Borrelia burgdorferi Complement Regulator-Acquiring Surface Protein 2 (CspZ) as a Serological Marker of Human Lyme Disease

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Serological diagnosis of Lyme disease may be complicated by antigenic differences between infecting organisms and those used as test references. Accordingly, it would be helpful to include antigens whose sequences are well conserved by a broad range of Lyme disease spirochetes. In the present study, line blot analyses were performed using recombinant complement regulator-acquiring surface protein 2 (BbCRASP-2) from Borrelia burgdorferi sensu stricto strain B31 and serum samples from human Lyme disease patients from throughout the United States and Germany. The results indicated that a large proportion of the patients had produced antibodies recognizing recombinant BbCRASP-2. In addition, Lyme disease spirochetes isolated from across North America and Europe were found to contain genes encoding proteins with high degrees of similarity to the B. burgdorferi type strain B31 BbCRASP-2, consistent with the high percentage of serologically positive patients. These data indicate that BbCRASP-2 may be valuable for use in a widely effective serological assay.

Lyme disease (Lyme borreliosis) is the most prevalent tick-borne disease affecting humans in the United States, Europe, and a broad swath across Asia (18, 46, 62). Symptoms of human Lyme disease generally include nonspecific “flu-like” complaints, such as fever and body aches, and may or may not include problems such as skin rashes and lesions, arthritis, and neurological or cardiac difficulties (62). Genetic analyses of the causative agent, the spirochete Borrelia burgdorferi sensu lato, have divided that species into a number of genovars or genospecies, including B. burgdorferi sensu stricto, B. garinii, B. afzelii, B. spielmanii, B. bissetti, and others (7, 49, 52, 62, 77). Results of several studies have indicated associations between certain borrelial genospecies and particular Lyme disease symptoms (4, 6, 8, 37, 47, 55, 68, 71, 75, 77, 81). The range of symptoms that may be seen with each patient often complicates clinical diagnosis of Lyme disease. In addition, genetic variations among Lyme disease spirochetes pose difficulties for the development of widely useful serological tools for identifying infected individuals. For example, the outer surface protein OspC is produced by B. burgdorferi sensu lato during the initial stages of mammalian infection and is the target of a strong humoral immune response, but ospC gene sequences differ considerably among Lyme disease borreliae, even among bacteria within the same geographic location (36, 59, 64, 69, 78, 79). Identification of highly antigenic borrelial proteins whose sequences are well conserved across genospecies and geographic areas will be very important for development of improved serological tools for diagnosis of Lyme disease.

Our laboratories, and others, have been investigating mechanisms used by B. burgdorferi sensu lato to avoid killing by its hosts’ immune systems during vertebrate infection. Resistance to the alternative pathway of complement activation appears to be facilitated, in part, by binding host complement regulatory proteins, such as factor H, factor H-like protein 1 (FHL-1), and factor H-related proteins to the bacterial outer surface (3, 25, 32–34, 39, 80). Borrelial proteins that serve those functions have been designated CRASPs (complement regulator-acquiring surface proteins) (34). Different borrelial genospecies appear to produce different CRASPs, so the proteins of B. burgdorferi sensu stricto are often referred to as BbCRASPs and those of B. afzelii are called BaCRASPs, etc. (27, 34). Intriguingly, very few isolates of B. garinii have been found to produce CRASPs during laboratory cultivation, and so little is known about how that bacterium evades clearance by its vertebrate hosts (1, 34).

Three genetically distinct classes of BbCRASPs have been identified. BbCRASP-1, encoded by the cspa gene, is produced by B. burgdorferi sensu stricto only during tick-to-mammal and mammal-to-tick transmission stages (12, 15, 19, 28, 35, 72). Presumably as a consequence of the short duration of BbCRASP-1 expression during mammalian infection, humans
**TABLE 1. Borrelia spp. strains used in this work**

| Genospecies and strain | IGS type | Presence of cspZ | Original source | Reference(s) |
|------------------------|----------|------------------|----------------|--------------|
| B. burgdorferi sensu stricto strains | | | | |
| B31 | 1 | Yes | Tick, NY | 13, 14 |
| BL206 | 1 | Yes | Human, NY | 68, 75 |
| CA15 | ND | Yes | Tick, CA | 60 |
| LW2 | ND | Yes | Human, Germany | 73 |
| ZS7 | 3 | Yes | Tick, Germany | 74 |
| 2307/5 | ND | Yes | Tick, Italy | 30 |
| Z25 | ND | Yes | Tick, Germany | 30 |
| JD1 | 5 | Yes | Tick, MA | 48, 68 |
| 93-0117 | ND | Yes | Human, WI | 41 |
| N40 | 9 | Yes/no | Tick, NY | 10, 13 |
| 124a | ND | Yes | Human, NY | 41 |
| 156a | 2 | Yes | Human, United States | 51 |
| 297 | 2 | Yes | Human, NY | 13, 63 |
| Sh-2-82 | 2 | Yes | Tick, NY | 58 |
| B. garinii strains | | | | |
| PB1 | Yes | Human, Germany | 79 |
| Ip89 | Yes | Tick, Russia | 82 |
| B. spielmanii strains | | | | |
| A145 | Yes | Human, The Netherlands | 76 |
| PC-Eq17 | No | Tick, France | 52 |
| B. bissetti strain | | | | |
| DN127 | Yes | Tick, CA | 11 |
| B. afzelii strains | | | | |
| PKo | No | Human, Germany | 50 |
| ACA-1 | No | Human, Sweden | 5 |

* B. burgdorferi sensu stricto intergenic spacer (IGS) type, as determined by sequence analysis of the 16S-23S rRNA gene spacer region (13).
* ND, not determined.
* Two cultures of B. burgdorferi sensu stricto strain N40 were examined. One contained cspZ, and the other lacked both cspZ and an lp28-3-related plasmid.

**TABLE 2. Oligonucleotides used in this study**

| Oligonucleotide name | Sequence (5’ to 3’) |
|----------------------|---------------------|
| CSPZ-1 | GTA GCA ATA TAC TTC TGG TAG AGG |
| CSPZ-2 | TCT TTG ATG ATT GTC CAT AAG |
| CSPZ-3 | TAA ATA AAG TTT TCT CCT TAA |
| CSPZ-4 | AGA AAT ATT RAG AGG |
| CSPZ-5 | GCT TGT AAA TCT GCC ATT AAG |
| CSPZ-6 | TCT TGT GTA TTA GCC TAC AAG AG |

* R indicates either pure urine at this position.

**MATERIALS AND METHODS**

*Bacteria.* Strains of *B. burgdorferi* sensu lato used in these studies are listed in Table 1. Borrelial strains were grown at 33°C to cell densities of approximately $1 \times 10^8$ in either modified Barbour-Stoenner-Kelly medium or Barbour-Stoenner-Kelly-H complete medium (Sigma-Aldrich, St. Louis, MO) as described previously (21, 31, 83).

*Serological analyses.* Preexisting human Lyme disease and control serum samples were kindly provided by Martin Schröfer (U.S. Centers for Disease Control and Prevention, Fort Collins, CO), Gary Wormser (New York Medical College, Valhalla, NY), the University Hospital of Frankfurt, or the blood bank of the blood bank of Frankfurt, Germany. Human control samples consisted of sera obtained from healthy blood donors ($n = 10$) and from patients with active or recent primary syphilis ($n = 10$), leptospirosis ($n = 15$), human immunodeficiency virus (HIV; $n = 15$), ankylosing spondylitis ($n = 15$), and rheumatoid arthritis ($n = 15$). Serum samples were from clinically diagnosed patients and have been used as positive controls.

**Recombinant BbCRASP-2 protein** was produced based on the sequence of *B. burgdorferi* sensu stricto strain B31 (24). For line blotting, recombinant *B. burgdorferi* was transferred to nitrocellulose membranes by a microdispensing method in amounts of $32, 16, 8, 4, 2, 1$, or 0.1 ng per stripe. Individual membranes were incubated with human sera ($r a t e d$ at 1:100 and U.S. sera at 1:200) or mouse sera (1:100). Serum dilutions were based upon serological studies with other borrelial antigens (see above). Binding of specific antibodies was detected by using alkaline phosphatase-conjugated goat anti-human IgG serum (1:100; Sigma, St. Louis, MO) as detection antibody. The detection of specific antibodies was detected by using alkaline phosphatase-conjugated goat anti-human IgG serum (1:100; Sigma, St. Louis, MO) as detection antibody.

**cspZ sequencing.** The *cspZ* gene sequence of *B. burgdorferi* sensu stricto type strain B31 (open reading frame BBH006) was previously determined as part of the genome sequencing project for that bacterium (16, 21). The *cspZ* genes from *B. burgdorferi* sensu stricto strains BL206, 93-0117, CA15, LW2, 2307/5, ZSS, N40, 124a, 297, and Sh-2-82 were amplified from total purified bacterial plasmids using oligonucleotides CSPZ-1 and CSPZ-2 (Table 2). Sequences of those two *cspZ* genes were also determined for a variety of borrelial strains of different genospecies. Our results indicate that *cspZ* sequences are well conserved among Lyme disease borreliae independently of their geographic distribution and that antibodies recognizing BbCRASP-2 are frequently produced by humans with Lyme disease.
of those bacteria. *B. afzelii* strains were examined for the presence of *cspZ* loci by BLAST-P and BLAST-N analyses of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and searches of the published partial genome of *B. afzelii* strain Pko (23) and of the completed, unpublished whole-genome sequences of *B. afzelii* strains Pko and ACA (S. Casjens, unpublished data). The *B. garinii* strain Pbi *cspZ* gene sequence was identified in GenBank by BLAST-P analysis using the strain B31 *BbCRASP-2* protein sequence as the query. The strain Pbi *cspZ* gene is within the deposited DNA segment GI1a4af6.fr1 (accession number AY722031), an unassembled plasmid fragment of strain Pbi that was sequenced during a partial sequence analysis of that strain (22).

DNA sequences were analyzed using the DNA Strider 1.4f8 (emarck@cea.fr) or DNAStar Lasergene 99 (Madison, WI) program. Predicted amino acid sequences were compared and aligned using Clustal X (70) with default parameter settings.

**Nucleotide sequence accession numbers.** The sequences of the *cspZ* loci from *B. burgdorferi* sensu stricto strains BL206, CA15, LW2, 2307/5, Z25, 93-0117, N40, 124a, 297, and Sh-2-82 have been assigned accession numbers EU272843 through EU272852, respectively. The *cspZ* locus of *B. garinii* strain Ip89 has accession number EU272853, and that of *B. spielmanii* strain A14S has accession number U272854.

(Parts of this work form parts of the M.D. thesis of Annekatrin Seling.)

## RESULTS

**Serological examination of human Lyme disease patient sera.** Our earlier investigations, using the mouse model of Lyme disease, discovered that *B. burgdorferi* produced elevated levels of *cspZ* transcript within 2 weeks of infecting mammals and that mice seroconverted within 4 weeks of infection (the earliest time points examined) (15, 24). Those data suggested that human patients are probably also exposed to *BbCRASP-2* during the early stages of Lyme disease. To evaluate that hypothesis and to explore the potential usefulness of *BbCRASP-2* as a serodiagnostic antigen, we examined serum samples from patients diagnosed with Lyme disease in Germany and from several locations across the United States. Patients had exhibited a range of symptoms, including erythema migrans, arthritis, acrodermatitis chronica atrophicans (ACA), and neuroborreliosis. As noted above, several of those Lyme disease manifestations are associated with specific *Borrelia* genospecies (4, 6, 8, 57, 47, 55, 68, 71, 75, 77, 81). In this manner, we sought to examine patients likely to have been infected with a range of different borrelial genospecies and varieties within those species. Moreover, sera were collected from some patients within 1 day of symptom appearance, allowing us to examine whether or not humans seroconvert during early stages of Lyme disease.

The human serum samples were analyzed by immunoblotting with nitrocellulose strips containing various amounts of recombinant *B. burgdorferi* strain B31 *BbCRASP-2*. The use of different amounts of *BbCRASP-2* allowed optimization of both sensitivity and specificity. All examined patients with erythema migrans, ACA, or Lyme arthritis yielded immunoblot signals when 16 ng recombinant *BbCRASP-2* was examined, and many patient samples provided strong signals from much less test antigen (Fig. 1A to F; Table 3). Sera from 80% of tested neuroborreliosis patients also gave a positive signal against 16 ng *BbCRASP-2*. Patients from both the United States and Germany produced *BbCRASP-2*-binding antibodies. Consistent with the observed early expression of *BbCRASP-2* during murine infection (15), strong immunoblot signals were obtained with sera taken from patients within 1 to 3 days of the appearance of symptoms (Fig. 1A). Mice experimentally infected with *B. burgdorferi* sensu stricto strain B31 via tick bite all produced high levels of *BbCRASP-2*-directed antibodies (Fig. 1G), while uninfected mice did not contain detectable levels of such antibodies (data not shown) (24).

Serum samples from control patients, which consisted of healthy blood donors and patients with either syphilis, leptospirosis, HIV, antinuclear antibodies, or rheumatoid arthritis, were almost uniformly negative for the presence of antibodies that bound *BbCRASP-2* (Fig. 1H to M; Table 3). Samples from one syphilis patient and one blood donor who were otherwise seronegative for exposure to Lyme disease contained antibodies that recognized some higher concentrations of *BbCRASP-2* (Fig. 1H and M; Table 3). These may represent undiagnosed prior exposure to Lyme disease spirochetes or cross-reacting antibodies directed against *Treponema pallidum* or other sources.

**Conservation of *cspZ* sequences among Lyme disease borreliae.** Earlier work from our laboratories identified *cspZ* of *B. burgdorferi* sensu stricto strain B31 as encoding *BbCRASP-2* (24). Pathogenic strains of *B. afzelii* and *B. spielmanii* also produce similarly sized factor H-binding proteins, known as BaCRASP-2 and BsCRASP-2, respectively, although the gene encoding those proteins had not previously been identified (27, 34). In the serological studies described above, it is probable that some of the examined patients were infected with borreliae other than *B. burgdorferi* sensu stricto, especially the European patients, who are likely to become exposed to other infectious genospecies, such as *B. garinii* (7, 77). The preponderance of human Lyme disease patients who produced antibodies recognizing *BbCRASP-2* suggested a high degree of *cspZ* sequence conservation among strains and genospecies of Lyme disease spirochetes. To assess that possibility, bacteria isolated from human and tick sources across North America and Eurasia were analyzed for *cspZ*, and gene sequences were determined. Examined borreliae included members of all the major species known to be infectious to humans.

The *B. burgdorferi* sensu stricto type strain B31 has been completely sequenced and carries its *cspZ* gene on a plasmid, lp28-3 (16, 21, 24). Genome sequencing of several additional Lyme disease borreliae is in the final stages of closure, and examination of those sequences reveals that the *cspZ* locus of those strains is also located on a plasmid related to lp28-3 (S. Casjens, unpublished results). An unavoidable difficulty with analyzing plasmidic bacterial strain does not necessarily mean that its wild parent lacked the gene.

Through a combination of whole-genome sequencing and PCR using oligonucleotide primer pairs based on DNA sequences flanking or within the *cspZ* open reading frame of *B. burgdorferi* sensu stricto strain B31, we identified *cspZ* genes in the majority of examined strains of *B. burgdorferi* sensu stricto, as well as in *B. spielmanii* and *B. bissetti* (Table 1). Unexpectedly, examination of a *B. garinii* strain, Ip89, revealed that it too contains a *cspZ* locus. BLAST analyses of GenBank unearthed the sequence of an unannotated DNA fragment of *B. garinii* strain PBI that contains a *cspZ* gene similar to that of strain Ip89. None of the *B. afzelii* strains we examined carried a *cspZ* locus. However, while the manuscript was being prepared, another research group reported detection of *cspZ* genes in two of four tested *B. afzelii* strains (53). Thus, all the major genospecies associated with human Lyme disease have been found to contain *cspZ* genes. Alignment of the predicted
protein sequences of these cspZ genes revealed extensive similarities throughout the proteins (Fig. 2). High levels of protein identities were evident both within regions determined to be involved with binding factor H and/or FHll-1 and within regions that probably serve more structural roles. Thus, cspZ-encoded proteins are predicted to exhibit conservation of both primary amino acid sequences and higher-order structural elements. These similarities suggest that antibodies targeting both linear and structural epitopes will bind cspZ-encoded proteins from other Lyme disease spirochetes, consistent with our serological studies described above (Fig. 1; Table 3).

**DISCUSSION**

The present study revealed that a large proportion of tested human Lyme disease patients produce antibodies that recog-
recognize the *B. burgdorferi* sensu stricto strain B31 BbCRASP-2 factor H/FHL-1-binding protein. Analyses of a wide variety of geographically dispersed isolates of Lyme disease *Borrelia* species revealed very high degrees of *cspZ* sequence conservation. The examined strains included two cultures of borreliae isolated from human Lyme disease patients who had produced detectable levels of antibodies recognizing the B31 BbCRASP-2 protein (Fig. 1A and B). Serum samples drawn from patients as early as the day of diagnosis contained detectable levels of antibodies that bound BbCRASP-2. Thus, we conclude that, early in human infection, a large proportion of Lyme disease patients mount a detectable humoral immune response to the *cspZ*-encoded protein produced by the infecting spirochetes. That antigenicity, plus the high degrees of similarities among *cspZ* genes between genetically and geographically diverse bacteria, suggests that BbCRASP-2, or a portion of that protein, may be a useful antigen for serodiagnosis of Lyme disease.

Some of the serum samples examined from syphilis patients...
or control blood donors contained antibodies that bound recombinant BbCRASP-2. This suggests that *T. pallidum* and other organisms may produce proteins having sequence or conformational similarities with BbCRASP-2. Studies are under way to extend our studies using various fragments of BbCRASP-2, to eliminate possible cross-reactive epitopes and increase the specificity of this serological test.

The strong humoral immune responses of infected humans and mice against cspZ-encoded CRASPs are somewhat paradoxical, given the high levels of cspZ expression during mammalian infection (15). Some other antigenic borrelial outer surface proteins are also produced at high levels during persistent infection, including the factor H-binding Erp proteins (44, 45, 65). It remains a mystery how Lyme disease spirochetes persistently infect humans and other mammals despite their production of antibodies against highly expressed borrelial outer surface proteins such as CRASP-2. Possibilities include characteristics of those proteins such that they preferentially elicit production of nonneutralizing antibodies, the adherence of host factors (such as factor H and FHL-1) blocking recognition by antibodies during infection, or bacterial inhabitation of host tissues that are privileged from the humoral immune system.

An unexpected finding from these studies was the discovery that bacteria of the genospecies *B. garinii* contain cspZ genes, encoding proteins that are highly similar to BbCRASP-2. This result was unexpected, in that no examined strain of *B. garinii* produces a factor H/FHL-1-binding protein of this size when grown in culture (1, 3, 24, 31, 32, 39). This may indicate that *B. garinii* cspZ-encoded proteins unable to bind human factor H, as recently suggested by Rogers and Marconi (53). Differences among regions known to be involved in factor H/FHL-1-binding support that hypothesis (Fig. 2) (24). As a second possibility, *B. garinii* and *B. burgdorferi* sensu stricto may control cspZ expression differently, such that *B. burgdorferi* sensu stricto produces its protein during cultivation in artificial laboratory media, while *B. garinii* does not. It has been demonstrated that *B. burgdorferi* sensu stricto significantly represses cspZ transcription during laboratory cultivation, compared to expression levels during mammalian infection (15). Further supporting that hypothesis, the 5′ noncoding regions of *B. burgdorferi* cspZ loci share little homology with those of *B. burgdorferi* sensu stricto (data not shown) and may therefore be transcriptionally regulated through distinct mechanisms. Studies are ongoing in our laboratories to evaluate each of these hypotheses.

In conclusion, a majority of tested human Lyme disease patients produced antibodies recognizing BbCRASP-2, while only a small portion of negative control serum samples contained reactive antibodies. Consistent with those serological data, cspZ gene sequences were found to be highly conserved across a broad range of Lyme disease spirochete genospecies. These results demonstrate that Lyme disease borreliae produce cspZ-encoded proteins during human infection and suggest that BbCRASP-2 has potential for use in sensitive and specific serological diagnosis of Lyme disease.

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