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Characterization of Multi-protein Complexes of the *Borrelia burgdorferi* Outer Membrane Vesicles

Xiuli Yang[1,¶], **Kamoltip Promnares**[1,a,¶], **Jinhong Qin**[1,b,¶], **Ming He**[2], **Deborah Y. Shroder**[1,c], **Toru Kariu**[1], **Yan Wang**[3], and **Utpal Pal**[1,*]

[1]Department of Veterinary Medicine, University of Maryland, College Park and Virginia-Maryland Regional College of Veterinary Medicine, Maryland 20742, USA

[2]Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[3]College of Chemical and Life Sciences, University of Maryland, College Park, Maryland 20742, USA

Abstract

Amongst bacterial cell envelopes, the *Borrelia burgdorferi* outer membrane (OM) is structurally unique in that the identities of many protein complexes remain unknown; however, their characterization is the first step towards our understanding of membrane protein interactions and potential functions. Here, we used two-dimensional blue native/SDS-PAGE/mass spectrometric analysis for a global characterization of protein-protein interactions as well as to identify protein complexes in OM vesicles isolated from multiple infectious sensu stricto isolates of *B. burgdorferi*. Although we uncovered the existence of at least 10 distinct OM complexes harboring several unique subunits, the complexome is dominated by the frequent occurrence of a limited diversity of membrane proteins, most notably P13, outer surface protein (Osp) A, -B, -C and -D and Lp6.6. The occurrence of these complexes and specificity of subunit interaction were further supported by independent two-dimensional immunoblotting and co-immunoprecipitation assays as well as by mutagenesis studies, where targeted depletion of a subunit member (P66) selectively abolished a specific complex. Although a comparable profile of the OM complexome was detected in two major infectious isolates, such as B31 and 297, certain complexes are likely to occur in an isolate-specific manner. Further assessment of protein complexes in multiple Osp-deficient isolates showed loss of several protein complexes but revealed the existence of additional complex/subunits that are undetectable in wild-type cells. Together, these observations uncovered borrelial antigens involved in membrane protein interactions. The study also suggests that the assembly process of OM complexes is specific and that the core or stabilizing subunits vary between complexes. Further characterization of these protein complexes including elucidation of their biological significance may shed new light on the mechanism of pathogen persistence and the development of preventative measures against the infection.

[*]Corresponding author: Utpal Pal, Department of Veterinary Medicine, Building 795, Room1341, University of Maryland, College Park, MD 20742. Phone: 301-314-2118, Fax: 301-314-6855, upal@umd.edu.
[¶]These authors contributed equally to the work
[a]Current address: Department of Molecular Biotechnology and Bioinformatics, Prince of Songkla University, Songkla Thailand, 90112
[b]Department of Medical Microbiology and Parasitology, Shanghai Jiao Tong University School of Medicine, Shanghai, China 200025
[c]Current address: Department of Biochemistry and Molecular Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Supporting Information
Table S1 – S6. This material is available free of charge via the Internet at http://pubs.acs.org.

Keywords

Borrelia burgdorferi; Lyme disease; microbial pathogenesis; outer membrane; protein complex

Introduction

Lyme disease is a common tick-borne illness in the United States and an emerging public health threat in many parts of the world. *Borrelia burgdorferi*, the spirochetal agent of Lyme disease, is maintained in nature via a complex enzootic life cycle involving wild rodents and *Ixodes* ticks [1, 2]. The spirochete alters its antigenic composition via intragenic recombination and regulation of gene expression when encountering new host or vector environments [3–6] (for details see a recent review [7]). This assists the pathogen to navigate between a diverse array of host microenvironments and to establish a persistent infection [8]. Although microarray studies have identified a large set of borrelial genes that are highly responsive to environmental cues [9–12], relatively limited information is available on the composition of or potential changes in the borrelial proteome [13–15], especially those contained in the outer membrane (OM). OM proteins are expected to play critical roles in *B. burgdorferi* persistence through the vector-host infection cycle. Therefore, characterization of OM complexes is important to our understanding of the intriguing biology of spirochetes and to the development of novel preventative and therapeutic measures against Lyme borreliosis.

In Gram-negative bacterial pathogens, OM proteins often contribute to various stages in the infection process including tissue adhesion, colonization, immune cell activation and evasion of the host immune system [16, 17]. The *B. burgdorferi* OM undergoes constant antigenic alterations induced by the surrounding environment. In contrast to other Gram-negative bacteria, the *B. burgdorferi* OM features an absence of usual lipopolysaccharide elements. Instead, the borrelial OM contains numerous surface lipoproteins that do not have membrane-spanning topology and are anchored to the membrane via amino-terminal lipid motifs [18, 19]. It is also likely that many soluble proteins tether to the membrane through non-covalent interactions with the constitutive lipids and proteins. Lastly, although *B. burgdorferi* OM retains much lower density of membrane-spanning proteins compared to that in other Gram-negative bacteria, it contains more proteins than that found in the OM of the related pathogenic spirochete Treponema pallidum [20, 21].

Generally, proteins often assemble into multi-protein complexes that carry out important biochemical processes [22] including specific roles in membrane biogenesis and function, such as energy generation, protein assembly, lipoprotein trafficking and small molecule transportation [23]. These functions, in turn, contribute to the microbial pathogenesis [24]. Two-dimensional (2D) blue native (BN)/PAGE technology has been widely applied for the isolation of protein complexes in native conditions and generation of global overviews of protein-protein interactions in biological membranes [23–27]. For better understanding of spirochete biology, we sought to use the 2D-BN/SDS-PAGE technology to identify protein complexes in the OM of multiple pathogenic isolates of *B. burgdorferi*. We also explored potential alterations in the dynamics of protein complexes in isogenic mutants lacking a single or multiple subunit members. Overall, we believe that further characterization of protein complexes will shed new light on the molecular mechanisms of *B. burgdorferi* enzootic cycle persistence and aid development of novel ways to prevent the infection.

Experimental section

Bacterial strains

The following *B. burgdorferi* isolates were used in the study: a clonal and low-passage infectious *B. burgdorferi* B31 isolate A3 [28], a non-infectious mutant B314 that lacks many linear endogenous plasmids [29], and an infectious wild-type isolate 297, clone BbAH130 [30]. All spirochetes were cultured in BSK-H medium supplemented with 6% rabbit serum at 33°C and grown until a density of $5\times10^7 – 10^8$ cells/ml.

Isolation of outer membrane vesicles

Isolation of the outer membrane (OM) vesicles of *B. burgdorferi* was performed as described [31]. Briefly $5 \times 10^{10} – 10^{11}$ cells were harvested by centrifugation and the pellets were washed twice with phosphate buffered saline pH 7.4 (PBS) supplemented with 0.1% bovine serum albumin (BSA). The cells were resuspended in ice-cold 25 mM citrate buffer (pH 3.2) containing 0.1% BSA and subsequently incubated on a rocker at room temperature for 2 hours. The OM vesicles were isolated from the protoplasmic cylinder by using sucrose density gradient centrifugation as detailed [32]. The OM vesicles were monitored for purity by immunoblotting using antibodies against OspA and FlaB as described [33]. Generation of polyclonal rabbit antiserum against isolated OM proteins was performed as described [32].

Generation of recombinant proteins and antisera

Recombinant proteins, BmpA, BB0028, OspA, -B, -C, -D, La7, FlaB and Lp6.6 were produced as described [32, 34–38]. Briefly, the coding region of genes without respective signal peptides were amplified as separate DNA inserts using published primers as detailed [32, 34–38], whereas primers specific for *bb0028* are indicated in Supplementary Table S6. DNA inserts were directionally cloned into *EcoRI* and *XhoI* sites of the expression vector pGEX-6P-1 (GE Healthcare). The expression, purification, and enzymatic cleavage of the glutathione-S-transferase (GST) fusion proteins were carried out as described previously [37]. To generate polyclonal antisera, the proteins (without the GST tag) produced in *Escherichia coli* were emulsified in complete Freund's adjuvant and injected into groups of 2 rabbits (100 μg/animal) or 5 mice (10 μg/animal). The animals were boosted twice at 3-week intervals with the same dose of antigen in incomplete Freund's adjuvant, and the serum samples were collected two weeks following the second boost. Development of antibodies was analyzed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting, as described [32].

Blue native-PAGE, two-dimensional SDS-PAGE and Western blotting

Analysis of OM complexes was performed under native conditions by BN/PAGE, as described [32]. The isolated OM vesicles were solubilized with n-dodecyl β-D-maltoside (DDM) (DDM/protein = 40/1 w/w) and subjected to first dimensional BN/PAGE at 4 °C in a 5–14% polyacrylamide gradient gel. Native protein markers (Invitrogen) were used to estimate the approximate size of protein complexes in the gel. The intact individual lanes from the BN/PAGE gel were excised and transferred onto a nitrocellulose membrane for immunodetection with anti-OM, anti-P66 or anti-OspA antibodies. Parallel BN/PAGE gels were incubated for 30 min in 25mM Tris/HCl, pH 7.5, containing 1% SDS (w/v) and 1% dithiothreitol (w/v), and subjected to a second dimensional SDS-PAGE for determination of subunit compositions of the protein complexes using 12–20% linear gradient polyacrylamide gels containing 7 M urea. Pre-stained protein markers (New England Biolabs) were used for estimation of molecular masses of proteins in SDS-PAGE gels. The proteins separated in 2D-BN/SDS-PAGE gel were either stained by SYPRO Ruby (Invitrogen) or transferred onto a nitrocellulose membrane. The membrane was blocked with

3% BSA-PBS overnight and incubated with subunit-specific primary antibodies. The signals were developed by the addition of horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG at a 1:10,000 dilution. Protein signals were visualized using chemiluminescence Western Blotting Detection Reagent (GE Healthcare).

Liquid chromatography-mass spectrometry and data analysis

Samples for tandem mass spectrometry were prepared by tryptic in-gel digestion of excised protein bands as described [32]. Briefly, protein complexes from gels were excised, chopped into pieces and transferred to tubes that were previously rinsed with 0.1% trifluoroacetic acid (TFA) and 60% acetonitrile (ACN). The gels were then serially washed with 50% ACN, a mixture of 50% ACN, 50 mM NH_4HCO_3, and 10 mM NH_4HCO_3. The gel pieces were dried, digested with trypsin (0.1 mg/ml) (Promega Corporation, Madison) and further extracted with 100 μl of 50% ACN and 5% TFA and concentrated to a final volume of 40 μl. Tryptic digests were injected onto a Zorbax SB300 C18 column (1.0 × 100 mm) (Agilent Technologies, Santa Clara, CA) connected to an Accela HPLC system (Thermo Electron, San Jose, CA) and interfaced to a Thermo Finnigan LTQOrbitrap XL mass spectrometer equipped with an Ion Max electrospray source. Separation of peptides was achieved by a linear gradient of 5–35% solvent B at 50 μl/min for 45 min (solvent A: 95% water, 5% ACN, 0.1% formic acid, solvent B: 5% water, 95% can, 0.1% formic acid). The LTQ Orbitrap XL mass spectrometer (Thermo Electron) was operated in positive ion mode with data dependent MS/MS acquisition. The instrument was set to complete a full mass scan from mass-to-charge ratio (m/z) 350–2000 with resolution 60,000 (m/z 400) in the Orbitrap followed by data dependent MS/MS analysis of up to five of the most intense ions with CID in the linear ion trap at unit mass resolution. Dynamic exclusion was turned on to exclude ions from being selected again for MS/MS analysis for 30 sec with a window of [−0.5 – +1.5] m/z. LC-MS/MS data files were searched using Sequest search engine through Bioworks (Thermo Electron) and Mascot search engine through the in-house Mascot Server (Matrix Science, London, U.K.) against a borrelial protein databases extracted from the UniProt database. Results were combined using Scaffold Distiller (Proteome Software, Portland, OR) for identification of proteins. Predicted localization of *B. burgdorferi* proteins to the spirochete membrane was determined according to the database annotation and PSORT Prediction, as detailed [39].

Protein cross-linking and co-immunoprecipitation assay

The chemical cross-linking using dithiobis(succinimidyl) propionate (DSP) was performed as described previously [32]. Briefly, 10^{10} *B. burgdorferi* cells were collected, washed with PBS, and further incubated in PBS containing 1 mM DSP and 4% dimethyl sufoxide for 2 hours on the ice. The reaction was terminated using Tris-HCl (pH 7.5) with a final concentration of 10 mM and centrifuged. The pellet was resuspended in PBS, sonicated and equally split into three tubes and incubated with mouse monoclonal antibodies against OspA, P66 and GST for 4 hours at 4°C. The reaction mixtures were further incubated with protein A-sepharose beads (Pierce Biotechnology Inc., Rockford, IL) overnight at 4°C. The protein bound antibody complexes were then washed five times with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40 substitute, 150 mM NaCl, 1 mM EDTA) and dissolved in Laemmli sample buffer for SDS-PAGE and immunoblot analysis.

PCR

The oligonucleotide primers used for the PCR or RT-PCR analysis of *B. burgdorferi* genes are listed in Supplementary Table S6. Total RNA was extracted from *B. burgdorferi* using Trizol reagent (Invitrogen), further digested with RNase-free DNaseI (New England Biolabs, Ipswich, MA), purified using the RNeasy kit (Qiagen, Valencia, CA) and then

converted to cDNA using the AffinityScript cDNA synthesis kit (Agilent Technologies). The gene transcripts were normalized to the *B. burgdorferi flaB* levels, as detailed [39].

Generation of p66-deficient B. burgdorferi B31

The infectious *B. burgdorferi* isolate A3 was used to create an isogenic *bb0603* (*p*66) deficient *B. burgdorferi* by an exchange of the *p66* open reading frame with a kanamycin-resistance cassette via homologous recombination. DNA fragments flanking up- and downstream regions of the *p66* gene were PCR-amplified using primers P1-P4 and inserted into two multiple-cloning sites flanking the kanAn cassette in plasmid pXLF10601, as detailed [39]. The recombinant plasmid was sequenced to confirm the identity of the insert and electroporated into *B. burgdorferi*. Transformants were then selected on kanamycin (350 μg/ml). Of the antibiotic resistant clones, one harboring the intended recombination and carrying the endogenous set of plasmids present in the wild-type isolate was selected by PCR [39] and used for additional experiments.

Generation of OspA/B/C-deficient B. burgdorferi 297

Inactivation of *ospAB* and *ospC* individually has been described previously [30, 38]. To construct an *ospAB* and *ospC* double mutant, an *ospAB* mutant was first re-constructed with an erythromycin-resistant (Erm^R) marker instead of a streptomycin resistant ($Strep^R$) marker that was reported previously. To do so, the previously described suicide plasmid pXT-OspAB that harbors 4 kb DNA fragment with *ospAB* and flanking sequence [30] was digested with *BstAPI* and *AccI* and replaced with a 1016bp erm^R cassette [40]. The resulting suicide plasmid DNA was transformed into an infectious clone of *B. burgdorferi* strain 297, BbAH130 [30], to produce the *ospAB* mutant BbXY326. To inactivate *ospC* in BbXY326, the previously described suicide vector pOspC-Strep used for disrupting *ospC* [38], was transformed into BbXY326. Erm^R- and $Strep^R$-positive clones were selected and the absence of OspA, OspB and OspC in the mutant clones was confirmed by immunoblot analysis (data not shown).

Results

Identification of B. burgdorferi outer membrane protein complexes and their subunit compositions

To characterize protein complexes in the OM of *B. burgdorferi*, we purified the OM vesicles from an infectious B31 isolate (Figure 1A) and performed BN/PAGE analysis. Assessment of BN gels reflected the presence of five major bands that migrated with approximate molecular masses between 148–480 kDa (Figure 1B, left panel, arrowheads). Further staining of BN/PAGE gels with Coomassie brilliant blue (Figure 1B, middle panel) identified additional less conspicuous high molecular weight complexes thereby revealing a total of 10 discrete complexes (complex I-X, middle panel, Figure 1B). Although immunoblotting of one-dimensional BN/PAGE gels typically yields poorly resolved protein bands [41], these efforts using anti-*B. burgdorferi* OM antibodies detected less abundant and lower molecular-weight complexes (Figure 1B, right panel, arrows). This likely represents protein monomers as revealed by mass spectrometry (MS) assays (detailed below) and thus were labeled as monomeric group (MG) I and II. To ascertain the subunit identity of the protein complexes, complexes I-X and MGI-II were separately excised from one-dimensional BN/PAGE gel and digested with trypsin. The enzymatic digests were subjected to MS-based protein identification using liquid chromatography (LC)-MS/MS analysis. The MS analyses were repeated in an independent experiment consistently identifying 20 unique proteins as participating members of 10 *B. burgdorferi* OM protein complexes whereas MGI and II reflect the existence of 27 proteins (Table S1). Notably, a few of the previously known abundant membrane proteins, OspA, -B and -C occurred in all protein complexes

(Table S1). P13, OspD and Lp 6.6 were detectable in all complexes except for one, whereas other proteins, such as P66, BB0405, BB0543, BB0553, BBA03, La7, ErpA, BBH37, BBI39 and BBK45 displayed intermittent distributions between multiple complexes or existed as monomers. At least 27 proteins including BmpA were identified exclusively in MGI-II, indicating their occurrence as free monomers or unstable subunits of protein complexes.

To corroborate subunit identification of OM protein complexes from one-dimensional BN/PAGE gels, individual proteins in each complex were further separated in second dimension SDS-PAGE and then identified by LC-MS/MS analysis. As complexes greater than 500 kDa (complex I-V, Figure 1B) were overlapping and difficult to resolve by second-dimensional gel (data not shown), we focused on characterizing the remaining complexes (complex VI-X) and monomeric groups (MGI-II, Figure 1B). The OM protein complexes were first resolved by one-dimensional BN/PAGE and then the excised gel strips were subjected to a second dimension SDS-PAGE and stained with SYPRO Ruby enabling visualization of individual subunits (Figure 1C). To identify the subunit composition of individual protein complexes visualized in 2D-BN/SDS-PAGE, the gel spots from each complex were excised (numbered 1–30, Figure 1C), and digested with trypsin, and proteins were identified by LC-MS/MS analysis (Table S2). Note that gel spot 22 contained a relatively high molecular weight (67-kDda) protein without identifiable membrane localization signal and thus likely represent a contaminating protein (Table S2). Both series of MS data, the one-dimensional BN/PAGE (Table S1) and the 2D-BN/SDS-PAGE (Table S2) were compared, and common subunits of complexes as well monomers were further tabulated (Table S3). We concluded that complexes VI-X contained at least 10 unique subunits. Among them, five membrane-anchored proteins, OspA, -B, -C, -D and Lp6.6, and three transmembrane proteins, P66, P13 and BB0405 were found to be the most common members of OM complexes. Note that the OspB protein detected in the current *B. burgdorferi* isolate migrates as a ~19-kDa protein, which represents a truncated version of the larger OspB protein, as reported earlier [28].

Specificity of protein-protein interactions amongst the selected members of outer membrane protein complexes

As MS-based identity of complex subunits revealed ubiquitous occurrence of a few Osps that are known to be abundant in the OM, we sought to obtain additional experimental data to corroborate the MS results. To achieve this, a series of immunoblot analyses were performed using antibodies available against selected subunits that were detected in one or multiple complexes. As presented in Figure 2, the results supported the MS-based subunit identity of protein complexes showing a common distribution of OspA, -B, -C, -D and Lp6.6 in most complexes. Similarly, a selective distribution was observed for other proteins, such as P66 (complex VI) or BB0405 (complex X).

To obtain further evidence of the specificity of protein-protein interaction between subunit members, we sought to perform co-immunoprecipitation analyses using selected antibodies. In order to stabilize the protein complexes, cultured *B. burgdorferi* cells were treated with DSP, a membrane-permeable, amine-reactive homobifunctional crosslinker with a spacer arm of 12Å. Subsequently, cells were subjected to co-immunoprecipitation analyses. As OspA and P66 are subunit members of multiple protein complexes, antibodies specific to these proteins were used to immunoprecipitate other associated subunits (Table S3 and Figure 2) and later identified by immunoblot analysis. OspA antibodies were able to pull-down OspA and associated subunits, such as P66, OspB, -C, -D and Lp6.6 (Figure 3A). Similarly, P66 antibodies were able to pull down P66, OspA, -B and -C (Figure 3B). The OspA or P66 antibodies did not immunoprecipitate other proteins, such as FlaB, that were not expected to be present in the protein complex (Figure 3A and 3B). Overall, as suggested

by BN/MS-based analysis, these results support the possible interaction of selected proteins showing their occurrence in common OM protein complexes.

P66 is essential for the occurrence of complex VI

Since certain integral membrane proteins are common members of OM complexes, we next assessed the occurrence of protein complexes using spirochetes deficient in a selected integral membrane protein. Based on the molecular weight, occurrence of P66 in MGI is likely monomeric, however, the protein is detectable in a high-molecular weight complex (complex VI, Table S3 and Figure 2B). We therefore, sought to generate an isogenic *p66* mutant using the infectious parental isolate and then assess the formation of OM protein complexes. As shown in Figure 4A, a mutant was created by replacing the entire open reading frame of *bb0603* (*p66*) with an antibiotic resistance gene via allelic exchange. The selected mutant clone retained the same set of plasmids as the wild-type spirochetes (data not shown) and failed to produce *p66* mRNA (Figure 4B) or protein (Figure 4C). The genetic manipulation did not introduce apparent polar effects, as the mutant expressed neighboring genes *bb0602* and *bb0604* at levels similar to the wild-type isolate (Figure 4B). Additionally, deletion of *p66* did not alter *B. burgdorferi* growth kinetics *in vitro* (Figure 4D). To assess if P66 deficiency affects the occurrence of *B. burgdorferi* OM protein complexes, wild-type spirochetes and *p66* mutants were grown to stationary phase, OM vesicles were isolated, and protein complexes were analyzed using BN/PAGE. Results showed that while *p66* mutants maintained a similar profile of protein complexes as parental spirochetes, complex VI was conspicuously absent (Figure 4E, left panel, arrow) where P66 either exists as homo-oligomer or alternatively as an essential member of a heteromeric complex. As expected, P66 is non-essential for the occurrence of MGI, which existed in the *p66* mutant (Figure 4E, right panel, arrowhead).

OM complexes are conserved in diverse B. burgdorferi isolates but dramatically altered in Osp-deficient cells

As certain abundant outer surface proteins (Osps) are common members of protein complexes I-X (Table S3), we next assessed possible alteration of complex profiles in diverse infectious isolates and in mutants where multiple Osps are absent. In addition, we wanted to identify less conspicuous complexes or subunits that are potentially hidden in wild-type *B. burgdorferi* but are detectable in spirochetes deficient in abundant Osp subunits. Analysis of OM vesicles in a linear plasmid-deficient *B. burgdorferi* mutant, isolate B314 [29] deficient in OspA, -B, and -D by BN/PAGE analysis indicated the loss of multiple complexes that are apparent in wild-type cells, such as complexes I-V, VII, VIII and IX (Figure 5A, left panel). MS analysis of detectable protein complexes in the B314 isolate (Figure 5A, right panel) identified a total of 11 unique proteins (Table S4). Similar to wild-type spirochetes, P13, OspC and P66 were detected in most complexes; BB0418, an OM efflux channel protein (BB0142) and a putative phosphomannomutase (BB0835) were intermittently distributed across the complexes. Immunoblot analysis of 2D-BN/SDS-PAGE gels indicated that while La7 is detectable only in MGII group; BB0405, P66, OspC and BmpA subunits are distributed in most protein complexes of the B314 isolate (Figure 5B. arrows). However, MS analysis identified new subunit members, such as BB0406, P83/100, BB0028 and BB0795 in the B314 isolate (Table S4) that were not detectable in wild-type cells (Table S1-S3).

We next assessed the conservation of protein complexes in other major *B. burgdorferi* infectious isolates, such as a human isolate 297. We also sought to assess the dynamics of OM complexes when three major Osps (OspA/B/C) were depleted in the wild-type 297 isolate, which is also naturally deficient in another major Osp (OspD). We expect that, similar to B314 isolate, Osp deficiency would allow us to identify less conspicuous

complexes/subunits. A mutant with serial deletions of *ospA*, *-B* and *-C* was created as described in the Materials and Methods section. Analysis of OM vesicles by BN/PAGE indicated that with the exception of complex IX, other complexes are conserved in both wild-type B31 and 297 isolates, (Figure 5C, left and central lanes). However, the Osp-deficient 297 isolate reflected an apparent loss of complexes I-V, VII, and VIII (Figure 5A, right panel), but harbors four inconspicuous but detectable complexes (Figure 5C, right panel, arrows) with 24 unique subunit members (Table S5) - of which 12 are non-identifiable in wild-type spirochetes or B314 isolates. However, immunoblot analysis of 2D-BN/SDS-PAGE gels indicated a comparable distribution of common subunits in Osp-deficient 297 (Figure 5D) and B314 (Figure 5B) isolates. Together, while these studies identified low abundance protein complex/subunits in Osp-deficient spirochetes, these data together indicated a high conservation of OM complexes in *B. burgdorferi* sensu stricto isolates, as well as possible existence of OM complexes that are specific to a strain.

Discussion

The outer membrane (OM) of Gram-negative bacteria houses typical β-barrel integral membrane proteins as well as membrane-anchored proteins, some of which exist as membrane protein complexes, serving vital roles in membrane function and biogenesis [42]. The *B. burgdorferi* genome [43, 44] encodes a large number of potential membrane proteins and our current study identified the existence of multiple OM complexes in infectious *B. burgdorferi* isolates. Although we do not know the stoichiometry of identified complexes or whether low molecular weight complexes simply represent protein monomers, our study has identified a number of unique subunit proteins that were detected in blue native (BN) analysis of wild type or Osp-deficient cells. These results demonstrate that these protein complexes could be involved in protein-protein interaction relevant to unknown function in spirochete biology. While other pathogenic Gram-negative bacteria, such as *Helicobacter pylori*, possess fewer OM protein complexes, mostly multimeric enzymes [24], borrelial OM harbors a larger number of complexes dominated by a few membrane-spanning proteins, such as P66, P13, BB0405 and membrane-anchored lipoproteins, such as OspA, -B, -C, -D, Lp6.6, which either have unknown function or are known to be involved in membrane transport, tissue adhesion and immune evasion.

BN/PAGE technology represents a reliable approach for the identification of protein complexes in native conditions as well as the assessment of novel protein-protein interactions [23]. We have previously reported the existence of seven protein complexes in *B. burgdorferi* OM [32]. Our current study extends the initial observation by showing the existence of at least 10 OM complexes and determining their subunit identity. The occurrence of a limited diversity of proteins, such as a few Osps as ubiquitous subunits raises the possibility that some of these complexes may be derived from unstable higher order complexes. However, we performed a detergent screen involving digitonin [45], n-octyl-β-D-glucoside [46], Triton-X-100 [25], IGEPAL CA-360 or n-dodecyl β-D-maltoside [23, 32] and determined that the latter detergent generates a reproducible profile of OM complexes (data not shown). These results suggest that the complexes I-X are unlikely to be derived from unstable higher order complexes and are further supported by independent LC-MS/MS analyses, BN/PAGE gel immunoblotting and co-immunoprecipitation analysis. Finally, analyses of OM complexes in isogenic mutants also supported the specificity of individual complexes. For example, loss of complex VI in *p66* deletion mutants suggests the involvement of P66 as an essential subunit. Although P66 is detected in multiple complexes by second-dimensional immunoblot and MS analysis, its participation is redundant in all complexes expect for the primary complex VI, which is detectable even by immunoblot analysis of one-dimensional BN/PAGE gel (Figure 2A) that has a limited capability of resolution of protein bands [41]. On the other hand, analysis of complex formation in a

targeted Osp-deficient isolate suggested that the missing subunit(s) OspA -B, -C are integral for the occurrence of several complexes, such as complex I-V, VII and VIII. Although occurrence of these limited Osps in multiple complexes is puzzling, previous studies [47] demonstrated that exogenous addition of certain Osps, such as OspA or -D, results in their insertion into borrelial OM, a process primarily attributed to the amino-terminal lipid moiety but also contributed to by protein-protein interaction. In addition, subunit interactions as observed in our study were also previously documented, such as OspA interaction with OspA [48], P66 association with OspA, -B and -C [49] and co-localization of OspA and -B in the spirochete membrane [50]. Overall, these studies suggested the existence of discrete OM complexes that are composed of a limited diversity of membrane proteins and that specific subunits are essential or redundant for OM complex formation.

P66 is shown to act as a *B. burgdorferi* adhesin, which can bind to β3-integrin [51, 52]. Additionally, P66 has also been shown to modulate the expression of a specific set of genes in cultured human cells [53]. However, similar to other transport proteins, such as P13 [54], DipA [55], BB0142 [56], MIP family proteins [57, 58] or oligopeptide permeases (Opp) complex [59, 60], P66 is also suggested to be involved in solute transport across the borrelial membrane [61, 62]. While we show that some of these proteins involved in formation of OM complexes, the function of most of the unique proteins (Table S1-S3) including their precise participation in a given complex warrants future investigations. The presence of common protein spots in the same vertical line of the 2D-BN/SDS-PAGE (Figure 1C) could be indicative of the protein's presence in the same complex. However, those proteins could also be part of separate complexes with similar electrophoretic mobility in the BN/PAGE [63]. The protein bands that migrated with approximate molecular masses between 20–66 kDa in BN/PAGE gels were labeled as monomeric protein groups (MGI and MGII) as they cannot contain so many full-length subunits, and therefore, largely represent collections of monomeric proteins. Although MGI and MGII could still house complexes of small molecular weight proteins, we speculate that majority of these proteins may exist as such in the membrane or were disrupted from larger unstable complexes during purification. Additionally, we could not exclude the potential occurrence of undetectable low abundance complexes, overlapping monomers or multiple complexes in a single complex. An unstable complex or subunit member could also be lost during membrane extraction procedures. For example, although we have detected one of the members of the *Borrelia* efflux system, BesC *(bb0142)*, two other members of the Bes protein complex [64], BesB and BesA remained undetectable. Finally, the occurrence of certain abundant subunits, such as Osps, could potentially interfere with the ability of MS to detect relatively scarce but unique peptides. For example, many unique proteins, some possessing identifiable transmembrane motifs (Table S4 and S5), remained detectable only in Osp-deficient isolates. This suggests their likely occurrence as low-abundance proteins yet highlighting their possible involvement in protein-protein interaction. The functions of some of them are either unknown (BB0028, BB0324, BB0406, BppA1, DegP ortholog BB0104), linked as channel forming (BBA01) [65], host-pathogen interaction (OspE paralog BBN38) [66], as a component of β-barrel assembly machine or BAM (BamA or BB0795) [67]. In contrast, others are known to be immunogenic and identified or speculated to play an important role in the borrelial enzootic cycle, such as BB0323 [68], surface lipoprotein BBA07 [69], BB0744 [70], MlpJ [71], surface protein RevA1 [70], virulent strain-associated repetitive antigen BBI16 [72], OM associated protein BBA74 [73]. Therefore, assessment of additional mutants lacking abundant Osps in BN/PAGE-based assays is likely to be useful to identify unknown subunits of *B. burgdorferi* OM complexes, such as BAM [67], or other low abundance protein complexes. Adoption of more recently described techniques, such as high-resolution clear native electrophoresis [74, 75] could be attempted for better separation of membrane protein complexes allowing more precise identification of the subunits.

In conclusion, our study uncovered the existence of discrete protein complexes in *B. burgdorferi* OM vesicles as well as identified a number of unique subunits that are members of the OM complexome. However, the biological significance of the occurrence of a limited diversity of membrane proteins, including few Osps, as members of OM complexes remains to be determined. Whether these enigmatic Osps perform additional redundant functions in pathogen biology, such as stabilization of the OM in addition to their stage-specific expression or function in the *B. burgdorferi* enzootic infection cycle [76–79], remains to be elucidated. Incidentally, a previous study also suggested redundant and interchangeable roles of at least some of these membrane proteins, such as OspA and -C in the stabilization of borrelial OM and pathogen protection from the hostile host immune environment [80]. Further characterization of the membrane protein complexes including their *in vivo* occurrence, function and the significance of protein-protein interaction will shed new light on understanding how *B. burgdorferi* evolved to survive in the complex enzootic cycle and in the development of new strategies to interfere with the infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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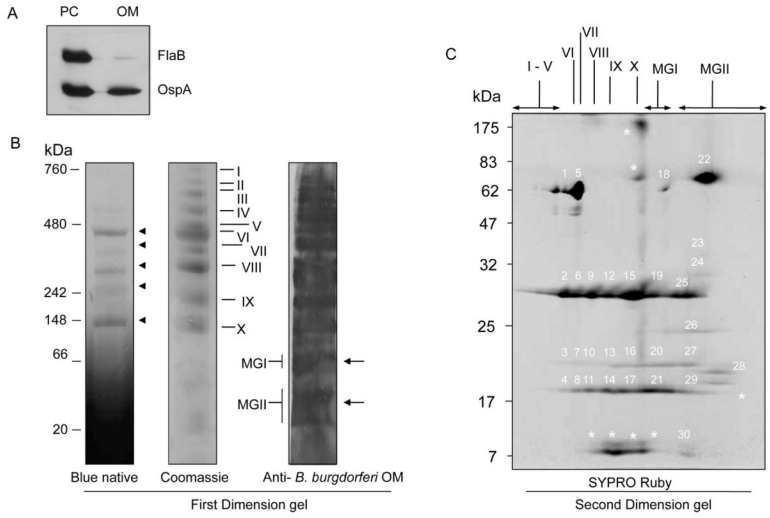

Figure 1. Identification of *B. burgdorferi* outer membrane (OM) complexes and constituent subunit members

(A) Assessment of the purity of isolated OM fraction of *B. burgdorferi*. The OM vesicles were isolated, and the purity of solubilized proteins was tested by immunoblotting using antibodies against known OM (OspA) and periplasmic cylinder (PC) protein (FlaB). **(B)** Separation of OM protein complexes by blue native/polyacrylamide gel electrophoresis (BN/PAGE). A 5–14% gradient BN/PAGE gel was used for the separation of protein complexes. The first dimensional gel (left panel), which detected five major bands (arrowheads) was either stained with Coomassie brilliant blue (middle panel) or transferred onto a nitrocellulose membrane and probed with antibodies against wild-type *B. burgdorferi* OM proteins (right panel). The identified complexes were labeled numerically from I to X while monomeric protein groups were indicated as MGI and MGII (arrows). **(C)** SYPRO Ruby staining of OM complexes resolved by second dimensional SDS-PAGE. The OM protein complexes and monomeric protein groups, as separated by 5–14% gradient BN/PAGE, were resolved by denaturing second dimension 12–20% gradient SDS-PAGE followed by staining with SYPRO Ruby. Protein markers are shown to the left. Thirty gel spots (indicated as white numbers) were excised for mass spectrometry and the proteins identified are tabulated in Table S2. Asterisks indicate gel spots where a precise identification was not possible.

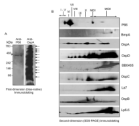

Figure 2. Assessment of OM protein complexes by immunoblot analysis using antibodies specific to selected subunits

(A) Detection of OM protein complexes containing OspA or P66. Protein complexes were separated by first dimensional BN/PAGE, transferred to a nitrocellulose membrane and blotted with anti-P66 (left panel) and anti-OspA (right panel). P66 antibody recognized a major band (arrowhead) that corresponds to complex VI (Figure 1B), while the OspA antibody detected multiple bands (arrows) comparable to the migration of complexes I-X and monomeric protein groups MGI-II. **(B)** Detection of subunit members of OM protein complexes in second dimensional SDS-PAGE by immunoblot analysis. Protein complexes, as separated by BN/PAGE, were further resolved into subunits by second dimensional SDS-PAGE, transferred to a nitrocellulose membrane and blotted with specific antibodies against P66, BmpA, OspA, -D, BB0405, OspC, La7, OspB and Lp6.6.

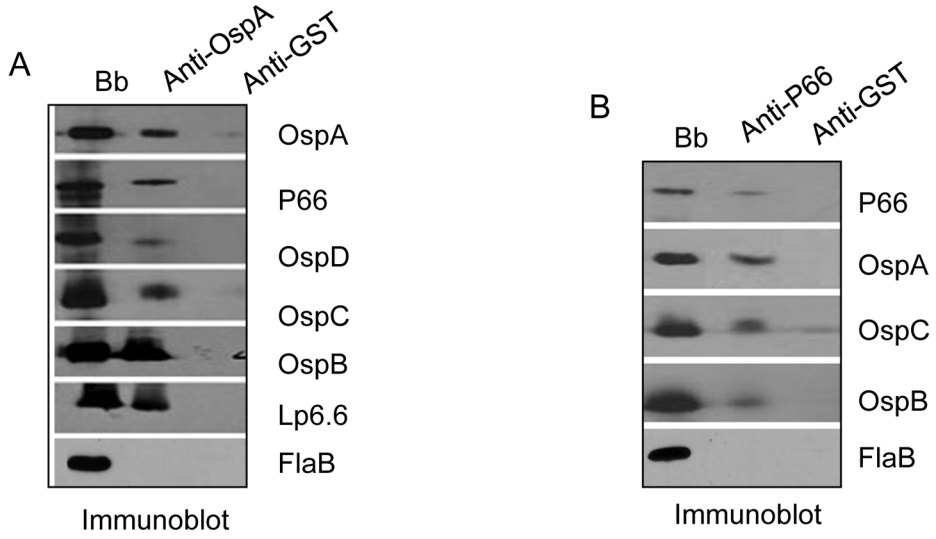

Figure 3. Interaction of subunits assessed by protein cross-linking/co-immunoprecipitation

B. burgdorferi (*Bb*) lysates were treated with a protein cross-linker (DSP) and immunoprecipitated either with OspA (panel A) or P66 (panel B) antibodies. Antibodies against glutathione-S-transferase (GST) were used as a control. Left lane (*Bb*) donates antibody recognition of target proteins in *B. burgdorferi* lysates without the immunoprecipitation step. Immunoprecipitated proteins (middle and right lanes) were identified by antibodies against OspA, P66, OspD, -C, -B and Lp6.6. As an additional control, immunoprecipitates were also probed with antibodies against the *B. burgdorferi* protein (FlaB), which was not expected to be present in the complex.

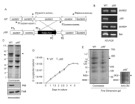

Figure 4. Generation of isogenic *B. burgdorferi p66* mutants and assessment of OM protein complexes

(A) Schematic representation of wild type (WT) and *p66* mutant (*p66*$^{-}$) *B. burgdorferi* at the *bb0603* (*p66*) locus. Genes *bb0601- bb0605* (white box arrows) and the kanamycin-resistance cassette driven by the *B. burgdorferi flaB* promoter (*flaBp-Kan*, black box arrow) are indicated. The regions up- and down-stream of the *p66* locus were amplified using primers P1-P4 (black arrow-heads) and cloned to *BamHI-SacII* and *XhoI-KpnI* sites flanking the *flaBp-Kan* cassette. **(B)** RT-PCR analysis for assessment of *p66* transcripts and polar effects of mutagenesis. Total RNA was isolated from wild type (WT) and *p66* mutant (*p66*$^{-}$) *B. burgdorferi*, converted to cDNA for detection of *p66*, *flaB*, *bb0602* and *bb0604* transcripts. **(C)** Protein analysis of wild type (WT) and *p66* mutant (*p66*$^{-}$). Equal amounts of protein were separated on SDS-PAGE gels and either stained with Coomassie blue (upper panel) or transferred onto a nitrocellulose membrane and probed with P66 and FlaB antibodies (lower panels). Protein standards are shown to the left in kDa. Arrow indicates the missing P66 band in mutant lysates. **(D)** *p66* mutant lacks detectable growth defects *in vitro*. Wild type (WT) and *p66* mutant (*p66*$^{-}$) spirochetes were diluted to a density of 10^5cells/ml, grown at 33°C in BSK-H medium and counted under a dark-field microscope. **(E)** Analysis of OM protein complexes in *p66* mutants. The OM fraction from wild type or *p66* mutant *B. burgdorferi* was isolated, and protein complexes were separated using first dimensional BN/PAGE (left panel). A parallel first dimensional BN/PAGE gel containing protein complexes of the *p66* mutants was transferred onto a nitrocellulose membrane and immunoblotted with anti-OM antibodies (right panel). Complex VI was absent in the *p66* mutant (arrow), while monomeric protein group MGI was present (arrowhead).

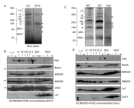

Figure 5. *B. burgdorferi osp* mutant isolates displays dramatic alterations of OM protein complexes

(A) Analysis of OM protein complexes in the *osp* mutant *B. burgdorferi*. The OM fractions were isolated from the wild type and the *osp* mutant isolate B314 and corresponding protein complexes were resolved by first dimensional BN/PAGE. Four protein complexes in *osp* mutant that likely migrated with similar molecular masses of wild-type complexes VI and X as well as monomeric protein groups MGI and MGII (arrowheads) were excised (B314 panel) and protein identification was performed by mass spectrometry, as presented in Table S4. **(B)** Subunit detection of OM protein complexes in B314 isolate by immunoblot analysis of gels resolved by second dimensional SDS-PAGE. OM protein complexes were resolved by BN/PAGE and second dimensional SDS-PAGE, transferred to a nitrocellulose membrane and blotted with specific antibodies against P66, BmpA, BB0028, BB0405, OspC and La7. Arrows indicate enhanced distribution of BmpA, BB0405 and OspC towards high-molecular-weight complexes, compared to that in wild-type cells (Figure 2B). **(C)** Comparison of OM protein complexes in the *B. burgdorferi* isolates B31, 297 and Osp-deficient 297. The OM fractions were purified from indicated spirochete isolates and corresponding protein complexes were resolved by first dimensional BN/PAGE. A few protein complexes in Osp-deficient 297 that likely migrated with comparable molecular masses of wild-type complexes VI, IX and X (arrows) were excised (Osp- panel) and protein identification was performed by mass spectrometry, as presented in Table S5. **(D)** Subunit detection of OM protein complexes in Osp-deficient 297 isolate by immunoblot analysis of gels resolved by second dimensional SDS-PAGE. OM protein complexes were resolved by BN/PAGE and second dimensional SDS-PAGE, transferred to a nitrocellulose membrane and blotted with specific antibodies against P66, BmpA, BB0028, BB0405, La7 and Lp6.6.