Genetic Analysis of *Borrelia garinii* OspA Serotype 4 Strains Associated with Neuroborreliosis: Evidence for Extensive Genetic Homogeneity

R. T. MARCONI,1 S. HOHENBERGER,2 S. JAURIS-HEIPKE,2 U. SCHULTE-SPECHTEL,2 C. P. LAVOIE,1 D. RÖSSLER,2 AND B. WILSKE2*

Medical College of Virginia at Virginia Commonwealth University, Richmond, Virginia 23298-0678,1 and Max von Pettenkofer Institut, Ludwig-Maximilians-Universität München, D-80336 Munich, Germany2

Received 27 May 1999/Returned for modification 22 July 1999/Accepted 19 August 1999

Infection with *Borrelia garinii* outer surface protein (Osp) A serotype 4 strains has been correlated with the development of neuroborreliosis in Lyme borreliosis patients in Europe. OspA serotype 4 isolates have been recovered primarily from human cerebrospinal fluid, suggesting a tropism for this environment. Previous studies with monoclonal antibodies directed against OspA and OspC demonstrated that OspA serotype 4 strains are antigenically closely related. In view of the pronounced antigenic and genetic variability that has been noted in the Osps of other *Borrelia* isolates, we sought to determine if OspA serotype 4 strains represent a recently emerged clonal lineage of *B. garinii*. Toward this goal, a representative group of OspA serotype 4 strains was analyzed for traits that typically exhibit hypervariability among isolates that cause Lyme borreliosis. The following criteria were assessed: (i) *ospC* sequences, (ii) plasmid composition, (iii) genomic restriction fragment length polymorphism (RFLP) patterns, and (iv) the RFLP patterns of the upstream homology box (UHB) element which flanks members of the UHB gene family at their 5′ end. Collectively, these analyses demonstrate genetic homogeneity, suggesting that OspA serotype 4 strains are a recently emerged clonal lineage with an apparent tropism for the central nervous system.

The genus *Borrelia* contains several spirochete species that are causative agents of important human diseases such as Lyme borreliosis and relapsing fever (for a review, see reference 7). Lyme borreliosis is caused by pathogenic species of the *Borrelia burgdorferi* sensu lato complex. While as many as nine species have been delineated in the *B. burgdorferi* sensu lato complex, disease in humans is attributed primarily to *B. burgdorferi*, *B. garinii*, and *B. afzelii* (2, 17, 18, 21, 23, 28, 29, 36, 37, 38, 51, 52, 57). Members of the genus exhibit several unique features which distinguish them from other eubacteria. One such feature is their genome, which is comprised of a linear chromosome and a variable series of linear and circular plasmids (6). Interestingly, the plasmids carry a large number of genes that appear to be completely unique to the genus *Borrelia* (15).

The proteins encoded by these *Borrelia*-specific genes likely play central roles in defining the unique aspects of *Borrelia* biology and pathogenesis. The plasmids also carry the majority of outer surface protein (Osp) genes including the *ospAB* operon, *ospC*, *ospD*, and the upstream homology box (UHB) gene family (5, 12, 32, 33, 35, 40). Like the plasmids that harbor them, the genes encoding surface-exposed proteins exhibit significant degrees of inter- and intraspecies variation (20, 30, 33, 48, 51, 57–59).

Analyses of phenotypic diversity of the Lyme disease spirochetes have centered primarily on the Osps, on which the focus of efforts to design *Borrelia* vaccines and diagnostic assays (43, 46). On the basis of variation in the Osps, isolates of the *B. burgdorferi* sensu lato complex can be antigenically subtyped (53, 56, 57). On the basis of immunoreactivity with monoclonal antibodies, seven OspA serotypes have been defined, and specific serotypes have been demonstrated to correlate with specific species of the *B. burgdorferi* sensu lato complex (57). *B. burgdorferi* strains are serotype 1, and *B. afzelii* strains are serotype 2. The OspA proteins of *B. garinii* are more antigenically diverse and are divided into five distinct OspA serotypes (serotypes 3 through 7). Another important Osp carried by species of the *B. burgdorferi* sensu lato complex is OspC (16). The *ospC* gene has been mapped to a highly stable 26-kb circular plasmid that is universally distributed among isolates (32, 40). The highly immunogenic OspC protein exhibits a high degree of inter- and intraspecies variation, with 16 distinct OspC types having been delineated (48, 56). OspE and OspF (22) are additional outer surface proteins that are genetically and antigenically diverse (34, 47). These immunogenic proteins are members of a large protein family called the UHB protein family (34, 47). UHB gene family members are flanked at their 5′ ends by a highly conserved upstream homology box (UHB) element and are carried by a series of closely related 32-kb circular plasmids (p32s) (12). A striking feature of this gene family is that it appears to be evolutionarily unstable and to undergo recombinational and mutation events at a high frequency during infection (25). It has been postulated that rearrangements in these genes may allow for the continual generation of new antigenic variants which could aid in evasion of the humoral immune response (34, 47).

Serotyping studies of isolates from Europe revealed a striking correlation between neuroborreliosis and infection with *B. garinii* OspA serotype 4 strains (16). Specifically, the majority of *B. garinii* isolates that have been recovered and cultured from cerebrospinal fluid from patients in Germany, The Netherlands, Denmark, and Slovenia are OspA serotype 4 (54, 55, 57). It is interesting that while strains of this serotype have been recovered from Lyme borreliosis patients, they have not been cultivated directly from ticks. Regarding the association between OspA serotype 4 strains, neuroborreliosis, and central...
nervous system invasion, it is possible that isolates of this serotype have a higher pathogenic potential than isolates of other OspA serotypes. Support for this comes from the observation that OspA serotype 4 strains exhibit a greater degree of resistance to serum than do other B. garinii OspA serotypes, which are serum sensitive (49).

To determine if OspA serotype 4 strains represent a genetically and antigenically homogeneous group of neurotropic organisms, we have conducted a comparative analysis of a representative group of strains. As a means for assessing homogeneity, we have characterized traits of B. burgdorferi sensu lato isolates that have been demonstrated to be highly variable among isolates. These analyses demonstrate that B. garinii OspA type-4 serotype strains are significantly more genetically homogeneous than any other defined group of isolates analyzed to date. The data suggest that B. garinii OspA serotype 4 strains are a recently emerged clonal lineage that appear to have a strong potential to disseminate and a higher tropism for the central nervous system than strains of other OspA serotypes.

**RESULTS AND DISCUSSION**

**Analysis of ospC genes in OspA type 4 strains.** To assess possible genetic variation among the ospC genes of B. garinii OspA serotype 4 strains, the genes were amplified with primers that target conserved segments of ospC. All isolates yielded ospC PCR amplicons that were approximately 650 bp in length, indicating size conservation among isolates. The ospC amplicons from B. garinii isolates PFfin, PBae, PFlik, PMue, PScf, PWa, and PHoe were sequenced in their entirety (data not shown), and surprisingly, all were identical at the nucleotide level. Comparative analyses were facilitated by the extensive number of ospC sequences in the databases. Alignment of the sequences determined here with the ospC sequences available in the databases revealed that they are nearly identical to the ospC genes of B. garinii PBI, PTrob, and DK6. These isolates are serotype 4 strains, and both PBl and DK6 were isolated from cerebrospinal fluid (55). The only difference among the sequences was in that of the ospC gene of B. garinii PBI, which has a single nucleotide difference from the other ospC gene sequences. This single point mutation results in the replacement of a Gln residue by a Glu residue. The observed high degree of ospC sequence identity among strains is extremely unusual since ospC genes typically exhibit a high degree of sequence variation (20, 48, 55). In addition, diversity among ospC sequences is further driven by the lateral transfer of the circular plasmid carrying the ospC gene (20, 24). However, the conservation of the ospC sequences suggests that lateral transfer of the ospC plasmid between OspA serotype 4 strains and other OspA serotypes has not occurred to any significant extent. This does not, however, rule out the possibility that transfer has occurred among OspA serotype 4 isolates.

**Plasmid and RFLP pattern analyses.** In recent years, the plasmid profiles of hundreds of B. burgdorferi sensu lato complex isolates have been determined and have been found to exhibit extensive variation. To compare the plasmid profiles of the OspA serotype 4 isolates, undigested genomic DNA was fractionated by pulsed-field gel electrophoresis (Fig. 1). Overall, the plasmid profiles were found to be strikingly similar. Previous studies of the plasmid profiles of Lyme disease spirochetes obtained from a defined geographic region have demonstrated that these patterns are rather variable (42). Three large plasmids of approximately 66, 57.5, and 50 kb were observed in each isolate. In addition, three to four plasmids between 20 and 30 kb were also noted. The similar plasmid profiles demonstrate an unusual degree of conservation of this component of the genome among B. garinii OspA serotype 4 strains.

To further assess the genetic relatedness among OspA serotype 4 strains, a variety of RFLP analyses were conducted. DNA in agarose plugs was digested with Apal, BssHII, MluI, or Smal. The digested DNA was then fractionated by pulsed-field gel electrophoresis. Although some minor variations in RFLP patterns were noted, the patterns exhibited high degrees of conservation among OspA serotype 4 strains, further demonstrating an unusual level of genetic homogeneity and sequence

**TABLE 1. B. garinii OspA serotype 4 strains analyzed in this study**

<table>
<thead>
<tr>
<th>B. garinii isolate</th>
<th>Biological source</th>
<th>Geographic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A01</td>
<td>Cerebrospinal fluid</td>
<td>The Netherlands</td>
</tr>
<tr>
<td>DK6</td>
<td>Cerebrospinal fluid</td>
<td>Denmark</td>
</tr>
<tr>
<td>PBaeII</td>
<td>Cerebrospinal fluid</td>
<td>Germany</td>
</tr>
<tr>
<td>PFin</td>
<td>Cerebrospinal fluid</td>
<td>Germany</td>
</tr>
<tr>
<td>PScf</td>
<td>Synovia</td>
<td>Germany</td>
</tr>
<tr>
<td>PWa</td>
<td>Cerebrospinal fluid</td>
<td>Slovenia</td>
</tr>
</tbody>
</table>

**Bacterial strains and DNA isolation.** The B. garinii OspA serotype 4 strains investigated in this study are described in Table 1. All strains except those specifically indicated in Table 1 were isolated at the Max von Pettenkofer-Institut, Munich, Germany. Strain AO1 was provided by A. van Dam, and strain DK6 was provided by K. Hansen. All bacterial isolates were cultivated in modified Kelly’s broth medium as described previously (59), and total genomic DNA was extracted as described previously (27).

**Amplification and sequence analysis of the ospC gene.** The ospC genes were amplified from isolated genomic DNA by PCR with the OspC1 (5′-GAG GGA AAT TGA AGG TAA ATT CTT GGT ATT G-3′) and OspC3 (5′-GAG CTG CAG TTA AGG TTT TTT TGG ACT TTC TGC) primers. Cycling conditions and PCR conditions and reagents were as described previously (20, 54, 55). The sequences that were determined were transcribed by the dideoxy chain termination method using a variety of oligonucleotide primers that target conserved segments of the ospC gene. The sequences that were determined were translated and were aligned with the OspC sequences of three B. garinii OspA serotype 4 strains published previously (strain PBl, EMBL accession no. X69595; strain PTrob, EMBL accession no. X63554; and strain DK6, EMBL accession no. X73624).

**Pulsed-field gel electrophoresis of borrelial DNA.** Borrelial strains were embedded in agarose plugs, and the DNA was released from the cells as described previously (9). To analyze the plasmid profiles, pulse times of 0.5 to 3.0 for 30 h were used. For restriction fragment length polymorphism (RFLP) analyses, the embedded bacterial DNA was digested with Apal, BssHII, MluI, and MboI under the conditions recommended by the supplier (Boehringer Mannheim). The products obtained from the Apal and MboI digests were separated for 30 h with pulse times of 1 to 20 and 1 to 30 s, respectively. The products obtained from the BssHII and MluI digestions were separated for 30 h with pulse times of 1 to 40 s.

**Hybridization analyses.** To assess the UHB RFLP patterns, the isolated DNA was digested to completion with HaeI, fractionated in a 0.8% GTG agarose gel, visualized with the VacuGene system (as described by the manufacturer; Pharmacia) onto a Hybond-N membrane (Amersham), and fixed to the membrane by UV cross-linking (BioRad Gene Linker). Prehybridization and hybridization were conducted in hybridization buffer consisting of 0.2% (wt/vol) bovine serum albumin, 0.2% (wt/vol) polyvinylpyrrolidone (molecular weight, 40,000), 50 mM Tris-HCl (pH 7.5), 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 10% (wt/vol) dextran sulfate, 100 μg of herring sperm DNA ml-1, and 1 M NaCl. The hybridization buffer consisted of [γ-32P]ATP (6,000 Ci mmol-1; DuPont-NEN) by using polynucleotide kinase under standard conditions. Hybridizations were conducted in a Hybird hybridization oven at 37°C overnight. Two 10-min washes with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS and a 1-h wash with 0.2× SSC-0.1% SDS were performed, followed by a final 5-min wash with 0.2× SSC-0.1% SDS at room temperature with vigorous shaking.

**Nucleotide sequence accession numbers.** The B. garinii ospC sequences determined in this study have been deposited in the EMBL database under the following accession numbers: AJ132793 through AJ132798 for isolates PBaeII, PWa, PHoe, PFfin, PFlik, and PMue, respectively, and AJ236907 and AJ236908 for isolates PScf and PSh, respectively.
conservation among the plasmids of these isolates. The results obtained for the BssHII digestion are shown in Fig. 2.

Hybridization analysis of UHB element. The UHB gene family is a large group of lipoprotein-encoding genes, all of which are flanked at their 5' end by a highly conserved, upstream, promoter-carrying sequence called the UHB element (34, 47). Although the sequence of the UHB element itself is highly conserved, previous analyses of the UHB-flanked genes have revealed that their coding sequences exhibit variability among isolates, and the RFLP patterns of the UHB element have been demonstrated to be hypervariable among isolates of the B. burgdorferi sensu lato complex even at the intraspecies level (34, 47). It has been hypothesized that the pronounced variation in the RFLP patterns of UHB elements among isolates has resulted from recent molecular rearrangements in the UHB-flanked genes and the plasmids that carry them (34, 47), and recent data suggest that immune pressures drive or select for rearrangements (25). To assess the degree of variability in the RFLP patterns of UHB elements of OspA serotype 4 strains, DNA isolated from each strain was digested with HaeIII, fractionated, and probed with the uhb(+) oligonucleotide. In contrast to that noted in other B. burgdorferi sensu lato complex isolates (34, 47), the RFLP patterns of the OspA serotype 4 strains differed from one another only in the number of hybridizing bands detected (from two to five) rather than in variations in the sizes of individual bands (Fig. 3). The nature of these differences is important because the observed different numbers of hybridizing bands suggest that differences in the RFLP patterns of UHB elements are due primarily to the loss of some UHB element-carrying plasmids among these isolates rather than to molecular rearrangement events. The absence of detectable recombination events in the UHB-flanked genes is consistent with the possibility that OspA serotype 4 strains represent a recently emerged clonal lineage.

Conclusions. It has been demonstrated that infection with particular species or subspecies (OspA types) of the B. burgdorferi sensu lato complex correlates with the development of certain clinical manifestations of Lyme borreliosis (1, 9, 10, 58, 60, 61). This has been demonstrated for the dermatological manifestation acrodermatitis chronica atrophicans, which is associated with B. afzelii. B. afzelii is OspA serotype 2. In contrast, the causative agents of neuroborreliosis and arthritis in Europe are heterogeneous, and the development of these manifestations cannot be attributed to infection with a specific species or OspA serotype. The diversity of the isolates associated with neuroborreliosis and arthritis is reflective of the diversity of isolates recovered from ticks in Europe. While OspA serotype 4 is not the only OspA serotype associated with
neuroborreliosis, it is striking that isolates of this serotype have been recovered almost exclusively from human cerebrospinal fluid (8, 13, 14, 50, 58, 60, 61). The goal of this study was to determine if OspA serotype 4 strains represent a recently emerged clonal lineage. If this is in fact the case, then one would expect genetic homogeneity in traits that have been widely demonstrated to exhibit variability among isolates. To assess this we analyzed features or components of the Borrelia genome that are known to be highly variable among isolates. These comparative analyses focused on four areas: (i) ospC gene sequences, (ii) plasmid profiles, (iii) RFLP patterns obtained with infrequently cutting enzymes, and (iv) the RFLP patterns of the UHB element. In summary, all four of these areas were found to be conserved among the OspA serotype 4 strains investigated in this study, suggesting that they represent a recently emerged clonal lineage.

Comparative analyses of the OspC amino acid sequences revealed sequence identities of greater than 99%. ospC sequence conservation of this extent has not been reported for any other OspA serotypes. In fact, comparative analyses of OspC amino acid sequences from isolates of different B. burgdorferi sensu lato species have revealed extensive divergence, with identity values dropping to as low as 62% (20). At the intraspecies level, OspC identities were found to be as low as 71, 76, and 68% in B. burgdorferi, B. afzelii, and B. garinii, respectively. When OspC sequences from isolates of OspA serotype 6 were analyzed, sequence identities were as low as 68% (20). Hence, the ospC sequences determined here provide a clear indication of the genetic homogeneity at this typically variable locus in OspA serotype 4 strains.

Pulsed-field gel electrophoresis fractionation of genomic DNA and analysis of the plasmid profiles revealed strong conservation of the plasmid composition among the OspA serotype 4 strains. As discussed above, although the plasmids are thought to be essential for survival, as inferred from their ubiquitous distribution among B. burgdorferi sensu lato complex isolates, it has been clearly demonstrated that plasmid composition can vary widely among B. burgdorferi sensu lato complex strains (3, 11, 26, 31, 42, 45). Mechanisms that contribute to plasmid heterogeneity include plasmid loss (35, 41), lateral transfer (20, 33), recombination (30, 33, 39, 47), and dimmer formation (19, 26). The presence of repeated sequences and gene families on plasmids may allow for interplasmid homologous recombination (4, 12, 34, 36, 44, 62).

To further analyze possible genetic homogeneity, RFLP patterns were assessed with the enzymes BsoHI, MluI, ApaI, and SmalI. The RFLP patterns exhibited an unusual degree of conservation, with only minor differences noted. This observation indicates that both the plasmids and the chromosomes of OspA serotype 4 strains have not undergone significant molecular rearrangement events. Similarly, analysis of the UHB-element RFLP patterns of the serotype 4 strains revealed that they are also highly conserved. Previous analyses of UHB-element RFLP patterns have demonstrated that they are variable even among closely related isolates of the same species (47). In the OspA serotype 4 strains, the differences in the UHB-element RFLP patterns were primarily due to the absence of some hybridizing bands from some isolates. This is in contrast to the case for other B. burgdorferi sensu lato isolates, which differ not only in the numbers of fragments but also in the sizes of the hybridizing fragments. Hence, it appears that in the OspA serotype 4 strains, differences in the UHB-element RFLP patterns are due to the loss of some of the UHB-element-carrying plasmids rather than to rearrangements among plasmids.

From the analyses presented here it can be concluded that OspA serotype 4 strains are a genetically homogeneous group of B. burgdorferi sensu lato complex strains. The observed genetic homogeneity indicates that this serotype is a recently emerged clonal variant of B. garinii. It is interesting that strains of this serotype have not been recovered from ticks but have been recovered only from humans. The basis for this observation is undefined and warrants further study. Furthermore, it remains to be determined whether this particular variant has established an apparent tropism for the central nervous system. Comparative analysis of type 4 strains with those of other OspA serotypes may allow the identification of specific factors that facilitate invasion of the central nervous system and persist in cerebrospinal fluid. Since serum resistance promotes dissemination and, therefore, by extension, may allow OspA serotype 4 strains to have greater pathogenic potential than strains of other OspA serotypes, comparative studies of the dissemination characteristics of OspA serotypes in ticks and mammals are warranted.

ACKNOWLEDGMENTS

R. T. Marconi and C. P. LaVoie are supported in part by grants from the Jeffress Trust and the National Institutes of Health.

REFERENCES


CLONALITY OF *B. GARINII* Ospa SEROTYPE 4


Marconi, R. T., and C. F. Garon.

Marconi, R. T., S. Casjens, U. G. Munderloh, and D. S. Samuels.

Marconi, R. T., and C. F. Garon.

Marconi, R. T., and C. F. Garon.

Marconi, R. T., R. T. Konkel, and C. F. Garon.

Marconi, R. T., D. S. Samuels, and C. F. Garon.


Marconi, R. T., S. Y. Sung, C. N. Hughes, and J. A. Carlyon.


