Lyme disease is a multisystemic disorder that leads to arthritis in 60% of cases, carditis in 10% of untreated adults, and other neurological symptoms. The causative agent of Lyme disease is the spirochetal pathogen *Borrelia burgdorferi*, which is transmitted to humans through the bite of an infected *Ixodes* sp. tick (1). There is currently very little information available on the tissue-specific host-pathogen interactions that lead to pathological manifestations of *B. burgdorferi* infection. This pathogen’s ability to colonize mammals is dependent on its capacity to rapidly alter gene expression in response to highly disparate environmental signals following transmission from infected ticks (2). The open reading frames that are upregulated upon infection include members of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) protein family, and they facilitate the adherence of *B. burgdorferi* to extracellular matrix (ECM) components of the host (3).

Comparative genome analysis has also identified a family of von Willebrand factor A (vWFA) domain-containing proteins in *B. burgdorferi*, including BB0172, BB0173, BB0175, and BB0325 (4–6). The vWFA domains present in ECM proteins and on eukaryotic cells are involved in cell adhesion and protein-protein interactions; they play key roles in the adhesion of platelets to areas of vascular damage by binding to glycoproteins on the platelet surface, to exposed ECM components (5, 7, 8), and to metalloproteases (ADAMTS 13) (7–12). Therefore, the vWFA domain-containing borrelial proteins might be involved in the adhesion of *B. burgdorferi* to eukaryotic cells, ECM components, and activated platelets, and they may thus play a role in the virulence mechanisms of *B. burgdorferi*. In humans, absence of vWF results in severe bleeding disorders due to the removal of blood clotting factor VIII from the circulation (12–15). vWF-binding proteins (vWbp) have been identified in several bacterial species, such as *Helicobacter pylori* and *Staphylococcus aureus* (16–19), and these secreted or surface-exposed proteins are involved in the binding of these pathogens to ECM components, platelets, and endothelial cells, thus playing an important role in pathogen colonization and dissemination in the mammalian host. *In silico* sequence analysis has shown that *B. burgdorferi* vWFA domain-containing proteins have a sequence domain (DXSXS) that is very similar to the metal ion-dependent adhesion site (MIDAS) found in integrins (20, 21). These proteins also show similarity to the *Plasmodium* spp. extracellular adhesion molecule TRAP and the LFA-1 integrin (Fig. 1) (6, 22).

The Lyme disease agent binds to a variety of ECM components and integrins, which are metal ion-dependent heterodimeric receptors that mediate cell-to-cell and cell-to-ECM interactions (23). The present study used a well-established *in vitro* model to investigate the localization and function of the vWFA domain-containing BB0172 protein of *B. burgdorferi* and to determine its function in adherence to different tissues during infection. Our findings established the topology of the BB0172 protein in biological membranes and its adherence to different ECM components and integrins, emphasizing the complexity of host-pathogen interactions in Lyme disease.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *Borrelia burgdorferi* B31 isolate A3 (24) was used throughout this study. To mimic the temperature and pH conditions during the transition of this bacterium from the unfed to the fed tick, the strain was grown in BSK-II medium pH 7.6 complemented with 6% inactivated normal rabbit serum at room temperature (RT) until reaching a density of 10⁷ cells/ml. Then an aliquot of this culture was transferred to BSK-II medium (pH 6.8) and incubated at 37°C with 1% CO₂ until reaching a density of 5 × 10⁷ cells/ml (25). *Escherichia coli* OneShot Top10 cells (Invitrogen, CA) were used for all cloning steps, and Rosetta-gami(DE3)pLysS cells (Novagen, Madison, WI) were used for expression in *E. coli*. Recombinant *B. burgdorferi* BB0172 was overexpressed in an *E. coli* host strain and purified from inclusion bodies (26). The protein was refolded in 50 mM Tris-HCl (pH 8.0) containing 500 mM dithiothreitol (DTT) and 5 mM of metal ions (EDTA and/or ZnCl₂). The refolded protein was incubated at 25°C and 200 rpm for 48 h before loading it on a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated in PBS (pH 7.4) containing 200 mM NaCl. The protein was then loaded onto a Hitrap Crude Protein HP column (GE Healthcare) equilibrated in PBS (pH 7.4) containing 200 mM NaCl. Fractions with similar size were pooled and dialyzed against PBS (pH 7.4) containing 150 mM NaCl with 50 mM NaCl and 500 mM DTT. Protein concentration was determined using the Bradford method (27) with bovine serum albumin as a standard. Purified recombinant protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (28) using a mouse monoclonal antibody against *B.burgdorferi* major surface protein (MSP) (29). BB0172 was used as a molecular biology marker in the different cloning steps, and Rosetta-gami(DE3)pLysS cells (Novagen, Madison, WI) were used for expression in *E. coli*. Recombinant *B. burgdorferi* BB0172 was overexpressed in an *E. coli* host strain and purified from inclusion bodies (26). The protein was refolded in 50 mM Tris-HCl (pH 8.0) containing 500 mM dithiothreitol (DTT) and 5 mM of metal ions (EDTA and/or ZnCl₂). The refolded protein was incubated at 25°C and 200 rpm for 48 h before loading it on a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated in PBS (pH 7.4) containing 500 mM NaCl. The protein was then loaded onto a Hitrap Crude Protein HP column (GE Healthcare) equilibrated in PBS (pH 7.4) containing 500 mM NaCl. Fractions with similar size were pooled and dialyzed against PBS (pH 7.4) containing 150 mM NaCl with 500 mM DTT. Protein concentration was determined using the Bradford method (27) with bovine serum albumin as a standard. Purified recombinant protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (28) using a mouse monoclonal antibody against *B. burgdorferi* major surface protein (MSP) (29).
Madison, WI) were used for BB0172 recombinant protein expression. All *E. coli* strains were grown in LB (Difco) broth with the appropriate antibiotics. RNA and genomic DNA purification for detecting *bb0172* transcripts by PCR. RNA was extracted as previously described (25, 26). Briefly, *B. burgdorferi* cultures were grown to a density of 2 to 3 × 10^7 spirochetes/ml under the shifting conditions outlined above. RNA was extracted by resuspending the bacterial pellets with 0.2 ml RNA-Bee (Tel-Test, Inc., Friendswood, TX) for every 10^6 cells. Following extraction with chloroform, RNA was precipitated with isopropanol, washed with 75% ethanol, air dried, and resuspended in RNase-free water. To remove contaminating DNA, the RNA was treated twice with DNase I at 37°C for 45 min. Then, the total RNA was quantified spectrophotometrically and reverse transcribed to cDNA by using TaqMan reverse transcription reagents (Applied Biosystems). From *B. burgdorferi* cultures growing under tick-feeding conditions (pH 6.8, 37°C) or regular growing conditions (pH 7.6, 32°C), genomic DNA was obtained by general phenol-chloroform extraction. RNA, cDNA, and genomic DNA (positive control) samples from each growing condition were used to detect when *bb0172* was expressed. A 500-bp fragment of *bb0172* was amplified using primers BB0172cDNA-F (nt 174705 to 174728) and BB0172cDNA-R (nt 174225 to 174249) (Table 1). Primers specific to the *flaB*, *ospC*, and *p66* genes were also included as controls for the temperature and pH shift (Table 1) (27, 28). PCR products were separated on 0.8% agarose gels and imaged using the Bio-Rad Gel Doc XR system.

**FIG 1** In silico analysis of BB0172. (A) Schematic representation of BB0172. TM1 and TM2, transmembrane domains 1 (amino acids 17 to 35) and 2 (amino acids 264 to 281), respectively. The vWFA domain includes amino acids 51 to 256. The recombinant BB0172 protein (BB0172_r) amino acid sequence spans residues 38 to 291. The anti-BB0172 antibody (Ab) was generated against a 30-mer peptide, from amino acids 150 to 180. The MIDAS motif is located at residues 57 to 61. (B) Hydrophobic amino acid sequences (HRs) were cloned to study their insertion into membranes. HR1, amino acids 16 to 38, HR2, amino acids 263 to 284. (C) Clustal W (v1.83) alignment of BB0172 of *B. burgdorferi* B31 (in bold) and ZS7 strains to its homologues in *Borrelia garinii* (NTL01BG0170), *Borrelia afzelii* (NTL07BA0169), and the relapsing fever species *Borrelia hermsii* (NT03BH0168) and *Borrelia tunicate* (NT06BT0168), as well as to the human adhesins LFA-1 and CD11b and the *Plasmodium falciparum* membrane protein TRAP. Bold text with gray shadowing indicates the MIDAS domain (DXXSXS) and the aspartic acid (D) necessary to complete the metal binding (20, 21). The threonine (T) that is highlighted in gray and underlined is also involved in the MIDAS motif function; it is present in a different location in the bacterial species compared to eukaryotic counterparts. MacVector version 12.6 (MacVector, Inc.) was used for sequence analyses and production of schematics.
SignalP 4.0 server (35; http://www.cbs.dtu.dk/services/SignalP/). All user-adjustable parameters were left at their default values.

**Cloning of transmembrane domain regions.** To study the insertion of BB0172 hydrophobic regions into membranes, the segments to be tested (Fig. 1A and B) were engineered into the luminal P2 domain of the integral membrane protein Lep from *E. coli* (leader peptidase), which was flanked by two N-glycosylation sites that were used as reporters (see Fig. 5, top). To further clone these regions, the two BB0172 putative TM domains were PCR amplified from total genomic DNA obtained from the B31A3 Borrelia strain by using the primers described in Table 1. PCR product size was verified on 2% agarose gels, and then amplicons were cleaned using the Wizard SV gel and PCR cleanup system (Promega, Madison, WI), following the manufacturer’s recommendations. The PCR product were then digested using SpeI/KpnI enzymes (NEB, Ipswich, MA) and cloned into pGEM-Lep as previously described (36–38).

Ligation reaction mixtures were precipitated overnight and electroporated into TOP10 cells. Positive clones were selected on ampicillin plates (100 μg/ml) and verified by sequencing (Eton Biosciences, San Diego, CA). Clones showing the hydrophobic TMs in frame with Lep were selected for the in vitro transcription-translation experiments.

**In vitro transcription-translation.** In vitro transcription of in vitro-transcribed mRNA was performed in the presence of reticulocyte lysate, [35S]Met-Cys, and dog pancreas microsomes, as described previously (39). Lep constructs with hydrophobic region (HR)-tested segments from the BB0172 sequence (residues 16 to 38 and 263 to 284) were transcribed and translated as previously reported (36). After translation, membranes were collected by ultracentrifugation and analyzed by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were visualized on a Fuji FLA3000 PhosphorImager with ImageGauge software.

The proteinase K protection assay was performed as previously described (40). Briefly, the translation mixture was supplemented with 1 μl CaCl2 (50 mM) and 1 μl proteinase K (4 mg/ml) and then digested for 40 min on ice. The reaction was stopped by adding 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by SDS-PAGE analysis. This process is shown schematically in Fig. 5, below.

**Expression and purification of BB0172.** For this experiment, a truncated BB0172 (BB01722) protein was purified to avoid insertion of the protein into *E. coli* membranes and to ensure purification of BB0172 as a soluble protein. Total genomic DNA obtained from the B31A3 strain (Table 2) was used as a template to PCR amplify bb0172 from nucleotides 150 to 873 (amino acids 50 to 290), using the primers listed in Table 1. The amplicons were cloned into the pCR2.1 TOP 10 vector (Invitrogen), transformed into *E. coli* TOP 10 cells, and subjected to blue/white colony screening in the presence of ampicillin (100 mg/ml) and kanamycin (50 mg/ml). The insert was digested with Ndel/Xhol and ligated into the pET23a expression vector. The ligated products were electrotransformed into *E. coli* TOP10 cells and screened for the presence of the insert by restriction enzyme digestion. The junctions of plasmids containing inserts of the expected sizes were sequenced and used to transform the *E. coli* expression host. Recombinant BB0172 (rBB0172) with a C-terminal 6×His tag was overexpressed by inducing the *E. coli* strain containing pET23a-‐bb01722 with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. The bacterial pellets were disrupted by using a French press and denaturing lysis buffer (8 M urea, 20 mM imidazole; pH 7.4). The supernatants were collected, clarified by centrifugation, and subjected to affinity purification with a His60 Ni Superflow resin (Clontech, Mountain View, CA), following the manufacturer’s instructions. The bound 6×His-tagged proteins were eluted as 0.5–ml fractions with elution buffer (8 M urea, 300 mM imidazole; pH 7.4) and then analyzed by SD–12.5% PAGE. Select fractions with the largest concentrations of eluted proteins were further purified using dialysis against 50 mM sodium phosphate and 300 mM NaCl (pH 7.4; Slide-A Lyse G2 dialysis cassette; Thermo Scientific). After dialysis, Amicon centrifugal filters (Millipore) were used to concentrate the proteins. A 27.5-kDa protein was purified to homogeneity (data not shown).
B. burgdorferi to ensure separation of the outer membrane proteins without disruption 1% Triton X-114, and incubated overnight at 4°C with gentle rocking washed in phosphate-buffered saline (PBS), resuspended in PBS contain-
porated into Rosetta-gami and purification, 2 motif clones were stored at
Eton Biosciences, Ltd., San Diego, CA). Mutant MIDAS
characterizing their primer design tool for site-directed mutagenesis. Positive colonies
ble 1 (use of E. coli Cloning host, containing E. coli Lep, leader peptidase (Amp)’
82
This study
This study
This study
This study
This study

TABLE 2 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. burgdorferi B31A3</td>
<td>cp9 , wild type</td>
<td>Rocky Mountain Labs (1)</td>
</tr>
<tr>
<td>E. coli strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OneShot Top10</td>
<td>Cloning host; F− mcra A Δ(mrr-hsdRMS-mcrBC) &amp;80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu)7697 galU galK rpsL(300) endA1 napG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Rosetta-gami(DE3)pLyS</td>
<td>Expression host; Δ(ara leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 abpC galE galK rpsL DE3 F’ [lac’ F’pro] gor52:2 Tn10 trxB pLyS RARE2 (Cam’ Kan’ Str’ Tet’</td>
<td>Novagen</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-Lep</td>
<td>Cloning host, containing E. coli Lep, leader peptidase (Amp’)</td>
<td>82</td>
</tr>
<tr>
<td>pLE102</td>
<td>pGEM-1(bb0172TM1-Lep)</td>
<td>This study</td>
</tr>
<tr>
<td>pLE103</td>
<td>pGEM-1(bb0172TM2-Lep)</td>
<td>This study</td>
</tr>
<tr>
<td>pLE136</td>
<td>pET23a(bb0172sa-290)</td>
<td>This study</td>
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<tr>
<td>pECW1</td>
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<td>pECW3X</td>
<td>pET23a(bb0172sa-290-AXXAXA)</td>
<td>This study</td>
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</tbody>
</table>

shown), quantified in a bicinchoninic acid (BCA; Thermo Scientific, Inc.) assay, and stored at ~80°C until further use.

Site-directed mutagenesis of DXSXS metal-binding domain. BB0172 contains a MIDAS motif (Fig. 1C) comprising amino acids 57 to 61 (DXSXS). To determine which amino acids are essential for maintaining this protein’s function, we performed site-directed mutagenesis to change the motif-relevant amino acids to alanine, creating the mutants D57A, S59A, and S61A and the triple mutant (21). For this process, we used the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies), following the manufacturer’s recommendations, or transferred to polyvinylidene difluoride (PVDF) membranes to determine the BB0172 localization by immunoblot assay as described below. OMPs OspC and P66, the periplasmic protein superoxide dismutase A (SodA), and the cytosolic proteins carbon storage regulator A (CosA) and the Borrelia oxidative stress regulator (BosR) were used as controls.

SDS-PAGE gels and immunoblot analysis. Borrelia burgdorferi whole-cell lysates (prepared from cultures grown at RT and pH 7.6, then shifted to pH 6.8 and 37°C) were treated with proteinase K (20 μg/ml) and separated by SDS–12.5% PAGE. The separated proteins were either visualized by Coomassie brilliant blue staining or transferred onto a PVDF membrane (Hybond-P; GE Healthcare, Piscataway, NJ) as previously described (25, 26). Polyclonal mouse anti-P66 and anti-OspC (20), and rabbit anti-BB0172 sera were used as controls for proteinase K activity on outer membrane proteins and for the fractioning of outer membrane proteins in the Triton-X114 partitioning assay. Polyclonal mouse anti-SodA and rabbit anti-BB0172 sera were used as controls of cytoplasm-located proteins (44). The blots were developed following incubation with appropriate dilutions of horseradish peroxidase (HRP)-conjugated secondary antibodies and using enhanced chemiluminescence Western blotting reagents (GE Healthcare, Piscataway, NJ) as previously described (25, 26, 44, 45).

Binding assays. To understand the possible capability of BB0172 to bind to ECMs, we purified a truncated BB0172 protein lacking the N terminus and the first TM segment (BB01724) (Fig. 1), and we analyzed its binding to collagen type I, collagen type IV, laminin, and human fibronectin (BD BioCoat, Bedford, MA). All incubations were performed using BSA (1 mg/ml) containing 25 mM CaCl2, 1 mM MgCl2, and 1% bovine serum albumin (BSA) (46, 47). After blocking plates with 3% BSA in HBS containing 1% Tween 20 (HBS-T), we added 500 ng/well of rBB0172, or rBBK32 (positive binding control)
A conjugated antibody was applied for integrin antibody. The plates were washed again, and a secondary HRP-conjugated antibody was used as a negative control (–). (A) RNA samples from all purifications were separated on a 12% SDS-PAGE gel and stained with Coomassie blue (A) or transferred to PVDF membranes and probed with mouse anti-BB0172 antibody followed by anti-mouse HRP-conjugated antibody (B). OspC (outer membrane lipoprotein), SodA (intracellular), and P66 (outer membrane integral protein) were used as controls for the proteinase K treatment. T/pH shift denotes the shift from RT/7.6 to 37°C/pH 6.8.

RESULTS

B. burgdorferi expression and localization of BB0172 protein.

We found that the bb0172 gene was not expressed when B. burgdorferi was grown at RT and pH 7.6, while an mRNA product was detected when cultures were shifted from RT/pH 7.6 to either 37°C/pH 6.8 or 32°C/pH 7.6 (Fig. 2, compare lanes 1 and 3 in A to lanes 1 and 4 in B). Evaluation of flaB, P66, and ospC gene expression at the mRNA level showed the presence of flaB and P66 under both the RT/pH 7.6 and the temperature-pH (T/pH)-shifting conditions, while ospC was expressed only under T/pH-shifting conditions (Fig. 2A). These results suggested that bb0172 gene expression is selectively triggered by a change in temperature. Therefore, in subsequent studies, we adopted the temperature and pH shift strategy to induce BB0172 expression on borrelial cells and to detect the protein.

We next evaluated the cellular localization of the BB0172 protein in B. burgdorferi based on proteinase K sensitivity and Triton X-114 partitioning of OMPs, followed by immunoblot analysis (Fig. 3). The BB0172 protein was only present in cultures that were shifted to 37°C/pH 6.8, not in RT cultures (Fig. 3, compare lanes 1 and 3 in A to lanes 1 and 4 in B). This result correlates with the RNA data, supporting the hypothesis that this protein is expressed during the change from RT conditions to warmer conditions (32°C to 37°C). Furthermore, the protein was not observed after proteinase K treatment, suggesting that the WFWA domain of this protein (against which the antibodies were generated) is accessible from outside the cell.

We also evaluated the presence of BB0172 in the outer membrane fraction of B. burgdorferi by using Triton X-114 partitioning of OMPs after shifting the cultures from RT/pH 7.6 to 37°C/pH 6.8. BB0172 was only detected in the OMP fraction of the shifted B. burgdorferi cultures, not in the PC or OMP fractions extracted from cultures grown at RT/pH 7.6 (Fig. 4B). In addition, when B. burgdorferi was grown at 37°C/pH 6.8, the borrelial outer membrane lipoprotein OspC was detected at larger amounts in the OMP fraction than in the PC fraction. Very small amounts of OspC were detected in the OMP fraction isolated from cells grow-
ing at RT/pH 7.6, and it was only detectable after overexposing the immunoblot (data not shown). Moreover, SodA was only detected in the B. burgdorferi aqueous phase, regardless of the growing conditions used, while BosR was only detected in the PC fraction from cells growing at 37°C/pH 7.6. Altogether, these results confirmed the presence of the BB0172 protein in the outer membrane of B. burgdorferi, with its vWFA domain oriented toward the extracellular milieu.

BB0172 is anchored to biological membranes through two transmembrane segments. The BB0172 amino acid sequence was parsed to test the performance of several commonly used algorithms for predicting TM regions of integral membrane proteins. We submitted the BB0172 sequence to the most current online versions of six widely used prediction methods: DAS-TMfilter (29), ΔG Predictor (30), MEMSAT3 (31), OCTOPUS (32), SOSUI (33), and TMHMM (34). The predicted outcomes significantly coincided across the different methods used, with all algorithms predicting the presence of two TM domains at similar positions in the protein sequence (Table 3). It has been established that the reliability of a given prediction is very high when many different methods agree (40). Additionally, the SignalP 4.0 (35) results predicted that it is unlikely that BB0172 contains a signal sequence.

The membrane insertion of the two BB0172 hydrophobic regions was investigated by using an in vitro experimental system that accurately reports the integration of TM helices into microsomal membranes (Fig. 5, top) (37, 50). The translation of the chimeric constructs harboring the predicted BB0172 TM regions as HR-tested sequences mainly resulted in single glycosylated forms (Fig. 5, lanes 4 to 9), suggesting membrane integration of these two regions. Lanes 1 to 3 in Fig. 5 show the control construct (38, 40, 51) with a previously tested computer-designed translocated (nonintegrated) sequence. Proteinase K treatment of these samples rendered complete loss of detectable fragments in the case of the BB0172 tested sequences, while a clear protected band was observed in the translocated control construct (Fig. 5, compare lane 3 to lanes 6 and 9), confirming membrane insertion of the BB0172 hydrophobic regions. Although these in vitro experiments are not entirely representative of in vivo conditions, the results strongly suggest that the translocon machinery can recognize the assayed regions, ultimately ensuring their proper integration into the intended target membrane.

Binding of BB0172 to human ECM components and integrins. After characterizing the orientation in the membrane of the BB0172 vWFA domain, the recombinant BB0172 protein was used to perform a number of binding assays to different human ECM components and integrins. Recombinant expression of full-length BB0172 protein in Esherichia coli cells was not detected on Coomassie-stained gels or Western blots (data not shown). Since it was previously reported that the first TM segment can prevent translation of membrane proteins in E. coli cells (52), we then expressed a truncated version of the BB0172 protein lacking the N terminus and the first TM segment (BB0172_{T}) (Fig. 1). This strategy enabled expression and purification of the truncated BB0172 as a recombinant protein in sufficient concentrations for further characterization. The first set of binding experiments showed that

![Image](https://example.com/image.png)

**FIG 4** *B. burgdorferi* BB0172 anchors to the outer membrane. The *B. burgdorferi* B31A3 strain was cultured at RT/pH 7.6 and shifted to 37°C/pH 6.8, and the PCs and OMPs were separated by Triton X-114 partitioning. PC and OMP fractions from all treatments were separated on 12% SDS-PAGE gels and stained with Silver Staining Plus (Bio-Rad) (A) or transferred to PVDF membranes and probed with mouse anti-BB0172 antibody (B). Serum specific for OspC (outer membrane lipoprotein), SodA (periplasmic), BosR (cytoplasmic), and CsrA (cytoplasmic) were used as a controls. T/pH shift denotes the shift from RT/pH 7.6 to 37°C/pH 6.8. MW, molecular weight marker; lane 1, PCs from cultures at RT/pH 7.6; lane 2, OMPs from cultures at RT/pH 7.6; lane 3, PCs from cultures at 37°C/pH 6.8; lane 4, OMPs from cultures at 37°C/pH 6.8; lane a, aqueous phase from cultures at RT/pH 7.6; lane b, aqueous phase from cultures at 37°C/pH 6.8.

**TABLE 3** *In silico* analysis of the BB0172 amino acid sequence

<table>
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<tr>
<th>Algorithm</th>
<th>Membrane protein?</th>
<th>Resulting no. of TM segments (starting amino acids/ending amino acids)</th>
</tr>
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<tbody>
<tr>
<td>DAS-TMfilter</td>
<td>Yes</td>
<td>2 (17/35 and 264/281)</td>
</tr>
<tr>
<td>ΔG Prediction</td>
<td>Yes</td>
<td>2 (16/38 and 263/284)</td>
</tr>
<tr>
<td>MEMSAT3</td>
<td>Yes</td>
<td>2 (21/39 and 264/283)</td>
</tr>
<tr>
<td>OCTOPUS</td>
<td>Yes</td>
<td>2 (17/38 and 263/283)</td>
</tr>
<tr>
<td>SOSUI</td>
<td>Yes</td>
<td>4 (16/38, 177/198, 219/241, and 258/280)^a</td>
</tr>
<tr>
<td>TMHMM</td>
<td>Yes</td>
<td>2 (20/39 and 266/284)</td>
</tr>
</tbody>
</table>

^a Bold numbers denote “primary” helices.
the BB0172 protein was slightly bound to human plasma fibronectin in the presence of the metals Ca²⁺, Mn²⁺, and Mg²⁺; binding to other ECM components was not observed (Fig. 6A). The binding of the BB0172 protein to human plasma fibronectin was very weak and significantly lower than that observed for BBK32, an integrin-binding protein.

FIG 5 Hydrophobic regions of the BB0172 insert into microsomal membranes. (Top) Schematic representation of the Lep construct used to report insertion into the endoplasmic reticulum (ER) membrane of BB0172 hydrophobic regions. The TM segment under investigation (HR-tested) was inserted into the P2 domain of Lep, flanked by two artificial glycosylation acceptor sites (G1 and G2). Recognition of the tested sequence as a TM domain by the translocon machinery results in the location of only G1 in the luminal side of the ER membrane, preventing G2 glycosylation (left). The Lep chimera is doubly glycosylated when the sequence being tested is translocated into the lumen of the microsomes (right). (Bottom) In vitro translation of different Lep constructs containing BB0172 HR1 (residues 16 to 38; lanes 4 to 6) and HR2 (residues 263 to 284, lanes 7 to 9) sequences in the presence of membranes. A control construct was used to verify sequence translocation (lanes 1 to 3). Constructs were transcribed and translated in the presence or absence of rough microsomal membranes (RM) and proteasome K (PK), as indicated. Bands of nonglycosylated protein are indicated by a white dot; singly and doubly glycosylated proteins are indicated by one and two black dots, respectively. The arrowhead identifies protected a double-glycosylated P2 domain after PK treatment.

resides of the MIDAS motif (DXSXS) (Table 2) with alanine through site-directed mutagenesis. Each single-point mutant strain (AXXXS, DXAXS, and DXSXXA) plus a triple mutant strain harboring all three replacements (AXAXA) was used in a αβ₁ integrin-binding assay. The results showed that all three individual mutations significantly reduced the binding of BB0172 to αβ₁ integrin compared to the wild-type sequence (Fig. 6C), suggesting that all three amino acid residues are important for the coordination of the metal required to maintain the function of the BB0172 protein. As expected, the triple mutation induced the highest reduction of binding, indicating that these amino acids play a critical role in maintaining the coordination of metals in the BB0172 protein.

FIG 6A In vitro translation of different Lep constructs containing BB0172 HR1 (residues 16 to 38; lanes 4 to 6) and HR2 (residues 263 to 284, lanes 7 to 9) sequences in the presence of membranes. A control construct was used to verify sequence translocation (lanes 1 to 3). Constructs were transcribed and translated in the presence or absence of rough microsomal membranes (RM) and proteasome K (PK), as indicated. Bands of nonglycosylated protein are indicated by a white dot; singly and doubly glycosylated proteins are indicated by one and two black dots, respectively. The arrowhead identifies protected a double-glycosylated P2 domain after PK treatment.

DISCUSSION

vWFA domain-containing proteins are found in a broad array of systems, from integrins in mammalian organisms to bacteria (5, 13, 56–58). To be functional, these proteins must exist on the cell surface to interact with their receptors. The chromosome of Borrelia burgdorferi encodes a series of vWFA domain-containing proteins (BB0172, BB0173, BB0175, and BB0325). Of all these hypothetical proteins, in silico analysis only predicts those encoded by the bb0172 and bb0173 genes to be membrane proteins. The main objectives of the present work were to determine the BB0172 protein expression pattern, to demonstrate its membrane association, and to characterize its binding to potential host components. Furthermore, we evaluated the cellular localization of the BB0172 vWFA domain by using proteasome K treatment and Triton X-114 partitioning, together with immunoblot analyses. Altogether, the results showed that BB0172 is a helical membrane protein with two TM segments expanding from amino acids 16 to 38 and 263 to 284, suggesting a specific membrane disposition (Fig. 7). Immunoblot analysis with proteasome K-treated whole cells consistently revealed that the vWFA domain of BB0172 was not visible due to its degradation during treatment, indicating that the vWFA domain was exposed to the outer cell surface. Triton X-114 partitioning experiments also demonstrated the localization of BB0172 in the outer membrane of B. burgdorferi.

This is the first study demonstrating that a vWFA domain-containing borrelial protein is anchored to the cell surface through two TM helices. We could not obtain the full-length protein by in vitro translation techniques, probably due to differences in the codon usage between B. burgdorferi and the rabbit system utilized in these experiments. For instance, the codons for Arg (AGA), Gln (CAA), Ile (AUU), Leu (UAU) Pro (CCU), Ser (UCU), and Thr (ACU) that are preferentially used by B. burgdorferi are poorly used in rabbit (Oryctolagus cuniculus). In addition, previous experiments conducted in our laboratory showed that some borrelial proteins are only recombinantly expressed in E. coli strains (Rosetta strain from Novagen) that are engineered to recognize rare codons for Leu and Ile (data not shown). Due to these limitations, here we evaluated short polypeptide regions by using in vitro translocation/insertion of putative TM domains in the presence of canine pancreatic microsomes and rabbit reticulocytes. While the hydrophobic regions tested here came from prokaryotic proteins, the high degree of sequence conservation in the translocon components between prokaryotes and eukaryotes (59) suggests that general conclusions can be drawn based on studies using the microsomal assay system and that the present results are relevant to the study of the BB0172 surface-exposed regions of B.
This contention is further supported by previous observations of prokaryotic sequences based on measurements obtained using this system (60). Based on our present results, this strategy appears to be a valuable tool for studying complex membrane proteins from prokaryotic organisms that are difficult to culture in vitro but that have a sequenced genome. Further modifications of the current system utilizing a synthetic bb0172 gene recoded with a more appropriate codon combination are in progress.

In our study, both bb0172 RNA and protein were only detected when the pathogenic B. burgdorferi B31A3 strain was shifted from RT/pH 7.6 to 37°C/pH 6.8 or 32°C/pH 7.6. Additionally, bb0172 expression was inhibited when cells were adapted to any of the studied temperature/pH combinations. These results suggest that BB0172 is only present under the conditions found during the temperature shift that is part of the transition of B. burgdorferi from the unfed tick to the fed tick, indicating that BB0172 might be of great importance during the migration of this pathogen from the tick to the mammalian host. It should be noted that a recent proteomic analysis of B. burgdorferi in response to culture condition changes detected two BB0172-derived peptides at RT/pH 7.6 but not at 34°C/pH 6.6 (61). This discrepancy with the present data could have arisen from the differences in culture shifting conditions between studies and from the low total number of spectral counts found with the proteomic approach, which indicates a very low abundance of BB0172 under all the conditions studied. Taking all these observations together, we hypothesize...
that BB0172 plays an important role as an adhesive during the first hours postinfection. Therefore, we performed a number of binding assays to ECM components as well as to several commercially available integrins. Our findings suggested that the BB0172 protein is a metal-dependent adhesive to the human α3β1 integrin.

Integrins are heterodimeric receptors that mediate cell-to-cell and cell-to-ECM interactions (23, 62). Pathogenic *B. burgdorferi* can target integrins and ECM components to local sites of damage in host cells to promote bacterial dissemination (23, 63). For instance, the outer membrane protein P66 interacts with integrin αmβ2 on the surface of activated platelets, as well as with integrin αvβ3 on endothelial cells (47, 64, 65). Similarly, *B. burgdorferi* infection induces the expression of selected integrins in the C3H/HeN mouse model, which can influence the severity of Lyme disease-derived carditis and arthritis (66, 67). Moreover, different *B. burgdorferi* proteins can bind to specific ECM components; for example, the BBK32 protein interacts with fibronectin (48, 53–55), and decorin-binding proteins A and B (DbpA and DbpB) interact with decorin and chondroitin (68–71). However, analysis of borrelial mutants deficient in BBK32 or DbpA/B did not show complete abrogation of the binding to extracellular products, suggesting that this binding might be mediated by unknown functionally and structurally related determinants (49).

Behera and collaborators (72) previously described BB087, an integrin αβ1-binding protein of *B. burgdorferi*. They showed that the RGD-dependent binding of the BB087 protein to integrins specifically stimulated the generation of proinflammatory cytokines in primary human chondrocyte cultures. In the present study, we observed a similar level of binding between the BB0172 protein and the same α3β1 integrin, but in an RGD-independent manner, since BB0172 lacks this motif. Integrin α3β1 binds to collagen I and IV, laminin, fibronectin, and nidogen, and in some cell types to thrombospondin 1 (73–76). Integrin αβ1 is also critical for the formation of kidneys and epidermis, and also in wound healing, and it is significantly expressed by neurons in the brain (73–76).

Interestingly, we found that BB0172 has a MIDAS sequence motif at residues 57 to 61 (DXXXS), which is highly conserved among *Borrelia* species. Mutations of these amino acid residues reduced recombinant BB0172 binding to integrin α3β1, with the strongest impact seen with the triple mutant (AXAXA), which reduced the binding to near background levels. We hypothesized that these mutations reduced the ability of BB0172 to coordinate metals, thus diminishing capability of the protein to bind integrin αβ1. Consequently, we speculate that the vWFA domain of BB0172 includes a MIDAS domain similar to that found in the I-domain of integrins (77–81), which is essential to maintain the protein’s function. Further studies are in progress with the aim of deleting bb0172 from *B. burgdorferi* and complementing it with its native form and the mutated versions. We propose that this mutation might reduce the ability of *B. burgdorferi* to bind to tissues where integrin α3β1 is highly expressed, e.g., the basolateral membrane in epidermis, glomerular podocytes in the kidney, and collecting tubes and neurons in the brain (74).

In summary, the results of the present study prove that BB0172 is a surface-exposed membrane protein that displays a significant capacity to bind integrin α3β1. Furthermore, the *in vitro* transcription/translation strategy utilized here could be useful for further studies of the helical membrane proteins of fastidiously growing bacteria. The technique developed in this paper was demonstrated to correlate well with studies of whole-protein localization on the borrelial surface, validating the potential use of this technology with other hypothetical membrane proteins of *B. burgdorferi*. Finally, this is the first report of a metal-dependent borrelial adhesin with an active MIDAS motif that is essential for maintaining protein function.

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