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Essential protective role attributed to the surface lipoproteins of *Borrelia burgdorferi* against innate defenses

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Summary

To initiate infection, a microbial pathogen must be able to evade innate immunity. Here we show that the Lyme disease spirochete *Borrelia burgdorferi* depends on its surface lipoproteins for protection against innate defenses. The deficiency for OspC, an abundantly expressed surface lipoprotein during early infection, led to quick clearance of *B. burgdorferi* after inoculation into the skin of SCID mice. Increasing expression of any of the four randomly chosen surface lipoproteins, OspA, OspE, VlsE or DbpA, fully protected the *ospC* mutant from elimination from the skin tissue of SCID mice; moreover, increased OspA, OspE, or VlsE expression allowed the mutant to cause disseminated infection and restored the ability to effectively colonize both joint and skin tissues, albeit the dissemination process was much slower than that of the mutant restored with OspC expression. When the *ospC* mutant was modified to express OspA under control of the *ospC* regulatory elements, it registered only a slight increase in the 50% infectious dose than the control in SCID mice but a dramatic increase in immunocompetent mice. Taken together, the study demonstrated that the surface lipoproteins provide *B. burgdorferi* with an essential protective function against host innate elimination.

Like typical Gram-negative bacteria, the Lyme disease spirochete *Borrelia burgdorferi* possesses inner and outer membranes, between which is a periplasmic space (Cullen *et al.*, 2004; Steere, 2001). Gram-negative pathogens make a thick LPS coat to provide a broad array of crucial protection (Raetz and Whitfield, 2002). However, *B. burgdorferi* does not produce any LPS but instead abundantly expresses lipoproteins and anchors them to the outer membranous surface through lipidation (Cullen *et al.*, 2004; Radolf *et al.*, 1994; Takayama *et al.*, 1987).

B. burgdorferi appears to maintain its overall surface lipoprotein expression level during the enzootic life cycle traveling between the tick vector and a mammal, and the course of mammalian infection. The pathogen abundantly expresses outer surface proteins (Osps) A and B in the unfed tick (de Silva *et al.*, 1996; Ohnishi *et al.*, 2001; Schwan *et al.*, 1995; Schwan and Piesman, 2000), a fresh blood meal induces the down-regulation of OspA/B and the up-regulation of OspC and others, a process that prepares *B. burgdorferi* for infection of mammals (Fingerle *et al.*, 2007; Grimm *et al.*, 2004; Pal *et al.*, 2004b; Stewart *et al.*, 2006). Abundant OspC expression ultimately induces a robust early humoral response that imposes tremendous pressure on the pathogen (Fung *et al.*, 1994; Xu *et al.*, 2006). To evade the specific humoral response and cause persistent infection, *B. burgdorferi* down-regulates OspC and dramatically upregulates other surface lipoproteins, including VlsE and BBF01 (Crother *et al.*, 2004; Liang *et al.*, 2002a; Liang *et al.*, 2002b; Liang *et al.*, 2004). These changes in surface antigen expression certainly allow *B. burgdorferi* to better adapt to different environments, and

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potentially also provide a scheme for maintaining the overall surface lipoprotein expression level.

The various expression levels and diverse functions of different surface lipoproteins make investigation into their common role extremely challenging. For instance, deletion of the *ospAB* locus or even the *ospB* gene alone diminishes the ability of *B. burgdorferi* to persist in the tick vector but does not affect virulence in mammalian hosts (Neelakanta *et al.*, 2007; Yang *et al.*, 2004), while inactivation of the *ospD* gene does not reduce the viability either in the tick or a mammal (Li *et al.*, 2007). The fibronectin-binding protein BBK32 and decorin-binding proteins (Dbps) A and B are not expressed during the life cycle in ticks because their expression depends on the induction of *rpoS*, which is silent in the tick (Caimano *et al.*, 2007; He *et al.*, 2007; Hubner *et al.*, 2001), and thus should not be expected to have a role in the vector. In fact, none of the three surface lipoprotein adhesins is required for mammalian infection (Li *et al.*, 2006; Seshu *et al.*, 2006; Shi *et al.*, 2006), although both DbpA and DbpB are critical for the overall virulence of *B. burgdorferi* (Shi *et al.*, 2008). VlsE, the variable surface antigen identified in *B. burgdorferi* (Zhang *et al.*, 1997), is required for persistent infection of immunocompetent animals but does not significantly contribute to infectivity in SCID mice (Bankhead and Chaconas, 2007; Labandeira-Rey *et al.*, 2003; Xu *et al.*, 2005). OspE, a member of a large surface lipoprotein family called Erp (Lam *et al.*, 1994; Stevenson *et al.*, 1998), is one of five surface lipoproteins that are shown binding the complement regulator factor H (Bykowski *et al.*, 2007; Hartmann *et al.*, 2006; Hellwage *et al.*, 2001; Kraiczy *et al.*, 2004; McDowell *et al.*, 2004; Metts *et al.*, 2003; Stevenson *et al.*, 2002). Although remaining to be investigated, OspE is very unlikely to be critical for mammalian infection because other members of the families may compensate for its loss. To date, OspC has been the only surface lipoprotein shown to be essential for mammalian infection (Grimm *et al.*, 2004; Stewart *et al.*, 2006; Tilly *et al.*, 2006; Tilly *et al.*, 2007).

The essential role repeatedly demonstrated for OspC in mammalian infection led Rosa and colleagues to hypothesize that OspC is required for evasion of innate immunity during initial infection (Tilly *et al.*, 2007). However, given that OspC is probably the most abundantly expressed surface lipoprotein during early infection, deletion of the *ospC* gene may severely compromise the integrity of the surface lipoprotein layer, which may provide *B. burgdorferi* with protection against innate defenses. None of the other surface lipoproteins has been shown to be essential for mammalian infection, probably because they are expressed at relatively low levels so their absence does not significantly reduce the integrity of the lipoprotein layer. To explore the hypothesis that the surface lipoproteins collectively play an essential protective role against innate defenses, OspC-deficient *B. burgdorferi* was modified to increase expression of well-defined surface lipoproteins, OspA, OspE, VlsE and DbpA, and then examined for dissemination, tissue colonization, infectivity and persistence in the murine model.

Results

Generation of B. burgdorferi with increased expression of OspA, OspE, VlsE or DbpA

Five constructs, namely pBBE22-*ospC'*, pBBE22-*ospA'*, pBBE22-*ospE'*, pBBE22-*vlsE'* and pBBE22-*dbpA'*, and their parental vector, pBBE22, were electroporated into the *ospC* mutant, which was generated and characterized in our previous study (Xu *et al.*, 2007a). pBBE22-*ospA'*, pBBE22-*ospE'*, and pBBE22-*vlsE'* were constructed as illustrated in Fig. 1A; pBBE22-*ospC'* and pBBE22-*dbpA'* were generated in our previous studies (Xu *et al.*, 2006; Xu *et al.*, 2007b). All the five introduced genes would be expressed under control of the *flaB* promoter (Table 1). Because the *ospC* mutant had lost lp25, the plasmid that carries the gene *bbe22* coding for a nicotinamidase essential for survival of *B. burgdorferi* in the mammalian environment, the recombinant plasmid pBBE22, which harbors a copy of *bbe22*, was used as

the shuttle vector (Purser *et al.*, 2003). Between 8 and 15 transformants were obtained from transformation with each construct. Plasmid analyses identified two clones receiving each construct, namely *ΔospC*/E22/1, *ΔospC*/E22/2, *Δosp*/*ospC'*/1, *ΔospC*/*ospC'*/2, *ΔospC*/*ospA'*/1, *ΔospC*/*ospA'*/2, *ΔospC*/*ospE'*/1, *ΔospC*/*ospE'*/2, *ΔospC*/*vlsE*'/1, *ΔospC*/*vlsE*'/2, *ΔospC*/*dbpA*'/1, and *ΔospC*/*dbpA*'/2. These clones shared the same plasmid content as the *ospC* mutant, which had lost lp25, lp5, lp21, lp56 and cp9 (Xu *et al.*, 2007a). Increased Osp expression resulting from transformation was confirmed by immunoblot analysis. Introduction of pBBE22-*ospC'*, pBBE22-*ospE'*, pBBE22-*vlsE'*, and pBBE22-*dbpA'* led to dramatically increased expression of respective Osps (Fig. 1B). Unfortunately, overwhelming OspA expression resulting from the native *ospA* copy masked the potential contribution of the introduced pBBE22-*ospA'*.

Increasing expression of an outer surface protein overrides the essential role of OspC in protecting B. burgdorferi *from quick clearance in murine skin*

Groups of six SCID mice each received two intradermal/subcutaneous inoculations of 10^5 spirochetes of the clone *ΔospC*/E22/1, *ΔospC*/E22/2, *ΔospC*/*ospC'*/1, *ΔospC*/*ospC'*/2, *ΔospC*/*ospA'*/1, *ΔospC*/*ospA'*/2, *ΔospC*/*ospE'*/1, *ΔospC*/*ospE'*/2, *ΔospC*/*vlsE*'/1, *ΔospC*/*vlsE*'/2, *ΔospC*/*dbpA*'/1, or *ΔospC*/*dbpA*'/2. The two inoculation sites were at least 2 cm apart. Two animals from each group were euthanized at 24, 48 or 72 h later; inoculation site skin specimens were harvested for spirochete culture. As a positive control, the *ΔospC*/*ospC'*/1 and *ΔospC*/*ospC'*/2 bacteria were consistently grown from each of the 24 inoculation sites from all 12 inoculated mice (Table 2). In contrast, the *ΔospC*/E22/1 and *ΔospC*/E22/2 spirochetes were recovered from only two of the eight sites harvested within 24 hours, and from none of the 16 specimens collected after then, confirming the essential role of OspC in protecting *B. burgdorferi* from quick clearance in murine skin reported by Tilly *et al.* (Tilly *et al.*, 2007). Like the positive control, *ΔospC*/*ospA'*/1, *ΔospC*/*ospA'*/2, *ΔospC*/*ospE'*/1, *ΔospC*/*ospE'*/2, *ΔospC*/*vlsE*'/1, *ΔospC*/*vlsE*'/2, *ΔospC*/*dbpA*'/1, and *ΔospC*/*dbpA*'/2 spirochetes were consistently grown from each of the specimens harvested from all inoculated mice at all time points. Thus, the study also demonstrated that the essential protective role of OspC against early elimination can be overridden by increasing expression of any of the four Osps.

OspC is required for efficient dissemination and this function can be substituted to varying extents by other outer surface lipoproteins

Groups of six to 12 SCID mice each received a single intradermal/subcutaneous inoculation of 10^5 spirochetes of the clone *ΔospC*/*ospC'*/1, *ΔospC*/*ospC'*/2, *ΔospC*/*ospA'*/1, *ΔospC*/*ospA'*/2, *ΔospC*/*ospE'*/1, *ΔospC*/*ospE'*/2, *ΔospC*/*vlsE*'/1, *ΔospC*/*vlsE*'/2, *ΔospC*/*dbpA*'/1, or *ΔospC*/*dbpA*'/2. Three animals from each group were euthanized at 1-wk intervals; inoculation site and remote site skin, ear, heart, and joint specimens were harvested for spirochete isolation. Bacteria were injected into the dermis of the chest so the skin from the back was harvested as remote sites. As a positive control, the *ΔospC*/*ospC'*/1 and *ΔospC*/*ospC'*/2 bacteria were grown from all of the skin, joint and heart specimens but from none of the ear samples at first week; all sites became culture positive at 2 wk after initial inoculation (Table 3). The *ΔospC*/*ospA'*/1, *ΔospC*/*ospA'*/2, *ΔospC*/*ospE'*/1, *ΔospC*/*ospE'*/2, *ΔospC*/*vlsE*'/1, and *ΔospC*/*vlsE*'/2 bacteria were grown from all of the inoculation sites and joint specimens but from only one heart sample at a week post-inoculation; all hearts but only 3 ear specimens became positive at 2 wk; most ear samples were not colonized until 3 wk. Although the *ΔospC*/*dbpA*'/1 and *ΔospC*/*dbpA*'/2 spirochetes were consistently grown from all of the inoculation sites, they were not recovered from any distal tissues during the 4-wk period. These data demonstrated that OspC is required for efficient dissemination and that this function can be substituted to varying extents by other Osps.

All isolated spirochetes were grown to stationary phase and analyzed for OspC expression by immunoblotting. All recovered *ΔospC*/*ospC'*/1 and *ΔospC*/*ospC'*/2 spirochetes abundantly

expressed OspC but none of the *ΔospC*/*ospA*'/1, *ΔospC*/*ospA*'/2, *ΔospC*/*ospE*'/1, *ΔospC*/*ospE*'/2, *ΔospC*/*vlsE*'/1, *ΔospC*/*vlsE*'/2, *ΔospC*/*dbpA*'/1 or *ΔospC*/*dbpA*'/2 isolates produced the antigen (data not shown), indicating that all of the *ospC* mutant derivatives remained OspC-deficient.

OspC is not required for efficient colonization in the joint or skin but heart tissues of SCID mice

To examine the influence of OspC deficiency on tissue colonization, subgroups of five SCID mice each received a single intradermal/subcutaneous inoculation of 10^5 spirochetes of the clone *ΔospC*/*ospC*'/1, *ΔospC*/*ospC*'/2, *ΔospC*/*ospA*'/1, *ΔospC*/*ospA*'/2, *ΔospC*/*ospE*'/1, *ΔospC*/*ospE*'/2, *ΔospC*/*vlsE*'/1, or *ΔospC*/*vlsE*'/2. In 10 mice that were inoculated with the *ΔospC*/*ospC*'/1 or *ΔospC*/*ospC*'/2, joint swelling evolved around 10 d post-inoculation and developed into severe arthritis a wk later (data not shown). In the remaining mice, joint swelling did not become apparent until 3 wk post-inoculation and slowly developed after then, indicating that the OspC-deficient phenotypes with increased Osp expression cause delayed, less severe arthritis.

Ear biopsies were taken for bacterial culture at 2 and 3 wk post-inoculation. At 2 wk, all of the 10 mice that were inoculated with the *ΔospC*/*ospC*'/1 or *ΔospC*/*ospC*'/2 bacteria had a positive biopsy (data not shown). The remaining mice did not produce a positive biopsy until 3 wk post-inoculation. Again, the study demonstrated that OspC is not required for infection of immunodeficient mice but is important for efficient dissemination once the *ospC* mutant is modified with increased *osp* expression.

All the 40 mice were euthanized 1 mo post-inoculation; DNA was extracted from heart, joint and skin specimens and quantified for bacterial burden. In heart tissue, the *ΔospC*/*ospC*' spirochete burden was 84%, 89%, and 81% higher than those of the *ΔospC*/*ospA*' ($P = 4.5 \times 10^{-4}$), *ΔospC*/*ospE*' ($P = 3.5 \times 10^{-4}$), and *ΔospC*/*vlsE*' ($P = 9.1 \times 10^{-4}$), respectively, while the three genotypes with increased OspA, OspE or VlsE expression generated similar bacterial loads ($P > 0.05$) (Fig. 2). However, there was no significant difference in bacterial load among the four genotypes either in joint ($P > 0.05$) or skin tissues ($P > 0.05$). The study indicated that OspC is not required for efficient colonization in both joint and skin but in the heart tissue of SCID mice.

OspC-deficient B. burgdorferi with increased OspA expression registers a slight ID_{50} increase in SCID mice but a dramatic increase in immunocompetent mice

To further assess the contribution of OspC to infectivity, the ID_{50} value was determined. For this purpose, OspC-deficient *B. burgdorferi* was modified to express OspA under control of the *ospC* regulatory elements, including both promoter and operator (Xu *et al.*, 2007a). Because the *ospC* promoter is RpoS-dependent (Hubner *et al.*, 2001), the absence of the operator allows it to drive constitutive expression and, as a consequence, diminishes the ability of *B. burgdorferi* to evade humoral immunity during infection of immunocompetent mice (Xu *et al.*, 2007a). The inclusion of the operator may allow recombinant *B. burgdorferi* to down-regulate the introduced *ospA* copy in response to the development of specific humoral responses. OspA was chosen for this purpose because OspA and OspC are so unrelated. First, unlike OspC, OspA is abundantly expressed in the tick (Schwan *et al.*, 1995). Second, OspA is β-sheet dominant, while OspC is primarily composed of α-helices (Eicken *et al.*, 2001; Kumaran *et al.*, 2001; Li *et al.*, 1997).

The construct pBBE22-*CpospA* was created as illustrated in Fig. S1 (Supplemental Material). To examine whether it drove phase-dependent *ospA* expression, an *ospA* mutant was generated (Supplemental Material). pBBE22-*CpospA* was electroporated into the *ospA* mutant. Twelve

transformants were obtained; plasmid content analyses led to selection of two clones, namely, *ΔospA*/*CpospA*/1 and *ΔospA*/*CpospA*/2. Immunoblotting showed that both clones exhibited phase-dependent *ospA* expression, when grown *in vitro* (Fig. 3).

Next, pBBE22-*CpospA* was electroporated into the *ospC* mutant. Fifteen transformants were obtained; plasmid content analyses led to selection of two clones, namely, *ΔospC*/*CpospA*/1 and *ΔospC*/*CpospA*/2, which shared the same plasmid content as the *ospC* mutant (Xu *et al.*, 2007a). Groups of three SCID mice each received one single inoculation of 10^1 to 10^4 spirochetes of the clone *ΔospC*/*FL*/1, *ΔospC*/*FL*/2, *ΔospC*/*CpospA*/1, or *ΔospC*/*CpospA*/2. The clones *ΔospC*/*FL*/1 and *ΔospC*/*FL*/2 were generated via introduction of a full-length *ospC* gene including both the promoter and operator carried by the shuttle vector pBBE22 into the *ospC* mutant in our previous study (Xu *et al.*, 2007a). Ear biopsies were taken for bacterial culture at 2, 3 and 4 wk post-inoculation. At 2 wk, all of the 20 mice that were found to be infected with the *ΔospC*/*FL*/1 or *ΔospC*/*FL*/2 bacteria at the end of study had a positive biopsy (Table 4). In contrast, none of the mice inoculated with the clone *ΔospC*/*CpospA*/1 or *ΔospC*/*CpospA*/2 produced a positive biopsy at 2 wk. All the six mice that received 10^2 organisms of the clone *ΔospC*/*CpospA*/1 or *ΔospC*/*CpospA*/2 did not show a positive biopsy until 4 wk post-inoculation. Again, the study demonstrated that OspC deficiency severely impairs dissemination.

All animals were euthanized 1 mo post-inoculation; heart, joint and skin specimens were cultured for spirochetes. The ID_{50} values of both clones *ΔospC*/*CpospA*/1 and *ΔospC*/*CpospA*/2 were 32 organisms, compared to 18 organisms determined for both clones *ΔospC*/*FL*/1 and *ΔospC*/*FL*/2 (Table 4). Similar results were obtained in a separate experiment, in which the ID_{50} values of the clones *ΔospC*/*CpospA*/1 and *ΔospC*/*CpospA*/2 were 32 and 18 organisms, respectively, compared to 18 organisms for both clones *ΔospC*/*FL*/1 and *ΔospC*/*FL*/2. By combining the two experiments, the study indicated that the OspC deficiency led to only a 1.6-fold ID_{50} increase in SCID mice ($P = 0.05$) after modification with increased OspA expression.

Next, the influence of OspC-deficiency on spirochetal dissemination and the ID_{50} value was investigated in immunocompetent mice. In two separate experiments, all mice that were found to be infected with the *ΔospC*/*FL*/1 or *ΔospC*/*FL*/2 bacteria at the end of study produced a positive biopsy either at 2 or 3 wk post-inoculation, depending on inoculation doses (Table 5). In contrast, none of the mice inoculated with the clone *ΔospC*/*CpospA*/1 or *ΔospC*/*CpospA*/2 produced a positive biopsy within the first 3 wk; most of the inoculated mice produced a positive biopsy at 4 wk, and four infected mice did not develop a positive ear biopsy until wk 5. The study demonstrated that OspC deficiency severely impairs dissemination in immunocompetent mice.

The ID_{50} values for both clones *ΔospC*/*FL*/1 and *ΔospC*/*FL*/2 were 32 organisms in both experiments, in comparison to 1000, 1778, 1778 and 3162 organisms determined for the clones *ΔospC*/*CpospA*/1 and *ΔospC*/*CpospA*/2 (Table 5). Overall, OspC-deficient *B. burgdorferi* with OspA expression under control of the *ospC* regulatory elements registered a 60-fold ID_{50} increase than the mutant restored with wild-type OspC expression ($P = 0.006$).

Another defect noted from OspC-deficient *B. burgdorferi* with increased OspA expression was a reduced frequency in colonization of the heart and joint tissues of immunocompetent mice. The *ΔospC*/*CpospA*/1 and *ΔospC*/*CpospA*/2 spirochetes were grown only from 6 heart and 10 joint specimens of the 22 infected mice (Table 5); in contrast, the clones *ΔospC*/*FL*/1 and *ΔospC*/*FL*/2 colonized all the tissues of the 30 infected mice. The study indicated that the OspC deficiency leads to a 73% and 55% decrease in frequency of colonizing heart ($P = 7.1 \times 10^{-26}$) and joint tissues ($P = 1.2 \times 10^{-12}$), respectively, during early infection of

immunocompetent mice. As shown in Table 3, the joint was the first distal tissue to be colonized, followed by the heart, in SCID mice, it is possible that spirochetes might have colonized these tissues but subsequently been cleared by the specific humoral response during infection in immunocompetent mice. Alternatively, the specific humoral response might have blocked spirochetes from disseminating to these tissues.

OspC-deficient B. burgdorferi with increased OspA expression can persist in the skin but not heart or joint tissues during chronic infection of immunocompetent mice

Next, OspC deficient spirochetes with increased OspA expression were investigated for the ability to evade adaptive immunity and cause persistent infection. Subgroups of five BALB/c mice each received one single intradermal/subcutaneous injection of 10^4 spirochetes of the clone *ΔospC*/*FL*/1, *ΔospC*/*FL*/2, *ΔospC*/*CpospA*/1, or *ΔospC*/*CpospA*/2. Animals were euthanized 4 mo after initial inoculation; heart, joint, and skin samples were cultured for spirochetes. *B. burgdorferi* was grown from each skin specimen of all 20 mice, regardless of whether they received OspC-deficient or control spirochetes (Table 6). The *ΔospC*/*FL*/1 and *ΔospC*/*FL*/2 spirochetes were successfully grown from all of the 10 hearts and 9 of the 10 joint specimens; however, OspC-deficient *B. burgdorferi* was not recovered from 9 of the 10 hearts and 8 of the 10 joint specimens. The study indicated that OspC-deficient *B. burgdorferi* with increased OspA expression has a diminished ability to colonize or persist in heart and joint tissues of immunocompetent mice during chronic infection.

The anti-OspA humoral response was analyzed in the 20 infected mice. No or weak anti-OspA response was detected in the 10 mice that were infected with the genotype *ΔospC*/*FL* (Fig. 4). In contrast, mice infected with the genotype *ΔospC*/*CpospA* produced anti-OspA responses 382-fold higher than those that were inoculated with the *ΔospC*/*FL* spirochetes ($P < 0.05$).

Discussion

The current study took the advantage that inactivation of the *ospC* gene results in quick clearance of *B. burgdorferi* in the murine host, and clearly demonstrated that increasing expression of any of the four randomly chosen but well-studied surface lipoproteins can fully restore the ability of the *ospC* mutant to evade innate defenses. Because OspC is an abundantly expressed surface antigen and may contribute dominantly to the overall integrity of the outer lipoprotein layer during initial infection, the OspC deficiency may cause a severe compromise of the lipoprotein layer and, as a result, completely abrogate its protective function and lead to quick clearance of *B. burgdorferi* by the first line of host defenses. Increasing expression of OspA, OspE, VlsE or DbpA may successfully compensate for the loss of OspC and restore the integrity of the lipoprotein layer and, consequently, its protective function. Clearly, the study revealed one common function of the surface lipoproteins, which is to protect the pathogen from elimination by host innate defenses.

The current study showed different roles of individual surface lipoproteins, while demonstrating their common role. Although increasing expression of any of the four lipoproteins fully protected OspC-deficient *B. burgdorferi* from elimination, distinct phenotypes were observed during infection of SCID mice. Increased expression of OspA, OspE or VlsE allowed the *ospC* mutant to cause disseminated infection, albeit the dissemination process was much slower than that of the mutant restored with OspC expression. However, increasing DbpA expression protected OspC-deficient spirochetes in murine skin but could not make them disseminate to distal tissues. Analysis of bacterial loads revealed that increasing expression of OspA, OspE or VlsE restored the ability of the *ospC* mutant to effectively colonize both joint and skin tissues but not heart of SCID mice. During the course of infection of SCID mice, *ospC* is highly expressed in the heart tissue (Liang *et al.*, 2004), where host ligands may exist (Antonara *et al.*, 2007); potential interactions of *B. burgdorferi* with host

components mediated by OspC may facilitate tissue colonization. When OspA was selected for examining the ability to replace OspC for restoration of infectivity, increasing OspA expression provided the *ospC* mutant with an ID_{50} value comparable to that of the mutant restored with OspC expression in SCID mice. Given that most of the roles of OspC can be replaced with other surface lipoproteins, the primary function attributed to OspC should be to facilitate dissemination.

The overall surface architecture of *B. burgdorferi* is collectively determined by each of the individual lipoproteins and their expression levels on the surface, and directly interacts with the environment, thus largely influencing the behavior of the pathogen in a specific microenvironment. After the surface is decorated dominantly with OspA and OspB, *B. burgdorferi* probably fits best to the environment of the tick midgut (Neelakanta *et al.*, 2007; Yang *et al.*, 2004), where the two lipoproteins may mediate interactions with gut ligands (Pal *et al.*, 2004a). A fresh blood meal induces the down-regulation of OspA and OspB and the up-regulation of OspC as well as other surface antigens (Schwan *et al.*, 1995), reshaping the surface architecture, an event that prepares the pathogen for the mammalian environment. These modifications may also prompt *B. burgdorferi* to migrate to salivary glands and finally into the dermis of a mammal (Pal *et al.*, 2004b). Repression of OspA/B expression during infection of mammals is critical for the maintenance of the enzootic cycle because their expression would ultimately induce a strong humoral response and, as a result, may effectively block acquisition of *B. burgdorferi* by the vector (de Silva *et al.*, 1997; Tsao *et al.*, 2001; Tsao *et al.*, 2004), regardless of whether OspA/B can be targeted by borreliacidal antibodies in mammalian tissues (Strother *et al.*, 2007). As clearly shown in the current study, abundant OspC expression is critical for efficient dissemination to and quick colonization of distal tissues, and an establishment of systemic infection. Because OspC is a strong immunogen and an effective target of protective immunity, active OspC expression soon becomes a disadvantage as specific humoral immune responses are being elicited (Xu *et al.*, 2006; Xu *et al.*, 2007a). To evade the specific humoral response and proceed to persistent infection, *B. burgdorferi* down-regulates OspC and greatly up-regulates other surface antigens, such as VlsE (Liang *et al.*, 2004). As a variable surface antigen, VlsE vigorously undergoes antigenic variation and is required for persistent infection of immunocompetent mice (Bankhead and Chaconas, 2007; Zhang *et al.*, 1997; Zhang and Norris, 1998). Therefore, *B. burgdorferi* certainly benefits from these surface modifications during infection of immunocompetent hosts.

DbpA, a well-defined surface adhesin, binds both decorin and glycosaminoglycans (Fischer *et al.*, 2003; Guo *et al.*, 1998), and contributes significantly to the overall virulence of *B. burgdorferi* (Shi *et al.*, 2006). Increasing DbpA expression significantly reduces the ID_{50} value but severely impairs dissemination, probably because an abundant presence of DbpA on the spirochetal surface facilitates the interaction of the pathogen with host decorin (Xu *et al.*, 2007b). Consistent with these previous studies, the current study showed that increasing DbpA expression effectively protected OspC-deficient *B. burgdorferi* in murine skin but completely inhibited dissemination. In contrast, after OspC expression was restored, *B. burgdorferi* quickly disseminated to distal tissues. The identification of this extreme phenotype further underscores our notion that the overall surface architecture greatly determines the infectious behavior of *B. burgdorferi*. One may argue that increasing DbpA expression protected the *ospC* mutant only in skin so spirochetes might be quickly eliminated once they left the tissue. If this was the case, OspC-deficient *B. burgdorferi* with increased DbpA expression should be grown from remote skin specimens of infected SCID mice. As a matter of fact, however, bacteria with increased DbpA expression were not grown from any remote skin samples during the 4-wk period.

As the first surface lipoprotein that is shown binding the complement regulator factor H (Hellwage *et al.*, 2001), OspE is one of five complement regulator-acquiring surface proteins

that have been identified in *B. burgdorferi* to date (Hartmann *et al*., 2006; Kraiczy *et al.*, 2004; McDowell *et al.*, 2003; Metts *et al.*, 2003; Stevenson *et al.*, 2002), and one of the four that are persistently expressed during murine infection (Bykowski *et al.*, 2007). Although an *in vitro* study showed a critical role of the regulator-binding proteins in contributing to complement resistance in *B. burgdorferi* (Brooks *et al.*, 2005), a recent study indicated that binding of factor H does not significantly affect the infectivity of *B. burgdorferi* in mice (Woodman *et al.*, 2007). While it remains to be addressed whether OspE functions as a regulator-binding protein to provide protection against complement, the current study showed that OspE, just like other surface lipoproteins, is able to protect OspC-deficient *B. burgdorferi* against elimination by host innate defenses.

Naïve and immune statuses constitute two distinct environments to microbial pathogens. The ability of OspA to replace OspC was investigated in the two environments by using immunodeficient and immunocompetent mice. OspC-deficient *B. burgdorferi* modified with OspA expression under control of the *ospC* regulatory elements, including both the promoter and operator (Xu *et al.*, 2007a), registered only a 1.6-fold ID_{50} increase in SCID mice but an over 60-fold increase in immunocompetent mice than the mutant restored with OspC expression. The mutant with increased OspA expression colonized all tissues of infected SCID mice and generated similar bacterial loads in both joint and skin tissues as the mutant restored with OspC expression, but was grown only from less than 20% of the heart and joint samples of chronically infected immunocompetent mice. This reduced ability of OspA to replace OspC in evasion of adaptive immunity may result from their unequal effectiveness targeted by protective humoral responses. In fact, OspC is an effective target of protective immunity (Xu *et al.*, 2006); to evade the anti-OspC immune response, *B. burgdorferi* reduces OspC expression to a baseline level (Liang *et al.*, 2002a; Liang *et al.*, 2004), probably via the induction of a yet unidentified repressor to interact with the *ospC* operator (Xu *et al.*, 2007a). As the current study showed, the *ospC* promoter indeed drove phase-dependent *ospA* expression in cultured spirochetes. However, it remains to be addressed whether the *ospC* operator can effectively respond to environmental changes, including the development of a specific humoral response, when it controls *ospA* expression.

One unique function that was identified for OspC in the current study is to facilitate dissemination, which can be substituted to varying extents by other Osps. OspC is highly expressed during initial murine infection but is downregulated after the specific humoral response has developed (Liang *et al.*, 2002a; Liang *et al.*, 2004), consistent with this newly defined role. Given this unique role, the early abundant expression promotes bacterial dissemination; after distal tissues are colonized and specific humoral responses develop, OspC is downregulated. The downregulation allows *B. burgdorferi* not only to effectively evade humoral immunity but also to preserve the integrity of the critical gene for the subsequent enzootic cycle, because active *ospC* expression would result in either clearance of infection or selection of *ospC* mutants (Xu *et al.*, 2006). It would be interesting to examine the influence of OspC from different OspC genotypes of *B. burgdorferi* on spirochetal dissemination by cloning their *ospC* gene into the same *ospC* mutant, given that a study by Seinost *et al.* suggested an association of selective OspC genotypes with invasive infection (Seinost *et al.*, 1999), although such a correlation has been challenged by a recent report from Alghaferi *et al.* (Alghaferi *et al.*, 2005).

OspC is absolutely not required for tick colonization, but it remains controversial whether it is essential for transmission from the vector to a mammal (Fingerle *et al.*, 2007; Grimm *et al.*, 2004; Pal *et al.*, 2004b; Stewart *et al.*, 2006). Rosa and colleagues repeatedly showed that OspC is not required for the enzootic cycle of *B. burgdorferi* in the tick vector (Grimm *et al.*, 2004; Stewart *et al.*, 2006). In contrast, neither Pal *et al.* nor Fingerle *et al.* were able to make OspC mutants cross the tick salivary gland barrier to the murine host (Fingerle *et al.*, 2007;

Pal *et al.*, 2004b). The fact that the first group used fully competent *ospC* mutants in their studies while the *ospC* mutants generated by the latter groups were not restored with infectivity could be a reason causing the disparity. Because none of their OspC-deficient clones was infectious, they had to use artificial tick feeding, which may also be a contributing factor for the disparity. The strategy to overcome the essential role of OspC in mammalian infection developed in the current study may help resolve the controversy.

B. burgdorferi very actively expresses OspA and OspB during *in vitro* cultivation before inoculation into mice. Although the pathogen down-regulates OspA and OspB immediately after the contact of murine tissues, these already expressed on the surface should remain protective until they are degraded or diluted through spirochete replication. However, an entire inoculum of 10^5 organisms deficient for OspC was eradicated, in most cases, within 24 h after inoculation into the skin of SCID mice, indicating that *B. burgdorferi* with compromised surface lipoprotein expression is extremely vulnerable to the innate immune system, albeit it remains to be determined which components of the system, phagocytes, complement and/or others, play a major role in this regard. Increasing expression of any of the four randomly chosen but well- studied surface lipoproteins successfully protected the *ospC* mutant in SCID mice. Given that the four lipoproteins are completely unrelated to OspC or to each other but were able to protect the pathogen just as OspC, the study demonstrated one common function of the surface lipoproteins, which is to protect *B. burgdorferi* against elimination by host innate defense mechanisms. It would be interesting to address how these antigens can provide the pathogen with such protection while they serve as potent innate immune stimulators via Toll-like receptor-dependent signaling.

Experimental procedures

Strains and constructs that were generated previously and used in the current study

The *ospC* mutant, and the clones *ΔospC*/*FL*/1 and *ΔospC*/*FL*/2 were generated previously (Xu *et al.*, 2007a) (Table 1). The constructs pBBE22-*ospC′* and pBBE22-*dbpA′* were constructed previously (Xu *et al.*, 2006; Xu *et al.*, 2007b). The shuttle vector pBBE22 was a gift from S. Norris (Purser *et al.*, 2003).

*Construction of pBBE22-*ospA', *pBBE22-*ospE', *and pBBE22-vlsE′*

As illustrated in Fig. 1A, three primer pairs, P2F and P3R, P3F and P4R, and P4F and P5R (Table S1, Supplemental Materials), were used, respectively, to amplify 845-bp promoterless *ospA*, 1119-bp *vlsE*, and 593-bp *ospE* sequences from borrelial DNA. Two fragments, 254-bp and 256-bp, were amplified from the promoter region of *flaB* using a common forward primer, P1F, and two reverse primers, P1R and P2R, respectively. The 254-bp *flaBp* amplicon was pooled with the 845-bp *ospA* and 1119-bp *vlsE* amplicons, respectively, digested with *Nde*I, purified, then ligated to form *flaBp-ospA* and *flaBp-vlsE*. The 256-bp *flaBp* amplicon was pooled with the 593-bp *ospE* product, digested with *Nco*I and *Bsp*HI, purified, and ligated to create *flaBp-ospE*. The fragments *flaBp-ospA*, *flaBp-vlsE*, and *flaBp-ospE* were amplified with the use of a common forward primer, P5F, and three reverse primers, P6R, P7R and P8R, respectively, digested with *Bam*HI and *Xba*I, purified, and cloned into the shuttle vector pBBE22 (Purser *et al.*, 2003) after the vector was digested with the same enzymes. The inserts within pBBE22 were sequenced to ensure that the inserts and their flanking sequences were arranged as designed.

Generation of B. burgdorferi *with increased Osp expression*

The constructs pBBE22-*ospC′*, pBBE22-*ospA′*, pBBE22-*ospE′*, pBBE22-*vlsE′* and pBBE22-*dbpA′*, and their parental vector, pBBE22, were electroporated into the *ospC* mutant, which was generated and characterized in our previous study (Xu *et al.*, 2007a), as described

previously (Xu*et al.*, 2005). pBBE22-*ospC'* and pBBE22-*dbpA'* were generated in our previous studies (Xu *et al.*, 2006; Xu *et al.*, 2007b). Resulting transformants were first surveyed for the presence of lp28−1 because this plasmid is essential for persistent infection of immunocompetent hosts (Labandeira-Rey *et al.*, 2003; Purser and Norris, 2000; Xu *et al.*, 2005). Only clones containing lp28−1 were further analyzed for plasmid content as described previously (Xu *et al.*, 2005).

In vitro *characterization of transformants*

Transformants were grown in Barbour-Stoenner-Kelly H (BSK-H) complete medium (Sigma Chemical Co., St. Louis, MO) to late-log phase at 33°C, and harvested by centrifugation. Spirochete lysates were subjected to immunoblot analysis probed with FlaB (Barbour *et al.*, 1986), OspA (Sears *et al.*, 1991) or OspC mAb (Mbow *et al.*, 1999), or mouse anti-DbpA sera (Shi *et al.*, 2006), or mouse antisera raised against recombinant OspE or VlsE prepared as described below. Immunoblotting was performed as described previously (Xu *et al.*, 2006).

Preparation of recombinant proteins and generation of mouse antisera

The coding regions excluding the signal peptide-coding sequence of the genes *ospA*, *ospE* and *vlsE* were PCR amplified and cloned into the expression vector pET16b, and transformed into the *Escherichia coli* strain BL21(DE3) (Novagen, La Jolla, CA). Recombinant protein was purified using the Hi-Trap affinity column (Amersham-Pharmacia Biotech, Piscataway, NJ). The protein purity and concentration were determined using SDS-PAGE and the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), respectively. Approximately 70 μg of recombinant protein was dissolved in 100 μl of PBS (pH = 7.3) and emulsified with 30 μl of Freund's complete (first injection) or incomplete adjuvant (remaining injections), and subcutaneously administered into each BALB/c mouse (ages, 5 − 8 wk) at 3-wk intervals. The specific humoral response was monitored by immunoblotting with use of borrelial lysates as antigen. Mice were euthanized 3 wk after last immunization for antiserum preparation.

Inoculation of SCID mice

BALB/c SCID mice (ages of 4--8 wk, provided by the Division of LSU Laboratory Animal Medicine) were given two intradermal/subcutaneous injections of 10^5 spirochetes. The two inoculation sites were at least 2 cm apart. Animals were sacrificed 24, 48 or 72 h later; inoculation site skin tissues were harvested for spirochete isolation as described previously (Xu *et al.*, 2005).

In a second experiment, SCID mice each received a single intradermal/subcutaneous injection of 10^5 spirochetes and were euthanized at 1, 2, 3, and 4 wk post-inoculation. Inoculation site and remote site skin, ear, heart, and joint specimens were harvested for spirochete isolation as described previously (Xu *et al.*, 2005). Spirochetes were injected into the dermis of the chest so the skin from the back was harvested as remote sites. Isolated bacteria were grown to late-log phase, and were subjected to immunoblot analysis probed with a mixture of FlaB and OspC MAbs as described previously (Xu *et al.*, 2006).

In a third experiment, SCID mice each received a single intradermal/subcutaneous injection of 10^5 spirochetes. Ear biopsies were taken for bacterial culture at 2 and 3 wk post-inoculation. Animals were examined for the development of arthritis at 2-d intervals, starting at d 7, and sacrificed 1 mo post-inoculation. Joint, heart, and skin specimens were collected for DNA isolation. DNA was quantified by qPCR for copy numbers of *flaB* and murine actin genes (Xu *et al.*, 2005). The tissue spirochete burden was expressed as *flaB* DNA copies per 10^6 host cells (2×10^6 actin DNA copies).

Determination of ID_{50} values

Spirochetes were grown at 33°C to late-log phase (10^8 cells per ml) and 10-fold serially diluted with BSK-H complete medium. BALB/c or BALB/c SCID mice (age, 4 -- 8 wk; provided by the LSU Division of Laboratory Animal Medicine) each received a single intradermal/subcutaneous injection of 100 μl of spirochetal suspension. Ear biopsies were performed up to 5 wk post-inoculation, starting at wk 2, as described previously (Xu *et al.*, 2006). SCID and wild-type mice were euthanized 1 mo and 6 wk post-inoculation, respectively; heart, tibiotarsal joint, and skin (not from inoculation site) specimens were harvested for bacterial culture as described previously (Xu *et al.*, 2005). The ID_{50} value was calculated as described by Reed and Muench (Reed and Muench, 1938).

Persistent infection study in immunocompetent mice

BALB/c mice at ages of 4 -- 8 wk were given a single intradermal/subcutaneous injection of 10^4 spirochetes. All mice were sacrificed 4 mo after the initial inoculation; heart, tibiotarsal joint, and skin specimens were collected for spirochete culture as described previously (Xu *et al.*, 2005).

Measurement of anti-OspA humoral immune response

Specific OspA antibody end-point titers were determined by an ELISA. Ninety-six-well plates (Fisher Scientific, Pittsburgh, PA) were coated with 100 μl of 2.0 μg/ml recombinant OspA per well. Sera were 2-fold serially diluted, starting at 1/200. Five samples drawn from naive BALB/c mice were used as a control. The ELISA was performed as previously described (Xu *et al.*, 2006).

Statistical analysis

qPCR data and calculated ID_{50} values were analyzed by using a one-way analysis of variance (ANOVA), followed by a two-tailed Student *t* test to compare two treatments and calculate *P* values. Calculated *P* values of ≤ 0.05 were considered to be significant. Fisher's exact test was used to analyze tissue colonization data.

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Supplementary Material

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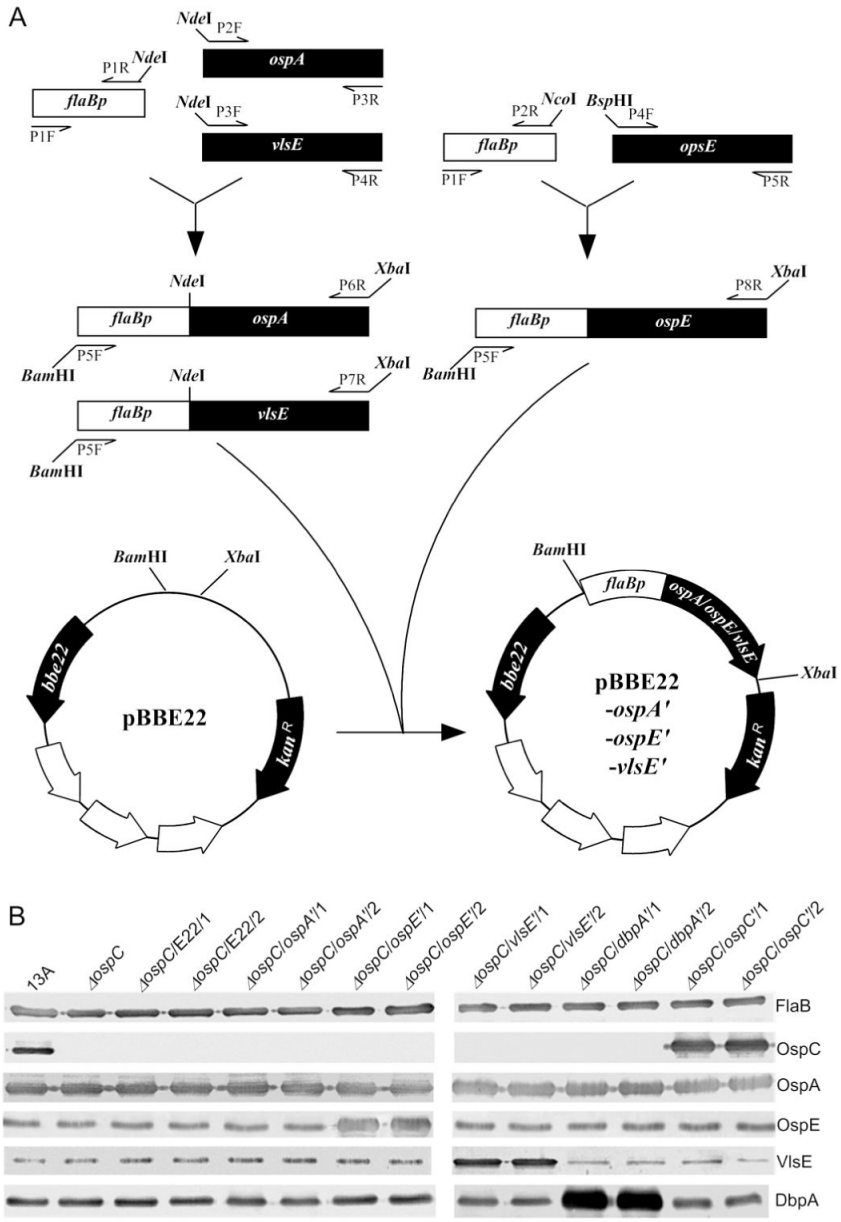

Fig. 1. Generation of *B. burgdorferi* with increased Osp expression

A. Construction of pBBE22-*ospA'*, pBBE22-*ospE'*, and pBBE22-*vlsE'*. The *flaB* promoter region (*flaBp*) and a promoterless *ospA*, *ospE*, or *vlsE* gene were PCR amplified, fused, and cloned into pBBE22.

B. Generation of OspC-deficient *B. burgdorferi* with increased OspC, OspA, OspE, DbpA or VlsE expression. pBBE22-*ospA'*, pBBE22-*ospA'*, pBBE22-*ospE'*, pBBE22-*dbpA'*, pBBE22-*vlsE'*, and pBBE22 were electroporated into the *ospC* mutant. pBBE22-*ospC'* and pBBE22-*dbpA'* were constructed in our previous studies (Xu *et al.*, 2006; Xu *et al.*, 2007b). The parental clone 13A, the *ospC* mutant, and transformants *ΔospC*/*ospC'*/1, *ΔospC*/*ospC'*/2, *ΔospC*/*ospA'*/1, *ΔospC*/*ospA'*/2, *ΔospC*/*ospE'*/1, *ΔospC*/*ospE'*/2, *ΔospC*/*vlsE'*/1, and *ΔospC*/*vlsE'*/2 were verified for Osp expression by immunoblots probed with FlaB, OspC and OspA MAbs, and mouse antisera raised against recombinant OspE, DbpA and VlsE.

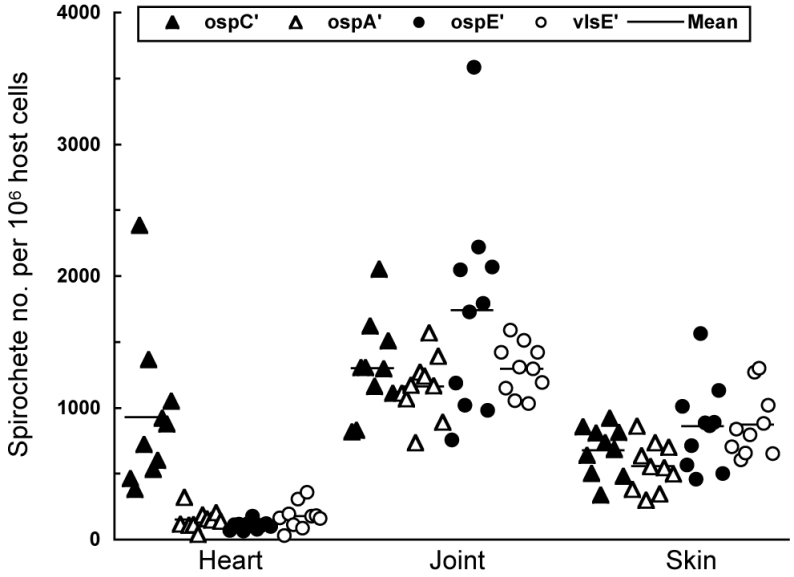

Fig. 2. OspC deficiency does not reduce the ability of *B. burgdorferi* to colonize joint or skin but heart tissues of SCID mice

Subgroups of five SCID mice were inoculated with 10^5 spirochetes of the clone *ΔospC/ospC'*/1, *ΔospC/ospC'*/2, *ΔospC/ospA'*/1, *ΔospC/ospA'*/2, *ΔospC/ospE'*/1, *ΔospC/ospE'*/2, *ΔospC/vlsE'*/1, or *ΔospC/vlsE'*/2, and euthanized a month later. DNA was prepared from heart, joint and skin specimens and analyzed for spirochete *flaB* and murine actin DNA copies by qPCR. Data are expressed as spirochete numbers per 10^6 host cells and presented in four groups by combining the subgroups *ΔospC/ospC'*/1 and *ΔospC/ospC'*/2, *ΔospC/ospA'*/1 and *ΔospC/ospA'*/2, *ΔospC/ospE'*/1 and *ΔospC/ospE'*/2, and *ΔospC/vlsE'*/1 and *ΔospC/vlsE'*/2.

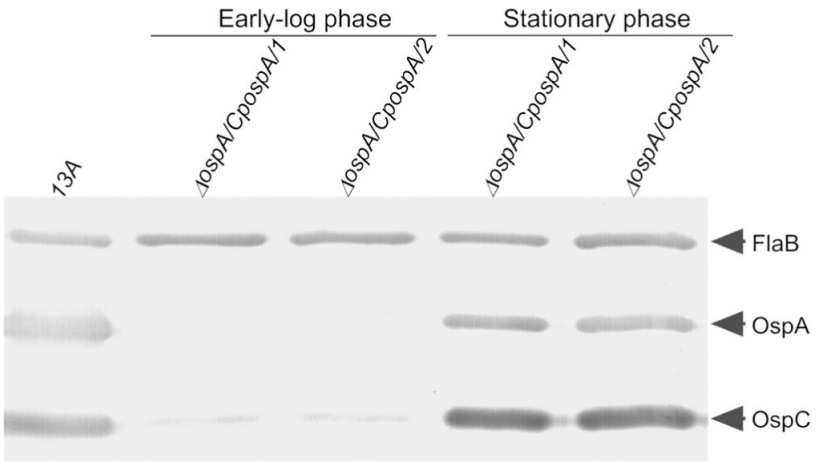

Fig. 3. The *ospC* promoter drives phase-dependent *ospA* expression

The 13A spirochetes harvested at stationary phase and the *ΔospA*/*CpospA*/1 and *ΔospA*/*CpospA*/2 bacteria grown to early-log and stationary phases were analyzed by immunoblot probed with a mixture of FlaB, OspA and OspC MAbs.

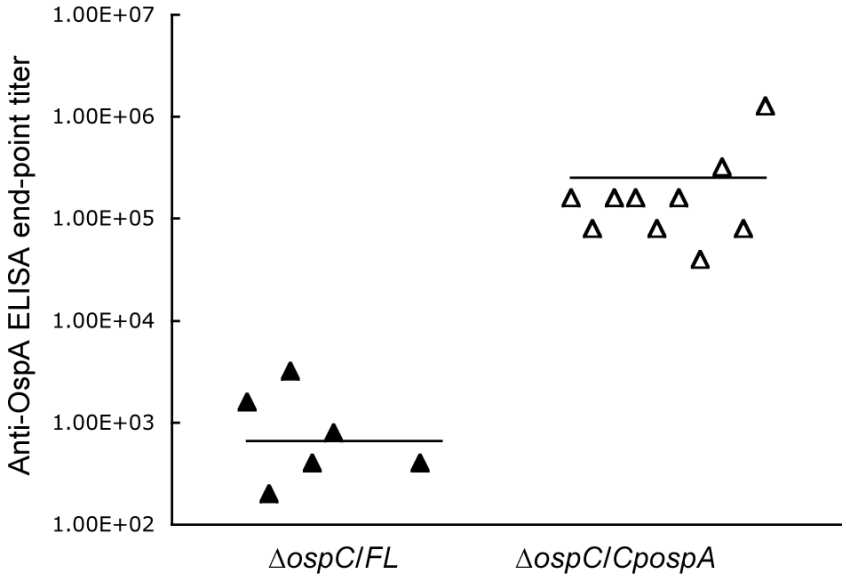

Fig. 4. Strong anti-OspA responses are induced by OspC-deficient *B. burgdorferi* with OspA expression under control of the *ospC* regulatory elements

Groups of 10 mice were infected with either *ΔospC/FL* or *ΔospC/CpospA* spirochetes for 4 mo. Serum samples were collected and analyzed for anti-OspA antibody titers by an end-point ELISA. No response was detected in four of the mice infected with the genotype *ΔospC/FL* so that only six datum points are shown for this group. The average titers (horizontal lines) for each group are also presented.

Table 1

Constructs and clones used in the study.

Construct or clone	Description	Source
pBBE22	pBSV2 carrying a *bbe22* copy	(Purser *et al.*, 2003)
pBBE22-*ospC′*	pBBE22 carrying promoterless *ospC* fused with *flaB* promoter	(Xu *et al.*, 2006)
pBBE22-*ospA′*	pBBE22 carrying promoterless *ospA* fused with *flaB* promoter	This study
pBBE22-*ospE′*	pBBE22 carrying promoterless *ospE* fused with *flaB* promoter	This study
pBBE22-*vlsE′*	pBBE22 carrying promoterless *vlsE* fused with *flaB* promoter	This study
pBBE22-*dbpA′*	pBBE22 carrying promoterless *dbpA* fused with *flaB* promoter	(Xu *et al.*, 2007b)
pBBE22-*CpospA*	pBBE22 carrying promoterless *ospA* fused with *ospC* regulatory elements[a]	This study
Δ*ospC*	*ospC* mutant	(Xu *et al.*, 2007a)
Δ*ospA*	*ospA* mutant	This study
Δ*ospC*/E22/1	*ospC* mutant without increased *osp* expression	This study
Δ*ospC*/E22/2	*ospC* mutant without increased *osp* expression	This study
Δ*ospC/ospC′*/1	*ospC* mutant expressing *ospC* driven by *flaB* promoter	This study
Δ*ospC/ospC′*/2	*ospC* mutant expressing *ospC* driven by *flaB* promoter	This study
Δ*ospC/ospA′*/1	*ospC* mutant expressing *ospA* driven by *flaB* promoter	This study
Δ*ospC/ospA′*/2	*ospC* mutant expressing *ospA* driven by *flaB* promoter	This study
Δ*ospC/ospE′*/1	*ospC* mutant expressing *ospE* driven by *flaB* promoter	This study
Δ*ospC/ospE′*/2	*ospC* mutant expressing *ospE* driven by *flaB* promoter	This study
Δ*ospC/vlsE′*/1	*ospC* mutant expressing *vlsE* driven by *flaB* promoter	This study
Δ*ospC/vlsE′*/2	*ospC* mutant expressing *vlsE* driven by *flaB* promoter	This study
Δ*ospC/dbpA′*/1	*ospC* mutant expressing *dbpA* driven by *flaB* promoter	This study
Δ*ospC/dbpA′*/2	*ospC* mutant expressing *dbpA* driven by *flaB* promoter	This study
Δ*ospC/FL*/1	*ospC* mutant expressing *ospC* controlled by *ospC* regulatory elements	(Xu et al., 2007a)
Δ*ospC/FL*/2	*ospC* mutant expressing *ospC* controlled by *ospC* regulatory elements	(Xu *et al.*, 2007a)
Δ*ospC/CpospA*/1	*ospC* mutant expressing *ospA* controlled by *ospC* regulatory elements	This study
Δ*ospC/CpospA*/2	*ospC* mutant expressing *ospA* controlled by *ospC* regulatory elements	This study
Δ*ospA/CpospA*/1	*ospA* mutant expressing *ospA* controlled by *ospC* regulatory elements	This study
Δ*ospA/CpospA*/2	*ospA* mutant expressing *ospA* controlled by *ospC* regulatory elements	This study

[a] The *ospC* regulatory elements include both operator and promoter.

Table 2

Increasing expression of an Osp protects OspC-deficient *B. burgdorferi* from quick elimination in murine skin.[a]

Clone	No. of sites positive/Total no. of sites examined at post-inoculation hours		
	24	48	72
Δ*ospC/ospC′*/1	4/4	4/4	4/4
Δ*ospC/ospC′*/2	4/4	4/4	4/4
Δ*ospC*/E22/1	1/4	0/4	0/4
Δ*ospC*/E22/2	1/4	0/4	0/4
Δ*ospC/ospA′*/1	4/4	4/4	4/4
Δ*ospC/ospA′*/2	4/4	4/4	4/4
Δ*ospC/ospE′*/1	4/4	4/4	4/4
Δ*ospC/ospE′*/2	4/4	4/4	4/4
Δ*ospC/vlsE′*/1	4/4	4/4	4/4
Δ*ospC/vlsE′*/2	4/4	4/4	4/4
Δ*ospC/dbpA′*/1	4/4	4/4	4/4
Δ*ospC/dbpA′*/2	4/4	4/4	4/4

[a]Groups of six BALB/c SCID mice each received two intradermal/subcutaneous injections of the clone Δ*ospC/ospC′*/1, Δ*ospC/ospC′*/2, Δ*ospC*/E22/1, Δ*ospC*/E22/2, Δ*ospC/ospA′*/1, Δ*ospC/ospA′*/2, Δ*ospC/ospE′*/1, Δ*ospC/ospE′*/2, Δ*ospC/vlsE′*/1, Δ*ospC/vlsE′*/2, Δ*ospC/dbpA′*/1, or Δ*ospC/dbpA′*/2. Approximately 10^5 organisms were administered in each inoculation; two inoculation sites were at least 2 cm apart. Two animals from each group were euthanized at 24, 48, and 72 h post-inoculation; skin specimens were harvested from inoculation sites and cultured for spirochetes in BSK-H complete medium.

Table 3

OspC-deficient *B. burgdorferi* with increased expression of OspA, OspE or VlsE but not DbpA causes disseminated infection in SCID mice. [a]

Clone	No. of specimens positive/Total specimens examined at post-inoculation weeks																			
	1					2					3					4				
	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint
Δ*ospC/ospC′*/1	3/3	3/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND
Δ*ospC/ospC′*/2	3/3	3/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Δ*ospC/ospA′*/1	3/3	0/3	0/3	1/3	3/3	3/3	3/3	1/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	ND	ND	ND	ND	ND
Δ*ospC/ospA′*/2	3/3	0/3	0/3	0/3	3/3	3/3	3/3	1/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	ND	ND	ND	ND	ND
Δ*ospC/ospE′*/1	3/3	0/3	0/3	0/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	ND	ND	ND	ND	ND
Δ*ospC/ospE′*/2	3/3	0/3	0/3	0/3	3/3	3/3	3/3	1/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	ND	ND	ND	ND	ND
Δ*ospC/vlsE′*/1	3/3	0/3	0/3	0/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	ND	ND	ND	ND	ND
Δ*ospC/vlsE′*/2	3/3	0/3	0/3	0/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	ND	ND	ND	ND	ND
Δ*ospC/dbpA′*/1	3/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Δ*ospC/dbpA′*/2	3/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3

[a] Groups of six --12 BALB/c SCID mice each received a single intradermal/subcutaneous injection of 10^5 organisms of the clone Δ*ospC/ospC′*/1, Δ*ospC/ospC′*/2, Δ*ospC/ospA′*/1, Δ*ospC/ospA′*/2, Δ*ospC/ospE′*/1, Δ*ospC/ospE′*/2, Δ*ospC/vlsE′*/1, Δ*ospC/vlsE′*/2, Δ*ospC/dbpA′*/1, or Δ*ospC/dbpA′*/2. Three animals from each group were euthanized at 1, 2, 3, and 4 wk post-inoculation; inoculation site (I.S.) and remote site (R.S.) skin, ear, heart, and joint specimens were harvested for spirochete isolation. The I.S. site was at the chest; therefore the R.S. site was at the back of mice.

[b] ND, not determined.

Table 4

OspC-deficient *B. burgdorferi* with increased OspA expression registers a slight ID_{50} increase in SCID mice.[a]

Expt, clone, and dose (no. of organisms)	No. of biopsies positive/Total ear biopsies conducted at post-inoculation weeks			No. of cultures positive/Total specimens examined				No. of mice infected/Total mice inoculated	ID_{50} (no. of organisms)
	2	3	4	Heart	Joint	Skin	All site		
I									
Δ*ospC/FL*/1									18
10^4	3/3	ND[b]	ND	3/3	3/3	3/3	9/9	3/3	
10^3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^2	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^1	1/3	ND	ND	1/3	1/3	1/3	3/9	1/3	
Δ*ospC/FL*/2									18
10^4	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^2	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^1	1/3	ND	ND	1/3	1/3	1/3	3/9	1/3	
Δ*ospC/CpospA*/1									32
10^4	0/3	3/3	ND	3/3	3/3	3/3	9/9	3/3	
10^3	0/3	3/3	ND	3/3	3/3	3/3	9/9	3/3	
10^2	0/3	0/3	3/3	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
Δ*ospC/CpospA*/2									32
10^4	0/3	3/3	ND	3/3	3/3	3/3	9/9	3/3	
10^3	0/3	3/3	ND	3/3	3/3	3/3	9/9	3/3	
10^2	0/3	0/3	3/3	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
II									
Δ*ospC/FL*/1									18
10^3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^2	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^1	1/3	ND	ND	1/3	1/3	1/3	1/9	1/3	
Δ*ospC/FL*/2									18
10^3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^2	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^1	1/3	ND	ND	1/3	1/3	1/3	3/9	1/3	
Δ*ospC/CpospA*/1									32
10^3	0/3	3/3	ND	3/3	3/3	3/3	9/9	3/3	
10^2	0/3	0/3	3/3	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
Δ*ospC/CpospA*/2									18
10^3	0/3	3/3	ND	3/3	3/3	3/3	9/9	3/3	
10^2	0/3	0/3	3/3	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	1/3	1/3	1/3	1/9	1/3	

[a] The Δ*ospC/FL*/1, Δ*ospC/FL*/2, Δ*ospC/CpospA*/1, and Δ*ospC/CpospA*/2 spirochetes were grown to late-log phase (10^8 cells per ml) and 10-fold serially diluted with BSK-H medium. Groups of three BALB/c SCID mice each received a single intradermal/subcutaneous dose of 100 μl of bacterial suspension; ear biopsies were performed up to four weeks post-inoculation, starting at week 2. Once all three animals of a dose group became positive, biopsies were no longer performed on the group. All animals were sacrificed 1 month post-inoculation; heart, tibiotarsal joint, and skin specimens were harvested for bacterial isolation. The ID_{50} values were calculated by the method of Reed and Muench (Reed and Muench, 1938), and determined in two separate experiments.

[b] ND, not determined.

Table 5

OspC-deficient *B. burgdorferi* with increased OspA expression registers a dramatic ID_{50} increases in immunocompetent mice.[a]

Expt, clone, and dose (no. of organisms)	No. of biopsies positive/Total ear biopsies conducted at post-inoculation weeks				No. of cultures positive/Total specimens examined				No. of mice infected/Total mice inoculated	ID_{50} (no. of organisms)
	2	3	4	5	Heart	Joint	Skin	All site		
I										
Δ*ospC/FL*/1										32
10^4	3/3	ND[b]	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^3	1/3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^2	0/3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
Δ*ospC/FL*/2										32
10^4	3/3	ND	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^3	1/3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^2	0/3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
Δ*ospC/CpospA*/1										1000
10^5	0/3	0/3	3/3	ND	1/3	3/3	3/3	7/9	3/3	
10^4	0/3	0/3	3/3	ND	1/3	2/3	3/3	6/9	3/3	
10^3	0/3	0/3	0/3	1/3	0/3	0/3	1/3	1/9	1/3	
10^2	0/3	0/3	0/3	1/3	0/3	0/3	1/3	1/9	1/3	
Δ*ospC/CpospA*/2										1778
10^5	0/3	0/3	3/3	ND	1/3	2/3	3/3	6/9	3/3	
10^4	0/3	0/3	3/3	ND	1/3	1/3	3/3	5/9	3/3	
10^3	0/3	0/3	0/3	1/3	0/3	0/3	1/3	1/9	1/3	
10^2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
II										
Δ*ospC/FL*/1										32
10^3	1/3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^2	0/3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
Δ*ospC/FL*/2										32
10^3	0/3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^2	0/3	3/3	ND	ND	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
Δ*ospC/CpospA*/1										1778
10^4	0/3	0/3	3/3	ND	1/3	1/3	3/3	5/9	3/3	
10^3	0/3	0/3	0/3	1/3	0/3	0/3	1/3	1/9	1/3	
10^2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
Δ*ospC/CpospA*/2										3162
10^4	0/3	0/3	3/3	ND	1/3	1/3	3/3	5/9	3/3	
10^3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	
10^2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/9	0/3	

[a] The Δ*ospC/FL*/1, Δ*ospC/FL*/2, Δ*ospC/CpospA*/1, and Δ*ospC/CpospA*/2 spirochetes were grown to late-log phase (10^8 cells per ml) and 10-fold serially diluted with BSK-H medium. Groups of three BALB/c mice each received a single intradermal/subcutaneous dose of 100 μl of bacterial suspension; ear biopsies were performed up to five weeks post-inoculation, starting at week 2. Once all three animals of a dose group became positive, biopsies were no longer performed on the group. All animals were sacrificed 6 week post-inoculation; heart, tibiotarsal joint, and skin specimens were harvested for bacterial isolation. The ID_{50} values were calculated by the method of Reed and Muench (Reed and Muench, 1938), and determined in two separate experiments.

[b] ND, not determined.

Table 6

OspC-deficient *B. burgdorferi* with increased OspA expression can persist in the skin but not heart or joint tissue of chronically infected immunocompetent mice.[a]

Clone	No. of cultures positive/Total no. of specimens examined			
	Heart	Joint	Skin	All sites
Δ*ospC/FL*/1	5/5	5/5	5/5	15/15
Δ*ospC/FL*/2	5/5	4/5	5/5	14/15
Δ*ospC/CpospA*/1	0/5	1/5	5/5	6/15
Δ*ospC/CpospA*/2	1/5	1/5	5/5	7/15

[a] Groups of five BALB/c mice were inoculated with 10^4 spirochetes of the clone Δ*ospC/FL*/1, Δ*ospC/FL*/2, Δ*ospC/CpospA*/1, or Δ*ospC/CpospA*/2. Mice were sacrificed 4 mo post-inoculation; heart, tibiotarsal joint and skin specimens were harvested for spirochete culture in BSK-H complete medium.