

OspC Is Potent Plasminogen Receptor on Surface of *Borrelia burgdorferi*^{*[5]}

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Background: Binding of the vertebrate protease plasminogen is critical during the infectious cycle of *Borrelia burgdorferi*, although the mechanism of immobilization is unknown.

Results: Only OspC-expressing spirochetes immobilize plasminogen.

Conclusion: OspC, a dominant surface protein during the tick-to-host transition, is a potent plasminogen receptor.

Significance: Determining the timing and location of specific protein partnerships is crucial to understanding the infectious cycle.

Host-derived proteases are crucial for the successful infection of vertebrates by several pathogens, including the Lyme disease spirochete bacterium, *Borrelia burgdorferi*. *B. burgdorferi* must traverse tissue barriers in the tick vector during transmission to the host and during dissemination within the host, and it must disrupt immune challenges to successfully complete its infectious cycle. It has been proposed that *B. burgdorferi* can accomplish these tasks without an endogenous extra-cytoplasmic protease by commandeering plasminogen, the highly abundant precursor of the vertebrate protease plasmin. However, the molecular mechanism by which *B. burgdorferi* immobilizes plasminogen to its surface remains obscure. The data presented here demonstrate that the outer surface protein C (OspC) of *B. burgdorferi* is a potent plasminogen receptor on the outer membrane of the bacterium. OspC-expressing spirochetes readily bind plasminogen, whereas only background levels of plasminogen are detectable on OspC-deficient strains. Furthermore, plasminogen binding by OspC-expressing spirochetes can be significantly reduced using anti-OspC antibodies. Co-immunofluorescence staining assays demonstrate that wild-type bacteria immobilize plasminogen only if they are actively expressing OspC regardless of the expression of other surface proteins. The co-localization of plasminogen and OspC on OspC-expressing spirochetes further implicates OspC as a biologically relevant plasminogen receptor on the surface of live *B. burgdorferi*.

Membrane-associated or secreted bacterial proteases are essential to many bacterial species during vertebrate infections.

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[5] This article contains supplemental Figs. S1–S9.

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Bacterial proteases can target host proteins to provide essential nutrients for the pathogen, to disarm components of the host immune response, and to remove obstructions to efficient dissemination within the host (1, 2). Many bacterial pathogens that do not encode or produce endogenous membrane-associated or secreted proteases appropriate host proteases to increase the probability of successful host colonization (3, 4). *Borrelia burgdorferi*, the spirochete bacterium that causes Lyme disease in North America, traverses many membranes *en route* from the bite of a tick to secondary infection sites deep within hosts, although it does not produce an extra-cytoplasmic protease (5). To overcome the absence of endogenous extra-cytoplasmic proteases necessary for efficient migration across host tissue barriers, *B. burgdorferi* is hypothesized to immobilize host-derived plasminogen to its outer membrane (6–8). However, no *B. burgdorferi* protein has yet been demonstrated to immobilize plasminogen to the surface of the bacterium.

The vertebrate plasminogen system, which has a central role in the extracellular matrix degradation essential for eukaryotic cell migration (9–11), is used by numerous pathogenic bacterial species during vertebrate infections (3–5, 12). Immobilization of plasmin, the enzymatically active form of plasminogen, to the surface of *Borrelia* species promotes invasiveness in tick vectors (7) and in laboratory animals (7, 13, 14) to enhance transmission and dissemination. Plasmin localized to the surface of *Borrelia* species turns the bacterium into a proteolytically active organism capable of degrading tissue barriers such as basement membranes and extracellular matrices *in vitro* (6, 8, 15–17) and enhances dissemination in ticks and vertebrates *in vivo* (6, 7, 14, 18). Bacteremia is significantly greater in animals with intact plasminogen systems than in plasminogen-deficient animals suggesting that plasminogen may enhance the capacity for invasion of the cardiovascular system (7).

The molecular mechanism by which *B. burgdorferi* immobilizes plasminogen to its surface has been elusive due in part to the inherent difficulties in genetically manipulating *B. burgdorferi* (19). Furthermore, experimentally eliminating a *B. burgdorferi* plasminogen receptor may be problematic as all bacterial plasminogen-binding proteins identified to date in other bacterial species perform other essential functions (3, 12). *In vitro*

binding assays employing soluble recombinant proteins have identified several proteins with plasminogen-binding potential (20–24). However, the *in vivo* relevance of *in vitro* protein-protein assays must be cautiously interpreted as membrane-associated proteins produced natively differ considerably from their soluble recombinant forms in structure and availability of binding sites (25, 26). A direct demonstration of physical interactions between plasminogen and native proteins embedded in the bacterial membrane is necessary to identify only the specific physiological associations between proteins that occur naturally. Deciphering where and when specific protein partnerships occur in or on living cells is critical to assess the biological relevance of membrane-associated proteins to plasminogen immobilization and utilization during infections.

Although active plasmin may be needed during multiple phases of the infectious cycle, plasmin use by *B. burgdorferi* has been best demonstrated during the transmission from ticks and during the early stages of vertebrate infections (7, 8). The expression of several proteins such as the outer surface protein C (OspC) are dramatically up-regulated during this period leading to the hypothesis that these proteins may be plasminogen receptors. OspC is a 21-kDa membrane-associated lipoprotein (27, 28) that is expressed on the surface of the bacterium as it migrates from the tick midgut to the vertebrate and remains expressed during the first weeks of infection (29–31), the time frame in which plasminogen is utilized by infecting *B. burgdorferi* (7, 8). OspC expression is also tightly correlated with the invasion of the tick salivary glands and host tissues suggesting a mechanistic role for OspC during these processes (32–36). However, the function of OspC remains the subject of considerable debate (37), and a native OspC-plasminogen interaction remains to be demonstrated.

Unraveling the molecular mechanism by which plasminogen is immobilized to the surface of *B. burgdorferi* requires a detailed understanding of plasminogen binding in the context of the membrane. This study focuses on the plasminogen-binding potential of the native OspC protein while embedded in the surface of live bacteria. Here, we employ genetically manipulated *B. burgdorferi* strains to test the hypothesis that OspC is a plasminogen receptor on the surface of *B. burgdorferi*. The results of quantitative proteome analyses, co-immunofluorescence microscopy, and whole-cell binding assays are all in agreement and strongly suggest that OspC is a potent plasminogen receptor on the surface of the Lyme disease bacterium. These analyses represent a significant methodological and conceptual step forward in dissecting the molecular mechanism of plasminogen utilization in a human pathogen of major public health importance.

EXPERIMENTAL PROCEDURES

Reagents—Polyclonal anti-OspC antibodies were produced at AnaSpec, Inc. (Fremont, CA), by subcutaneously injecting rabbits with 250 μ g of recombinant OspC protein in Complete Freund's adjuvant on four occasions. Recombinant OspC was produced by cloning a 560-bp gene fragment, amplified using forward primer OCF-BsrGI (5'-GCGGCATGTACACGAATAATTCAGGGAAAGATGGG-3') and reverse primer OCR-XhoI (5'-GCCGCACTCGAGTTAAGGTTTTTTTG-

GACTTTC-3') from *B. burgdorferi* strain B31, into a pET45(b) vector using the BsrGI and XhoI restriction sites. The plasmid insert was sequenced to confirm identity with previously published *ospC* sequences. Recombinant OspC proteins were expressed in *Escherichia coli* BL21 (DE3) and purified using nickel-nitrilotriacetic acid Superflow Resin for His₆-tagged proteins (Qiagen, 30410) and by ion exchange chromatography using Q-Sepharose Fast Flow (GE Healthcare). Anti-OspC antibodies were detected using goat anti-rabbit antibodies conjugated to AlexaFluor594 (Alexa, A11012). Native human plasminogen was purchased from Calbiochem (528178) and was detected using polyclonal FITC-conjugated sheep anti-plasminogen antibodies (Abcam Inc., Cambridge). α_2 -Antiplasmin and uPA⁴ were obtained from Calbiochem. Plasmin from human plasma was purchased from Sigma. Plasmin activity was assayed using the fluorometric plasmin substrate H-D-Val-Leu-Lys-7-amino-4-methylcoumarin (Bachem, Germany).

Bacterial Strains and Growth Conditions—*B. burgdorferi* strains were generously provided by P. Rosa (35). Briefly, strain *ospCK1* (hereafter *ospC*-ko) is an *ospC*-deficient mutant derived from the wild-type *B. burgdorferi* clone B31-A3. *ospCK1/pBSV2G-ospC* (hereafter *ospC*-comp) is the *ospCK1* strain complemented by homologous recombination using a shuttle vector carrying *ospC*. Assays used cultures grown to mid-log phase at 34 °C in BSK-II complete medium (38). In additional experiments, presented in the supplemental material, coumermycin (20 ng/ml), an antibiotic previously shown to up-regulate OspC (39), was added to cultures 24 h prior to experimental assays. *B. burgdorferi* densities were estimated using dark field microscopy with a Petroff-Hauser cell counter. Cultures were maintained at 34 °C and pH 7 prior to harvesting, conditions demonstrated to promote the expression of host-associated outer surface proteins (31, 40–43). For each culture, we harvested 5×10^8 bacteria by centrifugation at $2,000 \times g$ for 20 min. Cells were washed three times in 1 ml of Hanks' balanced salt solution (HBSS), and 10^8 cells were resuspended in 215 μ l of HBSS. The remaining cells were stored at -20 °C to assess plasmid content, as described previously (44), and protein expression by mass spectrometry (45).

Mass Spectrometry—The level of expression of all putative *B. burgdorferi* plasminogen receptors in the B31-A3, *ospC*-ko, and *ospC*-comp strains was assessed by mass spectrometry. Mass spectrometry is a high throughput technique that provides accurate quantification of all proteins in a sample. The mass spectrometric data were used to control for potential differences among the experimental strains that may affect plasminogen immobilization. To perform mass spectrometry, cells were first lysed by mechanical disruption using 0.1-mm diameter silica beads (Biospec Products, Inc.). Protein extracts were digested in-solution using trypsin (Promega, Madison, WI) at 37 °C overnight after DTT reduction and iodoacetamide alkylation. The resulting peptide mixtures were analyzed using an LCQ Deca XP Plus mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA) coupled to a nano-HPLC system (DIONEX) as described previously (45). Bioinformatics data

⁴ The abbreviations used are: uPA, urokinase-type plasminogen activator; HBSS, Hanks' balanced salt solution; ϵ -ACA, ϵ -aminocaproic acid.

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analysis was accomplished in the BioWorks 3.3.1 SP1 platform using the SEQUEST algorithm (Thermo Scientific) and the *B. burgdorferi* proteome database. Peptide identity was considered confident if ΔCn was greater than 0.1 and the cross correlation score (Xcorr) was greater than 1.5, 2.0, or 2.5 for peptides with charges (z) of +1, +2, or +3, respectively (45). Relative protein abundance was determined by a label-free mass spectral method based on precursor peak intensity measurement of individual peptides (*i.e.* the maximum ion count observed for a precursor ion) (46, 47). The precursor peak intensity was obtained directly from the raw data acquisition file. The mass-to-charge ratios (m/z), chromatographic retention times, and patterns of peaks near the focal peak were used to confirm that the chromatogram peak intensities compared among samples corresponded to identical peptides. Protein concentrations were determined by Bradford assay using BSA as standard.

Immunofluorescence Localization—Each strain was incubated on an uncoated cover glass for 1 h at 34 °C with 0.167 μ M human plasminogen, one-tenth the physiological concentration of plasminogen in vertebrate blood (48). Coverslips were washed five times in 1 ml of HBSS to remove weakly bound plasminogen and fixed using 16% paraformaldehyde. The coverslips were washed in PBS and incubated for 1 h in a 1:100 dilution of rabbit anti-OspC antibodies, followed by a 1:200 dilution of goat anti-rabbit antibodies conjugated to AlexaFluor594 (red), and finally a 1:100 dilution of FITC-conjugated sheep anti-plasminogen polyclonal antibodies (green) with the appropriate wash steps between. Coverslips were mounted using Mowiol with 2.5% 1,4-diazabicyclo-[2,2,2]-octane and examined at $\times 1,000$ magnification by epifluorescence microscopy (Eclipse TE2000; Nikon, Tokyo, Japan).

Plasmin Binding and Activity—Each strain (10^8 cells) was incubated with gentle agitation for 1 h at 34 °C with 1.67 μ M human plasmin or human plasminogen. Cells were then washed five times in 1 ml of HBSS to remove weakly bound plasmin or plasminogen. Cells were incubated for an additional 20 min with gentle agitation in 250 μ l of HBSS with 15 units of uPA (Calbiochem 672081) as determined by the manufacturer. Urokinase-type plasminogen activator was included as no plasminogen activator endogenous *B. burgdorferi* has been detected to date (8). Cells were washed three times, and the residual plasmin activity was determined. The number of cells was confirmed by measuring the absorbance at 210 nm after the final wash. Each well was supplemented with 50 μ l of the fluorogenic plasmin substrate D-Val-Leu-Lys-7-amino-4-methylcoumarin (1.2 mM in HBSS). Fluorescence intensity generated by the enzymatic degradation of the fluorogenic substrate was measured every 30 s for 30 min (excitation, 370 nm; emission, 430 nm read on a Analyst GT microplate reader, Molecular Devices). The number of active plasmin molecules in each well was determined by comparing fluorescence intensities to standards with known amounts of active plasmin. The retention of active plasmin bound by each strain was normalized by the density of spirochetes in each microplate well. To test for specific associations, α_2 -antiplasmin (100 μ g/ml) was added to the plasmin-loaded bacteria and allowed to react for 20 min at room temperature prior to measuring plasmin activity levels in some assays. Anti-OspC antibodies (1:100) or the lysine analog

ϵ -aminocaproic acid (ϵ -ACA; 10 mM) was added to the bacteria during the plasminogen or plasmin incubation in some assays to assess the role of OspC or lysine residues in plasminogen binding. Each strain was assayed in triplicate from six independent cultures per strain.

RESULTS

Expression and Relative Quantification of *B. burgdorferi* Surface Proteins—Surface protein expression was detected and quantified from whole-cell lysates of each strain using tandem mass spectrometry (Table 1). As expected, OspC peptides were observed only in wild-type (B31–A3) and *ospC*-complement (*ospC*-comp) strains at similar levels, but the knock-out strain, *ospC*-ko, did not have detectable OspC expression (Table 1 and supplemental Figs. S1 and S2). *Borrelia* plasminogen-binding protein, OspE-, and OspF-related proteins, and BbCRASP-1, each of which has been hypothesized as a potential plasminogen receptor (20, 22, 24), are expressed in all strains under the conditions studied (Table 1). Importantly, the expression levels of these proteins were similar among strains thus allowing us to isolate the effect of OspC on plasminogen immobilization from the effects caused by the other putative plasminogen receptors, *i.e.* no hypothesized plasminogen receptor aside from OspC can account for the observed differences in plasminogen binding among these strains. Additionally, any residual plasminogen detected in cultures of *ospC*-deficient strains may implicate one or more of the other surface proteins as alternative plasminogen receptors.

OspC and Plasminogen Co-localize on Surface of *B. burgdorferi*—Immunofluorescence microscopy revealed that OspC was expressed and accessible to anti-OspC antibodies on the surface of a subset of the spirochetes from B31–A3 (wild type) and *ospC*-comp (complement) cultures, but no spirochetes from the *ospC*-ko (knock-out) culture (Fig. 1A and supplemental Figs. S3–S8). OspC was distributed along the surface of the bacteria in a nonhomogeneous punctate manner (Fig. 1A, *OspC*). Immunofluorescence microscopy of cultures incubated with plasminogen and then anti-plasminogen antibodies revealed that plasminogen was only associated with spirochetes from the OspC expression strains B31–A3 and *ospC*-comp (Fig. 1A, *PLG*). Anti-plasminogen antibody staining of *ospC*-ko spirochetes that were incubated with plasminogen did not reveal plasminogen bound to any spirochetes (Fig. 1A, *PLG*). Co-immunofluorescence staining using rabbit anti-OspC antibodies, AlexaFluor594-conjugated goat anti-rabbit antibodies, and FITC-conjugated anti-plasminogen antibodies demonstrated that the plasminogen bound to spirochetes perfectly co-localized with OspC (Fig. 1A, *Merge*). Epifluorescence microscopy images taken of spirochetes at both 594 nm (Fig. 1A, *OspC*) and 488 nm (Fig. 1A, *PLG*) are overlaid on the phase contrast microscopy images (Fig. 1A, *Phase*) to estimate the proximity of OspC and bound plasminogen (Fig. 1B). The fluorescence intensity profiles of the double-stained spirochetes suggest that areas with high plasminogen staining have correspondingly high levels of OspC staining (Fig. 1B). The perfect co-localization of OspC and plasminogen suggests that OspC may be responsible for the immobilization of plasminogen to the sur-

TABLE 1

Expression and relative quantification of putative plasminogen receptors in strains B31–A3, *ospC*-ko, and *ospC*-comp

Borrelia plasminogen-binding protein (BPPB), BbCRASP-1 (*cspA*), and members of the OspE- and OspF-related proteins, all of which have been proposed as potential plasminogen receptors, are expressed at similar levels in all experimental strains as determined by the precursor peak intensities of the identified peptides in mass spectrometry analyses. As expected, OspC was detected only in strains B31–A3 and *ospC*-comp. Thus, no hypothesized plasminogen receptor aside from OspC can account for observed differences in plasminogen binding among these strains. Protein description, accession numbers, peptide sequences, precursor ion *m/z*, and the charge states (*z*) are listed. ND means not detected.

Protein peptide ^a	GenBank™ accession no.	Precursor <i>m/z</i> , <i>z</i>	Precursor peak intensities ^b		
			B31–A3	<i>ospC</i> -ko	<i>ospC</i> -comp
Outer surface protein C (OspC) GPNLTEISK EVEALLSSIDEIAAK ELTSPVVAESPK	NP_047005	480.2, 2 794.8, 2 629.3, 2	1.25 × 10 ⁸ 1.25 × 10 ⁷ 8.44 × 10 ⁷	ND ND ND	1.40 × 10 ⁸ 1.79 × 10 ⁷ 9.63 × 10 ⁷
BPPB NAQEYFDETVPESELGIK KLLAEAGYPDGK IRDDYYSGLK	NP_212462	1035.5, 2 631.7, 2 410.9, 3	8.36 × 10 ⁸ 1.23 × 10 ⁷ 2.84 × 10 ⁸	7.03 × 10 ⁸ 5.36 × 10 ⁷ 1.37 × 10 ⁸	4.60 × 10 ⁸ 8.88 × 10 ⁷ 5.28 × 10 ⁸
BbCRASP-1 (CspA) TLYSSLDYK DFDTLKPAPFY KITNPGENQNFEDK	NP_045741	545.6, 2 609.2, 2 579.5, 3	7.52 × 10 ⁷ 7.99 × 10 ⁷ 3.35 × 10 ⁷	6.05 × 10 ⁷ 8.89 × 10 ⁷ 4.58 × 10 ⁷	8.31 × 10 ⁷ 6.45 × 10 ⁷ 5.02 × 10 ⁷
OspE group ErpA (BbCRASP-5) IHTSYDEQSNGEVKVKK ErpP (BbCRASP-3) TAEYAIPLVLK ErpC (BbCRASP-4) KIEFSEFTVK	NP_051199 NP_051449 AAC34910	981.7, 2 737.1, 2 614.9, 2	4.68 × 10 ⁷ 3.45 × 10 ⁶ 1.28 × 10 ⁷	4.79 × 10 ⁷ 2.14 × 10 ⁶ 1.20 × 10 ⁷	2.59 × 10 ⁷ 8.41 × 10 ⁶ 1.31 × 10 ⁷
OspF group ErpG QNVKEKVEGFLDK EKEIQELK ErpL NSEQNLESSEQNVK NSEQNLESSEQNVKK	NP_051244 NP_051372	512.0, 3 1016.8, 1 803.2, 2 867.3, 2	5.94 × 10 ⁷ 1.01 × 10 ⁸ 1.14 × 10 ⁸ 1.40 × 10 ⁸	4.32 × 10 ⁷ 1.41 × 10 ⁸ 1.44 × 10 ⁸ 1.54 × 10 ⁸	9.46 × 10 ⁷ 1.34 × 10 ⁸ 8.95 × 10 ⁷ 2.98 × 10 ⁸
Elp ErpX LNKDNK ENVDVSEIKEDLEK	NP_051509	732.5, 1 549.5, 3	2.52 × 10 ⁷ 1.13 × 10 ⁸	1.54 × 10 ⁷ 6.37 × 10 ⁷	3.40 × 10 ⁷ 5.39 × 10 ⁷

^a The peptides listed from each protein were detected with high confidence by mass spectrometry ($\Delta Cn > 0.1$ and the cross-correlation score (Xcorr) is greater than 1.5, 2.0, or 2.5 for peptides with charges (*z*) of +1, +2, or +3, respectively) and checked visually to confirm correct peptide identification.

^b Precursor peak intensities of each peptide are listed for wild-type (B31–A3), OspC knockout (*ospC*-ko), and complement (*ospC*-comp) strains. Assays of 0.50 pm/μl bovine cytochrome *c* digest (Dionex) under similar conditions yielded peak intensities of (5–10) × 10⁸ for major peptides.

face of the bacterium. The OspC-deficient strain showed only background fluorescence on both channels.

OspC expression, and thus plasminogen-binding, was variable among spirochetes of strains B31–A3 and *ospC*-comp in cultures not induced with coumermycin. OspC could be detected by immunofluorescence on a fraction of individual spirochetes from strains B31–A3 and *ospC*-comp, similar to what has been observed in previous OspC expression experiments (31). Interestingly, OspC-expressing spirochetes had similar quantitative levels of OspC detectable by immunofluorescence, *i.e.* it appeared that variation in OspC expression among spirochetes is not continuous but that OspC expression is either on or off in each spirochete. By contrast, nearly all spirochetes of strains B31–A3 and *ospC*-comp expressed OspC at the maximally distinguishable level in cultures induced with coumermycin (supplemental Figs. S6–S8). The proportion of spirochetes expressing OspC decreases steadily as strains were continuously maintained in culture as noted previously (31). Regardless of the age of the culture, plasminogen binding was always restricted to OspC-expressing spirochetes in cultures of B31–A3 and *ospC*-comp, suggesting that OspC is a potent plasminogen receptor on the surface of the bacterium.

OspC Enhances the Ability of B. burgdorferi to Immobilize Active Plasmin and Plasminogen—The binding capacity and ability to use the enzymatic activity of plasmin was measured for each of the *B. burgdorferi* isolates by two methods. First, each strain was incubated with active plasmin and washed to remove weakly bound plasmin, and the activity of the remaining plasmin bound to the bacteria was measured using the fluorogenic plasmin substrate, D-Val-Leu-Lys-7-amino-4-methylcoumarin. Cultures of the OspC-expressing strains B31–A3 (B31) and *ospC*-comp (Comp) each retain over 4 × 10¹⁰ molecules of active plasmin per culture (10⁸ spirochetes), while cultures of the *ospC*-ko (KO) strain were associated with fewer than 0.25 × 10¹⁰ (10⁸ spirochetes) (Fig. 2A). The plasminogen binding capacity of the OspC-expressing strains was even greater when OspC expression was induced with coumermycin, whereas plasminogen binding by the OspC-deficient strain remained constantly low across culture conditions (supplemental Fig. S9). Preincubating B31–A3 with polyclonal anti-OspC antibodies (B31 + 1°Ab) prevented the spirochetes from binding active plasmin, suggesting that the reduction in binding observed in the OspC-deficient strain was not caused by a reduction of total surface protein or membrane integrity but was caused by the loss of available OspC (Fig. 2A). Incubating

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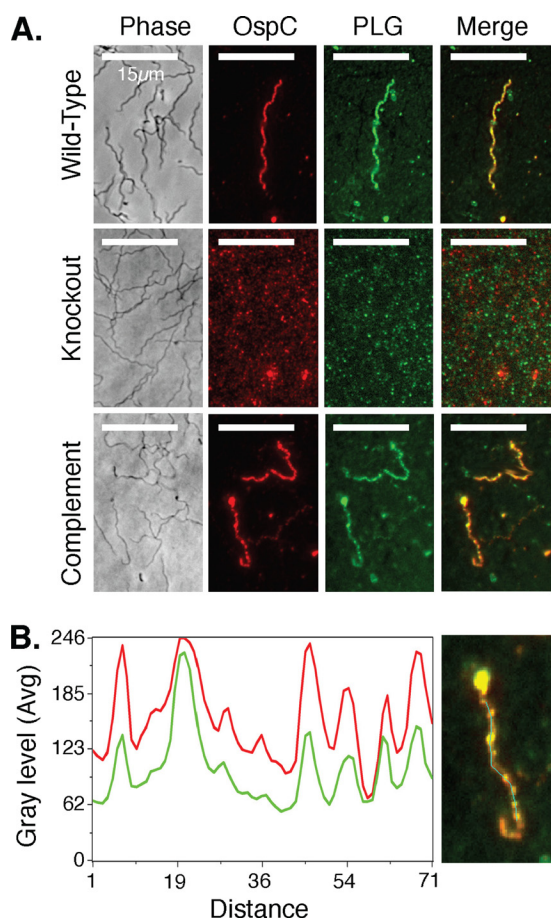


FIGURE 1. OspC and plasminogen co-localize on the surface of OspC-expressing spirochetes. *A*, OspC is expressed and distributed along the surface of a fraction of the spirochetes (OspC) from strains B31–A3 (*wild-type*) and *ospC-comp* (*Complement*) in a nonhomogeneous pattern but is not expressed in the OspC-deficient strain (*Knockout*). Spirochetes that express OspC (*wild-type* and *complement*) are associated with plasminogen (PLG), again in a nonhomogeneous pattern, whereas spirochetes that do not express OspC (*knock-out*) are not associated with plasminogen. Anti-plasminogen antibody staining of *ospC-ko* spirochetes that were incubated with PLG result in only background staining, similar to the pattern found after anti-OspC staining (*OspC*) of this strain. Each row (*Wild-type*, *Knock-out*, and *Complement*) represents $\times 1000$ images of paraformaldehyde-fixed slides taken with either phase contrast (*Phase*) or epifluorescence filters (594 nm, OspC; 488 nm, plasminogen). These images were overlaid (*Merge*) to estimate the proximity of OspC and bound plasminogen. *B*, importantly, bound plasminogen co-localizes with OspC on B31–A3 and *ospC-comp* spirochetes that were incubated with plasminogen. Fluorescence intensities of individual image pixels along the length of the double-stained spirochete (*cyan line* in right panel) were measured at both 594 nm (OspC) and 488 nm (plasminogen) and plotted against the distance in pixels (*left panel*). The fluorescence intensity profiles of double-stained spirochetes (*red*, OspC; *green*, plasminogen) demonstrate that areas with high plasminogen staining have correspondingly high levels of OspC staining, *i.e.* OspC and plasminogen are located in the exact same positions on the surface of the spirochetes (*right panel*, manifested by *yellow overlap regions* in the final merged image) suggesting that OspC is a potent plasminogen receptor.

B31–A3 with anti-OspA serum did not affect plasminogen binding (data not shown). Spirochetes did not show any increase in mortality or decrease in activity after a 3-h incubation with either anti-OspC or anti-OspA antibodies compared with spirochetes that were not incubated with antibodies. Binding of plasminogen to strain B31–A3 is reduced in the presence of ϵ -ACA, suggesting lysine residues are important in the OspC-plasminogen interaction, similar to what was previously observed in a protein-protein binding assay (23). The surface-

bound plasmin was protected against inactivation by the serum-derived inhibitor α_2 -antiplasmin (Fig. 2, *A* and *B*).

The OspC-expressing strains are also significantly better at capturing plasminogen than the *ospC-ko* strain or the B31–A3 parent strain preincubated with anti-OspC antibodies (Fig. 2*B* and supplemental Fig. S9*B*). The bound plasminogen could subsequently be converted to active plasmin by the addition of the exogenous activator uPA. Each strain was incubated with plasminogen, washed to remove weakly bound plasminogen, and then incubated with uPA before assessing the amount of active plasmin that remained bound to the spirochetes. No proteolytic activity was detected with any strain when the bacteria were incubated with plasminogen or uPA alone.

DISCUSSION

B. burgdorferi, the invasive bacterial pathogen that causes human Lyme disease in North America (49), completes its natural infectious cycle without expressing an endogenous extracytoplasmic protease (5). *B. burgdorferi* rapidly traverses many basement membranes and extracellular matrices as it migrates from the tick to the host dermis and subsequently enters and exits the vascular system *en route* to disseminated infection sites. Experimental infections of laboratory animals have shown that the plasminogen system is required for efficient transmission of *B. burgdorferi* from ticks to vertebrate hosts (7). However, the molecular mechanism by which *B. burgdorferi* immobilizes plasminogen to enhance dissemination was previously unknown. We used wild-type and genetically manipulated *B. burgdorferi* in immunofluorescence microscopy and live cell binding assays to test the hypothesis that the OspC is a plasminogen receptor on the surface of the bacterium. Immunofluorescence microscopy images demonstrate that only OspC-expressing spirochetes immobilize plasminogen to their surface, while plasminogen does not associate with OspC-deficient spirochetes (Fig. 1*A*). Furthermore, wild-type spirochetes that were not actively expressing OspC did not show measurable plasminogen binding (Fig. 1*A*). Importantly, plasminogen co-localizes with OspC on the cell surface, strongly implicating OspC as a biologically relevant plasminogen receptor (Fig. 1*B*). Plasminogen immobilization by live spirochetes was also much greater in cultures of OspC-expressing strains than in cultures of OspC-deficient strains (Fig. 2 and supplemental Fig. S9). Plasminogen binding is severely diminished in the presence of anti-OspC antibodies but not in the presence of anti-OspA antibodies suggesting a specific protein partnership between OspC and plasminogen. Plasmin bound to OspC is protected from inhibition by serum-derived α_2 -antiplasmin further indicating that this is a specific interaction that can provide the bacteria with active protease activity within a vertebrate host (Fig. 2, *A* and *B*). The data presented here demonstrate that OspC, a surface-exposed protein expressed during the critical tick-to-host transition, is a potent plasminogen receptor on the surface of living bacteria. These results are consistent with the hypothesis that OspC can enhance the efficacy of the infectious cycle in *B. burgdorferi* by immobilizing plasminogen.

Several lines of *in vitro* and *in vivo* evidence support the hypothesis that plasminogen enhances the efficacy of the infectious cycle of *B. burgdorferi* and related species. Specifically,

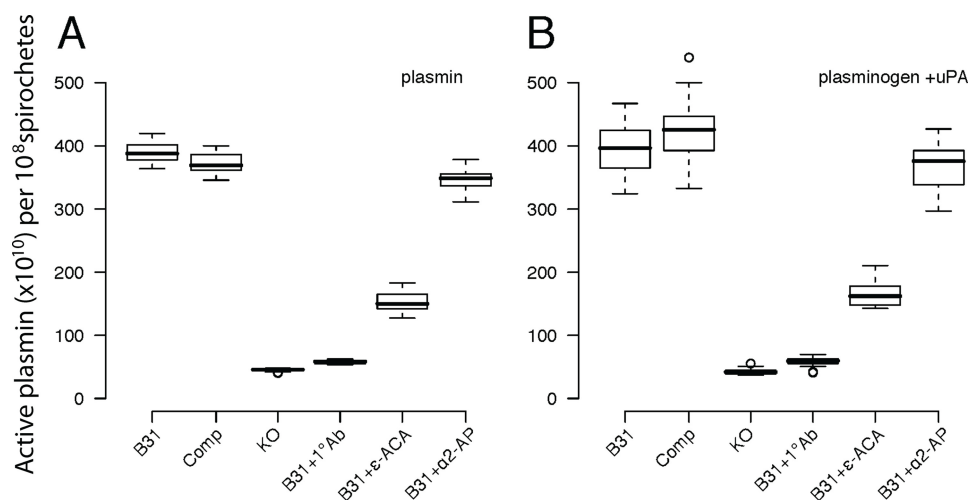


FIGURE 2. OspC-expressing strains bind significantly more plasmin and plasminogen than OspC-deficient strains. Cultures of the OspC-expressing strains B31–A3 (B31) and *ospC*-comp (Comp) each retain over 4×10^{10} molecules of active plasmin (A) or plasminogen (B) per culture containing 10^8 spirochetes, whereas the OspC-deficient strain, *ospC*-ko (KO) was associated with fewer than 0.25×10^{10} plasmin molecules per culture containing 10^8 spirochetes. Preincubating strain B31–A3 with polyclonal anti-OspC antibodies (B31 + 1°Ab) inhibits the spirochetes from binding active plasmin or plasminogen. The lysine analog ε-ACA inhibits binding of plasmin and plasminogen to the OspC-expressing spirochetes (B31 + ε-ACA) confirming the role of lysine molecules in this interaction. Active plasmin bound to the spirochetes is protected from inhibition by α₂-antiplasmin (α₂-AP), a potent serum-derived inactivator of unbound plasmin. The number of active plasmin molecules that remained in spirochete cultures after incubation and extensive washing was estimated by comparing plasmin activity levels associated with each culture to experimentally prepared standards. Plasmin activity data were normalized by the number of spirochetes remaining in each experimental culture after all washing steps.

extensive studies have demonstrated that the use of plasminogen by *B. burgdorferi* enhances invasiveness in *in vitro* assays through degradation of extracellular matrix components, penetration of biological barriers, and dissemination within ticks and into the vasculature of vertebrates (28–34). Furthermore, plasminogen increases the efficacy of internal organ invasion by relapsing fever *Borrelia* (6, 14). We propose that immobilization of plasminogen by the OspC surface receptor of *B. burgdorferi* increases the probability of a successful vertebrate infection from a tick bite at one or more of the following points in the infectious cycle. First, the probability of infection per tick bite may increase in the presence of plasminogen if more spirochetes can successfully migrate to the salivary glands and thus a greater number enter a host during tick feeding. Second, plasmin immobilized to the bacterial surface may increase the probability and efficacy of a bacterium entering the host vasculature. Finally, plasmin-coated bacteria in the host may be less likely to be destroyed by the host immune response as plasmin actively cleaves immunoglobulins and damages components of the complement pathway (50).

Although the data presented suggest that OspC-bound plasmin can enhance the infectivity of *B. burgdorferi* during the transmission from ticks to hosts, neither plasmin nor OspC is strictly required for successful infection (7, 51, 52). Overexpression of alternate surface-bound proteins can rescue the infectious phenotype of OspC-deficient strains, albeit with severely reduced infectiousness (51). The reduction of infectivity in wild-type *B. burgdorferi* observed in plasminogen-deficient laboratory animals is much less severe than the reduction in infectiousness observed in OspC-deficient and some OspC mutant strains infecting wild-type laboratory animals (33) suggesting that OspC has functions other than plasminogen immobilization. The pleiotropic nature of OspC is similar to the plasminogen receptors identified from all other plasminogen-utilizing

bacteria that have alternative functions ranging from glycolytic enzymes to cellular adhesion molecules, many of which are essential even in *in vitro* culture (3). Indeed, a recent study suggests that OspC functions as an immune modulator and is also required for efficient dissemination, functions that are accomplished by different parts of the molecule (53). Thus, it is likely that OspC functions as a plasminogen receptor facilitating efficient dissemination in addition to other molecular functions. OspC, like most proteins, is likely pleiotropic and assumes several functions during the tick-to-host transition (reviewed in Ref. 37).

Although overexpression of alternative outer membrane proteins can partially rescue the infectious phenotype of OspC-deficient *B. burgdorferi* (51), the data presented here suggest that these surface proteins cannot completely rescue the plasminogen immobilization capacity provided by OspC during the tick-to-host transmission. Indeed, OspC-deficient strains express the other putative plasminogen receptors (Table 1) yet bound very little plasminogen in these assays (Fig. 2, A and B). This is unlikely the result of simultaneous down-regulation of OspC and other putative plasminogen receptors (54–57) as all strains expressed the other surface proteins of interest at similar levels (Table 1). Nevertheless, the other putative plasminogen receptors may be responsible for the low level plasminogen binding observed by *ospC*-ko, and thus genetic modification to dramatically overexpress these genes (51) may partially rescue plasminogen immobilization. Furthermore, the expression of alternative surface proteins may be much greater in different *in vitro* and *in vivo* conditions than the culture conditions used here (however, see Refs. 56, 57), which could potentially restore plasminogen immobilization in spirochetes not actively expressing OspC. Further research into the binding capabilities of alternative surface proteins embedded in the bacterial membrane in alternative experimental conditions is needed to assess

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their roles in plasminogen binding during the infectious cycle. We expect that plasminogen interacts with several other *B. burgdorferi* proteins embedded in the membrane of living cells at different stages of the infectious cycle that could potentially be detected using different culture conditions (*i.e.* conditions that mimic the divergent microenvironments that *B. burgdorferi* may inhabit during its life cycle). Regardless of the role that proteins may play in plasminogen immobilization, the data presented here demonstrate that OspC, a dominant surface protein during the critical tick-to-host transition (51), readily immobilizes plasminogen to the bacterial surface thus providing the bacteria with proteolytic activity in the presence of host tissues.

The results presented are consistent with the hypothesis that the sequence differences among *ospC* alleles affect the dissemination potential of *B. burgdorferi* due to the effect on OspC-plasminogen binding affinity. Several previous studies demonstrate that *B. burgdorferi* lineages that differ at the *ospC* locus have significantly different probabilities of disseminating in human patients (58–60). Furthermore, a protein-protein binding assay demonstrated that OspC sequence variation affects binding affinity to human plasminogen (23). Interestingly, recombinant OspC variants from two *B. burgdorferi* lineages that regularly cause disseminated human infections bind to human plasminogen with significantly higher affinity than two variants from lineages that rarely infect humans (23). Future studies using *B. burgdorferi* strains with genetically modified *ospC* alleles in whole-cell assays are essential to determine the relevance of OspC sequence differences to plasminogen immobilization on the surface of live cells and to the probability of human infection.

The distribution of OspC and plasminogen along the length of the bacteria was not homogeneous in this study (Fig. 1). OspC exists naturally as a multimer (61, 62) that could cause the nonhomogeneous pattern observed and thus could concentrate the proteolytic activity of plasmin to promote degradation of intercellular membranes. However, the nonhomogeneous distribution of OspC may be a methodological artifact resulting from the fixation process. Previous reports demonstrate that the outer surface protein A (OspA) forms highly localized aggregates when the bacteria are antibody labeled prior to fixation but show a more even distribution when the bacteria are fixed prior to exposure to anti-OspA antibodies (63). Regardless of the microvariation in the distribution of OspC, the colocalization of OspC and plasminogen along the length of the bacteria can serve to focus proteolytic activity to the extracellular matrices during the stationary adhesions to intercellular junctions (64). Thus, the OspC-plasminogen association could act to increase the ability of spirochetes to traverse membranes in ticks or hosts and thus increase the efficacy of the tick-to-host transition.

Many infectious bacteria including *Yersinia pestis*, *Helicobacter pylori*, *Staphylococcus aureus*, and *Streptococcus pyogenes* use host-derived plasmin to eliminate barriers to migration across extracellular matrices and basement membranes (3, 12), closely resembling the process of eukaryotic cell migration during repair of damaged tissues and cancer metastasis (65). The use of a host-derived proteolytic enzyme, as opposed to an

endogenous bacterial enzyme, may allow the bacteria to efficiently migrate within a host without invoking an acute inflammatory response during early infections (16). In this study, we have identified OspC as a potent plasminogen receptor that is abundant on the surface of *B. burgdorferi* during the tick-to-host transition, a point in the enzootic cycle when proteolytic activity is critical. The approaches employed here, coupled with the developing genetic tool kit in *B. burgdorferi*, will allow further dissection of the quantitative effects of the OspC-plasminogen interaction on infectiousness and can be used to explore the specific regions or residues responsible for this interaction.

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