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Molecular Typing of *Borrelia burgdorferi* Sensu Lato by PCR-Restriction Fragment Length Polymorphism Analysis

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The etiologic agent of Lyme borreliosis, *Borrelia burgdorferi* sensu lato, has been isolated from many biologic sources in North America and Eurasia, and isolates have been divided into three distinct genospecies (*B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*). In order to explore the possible association of genospecies with disease manifestation, 60 isolates of *B. burgdorferi* sensu lato were subjected to 5S rDNA-linked restriction fragment length polymorphism (RFLP) analysis. The results confirmed earlier studies which indicated that virtually all North American isolates are *B. burgdorferi* sensu stricto, whereas Eurasian strains fall into all three genospecies. Thirty-five isolates were further characterized by PCR amplification of a region of the 16S-23S rDNA spacer and *Hin*fI digestion of the products. This method resulted in the subdivision of *B. burgdorferi* sensu stricto into two distinct PCR-RFLP types. In contrast, *B. garinii* isolates all displayed an identical pattern. Additionally, a number of previously unclassified North American isolates (25015, DN127, 19857, 24330) showed distinctively different PCR-RFLP patterns. The application of this method for the typing of uncultured *B. burgdorferi* directly in biologic samples was demonstrated by analysis of several field-collected *Ixodes scapularis* tick specimens. The described PCR-RFLP technique should allow for the direct and rapid molecular typing of *B. burgdorferi*-containing samples and facilitate studies of the relationship between spirochete genotype and clinical disease.

Lyme disease is the most common vector-borne disease in the United States (32); 9,677 provisional cases were reported to the Centers for Disease Control and Prevention in 1992 (13). Lyme disease is also common in other countries of the Northern Hemisphere, although accurate statistics on the overall incidence of the disease are not available. The etiologic agent of Lyme disease is the spirochete *Borrelia burgdorferi*, which is transmitted to humans by the bite of an infected tick (34). Lyme disease is initially manifested by mild constitutional symptoms often accompanied by an expanding skin rash (erythema migrans) and can progress to chronic neurologic, cardiac, cutaneous, and arthritic manifestations if it is left untreated (33). Arthritis appears to be the more common late manifestation among patients in the United States, whereas neuroborreliosis and chronic skin disease are more frequent among patients in Europe (28). Recent studies have provided evidence suggesting that different species (or subspecies) of *B. burgdorferi* which are prevalent in these geographic locations may be responsible for the observed differences in disease manifestations (11, 35).

A large number of investigations have indicated that there is significant genotypic variation among *B. burgdorferi* isolates (23, 29, 36, 37). These different isolate types have been variously referred to as phyletic groups, genotypes, serotypes, and genospecies. Recently, the species *B. burgdorferi* sensu lato has been separated into three distinct species, *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*, primarily on the basis of differences in DNA-DNA hybridization and 16S rRNA sequences (7, 11). A fourth species, *Borrelia japonica*, has also been identified in Japan (17). Common to all members of *B. burgdorferi* sensu lato is that they are causative agents of Lyme disease. *B. garinii* and *B. afzelii* are found exclusively in Europe and Asia, whereas *B. burgdorferi* sensu stricto has been isolated from ticks and patients in the United States as well (7). This has further reinforced the notion that different species of *B. burgdorferi* sensu lato are responsible for distinctive clinical manifestations of Lyme disease.

Elucidation of the relationship between species and disease is complicated by the difficulty in culturing *B. burgdorferi* sensu lato. While purified isolates have been obtained from a variety of biologic sources throughout the world (including ticks, vertebrate reservoir hosts, and human tissue), routine cultivation is limited by the fastidious nature of the organism, the complexity of the culture medium, and the time required for successful growth. *B. burgdorferi* isolates have been classified by serology, sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles, ribotyping, pulsed-field gel electrophoresis, and direct sequencing of cloned genes (8, 23, 31, 37, 39). A common shortcoming in all of these approaches is that the spirochetes to be studied must first be obtained in pure culture. A rapid and simple method for molecular typing of uncultured *B. burgdorferi* would greatly facilitate the large-scale analysis of patient-, tick-, and wildlife-derived *B. burgdorferi* samples. Such studies would provide a better understanding of the geographic and genetic variabilities of *B. burgdorferi* as well as provide insights into the possible roles that specific genotypes might play in infectivity and disease causation.

Previous studies from our laboratory have elucidated the complete sequence of the rRNA gene region of *B. burgdorferi* sensu stricto (15). One of the unusual properties of the rRNA gene cluster is the large (>3-kb) intergenic spacer between the 16S and 23S rRNA genes. These spacer regions accumulate higher degrees of sequence variation between related species than do coding regions because these sequences do not produce a functional gene product. The general utility of molecular analysis (e.g., restriction fragment length polymorphism [RFLP] analysis) of rDNA spacer regions has been demonstrated in the typing of a number bacterial species (16, 19). We

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have combined PCR of the 16S-23S intergenic spacer with restriction enzyme digestion of the amplified DNA in order to develop a rapid typing method for *B. burgdorferi* sensu lato. The method obviates the need for isolation and culture. The results indicate that this approach provides a rapid and sensitive method for typing both cultured and uncultured isolates of *B. burgdorferi.*

MATERIALS AND METHODS

Bacterial strains. The biologic and geographic sources of the *B. burgdorferi* isolates used in the study are listed in Table 1.

DNA preparation. *B. burgdorferi* genomic DNA was prepared from 25 ml of cultures grown at 34°C in BSKII medium as described by Lefebvre et al. (20). *B. burgdorferi* cell lysates for PCR amplification were prepared from 0.1-ml samples (approximately 10^7 cells) grown in BSKII at 34°C. Cultures were washed once in phosphate-buffered saline (pH 7.0), and the cells were resuspended in 100 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 0.5% Nonidet P-40, 0.5% Tween 20) containing 100 μg of proteinase K per ml. The mixture was incubated for 1 h at 55°C and was boiled for 15 min. A total of 5 μl of cell lysate was used for PCR amplification.

Field-collected *Ixodes scapularis* ticks were preserved in 70% ethanol. Each tick was sterilely transferred to 100 μl of 10 mM Tris-HCl–1 mM EDTA (pH 7.4) and was triturated in this solution. Aliquots of 10 μl were frozen at −20°C until further use. For PCR amplification, one aliquot was thawed and was added to a 50-μl PCR mixture.

Southern blotting. Genomic DNAs from various *B. burgdorferi* isolates were digested by overnight incubation with *Hpa*I at 37°C. Southern transfer and hybridization with ^{32}P-labeled 5S rDNA-specific probes were performed as described previously (31).

PCR. Oligonucleotide primers were synthesized on a Cyclone DNA synthesizer (Milligen/Biosearch). The sequences of the primers and their positions relative to the *B. burgdorferi* rDNA gene region are given in Fig. 1.

Samples were processed for PCR amplification in 50 μl of a buffer containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (wt/vol) gelatin, 100 μM (each) dATP, dGTP, dCTP, and TTP, 1.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.), and 20 pmol of each primer. The amplification was carried out for 35 cycles in a model 9600 thermocycler (Perkin-Elmer Cetus) with an amplification profile of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min.

RFLP analysis. A total of 10 μl of PCR mixture containing the amplified fragment was digested with 2.5 U of *Hin*fI in a total volume of 20 μl. The digested products were analyzed by electrophoresis on a 1.5% agarose gel (SeaKem LE agarose, FMC, Rockland, Maine), staining with ethidium bromide, and visualization of the DNA fragments by UV illumination.

RESULTS

RFLP analysis of *B. burgdorferi* sensu lato isolates. Previous studies have demonstrated that all of the genes encoding the rRNAs of *B. burgdorferi* are located within a single 10.9-kb region of the chromosome and revealed an unusual rRNA gene organization (15, 31). *B. burgdorferi* contains a single copy of the 16S rRNA gene and two copies each of the 23S and 5S rRNA genes which are tandemly duplicated. We have shown previously, using Southern blot analysis, that various isolates could be differentiated on the basis of an rDNA-linked RFLP (31). That earlier study has now been extended to 57 different North American and Eurasian isolates. Representative Southern blots are shown in Fig. 2, and the results are summarized in Table 1. All isolates yielded a 3.2-kb fragment in common and a second, species-specific fragment (indicative of the 23S-5S gene duplication). The observed RFLP patterns were completely consistent with the classification of *B. burgdorferi* sensu lato into three distinct species (*B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*).

There were several noteworthy exceptions to these three patterns. First, two North American isolates, 25015 and DN127, which have been characterized as "unusual" (2, 14, 36) each displayed a unique RFLP pattern. Second, strain IKA2, a Japanese isolate from *Ixodes ovatus* (25), was the only organism tested which did not yield two bands on the Southern blot analysis. This finding implies that IKA2 lacks the gene duplication. This has been confirmed by PCR analysis (22) and is consistent with the fact that isolates from *I. ovatus* are characterized as *B. japonica* (17).

These RFLP observations are important for several reasons. Since the 23S-5S gene duplication is unique to *B. burgdorferi* and was not observed for closely related species (31), this represents a simple method for the identification of new spirochete isolates as *B. burgdorferi* sensu lato. In addition, this assay can be used to differentiate between the various genotypes of *B. burgdorferi* sensu lato.

PCR-RFLP analysis of cultured *B. burgdorferi* sensu lato isolates. A weakness of the RFLP analysis described above is the requirement for relatively large amounts of purified genomic DNA which limits its application to cultured isolates of *B. burgdorferi*. Previous sequence analysis of the *B. burgdorferi* (B31) rRNA gene region demonstrated that a 3,052-bp spacer separates the 16S rRNA gene from the gene coding for 23S rRNA (15, 31). Since intergenic spacer regions accumulate higher degrees of sequence variation between related species than do coding sequences such regions make ideal targets for analysis of genetic variability between closely related species. The utility of this spacer region for rapid typing of *B. burgdorferi* isolates by a combination of PCR and restriction enzyme digestion was therefore explored.

The two primers chosen for amplification of the entire intergenic spacer are designated P_A and IS2 (Fig. 1). The expected size of the amplified DNA on the basis of the determined *B. burgdorferi* B31 sequence is 3,105 bp. PCR amplification of representative strains of *B. burgdorferi* yielded amplified products which differed in size. The *B. garinii* and *B. afzelii* isolates have spacers which are 1.2 to 1.5 kb larger than that of *B. burgdorferi* sensu stricto (data not shown). In addition, the reaction was specific for *B. burgdorferi*. Amplification of genomic DNAs from *Mycobacterium tuberculosis*, *Treponema pallidum*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, and *Escherichia coli* with P_A and IS2 did not result in any detectable product (data not shown).

The large fragment size produced by PCR with this primer combination (3.1 to 4.7 kb) resulted in variable amplification efficiencies. We therefore chose a second primer to use in combination with P_A. The sequence of this primer, P42, is complementary to a region 1,712 bp downstream of P_A (on the basis of the B31 sequence), within the intergenic spacer (Fig. 1). PCR amplification of 17 different isolates of *B. burgdorferi* sensu stricto (Fig. 3A), 7 isolates of *B. garinii* (Fig. 4A), and 2 of 4 isolates of *B. afzelii* (Fig. 5A) with this set of primers produced the expected 1,712-bp DNA fragment. The only exceptions were *B. afzelii* isolates VS461 and ACA1, which yielded a smaller fragment of approximately 1.4 kb (Fig. 5A).

*Hin*fI digestion of the PCR products described above resulted in several different RFLP patterns (Fig. 3B, 4B, and 5B). Pattern 1, consisting of fragments of 302, 372, and 1,038 bp, is found in the *B. burgdorferi* sensu stricto type strain B31, consistent with that predicted from the known sequence (Fig. 1 and 3B). Acquisition of an additional *Hin*fI site in the 372-bp fragment, yielding two smaller fragments of 241 and 131 bp, results in pattern 2, as shown by isolate 297 (Fig. 3B, lane 297). Of the 17 *B. burgdorferi* sensu stricto isolates analyzed, 5 had pattern 1, 11 had pattern 2, and one (isolate 24352) had a unique pattern (Fig. 3B, lane 24352). This latter RFLP pattern arises from the loss of the *Hin*fI site between the 372- and the 1,038-bp fragments.

All seven isolates of *B. garinii* tested were homogeneous with respect to the *Hin*fI RFLP pattern, yielding exclusively pattern 1 (B31-like) (Fig. 4B). PCR-RFLP of the *B. afzelii* isolates

TABLE 1. *B. burgdorferi* isolates studied by 5S rDNA RFLP and PCR-RFLP

Isolate	5S RFLP[a]	PCR-RFLP[b]	Origin		
			Biologic[c]	Geographic	Source or reference
B31	A	1	*Ixodes scapularis*	New York	American Type Culture Collection
N40	A	2	*Ixodes scapularis*	New York	D. Persing
24352	A	*	*Ixodes scapularis*	New York	J. Anderson
24330	A	*	*Ixodes dentatus*	New York	J. Anderson
IPS	A	ND[d]	*Ixodes pacificus*	California	R. Picken
Lake 339	A	ND	*Ixodes pacificus*	California	R. Picken
Son 328	A	2	*Ixodes pacificus*	California	R. Picken
CA287	A	ND	*Ixodes pacificus*	California	21
MMTI	A	ND	*Ixodes scapularis*	Minnesota	R. Picken
20001	A	1	*Ixodes ricinus*	France	J. Anderson
20004	A	1	*Ixodes ricinus*	France	J. Anderson
20063	A	ND	*Ixodes ricinus*	France	J. Anderson
Ip21	A	1	*Ixodes persulcatus*	Russia	E. Korenberg
21343	A	2	*Peromyscus leucopus*	New York	J. Anderson
MMI	A	2	*Peromyscus leucopus*	Minnesota	R. Picken
26815	A	2	Chipmunk	Connecticut	J. Anderson
297	A	2	CSF	Massachusetts	34
WI91-1828	A	ND	CSF	New York	G. L. Campbell
EK	A	ND	Blood	New York	C. Pavia
CA91-0808	A	ND	Blood	California	G. L. Campbell
CA91-0809	A	ND	Blood	California	G. L. Campbell
WI91-1223	A	ND	Skin (EM)	Wisconsin	G. L. Campbell
CD115	A	2	Skin (EM)	New York	C. Pavia
CD117	A	ND	Skin (EM)	New York	C. Pavia
CD126	A	2	Skin (EM)	New York	C. Pavia
CD132	A	2	Skin (EM)	New York	C. Pavia
CD134	A	1	Skin (EM)	New York	C. Pavia
CD135	A	2	Skin (EM)	New York	C. Pavia
CD137	A	2	Skin (EM)	New York	C. Pavia
Hb1	A	1	Skin (EM)	Austria	G. Stanek
20047	B	1	*Ixodes ricinus*	France	J. Anderson
NBS	B	1	*Ixodes ricinus*	Sweden	S. Bergström
NBS16	B	1	*Ixodes ricinus*	Sweden	S. Bergström
NBS23a	B	1	*Ixodes ricinus*	Sweden	S. Bergström
NBS23b	B	ND	*Ixodes ricinus*	Sweden	S. Bergström
VS3	B	ND	*Ixodes ricinus*	Switzerland	R. Picken
VS185	B	ND	*Ixodes ricinus*	Switzerland	R. Picken
Ip90	B	ND	*Ixodes persulcatus*	Russia	E. Korenberg
Ir210	B	ND	*Ixodes ricinus*	Russia	E. Korenberg
FujiP1	B	ND	*Ixodes persulcatus*	Japan	T. Masuzawa
G1	B	1	CSF	Germany	A. Barbour
57	B	ND	CSF	Austria	G. Stanek
60	B	1	CSF	Austria	G. Stanek
Sch	B	ND	CSF	Austria	G. Stanek
Las	B	ND	Skin (ACA)	Austria	G. Stanek
UO1	B	ND	Skin (EM)	Sweden	S. Bergström
ECM1	B	1	Skin (EM)	Sweden	5
VS461	ND	*	*Ixodes ricinus*	Switzerland	R. Picken
Ip3	C	ND	*Ixodes persulcatus*	Russia	E. Korenberg
Ip89	C	ND	*Ixodes persulcatus*	Russia	E. Korenberg
P/Sto	C	ND	Skin (ACA)	Germany	38
P/Gau	C	1	Skin (ACA)	Germany	38
ACA1	C	*	Skin (ACA)	Sweden	5
DK8	C	ND	Skin (ACA)	Denmark	A. Lebech
H2	C	1	Skin (EM)	Austria	G. Stanek
25015	*	*	*Ixodes scapularis*	New York	J. Anderson
DN127	*	*	*Ixodes pacificus*	California	9
19857	ND	*	Rabbit	New York	3
IKA2	*	*	*Ixodes ovatus*	Japan	T. Masuzawa
HO14	ND	*	*Ixodes ovatus*	Japan	26

[a] A, B, and C refer to RFLP patterns corresponding to *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively (Fig. 2); *, unique pattern.
[b] The numbers 1 and 2 refer to the major PCR-RFLP patterns observed (Fig. 3 to 5); *, unique pattern.
[c] CSF, cerebrospinal fluid; EM, erythema migrans lesion; ACA, acrodermatitis chronica atrophicans lesion.
[d] ND, not determined.

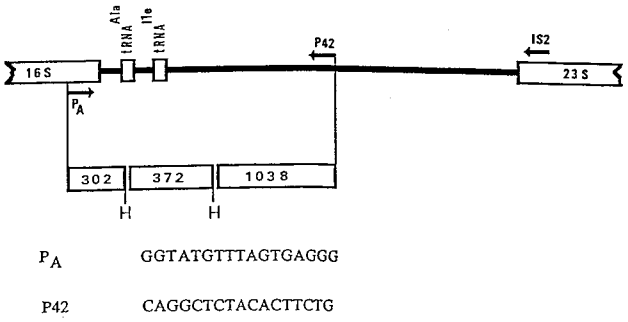

FIG. 1. *B. burgdorferi* 16S-23S rDNA spacer region. The relative positions of 16S rDNA, spacer tDNAs, and 23S rDNA (15) are indicated. H, *Hin*fI sites. The expected fragment sizes for *B. burgdorferi* B31 are shown. The positions of primers P_A, P42, and IS2 are shown, and the sequences for P_A and P42 are indicated. The sequence of IS2 has been described previously (30).

P/Gau and H2 also generated pattern 1. However, *B. afzelii* type strain VS461 and human skin isolate ACA1 yielded an atypical *Hin*fI RFLP pattern not observed in any other *B. burgdorferi* strains tested (Fig. 5B).

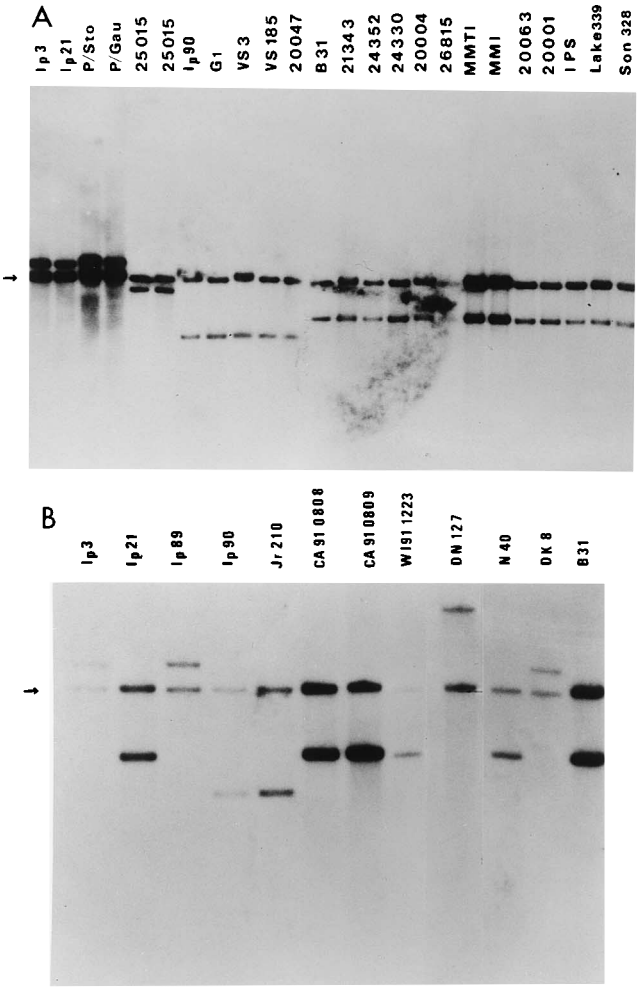

FIG. 2. RFLP analysis of *B. burgdorferi* sensu lato isolates. Genomic DNAs from the indicated *B. burgdorferi* isolates were digested with *Hpa*I and were analyzed by Southern blot hybridization as described in Materials and Methods. The blots were probed with ^{32}P-labeled *B. burgdorferi* 5S rDNA-specific probe. The arrows indicate the common 3.2-kb fragment.

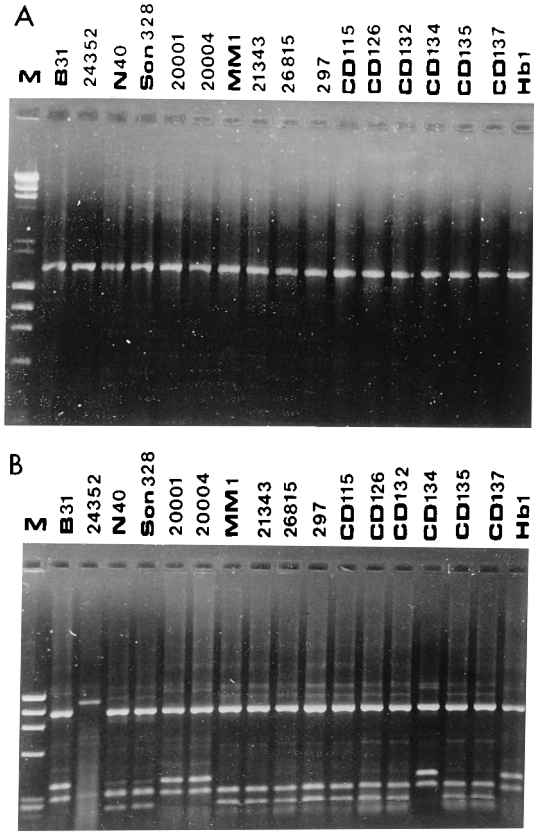

FIG. 3. PCR-RFLP analysis of *B. burgdorferi* sensu stricto isolates. Genomic DNA or cell lysates were prepared from various *B. burgdorferi* isolates. These were used as templates in PCR amplifications with P_A and P42 as primers. PCRs were carried out as described in Materials and Methods. A total of 10 μl of each reaction mixture was electrophoresed on a 1% agarose gel and was stained with ethidium bromide (A) or digested with restriction endonuclease *Hin*fI prior to electrophoresis on 1.5% agarose gels (B). Lanes M, DNA size marker (*Hae*III-digested ϕX174 DNA and *Hin*dIII-digested bacteriophage λ DNA).

Previous analyses have shown that a number of *B. burgdorferi* isolates are unusual, with properties different from those of other well-characterized members of *B. burgdorferi* sensu lato. Several of these were analyzed by PCR-RFLP, and the results are presented in Fig. 6. Three North American isolates (24330 from an *Ixodes dentatus* tick, 24352 from an *I. scapularis* tick on a cottontail rabbit [1], and 19857 from rabbit kidney [3]) yielded a PCR fragment identical in size to that from B31. However, each of these had unique restriction patterns on digestion with *Hin*fI. Two other unusual isolates, DN127 and 25015, produced a larger PCR product of approximately 2 kb and distinct *Hin*fI RFLP patterns. Finally, HO14 and IKA2, which have been characterized as *Borrelia japonica* (17), yielded distinctly different and unique PCR-RFLP results.

These findings indicate that PCR-RFLP analysis can be used for the rapid typing of *B. burgdorferi* isolates and allows discrimination between established genospecies and isolates with unusual genotypic properties.

Typing of *B. burgdorferi* isolates in infected ticks by PCR-RFLP. A primary advantage of this PCR-RFLP analysis is its potential applicability for typing of uncultured *B. burgdorferi* specimens. Theoretically, any material containing *B. burgdorferi* which is amenable to PCR amplification would be an appropriate starting material for testing by this technique. In order to evaluate the potential utility of this assay for molec-

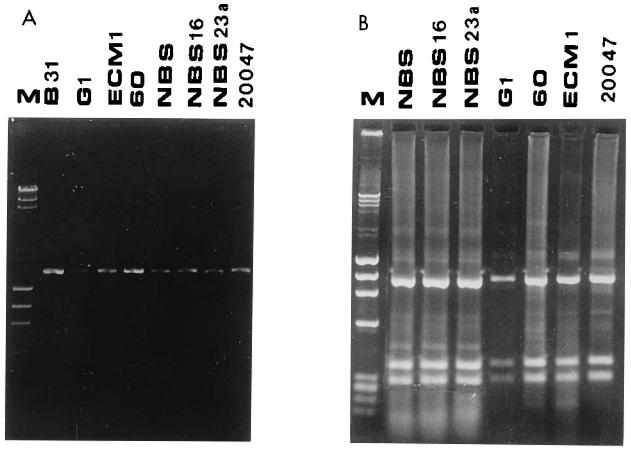

FIG. 4. PCR-RFLP analysis of *B. garinii* isolates. (A) PCR. Strain B31 is a representative of *B. burgdorferi* sensu stricto. (B) *Hin*fI digestion of PCR products. Lanes M, DNA size markers.

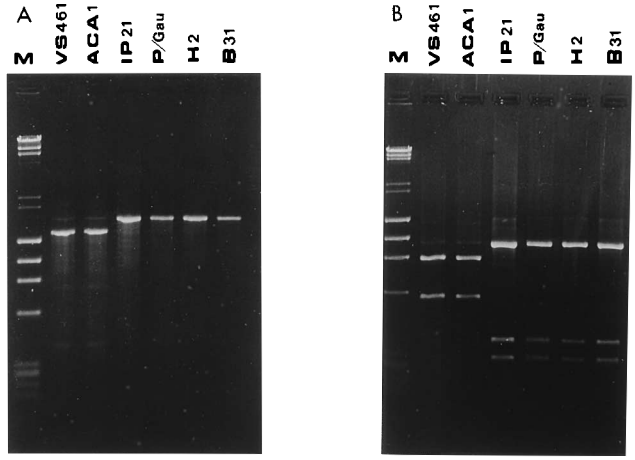

FIG. 5. PCR-RFLP analysis of *B. afzelii* isolates. (A) PCR. (B) *Hin*fI digestion of PCR products. Strains Ip21 and B31 are *B. burgdorferi* sensu stricto. Lanes M, DNA size markers.

ular characterization of uncultured *B. burgdorferi*, lysates of several *I. scapularis* ticks collected in New York State were tested. PCR amplification resulted in an amplified product of 1.7 kb in three of four specimens, and *Hin*fI digestion indicated that all had the RFLP pattern characteristic of strain B31. The results demonstrate that this method can be used for the molecular typing of *B. burgdorferi* isolates without the necessity for prior culture.

DISCUSSION

In an earlier report, we demonstrated the existence of an RFLP associated with the 23S-5S rRNA gene duplication in *B. burgdorferi* (31). In the present study this simple analysis has been extended to many additional isolates from a variety of geographic and biologic origins. In general, the results presented in Fig. 2 and Table 1 are in complete agreement with studies by others who have analyzed the same isolates by a variety of techniques (7, 10, 24, 27, 29). This confirms that simple RFLP analysis can be a useful tool for typing of *B. burgdorferi* isolates. Several points should be made with regard to these data. (i) Virtually all North American isolates tested, regardless of their biologic origins (i.e., tick, human, or animal), are *B. burgdorferi* sensu stricto. There were two exceptions, isolates 25015 and DN127, which produced unique RFLP patterns. The unusual characteristics of these isolates have been noted by others (4, 9, 36) and were also observed in the present study by PCR-RFLP. (ii) Eurasian isolates are more heterogeneous, falling into all three *B. burgdorferi* sensu lato genospecies. (iii) A number of other investigators have analyzed Russian isolates, particularly Ip3, Ip21, and Ip90, which were originally isolated by the group of E. Korenberg (18). Previous studies (including our earlier report) have indicated that Ip21 is *B. afzelii* (24, 31); Baranton et al. (7), however, found it to be *B. burgdorferi* sensu stricto. We reanalyzed this particular isolate after obtaining a new culture directly from Korenberg and have now found this to be *B. burgdorferi* sensu stricto (Fig. 2B). (iv) IKA2, a Japanese isolate from *I. ovatus* (25), is the only 1 of the 57 isolates tested which yielded a single band in the Southern blot analysis, which indicates the absence of the 23S-5S gene duplication. We have previously suggested that the presence of the gene duplication is a unique characteristic of *B. burgdorferi* sensu lato because it is not found in closely related *Borrelia* species (31). As already pointed out, IKA2 has recently been shown to be a member of a new species, *B. japonica* (17). Whether the gene duplication is absent from other members of this species or is unique to IKA2 awaits further investigation.

The molecular analysis of individual isolates of *B. burgdorferi* and correlation of particular bacterial characteristics with specific disease manifestations are limited to culturable isolates of *B. burgdorferi* sensu lato because of the difficulty in culturing the organisms from most biologic sources. It is formally possible, however, that those individual isolates of *B. burgdorferi* which are amenable to culture represent only a small portion of the diversity of *B. burgdorferi* sensu lato in nature. In order to clarify the relationship between spirochete genotype and disease it will be necessary to greatly expand our ability to type *B. burgdorferi* from patients, ticks, and reservoir hosts. In order to simplify genotyping analysis and develop a method which would not require prior culture, the capability of typing individual *B. burgdorferi* isolates by PCR-RFLP of the 16S-23S rDNA intergenic spacer was assessed. Thirty-five isolates of *B. burgdorferi* sensu lato were analyzed by this procedure, and most yielded a PCR product of the expected size. Digestion of the amplified DNA with *Hin*fI resulted in two predominant

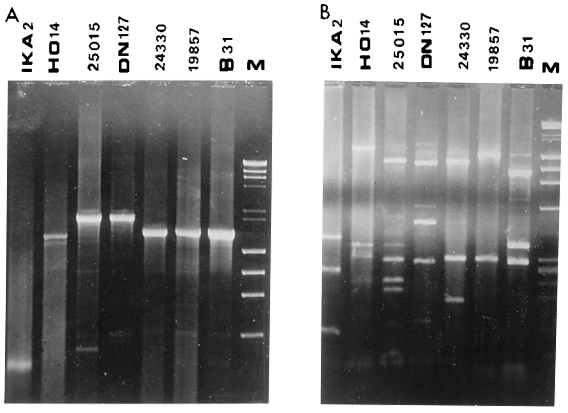

FIG. 6. PCR-RFLP analysis of atypical *B. burgdorferi* isolates. (A) PCR. (B) *Hin*fI digestion of PCR products. Strain B31 is *B. burgdorferi* sensu stricto. Lanes M, DNA size markers.

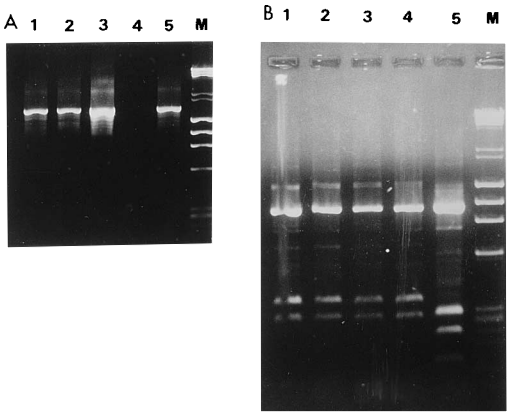

FIG. 7. PCR-RFLP analysis of extracts from field-collected *I. scapularis*. (A) PCR. Lanes 1 to 4, extracts from four individual ticks; lane 5, strain B31. (B) *Hin*fI digestion of PCR products. Lane 1, strain B31; lane 2 to 4, ticks 1 to 3, respectively; lane 5, strain 297. Lanes M, DNA size markers.

DNA fragment patterns (Fig. 3 to 6), one characteristic of strain B31 (pattern 1) and the other characteristic of strain 297 (pattern 2).

Most earlier studies had indicated that *B. burgdorferi* isolates from North America were a highly homogeneous group, with virtually all isolates characterized as *B. burgdorferi* sensu stricto (7, 27, 36, 37, 39). The present investigation reveals that North American isolates are, in fact, somewhat heterogeneous. Similar findings were recently reported by Assous et al. (6). Interestingly, the RFLP pattern of B31, the type strain of *B. burgdorferi* sensu stricto, was less frequently observed (6 of 19 isolates) among these isolates than was pattern 2. In contrast to the findings with *B. burgdorferi* sensu stricto a single RFLP profile (pattern 1; B31 type) was obtained for all seven *B. garinii* isolates analyzed (Fig. 4). The *B. afzelii* isolates tested were heterogeneous with respect to both PCR product size and RFLP profile (Fig. 5). VS461 (the type strain) and ACA1 had a characteristically smaller 1.4-kb fragment, whereas P/Gau and H2 had the B31-type RFLP profile.

It is perhaps noteworthy that six of seven North American patient isolates analyzed showed RFLP pattern 2. The significance of this observation at present is unclear. Most of these isolates were cultured from erythema migrans lesions of patients in Westchester County, N.Y., and may therefore reflect the predominance of RFLP pattern 2 in this geographic area. The fact that the field-collected ticks from New York State which were assayed by PCR-RFLP all showed pattern 1 (Fig. 7) suggests that this may not be the case. It is tempting to speculate that RFLP pattern 2 may be associated with clinically infectious isolates of *B. burgdorferi* sensu stricto in the northeastern United States, but this warrants further investigation.

A growing number of *B. burgdorferi* sensu lato isolates from the United States with properties which preclude their facile species classification have been described. Several such isolates have been analyzed in the present study. Isolate 25015 (a nonpathogenic isolate from an *I. scapularis* tick from New York State) and DN127 (an isolate from an *I. pacificus* tick from California) both produce a PCR fragment which is approximately 300 bp larger than that observed with other *B. burgdorferi* isolates. However, they can be distinguished from each other by *Hin*fI RFLP analysis. This supports other studies which indicated that these isolates may be phylogenetically distinct from *B. burgdorferi* sensu stricto and from each other (12). Three other atypical isolates, 19857 (from a cottontail rabbit in New York State), 24352 (from an *I. scapularis* tick on a cottontail rabbit in New York State), and 24330 (from an *I. dentatus* tick on a cottontail rabbit in New York State), yielded PCR products of the expected size, but all showed distinctive *Hin*fI RFLP patterns. It thus appears that a significant number of North American isolates are genetically distinct from *B. burgdorferi* sensu stricto, and additional studies will be necessary to ascertain whether atypical isolates are truly unique or only appear that way because of limited population analysis. Such investigations will be facilitated by the technique described here because large numbers of tick, wildlife, and patient specimens can be screened for *B. burgdorferi* by PCR and can readily be typed by RFLP analysis of the amplification products. The utility of this approach was demonstrated by typing of three field-collected tick specimens without the need for prior culture. This technique should provide for the direct, simple, and rapid typing of *B. burgdorferi* which is necessary to clarify the relationship between spirochete genotype and disease and the extent of genospecies diversity within *B. burgdorferi* sensu lato.

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