

Outer Surface Protein A Protects Lyme Disease Spirochetes from Acquired Host Immunity in the Tick Vector[∇]

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The Lyme disease spirochete *Borrelia burgdorferi* alters the expression of outer surface protein (*osp*) genes as the bacterium cycles between ticks and mammals. OspA is produced as borreliae enter the tick vector and remains a major surface antigen during midgut colonization. To elucidate the role of OspA in the vector, we created an insertional deletion of *ospA* in strain B31-A3. The *ospA* mutant infects mice when it is injected intradermally and is acquired by larval ticks fed on these mice, where it persists through the molt to the nymph stage. Bacterial survival rates in artificially infected tick larvae fed on naïve mice were compared with those in the vector fed on immune mice. The *ospA* mutant proliferates in larvae if it is exposed to blood from naïve mice, but it declines in density after larval feeding if the blood is from immune mice. When uninfected larvae are fed on B-cell-deficient mice infected with the *ospA* mutant, larvae show borrelial densities and persistence that are significantly greater than those fed on infected, immunocompetent mice. We conclude that OspA serves a critical antibody-shielding role during vector blood meal uptake from immune hosts and is not required for persistence in the tick vector.

The Lyme disease agent *Borrelia burgdorferi* is maintained in natural enzootic cycles involving small mammals and blood-feeding ticks (1, 21, 38, 40). When borrelia-infected *Ixodes scapularis* ticks feed on humans, infection may occur with protean clinical manifestations. Outer surface protein A (OspA) was the first surface protein identified in this bacterium (5, 32). The vector-restricted pattern of OspA production was exploited in its development as a transmission-blocking vaccine (24), and in a purified recombinant form, it was the specific immunogenic component of the first Lyme disease vaccine for humans (61, 63, 66). OspB is the product of a gene cotranscribed with the OspA-encoding gene (31); it is homologous to OspA by inferred amino acid sequence (9) and by structural analysis (7). OspA and -B are differentially produced as borreliae alternate between vector and host, synthesized in ticks but only rarely in mammals (6, 60). During the uptake of an infected blood meal, antibodies to OspA elicited by vaccination appear to block the acquisition of borreliae by the tick (20). A significant portion of borreliae in the tick midgut down-regulates transcription of the *ospAB* operon (49) and synthesis of the encoded proteins (16, 19, 59) during the 3- to 7-day feeding. Concurrent with the loss of OspA and -B, borreliae disseminate from the tick's midgut to the salivary glands (52).

Characterization of OspA as a predominantly tick-restricted surface protein prompted investigation of its function in the vector. OspA may function as a plasminogen binding protein

(28, 36), a function necessary for efficient dissemination in the tick (16). OspA may also function to shield other conserved borrelial surface antigens, such as P66 (12) and P13 integral membrane proteins (43, 56), from antibody recognition; P66 is immunogenic in the mouse reservoir of *B. burgdorferi* in North America (11). Borreliae producing OspA in the tick may be targeted for immune recognition by natural antibodies, based on the relative levels of the *B. burgdorferi* OspA-positive N40 strain in the salivary glands of ticks fed on control and B-cell-deficient mice (8). OspA may act as a midgut adhesin, based on binding of recombinant OspA and of intact borreliae to tick midgut extracts (45, 47). Mutation of *ospA* in *B. burgdorferi* strain 297 is reported to give rise to borreliae that lose tick colonization function (69). Screening of a tick cDNA expression library indicates a specific tick midgut receptor for OspA (46); this paper also reports that the absence of OspA reduces but does not abolish borrelial binding to tick midgut extract. Mutagenesis of the *ospB* gene in the strain B31 borreliae that retain production of OspA is also reported to block tick acquisition of borreliae from infected mice (42). Of the strains used in these studies, strain 297 is a human cerebrospinal fluid isolate, and N40 and B31 are tick isolates: all are infectious in mice (30) but have not been directly compared in the mouse-tick infectious cycle. The strain B31 chromosome (27) and its 21 extrachromosomal elements (14) comprise the only complete Lyme disease borrelia genome published to date. In summary, both vector colonization (attachment and/or dissemination) and host immune avoidance are suggested as reasons for the temporal pattern of OspA production.

To further elucidate the function of OspA in borrelial colonization and survival in the tick vector, we created an *ospA* mutant in an infectious wild-type clone of strain B31 and an *ospA*-reconstituted OspA-producing derivative of the mutant to confirm the single-locus nature of the mutant phenotype.

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TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Bacterium, plasmid, or oligonucleotide	Description or sequence	Reference or source
<i>B. burgdorferi</i> strains		
B31 MI	Medimmune B31; nonclonal, low-passage infectious strain	14
A3	Infectious, transformation-competent clone of B31 MI	23
6	Clonal derivative of A3 (WT)	This study
2E6	<i>ospA</i> mutant generated with pOKOSPAKO (<i>ospA1</i>)	This study
7A	<i>ospA</i> restored, <i>ospB</i> mutant generated with pOKOSPA-C' (<i>ospA</i> ⁺ <i>B1</i>)	This study
<i>E. coli</i> strain		
TOP10F'	Used for propagating plasmids	Invitrogen
Plasmids		
pOK12	P15A replicon, multiple cloning site <i>lacZ'</i> vector	67
pTakanA	<i>B. burgdorferi</i> <i>flaB_p-kan</i> cassette in pCR2.1-TOPO	10
pTAGmA	<i>B. burgdorferi</i> <i>flaB_p-aacC1</i> cassette in pCR2.1-TOPO	22
pOKLP54	pOK12 amplicon fused to LP54 amplicon	This study
pOKOSPAKO	<i>ospA</i> inactivation plasmid; pOKLP54 SpeI-EcoRI fragment ligated with NheI-EcoRI fragment of pTakanA	This study
pOKOSPA-C'	<i>ospA</i> restoration plasmid; pOKLP54 BclI-PvuII fragment ligated with BamHI-PvuII fragment of pTAGmA	This study
Oligonucleotides		
POK12-F1+AscI	5'-TGGCGCGCCTCGCCCTTCCCAACAGTTG	This study
POK12-B1+AvrII	5'-ACCTAGGGCGTATTGGAGCTTTTCGCG	This study
LP54-F2+AscI	5'-AGGCGCGCCTTGGTGGCAAGGAAAACATAC	This study
LP54-B3+AvrII	5'-TCCTAGGCCCTTGGCTTGTGTCTGCG	This study
OSP1A	5'-TAGGATCCAAGCTTAATTAGAACCAAAC	54
OSP2	5'-CTTCTACACTAGCTGATGC	54
KANA.5	5'-AGCCATATTCAACGGGAAACG	This study
KANA.3	5'-CAAGTCAGCGTAATGCTCTGCC	This study
flaB470F	5'-TGGTTTGCCTCCAACATGAACTC	This study
flaB547R	5'-TGCAAAAATTAACACACCAGCAT	This study
flaB523TR	5'-FAM-ACCTTTCAGGGTCTCAAGCGTCTTGGACTTT-TAMRA ^a	This study

^a FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

We introduced these clones and the parental clone into the mouse-tick infectious cycle and examined borrelial survival in ticks through the larval and nymphal stages of transmission.

MATERIALS AND METHODS

Construction of allelic replacement vectors for *ospA* and *ospB* mutagenesis. DNA from the *B. burgdorferi* strain B31 MI (14, 15, 27) was PCR amplified (Expand high-fidelity PCR system; Roche Applied Science, Indianapolis, IN) with the primers LP54-F2+AscI and LP54-B3+AvrII (Table 1), corresponding to sequences within open reading frames (ORFs) BBA1 1 and BBA19 of the lp54 plasmid (14), to yield a 5,226-bp product containing the *ospA* (BBA15) and *ospB* (BBA16) genes. Primers POK12-F1+AscI and POK12-B1+AvrII (Table 1) were likewise used to amplify a 1,737-bp portion of pOK12 (67) containing the kanamycin resistance gene (*kan*) and the p15A low-copy origin of replication. PCR products, isolated by QIAquick purification (Qiagen, Valencia, CA) were separately digested with AscI and AvrII and ligated (T4 ligase; New England Biolabs, Ipswich, MA). The resultant DNA was used to transform *Escherichia coli* (One Shot TOP10F'; Invitrogen Corp., Carlsbad, CA). Plasmid DNA from colonies selected on kanamycin LB plates (50 µg/ml) was screened for predicted restriction fragment sizes by agarose gel electrophoresis, using PacI digest to identify desired products. One clone, pOKLP54, was chosen to produce internally deleted versions of the *ospA* and *ospB* genes bearing selectable markers. The pTakanA vector, containing the *kan* resistance gene controlled by the *flaB* promoter of *B. burgdorferi* (*flaB_p-kan*) (10), was digested with EcoRI and NheI to produce a *flaB_p-kan* cassette fragment. This fragment and the 6,620-bp product of an EcoRI-SpeI restriction digest of pOKLP54 were isolated by agarose gel electrophoresis and QIAquick purification. The latter enzymes remove a 324-bp fragment from the *ospA* gene. The *flaB_p-kan* cassette was inserted into the deleted *ospA* gene of pOKLP54, and transformants of *E. coli* TOP10 plated on 50 µg/ml kanamycin LB were screened by PCR and restriction digestion, resulting in pOKOSPAKO.

To generate a plasmid to restore the functional *ospA* allele, we inserted a gentamicin resistance marker in the *ospB* gene of pOKLP54, leaving *ospA* intact. pOKLP54 was digested with PvuII and BclI to remove 567 bp of *ospB*. The resistance cassette vector pTAGmA, containing the aminoglycoside acetyltransferase (*aacC1*) gene of Tn1696 linked to the *flaB* promoter of *B. burgdorferi* (22), was digested with PvuII and BamHI. The pOKLP54 backbone fragment and the *flaB_p-aacC1* cassette were isolated by agarose gel electrophoresis with BlueView stain (Sigma-Aldrich, St. Louis, MO), purified with QIAquick, and ligated. Transformants of *E. coli* TOP10 selected on 50 µg/ml kanamycin-5 µg/ml gentamicin LB plates were screened for the expected construct by PCR and restriction digest to yield pOKOSPA-C'.

Transformation of *B. burgdorferi*. A single colony isolate of *B. burgdorferi* B31-A3 (23), designated the wild type (WT), was prepared for electroporation as described previously (53, 57), transformed with 25 µg of pOKOSPAKO and plated on Barbour-Stoener-Kelly (BSK) agarose containing 100 µg/ml kanamycin. Twelve kanamycin-resistant borrelia colonies were recovered after 7 to 10 days of growth for expansion in liquid BSK II medium (2, 33, 62) containing the same concentration of kanamycin. PCR amplification of DNA from the resistant clones with primers flanking the *ospAB* operon (Table 1) gave 2.8-kb products, as expected from the excision of 324 bp from *ospA* and the insertion of the *flaB_p-kan* cassette, while the untransformed WT gave a 1.9-kb product. All of the clones showed the expected product size of 857 bp when amplified with the *kan* gene primers KANA.5 and KANA.3 (Table 1). Five of twelve clones contained the WT complement of plasmids, detected with 29 plasmid-specific primer pairs as described previously (23); one, designated the *ospA1* clone, was chosen for use in further work.

To restore OspA production, *ospA1* was transformed with 30 µg of pOKOSPA-C' (as described above) and plated on BSK medium containing 10 µg/ml gentamicin. Twelve colonies were expanded in liquid medium with gentamicin and screened by PCR with OSP1A and OSP2 to identify the *ospAB* locus products with the expected size of 2.48 kb and with KANA.5 and KANA.3 to test for the loss of the *flaB_p-kan* cassette from *ospAB*; five of these products were then screened with strain B31

plasmid-specific PCR primer pairs, and two of them retained the full plasmid complement; one of these products was designated *ospA⁺B1*. Passages of the WT and mutant clones used for experiments were monitored for plasmid content by PCR with plasmid-specific primer pairs (23).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. Lysates of low-passage, uncloned MI B31 and B31-A3 WT and the *ospA1* and *ospA⁺B1* clones, grown at 35°C to stationary phase were prepared as described previously (58), except that 50 mM dithiothreitol (Boehringer Mannheim, Indianapolis, IN) was substituted for 2-mercaptoethanol as the reducing agent in lysates prepared for analysis of the OspA protein (5). Lysates were subjected to SDS-PAGE (12.5% separating gel; acrylamide/bisacrylamide, 30:0.8) (37). Proteins in replicate gel panels were stained with Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA) or transferred to nitrocellulose membranes (64) as described previously. Membranes were blocked and reacted with monoclonal antibodies H5332 (anti-OspA) (5), H6831 (anti-OspB) (4), H4610 (anti-OspB) (54), mouse sera, and rabbit polyclonal J5750 (anti-*B. hermsii* FlaB; C. Guyard M. Schrupf, and T. Schwan, unpublished data). Bound antibodies were reacted with horseradish peroxidase-conjugated protein A (Zymed, San Francisco, CA) and detected by enhanced chemiluminescence, as recommended by the manufacturer (Amersham ECL Western blotting detection reagents; GE Healthcare, Buckinghamshire, United Kingdom), using Hyperfilm ECL (Amersham).

Borrelial survival in ticks feeding on infected mice. Rocky Mountain Laboratories (RML) mice, an outbred strain of Swiss-Webster mice, were injected intradermally with the WT, *ospA1*, and *ospA⁺B1* clones at inoculating doses as described for individual experiments. C57BL/6J (B6) and B6.129S2-*Igh-6^{mlCgn}/J* (B6.Igh^{-/-}) (34) mice obtained from JAX (Bar Harbor, ME) were injected intradermally with 10⁴ to 10⁵ borreliae of the WT, *ospA1*, and *ospA⁺B1* clones. Mice were tested at 3 weeks postinjection by immunofluorescence assay (IFA), as described previously (50), to test for seroconversion. Healthy *Ixodes scapularis* larvae (Tick Rearing Facility, Oklahoma State University, Stillwater, OK) were fed on these mice. At the times indicated for each experiment, tick midguts were macerated with forceps against the wells of Teflon-coated slides in phosphate-buffered saline, homogenized by pumping with a micropipette prior to drying and fixation, and reacted for IFA with rabbit anti-*B. burgdorferi* and fluorescein-labeled goat anti-rabbit immunoglobulin G (IgG) (KPL, Gaithersburg, MD) as described previously (50). To reduce fluorescence interference from blood, 0.1 volume of homogenate was aliquoted to a separate well before IFA. Molted, unfed nymphs were also analyzed by IFA. Statistical analyses of enumerated borreliae were performed with GraphPad Prism software, version 4.0c for Macintosh (GraphPad Software, San Diego, CA). The RML animal facility is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Experiments using animals were carried out using protocols approved by the RML animal care and use committee, prepared according to National Institutes of Health guidelines.

In vitro serum killing assay. Based on a 24-h killing assay (8), but utilizing BSK II medium rather than glucose-supplemented phosphate-buffered saline as the assay medium, the WT, *ospA1*, and *ospA⁺B1* clones grown to late exponential stage were adjusted to 2 × 10⁶ to 5 × 10⁶ borreliae per ml of BSK II medium. Duplicate 100-μl aliquots of each culture were mixed with 100 μl of BSK II, BSK II containing naïve mouse serum, or BSK II containing immune mouse serum and all containing an antibiotic mixture for borrelia (Sigma-Aldrich). Mouse sera pooled from three naïve and three WT-infected RML mice (8 weeks postinfection) were added to BSK II at a dilution of 1 part per 7 parts of medium (a final serum titer of 1:16) and filtered through a 0.22-μm syringe (Millex-GS; Millipore Corp., New Bedford, MA) before the addition to borreliae. Sealed tubes were incubated for 24 h, and aliquots of each culture were plated in BSK II agarose and counted after 10 days of incubation at 35°C in 2.5% CO₂ to determine CFU.

Artificial infection of ticks and feeding on mice. *I. scapularis* larvae were infected with the WT, *ospA1*, and *ospA⁺B1* clones by 1.5-h immersion in culture suspensions of 1 × 10⁷ to 3 × 10⁷ borreliae per ml (50), with the following modification. Larvae were preexposed to a relative humidity of 80.5% maintained by a saturated solution of (NH₄)₂SO₄ (68) immediately before immersion to stimulate rehydration behavior (35, 55). Exposure to 80.5% relative humidity was adjusted to 4 days for 1- to 2-month-old larvae, 3 days for 3- to 4-month-old larvae, and 1 day for 5- to 6-month-old larvae (larvae of >6 months postemergence were not used for immersion). Calibrated dehydration reproducibly increased the percentage of infected larvae (data not shown). At the times pre- and postfeeding as indicated in each experiment, infected tick midguts were examined by IFA as described above.

Borrelial survival in infected larvae fed on immune versus naïve mice. “Immune” RML mice were first infected by tick bite using *I. scapularis* larvae artificially infected with the WT clone; “naïve” control mice were infested with

noninfected larvae. At 4 weeks postfeeding, mice were tested by IFA for reactivity to B31. Both the immune and the naïve groups were then treated with ceftriaxone (50 mg/kg of body weight/dose; Sigma-Aldrich) intramuscularly for 5 days (41, 48) and tested for transmissible borrelia at 1 week posttreatment by xenodiagnosis (feeding of healthy ticks and examining midguts by IFA) to verify clearance of borrelial infection. Groups of tick larvae infected by immersion with the WT, *ospA1*, and *ospA⁺B1* clones were then applied to immune and naïve mice (approximately 90 days after the initial infection). Dissected midguts of larvae were examined at 7 days postrepletion and after molt by IFA for the enumeration of borreliae.

Detection of mouse infection. Mice that were fed upon by larval and nymphal stage ticks were held for 3 to 4 weeks before IFA to determine their exposure to B31 as described previously (50). Mice were sacrificed, and bladder and joint tissues were cultured at 35°C in BSK II medium containing an antibiotic mixture for borrelia (Sigma-Aldrich) to control potential contaminating bacteria. Cultures containing borreliae detected by dark-field microscopy at 2 weeks postinoculation were extracted for DNA (DNeasy; Qiagen). DNA was amplified by PCR (Roche Corp., Indianapolis, IN) with primers OSP1A and OSP2 (Table 1), and products were analyzed by agarose gel electrophoresis to identify the size of the *ospAB* locus. To detect levels of borrelial DNA in infected tissue, DNA from mouse ears was extracted (DNeasy) and further purified by Qiaquick PCR purification (Qiagen). Aliquots of 100 ng were analyzed in triplicate for *B. burgdorferi* *flaB* DNA, using the primers *flaB470F* and *flaB547R* and the probe *flaB523TR* (Table 1), designed with ABI Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA), by real-time quantitative PCR with an ABI Prism 7900HT sequence detection system (Applied Biosystems), using Brilliant II QPCR Master Mix (Stratagene, La Jolla, CA), as recommended by the manufacturer. DNA from strain B31 MI served as the standard for quantification of the *flaB* copies.

RESULTS

Inactivation and reconstitution of *ospA*. A segment of lp54 containing the *ospAB* locus from *B. burgdorferi* strain B31 cloned in a shuttle vector (pOKLP54) was modified by the placement of a *flaB_P-kan* antibiotic resistance marker within an internally deleted *ospA* coding region. The resultant construct, pOKOSPAKO, was used to transform a clonal derivative of the B31-A3 WT. Five kanamycin-resistant transformants retained all plasmids found in the WT clone. The sequencing (data not shown) of one clone, 2E6, at the *ospAB* locus was compatible with a double crossover insertion of the *flaB_P-kan* element from the transforming vector DNA into the *ospA* ORF (Fig. 1) located on the 54-kb linear plasmid (3). This is the *ospA1* clone used in all experiments.

B31-A3 is a clone derived from strain B31 MI with a uniform extrachromosomal DNA profile that is competent for transformation, infectious when injected in mice, acquired by *I. scapularis* larvae from mice, and transmitted to mice by nymphal ticks (23, 29). Although it is designated WT for comparison to the *ospAB* mutants described here, it produces a full-length OspA protein and a truncated OspB protein (23). To identify *ospAB* products in the WT and the *ospA1* mutant, we examined borrelial lysates by SDS-PAGE and immunoblotting (Fig. 2). B31 MI produced full-length OspA and -B proteins, the WT clone contained full-length OspA and truncated OspB, and the *ospA1* mutant lacked both OspA and OspB. Both the full-length and truncated forms of OspB (Fig. 2D) are recognized by monoclonal antibody H4610 (54). The *flaB_P-kan* insertion in the *ospA* ORF eliminates both OspA production and truncated OspB production, the latter by apparent polar effect on the *ospB* gene. As a control, FlaB presence was examined and found at equivalent levels in all three extracts (Fig. 2E).

An isogenic *ospA* restoration strain was constructed in *ospA1* by a similar allelic exchange strategy. A *flaB_P-aacC1* resistance

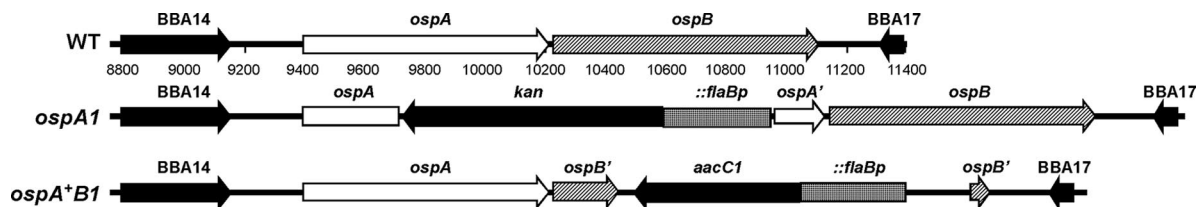


FIG. 1. Physical maps of the lp54 *ospAB* locus in the B31-A3 WT, the *ospA* mutant, and the *ospA* restored strains. WT, segment of lp54 from bp 8750 to 11400, as indicated by numbers below the map (27), from locus tag BBA14 to BBA17 in clonal isolate A3-6. *ospA1*, mutation of *ospA* by insertion of a *flaB_p-kan* marker (10) inserted in an internally deleted *ospA* gene via transformation of A3-6 with pOKOSPAKO (described in Materials and Methods), resulting in clone 2E6. *ospA+B1*, restoration of the *ospA* mutation by insertion of a *flaB_p-aacC1* marker (22) in an internally deleted *ospB* gene on the 3' end of an intact *ospA* gene via transformation of 2E6 with pOKOSPA-C' (described in Materials and Methods), resulting in clone 7A.

marker was inserted within *ospB* in pOKLP54 to create pOKOSPA-C', which was electroporated into *ospA1*. The resultant gentamicin-resistant clones were screened by PCR analysis of the *ospAB* locus to identify those transformants that contained a single product of the expected size. Sequence analysis (data not shown) of clone 7A verified that an intact *ospA* gene, in tandem with an internally deleted *ospB* gene bearing the *flaB_p-aacC1* marker, had inserted in lp54 at the *ospAB* locus (Fig. 1). Immunoblot analysis of this clone (Fig. 2B) showed OspA production, and immunofluorescent staining of live borreliae (data not shown) verified that OspA was exposed on the borrelia surface; no OspB was detected by immunoblotting analysis (Fig. 2C and D). Clone 7A is the *ospA+B1* clone in all experiments.

The tick vector acquires OspA-deficient borreliae from infected mice. A previous study indicated that borreliae lacking

OspA are not stably acquired by *I. scapularis* nymphs when they feed upon mice infected with an *ospAB* mutant of *B. burgdorferi* strain 297 or when ticks are inoculated via the rectal aperture with this mutant (69). We tested the ability of the B31-derived *ospA1* strain to establish infection in mice and in *I. scapularis* larvae. Two mice per group were injected intradermally with 5×10^3 borreliae of the WT, *ospA1*, and *ospA+B1* clones. At 3 weeks postinjection, we observed seroconversion in one WT-injected mouse, in both *ospA1*-injected mice, and in one *ospA+B1*-injected mouse (data not shown). Larvae fed on seropositive mice were dissected for IFA at 0, 3, and 10 days postrepletion. IFA is routinely used as an appropriate and accurate method for the evaluation of individual tick midguts for borrelial density (17, 20, 42, 44, 46, 47, 59, 69). As a test of viability of acquired borreliae, larvae fed on mice infected with the three *ospA* genotypes were homogenized and plated in BSK-agarose at day 0 postrepletion; all cultures gave rise to borrelia colonies. Larvae fed on mice infected with the *ospA1* and *ospA+B1* borreliae showed significantly lower borrelial densities than the larvae fed on mice infected with the WT at day 0, but at day 3 only the *ospA1* group was significantly different from the WT (Fig. 3). At day 10 postrepletion, we found no borreliae in 12 midguts examined from the two *ospA* mutant-infected mouse feedings; the *ospA+B1* clone was not significantly different from the WT at day 10. The absence of detectable *ospA1* borreliae at day 10 appears to reflect a limit of detection by the IFA, because molted, unfed nymphs from all three groups showed the presence of midgut borreliae, including 4 of 10 midguts examined for *ospA1*. The densities of *ospA1* and *ospA+B1* borreliae infections in unfed nymphs were not statistically different from that of the WT. Acquisition of the mutant was not abolished by the deficiency of OspA and OspB, in contrast to results with the strain 297 *ospAB* mutant (69). The WT clone, producing a truncated OspB protein, and the *ospA+B1* clone, lacking OspB, were both capable of establishing tick midgut infections that showed no population decline during the 10-day postengorgement period and persisted transstadially, in contrast to results with another strain B31 *ospB* mutant (42).

Density of mouse infection and immune response to the OspA-deficient clone. Since the lack of OspA impaired but did not abolish acquisition of borreliae by tick larvae, we asked if aspects of mouse infection by the mutant, including relative levels of borreliae in mouse tissue and the quality of the humoral immune response to infection, might influence borrelial

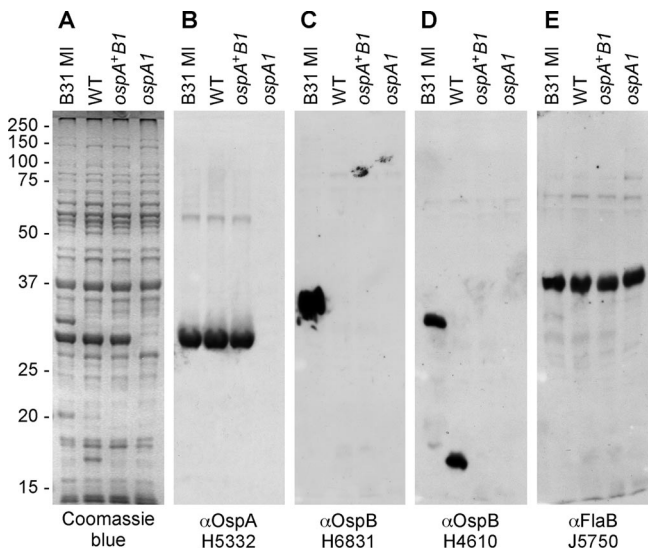


FIG. 2. Protein profiles of strain B31 MI and the WT and isogenic *ospAB* clones; detection of OspA, OspB, and FlaB. (A) Coomassie blue gel shows whole-cell lysates from 35°C cultures of B31 MI, WT, *ospA+B1*, and *ospA1* strains separated by SDS-PAGE and stained with Coomassie blue. (B to E) Immunoblots of replicate SDS-PAGE separations reacted as described in Materials and Methods with OspA-specific monoclonal antibody H5332 (B), OspB-specific monoclonal antibody H6831 (C), OspB-specific monoclonal antibody H4610 (D), and FlaB-specific rabbit polyclonal antiserum J5750 (E). Migration of molecular mass standards in kDa is indicated at left. α OspA, anti-OspA.

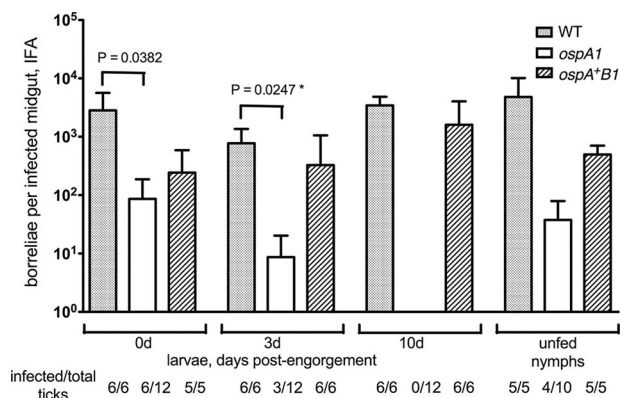


FIG. 3. Acquisition of the WT and *ospAB* clones from infected mice. RML mice were injected intradermally with 5×10^3 bacteria of the WT, *ospA1*, and *ospA⁺B1* clones, as designated, and normal *I. scapularis* larvae were allowed to feed on seropositive mice at 3 weeks postinjection. Borreliae in dissected midguts from each group were enumerated by IFA at 0, 3, and 10 days postengorgement and after they molted to nymphs (unfed nymphs); values represent means plus standard deviations (error bars) for infected midguts. Infection frequency (no. of infected/total ticks) of assayed tick groups is shown. The *ospA1* and *ospA⁺B1* groups were subjected to a *t* test comparison with the WT group at each time point; a significant *t* test *P* value is shown above bracket. *, the value for the WT and *ospA1* clones at 3 days postengorgement was significant ($P = 0.0247$) compared by an unpaired *t* test with Welch's correction.

density upon vector acquisition. We repeated the injection of mice with the WT and *ospA1* isolates, at an inoculating dose of 5×10^4 borreliae and observed seroconversion for all three animals in each group at 3 weeks postinjection. At 8 weeks postinfection, animals were sacrificed, and sera and tissue were recovered. All animals were culture positive. We assayed mouse ear DNA for *B. burgdorferi flaB* DNA copies by quantitative PCR (Table 2) to avoid variable efficiency in the recovery of viable borreliae from tissue samples. Values for *flaB* were significantly different between animals within the WT group and those within the *ospA1* groups, but there were no statistically significant differences between the WT and *ospA1* groups.

To compare the patterns of borrelial antigen recognition between mice infected with WT and those infected with the *ospA1* clones, we reacted immunoblots of the WT and the *ospA1* lysates with each serum sample (Fig. 4). With minor

TABLE 2. Copies of *flaB* DNA in mouse tissue

Clone	Mouse	Mean no. \pm SD of copies of <i>flaB</i> /100 ng DNA ^a
WT	1	107.1 \pm 15.5
	2	235.7 \pm 12.0
	3	4.9 \pm 2.1
<i>ospA1</i>	1	9.9 \pm 3.0
	2	265.4 \pm 39.6
	3	12.1 \pm 4.8

^a Quantitative PCR was performed with triplicate 100-ng aliquots of mouse ear DNA for each sample to determine the mean number of copies of *flaB* DNA \pm standard deviation (SD) relative to that of the B31 MI DNA standard, as described in Materials and Methods.

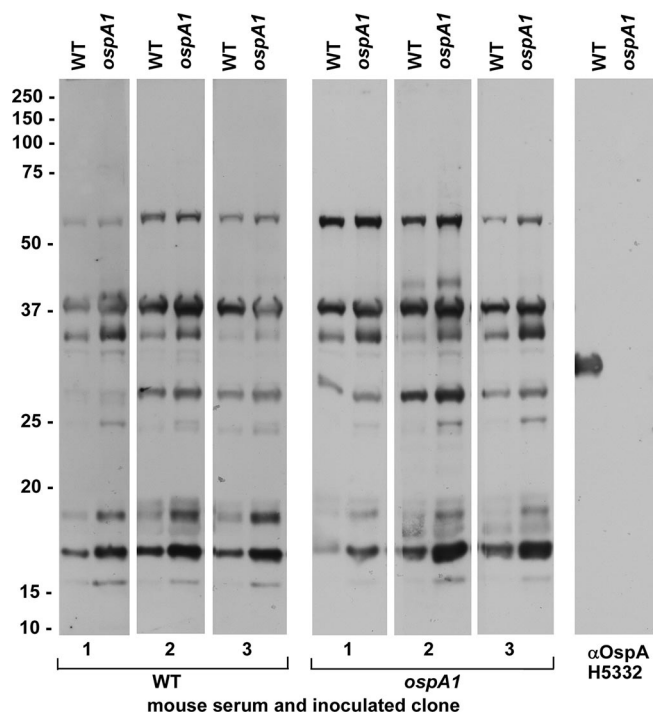


FIG. 4. Recognition of *B. burgdorferi* antigens by sera from infected mice. RML mice were injected intradermally with 5×10^4 bacteria of the WT or *ospA1* clone, as designated, and serum was recovered from blood collected 8 weeks postinjection. These infected mouse sera (1:200 dilution) and OspA-specific monoclonal antibody H5332 (1:500 dilution) were reacted with immunoblots of the WT and *ospA1* clones as described in Materials and Methods. Migration of molecular mass standards in kDa is indicated at left. α OspA, anti-OspA.

differences between the intensities of reactions, the two borrelial clones produced antibody responses in mice that resulted in recognition of similar antigens in the WT and *ospA1* lysates. Recognition of OspA, as defined here and in Fig. 2 by reactivity to monoclonal antibody H5332, is absent in the mouse serum samples. Thus, similar antigens may be targeted for humoral immune recognition when ticks acquire borreliae from a blood meal, whether the mouse infection involves OspA-producing or OspA-deficient borreliae.

OspA-deficient borreliae persist through larval and nymphal feeding on naïve mice. Since the *ospA1* mutant was less effective than the controls at colonizing larvae when acquired from infected mice, we compared colonization and replication of the *ospA1* isogenic clones in ticks by infecting larvae before blood meal exposure. We used calibrated dehydration of larvae (see Materials and Methods) to enhance the frequency of artificial infection by immersion. The *ospA1* mutant was present in larval midgut samples before and after feeding on naïve mice, as were the WT and *ospA⁺B1* clones (Fig. 5). There were no significant differences in relative densities between clones in unfed larvae, suggesting an ability of the *ospA1* mutant to colonize the midgut in the absence of the blood meal. After the uptake of blood, the *ospA1* colony density was reduced relative to that of the WT in 7-day fed larvae, while the *ospA⁺B1* restored clone density showed no significant difference from that of the WT. Importantly, the blood meal from these naïve mice resulted in an average density of 4.3×10^3 *ospA1* borre-

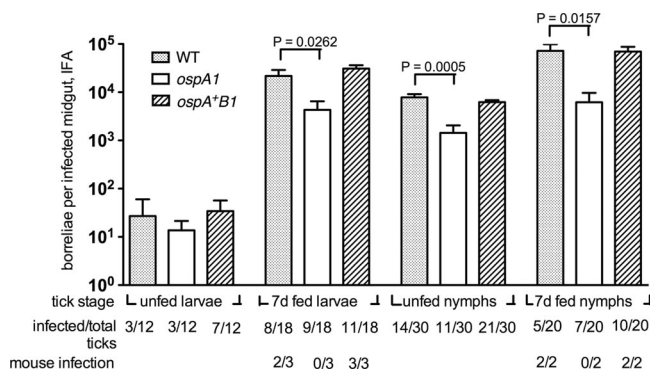


FIG. 5. Replication of the WT and *ospAB* borreliae in artificially infected larvae fed on naïve mice. *I. scapularis* larvae infected by immersion in the WT, *ospA1*, and *ospA⁺B1* clones as indicated were fed on RML mice at larval and nymphal stages. Borrelial density in infected midguts from larvae and nymphs before feeding or at 7 days postengorgement was enumerated by IFA; values represent means plus standard deviations (error bars) for infected midguts. The infection frequency of assayed tick groups is shown as in Fig. 3. The transmission frequency of borreliae to mice (mouse infection) was determined as described in Materials and Methods. Ticks infected with the *ospA1* and *ospA⁺B1* clones were subjected to *t* test comparison with the WT at each time point; significant *t* test *P* values are shown above the brackets.

liae per infected midgut at 7 days postengorgement, whereas the same clone was not detectable in 10-day-engorged larvae acquired from infected mice (Fig. 3). The densities of midgut infections at 7 to 10 days postengorgement for the WT and *ospA⁺B1* clones were similar, whether infection arose from the blood meal (Fig. 3) or from preliminary inoculation by immersion (Fig. 5). The results of these two experiments suggest that the density of midgut infection by the *ospA1* mutant is influenced by the immune status of the mouse providing the blood meal. The *ospA1* mutant replicates in larval midguts when exposed to blood from a naïve mouse but may not achieve the same midgut density as controls because of a natural antibody toward other borrelial antigens (8), a lack of plasminogen binding (16), or reduced midgut adhesion (46). Transmission of these clones to the naïve mice was examined by IFA and culture of mouse tissue. The WT clone infected two of three mice, the *ospA1* clone infected none of three mice, and the *ospA⁺B1* clone infected all three mice. These values were not subjected to statistical analysis but suggest that the *ospA1* clone may not be transmitted from infected larvae.

After they molted to the nymphal stage, WT, *ospA1*, and *ospA⁺B1* clone-infected nymphs were tested by IFA to determine midgut borrelial densities before feeding (Fig. 5). Unfed nymphs infected with the *ospA1* clone had significantly lower midgut borrelial densities than nymphs infected with the WT. After nymphs fed on naïve mice, the density of borreliae increased, but the *ospA1* clone density was, again, lower than that of the WT. Each pair of mice in the WT and *ospA⁺B1* clone-infected groups gave serological and cultural evidence of infection, but neither of the mice fed upon by nymphs infected with the *ospA1* clone became infected. Therefore, the *ospA1* mutant survived transstadially from larvae to nymphs and survived the nymphal blood meal but may not be competent to infect mice during nymphal feeding. Truncation of OspB in the

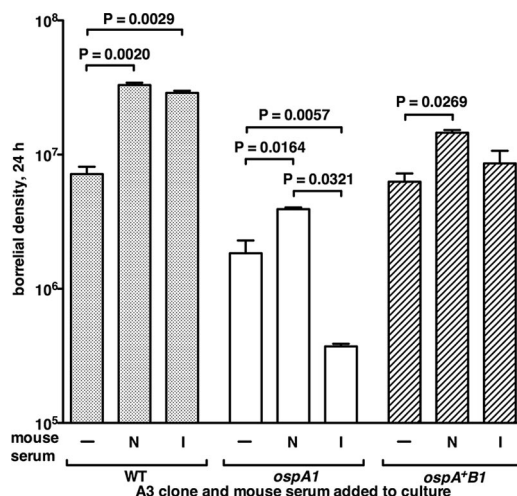


FIG. 6. Survival of the OspA-deficient and control clones with mouse serum in vitro. The WT, *ospA1*, and *ospA⁺B1* clones grown in BSK II (-) or BSK II containing naïve (N) or borrelia-infected (I) mouse sera, as described in Materials and Methods. Values are mean borrelial densities plus standard deviations (error bars) from colony counts of dilutions after 24 h of incubation (duplicate incubations for each condition). One-way analysis of variance posttest values for significant differences between conditions are shown above the brackets.

WT and its absence in the *ospA⁺B1* reconstituted clone did not prevent tick colonization or transmission to mice.

Serum killing of the OspA-deficient clone in vitro. Since the *ospA1* clone was found in larval midguts at a lower density than the WT after engorgement on infected mice (Fig. 3) but replicated in larvae after a naïve blood meal (Fig. 5), we compared the WT, *ospA1*, and *ospA⁺B1* clones in vitro for their abilities to survive exposure to serum from naïve mice and mice infected with the WT borreliae, independent of the larval midgut environment. The WT-immune sera were identical to those collected and tested by immunoblotting in Fig. 4, as described above. We assayed viability in serum that was not heat treated to allow antibody interaction with complement activity in killing of borreliae. Incubation of the WT in BSK II supplemented with naïve or immune serum resulted in statistically significant increases in density relative to that of incubation in BSK II alone (Fig. 6). The OspA-deficient *ospA1* clone also showed a significant increase in density in naïve serum but a significant decrease in immune serum; it declined 10-fold in viable count relative to that of naïve serum. The *ospA⁺B1* clone showed enhanced survival in untreated naïve serum compared to that in BSK II alone, but immune serum did not significantly affect its growth. This test indicates that OspA deficiency can alter the viability of in vitro-grown borreliae upon exposure to mouse serum, consistent with previous results of serum sensitivity of borreliae lacking Osps (12, 56). The BSK II medium used in this assay allow continued growth of borreliae, which may obscure natural antibody killing, which has been detected in medium that does not support borrelial growth (8).

OspA-deficient borreliae are depleted in ticks after feeding on an immune mouse. We wanted to determine whether the *ospA1* mutant, in preinfected ticks, was selectively cleared from the midgut by a blood meal from an immune mouse. Such

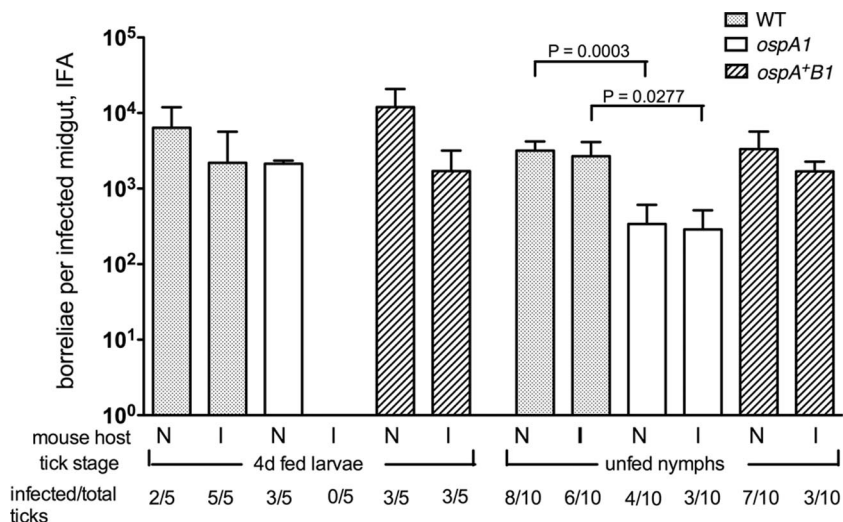


FIG. 7. Effect of acquired immunity to *B. burgdorferi* on the survival of OspA-deficient borreliae. Naïve, mock-infected RML mice (N) and immune mice (I) infected via larval tick bite with the A3-6 wild type, identified as seropositive and cured by ceftriaxone treatment, were used as hosts for feeding by larvae artificially infected with the WT, *ospA1*, and *ospA^{+B1}* clones. Borrelial densities in infected midguts from larvae 4 days postengorgement and in molted, unfed nymphs were enumerated by IFA; values represent means plus standard deviations (error bars) for infected midguts. The infection frequencies of assayed tick groups are shown as in Fig. 3. Student's *t* test values for significant differences between naïve- and immune-fed ticks at each tick stage are shown above the brackets.

clearance could be inferred from the results shown in Fig. 3 and 5, but a more direct comparison of the effects of immune and naïve blood on resident borreliae was needed. Mice in an “immune” group were infected with WT borreliae and cured by antibiotic treatment, as verified by xenodiagnosis (see Materials and Methods). “Naïve” host mice were fed upon by uninfected larvae but also received antibiotic and xenodiagnosis. Test larvae were artificially infected with the WT, *ospA1*, and *ospA^{+B1}* clones, as in the previous experiment, and divided into groups fed upon the immune and naïve mice. At day 4 postengorgement, the densities of borreliae in ticks infected with the WT and *ospA^{+B1}* clones were not significantly different between the larvae fed on immune mice and those fed on naïve mice (Fig. 7). The density of borreliae in ticks infected with the *ospA1* clone 4 days after naïve blood intake was not significantly different from the WT density. After immune blood meal uptake, however, there were no *ospA1* borreliae detected in larval midguts at day 4. After molting to nymphs, ticks from these groups were examined by IFA, at which time the *ospA1* mutant borreliae densities detected in ticks that had fed on naïve mice were similar to those that fed on immune mice at the larval stage, indicating they were not eliminated by the immune blood and were capable of replicating to comparable levels. These levels were still significantly reduced, relative to those of the WT borreliae, indicating an effect of OspA deficiency independent of the blood meal source. This experiment showed a significant if transient effect of an immune blood meal on the *ospA1* clone and corroborates earlier findings that borreliae producing OspA show significant resistance to acquired immunity to other surface antigens, both in vitro (56) and in vivo (18).

The *ospA1* mutant proliferates in larvae after acquisition from infected B-cell-deficient mice. Since survival of the *ospA1* mutant in larvae was diminished by specific immunity in the mouse host, we asked whether intake of borreliae

from infected mice lacking humoral immunity would show enhanced *ospA1* survival relative to that of immunocompetent mice. C57BL/6J (B6) control mice and B6.129S2-*Igh-6^{tm1Cgn}*/J (B6.*Igh^{-/-}*) B-cell-deficient congenic mice were injected intradermally with the WT, *ospA1*, and *ospA^{+B1}* borrelial clones. When mice were tested at 3 weeks postinjection, all B6 mice had seroconverted. B6.*Igh^{-/-}* mice were not tested for serological response, but all mice gave evidence of infection upon culture for borreliae after tick feed; a single B6.*Igh^{-/-}* mouse injected with the *ospA1* clone died before culture but was positive when tested for infection by xenodiagnosis. Uninfected larvae were fed on these infected mice, and midguts were examined by IFA at 0, 3, and 10 days postengorgement for density of borrelial infection. The *ospA1* clone was initially detected (at 0 and 3 days) in larval midguts after ticks fed on infected B6 mice (Fig. 8), but they were not observed at 10 days postengorgement, as previously observed with acquisition from RML mice (Fig. 3). After feeding on B6.*Igh^{-/-}* mice, the *ospA1* clone was detected at day 0 and remained at an elevated density up to 10 days postengorgement. It survived larval acquisition from B6.*Igh^{-/-}* mice at a level significantly greater than the same clone acquired from B6 mice, assessed at day 0 postengorgement. Densities were not significantly different at day 3 postengorgement. The lack of any sign of infection in larvae 10 days after feeding on B6 mice did not allow statistical comparison but clearly demonstrated a difference in outcome relative to the *ospA1* density in larvae feeding on B6.*Igh^{-/-}* mice. We observed significant increases at 0, 3, and 10 days postengorgement in the WT and *ospA^{+B1}* borreliae densities in ticks fed on B6.*Igh^{-/-}* mice compared to that of ticks fed on B6 mice, likely an effect of antibody on survival of borreliae that are competent for OspA production.

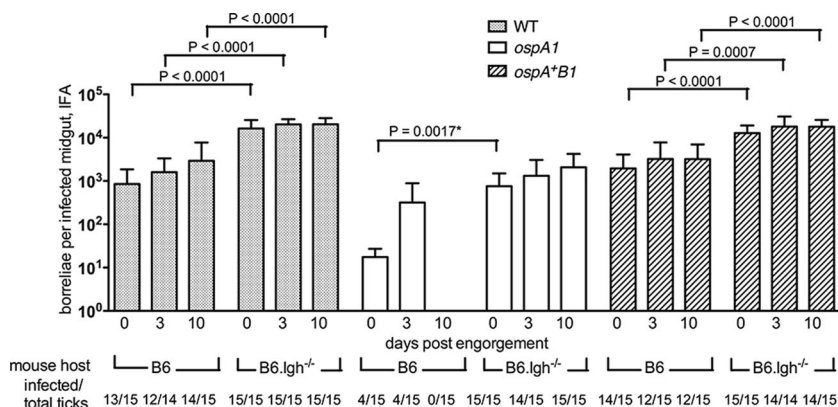


FIG. 8. Relative survival of OspA-deficient borreliae acquired from intact and B-cell-deficient infected mice. C57BL/6J (B6) and C57BL/6J *Igh*^{-/-} (B6.*Igh*^{-/-}) congenic mice were injected with the WT, *ospA1*, and *ospA*^{+B1} clones, and B6 mice were tested at 3 weeks for serological response; normal *I. scapularis* larvae were fed at 4 to 5 weeks postinfection. Borrelial density in infected midguts from larvae at 0, 3, and 10 days postengorgement was enumerated by IFA; values represent means plus standard deviations (error bars) for infected midguts. The infection frequencies of assayed tick groups are shown as in Fig. 3. Statistically significant *t* test values of borrelial midgut density for ticks fed on B6 mice relative to ticks fed on the B6.*Igh*^{-/-} mouse host genotype at each time point are shown above the brackets; *, the value for the *ospA1* clone at day 0 postengorgement, with B6 versus B6.*Igh*^{-/-} mice, was significant (*P* = 0.0017) compared by an unpaired *t* test with Welch's correction.

DISCUSSION

We created an *ospA* mutant from strain B31 to examine the relevance of OspA lipoprotein to borrelial colonization of the tick vector. The mutant declined to undetectable levels in the larval midgut when acquired from an immunocompetent mouse host (Fig. 3). However, when it was introduced by artificial infection by immersion, followed by larval engorgement on naïve mice, the *ospA1* mutant proliferated in larval midguts (Fig. 5). The relative survival of the *ospA1* mutant upon ingestion of naïve versus that on immune mouse blood is similar to the effects of naïve and immune serum on the *ospA1* mutant in vitro (Fig. 6) and suggests that mouse humoral immunity, in the absence of OspA, recognized borrelial antigens that are conserved between in vitro and in vivo (tick midgut) conditions. The data for the *ospA1* mutant midgut survival in naïve versus immune blood led us to propose that a role for OspA, which is not typically produced in the mammalian host and thus not recognized by immune serum, is to shield borreliae as they enter the midgut from specific antibodies in the mouse blood meal that recognize conserved borrelial proteins. We tested this hypothesis with immune mice that were cured of borrelial infection before feeding infected larvae (Fig. 7) and with normal larvae fed on infected mice that were deficient in humoral immune response (Fig. 8); the results of these experiments are consistent with the hypothesis that OspA provides an immunity-shielding function. The findings extend from previous observations with a strain B31 variant that lacks expression of OspA, -B, -C, and -D (12, 56). Those authors demonstrated in vitro growth inhibition of the mutant compared to that of the parental B31 by monoclonal antibodies against surface proteins P13 and P66, respectively, and proposed that OspA may act in vivo to shield borreliae from immunoglobulin recognition of these integral outer membrane proteins. Since OspA-deficient borreliae survived the host-vector transition at a significantly higher rate in the absence of the B-cell response, we conclude that borrelial antigens recognized by an intact immune system are shielded from antibody recog-

nition in the tick vector by the presence of OspA. Antigens conserved between the vector and host stages of the borrelial infectious cycle were shielded from recognition in the WT and *ospA*^{+B1} strains because of the abundance of OspA on the surface of these clones. Additionally, OspA interaction with midgut receptors (46) or plasminogen (16, 28) may indirectly shield borreliae from exposure to antibodies in the blood meal. The midgut density of the *ospA1* clone in the absence of mouse host B-cell function (Fig. 8) is particularly relevant to the separate function of OspA as an adhesin or a plasminogen binding factor; the mutant reached a density that was only a fraction of that of the control clones and therefore is still subject to growth restriction in the midgut.

The data are in agreement with a protective role for OspA against the acquired immunity of hosts repeatedly fed upon by infected ticks (8, 13). When *I. scapularis* larvae feed on a host infected with *B. burgdorferi*, borreliae from the epithelium mix with blood components as they enter the tick. In areas where Lyme disease is endemic, the majority of the reservoir host population has developed humoral immunity to borreliae infection (13, 40). The influx of antibody in the blood meal represents a deterrent to multiplication and transmission for any borreliae that do not shield constitutively produced surface antigens. Antibody shielding occurs in other bacterial pathogens in the context of serum resistance (25) but rarely seems to involve protein as the block to antigen recognition. *I. scapularis* anticomplement factor (65) may act to inhibit complement function in the tick midgut, but in vitro killing (Fig. 6) and in vivo midgut reduction in the density of the OspA-deficient clone (Fig. 3 and 8) may depend in part on complement activity.

Our findings for OspA-deficient borrelial survival in the vector are not consistent with previous research indicating that an OspA- and B-deficient strain 297 derivative does not colonize tick midgut (69). In that report, nymphs fed on infected mice and nymphs infected by rectal microinjection and fed on naïve mice did not show midgut survival of the *ospAB* mutant. The difference between the fate of OspA-deficient borreliae in

that study and the present work may result from the mode of tick infection. The rectal microinjection method (51) was reported to result in significant attenuation of *B. burgdorferi* strain JD-1 tick infection compared to mouse-acquired infection. Delivery of borreliae to the rectal sac may not provide adequate signals for borrelial adaptation to the midgut environment. Borreliae appear to enter the midgut via the oral cavity during infection by immersion, as they are observed in the midgut directly after this exposure (29, 50). A more recent report indicates that the strain B31 *ospB* insertion mutant, which retains OspA production, is also defective in colonization of the tick midgut when borreliae are introduced via rectal injection (42). As in previous experiments (23, 29), we found that the truncated form of OspB in the B31-A3 WT clone did not prevent host transmission or vector acquisition. The absence of OspB in the *ospA*⁺B1 clone likewise did not prevent the transmission-acquisition cycle. We propose that this inconsistency between the *ospB* mutant phenotypes may also stem from differential survival between the rectal and oral modes of tick infection. Importantly, our measurement of midgut colonization differs from those of the studies by Yang et al. (69) and Neelakanta et al. (42); these authors evaluated borrelial density after washing dissected midguts, whereas we evaluated the entire midgut borrelial population after dispersion, as have previous studies (17, 20, 44, 47, 59).

The phenotype of the *ospA1* mutant in relation to tick midgut epithelial cells and to components of the blood meal may result in a significant decrease in transmission to the mouse host at the nymphal feeding stage. Our data for feeding by the *ospA1* mutant-infected nymphs (Fig. 5) provide insufficient sample numbers for a statistically valid test of this potential defect. However, the data presented here for the effects of humoral immunity on vector acquisition of borreliae provide a basis for future studies, including localization of the *ospA1* mutant in the tick and the effects of OspA deficiency on the induction of borrelial RpoN-RpoS-dependent gene transcription, which is essential to transmission (26). The ability of the OspA-deficient strain to survive tick uptake of a naïve mouse blood meal provided a demonstration of the critical role for OspA as a shield for constitutively expressed surface components during the uptake of immune blood. It may also be possible to demonstrate by mutagenesis of the putative binding site at the C-terminal portion of OspA (39) that the adhesion function of OspA can be dissociated from the antibody-shielding function.

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