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Evaluation of Genetic Divergence among *Borrelia burgdorferi* Isolates by Use of OspA, *fla*, HSP60, and HSP70 Gene Probes

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In order to assess the genetic variation of immunologically relevant structures among isolates of the Lyme disease spirochete, *Borrelia burgdorferi*, three chromosomal genes encoding flagellin (*fla*) and the heat shock proteins HSP60 and HSP70, as well as the plasmid gene encoding outer surface protein A (OspA), from 55 different European and North American strains obtained from ticks and mammal hosts have been investigated by restriction fragment length polymorphisms (RFLPs). RFLPs of *fla* and the HSP60 and HSP70 genes revealed two distinct banding patterns (A and B) for each of the three genes and allowed the definition of four genomic groups [AAA, BBB, BBA, and B(A/B)A] for the three chromosomal genes. On the other hand, RFLPs of the OspA gene revealed six distinct banding patterns (types I to VI) making up six independent genomic groups for the plasmid-encoded gene. Furthermore, we have sequenced the chromosomal HSP60 gene from *B. burgdorferi* ZS7 and the plasmid-encoded OspA gene from two strains, ZQ1 and 19857. Alignment of the deduced HSP60 amino acid sequence from *B. burgdorferi* ZS7 (genomic group AAA) to a previously published HSP60 sequence derived from strain ACA-1, which according to the proposed classification is in a different genomic group (BBA), revealed a sequence identity of >99%. Similar alignments of the OspA sequence of strain ZQ1 to those of other isolates that were published previously revealed sequence identities of between 70 and 94% among strains of distinct OspA genomic groups. These data indicate the existence of a restricted number of species-specific subgroups and clearly show that genotypic variation is much more pronounced for the OspA gene than for *fla* and the HSP60 and HSP70 genes. A phylogenetic tree constructed on the basis of distance matrix analyses of 12 OspA sequences supports the proposed classification of genomic groups of *B. burgdorferi*.

The spirochete *Borrelia burgdorferi* is the etiological agent of Lyme borreliosis, an infectious disease distributed primarily throughout the Northern Hemisphere (8, 38). Clinical symptoms of Lyme borreliosis in humans vary from an acute skin rash (erythema chronicum migrans) to serious late manifestations including arthritis, encephalitis, acrodermatitis chronica atrophicans, and carditis (37).

Although serology is valuable for the diagnosis of Lyme borreliosis, standardized test systems are not available. Currently, routine laboratory tests are based on enzyme-linked immunosorbent assays but are difficult to evaluate because of their lack of specificity and sensitivity and because of interlaboratory variations (5, 17, 26, 30). The application of subfractions of *B. burgdorferi* lysates for serological analyses suggested that individual proteins such as flagellin, outer surface proteins A and B (OspA and OspB), heat shock proteins HSP60 (*Escherichia coli* GroEL homolog [32]) and HSP70 (*E. coli* DnaK homolog [22]), and structures with molecular masses of 22 (pC), 39 (p39), 80, and 110 kDa offer more reliable tools for diagnosis (9, 16, 18, 35, 36, 41, 42). However, in spite of their immunogenicity in humans, the applicability of flagellin, HSP70, and HSP60 as test antigens is doubtful because of their high homology with related proteins from other prokaryotic and eukaryotic species (10, 24). On the other hand, the outer surface proteins OspA and OspB do not elicit early and consistent antibody responses in humans and in addition show considerable variability (1, 3, 4, 7, 12, 31, 45). The application of a variety of techniques and approaches, including restriction endonuclease analysis, DNA hybridization, polymerase chain reaction, and analysis of plasmid profiles, in previous studies had already disclosed the genetic heterogeneity of different *B. burgdorferi* isolates (2, 21, 27, 29). Related studies using monoclonal antibodies (MAbs) to various *B. burgdorferi* structures have also shown remarkable heterogeneity in various *B. burgdorferi* isolates, especially for OspA and OspB (1, 3, 4, 7, 20, 41, 45, 46). In order to obtain more information on the suitability of spirochetal structures to be used as unequivocal antigens in serodiagnosis and/or vaccine development, we have analyzed three chromosomal genes (the *fla*, HSP60, and HSP70 genes) and one plasmid-encoded gene (the OspA gene) of a collection of 55 *B. burgdorferi* isolates from different geographic areas and sources (Table 1) by restriction fragment length polymorphism (RFLP) and sequence analyses of genomic DNA. In this article, the genomic grouping of *B. burgdorferi* strains elicited by these analyses is compared with genomic classifications proposed by others (1, 3, 23, 25, 27). The implications of these data for the development of diagnostic tools and vaccine candidates are discussed.

MATERIALS AND METHODS

***Borrelia* strains.** *B. burgdorferi* B31 (ATCC 35210), *B. coriaceae* Co53 (ATCC 43381), and *B. hermsii* (ATCC

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

Isolate	Biological origin[a]	Geographical origin	Genogroup (*fla*, HSP60, HSP70)	OspA genotype
B31 (ATCC 35210)	Tick (*Ixodes dammini*)	United States	AAA	I
ZS7	Tick (*I. ricinus*)	Germany	AAA	I
Z37	Tick (*I. ricinus*)	Germany	AAA	I
GeHo	Skin (ECM)	Germany	AAA	I
B1	Skin (ECM)	Germany	AAA	I
B2	Skin (ECM)	Germany	AAA	I
B3	Skin (AD)	Germany	AAA	I
20004	Tick (*I. ricinus*)	France	AAA	I
19535	Mouse (*Peromyscus* sp.)	United States	AAA	I
26816	Vole (*Microtus* sp.)	United States	AAA	I
28691	Tick (*I. dammini*)	United States	AAA	I
21305	Mouse (*Peromyscus* sp.)	United States	AAA	I
21343	Mouse (*Peromyscus* sp.)	United States	AAA	I
26815	Chipmunk	United States	AAA	I
297	Cerebrospinal fluid	United States	AAA	I
Mac3	Skin	United States	AAA	I
20001	Tick (*I. ricinus*)	France	AAA	I
CTiP7	Dog tick	United States	AAA	I
SH-2-82	Tick (*I. dammini*)	United States	AAA	I
CA-2-87	Tick (*I. pacificus*)	United States	AAA	I
S12/14	Tick (*I. ricinus*)	Germany	AAA	I
Z25	Tick (*I. ricinus*)	Germany	AAA	I
Z118	Tick (*I. ricinus*)	Germany	AAA	I
Z136	Tick (*I. ricinus*)	Germany	AAA	I
Z160	Tick (*I. ricinus*)	Germany	AAA	I
IP1	Cerebrospinal fluid	France	AAA	I
NE2	Tick (*I. ricinus*)	Switzerland	AAA	I
R7NE4	Tick (*I. ricinus*)	Switzerland	AAA	I
LW2	Skin	Germany	AAA	I
LW2.4	Skin	Germany	AAA	I
19857	Rabbit kidney	United States	B(A/B)A	III
21038	Larva (*I. dentatus*)	United States	B(A/B)A	III
ACA-1	Skin (ACA)	Sweden	BBA	IV
Bo23	Skin (ECM)	Germany	BBA	IV
So2	Tick (*I. ricinus*)	Great Britain	BBA	IV
PKo	Skin (ECM)	Germany	BBA	IV
ZQ1	Tick (*I. ricinus*)	Germany	BBB	II
NE4	Tick (*I. ricinus*)	Switzerland	BBB	II
NE58	Tick (*I. ricinus*)	Switzerland	BBB	II
NE11H	Tick (*I. ricinus*)	Switzerland	BBB	II
R3NE2	Tick (*I. ricinus*)	Switzerland	BBB	II
N34	Tick (*I. ricinus*)	Germany	BBB	II
IP3	Cerebrospinal fluid	France	BBB	II
IRS Us	Tick (*I. ricinus*)	Germany	BBB	II
Frst1	Skin	Germany	BBB	II
Frst2	Skin	Germany	BBB	II
So1	Tick (*I. ricinus*)	Great Britain	BBB	II
42/87		Sweden	BBB	II
152/86		Sweden	BBB	II
MK5	Tick (*I. ricinus*)	Hungary	BBB	II
MK6	Tick (*I. ricinus*)	Hungary	BBB	II
387	Cerebrospinal fluid	Germany	BBB	II
50	Cerebrospinal fluid	Germany	BBB	II
20047	Tick (*I. ricinus*)	France	BBB	V
S90	Tick (*I. ricinus*)	Germany	BBB	VI

[a] ECM, erythema chronicum migrans; ACA, acrodermatitis chronica atrophicans; AD, atrophodermia.

35209) were obtained from the American Type Culture Collection (Rockville, Md.). Other *B. burgdorferi* strains were isolated and kindly provided by our colleagues R. Ackermann, Cologne, Germany (strain N34), J. F. Anderson, New Haven, Conn. (CTiP7, 21343, 19535, 19857, 20004, 21038, 21305, 26815, 26816, and 28691), E. Åsbrink, Stockholm, Sweden (ACA-1), G. Baranton, Paris, France (IP1 and IP3), G. Bigaignon, Brussels, Belgium (20001 and 20047), E. C. Guy, Southampton, United Kingdom (So1 and So2), L. Gern, Neuchâtel, Switzerland (NE2, R7NE4, NE4, NE58, NE11H, and R3NE2), T. Häupl, Erlangen, Germany (LW2 and LW2.4), H. Hofmann, Munich, Germany (B1, B2,

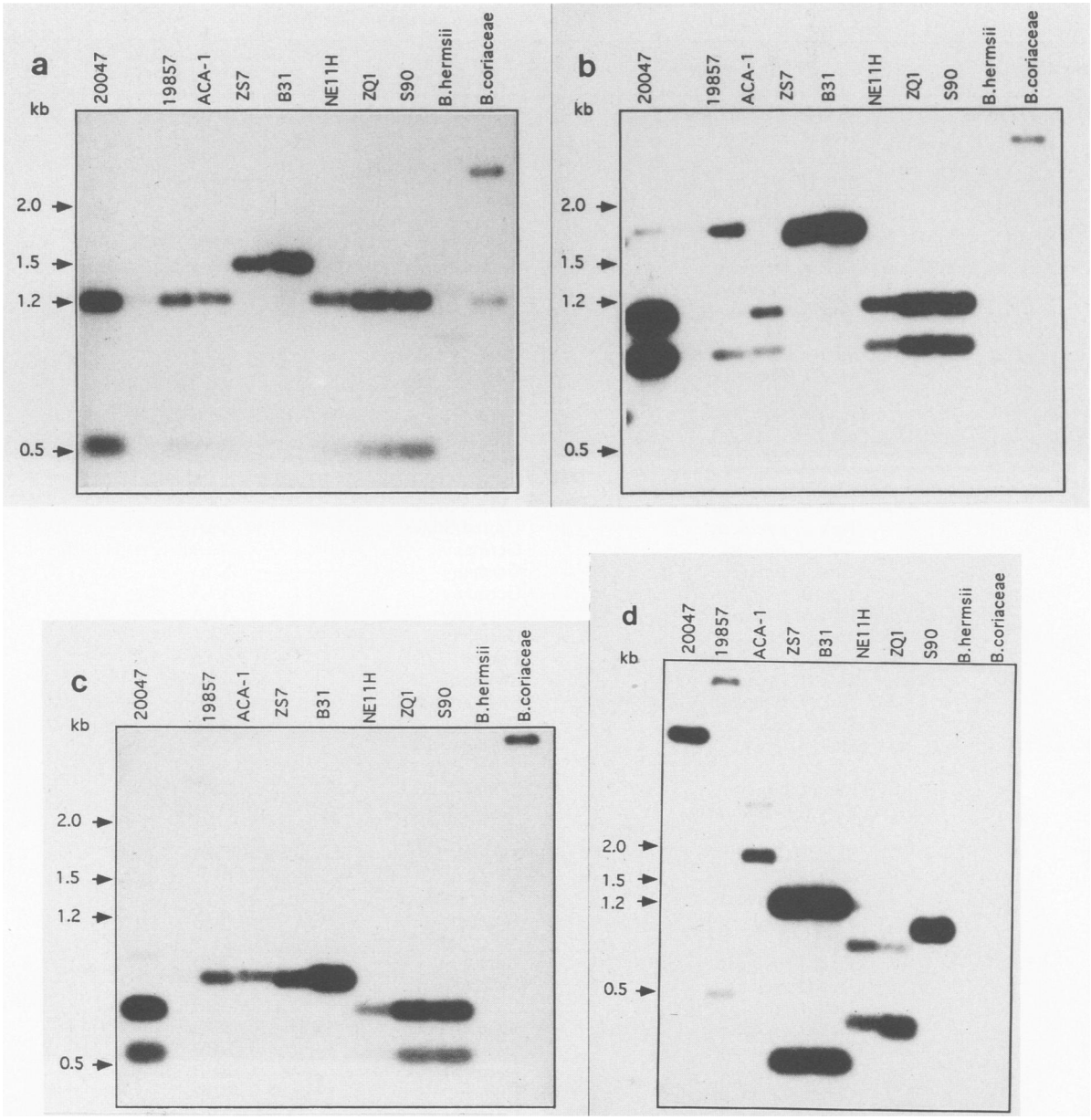

FIG. 1. DNA blot hybridization analysis. Whole-cell DNA (5 μg) derived from *B. burgdorferi* isolates was digested with *Hin*dIII, separated on a 0.7% agarose gel by electrophoresis, and transferred to a nylon membrane (Hybond-N). Sources of DNA are indicated above the lanes. B31 and ZS7 represent OspA genotype I, ZQ1 and NE11H represent OspA genotype II, 19857 represents OspA genotype III, ACA-1 represents OspA genotype IV, 20047 represents OspA genotype V, and S90 represents OspA genotype VI. Southern blots were probed with either the *fla* (a), HSP60 (b), HSP70 (c), or OspA (d) gene probe.

B3, and S90), K. Kurtenbach, Bonn, Germany (S12/14), A. Lakos, Budapest, Hungary (MK5 and MK6), K. Pelz, Freiburg, Germany (Bo23, GeHo, Z25, Z37, Z118, Z136, and Z160), H. Lotter, Göttingen, Germany (Frst1, Frst2, and IRS Us), V. Preac-Mursic, Munich, Germany (PKo), T. G. Schwan, Hamilton, Mont. (Mac3, SH-2-82, and CA-2-87), A. C. Steere, Boston, Mass. (297), V. Sticht-Groh, Würzburg, Germany (50 and 387), and B. Wretlind, Danderyd, Sweden (42/87 and 152/86). The geographic origins and sources of the *B. burgdorferi* strains used in this study are shown in Table 1.

Southern blot hybridization. Total genomic DNA was extracted from *Borrelia* species as described previously (42). Approximately 5 μg of DNA was digested with 100 U of restriction nuclease according to the manufacturer's recommendations (Boehringer, Mannheim, Germany). Samples were subjected to electrophoresis on a 0.7% agarose gel. DNA fragments were transferred to a nylon membrane (Hybond-N; Amersham) and then UV cross-linked and hybridized according to the procedure described by Church and Gilbert (11). Briefly, hybridization with ^{32}P-labeled probes was done overnight at 65°C in 0.5 M $NaHPO_4$–7% sodium dodecyl sulfate, pH 7.2. After being washed in 40 mM $NaHPO_4$–1% sodium dodecyl sulfate, pH 7.2, at room temperature for 30 min and then dried, the membrane was autoradiographed on Kodak XAR-5 film with intensifying

TABLE 2. Characteristic features of representative subgroup-specific *B. burgdorferi* strains

Strain(s)	Origin(s)	Source(s)	Southern blot results[a]							
			OspA		*fla*		HSP60		HSP70	
			Type	Fragment size(s)	Type	Fragment size(s)	Type	Fragment size(s)	Type	Fragment size(s)
B31, ZS7	United States, Germany	*Ixodes* ticks	I	1.2, 0.3	A	1.5	A	1.7	A	0.8
19857	United States	Rabbit	III	4, 0.5	B	1.2, 0.5	A/B	1.7, 0.9	A	0.8
ACA-1	Sweden	Human	IV	1.7	B	1.2, 0.5	B	1.1, 0.9	A	0.8
ZQ1	Germany	*Ixodes* tick	II	0.9, 0.4	B	1.2, 0.5	B	1.1, 0.9	B	0.7, 0.6
20047	France	*Ixodes* tick	V	3	B	1.2, 0.5	B	1.1, 0.9	B	0.7, 0.6
S90	Germany	*Ixodes* tick	VI	1.1	B	1.2, 0.5	B	1.1, 0.9	B	0.7, 0.6

[a] *Hin*dIII fragment sizes are given in kilobases.

screens at −80°C for 1 to 12 h. After each hybridization, DNA blots were stripped of ^{32}P-labeled probe by incubation in 300 ml of 10 mM Tris–1 mM EDTA–1% sodium dodecyl sulfate, pH 8.2, for 20 min at 95°C; blots were reused 5 to 10 times.

To prepare a hybridization probe for *B. burgdorferi ospA*, we subcloned a 950-bp fragment into pGem-3Z (43). A truncated *B. burgdorferi* flagellin gene derived from plasmid pB31/41-1 was used as a probe (42). According to a cloning strategy described for identification of the *fla* gene, the HSP60 and HSP70 genes were isolated with MAbs LA18 and LA3, respectively (20, 42). A *Hin*dIII fragment of 1.8 kb (22, 32) was used as an HSP60 gene hybridization probe, and a 0.7-kb *Hin*dIII fragment encompassing the C-terminal half of the coding region was used as an HSP70 gene probe; both were derived from *B. burgdorferi* ZS7 (unpublished data). Gene fragments of interest were recovered from low-melting-point agarose gels, ethanol precipitated, and radiolabeled as described previously (42).

DNA sequencing. *B. burgdorferi* genomic DNA fragments cloned in pUEX1 plasmids (Amersham) were sequenced by using a ^{T7}Sequencing kit (Pharmacia) according to the manufacturer's recommendations (42).

Amino acid sequence analyses. Simultaneous alignment for protein sequences and phylogenetic tree construction were performed by using HUSAR software (13, 39).

Nucleotide sequence accession numbers. The HSP60 gene sequences have been entered in the EMBL/GenBank data bases with accession numbers X54059 and X65139 for *B. burgdorferi* ACA-1 and ZS7, respectively. The OspA gene sequences have been assigned accession numbers X16467, X14407, M57248, X60300, M88764, X66065, and X68059 for *B. burgdorferi* ZS7, B31, N40, Goe2, B29, ZQ1, and 19857, respectively. The OspA amino acid sequences of *B. burgdorferi* 25015, ACA-1, IP90, PKo, and PBi were derived from previously published sequences (15, 19, 46). Accession number X67646 has been assigned to the HSP70 gene sequence.

RESULTS

RFLPs of the chromosomal *B. burgdorferi fla*, HSP60, and HSP70 genes. The three chromosomal genes encoding the *fla* protein, HSP60 (*E. coli* GroEL homolog [32]), and HSP70 (*E. coli* DnaK homolog [22]), as well as the plasmid-associated gene for OspA, of the 55 *B. burgdorferi* isolates listed in Table 1 were investigated by RFLP analysis with radiolabeled DNA probes derived from *B. burgdorferi* B31 (*fla* probe) and ZS7 (HSP60, HSP70, and OspA gene probes). Of the several restriction endonucleases used to digest *B. burgdorferi* genomic DNA, *Hin*dIII was found to generate the most decisive banding patterns.

Figure 1 displays representative banding patterns of *Hin*dIII-digested genomic *B. burgdorferi* DNA following hybridization with the radiolabeled *fla* (Fig. 1a), HSP60 (Fig. 1b), or HSP70 (Fig. 1c) gene probe. Accordingly, two genogroups (A and B) can be identified by using the *fla* gene probe: *fla* genogroup A strains (e.g., B31 and ZS7) give rise to a single hybridization band of about 1.5 kb, whereas *fla* genogroup B strains (e.g., ZQ1) exhibit two fragments of about 1.2 and 0.5 kb (Fig. 1a and Table 2). Because of the short exposure of the nylon membranes, the 0.5-kb band of strains 19857, ACA-1, and NE11H is rather weak, but it becomes obvious after prolonged exposure (data not shown). With the HSP60 gene-specific probe, two groups coinciding with *fla* genogroup A and B can again be distinguished; they are characterized either by a single 1.7-kb fragment (HSP60 genogroup A) or by two fragments of 1.1 and 0.9 kb (HSP60 genogroup B), with two exceptions. The North American strain 19857 exhibits the fragment of 1.7 kb typical for genogroup A and the 0.9-kb band but not the 1.1-kb band of genogroup B (Fig. 1b). Strain 20047 shows a weak 1.7-kb fragment in addition to the two bands at 1.1 and 0.9 kb; this isolate may represent a mixture of two strains of HSP60 genogroups A and B. Furthermore, the HSP70 probe again revealed two distinct groups characterized by either one 0.8-kb fragment (HSP70 genogroup A) or two fragments of 0.7 and 0.6 kb (HSP70 genogroup B). These groups coincide with the *fla* and HSP60 genogroups A and B, with the exception of the members of the ACA-1 group, which are of genogroup BBA (*fla*, HSP60, and HSP70, respectively [Fig. 1c and Tables 1 and 2]).

*Hin*dIII digests of DNAs from other *Borrelia* species (*B. hermsii* and *B. coriaceae* Co53) showed either only marginal signals at the relevant positions or banding patterns clearly distinguishable from those of *B. burgdorferi* strains with any one of the three gene probes used (Fig. 1).

RFLPs of the plasmid-encoded OspA gene. As shown in Fig. 1D, RFLP analysis for OspA using *Hin*dIII revealed at least six distinct hybridization patterns among the *B. burgdorferi* isolates tested. All of the *B. burgdorferi* strains tentatively characterized as group AAA strains are characterized by two hybridization fragments of 1.2 and 0.3 kb (OspA type I), and the majority of group BBB strains (17 of 19) express two fragments of 0.9 and 0.4 kb (OspA type II). Strains 20047 and S90 (group BBB) exhibit one fragment of 3 kb (OspA type V) and one fragment of 1.1 kb (OspA type VI), respectively. *B. burgdorferi* 19857 and 21038 [group B(A/B)A] exhibit two hybridizing fragments of 4 and 0.5 kb (OspA genotype III), and all ACA-1-like strains (group BBA)

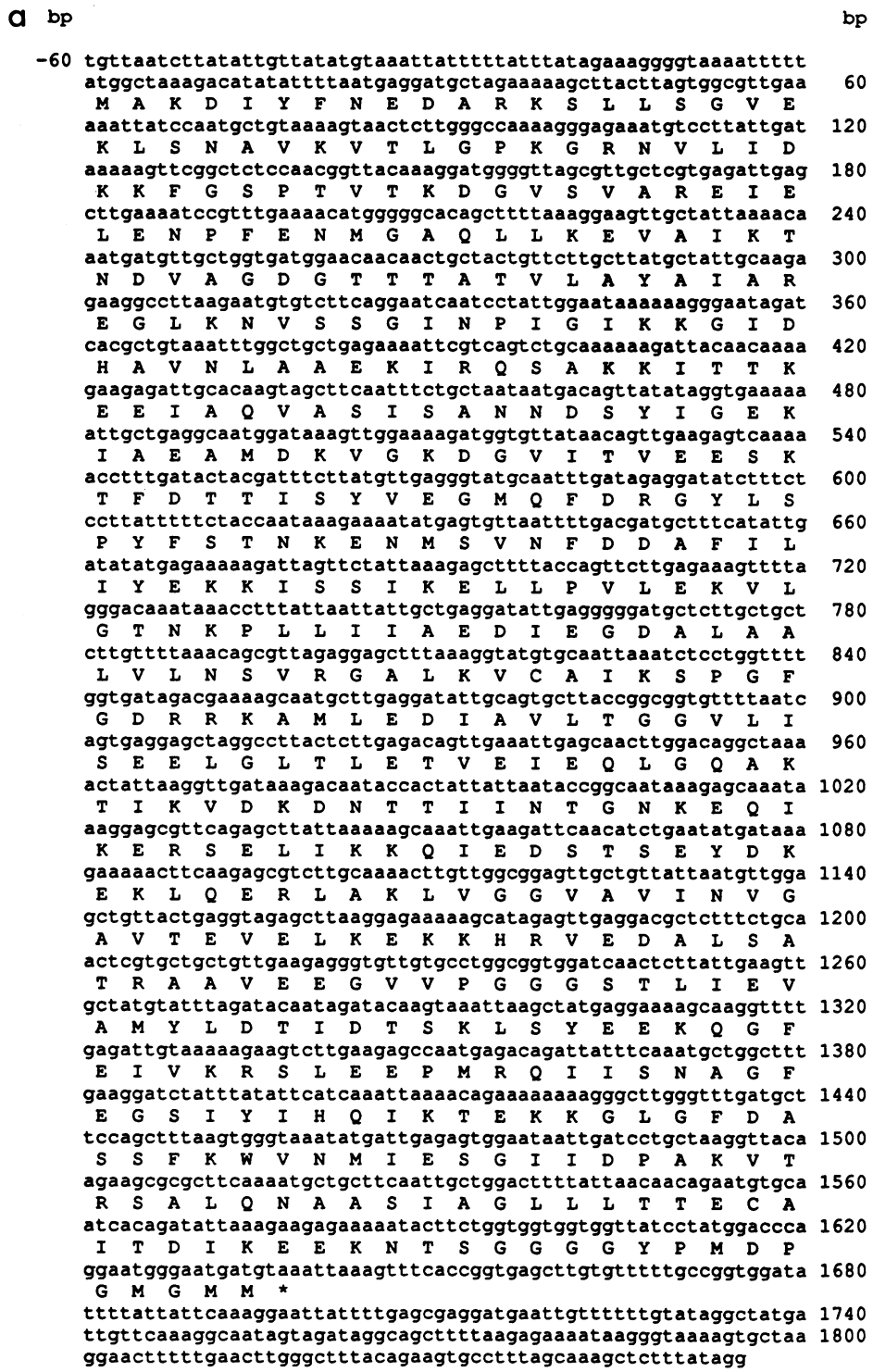

FIG. 2. (a) Complete nucleotide sequence and deduced amino acid sequence of the *B. burgdorferi* ZS7 HSP60 gene. (b) Deduced amino acid sequences of the HSP60 gene products of *B. burgdorferi* ZS7 (upper sequence) and ACA-1 (lower sequence).

express only one fragment of about 1.7 kb (OspA type IV) (Table 1). DNAs isolated from members of other *Borrelia* species did not hybridize to the OspA gene-specific probe (Fig. 1d). In Table 2, the data on the hybridization patterns are summarized for one representative strain of each of the six distinct OspA genogroups. The frequencies of individual OspA genotypes among the 55 *B. burgdorferi* isolates tested are as follows: 30 (55%) OspA type I, 17 (31%) type II, 2 (4%) type III, 4 (7%) type IV, 1 (2%) type V, and 1 (2%) type VI. Note that the American *B. burgdorferi* isolates analyzed

FIG. 2—*Continued.*

to this end belong to only one OspA genotype, i.e., type I, with the exception of two strains, 19857 and 21038, which express OspA genotype III. In contrast, among European isolates there are five OspA genotypes, i.e. I, II, IV, V, and VI (Table 1).

DNA and amino acid sequence analyses of HSP60 and OspA. The HSP60 gene nucleotide sequence derived from *B. burgdorferi* ZS7 (group AAA) was determined and compared with a previously published HSP60 gene sequence derived from strain ACA-1 (group BBA) (32) (Fig. 2). Sequence homologies observed for both structures were >95% at the DNA and >99% at the protein level, i.e., the HSP60 protein sequences of strains ZS7 and ACA-1 differed by only three amino acid substitutions at positions 124, 200, and 213 (Fig. 2b). OspA gene sequences derived from the German *B. burgdorferi* strain ZQ1 and the American strain 19857 are depicted in Fig. 3a and b, respectively. Progressive alignment of OspA protein sequences obtained from 12 *B. burgdorferi* isolates (Fig. 4) demonstrates that those strains assigned before by Southern blot hybridization to one particular OspA genotype, such as ZS7, B31, and N40 (OspA genotype I), were 99% identical to each other (Table 3). As shown in Table 3, a lower degree of sequence identity was observed by comparing OspA polypeptides derived from *B. burgdorferi* strains assigned to distinct OspA genotypes; namely, sequence identities were 81% between OspA from genogroup I (ZS7) and OspA from genogroup II (ZQ1), 70% between OspA from genogroup II (ZQ1) and OspA from genogroup III (19857), and 71% between OspA from genogroup III (19857) and OspA from genogroup IV (ACA-1). The 12 OspA sequences derived from independent *B. burgdorferi* isolates were subjected to the progressive alignment procedure, and the phylogenetic tree that emerged from this analysis indicates the existence of several OspA genogroups (Fig. 5) which coincide with the RFLP-based classification of the 55 *B. burgdorferi* isolates described herein (Table 1).

DISCUSSION

In the past, several attempts to classify *B. burgdorferi* isolates on the basis of genetic and phenotypic traits have been made. These studies were mainly led by the consider-

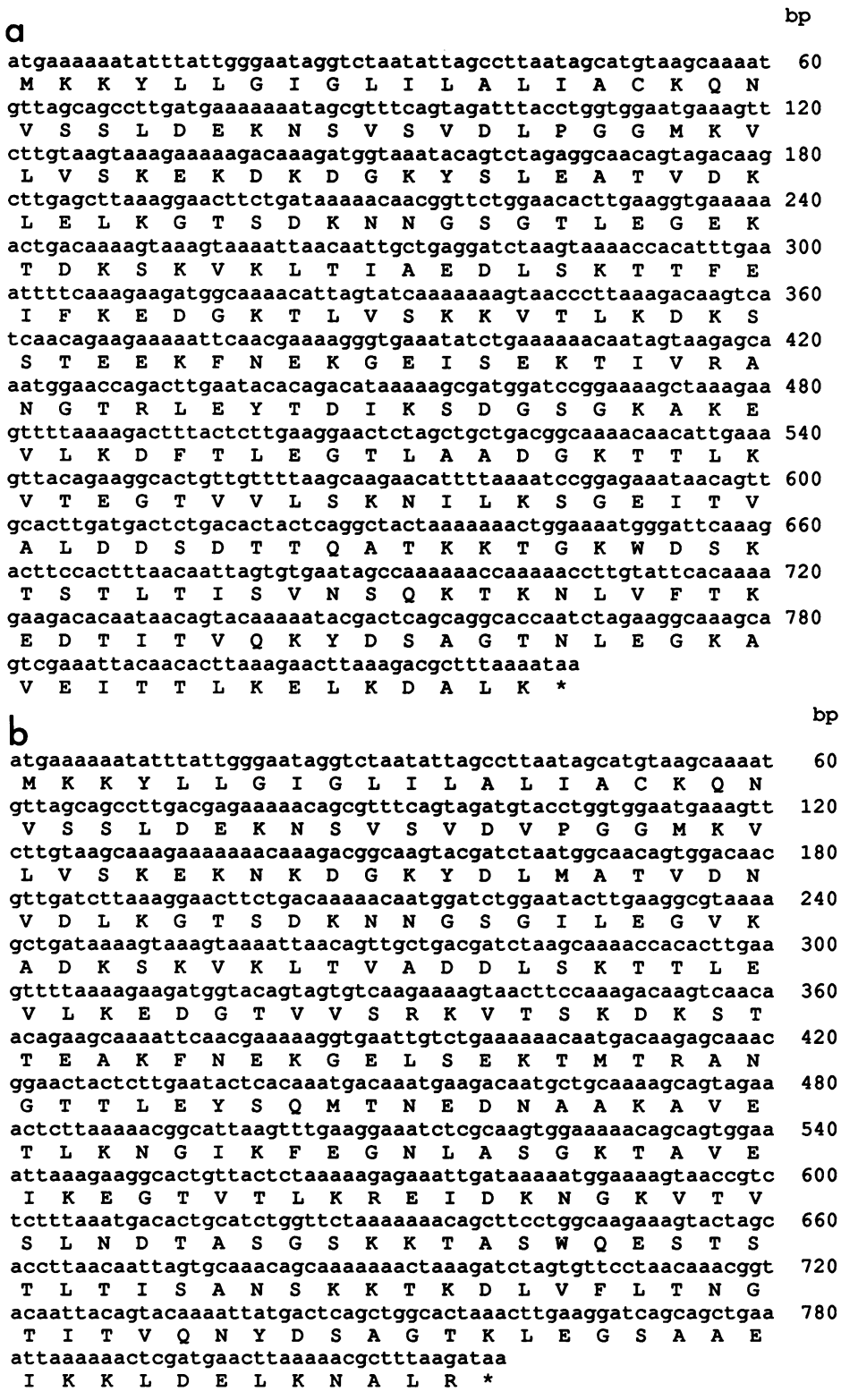

FIG. 3. Complete nucleotide sequences of the OspA genes of strains ZQ1 (a) and 19857 (b).

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Isolate
          **************************** *** ** * ******* ***** * ****
PKo       MKKYLLGIGLILALIACKQNVSSLDEKNSASVDLPGEMKVLVSKEKDKDGKYSLKATVDK
ACA-1     MKKYLLGIGLILALIACKQNVSSLDEKNSASVDLPGEMKVLVSKEKDKDGKYSLKATVDK
Goe2      MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDLPGGMTVLVSKEKDKDGKYSLEATVDK
B29       MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDLPGGMTVLVSKEKDKDGKYSLDATVDK
ZQ1       MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDLPGGMKVLVSKEKDKDGKYSLEATVDK
IP90      MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDLPGGMQVLVSKEKDKDGKYSLMATVDK
PBi       MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDLPGEMKVLVSKEKDKDGKYSLMATVDK
ZS7       MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDLPGEMNVLVSKEKNKDGKYDLIATVDK
N40       MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDLPGEMNVLVSKEKNKDGKYDLIATVDK
B31       MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDLPGEMKVLVSKEKNKDGKYDLIATVDK
25015     MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDLPGEMKVLVSKEKDKDGKYSLMATVDK
19857     MKKYLLGIGLILALIACKQNVSSLDEKNSVSVDVPGGMKVLVSKEKNKDGKYDLMATVDN

            ******* **** *** * **** ***   **  *  *  **** * ** *   ***
PKo       IELKGTSDKDNGSGVLEGTKDDKSKAKLTIADDLSKTTFELFKEDGKTLVSRKVSSKDKT
ACA-1     IELKGTSDKDNGSGVLEGTKDDKSKAKLTIADDLSKTTFELFKEDGKTLVSRKVSSKDKT
Goe2      LELKGTSDKNNGSGTLEGEKTDKSKVKLTIADDLSQTKFEIFKEDGKTLVSKKVTLKDKS
B29       LELKGTSDKNNGSGTLEGEKTDKSKVKLTIADDLSQTKFEIFKEDGKTLVSKKVTLKDKS
ZQ1       LELKGTSDKNNGSGTLEGEKTDKSKVKLTIAEDLSKTTFEIFKEDGKTLVSKKVTLKDKS
IP90      LELKGTSDKNNGSGTLEGEKTDKSKAKLTIAEDLSKTTFEIFKEDGKTLVSKKVTLKDKS
PBi       LELKGTSDKSNGSGTLEGEKSDKSKAKLTISEDLSKTTFEIFKEDGKTLVSKKVNSKDKS
ZS7       LELKGTSDKNNGSGVLEGVKADKSKVKLTISDDLGQTTLEVFKEDGKTLVSKKVTSKDKS
N40       LELKGTSDKNNGSGVLEGVKADKSKVKLTISDDLGQTTLEVFKEDGKTLVSKKVTSKDKS
B31       LELKGTSDKNNGSGVLEGVKADKSKVKLTISDDLGQTTLEVFKEDGKTLVSKKVTSKDKS
25015     LELKGTSDKNNGSGVLEGVKADKSKVKLTVSDDLSTTTLEVLKEDGKTLVSKKRTSKDKS
19857     VDLKGTSDKNNGSGILEGVKADKSKVKLTVADDLSKTTLEVLKEDG TVVSRKVTSKDKS

               ** ***   *   *  ** ***      *   ** * **     **      *
PKo       STDEMFNEKGELSAKTMTRENGTKLEYTEM KSDGTGKAKEVLK NFTLEGKVAND KVT
ACA-1     STDEMFNEKGELSAKTMTRENGTKLEYTEM KSDGTGKAKEVLK NFTLEGKVAND KVT
Goe2      STEEKFNEKGETSEKTIVRANGTRLEYTDI KSDGSGKAKEVLK DFTLEGTLAADGKTT
B29       STEEKFNEKGETSEKTIVRANGTRLEYTDI KSDGSGKAKEVLK DFTLEGTLAADGKTT
ZQ1       STEEKFNEKGEISEKTIVRANGTRLEYTDI KSDGSGKAKEVLK DFTLEGTLAADGKTT
IP90      STEEKFNAKGEASEKTIVRANGTRLEYTDI KSDKTGKAKEVLK DFALEGTLAADGKTT
PBi       SIEEKFNAKGELSEKTILRANGTRLEYTEI KSDGTGKAKEVLK DFALEGTLAAD KTT
ZS7       STEEKFNEKGEVSEKIITRADGTRLEYTEI KSDGSGKAKEVLK SYVLEGTLTAE KTT
N40       STEEKFNEKGEVSEKIITRADGTRLEYTEI KSDGSGKAKEVLK GYVLEGTLTAE KTT
B31       STEEKFNEKGEVSEKIITRADGTRLEYTGI KSDGSGKAKEVLK GYVLEGTLTAE KTT
25015     STEEKFNEKGELVEKIMARANGTILEYTGI KSDGSGKAKETLK EYVLEGTLTAE KAT
19857     TTEAKFNEKGELSEKTMTRANGTTLEYSQMTNEDNAAKAVETLKNGIKFEGNLASG KTA

              **** *   *   *     * *       ***  *    *****  *  **   **
PKo       LEVKEGTVTLSKEIAKSGEVTVALNDTNTTQATKKTGAWDSKTSTLTISVNSKKTTQLVF
ACA-1     LEVKEGTVTLSKEIAKSGEVTVALNDTNTTQATKKTGAWDSKTSTLTISVNSKKTTQLVF
Goe2      LKVTEGTVVLSKNILKSGEITVALDDSDTTQATKKTGKWDSKTSTLTISVNSQKTKNLVF
B29       LKVTEGTVVLSKNILKSGEITVALDDSDTTQATKKTGNWDSKSSTLTISVNSQKTKNLVF
ZQ1       LKVTEGTVVLSKNILKSGEITVALDDSDTTQATKKTGKWDSKTSTLTISVNSQKTKNLVF
IP90      LKVTEGTVVLSKHISNSGEITVELNDSDTTQATKKTGTWDSKTSTLTISVNSRKTKNLVF
PBi       LKVTEGTVVLSKHIPNSGEITVELNDSNSTQATKKTGKWDSNTSTLTISVNSKKTKNIVF
ZS7       LVVKEGTVTLSKNISKSGEVSVELNDTDSSAATKKTAAWNSGTSTLTITVNSKKTKDLVF
N40       LVVKEGTVTLSKNISKSGEVSVELNDTDSSAATKKTAAWNSGTSTLTITVNSKKTKDLVF
B31       LVVKEGTVTLSKNISKSGEVSVELNDTDSSAATKKTAAWNSGTSTLTITVNSKKTKDLVF
25015     LLVKEGTVTLSKHISKSGEVTAELNDTDSTQATKKTGKWDAGTSTLTITVNNKKTKALVF
19857     VEIKEGTVTLKREIDKNGKVTVSLND  TASGSKKTASWQESTSTLTISANSKKTKDLVF

              *** * *** ** *** * **  * * * **
PKo       TKQDTITVQKYDSAGTNLEGTAVEIKTLDELKNALK
ACA-1     TKQDTITVQKYDSAGTNLEGTAVEIKTLDELKNALK
Goe2      TKEDTITVQKYDSAGTNLEGKAVEITTLKELKDALK
B29       TKEDTITVQKYDSAGTNLEGKAVEITTLKELKAALK
ZQ1       TKEDTITVQKYDSAGTNLEGKAVEITTLKELKDALK
IP90      TKEDTITVQKYDSAGTNLEGKAVEITTLKELKDALK
PBi       TKEDTITVQKYDSAGTNLEGNAVEIKTLDELKNALK
ZS7       TKENTITVQQYDSNGTKLEGSAVEITKLDEIKNALK
N40       TKENTITVQQYDSNGTKLEGSAVEITKLDEIKNALK
B31       TKENTITVQQYDSNGTKLEGSAVEITKLDEIKNALK
25015     TKQDTITSQKYDSAGTNLEGTAVEIKTLDELKNALR
19857     LTNGTITVQNYDSAGTKLEGSAAEIKKLDELKNALR
```

FIG. 4. Comparison of deduced OspA amino acid sequences of the 12 *B. burgdorferi* strains indicated on the left. Asterisks denote locations where all residues are identical.

TABLE 3. Sequence similarity of OspA proteins

B. burgdorferi isolate	% Amino acid similarity with OspA of isolate:											
	PKo	ACA-1	Goe2	B29	ZQ1	IP90	PBi	ZS7	N40	B31	25015	19857
PKo		100.00	81.32	80.95	82.05	80.95	83.15	77.29	77.29	77.29	80.22	71.48
ACA-1	100.00		81.32	80.95	82.05	80.95	83.15	77.29	77.29	77.29	80.22	71.48
Goe2	81.32	81.32		98.54	98.18	93.43	87.91	80.95	80.95	80.95	80.59	69.26
B29	80.95	80.95	98.54		96.72	92.70	87.18	80.59	80.59	80.59	79.85	68.89
ZQ1	82.05	82.05	98.18	96.72		94.53	89.38	80.59	80.59	80.95	80.95	70.00
IP90	80.95	80.95	93.43	92.70	94.53		91.58	79.49	79.49	79.49	80.22	69.26
PBi	83.15	83.15	87.91	87.18	89.38	91.58		80.22	80.22	80.22	82.42	69.26
ZS7	77.29	77.29	80.95	80.59	80.59	79.49	80.22		99.63	98.90	84.62	72.96
N40	77.29	77.29	80.95	80.59	80.59	79.49	80.22	99.63		99.27	84.62	73.33
B31	77.29	77.29	80.95	80.59	80.95	79.49	80.22	98.90	99.27		85.35	73.70
25015	80.22	80.22	80.59	79.85	80.95	80.22	82.42	84.62	84.62	85.35		72.96
19857	71.48	71.48	69.26	68.89	70.00	69.26	69.26	72.96	73.33	73.70	72.96	

ations that either most-conserved structures such as rRNA (1, 23, 25) or randomly chosen spirochete-associated genes (27) would be suitable to uncover a prevailing heterogeneity within this species. However, in search of relevant structures for the development of optimal diagnostic tools and/or vaccine candidates, we felt it appropriate to analyze immunologically relevant *B. burgdorferi* structures. For this reason, we have employed four hybridization probes encoded either by chromosomal (*fla*, HSP60, and HSP70) or by plasmid-associated (OspA) genes. For each of the three chromosomal genes (the *fla*, HSP60, and HSP70 genes), two independent hybridization patterns (A and B) were obtained. By combining hybridization patterns for the *fla*, HSP60, and HSP70 genes, four groups of *B. burgdorferi* appeared: group AAA (with the representative strains B31 and ZS7), group B(A/B)A (strain 19857), group BBA (strain ACA-1), and group BBB (strain ZQ1). By comparing identical strains analyzed in independent studies, the classification proposed herein is in agreement with that of Marconi and Garon (23), who found by using 16S RNA sequence alignment that the isolates SH-2-82, B31, and 20004, all classified as AAA by our criteria, are tightly clustered. On the other hand, our scheme differs from that proposed by Postic et al. (25) for the two *B. burgdorferi* isolates IP3 and PKo, both of which were typed to the *B. burgdorferi* sensu stricto subgroup according to their rRNA gene restriction pattern analysis but are classified in genogroups BBB and BBA, respectively, by our criteria. Furthermore, our data corroborate results of Adam et al. (1). On the basis of Southern hybridization with probes for *fla* and rRNA as well as serological analyses, they described the existence of three subgroups (I, II, and III) of *B. burgdorferi* which coincide with genogroups BBB, AAA, and BBA, respectively. Despite the discrepancies revealed by the independent studies which may be due to the different target structures used or the different combination thereof, there is a considerable accordance with respect to the classification of *B. burgdorferi* subgroups.

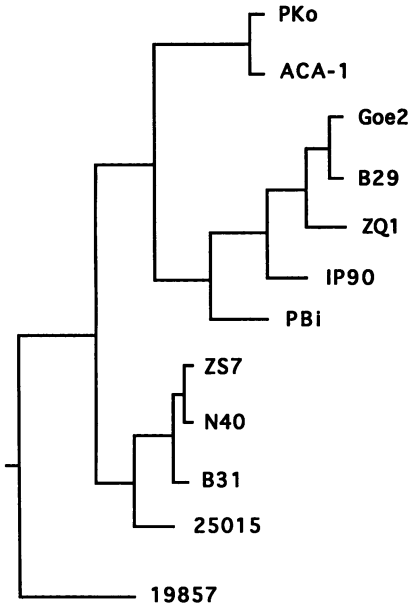

FIG. 5. Phylogenetic tree for 12 OspA proteins derived from individual *B. burgdorferi* isolates. The deduced OspA amino acid sequences were aligned by using the Tree alignment and phylogenetic tree construction program of the HUSAR software package (13, 39).

On the basis of different RFLPs for the more conserved *fla* protein, HSP60, and HSP70, the *B. burgdorferi* genogroup (AAA) represented by strain B31 is the most distant from the genogroup (BBB) represented by strain ZQ1. The ACA-1 genogroup (BBA) seems to be more closely related to the ZQ1 genogroup (BBB), since the RFLPs obtained for these genogroups differed only with respect to the HSP70 gene probe. Furthermore, isolates 19857 and 21038 [B(A/B)A] reside at similar distances from both groups AAA and BBB. Interestingly, these two *B. burgdorferi* isolates derived from *Ixodes dentatus* (21038) and the cottontail rabbit (19857) are likely to be representatives of an independent enzootic cycle, as recently suggested for cottontail rabbits and wood rats (6, 40).

Our finding that RFLPs of the plasmid-encoded OspA gene exhibit a higher degree of heterogeneity than those of the *fla*, HSP60, and HSP70 genes corroborates previous serological studies showing that individual *B. burgdorferi* isolates may express distinct arrays of OspA-associated epitopes (1, 3, 45, 46). This finding is further supported by serological analyses of those 55 isolates analyzed in this study with various MAbs to OspA of strain B31. It was found that these MAbs preferentially react with OspA proteins from homologous strains (type I) such as ZS7 and react much less with those of heterologous strains (types II to VI) (unpublished data).

According to the observed distinct OspA gene hybridization patterns, our RFLP analysis allowed classification of all *B. burgdorferi* strains into six subgroups, three of which (OspA types I, II, and IV) are similar to those reported previously by Barbour et al. (3). The classification of OspA subgroups was further substantiated by analyses of OspA protein sequences derived from various strains representing four of the six subtypes, i.e., OspA type I (ZS7 and B31), type II (ZQ1), type III (19857), and type IV (ACA-1). OspA species associated with one particular OspA subtype, e.g., OspA type I (ZS7, B31, and N40) exhibited sequence homologies of >99%, whereas those from different OspA genotypes showed much less homology (≥69%).

With two exceptions, strains 21038 and 19857 [B(A/B)A], all North American *B. burgdorferi* isolates can be assigned to the genotypic subgroup AAA (prototype strains B31 and ZS7) and express one OspA type, whereas European isolates were found to be associated with three genotypic subgroups (AAA, BBA, and BBB) and five different OspA types (I, II, IV, V, and VI), indicating a higher degree of heterogeneity among European isolates. One could speculate that the species *B. burgdorferi* evolved in the Old World and that only one or two types reached North America more recently.

Interestingly, all spirochetes belonging to genogroup BBB are characterized by the expression of OspA genotype II except two strains, 20047 and S90, which express individual RFLPs (genotype V and VI). Whether these two genotypes represent independent OspA types or mixtures of OspA types or have been generated by molecular events such as internal deletions remains to be elucidated.

The existence of *B. burgdorferi* strains expressing a restricted number of genotypic and phenotypic variants of immunologically relevant structures may have important implications on the development of diagnostic assays as well as on development of vaccines. For the development of a standard assay for serodiagnosis, it appears mandatory to use the relevant proteins from all genomic groups, e.g., OspA from genotypes I to VI and flagellin from genotypes A and B. This claim is also supported by the finding that despite marginal heterogeneity among flagellin proteins (>99% homology) these molecules can express distinct epitopes, as revealed by MAbs (44). Furthermore, the previous finding that OspA is able to induce antibodies which protect against challenge with the corresponding strain (14, 28, 33, 34) but protect only partially or not at all against challenge with *B. burgdorferi* isolates of distinct OspA genotypes implies that an effective vaccine should combine a restricted number of isoforms of relevant *B. burgdorferi* structures which cover all subtypes of this species (15, 27a).

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ADDENDUM IN PROOF

Since this paper was submitted, Anzola et al. (J. Anzola, B. J. Luft, G. Gorgone, R. J. Dattwyler, C. Soderberg, R. Lahesmaa, and G. Peltz, Infect. Immun. **60**:3704–3713, 1992) have reported data for a *B. burgdorferi* HSP70 homolog. The predicted amino acid sequences for both HSP70 homologs are identical.

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