Lipoprotein succession in Borrelia burgdorferi: similar but distinct roles for OspC and VlsE at different stages of mammalian infection

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Summary

Borrelia burgdorferi alternates between ticks and mammals, requiring variable gene expression and protein production to adapt to these diverse niches. These adaptations include shifting among the major outer surface lipoproteins OspA, OspC, and VlsE at different stages of the infectious cycle. We hypothesize that these proteins carry out a basic but essential function, and that OspC and VlsE fulfill this requirement during early and persistent stages of mammalian infection respectively. Previous work by other investigators suggested that several B. burgdorferi lipoproteins, including OspA and VlsE, could substitute for OspC at the initial stage of mouse infection, when OspC is transiently but absolutely required. In this study, we assessed whether vlsE and ospA could restore infectivity to an ospC mutant, and found that neither gene product effectively compensated for the absence of OspC during early infection. In contrast, we determined that OspC production was required by B. burgdorferi throughout SCID mouse infection if the vlsE gene were absent. Together, these results indicate that OspC can substitute for VlsE when antigenic variation is unnecessary, but that these two abundant lipoproteins are optimized for their related but specific roles during early and persistent mammalian infection by B. burgdorferi.

Introduction

Borrelia burgdorferi cycles between mammalian and tick environments, each of which varies over time (see Radolf et al., 2012 for a recent review). When the spirochaetes are deposited in mammalian skin by a feeding tick, they must combat the host innate immune defences while replicating and spreading throughout the body of the mammal. As the mammal mounts an acquired immune response against B. burgdorferi, the spirochaete evades immune surveillance, at least in part, by changing components of its lipoprotein coat (Zhang et al., 1997; Crother et al., 2004; Liang et al., 2004). When a naïve tick feeds on an infected mammal, the spirochaetes are acquired by the tick and establish infection in the midgut. As the tick digests the blood meal, the nutritional environment changes for the bacteria.

Borrelia burgdorferi genes important for nutrient acquisition and host adaptation are distributed throughout the complex B. burgdorferi genome, which is composed of a linear chromosome and multiple linear and circular plasmids (Fraser et al., 1997; Casjens et al., 2000; 2012). In addition to essential chromosomal genes, many genes encoding products important for growth in the mouse-tick infectious cycle are located on plasmids (Purser et al., 2003; Byram et al., 2004; Jewett et al., 2007b; 2009). Plasmid genes of unknown function are also important for the natural spirochaete life cycle (Labandeira-Rey et al., 2003; Grimm et al., 2004; Bankhead and Chaconas, 2007; Jewett et al., 2007a), but some entire plasmids are dispensable for bacterial growth in culture (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001) and the loss of others may lead to small or negligible effects on infectivity in mice and ticks (Elias et al., 2002; Dulebohn et al., 2011).

Survival in the diverse mouse and tick environments requires adaptation by altering the gene expression and protein composition of the bacteria (Hodzic et al., 2002; 2003). Among the adaptations observed is the characteristic succession of outer surface lipoproteins (Schwan et al., 1995; Ohnishi et al., 2001; Liang et al., 2002b; Crother et al., 2004), whose genes are located on circular and linear plasmids (cp and lp respectively). Within infected ticks, the spirochaetes produce OspA (outer surface protein A) (Fingerle et al., 1995; Schwan et al., 1995), which contributes to maintaining tick colonization (Pal and Fikrig, 2003; Yang et al., 2004; Battisti et al., 2008) and is encoded on the 54 kb linear plasmid lp54. When nymphal ticks feed, the spirochaetes begin to synthesize OspC and reduce OspA synthesis (Schwan et al.,...
The ospC gene is located on the circular plasmid cp26 in the *B. burgdorferi* genome, and is required for the bacteria to initiate infection of a naive mammal (Grimm *et al.*, 2004; Stewart *et al.*, 2006; Tilly *et al.*, 2006). OspC, however, is immunogenic and targeted by mammalian antibodies (Wilske *et al.*, 1986; 1988), so bacteria that persist within an immunocompetent mammal must subsequently downregulate OspC (Liang *et al.*, 2002a; 2004; Crother *et al.*, 2004). Concomitant with the development of the host acquired immune response and OspC downregulation is increased synthesis of VlsE by *B. burgdorferi* (Hodzic *et al.*, 2003; Crother *et al.*, 2004). VlsE is a third lipoprotein, whose amino acid sequence does not resemble either OspC or OspA, but is abundantly present on the spirochaete surface during persistent infection (Crother *et al.*, 2003). The vlsE locus is found on the 3’ end of the linear plasmid lp28-1, and has the key characteristic of undergoing antigenic variation by an error-prone gene conversion-like mechanism that utilizes the 15 silent vlsE cassettes found upstream of the vlsE locus on lp28-1 (Zhang *et al.*, 1997; Zhang and Norris, 1998; Coute *et al.*, 2009). Because it is antigenically variable, VlsE protein can be present on the spirochaete surface during persistent infection of an immunocompetent host. Spirochaetes acquired by a larval tick feeding on a persistently infected small mammal reduce VlsE production in the tick midgut (Indest *et al.*, 2004; Bykowski *et al.*, 2006) and resume synthesis of OspA (Schwan *et al.*, 1995; Hodzic *et al.*, 2002).

Although this pattern of lipoprotein succession has been described, the functions of these outer surface proteins remain undefined. OspA plays a role in tick colonization by *B. burgdorferi* (Yang *et al.*, 2004), and studies suggest that it is a tick midgut adhesin (Pal *et al.*, 2000) and shields spirochaetes from mammalian antibodies in the incoming blood meal of feeding ticks (Battisti *et al.*, 2008). OspC has been suggested to have roles in host selectivity (Brisson and Dykhuizen, 2004), plasminogen binding (Lagal *et al.*, 2006; Onder *et al.*, 2012), invasion (Lagal *et al.*, 2006; Wormser *et al.*, 2008), dissemination (Seemanapalli *et al.*, 2010), salivary gland migration in the tick (Pal *et al.*, 2004), evasion of innate immunity (Xu *et al.*, 2008a), and recognition of the mammalian environment (Earnhart *et al.*, 2010). Conflicting data exist as well for each of these proposed roles, and no well-supported function for OspC has emerged. ospC mutants have the clear phenotype of being defective at the initial phase of mammalian infection following needle inoculation or tick bite (Tilly *et al.*, 2007; Dunham-Ems *et al.*, 2012). An extensive study involving complementation of an ospC mutation with the genes for lipoproteins VlsE, OspA, and DbpA suggested that OspC provides a somewhat nonspecific function, since substituting ospC with the other (unrelated) lipoprotein genes allowed spirochaetes to persist for several weeks after inoculation into SCID mice (Xu *et al.*, 2008a). Further studies in immunocompetent mice suggested that the functions of these lipoproteins, while overlapping, may be optimized to the times at which they are naturally expressed, as constitutive expression of OspA and VlsE reduced infectivity of *B. burgdorferi* in mice (Xu *et al.*, 2008b).

A simple model to explain these and other results is that these major *B. burgdorferi* outer surface lipoproteins serve a common function at different stages of the bacterial mouse-tick life cycle. In this model, that function is fulfilled by OspC during initial mammalian infection, by VlsE during persistent infection, and by OspA during the tick stage of the life cycle. The nature of that function may be protection against mammalian and tick innate immune defences. The present study describes experiments designed to further test the model that OspC and VlsE play similar roles at different stages of mammalian infection.

## Results

### Determining if OspC can substitute for VlsE

#### Mouse infection for assessing pC<sub>ospC</sub> retention

The first set of experiments was designed to test whether OspC production must be sustained during infection when VlsE production is not possible. We pursued this question with an experimental design that was based on our previous studies with spirochaetes that contain lp28-1 and, therefore, produce VlsE (Tilly *et al.*, 2006). In those studies, initial infection with an ospC mutant spirochaete required complementation with the ospC gene on a shuttle vector (which we call pC<sub>ospC</sub>, to denote that it carries the ospC gene transcribed from the ospC promoter). The complementing plasmid was lost during persistent infection, indicating that ospC expression was not required at later times. In immunodeficient SCID mice, which lack B and T cells, the ospC-containing shuttle vector was more stable, most likely because these mice do not produce antibodies against OspC that could lead to clearance of spirochaetes that retained the plasmid and continued to express ospC. Nevertheless, significant shuttle vector loss was detected, indicating that sustained OspC production was not necessary during persistent infection of SCID mice either. In the present study, we reasoned that if VlsE normally fulfills the role of OspC during persistent infection, spirochaetes lacking VlsE would be unable to tolerate the absence of OspC during persistent infection. A measure of this intolerance would be strong positive selection for shuttle vector maintenance during persistent infection with a *B. burgdorferi* mutant lacking both the endogenous vlsE and ospC genes, but complemented with a shuttle vector carrying
the ospC gene. Consequently, we screened for retention of the ospC-containing shuttle vector at a time at which we had previously found significant loss of the same shuttle vector by ospC mutant spirochaetes that retained lp28-1 and, therefore, vlsE. We predicted that spirochaetes lacking VlsE would retain the ospC-containing shuttle vector, in order to continue producing OspC in the absence of VlsE. Consistent with this hypothesis, a previous study with lp28-1-deficient spirochaetes found that these spirochaetes maintained OspC production during persistent infection of an immunodeficient host (Embers et al., 2008).

Wild-type (WT, C3H) and C3H-SCID mice were inoculated with 104 spirochaetes per mouse of a strain lacking vlsE. We predicted that spirochaetes lacking VlsE would retain the ospC-containing shuttle vector, in order to continue producing OspC in the absence of VlsE. Consistent with this hypothesis, a previous study with lp28-1-deficient spirochaetes found that these spirochaetes maintained OspC production during persistent infection of an immunodeficient host (Embers et al., 2008).

Wild-type (WT, C3H) and C3H-SCID mice were inoculated with 104 spirochaetes per mouse of a strain lacking lp28-1 (vlsE), a double mutant lacking both vlsE and ospC (vlsE ospC), and the same two strains containing the ospC shuttle vector pCp ospC (Table 1A). Six weeks post-inoculation, the mice were euthanized and spirochaete isolation from tissues was attempted. As expected, all strains were highly attenuated for persistent infection in WT mice (Table 2). Isolates were obtained from ankle joints from only two WT mice infected with vlsE ospC and one WT mouse infected with vlsE ospC ospC (Table 2). However, all 10 WT mice inoculated with spirochaetes containing pCp ospC were seropositive (Table 2), indicating that they had been at least transiently infected. These data are consistent with previous studies showing that lp28-1 (carrying vlsE) is required for persistent infection of WT mice.

In contrast, spirochaetes lacking vlsE but containing ospC either at its normal location or on a shuttle vector (vlsE ospC, vlsE ospC ospC, and vlsE ospC ospC) persisted in all tissues in all SCID mice inoculated, whereas those lacking both ospC and vlsE (vlsE ospC) were not isolated from any SCID mouse tissues (Table 2). These findings were also consistent with previous studies, which demonstrated that lp28-1 was dispensable for persistent infection by B. burgdorferi in SCID mice (Lawrenz et al., 2004; Bankhead and Chaconas, 2007). Surprisingly, in this experiment, spirochaetes lacking just ospC (ospC) were isolated from most SCID mice in which they were inoculated. This result differed from previous work and other experiments in this study, in which ospC was found to be an essential gene for normal infection of both immunocompetent and immunodeficient mice (Grimm et al., 2004).

Retention of pCp ospC in spirochaetes lacking vlsE during mouse infection. If sustained OspC production is required when VlsE production is not possible, spiro-

### Table 1A. *Borrelia burgdorferi* strains used in this study.

| Strain | Description | Reference |
|--------|-------------|-----------|
| WT (B31-A3) | Wild type, infectious B31 clone lacking cp9 | Elias et al. (2002) |
| ΔospC (ospC1) | B31-A3 derivative with ospC deletion, kanamycin resistance cassette inserted in place of ospC locus (kan') | Elias et al. (2002) |
| ΔB31-A3 | Non-infectious B31 clone lacking lp5, lp25, lp28-1, lp28-4, lp56, cp-9, and cp32-6 | Jewett (2007a) |
| B31-A3 | B31-A3 derivative with the vlsE gene coding sequence replacing the ospC ORF at the ospC locus on cp26 (kan') | This study |
| vlsE (B31-A1) | Low passage B31 clone lacking lp28-1 (and vlsE) | Jewett (2007a) |
| vlsE ΔospC | B31-A1 derivative with ospC deletion, kanamycin resistance cassette inserted in place of ospC locus (kan') | Jewett (2007a) |

Table 1B. Shuttle vector constructs used in this study.

| Plasmid | Bb promoter | Gene expressed | Reference |
|---------|-------------|----------------|-----------|
| pCp vlsE | ospC | vlsE | This study |
| pCp vlsE2 | ospC | ospC signal sequence-vlsE fusion | This study |
| pF vlsE | flaB | vlsE | This study |
| pF ospA | flaB | ospA | This study |
| pCp ospA | ospC | ospC | Tilly et al. (2006) |
| pCp ospC (pBSV2GospC) | ospC | ospC | Frank et al. (2003) |
| pKFSS1 | N/A | N/A | N/A |

a. pBSV2G, conferring gentamicin resistance, is the shuttle vector backbone used for all constructs, except for pKFSS1, which confers streptomycin resistance.

b. N/A, not applicable.
Infectivity of the presence of pCp single colonies and colonies were screened by PCR for 

Xu typically produced during mammalian infection, studies by 

Although OspA is neither required for nor expression.

outer-surface lipoproteins, including VlsE and OspA (Xu et al., 2008a,b). To confirm these results, we constructed several plasmids to determine if VlsE or another unrelated major outer-surface protein, OspA, could complement ospC mutant spirochaetes when establishing mammalian infection. Shuttle vectors pCpVlsE1 (ospC promoter-vlsE1) and pCpOspA (ospC promoter-ospA; Table 1B) encode vlsE and ospA expression from the ospC promoter. Shuttle vectors pFvlsE1 (flaB promoter-vlsE1) and pFvlsE1 (flaB promoter-ospA) encode constitutive vlsE and ospA expression from the flaB promoter. Finally, shuttle vector pCplspE2 (ospC promoter-vlsE2) has the ospC promoter and signal sequence fused to the VlsE coding sequence (lacking its native signal sequence), to test if lipoprotein processing and membrane localization affects how well VlsE can fulfil the role of OspC (Table 1B). These shuttle vectors were introduced into ospC mutant B. burgdorferi (ospC) to determine if OspA or VlsE, appropriately regulated or constitutively expressed, could fulfil the requirement for OspC in establishing mammalian infection. The shuttle vectors were also introduced into WT B. burgdorferi to investigate if inappropriate expression of these lipoproteins in the presence of native ospC expression would be deleterious for the spirochaetes. Finally, we verified that expression of the CvlsE and CospA genes on the shuttle vector paralleled that of the endogenous ospC gene by introducing these constructs into the high-passage strain B313 (Table 1A) (Sadziene et al., 1993), which lacks native copies of the ospA and vlsE genes and constitutively produces OspC (Fig 2). We also confirmed the expression

| Table 2. Infectivity of B. burgdorferi with various combinations of WT and mutant ospC and vlsE genes. |
|-----------------------------------------------|
| B. burgdorferi strain | No. of persistently infected mice/no. of mice injected* |
|-----------------------|------------------------------------------------------|
| vlsE                  | 0/5 (5/5) 10/10                                       |
| vlsE/pCp ospC         | 2/5 (5/5) 5/5                                        |
| ΔospC                 | ND 4/5                                               |
| vlsEΔospC             | 0/5 (0/5) 0/10                                       |
| vlsEΔospCΔCp ospC     | 1/5 (5/5) 10/10                                       |

a. Both immunocompetent (WT, C3H) and immunodeficient (C3H-SCID) mice were injected with 10^4 spirochaetes of the indicated B. burgdorferi strain. Six weeks post-inoculation, WT mice were retro-orbitally bled and all mice were euthanized to assess infection by isolation of spirochaetes from ear, bladder, and ankle joints. Seroconversion of WT mice toward B. burgdorferi proteins is shown in parentheses, and indicates at least transient infection.

b. Isolation of these strains from WT mice was only successful from ankle joints.

c. ND, not determined, because the infection was not attempted in this experiment.

chaetes lacking both vlsE and ospC but containing the ospC-carrying shuttle vector pCp ospC should retain that shuttle vector, even during infection of SCID mice. To ascertain if this were true, isolates obtained from mice infected with bacteria containing pCp ospC were plated for single colonies and colonies were screened by PCR for the presence of pCp ospC. In SCID mice, we obtained isolates from all tissues of all mice infected with vlsE-deficient or vlsE- and ospC-deficient spirochaetes containing pCp ospC, and compared shuttle vector retention in the presence and absence of the native ospC gene in bacteria lacking vlsE. We found significant loss of pCp ospC in SCID mice infected with spirochaetes lacking VlsE alone, but complete retention of the shuttle vector when the vlsE spirochaetes also lacked the endogenous ospC gene (Fig 1) (P < 0.0001, as assessed by a two-tailed Mann–Whitney test or one-way ANOVA). This result supports our hypothesis that continued OspC production would be required by bacteria lacking VlsE, if these two outer surface proteins fulfil the same essential function during infection of the mammalian host.

**Determining if VlsE or another outer-surface protein, OspA, can substitute for OspC**

**Generating B. burgdorferi with increased vlsE and ospA expression.** Although OspA is neither required for nor typically produced during mammalian infection, studies by Xu et al. suggested that an ospC mutant could be partially complemented for mouse infectivity by several major outer-surface lipoproteins, including VlsE and OspA (Xu et al., 2008a,b). To confirm these results, we constructed several plasmids to determine if VlsE or another unrelated

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Fig. 1. Shuttle vector retention in isolates from SCID mice. Percent retention of pCp ospC by vlsE vs vlsEΔospC isolates from SCID mouse tissues 6 weeks post-inoculation. Each symbol denotes percent shuttle vector retention among 22–24 colonies screened per mouse isolate. All isolates of vlsEΔospC retained pCp ospC in all colonies screened. The differences between the shuttle vector retention results for isolates of single vlsE mutant vs double vlsE and ospC mutant spirochaetes (vlsE/pCp ospC vs vlsEΔospC/pCp ospC) pooled from all tissues are statistically significant (P < 0.0001, as determined by the Mann–Whitney test). For one vlsEΔospC/pCp ospC ear isolate, only 6 colonies were screened, all of which were shuttle vector-positive.
OspA and VlsE do not fulfill the role of OspC in establishing mammalian infection in immunocompetent or immuno-deficient SCID mice. Since the above results suggest that OspC is able to fulfill the role of VlsE (in the absence of an acquired immune response), we tested whether the converse were true, and VlsE could fulfill the role of OspC in establishing mammalian infection. We first used shuttle vectors carrying the VlsE or OspA open reading frames (ORFs) under the regulation of the ospC promoter. Groups of WT and C3H-SCID mice were intradermally injected with an inoculum of 10^4 spirochaetes of WT or ΔospC strains harbouring these shuttle vectors (Tables 1A and B). Immunocompetent mice were bled and all were euthanized three weeks later, and tissues were cultured for isolation of spirochaetes (Table 3). All WT mice that were positive by serology were also positive for B. burgdorferi isolation from all harvested tissues. Neither VlsE nor OspA was able to complement the ΔospC mutant (Table 3). Overproduction of VlsE and OspA was inadequate for even transient infection, since uninfected animals were also seronegative. Furthermore, we found that inappropriate expression of vlsE (i.e. from an additional copy on the shuttle vector and under control of the ospC promoter) attenuated WT B. burgdorferi at a dose of 10^4 in both WT and SCID mice (Table 3). This finding was similar to that of Xu et al., in which increased expression of vlsE led to clearance of the spirochaetes in immunocompetent mice (Xu et al., 2008b). To confirm that the reduced infectivity of spirochaetes carrying the ospC promoter-vlsE fusion on a shuttle vector was caused by inappropriate vlsE expression, we displaced the pCp-VlsE1 shuttle vector with the incompatible plasmid pKF131 (Frank et al., 2003). The resultant WT/pKF131 strain exhibited WT infectivity (Table 3), indicating that the attenuated infectivity of WT/pCp-vlsE1 was due to the ospC promoter-driven expression of vlsE from the shuttle vector.

Although neither VlsE nor OspA was capable of fulfilling the role of OspC when under control of the ospC promoter, the previous study of Xu et al. found that constitutive expression of these proteins in an ospC mutant background could restore infectivity to varying degrees in both WT and SCID mice (Xu et al., 2008a). Since our previous experiments utilized constructs with expression from the ospC promoter, we attempted to replicate the Xu et al. studies by introducing shuttle vector constructs with vlsE and ospA under control of the constitutive flaB promoter into both WT and ΔospC B. burgdorferi (Table 4). VlsE production did not complement the ΔospC mutant in either WT or SCID mice (Table 4), and uninfected animals were seronegative also, indicating that they had not been even transiently infected. OspA production was not able to complement the ΔospC mutant in WT mice (0/5 mice infected and seronegative), as was observed by Xu et al. (2008a,b), but we did find a significant difference between the ΔospC and the ΔospC/pFlΔospA strains in SCID mice (3/10 vs 5/5 mice infected respectively) (Table 4). However, in a separate experiment (Table 2), surprisingly, we found infection with the ΔospC strain in 4 out of 5 SCID mice at a dose of 10^4, so infection by ΔospC/pFlΔospA may not be a consequence of OspA production. Constitutive expression of vlsE did not attenuate WT B. burgdorferi, as we had seen with ospC promoter-driven expression of vlsE (Table 3). This may be the result of a lower expression level of VlsE from the pFlΔospA construct, since the ospC promoter is highly induced during early infection as compared with the flaB promoter (Tokarz et al., 2004).

Assaying the effect of replacing the endogenous ospC gene on cp26 with the vlsE gene expressed from the ospC promoter. In the experiments described above, complementation of an ospC mutant was attempted with genes located on shuttle vectors, which generally have slightly higher copy number than the endogenous genomic plasmids of B. burgdorferi (Tilly et al., 2006).
address the possibility that the lack of complementation (or attenuation of infection by WT *B. burgdorferi*) was a result of excess protein production due to higher copy number, we constructed a strain in which the ospC gene located on cp26 was replaced with the *vlsE* coding sequence, with the fusion between the ospC promoter at

The number of SCID mice infected by the ΔospC::pFp::ospA strain was significantly different from that of ΔospC (P = 0.026, as determined by Fisher’s two-tailed exact probability test).

### Table 4. Infectivity of *B. burgdorferi* expressing *vlsE* and *ospA* from the *flaB* promoter.

| *B. burgdorferi* strain | No. of infected mice/ no. of mice injected* |
|------------------------|-------------------------------------------|
| WT                     | 9/10                                      |
| WT/pF::vlsE            | 4/5                                       |
| WT/pF::ospA            | 2/5                                       |
| ΔospC                  | 0/10                                      |
| ΔospC::pF::vlsE        | 0/5                                       |
| ΔospC::pF::ospA        | 0/5                                       |
| a. Both immunocompetent WT mice and immunodeficient C3H-SCID mice were injected intradermally with 10⁵ of the designated *B. burgdorferi* strain. At three weeks post-injection, WT mice were retro-orbitally bled to test for infection by immunoblot for *B. burgdorferi* proteins, and all mice were euthanized for attempted isolation of spirochaetes from the ear, bladder, and tibiotarsal joint. All WT mice positive by serology were also positive by isolation from all three tissues. b. The number of SCID mice infected by the ΔospC::pF::ospA strain was significantly different from that of ΔospC (P = 0.026, as determined by Fisher’s two-tailed exact probability test). 

### Table 5. Infectivity of *B. burgdorferi* with the *vlsE* ORF replacing the *ospC* ORF on cp26.

| *B. burgdorferi* strain | No. of persistently infected mice/no. of mice injected* |
|------------------------|--------------------------------------------------------|
| WT                     | 5/5                                                    |
| ΔospC                  | 1/5                                                    |
| ΔospC::Cp::vlsE        | 0/5                                                    |
| a. Both immunocompetent (IRW) and immunodeficient C3H-SCID mice were injected with ~10⁵ organisms of the indicated *B. burgdorferi* strain. At three weeks post-inoculation, WT mice were retro-orbitally bled and tested for infection by immunoblot for seroconversion to *B. burgdorferi* proteins. All mice were euthanized and isolation of spirochaetes attempted from the ear, bladder, and ankle joint. 

### Discussion

Although OspC has been shown to fulfill a critical role early in mammalian infection by *B. burgdorferi*, its function has remained undefined. This paper presents data addressing the model in which the function that OspC provides initially is required throughout infection by *B. burgdorferi*, and *VlsE* subsequently fulfills that function during persistent infection. We provide strong evidence that OspC production can compensate for *VlsE* deficiency in an immunodeficient host. We demonstrate that the *ospC*
gene must be retained, and presumably expressed, when
vlsE is missing. These data imply that OspC can carry out
the function of VlsE during persistent infection, provided it
is not targeted by acquired immunity. However, although
OspC is able to substitute for VlsE in an immunodeficient
host, we found that VlsE could not substitute for OspC in
any host background. Therefore, our data indicate that the
two proteins are not strictly interchangeable, so the sim-
plest model of redundant function with reciprocal expres-
sion is not correct.

Although this study does not support the idea that
VlsE can also substitute for OspC, some data do suggest that other
B. burgdorferi proteins can take the place of OspC during initiation of mammalian infection.
One of the strongest pieces of evidence is apparently
normal infection of mice by an ospC deletion mutant
when tissue from a persistently infected animal was
transferred to a naive animal (Tilly et al., 2006). We pre-
viously speculated that VlsE, which is made by the host-
adapted spirochaetes, allows infection of the naive
animal in this scenario. Xu et al. (2008a) partially
restored infectivity of an ospC mutant by complementa-
tion with VlsE, and also suggested that the proteins
have similar functions. However, we were not able to
duplicate their findings (A. Bestor, unpubl. results), even
when using the same vlsE allele as Xu et al.

We also found that ospA was unable to fully comple-
ment an ospC mutation. Although we found apparent
complementation of the ospC mutation by ospA driven by
the flaB promoter in SCID mice, the ability of ΔospC
spirochaetes to infect SCID mice on occasion (see, e.g.
Table 2 and below) means that this result may have been
spurious. Clearly, there are differences between our
system and that of Xu et al., who did find complementa-
tion of an ospC mutation by overexpression of the vlsE
and ospA genes (Xu et al., 2008a). For example, the
typical ID50 of in vitro-grown WT B. burgdorferi is almost
100-fold higher in our experiments than in theirs. Also, the
ospC mutant used by Xu et al. (2008a) is an insertion,
rather than a deletion of the entire coding sequence, and
it lacks lp25 but carries the essential gene pncA on the
ospC-complementing plasmid, which requires that the
shuttle vector be retained during mammalian infection.
Nevertheless, in our experiments, VlsE and OspA did not
fulfil the role of OspC for initiation of infection.

It remains unclear why OspC can substitute for VlsE but
not vice versa. OspC may perform a unique role early in
infection, in addition to a common function that VlsE typi-
cally assumes as the spirochaetes persist in a mammal.
Without invoking an additional unknown role, VlsE could
be unable to take the place of OspC because the lipopro-
tein requirement during initial infection by B. burgdorferi is
more stringent, since few bacteria are present and they
are all located in the tick bite (or needle inoculation) site.

Because it appears that OspC and VlsE are not fully
interchangeable, we propose that these two proteins
require both appropriate context and appropriate timing to
be fully functional. By context, we encompass the roles of
other proteins produced at the same time and also vari-
ations in membrane composition and arrangement, which
are influenced by lipid availability and temperature.
Although the OspC and VlsE sequences are not related,
the tertiary structures of the OspC dimer and VlsE
monomer are similar in size and shape, with variable
regions forming surface-exposed loops (Eicken et al.,
2001; 2002; Kumaran et al., 2001), which may allow them
to carry out a common function. As to the question of what
their common function could be, OspC and VlsE might
serve to protect the bacterium from the particular host
environment in which it finds itself, or they might stabilize
the bacterial structure, again in the particular host context.

If OspC and VlsE protect B. burgdorferi against host
defences, which defences those might be remains unde-
fined. We have looked without success for differences
between ospC mutant and WT spirochaetes with respect
to phagocytosis by mouse and human neutrophils, phago-
ctosis by mouse macrophages, susceptibility to human
or mouse complement, or susceptibility to mouse natural
killer cells (K. Tilly, A. Porter, F. DeLeo, C. Checroun, and
P. Rosa, unpubl. data). Although these studies were not
exhaustive, they emphasized the complexity of the inter-
action between host and bacterium and the inherent limi-
tations of investigating that interaction either with isolated
components or in vivo. If OspC and VlsE stabilize the
bacteria in the host environment, large amounts of a par-
ticular lipoprotein may be an essential component of the
recently described B. burgdorferi lipid rafts (LaRocca
et al., 2010). Given that the B. burgdorferi membrane
structure and composition vary depending on the host
environment, it would not be surprising if an essential
lipoprotein component would also need to vary.

Although we did not succeed in heterologous comple-
mentation of the ospC deletion mutation, we did occa-
sionally observe infection of SCID mice by ΔospC spi-
rochaetes. Furthermore, in recent experiments we have
sometimes obtained infection in both WT and SCID mice
with ΔospC spirochaetes when inoculating at a dose ≥ 10^7
spirochaetes, confirmed by isolation of the mutant from all
cultured tissues (A. Bestor, K. Tilly and P. Rosa, unpubl.
results). This is in contrast to the complete lack of infectivity
by ospC mutant spirochaetes that we found in earlier
studies (Grimm et al., 2004; Stewart et al., 2006; Tilly et al.,
2006). ospC mutant spirochaetes grown directly from
stock frozen since the strain was originally isolated exhibit
a similar phenotype (K. Tilly, unpubl. results), so we do not
think that the ΔospC mutant used in this study has under-
gone a compensatory mutation. Since the earlier tissue
transfer studies suggest that a B. burgdorferi protein pro-
duced during persistent infection, when appropriately regulated and in the correct context, can successfully fulfil the ospC requirement (Tilly et al., 2009), our culture conditions may have shifted subtly to increase production of that product and its appropriate context by ΔospC spirochaetes. We have tested several batches of medium, and growth to different densities, but have not pinpointed a tangible variable that affects infectivity of the ospC mutant. Despite occasional infection by the ospC mutant at high dose, we have been unable to demonstrate consistently increased infectivity at a standard inoculum when the mutant was complemented with either vlsE or ospA.

This and other studies are delineating a succession of lipoproteins that coat B. burgdorferi and provide essential functions in the mouse and tick hosts at various stages of the bacterial life cycle. OspC is typically on the bacterial surface when spirochaetes initiate mammalian infection, and then subsequently downregulated. VlsE is produced and undergoes antigenic variation during persistent infection. OspA is required during bacterial colonization of ticks. Each has unique expression and protein characteristics, yet their roles may be related. The bacteria are exposed to different degrees of immune surveillance throughout the infectious cycle, from dermal inoculation of the mammal, to blood stream dissemination and peripheral tissue colonization, and acquisition with the blood of a seropositive host by the feeding tick. The spirochaetes also will have different protein and lipid composition at these stages, which could affect bacterial stability and survival in the host environment. The changing spirochaete surface, of which the major lipoproteins are essential components, could be considered to be analogous to the more complex developmental changes found in vector-borne parasites during their mammalian and arthropod phases. Further defining the shared or specific roles of major outer surface lipoproteins will help elucidate the details of the B. burgdorferi infection process and, in so doing, reveal potentially widespread adaptations to the host and vector environments.

**Experimental procedures**

**Bacterial strains and culture conditions**

*Borrelia burgdorferi* strains were derived from clones B31-A3 or B31-A1 (Elias et al., 2002), which we refer to as WT and vlsE respectively. Both B31-A3 and B31-A1 are clones derived from non-clonal B31 MI (Fraser et al., 1997), but B31-A3 lacks cp9, whereas B31-A1 lacks both cp9 and lp28-1 (see Table 1A for strain descriptions). Strain ospCK1 (referred to as ΔospC) (Tilly et al., 2006) is a derivative of WT B31-A3 in which the ospC gene is deleted and replaced with a flgBp-kan fusion. *B. burgdorferi* liquid cultures were grown in BSKII medium at 35°C. Electroporation was performed as described (Samuels, 1995), using 5–10 μg DNA. Selection for transformants was in solid BSK medium containing 200 μg ml⁻¹ kanamycin, 50 μg ml⁻¹ streptomycin, or 40 μg ml⁻¹ gentamicin. DNA manipulations in *Escherichia coli* were performed with TOP10 cells (Invitrogen, Carlsbad, CA) or NEB5α (New England Biolabs, Ipswich, MA).

To make strains for testing whether OspC could fulfil the role of VlsE, the ospC gene in vlsE, which lacks lp28-1 and the encoded vlsE locus (Table 1A), was inactivated by allelic exchange using plasmid pJK109 (Tilly et al., 2006). This plasmid includes ospC flanking sequences surrounding flgBp-kan, which replaces the ospC gene. We chose a transformant (vlsE-ΔospC) that had the same plasmid content as vlsE, but lacked the ospC ORF. vlsE and vlsE-ΔospC were transformed by electroporating with pBSV2GospC (referred to as pCv_ospC), a shuttle vector carrying the ospC gene and its own promoter (Table 1B) that had been methylated (Rego et al., 2011), and selecting for gentamicin-resistance. Transformant clones with the same plasmid content as vlsE and containing pCv_ospC were used for subsequent experiments.

**Construction of shuttle vectors carrying lipoprotein genes fused to various promoters**

The pCv_vlsE1 shuttle vector, expressing vlsE under control of the ospC promoter, was constructed as follows. The ospC promoter was amplified from ospC7 (Grimm et al., 2004) genomic (g) DNA with Vent polymerase (New England Biolabs) using primers 1 and 2 (Table 6) and ligated into the KpnI and XbaI sites in the multiple cloning site (MCS) of pBSV2G (Elias et al., 2003), yielding pBSV2G-ospC_VlsE. The region of lp28-1 encompassing the ospC ORF was amplified with Tag polymerase, using primers 3 and 4 (Table 6), from WT gDNA that had been treated with Mung Bean nuclease (New England Biolabs) to nick hairpin ends, and ligated into BspHI-digested pBSV2G-ospC, yielding pBSV2G-ospC-VlsE (pCv_vlsE1). The pCv_vlsE2 shuttle vector, expressing and transporting VlsE under the control of the ospC promoter and carrying the ospC signal sequence, was constructed as follows. The region of cp26 from nucleotides 16647–16971, encompassing the ospC promoter and ospC signal sequence, was amplified from WT gDNA with Vent polymerase using primers 1 and 5 (Table 6), and ligated into the KpnI and XbaI sites found in the MCS of pBSV2G, yielding pBSV2G-ospC-ss. The vlsE locus was amplified without the predicted signal sequence from pCv_vlsE1 with Vent polymerase using primers 6 and 7 (Table 6), and ligated into XbaI/HindIII-digested pBSV2G-ospC-ss, yielding pBSV2G-ospC-ss-vlsE (pCv_vlsE2).

The pFv_vlsE1 shuttle vector, constitutively expressing VlsE from the flaB promoter, was constructed as follows. The flaB promoter was amplified from WT gDNA with Vent polymerase using primers 8 and 9 (Table 6), and ligated into the XbaI site in the MCS of pBSV2G, yielding pBSV2G-flaBp. The region of lp28-1 encompassing the flaB locus was amplified from pFv_vlsE1 with Vent polymerase using primers 3 and 10 (Table 6), and ligated into BspHI/SphI-digested pBSV2G-ospC-ss, yielding pBSV2G-ospC-ss-vlsE (pFv_vlsE1).

The pCv_ospA shuttle vector, expressing ospA under control of the ospC promoter, was constructed as follows. The region of lp54 from nucleotides 9457 to 10278, encompassing the ospA ORF, was amplified from WT gDNA with Vent polymer-
ase using primers 11 and 12 (Table 6), and ligated into BspHI/Sphl-digested pBSV2G-ospCp, yielding pBSV2G-ospCp-ospA (pCp ospA).

The pFp ospA shuttle vector, constitutively expressing ospA from the flaB promoter, was constructed as follows. The region of lp54 from nucleotides 9457 to 10278, encompassing the ospA locus, was amplified from WT gDNA with Vent polymerase using primers 11 and 12 (Table 6), and ligated into BspHI/Sphl-digested pBSV2G-flaBp, yielding pBSV2G-flaBp-ospA (pFp ospA).

All constructs were sequenced upon completion to confirm they were as designed and free of any mutations before transforming them into the WT and the ΔospC strains (see Table 1), and selecting with gentamicin. *B. burgdorferi* plasmid contents of the transformants were confirmed to be the same as the parental strains by PCR (Akins et al., 1998).

**Table 6.** Primers used in this study.

| Primer | Designation | Sequence a |
|--------|-------------|------------|
| 1      | ospCp 5’ Kpnl | GGTACC CGGGGTACCAAGTTTGGATTTGCAGATTC |
| 2      | ospCp 3’ BspHI | GGCGCGTCATAGAATTGGTCCTCATTTTAC |
| 3      | vlsE 5’ BspHI | CGCGCGTCATAGA AAAATTTCAGAHTGC |
| 4      | vlsE 3’ Xbal | GCTCTAGATCTCGACTATTTTCTCTACT |
| 5      | ospCp (ss) 3’ Xbal | TCTAGACCCCTGAACTTTACGAACGAGAA |
| 6      | vlsE HindIII | AAGCCTTACCTATTCAGACGAGAG |
| 7      | vlsE (– ss) 5’ Xbal | TCTAGAAAGCCCAAGTTTCGATGAA |
| 8      | flaBp 5’ Xbal | TCTAGATATTGTCGACACTCAGGG |
| 9      | flaBp 3’ Xbal/BspHI | TCTAGAGTTTACCTCCATGATAAA |
| 10     | vlsE 3’ Sphl | GCACTTGTTTCTTTTTACTAGCAGG |
| 11     | ospA 5’ BspHI | TCATGAAAAAAATATTGGAATAGTCTAAATTAGGCT |
| 12     | ospA 3’ Sphl | GGGCCATGCTATTATTTAAAAAGCTTTTTATTTTCATCAAGTTTTGAATT |
| 13     | vlsE NotI | GCGGCCGGCTCACTATTCAGGAGGAGGTGTTTTTT |
| 14     | flgS-Avrfl | CCTAGGGTTAATACCCGAGCTCAGAGAG |
| 15     | aacC13-Nhel | GCTAGCCGATTCGCTCGTGAACG |

a. Restriction enzyme recognition sequences are indicated in bold type.

have lost lp25. Accordingly, the clone was transformed with 10 μg of total genomic DNA from A3 lp25-Sm, which has a flgB-aadA fusion (Frank et al., 2003) inserted into lp25 at the site used in A3 lp25-Gm (Grimm et al., 2005). A strepR colony in which lp25 had been restored was picked and used for subsequent experiments.

**Mouse infections**

All animal experiments were performed using protocols approved by the Animal Care and Use Committee of the Rocky Mountain Laboratories and according to the guidelines of the National Institutes of Health. Rocky Mountain Laboratories is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). C3H/HeN and C3H/HeN SCID mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). RML mice are outbred, derived from Swiss-Webster mice, and bred at the Rocky Mountain Laboratories. IRW mice are inbred, derived from RML mice, and also bred at the Rocky Mountain Laboratories. For infection studies, mice were injected with 80% of the inoculum intraperitoneally and 20% of the inoculum subcutaneously. In some cases (as indicated in the Results), bacteria were inoculated into the intradermal-subcutaneous compartment, with the appropriate number of bacteria provided in a single 100 μl injection with a 27 ga needle. The mice were euthanized three to six weeks post-inoculation, and ears, bladders, and ankle joints harvested and cultured in 10 ml BSKII medium. Isolates from mice infected with vlsE/pCp ospC or vlsE ΔospC/pCp ospC were plated in solid medium and resultant colonies were screened by PCR with primers 14 and 15 (Table 6), to assess retention of pCp ospC (Tilly et al., 2009).

**SDS PAGE and immunoblot analysis**

*Borrelia burgdorferi* protein lysates (approximately 107 spirochaete-equivalents per well) were separated in 12.5%
SDS-polyacrylamide gels and immunoblotted as described (Bestor et al., 2012). Detection was with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL). Antibodies were as follows: mouse monoclonal anti-FlaB H9724 (Barbour et al., 1986), rabbit polyclonal anti-VlsE (Bykowski et al., 2006), and mouse monoclonal anti-OspA H5332 (Barbour et al., 1983). Sera from inoculated animals were diluted 1:200 for assessing seroreactivity.

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References

Akins, D.K., Bourell, K.W., Caimano, M.J., Norgard, M.V., and Radolf, J.D. (1998) A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state. *J Clin Invest* 101: 2240–2250.

Bankhead, T., and Chaconas, G. (2007) The role of VlsE antigenic variation in the Lyme disease spirochete: persistence through a mechanism that differs from other pathogens. *Mol Microbiol* 65: 1547–1558.

Barbour, A.G., Tessier, S.L., and Todd, W.J. (1983) Lyme disease spirochetes and ioxid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. *Infect Immun* 41: 795–804.

Barbour, A.G., Hayes, S.F., Heiland, R.A., Schrumpf, M.E., and Tessier, S.L. (1986) A *Borrelia*-specific monoclonal antibody binds to a flagellar epitope. *Infect Immun* 52: 549–554.

Battisti, J.M., Bono, J.L., Rosa, P.A., Schrumpf, M.E., Schwan, T.G., and Policastro, P.F. (2008) Outer surface protein A protects Lyme disease spirochetes from acquired host immunity in the tick vector. *Infect Immun* 76: 5228–5237.

Bestor, A., Rego, R.O., Tilly, K., and Rosa, P.A. (2012) Competitive advantage of *Borrelia burgdorferi* with outer surface protein BBA03 during tick-mediated infection of the mammalian host. *Infect Immun* 80: 3501–3511.

Brissin, D., and Dykhuizen, D.E. (2004) *ospC* diversity in *Borrelia burgdorferi*: different hosts are different niches. *Genetics* 168: 713–722.

Bykowski, T., Babb, K., von Lackum, K., Riley, S., Norris, S.J., and Stevenson, B. (2006) Transcriptional regulation of the *Borrelia burgdorferi* antigenically variable VlsE surface protein. *J Bacteriol* 188: 4879–4889.

Byram, R., Stewart, P.E., and Rosa, P.A. (2004) The essential nature of the ubiquitous 26 kb circular replicon of *Borrelia burgdorferi*. *J Bacteriol* 186: 3561–3569.

Casjens, S., Palmer, N., van Vugt, R., Huang, W.M., Stevenson, B., Rosa, P., et al. (2000) A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. *Mol Microbiol* 35: 490–516.

Casjens, S.R., Mongodin, E.F., Qiu, W.G., Luft, B.J., Schutzer, S.E., Gilcrease, E.B., et al. (2012) Genome stability of Lyme disease spirochetes: comparative genomics of *Borrelia burgdorferi* plasmids. *PLoS ONE* 7: e33280.

Coutte, L., Botkin, D.J., Gao, L., and Norris, S.J. (2009) Detailed analysis of sequence changes occurring during *vlsE* antigenic variation in the mouse model of *Borrelia burgdorferi* infection. *PLoS Pathog* 5: e1000293.

Crother, C.R., Champion, C.I., Wu, X.Y., Blanco, D.R., Miller, J.N., and Lovett, M.A. (2003) Antigenic composition of *Borrelia burgdorferi* during infection of SCID mice. *Infect Immun* 71: 3419–3428.

Crother, T.R., Champion, C.I., Whitelegge, J., Aguiera, R., Wu, X.-Y., Blanco, D.R., et al. (2004) Temporal analysis of the antigenic composition of *Borrelia burgdorferi* during infection in rabbit skin. *Infect Immun* 72: 5063–5072.

Dulebohn, D.P., Bestor, A., Rego, R.O., Stewart, P.E., and Rosa, P.A. (2011) The *Borrelia burgdorferi* linear plasmid lp38 is dispensable for completion of the mouse-tick infectious cycle. *Infect Immun* 79: 3510–3517.

Dunham-Emms, S.M., Caimano, M.J., Eggers, C.H., and Radolf, J.D. (2012) *Borrelia burgdorferi* requires the alternative sigma factor RpoS for dissemination within the vector during tick-to-mammal transmission. *PLoS Pathog* 8: e1002532.

Earnhart, C.G., Leblanc, D.V., Alix, K.E., Desrosiers, D.C., Radolf, J.D., and Marconi, R.T. (2010) Identification of residues within ligand-binding domain 1 (LBD1) of the *Borrelia burgdorferi* OspC protein required for function in the mammalian environment. *Mol Microbiol* 76: 393–408.

Eicken, C., Sharma, V., Klabunde, T., Owens, R.T., Pikas, D.S., Höök, M., and Sacchettini, J.C. (2001) Crystal structure of Lyme disease antigen outer surface protein C from *Borrelia burgdorferi*. *J Biol Chem* 276: 10010–10015.

Eicken, C., Sharma, V., Klabunde, T., Lawrenz, M.B., Hardham, J.M., Norris, S.J., and Sacchettini, J.C. (2002) Crystal structure of Lyme disease variable surface antigen *VlsE* of *Borrelia burgdorferi*. *J Biol Chem* 277: 21691–21696.

Elias, A.F., Stewart, P.E., Grimm, D., Caimano, M.J., Eggers, C.H., Tilly, K., et al. (2002) Clonal polymorphism of *Borrelia burgdorferi* strain B31 M1: implications for mutagenesis in an infectious strain background. *Infect Immun* 70: 2139–2150.

Elias, A.F., Bono, J.L., Kupko, J.J., Stewart, P.E., Krum, J.G., and Rosa, P.A. (2003) New antibiotic resistance cassettes suitable for genetic studies in *Borrelia burgdorferi*. *J Mol Microbiol Biotechnol* 6: 29–40.

Embers, M.E., Alvarez, X., Ooms, T., and Philipp, M.T. (2008) The failure of immune response evasion by linear plasmid 28-1-deficient *Borrelia burgdorferi* is attributable to persistent expression of an outer surface protein. *Infect Immun* 76: 3984–3991.

Fingerle, V., Hauser, U., Liegl, G., Petko, B., Preac-Mursic, V., and Wilske, B. (1995) Expression of outer surface pro-
teins A and C of *Borrelia burgdorferi* in *Ixodes ricinus*. *J Clin Microbiol* **33**: 1867–1869.
Frank, K.L., Bundle, S.F., Kresge, M.E., Eggers, C.H., and Samuels, D.S. (2003) *aadA* confers streptomycin resistance in *Borrelia burgdorferi*. *J Bacteriol* **185**: 6723–6727.
Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., *et al.* (1997) Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature* **390**: 580–586.
Grimm, D., Tilly, K., Byram, R., Stewart, P.E., Krum, J.G., Bueschel, D.M., *et al.* (2004) Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. *Proc Natl Acad Sci USA* **101**: 3142–3147.
Grimm, D., Tilly, K., Bueschel, D.M., Fisher, M.A., Policastro, P.F., Gherardini, F.C., *et al.* (2005) Defining plasmids required for *Borrelia burgdorferi* colonization of tick vector *Ixodes scapularis* (Acari: Ixodidae). *J Med Entomol* **42**: 676–684.
Hodzic, E., Cheng, S., Freet, K.J., Borjesson, D.L., and Barthold, S.W. (2002) *Borrelia burgdorferi* population kinetics and selected gene expression at the host-vector interface. *Infect Immun* **70**: 3382–3388.
Hodzic, E., Cheng, S., Freet, K.J., and Barthold, S.W. (2003) *Borrelia burgdorferi* population dynamics and prototype gene expression during infection of immunocompetent and immunodeficient mice. *Infect Immun* **71**: 5042–5055.
Indest, K.J., Howell, J.K., Jacobs, M.B., Scholl-Meeker, D., Norris, S.J., and Philp, M.T. (2001) Analysis of *Borrelia burgdorferi* *vlsE* gene expression and recombination in the tick vector. *Infect Immun* **69**: 7083–7090.
Jewett, M.W., Byram, R., Bestor, A., Tilly, K., Lawrence, K., Burtnick, M.N., *et al.* (2007a) Genetic basis for retention of a critical virulence plasmid of *Borrelia burgdorferi*. *Mol Microbiol* **66**: 975–990.
Jewett, M.W., Lawrence, K., Bestor, A.C., Tilly, K., Grimm, D., Shaw, P., *et al.* (2007b) The critical role of the linear plasmid *Ip36* in the infectious cycle of *Borrelia burgdorferi*. *Mol Microbiol* **64**: 1358–1374.
Jewett, M.W., Lawrence, K.A., Bestor, A., Byram, R., Gherardini, F., and Rosa, P.A. (2009) *GuaA* and *GuaB* are essential for *Borrelia burgdorferi* survival in the tick-mouse infectious cycle. *J Bacteriol* **191**: 6231–6241.
Kumaran, D., Eswaramoorthy, S., Luft, B.J., Koide, S., Dunn, J.J., Lawson, C.L., and Swaminathan, S. (2001) Crystal structure of outer surface protein C (OspC) from the Lyme disease spirochete, *Borrelia burgdorferi*. *EMBO J* **20**: 971–978.
Labandeira-Rey, M., and Skare, J.T. (2001) Decreased infectivity in *Borrelia burgdorferi* strain B31 is associated with loss of linear plasmid 25 or 28-1. *Infect Immun* **69**: 446–455.
Labandeira-Rey, M., Seshu, J., and Skare, J.T. (2003) The absence of linear plasmid 25 or 28-1 of *Borrelia burgdorferi* dramatically alters the kinetics of experimental infection via distinct mechanisms. *Infect Immun* **71**: 4608–4613.
Lagal, V., Portnoi, D., Faure, G., Postic, D., and Baranton, G. (2006) *Borrelia burgdorferi* sensu stricto invasiveness is correlated with OspC-plasminogen affinity. *Microbes Infect* **8**: 645–652.
LaRocca, T.J., Crowley, J.T., Cusack, B.J., Pathak, P., Benach, J., London, E., *et al.* (2010) Cholesterol lipids of *Borrelia burgdorferi* form lipid rafts and are required for the bactericidal activity of a complement-independent antibody. *Cell Host Microbe* **8**: 331–342.
Lawrenz, M.B., Wooten, R.M., and Norris, S.J. (2004) Effects of *vlsE* complementation on the infectivity of *Borrelia burgdorferi* lacking the linear plasmid *Ip28*-1. *Infect Immun* **72**: 6577–6585.
Liang, F.T., Jacobs, M.B., Bowers, L.C., and Philipp, M.T. (2002a) An immune evasion mechanism for spirochetal persistence in Lyme borreliosis. *J Exp Med* **195**: 415–422.
Liang, F.T., Nelson, F.K., and Fikrig, E. (2002b) Molecular adaptation of *Borrelia burgdorferi* in the murine host. *J Exp Med* **196**: 275–280.
Liang, F.T., Yan, J., Mbow, M.L., Sviat, S.L., Gilmore, R.D., Mamula, M., and Fikrig, E. (2004) *Borrelia burgdorferi* changes its surface antigenic expression in response to host immune responses. *Infect Immun* **72**: 5759–5767.
Ohnishi, J., Piesman, J., and de Silva, A.M. (2001) Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. *Proc Natl Acad Sci USA* **98**: 670–675.
Onder, O., Humphrey, P.T., McOmber, B., Korobova, F., Francella, N., Greenbaum, D.C., and Brisson, D. (2012) OspC is potent plasminogen receptor on surface of *Borrelia burgdorferi*. *J Biol Chem* **287**: 16860–16868.
Pal, U., and Fikrig, E. (2003) Adaptation of *Borrelia burgdorferi* in the vector and vertebrate host. *Microbes Infect* **5**: 659–666.
Pal, U., de Silva, A.M., Montgomery, R.R., Fish, D., Anguita, J., Anderson, J.F., *et al.* (2000) Attachment of *Borrelia burgdorferi* within *Ixodes scapularis* mediated by outer surface protein A. *J Clin Invest* **106**: 561–569.
Pal, U., Yang, X., Chen, M., Bockensted, L.K., Anderson, J.F., Flavell, R.A., *et al.* (2004) OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. *J Clin Invest* **113**: 220–230.
Purser, J.E., and Norris, S.J. (2000) Correlation between plasmid content and infectivity in *Borrelia burgdorferi*. *Proc Natl Acad Sci USA* **97**: 13865–13870.
Purser, J.E., Lawrenz, M.B., Caimano, M.J., Radolf, J.D., and Norris, S.J. (2003) A plasmid-encoded nicotine amidase (*PncA*) is essential for infectivity of *Borrelia burgdorferi* in a mammalian host. *Mol Microbiol* **48**: 753–764.
Radolf, J.D., Caimano, M.J., Stevenson, B., and Hu, L.T. (2012) Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. *Nat Rev Microbiol* **10**: 87–99.
Rego, R.O., Bestor, A., and Rosa, P.A. (2011) Defining the plasmid-encoded restriction-modification systems of the Lyme disease spirochete *Borrelia burgdorferi*. *J Bacteriol* **193**: 1161–1171.
Sadziene, A., Wilske, B., Ferdows, M.S., and Barbour, A.G. (1993) The cryptic *ospC* gene of *Borrelia burgdorferi* B31 is located on a circular plasmid. *Infect Immun* **61**: 2192–2195.
Samuels, D.S. (1995) Electrotransformation of the spirochete *Borrelia burgdorferi*. In Methods in Molecular Biology. Nickoloff, J.A. (ed.). Totowa, NJ: Humana Press, Inc, pp. 253–259.

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Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C., and Rosa, P.A. (1995) Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc Natl Acad Sci USA* 92: 2909–2913.

Seemanapalli, S.V., Xu, Q., McShan, K., and Liang, F.T. (2010) Outer surface protein C is a dissemination-facilitating factor of *Borrelia burgdorferi* during mammalian infection. *PLoS ONE* 5: e15830.

Stewart, P.E., Wang, X., Bueschel, D.M., Clifton, D.R., Grimm, D., Tilly, K., et al. (2006) Delineating the requirement for the *Borrelia burgdorferi* virulence factor OspC in the mammalian host. *Infect Immun* 74: 3547–3553.

Tilly, K., Krum, J.G., Bestor, A., Jewett, M.W., Grimm, D., Bueschel, D., et al. (2006) *Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection. *Infect Immun* 74: 3554–3564.

Tilly, K., Bestor, A., Jewett, M.W., and Rosa, P. (2007) Rapid clearance of Lyme disease spirochetes lacking OspC from skin. *Infect Immun* 75: 1517–1519.

Tilly, K., Bestor, A., Dulebohn, D.P., and Rosa, P.A. (2009) OspC-independent infection and dissemination by host-adapted *Borrelia burgdorferi*. *Infect Immun* 77: 2672–2682.

Tokarz, R., Anderton, J.M., Katona, L.I., and Benach, J.L. (2004) Combined effects of blood and temperature shift on *Borrelia burgdorferi* gene expression as determined by whole genome DNA array. *Infect Immun* 72: 5419–5432.

Wilske, B., Preac-Mursic, V., Schieriz, G., and Busch, K.V. (1986) Immunochemical and immunological analysis of European *Borrelia burgdorferi* strains. *Zentralbl Bakteriol Mikrobiol Hyg A* 263: 92–102.

Wilske, B., Preac-Mursic, V., Schieriz, G., Kuhbeck, R., Barbour, A.G., and Kramer, M. (1988) Antigenic variability of *Borrelia burgdorferi*. In *Lyme Disease and Related Disorders*. Benach, J.L., and Bosler, E.M. (eds). New York: New York Academy of Sciences, pp. 126–143.

Wormser, G.P., Liveris, D., Hanincova, K., Brisson, D., Ludin, S., Stracuzzi, V.J., et al. (2008) Effect of *Borrelia burgdorferi* genotype on the sensitivity of C6 and 2-tier testing in North American patients with culture-confirmed Lyme disease. *Clin Infect Dis* 47: 910–914.

Xu, Q., McShan, K., and Liang, F.T. (2008a) Essential protective role attributed to the surface lipoproteins of *Borrelia burgdorferi* against innate defenses. *Mol Microbiol* 69: 15–29.

Xu, Q., McShan, K., and Liang, F.T. (2008b) Modification of *Borrelia burgdorferi* to overproduce OspA or VlsE alters its infectious behaviour. *Microbiology* 154: 3420–3429.

Yang, X.F., Pal, U., Alani, S.M., Fikrig, E., and Norgard, M.V. (2004) Essential role for OspA/B in the life cycle of the Lyme disease spirochete. *J Exp Med* 199: 641–648.

Zhang, J.R., and Norris, S.J. (1998) Kinetics and *in vivo* induction of genetic variation of *vlsE* in *Borrelia burgdorferi*. *Infect Immun* 66: 3689–3697.

Zhang, J.R., Hardham, J.M., Barbour, A.G., and Norris, S.J. (1997) Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. *Cell* 89: 275–285.