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Genomic Fingerprinting of *Borrelia burgdorferi* Sensu Lato by Pulsed-Field Gel Electrophoresis

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A total of 46 *Borrelia burgdorferi* sensu lato isolates that were isolated from patients with Lyme borreliosis and infected animals or were extracted from ticks of the genus *Ixodes* were analyzed. Large restriction fragment patterns obtained after cleavage of genomic DNAs with *Mlu*I were analyzed by pulsed-field gel electrophoresis (PFGE). To eliminate the contribution of plasmid DNA, only fragments greater than 70 kb were used for the analysis. The results indicated that each of the 14 *B. burgdorferi* sensu stricto isolates were recognized by a band at 135 kbp, each of the 12 *Borrelia garinii* isolates by two bands (220 and 80 kbp), and each of the 20 *Borrelia afzelii* isolates by three bands (460, 320, and 90 kbp). Whereas differences in the PFGE patterns among *B. burgdorferi* sensu stricto isolates and *B. garinii* isolates were noted, *B. afzelii* isolates were all similar. Identification of isolates by PFGE correlates with their belonging to a given species within *B. burgdorferi* sensu lato.

Borrelia burgdorferi was identified in 1982 as the agent of Lyme borreliosis (11) and was recognized as a new species of the genus *Borrelia* in 1984 (16). Three species were recently delineated for *B. burgdorferi* sensu lato: *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (4, 20). This division into three species was based on DNA-DNA hybridizations, rRNA gene restriction patterns, and identification by monoclonal antibodies, all tools recognized currently by taxonomists (29). The results obtained by different methods of typing isolates such as polymerase chain reaction (21, 23), arbitrarily primed polymerase chain reaction (30), multilocus enzyme electrophoresis (10), 16S rRNA sequencing (1, 2, 18, 19, 25), *fla* gene sequencing (21, 28), and serotyping (31) are consistent with this new taxonomy. Heterogeneity among isolates has also been observed by analysis of the profiles of linear and circular plasmids (6, 27). Digestion of genomic DNAs with restriction endonucleases followed by conventional electrophoresis also showed differences among isolates. However, the thousands of bands obtained are difficult to interpret except when Southern analysis is used following hybridization with diverse probes (17, 27, 28). The latter technique yielded groupings of isolates along the same lines as described elsewhere (4, 20).

Pulsed-field gel electrophoresis (PFGE) was used for constructing the physical map of *B. burgdorferi* (12). This genome is unique, since it contains a 945-kbp linear chromosome as well as linear and circular plasmids which vary in size and number among the isolates (7, 9, 13). In the present article, we analyze several different isolates by PFGE. The results indicate that a given isolate can be readily identified as belonging to a specific species.

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MATERIALS AND METHODS

Bacterial isolates and media. The *B. burgdorferi* sensu lato isolates used in this study are listed in Table 1. The isolates were grown in BSKII medium at 30°C (5).

PFGE, digestion of DNA in agarose, and large restriction fragments patterns LRFP nomenclature. Previously described procedures were used for the preparation of high-molecular-weight genomic DNAs and PFGE (9, 12). Separation was achieved with a pulse time ramped from 3 to 40 s for 20 h with a contour-clamped homogeneous electric field-DRII apparatus (Bio-Rad laboratories, Richmond, Calif.).

Restriction endonucleases *Mlu*I (A/CGCGT) and *Sma*I (CCC/GGG) were purchased from Pharmacia. Genomic DNAs, in low-melting-temperature agarose, were digested with 20 to 40 U of restriction endonuclease for 20 h in 200 μl of the buffer recommended by the supplier. The lambda concatemers used as size markers for Fig. 2 were those used in reference 12 (monomer size, 44.3 kbp) and for Fig. 1 and 3 were those commercialized by Tebu (monomer size, 48.5 kbp).

An LRFP is defined as a unique PFGE pattern. We ignored bands lower than 70 kb, since these bands would be the results of uncut or cut plasmids, which could complicate the interpretation (24). The LRFPs obtained were designated ML or SM to identify the restriction endonuclease *Mlu*I or *Sma*I, respectively, with the suffix b, g, or a denoting *B. burgdorferi* sensu stricto, *B. garinii*, or *B. afzelii*, respectively. Different LRFPs of the same species are numbered 1 or 2, etc.

DNA-blot analysis. For blot analysis according to Southern (26), DNA fragments separated by PFGE were submitted to depurination and then transferred to Hybond-N nylon membranes (Amersham International, Amersham, England). To prepare the probe, the 135-kbp *Mlu*I band from isolate B31 was excised from a low-melting-temperature agarose pulsed-field gel and washed with 1 ml of distilled water for 1 h at room temperature. A reduced volume of 250 μl of the sample was heated at 100°C for 10 min. The tube was placed on ice, and 25 μl was labelled by random priming with digoxigenin-11-dUTP. The probe thus generated was used in hybridization experiments as described elsewhere (8).

TABLE 1. *B. burgdorferi* sensu lato isolates used in this study and their characteristics[a]

Isolate	Origin	Geographical location	ML LRFP	Ribotype	Serotype	Electrotype (ET)
B. burgdorferi						
B31	*I. dammini*	United States	b1	E1/H1/R1/P1	1	6
297	Human CSF[b]	United States	b2	E1/H2/R1/P1	1	3
HUM115	*I. pacificus*	United States	b3	E1/H2/R1/P1	ND[c]	ND
IRS	*I. ricinus*	Switzerland	b3	E2/H1/R1/P1	1	8
HUM7814	*I. pacificus*	United States	b4	E1/H2/R1/P1	ND	ND
20006	*I. ricinus*	France	b5	E1/H1/R1/P1	1	7
HUM3336	*I. pacificus*	United States	b6	E1/H1/R1/P1	ND	ND
MEN115	*I. pacificus*	United States	b6	E1/H1/R1/P1	ND	ND
Lake 339	*I. pacificus*	United States	b7	E1/H1/R1/P1	ND	ND
SON188	*I. pacificus*	United States	b7	E1/H1/R1/P1	ND	ND
SON335	*I. pacificus*	United States	b8	E1/H1/R1/P1	ND	ND
Z136	*I. ricinus*	Germany	b8	E1/H1/R1/P1	ND	ND
21305	*Peromyscus leucopus*	United States	b9	E1/H2/R1/P1	ND	ND
212	*I. ricinus*	France	b10	E1/H1/R1/P1	ND	ND
B. garinii						
20047	*I. ricinus*	France	g1	E3/H3/R3/P2	0	25
VS185	*I. ricinus*	Switzerland	g1	E3/H4/R3/P2	6	24
VS468	*I. ricinus*	Switzerland	g2	E4/H3/R2/P3	6	31
N34	*I. ricinus*	Germany	g2	E3/H4/R2/P4	ND	31
G25	*I. ricinus*	Sweden	g2	E3/H4/R2/P4	ND	33
PBi	Human CSF	Germany	g2	E3/H4/R2/P4	4	33
TN	*I. ricinus*	Germany	g2	E4/H3/R2/P3	ND	ND
PBr	Human CSF	Germany	g2	E3/H4/R3/P5	3	ND
IPer90	*I. persulcatus*	CIS	g2	E5/H3/R3/P6	ND	ND
T25	*I. ricinus*	Germany	g3	E3/H4/R3/P2	7	ND
VS286	*I. ricinus*	Switzerland	g4	E3/H4/R3/P4	ND	ND
153	*I. ricinus*	France	g4	E3/H3/R3/P4	ND	ND
B. afzelii						
VS461	*I. ricinus*	Switzerland	a1	E7/H5/R4/P7	ND	16
PGau	Human ACA[d]	Germany	a1	E7/H5/R4/P7	2	17
F1	*I. ricinus*	Sweden	a1	E7/H5/R4/P7	0	18
UMO1	Human ECM[e]	Sweden	a1	E7/H5/R4/P7	ND	19
IPer3	*I. persulcatus*	CIS	a1	E7/H5/R4/P7	ND	ND
I. persulcatus	*I. persulcatus*	Japan	a1	E6/H5/R4/P7	ND	ND
PSto	Human ACA[d]	Germany	a1	ND	2	ND
DK3	Human ACA[d]	Denmark	a1	ND	2	ND
DK8	Human ACA[d]	Denmark	a1	E7/H5/R4/ND	2	ND
ACA1	Human ACA[d]	Sweden	a1	ND	2	15
ECM1	Human ECM[d]	Sweden	a1	E7/ND/R4/ND	ND	ND
ECM8	Human ECM[e]	Sweden	a1	ND	ND	ND
SmS1	*Apodemus flavicollis*	Sweden	a1	E7/H5/R4/ND	ND	ND
Ip21	*I. persulcatus*	CIS	a1	E7/H6/R4/ND	ND	ND
J1	*I. persulcatus*	Japan	a1	E6/H5/R4/ND	ND	ND
P427a	*I. persulcatus*	Japan	a1	E7/H5/R4/ND	ND	ND
BFox	Fox	Japan	a1	ND	ND	ND
HFox	Fox	Japan	a1	ND	ND	ND
M7	*I. persulcatus*	China	a1	E6/H5/R4/ND	ND	ND
2246	*I. persulcatus*	China	a1	E7/H5/R4/ND	ND	ND

[a] Ribotypes are from references 20 and 22; they have been designated E, H, R, and P to identify the restriction endonucleases *Eco*RI, *Hin*dIII, *Eco*RV, and *Pst*I, respectively, that were used, followed by numbers to indicate subtypes. Electrotypes are from reference 10, and serotypes are from reference 31.
[b] CSF, cerebrospinal fluid.
[c] ND, not done.
[d] ACA, acrodermatitis chronica atrophicans.
[e] ECM, erythema chronicum migrans.

RESULTS

Choice of a rare cutting restriction endonuclease yielding an evaluable pattern. Of the eight restriction endonucleases used for the construction of the physical map of *B. burgdorferi* sensu stricto isolate 212 (12), we chose *Mlu*I to compare the different isolates. This enzyme cuts the DNA in sufficient numbers of fragments to allow easily interpretable comparisons. Only fragments greater than 70 kb were used to define an LRFP.

Fingerprinting with *Mlu*I and recognition of the three species. A total of 46 *B. burgdorferi* sensu lato isolates were analyzed by *Mlu*I digestion of genomic DNA followed by PFGE. The results showed that with *Mlu*I, 15 LRFPs were obtained (Table 2). The distribution of LRFPs was as follows: 10 LRFPs for *B. burgdorferi* sensu stricto (14 isolates), 4 LRFPs for *B. garinii* (12 isolates), and 1 LRFP for *B. afzelii* (20 isolates) (Table 2). Specific bands were found for each species. Analysis of the LRFPs obtained allowed the recog-

TABLE 2. Sizes of the bands obtained with *Mlu*I digestion of DNAs of the *B. burgdorferi* sensu lato isolates used in this study

LRFP	Isolate(s)	Presence of band with the following sizes (kbp):														
		460	410	400	380	320	280	220	170	160	145	135	110	100	90	80
MLb1	B31		+								+	+				
MLb2	297		+								+	+		+		
MLb3	IRS and HUM115			+							+	+		+		
MLb4	HUM7814				+						+	+		+		
MLb5	20006			+							+	+		+		+
MLb6	MEN115 and HUM3336			+						+		+		+		
MLb7	Lake 339 and SON188			+								+	+	+		
MLb8	Z136 and SON335					+					+	+		+	+	
MLb9	21305					+						+	+	+	+	
MLb10	212		+								+	+		+		
MLg1	20047 and VS185						+	+				+				+
MLg2	N34, PBi, G25, VS468, TN, PBr, and IPer90			+				+						+		+
MLg3	T25							+	+					+		+
MLg4	VS286 and 153			+				+								+
MLa1	*B. afzelii*[a]	+				+									+	

[a] All 20 *B. afzelii* isolates in Table 1.

nition of a specific band at 135 kbp for *B. burgdorferi* sensu stricto (Fig. 1A). Two bands at 220 and 80 kbp were specific to *B. garinii* (Fig. 2, lanes 1 to 12), while for *B. afzelii*, three bands (at 460, 320, and 90 kbp) were characteristic (Fig. 2, lanes 13 and 14; Table 2).

The specific bands noted above for identification of a species are identified by their size only. The question arises as to whether the bands characteristic of a species are indeed genetically similar. The 135-kbp *Mlu*I band of *B. burgdorferi* sensu stricto isolate B31 hybridizes to the same 135-kbp band in each of the 14 isolates of that species (Fig. 1B). A similar experiment showed that the 220-kbp *Mlu*I band characteristic of *B. garinii* is indeed the same for each of the 12 isolates tested (data not shown). The experiments were not performed for *B. afzelii* because of the unique and unambiguous MLa1 pattern consisting of most of the genome (Table 2).

Fingerprinting with *Sma*I. Another rare cutting restriction endonuclease was used to differentiate among isolates showing the same *Mlu*I profile. *Sma*I allowed the differentiation of some isolates from each other. For example, among the *B. garinii* isolates that were indistinguishable with *Mlu*I (Table 2 [LRFP MLg2]), one isolate (PBr) had a unique LRFP with *Sma*I that was distinct from those of the other four isolates N34, G25, VS468, and TN) (Fig. 3). On the other hand, isolates T25 and PBr, which had different *Mlu*I patterns, shared the same *Sma*I pattern.

Among the 20 *B. afzelii* isolates which were indistinguishable with *Mlu*I, 15 isolates shared the SMa1 pattern, with a characteristic quadruplet (Fig. 3, lane 9) while five isolates had LRFPs which varied from that of SMa1 by one additional or one missing band (LRFPs SMa2, SMa3, and SMa4 [Fig. 3, lanes 10 to 12]).

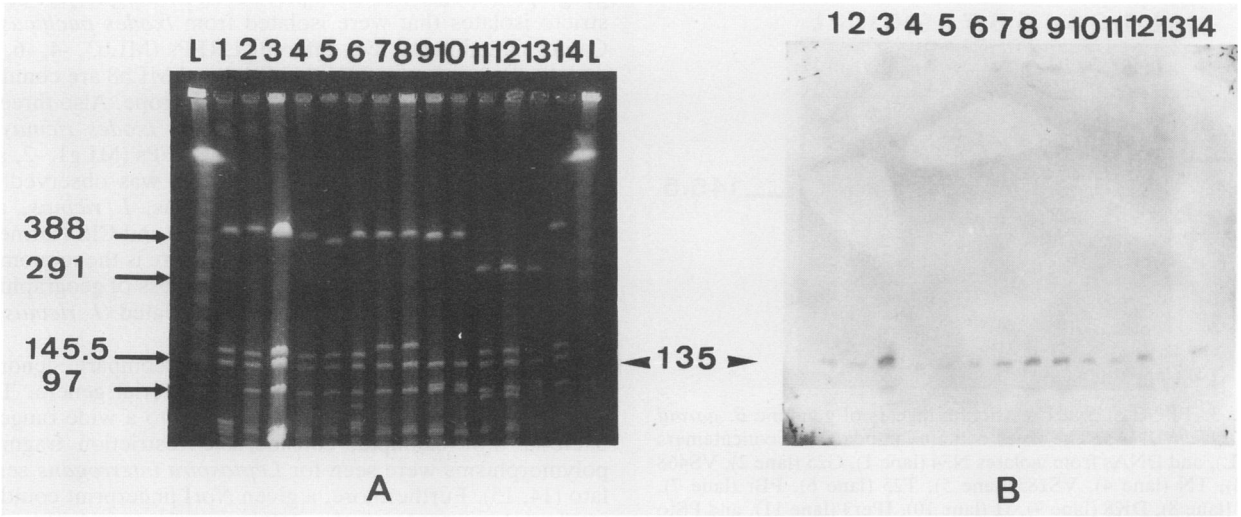

FIG. 1. (A) PFGE of *Mlu*I restriction digests of genomic *B. burgdorferi* sensu stricto DNAs. The lanes contain lambda DNA concatemers (lanes L) and DNAs from isolates B31 (lane 1), 297 (lane 2), IRS (lane 3), HUM115 (lane 4), HUM7814 (lane 5), 20006 (lane 6), MEN115 (lane 7), HUM3336 (lane 8), Lake 339 (lane 9), SON188 (lane 10), Z136 (lane 11), SON335 (lane 12), 21305 (lane 13), and 212 (lane 14). The 135-kbp band characteristic of *B. burgdorferi* sensu stricto is indicated by an arrowhead on the right. (B) Blot of the PFGE gel hybridized with the 135-kbp band from *B. burgdorferi* isolate B31 DNA.

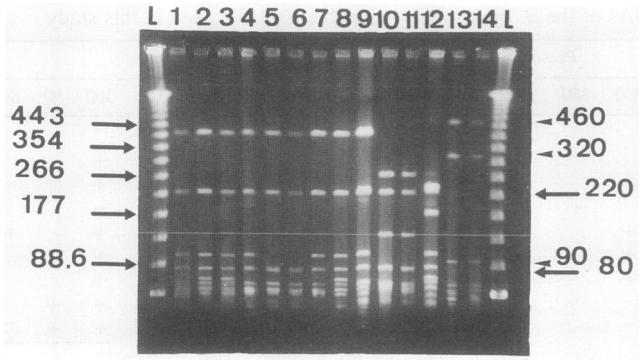

FIG. 2. PFGE of *Mlu*I restriction digests of genomic *B. garinii* DNAs and two representatives of *B. afzelii* DNA. The lanes contain lambda DNA concatemers (lanes L) and DNAs from isolates N34 (lane 1), PBi (lane 2), G25 (lane 3), VS468 (lane 4), VS286 (lane 5), 153 (lane 6), TN (lane 7), PBr (lane 8), IPer90 (lane 9), 20047 (lane 10), VS185 (lane 11), T25 (lane 12), PGau (lane 13), and VS461 (lane 14). The 220- and 80-kbp bands characteristic of *B. garinii* are indicated on the right by arrows. The arrowheads indicate the three bands (460, 320, and 90 kbp) characteristic of *B. afzelii*.

DISCUSSION

Species identification. Analysis of PFGE patterns after *Mlu*I digestion permitted the specific identification of each of the three *Borrelia* species associated with Lyme borreliosis. One band at 135 kbp was specific for *B. burgdorferi* sensu stricto, whereas two bands (220 and 80 kbp) were found in all *B. garinii* isolates tested. Three bands (460, 320, and 90 kbp) were characteristic of *B. afzelii*. Strong evidence that the bands characteristic of a given species were the same genetically strengthened our identification procedure.

Genetic diversity. Species identification by PFGE corresponded exactly to that obtained previously with rRNA gene restriction patterns (22). However, subgroups obtained with PFGE did not always correspond to those obtained with ribotyping. The PFGE method usually allowed further subdivision of isolates within a *Borrelia* species. For example, the 9 *B. burgdorferi* sensu stricto isolates tested, which had the same ribotype (E1/H1/R1/P1 [Table 1]) with enzymes *Eco*RI, *Hin*dIII, *Eco*RV, and *Pst*I (4), could be separated into six LRFPs (Table 1). On the other hand, the same MLb3 pattern was observed for two isolates showing two different ribotypes (Table 1). *B. burgdorferi* sensu stricto, which seemed homogeneous by ribotyping (4, 22) and serotyping (serotype 1 according to reference 31), appeared more heterogeneous with regard to the *Mlu*I PFGE patterns (MLb1 to MLb10). It should be noted that the type strain B31 is unique in lacking the 100-kbp *Mlu*I digestion product.

B. garinii was very heterogeneous in rRNA gene restriction patterns as well as in serotyping (Table 1) (31). In contrast, only four patterns (MLg1 to MLg4) for 12 *B. garinii* isolates were obtained, and among them, 7 isolates exhibited the MLg2 profile.

B. afzelii appeared very homogeneous whatever the method used. For 14 isolates with the same MLa1 pattern, only two ribotypes were observed with *Eco*RI (E6 and E7) and *Hin*dIII (H5 and H6), and a unique ribotype was observed with *Eco*RV (R4). Furthermore, Wilske et al. (31) found that six isolates, now known as *B. afzelii*, that had been isolated from cutaneous lesions all belonged to serotype 2 (31).

The results of multilocus enzyme electrophoresis (10), another technique used to type isolates, were incorporated in the present study (Table 1). No correlation between electrotype (obtained by multilocus enzyme electrophoresis) and either LRFP or ribotype could be shown.

A single *Mlu*I pattern was obtained for each of the 20 *B. afzelii* isolates tested. However, a quarter of them (ACA1, VS461, PGau, F1, and UMO1) had distinct electrotypes (ET15, ET16, ET17, ET18, and ET19, respectively) (10), and another quarter (J1, I. persulcatus, IPer3, ECM8, and PSto) had distinct *Sma*I patterns. Thus, the *B. afzelii* isolates are not entirely homogeneous, although the isolates that were tested had the same MLa1 pattern and *Eco*RV ribotype.

Epidemiological significance. Correlation of PFGE patterns with the geographical locations of isolation led us to conclude that a high genetic diversity exists within limited geographic areas. For example, seven *B. burgdorferi* sensu stricto isolates that were isolated from *Ixodes pacificus* in California exhibited five different LRFPs (MLb3, -4, -6, -7, and -8). Interestingly, LRFPs MLb3 and MLb8 are common to isolates from the United States and Europe. Also three *B. garinii* isolates that were isolated from *Ixodes ricinus* in Switzerland exhibited three different LRFPs (MLg1, -2, and -4). In contrast, a single LRFP (MLg2) was observed for isolates that were isolated from humans, *I. ricinus*, and *Ixodes persulcatus* in Sweden, Germany, and CIS (formerly USSR). However, the most striking feature is the monomorphism of the 20 *B. afzelii* isolates, regardless of geographical origin or host from which they were isolated (*I. ricinus*, *I. pacificus*, rodents, foxes, and humans).

Recently, PFGE has been used to compare genomic restriction patterns among different bacterial genera. This relatively simple technique is applicable to a wide range of bacteria. For example, considerable restriction fragment polymorphisms were seen for *Leptospira interrogans* sensu lato (14, 15). Furthermore, a given *Not*I fingerprint could be attributed to each of the 160 serovars tested (14, 15), allowing the identification of new isolates by LRFP. In contrast to the *L. interrogans* example, restriction analysis with *Xba*I could not distinguish among biovars in the genus *Brucella* (3).

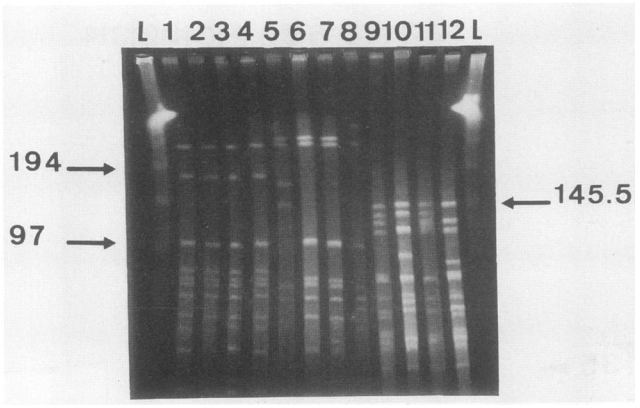

FIG. 3. PFGE of *Sma*I restriction digests of genomic *B. garinii* and *B. afzelii* DNAs. The lanes contain lambda DNA concatemers (lanes L), and DNAs from isolates N34 (lane 1), G25 (lane 2), VS468 (lane 3), TN (lane 4), VS185 (lane 5), T25 (lane 6), PBr (lane 7), VS286 (lane 8), DK8 (lane 9), J1 (lane 10), IPer3 (lane 11), and PSto (lane 12). LRFP SMa1, exemplified in lane 9 by isolate DK8, was shared by 15 *B. afzelii* isolates. LRFP SMa2, exemplified in lane 10 by isolate J1, was shared by isolate I. persulcatus. LRFP SMa3, exemplified in lane 11 by isolate IPer3, was shared by isolate ECM8. LRFP SMa4 was exemplified in lane 12 by isolate PSto.

PFGE analysis of *Borrelia* genomic DNAs provides a relatively simple means of characterizing Lyme borreliosis spirochetes. Examination of additional isolates would provide further evidence regarding the utility of this approach for taxonomic and epidemiologic purposes.

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