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Fibronectin-Binding Protein of *Borrelia hermsii* Expressed in the Blood of Mice with Relapsing Fever

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To identify and characterize surface proteins expressed by the relapsing fever (RF) agent *Borrelia hermsii* in the blood of infected mice, we used a cell-free filtrate of their blood to immunize congenic naive mice. The resultant antiserum was used for Western blotting of cell lysates, and gel slices corresponding to reactive bands were subjected to liquid chromatography-tandem mass spectrometry, followed by a search of the proteome database with the peptides. One of the immunogens was identified as the BHA007 protein, which is encoded by a 174-kb linear plasmid. BHA007 had sequence features of lipoproteins, was surface exposed by the criteria of *in situ* protease susceptibility and agglutination of Vtp$^-$ cells by anti-BHA007 antibodies, and was not essential for *in vitro* growth. BHA007 elicited antibodies during experimental infection of mice, but immunization with recombinant protein did not confer protection against needle-delivered infection. Open reading frames (ORFs) orthologous to BHA007 were found on large plasmids of other RF species, including the coding sequences for the CihC proteins of *Borrelia duttonii* and *B. recurrentis*, but not in Lyme disease *Borrelia* species. Recombinant BHA007 bound both human and bovine fibronectin with K_d (dissociation constant) values of 22 and 33 nM, respectively, and bound to C4-binding protein with less affinity. The distant homology of BHA007 and its orthologs to BBK32 proteins of Lyme disease species, as well as to previously described BBK32-like proteins in relapsing fever species, indicates that BHA007 is a member of a large family of multifunctional proteins in *Borrelia* species that bind to fibronectin as well as other host proteins.

Relapsing fever (RF) is an arthropod-borne disease that is caused by several species of the spirochete genus *Borrelia* (1). It is found in every geographic region except Antarctica and Oceania. At present, most cases of human RF in the world occur in sub-Saharan Africa, where it is underdiagnosed and often misdiagnosed as malaria (2, 3). It is a neglected tropical disease and affects predominantly rural poor and disadvantaged urban populations in Africa (3–5). The etiologic agents in Africa are the tick-borne species *B. duttonii* and *B. crocidurae* and the sole species transmitted by lice, *B. recurrentis*. Another region with a comparatively high incidence is Central Asia (6, 7). In North America, RF occurs mainly in the western United States and northern Mexico, and etiologic species for humans are *B. hermsii* and *B. turicatae* (8).

Comparatively little is known about the surface proteins expressed *in vivo* by RF *Borrelia* spirochetes during a mammalian infection. Besides the variable membrane proteins Vsp and Vlp, which are the basis of antigen variation during relapsing fever (9), as well as various subsurface structural proteins and enzymes common to many bacteria (10), proteins identified as being expressed during experimental or clinical relapsing fever include glycerophosphodiester phosphodiesterase (GlpQ) (11), factor H-binding protein A (FhbA) (12), and *Borrelia* immunogenic protein (BipA) (13). Alp is another *B. hermsii* protein that has been identified as being expressed in mice, although it is expressed mainly in ticks (14).

Our objective was to identify additional proteins expressed by RF *Borrelia* spirochetes in the blood of a mammalian host using a mouse model of *B. hermsii* infection. For a set of candidates of a manageable number and that might be enriched for outer membrane-associated proteins, we used an approach that was previously used to identify circulating antigens of the bacterial pathogens *Burkholderia pseudomallei* and *Francisella tularensis* in the serum of infected mice (15), namely, by immunizing immunocompetent mice with a cell-free filtrate of blood from immunodeficient mice bacteremic with *B. hermsii*. By this method, we identified a protein, BHA007, that is encoded by the large linear plasmid of this species and that has orthologs among other RF species but not Lyme disease *Borrelia* spp. We further characterized this protein with regard to surface exposure, immunogenicity, the elicitation of protective immunity, and binding activities for selected host proteins.

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MATERIALS AND METHODS

Organisms and cultivation. *B. hermsii* strains HS1 from Washington, CC1 from California, and LPO from Idaho (14); *B. parkeri* strain HR1

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from California; *B. turicatae* strain 91E135 from Texas (16); and *B. burgdorferi* strain B31 (ATCC 35210) were used. HS1 serotype 7 expresses Vlp7, and serotype 33 expresses Vtp (formerly Vsp33). A spontaneous Vtp$^-$ mutant of strain HS was previously described (14). Spirochetes were cultivated at 34°C in Barbour-Stoenner-Kelly II (BSK II) medium (17). Bacterial stocks in BSK II medium or infected mouse plasma were kept frozen at −80°C in 10% dimethyl sulfoxide. Cells were counted in a Petroff-Hausser chamber by phase-contrast microscopy. Spirochetes were harvested by centrifugation at 9,500 × *g* for 20 min and washed twice with phosphate-buffered saline (PBS)–5 mM $MgCl_2$ at pH 7.4 (PBS-Mg). *Escherichia coli* strains TOP10, BL21(DE3), and BL21(DE3)/pLysS were cultivated with Luria-Bertani (LB) broth or solid medium containing 50 μg/ml kanamycin or ampicillin at 37°C.

Generation of the Vtp$^-$ knockout mutant. The construction of the *vtp* inactivation plasmid pOKvtpKO (GenBank accession number EF488747), the preparation of electrocompetent HS1 serotype 33 cells, and the transformation of these cells with pOKvtpKO were carried out according to a procedure described previously by Battisti et al. (18). The insertion of the *flgB*-kanamycin resistance cassette into the *vtp* gene was confirmed by PCR with *Taq* DNA polymerase (New England BioLabs, Inc., Ipswich, MA) and primer sets A, B, and C. Conditions for PCR were 5 min of *Taq* activation at 95°C followed by 30 cycles of 30 s at 95°C, 60 s at 60°C, and 2.5 min at 72°C and then a final extension step at 72°C for 7 min, with the exceptions that annealing temperatures were 56°C and 63°C for primer sets B and C, respectively. Set A primers (5′-ATGAAGAAGA ATACATTAAGTGCGA-3′ and 5′-TTAACAGGGGTCGCTGG-3′) amplified a 623-bp product if *vtp* was intact or a 1,237-bp product if *vtp* was insertionally disrupted. Set B primers (5′-GCAAGATTAAGACTTTAAA TAAGACGAGAGAAG-3 and 5′-TTGGTTGTAACACTGGCAGAGCA-3′) yielded a 2,548-bp product if the disrupted *vtp* gene had replaced the intact *vtp* gene on the 53-kb linear plasmid of this strain. Set C primers (5′-TCGCCCTTCCCAACAGTTG-3′ and 5′-GGAGCTTTCGCGAGGG AA-3′) produced a 1,713-bp product if the transformed plasmid persisted extrachromosomally in *B. hermsii* cells. The PCR products obtained were those expected for a disrupted *vtp* gene, and there was no persistence of the transfected plasmid (see Fig. S1 in the supplemental material). The Vtp$^-$ phenotype of the transgenic cells was confirmed by polyacrylamide gel electrophoresis (PAGE) and Western blot (WB) analysis on whole-cell lysates and Vtp-specific monoclonal IgG antibody H4825 (see Fig. S1 in the supplemental material).

Mouse infections and immunizations. Mouse studies were carried out under University of California—Irvine Institutional Animal Care and Use Committee (IACUC)-approved protocol 1999-2080 and University of Nevada—Reno IACUC protocol 00024. Four- to six-week-old female CBySmn.CB17-*Prkdc*^{scid}/J mice (Jackson Laboratory, Bar Harbor, ME) with severe combined immunodeficiency (SCID) were used for infections and propagation of *B. hermsii* following intraperitoneal (i.p.) injection. Infection was monitored by phase-contrast microscopy of tail vein blood. Immunization of mice with a serum filtrate is described below. For preparation of infected mouse plasma stocks for infecting additional mice, blood was collected by terminal exsanguination with syringes coated with a 4% sodium citrate solution and then centrifuged at 1,000 × *g* for 1 min. For recombinant protein immunization, 4- to 6-week-old female wild-type BALB/cByJ mice (Jackson Laboratory) were inoculated i.p. with 100 μl of a solution containing 10 μg of recombinant protein in PBS emulsified with MPL+TPL RIBI adjuvant (Corixa, Seattle, WA) on day 0. Mice were boosted with the same preparation i.p. on day 24 and subcutaneously on day 42. Blood was obtained from the mice on day 58 right before they were challenged with ~100 serotype 7 cells i.p. For passive immunizations, the mice were injected i.p. with 100 μl of either undiluted preimmune serum or serum from rabbits immunized with recombinant protein 24 h before infection challenge.

Immunization with serum filtrate. Ten SCID mice were inoculated intraperitoneally with ~50 cells of serotype 7 on day 0, and their blood was monitored by phase microscopy. On day 8, when the density of spirochetes approached ~10^7 spirochetes/ml, intracardiac blood was collected from mice under terminal anesthesia with a 23-gauge needle, transferred into a polypropylene microcentrifuge tube, and allowed to clot for 30 min at 22°C. The serum fractions were obtained by centrifugation at 2,300 × *g* for 5 min, pooled, and then sequentially filtered through Millex polyvinylidene difluoride (PVDF) 0.45-μm and 0.1-μm low-protein-binding filters (Millipore Corporation, Billerica, MA). The presence of viable bacteria in the filtrate was assessed by two methods: (i) inoculation of 50 μl in BSK II medium and cultivation for 10 days and (ii) injection of two SCID mice with 50 μl of filtrate followed by monitoring of tail vein blood for the presence of spirochetes by phase microscopy after 10 days. In addition, the antibiotic carbenicillin, at a final concentration of 50 μg/ml, was incubated with the filtered sera for 30 min prior to injection of naive mice. Immunization of immunocompetent 4- to 6-week-old female wild-type BALB/cByJ mice (Jackson Laboratory, Bar Harbor, ME) was carried out essentially as described previously by Nuti et al. (15). Mice in groups of 5 were each injected subcutaneously with a mixture of 50 μl of the serum filtrate, 50 μl of PBS, and 100 μl of TiterMax gold adjuvant (TiterMax USA, Inc.). Approximately 200 μl of blood was collected retro-orbitally every 2 weeks for 2 months, and the mice were then exsanguinated terminally. Control sera were collected from mice that had been injected with MPL+TPL RIBI adjuvant emulsified in PBS without serum filtrate.

Production of rabbit antiserum. The production of rabbit polyclonal antibodies against recombinant BHA007 protein was carried out at Pacific Immunology (Ramona, CA). Two New Zealand White rabbits were immunized with 125 μg protein in complete Freund's adjuvant subcutaneously on day 0 and then immunized with the same dose in incomplete Freund's adjuvant on days 21, 42, and 70. Rabbits were bled on days 0 (before injection), 49, 63, 77, 91, 98, and 101.

PAGE and blot analysis. PAGE and WB analysis were carried out as described previously (14, 19). A PageRuler prestained protein ladder (Thermo Fisher Scientific, San Jose, CA) was used for PAGE and WB analysis of serum filtrate antisera and *Borrelia* whole-cell lysates, while a SeeBlue Plus2 prestained standard (Life Technologies, Carlsbad, CA) was used for other experiments. *B. hermsii* Vtp and *Borrelia* FlaB were identified by murine IgG monoclonal antibodies H4825, which is specific for Vtp (20), and H9724, which binds to FlaB of *Borrelia* spp. (21), respectively. Dilutions of filtrate antisera at 1:25, purified monoclonal antibody H4825 at 1:1,000, antibody H9724 in a hybridoma supernatant at 1:10, or rabbit antiserum at 1:100 were used. Bound mouse antibodies were detected with ReserveAP alkaline phosphatase-conjugated goat anti-mouse IgG and IgM (heavy and light chains) (Kirkegaard and Perry, Gaithersburg, MD) at a 1:10,000 dilution, and bound rabbit antibodies were detected with alkaline phosphatase-conjugated protein A/G (Pierce, Rockford, IL) at a 1:5,000 dilution. 1-Step nitroblue tetrazolium (NBT)–5-bromo-4-chloro-3-indolylphosphate (BCIP) substrate solution (Thermo Fisher Scientific, San Jose, CA) was used for colorimetric detection of both blots. Proteinase K treatment of whole cells prior to PAGE and WB analysis was carried out as described previously (22). In certain semiquantitative WB assays with recombinant protein, 3.15 μg total protein was used in each lane of the SDS-PAGE gel. Subsequent WB bands from bound antibody underwent ImageJ analysis to determine the number of pixels in each band (23). For dot blots, 5 μl of a solution containing 63 ng protein in 10 mM Tris-HCl (pH 8.0)–300 mM NaCl–2 mM EDTA–5 mM β-mercaptoethanol was spotted onto nitrocellulose paper and, when dried, underwent the same washing and antibody probing steps as those used for WB analysis.

Liquid chromatography-tandem mass spectrometry. *B. hermsii* cells were harvested by centrifugation from broth medium and resuspended at a final concentration of ~10^8 cells/ml in ice-cold PBS-Mg with 2% Triton X-114 (Sigma-Aldrich, St. Louis, MO), and detergent fractionation was performed as described previously (24, 25). After precipitation of proteins in the detergent phase in acetone and then centrifugation, the pellets were air dried before PAGE and WB analysis. Another preparation was done by using whole cells of *B. hermsii* serotype 7 isolated from the blood of in-

fected mice by differential centrifugation, as described previously (26), and then PAGE. Selected bands and regions in the gels of the detergent extract or whole-cell lysates were partially digested with trypsin and then subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) by the Arizona Proteomics Consortium at the University of Arizona, as described previously (27), on a ThermoFinnigan (San Jose, CA) linear quadrupole ion trap mass spectrometer equipped with a Michrom (Auburn, CA) Paradigm MS4 high-performance liquid chromatography system with a reversed-phased column, a SpectraSystems (San Jose, CA) AS3000 autosampler, and a nanoelectrospray source. Peptide spectra were analyzed with TurboSEQUEST, version 3.1 (28). All spectra were searched against a database of deduced open reading frames (ORFs) of the *B. hermsii* genome, including the chromosome (GenBank accession number CP00048), plasmid sequences reported previously by Dai et al. (29), and the 174-kb large plasmid reported previously by Campeau Miller et al. (GenBank accession number HM008709) (16).

DNA extractions and PCR. *Borrelia* genomic DNA was isolated from harvested cultures, blood, or tissue with a Qiagen (Valencia, CA) DNeasy Blood and Tissue kit, as described previously (30). The DNA target was amplified with Phusion high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA). For the partial or full-length *bha007* sequence, the forward and reverse primers were 5′-ATGCAATTGAAAAAACAATGTTT-3′ and 5′-CCTATGCCTGTTCTTTTTGC-3′ or 5′-ATGCAATTGAAAAAACAATG-3′ and 5′-TTATTTTAAGGCTTTCTTGAT-3′, respectively. The PCR conditions were as follows: 3 min at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 54°C (partial) or 56°C (full length), and 1 min at 75°C and then a final extension step for 7 min at 75°C. PCR products or cloned inserts in plasmid vector pCR2.1 in *E. coli* Top10F cells were sequenced over both strands by Genewiz (South Plainfield, NJ). Quantitative PCR using a Rotorgene 6000 thermal cycler (Qiagen, Valencia, CA) and primers and probe for the 16S rRNA gene of *B. hermsii* was performed as previously described (30). PCR results were normalized for total DNA concentrations of the samples; a positive result was ≥5 gene copies per μg tissue DNA.

Recombinant protein. The protein-encoding *bha007* sequence was modified by the replacement of the signal peptide with a histidine tag at the N terminus and substitution of *E. coli* codon usage. Gene synthesis, protein expression in *E. coli*, and protein purification of the *Borrelia* recombinant BHA007 protein were performed by GenScript (Piscataway, NJ). Protein purity was ≥70%, as estimated by using a Coomassie blue-stained PAGE gel. Recombinant Vlp7, FlaB, and factor H-binding protein A (FhbA) of *B. hermsii* strain HS1 were cloned with a replacement of their signal peptide by a 6× histidine tag, expressed in *E. coli*, and purified as described previously (31). Recombinant protein purity was estimated to be 80 to 90%. Protein concentrations were determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Microagglutination assay. Serum or antibody preparations were 2-fold serially diluted 1:5 in BSK II medium, with a final spirochete cell density of 10^8 spirochetes/ml. After incubation for 1 h at 37°C, 10 μl of the suspension was viewed under a coverslip by phase-contrast microscopy at a ×400 magnification. A positive agglutination reaction at a given dilution was >50% of the cells in the suspension in aggregates of ≥2 cells; the titer was the highest dilution of serum with a positive reaction. Monoclonal antibody H5332 for OspA (32) was a positive control for the agglutination of *B. burgdorferi*, and monoclonal antibody H4825 (see above) was a positive control for the agglutination of Vtp$^+$ *B. hermsii*.

Immunofluorescence assays. Thin smears on glass slides of blood from *B. hermsii*-infected mice were fixed in methanol, dried, and then blocked for 1 h at 22°C with PBS plus 2% bovine serum albumin (BSA) and 0.05% Triton X-100. Slides were then incubated with rabbit antiserum or preimmune rabbit serum diluted in 1:10 in the same buffer for 60 min at 37°C. The slides were washed with PBS and then incubated with fluorescein-labeled goat anti-rabbit IgG antibodies diluted 1:50 in buffer (Kirkegaard and Perry, Gaithersburg, MD). Fluorescein-conjugated goat anti-*Borrelia* species polyclonal antibodies (Kirkegaard and Perry) were used at a dilution of 1:30 as a positive control for spirochetes in the smears. After washing and drying, the slides were visualized under UV light at a magnification of ×400 on a BX60 microscope (Olympus, Center Valley, PA) with an attached QIClick digital charge-coupled-device (CCD) camera (QImaging, British Columbia, Canada). The fluorescence of spirochetes was scored on a scale from 0 to 3, where 0 was undetectable and 3 was strong.

Enzyme-linked immunosorbent assay (ELISA). Recombinant BHA007 or ovalbumin was used to coat a microtiter flat-bottom polystyrene MaxiSorp plate (Thermo Fisher Scientific, San Jose, CA) at 10 μg/ml with 100-μl volumes per well of 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 22°C for 1 h. After washing with 50 mM Tris (pH 7.4)–150 mM NaCl–0.05% (vol/vol) Tween 20 (TTBS), the wells were blocked with casein blocking buffer (Thermo Fisher Scientific, San Jose, CA) for 8 h. Mouse serum samples were diluted 1:100 in blocking buffer and incubated for 1 h at 22°C. After washing with TTBS, the wells were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Biomedia Corp., Foster City, CA) for 1 h in blocking buffer. After washing with TTBS, the wells were incubated with 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB)-containing liquid substrate (Bethyl Laboratories, Montgomery, TX) per well for 20 min before the reaction was stopped with H_2SO_4 stop solution. The optical density was read at 450 nm by using a Synergy 2 plate reader (Biotek, Winooski, VT).

Labeling proteins. Human plasma fibronectin was purchased from BD Biosciences (San Jose, CA); bovine plasma fibronectin and ovalbumin were purchased from Sigma-Aldrich (St. Louis, MO); human C4b-binding protein was purchased from Complement Technology, Inc. (Tyler, TX); and human C1 esterase inhibitor was purchased from EMD Millipore (Billerica, MA). Bovine plasma fibronectin biotinylated with 3 to 4 biotin moieties per protein molecule, according to the manufacturer's instructions, was purchased from Cytoskeleton, Inc. (Denver, CO). Unlabeled proteins were conjugated with biotin by using a ChromaLink biotin labeling kit (Solulink, Inc., San Diego, CA). The labeling resulted in 3.1, 3.8, 7.1, and 9.2 biotin moieties per molecule of ovalbumin, C1 esterase inhibitor, fibronectin, and C4b-binding protein, respectively. For the four proteins, this was a mean of 3.6 biotin moieties per 100 kDa of polypeptide, as calculated by linear regression of the assayed biotin moieties per protein molecule on molecular mass in kilodaltons ($y = 0.011x + 2.5$; $R^2 = 0.99$). The measures of moles of bound biotin were highly correlated with the moles of protein bound.

Protein binding assay. A total of 10 μg/ml of protein was used to coat wells of 96-well flat-bottom polystyrene MaxiSorp microtiter plates (Thermo Fischer Scientific, San Jose, CA) in 100 μl coating buffer per well for 1 h at 22°C. Plates were then washed and blocked as described above. For experiments in which there was an inhibition step, unlabeled proteins in casein blocking buffer were incubated for 1 h before incubation of the labeled proteins in the same buffer for 1 h at 22°C. After washes with TTBS, bound biotin was detected with horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Inc., Burlingame, CA) at 5 μg/ml in 100-μl volumes in blocking buffer for 1 h at 22°C. The wells were washed again with TTBS, as described above. After washing with TTBS, the wells were incubated with 100 μl of TMB per well, and the optical density at 450 nm was then read as described above. The dissociation constant (K_d) for protein-ligand binding in molar units was the concentration of the ligand at which the binding site on the protein was 50% occupied, as estimated by inhibition with unlabeled protein. Confidence intervals for K_d values were derived from the 95% confidence interval for the coefficient for the linear regression of the proportions of inhibition on the corresponding log-transformed molar protein concentrations.

DNA sequencing. Nucleotide sequencing of PCR products was performed by Genewiz (La Jolla, CA). For next-generation sequencing, DNA extracted from harvested cultures was first treated with RNase I (Fermentas, Burlington, Ontario, Canada), purified as described above, and then enzymatically sheared with an Ion Express Plus fragment library kit (Life Technologies, Grand Island, NY). Products were size selected by gel pu-

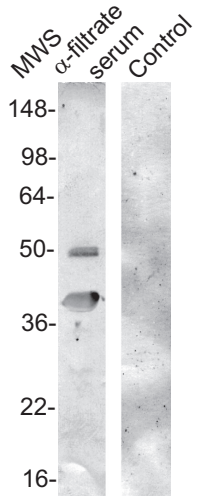

FIG 1 Western blot (WB) analysis with mouse antiserum to a cell-free filtrate of blood from congenic SCID mice infected with *Borrelia hermsii*. Proteins of a whole-cell lysate of serotype 7 of *B. hermsii* were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. Blots were incubated with either the antiserum or serum from an unimmunized, uninfected mouse (control). Bound antibodies were detected with alkaline phosphatase-labeled goat anti-mouse immunoglobulin. The locations of the molecular weight standards (MWS) (in thousands) are shown on the left.

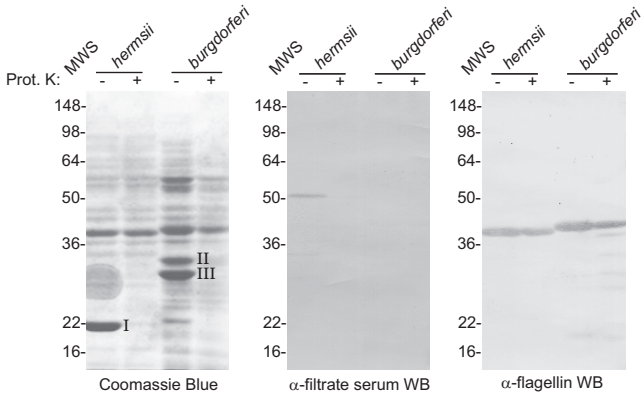

FIG 2 Effect of proteinase K (Prot. K) treatment on whole cells of *B. hermsii* serotype 33 or *B. burgdorferi* strain B31 with (+) or without (−) prior treatment. (Left) Coomassie blue-stained PAGE gel showing the location of the Vtp protein (I), OspA (II), and OspB (III), all of which are surface exposed. (Middle) WB with antiserum to the filtrate. (Right) WB with a monoclonal antibody to a flagellin protein of the periplasmic flagella of *Borrelia* spp. The locations of the molecular weight standards (MWS) (in thousands) are shown on the left of each panel.

rification with the E-Gel system (Invitrogen). Templates were prepared by emulsion PCR on an Ion Torrent OneTouch apparatus (Life Technologies), and these templates were sequenced on an Ion Torrent personal genome machine with Ion 316 chips (Life Technologies). The coverage was ≥20×. The RNA-Seq algorithm of clcBio Genome Workbench v. 6.3 software was used to count reads mapping to 100-nucleotide (nt)-long ordered segments of the full-length reference sequence, with criteria of a minimum length fraction of 0.8 and a minimum similarity fraction of 0.95.

Sequence analysis. Proteins were considered homologous if their E values in BLASTP searches were $\leq 10^{-4}$ over ≥90% of their lengths (33). Sequences of homologous genes in public databases were *B. turicatae bta001* (GenBank accession number KC845249), *B. recurrentis cihC* (accession number FN552439), *B. duttonii cihC* (accession number FN552440), and *B. crocidurae* "outer surface protein" (accession number NC_017778). Phylogenetic inference was carried out with the PhyML algorithm (34), using default settings and 1,000 bootstrap iterations of the SeaView version 4 suite (35). Comparison with protein families was carried out by using the Conserved Domain Database (36) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Secondary protein structure predictions were performed by using the PHD server (37) (http://www.predictprotein.org/). Subcellular localization predictions were performed with the CELLO v 2.5 program (38, 39) (http://cello.life.nctu.edu.tw/).

Nucleotide sequence accession numbers. The GenBank accession numbers assigned to *B. hermsii* strain HS1 *bha007* (*fbpC*) and orthologous genes in strains CC1 and LPO are HQ698268, KF537350, and KF537349, respectively. The GenBank accession numbers for the *bha007* ortholog (*fbpC*) *bpa001* of *B. parkeri*, BBK32-like genes (*fbpB*) of *B. turicatae* and *B. parkeri*, and the BBK32 ortholog (*fbpA*) of *B. turicatae* are KC845250, KC845251, KC845252, and KC845248, respectively. The GenBank accession number for the nucleotide sequence for the synthesis of a modified BHA007 gene is KJ599624.

RESULTS

Antiserum to the serum filtrate of infected blood. Our objective was to identify cell-free antigens of *B. hermsii* in the blood of infected immunodeficient mice by immunizing immunocompetent mice with a filtrate of sera obtained from the blood of infected syngeneic but immunodeficient mice. To this end, we infected five SCID mice with ~50 *B. hermsii* cells each on day 0. When the spirochete density in the blood had reached its peak on day 8, coagulable blood was collected. After centrifugation of clotted blood, sera were pooled and passed through a 0.1-μm filter. We confirmed the absence of infectious organisms by inoculating samples of the filtrate into culture medium and into SCID mice and monitoring these cultures and mice for infection. The pooled serum containing an antibiotic was then injected into naive immunocompetent mice. The filtrate plausibly contained secreted proteins and released membrane vesicles from the spirochetes (40, 41) but possibly also contained subcellular structures if some cells were disrupted during processing. Our reasoning was that this would provide a smaller set of proteins for eliciting antibodies than would be the case with either natural infection or whole-cell immunization.

The pooled antiserum, which was obtained from 5 mice beginning at 4 weeks after the initial immunization, was used at a 1:25 dilution to probe a Western blot (WB) of a whole-cell lysate of serotype 7 cells of *B. hermsii* HS1, the same clone that was used to infect the SCID mice. The negative control was pooled serum from mice immunized with PBS and adjuvant alone. Antibodies in the filtrate antiserum but not in the control serum bound to antigens with apparent molecular masses of about 50 kDa and 38 kDa (Fig. 1). We suspected that the 38-kDa band was the serotype-defining Vlp7 protein (29, 42), which was known to be expressed in the blood of mice (19). This was confirmed by the finding that the filtrate antisera but not the control antisera had antibodies that bound to recombinant Vlp7 and not ovalbumin by dot blotting (data not shown).

Our focus then turned to the ~50-kDa immunoreactive band. We applied the antifiltrate serum to a WB of whole-cell lysates of *B. hermsii* or *B. burgdorferi*, some samples of which had been treated with proteinase K before lysis (Fig. 2). The absence or diminution of a protein in the protease-treated sample is evidence

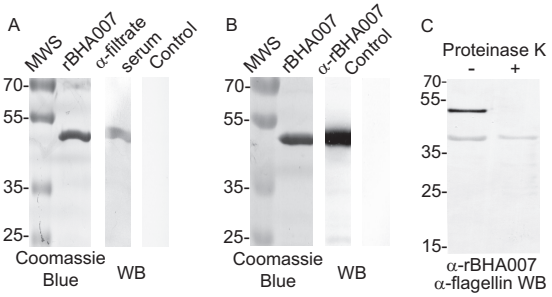

FIG 3 Analysis of recombinant BHA007 (rBHA007) and antisera. (A) Stained PAGE gel with purified recombinant protein as well as a WB of other lanes of this gel that was incubated with either the antifiltrate serum or control mouse serum. (B) Stained gel of recombinant BHA007 and a WB with rabbit antiserum to rBHA007. (C) WB of a PAGE gel of the lysate of *B. hermsii* cells with (+) or without (−) prior treatment with proteinase K. The blot was incubated with both antiserum to recombinant BHA007 and monoclonal antibody to flagellin. Detection of bound antibodies was done with alkaline phosphatase-labeled protein A/G for rabbit antibodies and alkaline phosphatase-labeled goat anti-mouse immunoglobulin. The locations of the molecular weight standards (MWS) (in thousands) are shown on the left of each panel.

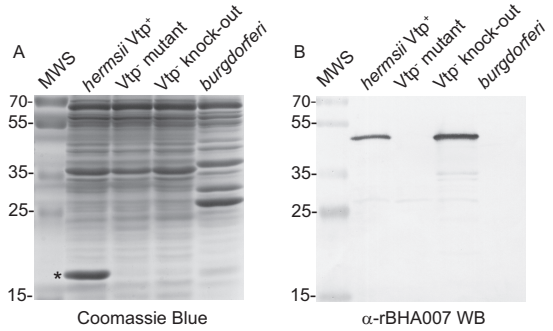

FIG 4 Expression of BHA007 by *B. hermsii* wild-type serotype 33 (Vtp^+), spontaneous Vtp^- mutant, or Vtp^- knockout mutant cells. (A) Stained gel of whole-cell lysates of the *B. hermsii* isolates as well as *B. burgdorferi*. The asterisk indicates the location of the Vtp protein. (B) WB of duplicate lanes from the same gel and with rabbit antiserum to recombinant BHA007. The locations of the molecular weight standards (MWS) (in thousands) are shown on the left of each panel.

of its surface exposure (22). To further confirm the identity of the 38-kDa band as Vlp7, we used serotype 33 cells, which express the surface-exposed 22-kDa Vtp protein but not Vlp7 (43). Antibody to FlaB, a major structural protein of the periplasmic flagella, provided confirmation of the action of the protease on surface proteins with sparing of the subsurface structures. *B. burgdorferi* with its surface-exposed OspA and OspB proteins provided another test of the specificity of the protease's actions. As shown in Fig. 2, the 50-kDa band was the only one of the two original bands present in the serotype 33 lysate, and it was undetectable in the lysate of protease-treated cells.

Identification of the components of the 50-kDa band. For further characterization of the immunoreactive band, we subjected culture-grown serotype 7 cells to Triton X-114 extraction (24) and applied the detergent fraction onto a PAGE gel. The contents of a slice containing the 50-kDa region of the gel were partially digested with trypsin, and the resultant peptides were subjected to LC-MS/MS analysis. The molecular masses of the peptides were applied against all possible ORFs of the chromosome and the linear and circular plasmids of *B. hermsii*. Four of the peptides, totaling 51 amino acids, had 100% matches to the amino acid sequence of the ORF BHA007 of the 174-kb large linear plasmid, lp174, of *B. hermsii* (16): LVQELKDLLEK (ORF positions 273 to 283), LTIDNAVEDFSK (positions 290 to 301), FSSETPQS NYVTEADK (positions 302 to 317), and FSEVENHLKDAK (positions 355 to 366). The peptide FSSETPQSNYVTEADK was also present in the library of spectra identified by LC-MS/MS analysis of a PAGE gel core containing proteins in the range of 28 to 55 kDa of a whole-cell lysate of serotype 7 cells grown in the blood of SCID mice. In the nonredundant GenBank database, of 3.8×10^7 proteins (as of 17 March 2014), the only proteins with E values of <10 by BLASTP v. 2.2.29 (http://www.ncbi.nlm.nih.gov/) with a word size of 2 and a PAM30 matrix were BHA007 of strain HS1 (E value of 4×10^{-7}) and its orthologs in other *B. hermsii* strains (E values of $<10^{-6}$). There was no significant similarity (E values of >10) by TBLASTN v. 2.2.29 (http://www.ncbi.nlm.nih.gov/) with a word size of 3, a BLOSUM62 matrix, and low-complexity filter of the 16-mer peptide with 7.5×10^7 nonredundant expressed sequence tags (ESTs) in GenBank (as of 10 March 2014); this also included a search restricted to the *Mus musculus* EST database.

Recombinant BHA007 and antiserum. BHA007 is a 374-amino-acid polypeptide (39 kDa) with a predicted lipoprotein leader sequence ending at a consensus signal peptidase II cleavage site, FISC, at residues 16 to 19. The *bha007* coding sequence was synthesized with *E. coli* codon usage, replacement of the signal peptide and the cleavage site cysteine with a His tag, and a molecular mass of 40.9 kDa. The recombinant protein migrated at an apparent molecular mass of ~50 kDa by PAGE and was bound by antibodies in the filtrate antiserum but not by those in the control serum (Fig. 3A). Antibodies in antiserum against recombinant BHA007, but not in preimmune serum, bound to this protein in a WB (Fig. 3B). A protein with the same electrophoretic migration was found in whole-cell lysates of *B. hermsii* (Fig. 4). Furthermore, the protein reactive with the anti-BHA007 antibodies was cleaved from the cells by the protease (Fig. 3C). The experiments demonstrated that BHA007 was at least one of the proteins constituting the 50-kDa immunoreactive band and that the protein has an anomalous migration in PAGE gels. This was not likely attributable to protein modification, because it was observed with both native and recombinant forms.

Further characterization of BHA007. Taken together, the *in situ* protease susceptibility and the lipoprotein features of the protein suggested that BHA007 is an outer membrane protein with surface exposure. We further investigated its location in spirochete cells by using the anti-BHA007 antiserum in a microagglutination assay with three isolates of serotype 33. One of these was the wild type, which expressed Vtp, and the other two were Vtp-less mutants, one of which was attributable to a spontaneous frameshift (14) and the other of which was a transgenic *vtp* knockout created here. Both Vtp-less mutants, which also lacked other variable membrane proteins, such as Vlp7, were used to assess the accessibility of BHA007 when an abundant outer membrane lipoprotein, such as Vtp or Vlp7, was present (22). *B. burgdorferi* cells, which were grown under the same *in vitro* conditions as those used for *B. hermsii* cells, were a negative control for spirochete cells, and antibody to OspA of *B. burgdorferi* was a positive control for an agglutinating antibody (32).

Table 1 summarizes the agglutination results. The only cells

TABLE 1 Microagglutination of *B. hermsii* and *B. burgdorferi*

Antibody	Agglutination result[a]			
	B. hermsii			
	WT	Mutant	KO	*B. burgdorferi*
Anti-BHA007 antiserum	−	−	+	−
Preimmune serum	−	−	−	−
Anti-Vtp antibody	+	−	−	−
Anti-OspA antibody	−	−	−	+

[a] A + indicates that >50% of the cells in the suspension were in aggregates of ≥2. WT, wild type; KO, knockout.

agglutinated by the anti-BHA007 antibodies were the Vtp knockout cells. In another experiment that confirmed these results, the anti-BHA007 serum agglutinated the knockout mutant cells out to a dilution of 1:480. The failure of the antibodies to discernibly agglutinate Vtp$^+$ wild-type cells was not unexpected, putatively because the highly abundant Vtp protein interfered with the access of antibody of BHA007 *in situ* (22). We were surprised by the absence of agglutination of the spontaneous Vtp$^-$ mutant cells. To determine whether this was attributable to the absence of BHA007, we performed WB analysis with a 1:100 dilution of anti-BHA007 serum and the different cell types of the agglutination assay. As shown in Fig. 4, the wild-type cells and the Vtp$^-$ cells had detectable BHA007 expression, but spontaneous mutant Vtp$^-$ cells did not. Besides providing an explanation for the lack of agglutination of this Vtp$^-$ isolate, this experiment also confirmed the expression of BHA007 by wild-type cells.

To investigate the genetic basis for the BHA007 null phenotype of spontaneous mutant Vtp$^-$ cells, we performed PCR with primers designed to amplify a 1,049-bp fragment corresponding to the full-length *bha007* gene from DNA isolated from wild-type *B. hermsii*, the two Vtp$^-$ mutants, and *B. burgdorferi*. Amplification products of the expected size were observed when PCR was performed with DNA from wild-type and knockout mutant cells but not spontaneous mutant Vtp$^-$ cells (Fig. 5A). As determined by mapping of next-generation sequence reads to the lp174 plasmid reference sequence, the absence of the *bha007* gene was attributable to a 37-kb truncation of the left end of lp174 (Fig. 5B). The knockout mutant had the full-length lp174 plasmid. These results also provided evidence that (i) the sequence encoding the immunoreactive protein was located at the left end of lp174 and (ii) the *bha007* gene as well as those within several kilobases of it are dispensable for growth *in vitro*.

Additional evidence of BHA007 expression *in vivo*. The presence of a peptide that was unique to BHA007 in spirochetes growing in the blood of mice was evidence of its *in vivo* as well as *in vitro* expression. To assess whether BHA007 expression was temperature associated, we used anti-BHA007 antiserum to probe a Western blot of a lysate of Vtp$^-$ cells grown *in vitro* at 23°C or 37°C. BHA007 expression was not discernibly different between the two conditions of this experiment for this isolate (see Fig. S2 in the supplemental material).

Immunofluorescence assays were performed on *B. hermsii* cells in thin smears of blood of infected SCID mice directly with labeled polyclonal anti-*Borrelia* species serum at a 1:30 dilution or indirectly with rabbit anti-BHA007 serum or preimmune serum at a 1:10 dilution (Fig. 6). The fluorescent spirochetes in individual, randomly chosen fields of the smears were counted. The mean numbers (95% confidence intervals) of fluorescent cells per 400× magnification were 25.5 (19.3 to 31.7) for the anti-*Borrelia* polyclonal antibodies as a positive control ($n = 9$), 17.4 (14.7 to 20.2) for the anti-BHA007 antibodies ($n = 13$), and 0.3 (0.0 to 0.6) for the preimmune rabbit sera as a negative control ($n = 7$). Under the null hypothesis of no difference between preimmune and postimmunization sera in reactivity with spirochetes in the blood, the probability of observing 17.4 fluorescent spirochetes per field by chance under the Poisson distribution, where the expected value per field was 0.3, was $\ll 10^{-6}$. While ~70% of the spirochetes in the blood smear were bound by the anti-BHA007 antibodies, the observed fluorescence was more variable in intensity, with scores ranging from 1 to 3, than for the positive-control antibody smear, in which the fluorescence scores for individual spirochetes ranged between 2 and 3.

Finally, sera from 10 BALB/c mice that had been infected with *B. hermsii* on day 0 were assayed for the presence of BHA007-specific antibodies in serum samples collected on days 7 ($n = 3$), 14 ($n = 3$), and 28 ($n = 4$). Sera from 3 uninfected mice served as controls. Antibodies that bound to purified recombinant BHA007 were measured by ELISA and also by densitometry of the band corresponding to BHA007 in a Western blot (Table 2). Anti-BHA007 antibodies were barely detectable by day 7 but were more clearly detectable by days 14 and 28.

Immunization with BHA007. The role of preexisting immunity to BHA007 in preventing infection by *B. hermsii* was also assessed. Active immunization of mice was performed with recombinant BHA007 combined with adjuvant in 3 doses ($n = 6$) and by passive immunization with rabbit anti-BHA007 serum

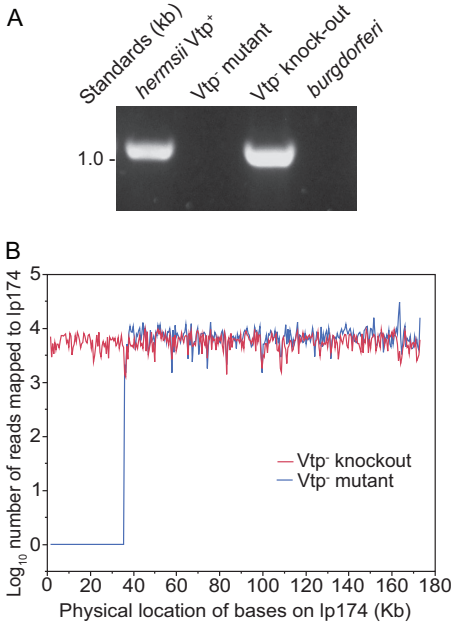

FIG 5 BHA007$^-$ mutant cells have a truncated large linear plasmid. (A) Ethidium bromide-stained 1% agarose gel of PCR products with primers for amplification of a 1,049-bp fragment containing the *bha007* gene from *B. hermsii* wild-type Vtp$^+$, Vtp$^-$ mutant, and Vtp$^-$ knockout cells and *B. burgdorferi* cells. (B) Plot of the number of next-generation DNA sequence reads with a mean size of ~100 nt that mapped with no mismatches or indels to 100-bp windows with a step size of 100 bp of the lp174 linear plasmids of cells of either the mutant or the knockout.

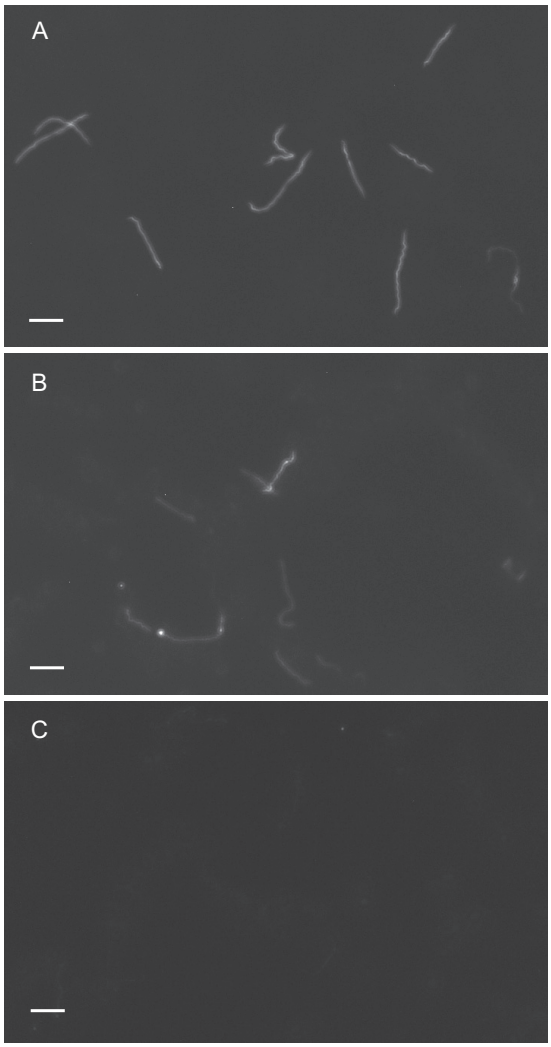

FIG 6 Direct and indirect immunofluorescence of *B. hermsii* serotype 7 cells in thin smears of blood from infected mice. (A) Slide incubated with fluorescein-labeled goat anti-*Borrelia* serum. (B) Slide incubated with rabbit antiserum to recombinant BHA007 and then fluorescein-labeled goat anti-rabbit IgG antibodies. (C) Slide incubated with preimmune rabbit serum before the secondary antibody. Bar, 5 μm.

($n = 6$) 24 h before infectious challenge. The negative-control antigen for active immunization was recombinant FlaB flagellin ($n = 4$), and the negative-control serum for the passive immunization was preimmune rabbit serum ($n = 4$). By dot blotting with sera at a 1:100 dilution, all BHA007-immunized mice had antibodies to BHA007 but not to FlaB by day 58. The reverse pattern of reactivity was found for sera from the FlaB-immunized mice. There was no greater protection afforded by BHA007 immunization against infection than that afforded by the subsurface flagellin protein (see Table S1 in the supplemental material). Four of six mice immunized with BHA007 were infected as determined by criteria of microscopic detection of *B. hermsii* in the blood during days 3 to 8 (3 of 4 mice) or PCR assay of the spleen and liver for *B. hermsii* DNA on day 18 (4 of 4 mice). In the passive immunization experiment, all 10 mice were bacteremic upon at least one microscopic examination of the blood through day 7.

TABLE 2 Antibody responses of mice infected with *B. hermsii* to recombinant BHA007, determined by ELISA and WB analysis[a]

Day of infection (no. of mice)	Mean OD (95% CI)	Mean no. of pixels in WB (95% CI)
0 (3)	0.07 (0.04–0.11)	0
7 (3)	0.14 (0.09–0.20)	0
14 (3)	1.10 (1.00–1.21)	9,391 (5,692–15,493)
28 (4)	1.07 (0.73–1.42)	6,447 (2,059–20,189)

[a] OD, optical density at 450 nm; CI, confidence interval.

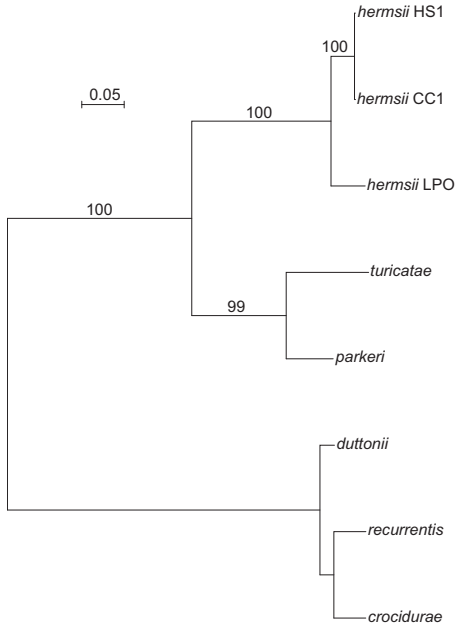

FIG 7 Distance phylogram of aligned protein sequences of BHA007 of three strains of *B. hermsii* (HS1, CC1, and LPO) and orthologous proteins of *B. turicatae*, *B. parkeri*, *B. duttonii*, *B. recurrentis*, and *B. crocidurae*. Nodes with ≥70% support in 1,000 bootstrap iterations of maximum likelihood inference are shown. The bar indicates amino acid distance as the proportion of nonidentity.

Orthologous proteins in other strains and species. The intraspecies diversity of *bha007* was evaluated by comparing the HS1 sequence to those of strains CC1 and LPO. Strains HS1 and CC1 are members of genomic group I of *B. hermsii*, and strain LPO belongs to genomic group II (44). The nucleotide and amino acid sequences of BHA007 of strains HS1 and CC1 differed at only 1 base out of 1,122 and 1 residue out of 374, while the corresponding sequences of HS1 and LPO differed at 77 nucleotide positions and 53 amino acid positions, including a gap of 4 residues in the HS1 protein.

No orthologous proteins were found among publicly available sequences of complete genomes for LD *Borrelia* species or other bacterial genera. ORFs orthologous to BHA007 were found in similar positions of the large linear plasmids of other North American species, namely, *B. turicatae* (16) and the closely related *B. parkeri* (GenBank accession number CP007036) (Fig. 7). Their ORF names are BTA001 and BPA001, respectively. BHA007, BTA001, and BPA001 were also noted to be orthologous to similarly positioned ORFs on the large plasmids of the African species *B. duttonii* and *B. recurrentis* (45) as well as in the deposited genome of *B. crocidurae* (46). With identities to BHA007 of only 42

to 44%, the ORFs of African species were more distantly related to BHA007 than were those of the other North American species, which were 56 to 60% identical in sequence (see Table S2 in the supplemental material). The lengths of the processed polypeptides ranged from 338 residues for *B. duttonii* to 356 residues for *B. hermsii*. With the exception of BHA007, which has a predicted pI of 8.3, the orthologous proteins had acidic pIs, ranging from 4.8 for *B. recurrentis* to 5.8 for *B. parkeri* for the processed proteins (see Table S3 in the supplemental material). All proteins had 19-residue signal peptides ending at a FISC signal peptidase II site. The predicted secondary structures for the 6 proteins were predominantly alpha-helical and coiled, with little beta-sheet content (see Table S4 in the supplemental material).

Function studies of BHA007. Previous studies of the orthologous proteins of *B. duttonii* and *B. recurrentis* by Grosskinsky et al. (45) provided guidance for our studies of the possible functions of BHA007. These authors named these proteins "CihC" for their ability to bind human C1 esterase inhibitor as well as C4b-binding protein. They also noted that the *B. duttonii* and *B. recurrentis* proteins appeared to bind fibronectin, but they did not further characterize this activity in that study. Given the considerable sequence diversity among this group of orthologous proteins, we did not assume that the same binding properties would be found for BHA007.

Equimolar amounts of recombinant BHA007 or ovalbumin were used to coat microtiter plate plates, which were then incubated with serial 2-fold dilutions of biotinylated human fibronectin, human C1 esterase inhibitor, human C4b-binding protein, or ovalbumin in two separate experiments. Bound biotinylated protein was detected with horseradish peroxidase-conjugated streptavidin, and the amount of binding was measured as the optical density at 450 nm for the subsequent substrate reaction. Fibronectin and, to a lesser extent, C4b-binding protein detectably bound to BHA007 on the plates (Fig. 8A). The binding of C1 esterase inhibitor to BHA007 on the plates was no greater than that of ovalbumin in these experiments. There was no detectable binding of any of biotinylated proteins to ovalbumin on the plates above the uncoated-well background level (data not shown). For the two experiments, the mean concentrations calculated by log-linear regression to give an optical density of 1.0, which was ~50% saturation under these conditions, were 1.8 nM (95% confidence interval, 1.5 to 2.1 nM) for fibronectin and 47 nM (95% confidence interval, 41 to 53 nM) for C4b-binding protein.

We further characterized the affinity of the binding of fibronectin to BHA007 by first exposing the protein on the plates to unlabeled proteins in solution for 1 h before washing and then incubating the plates with biotinylated human or bovine fibronectin at 5 μg/ml for 1 h. There was no detectable inhibition of binding of either fibronectin by ovalbumin (Fig. 8B). With biotinylated and unlabeled human fibronectin and 10 determinations from two separate experiments, the log-linear regression had a coefficient of 0.50 (95% confidence interval, 0.39 to 0.62) and an R^2 value of 0.93 ($F_{1,8} = 107$; $P < 0.0001$). The calculated K_d was 22 nM (95% confidence interval, 17 to 27 nM). With biotinylated and unlabeled bovine fibronectin and 10 determinations in a single experiment with replication, the log-linear regression had a coefficient of 0.41 (0.29 to 0.52) and an R^2 value of 0.89 ($F_{1,8} = 63$; $P < 0.0001$). The calculated K_d was 33 nM (95% confidence interval, 23 to 42 nM).

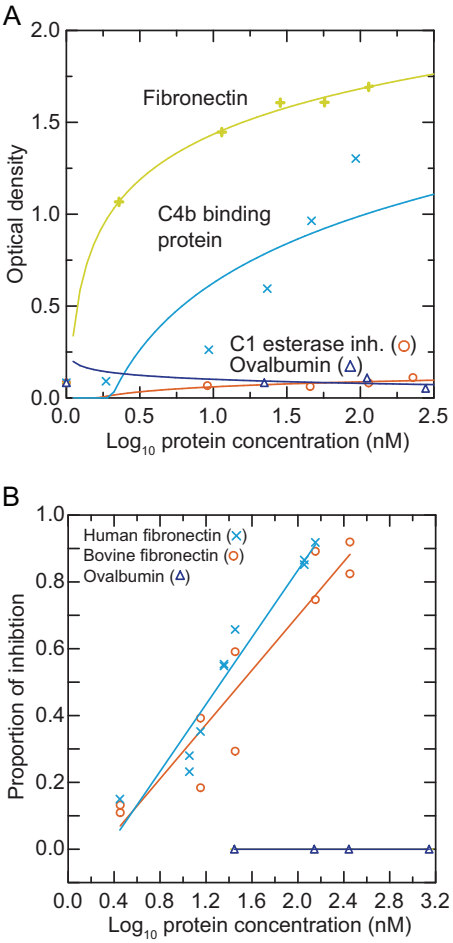

FIG 8 Fibronectin and C4b-binding protein bind to recombinant BHA007 protein. (A) Binding of biotinylated human fibronectin, human C4b-binding protein, human C1 esterase inhibitor (inh.), or chicken ovalbumin at different concentrations to microtiter plates coated with recombinant BHA007. Binding was measured as the optical density at 450 nm for the terminated reaction of horseradish peroxidase-conjugated streptavidin with the substrate. The correspondences of C1 esterase inhibitor or ovalbumin with different symbols are shown in parentheses. A logarithmic smoother filter was applied for each of the four proteins. (B) Inhibition of binding of biotinylated human or bovine fibronectin to recombinant BHA007 by prior incubation of plates with either unlabeled human or bovine fibronectin, respectively. These results were compared with the inhibition of binding of either human or bovine biotinylated fibronectin by prior incubation with unlabeled ovalbumin.

DISCUSSION

This study began as a broad screen for *B. hermsii* antigens in a cell-free filtrate of the blood of infected mice and concluded with the identification and fuller characterization of one of these constituents. To avoid anti-idiotype antibody production or immune-mediated bacterial lysis, we used immunodeficient mice as the sources for the sera for the immunizations of syngeneic immunocompetent animals. The samples were obtained early in infection (42), so the milieu for the spirochetes in SCID mice was not likely different from what the pathogen would encounter in wild-type animals. While we cannot rule out the presence of parts of lysed cells in the serum samples, care was taken in the collection and handling of the infected blood to minimize bacterial disruption and to select for components of <100 nm. Our aim was to

provide as immunogens those proteins that were in a cell-free state in the blood by virtue of being released, secreted, or, more likely for a *Borrelia* species, incorporated into membrane vesicles (40, 41, 47, 48). Carbohydrates or glycolipids might have been present in the filtrate and elicited an antibody response in the mice, as was the case for Nuti et al. with *Burkholderia pseudomallei* (15), but our subsequent protocol favored identification of proteins.

The antibodies to the Vlp7 protein in the antifiltrate sera provided evidence of the efficacy of the experimental approach. Variable membrane proteins, such as Vlp7, are the most abundant proteins expressed by *B. hermsii* and are localized at the surface of the outer membrane as lipoproteins, anchored there by their fatty acids (20, 49). Cleavage of this or another Vlp protein from its lipopeptide moiety and subsequent release into the surrounding medium or host fluid have not been observed. The presence of the highly polar Vlp7 protein in the cell-free serum fraction is most plausibly explained by carriage by micelle-like vesicles.

The second identified target of these antifiltrate antibodies was BHA007, another lipoprotein which is encoded by a gene at the far left end of the large linear plasmid of *B. hermsii* (16). Linear plasmids with a length of ≥150 kb are unique to RF species in the genus *Borrelia* but have been comparatively little studied. This replicon has a few biosynthetic genes, such as those for pyridines (50), but of the hundred or so other ORFs on the plasmid, the only ones that have been characterized to date are factor H-binding protein A (FhbA) (12, 51, 52), *Borrelia* immunogenic protein A (BipA) (13, 53), and arthropod-associated lipoprotein (Alp) (14). With the exception of the Alp protein, which appears to be expressed mainly in the tick vector (14), BHA007, FhbA, and BipA elicit antibody responses during infection of mice. Interestingly, the *bha007* and *fhbA* (or *bha008*) genes are adjacent on the same strand of lp174 and conceivably constitute a bicistronic operon.

Neither *bha007* nor *fhbA* was required for growth of an isolate of *B. hermsii* in a broth medium. A spontaneous Vtp$^-$ mutant lacking ~37 kb at the left end of lp174, which included both *bha007* and *fhbA*, as well as several other ORFs, was viable *in vitro* (Fig. 5). Lopez et al. reported that two *B. hermsii* strains retained infectivity for mice after extended serial passage *in vitro*, during which there was a truncation of ~30 kb from the left end of those strains' large plasmids (54). However, in that study, the inoculum was 10^4-fold larger than usually needed for a low-passage-number isolate of *B. hermsii* and was delivered by needle injection directly into the peritoneum. An assessment of the replication of these truncated plasmid mutants in a host animal or its transmission to and from a tick vector remains to be determined.

Regardless of whether BHA007 is required or not for maximum fitness, the protein's immunogenicity during mouse infection makes this protein a candidate antigen for an immunodiagnostic assay for RF that serologically distinguishes RF from Lyme disease. As discussed below, there are proteins in *B. burgdorferi* and other Lyme disease species that are homologous to BHA007, but these proteins are too divergent in sequence for cross-reactivity to be expected in immunoassays. The near identity of sequences of two different strains from one genomic group suggests that two proteins, one representative of each genomic group, would be sufficient as the basis of an assay.

However, immunogenicity of an antigen during infection does not imply that an immune response to it either hastens infection resolution or protects against challenge. In the present study, the 3-dose adjuvanted vaccination protocol did not provide BALB/c mice protection against a subsequent challenge by the needle route. There was also no apparent protection with passive immunization with rabbit antiserum that had a high titer of agglutinating antibodies for Vtp$^-$ cells. The failure of BHA007 as a vaccine in these two experiments may be attributable to the lack of access of anti-BHA007 antibodies to most cells in the population, through shielding by the abundant Vlp7 protein in this case, as occurs with the P66 outer membrane protein and the more abundant OspA protein in *B. burgdorferi* (22). This appears to be the explanation for the outcome of the microagglutination assays: the anti-BH007 serum agglutinated only cells lacking Vtp or other variable membrane proteins (Table 1).

Clues to the possible function of BHA007 were provided by a comparison with orthologous proteins in other species. Campeau Miller et al. identified an ortholog, *bta001*, in *B. turicatae* at the far left end of its large plasmid (16), and here we report another ortholog, *bpa001*, in another North American RF species, *B. parkeri*. Before this, Grosskinsky et al. reported a protein in *B. duttonii* and *B. recurrentis* that bound to the complement regulatory proteins human C1 esterase inhibitor and C4b-binding protein (45). Those authors named this protein CihC. The coding sequence for this protein, *cihC*, was synonymous with an ORF on the large linear plasmids of *B. duttonii* and *B. recurrentis* (55), which was in a large syntenic region corresponding to the left ends of the plasmids of *B. hermsii* and *B. turicatae* (16).

In the present study, recombinant BHA007 on a solid matrix was bound by fibronectin in solution with high affinity, was bound less well by C4b-binding protein, and was not detectably bound by a C1 esterase inhibitor (Fig. 8A). The binding affinities of human and bovine fibronectin for recombinant BHA007 of ~20 to 30 nM were comparable to those for fibronectin-binding proteins in Gram-positive cocci, including F2 of *Streptococcus pyogenes* and FnBPA of *Staphylococcus aureus* (56–59). Brenner et al. reported that BHA007 and its orthologs in *B. turicatae* and *B. parkeri* bound to fibronectin but neither C4b-binding protein nor C1 esterase inhibitor (60). They mapped the ligand-binding site for the *B. hermsii* protein by using blotting assays with human fibronectin in serum and RF *Borrelia* fibronectin-binding protein deletion mutants and made use of a spontaneous mutant of *bha007* that encoded a polypeptide lacking the C-terminal half of BHA007 (60). Depending on the RF *Borrelia* species, the fibronectin-binding domains within these proteins were mapped somewhere between amino acid positions 180 and 230 in the unprocessed polypeptides with the signal peptides (60). As for the protein that those authors identified in strain HS1 of *B. hermsii*, its protein was truncated at 191 residues and did not contain a predicted fibronectin-binding domain. The putative ligand-binding site identified in the CihC proteins appears not to have been well conserved in BHA007 (45).

As determined by a BLASTP search of *B. burgdorferi* ORFs, the protein most similar in sequence (E value = 5×10^{-6}) was BBK32, or fibronectin-binding protein, which is encoded by the lp36 plasmid of strain B31. BBK32 was also the top-ranking *B. burgdorferi* ORF out of ~1,700 by a BLASTP search with the CihC protein sequences of both *B. duttonii* (E value = 2×10^{-4}) and *B. recurrentis* (E value = 1.0×10^{-3}) as well as with the orthologous BTA001 protein of *B. turicatae* (E value = 5.0×10^{-8}). After processing, both the BHA007 and BBK32 proteins have actual masses of ~39 kDa, but their apparent masses determined by PAGE migration are ~50 kDa (61). Moreover, both BHA007 and

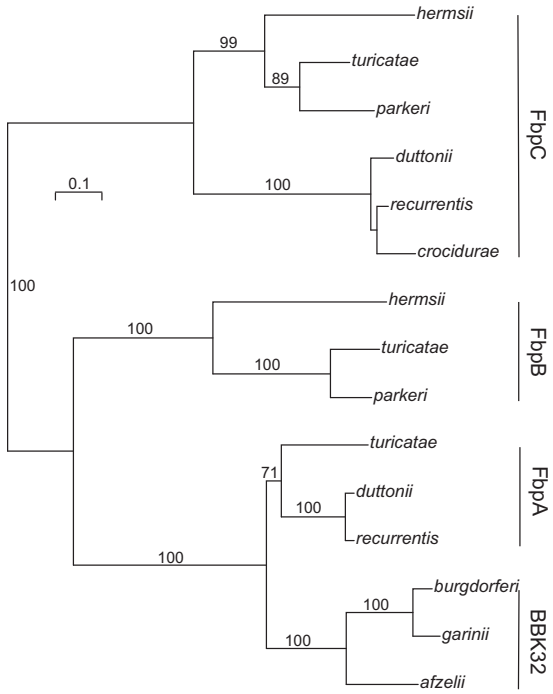

FIG 9 Distance phylogram of aligned protein sequences of three subfamilies (with proposed designations of "FbpA," "FbpB," and "FbpC") of homologous proteins of relapsing fever *Borrelia* spp. and their representation in *B. hermsii*, *B. turicatae*, *B. parkeri*, *B. duttonii*, *B. recurrentis*, and *B. crocidurae* as well as the counterparts of FbpA proteins among the BBK32-like proteins of the Lyme disease species *B. burgdorferi*, *B. garinii*, and *B. afzelii*. Nodes with ≥70% support in 1,000 bootstrap iterations of maximum likelihood inference are shown. The bar indicates amino acid distance.

BBK32 bind to other host macromolecules besides fibronectin (62).

B. hermsii also has a plasmid-borne coding sequence for a "BBK32-like" protein of 362 amino acids (GenBank accession number AAV88064). Orthologous genes are found in two other Nearctic species, *B. turicatae* (accession number KC845251) and *B. parkeri* (accession number KC845252), but not in deposited genome sequences of the Afrotropic species *B. duttonii* (accession number CP000976), *B. recurrentis* (accession number CP000993), and *B. crocidurae* (accession number CP003426). On the other hand, *B. duttonii* (accession number NC_011256), *B. recurrentis* (accession number NC_011258), and *B. turicatae* (accession number KC845248) encode what may be true orthologs of BBK32, with identity at 199 (51%) of 391 positions in the case of the *B. turicatae* ORF.

There is then relatedness among three sets of *Borrelia* proteins of ~40 kDa: (i) the BBK32 protein first observed in Lyme disease species and its orthologs in *B. turicatae*, *B. duttonii*, and *B. recurrentis*; (ii) the BBK32-like proteins found to date only in North American RF species; and (iii) the family comprising BHA007 and its orthologous counterparts in other RF species but not in Lyme disease species. We propose that these three groups constitute a larger cluster of homologous proteins. While the functional activities of the BBK32-like proteins have not been investigated, the other two sets of proteins feature binding to fibronectin and other host macromolecules. Figure 9 shows a phylogram of the aligned protein sequences of the three groups, with the BBK32 proteins of the Lyme disease species *B. burgdorferi*, *B. afzelii*, and *B. garinii* being distinguished from RF species orthologs by retention of the "BBK32" name. The fibronectin-binding domain of *B. burgdorferi* BBK32 (63), the C4b-binding protein and C1 esterase inhibitor-binding domain of *B. duttonii* CihC (45), and the fibronectin-binding domain of *B. hermsii* BHA007 (60) mapped to the same region of the protein (see Fig. S3 in the supplemental material). The RF species proteins in the 3 groups are named generically "fibronectin-binding proteins" and are further resolved into groups by the suffixes A, B, and C, assigned in order of discovery. We doubt that this will be the final word on nomenclature, but in the interim, it provides a conceptual framework for understanding similarities as well as differences between these proteins.

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