A Novel Surface Antigen of Relapsing Fever Spirochetes Can Discriminate between Relapsing Fever and Lyme Borreliosis

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In a previous immunoproteome analysis of Borrelia hermsii, candidate antigens that bound IgM antibodies from mice and patients infected with relapsing fever spirochetes were identified. One candidate that was identified is a hypothetical protein with a molecular mass of 57 kDa that we have designated Borrelia immunogenic protein A (BipA). This protein was further investigated as a potential diagnostic antigen for B. hermsii given that it is absent from the Borrelia burgdorferi genome. The bipA locus was amplified and sequenced from 39 isolates of B. hermsii that had been acquired from western North America. bipA was also expressed as a recombinant fusion protein. Serum samples from mice and patients infected with B. hermsii or B. burgdorferi were used to confirm the immunogenicity of the recombinant protein in patients infected with relapsing fever spirochetes. Lastly, in silico and experimental analysis indicated that BipA is a surface-exposed lipoprotein in B. hermsii. These findings enhance the capabilities of diagnosing infection with relapsing fever spirochetes.

The first outbreak of tick-borne relapsing fever in the United States was observed in Colorado in 1915 (39). Since then this disease continues to be under-reported and misdiagnosed, while regions where the disease is endemic continue to be identified in the Western and Southern United States (20, 51, 53). The three causative agents of relapsing fever in the United States are Borrelia hermsii, Borrelia turicatae, and Borrelia parkeri; the Borrelia spirochetes are maintained in enzoonotic cycles with their respective tick vectors Ornithodoros hermsi, Ornithodoros turicatae, and Ornithodoros parkeri (17, 22). Most reported cases of this disease and all human isolates collected in the United States are due to B. hermsii. Although B. parkeri and B. turicatae have not been isolated from a human, B. turicatae has been implicated in several outbreaks in Texas because of the association of its tick vector with an area where an outbreak occurred (46). B. turicatae has also been isolated from sick dogs (52, 58), further supporting the likelihood of infection to humans.

The spirochetemia during mammalian infection is cyclic with spirochetes escaping the immune response by genetic variation of surface proteins known as variable major proteins (Vmps) (5, 8, 16, 25, 47, 56). During episodes of spirochetemia, the bacteria can reach upwards of $10^7$ spirochetes per ml of blood (13, 15). However, spirochetes are cleared by IgM antibody primarily generated against these Vmps (1–5, 7, 10, 40, 56).

Given the ability to reach high spirochete densities in the blood (13, 15), infection can be diagnosed by microscopy. However, microscopic observation lacks sensitivity if spirochete densities are $<10^4$ spirochetes per ml of blood, for instance, when the examination is performed between high spirochetemic episodes (20). Further complicating diagnostic testing in the United States is the presence of B. burgdorferi in areas where relapsing fever spirochetes occur (19–21, 26, 32, 42, 49, 54, 59), with reports of serological cross-reactivity from patients infected with B. burgdorferi to other Borrelia species (35–38, 45).

Previously, a proteomic analysis identified 31 candidate antigens that reacted with IgM from mice and patients infected with B. hermsii (33). Searching GenBank using the basic local alignment search tool (BLAST) indicated that one candidate antigen with a molecular mass of 57 kDa (GenBank accession no. FJ446703) is unique to relapsing fever spirochetes; this has been designated Borrelia immunogenic protein A (BipA). The locus of this antigen was amplified and sequenced from 39 isolates to confirm the presence of bipA in B. hermsii isolates and to determine whether bipA segregated B. hermsii isolates into genomic group I (GGI) or genomic group II (GGII) isolates as previously reported for other loci (43, 52). Also, this gene was expressed in Escherichia coli as a recombinant fusion protein, and its antigenicity was determined using serum samples from mice and patients infected with B. burgdorferi or B. hermsii. Serum generated against this recombinant protein was tested with lysates from Borrelia species and GGI and GGII isolates of B. hermsii. Lastly, in silico and experimental analysis of the protein was performed to determine the surface local-
ization of this protein. These findings identify a new surface protein of B. hermsii that can aid in serodiagnosing infection by these spirochetes.

**MATERIALS AND METHODS**

*Borrelia* spirochetes. We used 23 GGI and 16 GGII isolates of *B. hermsii* in the present study; the origins of the isolates have previously been described (43, 54). Also, *B. turicatae* 9E135, *B. parkeri* RML, *Borrelia crocidurae* CR2A, *Borrelia recurrentis* 132, and *B. burgdorferi* B31 were used in the present study. Relapsing fever spirochetes were maintained in mBSK medium containing 12% autolyzate (34, 52).

BipA to amplify *bipA* as previously described (34).

Producing recombinant BipA (rBipA). The forward and reverse primers used to amplify *bipA* from *B. hermsii* DAH genomic DNA were 5′-CACCATGGATGTGGGAGTAAGAGAACCTCTTAGGAAAAGAATCC-3′ and 5′-GCTGGATTTGACGCCGCTATGCCTACGAGGACGTCGAC-3′, respectively.

**TABLE 1**. Primers used for amplifying, sequencing, and generating the Southern blot probe for *bipA*

| Primer            | Sequence (5′→3′)                          | Function                                      |
|-------------------|-------------------------------------------|-----------------------------------------------|
| FJ446703-R        | AGGACCTACCTCTAAGGCTTGG                   | Primer flanking *bipA* that was used to amplify the gene |
| FJ446703-F        | GGAATTTGATAGGAGTGG                      | Primer flanking *bipA* that was used to amplify the gene |
| FJ446703-R1       | GTCGGAAGCTCTCTTGGTTGG                   | Reverse internal sequencing primer             |
| FJ446703-R2       | CATTAGCAATCCATACAAGCAAC                 | Reverse internal sequencing primer             |
| FJ446703-R3       | CTCCACCGACGATCTAACTCC                   | Reverse internal sequencing primer             |
| FJ446703-F1       | GAGTTTCTGTTGTGTTCTGG                    | Forward internal sequencing primer             |
| FJ446703-F2       | ACTTTCGTACCGGCTACGCGGTCC               | Forward internal sequencing primer             |
| FJ446703-F3       | TGGAGATGATGATACTCTGGGTG                 | Primer used for generating the Southern blot probe |
| FJ446703-R4       | TCTCTATACAGCTACAACAGATATCATCA          | Primer used for generating the Southern blot probe |
| FJ446703-F4       | TTTAGGCAGTAAATAGAGAATTGACGGC           | Primer used for generating the Southern blot probe |

produced as previously described (34, 52).

The forward and reverse primers used to amplify *bipA* from *B. hermsii* DAH genomic DNA were 5′-CACCATGGATGTGGGAGTAAGAGAACCTCTTAGGAAAAGAATCC-3′ and 5′-GCTGGATTTGACGCCGCTATGCCTACGAGGACGTCGAC-3′, respectively. PCR amplification was performed by using a DNA Engine Tetrad (Bio-Rad) consisting of 1 cycle at 96°C for 5 min and 35 cycles with a denaturing temperature of 94°C for 30 s, an annealing temperature of 58°C for 30 s, and an extension temperature of 72°C for 2.5 min. Amplicons were cloned into the pET102/Directional TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, and correct orientation within the vector and nucleotide sequence were confirmed by using the Lasergene 8 software package (DNASTAR, Madison, WI). Chemically competent *E. coli* BL21 Star (DE3) cells (Invitrogen) were transformed with 10 ng of purified vector containing *bipA* according to the manufacturer’s instructions (Invitrogen). *E. coli* BL21 Star (DE3) cells were induced with a final concentration of 1 mM IPTG (isopropyl-1-thio-galactopyranoside) to produce rBipA. The recombinant-fusion protein was expected to be ~72 kDa and was purified by a His-Bind Quick Column (EMD, Gibbstown, NJ) following the manufacturer’s instructions.

Producing antisera to rBipA. Three Rocky Mountain Laboratory (RML) mice (a closed colony of Swiss-Webster mice used at the RML since 1937) were immunized twice intraperitoneally with 4 weeks between immunizations with 50 µg of rBipA that was excised from an acrylamide gel. Serum was collected prior to immunizations and 2 weeks after each immunization and used for reactivity with the 57-kDa protein in *B. hermsii* lysates and rBipA as described below. All animal handling was in compliance with the Rocky Mountain Laboratories Animal Care and Use Committee.

**RESULTS**

Plasmid mapping and sequence analysis of *bipA* from GGI and GGII isolates of *B. hermsii*. Originally BipA was identified by mass spectrometry in *B. hermsii* DAH as a 57-kDa protein with an unknown function (33). Searching the *B. hermsii* DAH chromosome (GenBank accession no. CP000048) and partially

Producing recombinant BipA (rBipA). The forward and reverse primers used to amplify *bipA* from *B. hermsii* DAH genomic DNA were 5′-CACCATGGATGTGGGAGTAAGAGAACCTCTTAGGAAAAGAATCC-3′ and 5′-GCTGGATTTGACGCCGCTATGCCTACGAGGACGTCGAC-3′, respectively. PCR amplification was performed by using a DNA Engine Tetrad (Bio-Rad) consisting of 1 cycle at 96°C for 5 min and 35 cycles with a denaturing temperature of 94°C for 30 s, an annealing temperature of 58°C for 30 s, and an extension temperature of 72°C for 2.5 min. Amplicons were cloned into the pET102/Directional TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, and correct orientation within the vector and nucleotide sequence were confirmed by using the Lasergene 8 software package (DNASTAR, Madison, WI). Chemically competent *E. coli* BL21 Star (DE3) cells (Invitrogen) were transformed with 10 ng of purified vector containing *bipA* according to the manufacturer’s instructions (Invitrogen). *E. coli* BL21 Star (DE3) cells were induced with a final concentration of 1 mM IPTG (isopropyl-1-thio-galactopyranoside) to produce rBipA. The recombinant-fusion protein was expected to be ~72 kDa and was purified by a His-Bind Quick Column (EMD, Gibbstown, NJ) following the manufacturer’s instructions.

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**SDS-PAGE** and immunoblotting. SDS-PAGE and immunoblotting were performed as described previously (50). Whole-cell lysates of uninduced or induced *E. coli* BL21 Star (DE3) cells, several *Borrelia* species, GGI and GGII isolates of *B. hermsii*, or 1 µg of purified rBipA were electrophoresed on 12% Tris-glycine gels (Invitrogen) and transferred onto nitrocellulose membranes by using the iBlot dry blotting system (Invitrogen). Membranes were probed with anti-poly-histidine peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:2,000, pooled murine preimmunization or preinfection sera at a dilution of 1:200, pooled sera from mice immunized with rBipA at a dilution of 1:200, or human serum samples at a dilution of 1:200. To determine titers to rBipA, serum samples from mice and patients infected with *B. hermsii* were diluted 5-fold starting at a dilution of 1:500. The secondary molecule used in immunoblotting was HRP-rec-protein A (Invitrogen) at 1:4,000. Immunoblots were performed three times, and images were captured by using the GelDoc imager (Bio-Rad).

**Proteinase K treatment of *B. hermsii*.** *B. hermsii* DAH was grown to 10^8 spirochetes per ml and treated with proteinase K as described previously (55). A final concentration of 5 mg of phenylmethylsulfonyl fluoride/ml in isopropanol was added to spirochetes immediately after incubating with proteinase K or after 30-, 60-, 90-, and 120-min incubations. Samples were electrophoresed and transferred to nitrocellulose membranes, and membranes were probed with pooled mouse immune sera generated to rBipA as described above. Assays were performed three times.

**Nucleotide sequence accession numbers.** Nucleotide sequences of *bipA* from GGI and GGII isolates of *B. hermsii* have been deposited in the GenBank database under accession numbers GU695876 to GU698524 and GU270942 for *B. turicatae*.
assembled plasmid sequences indicated that *bipA* was plasmid encoded. Also, Southern blotting of nine GGI and seven GGII isolates mapped *bipA* to the 200-kb linear plasmid (data not shown).

Amplifying the *bipA* locus from 23 GGI and 16 GGII isolates produced a product of the expected size (data not shown), indicating the probable presence of this gene in all isolates of *B. hermsii* tested. DNA sequencing of these amplicons confirmed the presence of the gene, and a phylogenetic tree differentiated *bipA* isolates into the two genomic groups (Fig. 1). These findings are consistent with most loci that have been sequenced in *B. hermsii* (34, 43, 53).

Phylogenetic analysis (Fig. 1) and determining the nucleotide identity of *bipA* between isolates (see Fig. S1 in the supplemental material) identified identical sequences from isolates with diverse geographical locations, an observation consistent with other genetic analyses of *B. hermsii* (43, 53). For example, CON, a GGI isolate originating from the Sierra Nevada Mountains in California, grouped more closely with HS1, MCN, CAR, and BYM isolates originating from Washington State and Idaho (Fig. 1 and Fig. S1 in the supplemental material). In addition, MAT and GAR had identical *bipA* sequences (Fig. 1 and Fig. S1 in the supplemental material); their origins were the Bitterroot Selway mountains, ID, and the Okanagan Valley, British Columbia, Canada, respectively.

The *bipA* locus demonstrated more genetic heterogeneity in GGI isolates with 13 alleles, compared to GGII isolates with only three alleles (Fig. 1). Sequence analysis also identified multiple indels (gaps resulting in insertions or deletions) between genomic groups that are reflected in the amino acid sequence alignments, with one isolate representing each allele (Fig. 2). The amino acid sequences shown from GGI isolates are HS1, MAT, DAH, MIL, WAD, ALL, EST, FRO, BRO, BAK, MAN, FRS, and SIS, and the sequences from GGII isolates are CMC, LAK-1, and MTW-2 (Fig. 2). Furthermore, BipA from GGII isolates was predicted to be larger with a molecular mass of ~62 kDa.

Expressing *bipA* in *E. coli* and reactivity of immune serum to rBipA. Coomassie stained gels of uninduced and induced *E. coli* lysates expressing *bipA* from the GGI isolate DAH produced a recombinant fusion protein of the expected molecular mass (see Fig. S2A in the supplemental material). Also, antipoly-histidine peroxidase MAb bound to the recombinant protein of the expected molecular mass as rBipA with minimal protein degradation (Fig. S2B in the supplemental material).

Immunoblotting using pooled serum samples from three mice infected with *B. hermsii* DAH (Fig. 3A) or serum samples from human patients (Fig. 3B to D) infected with unknown isolates of *B. hermsii* confirmed the immunogenicity of rBipA. Furthermore, preinfection serum samples from mice were negative to *B. hermsii* DAH lysates and rBipA (data not shown). Serum samples from patients infected with *B. burgdorferi* did not react with rBipA (Fig. 3E and F), indicating that rBipA has the potential to discriminate between infection by *B. burgdorferi* and *B. hermsii*. In addition, immunoblots from seven additional human patients infected with *B. hermsii* and five patients infected with *B. burgdorferi* displayed similar results (data not shown). The serum titers to rBipA from mice and human patients infected with relapsing fever spirochetes ranged between 1:2,500 to 1:12,500.

Detecting BipA in *Borrelia* species and GGI and GGII isolates of *B. hermsii*. Pooled immune sera from three mice immunized with rBipA reacted with the 57-kDa protein in spirochete lysates of *B. hermsii* DAH (Fig. 4A), while preimmunization sera did not (Fig. 4B). Also, the *B. recurrentis* (31) and *B. turicatae* genomes contain an orthologue to the *B. hermsii* DAH *bipA*, with amino acid identities of 28.1 and 39.7%, respectively. However, anti-rBipA immune sera did not bind to a protein in these spirochete lysates (Fig. 4C) or with lysates from *B. parkeri*, *B. crocidurae*, and *B. burgdorferi* (Fig. 4C, top panel). Interestingly, whereas anti-rBipA immune sera bound to the protein in lysates of GGI isolates, there was no reactivity with lysates from GGII isolates (Fig. 4D and E, top panels), even though the amino acid identity of BipA between the DAH and GGII isolates is ca. 68%. Probing immunoblots with an anti-flagellin MAb indicated that equal concentrations of protein were used from each species of *Borrelia* and GGI and GGII isolates (Fig. 4C to E, lower panels).

Serum reactivity to rBipA from mice infected with GGII isolates of *B. hermsii*. Immune sera generated against a GGI rBipA did not bind to a protein in GGII isolates; however, when mice were infected with YOR or MTW-4 they seroconverted to rBipA (Fig. 5A, B, D, and E). Also, preinfection serum samples from these mice were negative (Fig. 5C and F). These results indicate that BipA is produced in GGII isolates and is immunogenic during infection.

**In silico** analysis and cellular localization of BipA. Prosite Pattern and LipoP 1.0 predicted a signal sequence for the
FIG. 2. Amino acid alignments from isolates of *B. hermsii* that represent the 13 and 3 alleles in GGI and GGII isolates, respectively. Identical amino acids are shaded with a gray background.
first 22 amino acids of BipA, and Motif Scan predicted a lipid attachment site from amino acids 1 to 23 (data not shown). Also, scanning the amino acid sequence of BipA in the Motif Scan database detected the presence of the Mlp lipoprotein family motif (statistically significant E-value of 0.00064), pfam03304, for a section of 48 amino acids toward the C terminus of the protein (amino acids 392 to 440 of DAH BipA). These amino acids had 40.8% amino acid identity to a 43-amino-acid portion of a 23-kDa protein annotated as the Mlp lipoprotein family protein in *B. burgdorferi* (YP_002776332). Based on these in silico analyses suggesting the surface localization of BipA, the prediction that BipA was a surface protein of *B. hermsii* DAH was experimentally tested.

Intact spirochetes were incubated with proteinase K and immunoblotted with anti-rBipA immune sera, which demonstrated that the protein was digested within 30 min of enzymatic treatment (Fig. 6, top panel). Also, BipA did not degrade in untreated spirochetes incubated at 37°C for the duration of the assay (Fig. 6, top panel). Immunoblots were also probed for the periplasmic protein flagellin (Fig. 6, lower panel), indicating that the spirochetes were not permeabilized during the assay. These results suggest that spirochetes were intact during this assay and that BipA is exposed on the outer surface of *B. hermsii* DAH.

**DISCUSSION**

For the ecological niches where relapsing fever and Lyme disease causing spirochetes overlap (11, 12, 30, 32, 42, 60) and the serological cross-reactivity from infected patients occurs (37), identifying antigens that are species specific is important. We demonstrate here that a protein, which we have designated *Borrelia* immunogenic protein A (BipA), is surface localized and immunogenic in patients and mice infected with *B. hermsii*. Also, with no orthologue of bipA in *B. burgdorferi* (14, 24), lack of reactivity to rBipA from serum samples of patients infected with Lyme disease causing spirochetes was expected.

Interestingly, pooled sera from mice immunized with a GGI rBipA bound to the protein only in GGI isolates. Due to the regions of amino acid deletions in GGI isolates, the quaternary structure of the protein may differ between genomic groups. Thus, the mice used to produce antisera to rBipA may have generated antibodies against an epitope of the protein that is unique in GGI isolates. In addition, the amino acid alignments of BipA from GGI and GGII isolates

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**FIG. 3.** Immunoblotting to determine antibody reactivity to rBipA and borrelia lysates. The serum samples used included pooled immune sera from three mice (A) and three human patients infected with *B. hermsii* (B to D) or two human patients infected with *B. burgdorferi* (E and F). Arrows indicate protein bands of the expected size for rBipA, and the asterisk indicates the expected size of native BipA in *B. hermsii* DAH lysates. Molecular mass standards are indicated in kilodaltons.

**FIG. 4.** Immunoblotting with pooled sera from mice immunized with rBipA. Immune sera against rBipA (A) and preimmunization sera (B) were used to determine seroconversion to BipA in *B. hermsii* lysates. Five species of *Borrelia* (C), GGI (D), and GGII (E) isolates of *B. hermsii* were also probed with anti-rBipA sera (C to E, top panels) and a monoclonal antibody generated against *B. hermsii* flagellin (C to E, lower panels). Molecular mass standards are indicated on the left of each immunoblot in kilodaltons.
possibly explaining the lesser amount of nucleotide diversity of
may represent a more recent derivative from GGI isolates,
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implies a selective pressure on this gene. The surface localiza-

The nucleotide diversity of the
another plasmid-encoded gene, the variable tick protein (43).
transfer of
bipA
bipA
in the
locus, there was no evidence for the horizontal gene
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Although GGI isolates displayed more nucleotide diversity
in the
bipA
locus, there was no evidence for the horizontal gene transfer of
bipA between GGI and GGII isolates, as shown for
another plasmid-encoded gene, the variable tick protein (43).
The nucleotide diversity of the
bipA
locus in GGI isolates
implies a selective pressure on this gene. The surface localization
and immunogenicity of BipA supports the hypothesis that
immune pressure may be responsible for the observed genetic
diversity in GGI isolates. Furthermore, GGII isolates
may represent a more recent derivative from GGI isolates, possibly explaining the lesser amount of nucleotide diversity of
bipA in GGI isolates.

Further analysis of the
bipA
locus identified identical se-
quences from
B. hermsii
isolates spread over a large geographical area, a finding similar to that when the 16S-23S ribosomal DNA noncoding intergenic spacer region (IGS) was sequenced in these isolates (53). In addition, sequence analysis of
B. hermsii
detected in a squirrel in Montana was identical to that of
species having ca. 45 to 50% amino acid identity to
B. hermsii
GlpQ (data not shown), using
rBipA with
rGlpQ or the factor H binding protein in an
enzyme-linked immunosorbent assay could improve serodiagno-
sis. Furthermore, given the amino acid heterogeneity of BipA
between species of relapsing fever spirochetes, expressing
bipA orthologues in other bacteria, and human patients produce high
titer of antibody against
rBipA, this protein also has potential as a
diagnostic antigen. Also, with orthologues of
glpQ in
Yersinia,
Clostridium,
Salmonella,
and Klebsiella
species having ca. 45 to
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B. hermsii
GlpQ (data not shown), using
rBipA with
rGlpQ or the factor H binding protein in an
enzyme-linked immunosorbent assay could improve serodiagno-
sis. Furthermore, given the amino acid heterogeneity of BipA
between species of relapsing fever spirochetes, expressing
bipA orthologues as recombinant proteins may aid in determining the species causing infection.

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