A Phylogenetic Analysis of *Borrelia burgdorferi* Sensu Lato Based on Sequence Information from the *hbb* Gene, Coding for a Histone-Like Protein

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We describe a phylogenetic investigation of *Borrelia burgdorferi* sensu lato, the causative agent of Lyme disease, based on a DNA sequence analysis of the *hbb* gene, which encodes protein HBB, a member of the family of histone-like proteins. Because of their intimate contact with the DNA molecule, these proteins are believed to be fairly conserved through evolution. In this study we proved that the *hbb* gene is suitable for phylogenetic inference in the genus *Borrelia*. The *hbb* gene, which is 327 bp long and encodes 108 amino acids, was sequenced for 39 strains, including 37 strains of *B. burgdorferi* sensu lato, 1 strain of *Borrelia turicatae*, and 1 strain of *Borrelia parkeri*. Genetic variability was determined at the sequence level by computational analysis. Briefly, 81 substitutions were scored at the DNA level. Only 25 of these substitutions were responsible for amino acid substitutions at the translational level. The signature region for bacterial histone-like proteins was found in *hbb*. Although variable at the nucleotide level, it was highly conserved at the deduced amino acid level. A phylogenetic tree for the genus *Borrelia* that was generated from multiple sequence alignments was consistent with previously published data derived from DNA-DNA hybridization and multilocus enzyme electrophoresis analyses. The subdivision of *B. burgdorferi* sensu lato into five species (*B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia japonica*, and “*Borrelia andersonii*”) and at least four genomic groups (groups PotiB2, VS116, CA2, and DN127) was confirmed.

A large portion of our phylogenetic knowledge in the field of bacterial evolution is based on sequence data for the 16S rRNA subunit (56) common to all bacteria. An amazing amount of phylogenetic information, ranging from conserved spots to highly variable spots, is condensed in this genomic region. However, the usefulness of other genomic regions for evolutionary studies of microorganisms has been emphasized in several studies. Following this theme, phylogenies substantially different from the Woese concept have been proposed based on alignments of several housekeeping genes. These include the genes for the 70-kDa heat shock proteins (20), the 60-kDa heat shock proteins (homologs of *Escherichia coli* GroEL) (20, 52), elongation factor EF-Tu (40), glutamate dehydrogenase (7), glutamine synthetase (10, 29, 48), and RecA (27). These studies and a variety of others have clearly shown that phylogenies based on different genes may differ. Although there are virtually no rational grounds for choosing the right phylogeny, we speculate that a target genomic region for DNA sequence alignment can be more or less suitable for phylogenetic analysis of a particular group of organisms. While some genes might be appropriate for phylogenetic inference at the phylum level, other genes might be more appropriate at lower taxonomic levels, such as the family or genus level. In our phylogenetic approach we have focused on the use of bacterial histone-like proteins (also known as nucleoproteins) for inferring phylogenetic relationships of bacteria at the genus level. Because of the intimate interaction of these proteins with the DNA and a high degree of homology, it has been proposed that the sequences of this type of protein should be used as a tool to study the evolution of the organisms which produce them (21, 38). Histone-like proteins are small, basic, heat-

stable DNA-binding proteins. Among the most common histone-like proteins are major nucleoprotein HU and IHF (the integration host factor in *E. coli*). Rather than having properties of eucaryotic histones, these proteins occupy a central role in various gene regulation mechanisms (14, 17, 42). We used this approach to study the phylogenetic relationships of *Borrelia burgdorferi* sensu lato strains, the causative agents of Lyme disease, which have been the objects of intensive phylogenetic studies over the last few years. Our study was based on an analysis of the sequence of the histone-like *hbb* gene of reference strain ATCC 35210^T (= B31^T) (T = type strain), which was recently cloned by K. Tilly and coworkers. The recently cloned *hbb* gene (49) encodes protein HBB and complements both IHF- and HU-deficient *E. coli* strains by substituting for all IHF and HU activities required for λ growth (21, 36). In a comparative study performed with related genes in other organisms, the predicted amino acid sequence encoded by the *hbb* gene of *B. burgdorferi* sensu lato exhibited the highest degrees of similarity with the homologous proteins from *Thermus thermophilus* and *Bacillus subtilis* (about 36% of the amino acids were identical) (49). The HBB protein appears, therefore, to be a *B. burgdorferi* histone-like protein which has IHF and HU features.

B. burgdorferi, the original species involved in Lyme disease, was named in the early 1980s after its discoverer, W. Burgdorfer (11), and has been subdivided taxonomically. Several genomic species, including *B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia japonica*, have been validly described (6, 12, 28). An association with different chronic manifestations of Lyme disease was found for the first three of these genomic species (1, 2, 4, 51). However, it is possible to distinguish clearly additional genomic species with genetic distances comparable to those observed between the nomenclature species by using multilocus enzyme electrophoresis (MLEE) (5) and other molecular techniques (such as DNA-DNA hybridization [39] or PCR analysis of the *fla* gene [3]). We recently proposed a further subdivision of the Lyme spirochetes and

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TABLE 1. *Borrelia* strains used in this study

Strain	Division ^a	Species	Geographic origin	Accession no. ^b
ATCC 35210 ^T (= B31 ^T)	I	<i>B. burgdorferi</i> sensu stricto	United States	U48648
NY1387	I	<i>B. burgdorferi</i> sensu stricto	United States	U48649
A44S	I	<i>B. burgdorferi</i> sensu stricto	Holland	U48650
P1G	I	<i>B. burgdorferi</i> sensu stricto	Switzerland	U48651
IP1	I	<i>B. burgdorferi</i> sensu stricto	France	U48652
IP2	I	<i>B. burgdorferi</i> sensu stricto	France	U48653
IP3	I	<i>B. burgdorferi</i> sensu stricto	France	U48654
SIKA2	II	<i>B. garinii</i>	Japan	U48657
SIKA1	II	<i>B. garinii</i>	Japan	U48656
VSBP	II	<i>B. garinii</i>	Switzerland	U48658
P/Bi	II	<i>B. garinii</i>	Germany	U48659
VS102	II	<i>B. garinii</i>	Switzerland	U48660
NT29	III	<i>B. garinii</i>	Japan	U48661
Ip89	III	<i>B. garinii</i>	Russia	U48662
A19S	III	<i>B. garinii</i>	Holland	U48664
Poti B1	IV	NA ^c	Portugal	U48665
Poti B3	IV	NA	Portugal	U48667
Poti B2	IV	NA	Portugal	U48666
VS116	V	NA	Switzerland	U48668
UK	V	NA	England	U48669
CA2	VI	NA	United States	U48670
VS461 ^T	VII	<i>B. afzelii</i>	Switzerland	U48671
UO1	VII	<i>B. afzelii</i>	Sweden	U48672
DK8	VII	<i>B. afzelii</i>	Denmark	U48674
A26S	VII	<i>B. afzelii</i>	Holland	U48673
ECM1	VII	<i>B. afzelii</i>	Sweden	U48675
BO23	VII	<i>B. afzelii</i>	Germany	U48676
HO14 ^T	VIII	<i>B. japonica</i>	Japan	U48677
F63B	VIII	<i>B. japonica</i>	Japan	U48678
COW 611A	VIII	<i>B. japonica</i>	Japan	U48679
COW 611C	VIII	<i>B. japonica</i>	Japan	U48680
O612	VIII	<i>B. japonica</i>	Japan	U48681
DN127	IX	NA	United States	U48683
CA128	IX	NA	United States	U48684
CA55	IX	NA	United States	U48663
25015	X	NA	United States	U48685
19952	XI	" <i>B. andersonii</i> "	United States	U48686
M3001		<i>B. parkeri</i>	United States	U48655
OM2007		<i>B. turicatae</i>	United States	U48682
CO53		<i>B. coriaceae</i>	United States	
HS1		<i>B. hermsii</i>	United States	

^a *B. burgdorferi* sensu lato division as determined by MLEE (5).

^b GenBank accession number for the DNA sequence of the *hbb* gene.

^c NA, not assigned to a species at this time.

described seven new genomic groups in addition to the four accepted species (5) on the basis of the results of a MLEE analysis in which 12 enzyme loci were used. A name for the genomic group represented by strain 21123 (division XI in our previous study [5]), "*Borrelia andersonii*," has been proposed recently (34).

The aim of this study was to determine whether sequence data from a single gene locus encoding an histone-like protein are suitable for estimating phylogenetic relationships in the genus *Borrelia*.

MATERIALS AND METHODS

Bacterial strains. A total of 37 *B. burgdorferi* sensu lato strains from clinical samples and different tick species, as well as single strains of the relapsing fever spirochetes *Borrelia turicatae*, *Borrelia parkeri*, *Borrelia coriaceae*, and *Borrelia hermsii*, were used in this study (Table 1). The strains used were from many different geographic areas of the world and represented all of the divisions identified by MLEE (5). The *Borrelia* strains were grown in BSK-H medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 6% rabbit serum

(Sigma) at 32°C until the medium color turned from red to yellowish as previously described (4).

Construction of PCR primers. A 433-bp DNA fragment containing the 327-bp *hbb* gene was amplified by using flanking primers containing *Eco*RI-engineered restriction sites, which were designed by starting with the original sequence of strain ATCC 35210^T (= B31^T) (sequence kindly provided by K. Tilly, National Institutes of Health, Hamilton, Mont.). The primers used were primer B.I. (5'-GCGAAGAATTCATAAAAATAAGGCTGC-3'; -78 bp upstream of the *hbb* open reading frame [ORF]) and primer B.r. (5'-TATAAGAATTCACGA TATTAAGTGGC-3'; +28 bp downstream) (the underlined sequence is the *Eco*RI recognition sequence). A pair of nested primers was designed for conserved regions of the *hbb* gene by starting with the alignment for the 39 isolates. The nested primers used were primer B.I.nest (5'-AGATGCTTTTITTTGAAG AGC-3'; forward primer at positions 114 to 133 of the *hbb* ORF) and primer B.r.nest (5'-CAATCTTTGCTTGGACG-3'; reverse primer at positions 297 to 280 of the *hbb* ORF).

DNA extraction and PCR. DNA was extracted from 50 µl of a BSK-H liquid culture by using a commercial ion-exchange resin (InstaGene matrix; Bio-Rad Laboratories, Richmond, Calif.) according to the instructions of the manufacturer. A 10-µl portion of DNA extract was used for the PCR in a total reaction volume of 50 µl. Each reaction mixture also contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 200 µM, 0.01% gelatin type A (Sigma), each primer at a concentration of 0.5 µM, and 1 U of *Taq* polymerase (Boehringer, Mannheim, Germany). The

reaction mixtures were overlaid with paraffin oil (Merck, Darmstadt, Germany) to prevent evaporation. The thermal profile used was 35 cycles consisting of 94°C for 1 min, 50°C for 1 min, and 72°C for 30 s.

DNA sequencing. Templates for cycle sequencing were prepared by purifying PCR products with a Wizard PCR Preps DNA purification system (Promega, Madison, Wis.). Direct sequencing was done with an fmol DNA sequencing system (Promega) by using either primers B.I. and B.R. or a second set of nested primers (see above).

Analysis of nucleotide sequences. DNA nucleotide and amino acid sequences were stored and handled with the PC/GENE (version 6.8) sequence analysis program (IntelliGenetics, Inc., Geel, Belgium). A phylogenetic analysis of the sequence data was performed by using both CLUSTALW (a newer version of CLUSTAL [22]), which was based on the method of Higgins and Sharp (23), and DRAWTREE, which is included in the PHYLIP package (16). The conditions used for alignment were as follows: K-tuple value, 2; gap penalty, 10; gap extension penalty, 0.10 (although no gaps were present in the alignment); and window size, 10. In addition, transitions were weighted twice as likely as transversions.

Nucleotide sequence accession numbers. The original *hbb* sequence of reference strain ATCC 35210^T (= B31^T) of *B. burgdorferi* sensu stricto has been deposited in the GenBank database under accession number U35673. The accession numbers of the *hbb* gene sequences generated in this study are listed in Table 1.

RESULTS AND DISCUSSION

***hbb* gene.** A 433-bp fragment containing the *hbb* gene (length, 327 bp) was amplified for each isolate in a collection containing more than 60 *B. burgdorferi* sensu lato strains (data not shown). The same amplification product was generated from the relapsing fever agents *B. turicatae* and *B. parkeri*, but not from *B. coriaceae* and *B. hermsii*, suggesting that there was a lower degree of conservation of the flanking regions in isolates of the last two species. The length of the *hbb* gene (327 bp) was perfectly conserved in all of the isolates sequenced; no deletions or insertions were observed.

We computed the isoelectric points for the enzymes deduced from the amino acid sequences obtained for the collection of strains. This physicochemical parameter is an important feature of DNA-binding proteins; because of their function, these proteins are characterized by high isoelectric values. The values deduced for the *hbb* gene products matched the values found for other proteins of the same family and ranged from pH 10.47 to 10.95; the values for most of the strains were around pH 10.8. As expected, the G+C content of the *hbb* gene, as calculated from the DNA sequence data in Fig. 1A through C, accounted for 30.65% of the total nucleotides (average for the 39 strains sequenced), which mirrored the average content of 28 to 30.1% for the whole genome of *B. burgdorferi* sensu lato described elsewhere (24). The nucleotide at the wobble position of the preferentially used codons was more often an A or U (33.9 and 44%, respectively) than a G or C (15.5 and 6.4%, respectively), as has been postulated previously for organisms with low G+C contents (25).

In the strain collection studied (*B. burgdorferi* sensu lato, *B. turicatae*, and *B. parkeri*), overall, 81 nucleotide substitutions that were responsible for 25 substitutions at the deduced amino acid level were found within the 327 nucleotides composing the *hbb* gene.

The differences in nucleotide sequences between the *hbb* genes were not evenly distributed among all three codon positions; of the 81 substitutions, 17 were in the first codon position, 12 were in the second codon position, and 52 were in the third codon position. This result can be considered a natural outcome of degeneracy of the genetic code. When the *B. burgdorferi* sensu lato strains alone were considered and the sequences of the strains of the relapsing fever spirochetes *B. parkeri* and *B. turicatae* were not taken into account, the overall numbers of mutations dramatically decreased to 55 nucleotide substitutions and 15 amino acid substitutions. The two non-

Lyme disease spirochetes are therefore clearly distinct from the cluster represented by *B. burgdorferi* sensu lato strains (Fig. 2).

***hbb* sequence analysis versus MLEE and DNA-DNA hybridization.** From the alignment of the nucleotide sequences of 39 strains (Fig. 1) a distance matrix of identity (Fig. 3) and an unrooted phylogenetic tree (Fig. 2) were generated. In order to assess the robustness of the phylogenetic tree inferred from the DNA alignment (Fig. 2), data from the *hbb* sequence analysis were compared with data derived by using two additional methods, MLEE (5) and DNA-DNA hybridization (6). MLEE, whose results have been shown to match the species identification results obtained by DNA-DNA-hybridization (6, 8, 9), is widely considered one of the methods of choice for characterizing bacterial populations at the species level (43). The clustering obtained by aligning the *hbb* gene sequences was largely in agreement with information generated by the other two methods, particularly the dendrogram generated by MLEE data. Clusters containing *B. burgdorferi* sensu stricto, *B. afzelii*, *B. japonica*, group PotiB1, and group VS116 were clearly differentiated by all three methods. The results for strains CA2 and 25015 and the group formed by strains NT29, A19S, and Ip89 exhibited discrepancies if they were compared with the results of other typing methods. In this work, strain CA2 could be clearly separated from the group represented by strain 19952. This result is consistent with MLEE analysis results but is not consistent with DNA-DNA hybridization data. However, when the latter technique was used, strain CA2 had the lowest value in its group (level of DNA relatedness, 73%). Likewise, MLEE data and the results of this study showed that strain 25015 is the prototype of another genomic group, while hybridization data assigned this strain to the group containing strain DN127. When the classification of the group formed by strains NT29, A19S, and Ip89 was examined, our data confirmed previous findings of other authors who assigned these strains to the species *B. garinii* (18, 39); on the other hand, this result is not consistent with the MLEE data, which suggests that these organisms belong to a new genomic group. A more substantial difference between the dendrogram based on MLEE data and the dendrogram based on alignment of the *hbb* gene sequences is related to the classification of the relapsing fever spirochetes. Whereas on the MLEE-derived phylogenetic tree *B. parkeri*, *B. turicatae*, and *B. hermsii* were not separated clearly from the spirochetes associated with Lyme disease, on the tree derived from *hbb* sequence data *B. turicatae* and *B. parkeri* were a significant distance from the entire cluster of Lyme disease spirochetes. The classification of the relapsing fever spirochetes has been controversial in recent years. Noppa et al. (37) used *fla* gene sequences to classify these organisms in a group separate from the Lyme disease spirochetes, but Livesley et al. (30) classified them with the Lyme disease spirochetes on the basis of the results of an analysis of fatty acid methyl esters. Additional sequence analyses based on other genomic regions should be performed to explain these differences.

***hbb* gene product versus known histone-like proteins.** Protein HBB, which is encoded by the *hbb* gene, is larger (length, 108 residues) than other members of the family of histone-like proteins (length, roughly 90 residues). Several residues involved in the biological function of histone-like proteins, such as residues of the hydrophobic core and residues of the DNA-binding arm, are highly conserved across bacterial species (45). This feature is also found in *Borrelia* isolates; residues found to be involved in the hydrophobic core of the protein are conserved both between *Borrelia* and other species and also across all *Borrelia* isolates analyzed. In particular, seven of eight res-

FIG. 1. Alignments of the nucleotide (A through C) and deduced amino acid (D) sequences of the *hbb* gene and HBb protein from 39 *Borrelia* strains. A dash indicates that a position in the alignment is conserved. In panel D the underlined residues are residues involved in the hydrophobic core of the protein and the sequence enclosed in a box is the signature for histone-like proteins located on the putative DNA-binding arm.

C

B31	GAGTATGTTAAGGTCCTAGATCATCAGCTTGCCTATTTTCGTCAGGCAAGATTTGAAAGAGAGAGTGTGGGTATCAAAGGTAA	300	327
NY1387	-T-----		
A44S	-----		
P1G	-----		
IP1	-----		
IP2	-----		
IP3	-----		
BPAR	-----T--TGAT-----T---T-----A---CG-G--A-----T-----		
SIKA1	--A-----TT-----T-----T-----T-----		
SIKA2	--A-----TT-----T-----T-----T-----		
VSBP	--A-----TT-----T-----T-----T-----		
PBI	--A-----TT-----T-----T-----T-----		
VS102	--A-----TT-----T-----A-----T-----		
NT29	--A-----TT-----T-----G-----T-----		
IP89	--A-----TT-----T-----T-----T-----		
A19S	--A-----TT-----T-----G-----T-----		
POTIB1	-----TT-----T-----A-----T-----		
POTIB2	-----TT-----T-----A-----T-----		
POTIB3	-----TT-----T-----A-----T-----		
VS116	--A-----TT-----T-----A-----A-----T-----		
UK	--A-----TT-----T-----A-----A-----T-----		
CA2	--A-----TT-----T-----T-----T-----		
VS461	--A-----TT-----T-----T-----T-----		
UO1	--A-----TT-----T-----A-----T-----		
DK8	--A-----TT-----T-----T-----T-----		
ECM1	--A-----TT-----T-----T-----T-----		
A26S	--A-----TT-----T-----T-----T-----		
BO23	--A-----TT-----T-----T-----T-----		
HO14	--A-----TT-----T-----C-----T-----		
F63B	--A-----TT-----T-----C-----T-----		
COW611A	--A-----TT-----T-----C-----T-----		
COW611C	--A-----TT-----T-----C-----T-----		
O612	--A-----TT-----T-----C-----T-----		
BTUR	-----T--TGAT-----T---T-----A---A---AC---A---C---T---G---A---		
DN127	--A-----TT-G-----T---T-----G---C-----T-----		
CA128	--A-----TT-G-----T---T-----G---C-----T-----		
CA55	--A-----TT-G-----T---T-----G---C-----T-----		
Z5015	--A-----TT-G-----T---T-----G---C-----T-----		
19952	--A-----TT-G-----T---T-----G---C-----T-----		

D

B31	MSFSRRPKVTKSDIVDQIALNKKNNLKLEKKYIRLVIDAFFEELKSNLCSSNNVIEFHSFGTFEVRKRRKRLNARNPQTGEYVKVLDHHVAYFRPGKDLKERVWGIGK	50	100	108
NY1387	-----V-----			
A44S	-----S-----			
P1G	-----S-----			
IP1	-----S-----			
IP2	-----S-----			
IP3	-----S-----			
BPAR	-----N--S--S-E-----V-----NS--L-----Q-----N-D-----DG-----			
SIKA1	-----I-----FF-R-----G--L-----			
SIKA2	-----I-----FF-R-----G--L-----			
VSBP	-----I-----S-R-----G--L-----			
PBI	-----I-----S-R-----G--L-----			
VS102	-----I-----S-R-----G--L-----I-----			
NT29	-----I-----S-R-----G--L-----			
IP89	-----I-----S-R-----G--L-----			
A19S	-----I-----S-R-----G--L-----			
POTIB1	-----V-N--S--R-----			
POTIB2	-----V-N--S--R-----			
POTIB3	-----V-N--S--R-----			
VS116	-----P-----I-----S--R-----			
UK	-----P-----I-----S--R-----			
CA2	-----P-----S--R-----			
VS461	-----P-----S--R-----			
UO1	-----P-----S--R-----N-----			
A26S	-----P-----S--R-----			
DK8	-----P-----S--R-----			
ECM1	-----P-----S--R-----			
BO23	-----P-----S--R-----			
HO14	-----S--R-----G-----			
F63B	-----S--R-----G-----			
COW611A	-----S--R-----G-----			
COW611C	-----S--R-----G-----			
O612	-----S--R-----G-----			
BTUR	-----A-----N--S--S-E-----V-----NS--L-----L-----Q-----N-D-----			
DN127	-----S--R-----C-----R-----			
CA128	-----S--R-----C-----			
CA55	-----S--R-----C-----			
Z5015	-----S--R-----L-----R-----			
19952	-----S--R-----R-----			

FIG. 1—Continued.

FIG. 2. Unrooted phylogenetic tree based on nucleotide sequences of the *hbb* gene. The method used for drawing the tree was DRAWTREE, a program included in the software package PHYLIP. *B. burgdorferi* s.s., *B. burgdorferi* sensu stricto.

idues of the hydrophobic core (phenylalanine residues 41, 57, 60, 63, and 93 and leucine residues 45 and 49) are perfectly conserved, while leucine-20 is replaced by phenylalanine (also a hydrophobic amino acid) in two strains (SIKA1 and SIKA2).

We searched the ProDom protein domain database (which consists of an automatic compilation of homologous domains detected in the SWISS-PROT database) by using the DOMAINER algorithm (44) for a signature sequence for histone-like proteins. We found a signature sequence consisting of 20 residues which includes three perfectly conserved positions. This signature sequence is [GS]-F-X(2)-[LIVMF]-X(4)-[RKEQA]-X(2)-[RST]-X-[GA]-X-[KN]-P-X-T. It is predicted that this region forms the first half of the flexible DNA-binding arm according to the tertiary structure of one of the proteins proposed by Tanaka et al. (45) and, therefore, should be fairly well conserved. In all of the *Borrelia* strains analyzed in this study, the sequence of 21 residues (from position 59 to position 79) matched the signature sequence for histone-like proteins, with the exception of a single amino acid position (a gap was inserted in the original consensus sequence so that it would be aligned with *Borrelia* sequences). Particularly invariant amino acid residues were found in this region. Although rich in variation at the nucleotide level (14 substitutions in 63 nucleotides), this region was well conserved at the amino acid level (3 substitutions in 21 residues), which led to four unique sequences (one found in *B. parkeri* and *B. turicatae*, one found in strains 19952 and DN127, one found in strain 25015, and one found in all of the other *B. burgdorferi* sensu lato strains analyzed). All of the substitutions found in the *Borrelia* isolates at

the deduced amino acid level were located in variable regions of the signature (Table 2).

Furthermore, the signatures of 21 previously published sequences determined for histone-like proteins of different organisms (including gram-positive and gram-negative bacteria, as well as one species of cyanobacteria [*Anabaena* sp.], one chloroplast [from *Cryptomonas* sp.], and bacteriophage SPO1) were aligned with *Borrelia* sequences (Table 2). The dendrogram constructed from this alignment (Fig. 4) revealed that there is a clear difference between protein HBb of *Borrelia* strains and the histone-like proteins of other organisms. Whether HBb represents a new type of histone-like protein or reflects an evolutionarily early divergence from the family remains an open question. A possible explanation for this observation might be related to some peculiarities of the DNA structure found in these spirochetes. The predominant form of DNA found in borreliac (but not in most of the other organisms) is in fact linear, with covalently closed ends. Thus, the requirements for DNA condensation and replication might be different in *Borrelia* strains than in other organisms. The functions fulfilled by IHF and HU in other organisms (e.g., *E. coli*) might be accomplished by a single protein in these spirochetes.

***hbb* gene versus other *Borrelia* genes.** The robustness of DNA sequence analysis for phylogenetic purposes is guaranteed when the appropriate analysis tools (algorithms for data processing) and a representative collection of strains are carefully selected. However, the most crucial element for correct phylogenetic extrapolations is the choice of a suitable target genomic region. In the case of a coding region, the gene should

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1		328	325	318	313	312	311	311	310	314	310	310	308	311	310	308	311	308	306	305	306	308	305	310	282	277
2	99.7		324	317	312	311	310	310	308	313	308	308	308	310	308	307	310	305	305	304	305	305	304	308	281	278
3	98.4	99.1		320	313	313	313	312	312	318	312	312	311	313	312	309	312	308	308	307	307	307	306	312	283	278
4	97.2	96.9	97.9		314	312	314	313	315	317	313	313	312	314	313	310	315	311	311	310	308	310	309	311	284	281
5	95.7	95.4	95.7	96.0		319	321	320	322	308	308	307	307	311	310	309	308	307	307	308	304	306	306	306	279	274
6	95.4	95.1	95.7	95.4	97.6		325	326	324	310	310	309	308	313	312	313	310	309	309	308	308	310	310	308	279	274
7	95.1	94.8	95.7	96.0	98.2	99.4		326	326	310	310	309	308	313	312	311	308	309	309	308	308	308	308	308	279	274
8	95.1	94.8	95.4	95.7	97.9	99.7	99.7		325	309	308	308	308	312	311	312	309	308	308	307	307	308	307	308	280	275
9	94.8	94.5	95.4	96.3	98.5	99.1	99.7	99.4		311	311	310	310	314	313	312	309	310	310	309	307	308	308	308	280	275
10	96.0	95.7	96.6	96.9	94.2	94.8	94.8	94.5	95.1		315	315	316	315	314	310	311	310	310	307	305	307	306	309	283	278
11	94.8	94.5	95.4	95.7	94.2	94.8	94.8	94.5	95.1	98.3		321	320	315	314	314	315	311	311	309	308	308	308	311	284	279
12	94.8	94.5	95.4	95.7	93.9	94.5	94.5	94.2	94.8	96.3	98.2		325	315	314	312	313	313	310	308	310	309	309	308	283	278
13	94.5	94.2	95.1	95.4	93.9	94.5	94.5	94.2	94.8	96.6	97.9	99.4		314	313	311	312	311	311	308	306	308	308	308	283	278
14	95.1	94.8	95.7	96.0	95.1	95.7	95.7	95.4	96.0	96.3	96.3	96.3	96.0		326	315	314	314	314	313	311	313	312	314	283	278
15	94.8	94.5	95.4	95.7	94.8	95.4	95.4	95.1	95.7	96.0	96.0	96.0	95.7	99.7		314	313	313	313	312	310	312	311	313	282	277
16	94.2	93.9	94.5	94.8	94.5	95.7	95.1	95.4	95.4	94.8	96.0	95.4	95.1	96.3	96.0		322	314	312	312	311	313	312	311	278	273
17	95.1	94.8	95.4	96.3	94.2	94.8	94.2	94.5	94.5	95.1	96.3	95.7	95.4	96.0	95.7	98.5		313	311	311	310	312	311	308	279	274
18	93.6	93.3	94.2	95.1	93.9	94.5	94.5	94.2	94.8	94.8	95.1	95.7	95.1	96.0	95.7	96.0	95.7		325	322	320	322	320	307	282	277
19	93.6	93.3	94.2	95.1	93.9	94.5	94.5	94.2	94.8	94.8	95.1	95.7	95.1	96.0	95.7	95.4	95.1	96.4		324	322	324	320	307	282	277
20	93.3	93.0	93.9	94.8	93.6	94.2	94.2	93.9	94.5	93.9	94.5	94.8	94.2	95.7	95.4	95.4	95.1	98.5	99.1		321	323	319	306	283	278
21	93.6	93.3	93.9	94.2	93.0	94.2	93.6	93.9	93.9	93.3	93.6	94.2	93.6	95.1	94.8	95.1	94.8	97.9	98.5	98.2		323	321	304	282	277
22	93.6	93.3	93.9	94.8	93.6	94.8	94.2	94.5	94.5	93.9	94.2	94.8	94.2	95.7	95.4	95.7	95.4	98.5	99.1	98.8	98.8		321	306	285	280
23	93.3	93.0	93.6	94.5	93.6	94.8	94.2	94.5	94.5	93.6	94.2	94.5	94.5	95.4	95.1	95.4	95.1	97.9	97.9	97.6	98.2	98.2		305	285	280
24	94.8	94.5	95.4	95.1	93.6	94.2	94.2	93.9	94.5	94.5	95.1	94.5	94.2	96.0	95.7	94.8	94.5	93.9	93.9	93.6	93.0	93.6	93.3		285	280
25	86.2	85.9	86.5	86.9	85.3	85.3	85.3	85.6	86.5	86.5	86.5	86.2	85.0	85.3	86.2	86.2	86.2	86.5	86.2	87.2	87.2	87.2	87.2		87.2	316
26	84.7	84.4	85.0	85.9	83.8	83.8	83.8	84.1	84.1	85.0	85.3	85.0	85.0	85.0	84.7	83.5	83.8	84.7	84.7	85.0	84.7	85.6	85.6	85.6	85.6	

B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
1		107	107	106	106	105	105	105	105	105	105	105	104	104	104	105	105	103	103	103	102	103	102	104	94	95	
2	99.1		106	105	105	104	104	104	104	104	104	104	103	103	103	104	104	102	102	102	101	102	101	103	93	94	
3	99.1	98.1		107	105	106	106	106	106	106	106	106	105	105	105	106	106	104	104	104	103	104	102	105	95	96	
4	98.1	97.2	98.1		104	107	107	107	107	107	107	107	106	106	106	107	107	105	105	105	104	105	103	106	94	95	
5	98.1	97.2	97.2	96.3		105	105	105	105	103	103	103	102	104	104	103	103	101	101	101	100	101	100	102	92	93	
6	97.2	96.3	96.1	99.1	97.2		108	108	108	106	106	106	105	107	107	106	106	104	104	104	103	104	102	105	93	94	
7	97.2	96.3	96.1	99.1	97.2	100		108	108	106	106	106	105	107	107	106	106	104	104	104	103	104	102	105	93	94	
8	97.2	96.3	96.1	99.1	97.2	100	100		108	106	106	106	105	107	107	106	106	104	104	104	103	104	102	105	93	94	
9	97.2	96.3	96.1	99.1	97.2	100	100	100		106	106	106	105	107	107	106	106	104	104	104	103	104	102	105	93	94	
10	97.2	96.3	96.1	99.1	95.4	98.1	98.1	98.1	98.1		106	106	107	105	105	106	106	104	104	104	103	104	102	105	93	94	
11	97.2	96.3	96.1	99.1	95.4	98.1	98.1	98.1	98.1	98.1		106	105	105	105	106	106	104	104	104	103	104	102	105	95	96	
12	97.2	96.3	96.1	99.1	95.4	98.1	98.1	98.1	98.1	98.1	98.1		107	105	105	106	106	104	104	104	103	104	102	105	93	94	
13	96.3	95.4	97.2	98.1	94.4	97.2	97.2	97.2	97.2	99.1	97.2	99.1		104	104	105	105	103	103	103	102	103	101	104	92	93	
14	96.3	95.4	97.2	98.1	96.3	99.1	99.1	99.1	99.1	99.1	97.2	97.2	97.2	96.3		108	105	105	105	105	104	105	103	104	92	93	
15	96.3	95.4	97.2	98.1	96.3	99.1	99.1	99.1	99.1	99.1	97.2	97.2	97.2	96.3	100		105	105	105	105	105	104	105	103	104	92	93
16	97.2	96.3	98.1	99.1	95.4	98.1	98.1	98.1	98.1	98.1	98.1	98.1	97.2	97.2	97.2		108	106	106	106	105	106	104	105	94	95	
17	97.2	96.3	98.1	99.1	95.4	98.1	98.1	98.1	98.1	98.1	98.1	98.1	97.2	97.2	97.2	100		106	106	106	105	106	104	105	94	95	
18	95.4	94.4	96.3	97.2	93.5	96.3	96.3	96.3	96.3	96.3	96.3	96.3	95.4	97.2	97.2	98.1	98.1		108	108	107	108	106	103	94	95	
19	95.4	94.4	96.3	97.2	93.5	96.3	96.3	96.3	96.3	96.3	96.3	96.3	95.4	97.2	97.2	98.1	98.1	100		108	107	108	106	103	94	95	
20	95.4	94.4	96.3	97.2	93.5	96.3	96.3	96.3	96.3	96.3	96.3	96.3	95.4	97.2	97.2	98.1	98.1	100	100		107	108	106	103	94	95	
21	94.4	93.5	95.4	96.3	92.6	95.4	95.4	95.4	95.4	95.4	95.4	95.4	94.4	96.3	96.3	97.2	97.2	99.1	99.1	99.1		107	105	102	93	94	
22	95.4	94.4	96.3	97.2	93.5	96.3	96.3	96.3	96.3	96.3	96.3	96.3	95.4	97.2	97.2	98.1	98.1	100	100	100	99.1		106	103	94	95	
23	94.4	93.5	94.4	95.4	92.6	94.4	94.4	94.4	94.4	94.4	94.4	94.4	93.5	95.4	95.4	96.3	96.3	98.1	98.1	98.1	97.2	98.1		101	92	93	
24	96.3	95.4	97.2	98.1	94.4	97.2	97.2	97.2	97.2	97.2	97.2	97.2	96.3	96.3	96.3	97.2	97.2	95.4	95.4	95.4	94.4	95.4	93.5		94	95	
25	87	86.1	88	87	85.2	86.1	86.1	86.1	86.1	86.1	88	86.1	85.2	85.2	85.2	87	87	87	87	87	87	86.1	87	85.2	87		105
26	88	87	88.9	88	86.1	87	87	87	87	87	88.9	87	86.1	86.1	86.1	88	88	88	88	88	88	87	88	86.1	88	97.2	

FIG. 3. Matrix of pairwise identity scores for 26 unique nucleic acid sequences (A) and deduced amino acid sequences (B). 1, reference strain ATCC 35210^T (= B31^T) and A44S; 2, NY1387; 3, P1

TABLE 2. Alignment of the amino acid sequences of the signature region characterizing the histone-like DNA-binding proteins of various organisms

Organism(s)	Protein	Signature sequence ^a
<i>Rhizobium leguminosarum</i>	HRL18	GFGSFSVSRREAS-KGRNPST
<i>Rhizobium leguminosarum</i>	HRL53	GFGSFTVSHRAAT-KGRNPST
<i>Rhizobium meliloti</i>	HRM	GFGNFSVSREASK-GRRNPST
<i>Escherichia coli</i>	HU	GFGTFKVNHRAER-TGRNPQT
<i>Salmonella typhimurium</i>	HU	GFGTFKVNHRAER-TGRNPQT
<i>Vibrio proteolyticus</i>	HU	GFGTFKVNHRAER-TGRNPQT
<i>Pseudomonas aeruginosa</i>	HU	GFGTFKVNHRAER-TGRNPQT
<i>Anabaena</i> sp.	HU	GFGSFESRERKAR-EGRNPST
<i>Cryptomonas</i> (chloroplast)	HU	GFGSFESRERKAR-EGRNPST
<i>Clostridium pasteurianum</i>	HU	GFGTFETRERAAR-EGRNPST
<i>Bacillus stearothermophilus</i>	HU	GFGNFEVRERSAR-KGRNPQT
<i>Bacillus subtilis</i>	HU	GFGNFEVRERSAR-KGRNPQT
<i>Thermus aquaticus</i>	II	GFGTFEVRKRKAR-TGVKPKT
<i>Escherichia coli</i>	IHF-HIMD	GFGSFSLSHYRAPR-TGRNPST
<i>Serratia marcescens</i>	IHF-HIMD	GFGSFSLSHYRAPR-VGRNPST
<i>Escherichia coli</i>	IHF-HIMA	GFGNFDLRDKNQR-PGRNPST
<i>Serratia marcescens</i>	IHF-HIMA	GFGNFDLRDKNQR-PGRNPST
<i>Salmonella typhimurium</i>	IHF-HIMA	GFGNFDLRDKNQR-PGRNPST
<i>Rhodobacter capsulatus</i>	IHF-HIMA	SFGTFESVRDKTSR-MGRNPST
<i>Thermoplasma acidophilum</i>	HTA	GFGIFERRTQGPQ-KARNPQT
Bacteriophage SPO1	TF1	GFLNIKPVARQAR-KGFNPQT
<i>B. burgdorferi</i> sensu lato	HBb	SFGTFEVRKRKGRNLARNPQT
19952 and DN127	HBb	SFGTFEVRKRKGRNLARNPQT
25015	HBb	SFGTFELKRKGRNLARNPQT
<i>B. parkeri</i> and <i>B. turicatae</i>	HBb	SFGTFELKRKGRNLARNPQT

^a Positions 2, 19, and 21 are perfectly conserved.

lial structures related to the host-pathogen interaction and, therefore, presumably are under specific and significant selective pressure from the host. Although knowledge of the diversity of these genes is crucial for vaccine development, their use in phylogenetic studies is less suitable. Furthermore, lateral transfer among members of the *osp* family seems to occur quite often. Evidence showing that recombinational events occur with *ospD* and *ospC* has been reported recently (26, 31, 35, 55). While intragenic recombination seems to be rare for *ospA* (15), this phenomenon has not been observed with the remaining *osp* genes. In contrast to the *osp* genes, the *hbb* gene is encoded within the chromosome and does not seem to be subject to lateral transfer. In addition, in most reported studies, either a limited number of isolates or isolates that are not representative of the whole range of *B. burgdorferi* sensu lato species have been used in the sequence analysis. The advantage of this study is that we used a broad array of isolates (39 strains), including strains from a wide geographic area and different vector species and strains which are responsible for the complete spectrum of symptomatic manifestations of Lyme disease (erythema chronicum migrans, neurological and cardiac disorders, arthritis, acrodermatitis chronica atrophicans, and neuropathies of the central nervous system) (4).

In general, the variation in *hbb* is significantly lower than the variation in the *osp* genes. The length of the *hbb* gene is perfectly conserved (327 bp, with no gaps), while all other genomic regions described in other alignment studies are not. Moreover, the overall identities, as measured at the deduced amino acid level, of 74.4% for *ospA* (among 19 isolates of *B. burgdorferi* sensu lato), 60% for *ospC* (among 34 isolates of *B. burgdorferi* sensu lato), and 80% for *ospD* (among 15 isolates of *B. burgdorferi* sensu lato) are significantly lower than the values

FIG. 4. Dendrogram generated with the amino acid sequences of the signature region characterizing the family of bacterial histone-like DNA-binding proteins from various organisms. It is predicted that this region forms the first half of the flexible DNA-binding arm.

found for the *hbb* gene among 37 isolates of *B. burgdorferi* sensu lato (more than 93%). If non-Lyme disease spirochetes were considered, the identity dropped to 85%.

Significance of this study. One might question whether this work was useful for any purpose other than fundamental research purposes. Besides providing new insights into the genetic structure of bacterial populations of the genus *Borrelia*, this study allowed us to design a specific and sensitive nested PCR for the detection of a wide spectrum of *Borrelia* strains, including all of the species of *B. burgdorferi* sensu lato, as well as *B. parkeri* and *B. turicatae* (50). This new PCR assay might have applications both in clinical diagnosis and in fundamental research, allowing workers to detect and phylogenetically characterize strains without having to perform fastidious and often unsuccessful cultivation procedures.

Conclusions. We believe that the sequence of the *hbb* gene provides valuable information for phylogenetic studies in the genus *Borrelia*. The sequence analysis of this gene confirmed the subdivision of *B. burgdorferi* sensu lato into five species (*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. japonica*, and "*B. andersonii*") and at least four genomic groups (groups PotiB1, CA2, DN127, and VS116). Although our data originated from a single gene locus, they are consistent with information acquired by methods involving the whole genome, such as MLEE and DNA-DNA hybridization. In conclusion, our original hypothesis that histone-like proteins might be suitable targets for molecular phylogenetic investigations in bacterial species was confirmed in this work. The use of sequence analysis of genes encoding such proteins might be adequate for the study of the genetic structure of other bacterial populations as well.

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