Immunological and Molecular Analyses of the *Borrelia hermsii* Factor H and Factor H-Like Protein 1 Binding Protein, FhbA: Demonstration of Its Utility as a Diagnostic Marker and Epidemiological Tool for Tick-Borne Relapsing Fever

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It has been demonstrated that *Borrelia hermsii*, a causative agent of relapsing fever, produces a factor H (FH) and FH-like protein 1 (FHL-1) binding protein. The binding protein has been designated FhbA. To determine if FH/FHL-1 binding is widespread among *B. hermsii* isolates, a diverse panel of strains was tested for the FH/FHL-1 binding phenotype and FhbA production. Most isolates (23/24) produced FhbA and bound FH/FHL-1. Potential variation in FhbA among isolates was analyzed by DNA sequence analyses. Two genetically distinct FhbA types, designated *fhbA1* and *fhbA2*, were delineated, and type-specific PCR primers were generated to allow for rapid differentiation. Pulsed-field gel electrophoresis and hybridization analyses demonstrated that all isolates that possess the gene carry it on a 200-kb linear plasmid (lp200), whereas isolates that lack the gene lack lp200 and instead carry an lp170. To determine if FhbA is antigenic during infection and to assess the specificity of the response, recombinant FhbA1 (rFhbA1) and rFhbA2 were screened with serum from infected mice and humans. FhbA was found to be expressed and antigenic and to elicit a potentially type-specific FhbA response. To localize the epitopes of FhbA1 and FhbA2, truncations were generated and screened with infection serum. The epitopes were determined to be conformationally defined. Collectively, these analyses indicate that FH/FHL-1 binding is a widespread virulence mechanism for *B. hermsii* and provide insight into the genetic and antigenic structure of FhbA. The data also have potential implications for understanding the epidemiology of relapsing fever in North America and can be applied to the future development of species-specific diagnostic tools.

Tick- and louse-borne relapsing fever are significant health concerns in regions of endemicity (4). The impact of relapsing fever on human health can be staggering. In some districts of Tanzania and Ethiopia, approximately 40% of children under the age of 1 develop tick-borne relapsing fever (TBRF), with this infection being one of the top 10 killers of children under the age of 5 (7, 38). In North America, three closely related *Borrelia* species associated with TBRF exist (*Borrelia hermsii*, *Borrelia turicatae*, and *Borrelia parkeri*) (4). Several outbreaks of TBRF with serious illness have been reported in the United States (5, 14, 36, 40, 41). However, the true incidence of infection is not known because a definitive diagnosis is typically not obtained and the disease is not frequently reported. In addition, it remains to be determined if there is a correlation between severity of disease and the species of the infecting isolate. The lack of well-characterized species-specific antigens has slowed the development of diagnostic assays, epidemiological tools, and vaccines that could be used to diagnose, track, and prevent relapsing fever.

We have identified a factor H (FH) and FH-like protein 1 (FHL-1) binding protein expressed by *B. hermsii* designated

FhbA (17, 25). The ability to bind FH and FHL-1 has important implications for the host-pathogen interaction. Pathogens that bind FH/FHL-1 exploit the regulatory activity of these proteins which serve to increase the efficiency of factor I-mediated C3b cleavage, and thus, binding FH/FHL-1 contributes to evasion of opsonophagocytosis (1, 8, 13, 15–18, 25, 27–29, 33). In this study, we demonstrate that FhbA production and the FH/FHL-1 binding phenotype is common to and shared by most *B. hermsii* isolates. FhbA sequence analyses demonstrated the existence of two distinct phyletic clusters of FhbA designated FhbA1 and FhbA2. DNA hybridization analyses established that *fhbA* is carried by lp200. Immunological analyses revealed that FhbA is antigenic during infection in mice and humans and elicits an early and potentially type-specific antibody response. Through truncation analyses, the epitopes of FhbA were determined to be conformationally defined. The analyses presented here provide insight into the genetic and antigenic structure of FhbA and indicate that the antibody response to FhbA can be of potential utility as a diagnostic marker for TBRF caused by *B. hermsii*.

MATERIALS AND METHODS

Bacterial isolates and cultivation. Table 1 lists and describes the *Borrelia hermsii* isolates analyzed in this report (kindly provided by Tom Schwan, Rocky Mountain Laboratories, NIAID, NIH). The original isolation of these isolates is described in earlier publications (3, 14, 20, 31, 36, 37). All isolates were cultivated at 33°C in Barbour-Stoenner-Kelly H complete medium supplemented to 12%

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TABLE 1. Description of *B. hermsii* isolates and data summary

Isolate	Geographic origin ^a	Source	FhbA type ^b	<i>flaB-glpQ-gyrB</i> ^c
YOR _{LP}	Siskiyou Co., Calif.	Human	2	GGII
YOR _{HP}	Siskiyou Co., Calif.	Human	2	GGII
CMC	Stevens Co., Wash.	Human	2	GGII
OKA-1, -2, -3	Okanagan Valley, BC	Human	2	GGII
REN _{HP}	Okanogan Co., Wash.	Human	<i>fhbA</i> (-)	GGII
DAH	Spokane Co., Wash.	Human	2	GGI
FRO _{LP}	Eastern Washington	Human	1	GGI
FRO _{HP}	Eastern Washington	Human	<i>fhbA</i> (-)	GGI
SWA	Kootenai Co., Idaho	Human	2	GGI
RAL	Siskiyou Co., Calif.	Human	2	GGI
GMC	Stevens Co., Wash.	Human	2	GGII
HAN	Siskiyou Co., Calif.	Human	2	GGII
SIL	Boundary Co., Idaho	Human	2	GGII
BAK	Okanogan Co., Wash.	Human	2	GGI
BYM	Kootenai Co., Idaho	Human	2	GGI
MAN	Sierra Nevada Mtns., Calif.	Human	1	GGI
FRE	Pend Oreille Co., Wash.	Human	1	GGI
EST	Larimer Co., Colo.	Chipmunk	2	GGI
GAR	Okanagan Valley, BC	Human	1	GGI
WAD	Placer Co., Calif.	Human	1	GGI
BRO	Kootenai Co., Idaho	Human	1	GGI
CON _{LP}	Sierra Nevada Mtns., Calif.	Human	1	GGI
CON _{HP}	Sierra Nevada Mtns., Calif.	Human	<i>fhbA</i> (-)	GGI
SIS	Siskiyou Co., Calif.	Tick	1	GGI
LAK	Human Lake Co., Mont.	Human	2	GGII

^a Co., county; Mtns., mountains; BC, British Columbia.

^b 1, FhbA type 1; 2, FhbA type 2; *fhbA*(-) indicates that the *fhbA* gene and FhbA protein were not detected.

^c GGI, genomic group 1; GGII, genomic group 2. Designations were assigned by Porcella et al. (31) based on multilocus sequence typing.

with rabbit serum (Sigma-Aldrich, St. Louis, Mo.) and harvested by centrifugation. Note that two different stocks of both the CON and FRO isolates were analyzed in this report. The CON_{HP} and FRO_{HP} cultures were originally obtained from Rocky Mountain Laboratories in 1993 and have since been extensively passaged. While the exact passage history of these isolates is not known, the HP subscript was added to indicate high passage. The CON_{LP} and FRO_{LP} stocks have recently been acquired from Rocky Mountain Laboratories but have not been subjected to long-term passage. The LP designation indicates low passage. The REN isolate is high passage, and it has been continuously passaged in the laboratory at least 100 times since its original isolation (Tom Schwan, personal communication).

PCR and DNA sequence analysis of *fhbA*. A 100-μl aliquot of an actively growing culture was collected and centrifuged to harvest the cells. The cells were suspended in 100 μl of water, boiled for 10 min, and briefly centrifuged, and 1 μl of the supernatant was used as a template in a 30-μl PCR. To amplify the full-length gene from all isolates, the FhbA20(+)/LIC/FhbA192(-)/LIC primer set was used. Note that the numbering used in the primer designation for *fhbA2*-targeting primers are based on the YOR sequence, while those targeting *fhbA1* genes are based on the FRE isolate sequence (Table 2). All PCRs were performed using *Taq* polymerase with reagents supplied by the manufacturer (Promega). The resulting amplicons were analyzed by agarose gel electrophoresis in 1% GTG-agarose gels with Tris-acetate-EDTA (TAE) buffer, cloned, and sequenced on a fee-for-service basis (MWG Biotech). Based on the initial sequence analyses, additional primers that would amplify in a type-specific fashion were designed. The resulting amplicons were analyzed in 2.5% Metaphor agarose gels in TAE buffer and visualized by ethidium bromide staining.

Generation of infection serum to the *B. hermsii* YOR and FRE isolates and collection of human serum samples from patients with tick-borne relapsing fever. C3H-HeJ mice were infected with the *B. hermsii* YOR or FRE isolates by intradermal inoculation between the shoulder blades (10³ spirochetes in phosphate-buffered saline). The proliferation of spirochetes in the blood (i.e., spirochetemia) was assessed by dark-field microscopic analysis of blood smears collected by tail snip at 2 and 4 days. For immunological analyses, blood was collected by tail snip at weeks 0, 4, 6, 8, and 10, and the serum was recovered. We refer to the serum recovered from all actively infected mice or humans as "infection serum." Human sera were remnants of samples submitted to the Diagnostic and Reference Laboratory (CLIA identification no. 06D0880233) of the Bacterial Zoonoses Branch, Division of Vector-Borne Infectious Diseases,

Centers for Disease Control and Prevention, Fort Collins, Colorado, for laboratory confirmation of tick-borne relapsing fever.

Immunoblot analyses. *B. hermsii* cell lysates or recombinant proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% or 15% precast criterion gels (Bio-Rad), transferred to poly-

TABLE 2. Oligonucleotide sequences

Primer	Sequence (5'-3') ^a
FhbA20(+)/LIC	<u>GACGACGACAAGATTAGCTGTGATT</u> TATTCAATAAAAAAC
FhbA48(+)/LIC	<u>GACGACGACAAGATTCAAAAACAA</u> GCTCTCATTTAC
FhbA79(+)/LIC	<u>GACGACGACAAGATTTTACAGAAA</u> AAGAAAGAAGATCC
FhbA106(+)/LIC	<u>GACGACGACAAGATCAATAAATTAC</u> TTAATGAACTTGG
FhbA135(-)/LIC	<u>GAGGAGAAGCCCGGTTTACGTGCC</u> GTCTTTAATAGACTGTAGC
FhbA165(-)/LIC	<u>GAGGAGAAGCCCGGTTTATAATTTA</u> CCATTGATATATTGCAATGC
FhbA192(-)/LIC	<u>GAGGAGAAGCCCGGTCAACTTAAG</u> TTTTTAAATATTCC
FhbA ₁ 72(+)/LIC	<u>GACGACGACAAGATTTTAGAGAAA</u> AATAAAAAAGCTCC
FhbA ₁ 128(-)/LIC	<u>GAGGAGAAGCCCGGTTTACGTGCC</u> GTCTTTGATAGACTGTAGC
FhbA ₁ 158(-)/LIC	<u>GAGGAGAAGCCCGGTTTATAATTCA</u> CCATTGATAGATTGCAATGC
FhbA ₁ specific(+)	AAACAGAAACTCAAATGCTA
FhbA ₁ specific(-)	ATTGCAAACAGGAGCTTT
FhbA ₂ specific(+)	AAAACAAAAAATTAGATGCTG
FhbA ₂ specific(-)	CTTGCAAATCAGGATCTTC

^a Underlining indicates the tail sequences incorporated into each primer to allow for annealing with LIC vectors as described in the text.

FIG. 1. Demonstration that FhbA production and the FH/FHL-1 binding phenotype are shared by most *B. hermsii* isolates. Whole-cell lysates (indicated above each lane) were generated, separated by SDS-PAGE, transferred to membranes, and tested for FH/FHL-1 binding using the ALBI approach as described in the text. Expression of FhbA was also assessed by immunoblot analyses using anti-FhbA antisera. In panel B, the ability of high-passage (HP) and low-passage (LP) cultures of some isolates to bind FH/FHL-1 and produce FhbA was assessed (as described in the text). Molecular mass markers are indicated.

vinylidene difluoride by electroblotting (19), and screened as previously described (9). Mouse anti-FhbA antiserum (17) was used at a dilution of 1:2,000. All murine and human infection sera were used at a dilution of 1:1,000. Goat anti-mouse immunoglobulin G (IgG) or goat anti-human IgG (1:40,000; Pierce) served as the secondary antibody, respectively, with detection by chemiluminescence.

FH/FHL-1 ALBI analyses. Cell lysates of bacterial strains or recombinant proteins were separated by SDS-PAGE (15% acrylamide; Bio-Rad) and electroblotted onto an Immobilon-P membrane (Millipore) (26). To identify isolates or recombinant proteins that bind FH/FHL-1, affinity ligand binding immunoblot (ALBI) assays were conducted as previously described (30). Briefly, the immunoblots were incubated with purified human FH/FHL-1 (5 ng μl^{-1} ; Calbiochem), and bound FH/FHL-1 was detected using goat anti-human FH/FHL-1 antiserum (1:1,000; Calbiochem). Rabbit anti-goat IgG (1:40,000; Pierce) served as the secondary antibody, and detection was by chemiluminescence. As a control, additional blots were screened with primary and/or secondary antibody with no exogenous FH/FHL-1 added.

PFGE and hybridization analysis. Agarose plugs containing bacterial cells from 50-ml *B. hermsii* cultures were prepared as previously described for pulsed-field gel electrophoresis (PFGE) (17). Electrophoresis was conducted using the Bio-Rad contour-clamped homogenous electric field mapper system with 1% GTG-agarose gels in 0.5 $\times$ Tris-boric acid-EDTA buffer at 14°C as previously described (17). The DNA was then transferred onto a Hybond-N membrane

using the VacuGene vacuum blotting system (Pharmacia) and hybridized as previously described (22). The hybridization probe was generated by PCR amplification of *fhbA* from *B. hermsii* YOR and radioactively labeled using the Prime-A-Gene labeling system (Promega) with [α - ^{32}P]dATP (6,000 Ci mM^{-1}) as instructed by the manufacturer.

Production of recombinant proteins using LIC methodologies. All recombinant proteins were generated using a PCR-based strategy and ligase-independent cloning (LIC) methodologies. To generate a template for PCR, 100 μl of *B. hermsii* cells was recovered from actively growing cultures by centrifugation, washed, suspended in water, and boiled to lyse the cells. One microliter of the supernatant was used as a template in each PCR. *fhbA1* and *fhbA2* genes were amplified from the *B. hermsii* FRE and YOR isolates, respectively, using the primers described in Table 2. All primers were synthesized with extensions that allow for annealing into the pET32-Ek/LIC vector (Novagen). PCR was performed using *Taq* polymerase (Promega), and the resulting amplicons were treated with T4 DNA polymerase to generate single-stranded overhangs and annealed into the pET32-Ek/LIC vector as instructed by the supplier (Novagen). The N-terminal tag (which contains both S and His tags) adds approximately 17 kDa to the mass of the resulting fusion proteins. The resulting recombinant plasmids were propagated in *Escherichia coli* NovaBlue(DE3) cells and screened for the desired insert by PCR. Selected colonies were cultivated overnight at 37°C in LB broth (containing ampicillin at 50 $\mu\text{g ml}^{-1}$) with shaking (200 rpm). Protein production was induced without the addition of isopropyl- β -D-thiogalacto-

A.

B.

FIG. 2. Sequence analysis of FhbA in diverse strains: demonstration of two phyletic groups. *fhbA* sequences were determined as described in the text, and the corresponding amino acid sequences were aligned (A). The isolate origin for each sequence is indicated by a subscript. Identical positions are denoted by dots, and gaps introduced by alignment are shown by dashes. For reference, the putative FH/FHL-1 binding loop domain is indicated by underlining. A dendrogram was constructed using the translated sequences and is presented in panel B. The isolate of origin for each sequence is indicated at the end of the branch. Branch length values are given above each branch (amino acid substitutions per 100 residues), and bootstrap values (1,000 trials) are indicated at the nodes.

TABLE 3. Pairwise sequence comparison

Isolate	% Identity or similarity to ^a :									
	YOR	CMC	EST	OKA1	OKA2	DAH	SIL	BAK	FRE	GAR
YOR		100	100	99.4	99.4	98.8	93.6	81.5	80.3	76.9
CMC	100		100	99.4	99.4	98.8	93.6	81.5	80.3	76.9
EST	100	100		99.4	99.4	98.8	93.6	81.5	80.3	76.9
OKA1	100	100	100		98.8	98.3	93.1	80.9	79.8	76.3
OKA2	100	100	100	100		98.3	93.1	80.9	79.8	76.3
DAH	100	100	100	100	100		93.1	82.1	80.9	77.5
SIL	95.4	95.4	95.4	95.4	95.4	95.4		83.7	82.5	79.5
BAK	87.9	87.9	87.9	87.9	87.9	87.9	91.6		98.8	94.6
FRE	86.7	86.7	86.7	86.7	86.7	86.7	90.4	98.8		93.4
GAR	83.2	83.2	83.2	83.2	83.2	83.2	86.7	94.6	93.4	

^a Percent amino acid identity values are presented in the upper half of the table, and percent amino acid similarity values are given in the lower half of the table.

pyranoside (IPTG). Production of the proteins was verified by screening immunoblots with horseradish peroxidase (HRP)-conjugated S protein (Novagen) (9). The sequence of all constructs was confirmed through automated DNA sequencing (MWG Biotech) of one to three *E. coli* clones for each gene sequenced.

Nucleotide sequence accession number. The sequences determined as part of this report have been deposited in the NCBI databases. The FhbA2 sequences for the CMC, EST, OKA-1, OKA-2, DAH, and SIL isolates are DQ630545, DQ630542, DQ630543, DQ630544, DQ630546, and DQ630547, respectively. The FhbA1 sequences for the BAK, FRE, and GAR isolates are DQ630541, DQ630548, and DQ630540, respectively.

RESULTS

Demonstration that FhbA production and FH/FHL-1 binding is common to most *B. hermsii* isolates. Cell lysates of 24 *B. hermsii* isolates were immunoblotted and tested for FH/FHL-1 binding and production of FhbA. FH/FHL-1 binding was determined using an ALBI assay format. Of the 24 isolates tested in Fig. 1A, all except the REN isolate are considered to be low passage. All of the low-passage isolates bound FH/FHL-1 and produced FhbA, while the REN isolate did not (Fig. 1A). To determine if the FH/FHL-1 binding phenotype can change with prolonged cultivation, high- and low-passage cultures of the CON, FRO, and YOR isolates were assessed for binding (Fig. 1B). The high-passage CON and FRO cultures lost the ability to bind FH/FHL-1 and, consistent with that, did not produce detectable FhbA. The high-passage YOR isolate retained the FH/FHL-1 binding phenotype and produced FhbA. It can be concluded from these analyses that the FH/FHL-1 binding phenotype and FhbA production is a feature common to low-passage isolates of *B. hermsii* and that this ability can be lost with long-term cultivation.

Evolutionary analyses of FhbA sequences: delineation of two distinct phyletic types. In an earlier study, we identified two distinct variants of FhbA (18). To compare the relationships among FhbA sequences of different strains, PCR and subsequent DNA sequence analyses were performed. The amplicons from 10 isolates were cloned into the pET-32 Ek/LIC vector, transformed, and propagated in *E. coli* NovaBlue(DE3) cells. The inserts were sequenced, and the determined sequences were translated and aligned (Fig. 2A). Dendrogram construction revealed two distinct FhbA phyletic groups (Fig. 2B) that we designate the FhbA1 and FhbA2 groups. Proteins of the FhbA2 group (typified by the YOR isolate) have a greater mass than FhbA1 proteins (typified by the FRE isolate) due to

the presence of a tandem repeat (LLKTLDN) in the N-terminal domain of the FhbA2 proteins. The SIL isolate is an exception. While its FhbA sequence clearly lies in the FhbA2 group, it lacks the repeat. Pairwise sequence comparisons revealed that within a phyletic cluster, FhbA is highly conserved (Table 3). The amino acid identity values and similarity values among the FhbA2 proteins were $\geq 93.1\%$ and $\geq 95.4\%$, respectively. The identity and similarity values for FhbA1 proteins were $\geq 93.4\%$. Throughout this report, proteins belonging to these groups are referred to simply as FhbA1 or FhbA2, and where necessary, the isolate of origin is indicated by a subscript. The numerical designations assigned to each were selected to be as consistent as possible with the genomic group nomenclature proposed by Porcella et al., who recently identified two *B. hermsii* genomic groups, designated groups I and II (31).

The evolutionary relationships drawn from the *fhbA* sequence analyses are generally in good agreement with the phyletic groups inferred from the analysis of chromosomal loci, but some exceptions were noted (31). Of the nine sequences determined, the EST and DAH isolates which have FhbA2 type sequences, and thus would be predicted to belong to genomic group II, were classified by Porcella and colleagues as genomic group I isolates. The possible basis for the difference in clustering patterns collected from the *fhbA* plasmid-carried locus and the chromosomal loci analyzed by Porcella et al. is discussed in detail below.

The *fhbA* gene is carried by a linear plasmid of 200 kb. We previously demonstrated that the YOR, MAN, and DAH isolates carry the *fhbA* gene on lp200 (17). To determine if the genomic location of *fhbA* may differ among strains, PFGE was performed on 13 isolates and the fractionated DNA was transferred onto membranes for hybridization analyses. The membrane was screened with a full-length gene probe generated by PCR of *fhbA2* from the YOR isolate (Fig. 3). Based on the overall nucleotide identity between *fhbA1* and *fhbA2*, it was expected that this probe would hybridize with both *fhbA* types, and consistent with this, the probe detected an *fhbA*-related sequence in all isolates except the REN isolate. Analysis of the ethidium bromide-stained gels revealed that the REN isolate lacked lp200 and instead carried an lp170. In an earlier study in which high-passage cultures of the CON and FRO isolates were analyzed, we noted that these isolates lacked lp200 and instead carried lp170 (17). However, it is clear that the low-passage cultures of the CON and FRO isolates carry lp200 and

FIG. 3. Plasmid analysis of *B. hermsii* isolates and demonstration that *fhbA* is carried by lp200. DNA from representative *B. hermsii* isolates (indicated above each lane) were fractionated by PFGE, the gel was stained with ethidium bromide (left panel), and the DNA was transferred onto membranes for hybridization analysis with a labeled *fhbA2* probe (right panel). All methods are described in the text. DNA size markers (in kilobases) are indicated.

lack lp170. The data suggest that rearrangement has occurred during extended cultivation and is thus the most likely basis for the lack of FH/FHL-1 binding exhibited by high-passage cultures of REN, CON, and FRO. The loss of the *fhbA* coding region of lp200 in multiple strains upon extended cultivation suggests that the molecular events that led to truncation of lp200 are not random.

Analysis of the specificity and temporal development of the antibody response to FhbA during infection in mice. To determine if FhbA is expressed and elicits an antibody response during experimental infection of mice, serum from mice infected with the FhbA1- and FhbA2-producing *B. hermsii* FRE and YOR isolates, respectively, were assayed for anti-FhbA antibody. The mice were infected by needle inoculation, and infection was confirmed by analysis of blood smears collected at days 2 and 4. All mice were found to harbor spirochetes in the blood, with most spirochetes attached end-on to red blood cells. The interaction of *B. hermsii* and other relapsing fever spirochetes with red blood cells has also been reported by others (2, 6). Blood samples were recovered from each mouse at weeks 4, 6, 8, and 10 postinoculation and assayed via immunoblotting for an antibody response to FhbA. The test antigens for these analyses consisted of rFhbA1_{FRE} and rFhbA2_{YOR}. A truncated rBBN39, a member of paralogous protein family 163 of the Lyme disease spirochetes, served as a negative control. The integrity, quality, and relative loading of the test antigens were demonstrated by screening an immunoblot with HRP-conjugated S protein (Fig. 4). In addition, an immunoblot was screened with anti-FhbA antiserum. This antiserum, which was generated using the FhbA2_{YOR} protein, reacted with both proteins but displayed preferential reactivity with rFhbA2 over rFhbA1. A series of immunoblots were then screened with serum harvested at weeks 4, 6, 8, and 10 from mice infected with either the FRE or YOR isolates which produced FhbA1 and FhbA2 proteins, respectively. A strong IgG response was detected to FhbA that was readily apparent by week 4 and that

persisted through week 10. The antibody response to FhbA elicited in the YOR-infected mice was FhbA2 type specific. In contrast, the response elicited by the FRE isolate recognized both FhbA1 and FhbA2. Analysis of the FhbA sequences revealed that the FhbA proteins of these isolates may differ in structure (as inferred from coiled-coil analyses) and thus potentially present different sets of epitopes. The specificity of the response elicited by the YOR isolate suggests that the residues forming the epitopes exposed during infection most likely reside in the N-terminal, type-specific domain of the FhbA protein (see the sequence alignments presented in Fig. 2), while at least some of the epitopes of FhbA1 reside within conserved domains of the protein. However, it is equally plausible that the specificity of the response seen in the YOR-infected mice simply reflects differences in the antibody response elicited by individual mice.

FhbA elicits a potentially diagnostic antibody response during natural infection in humans. To determine if an antibody response is elicited to FhbA in naturally infected humans, rFhbA1_{FRE} and rFhbA2_{YOR} were screened with serum from all human tick-borne relapsing fever patients available to us ($n = 10$). All serum samples were immunoreactive with one or both rFhbA proteins (Fig. 5). No reactivity was observed with the rBBN39 negative control or with serum samples from Lyme disease patients (data not shown) or healthy individuals (control serum). One patient serum (052837) was immunoreactive only with FhbA1, while other patient sera displayed preferential immunoreactivity with FhbA1 over FhbA2 (003584, 041822, and 002996), preferential immunoreactivity with FhbA2 over FhbA1 (041556 and 011830), or equal detection of both (010159, 0032224, 003385, and 031960). None of the serum samples tested displayed specific immunoreactivity with FhbA2 alone.

Identification of the immunodominant epitopes of FhbA presented during infection of mice and humans. To localize the epitopes of FhbA1 and FhbA2 that are presented during

FIG. 4. Demonstration of an FhbA type-specific antibody response in infected mice. rFhbA1_{FRE}, rFhbA2_{YOR}, and rBBN39 (negative control) were fractionated by SDS-PAGE, immunoblotted, and screened with HRP-conjugated (conj.) S protein, anti-FhbA antiserum, or serum collected from mice infected with either the *B. hermsii* YOR or FRE isolate at different time points during infection (as indicated on the figure). Blots screened with HRP-conjugated S protein and mouse anti-FhbA antiserum verify expression, integrity, and relative loading of the recombinant proteins. All methods are described in the text. Molecular mass markers are indicated.

infection and to determine if these epitopes differ in mice versus humans, a panel of N- and C-terminal truncations of each protein were generated and screened with either infection sera from mice or humans. Data pertaining to FhbA2 are presented in Fig. 6. Full-length FhbA2 (residues 20 to 192) and the fragment consisting of residues 48 to 192 were detected by the *B. hermsii* YOR infection serum and by human infection serum shown in Fig. 5 to harbor antibody to FhbA2. Serum sample 052837, which we demonstrated was specific for FhbA1, did not react with any of the FhbA2 fragments. Further truncation of the N-terminal domain led to the loss of antibody binding. Truncation of the C terminus of FhbA2 also led to the elimination of infection antibody binding. The same pattern of reactivity with analogous FhbA1 fragments was observed in identical analyses (data not shown). Collectively, these analy-

ses revealed that all patient sera analyzed harbor antibody to FhbA and that FhbA N- and C-terminal determinants are required for antibody recognition. This suggests that the immunodominant epitopes are conformationally defined.

Development of *fhbA1*- and *fhbA2*-specific primers. To develop a rapid approach for determining *fhbA* type in newly identified strains, type-specific PCR primer sets were developed. To verify the specificity of the primers, PCR was performed using cloned copies of *fhbA1* and *fhbA2* (pET32-*fhbA1* and pET32-*fhbA2*, respectively) as templates. With these templates, the primer sets amplified in a completely type-specific fashion (Fig. 7). The primers were then used to screen the panel of isolates employed in this study. Several isolates were PCR positive for *fhbA1* or *fhbA2*, while others yielded amplicons with both primer sets. The fact that multiple FhbA pro-

FIG. 5. FhbA elicits an FhbA type-specific response during infection in humans. Recombinant proteins were fractionated by SDS-PAGE, immunoblotted, and screened with HRP-conjugated S protein, control serum, or serum samples collected from patients with tick-borne relapsing fever (as indicated). rBBN39 served as a negative control. All methods are described in the text. Molecular mass markers are indicated.

FIG. 6. Demonstration that the immunodominant epitopes of FhbA that are presented during infection of mice and humans are conformationally defined. rFhbA_{YOR} and recombinant proteins spanning the residues indicated above each lane were generated and analyzed by SDS-PAGE. Immunoblots of the proteins were screened with HRP-conjugated S protein, mouse infection serum, and human serum samples (as indicated on the figure). All methods are described in the text. Molecular mass markers are indicated.

teins (i.e., FhbA1 and FhbA2) are not observed to be produced by any of the isolates analyzed in this report (as inferred from the immunoblots presented in Fig. 1), suggests that if the populations are mixed, then one subpopulation dominates over the other in the cultured spirochetes. These data raise the possibility that some isolates recovered from humans are genetically heterogeneous, at least in regard to the *fhbA* locus and lp200.

DISCUSSION

The ability of *B. hermsii* to bind FH/FHL-1 to its surface and cleave C3b suggests that this activity is an important virulence mechanism that may contribute to immune evasion (17, 18, 25). However, prior to this report, FH/FHL-1 binding by *B. hermsii* had been examined in only a small number of isolates (17), some of which were high-passage strains that appear to

FIG. 7. Development of type-specific *fhbA* primers and evidence that some isolates represent mixed populations. Recombinant plasmids carrying *fhbA1* (pET32-*fhbA1*) or *fhbA2* (pET32-*fhbA2*) inserts were used to establish the specificity of the primer sets (indicated to the right of each panel). Once established, these primers were used to amplify *fhbA* from various *B. hermsii* isolates (indicated above each lane). The resulting amplicons were analyzed by electrophoresis in 2.5% Metaphor agarose gels and visualized by ethidium bromide staining. All methods are described in the text. Molecular size markers are indicated.

have undergone genetic changes during long-term cultivation. Genetic rearrangements in linear plasmids of *B. hermsii* (12) and the closely related species *B. turicatae* have been demonstrated with laboratory cultivation (34). Similarly, changes in plasmid profile both during *in vitro* cultivation (21, 32, 35) and during passage of clonal populations through mice (24) have been demonstrated for the Lyme disease spirochetes. Hence, in this report we investigated FH/FHL-1 binding and FhbA production by an extensive panel of *B. hermsii* isolates from different geographical regions and biological sources. Of the 24 different isolates screened, the only isolates that did not bind FH/FHL-1 and produce FhbA were the REN isolate and high-passage cultures of the CON and FRO isolates. While the specific passage number of the CON and FRO isolates is not known, the REN isolate had been passaged 100 times. While a low-passage culture of REN was not available for analysis, low-passage cultures of CON and FRO were, and both bound FH/FHL-1 and produced FhbA. In summary, it can be concluded that FhbA-mediated FH/FHL-1 binding is a phenotype shared by the vast majority of *B. hermsii* isolates and that this phenotype can change upon prolonged cultivation.

Earlier FhbA sequence analyses revealed that variation was localized primarily within the N-terminal domain of the protein (18). To further define the relationship among FhbA sequences, the *fhbA* gene was PCR amplified from several strains and sequence analyses were conducted. Two distinct *fhbA* phyletic groups or FhbA protein types were identified that we designate FhbA1 and FhbA2. Based on an analysis of three chromosomal loci (*flaB*, *glpQ*, and *gyrB*), Porcella et al. recently demonstrated the existence of two distinct genomic groups of *B. hermsii* (31). The *fhbA* clustering patterns delineated in this report were in most cases consistent with the genomic relationships inferred from analysis of the *flaB*-*glpQ*-*gyrB* loci (see Table 1); however, exceptions were noted for 8 of the strains. It is most likely that the chromosomal loci, which are more stable than that of plasmid-carried loci, are better predictors of evolutionary relationships. One possibility to explain the differences in predicted relationships observed in plasmid versus chromosomal loci could be lateral transfer of the *fhbA*-carrying plasmid or a portion thereof. This possibility is discussed below.

As discussed above, high-passage derivatives of the CON and FRO isolates do not produce FhbA and lack FH/FHL-1 binding ability (24). We postulated that long-term propagation in the laboratory may have led to genetic changes that resulted in the loss of *fhbA* and thus FH/FHL-1 binding. Comparison of the previously published plasmid profiles of the high-passage derivatives of the CON and FRO isolates (17) with the low-passage derivatives analyzed here revealed that plasmid rearrangement has occurred. The *fhbA*-carrying lp200 present in the low-passage CON and FRO isolates is absent from the high-passage derivatives, which instead carry an lp170 (17). Similarly, the high-passage REN isolate, which does not produce FhbA, lacks lp200 but carries lp170. While the molecular basis for these plasmid changes are yet to be determined, it is interesting that the genes encoding the FH-binding OspE proteins of the Lyme disease spirochetes are present on multicopy 32-kb circular DNA elements (23, 39) that are prophage (10, 11, 42). At least one of these prophage has integrated into a linear plasmid of *Borrelia burgdorferi*. It is possible that *fhbA*,

like the *ospE* paralogs, is carried by an ~30-kb prophage that has integrated into lp170 to form the *fhbA*-carrying lp200. Thus, lateral transfer of a putative *fhbA*-carrying bacteriophage could explain the difference in evolutionary relationships inferred from the analysis of plasmid-carried *fhbA* sequences with that of more stable chromosomal loci. Future analyses will seek to assess this hypothesis and test for the possible existence of bacteriophage in the relapsing fever spirochetes.

While the molecular basis of the interaction between FH/FHL-1 and FhbA has been investigated (18), little is known regarding the antibody response to FhbA and the determinants required for immune recognition. It is clear that both FhbA1 and FhbA2 are expressed during infection and are antigenic, as a strong and specific IgG response was readily detected in infected mice by week 4. Similarly, all serum samples collected from individuals with tick-borne relapsing fever had antibody to either FhbA1, FhbA2, or both. A noteworthy observation was the apparent specificity of the response to FhbA2 in mice. In spite of extended regions of homology between FhbA1 and FhbA2, the mouse infection sera generated upon infection with the YOR isolate reacted only with the corresponding FhbA2 protein. However, the converse was not observed, in that serum generated by infection with the FhbA1-producing FRE strain detected both FhbA1 and FhbA2. Most human patient serum displayed immunoreactivity with both FhbA1 and FhbA2, with the notable exception of serum sample no. 052837, which detected FhbA1 but not FhbA2. There are several possibilities to explain these observations. One is that the differences in structure of FhbA1 and FhbA2 result in differential presentation of epitopes. One of the notable differences in the predicted structure of FhbA2 is the potential for the formation of multiple coiled-coil domains, including two strong coiled-coil regions in the N terminus of the protein that are not predicted in the FhbA1 sequence. However, this suggestion is speculative, and it is equally plausible that the differences seen are simply due to differences in the antibody response among individual mice and human patients. In the human patients, it is possible that these individuals were infected with mixed populations of spirochetes that consist of a subset expressing FhbA1 and a subset expressing FhbA2. In any event, the central point is that, in all cases, an antibody response to FhbA was elicited during infection, supporting the hypothesized functional role of FhbA in the mammalian host. It remains to be determined if FhbA is expressed in ticks.

While the determinants of FhbA that are involved in FH/FHL-1 binding have been partially defined (18), nothing is known concerning the antigenic structure of FhbA. A prominent loop structure, formed by the interaction of two antiparallel coiled-coil domains, is required for FH/FHL-1 binding and appears to be the contact point for FhbA's interaction with FHL-1 and FH (18). To identify the immunodominant epitopes of FhbA1 and FhbA2 presented during infection, N- and C-terminal truncations of these proteins were generated and screened with serum from infected mice and humans. Consistent with that observed for FH/FHL-1 binding to FhbA, domains in both the N-terminal half of the protein and the C terminus were required for antibody binding. The fact that widely separated domains of FhbA are required for antibody recognition indicates that the immunodominant epitope(s) of

FhbA is conformationally defined. This is consistent with studies of the immunodominant epitopes of the FH-binding OspE protein family of the Lyme disease spirochetes which have been demonstrated to be conformational (26, 30). With both FhbA and OspE, antibody binding was found to be highly sensitive to relatively short C-terminal truncations. FhbA and OspE are both predicted to have C-terminal domains that form coiled coils, and hence, this structural element may be a common feature involved in epitope presentation in FH/FHL-1 binding proteins.

The antibody response to FhbA may be of diagnostic utility. Although specific titers to FhbA elicited during infection in humans were not determined as part of this report (due to the limited volume of serum available for analysis), the level of detection observed using human serum samples collected during early infection and diluted 1:1,000 in the immunoblot assays suggests that the response is robust and that FhbA is a dominant antigen. This coupled with the apparent specificity of the anti-FhbA response, which is not cross-immunoreactive with *B. turicatae*, *B. parkeri*, or other spirochetes (17), suggests that an FhbA-based serological assay would be specifically diagnostic for *B. hermsii*-induced tick-borne relapsing fever. We have also developed a PCR-based approach for differentiating the FhbA types which could be of epidemiological or diagnostic utility. These type-specific assays could also be applied in determining which specific species of *Borrelia* are predominantly associated with human disease in North America and in assessing potential correlations between *B. hermsii* FhbA type and incidence or severity of disease. Future analyses will explore such potential correlations and seek to exploit the diagnostic utility of FhbA.

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