence and another cloned fragment contained both *rrl* and *rrf* sequences. The results revealed that the *rrs* gene is located separately from the set of *rrl/rrf* genes, suggesting that these rRNA genes are expressed independently in *B. burgdorferi*.

Introduction

Borrelia burgdorferi, the causative agent of Lyme disease, was discovered about 10 years ago (Burgdorfer *et al.*, 1982), and the organisms have been isolated from humans, animals and ticks in different areas of the world (Hughes & Johnson, 1990; Postic *et al.*, 1990). Lyme borreliosis is a widespread zoonanthroposis which is transmitted by ticks (Steere *et al.*, 1983; Johnson *et al.*, 1984). Since the first isolation of the organism, immunological, biochemical and molecular biological studies have been reported (Barbour & Hayes, 1986; Barbour & Garon, 1987; Bergstrom *et al.*, 1989; Howe *et al.*, 1985; Hyde & Johnson, 1984). *B. burgdorferi* has a small (about 1000 kb) linear chromosome and linear plasmids (Baril *et al.*, 1989; Ferdows & Barbour, 1989).

We are interested in the organization and regulation of the genes coding for the rRNAs of spirochaetes. The organization of the rRNA genes in leptospirae is unique (Fukunaga & Mifuchi, 1989*a, b*; Fukunaga *et al.*, 1990*a, b*). Organization and transcription of genes for rRNA have been highly conserved among prokaryotes because the synthesis of rRNA is an essential process (Nomura & Morgan, 1977; Nomura *et al.*, 1984).

The number of rRNA genes in *B. burgdorferi* has been deduced from the results of genomic hybridization using *Escherichia coli* rRNA as a probe, and DNA relatedness among *Borrelia* strains has also been examined by DNA–DNA hybridization (Postic *et al.*, 1990). It was of interest to determine the number and organization of rRNA genes in *B. burgdorferi* and to compare the structures of these genes in various spirochaetes. Analysis of the genetic organization and nucleotide sequences of the rRNA genes in comparison with those of other organisms are very useful from the evolutionary and taxonomic viewpoints. In this paper we describe the cloning of rRNA genes of *B. burgdorferi*, physical maps, and structural relationship to other spirochaetal rRNA genes.

Methods

Bacterial strains and media. *B. burgdorferi* strain B31 was used. Cells were taken from a late exponential culture in 100 ml of BSKII medium (Barbour, 1984). *E. coli* strains HB101 and NM539 were grown in Luria medium, either in liquid form or solidified with 2% (w/v) agar (Miller, 1972). L-Agar plates supplemented with 100 μ g ampicillin ml⁻¹ were used to isolate *E. coli* transformants.

Preparation of DNA, and genomic- and plaque-hybridization. Total cellular DNA was extracted as described previously (Fukunaga & Mifuchi, 1989*a*). DNA was partially digested with *Sau*3AI and cloned into the *Bam*HI sites of bacteriophage λ EMBL3 as described by Fukunaga & Mifuchi (1989*b*). DNAs were cloned by the standard method of Sambrook *et al.* (1989) by using plasmid vector pUC18 or

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* Author for correspondence. Tel. 849 36 2111 ext. 5216; fax 849 36 2213.

Abbreviations: *rrl*, *rrs*, *rrf*, genes for 23S, 16S and 5S rRNA, respectively.

Fig. 1. Pattern of hybridization and physical map of the DNA fragment containing the *rrs* gene. Recombinant λ phage DNA containing the *rrs* gene was digested with the enzymes indicated, electrophoresed in 1% agarose gels, transferred and hybridized to the treponemal rRNA probe (a). The 6.2 kb *Bam*HI fragment was isolated and ligated into pUC18 DNA. The leptospiral *rrs* gene probe (*Ban*II fragment) was used to determine the orientation of the gene in the fragment (b). The enzymes used were: (a) *Bam*HI (lane 1), *Bam*HI + *Eco*RI (lane 2), *Bam*HI + *Mlu*I (lane 3), *Bam*HI + *Sac*II (lane 4), *Hind*III (lane 5), *Hind*III + *Bam*HI (lane 6), *Hind*III + *Mlu*I (lane 7), and *Hind*III + *Sac*II (lane 8); (b) *Mlu*I + *Sal*I (lane 1) and *Sac*I + *Sac*II (lane 2). Restriction fragment sizes were estimated by using size marker DNA. The location of the *rrs* gene was determined and is indicated together with restriction cleavage sites (c). The leptospiral *rrs* gene probe used in this experiment is also indicated. All of the other experimental conditions are described in the text.

bacteriophage λ EMBL3 arm DNA. DNA fragments in agarose gels or the phage plaques on the plates were transferred to a nylon membrane and hybridized with the probe. Experimental conditions were as described in our previous papers (Fukunaga & Mifuchi, 1989a, b; Fukunaga *et al.*, 1991). Ribosomal RNA of *Treponema phagedenis* strain Reiter was extracted by the method described previously (Fukunaga *et al.*, 1990a). All other experimental conditions were as described by Fukunaga *et al.* (1990a, b).

Probes used in this study. The DNA fragment generated by *Ban*II digestion of the *rrs* gene of *Leptospira interrogans* strain Moulton (nucleotides 220 to 590, '*rrs* gene probe': Fukunaga *et al.*, 1990c) was used as an *rrs* gene probe. The fragment including the 3' part of the *rrl* gene of the same organism generated by *Bam*HI + *Bgl*II digestion (nucleotides 1587 to 2839, '*rrl* gene probe': Fukunaga *et al.*, 1989) was

used as an *rrl* gene probe. The DNA fragment including the entire sequence of the *rrf* gene from strain Moulton (*Eco*RI–*Hinc*II fragment, '*rrf* gene probe': Fukunaga *et al.*, 1990a) was used as an *rrf* gene probe. For the genomic hybridization, the DNA fragment generated by *Mlu*I + *Sac*II digestion of the *rrs* gene (Fig. 1) and the DNA fragment generated by *Sty*I digestion of the *rrl* gene of *B. burgdorferi* (Fig. 3) were used as probes to determine the number of rRNA genes in this organism. Each DNA fragment was labelled with deoxycytidine 5'-[α - 32 P]triphosphate ([α - 32 P]dCTP) [6000 Ci mmol $^{-1}$; (222 TBq mmol $^{-1}$); New England Nuclear] and a random primer labelling kit (Takara Shuzo Co.). Treponemal rRNA (a mixture of *rrs* and *rrl*) was partially hydrolysed as described by Suzuki *et al.* (1987) and labelled with T4-polynucleotide kinase and adenosine 5'-[γ - 32 P]triphosphate ([γ - 32 P]ATP) [6000 Ci mmol $^{-1}$; (222 TBq mmol $^{-1}$); New England Nuclear] and used as an rRNA probe.

Fig. 2. Pattern of hybridization and physical map of the recombinant phage DNA including the *rrl* and *rrf* genes. Phage DNA was digested and electrophoresed in a 1% agarose gel, blotted and hybridized with the treponemal rRNA probe (a). The enzymes used were: *BlnI* (lane 1), *BlnI* + *SalI* (lane 2), *EcoRV* (lane 3), *EcoRV* + *SalI* (lane 4), *PstI* (lane 5), *PstI* + *SalI* (lane 6), *NheI* (lane 7), and *NheI* + *SalI* (lane 8). Summarized restriction sites and location of the rRNA genes are indicated in (b).

Results

Southern hybridization patterns and physical maps of λ recombinant DNA containing the rrs gene for B. burgdorferi

The recombinant λ EMBL3 phages were obtained by plaque hybridization with a treponemal rRNA probe. Clone b2, carrying the *rrs* gene of *B. burgdorferi*, was selected by using the leptospiral *rrs* gene probe and used for further study. Recombinant phage DNA was purified, digested with several restriction enzymes, and electrophoresed in an agarose gel. The pattern of Southern hybridization for clone b2 is shown in Fig. 1(a). The physical map is shown in Fig. 1(c). This physical map was constructed by digestion, partial digestion and double digestion of recombinant DNA with each enzyme. The location of the *rrs* gene was determined by Southern hybridization using the treponemal rRNA probe.

The *Bam*HI fragment (6.2 kb) which carries the *rrs* gene was isolated and ligated into the pUC18 *Bam*HI site. The orientation of the *rrs* gene was determined by using the leptospiral *rrs* gene (*Ban*II fragment) as a probe. The *rrs* gene probe hybridized solely with 3.1 kb (right half of the *Bam*HI fragment) fragment generated by digestion with *Mlu*I + *Sal*I, but the same probe hybridized with both the 4.1 kb (left 2/3 of the *Bam*HI fragment) and the 5.4 kb (right 1/3 of the *Bam*HI

fragment + pUC18 DNA) fragments by *Sac*I + *Sac*II double digestion (Fig. 1b). All of these results clearly indicate the location and orientation of the *rrs* gene.

Southern hybridization pattern and physical map of insert DNA containing rrl and rrf genes

Recombinant phages which hybridized with the *rrl* gene probe were isolated. Clone 16.1 was selected and used for further experiments. DNA was digested, electrophoresed, blotted and hybridized with the treponemal rRNA probe. The pattern of hybridization and the location of the *rrl* gene are shown in Fig. 2(a, b). Genomic Southern hybridization with the *rrf* gene probe after *Hind*III digestion gave a single radioactive band of 2.0 kb (data not shown). Digestion of recombinant phage DNA with *Hind*III generated a 2.0 kb fragment that hybridized with both *rrl* and *rrf* probes. To determine the location and linkage of the *rrl* and *rrf* genes in *B. burgdorferi*, the 2.0 kb *Hind*III fragment of clone 16.1 was electroeluted from an agarose gel slice, purified, and subcloned into plasmid pUC18 (Fig. 2b).

Linkage of the rrl and rrf genes

The DNA fragment which carries the *rrl* gene and the entire *rrf* gene(s) for *B. burgdorferi* was ligated into the pUC18 *Hind*III site. Bacterial clones harbouring recom-

Fig. 3. Patterns of hybridization and physical maps of plasmid DNA containing the 2.0 kb *Hind*III fragment that carries the *rrl* and *rrf* genes of *B. burgdorferi*. The fragment was subcloned into the *Hind*III site of pUC18. Recombinant plasmid DNA was digested with the enzymes indicated, electrophoresed in 2% agarose gels, blotted, and hybridized with the leptospiral *rrf* gene probe (a, clone 16.1H3b; the pUC18 multicloning site is the right end of the fragment in c) and the leptospiral *rrl* gene probe (b, clone 16.1H5a; the pUC18 multicloning site is the left end). The enzymes used were: (a) *Eco*RI + *Bln*I (lane 1), *Eco*RI + *Bst*PI (lane 2), *Eco*RI + *Eco*RV (lane 3), and *Eco*RI + *Sty*I (lane 4); (b) *Eco*RI + *Hinc*II (lane 1), *Eco*RI + *Eco*RV (lane 2), *Eco*RI + *Sty*I (lane 3), and *Eco*RI + *Bln*I (lane 4). All of these hybridization results are summarized in (c).

binant plasmid DNA were selected and used for further experiments. Recombinant plasmid DNA was extracted and digested with several restriction enzymes, electrophoresed, blotted and hybridized with the probe. The pattern of hybridization of clone 16.1H3b with the *rrf* gene probe is shown in Fig. 3(a). The hybridization pattern with the *rrl* gene probe for clone 16.1H5a, which contains the same 2.0 kb *Hind*III fragment but in the opposite direction, is shown in Fig. 3(b). The restriction cleavage map and the results of hybridization are shown in Fig. 3(c). The results clearly show that the *rrf* gene(s) is located between the *Eco*RV and *Eco*RI restriction sites of the fragment. For analysis of the 3' end of the *rrl* gene, a 1.2 kb *Bam*HI–*Bgl*III fragment (nucleotides 1587 to 2839, leptospiral *rrl* gene) was labelled and used as a probe. As shown in Fig. 3(b), the largest fragment which solely hybridized with the probe was the 1.5 kb fragment generated by *Eco*RI + *Eco*RV digestion. The results indicate that the 3' end of the *rrl* gene exists around the

*Eco*RV cleavage site. Thus, all of these results indicate that the sequences for the *rrl* and *rrf* genes are close to each other.

Number of rRNA genes in *B. burgdorferi*

We determined the number of *rrs* and *rrl* genes in *B. burgdorferi* by hybridization of several restriction endonuclease digests of genomic DNA to the *rrs* and/or *rrl* gene probes. (Fig. 4). There are two radioactive bands in the *rrs* probe hybridization with the *Hind*III digest (lane 3). The results indicate that there are *Hind*III sites inside the gene, and that the *rrs* probe (*Mlu*–*Sac*II fragment, Fig. 1c) hybridizes with both *Hind*III fragments. Only one radioactive band appeared in all the other digests using the *rrs* or *rrl* probes. These results indicate that there is one gene for both *rrs* and *rrl* in this organism. The number of *rrf* genes is estimated as one because the *rrf*

Fig. 4. Genomic hybridization with the rRNA gene probe. Genomic DNA from *B. burgdorferi* was digested with *Bgl*II (lanes 1 and 5), *Eco*RI (lanes 2 and 6), *Hind*III (lanes 3 and 7), *Hinc*II (lane 4) and *Sty*I (lane 8). DNA digests were electrophoresed in a 1% agarose gel, blotted and hybridized to the *rrs* gene probe (*Mlu*I–*Sac*II fragment, Fig. 1c) (lanes 1–4) or to the *rrl* gene probe (*Sty*I fragment, Fig. 3c) (lanes 5–8). All other experimental conditions are described in Methods.

gene probe hybridized to the 0.5 kb *Eco*RV–*Hind*III fragment, but the exact number will not be known until further experiments, such as nucleotide sequencing, have been done.

Discussion

In most eubacterial species so far examined, the sequences for the three rRNA genes are closely linked in gene sets, in the order *rrs*–*rrl*–*rrf* (Krawiec & Riley, 1990). Some exceptions to this rule have been reported (Chen & Finch, 1989; Taschke *et al.*, 1986; Ulbrich *et al.*, 1984).

The location, linkage and number of rRNA genes in leptospirae were determined previously (Fukunaga & Mifuchi, 1989a, b; Fukunaga *et al.*, 1990a, b). We found that the leptospiral genome has two genes for *rrs* and *rrl*; there are two *rrf* genes in saprophytic leptospirae but one *rrf* gene in parasitic leptospirae (Fukunaga *et al.*, 1990a, b). Furthermore, there is no linkage among those rRNA genes.

In one of the rRNA gene sets for *T. phagedenis* strain Reiter, three rRNA genes are arranged in the order *rrs*–

rrl–*rrf* (Fukunaga *et al.*, 1992). The sequences corresponding to the three rRNAs are all contained in a 5.3 kb fragment, implying that the three rRNA genes are closely linked. The other gene sets, including the genes of the pathogenic treponema *T. pallidum*, also contain at least one copy of the *rrs*, *rrl* and *rrf* gene sequences arranged in the same order. This arrangement is identical to that found in other common eubacteria.

The organization of the rRNA genes in *B. burgdorferi*, described here, is different from that in any other spirochaete. There is no linkage between the *rrs* gene and the *rrl/rrf* genes in this organism. Recent reports have given evidence of an rRNA gene organization similar to that of *B. burgdorferi* in the thermophilic eubacterium *Thermus thermophilus* and the planctomycete *Pirellula marina*. In these organisms, the *rrs* gene has been shown to be locally and transcriptionally separate from the *rrl/rrf* genes (Hartmann & Erdmann, 1989; Hartmann *et al.*, 1991; Liesack & Stackebrandt, 1989). The coincidence of the organization of rRNA genes in these organisms is surprising because they are members of three different eubacterial phyla (Woese, 1987).

Spirochaetes are identified by their characteristic spiral shape and axially coiled fibrils. Despite these morphological similarities, there are differences among them regarding habitat, physiological and biochemical characters, and DNA base composition (Canale-Perola, 1984; Johnson, 1981; Johnson & Faine, 1984). Molecular methods have given new insight into the taxonomy of spirochaetes. Oligonucleotide cataloguing using a partial *rrs* sequence has shown that the *Leptospiraceae* and other spirochaetes, such as the genera *Treponema* and *Borrelia*, constitute a discrete phylogenetic branch (Fox *et al.*, 1980; Paster *et al.*, 1984; Woese *et al.*, 1983; Woese, 1987). Paster *et al.* (1991) recently showed that the members of the genus *Borrelia* are more closely related to the spirochaetes than to the treponemes.

The results reported here and our earlier work, therefore, confirm the phylogenetic situation of *Borrelia* and *Treponema* in the evolution of the spirochaetes. In this study, we found that *B. burgdorferi* has single *rrl* and *rrs* genes and that the usually closely linked *rrs* and *rrl* genes are separate in this organism. For *T. thermophilus* the existence of two different transcription units for *rrs* and *rrl/rrf* has been proven (Hartmann & Erdmann, 1989; Hartmann *et al.*, 1991); our results suggest that this is also likely for *B. burgdorferi*.

We have cloned the *rrs* and *rrl* genes of *B. burgdorferi* in different λ clones. The recombinant DNA fragment including the *rrs* gene hybridized with the *rrs* gene probe but not with the *rrl* gene probe, and the *rrl*-containing fragment hybridized only with the *rrl* gene probe. The results described in this paper, therefore, indicate that the *rrs* gene and the *rrl* gene are located separately on the

genome. Genomic hybridization of the same organism using *E. coli* rRNA as a probe has been reported (Postic *et al.*, 1990). In that study, both *rrs* and *rrl* probes hybridized to a single 9 kb *EcoRI* fragment. Our experiments also showed that the *rrs* and *rrl* gene probes hybridized with a single 9.5 kb *EcoRI* fragment. All of these results indicate that the distance between the *rrs* and *rrl* genes is not clear but each gene in *B. burgdorferi* is located separately in different *EcoRI* fragments of similar size.

Very little is known about gene organization in spirochaetes. Further studies on the expression and regulation of rRNA genes and comparison of their nucleotide sequences with those of other organisms should provide insight into the evolution of eubacteria.

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