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Hemin-Binding Surface Protein from *Bartonella quintana*

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*Bartonella quintana*, the agent of trench fever and a cause of endocarditis and bacillary angiomatosis in humans, has the highest reported in vitro hemin requirement for any bacterium. We determined that eight membrane-associated proteins from *B. quintana* bind hemin and that a ~25-kDa protein (HbpA) was the dominant hemin-binding protein. Like many outer membrane proteins, HbpA partitions to the detergent phase of a Triton X-114 extract of the cell and is heat modifiable, displaying an apparent molecular mass shift from approximately 25 to 30 kDa when solubilized at 100°C. Immunoblots of purified outer and inner membranes and immunoelectron microscopy with whole cells show that HbpA is strictly located in the outer membrane and surface exposed, respectively. The N-terminus of the mature HbpA was determined and used to clone the HbpA-encoding gene (*hbpA*) from a lambda genomic library. The *hbpA* gene is 816 bp in length, encoding a predicted immature protein of approximately 29.3 kDa and a mature protein of 27.1 kDa. A Fur box homolog with 53% identity to the *Escherichia coli* Fur consensus is located upstream of *hbpA* and may be involved in regulating expression. BLAST searches indicate that the closest homologs to HbpA include the *Bartonella henselae* phage-associated membrane protein, Pap31 (58.4% identity), and the OMP31 porin from *Brucella melitensis* (31.7% identity). High-stringency Southern blots indicate that all five pathogenic *Bartonella* spp. possess *hbpA* homologs. Recombinant HbpA can bind hemin in vitro; however, it does not confer a hemin-binding phenotype upon *E. coli*. Intact *B. quintana* treated with purified anti-HbpA Fab fragments show a significant (*P < 0.004*) dose-dependent decrease in hemin binding relative to controls, suggesting that HbpA plays an active role in hemin acquisition and therefore pathogenesis. HbpA is the first potential virulence determinant characterized from *B. quintana*.

Trench fever is an arthropod-borne disease caused by *Bartonella quintana* and occurs about 10 days following the bite of an infected body louse (*Pediculus humanus*) (14). The morbidity impact of trench fever was second only to influenza in terms of lost man-hours during World War I, and thousands of troops were debilitated by the disease (56). Following a period of quiescence, trench fever reappeared during World War II (32) and appeared sporadically for the next four decades. Although the symptoms of trench fever can vary, the disease usually presents with mild to moderately severe fever, chills, malaise, myalgia, and bone pain that is prominent in the tibia (hence the nickname “shinbone fever”) (65). Occasionally, patients develop splenomegaly and a maculopapular rash resembling the rose spots of typhoid fever (56). Trench fever generally lasts about 1 week, but some cases can persist for up to 12 weeks with recurrent febrile episodes and protracted bacteremia (39).

*B. quintana* is currently reemerging as an etiologic agent primarily afflicting homeless, alcoholic males that live within the inner cities of the United States and Europe (28). Although many cases of “urban trench fever” present with symptoms of the classical disease (27), the pathogen can also cause potentially fatal bacillary angiomatosis (31), endocarditis (53), lymphadenopathy (31), infections of the central nervous system (45), and lytic bone lesions (30, 31). In addition, *B. quintana* infections have been reported in both immunocompro-

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* was grown overnight at 37°C in Luria-Bertani (LB) medium using standard antibiotic supplements when re-
**B. QUINTANA HEMIN-BINDING PROTEIN**

TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
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<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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</thead>
<tbody>
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<td><strong>Strains</strong></td>
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<td>Bartonella spp.</td>
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<td>CDC*</td>
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<td><strong>Plasmids</strong></td>
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<td>pBK-CMV</td>
<td>Excised vector from λ Zap</td>
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<td>pHBP-CMV</td>
<td>pBK-CMV plus 3.5-kbp Sau3A1 insert with hbpA</td>
<td>This study</td>
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* CDC, Centers for Disease Control and Prevention, Atlanta, Ga.

**Identification of *B. quintana* HBPs.** Eight HBPs of 11, 12, 19, 25 or 30 (depending upon solubilization temperature), 29, 36, 42, and 87 kDa were identified on hemin blots containing total cell lysates of *B. quintana* (Fig. 1A). HBPs with identical molecular masses were also observed in hemin blots of total membranes purified from *B. quintana* (Fig. 1B). Of the eight HBPs, a prominent HBP band of 25 kDa (termed HbPA) was observed to shift from approximately 25 to 30 kDa when heated, thus displaying a common characteristic of outer membrane proteins (see Discussion). The HbPA band also demonstrated the highest affinity for hemin relative to the seven other HBPs, based upon its unique ability to retain bound hemin after a 24-h wash with TBS (data not shown). In addition, the HbPA band was the only protein that was visibly brown on blots probed with hemin, prior to ECL detection (data not shown). Taken together, these data formed the basis of our hypothesis that HbPA was an HBP located in the outer membrane of *B. quintana*.

**Anti-HbpA antibodies and localization of HbpA in the *B. quintana* cell.** To characterize HbPA, whole-cell lysates were subjected to phase partitioning with Triton X-114. HbPA was observed predominantly in the detergent phase, which is typ-
ical of integral membrane proteins (9, 18). This phase was found to contain two dominant proteins, HbpA and a 36-kDa polypeptide, plus minor proteins of 33, 42, 46, and 87 kDa (Fig. 2A, lane 2). The Triton X-114 detergent fraction was further separated by SDS-PAGE and HbpA bands were excised from unfixed, Coomassie blue-stained gels to generate rabbit polyclonal antibody. Specificity of the anti-HbpA antibody for HbpA was verified using immunoblots (Fig. 2B). The antibody was able to recognize both the 25- and the 30-kDa forms of the molecule (Fig. 2C).

To verify HbpA’s suspected outer membrane location in the cell, inner and outer membranes were isolated from B. quintana in a manner similar to that described for B. bacilliformis (39) using sucrose density gradient centrifugation (44). The average buoyant density values (ρ) were determined from three membrane preparations and were calculated as 1.08 for the inner membrane and 1.2 for the outer membrane. These density values are very close to those reported for E. coli (42) and Salmonella spp. (44). Likewise, the characteristic tea-color of the B. quintana inner membrane, due to cytochromes, and white flocculence of the outer membrane was in keeping with characteristics of the respective membranes from E. coli. The protein profiles of the outer and inner membranes were distinct on SDS-PAGE (Fig. 3A). Immunoblot analysis using anti-HbpA antiserum detected HbpA in the outer membrane but not in the inner membrane (Fig. 3B). To corroborate these data, immunogold analyses were done using anti-HbpA and intact bacteria to determine if HbpA is surface exposed. The data clearly show that HbpA is both exposed and abundant on the surface of the B. quintana cell (Fig. 4). Immunogold controls prepared with an equal volume of PBS or preimmune rabbit serum showed insignificant protein A-gold binding (not shown).

In vivo hemin binding by HbpA and inhibition with Fabs. A standard liquid hemin-binding assay was done with freshly harvested B. quintana. Untreated B. quintana cells bound approximately 22% of exogenous hemin (1.4 μg of hemin/mg of

FIG. 1. Identification of hemin-binding proteins of B. quintana using hemin blots. (A) Total cell lysates. (B) Total membranes. Samples were solubilized in LSB at 24 or 100°C as indicated and then separated by SDS-PAGE, transferred to nitrocellulose, probed with hemin, and developed using ECL reagents (Amersham Pharmacia) as described in Materials and Methods. The position of the two forms of HbpA are indicated by asterisks. Molecular mass standards are given to the left in kilodaltons.

FIG. 2. Monospecificity of the anti-HbpA antibody and reactivity against both molecular mass forms of HbpA. (A) Coomassie blue-stained SDS-PAGE gel containing a B. quintana cell lysate (lane 1) and a phase-separated Triton X-114 cell extract of the bacterium (lane 2). Both samples were solubilized at 100°C in LSB. HbpA is arrowed. (B) Immunoblot corresponding to panel A but developed with rabbit anti-HbpA antisera showing monospecificity of the anti-HbpA antibody. (C) Immunoblot showing anti-HbpA antibody reactivity to phase-separated Triton X-114 cell extracts of B. quintana solubilized at 25°C (lane 1) and 100°C (lane 2). Note that the antibody recognizes both molecular mass forms of HbpA in panel C. Molecular mass standards are given to the left in kilodaltons.

FIG. 3. Localization of HbpA in the outer membrane of B. quintana. Lanes: 1, cell lysate; 2, purified inner membrane; 3, outer membrane. (A) Coomassie blue-stained SDS-PAGE gel. (B) Corresponding immunoblot developed with anti-HbpA showing HbpA in the outer membrane (arrowed). All samples were solubilized at 100°C in LSB. Molecular mass standards are given to the left in kilodaltons.
B. quintana protein) during a 60-min assay; this percentage falls within the range of hemin binding exhibited by several human bacterial pathogens (20, 26, 43, 51). B. quintana pretreated with anti-HbpA Fab fragments showed a very significant (P < 0.004), dose-dependent decrease in hemin binding, relative to controls treated with an equal volume of PBS. For example, B. quintana treated with 0.2 mg of Fab per ml showed a 26% decrease in hemin binding relative to its respective PBS control, whereas B. quintana treated with 0.4 mg of Fab per ml exhibited a 41% decrease in hemin binding relative to PBS controls (Fig. 5). These data show a dose-dependent decrease in hemin binding by B. quintana when cells are treated with anti-HbpA Fabs prior to the liquid hemin-binding assay.

Cloning the hbpA gene. The N terminus of mature HbpA was determined from two separate samples and was found to be ADVIATHEAAPVITTPNF. BLASTp searches with this amino acid sequence showed a high probability hit (63% identity) with the Pap31 protein from B. henselae (10). This discovery prompted us to design PCR primers based upon the first 31 nucleotides and the last 25 nucleotides (inverse complement) of the pap31 open reading frame (ORF) (GenBank accession no. AF001274). The pap31 primers produced PCR products of identical size (~840 bp) from both B. henselae and B. quintana DNA templates (data not shown). The B. quintana amplicon was subsequently used to screen a lambda ZAP Express (Stratagene) genomic library of B. quintana for hbpA. A set of positive plaques was identified, isolated, and tested by PCR to determine if a full-length copy of hbpA was present. A positive lambda clone that contained the full-length hbpA gene was excised in vivo to produce pHPB-CMV. This plasmid contains a Sau3AI insert of approximately 3.5 kbp.

Nucleotide sequence of hbpA. The sequence of hbpA was determined from both strands of pHPB-CMV (Fig. 6). The B. quintana hbpA gene is 816 bp long and has a 39.1 mol% G+C, a level in close agreement with the 38.5 mol% G+C for the B. quintana genome (64). The region upstream of the start codon of hbpA contains a consensus promoter sequence (0.92 score by prokaryotic promoter neural network prediction) and a potential ribosome-binding site with perfect identity to the E. coli consensus sequence, AGGA (23). A Fur box homolog is nested within the predicted promoter sequence of hbpA and has 53% identity to the E. coli Fur consensus (21). The hbpA ORF is followed 37 bp downstream by a 6-bp inverted repeat that may act as a rho-independent transcriptional terminator.

Computer analysis of the encoded HbpA protein predicts that it has 272 amino acids, a molecular mass of 29,270 Da, and a pI of approximately 9.5. The predicted protein also contains an internal 18-amino-acid stretch that is identical to the N terminus as determined from HbpA protein (Fig. 6). A predicted secretory signal cleavage site (67) was found between residues 21 and 22; one residue upstream of the sequence that was determined by Edman degradation (Fig. 6). Proteolytic cleavage at the actual peptidase cleavage site would yield a mature, secreted protein of 27,098 Da. Transmembrane computer predictions (TMPred program; ISREC server) identified three potential transmembrane helices in mature HbpA, encompassing amino acid residues 34 to 55, 74 to 101, and 218 to 236, that may serve to anchor HbpA to the outer membrane. Finally, the predicted HbpA sequence has a C-terminal phenylalanine, a characteristic of most integral outer membrane proteins (57).

Further analysis of the predicted full-length HbpA protein was done using BLAST 2.0. The highest-probability alignments resulting from this search included the Pap31 phage-associated membrane protein from B. henselae (10) with 58.4% identity, followed by the OMP31 porin protein of Brucella melitensis (66) with 31.7% identity. Alignments of these three protein

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**FIG. 4.** Immunoelectron microscopy showing surface localization of HbpA. Immunogold analysis and negative staining were done as previously described (50), using anti-HbpA and intact bacteria. Bar, 0.5 μm.

**FIG. 5.** Anti-HbpA Fab fragments inhibit hemin binding by B. quintana. The percent hemin uptake as a function of treatment volumes is shown. B. quintana was pretreated with 0, 40, or 80 μl of PBS or anti-HbpA Fab (0.2 and 0.4 mg/ml, respectively) and then assayed by a standard liquid hemin-binding assay. The values are the average of three determinations ± the SEM.
the gene is in opposite orientation to its own promoter, as the gene is in opposite orientation to the lac promoter in the pBK-CMV vector. E. coli XLOR or XLOLR containing the cloning vector pBK-CMV (Fig. 8A, lanes 1 and 2, respectively) did not produce rHbpA.

Liquid hemin-binding assays using XLOLR, XLOLR(pBK-CMV), or XLOLR(pHBP-CMV) did not reveal significant differences in hemin binding, indicating that rHbpA does not confer a hemin-binding phenotype upon E. coli (data not shown). However, in vitro analyses with rHbpA clearly show that it can bind hemin on blots (Fig. 8B, lane 1), as observed with B. quintana HbpA (Fig. 1). Taken as a whole, these results suggest that rHbpA, although capable of binding hemin, cannot do so in E. coli, perhaps because of improper folding or lack of surface exposure.

DISCUSSION

A number of bacterial pathogens have evolved systems for accumulating hemin in order to satisfy their iron (43, 60), protoporphyrin ring (15, 17), or cytochrome cofactor (40) requirements. In addition, hemin has been shown to facilitate entry of certain bacterial pathogens into their respective host cells (20, 58). Early studies with B. quintana showed that the pathogen has the highest in vitro hemin requirement for any known bacterium: 20 to 40 μg/ml (41). The reasons for this extraordinary need and the mechanism(s) whereby hemin is acquired are unknown. The unusual strategy of erythrocyte parasitism (hemotrophy) practiced by all Bartonella species may have evolved to meet the bacterium’s tremendous need for this molecule. Early studies with B. quintana indicated that iron, not the protoporphyrin ring, is the critical component provided by hemin supplements (41). In either case the bacterium would require the synthesis of a hemin receptor on the outer surface to facilitate the acquisition of the iron needed for growth.

We have identified and characterized a gene, hbpA, in B. quintana that encodes a protein, designated HbpA, that retained the ability to bind hemin after SDS-PAGE and electrophoretic transfer to nitrocellulose. Initial analyses suggested HbpA was a membrane protein with an apparent molecular mass of 30 kDa. Triton X-114 phase partitioning and heat modification studies strongly suggested that the protein was an integral membrane protein that localized to the outer envelope of B. quintana. These results were confirmed by immunoblot, comparing inner and outer membrane preparations probed with polyclonal antibody to HbpA, and immunoelectron microscopy, which indicated that the protein was not only found in the outer membrane but was also surface exposed. The observed phenomenon of heat modification has been associated with numerous outer membrane proteins from other bacteria and may be a reflection of HbpA’s interaction with lipopolysaccharide (2), interaction with peptidoglycan (4, 49), or tertiary structure (25). Interestingly, the increase in the apparent molecular mass of HbpA when heated (heated 30 kDa (29) and from 32 to 19 kDa (11) when heated.

Expression of hbpA in E. coli and characteristics of rHbpA. E. coli XLOR containing pHBP-CMV were found to synthesize recombinant HbpA (rHbpA) (Fig. 8). Although the protein was not apparent on Coomassie blue-stained gels (data not shown), it was clearly detected on immunoblots (Fig. 8A). The cloned hbpA gene is apparently expressed in E. coli from its own promoter, as the gene is in opposite orientation to the lac promoter in the pBK-CMV vector. E. coli XLOR or
demonstrated with other bacterial pathogens, where antibodies to components of the iron scavenging mechanism were shown to inhibit ligand-receptor interactions in vitro (24, 68). Hemin binding was not fully abolished in the presence of anti-HbpA Fab fragments, implying that *B. quintana* has multiple receptors that bind hemin, as is the case for *P. gingivalis* (52, 62) and *Haemophilus influenzae* (26, 48). This possibility is supported by the hemin blot (Fig. 1), which demonstrates that HbpA is only one of eight membrane-associated proteins in *B. quintana* that has an affinity for hemin. The exact localization of the additional hemin-binding membrane proteins and their involvement in iron acquisition are currently under investigation.

Partially purified HbpA was subjected to N-terminal sequence analysis, where we were able to reproducibly determine the first 18 amino acids of the mature protein. A BLASTp search of the NCBI database indicated a close match (63% identity) to the N terminus of a phage-associated protein (Pap31) identified in *B. henselae* (10). Pap31 was previously reported to be a membrane protein of *B. henselae* that copurifies with bacteriophage during phage isolation and purification (3). Due to the similarity between the N terminus of mature Pap31 and HbpA, the deduced nucleotide sequence of *pap31* was utilized as a template to develop primers in order to PCR amplify *hbpA* from *B. quintana*. A PCR amplicon was obtained, cloned, and used to probe a *B. quintana* genomic library, where the full-length gene and accompanying flanking sequences were cloned and analyzed.

Southern analysis showed that all pathogenic *Bartonella* spp. harbor *hbpA* homologs, implying a correlation between the presence of *hbpA* and pathogenicity. It was also evident that HbpA is synthesized as a preprotein with a 22-amino-acid signal sequence and a signal peptidase cleavage site. The HbpA signal sequence was strikingly similar (90% identity, 100% similarity) to the signal sequence of Pap31 (10), suggesting that Pap31 may also localize to the outer membrane in *B. henselae*.

A BLASTp search of the NCBI database using the full-length HbpA indicated that it was related not only to Pap31 of *B. henselae* but also to Omp31 of *Brucella melitensis*, which encodes for a 31- to 34-kDa outer surface protein proposed to be a porin (66). Interestingly, neither Pap31 nor Omp31 have been implicated in hemin binding or iron acquisition. Yet, within the putative promoter regions of *hbpA*, *pap31*, and *omp31* we identified an imperfect palindrome overlapping their putative –10 regions that closely resembles the ferric uptake regulator (Fur) consensus sequence found in *E. coli* (21). Similar Fur consensus sequences in *Yersinia* (55), *Neisseria* (36), and *Shigella* spp. have been observed upstream of genes that encode proteins that are involved in, or associated with, iron acquisition. Due to the presence of a Fur consensus sequence (53% identity to the *E. coli* Fur consensus) and the recent identification of *fur* homologs in *B. bacilliformis* and *B. henselae* (L. Hendrix, Abstr. 15th Sesquiannu. Meet. Am. Soc. Rickettsiol. abstr. 10, 2000), *hbpA* (as well as *pap31* and *omp31*) may be regulated by Fur in response to fluctuating cellular iron levels. We have yet to demonstrate any regulation

![FIG. 7. Multiple sequence alignment of *B. quintana* HbpA with *B. henselae* Pap31 and *B. melitensis* Omp31. Identical amino acid residues are noted in black, conserved residues in gray and introduced gaps by hyphens. The GenBank accession numbers for the Pap31 and Omp31 homologs are AF001274 and U39453, respectively.](image)

![FIG. 8. Expression of hbpA in *E. coli* and ability of recombinant HbpA to bind hemin in vitro. (A) Immunoblot developed with anti-HbpA antibody, showing lysses of *E. coli* XLOLR containing no plasmid (lane 1), pBK-CMV cloning vector (lane 2), or pHBP-CMV (lane 3). (B) Blots of an *E. coli* XLOLR(pHBP-CMV) lysate probed with hemin showing HbpA binding hemin (indicated by an asterisk) (lane 1). The corresponding immunoblot developed with anti-HbpA antiserum and ECL reagents (Amersham), identifying the recombinant HbpA (arrowed) (lane 2), is also shown. All samples were solubilized at 100°C in LSB. Molecular mass standards are given to the left in kilodaltons.](image)
of HbpA as a result of hemin or iron availability in vitro, but the abundance of HbpA on the cell surface of B. quintana grown on blood agar would suggest that cells grown under typical plating conditions (presumably iron replete) express HbpA.

The similarities between pap31 and hbpA suggest that they are homologs, and this brings into question the function of Pap31 in B. henselae. While this study does not directly address this question, we propose that both Pap31 of B. henselae and Omp31 of B. melitensis are involved in iron acquisition. Pap31’s original designation as a phage-associated membrane protein in B. henselae (3, 10) could be explained by the hypothesis that the bacteriophage may use Pap31 as a receptor on the outer surface of the bacterium. Thus, the receptor (presumably Pap31) may copurify with the phage.

Recombinant HbpA did not confer a hemin-binding phenotype to E. coli but instead retained the ability to bind hemin after SDS-PAGE and electrophoretic transfer to blots. This observation suggests that rHbpA is either improperly folded or not localized to the outer surface in E. coli. The signal sequence preceding HbpA may not be recognized or properly translocated by the secretory machinery of E. coli. Similar results were reported for the cloning and expression of B. melitensis omp31, where recombinant Omp31 was not surface exposed but still maintained the ability to form SDS-resistant oligomers (characteristic of bacterial porins) in E. coli (66).

HbpA is the first potential virulence determinant characterized from B. quintana. This pathogen’s high in vitro requirement for hemin makes it an ideal model to study iron acquisition and hemin binding in this genus. Obtaining iron needed for growth from hemin is typically a TonB-dependent process that entails the synthesis of a surface receptor to facilitate the binding of the ligand, a protein in the periplasmic space to ferry the hemin to the cytoplasmic membrane, and finally a permease to bring the molecule into the bacterial cell (35). We have cloned and characterized the first component, a gene encoding an HBP, in this choreographed chain of events. The remaining components must be identified and characterized in order to fully understand the role that hemin binding plays in the pathogenesis of Bartonella.

ACKNOWLEDGMENTS

We thank Elizabeth Fischer and Christian Eggers for their help with the electron microscopy; Russ Regnery for B. quintana; AmphiCon Express, Inc., for sequencing service; and J. M. Battisti and D. S. Samuels for critical reviews of the manuscript. Obtaining iron needed for growth from hemin is typically a TonB-dependent process that entails the synthesis of a surface receptor to facilitate the binding of the ligand, a protein in the periplasmic space to ferry the hemin to the cytoplasmic membrane, and finally a permease to bring the molecule into the bacterial cell (35).

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