

# Constitutive Expression of Outer Surface Protein C Diminishes the Ability of *Borrelia burgdorferi* To Evade Specific Humoral Immunity

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**The Lyme disease spirochete *Borrelia burgdorferi* reduces the expression of outer surface protein C (OspC) in response to the development of an anti-OspC humoral response, leading to the hypothesis that the ability to repress OspC expression is critical for the pathogen to proceed to chronic infection. *B. burgdorferi* was genetically modified to constitutively express OspC by introducing an extra *ospC* copy fused with the borrelial flagellar gene (*flaB*) promoter. Such a genetic modification did not reduce infectivity or pathogenicity in severe combined immunodeficiency mice but resulted in clearance of infection by passively transferred OspC antibody. Spirochetes with constitutive *ospC* expression were unable to establish chronic infections in immunocompetent mice unless they had undergone very destructive mutations in the introduced *ospC* copy. Two escape mutants were identified; one had all 7 bp deleted between the putative ribosome-binding site and the start codon, ATG, causing a failure in translational initiation, and the other mutant had an insertion of 2 bp between nucleotides 315 and 316, resulting in a nonsense mutation at codon 108. Thus, the ability of *B. burgdorferi* to repress *ospC* expression during mammalian infection allows the pathogen to avoid clearance and to preserve the integrity of the important gene for subsequent utilization during its enzootic life cycle.**

The genome of the Lyme disease spirochete *Borrelia burgdorferi* encodes more than 100 lipoproteins (6, 15). Many lipoproteins have been identified as targets of protective antibodies, including outer surface protein A (OspA) and OspC (14, 16, 34). Lipoproteins stimulate innate responses via Toll-like receptors, enhancing both humoral and cellular immune responses (1, 48). However, *B. burgdorferi* is able to cause persistent infection despite the development of vigorous immune responses against the pathogen (40).

Deliberate regulation of antigen expression is essential for spirochete survival in various environments. *B. burgdorferi* is maintained within a complex enzootic life cycle involving the tick vector *Ixodes scapularis* and a mammal (5). The up-regulation of OspA is crucial for colonization of ticks by *B. burgdorferi* (10, 39, 50), while the down-regulation of this lipoprotein during mammalian infection is important for continuation of the enzootic cycle, as its expression triggers an anti-OspA immune response effectively blocking acquisition by the vector (9, 46, 47).

*B. burgdorferi* up-regulates OspC when induced by a fresh blood meal, a response that may be essential for the organism to migrate into tick salivary glands and initiate a mammalian infection (17, 33). However, its expression is also affected by the host immune status. The antigen is abundantly expressed only in the absence of the adaptive immune response (7, 8, 24, 26, 28). *ospC* expression is dramatically reduced either by passive OspC antibody transfer in SCID mice or by the specific immune response induced during infection of an immunocompetent host (7, 24, 26, 28). We hypothesized that the down-regulation of OspC in response to the development of specific

immune pressure is an effective strategy for *B. burgdorferi* to proceed to chronic infection. To test this hypothesis, the *ospC* coding region was fused with the flagellin gene (*flaB*) promoter, and the consequence of constitutive *ospC* expression was explored in both immunodeficient and immunocompetent murine models.

## MATERIALS AND METHODS

**Construction of pBBE22-*ospC*'.** A 251-bp fragment of the *flaB* promoter region was amplified by PCR with the use of the primer pair 5'-AGAAGTACG AAGATAGAGAGAGAAA-3 (forward) and 5'-AACACATATGTCATTCTCCATGATAAA-3 (reverse). An 895-bp fragment extending from the ATG translational start codon to the 262-bp sequence downstream of the stop codon of the *ospC* gene was amplified with the use of another primer pair (forward, 5'-CCACCATATGAAAAAGAATACATTAAGT-3'; reverse, 5'-CCGTTTAA GCCTACTTAAAGTCT-3'). (The underlined sequences are NdeI restriction sites.) The two PCR products were pooled, purified by using a QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA), digested with NdeI, repurified, and ligated. The resultant product was used as a template and amplified by nested PCR with the use of a third primer pair (forward, 5'-ATAGGATCCAA GATAGAGAGAGAAAAGT-3'; reverse, 5'-TTCCTCTAGAGAAGAGCTTA AAGTTAA-3') (the underlined sequences are BamHI [forward] and XbaI [reverse] sites). The PCR product was purified, digested with BamHI and XbaI, and cloned into the recombinant plasmid pBBE22 (a gift from S. Norris) (35). The insert and flanking regions within the recombinant plasmid were sequenced to ensure that the construct was as designed. The generated plasmid was designated pBBE22-*ospC*'.

**Generation of transformants.** *B. burgdorferi* B31 5A13 spirochetes (a gift from S. Norris) were transformed with the recombinant plasmid pBBE22 or pBBE22-*ospC*' as described previously (38, 43, 49). Transformants were identified by PCR using a primer pair specific for the kanamycin cassette (49). The plasmid contents of transformants were surveyed by PCR and confirmed by microarray hybridization using previously reported primers and protocols (49).

**Replacement of pBBE22-*ospC*' with pGE22.** After the recombinant plasmid pBBE22 was digested with Acc65I, the BB22 insert was purified and cloned into the shuttle vector pBSV2G (a gift from P. Rosa and P. Stewart), which carries a gentamicin cassette (11), to construct the recombinant plasmid pGE22. A transformant that contained the recombinant plasmid pBBE22-*ospC*' was transformed with pGE22. Resultant transformants were screened in gentamicin-supplemented Barbour-Stoenner-Kelly H (BSK-H) complete medium (Sigma Chemical Co., St. Louis, MO) as described previously (49). Gentamicin-resistant

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clones were analyzed for the absence of pBBE22-*ospC'* as described above. Only transformants that contained pGE22 but had lost pBBE22-*ospC'* were examined for plasmid content by PCR as previously described (49).

**Preparation of recombinant OspC and generation of mouse anti-OspC sera.** The coding region, excluding the signal peptide coding sequence, of the *ospC* gene was PCR amplified, cloned into the expression vector pET16b, and transformed into *Escherichia coli* strain BL21(DE3) (Novagen, La Jolla, CA). The recombinant protein was purified using a Hi-Trap affinity column (Amersham Pharmacia Biotech, Piscataway, NJ). The protein purity and concentration were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a protein assay kit (Bio-Rad Laboratories, Richmond, CA), respectively. Approximately 70 µg recombinant protein was dissolved in 100 µl of phosphate-buffered saline (PBS; pH 7.3), emulsified with 30 µl of Freund's complete (first injection) or incomplete (remaining injections) adjuvant, and subcutaneously administered into each BALB/c mouse (age, 5 to 8 weeks) at 3-week intervals. Mice were euthanized 3 weeks after the last immunization for antiserum preparation.

**In vitro characterization of transformants and isolates recovered from infected mice.** Transformants or isolates were grown in BSK-H complete medium to late exponential phase at 23°C, 33°C, or 37°C and harvested by centrifugation. RNA samples were quantified for cDNA copy numbers of *flaB* and *ospC* by reverse transcription-quantitative PCR (RT-qPCR) as previously described (28). Spirochetes were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, separated by electrophoresis, and electrotransferred onto a nitrocellulose membrane (23). Blots were probed with a mixture of FlaB and OspC monoclonal antibodies (MAbs) or a mixture of FlaB MAb and mouse antiserum raised against a recombinant OspC antigen. The FlaB and OspC MAbs were developed by Barbour et al. (3) and Mbow et al. (30), respectively.

**Infectivity and pathogenicity study.** Groups of 10 SCID mice in the BALB/c background (Harlan, Indianapolis, IN) were given single intradermal/subcutaneous injections of 10<sup>4</sup> transformed spirochetes. Animals were examined for the development of arthritis at 2-day intervals, starting at 2 weeks, and sacrificed at 1 month postinoculation. Joint, heart, and skin specimens (not from the inoculation site) were used for DNA and RNA preparation. DNAs were quantified for copy numbers of the *flaB* and murine actin genes by qPCR as previously described (49). The tissue spirochete burden was expressed as *flaB* DNA copies per 10<sup>6</sup> host cells (2 × 10<sup>6</sup> actin DNA copies). RNAs were converted to cDNAs and quantified for mRNA copy numbers of *flaB* and *ospC* by qPCR as described above.

**Passive immunization of infected SCID mice with OspC MAb.** Groups of 10 SCID mice were inoculated with transformants as described above and received six subcutaneous injections of 100 µg OspC MAb (30) or mouse immunoglobulin G2a (IgG2a; Sigma) as a control on days 12, 14, 16, 18, 20, and 22. Mice were sacrificed 3 days after the last passive immunization. Heart, tibiotarsal joint, and skin specimens were collected aseptically for spirochete culture as previously described (49).

**Acute infectivity study.** Groups of five BALB/c mice (provided by the Division of Laboratory Animal Medicine, Louisiana State University) were inoculated with transformants as described above. Retro-orbital blood was drawn to monitor the anti-OspC response on day 10 by an enzyme-linked immunosorbent assay (ELISA), as described below; mice were sacrificed at 19 days postinoculation. Joint, heart, and skin specimens (not from the inoculation site) were used for DNA and RNA preparation; sera were collected for ELISA. The tissue bacterial burden and *ospC* expression level were analyzed by qPCR and RT-qPCR as described above.

**Chronic infectivity study.** Groups of three to eight BALB/c mice were inoculated with transformants as described above. In some experiments, ear biopsies were taken for spirochete culture by using a 2.0-mm punch, and retro-orbital blood was drawn to monitor the anti-OspC response at intervals of 2 to 4 weeks, starting on day 14 postinoculation. Mice were sacrificed 5 months later; heart, tibiotarsal joint, and skin specimens were collected aseptically for spirochete culture as previously described (49).

**Measurement of anti-OspC humoral immune response.** Specific OspC antibody end-point titers were determined by ELISA. Ninety-six-well plates (Corning Inc., Corning, NY) were coated with 100 µl of 2.0-µg/ml recombinant OspC per well. Sera were serially diluted twofold, starting at 1/200. Five samples drawn from naive BALB/c mice were used as a control. ELISA was performed as previously described (23).

**Identification of escape mutations.** Spirochetes were recovered from mice that had been inoculated with a transformant with constitutive *ospC* expression, grown to late exponential phase in BSK-H complete medium at either 23°C or 37°C, and harvested by centrifugation. Lysates were subjected to an immunoblot analysis of OspC expression as described above. Selected isolates were grown to

TABLE 1. Constitutive *ospC* expression results in spirochetal clearance by passively transferred OspC MAb in SCID mice<sup>a</sup>

Transformant	Missing plasmids	Antibody received	No. of positive cultures/total no. of specimens examined			
			Heart	Joint	Skin	All sites
5A13/pBBE22	cp9, lp25, lp56, lp21	IgG2a	5/5	5/5	5/5	15/15
		OspC MAb	5/5	5/5	5/5	15/15
5A13/pBBE22- <i>ospC'</i>	cp9, lp25, lp56, lp21, lp5	IgG2a	5/5	5/5	5/5	15/15
		OspC MAb	0/5	0/5	0/5	0/15

<sup>a</sup> Groups of 10 BALB/c SCID mice were inoculated with transformant 5A13/pBBE22 or 5A13/pBBE22-*ospC'*, passively immunized with either six doses of OspC MAb or mouse IgG2a as a control, and sacrificed 3 days after the last passive transfer. Heart, tibiotarsal joint, and skin specimens were harvested and cultured for spirochetes in BSK-H complete medium.

stationary phase at 33°C, and total DNA was extracted by using a DNeasy tissue kit (QIAGEN). *E. coli* DH5α competent cells (Invitrogen Life Technologies, Carlsbad, CA) were transformed with spirochetal DNA by heat shock. Three to five kanamycin-resistant colonies were randomly selected from each transformation experiment and sequenced for the introduced *ospC* copy and the fused *flaB* promoter.

**Statistical analysis.** Data were analyzed using Microsoft Excel (Redmond, WA) software. A two-tailed Student *t* test was used to analyze RT-qPCR and qPCR data. *P* values of ≤0.05 were considered significant.

## RESULTS

**Generation and in vitro characterization of transformants with constitutive *ospC* expression.** *B. burgdorferi* B31 5A13 spirochetes were transformed with the recombinant plasmid pBBE22 or pBBE22-*ospC'*; 12 and 9 transformants, respectively, were obtained from transformation experiments with the plasmids. Plasmid content analyses by PCR and microarray hybridization identified transformants 5A13/pBBE22, from the 12 clones that received pBBE22, and 5A13/pBBE22-*ospC'*, from the 9 clones that were transformed with pBBE22-*ospC'*, for further analysis because these two clones harbored all of the plasmids important for infection and also shared similar plasmid profiles. Both had lost cp9, lp25, lp56, and lp21; 5A13/pBBE22-*ospC'* was also lacking lp5 (Table 1).

Next, we examined whether the fused *flaB* promoter functioned properly. It is well documented that the activity of the native *ospC* promoter is temperature dependent when spirochetes are grown in vitro (2, 37, 42), providing a scheme for assessing the contribution of the fused *flaB* promoter to lipoprotein expression. 5A13/pBBE22 and 5A13/pBBE22-*ospC'* spirochetes were grown to late exponential phase at 23°C and 37°C; the accumulation of *ospC* and *flaB* transcripts was then analyzed by RT-qPCR. The 5A13/pBBE22 spirochetes matched every 1,000 *flaB* transcripts with only 1.5 *ospC* mRNAs on average, indicating that the native promoter is essentially silent, at 23°C, but the number increased to 45 *ospC* transcripts when spirochetes were grown at 37°C (Fig. 1A). In contrast, 5A13/pBBE22-*ospC'* spirochetes matched every 1,000 *flaB* mRNAs with 17,800 and 5,300 *ospC* transcripts at 23°C and 37°C, respectively, indicating that the fused *flaB* promoter is very active at both temperatures (Fig. 1A). The addition of the *flaB* promoter increased *ospC* mRNA copy numbers 12,000- and 120-fold (*P* values were 0.01 and 0.02, respectively) when spirochetes were grown at 23°C and 37°C, respectively. OspC

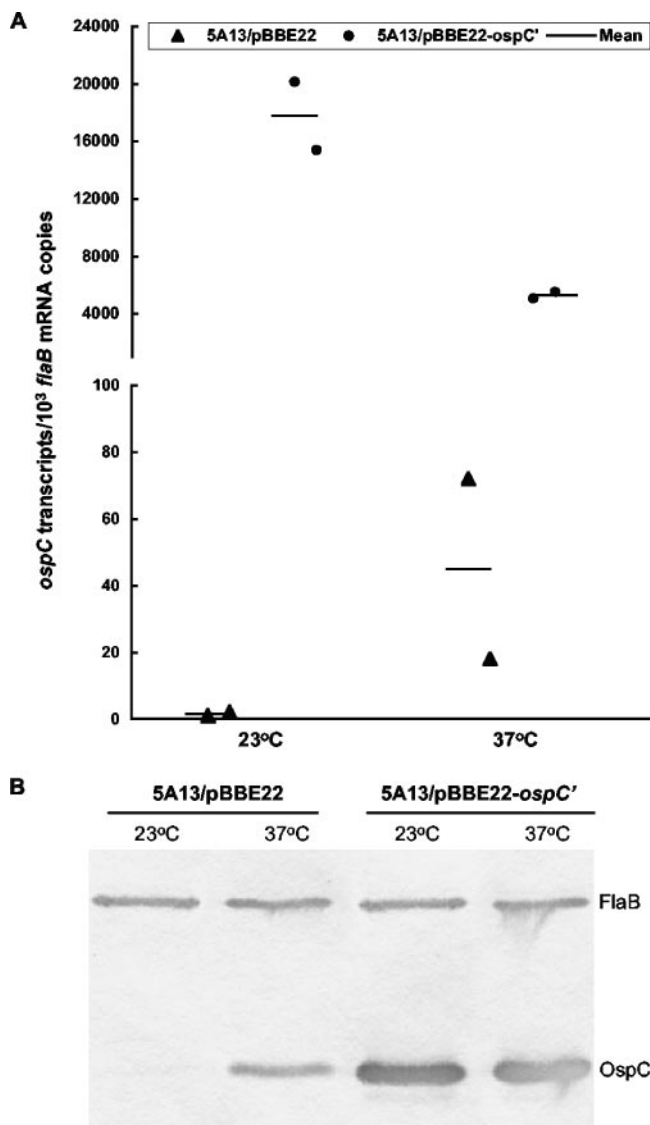


FIG. 1. Fused *flaB* promoter leads to constitutive *ospC* expression in cultured *B. burgdorferi*. (A) Constitutive *ospC* expression at the transcriptional level. Transformants 5A13/pBBE22 and 5A13/pBBE22-*ospC'* were grown to late exponential phase at 23°C and 37°C. RNA samples were analyzed by RT-qPCR for *flaB* and *ospC* mRNA copy numbers. Each datum point was calculated from a triplicate experiment and is presented as the mean *ospC* mRNA copy number per 1,000 *flaB* transcripts. (B) Constitutive *ospC* expression at the translational level. Spirochetes were prepared as described for panel A, and lysates were subjected to immunoblotting with a mixture of FlaB and OspC MAbs.

expression was also analyzed at the translational level by immunoblotting. When grown at 23°C, 5A13/pBBE22 produced too few OspC molecules for immunoblot detection; however, when the temperature was increased to 37°C, expression increased dramatically (Fig. 1B), consistent with the RT-qPCR data (Fig. 1A). 5A13/pBBE22-*ospC'* accumulated a significant amount of the antigen at both temperatures, a result that not only is consistent with the RT-qPCR data but also indicates that mRNAs transcribed from the introduced *ospC* copy are appropriately translated into protein.

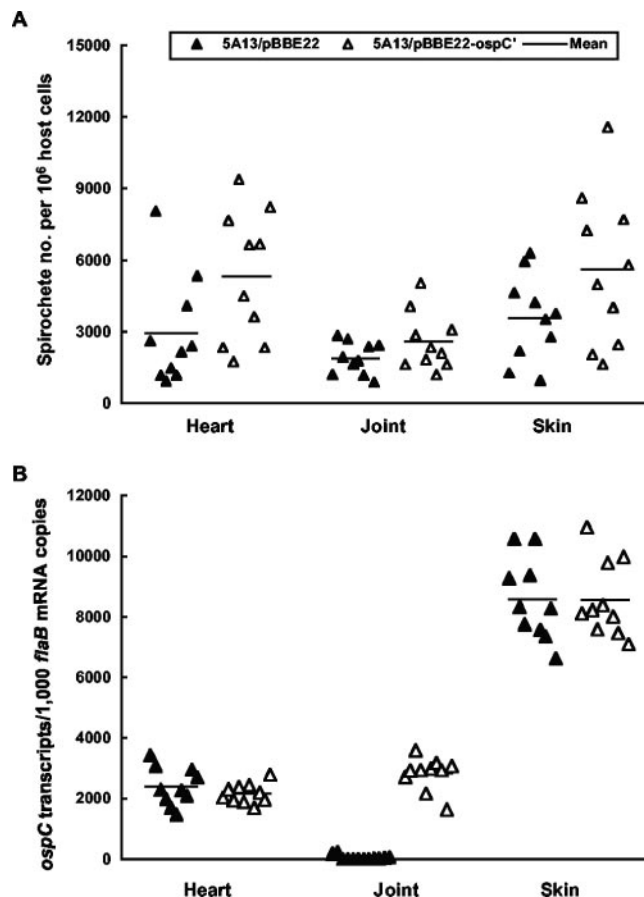


FIG. 2. Infectivity and *ospC* expression of transformants in SCID mice. (A) Groups of 10 BALB/c SCID mice were infected with either transformant 5A13/pBBE22 or 5A13/pBBE22-*ospC'* for 1 month. DNA samples were prepared from heart, joint, and skin specimens and analyzed for spirochete *flaB* and murine actin DNA copies by qPCR. The data are expressed as spirochete numbers per  $10^6$  host cells. (B) RNA samples were prepared from the heart, joint, and skin tissues and quantified for *flaB* and *ospC* expression by RT-qPCR. The data are presented as *ospC* mRNA copy numbers per 1,000 *flaB* transcripts.

**Constitutive *ospC* expression does not reduce infectivity or pathogenicity in SCID mice.** An immunodeficient host was used to address the specific issue of whether *ospC* expression reduces infectivity or pathogenicity because 5A13/pBBE22-*ospC'* spirochetes might not be able to effectively evade adaptive immunity. Groups of 10 SCID mice were challenged with either 5A13/pBBE22 or 5A13/pBBE22-*ospC'* spirochetes. Severe joint swelling evolved in each of the 20 mice between 2 and 3 weeks postinoculation, regardless of whether they received 5A13/pBBE22 or 5A13/pBBE22-*ospC'* spirochetes (data not shown). The tissue spirochete burdens were determined as an indication of infectivity. DNAs were prepared from heart, joint, and skin specimens from the 20 mice and quantified by qPCR (Fig. 2A). The 5A13/pBBE22-*ospC'* spirochete load was 40% higher in the heart tissue than the 5A13/pBBE22 load ( $P = 0.05$ ), while there was no significant difference in the joint or skin tissues from the two groups ( $P$  values were 0.14 and 0.10, respectively). This study indicates that constitutive *ospC* expression does not reduce infectivity

in either joint or skin tissues, but rather increases the spirochete load in the heart when the adaptive immune response is absent.

The *in vivo* activity of the fused *flaB* promoter was also assessed by analyzing the relative copy numbers of *ospC* and *flaB* mRNAs by RT-qPCR. Transformant 5A13/pBBE22 exhibited differential tissue expression of the lipoprotein gene (Fig. 2B). The native *ospC* promoter was very active in both heart and skin tissues, resulting in ratios of *ospC* to *flaB* transcripts of approximately 2.4:1 to 8.5:1. The native *ospC* promoter's low activity in the joint tissue, where the ratio of *ospC* to *flaB* transcripts measured in 5A13/pBBE22 was only 0.05:1, showed a significant contribution of the fused *flaB* promoter to *ospC* expression. In the same type of tissue, the amount of *ospC* mRNA accumulated by 5A13/pBBE22-*ospC'* spirochetes was 56-fold more than that accumulated by 5A13/pBBE22 spirochetes ( $P < 1.6 \times 10^{-13}$ ), indicating that the fused promoter functions well. Due to the high activity of the native *ospC* promoter, however, no significant difference in the amounts of *ospC* mRNA produced in the two transformants could be detected for either the heart or skin tissue ( $P$  values were 0.30 and 0.99, respectively).

**5A13/pBBE22-*ospC'* spirochetes are unable to evade clearance by passively transferred OspC MAb in SCID mice.** The consequence of constitutive *ospC* expression was first investigated in a passive transfer study. Two groups of 10 SCID mice each were inoculated with either transformant 5A13/pBBE22 or 5A13/pBBE22-*ospC'*. Two weeks later, after spirochetes disseminated and infection was established, five mice from each group were administered either OspC MAb or mouse IgG2a as a control. Spirochetes were recovered from each specimen from all 10 mice that had been inoculated with 5A13/pBBE22, regardless of whether they received the OspC MAb or IgG2a, but were only recovered from the specimens of the five 5A13/pBBE22-*ospC'*-infected mice that were given the isotype control (Table 1). Transferred OspC antibody cleared the 5A13/pBBE22-*ospC'* infection in each tissue of all five mice, indicating that constitutive *ospC* expression abrogates the ability of spirochetes to evade clearance by specific antibody.

**5A13/pBBE22-*ospC'* spirochetes evade the immune system less effectively than 5A13/pBBE22 spirochetes during early infection of immunocompetent mice.** Ten BALB/c mice were inoculated with either 5A13/pBBE22 or 5A13/pBBE22-*ospC'* to examine the influence of constitutive *ospC* expression on early infection in immunocompetent mice. Retro-orbital blood was drawn for assessment of the immune response on day 10; mice were sacrificed at 19 days postinoculation. 5A13/pBBE22-*ospC'* elicited a 2- to 4-fold stronger anti-OspC humoral response than did 5A13/pBBE22 within 10 days postinoculation, which increased to a 16-fold difference at the end of the experiment (Fig. 3A), suggesting that increased OspC expression resulted from the introduction of the extra *ospC* copy. Consistent with this explanation, RT-qPCR showed that 5A13/pBBE22-*ospC'* spirochetes maintained constitutive *ospC* expression (Fig. 3B). In contrast, *ospC* transcription in 5A13/pBBE22 was dramatically reduced by the immune response elicited during infection. 5A13/pBBE22-*ospC'* spirochetes accumulated *ospC* transcripts 140, 48, and 119 times more than did 5A13/pBBE22 spirochetes in the heart ( $P = 4.4 \times 10^{-5}$ ),

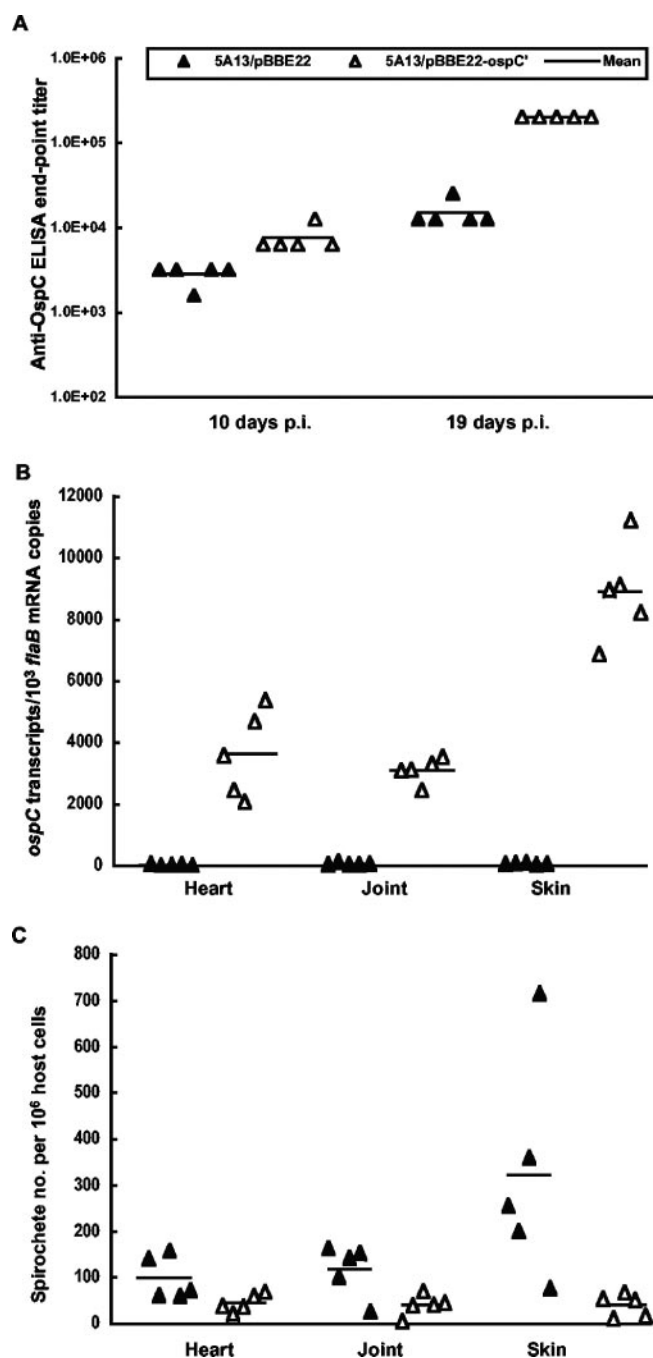


FIG. 3. 5A13/pBBE22-*ospC'* spirochetes evade the immune system less effectively than 5A13/pBBE22 spirochetes during early infection. (A) Groups of 10 BALB/c mice were inoculated with either transformant 5A13/pBBE22 or 5A13/pBBE22-*ospC'* and sacrificed on day 19 postinoculation. The anti-OspC humoral response was titrated by an end-point ELISA, using sera collected on days 10 and 19 postinoculation (p.i.). (B) RNA samples were prepared from heart, joint, and skin specimens from mice sacrificed on day 19 postinoculation and quantified for *flaB* and *ospC* expression by RT-qPCR. The data are presented as *ospC* mRNA copy numbers per 1,000 *flaB* transcripts. (C) DNA samples were prepared from heart, joint, and skin specimens and analyzed for spirochete *flaB* and actin DNA copies by qPCR. The data are expressed as spirochete numbers per 10<sup>6</sup> host cells.

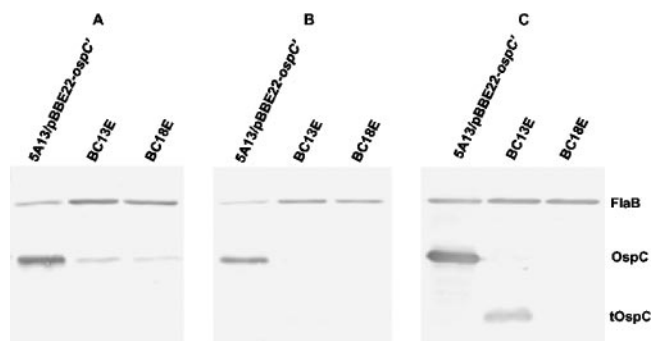


FIG. 4. Recovered spirochetes no longer produce an antigen reactive with OspC MAb from the introduced *ospC* copy. Spirochetes were isolated from ear biopsies of mice BC13 and BC18, which had been infected with transformant 5A13/pBBE22-*ospC'* for 17 weeks, designated BC13E and BC18E, respectively, and grown to late exponential phase at either 37°C (A) or 23°C (B and C). The initial inoculum, 5A13/pBBE22-*ospC'*, was used as a control. Lysates were subjected to immunoblot analysis with either a mixture of FlaB and OspC MAbs (A and B) or a mixture of FlaB MAb and mouse antisera raised against recombinant OspC (C). tOspC, truncated OspC antigen.

joint ( $P = 1.7 \times 10^{-7}$ ), and skin ( $P = 1.6 \times 10^{-6}$ ) tissues, respectively (Fig. 3B). As a result of constitutive *ospC* expression, the 5A13/pBBE22-*ospC'* spirochete burden was 54%, 66%, and 87% lower than the 5A13/pBBE22 burden in the heart ( $P = 0.047$ ), joint ( $P = 0.021$ ), and skin ( $P = 0.033$ ) tissues, respectively (Fig. 3C).

**Spirochetes with constitutive *ospC* expression either are cleared or have undergone escape mutations.** Two groups of eight BALB/c mice each were inoculated with either 5A13/pBBE22 or 5A13/pBBE22-*ospC'* to further examine the consequence of constitutive *ospC* expression in immunocompetent mice. Ear biopsies were taken for bacterial culture, and retro-orbital blood was drawn for assessment of the anti-OspC humoral response every 2 to 4 weeks postinoculation. 5A13/pBBE22-*ospC'* elicited an 8- to 16-fold stronger anti-OspC humoral response than did 5A13/pBBE22 at each time point (data not shown). Spirochetes were successfully isolated from all of the mice that received 5A13/pBBE22 at each of the time points; however, from the mice that were inoculated with 5A13/pBBE22-*ospC'*, spirochetes were recovered consistently only during the first 8 weeks (data not shown). Six of the eight mice that had been inoculated with the transformant became negative by culture after 11 weeks of infection. However, *B. burgdorferi* was consistently recovered from the remaining two mice, designated BC13 and BC18, for the remaining time points, indicating that chronic infection had been established.

To determine whether escape mutations might have occurred in the introduced gene copy, the OspC expression of spirochetes isolated from the ear biopsies of mice BC13 and BC18 was analyzed by immunoblotting. When grown at 37°C, both isolates BC13E and BC18E, recovered from mice BC13 and BC18, respectively, produced much less antigen reactive with OspC MAb than the original inoculum (Fig. 4A), suggesting that mutations may have occurred in the extra *ospC* copy. To eliminate the contribution to OspC expression by the native gene copy, cells were grown to late exponential phase at 23°C. As presented in Fig. 4B, in contrast to the initial inoculum,

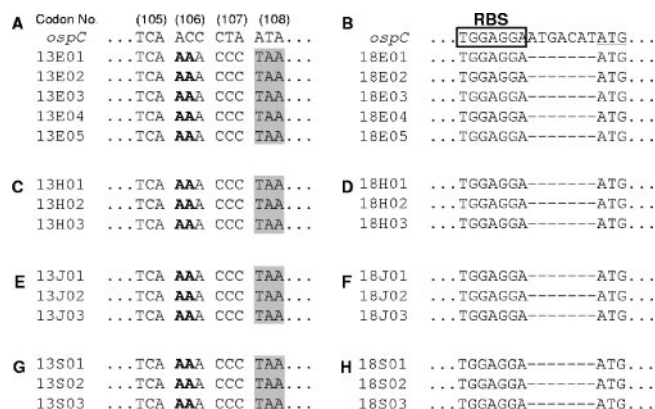


FIG. 5. Escape mutants have destructive mutations in the introduced *ospC* copy. Spirochetes were isolated from ear biopsies of mice BC13 and BC18 at 17 weeks postinoculation. The two mice were sacrificed after 5 months of infection; spirochetes were recovered from the heart, joint, and skin specimens. DNAs were extracted from these eight isolates and used to transform *E. coli*. Three to five kanamycin-resistant clones transformed with each DNA preparation were randomly selected and sequenced. In the four panels of the left column, the sequences represent isolates recovered from the ear biopsy (A) and the heart (C), joint (E), and skin (G) specimens of mouse BC13. All isolates obtained from this mouse had a 2-bp insertion (in bold) between nucleotides 315 and 316, producing a nonsense mutation, TAA (shaded), at codon 108. In the four panels of the right column, the sequences represent isolates from the ear biopsy (B) and the heart (D), joint (F), and skin (H) specimens of mouse BC18. All isolates recovered from this animal had 7 bp deleted between the putative ribosome-binding site (RBS) and the initial start codon, ATG (underlined).

both isolates lacked an OspC antigen reactive with the MAb, indicating that mutations had occurred. Since a MAb recognizes a single epitope, a mild mutation, such as an amino acid substitution, insertion, or deletion, may completely abolish antigenic reactivity. To assess the extent of mutation, lysates prepared from the isolates grown at 23°C were probed with antisera raised against a recombinant full-length OspC protein. Isolate BC13E, but not BC18E, did indeed abundantly produce a short version of the OspC antigen (Fig. 4C).

To determine whether the mutant gene copies were actively transcribed, total RNA was extracted from spirochetes grown at 23°C, and *ospC* mRNA transcripts were quantified by RT-qPCR. Both isolates and the original inoculum accumulated the transcripts at comparable levels (data not shown), indicating that the mutations did not affect the transcriptional process. The failure of the mutant *ospC* mRNA to be translated into an antigen reactive with OspC antibodies could be due to a lack of the ribosome-binding site and/or initial start codon, or the mutant mRNA might be translated into an unrelated polypeptide that does not share any antigenicity with OspC.

To identify mutations present in the pBBE22-*ospC'* plasmids of BC13E and BC18E, total DNA was prepared and used to transform *E. coli*. Five clones transformed with each of the DNA preparations were randomly selected, and DNA sequencing showed that both BC13E and BC18E had undergone destructive yet different mutations (Fig. 5A and B), consistent with the interpretation of the immunoblotting results. The BC13E mutant contained a 2-bp insertion between nucleotides 315 and 316, resulting in a nonsense mutation at codon 108,

TABLE 2. Constitutive *ospC* expression diminishes the ability of *B. burgdorferi* to establish chronic infections in immunocompetent mice<sup>a</sup>

Transformant	No. of positive cultures/total no. of specimens examined			
	Heart	Joint	Skin	All sites
5A13/pBBE22	8/8	8/8	8/8	24/24
5A13/pBBE22- <i>ospC'</i>	2/8	2/8	2/8	6/24

<sup>a</sup> Groups of eight BALB/c mice were inoculated with transformant 5A13/pBBE22 or 5A13/pBBE22-*ospC'* and sacrificed at 5 months postinoculation. Heart, tibiotarsal joint, and skin specimens were harvested and cultured for spirochetes in BSK-H complete medium.

and it encoded a polypeptide of only 107 amino acids, including the 19-amino-acid signal peptide. After the leader sequence was cleaved, the mutated OspC protein was less than one-half the normal length of 191 amino acids. All 7 bp between the putative ribosome-binding site and the start codon were deleted in BC18E, resulting in a mutation sufficient to abrogate translational initiation, although mutated *ospC* mRNA was abundantly accumulated. These changes apparently allowed the spirochetes to effectively avoid immune clearance and to establish chronic infection. All 16 mice were sacrificed at 5 months postinoculation. *B. burgdorferi* was recovered from each of the heart, joint, and skin specimens from all eight 5A13/pBBE22-inoculated mice but was recovered from the tissues of only mice BC13 and BC18 of the 5A13/pBBE22-*ospC'* group (Table 2), consistent with the ear biopsy results. DNA sequencing indicated that all of the isolates from either BC13 or BC18 might be derived from an individual mutant (Fig. 5C to H).

**Restoration of chronic infectivity by abolishing constitutive *ospC* expression.** 5A13/pBBE22-*ospC'* spirochetes were transformed with the recombinant plasmid pGE22 to replace pBBE22-*ospC'*. pGE22 was constructed from the shuttle vector pBSV2G by inserting a copy of the BBE22 gene. Eighteen transformants were obtained from a single experiment; eight of them contained both recombinant plasmids and were discarded. A plasmid content survey of the remaining 10 transformants that had lost pBBE22-*ospC'* revealed the following results: 5 maintained the same plasmid profile as the parent, 5A13/pBBE22-*ospC'*; 3 had lost lp28-1 and were discarded because they were not expected to establish infection in immunocompetent mice (21, 36); and the remaining 2 lacked either lp28-4 or cp32-3 and were designated 5A13/pGE22/07 and 5A13/pGE22/16, respectively. Three transformants, including 5A13/pGE22/07, 5A13/pGE22/16, and one clone that was randomly selected from the five clones that shared the same plasmid profile as the parental clone and designated 5A13/pGE22/03, were each inoculated into five BALB/c mice. Spirochetes were recovered from all 45 heart, joint, and skin specimens from the 15 mice after 5 months of infection (Table 3), indicating a full restoration of chronic infectivity once constitutive *ospC* expression had been abolished.

## DISCUSSION

*B. burgdorferi* causes persistent infection despite the development of vigorous immune responses against the pathogen.

TABLE 3. Full restoration of chronic infectivity once constitutive *ospC* expression is abolished<sup>a</sup>

Transformant	Missing plasmids	No. of positive cultures/total no. of specimens examined			
		Heart	Joint	Skin	All sites
5A13/pGE22/03	cp9, lp25, lp56, lp21, lp5	5/5	5/5	5/5	15/15
5A13/pGE22/07	cp9, lp25, lp28-4, lp56, lp21, lp5	5/5	5/5	5/5	15/15
5A13/pGE22/16	cp9, lp25, lp56, lp21, lp5, cp32-3	5/5	5/5	5/5	15/15

<sup>a</sup> Groups of five BALB/c mice were inoculated with transformant 5A13/pGE22/03, 5A13/pGE22/07, or 5A13/pGE22/16 and sacrificed at 5 months postinoculation. Heart, tibiotarsal joint, and skin specimens were harvested and cultured for spirochetes in BSK-H complete medium.

One potential strategy *B. burgdorferi* uses to effectively evade the immune system is to selectively reduce the expression of antibody-targeted antigens, exemplified by OspC, in response to immune pressure (7, 24, 26, 28). To explore this hypothesis, *B. burgdorferi* was genetically modified to constitutively express OspC by introducing an extra *ospC* copy fused with a *flaB* promoter. Such a modification did not reduce infectivity or pathogenicity but completely abrogated the ability of *B. burgdorferi* to avoid clearance by passively transferred OspC antibody in SCID mice. Spirochetes with constitutive *ospC* expression were unable to establish chronic infection unless they had undergone escape mutation during the infection of immunocompetent mice.

*B. burgdorferi* 5A13 harbors 20 of the 21 borrelial plasmids (36). The lack of lp25 makes spirochetes highly transformable because the plasmid carries BBE02, a putative restriction-modification gene (20, 22). The plasmid also harbors BBE22, which encodes a nicotinamidase essential for mammalian infection (21, 35). Transformation of 5A13 spirochetes with a shuttle vector containing a copy of BBE22 restores infectivity and pathogenicity (35). Because of the essential role of BBE22, any gene associated with the shuttle vector would be maintained in the mammalian environment. By using this system, we generated a constitutively *ospC*-expressing variant with full infectivity and pathogenicity in the absence of adaptive immune responses. The activity of the fused *flaB* promoter was first shown in vitro. The introduction of the extra copy increased the *ospC* mRNA copy number 120- and 12,000-fold at 37°C and 23°C, respectively, consistent with the results of immunoblot analysis, which showed a dramatic increase in the OspC antigen's accumulation.

The introduction of an extra gene copy significantly increased *ospC* mRNA accumulation in the joint tissues only but elevated the tissue spirochete burden in the hearts of infected SCID mice. Our previous study showed that OspC antibody represses *ospC* expression in all tissues but significantly reduces the bacterial load only in the heart, suggesting that the antigen may play an important role in spirochetal colonization in this specific tissue (28). We have not been able to assess in vivo gene expression at the translational level; it remains to be determined whether the presence of the fused *flaB* promoter increases OspC antigen synthesis in the heart. The addition of an extra *ospC* copy may increase the lipoprotein's expression,

even though RT-qPCR did not show a substantial increase in *ospC* mRNA accumulation, enhancing spirochetal colonization in the heart.

OspC antibody is able to effectively prevent initial infection (16, 30) but is unable to clear an established infection (4) because *B. burgdorferi* has the ability to repress the antigen's expression (7, 24, 26, 28). With the introduction of an extra *ospC* copy fused with the *flaB* promoter, *B. burgdorferi* was forced to constitutively express the antigen regardless of the immune status of the host. This modification resulted in clearance of infection when the passive transfer of OspC antibodies occurred in SCID mice. The immune response induced during infection of immunocompetent mice cleared most of the infections. Although spirochetes with constitutive *ospC* expression triggered a strong humoral response to the lipoprotein, this response did not appear to be the reason why chronic infection did not develop in the majority of infected mice. Once spirochetes had undergone escape mutation in the introduced *ospC* copy, persistent infection was readily established in the same strong immune environment. It is unknown whether recombination occurred between the introduced and native *ospC* copies.

The 5A13/pBBE22-*ospC'* clone was selected from a total of nine transformants that received the recombinant plasmid pBBE22-*ospC'*. It was first shown to be fully infectious and pathogenic in the absence of adaptive immune responses. The fact that all infections of SCID mice initiated with this clone were cleared by passively transferred OspC MAb could potentially be attributed to unnoted genetic defects that had been introduced specifically into this clone and that abolished its ability to avoid immune clearance. However, only two escape mutants were selected during infections of immunocompetent mice; both had a very destructive mutation in the constitutively expressed copy, which clearly associated the failure to evade specific humoral immunity with the presence of the introduced *ospC* copy. Moreover, once pBBE22-*ospC'* was replaced with the recombinant plasmid pGE22, all three transformants derived from clone 5A13/pBBE22-*ospC'* established chronic infections in immunocompetent mice, confirming the consequence of constitutive *ospC* expression.

*B. burgdorferi* dynamically modifies its surface antigenic architecture in response to environmental changes, including the development of specific immune responses. It actively expresses OspA in unfed ticks (10, 31, 39), consistent with an essential role of the lipoprotein in colonization of the vector by *B. burgdorferi* (32, 50). A fresh blood meal induces the down-regulation of OspA and the up-regulation of OspC (31, 39). It is crucial for maintaining the enzootic cycle to repress OspA expression during mammalian infection because its strong immunogenicity would elicit an immune response, effectively blocking acquisition of the spirochetes by the tick (9, 46, 47). In contrast, active OspC expression is required only for initial mammalian infection (17, 45). To avoid clearance by OspC-specific humoral immunity induced during infection, *B. burgdorferi* represses *ospC* expression once an infection has been established (7, 24, 28). In the immune environment, after *ospC* expression is repressed, the pathogen abundantly expresses a group of surface-exposed lipoprotein antigens (28), including VlsE, which undergoes antigenic variation (51), DbpA, which potentially interacts with host decorin (18), and BBF01 (12),

whose function remains to be identified. Unlike OspC, these antigens are allowed to be persistently expressed or even dramatically up-regulated during chronic infection, probably because specific immune responses to any of them are not able to affect spirochete infection in the mammalian host (13, 19, 23, 25, 27).

Nothing is known about the molecular mechanisms governing antibody-induced antigen down-regulation. *B. burgdorferi* may sense a specific antibody and selectively down-regulate an antigen via an as yet unidentified signaling pathway(s) or generate multiple phenotypes, such as cells that abundantly express OspC and others that do not. OspC antibodies may eliminate phenotypes actively expressing the antigen but allow others to persist. Although the *ospC* gene is polymorphic among *B. burgdorferi* isolates (29, 44), its sequence is well maintained during chronic infections of immunocompetent mice (41). While mutation is an effective way to evade the immune system, it may cause the functional loss of affected genes. Regardless of how the specific immune response represses *ospC* expression, our current study clearly shows that *B. burgdorferi* must undergo escape mutation in order to establish chronic infection if it is unable to repress *ospC* expression in the event of the immune response being mounted. By down-regulating *ospC* rather than mutating, *B. burgdorferi* is able to avoid clearance and to preserve the gene's integrity for subsequent utilization during its enzootic life cycle.

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