

# BB0172, a *Borrelia burgdorferi* Outer Membrane Protein That Binds Integrin $\alpha_3\beta_1$

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**Lyme disease is a multisystemic disorder caused by *Borrelia burgdorferi* infection. Upon infection, some *B. burgdorferi* genes are upregulated, including members of the microbial surface components recognizing adhesive matrix molecule (MSCRAMM) protein family, which facilitate *B. burgdorferi* adherence to extracellular matrix components of the host. Comparative genome analysis has revealed a new family of *B. burgdorferi* proteins containing the von Willebrand factor A (vWFA) domain. In the present study, we characterized the expression and membrane association of the vWFA domain-containing protein BB0172 by using *in vitro* transcription/translation systems in the presence of microsomal membranes and with detergent phase separation assays. Our results showed evidence of BB0172 localization in the outer membrane, the orientation of the vWFA domain to the extracellular environment, and its function as a metal ion-dependent integrin-binding protein. This is the first report of a borrelial adhesin with a metal ion-dependent adhesion site (MIDAS) motif that is similar to those observed in eukaryotic integrins and has a similar function.**

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 (vWFBp) 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  $10^7$  cells/ml. Then an aliquot of this culture was transferred to BSK-II medium (pH 6.8) and incubated at 37°C with 1% CO<sub>2</sub> until reaching a density of  $5 \times 10^7$  cells/ml (25). *Escherichia coli* OneShot Top10 cells (Invitrogen, CA) were used for all cloning steps, and Rosetta-gami(DE3)pLysS cells (Novagen,

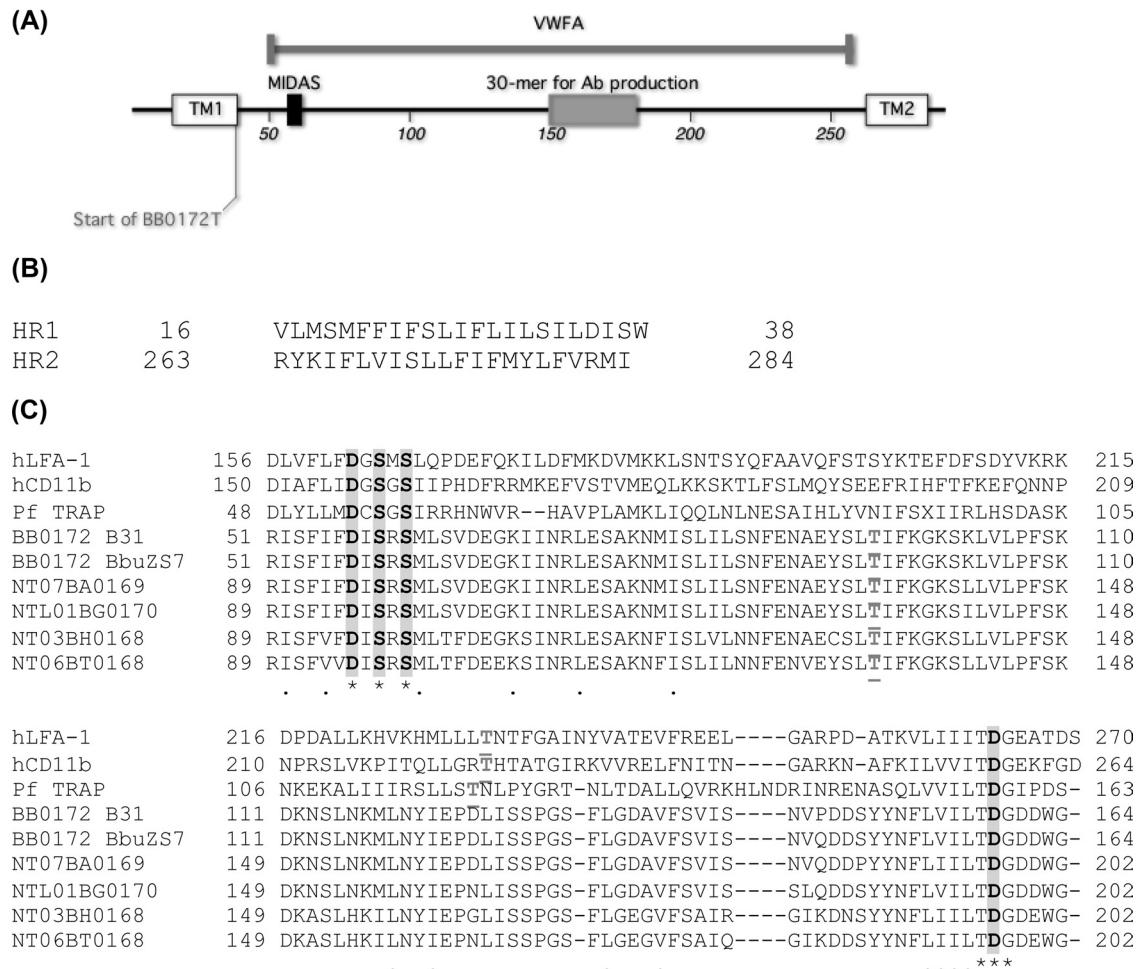
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**FIG 1** *In silico* analysis of BB0172. (A) Schematic representation of BB0172. TM1 and TM2, transmembrane domains 1 (amino acids 17 to 35) and 2 (amino acids 264 to 281), respectively. The vWFA domain includes amino acids 51 to 256. The recombinant BB0172 protein (BB0172<sub>T</sub>) amino acid sequence spans residues 38 to 291. The anti-BB0172<sub>pep</sub> 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 tunicata* (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 (DXSXS) 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.

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  $2 \times 10^7$  to  $3 \times 10^7$  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, Foster City, CA). 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 (*B. burgdorferi* nucleotides 174705 to 174728) and BB0172cDNA-R (*B. burgdorferi* nucleotides 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.

**Computer-assisted analysis of BB0172 transmembrane (TM) regions.** Membrane-spanning regions in the BB0172 sequence were predicted by using six of the most commonly used prediction methods available online: DAS-TMfilter (29; <http://mendel.imp.univie.ac.at/sat/DAS/DAS.html>),  $\Delta G$  Prediction (30; <http://dgpred.cbr.su.se/>), MEMSAT3 (31; <http://bioinf.cs.ucl.ac.uk/psipred/submit>), OCTOPUS (32; <http://octopus.cbr.su.se/index.php>), SOSUI (33; [http://bp.nuap.nagoya-u.ac.jp/sosui/sosui\\_submit.html](http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html)), and TMHMM (34; <http://www.cbs.dtu.dk/services/TMHMM/>). The potential presence of a putative signal sequence was also investigated using the

TABLE 1 Oligonucleotide primers used in this study

Primer pair	RS <sup>a</sup>	Sequence (5'→3') <sup>b</sup>	Application
BB0172cDNA-F		GCTTGATTTTTTAATTTTATCC	Amplification of <i>bb0172</i> 500-bp fragment from <i>B. burgdorferi</i> cDNA
BB0172cDNA-R		CGGGATTACTCCCGCCAATCCCAA	
FlaBcDNA-F		AACACACCAGCATCAGTTTCAGG	Amplification of <i>flab</i> 234-bp fragment from <i>B. burgdorferi</i> cDNA
FlaBcDNA-R		GAGAATTAACCTCCGCTTGAGAAGG	
P66cDNA-F		CAAAAAAGAAACACCCCTCAGATCC	Amplification of <i>p66</i> 683-bp fragment from <i>B. burgdorferi</i> cDNA
P66cDNA-R		CCTGTTTTAAATAAAATTTTGTAGCATC	
OspCcDNA-F		TATTAATGACTTTATTTTATTTATATCT	Amplification of <i>ospC</i> 593-bp fragment from <i>B. burgdorferi</i> cDNA
OspCcDNA-R		TTGATTTTAATTAAGGTTTTTTTGG	
BB0172 <sub>T</sub> -F	NdeI	ACGCCATATGTGGGGCAAAGAGCTGTGAGGA	Amplification of <i>bb0172</i> for cloning into pCR2.1 and pET23a for protein expression and purification
BB0172 <sub>T</sub> -R	XhoI	ACGCCTCGAGCTAAAAAGTTTCGTCCTCA	
BB0172TM1-F	SpeI	ACGCACTAGTGGAGGACCAGGAGTTCTAATGTCAATGTTT	Amplification of the first <i>bb0172</i> hydrophobic region in frame with Lep sequence
BB0172TM1-R	KpnI	ACGCGGTACCCCTCCTGGTCCCAAGAAATATCCAAAAAT	
BB0172TM2-F	SpeI	ACGCACTAGTGGAGGACCAGGAAGATATAAAATTTTTTTG	Amplification of the second <i>bb0172</i> hydrophobic region in frame with Lep sequence
BB0172TM2-R	KpnI	ACGCGGTACCCCTCCTGGTCTATCATCTGACAAACAA	
BB0172-T		ACAGATGGCGATGATTGGGGGGAA	Sequencing
SP6		ATTTAGGTGACACTATAG	Sequencing
BB0172 SDM MIDAS-1F		GAGAATTTCTTTTATTTTGTCTATTTCTCGTAGCATG	Site-directed mutagenesis of the first amino acid of the MIDAS motif, DXSXS→AXSXS
BB0172 SDM MIDAS-1R		CATGCTACGAGAAATAGCAAAAATAAAAGAAATTTCTC	
BB0172 SDM MIDAS-2F		GAGAATTTCTTTTATTTTGTATTTGCTCGTAGCATGTTAAG	Site-directed mutagenesis of the second amino acid of the MIDAS motif, DXSXS→DXAXS
BB0172 SDM MIDAS-2R		CTTAACATGCTACGAGCAATATCAAAAATAAAAGAAATTTCTC	
BB0172 SDM MIDAS-3F		GAGAATTTCTTTTATTTTGTATTTCTCGTGCTATGTTAAGTGTAGATG	Site-directed mutagenesis of the third amino acid of the MIDAS motif, DXSXS→DXSXA
BB0172 SDM MIDAS-3R		CATCTACACTTAACATAGCAGGAGAAATATCAAAAATAAAAGAAATTTCTC	
BB0172 SDM MIDAS-3X-F		GAGAATTTCTTTTATTTTGTCTATTTGCTCGTGCTATGTTAAGTGTAGATG	Site-directed mutagenesis of the three amino acids of the MIDAS motif, DXSXS→AXAXA
BB0172 SDM MIDAS-3X-R		CATCTACACTTAACATAGCAGGAGCAATAGCAAAAATAAAAGAAATTTCTC	

<sup>a</sup> Restriction sites (RS), when present, are indicated by bold text in the sequence.

<sup>b</sup> Site-directed mutations are underlined in the sequence.

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), where it 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 products were then double 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 used for the *in vitro* transcription-translation experiments.

**In vitro transcription-translation.** *In vitro* translation of *in vitro* transcribed mRNA was performed in the presence of reticulocyte lysate, [<sup>35</sup>S]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 CaCl<sub>2</sub> (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<sub>T</sub>.** For this experiment, a truncated BB0172 (BB0172<sub>T</sub>) 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-TOPO 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 NdeI/XhoI 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-*bb0172<sub>T</sub>* 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 SDS-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 Lyze 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

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source
<i>Borrelia burgdorferi</i> B31A3	cp9 <sup>-</sup> , wild type	Rocky Mountain Labs (1)
<i>E. coli</i> strains		
OneShot Top10	Cloning host; F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ( <i>ara leu</i> )7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str <sup>r</sup> ) <i>endA1</i> <i>nupG</i>	Invitrogen
Rosetta-gami(DE3)pLysS	Expression host; Δ( <i>ara leu</i> )7697 Δ <i>lacX74</i> Δ <i>phoA</i> <i>PvuII</i> <i>phoR</i> <i>araD139</i> <i>ahpC</i> <i>galE</i> <i>galK</i> <i>rpsL</i> DE3 F' [ <i>lac</i> <sup>+</sup> <i>lacI</i> <sup>q</sup> <i>pro</i> ] <i>gor522::Tn10</i> <i>trxB</i> pLysS RARE2 (Cam <sup>r</sup> Kan <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup> )	Novagen
Plasmids		
pGEM-Lep	Cloning host, containing <i>E. coli</i> Lep, leader peptidase (Amp <sup>r</sup> )	82
pLE102	pGEM-1( <i>bb0172</i> TM1-Lep)	This study
pLE103	pGEM-1( <i>bb0172</i> TM2-Lep)	This study
pLE136	pET23a( <i>bb0172</i> <sub>50-290</sub> )	This study
pECW1	pET23a ( <i>bb0172</i> <sub>50-290</sub> -AXSXS)	This study
pECW2	pET23a ( <i>bb0172</i> <sub>50-290</sub> -DXAXS)	This study
pECW3	pET23a ( <i>bb0172</i> <sub>50-290</sub> -DXSXA)	This study
pECW3X	pET23a ( <i>bb0172</i> <sub>50-290</sub> -AXAXA)	This study

shown), quantified in a bicinchoninic acid (BCA; Thermo Scientific, Inc.) assay, and stored at  $-80^{\circ}\text{C}$  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. Primers (Table 1) were designed as recommended by the manufacturer and using their primer design tool for site-directed mutagenesis. Positive colonies were screened by sequencing using the universal T7 promoter and terminator primers (Eton Biosciences, Ltd., San Diego, CA). Mutant MIDAS motif clones were stored at  $-80^{\circ}\text{C}$  for further use. For their expression and purification, 2  $\mu\text{l}$  of purified plasmid for each mutation was electroporated into Rosetta-gami *E. coli* strains (Novartis). Further binding assays were performed with all isolated mutants, as described below.

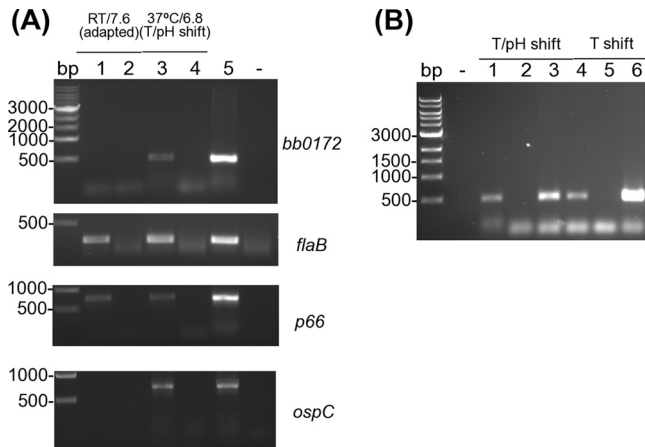
**Generation of specific BB0172 antibodies.** To generate antibodies specific to the extracellular domain of BB0172, we design a 30-mer-long peptide corresponding to amino acids 150 to 180 (Fig. 1A) with the sequence YNFLVILTDG DDWGENNYR FSKFVNLLKL conjugated to KLH (Peptide 2.0, Chantilly, VA). The synthetic peptide was dissolved in 10% dimethyl sulfoxide until reaching homogeneity and administered to three BALB/c mice at a dose of 50  $\mu\text{g}/\text{mouse}$  in conjugation with TiterMax Gold (Sigma-Aldrich, St. Louis, MO) on days 0, 14, and 21. At 28 days, the mice were euthanized and exsanguinated. Serum was collected and used in the following immunoblot and binding assays; this specific antiserum is henceforth referred to as anti-BB0172<sub>pep</sub>. Specific serum to the whole BB0172 protein was obtained following the same immunization protocol and using 50  $\mu\text{g}$  of rBB0172<sub>T</sub> per mouse, but we detected no BB0172 in the *B. burgdorferi* whole-cell lysates or from purified rBB0172<sub>T</sub>. Therefore, we used the anti-BB0172<sub>pep</sub> developed in this study.

**Triton X-114 partitioning of *B. burgdorferi* outer membrane proteins.** To evaluate the localization of BB0172 in *B. burgdorferi*, we separated the outer membrane proteins (OMPs) from the protoplasmic cylinders (PCs) by Triton X-114 partitioning as previously described (40–43). Briefly, *B. burgdorferi* was grown at RT and pH 7.6 and then shifted to 37°C, pH 6.8 and grown to a density of  $5 \times 10^7$  spirochetes/ml. Cells were washed in phosphate-buffered saline (PBS), resuspended in PBS containing 1% Triton X-114, and incubated overnight at 4°C with gentle rocking to ensure separation of the outer membrane proteins without disruption of the protoplasmic cylinders. After the overnight partitioning, PCs were

pelleted by centrifugation at  $15,000 \times g$  for 15 min at 4°C. Supernatants containing OMPs were incubated at 37°C for 15 min in the presence of 2% Triton X-114. The OMP-containing organic phase was separated by centrifugation at  $2,000 \times g$  for 10 min at RT and then washed three times with PBS. Finally the OMPs were precipitated out of the organic phase by adding  $10 \times$  ice-cold acetone at  $-20^{\circ}\text{C}$  for 2 h, followed by centrifugation at  $15,000 \times g$  for 30 min at 4°C. PCs, the aqueous phase, and the detergent phase containing the OMPs from different growing conditions were separated in SDS–12% PAGE gels and silver stained (Silver Staining Plus; Bio-Rad Laboratories, Inc., Hercules, CA), 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 (CsrA) 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  $\mu\text{g}/\text{ml}$ ) 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) and subjected to immunoblot analysis. The PVDF membranes were blocked overnight at 4°C in Tris-buffered saline containing 0.2% Tween 20 (TBS-T) and 10% skim milk. After blocking, membranes were probed with mouse anti-BB0172<sub>pep</sub>. Polyclonal mouse anti-P66 and anti-OspC (25) sera were used as controls for proteinase K activity on outer membrane proteins and controls for the fractionation of outer membrane proteins in the Triton-X114 partitioning assay. Polyclonal mouse anti-SodA and rabbit anti-BosR 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 (BB0172<sub>T</sub>) (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 HBS buffer (HEPES-buffered saline) containing 0.25 mM Ca<sup>2+</sup>, 1 mM Mn<sup>2+</sup>, 1 mM Mg<sup>2+</sup>, 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<sub>T</sub> or rBBK32 (positive binding control)

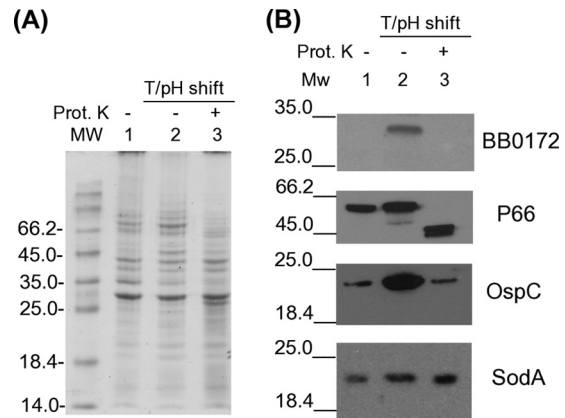


**FIG 2** BB0172 expression upon temperature shift. *B. burgdorferi* B31A3 strain was grown at RT/pH 7.6 and shifted to 37°C/pH 6.8 (T/pH shift) or to 32°C/pH 7.6 (T shift). The purified mRNA was reverse transcribed to cDNA, and PCR was performed to detect *bb0172*, *flaB*, *p66*, and *ospC*. Water was used as a negative control (-). (A) RNA samples from all purifications were checked for DNA contamination and applied in lanes 2 and 4. Genomic DNA was included as a positive control in lane 5. cDNA results are shown in lanes 1 and 3. (B) cDNA samples of cultures growing after T/pH shift or T shift are represented in lanes 1 and 4, RNA is in lanes 2 and 5, and genomic DNA is in lanes 3 and 6. The DNA ladder is shown on the left side of each panel, with the sizes indicated in base pairs.

to the plates in quadruplicate wells and incubated them at RT for 1 h (47–49). The plates were washed three times with HBS-T to remove unbound BB0172<sub>T</sub> protein. Then, plates were incubated with a 1:1,000 dilution of mouse-anti-rBB0172<sub>pep</sub> or 1:2,000 dilution of the mouse anti-rBBK32 antibody for 1 h at RT, followed by a 1-hour incubation with a 1:5,000 dilution of anti-mouse HRP-conjugated antibody (GE Healthcare, Piscataway, NJ). Plates were washed and incubated with *o*-phenyldiamine dihydrochloride (OPD) tablets (Thermo Scientific), and the absorbency at 450 nm was measured by using a FLUOstar Omega plate reader (BMG LabTech, Cary, NC). A blank control (BK) was included in quadruplicate in all plates.

To evaluate the binding of rBB0172<sub>T</sub> to a wider range of ECMs and integrins, and to prevent any interference of the anti-rBB0172 antibody with binding capabilities to the substrates, 96-well flat-bottom plates (Nunc) were coated with 500 ng/well of each of the recombinant proteins: rBB0172, rBBK32, and rP66. All incubations used HBS buffer containing 0.25 mM Ca<sup>2+</sup>, 1 mM Mn<sup>2+</sup>, and 1 mM Mg<sup>2+</sup> (46, 47). After coating overnight at 4°C in carbonate buffer, pH 9.2, plates were washed and blocked using HBS-T with 3% BSA buffer for 2 h at RT. Blocked plates were washed with HBS-T and incubated for 1 h at RT with 500 ng/well of each of the ECM components (human plasma fibronectin, human tissue fibronectin, aggrecan, decorin, vitronectin, elastin, and fibrinogen; Sigma-Aldrich, Co., LLC) and integrins ( $\alpha_1\beta_1$ ,  $\alpha_3\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ , and  $\alpha_5\beta_1$ ; EMD Millipore Corporation) (46, 47). After washing, plates were incubated for 1 h at RT with each respective anti-ECM component or anti-integrin antibody. The plates were washed again, and a secondary HRP-conjugated antibody was applied for 1 h at RT, followed by development with OPD tablets (Thermo Scientific) and measurement of the A<sub>450</sub> as described above. Wells without ECM components or integrins were used as blanks. The effects of the mutations in the MIDAS motif of BB0172 were also evaluated by analyzing their binding to  $\alpha_3\beta_1$  integrin following the same protocol described above.

**Statistical analysis.** Two-way analysis of variance was used to determine significant differences in the binding of BB0172<sub>T</sub> to the different integrins and extracellular matrix components, along with the Sidak multiple comparison test with a 95% confidence interval. Analyses were performed using Prism 6.0d (GraphPad Software, Inc.).



**FIG 3** BB0172 localizes on the *B. burgdorferi* surface. *B. burgdorferi* B31A3 was cultured at RT/pH 7.6 (lane 1), shifted to 37°C/pH 6.8 (lane 2), and shifted to 37°C/pH 6.8 and treated with proteinase K (lane 3). Whole-cell lysates of all treatments 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<sub>pep</sub> 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/pH 7.6 to 37°C/pH 6.8.

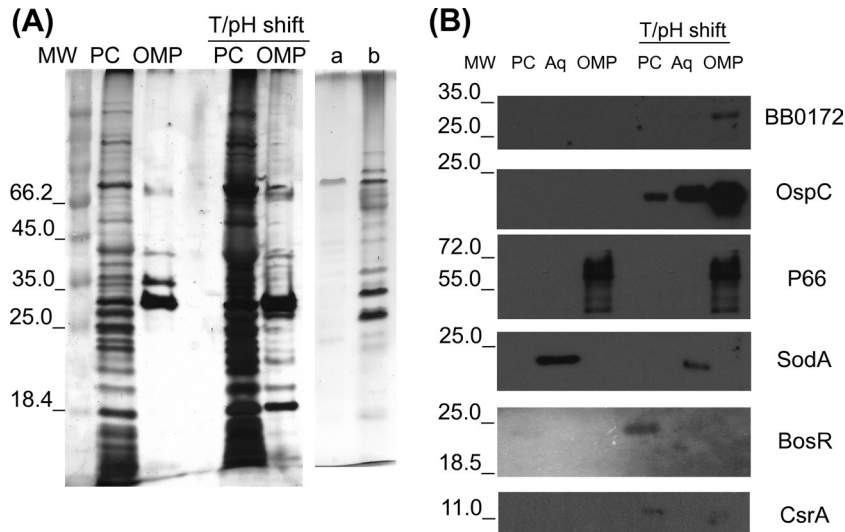
## RESULTS

### *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. 3B, lane 1). 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 vWFA 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-



**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 OPM 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<sub>pep</sub> followed by anti-mouse HRP-conjugated 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.

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 *Borrelia* 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),  $\Delta$ G 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.

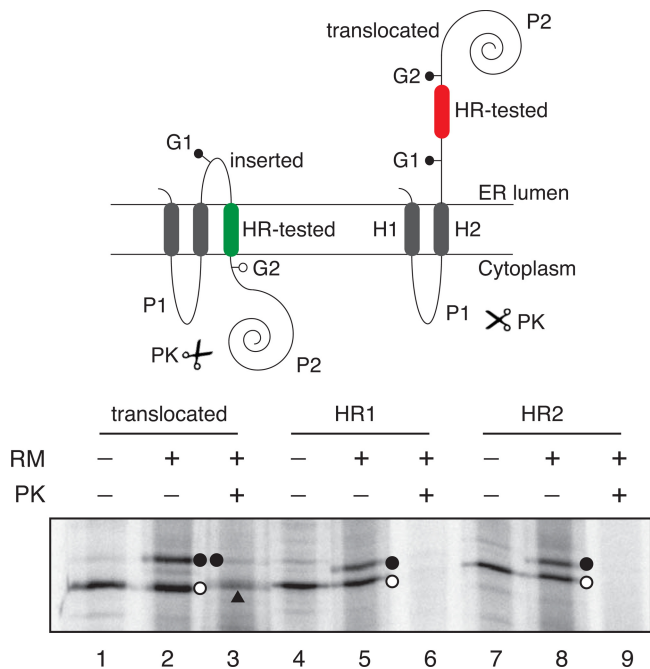
**TABLE 3** *In silico* analysis of the BB0172 amino acid sequence

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

<sup>a</sup> Bold numbers denote "primary" helices.

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 *Escherichia 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<sub>-</sub>) (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



**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 proteinase 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.

the BB0172 protein was slightly bound to human plasma fibronectin in the presence of the metals  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Mg}^{2+}$ ; 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 extensively characterized plasma fibronectin-binding protein of *B. burgdorferi* (48, 53–55). We next evaluated the binding capability of the BB0172 protein to integrins. The BB0172 protein significantly bound to  $\alpha_3\beta_1$  integrin in the presence of divalent cations, while P66 (a  $\beta_3$  integrin-binding protein from *B. burgdorferi*) showed very low binding (Fig. 6B). This result was consistently observed, suggesting that the BB0172 protein could be a  $\alpha_3\beta_1$  integrin-binding protein.

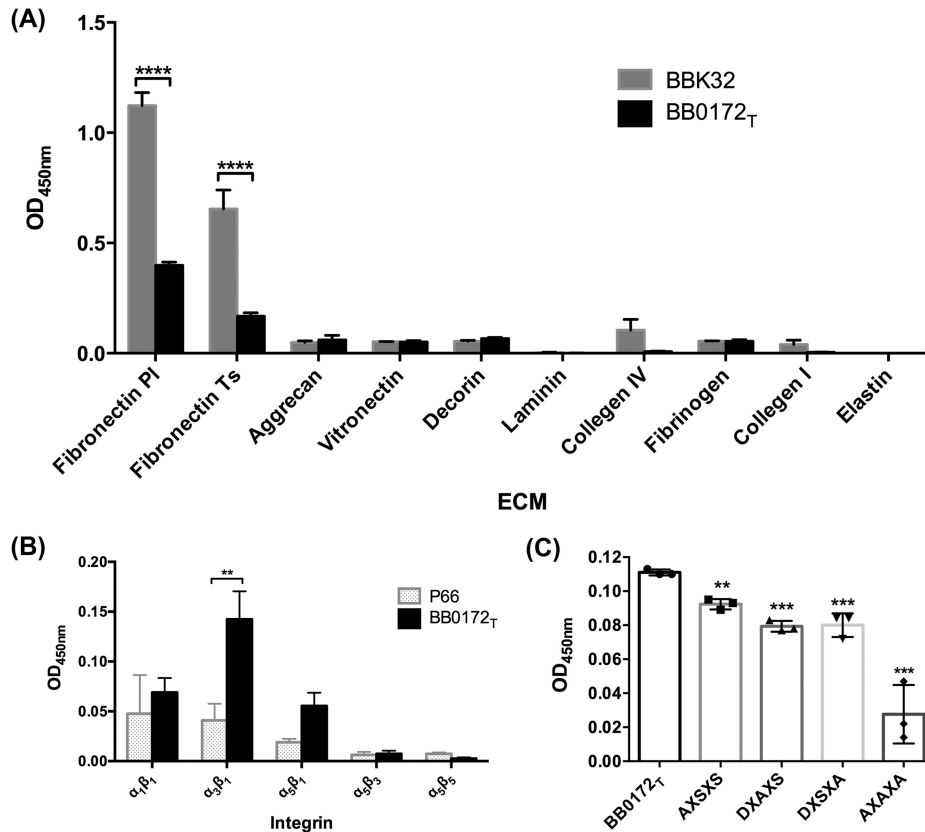
Ligand binding to integrins is usually dependent upon divalent cations. The presence of a MIDAS motif in the BB0172 amino acid sequence (Fig. 1C) suggests that this region may accomplish this function by adopting a specific fold. To investigate whether this region of BB0172 is functionally equivalent to a MIDAS domain, we performed an alanine-scanning analysis by replacing critical

residues of the MIDAS motif (DXSXS) (Table 2) with alanine through site-directed mutagenesis. Each single-point mutant strain (AXSXS, DXAXS, and DXSXA) plus a triple mutant strain harboring all three replacements (AXAXA) was used in a  $\alpha_3\beta_1$  integrin-binding assay. The results showed that all three individual mutations significantly reduced the binding of BB0172 to  $\alpha_3\beta_1$  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.

## DISCUSSION

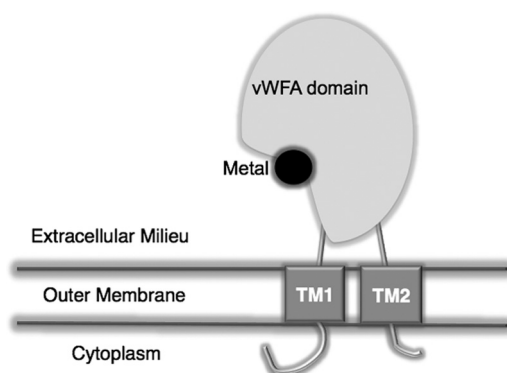
vWFA domain-containing proteins are found in a broad array of systems, from integrins in mammalian organisms to bacteria (5, 13, 56–58). To be functional, these proteins must exist on the cell surface to interact with their receptors. The chromosome of *Borrelia burgdorferi* encodes a series of vWFA 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 proteinase 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 proteinase 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 (UUA) 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.*



**FIG 6** BB0172 is an integrin-binding protein. (A) Purified BB0172<sub>T</sub> was incubated with different ECM components: aggrecan, collagen I and IV, decorin, elastin, laminin, fibrinogen, human plasma fibronectin, human tissue fibronectin, and vitronectin. Recombinant BBK32 was used as a borrelial positive-control protein for the binding to human plasma fibronectin. (B) The binding of BB0172 to different integrins by using HBS buffer containing Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Mn<sup>2+</sup>. Recombinant P66 was used as a positive control for the binding to human integrins. BB0172 bound more strongly to  $\alpha_3\beta_1$  integrin compared with the P66 binding. (C) Purified recombinant BB0172 proteins containing mutations in the MIDAS motif (AXSXS, DXAX, DXSXA, and AXAXA) were incubated with  $\alpha_3\beta_1$  integrin. BB0172<sub>T</sub> corresponds to the wild-type sequence. Each mutation of the MIDAS motif altered efficacy of the binding of BB0172 to  $\alpha_3\beta_1$  integrin. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (compared with the control protein). All binding assays entailed four replicates for each protein in each ECM component or integrin.

*burgdorferi*. 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 mem-



**FIG 7** Schematic representation of *B. burgdorferi* BB0172 topology. We propose that BB0172 is anchored to the borrelial outer membrane through two TM segments located at the N terminus and C terminus of the protein and that the vWFA domain will be exposed to the exterior of the cell. The MIDAS motif is also present in the external segment of this protein, actively participating in the adhesion of BB0172 to  $\alpha_3\beta_1$  integrin.

brane 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 adhesin 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 adhesin to the human  $\alpha_3\beta_1$  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  $\alpha_{IIb}\beta_3$  on the surface of activated platelets, as well as with integrin  $\alpha_v\beta_3$  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 BBB07, an integrin  $\alpha_3\beta_1$ -binding protein of *B. burgdorferi*. They showed that the RGD-dependent binding of the BBB07 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  $\alpha_3\beta_1$  integrin, but in an RGD-independent manner, since BB0172 lacks this motif. Integrin  $\alpha_3\beta_1$  binds to collagen I and IV, laminin, fibronectin, and nidogen, and in some cell types to thrombospondin 1 (73–76). Integrin  $\alpha_3\beta_1$  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 (DXSXS), which is highly conserved among *Borrelia* species. Mutations of these amino acid residues reduced recombinant BB0172 binding to integrin  $\alpha_3\beta_1$ , 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  $\alpha_3\beta_1$ . 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  $\alpha_3\beta_1$  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  $\alpha_3\beta_1$ . 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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