Structural Modeling and Physicochemical Characterization Provide Evidence that P66 Forms a β -Barrel in the *Borrelia burgdorferi* Outer Membrane

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The *Borrelia burgdorferi* outer membrane (OM) contains numerous surface-exposed lipoproteins but a relatively low density of integral OM proteins (OMPs). Few membrane-spanning OMPs of *B. burgdorferi* have been definitively identified, and none are well characterized structurally. Here, we provide evidence that the borrelial OMP P66, a known adhesin with pore-forming activity, forms a β -barrel in the *B. burgdorferi* OM. Multiple computer-based algorithms predict that P66 forms a β -barrel with either 22 or 24 transmembrane domains. According to our predicted P66 topology, a lysine residue (K487) known to be sensitive to trypsin cleavage is located within a surface-exposed loop. When we aligned the mature P66 amino acid sequences from *B. burgdorferi* and *B. garinii*, we found that K487 was present only in the *B. burgdorferi* P66 protein sequence. When intact cells from each strain were treated with trypsin, only *B. burgdorferi* P66 was trypsin sensitive, indicating that K487 is surface exposed, as predicted. Consistent with this observation, when we inserted a c-Myc tag adjacent to K487 and utilized surface localization immunofluorescence, we detected the loop containing K487 on the surface of *B. burgdorferi*. P66 was examined by both Triton X-114 phase partitioning and circular dichroism, confirming that the protein is amphiphilic and contains extensive (48%) β -sheets, respectively. Moreover, P66 also was able to incorporate into liposomes and form channels in large unilamellar vesicles. Finally, blue native PAGE (BN-PAGE) revealed that under nondenaturing conditions, P66 is found in large complexes of ~ 400 kDa and ~ 600 kDa. Outer surface lipoprotein A (OspA) and OspB both coimmunoprecipitate with P66, demonstrating that P66 associates with OspA and OspB in *B. burgdorferi*. The combined computer-based structural analyses and supporting physicochemical properties of P66 provide a working model to further examine the porin and integrin-binding activities of this OMP as they relate to *B. burgdorferi* physiology and Lyme disease pathogenesis.

Lyme disease is currently the most common arthropod-borne infection in the United States and is also prevalent throughout Europe and Asia (1). The disease is caused by pathogenic spirochetes belonging to the *Borrelia burgdorferi sensu lato* complex. The three major genospecies associated with Lyme disease include *B. burgdorferi sensu stricto* (here referred to as *B. burgdorferi*), *B. garinii*, and *B. afzelii* (2). *Borrelia* spirochetes are maintained in nature through an enzootic cycle that includes *Ixodes* ticks and a mammalian host (1, 3). In humans, Lyme disease typically manifests as an expanding skin rash, termed erythema migrans, which can be followed by cardiac symptoms, nervous system abnormalities, and arthritis (4, 5).

B. burgdorferi is a dual-membrane (diderm) organism with both an outer membrane (OM) and a cytoplasmic or inner membrane (IM). The borrelial OM differs markedly from the OMs of typical Gram-negative enteric organisms, such as *Escherichia coli* (3, 6). For example, the borrelial OM lacks lipopolysaccharide (LPS) (7, 8), the highly inflammatory glycolipid found in Gram-negative bacteria. Furthermore, the surface of *B. burgdorferi* is decorated with numerous lipid-modified, membrane-anchored lipoproteins, whereas surface-exposed lipoproteins are uncommon in typical Gram-negative bacteria (6, 9–11). Most importantly, with respect to the current study, freeze fracture electron microscopy, which visualizes integral OM proteins (OMPs) as intramembranous particles, revealed that the OM of *B. burgdorferi* also contains at least 10-fold fewer integral OMPs per μm^2 than that of *E. coli* (12, 13). Few of these outer membrane-spanning proteins have been identified, and none has been structurally

characterized to any extent (9). Given that OMPs identified in other diderm organisms, as well as eukaryotic organelles of bacterial origin (e.g., mitochondria and chloroplasts), consist of amphipathic β -strands that form β -barrels (14, 15), one would expect that *B. burgdorferi* OMPs form β -barrels as well. In diderms, the amphipathic nature of the β -barrel OMP precursors allows the translocation of these polypeptides across the hydrophobic IM. In contrast, IM proteins contain α -helical transmembrane domains that serve as stop transfer sequences that result in proteins being localized to the IM (16). Furthermore, as with Gram-negative organisms, nutrients must be transferred across the borrelial OM for the spirochete to survive within the host; thus, channels and pores must be present in the borrelial OM to facilitate nutrient acquisition. Moreover, we now know that *B. burgdorferi* has the machinery necessary to locate and fold β -barrel proteins into the borrelial OM. Recent studies have revealed that β -barrel OMPs from *E. coli*, *Neisseria meningitidis*, and all other diderm bacteria characterized to date are chaperoned into the OM via the multiprotein β -barrel assembly machine (BAM) complex (17–20). The central component of the BAM complex, BamA, is

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conserved among diderm organisms (21). Importantly, it has been shown that *B. burgdorferi* has a functional BamA ortholog and at least two Bam accessory proteins (22, 23). Similarly, *Treponema pallidum*, the spirochete that causes syphilis, also has been shown to contain a BamA ortholog (24).

Of the *B. burgdorferi* OMPs identified to date, P66, encoded by open reading frame *bb0603*, has been the most extensively studied. BamA is required for efficient transport of P66 into the borrelial OM (22). Proteolysis studies have confirmed that P66 has surface-exposed domains, including a putative surface-exposed loop (residues 459 to 502) near the C terminus (25–29). Among the *Borrelia* genospecies, sequence variation in the predicted surface-exposed loop is greater than that found throughout the rest of the P66 sequence, indicating that the loop may be under immune selection pressure during mammalian infection (28, 29). Furthermore, Skare et al. demonstrated that native P66 forms pores in lipid bilayer assays (30), and similar properties have been described for P66 from *B. garinii* and *B. afzelii* (31). *In vitro* analyses have implicated P66 as an adhesin that binds specifically to β_3 -chain integrins (32–34). Although the tertiary structure and final structural conformation of P66 are likely required for interaction with integrins (33), a region encompassing amino acids 150 to 343 is sufficient for ligand binding (32, 34, 35). Interestingly, while P66 is required for infection of mice by tick inoculation (36), a P66 mutant replicates normally within dialysis membrane chambers implanted into the peritoneal cavities of rats (36). Thus, the molecule's contribution to growth and nutrient acquisition during the mammalian phase of the spirochete's enzootic cycle is unclear.

While the functional studies described above have provided insight into the role of P66 *in vitro* and *in vivo*, little is still known about the protein's structure. Herein, we report that P66 is predicted to form a β -barrel and displays the properties expected of an OMP with an amphipathic β -barrel structure. Additionally, we confirm that lysine K487 is located in a surface-exposed loop that is protease sensitive and corroborate prior reports that P66 specifically interacts with OspA and OspB. These data support the contention that the *B. burgdorferi* P66 protein forms a β -barrel despite its lack of sequence homology with known OMPs of Gram-negative organisms. Our results provide a working model to further examine the porin and integrin-binding activities of this OMP as they relate to *B. burgdorferi* physiology and Lyme disease pathogenesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Borrelia* organisms, including *B. burgdorferi* B31, *B. burgdorferi* JD1, *B. garinii* Pbi, and *B. garinii* IP90, were cultivated at 34°C in BSK-II medium containing 6% heat-inactivated rabbit serum (BSK-II complete, pH 7.6) (37). For surface localization immunofluorescence and Triton X-114 phase partitioning experiments, we utilized the avirulent, high-passage-number strain *B. burgdorferi* clone F (cF), which was described previously (38, 39).

P66 sequence alignment and modeling. The membrane topology of the mature *B. burgdorferi* P66 protein (GenBank accession number NP_212737) was predicted using the PRED-TMBB server (<http://biophysics.biol.uoa.gr/PRED-TMBB/>) (40, 41). OM localization and β -barrel predictions were made using the following servers: CELLO (<http://cello.life.nctu.edu.tw/>) (42), PSORTb (<http://www.psort.org/psortb/>) (43), HHOMP (<http://toolkit.tuebingen.mpg.de/hhomp/>) (44), TMBETADISC-AAC (<http://rbf.bioinfo.tw/~sachen/OMPPredict/TMBETADISC-RBF.php>) (45), and BOMP (<http://services.cbu.uib.no/tools/bomp/>) (46). To predict the structural properties of *B. burgdorferi* P66, the mature P66 amino acid sequence was analyzed using the

TMBpro server (<http://tmbpro.ics.uci.edu/>) (47), and the resulting Protein Data Bank file was further analyzed by use of the Swiss-PdbViewer (v4.0.2) (48). Amino acid sequence alignments of P66 from *B. burgdorferi* B31, *B. burgdorferi* JD1, *B. garinii* Pbi, and *B. garinii* IP90 (GenBank accession numbers NP_212737, YP_005805693, AAU07452, and CAA61026, respectively) were generated using the MacVector (v10.0) program (MacVector, Inc., Cary, NC).

Cloning, purification, and folding of recombinant P66. P66 was amplified from *B. burgdorferi* B31 genomic DNA using primers P66 5' (GC GGCTAGCTTAAAGGAAAAAGATATATTTAAATA) and P66 3' (GC GCTCGAGGCTTCGCTGTAGGCTATTT) (restriction enzyme sequences are in bold). *B. burgdorferi* BamA was amplified from genomic DNA with primers BamA 5' (GCGGCTAGCGTTGAAAATTACAAGGGGA AAA) and BamA 3' (GCGCTCGAGATATCTCATCTCAATTCCTAA GA). *Escherichia coli* OmpA was amplified with primers OmpA 5' (GCG GCTAGCGCTCCGAAAGATAACACCTG) and OmpA 3' (GCGCTCGA GAGCCTGCGGCTGAGTTACA). For amplification of *B. burgdorferi* BamA Potra 1, primers BamA P1 5' (GCGGCTAGCAAGGGGAAAAATA AGGGTAT) and BamA P1 3' (GCGCTCGAGTTCTTTACAATAAAAT GTAATAAAAAAG) were used. The amplicons were subsequently digested and cloned into the NheI and XhoI sites of pET23a (EMD Millipore, Billerica, MA), which has a C-terminal His tag. The constructs were transformed into the *E. coli* strain Rosetta 2 DE3 (EMD Millipore, Billerica, MA), and DNA sequencing was performed to verify that the sequence remained unaltered throughout the cloning process.

For batch purification of recombinant P66, OmpA, and BamA, 1 liter of lysogeny broth was inoculated with 25 ml of an overnight culture, which was then grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.6. Protein expression was then induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and the culture was grown for an additional 3 h. After induction, the cells were pelleted at $10,000 \times g$ for 15 min at 4°C and resuspended in 25 ml of resuspension buffer (50 mM Tris, 100 mM NaCl, pH 7.6) with 25 μ l of protease inhibitor cocktail. The cells were then lysed by sonication and pelleted for 20 min at $20,000 \times g$. The pellet was resuspended in 10 ml of binding buffer (100 mM NaH_2PO_4 , 10 mM Tris, 8 M urea) at pH 8.0. The suspension was then rotated at room temperature for 30 min and subjected to centrifugation for 30 min at $20,000 \times g$. After centrifugation, the supernatant was applied to a column containing a 5-ml volume of nickel-nitrilotriacetic acid agarose (Qiagen, Valencia, CA) that had previously been equilibrated with binding buffer. After rocking the resin and supernatant for 20 min, the column was washed with 100 ml of wash buffer (100 mM NaH_2PO_4 , 10 mM Tris, 8 M urea) at pH 6.3. The protein was next eluted in 30 ml of elution buffer 1 (100 mM NaH_2PO_4 , 10 mM Tris, 8 M urea) at pH 5.8 and elution buffer 2 (100 mM NaH_2PO_4 , 10 mM Tris, 8 M urea) at pH 4.5. SDS-polyacrylamide gel electrophoresis (PAGE) was used to analyze the purified recombinant protein. After protein purification, the protein was concentrated using Amicon-Ultra centrifugal filters (EMD Millipore, Billerica, MA). *Treponema pallidum* protein TP0453 and *E. coli* OmpF were purified as described elsewhere (49, 50).

To fold recombinant P66, BamA, and OmpA, each protein was incubated in DDM buffer (50 mM Tris, pH 7.6, 100 mM NaCl, 2.0% dodecyl- β -D-maltopyranoside [DDM; Affymetrix, Santa Clara, CA]) for 24 h at 4°C, and the insoluble material was pelleted by centrifugation at $20,000 \times g$ for 30 min at 4°C. The supernatant was removed and analyzed by SDS-PAGE.

Immunoblotting and antibody production. SDS-PAGE and immunoblotting procedures were performed as previously described (22, 51). Rat polyclonal antibodies recognizing *B. burgdorferi* P66 and *E. coli* OmpA were generated by Harlan Bioproducts for Science, Inc. (Madison, WI), and were used at a 1:5,000 dilution for enhanced chemiluminescence. OspA, FlaB, BamA, BB0796, and CspA antibodies were described previously (22, 39, 52–54). Monoclonal mouse OspB antibodies (CB2) have been described elsewhere (55) and were provided by Jorge Benach (Stony Brook University, Stony Brook, NY).

Trypsin surface accessibility assays. To digest surface proteins with trypsin, 2×10^8 *B. burgdorferi* B31 cells were subjected to centrifugation at $4,000 \times g$ for 4 min and washed three times in phosphate-buffered saline (PBS; pH 7.4). The final pellet was resuspended in 1 ml of PBS, and samples were either treated or mock treated with trypsin (Sigma Chemical Company, St. Louis, MO) resuspended in 0.001 N HCl for 1 h at room temperature. Phenylmethylsulfonyl fluoride (0.4 mM; Sigma Chemical Company) was added to each sample to stop the protease activity, and the samples were pelleted by centrifugation at $10,000 \times g$ for 10 min. The final pellets were prepared for SDS-PAGE and immunoblot analysis using rat anti-P66 antibodies. Membranes were also subjected to immunoblotting with antibodies to OspB and FlaB for surface and subsurface controls, respectively.

Generation of c-Myc *B. burgdorferi* clones. To express P66 with a c-Myc tag in the K487 predicted surface loop, we generated the P66–c-Myc^{K487} construct by cloning *p66* and the *c-myc* tag sequence into pBSV2G (56). To clone the *flgB* promoter into pBSV2G, the *flgB* promoter was amplified from pBSV2 with primers *flgB* F (GCGGGTACCTACCCGAGCTTCAAGGAAGA) and *flgB* R (GCGGGATCCATGGAACCTCCC TCATTTAAA). The amplicon was cloned into the KpnI and BamHI sites of pBSV2G. Next, the portion of *p66* including the sequence encoding K487 and the upstream region was amplified from *B. burgdorferi* B31 genomic DNA with primers P66 F (GCGGGATCCATGAAAGCCATA TTTTATATAAATT) and P66 us R (GCGTCTAGACTTTGTGCTTGT GAACCTTTGT) and inserted into the BamHI and XbaI sites of the vector. The region of *p66* downstream of K487 was amplified with primers P66 ds F (GCGGTGCGACACCACAACCCCTAATCTGAC) and P66 R (GCGGT CGACTTAGCTTCCGCTGTAGGCTA) and cloned into the SalI site. To generate the *c-myc* tag, the *c-myc* tag sequence (GCGTCTAGAGAACA AAACCTATTCTGAAGAAGATCTGGTTCGACGCG) and the reverse complement sequence were annealed before ligating and cloning the product into the XbaI and SalI sites of the vector. As a control, we also generated a vector from which P66 would be expressed with a c-Myc tag at the P66 C terminus and termed the construct P66–c-Myc^{C-terminal}. *flgB* was inserted into the pBSV2G vector as described above, and the *p66* sequence without the stop codon was amplified with primers P66 F and P66 ns R (GCGTCTAGAGCTTCCGCTGTAGGCTATT) and cloned into the BamHI and XbaI sites of pBSV2G. Next, the c-Myc tag was inserted as described above; however, a stop codon was added to the *c-myc* sequence (GCGTCTAGAGAACAACCTATTCTGAAGAAGATCT GTAAGTCGACGCG). Finally, a control vector, P66–c-Myc[−], that expressed P66 without a c-Myc tag was generated by amplifying *p66* with primers P66 F and P66 R and inserting the amplicon into the BamHI and XbaI sites of pBSV2G, in which the *flgB* promoter had previously been inserted into the vector, as described above. The three vectors were electroporated into competent *B. burgdorferi* cF cells as described previously (57). After electroporation, spirochetes were selected with BSK-II complete medium containing gentamicin (40 µg/ml).

Surface localization immunofluorescence. Spirochetes were grown to mid-exponential phase and diluted to a final concentration of 5×10^6 cells. Cell suspensions were cocubated for 1 h with rabbit anti-c-Myc antibodies (Sigma Chemical Company) at a dilution of 1:10 and rat anti-FlaB antibodies at a dilution of 1:1,000. The cells were then washed three times in PBS. The final pellet was resuspended in 100 µl PBS, and 10 µl of the sample was spotted onto microscope slides and fixed for 10 min with acetone. Samples were subsequently blocked with PBS containing 1% bovine serum albumin (BSA). Samples were then incubated for 45 min with Alexa Fluor 488-conjugated goat antirabbit antibodies (1:500; Invitrogen, Carlsbad, CA) and Alexa Fluor 568-conjugated goat antirat antibodies (1:500; Invitrogen) before being washed three times with PBS containing BSA. Samples were then mounted in buffered glycerol containing DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA) and sealed with a coverslip, and images were visualized and captured with an Olympus BX-60 fluorescence microscope (Olympus America Inc., Center Valley, PA). As a control, samples were also spotted onto

microscope slides and fixed with acetone prior to incubation with rabbit anti-c-Myc antibodies and rat anti-FlaB antibodies. After these samples were washed three times with PBS containing 1% BSA, they were incubated with secondary antibodies and prepared as described above.

Tryptophan fluorescence. Tryptophan emission spectra were measured using a Hitachi F-2500 fluorescence spectrophotometer. Protein samples that were either denatured in urea or folded in DDM buffer (described above) were inserted into a 5-mm-path-length quartz cell at 25°C. The excitation wavelength and bandwidth were 295 nm and 2.5 nm, respectively. The tryptophan emission spectra were measured between 300 and 400 nm. Background spectra were also measured using DDM buffer without protein, and the background measurements were subtracted to obtain the final emission curves.

CD spectroscopy. All circular dichroism (CD) experiments were performed using a Jasco J-715 spectropolarimeter (Jasco, Easton, MD). Far-UV CD spectra were recorded at 20°C using a 1-mm-path-length cuvette, a bandwidth of 1 nm, a time response of 8 s, and a scan rate of 20 nm/min. CD spectra for each protein, representing the average of nine scans, were corrected by subtracting the background spectrum of the buffer. To assess the secondary structure contents of each protein, we analyzed the spectra using the DICHROWEB server (58, 59).

Triton X-114 phase partitioning. The amphiphilic properties of P66 from *B. burgdorferi* cF cells or recombinant protein (10 µg) were assessed by Triton X-114 phase partitioning as described elsewhere (22, 53, 60). The resulting aqueous- and detergent-enriched proteins were precipitated with acetone, and the protein was analyzed by SDS-PAGE and immunoblotting using rat anti-P66, rat anti-BamA, rat anti-OspA, and rat anti-BB0796 antibodies.

Preparation of liposomes. Large unilamellar vesicles (LUVs) were prepared as described previously (61). To simulate the phospholipid content of the *B. burgdorferi* outer membrane, a mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (70:30 mol%, respectively; Avanti Polar Lipids, Inc., Alabaster, AL) was used. Preparation of liposomes containing the terbium-dipicolinic acid complex [Tb(DPA)₃^{3−}] fluorophore was performed as previously described (61).

Liposome flotation assay. Recombinant P66 (400 ng) in DDM buffer was incubated with 750 µg of LUVs at room temperature for 1 h in 50 mM acetate buffer. After incubation, 200 mg of sucrose was added to the reaction mixture, and the sample was mixed thoroughly before being transferred to an ultracentrifuge tube. Discontinuous sucrose gradients were made by layering 40% and 6% sucrose on top of the sample in the ultracentrifuge tubes. Samples were centrifuged at 90,000 rpm for 1 h at 4°C. The samples were removed from the centrifuge, and three fractions were carefully collected from the tube: the liposome (top) layer and two non-liposome (middle and bottom) layers. Each fraction was separated by SDS-PAGE and subjected to immunoblot analysis using rat anti-P66 antibodies. Control experiments were performed using OmpA unfolded protein as well as OmpA protein folded in DDM buffer. Fractions from the control experiments were immunoblotted with rat anti-OmpA antibodies.

Pore formation assay. Pore formation assays were performed as described previously (50, 62). Liposomes loaded with Tb(DPA)₃^{3−} were diluted in 50 mM Tris (pH 7.5), 100 mM NaCl supplemented with 5 mM EDTA to a concentration of 100 µM total lipids. The sample was equilibrated at 25°C for 5 min, and the net initial emission intensity (F_0) was determined. Recombinant P66, OmpF, or TP0453 (final concentration, 100 nM) was incubated with the liposome suspension for 30 min at 37°C. Samples were then reequilibrated to 25°C. The final net emission intensity (F_f) of the sample was determined after subtracting the value for the blank and correcting for dilutions. The fraction of Tb(DPA)₃^{3−} quenched was estimated using F_f/F_0 .

Heat modifiability. *B. burgdorferi* whole-cell lysates or recombinant P66 and OmpA folded in DDM buffer as described above were boiled in sample buffer (62 mM Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 100 mM

dithiothreitol, 2% sodium dodecyl sulfate, 0.001% bromophenol blue) or incubated at room temperature prior to SDS-PAGE and subsequent immunoblot analysis with P66 or OmpA antibodies.

BN-PAGE analysis of native and recombinant P66. *B. burgdorferi* organisms were pelleted by centrifugation at $20,000 \times g$ for 20 min at 4°C. The subsequent pellet was resuspended and incubated overnight at 4°C in 50 mM Tris (pH 7.0), 2% DDM, and 5% protease inhibitor cocktail (PIC). Detergent-insoluble material was removed from the sample by centrifugation at $20,000 \times g$ for 20 min at 4°C. *B. burgdorferi* lysates (1×10^8 to 5×10^9 spirochetes) were analyzed using blue native PAGE (BN-PAGE) (63, 64). Lysates were resolved at 4°C in a 4 to 12% bis-Tris acrylamide gel (Bio-Rad). For the first 1/3 of the gel run, 0.02% Coomassie brilliant blue G-250 (CBB-G250) was added to the cathode buffer (50 mM Tricine [pH 7.0], 15 mM bis-Tris). Fresh cathode buffer that did not contain CBB-G250 was then used for the remaining time. The anode buffer consisted of 50 mM bis-Tris (pH 7.0) throughout the duration of the run time. After the lysates were resolved, the samples were transferred to a nitrocellulose membrane in 50 mM Tricine (pH 7.0). Membranes were next subjected to immunoblotting using rat anti-P66 antibodies or anti-OspA antibodies. Analysis of recombinant P66 was performed as described above, except that 1 µg of protein was incubated on ice for 30 min prior to BN-PAGE. To assess protein migration, the retardation factor (R_f) values were calculated according to the manufacturer's instructions (Invitrogen).

Co-IP. Cell lysates for coimmunoprecipitation (co-IP) experiments were prepared by harvesting mid-log-phase cultures of *B. burgdorferi* strain B31 5A4NP1 (65) by centrifugation at $5,000 \times g$ for 20 min. The cells were washed four times in PBS (pH 7.4), and the final pellet was solubilized in 2.5 ml per gram of wet cell weight 1× BugBuster reagent (EMD Biosciences, Inc., Darmstadt, Germany) supplemented with 10 µl per gram wet cell weight Lysonase bioprocessing reagent (EMD Biosciences, Inc.) and 20 µl of protease inhibitor cocktail (Sigma Chemical Company, St. Louis, MO). Samples were then incubated at room temperature for 20 min and subsequently pelleted by centrifugation at $15,000 \times g$ for 15 min at 4°C. Co-IPs were performed with the prepared lysates using a Pierce cross-link immunoprecipitation (IP) kit (Pierce Biotechnologies, Rockford, IL) according to the manufacturer's instructions. The lysates were precleared and then applied in IP/lysis buffer to protein A/G columns treated and cross-linked with 10 µl of rat antiserum to P66. After incubation at 4°C for 3 h, the columns were washed and the bound protein was eluted in low-pH elution buffer. The eluted protein was subjected to SDS-PAGE and analyzed by immunoblotting using rat anti-P66, rat anti-OspA, rat anti-CspA, and monoclonal OspB antibodies.

RESULTS

P66 is predicted to form a 22- or 24-stranded β-barrel in the *B. burgdorferi* OM. To assess whether *B. burgdorferi* P66 is predicted to form a β-barrel in the borrelial OM, we analyzed its sequence using computational algorithms that can predict the cellular location of proteins as well as their propensity to form a β-barrel. P66 was predicted by five out of the six computational programs utilized (CELLO [42], PSORTb [43], HHOMP [44], PRED-TMBB [40, 41], and TMBETADISC-AAC [45]) to be in a β-barrel conformation and located in the OM. To generate a model of P66 membrane topology, we analyzed the mature P66 amino acid sequence using PRED-TMBB (40, 41), which utilizes three different prediction algorithms (Viterbi, N-Best, and Posterior decoding) to identify putative transmembrane domains in protein sequences. As expected of a β-barrel OMP, P66 was predicted to have either 24 membrane-spanning regions according to the Viterbi and N-Best algorithms or 22 transmembrane strands according to the Posterior decoding algorithm (Fig. 1A). Additionally, the N- and C-terminal regions of P66 were predicted by all three PRED-TMBB algorithms to be located in the periplasm (Fig. 1A, highlighted in green), which also is

typical of β-barrel-forming OMPs (66). To predict the tertiary structure of P66, we submitted the mature sequence to the TMBpro server (47), which predicted that P66 forms a β-barrel structure with 24 transmembrane strands (Fig. 1B). Previous proteolysis assays have revealed that P66 has a surface-exposed, trypsin-sensitive lysine at position 487 (K487) (28). Consistent with this prior observation, the K487 residue was predicted by all PRED-TMBB algorithms to be in an extracellular domain spanning amino acids 459 to 498 (Fig. 1A). While TMBpro also predicted K487 to be located in an extracellular loop, the loop in this analysis was composed of residues 462 to 502 (Fig. 1B).

Lysine residue K487 is surface exposed and the target of trypsin degradation. Among the Lyme disease *Borrelia* isolates, the P66 amino acid sequence is well conserved (28). However, previous reports have shown that greater variability exists within a predicted surface loop of P66 containing the K487 residue (28). When the mature P66 amino acid sequences of strains *B. burgdorferi* B31, *B. burgdorferi* JD1, *B. garinii* IP90, and *B. garinii* Pbi were aligned, the sequences shared approximately 90% sequence identity between the two borrelial genospecies. However, sequence identity in the surface-exposed loop (amino acids 459 to 502) (Fig. 2A, boxed in red) was only approximately 70%. Interestingly, we observed that K487, the lysine thought to be the target of trypsin digestion, was detected only in the *B. burgdorferi sensu stricto* strains (Fig. 2A, red arrow). Therefore, we sought to confirm that K487 is the surface-exposed residue targeted by trypsin in the protease experiments. First, we confirmed that when intact *B. burgdorferi* cells were digested with increasing amounts of trypsin, which specifically cleaves on the carboxyl side of lysine and arginine residues, the 66-kDa full-length protein was reduced to ~52 kDa, the size predicted if cleavage occurs at K487 (Fig. 2B) (28). Next, we incubated *B. burgdorferi* B31, *B. burgdorferi* JD1, *B. garinii* IP90, and *B. garinii* Pbi cells with trypsin. Consistent with K487 being the trypsin cleavage site, only the *B. burgdorferi* B31 and JD1 strains yielded the 52-kDa truncated forms (Fig. 2C). Conversely, the *B. garinii* IP90 and Pbi strains that lacked K487 were resistant to trypsin digestion (Fig. 2C). Control immunoblots for the trypsin sensitivity experiments (Fig. 2B and C) demonstrated that the surface-exposed lipoprotein OspB was degraded by trypsin, while the periplasmic FlaB protein remained intact.

To verify the surface localization of the loop containing K487, we inserted a c-Myc tag into the P66 sequence adjacent to K487 and expressed P66 with the c-Myc tag in *trans* from pBSV2G (P66–c-Myc^{K487}; Fig. 3A). We next performed surface localization immunofluorescence assays with anti-c-Myc antibodies to determine if the c-Myc tag could be detected on the surface of *B. burgdorferi* cells. As a control, we also expressed the c-Myc tag at the C terminus of P66 (P66–c-Myc^{C-terminal}; Fig. 3A). Given that P66 is predicted to be folded into an amphipathic β-barrel with its extreme C terminus located in the periplasm (Fig. 1A), we would not expect the C terminus of the protein to be detected on the surface of *B. burgdorferi*. We also included a control in which P66 was expressed from pBSV2G but without a c-Myc tag (P66–c-Myc[−]; Fig. 3A). We first confirmed by immunoblotting using anti-c-Myc antibodies that the P66–c-Myc^{K487} and P66–c-Myc^{C-terminal} strains but not the P66–c-Myc[−] strain expressed c-Myc (Fig. 3B) and that all strains, as expected, expressed P66 (Fig. 3B). Surface localization immunofluorescence assays were next performed by incubation of intact cells with anti-c-Myc and anti-FlaB antibodies. By including the periplasmic FlaB pro-

FIG 1 P66 is predicted to form a β -barrel. (A) The *B. burgdorferi* P66 mature amino acid sequence was analyzed using the PRED-TMBB server. Topology results from algorithms used by PRED-TMBB include the Viterbi (#1), N-Best (#2), and Posterior decoding (#3) methods. Predicted transmembrane regions (red), extracellular domains (blue), and periplasmic domains (green) are indicated for each algorithm. The predicted surface-exposed loop containing K487 is noted with a black bar under the sequence, while K487 is indicated with an asterisk above the residue. (B) TMBpro (<http://tmbpro.ics.uci.edu/>)-predicted tertiary structure of *B. burgdorferi* P66 depicted as a ribbon model. Both side (left) and top (right) views of the predicted P66 model are shown. The lysine residue (K487) predicted to be sensitive to trypsin cleavage is labeled.

tein in the assays, we verified that the borrelial OM remained intact throughout the experiments. DAPI staining of cells also was included for visualization of spirochetes. As shown in Fig. 3C, c-Myc was detected on the surface of *B. burgdorferi* when expressed in the putative surface loop containing K487 (P66–c-Myc^{K487}) but not when expressed at the C terminus of P66 (P66–c-Myc^{C-terminal}). As expected, c-Myc antibodies did not detect protein in controls expressing P66 without the c-Myc tag (P66–c-Myc[–]; Fig. 3C). Additionally, the surface localization immuno-

fluorescence assays did not detect FlaB on the surface of the spirochetes (Fig. 3C). To verify that the c-Myc antibodies could bind to c-Myc expressed at the C terminus of P66, immunofluorescence assays also were performed by coincubating samples with anti-c-Myc and anti-FlaB antibodies after cells had been disrupted and fixed to microscope slides. Under these conditions, the P66–c-Myc^{K487} and P66–c-Myc^{C-terminal} strains, but not the P66–c-Myc[–] strain, were immunolabeled (Fig. 3C). FlaB antibodies labeled all organisms in the fixed samples, indicating that their outer

FIG 2 K487 is a target for trypsin degradation. (A) Amino acid sequence alignments of P66 from *B. burgdorferi* B31, *B. burgdorferi* JD1, *B. garinii* IP90, and *B. garinii* Pbi were generated using the MacVector (v10.0) program. The variable region predicted to be surface exposed is boxed in red, with an arrow indicating the lysine residue responsible for trypsin degradation (K487). (B) *B. burgdorferi* cells were washed and incubated with 0 to 300 µg/ml of trypsin. Samples were then immunoblotted with P66 antibodies to assess surface degradation. Equivalent samples also were immunoblotted with monoclonal OspB antibodies to control for protease activity and with antibodies that recognize the periplasmic protein FlaB to ensure that the OM remained intact throughout the proteolysis experiments. (C) *B. burgdorferi* B31, *B. burgdorferi* JD1, *B. garinii* IP90, and *B. garinii* Pbi cells were collected and incubated with 200 µg/ml trypsin before immunoblotting with rat anti-P66 antibodies. Samples were also immunoblotted with antibodies against the periplasmic FlaB protein (bottom) to ensure membrane integrity and outer membrane lipoprotein OspB antibodies (middle) as a positive control for protease activity. Molecular mass standards (in kDa) are shown at the left.

membranes had been disrupted (Fig. 3C). The combined immunofluorescence assays demonstrate unequivocally that K487 is located in a surface-exposed loop of P66.

***B. burgdorferi* P66 is amphiphilic and forms a β -barrel pore.** We next sought to determine whether P66 possesses the properties expected of an amphiphilic, β -barrel protein. To this end, we expressed P66 with a C-terminal His tag in *E. coli*, purified the recombinant protein, and folded it in detergent (2% DDM). Since P66 contains six tryptophan residues, folding of P66 could be monitored using tryptophan fluorescence. This technique utilizes the intrinsic fluorescence properties of the tryptophan residues within the protein (67). As an integral membrane protein folds, a blue shift in the fluorescence emission maximum can be measured as tryptophan residues move from an aqueous environment to a more hydrophobic environment. As shown in Fig. 4A, the expected shift was observed during incubation of unfolded P66 in DDM. We next assessed the β -sheet content of folded P66 using circular dichroism (CD) spectroscopy. CD analysis revealed a broad minimum at 211 nm, consistent with an extensive β -sheet structure (Fig. 4B). As a control, we also included the *B. burgdorferi* BamA protein, a bipartite protein with N-terminal periplasmic and C-terminal membrane-spanning β -barrel domains (22). Similar to P66, BamA also contained a broad minimum at 211 nm, as expected for a protein containing an extensive β -sheet (Fig. 4B). To assess the secondary structure contents of P66 and BamA, we

further analyzed the spectra using the DICROWEB server (58, 59). Both P66 and the BamA proteins were composed of ~48% β -sheet (data not shown).

We also performed Triton X-114 phase partitioning assays to determine whether P66 is amphiphilic. Assays were performed with both recombinant P66 and *B. burgdorferi* lysates. Both recombinant and native P66 associated with the detergent-enriched phase (Fig. 4C and D, respectively). As a control, recombinant BamA Potra 1, a soluble, periplasmic domain of BamA, also was subjected to phase partitioning with Triton X-114; as expected, it partitioned exclusively into the aqueous phase (Fig. 4C). Triton X-114 phase partitioning using borrelial whole-cell lysates resulted in partitioning of the amphiphilic, membrane-bound lipoprotein OspA into the detergent-enriched phase, as expected (Fig. 4D). BB0796, the periplasmic *B. burgdorferi* Skp ortholog, partitioned with the aqueous phase (Fig. 4D), as previously described (23).

To assess whether the folded recombinant *B. burgdorferi* P66 protein could incorporate into lipid bilayers, we performed liposome flotation assays. Liposomes were generated from a phospholipid mixture based upon the known phospholipid content of the *B. burgdorferi* OM (68) and subsequently incubated with folded P66 before being separated on discontinuous sucrose gradients. Fractions then were subjected to SDS-PAGE, followed by immunoblot analysis with P66 antiserum. P66 was detected only in the

FIG 3 K487 is located on the surface of *B. burgdorferi*. (A) Schematic of c-Myc constructs and controls generated in pBSV2G for surface localization immunofluorescence experiments. P66 expressed in *trans* from pBSV2G is indicated with a gray bar, and the location of the c-Myc tag is noted with a dark gray bar as well as the c-Myc sequence (EQKLISEEDL). (B) Equivalent whole-cell lysates from P66-c-Myc^{K487}, P66-c-Myc^{C-terminal}, and P66-c-Myc⁻ strains were subjected to immunoblot analysis with rabbit anti-c-Myc and rat anti-P66 antibodies. (C) For surface localization assays, P66-c-Myc^{K487}, P66-c-Myc^{C-terminal}, and P66-c-Myc⁻ cells were coincubated with rabbit anti c-Myc antibodies and rat anti-FlaB antibodies before being fixed to slides and incubated with the appropriate Alexa Fluor-conjugated secondary antibodies. Samples were also fixed to slides prior to coincubation with rabbit anti c-Myc antibodies and rat anti-FlaB antibodies. All spirochetes were also counterstained with DAPI.

liposome-containing top fraction (TF), as opposed to the middle fraction and bottom fraction (MF and BF, respectively), which contain unincorporated material (Fig. 4E). Parallel experiments also were performed with *E. coli* OmpA, an extensively characterized bacterial porin with a β -barrel structure (69). Similar to the P66 result, OmpA folded in detergent was detected in the liposome-enriched top fraction (Fig. 4E, bottom). Furthermore, we examined the pore-forming capabilities of P66 using pore formation assays in which we measured the efflux of the fluorophore Tb(DPA)₃³⁻ from liposomes (50, 61, 62). The fluorophore escaped from liposomes loaded with P66 (Fig. 4F), indicating that the folded, recombinant protein forms a pore. Fluorophore efflux also was detected in liposomes incubated with the *E. coli* pore-

forming protein OmpF (Fig. 4F). Previous studies have demonstrated that the *Treponema pallidum* protein TP0453 does not permit fluorophore efflux at neutral pH (50). Therefore, we utilized TP0453 as a non-pore-forming control; as expected, fluorophore efflux was not observed in TP0453-loaded liposomes (Fig. 4F). The combined physicochemical data strongly suggest that P66 forms a β -barrel pore in the OM of *B. burgdorferi*.

Canonical β -barrel OMPs remain folded when solubilized in SDS at room temperature; as a result, when separated by SDS-PAGE, they migrate at an apparent molecular mass lower than that of the boiled (i.e., fully denatured) protein, a property termed heat modifiability (14, 70). Having established that P66 has an extensive β -structure, is amphiphilic, can incorporate into artifi-

FIG 4 *B. burgdorferi* P66 is amphiphilic and forms a β -barrel pore. (A) Tryptophan fluorescence emission spectra of recombinant *B. burgdorferi* P66 in DDM buffer (folded P66) or in denaturation buffer (unfolded P66). A.U., absorbance units. (B) Circular dichroism spectra of recombinant *B. burgdorferi* P66 and BamA in DDM buffer. (C) Recombinant P66 and recombinant *B. burgdorferi* BamA Potra domain 1 were subjected to Triton X-114 phase partitioning. The detergent-enriched (Det) and aqueous (Aq) phases were immunoblotted with P66 or BamA antibodies. (D) *B. burgdorferi* whole-cell lysates were subjected to phase partitioning. The detergent-enriched and aqueous-enriched phases were immunoblotted with P66 antibodies. Equivalent amounts of the detergent-enriched and aqueous phases were also immunoblotted with OmpA antibodies to provide a detergent-enriched fraction control and BB0796 antibodies to provide an aqueous-enriched fraction control. (E) Liposomes simulating the *B. burgdorferi* OM were incubated with folded P66 or folded OmpA before separation on discontinuous sucrose gradients. Fractions were collected from the top (TF), middle (MF), and bottom (BF) of the gradient before immunoblotting with rat anti-P66 antibodies or rat anti-OmpA antibodies. (F) Liposomes containing the fluorophore Tb(DPA)₃³⁻ were incubated with recombinant *B. burgdorferi* P66, *E. coli* OmpF, or *T. pallidum* TP0453 in buffer supplemented with EDTA, and fluorophore efflux was measured as quenched fluorescence. (G) Folded P66 and OmpA were incubated at room temperature (– boil) or boiled (+ boil) for 20 min in final sample buffer. The samples were then separated by SDS-PAGE and subjected to immunoblot analysis with either P66 or OmpA antibodies. (H) *B. burgdorferi* whole-cell lysates were incubated at room temperature (– boil) or boiled (+ boil) for 10 min in final sample buffer prior to SDS-PAGE and immunoblot analysis with P66 antibodies.

cial membranes, and can form pores, we also sought to determine if it is heat modifiable. While folded, recombinant OmpA displayed heat modifiability; recombinant P66 did not (Fig. 4G). Heat modifiability assays were also performed with *B. burgdorferi* whole-cell lysates, and, like recombinant P66, native P66 was not modified by heat (Fig. 4H).

P66 is part of a higher-order complex and associates with

OspA and OspB in the *B. burgdorferi* OM. To examine the oligomeric state of *B. burgdorferi* P66, *B. burgdorferi* lysates and recombinant P66 were subjected to blue native PAGE (BN-PAGE), followed by immunoblot analysis (Fig. 5A and B). The recombinant P66 migrated at 112 kDa (R_f value = 4.6) (Fig. 5B and C). After subtracting the molecular mass of the detergent micelle (~50 kDa), the size of the recombinant P66 was estimated to be 62

FIG 5 P66 forms a large protein complex and interacts with OspA and OspB in *B. burgdorferi*. (A and B) Immunoblot analysis of *B. burgdorferi* whole-cell lysates (A) and recombinant P66 subjected to BN-PAGE (B). Immunoblot analyses were performed using anti-P66 or anti-OspA antibodies. Molecular mass standards (kDa) are shown at the left of the panels. (C) The R_f values for the stained bands of molecular mass standards, P66 complexes I and II, and recombinant P66 were calculated and plotted versus molecular masses (in Da) using a second-order polynomial best fit according to the manufacturer's instructions (Invitrogen). (D) *B. burgdorferi* cell lysates were prepared and subjected to coimmunoprecipitation experiments using rat anti-P66 antibodies. The eluate from the coimmunoprecipitation was analyzed by SDS-PAGE and immunoblotting using OspA, OspB, CspA, and P66 antibodies. Whole-cell lysates (WCL) were also included in each immunoblot as a positive control.

kDa, indicating that it is monomeric. In contrast, native P66 migrated in complexes of ~400 kDa (band I; R_f value = 2.0) and ~600 kDa (band II; R_f value = 1.0) (Fig. 5A and C). Previous studies have shown that OspA and P66 can be coimmunoprecipitated after cross-linking of *B. burgdorferi* cells (71, 72). Consistent with these results, OspA was detected in a high-molecular-mass complex that migrated similarly to the large ~600-kDa P66-band II complex (Fig. 5A; compare the left and right panels). Interactions between P66 and OspB have also been reported (72, 73). Therefore, we next assessed whether P66 interacts with both OspA and OspB in *B. burgdorferi* cells by performing coimmunoprecipitation studies using P66 antisera. The eluate from the coimmunoprecipitation experiment was analyzed by immunoblotting with OspA and OspB antibodies. As shown in Fig. 5D, both OspA and

OspB coimmunoprecipitated with P66, suggesting that P66 interacts with OspA and OspB in *B. burgdorferi* cells. The factor H binding surface lipoprotein CspA, however, did not coimmunoprecipitate with P66, suggesting that the interaction between P66 and both OspA and OspB is specific and that P66 does not interact with all lipoproteins found in the borrelian OM (Fig. 5D).

DISCUSSION

While it has been well established that the surface of *B. burgdorferi* is decorated with numerous lipid-modified, membrane-anchored proteins (6, 8, 74), integral OMPs from this pathogenic spirochete are still only poorly characterized. In fact, borrelian OMPs have proved difficult to identify, which is due in part to the fragile OM of *B. burgdorferi*, the lack of sequence homology to known OMPs

from other Gram-negative bacteria, and the poor immunogenicity of integral OMPs compared to the highly immunogenic surface lipoproteins of *B. burgdorferi* (8, 74, 75). Despite the lack of structural data, the existence of *B. burgdorferi* membrane-spanning OMPs was clearly demonstrated by freeze fracture electron microscopy (12). While it is tempting to assume that these OMPs form β -barrels in the borrelial OM, as is the case for OMPs from Gram-negative bacteria, this has never been examined in *B. burgdorferi*. Although some *B. burgdorferi* proteins have been shown to be integral OMPs (P66 [BB0603] [30], P13 [BB0034] [76], BamA [BB0795] [22], BesC [BB0142] [77], BB0405 [53], Lmp1 [BB0210] [78], and DipA [BB0418] [79]), the native structures of these proteins are still unknown. A better understanding of the conformation of these proteins in the borrelial OM will provide important insight into the structure and biogenesis of the borrelial OM and could provide clues as to how these OMPs interact with host proteins. Given that prior studies have shown that P66 forms pores similar to β -barrel porins from other organisms and also is a known host adhesin protein (30, 32, 36), we chose to characterize the structure and physicochemical properties of P66 using multiple techniques to develop a working model of the P66 tertiary structure. This is an important first step that will provide working hypotheses for future studies aimed at examining the structure-function properties of P66, especially since crystal structures of membrane proteins, such as P66, are inherently difficult to obtain. The combined structural and physicochemical analyses performed here provide compelling evidence that P66 is folded into an amphipathic β -barrel protein that forms a pore and contains several surface-exposed loops that could interact with host proteins during infection.

P66, like other known OMPs, incorporates into liposomes, forms pores in large unilamellar vesicles, and has amphiphilic properties, all of which are expected of a β -barrel protein. Furthermore, circular dichroism indicated that the recombinant P66 is composed of 48% β -sheet structure, which was consistent with the computer-based structural analyses. Five out of the six secondary structure models used, along with the structural model prediction, indicated that P66 forms a β -barrel with 22 or 24 transmembrane strands. To date, bacterial OMPs with 8 to 24 transmembrane strands have been identified (14, 66), which indicates that P66 is predicted to contain a relatively large number of putative transmembrane domains. Other bacterial OMPs with 22 and 24 transmembrane domains include the *E. coli* TonB-dependent transporters (22 β -strands), the *Pseudomonas aeruginosa* siderophore receptors FpvA and FptA (22 β -strands), and *E. coli* PapC (24 β -strands) (14, 80). Previous reports have also indicated that P66 has porin activity with a large pore diameter of 2.6 nm (30). Given that other spirochetal proteins reported to be OM-spanning, pore-forming proteins in planar lipid bilayer assays were later revealed to be periplasmic proteins (81–83), we recognized the importance of examining pore formation by P66 using a technique other than the planar lipid bilayer assay, especially since the planar lipid bilayer assay has been misleading in the past. Therefore, we utilized the fluorophore Tb(DPA)₃^{3−} and assessed efflux of the fluorophore from liposomes as a measure of pore formation. Moreover, using the fluorophore allowed us to assess pore size. The fluorophore used in our studies, Tb(DPA)₃^{3−}, has been shown to have a diameter of approximately 1 nm (62). Thus, we can conclude that P66 does form pores larger than 1 nm, consistent with the previously published report (30). Importantly, the

size of the P66 pore is large enough to allow the influx of many nutrients and amino acids that are required for *B. burgdorferi* survival. Interestingly, although we found that P66 was not heat modifiable like many other bacterial porin proteins, this observation is consistent with the characteristics of β -barrel proteins BamA and Msp from the spirochetes *T. pallidum* and *T. denticola*, respectively, which also are not heat modifiable (24, 84). Although we used recombinant protein in our studies and cannot rule out the possibility that the protein was not in its native conformation, recombinant P66 formed pores in membrane vesicles, suggesting that the protein was, indeed, folded correctly. The lack of heat modifiability was the only biochemical property that was found to be different between P66 and other β -barrel proteins and may represent a unique property of spirochetal OMPs.

P66 was predicted to contain a surface-exposed loop from amino acids 459 to 502, which contains the trypsin-sensitive lysine residue at position 487 (K487) (27). We have now confirmed that this loop is located on the surface of *B. burgdorferi* by inserting a c-Myc tag adjacent to K487 and demonstrating that the predicted surface loop is in fact extracellular by surface localization immunofluorescence. To our knowledge, this is the first example in which an epitope tag has been inserted into a surface-exposed loop of a borrelial OMP and subsequently used to examine surface exposure of a protein or protein loop in *B. burgdorferi*. Furthermore, when we compared *B. burgdorferi* and *B. garinii*, only the P66 of *B. burgdorferi* contained a lysine residue at position K487. Consistent with this, only the *B. burgdorferi* P66 protein was observed to be susceptible to treatment with trypsin. Interestingly, within the surface-exposed loop containing the trypsin-accessible K487 (28), there also are lysine residues at positions 461 and 500 that are conserved among *B. burgdorferi* and *B. garinii* strains. Given that only the *B. burgdorferi* P66 is cleaved by trypsin, it would suggest that K461 and K500 are clearly not accessible to trypsin for degradation. Actually, all of the strains that we analyzed have numerous lysine residues that the computer models predict are located within various extracellular domains connecting adjacent transmembrane β -strands. Thus, it seems that lysine residues in other extracellular loops are protected from trypsin cleavage. While it may seem paradoxical that some lysines predicted to be surface exposed are susceptible to trypsin cleavage while others are not, complete and partial protease resistance has been described for other β -barrel OM proteins (15, 22, 85–89). In fact, bacterial OMPs, such as FomA from *Fusobacterium* and BamA from numerous organisms, have been shown to be at least partially trypsin resistant, despite the presence of numerous lysine and arginine residues throughout their primary sequence and predicted surface loops (17, 22, 24, 87, 90). According to three PRED-TMBB algorithms, the predicted loop in which K487 is located is one of the larger extracellular loops (Fig. 1A), and the lysine residue is predicted to be on the most distal portion of the loop in relation to the β -barrel in the TMBpro structural model (Fig. 1B). It is tempting to speculate that the distal position of K487 in the extracellular loop could make this residue more accessible to trypsin than the other lysine residues.

Previous studies have shown that P66-integrin binding requires amino acids 150 to 343 (33, 35); thus, the surface loop of P66 containing the K487 residue does not appear to be required for P66-integrin binding interactions. The region of P66 necessary for integrin binding contains several predicted extracellular loops that can now be examined to better define the integrin-binding

domain(s) that is surface exposed in *B. burgdorferi*. In this regard, the structural model predicted a large extracellular loop (amino acids 307 to 343) in the integrin-binding region that could be important in the P66-integrin interaction during infection. The structural predictions have provided a new working model that can guide the construction of specific mutants to examine in further detail the interaction between P66 and mammalian integrins.

Previous experiments have revealed an interaction between P66 and borrelial surface lipoproteins (71–73). According to the results of the BN-PAGE experiments reported here, recombinant P66 migrates at a much lower molecular mass than native P66, indicating that P66 is a member of a large protein complex in the borrelial OM. We also observed that P66 and OspA both migrated in a complex of similar size, which may indicate that the two proteins are members of the same multicomponent protein complex in the borrelial OM. This conclusion is further supported by Yang and colleagues, who observed that P66, OspA, and OspB can be detected in the same *B. burgdorferi* OM complex and that formation of the complex is dependent on the presence of P66 (72). Furthermore, we have shown that P66 coimmunoprecipitates specifically with OspA and OspB. According to the solved crystal structures of OspA and OspB, both proteins have a C-terminal, positively charged cleft with an adjacent cavity that is lined with hydrophobic residues (91, 92). These cavities have been proposed to possibly bind potential extracellular loops of surface proteins. Therefore, it is possible that the cavities of OspA and OspB bind one or more of the P66 extracellular loops. Although it is not known what function a potential OspA/B-P66 complex might play in the life cycle of this spirochete, one would predict that the interaction is most relevant during the tick phase or during transmission from the tick to mammal, since OspA and OspB are both dramatically downregulated during or soon after transmission to the mammal (93–98). For instance, OspA and OspB may bind P66 and protect the extracellular loops of the protein from degradation in the tick environment. As OspA and OspB are downregulated and the spirochete migrates to the mammalian host, however, P66 would no longer bind OspA or OspB, and the surface of P66 would then become accessible for binding other borrelial outer membrane proteins and/or proteins in the mammalian host, such as integrins, as has been previously reported (32). Given that recombinant P66 was able to form pores, it is clear that P66 porin activity does not require interactions with other proteins.

A recent report has shown that P66 is essential for infection of mice (36). While the ability of P66 to bind β_3 -chain integrins and to form pores has been well established (30, 32–34, 99–101), the relative contributions of the pore-forming and integrin-binding capabilities of P66 to spirochetal virulence are not clear. Although P66 is required for establishing an infection in mice, it is not essential for the growth of *B. burgdorferi* in dialysis membrane chambers (36). The latter observation could indicate that the porin activity of P66 is not required for nutrient acquisition in the mammalian environment. Alternatively, it is possible that the porin function of P66 is redundant with that of another OMP within the restricted environment of the dialysis membrane chamber, which precludes contact between the spirochete and immune effector cells as well as any molecule or nutrient larger than the 8-kDa membrane cutoff (98). Therefore, further studies will be required to elucidate the importance of the porin activity versus that of the integrin-binding activity of P66 as it relates to Lyme disease pathogenesis.

As mentioned above, few integral OMPs have been identified or characterized in *B. burgdorferi*. There are at least 10-fold fewer integral OMPs per μm^2 in the *B. burgdorferi* OM than the *E. coli* OM, as estimated by freeze fracture electron microscopy (12, 13). Although there is a dearth of information about the structures of borrelial integral OMPs even after more than 2 decades of study, the seminal observation made here, that P66 forms a β -barrel structure, is of particular importance. Not only is this the first empirical evidence that borrelial OMPs do, in fact, form β -barrels, but also the experiments outlined in this study establish methodological precedents by which other potential borrelial OMPs can be examined. Finally, future P66 studies can now use the framework and structural model provided in this study to examine in further detail the role of the porin/integrin binding of this protein either together or separately in *B. burgdorferi* virulence and Lyme disease pathogenesis.

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