

## Role of Outer Surface Protein D in the *Borrelia burgdorferi* Life Cycle<sup>∇</sup>

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***Borrelia burgdorferi* preferentially induces selected genes in mice or ticks, and studies suggest that *ospD* is down-regulated in response to host-specific signals. We now directly show that *ospD* expression is generally elevated within *Ixodes scapularis* compared with mice. We then assessed the importance of OspD throughout the spirochete life cycle by generating OspD-deficient *B. burgdorferi* and examining the mutant in the murine model of tick-transmitted Lyme borreliosis. The lack of OspD did not influence *B. burgdorferi* infectivity in mice or the acquisition of spirochetes by *I. scapularis*. OspD adhered to tick gut extracts *in vitro*, and the OspD-deficient *B. burgdorferi* strain had a threefold decrease in colonization of the tick gut *in vivo*. This decrease, however, did not alter subsequent spirochete transmission during a second blood meal. These data suggest that *B. burgdorferi* can compensate for the lack of OspD in both ticks and mice and that OspD may have a nonessential, secondary, role in *B. burgdorferi* persistence within *I. scapularis*.**

The etiologic agent of Lyme disease, *Borrelia burgdorferi*, is maintained in nature in an enzootic cycle that involves *Ixodes* sp. ticks and a vertebrate host such as *Peromyscus leucopus* (4, 34). *B. burgdorferi* adapts to these diverse situations, at least in part, through differential gene expression. More than 10% of the *B. burgdorferi* genome is predicted to encode surface lipoproteins (5, 7). Microarray studies have shown that many of these lipoproteins are differentially expressed in response to host-specific signals or conditions that mimic those of unfed or fed ticks (3, 13, 16, 19, 29, 35). Indeed, detailed analyses have demonstrated that outer surface proteins OspA, OspB, and OspC play important roles at the stages of the spirochete's life cycle where their expression is turned on; OspA and OspB are critical for *B. burgdorferi* colonization of the tick gut, and OspC plays a role in early infection of the mammalian host and spirochete migration within the engorging vector (8, 17, 21, 23, 28, 33, 37).

*B. burgdorferi* OspD is a surface-exposed lipoprotein with a molecular mass of 28 kDa (18). The gene encoding OspD, BBJ09, is located on linear plasmid 38 (lp38) (5, 7, 18). Although lp38 is not among the plasmids that are easily lost during *in vitro* passages of *B. burgdorferi* B31 (10, 27), it is absent in some natural *B. burgdorferi* isolates (9, 24). Microarray studies have shown that *ospD* expression varies significantly in response to different environmental cues. Upon a temperature shift from 23°C to 35°C, *ospD* expression in *B. burgdorferi* decreased 13-fold (19), and *ospD* was also down-regulated in response to mammalian host-specific signals when *B. burgdorferi* was cultured in a rat dialysis membrane chamber (3). *ospD* expression also declined in response to the combined effects of

blood and a temperature shift (35). These studies suggest that the *ospD* gene is differentially expressed by *B. burgdorferi* and conditions in the mammalian host and the engorging tick down-regulate *ospD*. The promoter region of the *ospD* gene contains 1 to 12 direct repeats of a 17-bp sequence that contains consensus –35 and –10 sequences recognized by  $\sigma^{70}$  (15, 18); however, its contribution to *ospD* expression has not yet been delineated.

Several hypotheses about the role of OspD are suggested in the existing literature. First, OspD is not likely to be involved in mammalian infection because (i) *ospD* expression is decreased in response to host-specific signals (3), (ii) many *B. burgdorferi* strains isolated directly from human patients do not contain the *ospD* gene (9, 24), and (iii) immunization with OspD does not protect mice from *B. burgdorferi* infection (14, 25). Second, OspD may not be essential for spirochete transmission during tick feeding because conditions mimicking an engorging tick—blood and a temperature shift from 23°C to 35°C—down-regulate *ospD* expression (35). Third, OspD may play a role within the tick because *ospD* expression is much greater at 23°C than at 35°C (19). Recent technical advances in the targeted mutagenesis of *B. burgdorferi* (30) allow us to test these hypotheses. Here, to better understand the importance of OspD, we constructed an isogenic *ospD* mutant from an infectious clone of *B. burgdorferi* strain B31 and investigated how the loss of OspD affects the spirochete in a laboratory tick-mouse model of Lyme borreliosis.

### MATERIALS AND METHODS

**Bacterial strains and medium.** Clone 5A11, a fully infectious isolate of *B. burgdorferi* strain B31 that lacks only lp5, was used as the wild-type *B. burgdorferi* strain throughout this study (11, 26). An isogenic *ospD* mutant was constructed from clone 5A11 through targeted mutagenesis as described below. Spirochetes were routinely cultured in BSK-H complete medium (Sigma) at 34°C unless otherwise stated. *Escherichia coli* strain TOP10 (Invitrogen) was used as the host strain for the transformation and maintenance of all plasmids. Luria broth was used to culture all *E. coli* strains.

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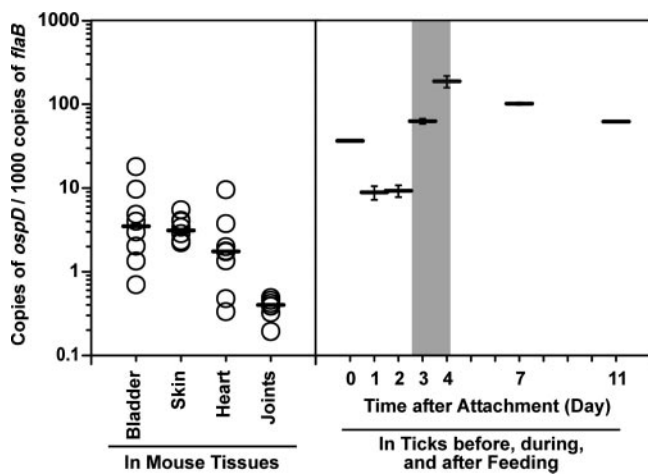


FIG. 1. Differential *ospD* expression in *B. burgdorferi* during its life cycle in mice and ticks. The relative *ospD* expression level in each cDNA sample was calculated as the number of copies of the *ospD* transcript per 1,000 copies of the *flaB* transcript. In the left panel, the open circles represent values of individual mouse tissue samples and a black bar represent the mean value of each column. Shown in the right panel are the means and standard deviations of duplicate measurement of pooled tick samples (five ticks per time point), and the shaded area represents the time window during which the majority (>95%) of the ticks feed to repletion.

**DNA and RNA preparation and cDNA synthesis.** DNA was extracted from mouse, tick, and spirochete samples with the DNeasy tissue kit (QIAGEN) by following the manufacturer's protocol. The mouse and tick samples used to measure *ospD* expression (Fig. 1) were the same as we had used previously (11). Briefly, bladder, heart, skin, and joint samples were collected at 21 days after the mice were each infected subcutaneously with  $10^5$  wild-type *B. burgdorferi* spirochetes. Nymphs infected with wild-type *B. burgdorferi* were allowed to feed on naive mice, and ticks were collected (five ticks per group) before, during, and after tick feeding (on days 0, 1, 2, 3, 4, 7, and 11 after tick attachment). The time window for ticks to feed to repletion was between 66 and 96 h after attachment. Thus, the 1- and 2-day ticks were pulled off of mice before they fed to repletion. The collected mouse and tick samples were immediately frozen in liquid nitrogen and ground thoroughly prior to RNA extraction. Total RNA was extracted from mouse and tick samples with the TRIZOL reagent (Invitrogen) by following the manufacturer's protocol. All RNA samples were digested with RNase-free DNase I (Roche) and then cleaned up on RNeasy mini spin columns (QIAGEN). Total RNA was converted into first-strand cDNA with random hexamers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

**PCR and quantitative PCR (Q-PCR).** The nucleotide sequences of the PCR primers used in this study are listed in Table 1. PCRs with primers P1 and P2, P3 and P4, and P13 and P14 were for cloning and were performed with Vent DNA polymerase (New England BioLabs Inc.). All cloned DNA fragments were sequenced to confirm that no mutations were introduced during PCR amplification. PCRs with primers P5 and P6, P7 and P8, P9 and P6, P9 and P8, and P15 and P16 were for detection and were performed with the *Taq* DNA polymerase (Roche). Q-PCR assays of DNA and cDNA samples were performed in an iCycler (Bio-Rad Laboratories) with high-fidelity Platinum *Taq* DNA polymerase (Invitrogen). The primers and probes used for Q-PCR analysis of *B. burgdorferi flaB*, mouse  $\beta$ -actin, and *Ixodes scapularis actin* were described in a previous study (11). The forward and reverse primers and probe used for Q-PCR analysis of the *B. burgdorferi ospD* gene were P10, P11, and P12, respectively. All Q-PCR probes were labeled at the 5' end with 6-carboxyfluorescein and at the 3' end with 5-carboxytetramethylrhodamine. Gene copy numbers of unknown DNA and cDNA samples were determined by the threshold cycle method according to 10-fold serial dilutions of standards. Genomic DNA of *B. burgdorferi* B31 clone 5A11 was used as the standard for Q-PCR analyses of the *flaB* and *ospD* genes. Standards for Q-PCR analysis of the mouse  $\beta$ -actin and *I. scapularis actin* genes were prepared from plasmid DNA containing the specific genes. The gene copy number in a solution of the standard was calculated on the basis of the molecular weight of the DNA molecule, and the DNA concentration was determined by

sample absorbance at 260 nm. In each 96-well Q-PCR plate, six 10-fold serial dilutions of the standards were assayed in duplicate and used to calculate gene copy numbers of unknown samples. The theoretical limit of detection was one gene copy per reaction. Thus, we assigned a value of 1 instead of 0 to any samples that did not yield a positive Q-PCR result. This allowed us to plot all of the data points, including those below the detection limit, on a log scale.

**Construction of the *ospD* mutant.** An approximately 1.4-kb DNA fragment upstream of the *ospD* gene, termed the 5' arm, was PCR amplified with primers P1 and P2 (Fig. 2A). An approximately 1.5-kb DNA fragment downstream of the *ospD* gene, termed the 3' arm, was PCR amplified with primers P3 and P4 (Fig. 2A). The suicide plasmid used for homologous recombination to replace the *ospD* gene with a kanamycin resistance cassette was constructed as follows. First, the *Borrelia*-adapted kanamycin resistance cassette *kanAn* was excised from pTAKanAn (2) by *EcoRI* digestion and then cloned into the *EcoRI* site of pBluescript (Stratagene). Subsequently, the 5' and 3' arms were double digested with *XbaI*-*BamHI* and *XhoI*-*KpnI*, respectively, and then cloned sequentially into the corresponding sites of pBluescript. Therefore, in the final construct, designated pXLF10601-*ospD*, the *kanAn* cassette was flanked upstream by the 5' arm and downstream by the 3' arm. Electroporation-competent spirochetes of clone 5A11 were prepared as previously described (31, 32). Five microliters (25  $\mu$ g) of the suicide plasmid was mixed with 50  $\mu$ l of approximately  $10^9$  competent spirochetes, placed in a 0.1-cm cuvette, and pulsed in a Gene Pulser apparatus (Bio-Rad) at settings of 1.8 kV, 25  $\mu$ F, and 400  $\Omega$ . A total of 12 kanamycin-resistant transformants were obtained after selection in BSK-H medium containing kanamycin (350  $\mu$ g/ml). Western blot analysis with murine sera raised against recombinant OspD (described below) confirmed that of these 12 transformants, 3 were negative for OspD expression. PCR analysis with *kanAn*-specific primers (P7 and P8) indicated that the nine OspD-positive transformants were negative for the *kanAn* cassette and therefore likely to be spontaneous kanamycin-resistant mutants. The three OspD-deficient kanamycin-resistant transformants were all PCR positive for the *kanAn* cassette. The plasmid content of these three OspD-deficient mutants was analyzed by a microarray assay (36),

TABLE 1. Primers and probe used in this study

Name	Sequence (5'→3') <sup>a</sup>	Application
P1	GCTCTAGAGGTTAGTAGTGTTC AAGAAAATGC	Cloning of 5' arm
P2	CGGGATCCATTTATACTCCTTA AATATAATGTC	Cloning of 5' arm
P3	CCGCTCGAGATAAAATAAAGA TGGAAG	Cloning of 3' arm
P4	GGGGTACCTGTAGAAGCTTTG AGAAGC	Cloning of 3' arm
P5	GCATCAAATGAGGCCAAATAA	Detection of <i>ospD</i>
P6	TACGGCCTCTTTAGCTTGTT	Detection of <i>ospD</i>
P7	GTAATCCTGATGATGCATGG	Detection of <i>kanAn</i>
P8	TGCATTTCTTTCCAGACTTG	Detection of <i>kanAn</i>
P9	TGTTTTCTTAGCCTTAAGCTAG CCAA	Detection of junction
P10	CTGACTATTCTGATTCTGCTGA	Q-PCR of <i>ospD</i>
P11	TATCCAATGTCTCTTTGACTGT	Q-PCR of <i>ospD</i>
P12	TCCAAGCTATTTCTACGGCC TCTT	Q-PCR of <i>ospD</i>
P13	CGGGATCCGTTTCATGATAAAC AAGAATTATC	Cloning of <i>ospD</i>
P14	CCGCTCGAGTCAAGTATTTAA CAAGGCCAC	Cloning of <i>ospD</i>
P15	GCTCAAATAAGAGGTTTGTC	Detection of <i>flaB</i>
P16	ATTCCAAGCTCTTCAGCTG	Detection of <i>flaB</i>

<sup>a</sup> The underlined sequences are restriction sites engineered into the primers for directional cloning of PCR-amplified DNA fragments. Primers P1 to P6 and P9 to P14 were designed according to the nucleotide sequence of *B. burgdorferi* lp38 (GenBank accession no. AE000787), P15 and P16 were designed according to the nucleotide sequence of the *B. burgdorferi* chromosome (GenBank accession no. AE000783), and P7 and P8 were designed according to the nucleotide sequence of *B. burgdorferi* shuttle vector pBSV2 (GenBank accession no. AY1187276).

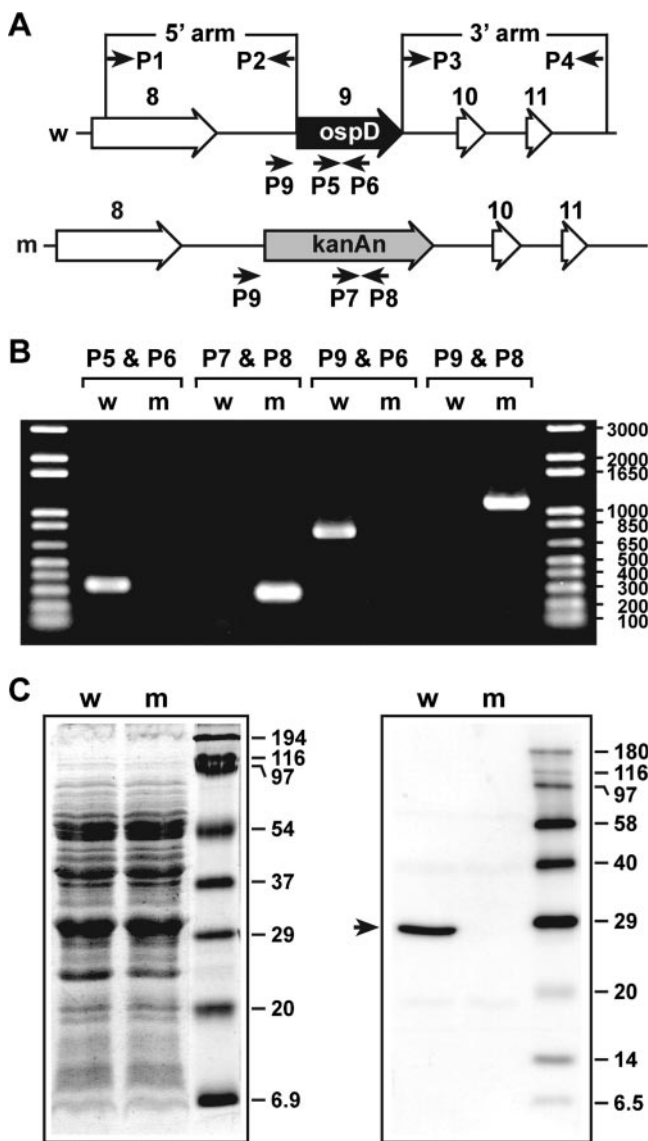


FIG. 2. Construction and characterization of the *ospD* mutant. (A) Schematic drawings of the wild-type *B. burgdorferi* strain (w) and the *ospD* mutant (m) near the *ospD* locus. The *ospD* (BBJ09) gene and its surrounding open reading frames, BBJ08 (8), BBJ10 (10), and BBJ11 (11), are illustrated as box arrows, the lengths and directions of which reflect the lengths and directions of the open reading frames. In the mutant, the *ospD* gene is replaced with the kanAn cassette. Primers P1 to P9 are illustrated as arrows with their positions and directions representing those of the primers. The positions of the two DNA fragments mediating the homologous recombination event (the 5' and 3' arms) are shown. (B) PCR analysis of the wild-type *B. burgdorferi* strain (w) and the *ospD* mutant (m). The primers used in each PCR are indicated above the gel. The molecular masses of the DNA standards are indicated on the right in base pairs. (C) Protein analysis of whole-cell lysates of the wild-type *B. burgdorferi* strain (w) and the *ospD* mutant (m). *Borrelia* lysates were separated by SDS-PAGE and then stained with Coomassie brilliant blue (left panel) or transferred to a membrane and probed with antibodies against OspD (right panel). An arrow points to the OspD protein. The molecular masses of the protein standards are shown on the right in kilodaltons.

which indicated that one of the mutants had the same plasmid profile as wild-type clone 5A11. This OspD-deficient mutant was selected for all further studies and is referred to as the *ospD* mutant hereafter.

**Expression and purification of recombinant OspD, OspA, OspB, OspC, and Dps.** The DNA sequences encoding the mature OspD peptide (minus the first 20 amino acid residues) were PCR amplified with primers P13 and P14. The PCR fragment was digested with BamHI and XhoI and cloned into the corresponding restriction sites of the expression vector pGEX-6P-2 (Amersham Biosciences). OspD was synthesized in *E. coli* TOP10 as a C-terminal fusion to glutathione S-transferase (GST). The fusion proteins were affinity purified with glutathione Sepharose 4B resin (Amersham Biosciences), and OspD was cleaved off of the GST tag with PreScission protease (Amersham Biosciences) by following the manufacturer's protocol. Recombinant OspD was further purified on a Superdex 200 column (Amersham Biosciences) and used to immunize mice to generate antisera. Recombinant OspA, OspB, OspC, and Dps proteins (6, 12, 20) were also expressed as GST fusions with the expression vector pGEX6p2 (Amersham Biosciences). Similarly, the GST tag was removed from the recombinant proteins during purification as described above.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.** *B. burgdorferi* whole-cell lysates, prepared as previously described (11), were separated on a two-layer (3.75% stacking and 12.5% resolving) polyacrylamide gel. The separated proteins were either viewed directly by staining the gel with Coomassie brilliant blue or transferred to a polyvinylidene difluoride membrane and subjected to Western blot analysis. The membrane was incubated with a 1:5,000 dilution of the murine sera against recombinant OspD (see above), followed by a 1:2,000 dilution of anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate (Sigma), and developed with ECL Western blotting detection reagents (Amersham Biosciences).

**Experimental mouse-tick model of Lyme disease.** Four- to 6-week-old female C3H/HeN and C3H/HeJ mice were purchased from the National Cancer Institute and the Jackson Laboratories, respectively, and housed in the Yale Animal Resources Center according to the institutional guidelines for the care and use of laboratory animals. Larval, nymphal, and adult *I. scapularis* ticks were routinely maintained in our laboratory as described previously (11). Mice were subcutaneously inoculated with in vitro-grown *B. burgdorferi* at a dose of  $10^5$ ,  $10^3$ , or  $10^2$  spirochetes per mouse. Fourteen days after inoculation, ear punch samples were collected and examined for the presence of spirochetes by *flaB* PCR with primers P15 and P16. When necessary, between 16 and 21 days after inoculation, larvae (200 to 300 per mouse) or naive nymphs (30 to 50 per mouse) were allowed to feed on spirochete-positive mice. Spirochete acquisition by ticks was studied by quantitative measurement of the spirochete burdens in ticks by both Q-PCR and immunofluorescence microscopy (described below). Twenty-one days after the initial spirochete inoculation, mice were sacrificed and examined for *Borrelia* infection by serology (serum IgG response to whole-cell lysates of in vitro-grown *B. burgdorferi*), by in vitro culturing of the bladder and the spleen, and by quantitative measurement of the spirochete burdens in the bladder, heart, skin, and joints. To study spirochete transmission, infected nymphs (obtained by feeding larvae on infected mice and letting them molt into nymphs) were allowed to feed on naive mice (five ticks per mouse). Twenty-one days after tick attachment, mice were sacrificed and assessed for *Borrelia* infection as described above. Q-PCR quantification of spirochete burdens in mice and ticks was performed on individual DNA samples.

**Immunofluorescence microscopy.** Spirochete burdens in ticks were also analyzed by immunofluorescence confocal microscopy as described previously (22, 37). Briefly, three to five guts from each group of nymphs were microscopically dissected in 20  $\mu$ l of phosphate-buffered saline (PBS). Guts containing blood were isolated, placed on Silane-Prep slides (Sigma), and washed with PBS to remove luminal contents and unbound bacteria. The washed gut samples and luminal contents were isolated separately and processed for immunofluorescence confocal microscopy. Gut and luminal content samples were blocked with PBS containing Tween 20 (0.05%) and goat serum (5%) for 1 h at 37°C and then incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-labeled anti-*Borrelia* antibody (KPL). Samples were subsequently stained with propidium iodide (20  $\mu$ g/ml) for 3 min at 37°C and then mounted with the SlowFade-Antifade kit (Invitrogen). The samples were viewed with a Zeiss LSM 510 scanning laser confocal microscope equipped with an argon-krypton laser (Zeiss Incorporated).

**In vitro tick gut extract (TGE) binding assay.** Guts free of luminal content from flat nymphal ticks (40 ticks) and free of blood meal from fed nymphal ticks (25 ticks) were dissected in PBS as described above and homogenized on ice with a Kontes tissue grinder (VWR Scientific Products) in 100  $\mu$ l of PBS containing protease inhibitor cocktail (Roche). Total protein concentrations in the TGE were determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc.).

One hundred microliters of unfed TGE (5  $\mu\text{g/ml}$ ) or fed TGE (5  $\mu\text{g/ml}$ ) in PBS was used to coat the wells of 96-well plates (Nunc). Control wells were coated with 100  $\mu\text{l}$  of a mouse b.End3 cell line extract (5  $\mu\text{g/ml}$ ). Mouse cell line extract was prepared by resuspending the b.End3 cell pellet in 100  $\mu\text{l}$  of Cell Lytic reagent (Sigma) and processed as mentioned above. Coated plates were tightly covered with cellophane tape to prevent evaporation and incubated overnight at 4°C. Plates were then incubated with 100  $\mu\text{l}$  of FITC-labeled OspD, OspA, OspB, OspC, or Dps (10  $\mu\text{g/ml}$ ) at 37°C for 1 h. FITC labeling of the individual proteins was performed with an EZ-label FITC labeling kit (Pierce) according to the manufacturer's instructions, and any unlabeled or residual FITC molecules were removed by centrifuging the samples in Amicon Ultra 10k filters (Millipore). Plates were then washed three times with PBS containing Tween 20 (0.05%). Binding was detected with anti-FITC IgG-horseradish peroxidase (Amersham Pharmacia Biotech), and TMB microwell peroxidase substrate (KPL) was used for color development. The reactions were stopped after 15 min of incubation with TMB stop solution (KPL), and optical density was read at a 450-nm wavelength.

**Statistics.** Statistical significance of differences observed in data sets was analyzed with GraphPad Instat version 3.05. In general, for data with small variation and normal distribution, the Student *t* test was performed to compare two means and one-way analysis of variance with Tukey's posttest was performed to compare multiple means; for data with large variation and/or nonparametric distribution, the Mann-Whitney test was performed to compare two medians and a Kruskal-Wallis test with Dunn's posttest was performed to compare multiple medians. Wherever necessary, two-tailed *P* values, along with the statistical test used, are reported.

## RESULTS

### *ospD* expression throughout the *B. burgdorferi* life cycle.

Previous microarray studies showed that *ospD* expression in *B. burgdorferi* varied significantly in response to different culturing conditions (3, 19, 35). Here, we used Q-PCR to systematically analyze *ospD* expression by the spirochete during its life cycle through the murine host and the tick vector (Fig. 1). The copy numbers of the *ospD* and *flaB* transcripts were quantified for each cDNA sample (see Materials and Methods for details) and the relative expression level of *ospD* was calculated as the number of copies of *ospD* per 1,000 copies of *flaB*. Our data indicated that *ospD* expression was highly variable during the spirochete's life cycle, with an approximately 500-fold difference between the lowest level observed in the murine joint tissue and the highest level observed in the tick shortly after the blood meal (Fig. 1). The Tukey-Kramer multiple-comparison test was used to compare *ospD* expression levels in various murine tissues and in ticks at different feeding stages. The *ospD* expression levels in murine bladder, skin, heart, and joints were comparable to those in the feeding ticks (1 and 2 days after tick attachment) ( $P > 0.05$ ) but were significantly lower than those in the unfed and fed ticks (0, 3, 4, 7, and 11 days after tick attachment) ( $P < 0.001$ ). During tick feeding, we observed a significant decrease in *ospD* expression ( $P < 0.01$ ), which is consistent with a previous finding that *ospD* expression decreases in response to the combined effects of blood and a temperature shift from 23°C to 35°C (35).

**Construction and characterization of an *ospD* mutant in vitro.** We constructed an isogenic *ospD* mutant from *B. burgdorferi* B31 clone 5A11 by replacing the *ospD* gene with the *Borrelia*-adapted kanamycin resistance cassette kanAn (2) through homologous recombination (Fig. 2A; see Materials and Methods for details). To verify the mutation, a series of PCRs were performed (Fig. 2B). The PCRs with primers P5 and P6 and primers P7 and P8 indicated the absence of the *ospD* gene and the presence of the kanamycin resistance cas-

sette, respectively, in the *ospD* mutant. The location of the kanamycin resistance cassette in the *ospD* mutant was confirmed by PCR with primers P9 and P8. The migration of these PCR-amplified DNA fragments in the agarose gel is consistent with their predicted sizes (321, 283, 765, and 1,130 bp for DNA fragments amplified with primers P5 and P6, P7 and P8, P9 and P6, and P9 and P8, respectively). These PCR results support the idea that a double-crossover event had likely occurred in the mutant and resulted in replacement of the *ospD* gene with the kanamycin resistance cassette. SDS-PAGE analysis of whole-cell lysates indicated that there was no apparent difference between the overall protein profiles of the mutant and the wild-type strain (Fig. 2C, left side). Western blotting with murine sera against recombinant OspD showed that the most prominent immunoreactive protein in the wild-type *B. burgdorferi* strain was missing from the *ospD* mutant (Fig. 2C, right side). The apparent molecular mass of this protein is 28 kDa, the same as previously reported for OspD (18). Our data indicate that the *ospD* mutant is indeed OspD deficient.

**Spirochete infectivity in mice.** To determine whether the loss of OspD expression in the mutant had any adverse effect on its infectivity, we examined both the wild-type strain and the mutant in a laboratory mouse model of Lyme borreliosis originally described by Barthold and colleagues (1). Groups of C3H/HeN mice (five per group) were subcutaneously inoculated with in vitro-grown spirochetes, either the wild-type strain or the *ospD* mutant, at a dose of  $10^5$  spirochetes per mouse (see Materials and Methods for details). On day 21 postinoculation, mice were sacrificed and assessed for spirochete infection by serology, by in vitro culturing of the bladder and the spleen, and by quantitative measurement of the spirochete burdens in various tissues. Results of two independent mouse experiments with a total of 20 mice, 10 infected with the wild-type strain and the other 10 infected with the mutant strain, indicated that all of the infected mice seroconverted and were culture positive for spirochetes in the bladder and the spleen, regardless of which spirochete strain they were infected with. Western blot analysis of spirochete cultures from mouse tissues indicated that the *ospD* mutant remained OspD deficient (data not shown). As shown in Fig. 3A, the spirochete burdens in a specific mouse tissue were comparable between mice infected with the wild-type strain and mice infected with the *ospD* mutant ( $P > 0.05$ , Dunn's multiple-comparison test). We also conducted a dose study to compare the infectivity of the *ospD* mutant and that of the wild-type strain in C3H/HeJ mice. As summarized in Table 2, there was no significant loss of infectivity of the *ospD* mutant even at the low inoculation dose of  $10^2$  spirochetes per mouse ( $P = 1$ , Fisher's exact test). In summary, our data demonstrate that the *ospD* mutant is fully infectious in mice.

**Spirochete acquisition by ticks.** To determine whether the deletion of *ospD* in the mutant had any adverse effect on spirochete acquisition by ticks, we fed larvae and naive nymphs on infected mice and quantified the spirochete burdens in fed ticks by Q-PCR analysis of individual ticks (see Materials and Methods for details). At 1 week after the larvae were fed to repletion, the spirochete burden was threefold greater in the larvae fed on mice infected with the wild-type strain than in those fed on mice infected with the *ospD* mutant ( $P = 0.007$ , Mann-Whitney test) (Fig. 3B). The spirochete burden in fed

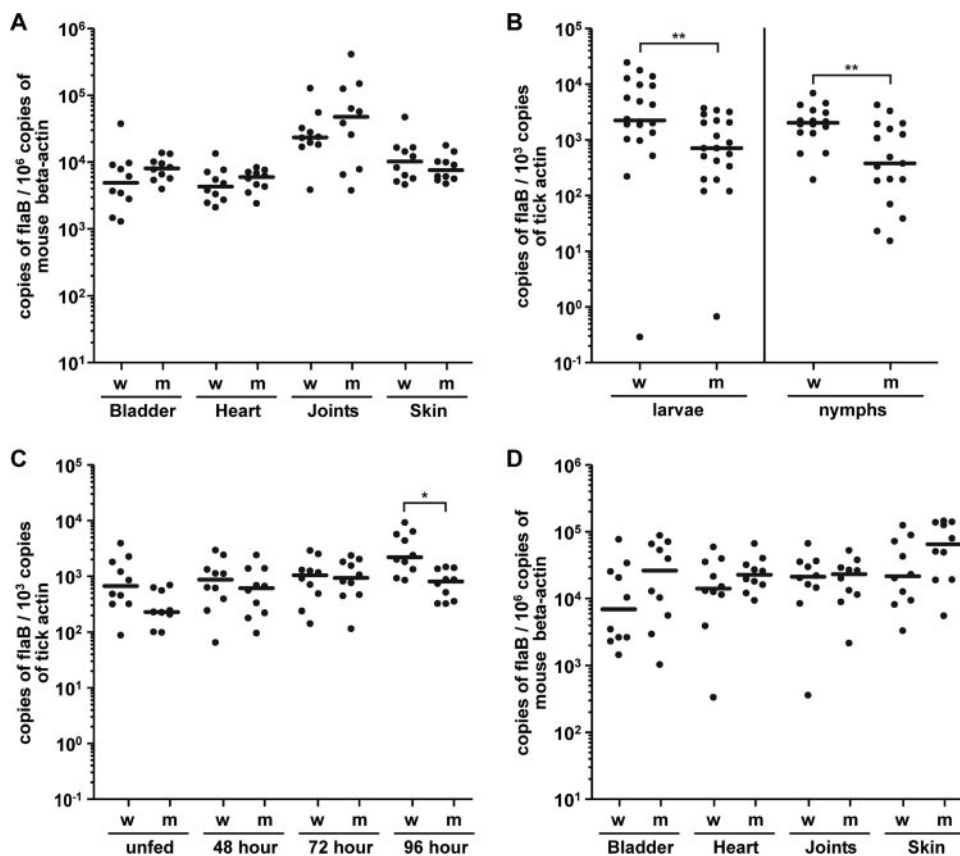


FIG. 3. Quantification of spirochete burdens in mice and ticks. DNA samples from mice and ticks infected with the wild-type *B. burgdorferi* strain (w) or the *ospD* mutant (m) were subjected to Q-PCR analyses to determine the copy number of the *B. burgdorferi flaB* gene and that of the mouse or tick *actin* gene. The relative spirochete burden is shown as the number of copies of the *B. burgdorferi flaB* gene per  $10^6$  copies of the mouse  $\beta$ -actin gene or per  $10^3$  copies of the tick *actin* gene. (A) Spirochete burdens in bladder, heart, joint, and skin samples of mice at 21 days after syringe inoculation with spirochetes at a dose of  $10^5$  spirochetes per mouse. (B) Spirochete burdens in larvae and nymphs after feeding on infected mice. (C) Spirochete burdens in infected nymphs before feeding (unfed) and during and after feeding (at 48, 72, and 96 h after tick attachment). (D) Spirochete burdens in bladder, heart, joint, and skin samples of mice at 21 days after being fed upon by infected nymphs. Individual circles indicate spirochete burdens in individual mouse tissue or tick samples, and a black bar represents the median of each column. The statistical significance of comparison of data is indicated by \* ( $P < 0.005$ ) or \*\* ( $P < 0.01$ ).

nymphs was analyzed shortly after the nymphs were fed to repletion (at 96 h after tick attachment), and it showed that the ticks fed on mice infected with the wild-type strain had fivefold greater spirochete burdens than the ticks fed on mice infected with the *ospD* mutant (Fig. 3B,  $P = 0.004$ , Mann-Whitney test).

We also examined spirochete colonization of the tick midgut by immunofluorescence microscopy. Representative confocal images of the blood meal and the midgut samples of the wild-type *B. burgdorferi* strain-infected and the *ospD* mutant-infected ticks are shown in Fig. 4A. The relative spirochete burdens in these samples were further quantified as the num-

TABLE 2. Infectivity of the wild-type strain and the *ospD* mutant in C3H mice

Strain	No. of infected mice/total at infection dose <sup>a</sup> of:			
	$10^5$	$10^4$	$10^3$	$10^2$
Wild type	9/9	9/9	8/9	7/9
<i>ospD</i> mutant	9/9	9/9	9/9	6/9

<sup>a</sup> Number of spirochetes per mouse.

ber of spirochetes per field (Fig. 4B), and our data indicated that whereas there were comparable numbers of spirochetes in the blood meals of these two groups of ticks ( $P > 0.05$ , Tukey-Kramer multiple-comparison test), there were threefold higher numbers of spirochetes in the midguts of ticks infected with the wild-type *B. burgdorferi* strain ( $P < 0.001$ , Tukey-Kramer multiple-comparison test). These results suggest that the loss of OspD expression may affect the spirochete's ability to fully colonize the tick midgut. We next examined whether OspD bound to TGE. The OspA and OspB proteins (6, 20) were used as positive controls, and the OspC and Dps proteins (12, 20) were used as negative controls. As shown in Fig. 5, similar to the OspA and OspB proteins, the OspD protein bound to unfed TGE and fed TGE at a level two- to threefold higher than its level of binding to a mouse cell line extract, whereas the OspC and Dps proteins did not preferentially bind to TGEs.

**Spirochete transmission by ticks.** We next examined whether the reduced level of spirochete colonization of the tick midgut would affect spirochete transmission in a subsequent feeding. The remaining larvae from the acquisition experiment described

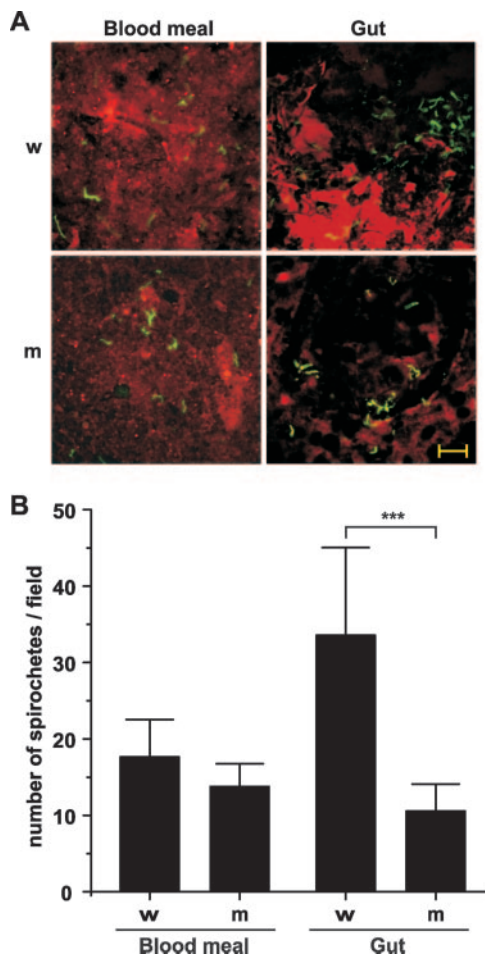


FIG. 4. Immunofluorescence microscopy analysis of ticks. Naive nymphs were fed on mice infected with the wild-type *B. burgdorferi* strain (w) or the *ospD* mutant (m). Shortly after tick feeding (at 96 h after tick attachment), ticks were dissected and the blood meal and tick midgut samples were separately isolated and processed for immunofluorescence microscopy as described in Materials and Methods. (A) Representative confocal images of gut and blood meal samples. Both samples were stained for *B. burgdorferi* with FITC-labeled anti-*Borrelia* antibody (green) and for tick cell nucleic acid with propidium iodide (red). Scale bar, 20 μm. (B) Quantitative assessment of the number of spirochetes from 15 random microscopic field observations. Values on the y axis are the numbers of spirochetes per microscopic field. Error bars define standard deviations from the mean. The statistical significance of comparison of data is indicated by \*\*\* ( $P < 0.001$ ).

above were allowed to molt into nymphs. Q-PCR analysis of individual ticks from these two groups of unfed nymphs indicated that the ticks infected with the wild-type *B. burgdorferi* strain had a threefold greater spirochete burden than the ticks infected with the *ospD* mutant (Fig. 3C,  $P = 0.05$ , Mann-Whitney test). To study spirochete transmission, we fed these infected nymphs on naive mice (5 to 12 ticks per mouse and 10 mice per group). Spirochete burdens in ticks were analyzed at 48, 72, and 96 h after tick attachment (Fig. 3C). Interestingly, the difference in spirochete burden observed between these two groups of ticks diminished during tick feeding; the spirochete burdens of these two groups of ticks were comparable at the 48- and 72-h time points ( $P > 0.05$ , Dunn's multiple-comparison test). Shortly after tick

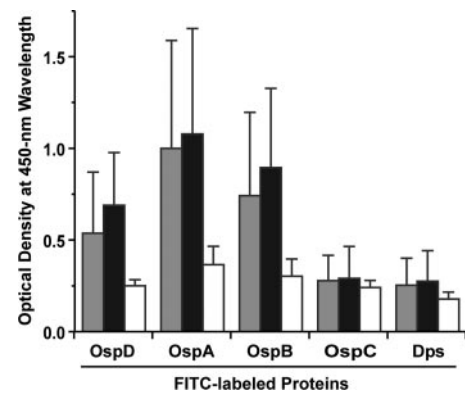


FIG. 5. In vitro TGE binding assay. The binding of FITC-labeled OspD, OspA, OspB, OspC, and Dps to TGE prepared from unfed (gray bar) and fed (black bar) ticks and to a mouse cell line extract (open bar) was assayed in triplicate or quadruplicate. The bars and error bars represent the means and standard deviations of three or four readings of optical density at a 450-nm wavelength.

feeding, at the 96-h time point, ticks infected with the wild-type *B. burgdorferi* strain had a 2.7-fold greater spirochete burden than ticks infected with the *ospD* mutant ( $P < 0.05$ , Dunn's multiple-comparison test). We also examined whether the mice fed on by these infected nymphs were infected with spirochetes at 21 days after tick attachment and found that all 20 mice seroconverted and were culture positive for *B. burgdorferi* (data not shown). The spirochete burdens in a specific mouse tissue, quantified by Q-PCR, were comparable between these two groups of mice (Fig. 3D,  $P > 0.05$ , Dunn's multiple-comparison test). These results showed that although the *ospD* mutant colonized the tick midgut at a threefold reduced level compared with the wild-type *B. burgdorferi* strain, such a defect did not affect spirochete transmission to a new host during a subsequent blood meal.

## DISCUSSION

Our data indicate that *ospD*, a gene encoding a surface lipoprotein of *B. burgdorferi*, undergoes differential expression during the spirochete's life cycle through mice and ticks (Fig. 1). The expression pattern of *ospD*, decreased in mice and feeding ticks and increased in fed ticks, is very similar to that of the gene encoding OspA, a crucial tick colonization factor (21, 37). To define the function of OspD, we constructed an isogenic *ospD* mutant from an infectious clone of *B. burgdorferi* strain B31 and examined both the mutant and parent strains in a laboratory mouse-tick model of Lyme borreliosis. In summary, our data show that the phenotype of the *ospD* mutant is consistent with the expression pattern of the *ospD* gene, as detailed below.

First, the loss of OspD had no adverse effect on the spirochete's infectivity in mice; this is consistent with the low *ospD* expression level in mice. The *ospD* mutant is as infectious in mice as is the parental wild-type *B. burgdorferi* strain, as determined by three independent experiments. When mice were syringe inoculated with a large dose of  $10^5$  spirochetes per mouse, the groups of mice inoculated with either the wild-type *B. burgdorferi* strain or the *ospD* mutant were all infected and had similar spirochete burdens in all of the tissues examined

(Fig. 3A). In a second experiment, groups of mice were syringe inoculated with the wild-type *B. burgdorferi* strain or the *ospD* mutant at a dose of  $10^5$ ,  $10^4$ ,  $10^3$ , or  $10^2$  spirochetes per mouse, and the result clearly showed that these two strains were comparable in infectivity (Table 2). Finally, when mice were naturally infected through tick bites, the spirochete burdens in the two groups of mice were also comparable (Fig. 3D). Therefore, OspD is dispensable for spirochete infection of mice.

Second, OspD is involved in spirochete colonization of the tick gut; this is consistent with a significant increase in *ospD* expression immediately after tick feeding (Fig. 1). Both larval and nymphal ticks fed on mice infected with the *ospD* mutant had three- to fivefold lower spirochete burdens compared to those fed on mice infected with the wild-type *B. burgdorferi* strain (Fig. 3B). Immunofluorescence microscopy of the fed nymphs revealed that the spirochete burdens in blood meal samples of these two groups of ticks were comparable whereas in the tick gut samples, there were threefold lower numbers of *ospD* mutant spirochetes (Fig. 4). This suggests that the reduced level of the *ospD* mutant in the ticks is more likely due to a reduction in colonization rather than a reduction in acquisition. Purified recombinant OspD protein also bound in vitro to TGE (Fig. 5).

Third, the lack of OspD had no adverse effect on spirochete transmission from the tick to a new host; this is consistent with decreased *ospD* expression during tick feeding. Although the unfed nymphs infected with the *ospD* mutant had an approximately threefold reduction in spirochete burden compared with those infected with the wild-type *B. burgdorferi* strain, this difference between these two groups of nymphs quickly diminished when ticks began to feed (Fig. 3C). Mice fed upon by either wild-type *B. burgdorferi* strain-infected or *ospD* mutant-infected ticks all became infected and had comparable spirochete burdens in tissues (Fig. 3D).

Although *ospD* has an expression pattern that is very similar to that of *ospA*, the phenotype of the *ospD* mutant suggests that OspD plays a much less important role than OspA and OspB in tick colonization. *B. burgdorferi* lacking OspA and/or OspB exhibited a much more severe defect in the colonization of and persistence in ticks (17, 37). Although not directly tested, the OspA and/or OspB deficiency would affect subsequent spirochete transmission to a new host because the *ospA/B* mutant spirochete completely failed to colonize the tick gut (37) and the *ospB* mutant failed to persist in the tick after molting (17). Our study showed that the reduced tick colonization by the *ospD* mutant had no effect on the subsequent transmission of the spirochete to a new host. This limited role for OspD in the Lyme disease spirochete's life cycle is consistent with the fact that the *ospD* gene is absent in many natural *B. burgdorferi* isolates (9, 15, 24).

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