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Identification of Novel Insertion Elements, Restriction Fragment Length Polymorphism Patterns, and Discontinuous 23S rRNA in Lyme Disease Spirochetes: Phylogenetic Analyses of rRNA Genes and Their Intergenic Spacers in *Borrelia japonica* sp. nov. and Genomic Group 21038 (*Borrelia andersonii* sp. nov.) Isolates

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***Borrelia* spp. associated with Lyme disease possess an rRNA gene organization consisting of a single 16S rRNA gene followed by a spacer of several kilobases and a tandem repeat of a 23S (*rrl*)-5S (*rrf*) rRNA gene cluster. The restriction fragment length polymorphism (RFLP) patterns for these genes have been widely used to classify Lyme disease spirochete isolates. We analyzed the rRNA gene organization and sequences for two *Ixodes ovatus* isolates from Japan (IKA2 and HO14) and two group 21038 isolates associated with *Ixodes dentatus* ticks or rabbits from North America (isolates 21038 and 19857). This analysis revealed unique polymorphisms not previously described in other Lyme disease spirochete isolates. The molecular basis of these polymorphisms was determined by Southern blotting and PCR analyses. Only one continuous copy of the *rrl-rrf* gene cluster was identified in isolates IKA2, 19857, and 21038. The second *rrl-rrf* gene cluster is entirely absent from the IKA2 genome. In isolates 19857 and 21038, an intervening sequence is present, resulting in a fragment *rrlB* gene. The insertion site of this intervening sequence element differed in each isolate. While isolates 19857 and 21038 were found to carry a fragmented *rrlB* gene, they lacked *rrfB*. To determine if these rRNA polymorphisms were indicative of an underlying phylogenetic divergence, sequence analysis of the 16S rRNA (*rrs*) genes was conducted. The phylogenies inferred from *rrs* sequence analysis suggest that the polymorphisms resulted from recent mutational events. In addition, the phylogenetic analyses also support the proposed species status of *Borrelia japonica* sp. nov. and indicate that isolates of genomic group 21038 belong to a previously undescribed species for which we propose the nomenclature *Borrelia andersonii* sp. nov.**

Lyme disease, a multisystem disorder, was first recognized in North America in the 1970s (34). The causative agent was found to be a previously uncharacterized spirochete (5, 7) now known as *Borrelia burgdorferi*. Lyme disease results when the causative agent is transmitted to humans via the bite of infected ticks belonging predominantly to the *Ixodes ricinus* complex. Recent studies have shown that other tick species, including *Dermacentor variabilis*, *Dermacentor andersonii*, and *Amblyoma americanum*, may also play a role in Lyme disease transmission in North America (11). Prompted by the high degree of variability observed in the properties of *B. burgdorferi* isolates, several groups have sought to assess the phylogenetic diversity among isolates. Analysis of the 16S rRNA sequence (18–20, 22), DNA-DNA hybridization (4, 29, 30), arbitrarily primed PCR (35), and multilocus enzyme electrophoresis (6) studies have demonstrated that *B. burgdorferi* isolates fall into distinct phyletic clusters which are separated by significant evolutionary distances. Subsequently, *B. burgdorferi* has been subdivided into three species, *B. burgdorferi*, *B. garinii* (4), and *B. afzelii* (9). The distributions of these species differ, with *B. burgdorferi* occurring worldwide, while *B. garinii* and *B. afzelii* are confined to Europe and Asia (18).

As the analysis of spirochete isolates from *Ixodid* ticks has intensified, numerous isolates which are closely related to yet distinct from the *Borrelia* species associated with Lyme disease have been identified. Of interest to us have been isolates recovered from *Ixodes dentatus* ticks or their wildlife hosts in North America (1, 2) and *Ixodes ovatus* ticks in Japan (14). These differ from characterized Lyme disease spirochetes (LDS) in their immunoreactivity patterns (25), chromosome physical maps (10), and restriction fragment length polymorphism (RFLP) patterns for various genes (14). It has been proposed by Kawabata et al. (14) that isolates from *I. ovatus* be designated *Borrelia japonica* sp. nov. *B. japonica* isolates exhibit DNA-DNA homology values ranging from 51 to 65% when compared with other LDS isolates (29). In addition, group 21038 isolates from *I. dentatus* ticks in North America also appear to be a divergent group of spirochetes (3). Studies by Anderson et al. (1, 2) have shown that these isolates are antigenically variable, and DNA-DNA hybridization studies indicate that they are distinct, exhibiting homology values of 59 to 66% when their sequences are compared with those of other LDS isolates (3). Recently, Casjens et al. (10) have shown that isolates of this group exhibit unique chromosomal physical

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TABLE 1. PCR primers and probes

Probe or primer[a]	Sequence (5′–3′)	Target site[b]
1. 16S-F	TTGATCCTGGCTTAGAACT	16S bases 14–31
2. 16S-R	CGGGTTAGAATAATAGCTT	16S bases 1433–1451
3. 16S-F3	ACACCACCCGAGTTGAGGAT	16S bases 1401–1420
4. IS1(F)	GGGAAGCCTTCCTCAAGA	23S bases 2801–2818
5. IS2(R)	CTAGGCATTCACCATAG	23S bases 19–36
6. 23SF1	GAGTCTATGGTGAATGCCTAG	23S bases 15–35
7. 23SF2	CTAATACTAATTACCCGTATC	23S bases 2897–2915
8. 23SF3	AGGTTAGAAGTGTAAGTATAGC	23S bases 2860–2881
9. 23SR1	CTTGAGGAAGGCTTCCACTTA	23S bases 2796–2816
10. 23SR2	ACTCTTATTACTGGCACC	23S bases 1–18
11. 23SR3	CTTACCACGACCTTCTTCGCC	23S bases 45–65
12. JS2-R	TATGTGCTCTACTCCCTCTATTAA	23S bases 928–948
13. P11-R	GGCTGCTTCTAAGCCAA	23S bases 1134–1151
14. P5-R	CTTTCCAGTTTAACTTCTCTG	23S bases 1282–1302
15. P2-R	AACCCGTTTCCCATCGA	23S bases 1448–1464
16. P30-R	CGTACTTATCCCGAAGT	23S bases 1768–1785
17. 2615-R	CACGACGTTCTAAACCCAGCT	23S bases 2615–2635
18. PLF-R	ACCCAAACTGTCTCACGACG	23S bases 2630–2648
19. 5S19-R	CTCTTTTCTTTAACCACCA	5S bases 1–19
20. 5S-94F	GAGTAGGTTATTGCCAGGG	5S bases 94–112

[a] The letters F or R have been included in the primer names to indicate whether they are forward (sense) or reverse (antisense) primers, respectively. All primer sequences are presented 5′ to 3′.

[b] The numbering of the target sequences is based on the rRNA sequences of *B. burgdorferi* B31 (13).

maps. It should be noted that the designations group 21038 (10) and group 21123 are synonymous (3, 23). While *B. japonica* and group 21038 isolates are closely related to LDS, it is unclear if they are human pathogens. To date, isolates of these species have not been recovered from humans.

In the study described here we assessed the relationship of group 21038 and *B. japonica* isolates with other LDS through an analysis of rRNA gene arrangement and sequences. We have identified unusual and previously undescribed rRNA gene arrangements consisting of various insertions and deletions. The nature of the polymorphisms suggests that the rRNA gene RFLP patterns may be unsuitable for inferring the species identities of these isolates. Isolate 21038 was found to carry an interrupted 23S rRNA gene (*rrlB*) which is transcribed, yielding fragmented 23S rRNA. Sequence analysis of the 16S rRNA gene (*rrs*) and the *rrlA-rrlB* intergenic spacers demonstrated that group 21038 isolates (19857 and 21038) and *B. japonica* isolates (IKA2 and HO14) comprised different and distinct phyletic clusters separated by sufficient evolutionary distances such that species status is warranted. We propose that group 21038 isolates be designated *Borrelia andersonii* sp. nov.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Isolates HO14 and IKA2 were isolated from *I. ovatus* ticks in Hokkaido and Shizuoka, Japan, respectively (14). Isolates 19857 and 21038 were isolated by Anderson et al. (2) in New York from the kidney of a cottontail rabbit (*Sylvilagus floridanus*) and *I. dentatus* larvae, respectively (2). Comparative reference strains for phylogenetic analyses included *B. burgdorferi* B31 and Sh-2-82; *B. garinii* VSBP, G1, and G2; *B. afzelii* VS461, J1, and R-IP3; and *B. anserina*; all strains have been described previously (23). Isolates were cultivated in BSK-H media (Sigma) supplemented with rabbit serum (Sigma) to 6% at 34°C.

PCR analyses of rRNA gene organization. Thirty cycles of PCR were performed in a volume of 25 μl by using the GeneAmp kit (Perkin-Elmer Cetus) and the primers described in Table 1. The relative binding sites of these primers are shown in schematic form in Fig. 1. Amplification templates were obtained by boiling 100 μl of cell culture as described previously (16) or from isolated DNA (23). Cycle conditions were 95°C for 45 s, 50°C for 30 s, and 72°C for 60 s. To amplify fragments larger than 2 kb, extension times of 1.5 to 2 min were used. Ten microliters of each reaction mixture were analyzed by electrophoresis in 1% agarose gels with TAE (40 mM Tris-acetate, 2 mM EDTA, pH 8.5) buffer.

Analysis of rRNA gene RFLP patterns. Purified DNA (23) was digested, fractionated by electrophoresis in 0.8 or 1.0% agarose gels, vacuum blotted onto Hybond N membranes (Amersham), fixed by UV cross-linking, and probed with a variety of rRNA directed probes (Table 1; Fig. 1). Probes were 5′ end labeled with polynucleotide kinase and [γ-^{32}P]ATP (6,000 Ci/mmol; DuPont-NEN). Hybridizations were conducted at 37°C in a Hybaid hybridization oven (Labnet) by using the buffers and wash conditions described previously (24). Prehybridizations were carried out for 5 min, and hybridizations were carried out for 4 h.

DNA sequencing and phylogenetic analysis. Two approaches were used to determine the 16S rRNA sequences: reverse transcriptase primer extension in the presence of dideoxynucleotides and cycle sequencing of PCR-generated templates. Reverse transcriptase primer extension methods, including RNA isolation, have been described previously (20). PCR-generated templates were purified with the Magic PCR Prep system (Promega) and were sequenced with the double-stranded DNA Cycle Sequencing System (Gibco-BRL) as recommended by the manufacturer. Reactions were electrophoresed in 6% acrylamide–8 M urea gels at 85 W.

Phylogenetic analyses were performed with masked 16S rRNA sequences by using the PHYLIP package as described previously (20). Briefly, the percent 16S rRNA sequence similarity values, calculated by the DNADIST program, were used to construct a gene tree with the FITCH program. Confidence intervals were assessed with the DNABOOT program.

Northern blot analyses. Total cellular RNA (0.5 μg) was fractionated in a 1.2% formaldehyde agarose gel, transferred onto a Hybond N membrane by vacuum blotting, and hybridized with either the 23SR3 or the P2 probe at 42°C under the conditions described previously (21). Prehybridizations were performed for 10 min, and hybridizations were carried out for 4 h. Reagents were prepared with diethylpyrocarbonate-treated water or were treated directly with diethylpyrocarbonate.

Nucleotide sequence accession numbers. All nucleotide sequences reported here have been deposited in GenBank.

RESULTS AND DISCUSSION

Analysis of rRNA gene polymorphisms in *B. japonica* and group 21038 isolates. Schwartz et al. (32) and Fukunaga et al. (12) have demonstrated that LDS possess an unusual rRNA gene organization composed of a single 16S rRNA (*rrs*) gene, followed by a spacer of several kilobases and then a tandem repeat of a 23S (*rrl*)-5S (*rrf*) gene cluster. Initial rRNA gene RFLP pattern analyses with a probe carrying the *rrnB* operon from *Escherichia coli*(pKK3535) indicated that alternative rRNA gene arrangements are present in some of the isolates investigated here and that these patterns may not be species specific. Although closely related, *B. japonica* HO14 and IKA2 each exhibited different patterns with *Eco*RV-, *Hin*dIII-, or *Hae*III-restricted DNA. The HO14 pattern was the same as that seen in other *B. japonica* isolates (29), whereas the pattern for IKA2 was distinctly different (data not shown). The patterns for the *I. dentatus*-associated isolates differed from each other, as well as from those of other LDS species (data not shown).

To characterize the molecular basis of these atypical RFLP patterns, Southern blotting was performed with probes specific for different regions of the *rrl*, *rrf*, and *rrs* genes (Fig. 1; Table 1). The 16S rRNA-specific probe (16SF3) hybridized with a single band in all isolates regardless of the restriction enzyme used, indicating the presence of a single *rrs* gene (Fig. 2). This is consistent with that described by Schwartz et al. (32) for other LDS isolates. With *Eco*RV-cut DNA, the hybridizing band was ~6,400 bp in isolates HO14 and IKA2 and ~6,000 bases in 19857 and 21038. Since a probe directed at the 5′ end of the *rrl* gene (23SF1) also bound to this fragment (Fig. 2), it can be concluded that this fragment carries the complete *rrs-rrl* intergenic spacer. The 23SF1 probe also bound to a second fragment in isolates HO14, 19857, and 21038 (Fig. 2) consistent with that expected in view of the tandem repeat of the *rrl-rrf* gene cluster. However, in IKA2 only one hybridizing band was observed (Fig. 2), suggesting a lack of the *rrl-rrf* gene duplication.

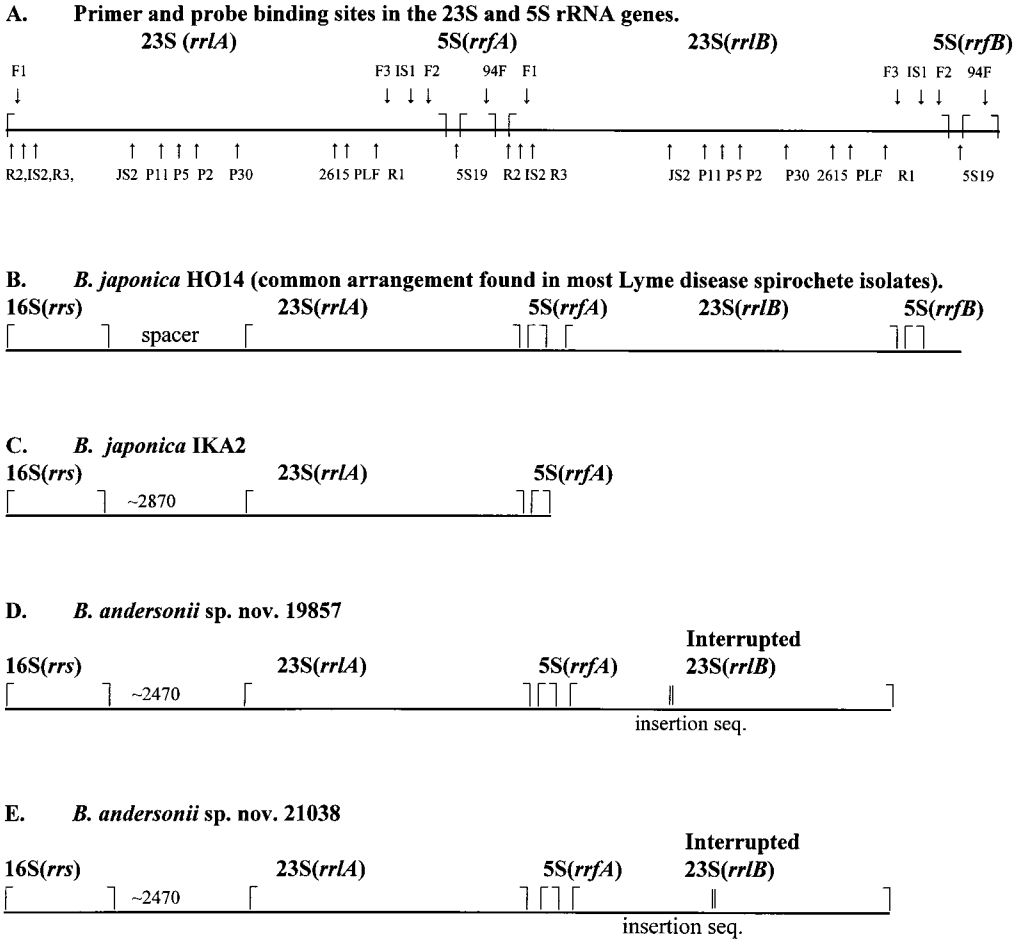

FIG. 1. Probe and primer binding sites and rRNA gene organization in *B. japonica* (HO14 and IKA2) and *B. andersonii* sp. nov. (19857 and 21038). (A) Arrangement of the 23S and 5S rRNA genes observed in isolates HO14. This arrangement is typical of that in other LDS isolates. Forward or sense primers are indicated above the line, while reverse primers are indicated below the line. Note that the primer names were abbreviated slightly from those listed in Table 1 in order to fit into the figure. The precise binding sites of all primers are listed in Table 1. (B through E) rRNA gene organizations determined for the listed isolates. The data used to determine these arrangements are described throughout the Results and Discussion section.

The locations of the *Eco*RV sites responsible for the generation of the 6.4- and 6.0-kb fragments discussed above were identified by sequence analysis. This allowed for the determination of the size of the *rrs-rrlA* intergenic spacer since the number of nucleotides contributed by *rrs* and *rrlA* to this fragment could be inferred. We estimate the spacer to be 2,870 and 2,470 bp in *B. japonica* and group 21038, respectively. While spacer size was the same in isolates within each genomic group, it differed between groups and from that reported in other LDS species. Sequence analysis by Gazumyan et al. (13) demonstrated that the spacer is 3,052 bp long in *B. burgdorferi*, while in *B. garinii* and *B. afzelii* isolates it is 5,250 bp (27). Thus, variations in the spacer likely have arisen after the divergence of the LDS into several species.

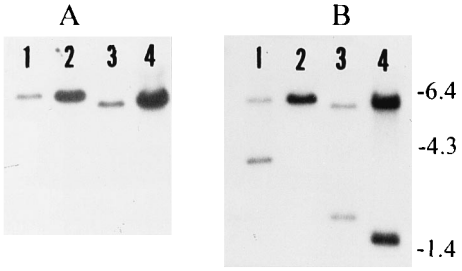

FIG. 2. Southern blot analysis of *Eco*RV-restricted DNA probing for the 16S and 23S rRNA genes. All methods are described in the text. (A) The blot was screened with 100 ng of radioactively labeled 16SF3 probe which targets the 3′ end of the *rrs* gene. (B) The blot was screened with the 23SF1 probe which targets the 5′ end of the *rrlA* and *rrlB* genes. Restricted DNAs from isolates HO14, IKA2, 19857, and 21038 were electrophoresed in lanes 1 through 4, respectively. DNA size standards are indicated on the right (in kilobases).

To assess the copy numbers of the 23S and 5S rRNA genes, restricted DNA was probed with gene-specific probes. Probes specific for the 5S rRNA gene hybridized with a single band in isolates IKA2, 19857, and 21038 and with two bands in HO14, irrespective of the restriction enzyme used (for brevity, Southern blot results obtained with *Eco*RV-restricted DNA are presented in schematic form in Fig. 3). These data indicate the presence of a single *rrf* copy in 19857, 21038, and IKA2 and two copies in HO14. The occurrence of a single *rrf* is unique and has not been reported for any other LDS isolate. Southern blot analysis of the *rrl* genes revealed additional features unique to IKA2. In this isolate, all *rrl* probes detected only a single hybridizing fragment, suggesting that IKA2 may be lacking a second *rrl* gene as well. Interestingly, while two hybridizing

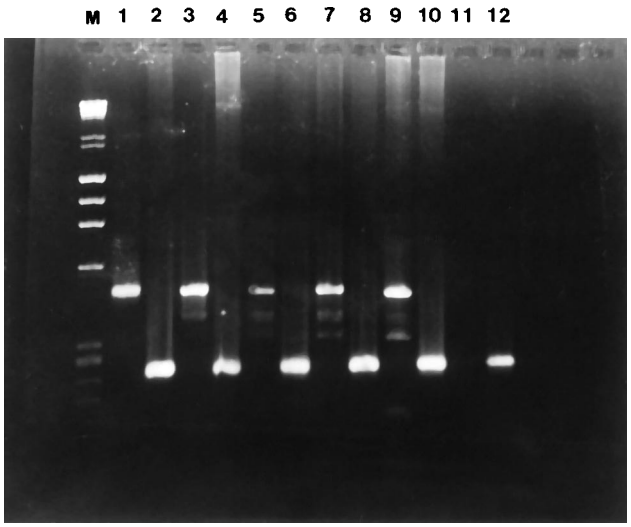

FIG. 3. PCR analysis of rRNA gene organization. DNAs from isolates *B. burgdorferi* B31 (lanes 1 and 2), *B. garinii* IP90 (lanes 3 and 4), *B. afzelii* VS461 (lanes 5 and 6) and J1 (lanes 7 and 8), and *B. japonica* HO14 (lanes 9 and 10) and IKA2 (lanes 11 and 12) were amplified with primer set IS1-IS2 (lanes 1, 3, 5, 7, 9, and 11) or IS1-5S19 (lanes 2, 4, 6, 8, 10, and 12), as described in the text. The DNA molecular weight markers (lane M) are a mix of bacteriophage lambda *Hin*dIII and ϕX174 *Hae*III-digested DNAs.

bands were observed in isolates HO14, 19857, and 21038, there was variation in the sizes of the hybridizing fragments. Those for HO14 were typical of those observed in other *B. japonica* isolates (29), whereas the patterns observed for 19857 and 21038 were different from each other and from those found in other group 21038 isolates.

Anomalies in the *rrl* genes in isolates 19857, 21038, and IKA2 were further evident upon PCR analysis. Attempts to amplify a fragment extending from the 3′ end of *rrlA* into the 3′ end of *rrlB* in these isolates were unsuccessful (for PCR data, see Table 2). However, when the same forward primer was used with reverse primers targeting the 5′ region of *rrlB*, product was obtained with 19857 and 21038, but not with IKA2 (Fig. 4). These data suggest that (i) isolate IKA2 lacks the *rrl-rrf* duplication and (ii) while 19857 and 21038 carry both the 5′ and 3′ regions of the *rrlB* gene, the gene may be interrupted by an insertion element, the size of which is large enough to prevent PCR amplification under the conditions used. It is possible that significant sequence divergence between *rrlA* and *rrlB* could be responsible for our failure to achieve amplification with certain primers and may have generated unique *Eco*RV sites in *rrlB*. However, it is unlikely that sequence divergence in the *rrlB* gene itself could account for the observed PCR and Southern blot results for the following reasons. Since reverse primers which did not yield PCR product hybridized with two fragments in Southern blots, we can conclude that these primers can bind to the intended target sites in both *rrlA* and *rrlB*. While failure to be amplified by PCR can be due to a single-base mismatch at the 3′ end of the primer, the likelihood of this occurring in all primers tested is very low. In addition, Gazumyan et al. (13) have previously demonstrated that in *B. burgdorferi* B31 there is no microheterogeneity between *rrlA* and *rrlB*. On the basis of these considerations, we favor the hypothesis that *rrlB* harbors an insertion. If this is the case the insertion sequence must possess internal *Eco*RV sites in order to account for the hybridization patterns seen in Fig. 3. This suggestion is supported by the observation that in 21038 the sum of the sizes of the *Eco*RV fragments carrying the 5′ and 3′ regions of *rrlB* (after subtracting the size of the fragment contributed by the intergenic region) is greater than the size of the gene itself. The different hybridization patterns seen in 19857 and 21038 would also suggest that the insertions map at different locations in these isolates. This point is highlighted by the fact that while probes P5 and P2 bound to the same 1.6-kb fragment in 19857, they bound to two different fragments (of 2.8 and 1.5 kb) in 21038 (Fig. 3). Hence, in contrast to isolate IKA2, which appears to lack the second 23S-5S rRNA gene cluster, the data suggest that the *rrlB* polymorphisms in isolates 19857 and 21038 are the result of an insertion or recombination event.

TABLE 2. Summary of PCR analyses of rRNA gene arrangement in LDS isolates[a]

Primer pair	*B. japonica* sp. nov		Genospecies 21038		Predicted amplicon size (bp)
	HO14	IKA2	19857	21038	
IS1-5S19	+	+	+	+	166
IS1-IS2	+	−	+	+	478
IS1-JS2	+	−	+	+	1,390
IS1-P11	+	−	+	+	1,593
IS1-P5	+	−	−	+	1,744
IS1-P2	+	−	−	−	1,906
IS1-P30	+	−	−	−	2,227
IS1-PLF	+	−	−	−	3,090
16SF-16SR	+	+	+	+	1,437

[a] The amplification products, obtained with primer pairs designed to amplify from the first 23S rRNA gene with the IS1 primer into the second 23S rRNA gene, contain approximately 450 bases derived from 23S gene 1, the 5S rRNA gene, and the intergenic spacer. Minus or plus signs indicate that amplification products either were not or were detectable in agarose gels upon staining with ethidium bromide, respectively.

Mapping of IS sites in *rrlB* of isolates 21038 and 19857. PCR analyses were used to define the location of the insertions in *rrlB* in 19857 and 21038. A forward primer (IS1) which hybridizes with the 3′ end of *rrlA* was used in combination with a series of reverse primers which progress through the 23S rRNA genes, 5′ to 3′. If the insertion sequence (IS) elements are within a size range that will allow for PCR amplification across the region, then PCR products larger than the size predicted for an uninterrupted 23S rRNA gene should be observed. Alternatively, that product would be absent if the IS element is too large to allow for efficient amplification under the PCR conditions used. Isolates 19857 and 21038 yielded fragments of the expected size when forward primer IS1 was used in conjunction with reverse primers complementing sequences up to base 1150. However, isolate 19857 did not yield a PCR product with reverse primer P5, which binds to bases 1282 to 1303. In contrast, isolate 21038 produced a product with P5 but not with P2, which targets bases 1448 to 1464. These PCR data are consistent with the hybridization data described above and support the idea that the *rrlB* genes of isolates 21038 and 19857 contain insertions. The insertion sites are unique in each isolate, occurring between bases 1151 and 1302 in 19857 and between bases 1300 and 1450 in 21038. Figure 1 presents a schematic depiction of the deduced rRNA gene arrangements.

Transcription of discontinuous 23S rRNA. The occurrence of fragmented rRNA has been demonstrated previously in some isolates of *Campylobacter jejuni* (15), *Salmonella* spp. (8), *Yersinia enterocolitica* (33), and *Leptospira* spp. (31). In these organisms the discontinuous rRNA genes are transcribed, with subsequent excision of the intervening sequence element from the transcript. As a result, the rRNA species present are frag-

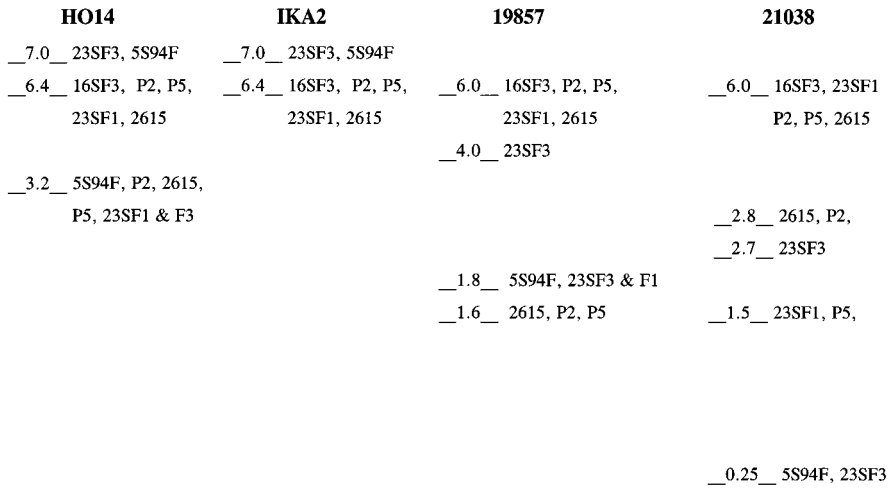

FIG. 4. Summary of Southern blot results in schematic form. The combined results of RFLP pattern analyses with a variety of rRNA gene probes (described in Table 1) are depicted. The schematic depicts hybridization fragments, with their approximate sizes (in kilobases) indicated. Probes which bind to a given fragment are indicated to the right of the fragment, and the isolates probed are listed above.

mented into smaller species, with their termini defined by the point of excision or processing of the intervening sequence. In contrast to the situation seen in *C. jejuni*, in which all of the 23S rRNA gene copies are fragmented, in 19857 and 21038 only the *rrlB* genes are interrupted. Agarose gel electrophoresis of the isolated RNA revealed the presence of abundant RNA molecules in 21038 of approximately 1,600 and 1,300 bases. Similar species were not observed in 19857 or any other LDS isolate tested. The nature of these RNA species in 21038 was assessed by Northern blotting with probes directed at the 5′ (23SR3) and 3′ (P2) ends of the 23S rRNA (Fig. 5). Both probes hybridized with a 2,900-base RNA molecule which corresponds to typical unfragmented 23S rRNA. However, probes P2 and 23SR3 also hybridized with RNAs of approximately 1,600 and 1,300 bases, respectively (Fig. 5). In all other isolates tested these probes hybridized only with a 2,900-base RNA molecule. It can be concluded that the 1,300-base fragment carries the 5′ portion of the 23S rRNA molecule, while the 1,600-base fragment carries the 3′ portion of the molecule. This is consistent with the PCR data described above. Furthermore, the sum of the sizes of these fragments suggests that the mature processed, fragmented rRNA molecules carry little, if any, sequence derived from the insertion element. This is the first demonstration of fragmented rRNA in *Borrelia* species. In addition, the occurrence of fragmented rRNA in 21038, which is derived from *rrlB*, provides evidence that both *rrlA* and *rrlB* are transcribed in LDS.

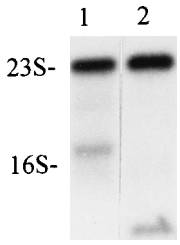

FIG. 5. Northern blot analyses demonstrating the presence of discontinuous 23S rRNA in isolate 21038. All methods were as described in the text. Lanes 1 and 2, total RNA isolated from 21038 probed with the P2 and 23SR3 probes, respectively, for 4 h and exposed to film for 15 min.

Analysis of rRNA intergenic spacer sequences. The organization of the intergenic region between *rrlA* and *rrlB* was evaluated by sequencing. It has been suggested that the *Dra*I and *Mse*I RFLP patterns of PCR-amplified fragments of this spacer region may be of utility in epidemiological surveys (28). The *Mse*I restriction map of this region indicates that isolates 19857 and 21038 would yield the type L RFLP pattern described by Postic et al. (28). These isolates do not possess any *Dra*I sites. These inferred RFLP patterns are consistent with those observed for other isolates of group 21038. Since isolate IKA2 could not be amplified because it lacks the *rrlB* gene, RFLP data for the intergenic region could not be obtained for this isolate. The RFLP patterns inferred from the HO14 sequence determined here are consistent with that reported by Postic et al. (28) for other *B. japonica* isolates.

The *rrlA-rrlB* intergenic sequences were compared with those from *B. burgdorferi* B31 (13), *B. japonica* HO14, and the group 21038 isolate, 21133 (Fig. 6). The *rrlA-rrfA* spacer was found to be 22 nucleotides in length, consistent with that reported for the B31 sequence (13). This spacer was not sequenced by Postic et al. (28) in their analysis of the rRNA intergenic regions. In the isolates analyzed here this spacer exhibited only minor sequence variation and was conserved in size. The close juxtaposition of the *rrl* and *rrf* genes in these isolates supports the suggestion that these genes are probably cotranscribed (13).

The *rrfA-rrlB* intergenic spacer was found to be variable among LDS species. With the exception of small insertions and deletions, the sequences of this spacer in isolates 19857 and 21038 were generally similar to those reported for other group 21038 isolates such as 21133 and 19952 (28). A unique feature of the intergenic spacer in isolate 19857 is the presence of the sequence AGAT, which is tandemly repeated eight times. Upon alignment these repeats appear to represent an insertion. More than one copy of this sequence, tandemly repeated, also occurs in 21133 and 19952. A second repeated sequence, GGAAGAT, occurs in the intergenic region of isolate 19857, 8 nucleotides 5′ of the *rrlB* gene. This sequence is repeated twice and occurs in a region which is conserved in other isolates.

Determination and phylogenetic analysis of 16S rRNA sequence. To determine if the observed RFLP patterns were reflective of phylogenetic relationships, we performed 16S

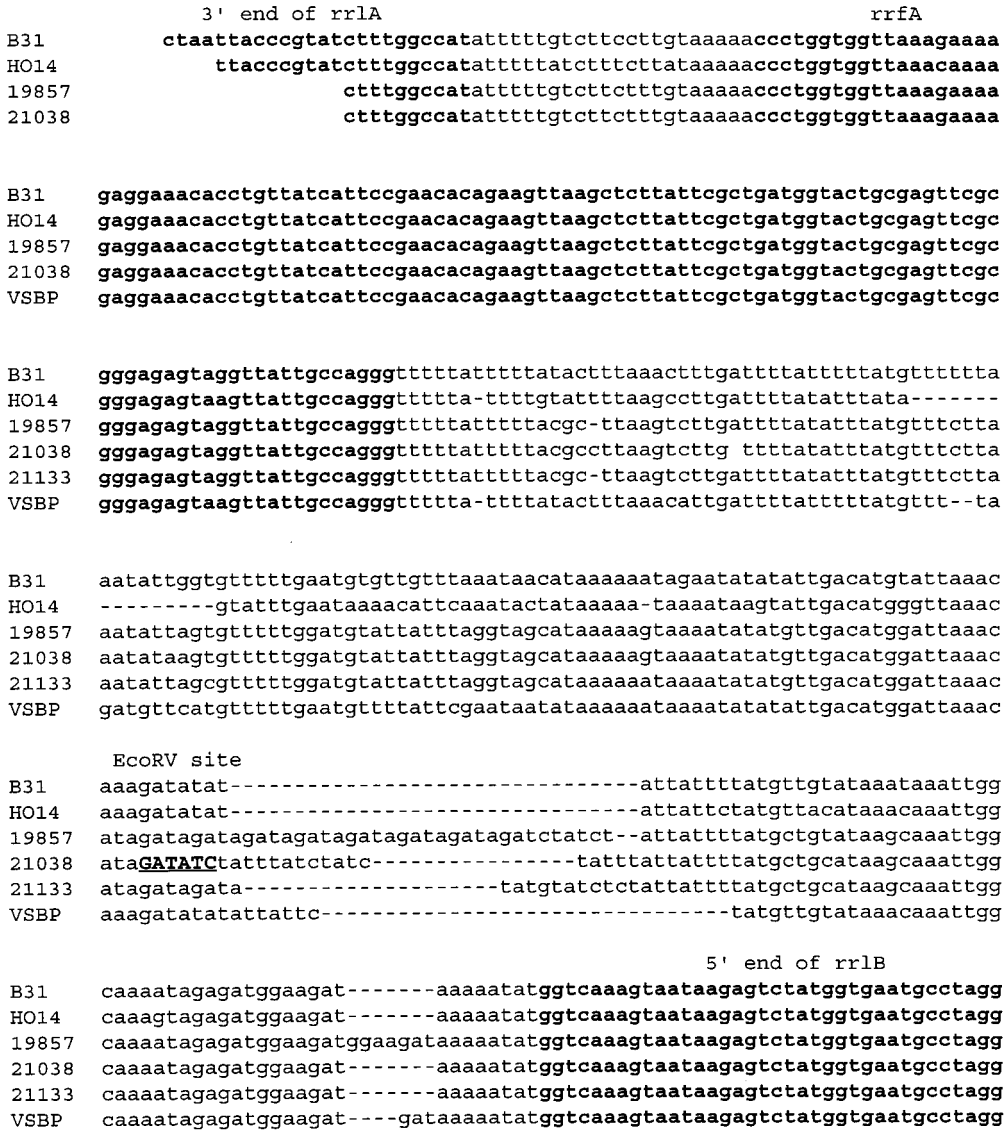

FIG. 6. Alignment of the intergenic sequence between the *rrlA* and *rrlB* genes. Sequences were determined as described in the text and were aligned. The 3′ region of the *rrlA*, the *rrfA* gene, and the 5′ end of *rrlB* are indicated in boldface type. A unique *Eco*RV site in the sequence from isolate 21038 is indicated in boldface type and is underlined. Gaps, introduced upon alignment, are indicated by dashes. For comparative purposes, the sequence from *B. garinii* VSBP was included.

rRNA sequence analysis. *rrs* sequences were determined and compared with those from other representative LDS isolates, and percent sequence similarity values were obtained (Table 3). The *B. japonica* isolates HO14 and IKA2 and the group 21038 isolates 19857 and 21038 were found to have sequence similarity values of 99.4 and 99.8%, respectively, for each pair, indicating close relationships. When compared with 16S rRNA sequences from representative LDS isolates, both groups were found to be significantly divergent. The Japanese isolates HO14 and IKA2 were also found to be distinct from *B. afzelii* J1 (18), an isolate from an *Ixodes persulcatus* tick from Japan, demonstrating phylogenetic diversity in LDS isolates originating from Japan. The calculated evolutionary distances for both of these groups of isolates from other LDS species are of a magnitude similar to the interspecies evolutionary distances between *B. burgdorferi*, *B. garinii*, and *B. afzelii* (18–20) and are consistent with those obtained by DNA-DNA hybridization (14, 28, 29).

A phylogenetic tree was constructed by using the calculated evolutionary distances as described previously (20) (Fig. 7). The branching patterns of the tree were evaluated by bootstrap analysis and were found to be highly supported. In general, proposals for the recognition of isolates as distinct species should be preceded by multiple independent analyses by the major approaches for species differentiation. These criteria have clearly been met with regard to *B. japonica* sp. nov and genospecies 21038. As discussed earlier, numerous isolates of these genetic groups have been investigated by a variety of approaches by other laboratories (1, 14, 16, 23, 25, 26, 28, 29). The interpretations of these data are in excellent and complete agreement and provide strong support for the recognition of these two phyletic clusters as distinct *Borrelia* species. We propose that isolates belonging to the genospecies 21038 be reclassified as a distinct species, *Borrelia andersonii* sp. nov. This name was chosen in honor of John Anderson, who was the first person to isolate and characterize this species.

TABLE 3. 16S rRNA percent sequence similarity values

Species[a]	% Sequence similarity										
	IKA2	19857	21038	G1	G2	461	IP3	J1	B31	SH2	BA
1. HO14	99.4	98.4	98.5	98.6	98.6	99.0	98.5	99.0	98.5	98.3	95.4
2. IKA2		98.7	98.8	98.8	98.8	98.6	98.8	98.6	98.6	98.4	95.7
3. 19857			99.8	98.8	98.8	98.7	99.0	98.8	98.6	98.6	96.0
4. 21038				98.9	98.9	99.0	99.2	99.0	98.9	98.7	96.3
5. G1					100	98.5	98.9	98.7	99.0	98.9	95.7
6. G2						98.5	98.9	98.7	99.1	99.0	95.7
7. 461							99.6	99.4	98.3	98.1	95.8
8. IP3								99.5	98.7	98.6	96.3
9. J1									98.4	98.2	95.8
10. B31										99.3	95.8
11. SH2											95.7
12. BA											

[a] Species identities for each isolate are as follows: HO14 and IKA2 are *B. japonica*; 19857 and 21038 are genomic group 21038 (or *B. andersonii*), G1 and G2 are *B. garinii*; 461, IP3, and J1 are *B. afzelii*; and B31 and SH2 are *B. burgdorferi*. Abbreviations are as follows: 461 is for VS461, IP3 is for R-IP3, and BA is for *B. anserina.*

Conclusions. The study presented here was undertaken as part of a continuing effort to assess the phylogenetic diversity of spirochete isolates associated with Lyme disease. An understanding of this diversity is indispensable in efforts to develop better diagnostic assays and potential vaccines. These studies have led to the identification of three isolates with highly unusual rRNA gene RFLP patterns. The molecular basis of these polymorphisms, which have not previously been observed in any other LDS isolates, was found to be the result of insertions and deletions in the region of the chromosome carrying the rRNA genes. The presence of the insertions in isolates 21038 and 19857 predicts that if these interrupted genes are transcribed, a fragmented 23S rRNA would be produced. The presence of such a fragmented molecule was demonstrated by Northern blot analyses in isolate 21038. That this fragmented 23S rRNA was derived from transcription of the second 23S rRNA gene is strongly supported by the Southern blotting and PCR analysis. These data allow us to speculate that both copies of the tandem 23S-5S rRNA gene cluster are likely active in other LDS isolates.

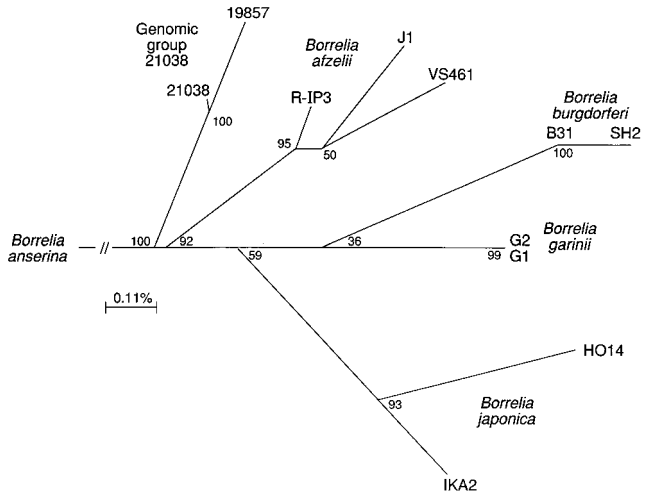

FIG. 7. Phylogenetic tree of 16S rRNA derived from LDS isolates. The phylogenetic tree was constructed as described in the text. Numbers at the branch nodes indicate the results of bootstrap analysis. The 16S rRNA sequence from *Borrelia anserina* served as an outgroup.

The presence of the tandem duplication in all LDS species suggests that the duplication arose prior to the divergence of the multiple species from a common ancestor. In contrast, the polymorphisms characterized here likely arose relatively recently. This suggestion is supported by the sequence analyses performed on the intergenic spacer regions and the 16S rRNA gene. These analyses revealed that the observed polymorphisms, and hence the RFLP patterns, for these isolates are not reflective of species identity. This finding has practical implications since rRNA gene RFLP patterns have been widely used to differentiate LDS species. Overall, RFLP patterns have proven to be generally reliable for this purpose. However, unusual patterns must be interpreted with care. The phylogenetic analyses presented here support the designation of *B. japonica* and *B. andersonii* sp. nov. as distinct species. To date neither of these species has been recovered from humans; hence, their role in Lyme disease remains unclear at this time. In recent infectivity studies using mouse models, we were unable to reisolate these organisms from mice infected in the laboratory (17). While more rigorous studies are required, this suggests that these species may not be capable of infecting mice. The identification of noninfective species may prove useful in defining and identifying which factors correlate with infectivity.

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