Analysis of the Genetic Polymorphism of *Borrelia burgdorferi* Sensu Lato by Multilocus Enzyme Electrophoresis

TIZIANO BALMELLI AND JEAN-CLAUDE PIFFARETTI*

Istituto Cantonale Batteriosierologico, 6904 Lugano, Switzerland

In recent years, *Borrelia burgdorferi* sensu lato has been subdivided into three species, *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*, and a new species restricted to Japan, *Borrelia japonica*, has been isolated from *Ixodes ovatus*. In addition, members of several new genomic groups have been found in America and in Europe, suggesting that there are additional genospecies. In order to study the diversity of *B. burgdorferi* sensu lato, we analyzed 54 isolates cultured from humans and from different tick species and obtained from diverse geographic areas, including Europe, the United States, Japan, and the People's Republic of China. In order to investigate the genetic relationship between microorganisms that are transmitted by soft ticks and microorganisms that cause Lyme disease, we also included three strains of relapsing fever spirochetes. The method which we used was multilocus enzyme electrophoresis; 12 genetic loci were characterized on the basis of the electrophoretic mobilities of their products, and 50 distinct allele profiles (electrophoretic types) were distinguished. The mean genetic diversity per locus was 0.747. A cluster analysis of a matrix of genetic distances for pairs of electrophoretic types revealed 11 divisions that were separated at genetic distances greater than 0.65. Five of these divisions corresponded to *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. japonica*, and the newly proposed species "*Borrelia andersonii*." Our results also confirmed that there are two additional genomic groups in Europe and at least one additional group in the United States. The relapsing fever spirochetes were not clearly separated from the spirochetes associated with Lyme disease. In conclusion, we believe that the previously proposed subdivision of *B. burgdorferi* sensu lato into only four species should be reconsidered.

Lyme borreliosis is a multisystem disease that is caused by the spirochete *Borrelia burgdorferi*, which is transmitted to humans by the following ticks: *Ixodes ricinus* in Europe, *Ixodes persulcatus* in Asia, and *Ixodes scapularis* and *Ixodes pacificus* in North America. In about 60 to 80% of the infected patients, the illness begins with a local proliferation of microorganisms at the site of the tick bite, which leads to the first symptom of the disease, erythema chronicum migrans. Neurological or cardiac abnormalities, skin manifestations, or intermittent arthritis may follow erythema chronicum migrans. The late stage of the disease is characterized by chronic manifestations in joints, skin, and brains, such as arthritis, acrodermatitis chronica atrophicans, and neuroborreliosis, respectively (for a review see reference 45).

Since the first isolation of *B. burgdorferi* from the midgut of *I. scapularis* (18), a large number of strains have been recovered from both humans and ticks. The bacteria identified as *B. burgdorferi* constitute a heterogeneous group that is composed of several genomic species. Analyses of strains originating from various geographic areas resulted in subdivision of the former species *B. burgdorferi* (*B. burgdorferi* sensu lato) into the following three species: *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (12, 15, 19). Several groups of workers, including our group, have demonstrated that there is an association between these three species and the different chronic manifestations of Lyme disease. *B. afzelii* and *B. garinii* have been found to be associated with acrodermatitis chronica atrophicans and neuroborreliosis, respectively, and arthritis, which is the predominant late complication of the disease in

the United States, has been found to be associated with *B. burgdorferi* sensu stricto (7, 9, 47).

Recently, it has been suggested that *B. burgdorferi* sensu lato includes additional species. A new, apparently nonpathogenic species, *Borrelia japonica*, was isolated from *Ixodes ovatus* and was found to be restricted to Japan. This species has been characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, pulsed-field gel electrophoresis, total DNA-DNA hybridization, ribotyping, and restriction fragment length polymorphism (RFLP) of the flagellin structural gene and the 16S rRNA gene (27, 38). Until very recently, all three species described by Baranton et al. (12) were found in Europe, while in the United States *B. burgdorferi* sensu stricto was found almost exclusively, suggesting that the North American population is homogeneous compared with the heterogeneous European population. However, several atypical strains have now been isolated on both the east and west coasts of the United States and characterized (4, 5, 17, 31). The results of a PCR analysis of the *fla* gene suggested that some of these atypical strains belong to two new American genomic groups (8). This suggestion was confirmed by the results of an RFLP analysis of the intergenic space between the tandemly repeated 23S (*rrl*) and 5S (*rrf*) rRNA genes (37). In the same report, several European strains were also identified as members of two additional genomic groups.

The purpose of this study was to determine the overall genetic relationships of *B. burgdorferi* sensu lato strains isolated in different parts of the world. Instead of the methods used most of the time to classify *B. burgdorferi* sensu lato strains, we used multilocus enzyme electrophoresis (MLEE), which is based on the polymorphism of a significant number of genes that encode housekeeping enzymes (and thus are selectively neutral) and not on a single chromosomal region. In this technique bacterial isolates are differentiated on the basis of variations in the electrophoretic mobilities of a large number of metabolic enzymes. The different electromorphs of each en-

* Corresponding author. Mailing address: Istituto Cantonale Batteriosierologico, via Ospedale 6, 6904 Lugano, Switzerland. Phone: (004191) 923 25 22. Fax: (004191) 922 09 93. Electronic mail address: piffaret@cmu.unige.ch.

zyme reflect different alleles at the corresponding gene locus of the enzyme, and the electromorph profiles for a number of enzymes are identified as multilocus phenotypes (electrophoretic types [ETs]) which reflect chromosomal genotypes. Therefore, MLEE is a suitable tool for analyzing genetic diversity and for determining genetic distances between genotypes (44). Moreover, when MLEE is used to identify genomic groups, the results correlate well with the results of DNA-DNA hybridization (12, 16), which is considered the standard reference technique (48). In this paper we describe the results of an MLEE analysis of 54 *B. burgdorferi* sensu lato strains and three strains of relapsing fever spirochetes.

MATERIALS AND METHODS

Bacterial isolates. We analyzed 50 *B. burgdorferi* sensu lato strains (28 clinical isolates and 22 strains cultivated from different *Ixodes* species) and five genetically characterized strains, including *B. burgdorferi* sensu stricto B31^T (T = type strain), *B. afzelii* VS461^T, *B. garinii* VS102 (16, 20, 36), and *B. japonica* HO14^T. In addition, we included in this study *Borrelia parkeri* M3001, *Borrelia hermsii* HSI, and *Borrelia turicatae* M2007 (Table 1). The origins of the strains were diverse and included Europe, the United States, Japan, and the People's Republic of China.

Cultures and preparation of lysates. Each *Borrelia* strain was grown in 2 liters of BSK-H medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 6% rabbit serum (Sigma) at 32°C until the color of the medium changed from red to yellowish. In order to avoid artifacts due to enzymatic proteolysis of dead cells that precipitated to the bottoms of the flasks, only the bacteria in the upper nine-tenths of each culture were harvested and centrifuged (17,000 × g, 4°C, 20 min). The cells were washed twice in lysate buffer (10 mM Tris, 1 mM EDTA, 0.5 mM NADP [pH 6.8]), resuspended in 2 ml of the same buffer, and disrupted by ultrasound treatment while they were cooled in ice (44). Cellular debris was removed by centrifugation at 20,000 × g and 4°C for 30 min, and the resulting supernatant was frozen and stored in 150-μl aliquots at -70°C until it was used.

Electrophoresis and enzyme staining. Bacterial lysates were thawed and subjected to gel electrophoresis under nondenaturing conditions in 10% starch gels as described by Selander et al. (44). Enzyme activities were detected on gels by using specific staining methods. We used the following 12 enzymes which exhibited demonstrable and interpretable activities for the MLEE analysis: Phe-L-Leu peptidase (PEP1), Leu-Leu-L-Leu peptidase (PEP2), Phe-L-Pro peptidase (PEP3), glyceraldehyde phosphate dehydrogenase (GP1), hexokinase (HEX), phosphoglucose isomerase (PGI), pyruvate kinase (PYK), triose phosphate isomerase (TPI), lactate dehydrogenase (LDH), mannose phosphate isomerase (MPI), 6-phosphogluconate dehydrogenase (6-PG), and esterase (EST). Electrophoresis for PEP1, PEP2, PEP3, GP1, HEX, PGI, PYK, TPI, and EST was performed with buffer system F (Tris-malate, pH 8.2), and electrophoresis for LDH, MPI, and 6-PG was performed with buffer system A (Tris-citrate, pH 8.0). The staining methods used for PEP1, PEP2, PEP3, GP1, HEX, PGI, EST, LDH, MPI, and 6-PG were the staining methods described by Selander et al. (44), and the staining methods used for PYK and TPI were the methods described by Harris and Hopkinson (24).

Statistical analysis. A statistical analysis of the data was performed by using a computer program designed by T. S. Whittam and R. K. Selander. The genetic diversity (*h*) at each enzyme locus was calculated as follows: $h = (1 - \sum x_i^2) / [n(n-1)]$, where x_i is the frequency of the *i*th allele and *n* is the number of ETs. The mean genetic diversity per locus was the arithmetic average of the *h* values obtained for the locus. The genetic distance between two ETs was expressed as the proportion of loci at which dissimilar alleles occurred. The ET clusters were determined by using the average-linkage method and a matrix of pairwise coefficients of genetic distance (44).

RESULTS

Genetic diversity. All of the enzyme loci analyzed in this study except the EST locus were polymorphic; the EST locus was monomorphic (Tables 2 and 3). For the 12 enzymes analyzed, the number of alleles per locus ranged from 1 (EST) to 22 (PEP1), and the average number of alleles was 10.6. The genetic diversity at each locus was between 0.000 (the value for EST) and 0.925 (the value for PEP1) (Table 3). The mean genetic diversity for all of the loci was 0.747. A total of 50 ETs were identified for the 57 strains which we studied (four strains exhibited ET 5, two strains exhibited ET 15, and four strains exhibited ET 42 [Table 1]). It should be pointed out that the ET numbers used in this study do not correspond to the num-

bers used in our previous studies (9, 15) because one enzyme locus was replaced by two other loci and a different collection of strains was studied. The allele profiles of the ETs are shown in Table 2.

Cluster analysis and dendrogram. A cluster analysis of the 50 ETs in which the average-linkage method was used revealed 11 divisions (divisions I to XI [Fig. 1]) that were separated by genetic distances greater than 0.65. Divisions I, II, VII, and VIII each contained a previously characterized strain of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica* (strains B31^T [ET 2], VS102 [ET 18], VS461^T [ET 28], and HO14^T [ET 41], respectively).

We found that, as determined by genetic distances, two strains of the relapsing fever spirochetes *B. hermsii* and *B. parkeri* (ETs 9 and 10) were more closely related to *B. burgdorferi* sensu stricto (genetic distances, 0.7 to 0.8) than to *B. garinii* and *B. afzelii* (genetic distances, >0.8). On the other hand, *B. turicatae* (ET 44), another relapsing fever spirochete, was more closely related to *B. afzelii* (genetic distance, 0.7 to 0.8) than to *B. burgdorferi* sensu stricto and *B. garinii* (genetic distances, >0.8).

DISCUSSION

Genetic structure of the *B. burgdorferi* sensu lato population.

In a previous study, a collection of *B. burgdorferi* sensu lato strains was separated into three genomic groups by MLEE analysis (15). This result, together with the results of other genetic studies, contributed to the identification of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (12). In this study, our analysis of a different population of strains revealed that there are additional groups. Our data are consistent with the results of a recent study in which the molecular polymorphism of a ribosomal intergenic space and total DNA-DNA hybridization were used; this study showed that there were four other genomic groups in addition to the four species described previously (37). Divisions I, II, VII, and VIII represent the four established species (*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica*, respectively), whereas the four genomic groups of Postic et al. (37) correspond to our divisions IV, V, IX, and XI. In addition, we identified division III containing ETs 19 through 21 (represented by three strains obtained from Japan, Russia, and Holland), division VI (represented by ET 27), and division X (represented by ET 48).

***B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*.** *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* are represented by divisions I, II, and VII, respectively. The genetic distances that separate these groups are consistent with data that were obtained by using a different collection of strains and a smaller number of enzyme loci (15). As reported previously, *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* are preferentially associated with different chronic manifestations of Lyme disease (7, 9, 47). Briefly, it appears that the first symptom of the disease, erythema chronicum migrans, is associated with all three of these species, whereas arthritis and acrodermatitis chronica atrophicans are caused by *B. burgdorferi* sensu stricto and *B. afzelii*, respectively, and neuroborreliosis occurs after infection by *B. garinii* or *B. burgdorferi* sensu stricto.

***B. japonica*.** We confirmed that the recently described Japanese species *B. japonica* does exist (division VIII, ETs 41 through 43, in Fig. 1); this species is represented by six strains isolated from *I. ovatus* (27, 38).

None of the strains isolated from the patients with Lyme disease was identified as *B. japonica*, which is consistent with the apparent lack of pathogenicity in this species. Experimental infections of mice showed that *B. japonica* O612 was less

TABLE 1. *B. burgdorferi* sensu lato strains studied by the MLEE method

Strain	ET	Division	Species	Biological origin	Geographic origin	Reference or source
NY 13-87	1	I	<i>B. burgdorferi</i> sensu stricto	Skin	New York	42
B31 ^T (= ATCC 35210 ^T)	2	I	<i>B. burgdorferi</i> sensu stricto	<i>Ixodes dammini</i>	New York	18
A44S	3	I	<i>B. burgdorferi</i> sensu stricto	Skin	Holland	47
VS219	4	I	<i>B. burgdorferi</i> sensu stricto	<i>I. ricinus</i>	Switzerland	36
P1G	5	I	<i>B. burgdorferi</i> sensu stricto	Knee	Switzerland	41
IP2	5	I	<i>B. burgdorferi</i> sensu stricto	CSF ^a	France	11
Pka	5	I	<i>B. burgdorferi</i> sensu stricto	Skin	Germany	39
BE1	5	I	<i>B. burgdorferi</i> sensu stricto	Knee	Switzerland	O. Péter
IP3	6	I	<i>B. burgdorferi</i> sensu stricto	CSF	France	12
IP1	7	I	<i>B. burgdorferi</i> sensu stricto	CSF	France	2
GeHo	8	I	<i>B. burgdorferi</i> sensu stricto	Skin	Germany	1
HS1	9		<i>B. hermsii</i>	<i>O. hermsi</i>	Washington	R. C. Johnson
M3001	10		<i>B. parkeri</i>	<i>O. parkeri</i>	United States	R. C. Johnson
Sika2	11	II	<i>B. garinii</i>	<i>I. persulcatus</i>	Japan	37
Sika1	12	II	<i>B. garinii</i>	<i>I. ovatus</i>	Japan	37
ECM8 ^b	13	II	<i>B. garinii</i>	Skin	Sweden	5
PD89	14	II	<i>B. garinii</i>	Blood	People's Republic of China	G. Baranton
387	15	II	<i>B. garinii</i>	CSF	Germany	1
VSBP	15	II	<i>B. garinii</i>	CSF	Switzerland	36
P/Bi	16	II	<i>B. garinii</i>	CSF	Germany	39
A76S	17	II	<i>B. garinii</i>	Skin	Holland	47
VS102	18	II	<i>B. garinii</i>	<i>I. ricinus</i>	Switzerland	36
NT29	19	III	NA ^c	<i>I. persulcatus</i>	Japan	23
Ip89	20	III	NA	<i>I. persulcatus</i>	Russia	23
A19S	21	III	NA	Skin	Holland	47
PotiB1	22	IV	NA	<i>I. ricinus</i>	Portugal	37
PotiB3	23	IV	NA	<i>I. ricinus</i>	Portugal	37
PotiB2	24	IV	NA	<i>I. ricinus</i>	Portugal	37
VS116	25	V	NA	<i>I. ricinus</i>	Switzerland	36
UK	26	V	NA	<i>I. ricinus</i>	England	37
CA2	27	VI	NA	<i>Ixodes neotomae</i>	California	43
VS461 ^T	28	VII	<i>B. afzelii</i>	<i>I. ricinus</i>	Switzerland	36
UO1	29	VII	<i>B. afzelii</i>	Skin	Sweden	S. Bergström
DK8	30	VII	<i>B. afzelii</i>	Skin	Denmark	29
A40S	31	VII	<i>B. afzelii</i>	Skin	Holland	47
P/Sto	32	VII	<i>B. afzelii</i>	Skin	Germany	V. Preac-Mursic
A45S	33	VII	<i>B. afzelii</i>	Skin	Holland	47
A26S	34	VII	<i>B. afzelii</i>	Skin	Holland	47
pko 2.85	35	VII	<i>B. afzelii</i>	Skin	Germany	39
ECM1	36	VII	<i>B. afzelii</i>	Skin	Sweden	S. Bergström
DK3	37	VII	<i>B. afzelii</i>	Skin	Denmark	30
PGAU	38	VII	<i>B. afzelii</i>	Skin	Germany	1
ACA1	39	VII	<i>B. afzelii</i>	Skin	Sweden	6
BO23	40	VII	<i>B. afzelii</i>	Skin	Germany	1
HO14 ^T	41	VIII	<i>B. japonica</i>	<i>I. ovatus</i>	Japan	27
F63B	42	VIII	<i>B. japonica</i>	<i>I. ovatus</i>	Japan	27
COW 611A	42	VIII	<i>B. japonica</i>	<i>I. ovatus</i>	Japan	27
COW 611C	42	VIII	<i>B. japonica</i>	<i>I. ovatus</i>	Japan	27
O612	42	VIII	<i>B. japonica</i>	<i>I. ovatus</i>	Japan	27
Ika2	43	VIII	<i>B. japonica</i>	<i>I. ovatus</i>	Japan	27
M2007	44		<i>B. turicatae</i>	<i>O. turicata</i>	United States	R. C. Johnson
DN127	45	IX	NA	<i>I. pacificus</i>	California	14
CA128	46	IX	NA	<i>I. neotomae</i>	California	17
CA55	47	IX	NA	<i>I. neotomae</i>	California	17
25015	48	X	NA	<i>I. dammini</i>	New York	4
21123	49	XI	NA	<i>Ixodes dentatus</i>	New York	3
19952	50	XI	NA	<i>I. dentatus</i>	New York	3

^a CSF, cerebrospinal fluid.^b This strain was described as a *B. afzelii* strain by Belfaiza et al. (13). However, these authors received the strain from two sources. By studying the RFLP of the intergenic space between the tandemly repeated 23S-5S rRNA genes, Baranton confirmed that the original isolate (which was the strain which we used) was a *B. garinii* strain (10).^c NA, not associated with any species.

infective than *B. garinii* and *B. afzelii* (26). Thus, the laboratory data support the hypothesis that the pathogenicity of *B. japonica* is limited rather than the hypothesis that *I. ovatus* cannot transmit pathogenic borreliae to humans.

Is division III a subspecies of *B. garinii*? In a previous study, we examined different *B. burgdorferi* sensu lato strains isolated from patients having different symptoms of Lyme disease. One of the strains, strain A19S (isolated in Holland from the skin of

TABLE 2. Allele profiles of 50 *B. burgdorferi* sensu lato ETs

ET	No. of alleles at the following enzyme loci:											
	PEP1	PEP2	PEP3	GPI	PYK	PGI	HEX	TPI	LDH	MPI	6-PG	EST
1	10	11	2	3	5	2	7	4	7	16	4	1
2	10	11	9	3	5	2	7	3	7	14	4	1
3	13	4	1	3	5	1	7	3	7	14	4	1
4	15	10	2	3	2	1	7	4	7	14	4	1
5 ^a	15	10	2	3	5	1	7	4	7	14	4	1
6	15	10	9	3	5	1	7	4	7	14	4	1
7	6	10	2	3	5	1	7	4	7	14	4	1
8	15	10	2	3	5	3	6	4	7	14	4	1
9	3	11	11	0	8	1	6	7	7	17	7	1
10	9	8	10	5	7	1	6	10	12	16	8	1
11	4	5	7	2	3	5	3	5	2	5	2	1
12	4	5	7	2	3	5	3	5	7	5	2	1
13	5	5	9	2	3	5	3	4	6	5	2	1
14	5	6	6	2	3	2	3	6	6	5	2	1
15 ^a	8	5	8	2	1	2	3	5	2	7	2	1
16	8	5	9	2	1	2	3	5	2	7	2	1
17	8	5	8	2	1	2	8	5	2	7	2	1
18	6	1	4	2	1	2	3	4	6	13	4	1
19	5	6	9	2	4	1	2	3	5	2	2	1
20	1	6	9	2	4	1	2	3	5	3	2	1
21	5	6	4	2	5	1	2	6	10	1	2	1
22	2	4	2	1	2	1	1	5	6	10	3	1
23	2	4	4	1	2	1	1	4	6	10	3	1
24	2	4	2	1	2	1	4	5	6	11	7	1
25	6	4	5	5	2	1	9	4	5	14	3	1
26	17	1	4	5	1	1	9	4	5	9	3	1
27	22	7	6	3	1	4	5	4	8	12	3	1
28	17	4	3	3	4	1	6	2	3	7	3	1
29	17	4	3	3	4	1	6	2	3	7	1	1
30	17	4	3	3	4	1	6	2	3	7	2	1
31	20	4	3	3	4	2	6	2	3	7	3	1
32	17	4	4	3	4	1	6	2	3	9	3	1
33	17	4	4	3	4	1	6	2	3	9	2	1
34	20	4	2	3	4	1	6	2	3	9	3	1
35	17	4	5	3	4	1	6	2	3	7	1	1
36	17	4	5	3	4	1	6	2	3	7	3	1
37	17	10	5	3	4	1	6	2	3	7	3	1
38	17	4	5	3	4	1	6	2	1	7	3	1
39	17	4	5	3	4	1	6	2	2	6	3	1
40	17	4	4	3	4	1	6	0	3	8	4	1
41	19	3	1	3	4	6	4	0	3	11	5	1
42 ^a	19	3	1	3	4	6	0	8	3	13	5	1
43	18	1	1	3	4	4	4	1	3	7	5	1
44	7	9	9	3	6	2	4	9	11	11	9	1
45	11	8	1	4	6	1	4	2	8	14	5	1
46	14	8	1	4	6	1	2	2	8	12	5	1
47	14	12	9	4	0	2	4	2	8	15	5	1
48	21	2	4	4	4	3	5	2	9	4	5	1
49	16	11	9	4	3	2	0	0	6	14	0	1
50	12	11	1	4	3	2	0	2	4	14	6	1

^a More than one isolate exhibited this ET.

a patient), could not be included in any of the three known pathogenic species. Therefore, we suggested that a new pathogenic group exists (9). In this study, we found that two strains isolated from *I. persulcatus* (strains NT29 [ET 19] and Ip89 [ET 20]) belonged to the same division as strain A19S (ET 21), division III, which was separated from the *B. garinii* division by a genetic distance of 0.74. The position of two strains NT29 and Ip89 is controversial. While Postic et al. (37) identified these organisms as *B. garinii* strains on the basis of DNA-DNA hybridization data, Fukunaga et al. (23) showed that they exhibited a different rRNA gene RFLP pattern than the other *B. garinii* isolates, which supports our observations. In two addi-

tional studies, workers in Fukunaga's group (22, 34) showed that other Japanese isolates obtained from patients with Lyme disease clustered in the same RFLP group as strains NT29 and Ip89. Since DNA hybridization is considered the standard reference technique for bacterial classification (48), division III strains must be considered members of *B. garinii*. However, there may be sufficient evidence to give division III subspecies status.

Two genomic species restricted to Europe. The five division IV and V strains (ETs 22 through 26) were all isolated from the European tick *I. ricinus*. The genetic distances (>0.75) that separate these divisions from the other divisions are consistent with the results of the DNA-DNA hybridization experiments performed by Postic et al. (37); the strains belonging to groups PotiB2 (division IV) and VS116 (division V) of Postic et al. were found to exhibit less than 70% DNA relatedness and ΔT_m values of 7.0°C or higher with the four established *B. burgdorferi* sensu lato species (ΔT_m is the difference between the melting temperature of a homologous hybrid and the melting temperature of a heterologous hybrid). It has been proposed that strain VS116 (ET 25) should be the reference strain for a new group on the basis of the polymorphism of its outer surface proteins (OspA and OspB) (36).

Heterogeneity of American isolates. In contrast to European isolates, American *B. burgdorferi* sensu lato strains have been considered homogeneous for a long time (12, 15). However, several authors have described atypical strains isolated on the west and east coasts of the United States. The differences between these strains and other American isolates have been assessed by different methods (14, 29, 38, 40, 49, 50). On the basis of the results of a *fla* gene PCR assay, an RFLP analysis of a 5S-23S rRNA intergenic spacer amplicon, and DNA-DNA hybridization, a large number of American isolates that differ from *B. burgdorferi* sensu stricto have been included in two new genomic species, designated groups DN127 and 21123 (8, 37). Recently, on the basis of the results of rRNA studies, the name "*Borrelia andersonii*" was proposed for genomic group 21123 (33). Our results support the hypothesis that these two genomic species, represented in our study by divisions IX (group DN127) and XI (group 21123, "*B. andersonii*"), are present in the United States.

In our study, two other American strains (strains CA2 and 25015 [ETs 27 and 48, respectively]) appeared to represent two distinct divisions (divisions VI and X, respectively [Fig. 1]). Although the level of relatedness between strains CA2 (division VI) and 21123 (division XI) was borderline (level of DNA

TABLE 3. Numbers of alleles and levels of genetic diversity at 12 enzyme loci of *Borrelia* species

Enzyme locus	No. of alleles	Genetic diversity
PEP1	22	0.925
PEP2	12	0.846
PEP3	11	0.888
GPI	6	0.673
PYK	9	0.802
PGI	6	0.610
HEX	10	0.846
TPI	11	0.810
LDH	12	0.854
MPI	17	0.886
6-PG	10	0.827
EST	1	0.000
Mean	10.6	0.747

FIG. 1. Genetic relationships among 50 ETs of *B. burgdorferi* sensu lato and relapsing fever spirochetes, based on electrophoretically demonstrable allelic variations at 12 enzyme loci. The dendrogram was generated by using the average-linkage method of clustering and a matrix of pairwise coefficients of genetic distance.

relatedness, 73%; ΔT_m , 5.0°C), Postic et al. (37) classified both of these strains in the same genomic group (group 21123). In contrast, we estimated that the genetic distance between these divisions was 0.95. This finding is supported by other data obtained in our laboratory, which, on the basis of the results of a nucleotide sequence alignment of the *hbb* histone-like protein, identified strain CA2 as a member of a new genomic group that is clearly separated from representatives of group 21123 (division XI) (46).

Although the results of DNA hybridization experiments placed strain 25015 (division X, ET 48) in group DN127 (division IX) (37), our results showed that the genetic distance between these divisions was 0.67. Data obtained with other genetic techniques (46) placed strain 25015 at the border of division IX.

Relapsing fever spirochetes. The taxonomy of *Borrelia* species associated with relapsing fever has not been properly investigated yet. In humans, Lyme borreliosis and relapsing fever present different clinical pictures and are transmitted by members of two different tick families, the hard ticks (Ixodidae, mostly *Ixodes* spp.) and the soft ticks (Argasidae, mostly *Ornithodoros* spp.), respectively (28). However, such a subdivision might be arbitrary. Fukunaga has isolated a relapsing fever *Borrelia* strain (*Borrelia miyamotoe* sp. nov.) from the hard tick *I. persulcatus* (21). In *Bergey's Manual of Systematic Bacteriology*

(28), the borreliae were classified into species on the basis of their arthropod vectors. For instance, *B. hermsii*, *B. turicatae*, and *B. parkeri* are transmitted only by the specific tick vectors *Ornithodoros hermsi*, *Ornithodoros turicata*, and *Ornithodoros parkeri*, respectively. However, on the basis of the results of DNA-DNA hybridization experiments, it has been suggested that these three species constitute a single species (25). Our MLEE data suggest that these three relapsing fever spirochetes represent three distinct species that are separated at genetic distances greater than 0.75.

Surprisingly, on our dendrogram the relapsing fever spirochetes clustered with the different genomic groups and species of Lyme disease agents (*B. burgdorferi* sensu lato) (Fig. 1). This finding is supported by the results of a recent study (32) that was based on an analysis of fatty acid methyl esters and showed that borreliae associated with Lyme disease could not be distinguished as a group from the relapsing fever spirochetes. However, Noppa et al. (35) sequenced the flagellin gene of different *Borrelia* species and showed that the Lyme disease species were evolutionarily more closely related to each other than to relapsing fever *Borrelia* species (*B. hermsii* and *Borrelia crocidurae*). In order to assess the exact relationship between these two groups of borreliae (the soft- and hard-tick spirochetes), more strains will have to be analyzed by different molecular genetic methods.

Conclusions. The main purpose of this study was to investigate the genetic structure of the *B. burgdorferi* sensu lato population. It seems clear that the previous subdivision of these microorganisms into only four species should be reconsidered. Our results confirmed the existence of the recently described species *B. japonica* and the recently proposed species "*B. andersonii*." Furthermore, they suggested that there are two additional genomic groups in Europe and at least one additional genomic group in the United States. We believe that promotion of these new genomic groups to *Borrelia* species status will be supported by the discovery of specific phenotypic characteristics for each of them. One may wonder whether the results generated by MLEE or other similar analyses faithfully reflect evolutionary history or whether they are the consequence of a genetic method that is far too sensitive. MLEE has been used successfully for many years to analyze the genetic structures of eukaryotic and bacterial populations (24, 44). An important property of MLEE is that it takes into account many chromosomal loci that encode housekeeping enzymes, which are considered selectively neutral. Thus, the conclusions inferred from MLEE results are based on a panel of genes that represent the chromosome as a whole and not on a single gene or a unique region of the genome. In general, MLEE results are compatible with the results of DNA-DNA hybridization, a method still considered the standard reference technique in bacterial species identification (12, 16, 48).

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