Modification of *Borrelia burgdorferi* to overproduce OspA or VlsE alters its infectious behaviour

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The surface lipoproteins of the Lyme disease spirochaete *Borrelia burgdorferi* directly interact with tissue microenvironments during mammalian infection, and thus potentially affect various aspects of infection. To investigate the influence of surface antigen synthesis on infectious behaviour, *B. burgdorferi* was modified to constitutively produce the well-characterized surface lipoproteins OspA and invariant VlsE. Although increasing OspA or VlsE production did not significantly affect synthesis of other surface lipoproteins or spirochaetal growth in *vitro*, overexpressing vlsE resulted in increased *ospA* but decreased *ospC* expression, and overexpressing *ospA* led to decreased *ospC* and vlsE expression in severe combined immunodeficient (SCID) mice. Increasing the expression of either *ospA* or vlsE did not alter the ID$_{50}$, but affected spirochaetal dissemination and significantly reduced tissue spirochaete loads in SCID mice. In immunocompetent mice, increased vlsE expression resulted in quick clearance of infection, while constitutive *ospA* expression led to a substantial ID$_{50}$ increase and severely impaired dissemination. Furthermore, *B. burgdorferi* with constitutive *ospA* expression persisted in the skin tissue but was cleared from both heart and joints of chronically infected immunocompetent mice. Taken together, the study indicates that increasing production of OspA or invariant VlsE influences lipoprotein gene expression in the murine host and alters the infectious behaviour of *B. burgdorferi*.

**INTRODUCTION**

The Lyme disease spirochaete *Borrelia burgdorferi* vigorously modifies its surface antigen expression to adapt to various environments during the enzootic cycle. It 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 & Piesman, 2000), consistent with a critical role for these lipoproteins in spirochaetal persistence in the vector (Neelakanta *et al.*, 2007; Yang *et al.*, 2004). A fresh blood meal downregulates OspA/B and upregulates OspC and others, a process that prepares *B. burgdorferi* for infection of mammals (Fingerle *et al.*, 2007; Grimm *et al.*, 2004; Pal *et al.*, 2004; Stewart *et al.*, 2006). The downregulation of OspA and OspB during mammalian infection is critical for the maintenance of the enzootic cycle because their expression would ultimately induce strong humoral responses to effectively block acquisition by the vector (de Silva *et al.*, 1997; Tsao *et al.*, 2001, 2004), regardless of whether OspA or OspB can be effectively targeted by borreliacidal antibodies in mammalian tissues (Strother *et al.*, 2007). *B. burgdorferi* abundantly expresses *ospC* only during early infection when the antigen may be crucial (Grimm *et al.*, 2004; Stewart *et al.*, 2006; Tilly *et al.*, 2006). However, its expression induces a robust humoral response that imposes tremendous pressure on the pathogen (Fung *et al.*, 1994; Xu *et al.*, 2006). To cause persistent infection, *B. burgdorferi* must downregulate *ospC* as the specific humoral immune response is developing (Liang *et al.*, 2002a, b, 2004b). It is also crucial for *B. burgdorferi* to downregulate *ospC* after it is acquired by the tick, as OspC antibodies in the blood meal may kill spirochaetes that express the antigen in the vector (Gilmore & Piesman, 2000), leading to discontinuation of the enzootic cycle.

*B. burgdorferi* also constantly modifies its surface antigenic expression in response to tissue microenvironmental changes, including the development of humoral responses, during the course of mammalian infection (Bykowski *et al.*, 2007; Crother *et al.*, 2004; Gilmore *et al.*, 2007; Liang *et al.*, 2002a, b, 2004b). The pathogen does not actively express VlsE, the surface variable antigen identified in *B.
*B. burgdorferi*, during early infection (Liang *et al.*, 2004b). After dissemination into the joint, the pathogen upregulates *vlsE*, while maintaining low expression levels in the skin and heart tissues in the absence of humoral immune responses (Liang *et al.*, 2004b). As the specific humoral response develops, *B. burgdorferi* downregulates OspC and many other surface antigens but dramatically upregulates VlsE and BBF01 (Crother *et al.*, 2004; Liang *et al.*, 2002a, b, 2004b), a process that probably allows the pathogen to more effectively evade the immune system and cause persistent infection.

As an extracellular bacterium, *B. burgdorferi* abundantly produces outer surface lipoproteins, which are directly involved in the interplay between the pathogen and tissue microenvironments and are likely to influence its infectious behaviour. To investigate the issue, *B. burgdorferi* was modified to overexpress either *ospA* or an invariant *vlsE* gene, and then examined for the ID50 dissemination, tissue colonization and persistence in the murine model.

**METHODS**

**Strains and constructs generated previously and used in the current study.** The *B. burgdorferi* B31 clone 13A, the transformants 13A/E22/C and 13A/E22/D, and the *ospA* mutant ΔospA were generated previously (Xu *et al.*, 2007a, b, 2008). The constructs pBBE22-*ospA’* and pBBE22-*vlsE’* were generated in a previous study (Xu *et al.*, 2008). The features of these clones and constructs are summarized in Table 1.

**Generation of transformants.** The clones 13A and ΔospA were transformed with pBBE22-*ospA’* or pBBE22-*vlsE’*, as described previously (Xu *et al.*, 2007a). Transformants were identified and plasmid content was analysed as described previously (Xu *et al.*, 2005). Selected transformants were analysed for OspA, OspC and VlsE expression by immunoblotting probed with a mixture of FlaB, OspA, and OspC mAbs, or mouse antisera raised against a recombinant VlsE, as described previously (Xu *et al.*, 2008).

**In vitro growth kinetics and serum resistance.** Spirochaetes were grown to late-exponential phase (∼10⁶ cells ml⁻¹), diluted in Barbour–Stoenner–Kelly H (BSK-H) complete medium (Sigma) or BSK-H medium supplemented with mouse sera at the ratio of 1:1 (v/v) to a density of ∼10⁶ cells ml⁻¹ and cultured at 33 °C. Mouse sera were prepared from blood collected from 10 severe combined immunodeficient (SCID) mice. The bacteria grown in BSK-H medium were counted every 24 h until reaching late-exponential phase. The organisms grown in the mixture of BSK-H and sera were assessed for viability every 12 h for 3 days under a darkfield microscope.

**Infectivity and pathogenicity in SCID mice.** BALB/c SCID mice (age 4 to 8 weeks; provided by the LSU Division of Laboratory Animal Medicine) were given one single intradermal/subcutaneous injection of 10⁵ spirochaetes. Animals were examined for the development of arthritis at 2-day intervals, starting at 10 days; animals were sacrificed 1 month post-inoculation. Tibiotarsal joint, heart and skin (not from the inoculation site) specimens were used for spirochaete isolation, and DNA and RNA preparation. DNA was quantified for bacterial loads by quantitative PCR (qPCR), as previously described (Xu *et al.*, 2005). RNA was quantified for the mRNA copy numbers of *flaB*, *ospA*, *ospC* and *vlsE* by reverse-transcription qPCR (RT-qPCR), as described previously (Liang *et al.*, 2004a). The expression levels were presented as *ospA*, *ospC* or *vlsE* mRNA copies per 10,000 *flaB* transcripts.

**Infectivity in immunocompetent mice.** BALB/c mice each received one single intradermal/subcutaneous injection of 10⁵ spirochaetes. Some animals were euthanized 1 month post-inoculation; heart, tibiotarsal joint and skin specimens were aseptically collected for spirochaete culture as previously described (Xu *et al.*, 2005), and blood was collected for ELISAs. Other inoculated mice were euthanized 1, 2, 3 and 4 weeks post-inoculation; the inoculation site and remote skin, ear, heart and joint specimens were aseptically harvested for spirochaete isolation.

**Measurement of anti-ospA and -vlsE humoral immune response.** Specific OspA and VlsE antibody end point titres were determined by ELISAs. Ninety-six-well plates (Fisher) were coated with 100 µl of 2.0 µg ml⁻¹ recombinant OspA or VlsE per well. Sera were twofold serially diluted, starting at 1/200. Five samples drawn from naïve BALB/c mice were used as a control. The ELISA was performed as previously described (Xu *et al.*, 2006).

**Chronic infectivity.** Subgroups of five BALB/c mice were given one single intradermal/subcutaneous injection of 10⁵ spirochaetes. Mice were euthanized 4 months post-inoculation; heart, tibiotarsal joint and skin specimens were aseptically collected for spirochaete culture, as previously described (Xu *et al.*, 2005).

**Table 1.** Constructs and clones used in the study

<table>
<thead>
<tr>
<th>Construct or clone</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>pBBE22-<em>vlsE’</em></td>
<td>pBBE22 carrying promoterless <em>vlsE</em> fused with <em>flaB</em> promoter</td>
<td>Xu <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>13A</td>
<td>Cloned from <em>B. burgdorferi</em> B31 A13</td>
<td>Xu <em>et al.</em> (2007a)</td>
</tr>
<tr>
<td>ΔospA</td>
<td><em>ospA</em> mutant generated from 13A</td>
<td>Xu <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>13A/E22/C</td>
<td>13A transformed with pBBE22</td>
<td>Xu <em>et al.</em> (2007b)</td>
</tr>
<tr>
<td>13A/E22/D</td>
<td>13A transformed with pBBE22</td>
<td>Xu <em>et al.</em> (2007b)</td>
</tr>
<tr>
<td>ΔospA/ospA’/1</td>
<td><em>ospA</em> mutant expressing <em>ospA</em> driven by <em>flaB</em> promoter</td>
<td>This study</td>
</tr>
<tr>
<td>ΔospA/ospA’/2</td>
<td><em>ospA</em> mutant expressing <em>ospA</em> driven by <em>flaB</em> promoter</td>
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<td>This study</td>
</tr>
<tr>
<td>13A/vlsE’/2</td>
<td>13A expressing <em>vlsE</em> driven by <em>flaB</em> promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>
Statistical analysis. A one-way analysis of variance (ANOVA) was used, followed by a two-tailed Student’s t test to calculate a P value for each pair of treatments. Fisher’s exact test was used to analyze data on ear biopsy and frequency of tissue colonization. A P value ≤0.05 was considered to be significant.

RESULTS

Generation of B. burgdorferi with increased OspA or VlsE production

The constructs pBBE22-ospA’ and pBBE22-vlsE’ were electroporated into the 13A spirochaetes. Eleven and 19 transformants were obtained from transformation with each construct. Because clone 13A lacks lp25, a plasmid that harbours bbe22, a gene encoding a nicotinamidase essential for survival of B. burgdorferi in the mammalian environment (Purser et al., 2003), both constructs were derived from pBBE22 (Xu et al., 2008) and thus carried a copy of bbe22, and should restore the infectivity of clone 13A. Plasmid content analyses identified two clones that received each construct, namely 13A/ospA’/1, 13A/ospA’/2, 13A/vlsE’/1 and 13A/vlsE’/2. These clones had identical plasmid content: all lost cp9, lp21 and lp5, in addition to lp25 and lp56. Increased VlsE expression resulting from the introduction of pBBE22-vlsE’ was easily confirmed by immunoblotting probed with mouse anti-VlsE sera (Fig. 1a). Unfortunately, due to overwhelming expression resulting from the native ospA copy, the potential contribution of pBBE22-ospA’ to OspA production could not be demonstrated in clone 13A/ospA’/1 or clone 13A/ospA’/2 (Fig. 1a). The immunoblot also showed that introduction of the constructs did not influence OspC production.

To examine whether pBBE22-ospA’ drove ospA expression, the construct was electroporated into ΔospA. Twelve transformants were obtained; two clones, namely ΔospA/ospA’/1 and ΔospA/ospA’/2, were selectively analysed by immunoblotting. Both clones abundantly produced OspA antigen (Fig. 1b).

OspA or VlsE overproduction does not affect spirochaete growth or serum resistance in vitro

The influence of lipoprotein overproduction on spirochaete growth and serum resistance was investigated. The 13A/E22/C, 13A/ospA’/1 and 13A/vlsE’/1 spirochaetes completed a generation approximately every 7 h when grown in BSK-H, and all three genotypes survived equally well in BSK-H/sera (data not shown), indicating that overproduction of OspA or VlsE does not influence growth or serum resistance in vitro.

Constitutive ospA expression represses vlsE and ospC expression, and increased vlsE expression enhances ospA but represses ospC expression during infection of SCID mice

To confirm in vivo increased ospA and vlsE expression, subgroups of five SCID mice were challenged with 10^4 spirochaetes of the clones 13A/ospA’/1, 13A/ospA’/2, 13A/vlsE’/1 or 13A/vlsE’/2. As a control, an additional 10 mice were inoculated with the clones 13A/E22/C or 13A/E22/D. The two clones were previously generated by transforming the 13A spirochaetes with pBBE22 (Xu et al., 2007b). Joint swelling started to develop in all mice at approximately 10 days, and quickly developed to severe arthritis (data not shown), indicating that introduction of pBBE22-ospA’ or pBBE22-vlsE’ did not affect arthritis virulence.

Infected mice were euthanized 1 month post-inoculation; RNA was prepared from heart, joint and skin specimens and assessed for the relative copy numbers of ospA, ospC, vlsE and flaB mRNAs by RT-qPCR. The 13A/ospA’ bacteria accumulated ospA mRNA 3189-, 1034- and 180-fold more...
than the genotype 13A/E22 in heart ($P = 6.8 \times 10^{-12}$), joint ($P = 4.4 \times 10^{-12}$) and skin tissues ($P = 5.1 \times 10^{-15}$), respectively (Fig. 2a). However, they reduced ospC expression 49 and 22% in heart ($P = 4.9 \times 10^{-6}$) and skin ($P = 0.002$), respectively, but increased expression 10.3-fold in joint tissue ($P = 2.9 \times 10^{-9}$) (Fig. 2b). The genotype 13A/ospA' also reduced vlsE mRNA accumulation 4.9-, 5.8- and 2.1-fold in heart ($P = 5.7 \times 10^{-3}$), joint ($P = 2.7 \times 10^{-10}$) and skin tissues ($P = 0.03$), respectively (Fig. 2c).

Introduction of pBBE22-vlsE increased vlsE expression 10.2-, 2.1- and 16.7-fold over the genotype 13A/E22 in heart ($P = 3.4 \times 10^{-9}$), joint ($P = 1.4 \times 10^{-6}$) and skin tissues ($P = 2.3 \times 10^{-9}$), respectively (Fig. 2c). The pBBE22-vlsE spirochaetes increased ospA expression 62-, 39- and 4.8-fold in heart ($P = 4.4 \times 10^{-6}$), joint ($P = 6.0 \times 10^{-5}$) and skin tissues ($P = 0.001$), respectively (Fig. 2a), but reduced ospC expression 6.4-, 10.1- and 13.2-fold in heart ($P = 3.7 \times 10^{-11}$), joint ($P = 1.6 \times 10^{-5}$) and skin tissues ($P = 1.2 \times 10^{-13}$), respectively (Fig. 2b).

**Increasing expression of either ospA or vlsE shows a tissue-dependent influence on colonization in SCID mice**

DNA was prepared from the heart, joint and skin specimens harvested from the 30 infected mice described above, and quantified for tissue spirochaete burden by qPCR as an indication of tissue colonization. As shown in

![Image](http://mic.sgmjournals.org/3423)

**Fig. 2.** The influence of overexpression of ospA and vlsE on gene expression and tissue bacterial loads during infection of SCID mice. Overexpressing vlsE results in increased ospA (a) but decreased ospC expression (b), but overexpressing ospA leads to decreased ospC and vlsE expression (c). Subgroups of five BALB/c SCID mice were inoculated with $10^9$ spirochaetes of the clones 13A/E22/C, 13A/E22/D, 13A/ospA'/1, 13A/ospA'/2, 13A/vlsE'/1 and 13A/vlsE'/2. Infected mice were euthanized 1 month later. RNA samples were prepared from heart, joint and skin specimens and analysed for flaB, ospA, ospC and vlsE expression by RT-qPCR. The data are presented as ospA (a), ospC (b) or vlsE (c) mRNA copy number per 10000 flaB transcripts and in three groups by combining the subgroups 13A/E22/C and 13A/E22/D (E22), 13A/ospA'/1 and 13A/ospA'/2 (ospA'), and 13A/vlsE'/1 and 13A/vlsE'/2 (vlsE'). (d) Increasing expression of ospA or vlsE leads to a tissue-dependent effect on spirochaetal load in SCID mice. DNA samples were prepared from the heart, joint and skin specimens of the 30 mice and analysed for spirochaetal flaB and murine actin DNA copies by qPCR. The data are expressed as spirochaete numbers per $10^6$ host cells and presented in three groups by combining the subgroups 13A/E22/C and 13A/E22/D, 13A/ospA'/1 and 13A/ospA'/2, and 13A/vlsE'/1 and 13A/vlsE'/2.
Fig. 2(d), constitutive ospA expression led to a 2.2- and 1.3-fold decrease in spirochaete load in heart (P=0.03) and joint tissues (P=0.03), respectively, but a 1.7-fold increase in skin (P=1.2 × 10^{-3}). Increasing vlsE expression resulted in a 5.6-, 1.6-, and 2.2-fold decrease in spirochaete load in heart (P=0.007), joint (P=0.009) and skin tissues (P=1.8 × 10^{-6}), respectively. When the two genotypes with increased osp expression were compared, the 13A/ospA’ spirochaetes generated bacterial loads 1.6- and 3.8-fold higher than the 13A/vlsE’ bacteria in heart (P=0.005) and skin tissue (P=1.2 × 10^{-5}), but registered similar loads to those of the latter in joint tissue (P=0.16).

Increasing expression of either ospA or vlsE affects dissemination but does not alter ID50 in SCID mice

The influence of increasing ospA or vlsE expression on the ID50 value and dissemination was first investigated in immunodeficient mice. Groups of three animals each received a single inoculation of 10^{1}–10^{3} spirochaetes of the clones 13A/E22/C, 13A/E22/D, 13A/ospA’/1, 13A/ospA’/2, 13A/vlsE’/1 and 13A/vlsE’/2. Ear biopsies were taken for bacterial culture at 2 and 3 weeks post-inoculation. Animals were euthanized 1 month post-inoculation; heart, joint and skin samples were cultured for ID50 determination. No difference in dissemination was observed between the genotypes 13A/E22 and 13A/ospA’ when the doses were 10^{2} or 10^{3} organisms, as all inoculated mice in these groups produced a positive ear biopsy at 2 weeks (Supplementary Table S1). When the 10^{2} dose groups were compared, the genotype 13A/vlsE’ disseminated at a slower pace than the 13A/E22 spirochaetes, as all the 12 mice that had been inoculated with the clones 13A/E22/C or 13A/E22/D in the dose group produced an ear biopsy at 2 weeks, but only two of the 12 mice in the same dose group of the clones 13A/vlsE’/1 and 13A/vlsE’/2 gave a positive biopsy within this time frame, indicating that increasing vlsE expression impairs dissemination (P=6.7 × 10^{-5}).

The ID50 values for the six clones were measured from six to 18 organisms in repeated experiments (Supplementary Table S1). These results allowed us to conclude that constitutive expression of either ospA or vlsE does not affect the ID50 value in immunodeficient mice (P>0.05).

Increasing the expression of vlsE but not ospA leads to clearance of spirochaetes during early infection of immunocompetent mice

Subgroups of five BALB/c mice each received one single intradermal/subcutaneous injection of 10^{5} spirochaetes of the clones 13A/ospA’/1, 13A/ospA’/2, 13A/vlsE’/1, 13A/vlsE’/2, 13A/E22/C and 13A/E22/D. Animals were euthanized 1 month post-inoculation; heart, joint and skin samples were harvested for bacterial culture and blood samples collected for ELISAs. B. burgdorferi was grown from each specimen of the 10 mice that had received the clones 13A/E22/C and 13A/E22/D (Table 2). Similarly, the 13A/ospA’/1 and 13A/ospA’/2 spirochaetes were consistently recovered from all the skin samples as well as most of the heart and joint specimens of the 10 inoculated mice. However, no spirochaetes were recovered from the 10 mice that had been inoculated with the clones 13A/vlsE’/1 and

Table 2. Constitutive expression of vlsE but not ospA abrogates the ability to infect immunocompetent mice

<table>
<thead>
<tr>
<th>Clone</th>
<th>Number of cultures positive/total number of specimens examined</th>
<th>Number of mice infected/total number of mice inoculated</th>
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<tbody>
<tr>
<td></td>
<td>Heart</td>
<td>Joint</td>
</tr>
<tr>
<td>13A/E22/C</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>13A/E22/D</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>13A/ospA’/1</td>
<td>3/5</td>
<td>4/5</td>
</tr>
<tr>
<td>13A/ospA’/2</td>
<td>4/5</td>
<td>4/5</td>
</tr>
<tr>
<td>13A/vlsE’/1</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>13A/vlsE’/2</td>
<td>0/5</td>
<td>0/5</td>
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</table>

Groups of five BALB/c mice were inoculated with 10^{5} spirochaetes of the clones 13A/E22/C, 13A/E22/D, 13A/ospA’/1, 13A/ospA’/2, 13A/vlsE’/1 and 13A/vlsE’/2, and sacrificed 1 month later. Heart, tibiotarsal joint and skin specimens were harvested for spirochaete culture.
13A/vlsE'/2, suggesting that increasing vlsE expression abrogates the ability of *B. burgdorferi* to infect immunocompetent mice.

Serum samples collected from the 30 mice were analysed for humoral responses to OspA and VlsE by ELISAs. As shown in Fig. 3(a), infection with the genotype 13A/ospA' elicited an anti-OspA response 817-fold stronger than the 13A/E22 spirochaetes (*P*=0.003). The anti-OspA titre resulting from infection with the 13A/vlsE' spirochaetes was also 7.7-fold higher than that measured in the mice infected with the genotype 13A/E22 (*P*=0.02). The 13A/E22 spirochaetes elicited an anti-VlsE response 4.5- and 7.8-fold higher than that of the genotypes 13A/ospA' (*P*=1.9×10^{-4}) and 13A/vlsE' (*P*=2.2×10^{-5}), respectively.

Although the 13A/vlsE'/1 and 13A/vlsE'/2 bacteria were not grown from any inoculated mice, the significant humoral responses to OspA and VlsE resulting from inoculation with these clones suggested that the animals might have been infected and subsequently cleared infection as the specific immune responses developed. To clarify the issue, groups of 12 BALB/c mice each received a single intradermal/subcutaneous inoculation of 10^5 spirochaetes of the clones 13A/E22/C, 13A/E22/D, 13A/ospA'/1, 13A/ospA'/2, 13A/vlsE'/1 and 13A/vlsE'/2. Three animals from each group were euthanized at 1-week intervals; the inoculation site and remote skin, ear, heart and joint specimens were harvested for spirochaete isolation. As a positive control, the 13A/E22/C and 13A/E22/D bacteria disseminated quickly, and colonized all tissues within 2 weeks (Table 3). The 13A/ospA'/1 and 13A/ospA'/2 spirochaetes disseminated slowly, and all ear tissue cultures did not become positive until 4 weeks post-inoculation, although all joint samples were positive within the first week. The 13A/vlsE'/1 and 13A/vlsE'/2 bacteria were recovered from at least one tissue of each inoculated mouse within the first 3 weeks, but none of the tissues was positive at 4 weeks post-inoculation, indicating that *B. burgdorferi* with increased vlsE expression can initiate infection but is subsequently eliminated.

**Constitutive ospA expression dramatically increases ID<sub>50</sub> and severely impairs dissemination in immunocompetent mice**

The influence of constitutive ospA expression on the ID<sub>50</sub> value and dissemination was investigated in immunocompetent mice. In experiment I, groups of three animals each received one single inoculation of 10^6–10^7 spirochaetes of the clones 13A/E22/C, 13A/E22/D, 13A/ospA'/1 and 13A/ospA'/2. Ear biopsies were taken for bacterial culture at 2, 3, 4 and 5 weeks post-inoculation. Animals were euthanized 6 weeks post-inoculation; heart, joint and skin samples were cultured for spirochaetes. At 2 weeks, almost all mice that had been challenged with 10^7 or more organisms of the clones 13A/E22/C and 13A/E22/D produced a positive biopsy; the remaining mice that were found to be infected at the end of the experiment became positive earlier (Supplementary Table S2). In contrast, none of the mice inoculated with the clones 13A/ospA'/1 and 13A/ospA'/2 produced a positive biopsy at 2 weeks; most of the mice that were found to be infected at the end of the experiment did not develop a positive ear biopsy until week 4. The study allowed us to conclude that increasing ospA expression severely delays dissemination during infection of immunocompetent mice.

The ID<sub>50</sub> values of the clones 13A/E22/C and 13A/E22/D were 18 and 32 organisms, compared to 178 and 56 for the clones 13A/ospA'/1 and 13A/ospA'/2 in experiment I (Supplementary Table S2). The values were 18 and 32 for the clones 13A/E22/C and 13A/E22/D, and 178 and 178 for the clones 13A/ospA'/1 and 13A/ospA'/2, respectively, in experiment II. Taken together, the study indicated that increasing ospA expression results in a 24-fold increase in ID<sub>50</sub> in immunocompetent mice (*P*=0.007).

Another defect resulting from constitutive ospA expression was a reduced frequency in colonizing heart and joint...

![Fig. 3. Humoral responses to OspA (a) and VlsE (b). Subgroups of five BALB/c mice were inoculated with the clones 13A/E22/C, 13A/E22/D, 13A/ospA'/1, 13A/ospA'/2, 13A/vlsE'/1 and 13A/vlsE'/2, and euthanized 1 month later. Serum samples were analysed for anti-OspA and -VlsE antibody titres by end-point ELISAs. The data are presented in three groups by combining the subgroups 13A/E22/C and 13A/E22/D, 13A/ospA'/1 and 13A/ospA'/2, and 13A/vlsE'/1 and 13A/vlsE'/2.](http://mic.sgmjournals.org)
tissues. The 13A/ospA'/1 and 13A/ospA'/2 spirochaetes were grown from only 10 heart and 15 joint specimens of the 23 infected mice; in contrast, the clones 13A/E22/C and 13A/E22/D were recovered from each specimen from all of the 26 infected mice (Supplementary Table S2). Overall, constitutive ospA expression led to a 67 and 35 % decrease in frequency of colonizing heart \((P=4.4 \times 10^{-6})\) and joint tissues \((P=1.1 \times 10^{-7})\), respectively.

**DISCUSSION**

In the current study, *B. burgdorferi* was modified to increase expression of two well-characterized surface lipoproteins, OspA and VlsE, leading to dramatic changes in infectious behaviour. Increasing the expression of either ospA or vlsE did not alter the ID\(_{50}\) value, but affected spirochaetal dissemination and significantly reduced tissue spirochaete loads in SCID mice. In immunocompetent mice, increased vlsE expression resulted in quick clearance of infection, while constitutive ospA expression led to a substantial ID\(_{50}\) increase and severely impaired dissemination. The study also showed that *B. burgdorferi* with constitutive ospA expression persisted in skin tissue but was cleared from both heart and joints of chronically infected immunocompetent mice. Overall, the study highlights a relationship between the surface antigen expression and the infectious behaviour of *B. burgdorferi*.

Both ospA and vlsE use \(a^{70}\)-dependent promoters, while the ospC promoter is RpoS-dependent (Hubner et al., 2001). Within mammals, however, ospA expression is essentially repressed, while vlsE expression is upregulated, especially during chronic infection of immunocompetent hosts (Liang et al., 2004b). Nothing is known about how this could occur. Nevertheless, our RT-qPCR results showed that constitutive ospA expression repressed vlsE and ospC expression, and that increased vlsE expression upregulated ospA but down-regulated ospC in infected SCID mice. The same scenario also appeared to occur in infected immunocompetent mice, as *B. burgdorferi* with constitutive ospA expression elicited a weaker anti-VlsE response, but bacteria with increased vlsE expression stimulated a stronger anti-OspA response than

**Table 3.** *B. burgdorferi* with constitutive *vlsE* expression is cleared during early infection of immunocompetent mice

Groups of 12 BALB/c mice each received a single intradermal/subcutaneous injection of \(10^5\) organisms of the clones 13A/E22/C, 13A/E22/D, 13A/ospA'/1, 13A/ospA'/2, 13A/vlsE'/1 and 13A/vlsE'/2. Three animals from each group were euthanized at 1, 2, 3 and 4 weeks post-inoculation; inoculation site (I.S.) and remote site (R.S.) skin, ear, heart and joint specimens were harvested for spirochaete isolation. The I.S. site was at the chest; therefore, the R.S. site was at the back of mice.

**Table 4.** *B. burgdorferi* with increased *ospA* expression persists only in the skin tissue of chronically infected immunocompetent mice

Groups of five BALB/c mice were inoculated with the clones 13A/E22/C, 13A/E22/D, 13A/ospA'/1 and 13A/ospA'/2, and sacrificed 4 months later. Heart, tibiotarsal joint and skin specimens were harvested for spirochaete culture.
To initiate an infection, *B. burgdorferi* must be able to evade innate immunity and colonize local tissues of the inoculation site; this ability was measured by ID₅₀ values in immunodeficient mice. Apparently, increasing *ospA* or *vlsE* expression did not affect this ability, as increasing the expression of either did not change the value in SCID mice. However, increasing *vlsE* expression slightly impaired dissemination, and constitutive *ospA* expression apparently facilitated the process in the absence of adaptive immunity, underscoring the importance of surface antigen expression in spirochaetal dissemination. Our previous study showed that increasing expression of DpbA severely impairs dissemination (Xu *et al.*, 2007b). Even more dramatically, overproducing DpbA restraints OspC-deficient *B. burgdorferi* to tissues at the inoculation site for 4 weeks without dissemination to distal tissues, further highlighting the influence of surface lipoprotein expression on dissemination (Xu *et al.*, 2008). OspC may function as a dissemination-facilitating factor (Xu *et al.*, 2008), and has been found to bind plasminogen (Lagal *et al.*, 2006). Interestingly, OspA is a primary plasminogen receptor (Fuchs *et al.*, 1994), and *in vitro* evidence shows that OspA helps *B. burgdorferi* to acquire plasminogen (O'Connell *et al.*, 1995; Hu *et al.*, 1995; Klempern *et al.*, 1996). Plasmin, a trypsin-like serine protease, is generated by limited proteolysis from its serum-derived zymogen precursor plasminogen by the urokinase-type plasminogen activator or tissue-type plasminogen activator. An *in vitro* study has shown that *B. burgdorferi* coated with plasmin gains a proteolytic activity to effectively degrade components of the mammalian extracellular matrix (O'Connell *et al.*, 1999), and an animal study has indicated that plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochaetemia in mice (O'Connell *et al.*, 1997). It remains to be addressed whether facilitated dissemination due to constitutive *ospA* expression results from gaining host plasminogen.

As an extracellular pathogen, *B. burgdorferi* resides primarily in the extracellular matrix and connective tissues as well as between host cells during mammalian infection. The influence of surface antigen expression on this aspect was reflected by tissue bacterial load. Increasing expression of either *ospA* or *vlsE* negatively affected the ability of *B. burgdorferi* to colonize most tissues. This adverse effect of high *vlsE* expression underscores the biological significance of low expression during early infection. *B. burgdorferi* does not actively express *vlsE* but increases expression after dissemination into joint tissues in the absence of adaptive immune responses (Crother *et al.*, 2003; Liang *et al.*, 2004b). Low *vlsE* expression may also allow bacteria to more efficiently disseminate to distal tissues, as the current study showed that increasing *vlsE* expression led to slow dissemination in SCID mice. After dissemination, humoral responses greatly upregulate *vlsE* in all tissues (Crother *et al.*, 2004; Liang *et al.*, 2004b), an event consistent with the critical role of VlsE in immune evasion during chronic infection of immunocompetent hosts (Bankhead & Chaconas, 2007; Zhang *et al.*, 1997).

Naive and immune statuses constitute two distinct environments to microbial pathogens. The current study used immunodeficient and immunocompetent mice to provide these environments in order to study the influence of increased surface lipoprotein expression on infectious behaviour. Increasing the expression of *ospA* or *vlsE* did not affect the ID₅₀ value in SCID mice. In sharp contrast, increased *vlsE* expression diminished the ability to infect immunocompetent mice. Although a less significant effect attributed to increased *ospA* expression was observed in immunocompetent mice, *B. burgdorferi* with constitutive *ospA* expression registered a substantial ID₅₀ increase and disseminated at a much slower pace. Even more dramatically, constitutive *ospA* expression diminished the ability to persist in both heart and joint tissues during chronic infection of immunocompetent mice.

An earlier study has shown that simultaneous constitutive expression of OspA and OspB abrogates infectivity in immunocompetent mice (Strother *et al.*, 2007). In the current study, although constitutive *ospA* expression significantly increased the ID₅₀ value and severely impaired dissemination, recombinant spirochaetes were able to persist in the skin tissue of chronically infected immunocompetent mice. Co-expressing OspA and OspB gives the immune system two targets, so that recombinant spirochaetes may be quickly cleared by the humoral responses after inoculation into immunocompetent mice. Alternatively, OspB is more effectively targeted than OspA by protective immunity, so that the anti-OspB response alone is sufficient to clear infection.

Increasing the expression of a lipoprotein appears to be an efficient way to test whether the specific antigen is effectively targeted by protective immunity. To date, four surface lipoproteins, OspC, DpbA, OspA and VlsE, have been individually examined in this way (Xu *et al.*, 2006, 2007b). *B. burgdorferi* modified with increased expression of *dbpA* or *ospA* can persist in the skin tissue of chronically infected immunocompetent mice (Xu *et al.*, 2007b). Although constitutive *ospC* expression diminishes the ability to cause persistent infection, recombinant spirochaetes are consistently recovered from immunocompetent mice for at least a month after infection (Xu *et al.*, 2006). In contrast, *B. burgdorferi* with constitutive invariant *vlsE* expression is cleared within a month after inoculation into immunocompetent mice, although the production of invariant VlsE antigen under the control of its own promoter allows spirochaetes to cause persistent infection (Lawrenz *et al.*, 2004). Taken together, these studies indicate that VlsE, when no antigenic variation occurs, is the most effective target of protective immunity.

*B. burgdorferi* actively modifies its surface antigen expression in order to better adapt to the tissue microenvironment.
during the course of mammalian infection. The current study shows that artificially increasing surface antigen expression alters infectious behaviour in a negative way, highlighting the importance of the coordination of surface antigen expression in the pathogenesis of *B. burgdorferi*.

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