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Assessment of the potential contribution of the highly conserved C-terminal motif (C10) of *Borrelia burgdorferi* outer surface protein C (OspC) in transmission and infectivity

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Abstract

OspC is produced by all species of the *Borrelia burgdorferi* sensu lato complex and is required for infectivity in mammals. To test the hypothesis that the conserved C-terminal motif (C10) of OspC is required for function in vivo, a mutant *Borrelia burgdorferi* strain (B31: :*ospC*ΔC10) was created in which *ospC* was replaced with an *ospC* gene lacking the C10 motif. The ability of the mutant to infect mice was investigated using tick transmission and needle inoculation. Infectivity was assessed by cultivation, qRT-PCR, and measurement of IgG antibody responses. B31: :*ospC*ΔC10 retained the ability to infect mice by both needle and tick challenge and was competent to survive in ticks after exposure to the blood meal. To determine if recombinant OspC protein lacking the C-terminal 10 amino acid residues (rOspCΔC10) can bind plasminogen, the only known mammalian derived ligand for OspC, binding analyses were performed. Deletion of the C10 motif resulted in a statistically significant decrease in plasminogen binding. Although deletion of the C10 motif influenced plasminogen binding, it can be concluded that the C10 motif is not required for OspC to carry out its critical *in vivo* functions in tick to mouse transmission.

Introduction

Lyme disease is a tick transmitted infection caused by several *Borrelia* species including *Borrelia burgdorferi*, *B. garinii*, and *B. afzelii* (Benach *et al.*, 1983; Burgdorfer *et al.*, 1982). In nature, numerous species of birds, mammals and lizards serve as reservoirs (Clark *et al.*, 2005; Gern *et al.*, 1998). Newly released case numbers calculated by the Center for Disease Control and Prevention suggest that there are a minimum of 300,000 cases each year in the

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United States. The disease is also highly prevalent in Europe but the true incidence is not known due to the lack of a uniform reporting system. Humans are incidental hosts and are not significant in the enzootic cycle of the Lyme disease spirochetes. With the exception of the pathognomonic erythema migrans rash, early symptoms of Lyme disease are non-specific (e.g., malaise, low grade fever) (Steere, 2001; Wormser *et al.*, 2006). If Lyme disease goes untreated cardiac, arthritic, dermatological, or neurological sequelae can occur (Steere, 2001; Steere, 2006). Preventive strategies for Lyme disease (acaricides and tick avoidance) are ineffective and a vaccine for use in humans is not available. Outer surface proteins (Osp) OspA, OspC, and recombinant-chimeric OspC derivatives represent the primary focus in efforts to develop new Lyme disease vaccines (reviewed in Earnhart and Marconi, 2008; Marconi and Earnhart, 2010).

The Lyme disease spirochetes must adapt to radically different environmental conditions as they cycle between ticks and mammals. Differential production of outer membrane lipoproteins plays a key role in adaptive responses (Crandall *et al.*, 2006; Narasimhan *et al.*, 2002; Revel *et al.*, 2002; Rogers *et al.*, 2009; Schwan, 2003; Tokarz *et al.*, 2004). OspC, a 22 kDa lipoprotein (Fuchs *et al.*, 1992), is upregulated during tick to mammal transmission and remains expressed during early infection in mammals (Brooks *et al.*, 2003; Gilmore *et al.*, 2001; Schwan *et al.*, 1995; Schwan and Piesman, 2000). Although OspC is an essential virulence factor, its precise and full range of functions are not known (Grimm *et al.*, 2004; Stewart *et al.*, 2006; Tilly *et al.*, 1997; Tilly *et al.*, 2006; Tilly *et al.*, 2007). Structural and bioinformatic analyses of OspC have identified several putative ligand binding domains (Eicken *et al.*, 2001; Kumaran *et al.*, 2001). Several studies suggest that OspC binds mammalian derived plasminogen (Lagal *et al.*, 2006; Onder *et al.*, 2012). Plasminogen binding and activation may facilitate invasion of host tissues and endothelial barriers. The potential binding site for plasminogen on OspC remains undefined. OspC has also been reported to bind to a tick-derived protein, Salp15 (Anguita *et al.*, 2002; Lagal *et al.*, 2006). However, the significance of Salp15 binding is unclear since its interaction is not required for needle inoculated spirochetes to infect and disseminate in mammals (a route independent of Salp15) (Earnhart *et al.*, 2010; Earnhart *et al.*, 2011; Grimm *et al.*, 2004). A putative small ligand binding domain (designated as LBD1) formed along the surface of the dimeric interface of OspC has been demonstrated to be critical for *in vivo* function (Eicken *et al.*, 2001; Kumaran *et al.*, 2001). A single amino acid substitution within LBD1 (residue E61) eliminated infectivity of *B. burgdorferi* in mice while other mutations influenced dissemination (Earnhart *et al.*, 2010). While the ligand for LBD1 remains unknown, the E61Q substitution did not affect plasminogen binding indicating that OspC may bind other ligands and have multiple functions critical for transmission and infectivity of the Lyme disease spirochetes(Earnhart *et al.*, 2010).

Approximately 30 distinct OspC phyletic types having been defined (Brisson and Dykhuizen, 2004; Earnhart *et al.*, 2005; Wang *et al.*, 1999). OspC phyletic type identity is defined largely by the sequence of its variable surface-exposed regions. In spite of significant inter-type diversity some OspC regions are highly conserved suggesting a direct (ligand binding) or indirect (maintenance of critical structural determinants) role in its function. One of the most highly conserved regions of OspC is its C-terminal 10 amino

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acids, henceforth referred to as the C10 motif. To test the hypothesis that the C10 motif is required for OspC function *in vivo*, the wild type *ospC* gene of *B. burgdorferi* B31 was replaced with an *ospC* gene encoding a truncated protein lacking the C10 motif to yield B31: :*ospC*ΔC10). B31: :*ospC*ΔC10 efficiently infected mice by both needle and tick challenge. Recombinant OspCΔC10 demonstrated attenuated plasminogen binding. The results presented within revealed that the C10 is not required for OspC to function in ticks or mammals raising interesting question about the selective pressures that have maintained its sequence conservation. From a technical perspective, this study is only the second to employ targeted mutagenesis of OspC to assess its functions in vivo.

Materials and Methods

Bacterial strains, cultivation conditions, tick and animal studies

Borrelia were cultivated in BSK-H medium supplemented with 6% bovine serum albumin at 25, 33 or 37 °C in sealed bottles under 3% CO_2 (with antibiotics where appropriate). The parental strain employed for genetic manipulation, *B. burgdorferi* B31-clone 5A4 (henceforth referred to as B31), produces a type A OspC. All strains employed in this study are described in Table 1. *E. coli* strains NovaBlue and BL21-DE3 were used for plasmid propagation and recombinant protein production, respectively, with growth conditions detailed below. C3H-HeJ mice (Jackson Laboratories) were employed to assess the infectivity of the *Borrelia* strains. All animal studies were conducted under protocols reviewed and approved by the Virginia Commonwealth University and University of Maryland IACUCs. *Ixodes scapularis* nymphal stage ticks were obtained from a pathogen free tick colony maintained under 95% humidity at University of Maryland.

SDS-PAGE and immunoblot analyses

Recombinant proteins or cell lysates were solubilized and fractionated by SDS-PAGE using precast 12% Criterion gels (Biorad). Proteins were transferred to PVDF membranes by electroblotting using standard methods. Non-specific binding to the membranes was blocked with PBS-T (5% non-fat dry milk in PBS; 0.02% Tween-20), and the membranes were screened with primary and secondary antibodies as indicated below. Mouse anti-His mAb (Pierce), mouse anti-OspC (Earnhart *et al.*, 2011), and mouse anti-FlaA antiserum (Pierce), were used at dilutions of 1:10,000, 1:10,000, and 1:40,000, respectively. Antiserum from mice challenged with spirochetes by needle inoculation or by tick feeding were used at a dilution of 1:2000. Antibody binding was detected using goat anti-mouse IgG peroxidase conjugate (1:40,000; Pierce) and chemiluminescent substrate (SuperSignal West Pico; Pierce).

Proteinase K digestion

Cultures were grown at 33 °C and then shifted to 37 °C for 2 days to maximize OspC production (Stevenson *et al.*, 1995). Proteinase K digestion assays were performed as previously detailed (Earnhart *et al.*, 2010). Cells were harvested by centrifugation, washed, suspended in PBS (0.3 OD_{600} units mL^{-1}) and proteinase K or PBS (negative control) were added. The samples were incubated at 22°C (1 hr) and then the proteinase K was inactivated

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by the addition of PMSF. The cells were solubilized, separated by SDS-PAGE, and transferred to membranes for subsequent immunoblot analyses.

Immunofluorescence assays

Strains were cultivated at 33 EC, temperature shifted to 37 EC (as detailed above), harvested by centrifugation, and immobilized on slides (Superfrost Plus, Fisher) as previously described (Buckles *et al.*, 2006). Non-specific binding was blocked with PBS-T containing 3% BSA. The slides were screened with mouse anti-OspC antiserum (1:2,000) with secondary detection by Alexa-488 conjugated goat anti-mouse IgG (1:200). Slides were mounted with Prolong Gold (Invitrogen) and the cells were visualized by fluorescence microscopy (Olympus BX51).

Production of recombinant OspC proteins

Full length rOspC (rOspCwt) and a C-terminal (10 aa) truncation variant (rOspCΔC10) were generated by PCR amplification using the OspC20(+)/OspC210(-) and OspC20(+)/ OspC200(-) primer sets (Table 2). The final constructs were designed without signal sequences. The amplicons were annealed to the pET46 Ek/LIC vector (Novagen). The plasmids were propagated in NovaBlue cells and plasmids isolated using Qiagen kits. The purified plasmids were transformed into BL21(DE3) cells, protein expressed by IPTG induction, and then the r-proteins purified using nickel affinity chromatography (Earnhart and Marconi, 2007b). Sequences were verified for all constructs described in this study on a fee for service basis (MWG Biotech).

Allelic exchange replacement of wild-type ospC

A pCAEV1-based suicide allelic exchange vector was created that encodes an OspC truncation variant lacking the 10 terminal amino acids. This vector is designed to integrate into circular plasmid 26 (cp26). The sequence encoding amino acids 20 to 200 of B31 *ospC* was amplified using the Insert-typeA20(+)BspEI and Insert-typeA200(-) BspEI primers, ligated into the BspEI sites of pCAEV1, and the plasmid propagated in *E. coli* NovaBlue (Earnhart *et al.*, 2010). The purified, linearized plasmid was introduced into *B. burgdorferi* B31-5A4 by electroporation (Samuels *et al.*, 1994). Clonal populations of the transformants were obtained by subsurface plating with streptomycin selection (Earnhart *et al.*, 2010). Colonies were cored from the plates and transferred to BSK-H medium for expansion. The plasmid content of each clone was determined by PCR using plasmid-specific primer sets (Rogers *et al.*, 2009). An aliquot of each culture was boiled in 100 :l water, and 1 :l was used as template in each reaction. Integration of the *ospC* cassette into the circular plasmid cp26 of B31 was verified by PCR amplification and DNA sequencing (Earnhart *et al.*, 2010). The growth rates of all strains were determined by daily triplicate cell counts of cultures grown at 37°C in BSK-H medium (no antibiotics) using dark-field microscopy.

Assessment of plasminogen binding by wild-type and site-directed mutant proteins

Plasminogen binding to rOspC and rOspC)C10 was assessed by ELISA (Lagal *et al.*, 2006). ELISA plates (Costar 3590) were coated with each protein (1:g $well^{-1}$) in 100 :l carbonate buffer (pH 9.6; overnight). Immobilized BSA and blank wells served as negative controls

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and rOspA (an established plasminogen binding protein (Fuchs *et al.*, 1994)) served as a positive control. Non-specific binding was blocked with TBS-T (5% BSA). Plasminogen was added (0.1 :g well^{-1} in blocking buffer) and the plates incubated for 2 hr at room temperature. After washing with TBS-T, goat anti-human plasminogen was added (1:1000; 1 hr; room temperature; Pierce). After washing, antibody binding was detected with peroxidase-conjugated rabbit anti-goat IgG (1;20,000 dilution; 1 hr; room temperature; Calbiochem) and ABTS chromogen as described by the supplier (Pierce). Absorbance was measured at 405 nm (A_{405}).

Infectivity studies

Strains B31 (wild type strain not subjected to genetic manipulation), B31: :*ospC*wt (B31 which has a *ospC* wild type- type A-strep cassette inserted as a control), B31: :*ospC*ΔC10, or B31: :*ospC* were needle inoculated (subcutaneous; 10^4 spirochetes) into C3H-HeJ mice (n = 5 per group). After four weeks the mice were euthanized and ear punch biopsies (2 mm), urinary bladders, and blood were collected. To determine if the spirochetes established an infection, skin biopsies and bladders were placed in BSK-H complete medium supplemented with rifampicin, fosfomycin, and amphotericin B (Sigma); the cultures were maintained at 33°C and their growth monitored by dark field microscopy. To assess antibody responses, serum was harvested and IgG titers were determined by ELISA as previously described (Earnhart and Marconi, 2007c). In brief, B31 cells (0.01 OD_{600} well^{-1}), rOspC, and rOspCΔC10 were immobilized in ELISA plate wells overnight in carbonate buffer (pH9.6) to saturation. Non-specific antibody binding was blocked with 1% BSA in PBS-T. The immobilized antigens were screened with serial dilutions of mouse sera (1:50 - 1:109,150) infected with the appropriate strain and antibody binding detected using peroxidase conjugated goat anti-mouse IgG (1:20,000) and ABTS chromogen.

Transmission analyses

To assess the ability of each strain to transit from ticks into mice, naïve, unfed *I. scapularis* nymphs (15 ticks per group) were infected by microinjection as previously described (Zhang *et al.*, 2009). After a two day recovery period, the ticks were placed on naïve mice (5 ticks per mouse; 3 mice per group), fed to repletion, and collected. Fourteen days after the ticks dropped off, the mice were euthanized, and heart, blood, and skin biopsies were collected. The presence of actively growing spirochetes in mouse tissues and in the fed ticks was assessed using qRT-PCR as previously described (Zhang *et al.*, 2009). To assess the antibody response to infection, immunoblots of cell lysates were screened with serum from each mouse (1:2000), and anti-*B. burgdorferi* IgG titers determined as described above.

Results

Generation and analysis of a B. burgdorferi strain expressing a C-terminally truncated OspC protein

PCR analyses confirmed that the wild-type cp26 (circular plasmid 26 kb) carried *ospC* gene of strain B31 (clone 5A4) was successfully replaced with a cassette encoding antibiotic resistance and an *ospC* gene lacking the C-terminal ten amino acids (C10 motif) (data not shown). Clonal populations of the transformed strain, designated as B31: :*ospC*ΔC10, were

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obtained by subsurface plating (Sung *et al.*, 2001) and screened for plasmid content using plasmid-specific PCR primers (Labandeira-Rey and Skare, 2001; McDowell *et al.*, 2001). Several clones harboring all plasmids present in the parental strain, with the exception of linear plasmid lp21, were identified (data not shown). The absence of lp21 is not relevant to this study as it is not required to infect mice or ticks and its absence has no effect on dissemination in mammals (Earnhart *et al.*, 2010). The growth rate of B31::*ospC*ΔC10 was found to be similar to that of the control strains (Figure 1, panel A). Immunoblot analyses verified production of full length OspC by B31 and B31::*ospC*wt, production of truncated OspC by B31::*ospC*)C10, and no OspC production by B31::*ospC* (Figure 1, panel B). Proteinase K digestion assays (Figure 1, panel B) and IFA analyses (Figure 1, panel C) demonstrated that OspC is exported and presented on the cell surface by each recombinant strain in a manner similar to wild type. It can be concluded that deletion of C10 and expression of antibiotic resistance does not alter growth rate, or the expression, production and export of OspC to the outer membrane.

B31::ospCΔC10 retains the ability to infect mice when administered by needle inoculation

To determine if the C10 motif mediates a function that is required for *B. burgdorferi* to infect mice, B31::*ospC*ΔC10 and control strains (B31, B31Δ*ospC*, B31::*ospC*wt) were needle inoculated into mice. Cultivation of tissue biopsies and urinary bladder collected four weeks post infection revealed that all mice inoculated with B31::*ospC*ΔC10 became infected whereas mice inoculated with the *ospC* deletion mutant (B31Δ*ospC*; 10^{-4} spirochetes) did not. Twelve of the 15 mice inoculated with B31::*ospC*ΔC10 or the positive control strains (B31, B31::*ospC*wt) were culture positive from the bladder and skin with the remaining three mice being culture positive from the bladder only (Table 3). It can be concluded that the C-terminus of OspC is not required for spirochetes to infect mice, persist, and disseminate.

Tick transmission analyses

To determine if deletion of C10 prevents OspC from participating in functions required for transmission from ticks to mammals, ticks infected by microinjection were fed to repletion on naïve mice and collected. qRT-PCR was used to verify the presence of viable spirochetes in a sampling of the micro-injected tick. *B. burgdorferi flaB* mRNA (a constitutively expressed gene) was detected in all microinjected ticks (data not shown). The mice were euthanized 2 weeks post tick drop off and blood, tissue biopsies, and hearts were harvested. A portion of the heart was placed in BSK-H medium (with antibiotics) to assess the ability of each strain to establish infection via the tick transmission route. Positive cultures were obtained from all mice except those that were fed on by ticks that were infected with the B31Δ*ospC* (Table 4). To screen for the presence of viable spirochetes in tissue collected from around the tick bite site, *flaB* qRT-PCR was performed. Transcript was detected in mice infected with B31, B31::*ospC*ΔC10 and B31::*ospC*wt whereas *flaB* mRNA was not detected in mice infected with B31Δ*ospC* (Figure 2; ANOVA, $p<.0001$). These results demonstrate that deletion of C10 does not block transmission via natural tick feeding.

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Immune responses to B. burgdorferi infection via needle and tick delivery approaches

The IgG response of mice infected by tick bite or needle inoculation was assessed through determination of anti-*B. burgdorferi* IgG antibody titers. ELISA analyses using *B. burgdorferi* B31-5A4 as the immobilized antigen revealed that all infected mice, regardless of infection route, mounted a significant IgG response (Figure 3, panels A and B). As expected, and consistent with data published in previous studies, a significant IgG response was not detected in mice infected with B31Δ*ospC* (data not shown) (Earnhart *et al.*, 2010; Earnhart *et al.*, 2011). To determine if deletion of C10 influences the anti-OspC IgG response, the antibody titer in each mouse infected by needle inoculation was determined by ELISA using rOspC (full length) and rOspCΔC10 as immobilized antigens. Mice infected with B31, B31: :*ospC*wt, and B31: :*ospC*ΔC10 had comparable anti-OspC antibody titers for the rOspC and rOspCΔC10 proteins (data not shown). Serum harvested from mice infected by needle inoculation and by tick bite were used to screen cell lysates of *B. burgdorferi* B31. Representative results are presented in Figure 4 (panels A and B). Both B31 and B31: :*ospC*Δ10 elicited IgG responses to numerous antigens. As expected, an IgG response was not observed with B31Δ*ospC*. Hence, C10 does not appear to be an immunodominant epitope and its deletion does not significantly influence IgG responses directed at OspC.

Plasminogen binding to recombinant proteins

It was previously demonstrated that OspC binds plasminogen (Earnhart *et al.*, 2010; Lagal *et al.*, 2006; Onder *et al.*, 2012). However, the binding determinants within OspC that are required for plasminogen binding have not been identified. To determine if the C10 motif is directly or indirectly required for this interaction, recombinant proteins were tested for plasminogen binding using an ELISA format. rOspA, a known plasminogen binding protein (Fuchs *et al.*, 1994), served as a positive control. Deletion of the C10 motif resulted in a small but statistically significant decrease in binding (Figure 5). It remains to be determined if this decrease is a direct or indirect effect associated with the C1- motif.

Discussion

The OspC determinants that participate in its critical, yet largely undefined, *in vivo* functions are not fully known. To date, only two published studies have investigated the potential contribution of specific amino acid residues of OspC in its *in vivo* function (Earnhart *et al.*, 2010; Earnhart *et al.*, 2011). A solvent accessible, surface exposed region of OspC, designated as ligand binding domain 1 (LBD1), likely is a key determinant in OspC function as single or double amino acid substitutions introduced within LBD1 render *B. burgdorferi* non-infectious or alter its dissemination patterns (Earnhart *et al.*, 2010). The results obtained through site directed mutagenesis are important as they refute earlier suggestions that OspC serves a non-specific function membrane stabilizing function during infection that can be complemented by other divergent outer surface proteins (Xu *et al.*, 2008). In a second study, allelic exchange mutagenesis was performed (Earnhart *et al.*, 2011) to test the hypothesis that OspC's *in vivo* function is dependent on the formation of higher order protein arrays in the spirochetal membrane (Lawson *et al.*, 2006; Zuckert *et al.*, 2001). Residue C130 is the sole cysteine of OspC and its spatial location on the surface of the OspC dimer raises the possibility that it could form inter-dimeric disulfide bonds. Substitution of C130 with

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Alanine and subsequent allelic exchange replacement of wild type *ospC* (to yield strain B31: :C130A) had no effect on infectivity. These data indicate that higher order oligomerization (i.e., tetramers, etc) is not required for OspC function *in vivo*.

The goal of this study was to test the hypothesis that the highly conserved C10 motif of OspC (PVVAESPKKP) directly or indirectly plays a role in OspC's *in vivo* functions. It had previously been suggested that the C10 motif may assume a polyproline II helical conformation (Mathiesen *et al.*, 1998), a secondary structure motif that may participate in protein-protein interactions (Brady *et al.*, 2010). While the C10 motif has been demonstrated to be solvent accessible, NMR analyses suggest it to be relatively unstructured (Huang *et al.*, 1999). To assess the potential role of C10 in OspC in vivo activity, a *B. burgdorferi* mutant strain (B31: :*ospC*ΔC10) was generated that produces an OspC protein lacking the C10 motif. The B31: :*ospC*ΔC10 strain, generated through allelic exchange, was found to have similar growth kinetics and OspC production patterns as the parental wild type strain. Deletion of the C10 motif did not disrupt OspC trafficking and presentation on the cell surface.

The potential of B31: :*ospC*ΔC10 to infect mice by needle inoculation was assessed. Positive cultures of B31: :*ospC*ΔC10 were obtained from 5/5 bladder samples and 3/5 tissue biopsies. B31: :*ospC*ΔC10 also elicited anti-*B. burgdorferi* IgG titers that were equivalent to that observed for mice infected with B31 and B31: :*ospC*wt. The ability of B31: :*ospC*ΔC10 to transit from ticks to mammals was also assessed using *I. scapularis* ticks infected by micro-injection. The infected ticks were fed on naïve mice and the ticks were collected after feeding to repletion. Spirochetes were readily detected in tissue biopsies harvested near the tick bite site for all mice except those exposed to the B31Δ*ospC* strain. In addition, all mice that were culture positive also seroconverted as assessed by ELISA and immunoblot.

Antibody responses to several OspC epitopes including the loop 5, alpha helix 5, and C10 motifs have been demonstrated to develop during early infection (Buckles *et al.*, 2006; Gilmore and Mbow, 1999). The loop 5 and alpha helix 5 epitopes are linear epitopes that elicit OspC type specific antibody responses that are protective (Buckles *et al.*, 2006; Earnhart *et al.*, 2007; Earnhart and Marconi, 2007a; Earnhart and Marconi, 2007b; Earnhart and Marconi, 2007c; Gilmore and Mbow, 1999). The C10 motif is also a linear epitope, and it has been reported to be immunodominant (Mathiesen *et al.*, 1998). The C10 motif may also contribute to the formation of a conformational epitope that includes the N-terminus of OspC (Gilmore and Mbow, 1999). To determine if deletion of C10 influences antigenicity, anti-OspC IgG responses were measured using rOspC and rOspCΔC10 as immobilized antigens. The proteins were screened with serum from mice infected by needle challenge. Statistically insignificant differences in titer to rOspC and rOspCΔC10 were observed suggesting that the C10 motif is not an immunodominant epitope.

As mentioned above, plasminogen is the only mammalian derived ligand for OspC identified to date. Recent studies suggest that OspC is the primary mediator of the plasminogen-*B. burgdorferi* interaction (Onder *et al.*, 2012). The contact points for plasminogen on OspC have yet to be identified. To determine if deletion of the C10 motif impacts plasminogen binding, recombinant full length and the C-terminally truncated OspC

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proteins were tested for plasminogen binding. A reproducible, albeit minor, decrease in plasminogen binding was observed with the truncated protein. However, this decrease proved not to influence infectivity as the B31: :*ospC*ΔC10 mutant remained infectious. This does not imply that OspC is not important in plasminogen binding. It is possible that decreased plasminogen binding by OspC is compensated for by one of several other proteins produced by *B. burgdorferi* that have plasminogen binding activity.

The genetic stability of the C10 motif suggest that there has been significant positivie selective pressure to maintain this OspC C-terminal sequence. Sequence conservation of a given motif often indicates an important structural or functional role. Based on this general paradigm we speculated that deletion of C10 would adversely affect the ability of OspC to carry out its critical, yet unknown, in vivo function(s). However as detailed above, deletion the C10 motif slightly attenuated plasminogen binging but had no discernable effect on the ability of *B. burgdorferi* to survive in ticks, infect mice and disseminate. While the data suggest that C10 is dispensable, it is possible that C10 may directly or indirectly mediate functions that are necessary for survival in other hosts or in other environmental conditions not assessed in this study. Additional mutational analyses of OspC will serve to further define its functional determinants. The identification of critical functional residues or domains of OspC may ultimately allow for the definitive determination of its potential ligands and precise biological functions.

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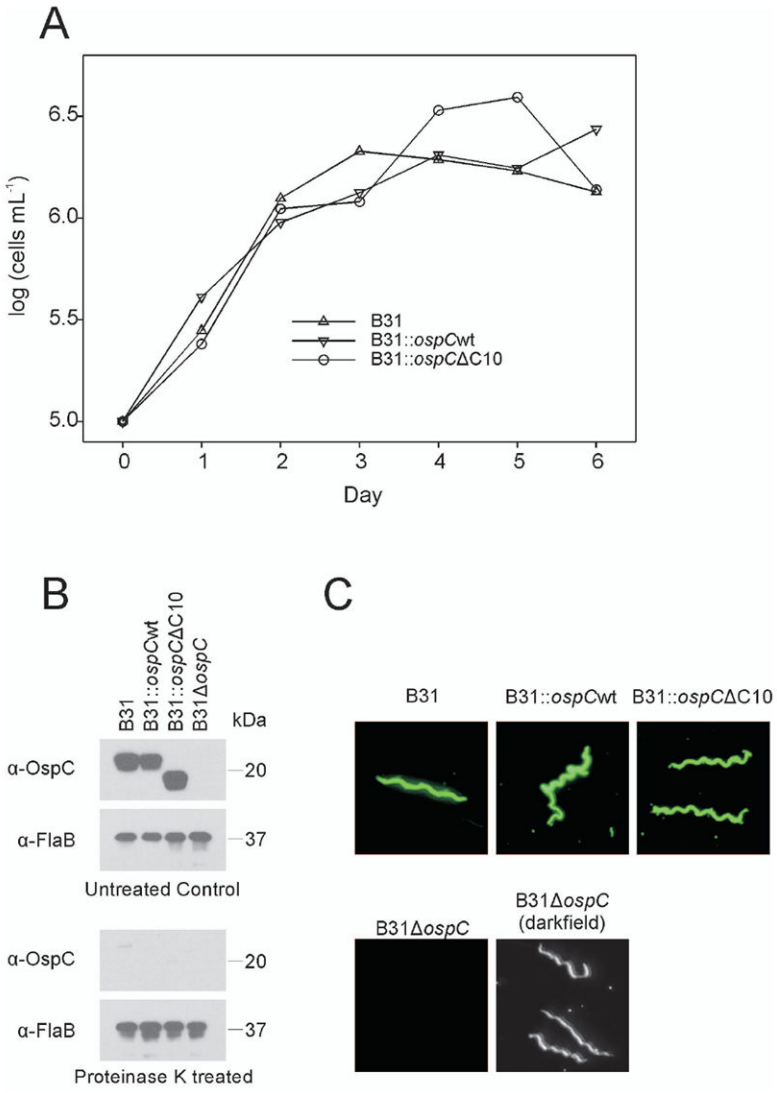

Figure 1.

Characterization of wild type and mutant *B. burgdorferi* strains. In panel A growth rates were determined by daily triplicate cell counts using dark field microscopy of cultures grown at 37°C in BSK-H medium (no antibiotics). In panel B the production and surface presentation of OspC by each strain was assessed by immunoblotting and proteinase K treatment, respectively. Proteinase K treated or untreated cells were fractionated by SDS-PAGE, immunoblotted and screened with polyclonal anti-OspC (phyletic type A) or anti-FlaB antiserum. Surface presentation was further demonstrated through indirect immunofluorescence assays using polyclonal anti-OspC antiserum (Panel D). All methods are detailed in text.

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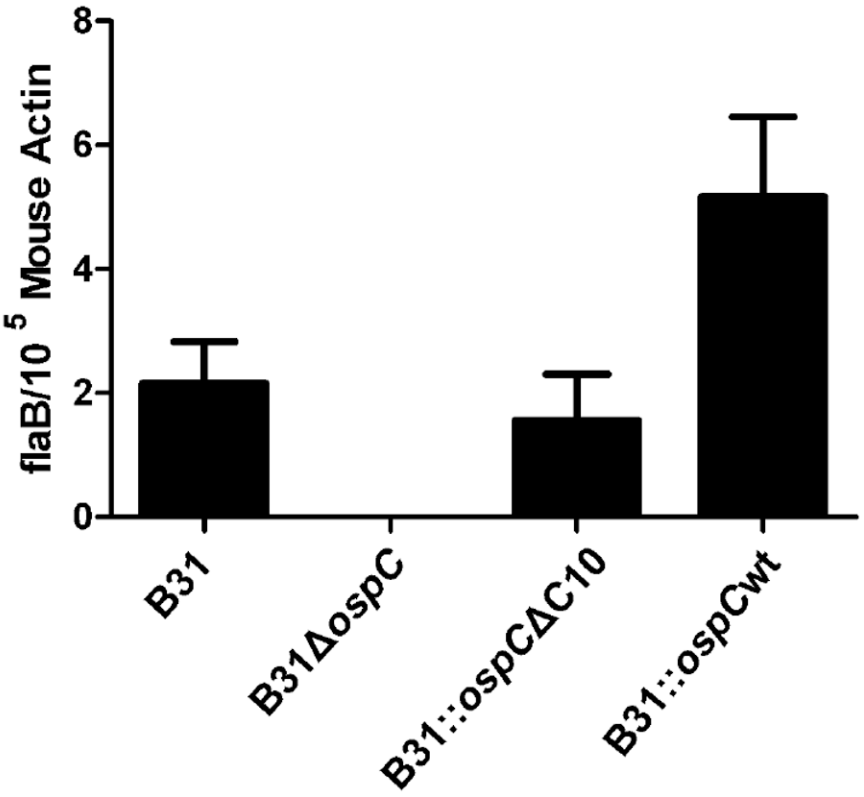

Figure 2.

Quantitative RT-PCR of spirochetes in mouse skin. The relative number of spirochetes in tissue biopsies was determined by qRT-PCR with primers specific for the *flaB* gene of *B. burgdorferi*. Normalization was achieved using primers that target the mouse actin gene. All methods are detailed in text.

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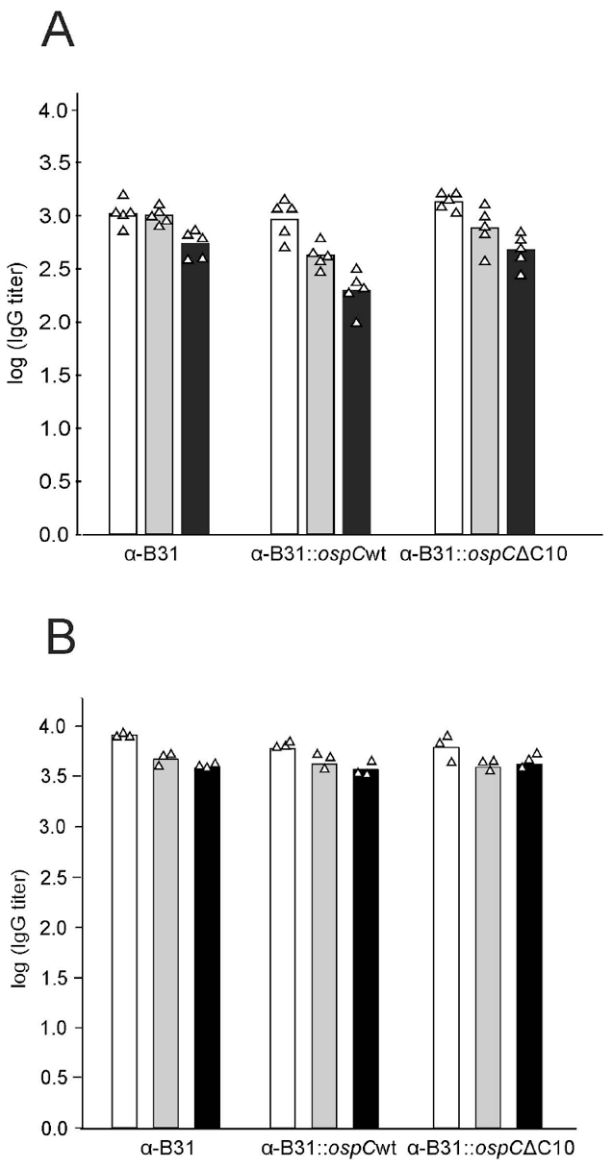

Figure 3.

Determination of IgG titer to *B. burgdorferi* and recombinant OspC proteins in mice infected with *B. burgdorferi* B31 and *ospC* mutant strains. Serum was harvested from mice infected by needle inoculation (panel A) or tick bite (panel B), serially diluted, and screened against immobilized whole *B. burgdorferi* B31 cells (white bar), rOspC (gray bar) and rOspCΔC10 (black bar). Individual mouse titers (indicated by triangles) were calculated as the log of the inverse of the dilution corresponding to 1/3 of the OD_{max} plateau on the sigmoidal curve. Bars denote the geometric mean titer. All methods are detailed in text.

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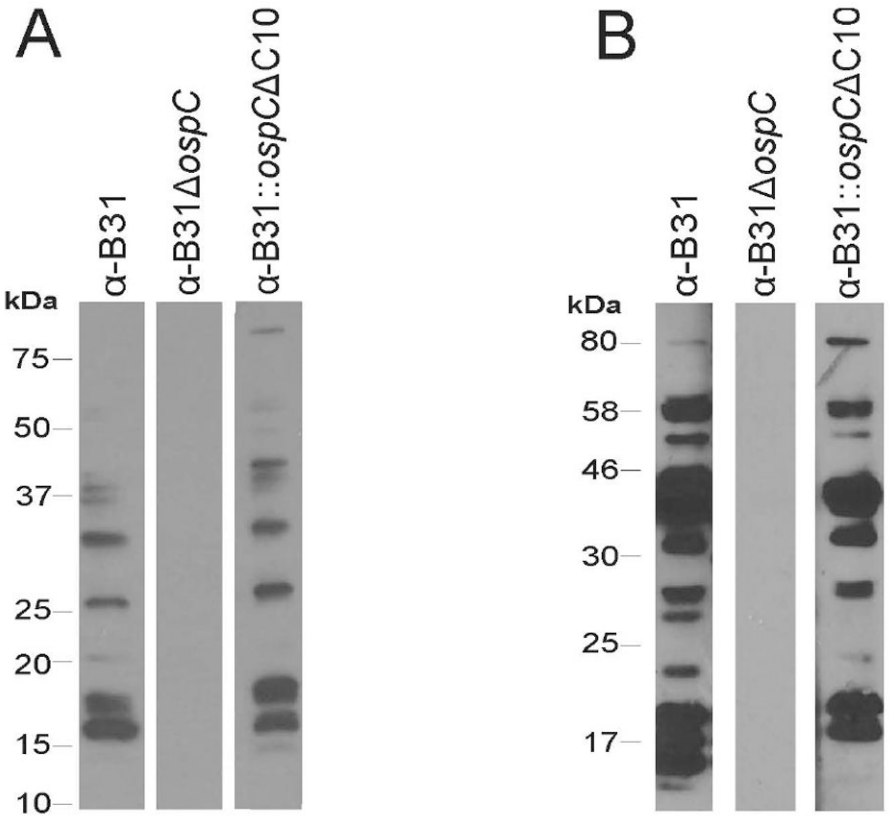

Figure 4.

The immunoreactive profiles of mice infected with control and mutant strains by tick bite and needle inoculation. In panel A, ticks were microinjected with each strain and then fed on naïve mice to repletion. Serum was collected after 3 weeks, diluted 1:2000, and used to screen immunoblot of *B. burgdorferi* B31 cell lysate. In panel B, antiserum from mice challenged with 10^4 spirochetes of each strain was diluted 1:2000 and used to screen immunoblot of *B. burgdorferi* B31 cell lysate. Representative data are shown. All methods are detailed in text.

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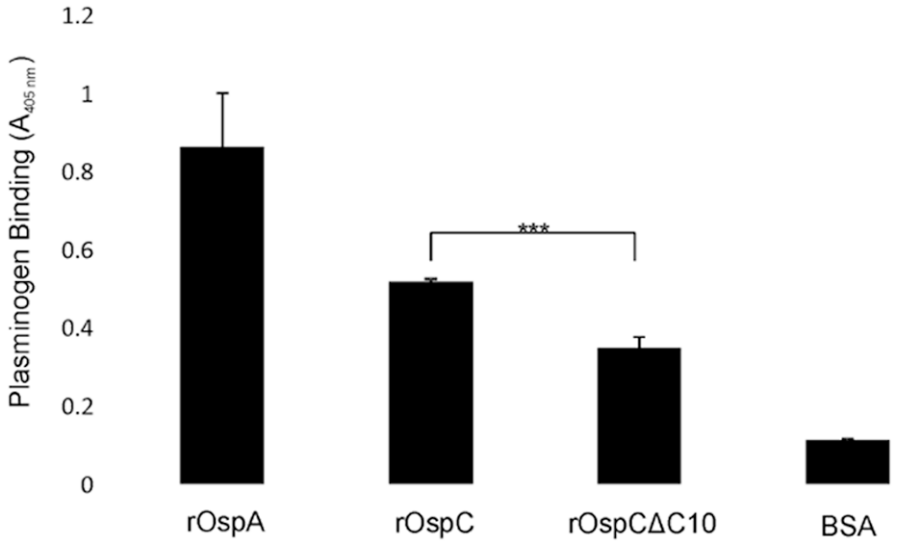

Figure 5.

Plasminogen binding to rOspC and rOspC)C10. rOspA (positive control), rOspC, rOspCΔC10, and BSA (negative control) were immobilized in ELISA plate wells and screened with 1μg mL^{-1} of human plasminogen. Binding was detected by goat anti-human plasminogen antibody and peroxidase-conjugated rabbit anti-goat IgG. Representative data is shown. All methods are detailed in text.

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Table 1

Strains used in this study

Strain	Description	Missing plasmids	Reference
B31 (5A4 clone)	Parental strain	None	(Kostick *et al.*, 2011)
B31::*ospC*wt	Transgenic control expressing wt OspC	lp21	(Earnhart *et al.*, 2010)
B31::*ospC*ΔC10	Transgenic expressing truncated OspC	lp21	(Earnhart *et al.*, 2010)
B31Δ*ospC*	Transgenic OspC knockout strain	None	This study

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Table 2

Summary of oligonucleotides used in this study

Primer	**Sequence (5' to 3')**
OspC 20 (+) LIC	GACGACGACAAGATTAATAATTCAGGGAAAGATGGG
OspC 210 (-) LIC	GAGGAGAAGCCCGGTTTAAGGTTTTTTTGGACTTTCTGC
OspC 200 (-) LIC	GAGGAGAAGCCCGGTTTAGCTTGTAAGCTCTTTAACTGAATT
Insert typeA20(+)BspEI	TCCGGAAAAGATGGGAATACATCTGCA
Insert type A200(-)BspEI	TCCGGATTAGCTTGTAAGCTCTTTAACTGAATTAG

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Table 3

Tissue culture results of mice inoculated with B31 and *ospC* mutant strains*

	Ear	Bladder
B31 (untransformed)	4/5	5/5
B31: :*ospC*wt	5/5	5/5
B31: :*ospC*ΔC10	3/5	5/5
B31Δ*ospC*	0/5	0/5

* ratios indicate the number of mice that were culture positive from a total of 5

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Table 4

Tissue culture results of mice infected by ticks microinjected with B31 and *ospC* mutant strains*

	Heart
B31 (untransformed)	3/3
B31::*ospC*wt	3/3
B31::*ospC*ΔC10	3/3
B31Δ*ospC*	0/3

* ratios indicate the number of mice that were culture positive from a total of 3