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Borrelia burgdorferi OspC Protein Required Exclusively in a Crucial Early Stage of Mammalian Infection

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This study demonstrates a strict temporal requirement for a virulence determinant of the Lyme disease spirochete *Borrelia burgdorferi* during a unique point in its natural infection cycle, which alternates between ticks and small mammals. OspC is a major surface protein produced by *B. burgdorferi* when infected ticks feed but whose synthesis decreases after transmission to a mammalian host. We have previously shown that spirochetes lacking OspC are competent to replicate in and migrate to the salivary glands of the tick vector but do not infect mice. Here we assessed the timing of the requirement for OspC by using an *ospC* mutant complemented with an unstable copy of the *ospC* gene and show that *B. burgdorferi*'s requirement for OspC is specific to the mammal and limited to a critical early stage of mammalian infection. By using this unique system, we found that most bacterial reisolates from mice persistently infected with the initially complemented *ospC* mutant strain no longer carried the wild-type copy of *ospC*. Such spirochetes were acquired by feeding ticks and migrated to the tick salivary glands during subsequent feeding. Despite normal behavior in ticks, these *ospC* mutant spirochetes did not infect naive mice. *ospC* mutant spirochetes from persistently infected mice also failed to infect naive mice by tissue transplantation. We conclude that OspC is indispensable for establishing infection by *B. burgdorferi* in mammals but is not required at any other point of the mouse-tick infection cycle.

Many vector-borne pathogens alternate between mammalian hosts and arthropod vectors. Some, such as *Borrelia burgdorferi*, a spirochete that causes Lyme disease, are unable to live outside the host or vector except under artificial laboratory conditions. Genetic studies are beginning to identify the mechanisms by which pathogens adapt to different host environments, as well as factors involved in transmission between hosts. In the case of *B. burgdorferi*, whose natural hosts are small mammals such as mice, and whose vectors are ixodid ticks, several genes have recently been shown to be required for either host or vector survival (14, 17, 34, 37, 40, 53). Among these is *ospC*, which is required for mammalian infection (18).

OspC was first identified as a seroreactive major outer surface protein (Osp) in a subset of *B. burgdorferi* strains (3, 49, 50). Subsequently, the *ospC* gene was mapped to cp26 (27, 41), a 26-kb circular plasmid that is a ubiquitous component of the segmented *B. burgdorferi* genome (5). Synthesis of OspC and that of another major outer surface protein, OspA, are often, but not always, inversely regulated (13, 31, 43, 44). During bacterial growth in ticks and in vitro, OspC protein levels are increased by stimuli, such as tick feeding and pH shift, that also lead to reduced OspA levels (6, 32, 43, 44).

During mammalian infection, *ospC* transcript is reduced and OspC protein disappears from the bacterial surface around 2 weeks after infection (9, 19, 25, 29). Because of this synthesis pattern, OspC was speculated to be required for some aspect of transmission, either migration of the spirochetes from the tick midgut to salivary glands and into the mammal or establishing an infection in the mammal (43). The related spirochete *Borrelia hermsii* has a gene homologous to *ospC*, called *vtp*, whose product is also present on the bacterial surface during transmission from tick to mammal (7, 42). Surprisingly, although the predicted OspC and Vtp products are only about 50% identical, the signal sequences are invariant (28, 36), raising the intriguing possibility that the cleaved signal sequences may have additional roles, perhaps as peptide pheromones.

Recently, we demonstrated that OspC is absolutely required for productive mammalian infection but not required for tick colonization or migration within ticks from midguts to salivary glands (18). The *ospC* mutant used in our initial study was created by inserting a selectable marker in the *ospC* gene, leaving the possibility of a partially functional fragment of OspC. A separate study concluded that a different *ospC* mutant was defective in migration from tick midguts to salivary glands (34), although mammalian infectivity was not addressed. The mutant in that study had a deletion of the 5′ end of the *ospC*

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TABLE 1. Oligonucleotides used in this study

No.	Name	Sequence (5′-3′)	Use	Reference
1	*ospC*169	TAATTTGTGCCTCCTTTTTATTTAT	Cloning *ospC5′*	This work
2	*ospC*170	GAGCTCCCATATTCTTGCTTAGAGT	Cloning *ospC5′*	This work
3	*ospC*171	TTAAGATCAATATTATAAGATTAAT	Cloning *ospC3′*	This work
4	*ospC*172	CTCTCCAGTAATGATAATACTGTGC	Cloning *ospC3′*	This work
5	PC33	ATAAGTCCTAGAATAAATTA	Analyzing *ospC* locus	18
6	PC34	GGATCCATAAGTCCTAGAATAAATT	Analyzing *ospC* locus	18
7	*ospC*33	CAAGATATTGAAGAATTTGA	Analyzing *ospC* locus	This work
8	*ospC*34	GACTTTATTTTTCCAGTTAC	Analyzing *ospC* locus	This work
9	flgPo.Not	GCGGCCGCTACCCGAGCTTCAAGGAAGATT	Cloning $flgB_p$-*kan*	5
10	KanTerm-Xho	ATCTCGAGCTAGCGCCGTCCCGTCAA	Cloning $flgB_p$-*kan*	5
11	*aacC13′*	CATATGTTACGCAGCAGCAACGATGTTACG	Screening for plasmid	11
12	*aacC15′*	GCTAGCCGATCTCGGCTTGAACG	Screening for plasmid	11

gene, so it presumably would make no portion of OspC. To further delineate the role of OspC and to address the possibility that the signal sequence or truncated protein retained activity adequate for transmission from the tick but not for mammalian infection, we have constructed a new mutant that lacks the entire *ospC* coding sequence and complemented this mutant in *trans* with a shuttle vector carrying the wild-type *ospC* gene. In the present study, we have again found that OspC is required for mammalian infection but not for tick colonization (including natural acquisition from the mammal) or transmission. Furthermore, we have established that the requirement of *B. burgdorferi* for OspC is limited to a crucial period early in infection of the mammalian host. Finally, we show that OspC is even required for mammalian tissue-derived spirochetes to establish infections in naive mice. These findings, together with previous observations regarding differential regulation of spirochetal gene expression in ticks and mammals (9, 26, 32, 44), indicate that host adaptation by *B. burgdorferi* not only occurs in response to the disparate arthropod and mammalian environments but also varies within each host at stages roughly corresponding to colonization, persistence, and transmission.

MATERIALS AND METHODS

Construction of plasmids and strains used in this study. The plasmid used to inactivate the *ospC* gene was constructed in several steps. First, about 500 bp 5′ of the start codon and 3′ of the stop codon were amplified (with primer pairs 1-2 and 3-4, respectively, both of which are described in Table 1), and KOD XL DNA polymerase (EMD Biosciences, San Diego, CA). Each fragment was cloned into pCR-Blunt-II-TOPO (Invitrogen, Carlsbad, CA), yielding pJK99b (5′) and pJK100 (3′), respectively. The 3′-flanking sequence fragment was excised from pJK100 with EcoRI, and the ends were filled in with T4 DNA polymerase. The fragment was ligated with pJK99b that had been digested with XbaI and filled in with T4 polymerase, yielding pJK101. A plasmid containing a $flgB_p$-*kan* fusion flanked by NotI and XhoI sites (pJK102) was constructed by amplifying the fusion with primers 9 and 10 (5) and Vent polymerase (New England Biolabs, Beverly, MA, from whom restriction enzymes and ligase were also purchased) and cloning the resulting fragment into plasmid TOPO-XL (Invitrogen). This $flgB_p$-*kan* fusion was excised by NotI and XhoI digestion and ligated with NotI-XhoI-digested pJK101. A plasmid with the correct nucleotide sequence and orientation of all fragments was named pJK109.

Plasmid pJK109 DNA was transformed into *B. burgdorferi* B31-A3 (12) that had been recloned recently and confirmed to have all plasmids except cp9, which is not required for the mouse-tick infection cycle. Since the sequenced *B. burgdorferi* strain B31 genome contains 12 linear and 9 circular plasmids (8, 15), at least several of which are required for full infectivity in mice and/or ticks, we monitored plasmid content after all genetic manipulations to ensure that strains were isogenic and that plasmid loss could not contribute to any phenotypes that we might detect. After electroporation and plating in the presence of 200 μg/ml kanamycin, potential transformants were screened by PCR for the presence of a mutant *ospC* locus. Because of the deletion-insertion event in the inactivation construct, PCR with primers 2 and 4 amplifies a fragment of 2.4 kb from the mutant locus, in contrast to a 1.6-kb fragment amplified from the wild-type locus. Several mutants identified by this method were subsequently confirmed to have all of the plasmids found in the parent clone, A3. One mutant, named *ospC*K1, was selected for further characterization (Fig. 1A).

The *ospC*K1 mutant was complemented with pBSV2G-*ospC*, a shuttle vector carrying a wild-type copy of the *ospC* gene (Fig. 1B). Plasmid pBSV2G-*ospC* was constructed by using NotI to excise the *ospC* gene with ~200-bp 5′ and 3′ flanking sequences from pGTEC-Δ*bla*2 (18) and cloning it into NotI-digested pBSV2G (11). This plasmid also carries the BBB20 locus (15), which is probably not a functional gene since it is very small (111 bp), there is no evidence for BBB20 expression (33), and the closely related spirochete *Borrelia garinii* does not have an open reading frame in that location (16). Plasmid pBSV2G-*ospC* was used to transform the *ospC*K1 mutant, and gentamicin-resistant transformants that retained the *B. burgdorferi* plasmid content of the parent strain were isolated. One such clone, *ospC*K1/pBSV2G-*ospC*#3, was used in further experiments.

Similarly, *B. burgdorferi* clone A3 (wild type) was transformed with pBSV2G or pBSV2G-*ospC*. Gentamicin-resistant transformants were screened for plasmid content, and clones A3/pBSV2G#1 and A3/pBSV2G-*ospC*#46, which retained all of the plasmids found in A3, were selected for further studies. We did not observe concomitant loss of essential plasmid lp25 during transformation with pBSV2G, which was found with the closely related shuttle vector pBSV2 (21, 23). This finding obviates the need to clone the essential gene *bbe22* into the complementing plasmid, which was required with pBSV2-mediated complementation (14, 24).

Sodium dodecyl sulfate gel and Western blot analysis. Samples were separated by electrophoresis through 12.5% sodium dodecyl sulfate gels and blotted to nitrocellulose membrane with a Bio-Rad Mini-Protean II system (Bio-Rad, Hercules, CA). Immunoblots were hybridized with monoclonal antibodies that recognized *B. burgdorferi* flagellin (1:200 dilution of H9724, a gift from T. Schwan, Rocky Mountain Laboratories [RML], Hamilton, MT), OspC (1:5,000 dilution; a gift from R. Gilmore, Centers for Disease Control and Prevention, Fort Collins, CO), and with infected mouse sera (1:200 dilution). Conditions, secondary antibodies, and detection were as previously described (18). Gels were silver stained with the Bio-Rad Silver Stain Plus kit.

Experimental mouse-tick-mouse infection cycle. The RML are accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for animal experiments were prepared according to the guidelines of the National Institutes of Health and approved by the RML Animal Care and Use Committee. In order to mimic the natural host population, most mice were from an outbred colony maintained at the RML and derived from Swiss-Webster mice. In one experiment, C3SnSmn.CB17-Prkdc<SCID>/J mice (hereafter designated SCID; Jackson Laboratories, Bar Harbor, ME) were used. When determining the infectious dose, inbred C3H-HeN mice (Harlan Sprague-Dawley, Indianapolis, IN) were used in order to have a uniform host population. In this experiment, 50% infectious doses (ID_{50}s, the doses required to infect half of the mice inoculated) of the A3 and *ospC*K1/pBSV2G-*ospC* strains were compared with a logit model and the logs of the doses. Data for the two strains were fitted jointly by assuming a common slope. This model was chosen because it directly tests for a difference between the ID_{50}s and is typically appropriate for such experiments. Modeling was performed with SAS version 9.1 (SAS Institute, Cary, N.C.). Standard infections were initiated by injecting 5×10^3 *B. burgdorferi*

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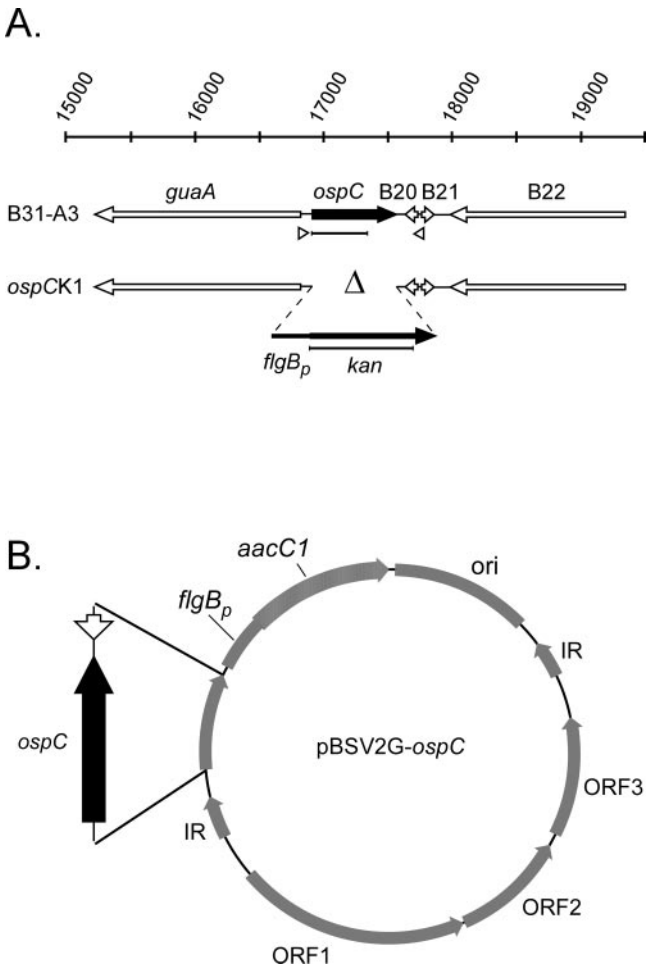

FIG. 1. Diagram of *ospC* wild-type, mutant, and complementing loci. (A) Structures of the wild type (B31-A3, top) and the Δ*ospC*::*flgB*$_p$-*kan* mutant (*ospC*K1, bottom). The scale bar shows the coordinates on cp26 in kilobases. Oligonucleotide binding sites used in constructing pBSV2G-*ospC* and screening for the *ospC* genotype are represented by arrowheads (5 or 7 [5′] and 6 or 8 [3′]; see Table 1). Probes used in Southern blot assays (Fig. 4B) are shown as lines below genes. (B) Structure of pBSV2G-*ospC* (not drawn to scale). The *flgB*$_p$-*aacC1* fusion confers gentamicin resistance on *B. burgdorferi* and *E. coli* (11). ori, colE1 origin of replication; IR, inverted repeat from cp9; ORF1 to ORF3, open reading frames allowing plasmid replication in *B. burgdorferi* (47).

bacteria into mice. Two tests were carried out on inocula to ensure that the majority of the cultures contained plasmids known to be required for infection, which are often unstable during in vitro culture. First, DNA was prepared from the inoculum cultures and screened by PCR with a panel of primer pairs for the presence of all *B. burgdorferi* plasmids (12). Second, a portion of the inoculum was plated for single colonies and 24 colonies were screened for the presence of lp25 and lp28-1 (22, 38). More than 75% of the colonies screened were positive for these plasmids in cultures used in the experiments described here. Three weeks after inoculation, the mice were bled and their sera were assessed by Western blot assay for reactivity with *B. burgdorferi* proteins (46).

Uninfected larval ticks (about 3 months old, from a colony maintained at the RML) were fed upon seropositive mice. Tick infection was assessed by immunofluorescence assay (IFA) of dissected midguts with rabbit anti-*B. burgdorferi* (a gift from T. Schwan) as the primary antibody and fluorescein isothiocyanate-labeled goat anti-rabbit (Kierkegaard & Perry Laboratories, Gaithersburg, MD) as the secondary antibody. After the molt to the nymphal stage, the ticks were fed upon naive mice to assess bacterial transmission. Replete ticks were assayed for infection 5 to 7 days postfeeding, and the serological responses of the mice to *B. burgdorferi* proteins were measured by Western blot assay 3 weeks after tick application. When migration to salivary glands was to be assessed, partially fed nymphal ticks were removed approximately 72 h after attachment. Salivary glands were rinsed sequentially five times in phosphate-buffered saline before fixation to eliminate midgut contamination. IFA on midguts was done as described above, whereas IFA on salivary glands was modified by using Alexa 488-labeled goat anti-rabbit (Kierkegaard & Perry Laboratories) as the secondary antibody with the DNA stain DRAQ5 (Biostatus Limited, Shepshed, United Kingdom) added at a 1:1,000 dilution (18). Salivary glands were visualized with a Bio-Rad model 1024 confocal microscope with LaserSharp 2000 software (Bio-Rad).

Artificial infection of ticks. Approximately 100 1- to 3-month-old larval *Ixodes scapularis* ticks were infected by immersion in exponential-phase cultures of various strains of *B. burgdorferi* at 32°C for 90 min as previously described (35). The tubes were spun briefly, and cultures were removed. After about 3 days of recovery at 98% humidity, the artificially infected ticks were fed upon naive mice. Infection of ticks and mice was assessed as described above.

In vitro shuttle vector stability. Stability of shuttle vectors pBSV2G and pBSV2G-*ospC* during bacterial growth in vitro was assessed by growing cultures from frozen stocks in the presence of antibiotic selection (40 μg/ml gentamicin) and then passaging duplicate or triplicate cultures by 1/1,000 dilution five times (approximately 50 doublings over the course of 2.5 weeks) without antibiotic selection. The cultures were plated without selection, and 24 colonies from each were screened for the presence of plasmids pBSV2G and pBSV2G-*ospC* with primers 11 and 12 (Table 1). Statistical tests were performed with StatXact version 6 (Cytesl Software Corporation, Cambridge, MA).

Infection by tissue transplantation. Donor mice were infected by inoculation with 5×10^3 A3, *ospC*K1, or *ospC*K1/pBSV2G-*ospC* bacteria as described above. Mice were bled, and their sera were tested for immunoreactivity with *B. burgdorferi* antigens. Eight weeks after inoculation, we attempted to isolate spirochetes from 3-mm ear punches placed in Barbour-Stoenner-Kelly II medium. Positive isolates from *ospC*K1/pBSV2G-*ospC*-inoculated mice were plated for single colonies, and colonies were screened for the presence of pBSV2G-*ospC*. None of the colonies screened (0/24) retained the shuttle vector. At 73 days postinoculation, donor mice were euthanized and two 3-mm ear punches per mouse were implanted beneath the dorsal lumbar skin of naive mice. Spirochete culture from ears, bladders, and ankle joints of donor mice was attempted, and ears, hearts, and ankle joints were frozen for DNA extraction and quantitative PCR analysis. Tissue DNA was extracted by a previously published method (30) that involves collagenase A (Roche, Indianapolis, IN), proteinase K (Invitrogen), and RNase digestions in combination with phenol-chloroform and chloroform extractions and ethanol precipitations. Real-time PCR to quantitate *B. burgdorferi* genomes with respect to mouse genomes was done with TaqMan primers and probes (Applied Biosystems, Foster City, CA) for the *flaB* gene (*B. burgdorferi* chromosome) and the mouse *nidogen* gene (30) in an Applied Biosystems 7900HT instrument. Recipient mice were analyzed in the same manner, and culture of bacteria from the transplantation site (dorsal lumbar skin) was also attempted.

RESULTS

***ospC* mutant and complemented strains.** Our previous study showed that OspC had the hallmarks of a virulence factor, being required for mammalian infection although dispensable for growth in a tick (18). However, our original mutation was a simple insertion two-thirds of the way into the *ospC* gene, which could potentially yield a truncated protein with partial activity. To eliminate this possibility, complete deletion of the *ospC* gene was done by using allelic exchange to replace the entire coding sequence with a *flgB*$_P$-*kan* fusion, which confers kanamycin resistance on *B. burgdorferi* (Fig. 1A). This mutation was complemented in *trans* with plasmid pBSV2G-*ospC* (Fig. 1B). PCR, Southern blot, and immunoblot analyses demonstrated that the *ospC* gene and the protein it encodes were absent in the mutant and restored in the complemented strain (Fig. 2A and data not shown).

***ospC* mutant phenotype in ticks.** An initial objective of this study was to address the differences between our previous study (18) and that conducted with another *ospC* mutant

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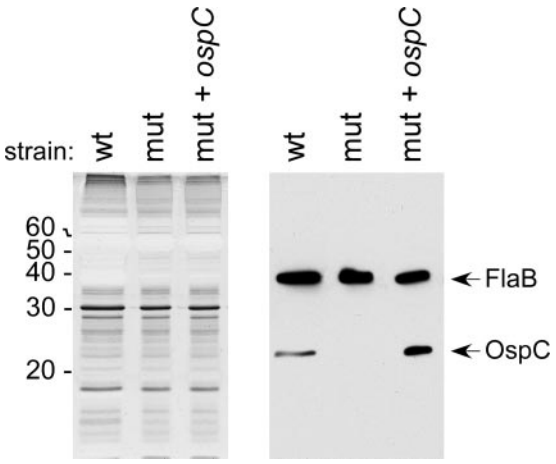

FIG. 2. OspC production by various *B. burgdorferi* strains grown in vitro. Left, silver-stained gel showing similar amounts of lysate loaded; right, Western blot probed with antibodies recognizing FlaB and OspC. wt, A3; mut, *ospC*K1; mut + *ospC*, *ospC*K1/pBSV2G-*ospC*. The values on the left are molecular sizes in kilodaltons.

(34). Specifically, we wished to examine the phenotype of the *ospC*K1 mutant during all stages of tick infection to determine if the entire *ospC* gene could be deleted without altering bacterial survival, replication, and migration to the salivary glands. To obtain uniform populations of infected ticks containing A3, *ospC*K1, or *ospC*K1/pBSV2G-*ospC*, we artificially infected ticks by immersion in bacterial cultures (35). Pools of larvae infected with these three strains were fed upon naive mice. IFA on three to five fed larvae from each pool demonstrated that all were infected. After the molt, nymphs were fed on naive mice. Transmission was assessed by Western blot assays of sera obtained 3 weeks after tick application and/or isolation at later times. In all cases, mice fed upon by either larvae or nymphs infected with A3 or *ospC*K1/pBSV2G-*ospC* were positive (7/7 positive), whereas those fed upon by *ospC*K1 mutant-infected ticks (at either life stage) were negative (0/3 positive).

To assess the ability of *ospC*K1 mutant and complemented spirochetes to carry out all stages of tick infection and transmission, cohorts of infected nymphs were fed upon mice for approximately 72 h, at which point the ticks were removed and salivary glands and midguts were dissected. Both groups of organs were subjected to IFA, and the salivary glands were examined for the presence of spirochetes by confocal microscopy. Low but similar numbers of wild-type, *ospC* mutant, and complemented spirochetes were observed within the salivary glands (Fig. 3 and data not shown), indicating that OspC is not required for migration of spirochetes from midguts to salivary glands, consistent with previous results obtained with a different *ospC* mutant (18). In all cases, large numbers of spirochetes were observed within the midguts (data not shown). The extensive washes of the salivary glands, coupled with the localization of spirochetes within glands by confocal microscopy, make it highly unlikely that contamination by midgut spirochetes accounted for our results. These results are identical to those previously obtained, confirming that the nature of the original mutation did not account for the normal migration to salivary glands that we observed (18).

***ospC* mutant phenotype in mice.** To examine the ability of the complete *ospC* deletion mutant to infect mice, we needle inoculated animals with 5×10^3 A3, *ospC*K1, and *ospC*K1/pBSV2G-*ospC* bacteria. We found that the mutant with a complete deletion of the *ospC* gene (*ospC*K1) did not infect mice, but both the wild-type (A3) and complemented (*ospC*K1/pBSV2G-*ospC*) strains successfully infected mice, as assessed by seroconversion 3 weeks after inoculation and bacterial reisolation from mouse tissue samples 7 weeks after inoculation (Fig. 4A and Table 2). These results were consistent with the inability of either larval or nymphal ticks infected with *ospC*K1 to infect mice by tick bite. Surprisingly, analysis of the *ospC* genotypes of reisolated spirochetes demonstrated that the majority of the isolates from *ospC*K1/pBSV2G-*ospC*-infected mice were no longer heterozygous at the *ospC* locus but only retained the mutant locus (Fig. 4B; see below), suggesting that the complementing plasmid had been lost. Among the many reisolates in which pBSV2G-*ospC* was undetectable by PCR, we selected two and confirmed by Southern blot analysis that they had lost the complementing plasmid (Fig. 4C). We were also unable to rescue the shuttle vector from these reisolates by transformation of *Escherichia coli* (data not shown). The loss of the complementing plasmid in these reisolates suggests that

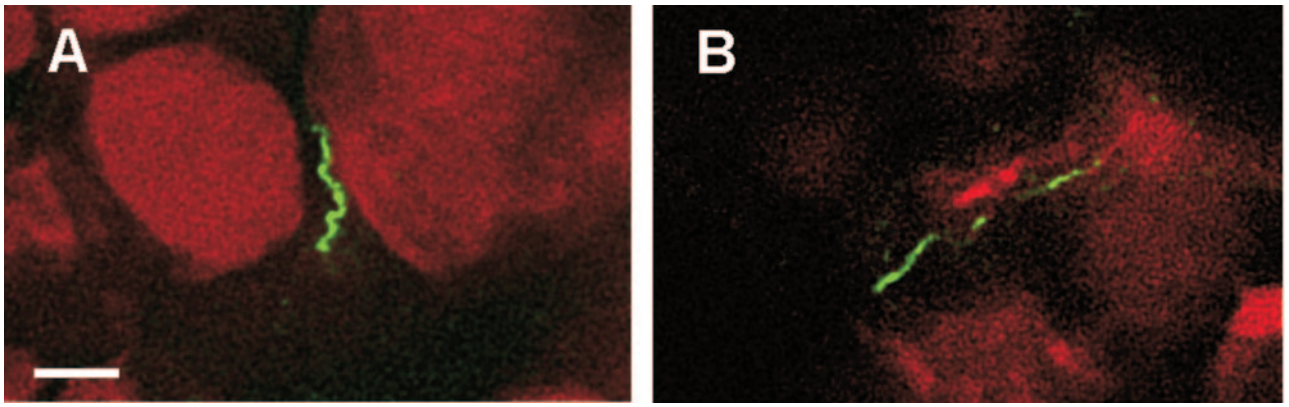

FIG. 3. Confocal images of spirochetes within tick salivary glands after 72 h of feeding. Panels: A, *ospC*K1 spirochete; B, *ospC*K1/pBSV2G-*ospC* spirochetes. Spirochetes were detected by immunofluorescence with rabbit anti-*B. burgdorferi* antibody and Alexa 488-labeled anti-rabbit antibody. Salivary gland cell nuclei were counterstained with DRAQ5. Scale bar, 10 μm.

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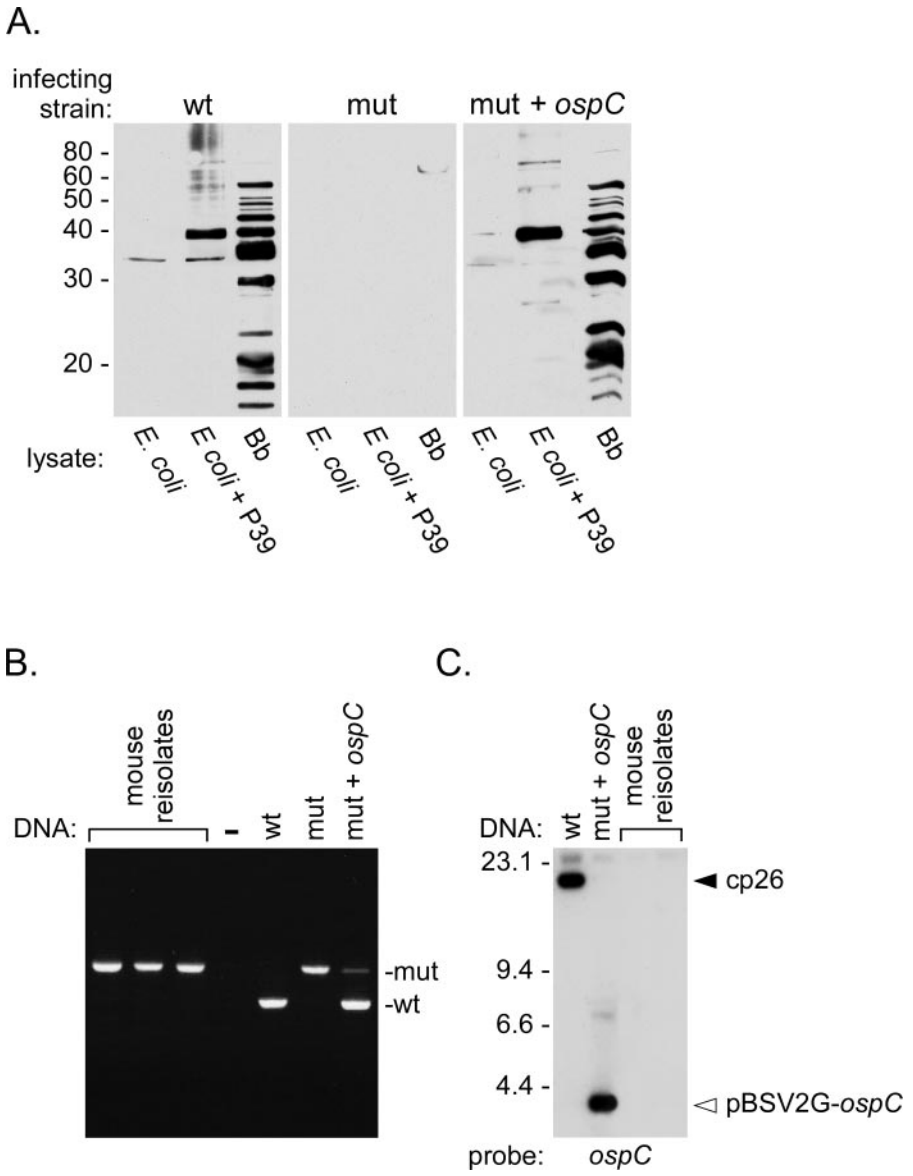

FIG. 4. Serological responses to infection with various strains of *B. burgdorferi* and assessment of maintenance of pBSV2G-*ospC* in *B. burgdorferi* mouse reisolates. (A) Western blot analysis of sera from mice injected with 5×10^3 spirochetes of various strains. Samples loaded on gels: *E. coli*, lysate of *E. coli* carrying cloning vector; *E. coli* + P39, lysate of *E. coli* carrying cloning vector encoding *B. burgdorferi* proteins P39 and P28; Bb, lysate of *B. burgdorferi*. Representative results obtained with serum from one mouse per inoculated strain are shown. wt, A3; mut, *ospC*K1; mut + *ospC*, *ospC*K1/pBSV2G-*ospC*. The values on the left are molecular sizes in kilodaltons. (B) PCR screening of *ospC* loci of three reisolates from mice injected with *ospC*K1/pBSV2G-*ospC* (mouse reisolates) and controls (−, no DNA; wt, A3 DNA; mut, *ospC*K1 DNA; mut + *ospC*, *ospC*K1/pBSV2G-*ospC* DNA). Primers 7 and 8 (Fig. 1 and Table 1), which amplify unique fragments from wild-type and mutant loci, as indicated on right of the panel, were used. (C) Southern blot assay of two *B. burgdorferi* mouse reisolate DNA samples and controls probed with the *ospC* gene. wt, DNA derived from wild-type *B. burgdorferi*; mut + *ospC*, DNA derived from in vitro-grown *ospC*K1/pBSV2G-*ospC* mutant bacteria; mouse reisolates, DNA of two *ospC*K1* reisolates from mice injected with *ospC*K1/pBSV2G-*ospC* mutant bacteria. The *ospC* probe does not hybridize to cp26 of *ospC*K1* because the *ospC* gene is completely deleted in this strain. The values on the left are molecular sizes in kilobase pairs.

OspC is only required for the early stages of mammalian infection and, possibly, that continual synthesis is deleterious. We will refer to *ospC* mutant bacteria derived by mammalian infection with *ospC*K1/pBSV2G-*ospC*, in which the complementing plasmid was subsequently lost, as *ospC*K1*.

Following inoculation of mice with A3, *ospC*K1, and *ospC*K1/pBSV2G-*ospC* bacteria, we obtained animals that were infected with wild-type *B. burgdorferi*, uninfected, or infected with *ospC*K1* spirochetes, respectively. The third group of mice presented us with the unique possibility of naturally infecting ticks with *ospC* mutant spirochetes. To determine the mutant phenotype at all stages of tick infection, including acquisition during feeding, larval ticks were fed upon mice that had been inoculated with A3 or *ospC*K1/pBSV2G-*ospC* bacte-

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TABLE 2. Infectivity and transmission of *B. burgdorferi* clones in mice and ticks

Clone	Infection route	Mouse infection[a]	Tick infection[b]
A3	Needle inoculation	3/3	6/6
A3	Nymphal tick feeding[c]	1/1	3/3
*ospC*K1	Needle inoculation	0/3	ND[d]
*ospC*K1/pBSV2G-*ospC*	Needle inoculation	3/3	17/18
*ospC*K1*	Nymphal tick feeding[c]	0/2	4/4

[a] Number of mice infected/number of mice tested by serology (at 3 weeks postinoculation or post tick application) and/or culture (at 52 days postinoculation or 18 weeks post tick application).
[b] Number of ticks infected/number of ticks tested by IFA or plating. Ticks were infected by feeding larvae on mice described in the corresponding line.
[c] Ticks acquired spirochetes by feeding as larvae on infected mice described in the preceding line.
[d] ND, not determined.

TABLE 3. Infectious dose determination

Dose (no. of spirochetes/mouse)	No. of mice positive/no. inoculated with strain:		
	A3	*ospC*K1/pBSV2G-*ospC*	*ospC*K1
20	0/6	0/6	ND[a]
100	0/6	1/6	ND
500	0/6	2/6	ND
2,000	3/6	3/6	ND
10,000	2/2	ND	0/5
1,000,000	2/2	ND	0/5

[a] ND, not determined.

ria (which contained *ospC*K1* bacteria). Both tick populations became infected with *B. burgdorferi* (Table 2) and contained similar numbers of spirochetes (as judged by IFA of tick midguts), suggesting that the loads of bacteria in the tissues of infected mice were similar. After molting to the nymphal stage, the ticks were fed upon naive mice. Ticks infected with A3 were able to transmit and cause infection, as expected. In contrast, ticks infected by feeding upon *ospC*K1*-infected mice contained spirochetes yet were not able to cause an infection, as we had found previously with ticks artificially infected with our *ospC* mutants (described above and in reference 18). To examine the *ospC* genotypes of the bacteria contained within the ticks, *B. burgdorferi* colonies were derived by crushing and plating individual *ospC*K1*-infected nymphs. The wild-type *ospC* locus was absent in all screened colonies derived from four ticks infected with *ospC*K1* (0/48 positive). These results demonstrated that only *ospC* mutant bacteria were detectable within the ticks and that the OspC protein was not required for acquisition of spirochetes during the tick blood meal or for infection of ticks.

A possible explanation for the survival of *ospC* mutant bacteria in mice, even after loss of the complementing plasmid, is that they had undergone a compensatory mutation. Such a mutation could allow another product to fulfill the function normally performed by OspC. Feeding *ospC*K1*-infected nymphs on naive mice did not result in infection, consistent with no compensatory mutation in the bacteria, but needle inoculation represents a different mode of infection (34, 39). To determine if a mouse reisolate of *ospC*K1* was able to initiate mammalian infection in the absence of OspC function, it was injected into two mice. Injected mice did not produce antibodies against *B. burgdorferi* proteins, and no bacteria were isolated from cultured tissues. In the same experiment, *ospC*K1 bacteria were noninfectious and *ospC*K1/pBSV2G-*ospC* bacteria were infectious (data not shown). Hence, although *ospC*K1* bacteria were recovered from infected mice, they did not appear to contain a compensatory mutation that enabled them to bypass the requirement for OspC protein to establish a mammalian infection since they did not infect.

Since the *ospC*K1/pBSV2G-*ospC* strain was clearly losing pBSV2G-*ospC* during growth in mice, it seemed possible that the ability of the complemented strain to infect mice was diminished. To address this possibility, we determined the ID_{50} relative to that of wild-type bacteria. In this experiment, groups of six mice were inoculated with four doses of spirochetes and infection was assessed by culturing tissue samples 4 weeks after inoculation (Table 3). The data were modeled as described in Materials and Methods, which yielded ID_{50} estimates of 2,750 and 1,150 spirochetes per mouse for A3 and *ospC*K1/pBSV2G-*ospC*, respectively, which were not significantly different ($P = 0.26$). We conclude that the instability of the complementing plasmid does not have an adverse effect on the ability of *ospC*K1/pBSV2G-*ospC* to infect mice.

At the time of bacterial isolation (28 days after inoculation), most of the reisolates from *ospC*K1/pBSV2G-*ospC*-inoculated mice had little to no pBSV2G-*ospC* remaining (as determined by screening of individual colonies and PCR from the uncloned isolates; data not shown). These experiments suggest that the subsequent plasmid loss found in the complemented strain is not reflected in a reduced ability to establish infection and that expression from the complementing copy of the *ospC* gene is sufficient to allow infection at a rate similar to that of the wild type. The results also show that the requirement for OspC ends before 28 days. In this experiment, we also inoculated five mice each with high doses of the *ospC*K1 mutant. No mice were infected, even at doses of 10^6 spirochetes per mouse, further demonstrating the importance of the OspC protein for establishing infection.

In vivo versus in vitro plasmid stability. Since analysis of isolates from mice infected with *ospC*K1/pBSV2G-*ospC* indicated that the complementing plasmid could be lost during mammalian infection and that *ospC* was only required for early stages of mouse infection, we wished to distinguish among several possible reasons for the plasmid loss. Although previous work had shown that pBSV2G appeared to be relatively stable during growth of bacteria in culture (11), this shuttle vector may be inherently unstable in bacteria during mouse infection. Alternatively, inserting the *ospC* gene into pBSV2G may have rendered it unstable. Finally, some aspect of the host environment may specifically select against bacteria carrying pBSV2G-*ospC*. To address these possible reasons for plasmid instability, we constructed wild-type strains carrying either the shuttle vector alone (A3/pBSV2G) or the shuttle vector containing *ospC* (A3/pBSV2G-*ospC*). We compared plasmid stability in these two strains with that of *ospC*K1/pBSV2G-*ospC* during bacterial growth in culture by measuring the proportion of bacteria retaining the shuttle vector after approximately 50 doublings in Barbour-Stoenner-Kelly II medium without selec-

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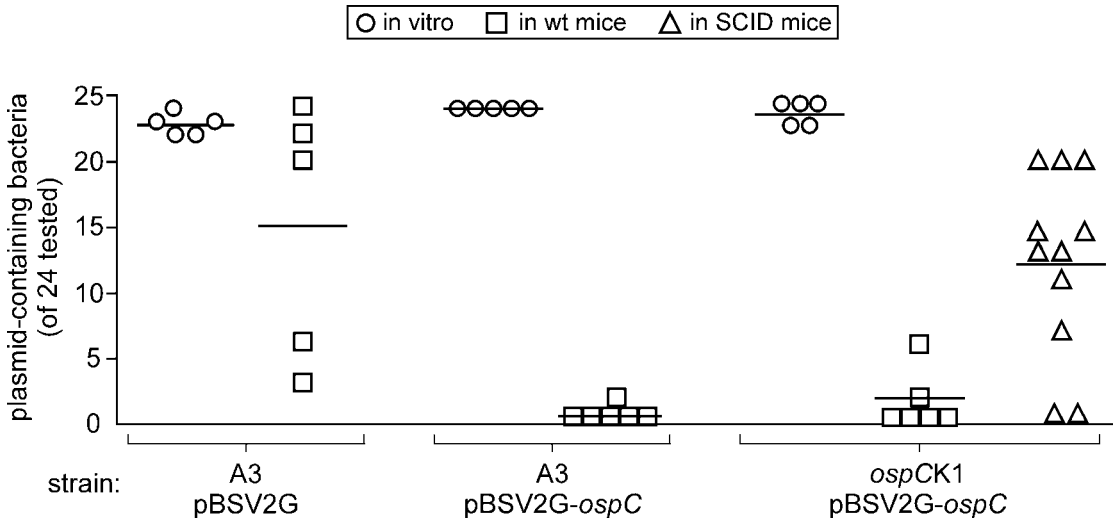

FIG. 5. Stability of pBSV2G and pBSV2G-*ospC* in *B. burgdorferi* during growth in vitro and in mice. Plotted is retention of plasmids by *B. burgdorferi* after growth in culture (circles), in wild-type (wt) mice (squares), and in SCID mice (triangles). In vitro, cultures were plated after 50 doublings without selection for plasmid retention. In wild-type mice, two tissue types per mouse from three mice per strain were cultured without selection for plasmid retention 6 weeks after inoculation with A3/pBSV2G, A3/pBSV2G-*ospC*, or *ospC*K1/pBSV2G-*ospC* bacteria. For testing of pBSV2G-*ospC* stability in SCID mice, four mice were infected and three tissue types per mouse were cultured 6 weeks after inoculation. Each symbol represents the number of colonies that retained pBSV2G or pBSV2G-*ospC* out of 24 colonies screened per culture or tissue type. Differences in plasmid retention during growth in wild-type mice between pBSV2G in A3 bacteria and pBSV2G-*ospC* in A3 or *ospC*K1 mutant bacteria were determined to be significant with an exact, two-sided Wilcoxon test, with $P \leq 0.01$. The stability of pBSV2G-*ospC* in SCID mice was also significantly greater than in wild-type mice ($P < 0.01$) but not significantly different from that of pBSV2G in wild-type mice ($P = 0.39$).

tion. Both shuttle vectors were stable in this environment in all of the strains tested (Fig. 5).

Shuttle vector stability during bacterial growth in vivo was assessed by injecting each strain into mice. All mice seroconverted to *B. burgdorferi* antigens after 3 weeks (data not shown). Mice were euthanized 6 weeks postinoculation, and culture of spirochetes from ear skin and ankle joints was attempted with and without the addition of gentamicin, which would select for the presence of the shuttle vector. Most culture attempts were successful (Table 4), showing that all of the mice were persistently infected and that at least some of the bacteria within each mouse retained the plasmid. When reisolates cultured without selection were plated and the resulting colonies were screened by PCR for the presence and absence of plasmid (Fig. 5), a more complex picture emerged. Whereas pBSV2G appeared to be quite stable during mouse infection, pBSV2G-*ospC* was not, in both the wild-type (A3) and *ospC*K1 mutant backgrounds, suggesting that insertion of *ospC* into pBSV2G correlated with plasmid loss in the mammalian environment.

To determine if the acquired immune response of the host to *B. burgdorferi* influenced pBSV2G-*ospC* stability, we inoculated *ospC*K1/pBSV2G-*ospC* into SCID mice and assessed plasmid retention after 6 weeks of infection (Fig. 5). The stability of pBSV2G-*ospC* in SCID mice was significantly greater than for the same plasmid in wild-type mice ($P < 0.01$), and it resembled that of pBSV2G in wild-type mice ($P = 0.39$). These results suggest that some aspect of acquired immunity in the host selected against *ospC* retention in infected mice.

Two results indicated that pBSV2G-*ospC* was stable during bacterial growth in ticks. First, bacteria isolated from two fed nymphs artificially infected as larvae with *ospC*K1/pBSV2G-*ospC* were assessed by PCR for retention of the complementing plasmid and 23/24 colonies per nymph retained pBSV2G-*ospC*. Second, the cohort that included those nymphs was able to transmit an infection to naive mice when they fed (data not shown). Taken together, these results suggest that only the mammalian environment, and specifically acquired immunity, selects against maintenance of pBSV2G-*ospC* in infecting bacteria.

Persistence of *ospC*K1* spirochetes in mice. Since we were able to obtain mice infected with *ospC*K1* bacteria by inoculating them with *ospC*K1/pBSV2G-*ospC*, we were interested in the ability of *ospC* mutant bacteria to persist for extended periods in infected mice. To address this question, tick larvae artificially infected with *ospC*K1/pBSV2G-*ospC* were fed upon two mice. Five months later, when the infecting bacteria had lost the complementing plasmid, the mice were used both for infection of naive larval ticks and for reisolation from mouse tissues. When the *ospC* genotypes of reisolates from the fed larval ticks were assessed by plating and screening the resulting colonies by PCR, only the mutant locus was present (data not shown), confirming that *ospC* was not required for acquisition

TABLE 4. In vivo plasmid stability upon isolation of bacteria from mouse tissues 6 weeks after inoculation with various strains

Infecting strain	Isolation with selection[a]	Isolation without selection[a]
A3/pBSV2G	6/6	5/6
A3/pBSV2G-*ospC*	6/6	6/6
*ospC*K1/pBSV2G-*ospC*	4/6	6/6

[a] For selection, gentamicin was present in the culture medium. The number of tissue samples positive/number of tissue samples tested is shown (three mice per strain infected and two tissue samples per mouse cultured).

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by the larval ticks. Similarly, the mouse tissue reisolates lacked the wild-type *ospC* locus, demonstrating that *ospC* mutant bacteria could persist in mice for at least 5 months once an infection was established by *ospC*-containing spirochetes. These findings further support the idea that OspC is only required at the initial stage of mammalian infection.

Infection by transplantation. Since our findings strongly suggested that functional OspC is required for establishing infection by either needle inoculation or tick bite, we wondered if mammalian host-derived spirochetes would be able to bypass that requirement. To address this question, we attempted infection by tissue transplantation with mouse skin from mice inoculated with A3, *ospC*K1, and *ospC*K1/pBSV2G-*ospC* bacteria after 73 days of infection. At this time, the complementing plasmid was expected to have been lost within mice inoculated with *ospC*K1/pBSV2G-*ospC*, so the mice were presumably infected with *ospC*K1*. As expected, we were able to culture spirochetes from the ear skin, bladders, and ankle joints of the donor mice that had been inoculated with A3 and *ospC*K1/pBSV2G-*ospC* but not from mice inoculated with *ospC*K1. We confirmed that the isolates from the *ospC*K1/pBSV2G-*ospC*-inoculated mice had lost the complementing plasmid by the following methods. First, we plated the isolates and screened individual colonies for plasmid presence, finding 0/24 positive for pBSV2G-*ospC* in every isolate. Second, we made DNA from the uncloned isolates and attempted to rescue pBSV2G-*ospC* by electroporation of *E. coli* but found no transformants for any genomic DNA preparation tested. Third, we plated 10^5 to 10^7 bacteria of the isolates on medium containing gentamicin, selecting for the presence of pBSV2G-*ospC*, but found no colonies for any isolate. These results show that no detectable spirochetes within the donor mice retained the complementing plasmid by ~10 weeks of infection, although plasmid-containing bacteria were still detectable at 6 weeks postinoculation.

We wished to assess approximate spirochete numbers in donor mouse tissues in order to determine if the recipient mice received similar doses by transplantation, so we extracted DNA from tissues of the donor mice and assessed their spirochete loads by quantitative PCR. We found similar low levels of spirochetes (for example, around 10 *B. burgdorferi* genome equivalents per 1,000 mouse genome equivalents in ear skin) in tissues from mice inoculated with the wild-type and complemented strains, showing that the numbers of bacteria transplanted to recipient mice were similar. As anticipated, we were unable to detect spirochetes in donor mice that were inoculated with the *ospC*K1 mutant.

Three weeks after transplantation of ear punches from donor mice under the dorsal lumbar skin of naive mice, the recipient mice were bled and their sera was assessed for reactivity with *B. burgdorferi* antigens. As expected, the mice that had received tissue transplants from wild-type-infected mice were seropositive and those that had received transplants from *ospC*K1-inoculated mice were seronegative (Table 5). Intriguingly, the mice receiving transplants from *ospC*K1*-infected mice were also seronegative, demonstrating that OspC function is required to establish infection, even with host-adapted *ospC*K1* spirochetes, which were able to persist indefinitely in their original mammalian hosts. At 6 weeks posttransplantation, recipient mice were sacrificed and culture of spirochetes from their ears, bladders, joints, and transplantation sites was attempted. As expected, only seropositive mice (A3 infected) were culture positive (Table 5).

TABLE 5. Tissue transplantation donor and recipient analysis

Strain inoculated	Donor serology[a]	Donor isolation[b]	*B. burgdorferi* strain recovered from donor	Recipient serology[a]	Recipient isolation[b]
A3	3/3	9/9	A3	3/3	11/12
*ospC*K1	0/3	0/9	NA[c]	0/3	0/12
*ospC*K1/pBSV2G-*ospC*	3/3	9/9	*ospC*K1*	0/3	0/12

[a] Number of animals positive/number of animals tested.
[b] Number of tissue samples positive/number of tissue samples tested (three sites per donor, four sites per recipient).
[c] NA, not applicable (no spirochetes recovered).

DISCUSSION

The experiments described herein establish that OspC is not required by *B. burgdorferi* beyond the initial stage of mammalian infection or for acquisition of spirochetes by feeding ticks. Earlier studies had demonstrated lack of *ospC* gene expression and loss of OspC protein on bacterial surfaces within a few weeks after transmission (9, 19, 25, 29), but the low numbers of bacteria present in infected mammals made it impossible to say that sporadic or time- or tissue-specific expression was not important. Our ability to isolate bacteria with no wild-type copy of the *ospC* gene from multiple mammalian tissues that had been colonized with *ospC*-positive spirochetes demonstrates that *ospC* function is dispensable by 3 to 4 weeks postinoculation. We have not defined the earliest point during mammalian infection after which *ospC* function is no longer required, but it might be possible to approximate that point by isolating bacteria at different times after injection and determining when *ospC*K1* mutant bacteria first appear. Detecting *ospC*K1*, however, requires two steps: first, passing the point at which OspC is no longer required, and second, losing the complementing plasmid. Since the majority of isolates from mice inoculated with *ospC*K1/pBSV2G-*ospC* mutant bacteria analyzed at 28 days postinoculation were *ospC*K1* mutants, OspC is dispensable before that time. The shuttle vector was still detectable in isolates obtained 6 weeks after inoculation, while no bacteria retained the complementing plasmid after about 10 weeks of infection. The nature of the mammalian selection against pBSV2G-*ospC* retention would determine how soon after the end of the requirement for OspC detectable numbers of bacteria lacking the complementing plasmid would be present.

Here we also show that OspC is not required for acquisition of spirochetes by ticks, since they acquire *ospC* mutant spirochetes by feeding on mice infected with *ospC*K1* bacteria (i.e., *ospC*K1/pBSV2G-*ospC* mutant bacteria that had subsequently lost the complementing plasmid). As with our previous mutant (18), we found no defect in *ospC* mutant replication in tick midguts or in migration to the salivary glands. We believe that transmission occurs for bacteria that enter the salivary glands, since residence in this tissue is transient and transfer to the mammal via tick saliva is most likely mechanical. In support of

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this idea, Pal et al. were able to detect *B. burgdorferi* DNA in mouse skin to which ticks infected with wild-type or *ospC* mutant bacteria had been attached, even 12 days after drop-off (34). Also, Ohnishi et al. detected OspC-negative spirochetes in skin fragments attached to the mouth parts of infected ticks that were removed when partially fed (32). Therefore, despite the requirement for OspC function to initiate mammalian infection, the protein is not essential for migration to the salivary gland or transmission to the mammal by a feeding tick. Recently, Ramamoorthi et al. found that OspC binds a tick salivary gland protein that has immunosuppressive activity, increasing the bacterial load in mammals, especially in immune hosts (39). This study shows that OspC interaction with this protein is not required for ticks to acquire spirochetes from infected animals. Also, our present and previous findings (18) show that OspC has an additional and distinct role in mammalian infection, since the protein appears to be essential for *B. burgdorferi* infection even by needle inoculation and tissue transplantation.

Determining the role of OspC in early mammalian infection remains a challenge. One possibility is that OspC serves a specific purpose that is only required early in infection. Alternatively, OspC may perform a function that is required throughout infection, but that role is fulfilled at later times by another protein that is produced after infection is established. OspC may be involved in evading some aspect of the mammalian immune system. Clearance of the *ospC* mutant from SCID mice (18), as well as from immunocompetent mice, at the initial stage of infection, preceding any detectable serologic response, indicates that a possible role in immune evasion would likely be directed at components of innate immunity or antibody-independent actions of complement. OspC does not appear to be solely involved in resisting immunity dependent on Toll-like receptor-mediated signaling, since we recently found that *ospC* mutant spirochetes are unable to infect mice defective in MyD88, an adapter required for most of that host response (48).

Our data are also consistent with OspC potentially being involved in recognizing the mammalian environment and initiating a developmental pathway required for survival. Such a pathway might lead to evasion of innate immune responses or dissemination. Previously, we found that injecting an *ospC* mutant directly into sites that normally become persistently infected (i.e., skin and joint) did not bypass the requirement for OspC (18), perhaps because the bacteria remained unable to recognize their host location and regulate gene expression appropriately. The present transplantation data and those found by Stewart et al. (48) indicate that either the bacterium or the host is "reset" when host-adapted spirochetes enter a new host, renewing the requirement for OspC. Furthermore, our inability to isolate *ospC*K1* bacteria from the site of tissue transplantation argues that OspC plays an essential role preceding dissemination.

Another aspect of infection in which OspC may be involved is host and tissue specificity. Several studies have identified correlations between OspC type (based on amino acid sequence) and productive infection of various hosts or localization to specific tissues (4, 45). Others, however, have found evidence contrary to this correlation (e.g., see references 1 and 10) suggesting either that a closely linked marker is the determining factor for host and tissue specificity or that there is another explanation for the correlations observed.

A remaining question is why pBSV2G-*ospC* is lost during mouse infection. The simplest explanation is that expression of *ospC* from the shuttle vector location leads to immune selection against vector-containing organisms that continue to produce OspC. If down-regulation of *ospC* is a somewhat stochastic process and plasmid loss occurs at a moderate frequency in the absence of immune selection (as shown by loss of pBSV2G alone and increased stability of pBSV2G-*ospC* in SCID mice), then the appearance of antibodies that recognize OspC would select for bacteria that have either shut down *ospC* expression or lost the plasmid that carries it. This model is consistent with the findings of Liang et al., in which OspC-synthesizing bacteria were eliminated in mice that had normal immune systems but not in SCID mice (25). We have determined by quantitative PCR that the copy numbers of pBSV2G and pBSV2G-*ospC* are ~5 to 10, relative to cp26 and the bacterial chromosome, which are present in 1 or 2 copies per cell (data not shown and reference 30). However, we have no direct evidence that this leads to aberrant *ospC* expression. The shuttle vector-borne *ospC* gene is properly regulated in ticks, as assessed by IFA (data not shown), so expression within mammals is probably also regulated appropriately. We have also found little difference in OspC production between A3 and *ospC*K1/pBSV2G-*ospC* bacteria grown in culture (Fig. 2). Finally, sera from mice inoculated with *ospC*K1/pBSV2G-*ospC* bacteria do not have abnormally high seroreactivities with OspC, as might be expected if there were inappropriate OspC synthesis in mice (unpublished results). Nevertheless, even a subtle difference in *ospC* expression could have profound effects on the survival of plasmid-containing bacteria.

This study further defines the time during which the OspC protein is required by *B. burgdorferi* for growth in the mouse-tick-mouse cycle. We demonstrate that the protein is essential in the mammal, but only for a period of days to weeks, whereas rodent hosts remain persistently infected. Upon acquisition, spirochetes are maintained for months within ticks, in which host there is no selection for *ospC* gene retention. As shown here, if *ospC* is located on a nonessential plasmid (e.g., pBSV2G), the mammalian acquired immune response selects for spirochetes that have lost the plasmid carrying *ospC*. Therefore, *B. burgdorferi* requires a mechanism to ensure *ospC* retention throughout the infection cycle in mice and ticks. The location of the *ospC* gene on cp26, which also carries the essential gene *resT* (5), guarantees its maintenance at all stages of the bacterial life cycle, even in the face of acquired immunity. Regulation of the *ospC* gene appears to have a stochastic component, since OspC synthesis continues in spirochetes infecting SCID mice (25), suggesting that the host immune response to OspC selects a bacterial population that has down-regulated expression of the *ospC* gene.

The *B. burgdorferi* life cycle involves two very different host environments to which the spirochete must adapt in order to survive in nature. This and other studies (e.g., reference 43) show that life within each host also involves several stages, which can be roughly described as establishment or acquisition, persistence, and transmission. Other vector-borne protozoan pathogens, such as *Plasmodium* spp., have well-defined developmental cycles within their hosts, with various morphological

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forms living at different times and in specific tissues within the arthropod and mammal. *B. burgdorferi* may undergo a primitive version of such a cycle, defined by changes in surface properties that correlate with or cause changes in gene expression that are required for establishing infection, disseminating to various tissues, and preparing for transfer to the alternate host. In this cycle, the surface protein OspC would be characteristic of and required for the initial stage of mammalian infection, with the variable surface antigen VlsE required for subsequent persistence in the mammal (38, 54) and OspA required for persistence in the tick (53). Although stimuli affecting *ospC* expression in vitro (2, 44) and genes involved in *ospC* regulation (14, 20, 51, 52) have been identified, the signals by which *B. burgdorferi* identifies its host location and carries out this cycle remain unknown. With the genetic tools currently available for studying *B. burgdorferi*, along with the ability to reproduce its natural infection cycle in the laboratory, we can test this model and further define the developmental changes required for colonization, persistence, and transmission of the spirochete both within and between the tick vector and mammalian host.

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