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# Diversity of *Borrelia burgdorferi* Sensu Lato Evidenced by Restriction Fragment Length Polymorphism of *rrf* (5S)-*rrl* (23S) Intergenic Spacer Amplicons

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**The organization of the ribosomal genes is unique in *Borrelia burgdorferi* in that the *rrl* (23S) and *rrf* (5S) genes are tandemly duplicated. We took advantage of this uniqueness to assess the restriction polymorphism of PCR products obtained with primers at the 3′ end of the first *rrf* gene and at the 5′ end of the second *rrl* gene. An amplicon that was 226 to 266 bp long was generated from 99 of 100 *B. burgdorferi* sensu lato strains. The nuclease *Mse*I restriction polymorphism of the amplicons provided a useful tool for identifying *B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii* (formerly group VS461), and *Borrelia japonica* (formerly group F63B). Furthermore, it allowed us to recognize four new genomic groups, which were confirmed by DNA-DNA hybridization data. Two of these genomic groups comprised European strains, and the other two groups contained American strains. The American genomic groups involved vectors with enzootic cycles quite different from those of *B. burgdorferi* sensu stricto, which previously was the only Lyme disease *Borrelia* species known to occur in the United States. Our method could be used for rapid screening of strain collections and for epidemiological and medical purposes.**

Since the first description of *Borrelia burgdorferi* in 1984 (20), numerous strains have been isolated throughout the world. Using the reference method for delineation of bacterial species, DNA-DNA hybridization, workers have described three species associated with Lyme borreliosis, *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (formerly group VS461). In addition, a new nonpathogenic species seems to be restricted to Japan; this species was described as group F63B (30) and has been named *Borrelia japonica* (21). Recently, two new American genomic groups were identified on the basis of the results of a *fla* gene-based PCR assay (6). One interesting aspect of the species classification described above is its correlation with epidemiological and clinical features (5, 41). *B. burgdorferi* sensu stricto is present both in the Old World and in the New World, but it seems to be absent from Asia. Moreover, *B. burgdorferi*, the only Lyme disease *Borrelia* species known to occur in the United States, is mainly associated with arthritic forms of Lyme disease. *B. garinii* and *B. afzelii* are present in Europe and Asia; the former is frequently associated with neurological manifestations, and the latter seems to be the exclusive agent of late cutaneous lesions of acrodermatitis chronica atrophicans (Pick-Herxheimer disease), which occurs mainly in northern Europe (13, 41, 44).

Genetic diversity among *Borrelia* isolates, both at the interspecific level and at the infraspecific level, has been investigated by different approaches, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1, 28), multilocus enzyme electrophoresis (11), DNA restriction enzyme analysis (23), ribotyping (7), plasmid profile analysis (8, 16, 39), and pulsed-field gel electrophoresis (9). The disadvantage of all of these methods is that they require large quantities of the bacteria, which are fastidious and difficult to grow. In contrast, PCR methods do not require DNA extraction or a large culture volume. Different templates have been used for these PCR (2, 25, 27, 29, 33, 37).

In this study our objective was to develop a simple epidemiological and diagnostic tool to assess rapidly the genetic diversity of *B. burgdorferi* sensu lato strains. Because of the organization of the rRNA genes in the genus *Borrelia* (18, 38) and the uniqueness of the tandemly repeated 23S (*rrl*)-5S (*rrf*) rRNA genes among bacteria, we used the intergenic spacer *rrf-rrl* as a template for amplification and for a restriction polymorphism analysis of the amplified sequence.

## MATERIALS AND METHODS

**Bacterial strains.** Table 1 lists the 100 *Borrelia* strains (which were mostly high-passage strains) that were used in this study and their origins. A total of 30 of these strains were *B. burgdorferi* sensu stricto, 28 were *B. garinii*, 19 were *B. afzelii*, and 5 were *B. japonica*, as previously identified by DNA-DNA hybridization experiments, reactivity with monoclonal antibodies, and ribotyping (7, 13, 30). Eighteen strains were unclassified. Ninety-nine strains were grown in BSKII medium at 34°C. A small amount of strain NE57 DNA was kindly provided by J. Meyer. The pellet from 2 ml of culture was washed and resuspended in 100 μl of distilled water before the preparation was boiled at 100°C for 10 min. This suspension was used for PCR. Strains of *Borrelia hermsii*, *Borrelia parkeri*, *Borrelia turicatae*, *Leptospira interrogans*, *Serpulina hyodysenteriae*, and *Escherichia coli* were used as controls.

**PCR.** A primer set (primer 1 [5′-CTGCGAGTTCGCGGGAGA-3′] and primer 2 [5′-TCCTAGGCATTCACCATA-3′]) was chosen on the basis of a previously published ribosomal DNA sequence (38) and was used to amplify the variable spacer region between two conserved structures, the 3′ end of the 5S rRNA (*rrf*) and the 5′ end of the 23S rRNA (*rrl*). The uniqueness of the *Borrelia* rRNA gene organization among bacteria made these primers suitable for specific identification of *Borrelia* strains despite the lack of primer specificity. The PCR was performed in 50 μl of a solution containing 10 mM

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TABLE 1. Origins and *rrl-rrf* RFLP patterns of *B. burgdorferi* sensu lato strains used in this study

| Isolate | Source | Country | RFLP patterns[a] | |
|---|---|---|---|---|
| | | | *Dra*I | *Mse*I |
| *B. burgdorferi* sensu stricto | | | | |
| IP1 | Human (CSF)[b] | France | ND | A |
| IP2 | Human (CSF) | France | A′ | A |
| IP3 | Human (CSF) | France | ND | A |
| 297 | Human (CSF) | United States | A | A |
| Z118 | *Ixodes ricinus* | Germany | ND | A |
| Z136 | *Ixodes ricinus* | Germany | ND | A |
| 212 | *Ixodes ricinus* | France | A′ | A |
| B31 | *Ixodes scapularis* | United States | A′ | A |
| Charlie tick | *Ixodes scapularis* | United States | ND | A |
| 19535 | *Peromyscus leucopus* | United States | A′ | A |
| 21305 | *Peromyscus leucopus* | United States | A′ | A |
| 27985 | *Ixodes scapularis* | United States | A′ | A |
| 26816 | *Microtus pensylvanicus* | United States | A′ | A |
| HUM 3336 | *Ixodes pacificus* | United States | A′ | A |
| SON 328 | *Ixodes pacificus* | United States | A′ | A |
| HUM 7814 | *Ixodes pacificus* | United States | A′ | A |
| MEN 115 | *Ixodes pacificus* | United States | A′ | A |
| SON 188 | *Ixodes pacificus* | United States | A′ | A |
| LAKE 339 | *Ixodes pacificus* | United States | A′ | A |
| CA5 | *Ixodes pacificus* | United States | ND | A |
| VS2 | *Ixodes scapularis* | United States | A′ | A |
| NY1-86 | Human (skin) | United States | A′ | A |
| Veery | Bird | United States | A′ | A |
| SV1 | *Ixodes ricinus* | France | A′ | A |
| SV3 | *Ixodes ricinus* | France | A′ | A |
| ESP1 | *Ixodes ricinus* | Spain | A′ | A |
| Cat Flea Texas | Cat flea | United States | A′ | A |
| Texas skin | Human (skin) | United States | ND | A |
| HII | Human (blood) | Italy | ND | A |
| GeHo | Human (skin) | Germany | ND | A |
| *B. garinii* | | | | |
| 20047 | *Ixodes ricinus* | France | B′ | B |
| G25 | *Ixodes ricinus* | Sweden | ND | B |
| N34 | *Ixodes ricinus* | Germany | B′ | B |
| 153 | *Ixodes ricinus* | France | B′ | B |
| VS185 | *Ixodes ricinus* | Switzerland | ND | B |
| VS286 | *Ixodes ricinus* | Switzerland | ND | B |
| VS468 | *Ixodes ricinus* | Switzerland | ND | B |
| PBi | Human (CSF) | Germany | B′ | B |
| PBr | Human (CSF) | Germany | ND | B |
| T25 | *Ixodes ricinus* | Germany | B′ | B |
| HP3 | *Ixodes persulcatus* | Japan | B′ | B |
| TN | *Ixodes ricinus* | Germany | ND | B |
| NBS16 | *Ixodes ricinus* | Sweden | B′ | B |
| G152 | *Ixodes ricinus* | Sweden | B′ | B |
| FujiP1 | *Ixodes persulcatus* | Japan | B′ | B |
| FujiP2 | *Ixodes persulcatus* | Japan | B′ | B |
| SV2 | *Ixodes ricinus* | France | B′ | B |
| AR-1 | *Ixodes ricinus* | The Netherlands | B′ | B |
| PD89 | Human (blood) | People's Republic of China | B′ | B |
| Ir210 | *Ixodes ricinus* | Russia | B′ | B |
| Sika1 | *Ixodes ovatus* | Japan | B′ | B |
| Sika2 | *Ixodes persulcatus* | Japan | B′ | B |
| P526d | *Ixodes persulcatus* | Japan | B′ | B |
| HP13 | *Ixodes persulcatus* | Japan | B′ | B |
| K48 | *Ixodes ricinus* | Slovakia | ND | B |
| BITS | *Ixodes ricinus* | Italy | ND | B |
| NT29 | *Ixodes persulcatus* | Japan | C′ | C |
| Ip89 | *Ixodes persulcatus* | Russia | C′ | C |
| *B. afzelii* | | | | |
| VS461 | *Ixodes ricinus* | Switzerland | D′ | D |
| IPer3 | *Ixodes ricinus* | Russia | D′ | D |
| Ip21 | *Ixodes persulcatus* | Russia | D′ | D |
| M7 | *Ixodes persulcatus* | People's Republic of China | D′ | D |
| 2246 | *Ixodes persulcatus* | People's Republic of China | D′ | D |

*Continued on following page*

TABLE 1—*Continued*

| Isolate | Source | Country | RFLP patterns[a] | |
|---|---|---|---|---|
| | | | *Dra*I | *Mse*I |
| IpF[c] | *Ixodes persulcatus* | Japan | D′ | D |
| P427a | *Ixodes persulcatus* | Japan | ND | D |
| PSto | Human (skin) | Germany | ND | D |
| BO23 | Human (skin) | Germany | D′ | D |
| UM01 | Human (skin) | Sweden | D′ | D |
| PGau | Human (skin) | Germany | ND | D |
| PKo-85 | Human (skin) | Germany | D′ | D |
| DK3 | Human (skin) | Denmark | ND | D |
| DK8 | Human (skin) | Denmark | D′ | D |
| ACA1 | Human (skin) | Sweden | ND | D |
| ECM1 | Human (skin) | Sweden | D′ | D |
| SmS1 | *Apodemus flavicollis* | Sweden | D′ | D |
| LABC | Human (skin) | Sweden | ND | D |
| Nancy | Human | Italy | ND | D |
| *B. japonica* | | | | |
| Ika2 | *Ixodes ovatus* | Japan | | |
| HO14 | *Ixodes ovatus* | Japan | NS | E |
| Cow611C | *Ixodes ovatus* | Japan | NS | E |
| O612 | *Ixodes ovatus* | Japan | NS | E |
| F63B | *Ixodes ovatus* | Japan | NS | E |
| Group VS116 | | | | |
| VS116 | *Ixodes ricinus* | Switzerland | B″ | F |
| UK | *Ixodes ricinus* | England | B″ | F |
| NE57[d] | *Ixodes ricinus* | Switzerland | ND | F |
| Group PotiB2 | | | | |
| PotiB1 | *Ixodes ricinus* | Portugal | G′ | G |
| PotiB2 | *Ixodes ricinus* | Portugal | G′ | G |
| PotiB3 | *Ixodes ricinus* | Portugal | H′ | H |
| Group DN127 | | | | |
| DN127 | *Ixodes pacificus* | United States | I′ | I |
| CA118 | *Ixodes neotomae* | United States | J′ | J |
| CA128 | *Ixodes neotomae* | United States | J′ | J |
| CA55 | *Ixodes neotomae* | United States | J′ | J |
| CA127 | *Ixodes neotomae* | United States | J′ | J |
| 25015 | *Ixodes scapularis* | United States | K′ | K |
| Group 21123 | | | | |
| 21123 | *Ixodes dentatus* | United States | NS | L |
| 21133 | *Ixodes dentatus* | United States | NS | L |
| 19952 | *Ixodes dentatus* | United States | ND | L |
| 19865 | Rabbit (kidney) | United States | NS | L |
| 19857 | Rabbit (kidney) | United States | ND | L |
| CA2 | *Ixodes neotomae* | United States | M′ | M |

[a] NS, no *Dra*I restriction site; ND, not done. The *Dra*I restriction fragment sizes were 204 and 49 bp for *B. garinii* (pattern B′) and 206 and 49 bp for group VS116 (pattern B″). Patterns B′ and B″ were indistinguishable on gels.
[b] CSF, cerebrospinal fluid.
[c] Strain IpF was also called IPF, I. persulcatus, and J1.
[d] For strain NE57, only a small amount of DNA was available, which was kindly provided by J. Meyer.

Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 1.25 U of *Taq* polymerase (Amersham International, Amersham, England), each of the primers at a concentration of 1 μM, and 5 μl of heated bacterial suspension or 10 ng of DNA. The amplification reaction was carried out for 30 cycles with the following profile: denaturation at 94°C for 1 min, annealing at 52°C for 1 min (56°C for the F63B group), and extension at 72°C for 2 min.

**Digestion with *Mse*I and *Dra*I endonucleases and electrophoresis.** Endonucleases *Mse*I (New England Biolabs, Beverly, Mass.) and *Dra*I (Amersham) were used to cleave PCR products (20 U/μg of DNA) as recommended by the manufacturers. Electrophoresis was carried out in a 16% acrylamide–0.8% bisacrylamide gel for 3 h at 100 V. Marker V (Boehringer, Mannheim, Germany) was used as a molecular weight marker.

**Cloning and DNA sequencing.** Each PCR product (60 ng) was ligated in *Sma*I-cleaved pUC18 DNA by using a Surclone ligation kit (Pharmacia, Uppsala, Sweden). Ligation mixtures were transformed into *E. coli* DH5α, and transformants were selected on Luria-Bertani agar (26) containing 100 μg of ampicillin per ml. Recombinant clones, each of which contained an insert of approximately 300 bp after restriction by *Eco*RI and *Pst*I, were kept for further use.

The DNA sequence of the insert was determined by using a T7 sequencing kit (Pharmacia), a reverse primer (5′-AACAG CTATGACCATG-3′), and $^{35}S$-labeled dATP (Amersham). For each strain, two independent clones were sequenced, and the sequences of both strands were determined. Sequences were aligned and a phylogenetic tree was constructed by the neighbor-joining method (34) by using Clustal V software (19) and njplot software.

**DNA-DNA reassociation.** DNA was prepared, DNA relat-

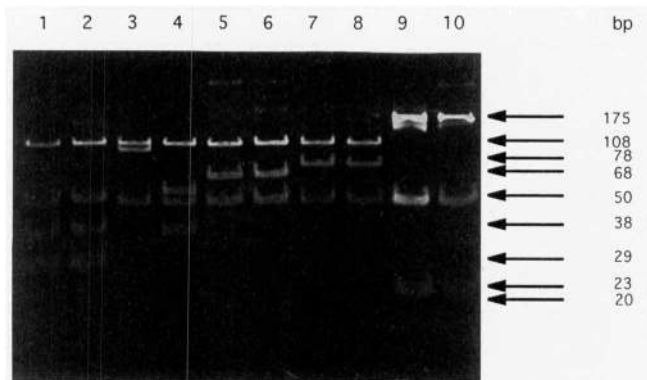


FIG. 1. *Mse*I restriction patterns of *Borrelia* sensu lato strains. DNA was electrophoresed on a 16% acrylamide gel, stained with ethidium bromide, and UV illuminated. Lanes 1 and 2 pattern A produced by *B. burgdorferi* sensu stricto (strains Men115 and B31$^T$); lanes 3 and 4, patterns B and C produced by *B. garinii* (strains P526d and Ip89); lanes 5 and 6, pattern D produced by *B. afzelii* (strains VS461$^T$ and IpF); lanes 7 and 8, pattern E produced by *B. japonica* (strains HO14$^T$ and 0612); lanes 9 and 10, pattern F produced by group VS116 (strains VS116 and UK). Small fragments were difficult to distinguish on gels. Exact sizes were determined from the sequences.

edness values were determined, and levels of DNA divergence were estimated from $\Delta T_m$ values (14) by using the S1 nuclease method as described previously (31) ($\Delta T_m$ is the difference between the melting temperature of a homologous hybrid and the melting temperature of a heterologous hybrid).

**Nucleotide sequence accession numbers.** The *rrf-rrl* spacer sequences of 18 *B. burgdorferi* sensu lato strains have been assigned GenBank accession numbers L30127 (strain B31$^T$ [T = type strain]), L30121 (212), L30119 (20047$^T$), L30130 (NT29), L30135 (VS461$^T$), L30129 (J1), L30128 (HO14$^T$), L30125 (Cow611C), L30131 (PotiB2), L30132 (PotiB3), L30134 (VS116), L30133 (UK), L30126 (DN127), L30122 (25015), L30124 (CA55), L30120 (21133), L30118 (19952), and L30123 (CA2).

## RESULTS

**PCR analysis.** PCR amplification of the spacer region located between the *B. burgdorferi* sensu lato *rrf* and *rrl* genes generated a fragment that was 226 to 266 bp long, depending on the strain group. All strains except Japanese strain Ika2 produced this fragment. *B. hermsii*, *B. parkeri*, *B. turicatae*, *L. interrogans*, *S. hyodysenteriae*, and *E. coli* gave no amplification product.

**RFLP of the *rrf-rrl* spacer amplicon.** After cleavage by *Mse*I, the 99 PCR products were classified into 13 different patterns, designated patterns A to M (Fig. 1). Table 2 lists these patterns and shows the sizes of the *Mse*I DNA fragments. All of the *B. burgdorferi* sensu stricto strains fell into pattern A. All 19 *B. afzelii* strains fell into pattern D, and the five *B. japonica* strains fell into pattern E, whereas the *B. garinii* strains exhibited two different patterns (patterns B and C). Similar results were obtained after restriction by *Dra*I (data not shown). The eight remaining *Mse*I patterns were produced by the 18 unclassified strains. The European strains could be divided into group VS116 (pattern F) and group PotiB2 (patterns G and H). Pattern H (strain PotiB3) was similar to pattern E (*B. japonica*); the greatest difference was the presence of a 16-bp restriction fragment which was difficult to visualize. Patterns E and H could be differentiated easily after restriction by *Dra*I. *B.*

TABLE 2. *Mse*I restriction fragments of *rrl-rrf* intergenic spacer amplicons

| Taxon(a) | Amplicon size (bp) | RFLP pattern | *Mse*I restriction fragment sizes (bp)[a] |
|---|---|---|---|
| *B. burgdorferi* | 254 | A | 108, 51, 38, 29, 28 |
| *B. garinii* | | | |
| 20047 | 253 | B | 108, 95, 50 |
| NT29 | 253 | C | 108, 57, 50, 38 |
| *B. afzelii* | 246 | D | 108, 68, 50, 20 |
| *B. japonica* | 236 | E | 108, 78, 50 |
| Group VS116 | 255 | F | 175, 50, 23, 7 |
| Group PotiB2 | | | |
| PotiB1 and PotiB2 | 257 | G | 108, 81, 39, 29 |
| PotiB3 | 255 | H | 108, 79, 52, 16 |
| Group DN127 | | | |
| DN127 | 257 | I | 108, 51, 38, 33, 27 |
| California strains | 226 | J | 108, 51, 38, 29 |
| 25015 | 253 | K | 108, 51, 34, 27, 17, 12, 4 |
| Group 21123 | | | |
| Group dentatus | 266 | L | 120, 67, 51, 28 |
| CA2 | 255 | M | 91, 50, 40, 28, 22, 17, 7 |

[a] Exact sizes were determined from the sequences.

*japonica* strains did not possess any *Dra*I restriction site, while there were two *Dra*I restriction sites in the PotiB3 amplicon, which produced three fragments (data not shown). The restriction fragment length polymorphism (RFLP) analysis revealed that there was broad heterogeneity in the unclassified American strains since five patterns (patterns I to M) were produced. Isolates DN127, 25015, and CA2 produced individual patterns (patterns I, K, and M), the four California isolates obtained from *Ixodes neotomae* produced pattern J, and the five strains obtained from *Ixodes dentatus* produced pattern L (Table 2).

**Comparison of the nucleotide sequences of the *rrf-rrl* spacer amplicon.** Figure 2 shows the nucleotide sequences of the *rrf-rrl* amplicons obtained from 18 isolates. To assess the levels of homology within and between the different RFLP groups, we selected two isolates from each RFLP group for sequencing. No differences were observed in different sequences from the same isolate, excluding possible errors resulting from the use of the *Taq* polymerase during PCR. The level of identity among the sequences of the *rrf-rrl* amplicons from the 18 *B. burgdorferi* sensu lato strains was 60%. It is interesting that the identity between two isolates belonging to each of the four previously described genomospecies was 99.2, 98, 100, and 99.6% for *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica*, respectively. The sequences of the two group VS116 isolates (strains VS116 and UK) were 99.2% identical. The sequences of the two strains isolated from *I. dentatus* were 99.6% identical. There was only 95.7% identity among the three group DN127 isolates, and there was also 95.7% identity among the three group PotiB2 isolates. A phylogenetic tree was deduced from the sequences (Fig. 3), and this tree showed the partition of the sequenced strains into nine branching groups.

**DNA homology.** The levels of DNA relatedness among *B. burgdorferi*, *B. garinii*, *B. afzelii*, and *B. japonica* have been published previously. None of the strains included in the four new RFLP groups (groups VS116, PotiB2, DN127, and 21123) were related to the type strains of *B. garinii*, *B. afzelii*, and *B. japonica* since the levels of relatedness were less than 70% and the $\Delta T_m$ values were more than 5°C (Table 3). None of the strains in groups VS116, PotiB2, and 21123 were related (<70% relatedness; $\Delta T_m$, >6°C) to strain B31, the type strain of *B. burgdorferi* sensu stricto.

Four of the unclassified strains (VS116, PotiB2, DN127, and 21123) were labelled for reverse hybridization experiments. The European strains constituted two homogeneous genomic groups that were not related to each other or to the other genomic species, as shown in Table 3. Group VS116 included strains VS116 and UK, which were 93 to 100% related to one another. Group PotiB2 included three Portuguese strains which were 92 to 100% related to each other. Among the American strains, two genomic groups could be distinguished. Genomic group DN127 included strain DN127 isolated from *Ixodes pacificus*, strain 25015 isolated from *Ixodes dammini*, and four California strains isolated from *I. neotomae*. These six strains were 79 to 100% related to each other, and the $\Delta T_m$ values were 0 to 3°C. They were not related to other groups, but the levels of divergence in comparisons with isolates belonging to the *B. burgdorferi* genomospecies were borderline ($\Delta T_m$, 5 to 7°C). Genomic group 21123 was composed of five strains isolated from *I. dentatus* and one strain isolated from *I. neotomae*. Strains in this group were 73 to 100% related to strain 21123 and less than 68% related to members of other groups.

## DISCUSSION

Among *B. burgdorferi* sensu lato strains four species have been described on the basis of DNA-DNA hybridization data. Reactivity with species-specific monoclonal antibodies provided phenotypic characteristics that were required for species descriptions. Ribotyping provided an identification system at the species level. DNA-DNA hybridization is presently the reference method used for species delineation (42). However, other less fastidious methods could be used to classify large strain collections, to identify *Borrelia* strains that occur in tick populations, or possibly in medical diagnosis. Methods based on PCR DNA amplification seem to be the methods of choice for these purposes. The organization of rRNA genes in *Borrelia* strains associated with Lyme borreliosis is unique among bacteria. There is a single *rrs* gene and two copies each of the *rrl* and *rrf* genes, which are tandemly duplicated (18, 38). The individual copies of the *rrl-rrf* duplicate genes are separated by a 182-bp spacer (38). We used this characteristic to design primers for the conserved ribosomal genes and to amplify the spacer between the two copies of the *rrl-rrf* tandem repeat. Thus, the amplification product was specific for *B. burgdorferi* sensu lato, despite the lack of primer specificity. Given the unusual *rrl-rrf* tandem genes, the duplicate genes attesting to gene rearrangements in the past, diversity among spacer sequences was expected. However, the results of ribotyping experiments performed with four distinct enzymes suggested that the flanking regions of ribosomal genes might be conserved enough to exhibit the level of variability that is mainly associated with genomic groups (7, 30). Otherwise, it has been shown that genetic transfers are very rare events in the genus *Borrelia* (15), and there is no insertion sequence which is known to introduce potential variability in the chromosome.

No amplification product was obtained from *B. japonica* Ika2, and this strain does not exhibit the same characteristic rRNA gene organization as other *B. burgdorferi* sensu lato strains (36). An analysis of the patterns obtained after restriction by *Mse*I allowed us to recognize 13 patterns. Strains belonging to *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. japonica* produced species-specific patterns A, D, and E, respectively. Strains belonging to *B. garinii* could be divided into two RFLP groups (patterns B and C). The group whose members produced pattern B included 26 of the 28 *B. garinii* strains, including type strain 20047, and the group whose members produced pattern C included only strains Ip89 and NT29, which were obtained from Russia and Japan, respectively. A sequence analysis revealed only 5 nucleotide differences in the 253-bp amplicon, with one mutation located in an *Mse*I site. It is interesting that *B. garinii* was also found to be the most heterogeneous species by ribotyping. Fukunaga et al. (17) found that Ip89 and NT29 produce an rRNA gene RFLP pattern distinct from the patterns produced by other *B. garinii* strains. However, the results of DNA relatedness studies allowed us to assign these strains to *B. garinii* (Table 3).

Since strains belonging to a previously defined species produced similar *Mse*I patterns, an analysis of the *Mse*I restriction polymorphism patterns of PCR-amplified products provided a simple tool for assigning unknown strains to genomic groups. On the basis of the results of an analysis of the patterns obtained after cleavage by *Mse*I and *Dra*I separately, two groups composed of European strains and two groups composed of American strains were identified. DNA-DNA hybridization results confirmed assignment of strains that produced the same RFLP pattern to the same new genomic group.

The first European genomic group, group VS116, included three strains, VS116, UK, and NE57. VS116 and NE57 have both been reported to be putative representatives of a new genomic group on the basis of the electrophoretic mobilities of OspA and OspB (28) and on the basis of chromosomal DNA RFLP patterns (16), respectively. The results of DNA relatedness and *rrf-rrl* spacer RFLP experiments allowed us to place these strains in the same genomic group. The second European group, group PotiB2, contained three strains which are closely related as determined by DNA-DNA hybridization but are quite different in their *rrf-rrl* nucleotides sequences. It is interesting that strain PotiB3, which differed in its restriction polymorphism pattern (pattern H), was isolated from the same individual *Ixodes ricinus* tick as strain PotiB2 (pattern G).

On the basis of DNA relatedness data, American strains could be divided into two additional genomic groups. The results of the *rrf-rrl* spacer RFLP experiment supported the heterogeneity of these organisms. Until now, American *B. burgdorferi* strains have been considered to be very homogeneous, and *B. burgdorferi* sensu stricto was the only Lyme disease species known to occur in the United States. However, some strains have been described as atypical. Strain DN127 was reported to be an unusual strain that did not react with monoclonal antibodies to OspA and OspB and produced an abundant 25-kDa protein (10, 22). This strain did not give an amplification product with primers flanking the *osp* operon, thus behaving differently than strains belonging to *B. burgdorferi* sensu stricto (35). The results of an arbitrarily primed PCR (43) and restriction site polymorphism in the 16S rRNA genes (32) also distinguished strain DN127 from other phyletic groups.

The results of a study of the variability of the *osp* operon led Marconi et al. to propose that there is a peripheral relationship between American strain 25015 and *B. burgdorferi* sensu stricto (24). The results of a comparison of *ospA* sequences led Dykhuizen et al. to not classify strains 25015 and 19857 as members of the three major species (15). An analysis of RFLP of the 83-kDa antigen gene revealed that two American strains, DN127 and 25015, did not cluster with other American isolates (45, 46). The OspA protein of these strains has a molecular weight of about 32,000, in contrast to a molecular weight of 31,000 for the OspA of *B. burgdorferi* sensu stricto (3, 10). Moreover, strain 25015 has been described as an infectious but nonpathogenic isolate (3). DNA-DNA hybridization results have shown that strains DN127 and 25015 belong to the

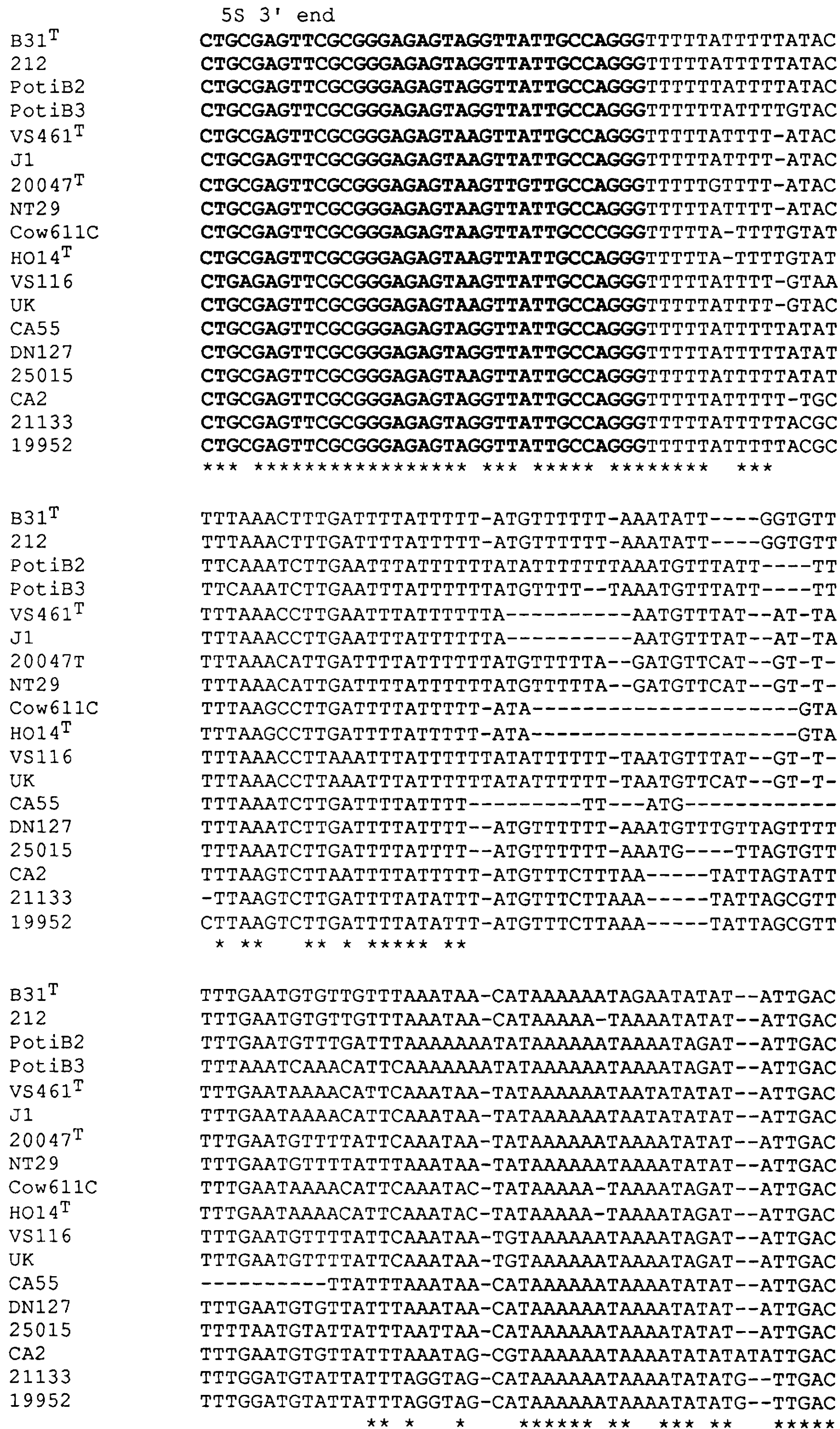


FIG. 2. Nucleotide sequences of the *rrf-rrl* spacer amplicons of *B. burgdorferi* sensu lato isolates. Gaps were introduced to obtain maximum levels of homology. The 3′ end of the *rrf* gene and the 5′ end of the *rrl* gene are indicated by boldface type.

```
B31T       ATGTATTAAACAAA----GATATAT--------ATTATTTTATGTTGTAT
212        ATGTATTAAACAAA----GATATAT--------ATTATTTTATGTTGTAT
PotiB2     ATGGATTAAACAAA----GATATAT--------ATTATTCTATGTTGCAT
PotiB3     ATGGATTAAACAAA----GATATAT--------ATTATTCTATGTTGCAT
VS461T     ATGGATTAAACAAA----GATATAT--------ATTATTCTATGTTGTAT
J1         ATGGATTAAACAAA----GATATAT--------ATTATTCTATGTTGTAT
20047T     ATGGATTAAACAAA----GATATAT--------ATTATTCTATGTTGTAT
NT29       ATGGATTAAACAAA----GATATAT--------ATTATTCTATGTTGCAT
Cow611C    ATGGGTTAAACAAA----GATATAT--------ATTATTCTATGTTACAT
HO14T      ATGGGTTAAACAAA----GATATAT--------ATTATTCTATGTTACAT
VS116      ATGGATTGAACAAA---AGATATAT--------ATTATTTTATGTTGCAT
UK         ATGGATTGAACAAA---AGATATAT--------ATTATTTTATGTTGCAT
CA55       ATGGATTAAACAAA----GATATAT--------ATTATTTTATGTTGTAT
DN127      ATGGATTAAACAAA----GATATAT--------ATTATTTTATGTTGCGT
25015      ACGGATTAAACAAA----GATATAT--------ATTATTTTATGTTGCAT
CA2        ATGGATTAAACAAAGATATATAT------------TAATTTATGTTGCAT
21133      ATGGATTAAACATAGATAGATATATGTATCTCTATTATTTTATGCTGCAT
19952      ATGGATTAAACATAGATAGATATATGTATCTCTATTATTTTATGCTGCAT
           * *  ** **** *     ****            ** * **** *   *

B31T       AAATAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
212        AAATAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
PotiB2     AAACAAATTGGCAAAGTAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
PotiB3     AAACAAATTGGCAAAGTAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
VS461T     AAACAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
J1         AAACAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
20047T     AAACAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
NT29       AAGCAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
Cow611C    AAACAAATTGGCAAAGTAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
HO14T      AAACAAATTGGCAAAGTAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
VS116      AAACAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
UK         AAACAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
CA55       AAACAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
DN127      AAACAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
25015      AAACAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
CA2        AAACAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
21133      AAGCAAATTGGCAAAATAGAGATGGAAGATAAAAAATATGGTCAAAGTAAT
19952      AAGCAAATTGGCAAAATAGAGCTGGAAGATAAAAAATATGGTCAAAGTAAT
           **  *********** ***** *****************************

B31T       AAGAGTCTATGGTGAATGCCTAGGA
212        AAGAGTCTATGGTGAATGCCTAGGA
PotiB2     AAGAGTCTATGGTGAATGCCTAGGA
PotiB3     AAGAGTCTATGGTGAATGCCTAGGA
VS461T     AAGAGTCTATGGTGAATGCCTAGGA
J1         AAGAGTCTATGGTGAATGCCTAGGA
20047T     AAGAGTCTATGGTGAATGCCTAGGA
NT29       AAGAGTCTATGGTGAATGCCTAGGA
Cow611C    AAGAGTCTATGGTGAATGCCTAGGA
HO14T      AAGAGTCTATGGTGAATGCCTAGGA
VS116      AAGAGTCTATGGTGAATGCCTAGGA
UK         AAGAGTCTATGGTGAATGCCTAGGA
CA55       AAGAGTCTATGGTGAATGCCTAGGA
DN127      AAGAGTCTATGGTGAATGCCTAGGA
25015      AAGAGTCTATGGTGAATGCCTAGGA
CA2        AAGAGTCTATGGTGAATGCCTAGGG
21133      AAGAGTCTATGGTGAATGCCTAGGA
19952      AAGAGTCTATGGTGAATGCCTAGGA
           ************************
             23S 5'end
```

FIG. 2—*Continued.*

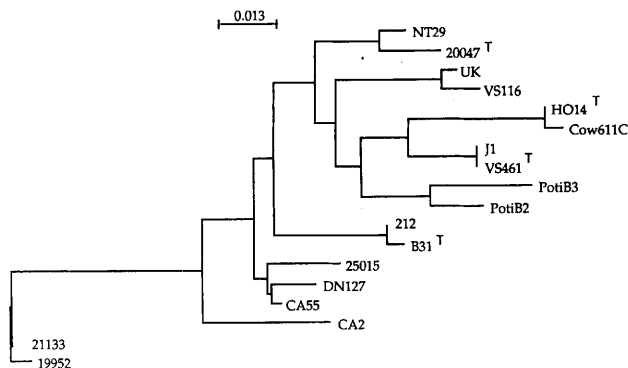


FIG. 3. Phylogenetic tree determined from the DNA sequences of *rrl-rrf* spacer amplicons.

same genomic group, group DN127, along with four California strains isolated from *I. neotomae* (6). The protein profiles of these California isolates differed markedly from the profiles of the eastern United States isolates (12). The enzootic cycle involving *I. neotomae* has been reported to be quite different from the cycles involving *I. pacificus* or *Ixodes scapularis* (12).

The second American genomic group characterized in this study, group 21123, comprised five strains isolated from cottontail rabbits and *I. dentatus* ticks. These strains did not react with *B. burgdorferi* sensu stricto specific monoclonal antibody H3TS (4). Despite differences in levels of DNA relatedness between strains 19865 and 19941 and *B. burgdorferi* sensu stricto reference strain B31$^T$ (61 and 64%, respectively), Anderson et al. did not conclude that these organisms diverged at the species level (4). Our previous results clearly showed that these strains belong to a genomic group that is distinct from *B. burgdorferi* sensu stricto (6). Group 21123 appears to be less closely related to *B. burgdorferi* sensu stricto than group DN127 is when both genetic and epidemiological features are considered. DNA-DNA hybridization experiments gave borderline results with the six strains belonging to group DN127,

TABLE 3. Levels of DNA relatedness for *B. burgdorferi* sensu lato strains used in this study

| Isolate | Level of DNA relatedness (relative binding ratio) with labelled DNA from[a]: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Strain B31$^T$ | Strain 20047$^T$ | Strain VS461$^T$ | Strain Ika2 | Strain VS116 | Strain PotiB2 | Strain DN127 | Strain 21123 |
| *B. burgdorferi* sensu stricto | | | | | | | | |
| IP1 | | | | | | | 57 (5) | 64 (8) |
| Z118 | | 46 | | | | | | |
| B31$^T$ | **100 (0)**[b,c] | 55 (8)[c] | 48 (9)[c] | | 54 (7) | 57 | 59 (7) | 70 (6) |
| *B. garinii* | | | | | | | | |
| 20047$^T$ | 51 (10)[c] | **100 (0)**[c] | 65 (7)[c] | | 60 | 62 (7) | 45 | 62 (8) |
| FujiP2 | 58 | 100 | 68 (8) | | | | | |
| HP13 | 60 | 78 (1) | 73 (7) | | | | | |
| NT29 | 58 | 86 (3) | 66 (6) | | | 62 | | |
| Ip89 | | 84 | 67 | | | | | |
| *B. afzelii* VS461$^T$ | 51[c] | 62 (7)[c] | **100 (0)**[c] | | 60 | 58 (8) | 39 (9) | 50 (9) |
| *B. japonica* | | | | | | | | |
| Ika2 | 58[c] | 58[c] | 65 (7)[c] | **100 (0)**[c] | | 64 (8) | 57 | |
| Cow611C | | | | | | | 53 | |
| Group VS116 | | | | | | | | |
| VS116 | 54 (7) | 56 (7) | 64 (8) | 58 | **100 (0)** | | 46 | 60 |
| UK | 65 (8) | 63 | 70 | | 93 (0) | 60 | | |
| Group PotiB2 | | | | | | | | |
| PotiB1 | 53 (9) | | | | 54 | 98 | 26 | |
| PotiB2 | | | | | 56 | **100 (0)** | | |
| PotiB3 | 44 | | | | 53 | 92 (0) | | 58 |
| Group DN127 | | | | | | | | |
| DN127 | 67 (6)[c] | 52 (8) | 61 | 52 (7) | | 54 | **100 (0)**[c] | 64 (7)[c] |
| CA118 | 72 (6)[c] | | | | | | 79 (0)[c] | |
| CA128 | 68 (5)[c] | | | | 52 | 54 | 80 (0)[c] | 63 (7)[c] |
| CA55 | 72 (6)[c] | | | | | | 80 (0)[c] | 62 (6)[c] |
| CA127 | 73 (7)[c] | | | | | | 95[c] | |
| 25015 | 68 (6)[c] | | | | | | 83 (3)[c] | 64[c] |
| Group 21123 | | | | | | | | |
| 21123 | | | | 51 (8) | | 55 | 66[c] | **100 (0)**[c] |
| 21133 | 63[c] | | | | | | 56 (8)[c] | 86 (2)[c] |
| 19952 | 59 (6)[c] | 52 | 54 | | | | 60[c] | 87 (1)[c] |
| 19865 | 68 (7) | 50 | 64 | | | | 59 | 86 (2) |
| 19857 | | | | | | | | 82 (2) |
| CA2 | 67 (7) | 57 | 60 | 56 (8) | | 56 | 64 (7) | 73 (5) |

[a] Relative binding ratios were determined at 60°C. The values in parentheses are $\Delta T_m$ values (in degrees Celsius). For more data on levels of DNA relatedness to *B. burgdorferi*, *B. garinii*, and *B. afzelii* (formerly group VS461) see reference 7, and for data on levels of DNA relatedness to *B. japonica* (formerly group F63B) see reference 30.

[b] The values in boldface type are homologous hybridization results.

[c] Previously published data (6, 7, 30).

since the levels of relatedness to B31$^T$ were 68 to 73% and the $\Delta T_m$ values were 5 to 7°C. *I. dentatus* ticks feed almost exclusively on rabbits and birds and rarely on humans. *Borrelia* strains similar to those obtained from *I. dentatus* have never been isolated from humans, which suggests that group 21123 strains may be nonpathogenic for humans. Our data confirm the two new American genomic groups proposed on the basis of the results of an *fla* gene-based PCR assay (6). Groups DN127 and 21123 were found to be related to *B. burgdorferi* sensu stricto, although they are distinct from this species as determined by DNA-DNA hybridization (6). The status of strain CA2, which was isolated from *I. neotomae*, remains unclear. This strain was not related to *B. burgdorferi* sensu stricto or to group DN127, in contrast to other strains obtained from *I. neotomae*. Strain CA2 was related to strain 21123 from *I. dentatus*, but the level of relatedness was borderline. Whereas the *rrl-rrf* DNA sequences of two other strains obtained from *I. dentatus* (strains 21133 and 19952) exhibited 99.6% identity, strain CA2 exhibited only 87.2% identity with these two strains and 79.6% identity with strain CA55 from *I. neotomae*. Complementary studies would be needed to assign strain CA2 definitively to genomic group 21123.

The results of comparison of *rrf-rrl* spacer sequences are not suitable for drawing phylogenetic conclusions. However, it is interesting that the branching pattern revealed, on one side, *B. burgdorferi* sensu stricto and the other two American genomic groups and, on the other side, five other branches, each of which corresponded to a genomic group. Interestingly, genomic group VS116, *B. japonica*, *B. afzelii*, and genomic group PotiB2 seem to have evolved from *B. garinii*. This hypothesis was deduced previously from a comparison of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii ospC* sequences (40).

The existence of two new genomic groups in the United States could be related to different enzootic maintenance cycles involving a broader vector and host spectrum. The importance of these new groups in terms of pathogenicity for humans is not known yet.

Our results indicate that *rrf-rrl* spacer RFLP analysis is a simple and useful tool for assessing the genetic diversity of *Borrelia* strains associated with Lyme borreliosis. This technique could have applications in epidemiological and medical fields.

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