Borrelia burgdorferi Sensu Stricto, a Bacterial Species “Made in the U.S.A.”?

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Among the three main species of *Borrelia burgdorferi* sensu lato associated with Lyme borreliosis, *B. burgdorferi* sensu stricto (*B. burgdorferi*) is the sole species present both in North America and in Europe, where *Borrelia garinii* and *Borrelia afzelii* also occur. The greater genetic diversity together with the greater clinical polymorphism observed in the Old World suggests that this is the birthplace of the complex *B. burgdorferi* sensu lato. However, the genetic proximity of some North American and European *B. burgdorferi* strains is quite mystifying. A previous study of the whole genome (M. Foretz, D. Postic, and G. Baranton, Int. J. Syst. Bacteriol. 47:11–18, 1997) compared the diversity of North American and European *B. burgdorferi* strains. To further investigate the geographical origin and the migration of *B. burgdorferi*, we have focused on the study of the single variable and highly adaptive gene *ospC*. Both approaches demonstrated the greater diversity of North American strains and the close relatedness between European strains and between some isolates from the two areas. We discuss the significance of these features and suggest that they might be evidence of the anteriority of North American *B. burgdorferi* strains.

Over the last few years, the agents of Lyme disease, *Borrelia burgdorferi* sensu lato bacteria, have been subdivided into several groups. Some of them have been validated as species, whereas others remain as DNA groups. Among the main species, *Borrelia garinii* and *Borrelia afzelii* constitute the most prevalent species in Eurasia and are absent from America. In contrast, *B. burgdorferi* sensu stricto (*B. burgdorferi*) is characterized by its expanding geographical distribution both on the North American continent and in Europe, although it is absent from Asia. The analysis of the whole genome by pulsed-field gel electrophoresis and arbitrarily primed PCR (5) had previously shown that the strains of *B. burgdorferi* isolated in North America were genomically more heterogeneous than those isolated in Europe. This fact suggested that the ancestor of this species was more probably North American than European, as was commonly accepted considering the greater genomic diversity of the complex *B. burgdorferi* sensu lato in Europe. The aim of this study was to confirm these data by the analysis of a single gene, *ospC*. The high degree of polymorphism of this gene has previously been reported, both at a phenotypic and at a genotypic level (8, 23, 25, 28). Although *ospC* is encoded on a plasmid, the sequence diversity of the gene was correlated with the phyletic distribution of *B. burgdorferi* sensu lato into different species (9, 13, 24, 25). However a mechanism of intragenic recombination, which increases the heterogeneity of the *ospC* gene, was demonstrated for *ospA* and *ospC* genes (3, 9, 13, 24). In contrast to *ospA*, which is more conserved between North American and European strains (9), the *ospC* gene appears to reflect the divergence between *Borrelia* strains. To evaluate the polymorphism within *B. burgdorferi*, we have sequenced the *ospC* genes of 13 European strains and compared them to 29 *ospC* sequences available in data banks. Half of the strains studied were isolated in Europe, and the other half were isolated in North America. The results allowed us to

confirm the hypothesis of the anteriority of North American strains of *B. burgdorferi*, which had recently been imported into Europe. A hypothesis for the migration route of *B. burgdorferi* is proposed.

MATERIALS AND METHODS

Bacterial strains. The *B. burgdorferi* strains used in this study are listed in Table 1. The *ospC* genes of 13 European *B. burgdorferi* strains, chosen on the basis of previous results (5) to represent the greater genomic diversity, were sequenced in this study. In contrast, the 29 other *ospC* sequences, and particularly those from the 21 North American isolates, were arbitrarily chosen among sequences available in data banks. All other *ospC* sequences from *B. burgdorferi* sensu lato were also taken from data banks. Strains were grown at 30°C, in BSK-H medium (Sigma) supplemented with 6% rabbit serum and 1% gelatin (2).

DNA preparation. DNA was extracted from 1.5 ml of culture by using the Dynabeads DNA direct kit (Dynal) and eluted in 30 µl of water. DNA samples were stored at –20°C until required for PCR experiments.

Solid-phase DNA sequencing of PCR products. The amplification of approximately 600 bp from the *ospC* gene was performed by using the primer set *OspC3-OspC4* (Table 2), deduced from sequences in data banks. During PCR, biotin was introduced into one of the strands by using alternatively one of the primers biotinylated at the 5' end. The PCR mixture (50 µl) contained 5 µl of DNA, 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 200 µM (each) four deoxynucleoside triphosphates, 1.25 U of *Taq* polymerase (Amersham International, Amersham, England), 0.5 µM biotinylated primer, and 1 µM second primer. The reaction mixtures were overlaid with mineral oil (50 µl). The amplification reaction was carried out for 35 cycles (denaturation at 93°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min), and finally, extension was carried out at 72°C for 9 min. Negative controls were included in all experiments. The PCR products were detected by electrophoresis in a 1% agarose gel in 1× Tris-borate-EDTA and then stained with ethidium bromide.

We used a solid-phase approach to sequence DNA from PCR products. The PCR product was immobilized through the interaction between biotin and streptavidin with the Dynabeads M-280 streptavidin kit (Dynal). The two DNA strands were separated, and the biotinylated strand was immobilized with Dynal MPC (Dynal) and finally eluted in 12 µl of water. The *ospC* gene was sequenced with the Cy5-AutoRead sequencing kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The sequencing primer (10 pmol) (Table 2) was annealed to the biotinylated single-stranded template in the appropriate buffer at 65°C for 10 min and then at 37°C for 10 min and finally cooled at room temperature for 10 min. In a second step, Cy5-dATP labelling mixture (Pharmacia) was incorporated, and the labelling reaction was started by adding T7 DNA polymerase (3 U). Six percent polyacrylamide gels in an ALF express automatic sequencer (Pharmacia) were used for sequencing reactions. *ospC* genes of all strains were sequenced at least twice on both strands.

***ospC* sequence analysis.** Nucleotide sequences and deduced amino acid sequences were aligned with sequences available in data banks for North American and European strains both with the Clustal V software (7) and manually on VSM software, version 2.0, written by B. Lafay and R. Christen as described in refer-

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TABLE 1. *B. burgdorferi* strains used in this study

Isolate	PFGE group ^a	Accession no. and/or reference	Country	Source
35B808	M	U91794 (this study)	Germany	<i>Ixodes ricinus</i>
HII	N	U91792 (this study)	Italy	Human blood
212	O	U91790 (this study)	France	<i>I. ricinus</i>
20006	O	U91796 (this study)	France	<i>I. ricinus</i>
61BV3	O	U91795 (this study)	Germany	Human skin
ESP-1	O	U91791 (this study)	Spain	<i>I. ricinus</i>
MIL	P	U91802 (this study)	Slovakia	<i>I. ricinus</i>
Z136	Q	U91793 (this study)	Germany	<i>I. ricinus</i>
NE56	R	U91800 (this study)	Switzerland	<i>I. ricinus</i>
L5	S	U91798 (this study)	Austria	Human skin
P1F	S	U91801 (this study)	Austria	Human synovial fluid
IP1	S	U91799 (this study)	France	Human CSF ^b
IP3	S	U91797 (this study)	France	Human CSF
IP2	S	L42887 (13)	France	Human CSF
PKa	S	X69589 (28)	Germany	Human CSF
ZS7	O	L42868 (13)	Germany	<i>I. ricinus</i>
VS219		12	Switzerland	<i>I. ricinus</i>
VS215		12	Switzerland	<i>I. ricinus</i>
PBre		X81522 (9)	Germany	Human skin
DK7	O	X73625 (25)	Denmark	Human skin
T255		X81524 (27)	Germany	<i>I. ricinus</i>
26816	S	12	United States (Shelter Island)	<i>Microtus pennsylvanicus</i>
B31	S	X69596 (8)	United States (New York)	<i>Ixodes scapularis</i>
CA4		L81131 (unpublished data)	United States (California)	<i>Ixodes pacificus</i>
TXGW		X84783 (24)	United States (Texas)	Human skin
CA-11 2A		L25413 (15)	United States (California)	<i>I. pacificus</i>
BUR		X84765 (24)	United States (New York)	Human skin
297	C	L42893 (13)	United States (Connecticut)	Human CSF
HB19	E	U04281 (23)	United States (Connecticut)	Human blood
272		X84785 (24)	United States (Connecticut)	Human skin
28354		L42895 (13)	United States (Maryland)	<i>I. scapularis</i>
MUL		X84779 (24)	United States (New York)	Human skin
KIPP		X84782 (24)	United States (New York)	Human skin
28691		L42894 (13)	United States (Pennsylvania)	<i>I. scapularis</i>
N40		U04240 (23)	United States (New York)	<i>I. scapularis</i>
2591		U01892 (19)	United States (Connecticut)	White-footed mouse
DUN		X84778 (24)	United States (New York)	Human skin
SON 188	G	L81130 (unpublished data)	United States (California)	<i>I. pacificus</i>
27579		L42896 (13)	United States (Connecticut)	<i>I. scapularis</i>
<i>Borrelia pacificus</i>		X83555 (9)	United States (California)	<i>I. pacificus</i>
26815		L42897 (13)	United States (Connecticut)	Chipmunk
21347		L42899 (13)	United States (Wisconsin)	White-footed mouse

^a PFGE, pulsed-field gel electrophoresis. Groups are defined in reference 5.^b CSF, cerebrospinal fluid.

ence 21. Evolutionary distances were computed with the Jukes-Cantor option in both the Phylogeny Inference Package (PHYLIP) written by Felsenstein (4) and the Molecular Evolutionary Genetics Analysis (MEGA) software (10). Phylogenetic trees were constructed with the Kitsch⁺ genetic distance algorithm from

distance matrix data and by both the neighbor-joining (22) and the unweighted-pair-group-with-mathematical-average methods in MEGA.

Nucleotide sequence accession numbers. The *ospC* sequences have been deposited in the GenBank database, and their accession numbers are listed in Table 1.

RESULTS AND DISCUSSION

***ospC* sequence analysis of *B. burgdorferi*.** To estimate the genetic variation within the species *B. burgdorferi*, isolates representing the range of known genetic diversity were selected on the basis of pulsed-field gel electrophoresis and AP-PCR polymorphism groups defined previously (5). A fragment of 606 to 618 bp of the *ospC* gene was amplified from 13 European *B. burgdorferi* isolates. Partial nucleotide sequences of approximately 500 bp, including mainly the variable part of the *ospC* gene, were determined. A common segment from nucleotides 88 to 568, corresponding to 53 amino acids in the conserved region and 101 amino acids in the variable region, was compared with *ospC* sequences from 8 other European and 21 North American strains available in the databases (Table 1). As previously reported (13, 24), the *ospC* gene showed great diversity. The level of similarity indicated by pairwise sequence

TABLE 2. Primers used for amplification and sequencing of the *ospC* gene

Primer	Sequence (5' to 3')	Position (strain pKo numbering [6])
PCR		
ospC3	AAGTGC(AG)ATATTAATGACTTTA	18–39
ospC4	TTTTTTGGACTTTCTGCCACA	632–612
Sequencing		
ospC1	TTGAAGCTTTGCTTTCATCTAT	188–209
ospC2	AGG(AG)CTTGTAAGCTCTTTA	609–591
ospC5	ATAAGCTCCCGCTAACAATG	309–290
ospC6	GTTACTGATGCTGATGCAAAA	454–474
ospC7	CCGCATCAATTTTTTCTTTA	385–365
ospC8	GCTAACAAATGAGGTGTTCT	299–281

FIG. 1. Unrooted tree showing the phylogenetic relationships of different *B. burgdorferi* sensu lato species. The tree, constructed by the neighbor-joining method with MEGA software, is based on a comparison of approximately 500 nucleotides of the *ospC* gene. *Borrelia* b, *B. burgdorferi* sensu stricto. The capital letter at the end of the isolate designation denotes its geographical origin (A, America; E, Europe). The arrowheads indicate the *B. burgdorferi* strains that have been subjected to a genetic transfer.

		<----- C1 ----->				
<i>B burgdorferi</i> 20006	GNASANS	ADE	SAKGPNLTEI	SKKITDSNAF	VLAVKEVETL	VSSIDELA-K
<i>B afzelii</i> DK1	-S..T.P	...	...	...	...	...
<i>B afzelii</i> Plud	-S..T.P	...	...	...	...	...
<i>B burgdorferi</i> ESP-1		.V.....A..	E...V		L.....	
<i>B burgdorferi</i> NE56		.V.....A..	E...V		L.....	
<i>B garinii</i> H13	-T..T.P--	.T.....I..	V	A..	I.....	
<i>B garinii</i> Tls1	-T..T.P--	.T.....I..	V	A..	I.....	
<i>B burgdorferi</i> Z136		.V.....		A..	I.....	
		><----- V1 -----><----- SV -----><-----				
<i>B burgdorferi</i> 20006	AIGKKIDNNN	GLSANANLNT	SLLAGA-YAI	STLIKQKLDG	--KGLEGLKE	
<i>B afzelii</i> DK1						
<i>B afzelii</i> Plud				E.....		
<i>B burgdorferi</i> ESP-1	T.....EAN-	..GNE.DK..	S..	S..TK..E.	LI.NSGE..T	
<i>B burgdorferi</i> NE56	T.....EAN-	..GNE.DK..	S..	S..TK..E.	LI.NSGE..T	
<i>B garinii</i> H13	VEAN-	..GNE.DR..	HE.	I..T...TA	...DSG...A	
<i>B garinii</i> Tls1	VEAN-	..GNE.DR..	HE.	I..T...TA	...DSG...A	
<i>B burgdorferi</i> Z136	VEAN-	..GNE.DR..	HE.	I..T...TA	...DSG...A	
		----- V2 -----				
<i>B burgdorferi</i> 20006	KIEKAKNASA	AFTNKLKNSH	VELGVAGNGA	TTDENAQKAI	LKTNA--NDK	
<i>B afzelii</i> DK1			A.....	...D.....		
<i>B afzelii</i> Plud			A.....A..G	A..AH.KE..	..S.P..K..	
<i>B burgdorferi</i> ESP-1	EV.....	T..	AQ..A.NG.-	-..AH.KE..	..S.P..K..	
<i>B burgdorferi</i> NE56	EV.....	T..	AQ..A.NG.-	-..AH.KE..	..S.P..K..	
<i>B garinii</i> H13	E.AE..KC.E	...K...DNN	AQ..IQNV--	--.VE.K...	...G..IS.	
<i>B garinii</i> Tls1	E.AE..KC.E	...K...DNN	AQ..IQNV--	--.VE.K...	...G..IS.	
<i>B burgdorferi</i> Z136	E.AE..KC.E	...K...DN	AQF.IQNV--	--.VE.K...	...K...	
		----->				
<i>B burgdorferi</i> 20006	GAKELKELFE	SVESLAKA				
<i>B afzelii</i> DK1						
<i>B afzelii</i> Plud	.VT..E...K	...G.P..				
<i>B burgdorferi</i> ESP-1	.VT..E...K	...G....				
<i>B burgdorferi</i> NE56	.VT..E...K	...G....				
<i>B garinii</i> H13						
<i>B garinii</i> Tls1						
<i>B burgdorferi</i> Z136	..ED.EK..K	A....S..				

FIG. 2. Amino acid sequences of OspC proteins deduced from the nucleotide sequences and aligned with MEGA software. The conserved amino-terminal (C1), variable (V1 and V2), and semivariable (SV) domains are indicated according to reference 13. The high identity (indicated by shading) between amino acid sequences of strains belonging to different *B. burgdorferi* sensu lato species evidences a lateral transfer of part of the *ospC* genes.

comparison ranged from 68.54 to 100%. Among European strains, three groups of strains were pairwise related at a level of 100% sequence identity, and another group of seven strains were related at a level of 76.5% sequence identity. Among the

seven remaining strains, four are artificially dispersed due to a mechanism of genetic transfer (see below). In contrast, North American strains, whose *ospC* sequence similarity ranged from 68.54 to 100%, appeared to be completely scattered.

Phylogenetic analysis of *B. burgdorferi* strains. Phylogenetic trees were constructed by the unweighted-pair-group-with-mathematical-average method, inferred from the *ospC* sequence similarity matrix from European and North American strains (data not shown). Phylogenetic trees constructed by the neighbor-joining method and the parsimony method (data not shown) were completely consistent with these results. A tree was constructed from the *ospC* sequences of all North American and European strains, together with sequences from other *B. burgdorferi* sensu lato strains available in data banks (Fig. 1). This tree showed that among 16 clusters comprising *B. burgdorferi* strains, 8 comprised only North American strains, 5 comprised only European strains, and 3 comprised both North American and European strains. In these three mixed clusters, the *ospC* sequence similarity ranged from 98.75 to 100%, and the deduced amino acid sequences had close to 100% identity. These data strongly suggested a common ancestor for strains isolated from each side of the Atlantic. According to the phylogenetic tree (Fig. 1), it seems that European strains ZS7, VS219, VS215, PBr, DK7, 61BV3, and 35B808 evolved from the North American strain BUR and that the North American strain CA4 together with European strains pKa, P1F, L5, IP1, IP2, IP3, and HII and North American strains B31 and 26816 evolved from the North American strain TXGW. A North American strain, 26815, could be the older ancestor.

The results of the present study in addition to the results of previous analysis of the whole genome (5) emphasize both the greater diversity of isolates from North America and the close genetic relatedness between some North American and European strains.

Lateral genetic transfer from *B. garinii* to other species. The phylogenetic tree (Fig. 1) showed a main branch comprising *B. burgdorferi* *ospC* sequences and a second branch divided into three main clusters comprising *Borrelia andersonii*, *B. afzelii*, and *B. garinii*. Three *B. burgdorferi* strains clustered with *B. andersonii*, and the strains belonging to the recently individualized DNA group DN127 clustered with *B. burgdorferi*. This is not surprising given the genetic proximity of *B. burgdorferi*, *B. andersonii*, and the DNA group DN127 (20). More surprising was the phylogenetic placement of the four *B. burgdorferi* strains ESP-1, NE56, 20006, and Z136, included in the *B. garinii* cluster together with some *B. afzelii* strains. The comparison of the *ospC* sequences of these four *B. burgdorferi* strains with the sequence of their nearest neighbor in the tree showed that lateral transfers occurred between strains belonging to *B. burgdorferi* and *B. garinii*. The analysis of the amino acid sequence deduced from *ospC* nucleotide sequences comprising the conserved domain C1 and the three variable domains (according to the terminology of Livey et al. [13]) is shown in Fig. 2. It reveals that large regions are common to European *B. burgdorferi* and *B. garinii* or *B. afzelii* strains. This interspecific genetic exchange considerably and artificially increased the heterogeneity of European *B. burgdorferi* strains. Indeed, the four strains ESP-1, NE56, 20006, and Z136 constituted three separate clusters in Fig. 1 likely because of genetic transfers. No such interspecific transfer was evidenced among North American *B. burgdorferi* strains studied in this work. Otherwise, *B. garinii* seems to behave as the sole donor (Fig. 1), whereas the other species are only recipients. The reason why all *ospC* sequences showing lateral transfers fell only into the cluster including sequences of *B. garinii* remains unclear.

Conclusion. *B. burgdorferi* is present on both the European and North American continents. Curiously, this species is absent from Asia. Recently, it was demonstrated that *B. andersonii* (14) and the DNA group DN127, both closely related to

B. burgdorferi, also occurred in North America (1, 20), whereas additional species, *Borrelia valaisiana* and *Borrelia lusitaniae*, were identified in Europe (11, 26). The question arose whether *B. burgdorferi* originated in America or in Europe. A previous study (5) concluded that the greater genetic diversity of North American *B. burgdorferi* strains compared to European ones suggested the anteriority of the former. Recently, a study based on pulsed-field gel electrophoresis patterns and 23S ribosomal DNA and *ospA* sequences of a large number of North American strains concluded that the genetic diversity was much greater than previously thought (16). The faster evolution of *ospC* compared to that of other genes made it appropriate for comparison of strains within a species which probably diverged only recently. Moreover, this gene, coding for a highly immunogenic protein, is therefore likely to be subjected to selective pressure (24), which might be one explanation for the *ospC* variability. Obviously, the respective effects of environmental constraints and of evolution time on the variability of the *ospC* gene are difficult to establish.

The present study strengthens our previous conclusions (5) and sharpens our hypothesis that *B. burgdorferi* originated in North America and later migrated to Europe. The close *ospC* relatedness between European strains and some North American strains, their putative ancestor, suggests that this migration has been recent and is possibly still in progress. Several European strains within a cluster showed a strict identity of the hypervariable *ospC* sequence, although these strains were isolated from distant areas. This fact suggests an undetectable evolution and is evidence of the unexpectedly short time of evolution of clones introduced in Europe.

The diversity of *B. burgdorferi* sensu lato in Eurasia is much greater than it is in North America; thus it is likely that the ancestor of this complex was located in Eurasia and was close to *B. garinii*, which has shown the greatest genetic heterogeneity, whatever the method used. It was an interesting feature that *B. garinii* was the main if not the single species isolated from transoceanic migratory birds or from their ticks, *Ixodes uriae*, in the higher latitudes of both hemispheres (17, 18). Our hypothesis is that *B. garinii* or a close ancestor species had first been introduced on the North American continent from Eurasia by way of migratory birds and had adapted to new ecological conditions and that a speciation process led to *B. burgdorferi*. This migration would have occurred millions of years ago, during which genetic diversity has been achieved and *B. burgdorferi* has diverged into the closely related species DNA group DN127 and *B. andersonii*. Since no *B. burgdorferi* strain was ever identified on seabird ticks, *B. burgdorferi* could have been introduced into Europe only by way of human communications, which means in the post-Columbian period.

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