

## Phenotypic and Genetic Characterization of a Novel *Borrelia burgdorferi* Sensu Lato Isolate from a Patient with Lyme Borreliosis

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***Borrelia burgdorferi* sensu lato A14S was cultured from a skin biopsy specimen of a patient with erythema migrans in The Netherlands. This isolate had a unique DNA fingerprint pattern compared to 135 other *B. burgdorferi* sensu lato isolates. In this study, the isolate A14S was further characterized by protein analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reactivity with various monoclonal antibodies. In addition, the 16S rRNA, *ospA*, and *ospC* genes, as well as the 5S-23S rRNA intergenic spacer DNA, were amplified by PCR, cloned, and sequenced. SDS-PAGE protein profiles and phylogenetic analysis based on all of the analyzed genes confirmed that *B. burgdorferi* sensu lato A14S was phenotypically and genetically different from the three human pathogenic species *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*, as well as from other *B. burgdorferi* sensu lato species. Our findings indicate that *Borrelia* genomic groups or isolates other than the three well-known human pathogenic species may also cause human Lyme borreliosis.**

Lyme borreliosis (LB) is a worldwide multisystemic infection transmitted to humans by the bite of an infected tick of the genus *Ixodes*. Erythema migrans, an early characteristic skin lesion, is relatively consistent and is found in about 50% of LB cases (17). *Borrelia burgdorferi* sensu lato, the causative bacterium of LB, is genetically divergent and has been divided into several species or genomic groups. These include *B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii* (1, 2), *Borrelia japonica* (6), *Borrelia valaisiana* (21), *Borrelia lusitaniae* (8), *Borrelia andersonii* (10), *Borrelia tanukii*, *Borrelia turdi* (5), and *Borrelia bissettii* (formerly genomic group DN127) (14). *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* have been cultured from patients and are thought to be responsible for human LB. Recently, Picken et al. (12, 18) reported that nine *B. burgdorferi* isolates from patients in Slovenia were closely related to the North American isolate 25015, a member of *B. bissettii*. Thus, strains belonging to *B. bissettii* may also cause human disease.

In 1992, we cultured a *B. burgdorferi* sensu lato isolate, A14S, from the skin biopsy specimen of a Dutch patient with erythema migrans who had contracted the disease in The Netherlands. This isolate could not be typed based on its reactivity with various monoclonal antibodies (MAbs) against *B. burgdorferi* sensu lato (20). Also, it showed a unique pattern in ribotyping, differing from *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (20). In addition, a striking genetic difference between this isolate and 135 other LB-related *Borrelia* isolates was noted in the randomly amplified polymorphic DNA (RAPD) fingerprinting analysis (22). Therefore, *Borrelia* isolate A14S could well represent a novel *B. burgdorferi* sensu lato strain causing human LB.

In the present study, isolate A14S at low passage (passages 5 to 10) was further analyzed by protein profiling with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reactivity with MAbs against different outer surface proteins of *B. burgdorferi* sensu lato. The 16S rRNA, *ospA*, and *ospC* genes, as well as the 5S-23S rRNA intergenic spacer from this isolate, were amplified by PCR, sequenced, and compared to those from *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, the three pathogenic *Borrelia* species for humans, and other species in the *B. burgdorferi* sensu lato complex.

SDS-PAGE was performed as described previously (20). The protein profile of isolate A14S was clearly different from those of the representative isolates of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. valaisiana* (Fig. 1). For example, isolate A14S contained at least three unique protein bands with molecular masses of 37, 49, and 72 kDa (Fig. 1, asterisks). Apart from its reactivity with *B. burgdorferi* sensu stricto-specific MAb H3TS (20), this isolate reacted with the *OspA*-specific MAb LA26, which recognizes both *B. burgdorferi* sensu stricto and *B. afzelii*. No reactivity was observed to the *OspB*-specific MAb 84C and the *OspC*-specific MAb L22 C11, two MAbs reactive with *OspB* and *OspC* proteins, respectively, from *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (data not shown).

The 5S-23S spacer PCR-restriction fragment length polymorphism (RFLP) analysis has been widely applied to assess the genetic diversity of *B. burgdorferi* sensu lato strains and to identify *Borrelia* genomic groups (13). In our study, a 225-bp 5S-23S intergenic spacer from *Borrelia* isolate A14S was amplified by PCR. The size of this spacer was notably different in comparison to those of other European isolates from five different *Borrelia* species, which ranged from 246 to 257 bp (13). The PCR amplicon of isolate A14S was sequenced directly as described previously (21). Digestion of this amplicon with *MseI* resulted in three fragments with sizes of 106, 68, and 51 bp, respectively (data not shown). This *MseI* restriction pattern was different from previously reported patterns A to Q from other LB-related *Borrelia* species (11, 13) and was designated

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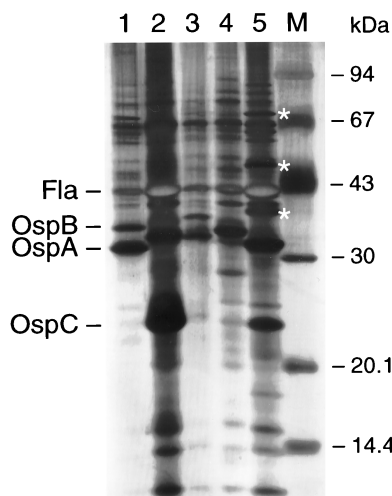


FIG. 1. Silver-stained SDS-PAGE gel of whole-cell lysates of *B. burgdorferi* sensu lato strains. Lanes 1 to 5, *B. burgdorferi* sensu stricto B31, *B. garinii* 20047, *B. afzelii* VS461, *B. valaisiana* AR-2, and *Borrelia* isolate A14S, respectively. The positions of flagellin (Fla), OspA, OspB, and OspC are indicated by arrows. The unique protein bands of isolate A14S are marked with asterisks. Molecular mass standards (Pharmacia) are shown on the right.

pattern R. Sequence analysis of the 5S-23S intergenic spacer DNA of isolate A14S showed only 91.6, 92.9, 92.4, and 93.3% identity to the type strains of *B. burgdorferi* sensu stricto B31, *B. garinii* 20047, *B. afzelii* VS461, and *B. bissettii* DN127, respectively. At 10 positions, nucleotides differing between all type strains of these *B. burgdorferi* sensu lato species and isolate A14S could be identified (Fig. 2).

For further evaluation of the phylogeny of *Borrelia* isolate A14S, the highly conserved 16S rRNA gene from this isolate was amplified by PCR with primer BRNA8 (5' ACGCTGGC AGTCGCTCTTA 3', positions 33 to 55, *B. burgdorferi* B31 numbering [3]) (22) and primer UniB (5' T[A/C]AAGGAGGTG ATCCAGC 3', positions 1540 to 1522) as described previously (22). The PCR amplicon was cloned directly into the PCR2.1 vector and transformed into *Escherichia coli* INV<sup>F</sup> cells by following the manufacturer's instructions (Invitrogen BV, Leek, The Netherlands). Subsequently, plasmid DNA from three recombinant colonies containing the amplified fragment was prepared by using a plasmid miniprep kit (Qiagen

GmbH, Hilden, Germany). DNA sequence was determined by the dideoxy chain-termination technique in an ABI 373A sequencer by using either the Dye terminator or the Dye primer cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) with additional custom-synthesized primers. A phylogenetic tree was constructed by using a neighbor-joining method with Kimura's two-parameter distance algorithm in the MEGA program (7).

A nearly complete (1,503-bp) 16S cDNA was amplified from isolate A14S by PCR. Sequence analysis showed that it possessed more than 98.5% similarity with the five LB *Borrelia* species occurring in Europe, *B. burgdorferi* sensu stricto (B31, 98.9%), *B. garinii* (20047, 99.2%), *B. afzelii* (DK1, 99.0%), *B. valaisiana* (VS116, 98.7%), and *B. lusitaniae* (99.1%), but less than 97% similarity with *B. japonica* (HO14, 95.5%), *B. andersonii* (19857, 96.6%), and *B. bissettii* (DN127, 96.3%), which have been isolated mainly in Japan (*B. japonica*) and North America (*B. andersonii* and *B. bissettii*). This finding suggested that *Borrelia* isolate A14S was closely related to the European *Borrelia* species. Only 13, 11, and 17 nucleotide substitutions in *Borrelia* isolate A14S were identified when its 16S rRNA gene was compared to the corresponding genes from *B. burgdorferi* sensu stricto (B31), *B. garinii* (20047), and *B. afzelii* (DK1), respectively. It is noteworthy that some of these nucleotide substitutions in the 16S rRNA gene of *Borrelia* isolate A14S occurred in the *Bfa*I restriction site, which would allow easy distinction of A14S from the three *Borrelia* species causing human LB (8). Like other newly described *Borrelia* species such as *B. lusitaniae* (8), *B. tanukii*, and *B. turdi* (5), isolate A14S constituted an independent branch in the phylogenetic tree based on its 16S rRNA gene sequence (Fig. 3), indicating that isolate A14S represents a new *Borrelia* genomic group in the *B. burgdorferi* sensu lato complex.

The *ospA* gene of *Borrelia* isolate A14S was amplified from genomic DNA by PCR with primer OspA1 (5' GGAGAATA TATTATGAAA 3', positions -12 to 6) and OspA2 (5' CTC CTTATTTTAAAGCG 3', positions 826 to 809) as described by Dykhuizen et al. (3). Five recombinant colonies were selected and sequenced. An open reading frame of 822 bp was identified from each of the recombinants. No sequence discrepancy was found among these colonies. The nucleotide sequence of the *ospA* gene of isolate A14S had 87.6, 88.0, 88.6, and 87.3% identity to the *ospA* sequences of *B. burgdorferi* sensu stricto B31, *B. garinii* PBI, *B. afzelii* VS461, and *B. bissettii* 25015, respectively. The protein in isolate A14S encoded by

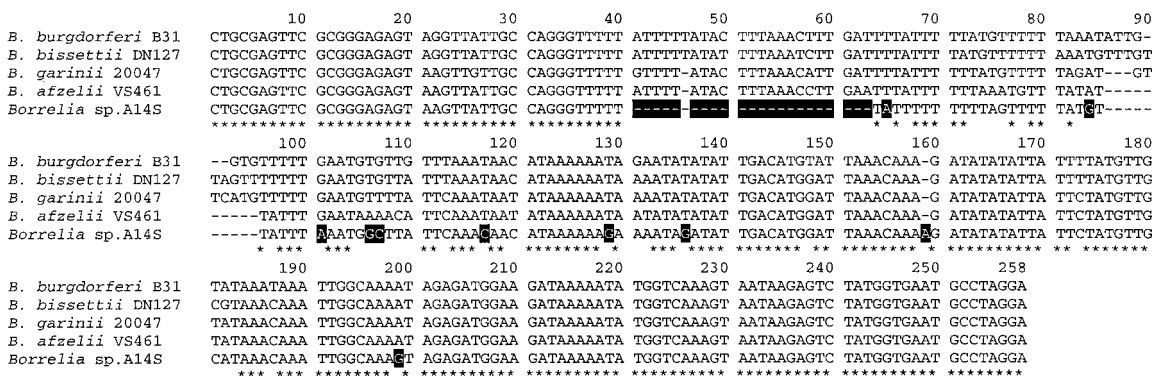


FIG. 2. Sequences of the 5S-23S rRNA intergenic spacers of *Borrelia* sp. strain A14S and the type strains of *B. burgdorferi* species known to be pathogenic to humans. Gaps were introduced to obtain maximum levels of homology. The nucleotides identical among all isolates are indicated with asterisks under the sequences, and the unique nucleotide positions of isolate A14S are shaded in black. The accession numbers of the 5S-23S rRNA intergenic spacer sequences of *Borrelia* strains used for comparison are L30127 (*B. burgdorferi* sensu stricto B31), L30119 (*B. garinii* 20047), L30135 (*B. afzelii* VS461), and L30126 (*B. bissettii* DN127).

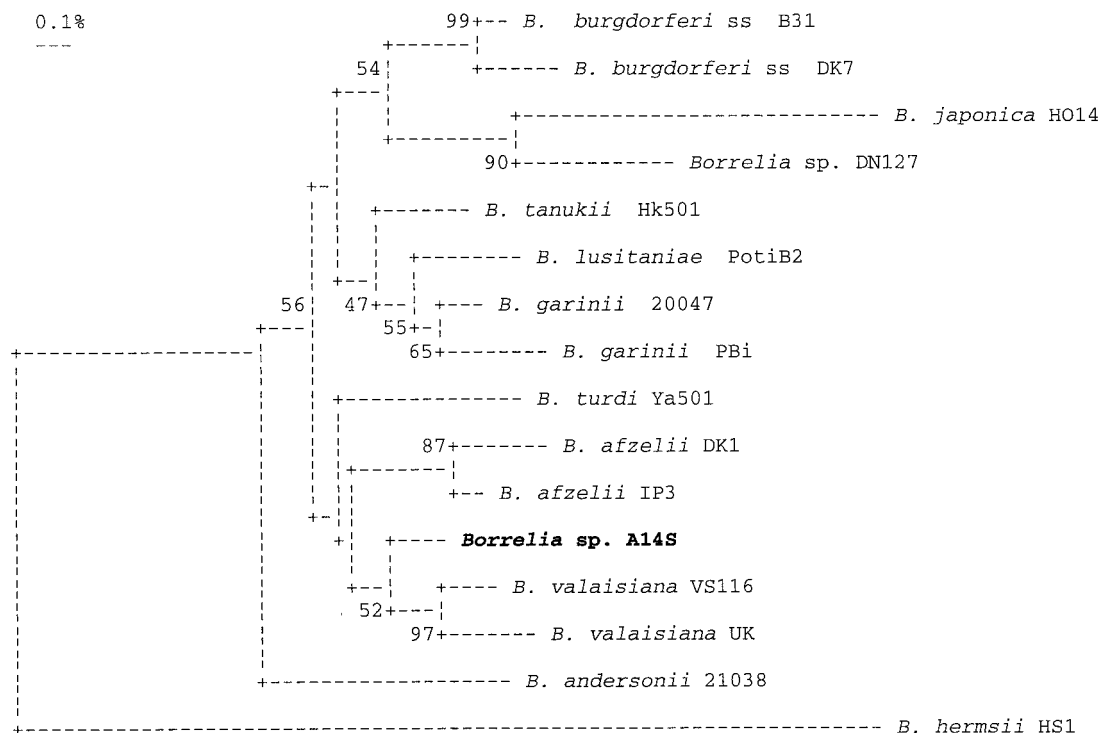


FIG. 3. Phylogenetic tree of *B. burgdorferi sensu lato* strains based on 1,330 bp of 16S rRNA gene sequences (positions 34 to 1364). *B. hermsii* HS1 was used as an out-of-group control. Numbers at each of the branch nodes indicate results from bootstrap analysis. The accession numbers (in parentheses) of the 16S rRNA gene sequences of *Borrelia* strains used for comparison are as follows: B31 (U03396), DK7 (X85195), 20047 (D67018), PBi (X85199), DK1 (X85190), IP3 (M84815), HO14 (L40597), VS116 (X98232), UK (X98233), PotiB2 (X98228), 21038 (L46701), DN127 (L40596), Hk501 (D67023), Ya501 (D67022), and HS1 (U42292).

this gene contained 273 amino acids and had a deduced molecular mass of 29.5 kDa. Phylogenetic analysis based on the deduced amino acid sequences of the OspA proteins of isolate A14S and isolates from other *B. burgdorferi sensu lato* species was well in accordance with results from 16S rRNA gene sequence analysis (data not shown). *Borrelia* isolate A14S constituted a separate branch in both phylogenetic trees. Each of the other clusters in these phylogenetic trees corresponded to one of the earlier-defined *Borrelia* species.

Previous studies showed that among *Borrelia* strains *ospC* is more polymorphic than *ospA* and could be divided into at least 35 different *ospC* RFLP patterns (9). Despite the high genetic heterogeneity of *ospC*, however, a species-specific motif has been identified in the deduced amino acid sequences of OspC from a number of *B. burgdorferi sensu lato* strains (4, 9). In this study, the *ospC* gene from *Borrelia* isolate A14S was amplified by PCR with primer OspC-N (5' CACAAATTAATGAAAA AGAATACA 3') and primer OspC-C (5' CCAGTTACTTTT TTAACAATAATTA 3'), which were predicted to yield an approximately 650-bp amplicon (9). Unexpectedly, a predominant large fragment with a size between 1.0 and 1.1 kb was obtained in our PCR, while a very weak band at 0.65 kb was also observed. Cloning and sequencing of the larger fragment confirmed that it consisted of a 633-bp complete *ospC* gene as well as a 403-bp flanking sequence at the 3' end. An 8-bp deletion occurring at the original primer binding site in the flanking sequence of *ospC* caused a mismatch of primer OspC-C with the sequence of the *ospC* gene of A14S. The nucleotide sequence of the *ospC* gene of isolate A14S showed 83.1, 83.3, 82.9, and 78.6% identity to the *ospC* sequences of *B. burgdorferi sensu stricto* B31, *B. garinii* 20047, *B. afzelii* VS461, and *B. bissettii* 25015, respectively. Digestion of the *ospC* gene

from *Borrelia* isolate A14S with *Dra*I yielded two fragments with sizes of 154 and 479 bp. This RFLP pattern, as well as the RFLP pattern after *Dpn*II digestion, differed from all of the *ospC* RFLP patterns from *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii* reported by Livey et al. (9).

The deduced translation product (OspC) from the 633-bp *ospC* gene of *Borrelia* isolate A14S contained 210 amino acids and had a molecular mass of 22.2 kDa. Its amino acid sequence from positions 23 to 34 of *Borrelia* isolate A14S differed from that of the species-specific motifs described for other *Borrelia* species or genomic groups. A nucleotide T-to-G conversion at position 90 of the *ospC* gene of isolate A14S would result in an amino acid change from an Asn to a Lys residue. Such a conversion has been reported previously for only one *B. garinii* isolate from Denmark (strain DK35) (19), but the difference in amino acids at positions 29 and 35 distinguishes the two strains.

It seems unlikely that the *Borrelia* isolate A14S is a mixture of multiple *Borrelia* species, since this isolate possessed some unique nucleotides in its 16S rRNA and *ospC* genes which were definitely different from those of *Borrelia* species in the *B. burgdorferi sensu lato* complex. It is also highly unlikely that the differences between isolate A14S and other *Borrelia* species are a result of in vitro passage. In vitro passage of *B. burgdorferi* can lead to plasmid loss, as well as changes in antigenic expression, and subsequently to differences in SDS-PAGE patterns and reactivities with MABs (15, 16). However, the 16S rRNA, *ospA*, and *ospC* genes, as well as the 5S-23S intergenic spacer sequences, are not subject to major alteration by in vitro passage. On the basis of our present study, as well as of findings obtained by ribotyping (20) and RAPD fingerprinting analysis (22), we conclude that *Borrelia* isolate A14S does not belong to *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii*, the three

*Borrelia* species pathogenic to humans. Based on the *ospA*, *ospC*, and 16S rRNA gene sequence analysis, 5S-23S rRNA intergenic spacer RFLP pattern (this study), and RAPD fingerprinting (22), isolate A14S also clearly differs from *B. bissettii*, the fourth genomic group recently shown to be able to cause human LB, as well as from other LB-associated *Borrelia* species. Therefore, this isolate most likely represents a new *Borrelia* genomic group, which is the fifth *Borrelia* genomic group with culture-confirmed pathogenic potential for causing human LB, besides *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. bissettii*.

**Nucleotide sequence accession numbers.** The 16S rRNA, *ospA*, *ospC*, and 5S-23S intergenic spacer sequences of isolate A14S that we determined in this study have been assigned GenBank accession no. AF102056, AF102057, AF102058, and U76616, respectively.

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