

rRNA Gene Organization in the Lyme Disease Spirochete, *Borrelia burgdorferi*

JOHN J. SCHWARTZ, ANNA GAZUMYAN, AND IRA SCHWARTZ*

*Department of Biochemistry and Molecular Biology, New York
Medical College, Valhalla, New York 10595*

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Lyme disease is the most common vector-borne disease in the United States. The causative agent is the spirochete *Borrelia burgdorferi*. The copy number and organization of the genes encoding the rRNAs of this organism were determined. There is a single gene for 16S rRNA and two copies each of the 23S rRNA and 5S rRNA genes. All of the genes are located within a chromosomal fragment of approximately 9.5 to 10.0 kb. The 23S and 5S rRNA genes are tandemly duplicated in the order 23S-5S-23S-5S and are apparently not linked to the 16S rRNA gene, which is situated over 2 kb upstream from the 23S-5S duplication. The individual copies of the 23S-5S duplication are separated by a 182-bp spacer. Within each 23S-5S unit, an identical 22-bp spacer separates the 23S and 5S rRNA sequences from each other. The genome organization of the 23S-5S gene cluster in a number of different *B. burgdorferi* isolates obtained at a number of different geographical locations, as well as in several other species of *Borrelia*, was investigated. All isolates of *B. burgdorferi* tested displayed the tandem duplication, whereas the closely related species *B. hermsii*, *B. anserina*, and *B. turicatae* all contained a single copy of each of the genes. In addition, different geographical isolates of *B. burgdorferi* can be differentiated on the basis of a restriction fragment length polymorphism associated with the 23S-5S gene cluster. This polymorphism can be a useful tool for the determination of genetic relatedness between different isolates of *B. burgdorferi*.

Lyme disease is characterized initially as a skin lesion usually caused by the bite of an infected tick of the genus *Ixodes* (36). Lyme disease is now the most common vector-borne disease in the United States, with cases having been reported from 46 states (9). The etiologic agent of the disease, which is transmitted by a tick bite, is the spirochete *Borrelia burgdorferi* (6, 35). The organism grows slowly and requires a complex culture medium (3), and these factors have limited the ability to analyze the metabolism of the spirochete and its regulation.

rRNA genes for many other prokaryotic organisms have been studied. Typically, the genes for the rRNAs are organized into operons with the general structure 16S-23S-5S (33). The operon is transcribed as a single, large precursor RNA from a promoter(s) in front of the 16S gene, and this primary transcript is processed to yield the mature, individual rRNA molecules (33). In many species, tRNA genes are located in the spacer between the 16S and 23S genes (33). Although rare, exceptions to this characteristic rRNA operon structure have been demonstrated, particularly for organisms in which the rRNA genes are completely unlinked (12, 28).

In order to begin to characterize the molecular physiology of *B. burgdorferi*, the cloning and sequencing of its rRNA genes were undertaken. We report here the copy number and organization for the rRNA genes of *B. burgdorferi*. This organism contains a single 16S rRNA gene and two copies each of the 23S and 5S genes. The two latter genes are tightly linked and tandemly duplicated. In contrast, the 16S rRNA gene is physically separated from the tandem 23S-5S gene cluster by more than 2 kb. The duplication of the 23S and 5S genes is unique to *B. burgdorferi* and was not observed for the closely related species *Borrelia hermsii*, *Borrelia anse-*

rina, or *Borrelia turicatae*. On the basis of this finding, a simple restriction fragment length polymorphism (RFLP) assay which can specifically differentiate *B. burgdorferi* from other species of *Borrelia* was developed.

MATERIALS AND METHODS

Bacteria and plasmids. The bacteria and plasmids used in this study are listed in Table 1.

Enzymes and biochemicals. Restriction endonucleases were purchased from GIBCO-BRL or New England Biolabs and used according to the manufacturer's instructions. The 5'-end labeling kit and terminal deoxynucleotidyl transferase were obtained from GIBCO-BRL. Modified T7 DNA polymerase (Sequenase) and the Sequenase sequencing kit were obtained from U.S. Biochemicals. All radiochemicals were obtained from Dupont-New England Nuclear. Oligonucleotides were synthesized by the phosphoramidite method on a Milligen/Bioscience Cyclone DNA synthesizer.

Growth of *B. burgdorferi*. *B. burgdorferi* cells were grown at 34°C in BSKII medium (3) to a density of 10^7 to 10^8 cells per ml. Cells were harvested by centrifugation at $10,000 \times g$ and washed once with TE buffer (10 mM Tris-HCl [pH 8.0], 1.0 mM EDTA). Cell pellets were frozen and stored at -20°C until further use.

Preparation of rRNA. *B. burgdorferi* cells (0.5 g) were ground with an equal weight of alumina in 2 ml of TMAI buffer (10 mM Tris-acetate [pH 7.8], 40 mM magnesium acetate, 30 mM ammonium acetate, 7 mM β -mercaptoethanol, 0.1 mM EDTA), and ribosomes were prepared by successive centrifugation at $10,000 \times g$ for 10 min, $30,000 \times g$ for 45 min, and 45,000 rpm for 2.5 h in a Beckman 50Ti rotor. The resultant pellet was resuspended in 0.5 ml of TMAI buffer. A typical yield of 200 μ g of ribosomes was obtained from 0.5 g (wet weight) of *B. burgdorferi* cells. rRNA was prepared from the ribosome preparation by

* Corresponding author.

TABLE 1. Bacterial strains, isolates, and plasmids

Organism or plasmid	Strain or isolate	Biological origin (<i>Ixodes</i> species)	Geographic location	Source	
Bacteria					
<i>B. burgdorferi</i>	B31	Tick (<i>I. dammini</i>)	New York	American Type Culture Collection (ATCC 35210)	
	IP21	Tick (<i>I. persulcatus</i>)	St. Petersburg, Russia	A. Barbour	
	IP3	Tick (<i>I. persulcatus</i>)	St. Petersburg, Russia	A. Barbour	
	IP90	Tick (<i>I. persulcatus</i>)	Khabarovsk, Russia	A. Barbour	
	20004	Tick (<i>I. ricinus</i>)	France	J. Anderson	
	20047	Tick (<i>I. ricinus</i>)	France	J. Anderson	
	24330	Tick (<i>I. dentatus</i>)	New York	J. Anderson	
	24352	Tick (<i>I. dammini</i>)	New York	J. Anderson	
	HK	Blood	New York	C. Pavia	
	G1	Cerebrospinal fluid	Germany	A. Barbour	
	297	Cerebrospinal fluid	Connecticut	C. Pavia	
	<i>B. hermsii</i>	Serotype C			A. Barbour
	<i>B. turicatae</i>				A. Barbour
<i>B. anserina</i>				A. Barbour	
<i>E. coli</i>	DH5 α			GIBCO-BRL	
Plasmids					
pUC19				GIBCO-BRL	
pRC2	3.2-kb <i>Pst</i> I fragment of <i>B. burgdorferi</i> DNA in pUC19			This study	

phenol extraction as previously described (20). ³²P-labeled rRNA probes were prepared by 5'-end labeling with T4 DNA kinase and [γ -³²P]ATP as previously described (31).

Genomic DNA isolation and cloning. Genomic DNA was isolated by established procedures (32) with minor modifications. High-molecular-weight *B. burgdorferi* DNA (60 ng) was digested with *Pst*I and ligated into 20 ng of *Pst*I-cleaved pUC19 DNA. The resultant recombinant plasmid bank was transformed into *Escherichia coli* DH5 α , and transformants were selected on LB agar plates containing 100 μ g of ampicillin per ml. Recombinant clones containing rRNA genes were identified by colony hybridization (21) by using ³²P-labeled *B. burgdorferi* rRNA as the probe.

DNA sequencing. Plasmid DNA was isolated from a positively hybridizing clone by a rapid alkaline lysis procedure (8). The DNA sequence of the insert was determined by double-stranded dideoxy sequencing with modified T7 DNA polymerase (Sequenase) and [³⁵S]dATP α S (10). The sequences of both strands were determined, and areas of band compression were resequenced with 7-deazaGTP.

Southern blot analysis. High-molecular-weight chromosomal DNA from *B. burgdorferi* B31 or 297 was isolated as described above. For the other *Borrelia* spp., genomic DNA was obtained from 10-ml cultures essentially as described by LeFebvre et al. (16). High-molecular-weight DNA (10 μ g) was digested with 10 to 20 units of each restriction endonuclease at 37°C overnight, with buffers provided by the manufacturers. Digested DNAs were resolved by electrophoresis through a 0.85% agarose gel containing 0.5 μ g of ethidium bromide per ml in 1 \times TBE buffer (0.09 M Tris-borate, 2 mM EDTA [pH 8]) at 100 V for 4 h. Gels were soaked in 0.4 N NaOH for 10 min, and the DNA was transferred to a nylon membrane (GeneScreen; DuPont-New England Nuclear, Boston, Mass.) by capillary action over-

night, with 0.4 N NaOH as the transfer solution. Membranes were neutralized with 6 \times SSPE (6 \times SSPE is 0.9 M NaCl, 0.06 M NaPO₄, 6.0 mM EDTA [pH 7.4]) and allowed to air dry. Dried membranes were rehydrated in 25 mM sodium phosphate (pH 6), placed DNA-side down on the surface of a long-wave UV light box, and exposed for 2 min to facilitate covalent linkage of DNA to the membranes.

Prehybridization was performed for 1 h at 42°C in a solution containing 5 \times Denhardt's solution, 5 \times SSPE, 5% sodium dodecyl sulfate (SDS), and 10 μ g of denatured salmon sperm DNA per ml for 1 h. Hybridizations were accomplished by the addition of the radiolabeled probe (10⁶ to 5 \times 10⁶ cpm/ml) to the same solution and overnight incubation at 42°C. Blots were washed in 6 \times SSPE-0.5% SDS at room temperature three times for 15 min each, with a final 15-min wash in this solution at 42°C. Hybridized bands were visualized by exposure to Kodak XAR-5 film for 24 to 72 h.

Oligonucleotide probes were labeled at their 3' ends with [α -³²P]dATP and terminal deoxynucleotidyl transferase essentially as described previously (11). Unincorporated nucleotide was removed by NACS column chromatography according to the supplier's instructions (GIBCO-BRL, Gaithersburg, Md.). Oligonucleotides labeled in this manner had specific activities of 10⁹ to 10¹⁰ cpm/ μ g as determined by trichloroacetic acid-precipitable radioactivity.

PCR. The polymerase chain reaction (PCR) amplification was carried out in a 50- μ l solution containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.1% (wt/vol) gelatin, 100 μ M (each) dATP, dGTP, dCTP, and TTP, 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus), 15 pmol each of the indicated primers, and 20 to 50 ng of chromosomal DNA. The amplification reaction was carried out for 35 cycles in a DNA thermal cycler (Perkin-Elmer

TABLE 2. Oligonucleotides employed as probes and PCR primers

Oligo-nucleotide	Sequence	rRNA	Position in rRNA
BS1R	CCATGATGATTTGACGT	16S	1180–1196 ^a (inverse complement)
FS1	AGTCTGTTTTAAAAAGGCA	23S	820–837 ^a
P1	TAGTCGATGGGAAACGG	23S	1446–1462 ^a
P19	CCCGCGAACTCGCAGTA	5S	76–92 ^b (inverse complement)
P12	ACTCTTATTACTTTGACC	23S	1–18 ^b (inverse complement)
P3	GGAATCGCTAGTAATCG	16S	1326–1342 ^a
P8-28	AGAGTTTGATCCTGGCTTAG	16S	8–28 ^a

^a 16S and 23S rRNA sequences have been deposited in GenBank with accession numbers M88329 and M88330, respectively (31a).

^b This study.

Cetus), with a profile of denaturation at 94°C for 1 min, annealing at 43°C for 1 min, and extension at 72°C for 1 to 2 min, depending on the expected size of the amplified product. Following amplification, 5 to 10 μ l of the reaction mixture was subjected to electrophoresis on 0.85% agarose gels as described above.

Nucleotide sequence accession number. The sequence shown in Fig. 2 has been assigned EMBL accession number X57791.

RESULTS

rRNA gene copy number. The number of copies of the 16S and 23S rRNA genes in *B. burgdorferi* were determined by Southern blot hybridization with oligonucleotide probes which were complementary to specific sequences in either the 16S or 23S gene. The sequences of these probes are given in Table 2. Chromosomal DNA was isolated from *B. burgdorferi* B31 and digested with either *Eco*RI, *Hpa*I, *Pst*I, *Ava*I, *Bgl*II, or *Eco*RV. The digests were subjected to electrophoresis and blot hybridization. The identical blot was hybridized with either a 16S or 23S rRNA gene probe. In all of the digests, a single major hybridizing band was observed for the 16S rRNA probe (Fig. 1B). A fragment of approximately 9.4 kb was the main band in the *Eco*RI digest (lane 1). A second, minor band migrating at 1.7 kb was observed in this experiment but was absent in other blots. Minor bands were also observed in the *Pst*I digest (lane 3), but the major band is the one migrating at approximately 23 kb. By contrast, the 23S rRNA-specific probe hybridized to two bands in the *Hpa*I, *Pst*I, *Ava*I, and *Eco*RV digests (Fig. 1A). A common fragment of 3.2 kb was observed for each of these digests, in addition to a second fragment of variable size. The additional fragment of approximately 6.4 kb in the *Eco*RV digest is barely visible in this figure (lane 6) but was observed consistently in other experiments. A hybridization pattern similar to that found with the 23S rRNA probe was observed when the same blot was probed with a 5S rRNA-specific probe (data not shown). The results strongly suggest that there is a single 16S rRNA gene in *B. burgdorferi* but two copies each of the genes for 5S and 23S rRNA. It should be noted that all three probes appeared to hybridize to the same *Eco*RI fragment, which migrated with an apparent size of approximately 9.4 kb. This finding implies that all the rRNA genes may be contained in a region of the *B. burgdorferi* chromosome spanning 9.4 kb.

The 23S-5S rRNA genes are tandemly duplicated. The

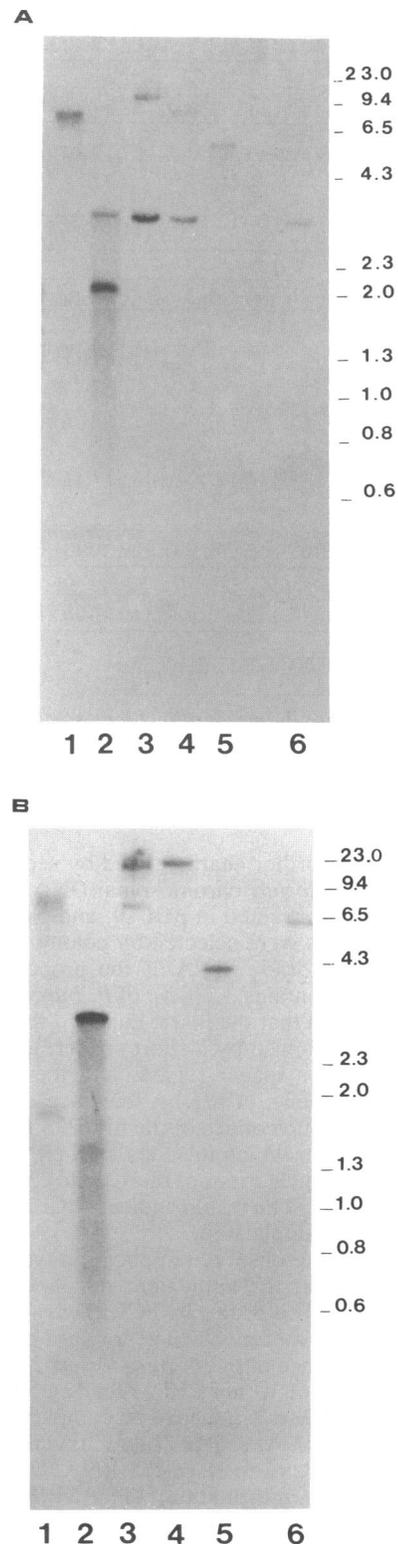


FIG. 1. rRNA gene copy number determination by Southern blotting. Total genomic DNA from *B. burgdorferi* B31 was digested with the indicated enzymes, and the digests were analyzed by Southern blot hybridization. Lanes 1 to 6 are digests with *Eco*RI, *Hpa*I, *Pst*I, *Ava*I, *Bgl*II, and *Eco*RV, respectively. Migration positions of DNA marker fragments (in kilobases) are indicated on the right. Blots were probed with the 23S (A) or the 16S (B) rRNA-specific oligonucleotide (FS1 or BS1R, respectively; Table 2).



FIG. 2. Sequence of *B. burgdorferi* chromosomal DNA fragment containing the 3' end of the 23S rRNA gene (copy 1) and the 5S rRNA gene and 5' end of the 23S rRNA gene (copy 2). The mature rRNA sequences are enclosed in boxes.

rRNA genes were further characterized by sequence analysis. A total *B. burgdorferi* chromosomal DNA library (from isolate 297) was constructed in pUC19, and clones containing rRNA sequences were selected by colony hybridization employing *B. burgdorferi* rRNA as the probe. One clone, designated pRC2, contains 3,240 bp of *B. burgdorferi* DNA. Sequencing revealed that the insert included the 3' 90% of a 23S rRNA gene, followed by a short spacer, a complete 5S rRNA gene, another spacer region, and the 5' 10% of a second 23S rRNA gene. A 616-bp sequence encompassing the 3'-terminal 129 nucleotides of the first 23S rRNA gene, a 316-nucleotide spacer which includes a 5S rRNA gene, and the 5'-terminal 171 nucleotides of the second 23S rRNA gene is presented in Fig. 2. The results indicate that the 23S rRNA genes are tandemly duplicated.

To confirm that the observed duplication was not caused by a cloning artifact, the equivalent region was amplified directly out of the chromosome by PCR. Two amplicon pairs were used in these experiments. The forward primer in both cases was the sequence of the *B. burgdorferi* 23S RNA from positions 1445 to 1461 (primer P1; Table 2). The 3' primer was either P19 (Table 2), which is complementary to a sequence in the 5S RNA, or P12 (Table 2), which is complementary to nucleotides 1 to 18 of 23S RNA. On the basis of the above sequence information, the P1-P19 primer pair should yield an amplified product of approximately 1.6 kb, and the P1-P12 primer pair should lead to the synthesis of a 1.8-kb product (see Fig. 5). An ethidium bromide-stained gel of the PCR reaction products is shown in Fig. 3 and indicates that the resulting amplification products are precisely those expected. It should be stressed that the only way for the P1-P12 amplicon pair to yield a discrete amplification product would be for there to be a direct tandem duplication of the 23S RNA gene. To our knowledge, this is the first

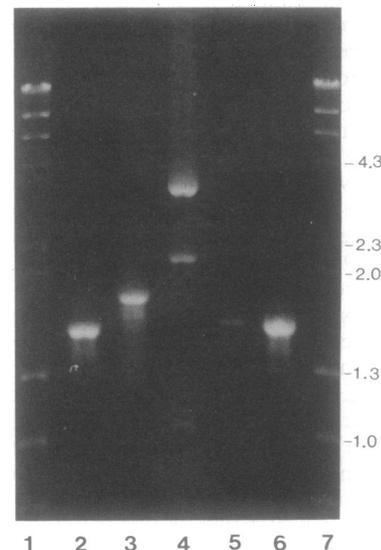


FIG. 3. Analysis of *B. burgdorferi* rRNA gene organization by PCR amplification. Genomic DNAs from *B. burgdorferi* B31, *B. hermsii*, or *B. anserina* were employed as templates in PCR amplification reactions with P1 and P19 (lanes 2, 5, and 6), P1 and P12 (lane 3), or P3 and P12 (lane 4) as primers. PCR amplification was carried out as described in Materials and Methods. Five microliters of the reaction mixture was electrophoresed on a 1% agarose gel and stained with ethidium bromide. Lanes 1 and 7 are a mixture of *Hae*III-digested ϕ X174 DNA and *Hind*III-digested λ DNA as molecular weight markers. DNA fragment sizes (in kilobases) are indicated on the right.

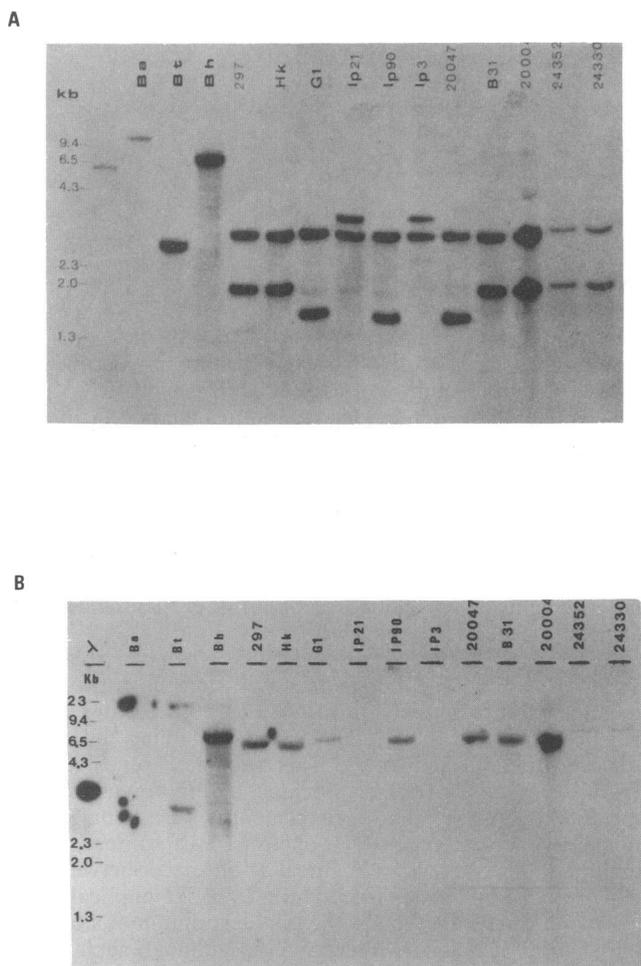


FIG. 4. RFLP analysis of *Borrelia* isolates. Total genomic DNA from the indicated *Borrelia* isolates (designated above each lane) was digested with *Hpa*I and analyzed by Southern blot hybridization. Migration positions of DNA fragment markers are indicated on the left. Ba, *B. anserina*; Bt, *B. turicatae*; Bh, *B. hermsii*. The blots were probed with a 5S (A) or 16S (B) rRNA-specific oligonucleotide (P19 or P8-28, respectively; Table 2).

description of such a direct rRNA gene duplication in prokaryotes.

Use of *B. hermsii* or *B. anserina* chromosomal DNA as the template in a PCR reaction with primers P1 and P19 resulted in a product with a size of approximately 1.6 kb (Fig. 3, lanes 5 and 6). This result indicates that these *Borrelia* species also contain a 23S-5S gene cluster. In contrast, use of the P1-P12 primer pair did not result in any amplification (data not shown). This finding is in agreement with the results of Southern blotting experiments (Fig. 4A), which indicated that there is only a single copy of both the 5S and 23S RNA genes in these *Borrelia* species.

Location of the 16S rRNA gene of *B. burgdorferi*. The results of the Southern blotting experiments in Fig. 1 indicate that there is a single copy of the 16S rRNA gene in *B. burgdorferi*. As already noted, oligonucleotide probes specific for each of the three rRNA species all apparently hybridized to the same *Eco*RI fragment with an approximate size of 9.4 kb. This finding suggests the possibility that all of the rRNA genes are physically associated in the *B. burgdor-*

feri genome. This likelihood was tested by the following PCR amplification experiment. Chromosomal DNA isolated from *B. burgdorferi* B31 was employed as the template in a reaction using an oligonucleotide with a sequence identical to nucleotides 1326 to 1344 of 16S RNA as the forward primer (P3, Table 2) and P12 as the 3' primer. The results are presented in Fig. 3 (lane 4) and indicate that an amplified product with an approximate size of 2.7 kb was obtained. This demonstrates that the 16S and 23S rRNA genes are, in fact, in physical proximity on the *B. burgdorferi* chromosome and that the 16S gene is located 5' to the 23S-5S duplication.

An RFLP characterizes different geographic isolates of *B. burgdorferi*. A large number of *B. burgdorferi* isolates have been obtained from tick vectors, animal hosts, and human clinical material from varied geographic locations. It was of interest to determine whether the unusual gene organization observed for *B. burgdorferi* B31 exists in other isolates of *B. burgdorferi*. Chromosomal DNA was isolated from 11 different *B. burgdorferi* isolates, as well as from *B. hermsii*, *B. anserina*, and *B. turicatae*. These DNAs were digested with *Hpa*I; the digests were separated on an agarose gel, blotted to nylon membranes, and probed with a 5S rRNA-specific oligonucleotide (P19, Table 2). The results are presented in Fig. 4A and reveal that all *B. burgdorferi* isolates contain two copies of the 5S rRNA gene. This finding is in contrast to those for the other *Borrelia* species, which have only one hybridizing band, suggesting that these species contain a single 5S RNA gene. When this blot was stripped and reprobed with an oligonucleotide specific for the 3' end of 23S RNA (P21, Table 2), an identical hybridization pattern was observed (data not shown).

Perhaps of greater interest is the RFLP associated with the 5S and 23S RNA gene cluster in *B. burgdorferi*. Three different RFLP patterns were observed. All the isolates share a common 3.2-kb band. This band represents a fragment which begins at nucleotide 1511 in the first gene copy of 23S RNA; includes the 23S-5S spacer, the 5S RNA gene, and the 5S-23S spacer; and ends at nucleotide 1510 in the second copy of the 23S RNA gene. In addition, there is a second band which contains the remainder of the second 23S RNA gene, the second copy of the 5S RNA gene, and the downstream region until the next *Hpa*I site. The nature of the second band appears to correlate with the geographic origin of the isolate. Thus, for all the North American isolates (HK, 297, B31, 24352, and 24330), the polymorphic band has an apparent size of 1.8 kb. The European isolates G1 and 20047 display a band of approximately 1.6 kb. Interestingly, three Russian isolates, which were initially obtained from the tick, *Ixodes persulcatus*, were included in this analysis. IP21 and IP3, which were isolated in the St. Petersburg area, share a common polymorphic band of approximately 3.6 kb. IP90, which was obtained from a tick in Russian Asia, has a pattern identical to that of the German (G1) and French (20047) isolates.

The blot shown in Fig. 4A was stripped and reprobed with a 16S rRNA-specific oligonucleotide (P8-28, Table 2). As expected, only one band hybridized to this probe in all the *B. burgdorferi* isolates tested (Fig. 4B). This hybridization pattern confirms the results presented in Fig. 1 and indicates that *B. burgdorferi* contains only one gene for 16S rRNA. Hybridization to the 16S rDNA-containing fragment in isolates IP21 and IP3 was quite weak; however, a band of >20 kb was observed. The reason for the weak hybridization is not known but is probably the result of removal of DNA from the filter by the stripping procedure. This result may

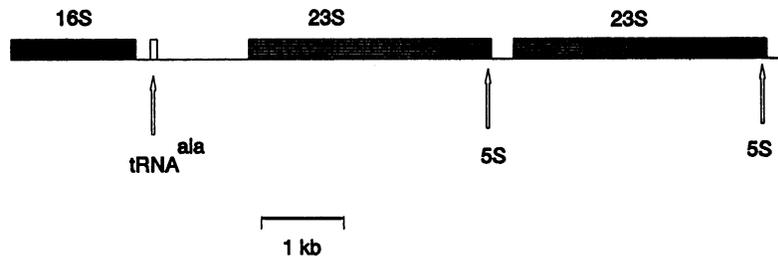


FIG. 5. Schematic diagram of chromosomal region containing *B. burgdorferi* rRNA genes.

represent an additional rDNA-linked polymorphism unique to these two isolates.

DISCUSSION

A schematic diagram of the organization for *B. burgdorferi* rRNA genes is presented in Fig. 5. The *B. burgdorferi* genome contains one gene for 16S rRNA and two genes each for both 23S and 5S rRNA. This conclusion is based on Southern blotting experiments (Fig. 1 and 4) which produced only one hybrid band with 16S rRNA probes but two bands with both 23S and 5S probes. Since the probes employed were relatively short oligonucleotides (17- to 20-mers), and none contained the restriction sites for the enzymes used in the digestion, the appearance of two bands in a blotting experiment is strong evidence for the existence of two separate genes for 23S and 5S rRNA. The arrangement depicted in Fig. 5 was confirmed by analysis of PCR amplification products produced by specific primer sets. Thus, the location of the 16S gene 5' to the 23S-5S duplication was demonstrated by PCR-directed synthesis of an approximately 2.7-kb amplicon when P3 (nucleotides 1326 to 1342 in 16S rRNA) and P12 (inverse complement of nucleotides 1 to 18 in 23S rRNA) were used as the 5' and 3' amplimers, respectively. The size of the amplicon indicates that the two genes are separated by approximately 2.4 kb.

The tandem duplication of the 23S-5S genes was confirmed in two ways. DNA sequencing of a cloned fragment containing a 5S rRNA sequence revealed that this sequence was flanked on either side by 23S rRNA-specific sequences (Fig. 2). That the arrangement detected in the cloned fragment was characteristic of the genomic organization, and not an artifact of the cloning itself, was also shown by PCR analysis. An amplified fragment of the expected size was obtained when P1 (nucleotides 1445 to 1461 in 23S rRNA) and P12 were used as the 5' and 3' primers, respectively. Only a gene arrangement such as that shown in Fig. 5 could result in the synthesis of this DNA fragment. It should be noted that physical mapping of the *B. burgdorferi* genome with several restriction enzymes, and probing for rRNA genes with total *E. coli* rDNA, yielded an rRNA gene organization essentially identical to that shown here (11a).

Two recent reports of Southern blotting experiments for several isolates of *B. burgdorferi*, including a number of isolates employed in the present study, have been presented (22, 26). Postic et al. utilized a mixture of *E. coli* 16S and 23S rRNA as the probes for the rRNA genes and presented schematic graphs for blots obtained with *EcoRI*-, *EcoRV*-, and *PstI*-digested *B. burgdorferi* DNA (26). Similarly, Marconi and Garon employed a single probe for all rRNA genes, in this case plasmid pKK3535, which includes the entire *rnmB* operon of *E. coli* (22). The *EcoRV* digests presented in these accounts show hybridizing bands of 6.4 and 3.2 kb. These

results are similar to those presented here. In the present study, oligonucleotide probes specific for each rRNA species were employed individually, and this method facilitated the determination of the copy number for each of the rRNA genes. The importance of this approach is indicated by the fact that Postic et al. concluded that all the rRNA genes are contained on a single large *EcoRI* fragment but also inferred that there are single copies of both the 16S and 23S rRNA genes (26). The data presented here demonstrate that the latter conclusion was not correct but rather that the 23S and 5S rRNA genes are duplicated.

In experiments not described here, we have isolated a series of overlapping clones encompassing the entire chromosomal region depicted in Fig. 5. Over 90% of this region has been sequenced, including 5' and 3' flanking and spacer regions (unpublished data). This sequence revealed that there is a gene for tRNA^{Ala} located 168 bp downstream of the 16S rRNA gene. This arrangement is a common feature in many other bacterial rRNA operons. Furthermore, it was confirmed that there is an identical 22-bp spacer between the 23S and 5S RNA genes in both copies of the duplication.

To our knowledge, this is the first description of a tandem duplication of this sort in eubacteria, although such rRNA gene organization has been described for eukaryotes (e.g., yeast cells) (34). rRNAs (and the genes encoding them) are a very highly conserved class of molecules, with respect to sequence, structure, genetic organization, and function (23, 33, 40, 41). In most eubacteria, the rRNA genes are arranged into single operons in the order 16S-23S-5S and the rRNA is usually synthesized as a single primary transcript (33, 34). The number and chromosomal location of the rRNA operons can vary greatly from species to species. For example, *E. coli* has seven copies of the rRNA operon, *Bacillus subtilis* has ten, and some species of *Mycoplasma* contain only one (33). There are, however, a number of exceptions to this characteristic operon organization. *Thermus thermophilus* and *Pirellula marina* have two copies of a 23S-5S operon and two copies of an unlinked 16S gene (14, 15, 18). *Leptospira interrogans* and *Thiobacillus acidophilum* contain separate operons for each of the different rRNA species (12, 28). In virtually all prokaryotes studied to date, regardless of operon organization and linkage, the number of gene copies for each rRNA species is identical. Presumably, this would ensure stoichiometric production of each of these RNA species for ribosome assembly, since one molecule of each RNA is present in functional ribosomes. Hence, *B. burgdorferi* rRNA operon organization is unique among eubacteria in two ways: it has two copies of the 23S and 5S genes but only one copy of the 16S gene, and the two copies of the 23S-5S gene cluster are arranged as a tandem repeat.

Transcription of the rRNA genes in *B. burgdorferi* is an open question. It seems highly likely that the 23S and 5S genes are transcribed to produce a single precursor RNA

French isolate 20004 yielded an RFLP pattern more characteristic of the North American isolates rather than of the European one. A similar grouping of 20004 with North American isolates has been reported by others (22, 29). It should also be noted that *B. burgdorferi* isolate IP90 displayed the same 5S rRNA RFLP pattern as the Western European G1 and 20047 (Fig. 4) as opposed to the distinct pattern observed with the other two Russian isolates (IP21 and IP3), which were isolated in St. Petersburg. Interestingly, Rosa et al. (29) included these two latter isolates in their PCR-based typing study and classified them with the other European isolates (including G1). This classification further demonstrates the increased capability of the RFLP analysis described here to differentiate closely related isolates.

The correlation of geographic isolates with a specific RFLP is particularly interesting in light of the reported differences in the clinical manifestations of Lyme disease in the United States and Europe. In the United States, there is a greater tendency for the disease to develop into arthritis, whereas neurological, cardiac, and cutaneous manifestations are more common in Europe (27). rRNA genes are certainly not likely to be determinants of bacterial pathogenesis. However, the presence of an RFLP which groups the American isolates together and separate from the European isolates may provide a helpful correlate to genetic relatedness which, in turn, may also be related to the pathogenicity of local populations of *B. burgdorferi*. While more isolates need to be tested, this RFLP should prove useful in epidemiologic studies of Lyme disease. Such investigations are currently in progress.

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