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Infection of Mice with Lyme Disease Spirochetes Constitutively Producing Outer Surface Proteins A and B[▽]

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Outer surface protein A (OspA) of the Lyme disease spirochete is primarily produced in the tick vector. OspA, which is a receptor for attaching spirochetes to the tick gut, is down regulated as the spirochetes leave the tick and enter the mammalian host. Although OspA is not a major antigen produced in the mammal, the protein appears to be produced under some conditions and production has been linked to more severe disease. A Lyme disease vaccine based on recombinant OspA has been approved for human use. However, the vaccine is no longer available, in part because of fears that OspA causes arthritis in people. To further understand the consequences of OspA production in the host, we created a *Borrelia burgdorferi* mutant that was unable to down regulate OspA. C3H/HeN mice infected with this mutant developed a specific anti-OspA immune response, and the spirochetes were unable to persist in these mice. In contrast, immunodeficient SCID mice were persistently infected with the mutant. We conclude that spirochetes producing OspA and B from the *flaB* promoter in immunocompetent mice stimulate an immune response that clear the bacteria without any signs of disease development in the mice.

The Lyme disease spirochete *Borrelia burgdorferi* produces an array of outer surface lipoproteins (Osps). While some of the lipoproteins are chromosomally produced, the majority are produced on extrachromosomal plasmids. As many as 91, or 14.5%, of the genes encoded on plasmids are putative lipoproteins (7). Many plasmid-encoded lipoproteins are differentially expressed in the tick vector or the vertebrate host, indicating that they function at specific stages in the life cycle of the spirochete. The function of several lipoproteins has been studied using genetic and biochemical approaches, but there still remains much to be learned about these proteins.

OspA and B are two proteins encoded by a single operon on linear plasmid 54 (lp54). These two surface proteins are produced in abundance by spirochetes grown in culture. OspA, in particular, has been the focus of study because the gene encoding OspA was among the first *B. burgdorferi* genes to be cloned and a recombinant OspA vaccine has been approved for use in people and animals. However, the vaccine is no longer available, in part because of fears that the protein or an immune response against the protein could induce arthritis. OspA is differentially produced during the natural transmission cycle of the spirochete. When spirochetes first enter a tick, OspA is upregulated and the protein is required for tick colonization. OspA serves as a ligand for tethering spirochetes to a receptor in the tick gut (20). When infected ticks feed again, the spirochetes multiply within the vector, downregulate the production of OspA and infect the host via the salivary glands of the tick. Nonspecific natural antibody in a host may be one signal that down regulates *ospA* expression (16). Mutants missing OspA and B are able to infect mice and cause disease (28).

There is conflicting data about the role of OspA in infection and disease in the murine model of Lyme disease. In rodents infected by a tick bite, OspA antibodies are rarely, if ever, detected, indicating lack of expression of this gene, and *ospA* transcripts are not found in the dermis at the site of attachment (15). Mice infected by injection of high doses of cultured organisms often develop anti-OspA antibodies, most likely because the inoculum contains many organisms producing the protein. However, *ospA* transcripts can only be detected up to 5 days postinfection in rabbits injected with spirochetes (8). Despite the apparent lack of OspA production in rodents, signals present at sites of inflammation in the murine model have been shown to increase OspA production (9). In addition, lipidated OspA injected into rat joints caused arthritis (5). In summary, a large body of work supports the view that *B. burgdorferi* OspA is primarily a protein that is expressed in the vector for attachment of spirochetes to the tick gut epithelium. There are also studies that point to a possible role for OspA in late stage disease under certain conditions, but the evidence is largely indirect.

Many studies on *B. burgdorferi* study gene function by creating genetic knockouts, whereby a selectable antibiotic marker is inserted into the gene of interest. Another approach is to cause the overexpression of a particular gene to see what phenotype the spirochete exhibits when it is unable to shut off the gene. In this manner, we have created a spirochete mutant that constitutively produces OspA and B and tested this mutant in the murine model of Lyme disease to study the role of OspA in murine infection and disease.

MATERIALS AND METHODS

Mice. Specific-pathogen-free C3H/HeN (C3H) and C3H/SmnCIcr-*scid* (SCID) mice (3 to 5 weeks old) were purchased from Frederick Cancer Research Center

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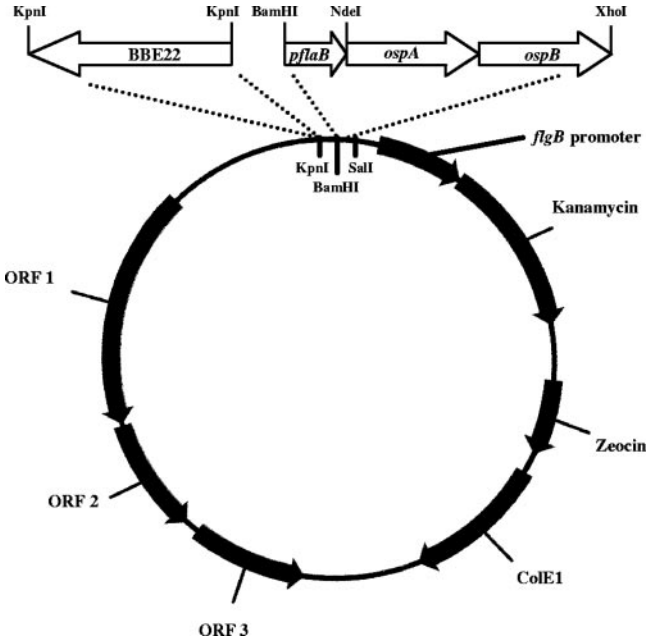

FIG. 1. Schematic of the creation of the pBSV2+22+FA shuttle vector. The shuttle vector consists of three open reading frames from *B. burgdorferi* cp9, the *E. coli* origin of replication, and the two antibiotic resistance cassettes. The inserts consisted of the *flaB* promoter attached to the *ospAB* genes as well as the BBE22 gene.

(Frederick, MD) and Harlan Sprague-Dawley, Inc. (Indianapolis, IN), respectively. Mice were maintained in individual isolator cages within an infectious disease containment room and fed commercial mouse diet and water ad libitum. Mice were euthanized by carbon dioxide asphyxiation, followed by exsanguination by cardiocentesis.

***Borrelia burgdorferi* strains and creation of DNA constructs.** *Borrelia burgdorferi* strain B31 MI-A3 was the parental strain used in the experiments. MI-A3, which was kindly provided by Patricia Rosa (Rocky Mountain Laboratories, NIAID-NIH, Hamilton, MT), is a clonal isolate of the nonclonal low-passage infectious strain B31 MI that is missing one circular plasmid, cp9. This strain also produces a truncated version of OspB that is 18 kDa as opposed to the normal 34-kDa size (10). Two mutants of MI-A3 were created by insertion of pBSV2 shuttle vector modifications into the spirochetes. The shuttle vector pBSV2, also kindly provided by Patricia Rosa, is derived from *B. burgdorferi* B31 cp9 and contains a kanamycin resistance cassette. The first modification of the shuttle vector was created by inserting the gene BBE22 into the vector to create the vector pBSV2+22 as outlined by Strother and de Silva (24). The second modified shuttle vector was created by insertion of the B31-C1 *ospAB* gene connected to the B31-C1 *flaB* promoter into the pBSV2+22 shuttle vector to create pBSV2+22+FA (Fig. 1). The *flaB* promoter was amplified using primers pFlaB-F and pFlaB-R, while the *ospAB* operon was amplified using primers ospAB-F and ospAB-R (Table 1). The *flaB* gene is constitutively produced in spirochetes, so putting its promoter in front of the *ospA* and *ospB* genes would cause constitutive production of these two genes. The pBSV2+22 shuttle vector was digested with BamHI and SalI into which the *flaB* promoter and *ospAB* operon construct was ligated. The 3′ end of the construct had an XhoI site, which joined with the SalI site of the vector.

Spirochete transformation and characterization. Spirochetes were electrically transformed with the two shuttle vector constructs following the methodology of Samuels (23), and transformants were selected using the methods of Yang et al. (28). Transformants were tested for the shuttle vector using M13 primers that spanned the insert region. Once confirmed as containing the shuttle vector, a plasmid profile was performed on the spirochetes using a set of 29 primers (10). By this method, two strains of spirochetes were created. The first strain, named A3-pB22, was created by insertion of the pBSV2+22 shuttle vector, while the pBSV2+22+FA shuttle vector was electroporated into spirochetes to create strain A3-pB22-FA.

The two spirochete strains were tested for overproduction of OspA through Western blotting. MI-A3, A3-pB22, and A3-pB22-FA were grown in BSK-H medium at 35°C for 1 week, after which samples were concentrated, boiled, run on a polyacrylamide gel, and analyzed by Western blotting. Blots were run against a FlaB monoclonal antibody to check for loading and against an OspB monoclonal antibody as indirect evidence of OspA production. The entire protein profile of the three strains was also visualized on a polyacrylamide gel using SimplyBlue stain (Invitrogen, Carlsbad, CA). To further confirm the activity of the *flaB* promoter and *ospA* gene insert, RNA was harvested from in vitro-grown spirochetes (RNeasy mini kit; QIAGEN, Valencia, CA) and reverse transcription (RT)-PCR was performed on the RNA using QIAGEN′s Omniscript RNA transcription kit (Valencia, CA). PCR was performed on the RT-PCR using primers that amplified a portion of the *ospA* gene (primers ospA-F/ospA-R) as well as primers that amplified specifically from the *flaB*/*ospA* insert in the A3-pB22-FA mutant (primers pFlaB-F2/ospA-R) (Table 1).

Mouse infection. Low-passage isolates of *B. burgdorferi* strains MI-A3, A3-pB22, and A3-pB22-FA were grown in modified Barbour-Stoenner-Kelly (BSK II) medium as described previously (3). Sixty C3H mice as well as 60 SCID mice were randomly divided into three groups of 20 C3H and 20 SCID mice in each. Mice in one group were infected with 10^4 spirochetes of the MI-A3, A3-pB22, or A3-pB22-FA strain at the mid-log phase in 0.1 ml of BSK II by intradermal

TABLE 1. Primers and probes used in this study

Gene	Primer or probe designation	Primer or probe sequence (5′–3′)	Source or reference
flaB	pFlaB-F	GCGGGATCCGTCTGTCGCCTCTTGTGGCTTC	This study
	pFlaB-R	GTATTATGATTGATAATCATATGTCATTCCTCCATG	This study
	FL-571F	GCAGCTAATGTTGCAAATCTTTTC	14
	FL-677R	GCAGGTGCTGGCTGTTGA	14
	FL-611P	AAACTGCTCAGGCTGCACCGGTTC	14
ospA	ospA-F	GCAGCCTTGACGAGAAAAAC	This study
	ospA-R	AACTGCTGACCCCTCTAATTTGGTGCC	This study
	ospAB-F	CATATGAAAAAATATTTATTGGGAATAGGTCTAAT ATTAGCCTT AATAGC	This study
	ospAB-R	GCTCGAGGGTTAAGGACCTAAATAGCTTGACTTATGC	This study
	OSPA-288F	TGAAGGCGTAAAAGCTGACAAA	14
	OSPA-369R	TTCTGTTGATGACTTGTCTTTGGAA	14
	OSPA-266P	CAATTTTGAACGATCTAGGTCAAACCACACTTGA	14
ospC	OSPC-146F	TTACGGATTCTAATGCGGTTTTACT	This study
	OSPC-235R	TACCAATAGCTTTAGCAGCAATTTC	This study
	OSPC-205p	TAGATGACAGCAACGCTTCAACCTCTTTCA	This study
flaB-ospAB	pFlaB-F2	AACAGGCAAAAGGATTTGCCAAAGTCAGAA	This study
	FLAB+OSPAB-F	TTTTTAATGCTATTGCTATTTGCGTTT	This study
	FLAB+OSPAB-R	AAGAACTTTCATTTCACCAGGCA	This study
	FLAB+OSPAB-p	AAACGCTGTTTTTCTCGTCAAGGCTGCTAA	This study

inoculation at the dorsal thoracic midline of each mouse. At days 4, 15, 30, and 60 postinoculation, necropsy was performed on each of 5 C3H mice and 5 SCID mice infected with the three strains at each time point. At necropsy, the ear, heart base, ventricular muscle, and left tibiotarsal joint were individually stored, snap-frozen, and kept at −70°C until they could be weighed and processed for DNA and RNA extraction. To confirm the infectious status of each mouse, the urinary bladder was cultured in modified BSK II medium. The right knee, right tibiotarsal joint, and the remainder of the heart were formalin fixed, paraffin embedded, and processed for histological analysis. Slides were coded and examined in a blinded fashion for prevalence and severity of arthritis and carditis.

Quantitative analysis of *B. burgdorferi* DNA. DNA was extracted from tissue samples (ear, heart base, ventricular muscle, and tibiotarsal joint) using DNeasy kits (QIAGEN, Valencia, CA) according to the manufacturer's instructions for tissue and insects. Before DNA extraction, all tissue samples were weighed, snap-frozen in liquid nitrogen, pulverized, homogenized using a QIAshredder kit (QIAGEN, Valencia, CA), and then split equally for DNA and RNA extraction. The *B. burgdorferi flaB* (flagellin) target gene was expressed per 1 mg of tissue weight. For real-time PCR amplification, primers and a labeled probe of *flaB* gene from published sequences (14) were synthesized to amplify a 107-bp fragment. Real-time PCR was made quantitative by generating a *flaB* plasmid standard that was used to create an absolute standard curve as described previously (13).

Extracted DNA from SCID mice given the A3-pB22-FA strain was also sequenced to test for mutations in the inserted plasmid. The entire *flaB* promoter and *ospAB* insert were PCR amplified from DNA that came from the heart tissue of 1 mouse at 15 days, 2 mice at 30 days, and 1 mouse at 60 days postinfection. The product of interest was gel purified (QIAGEN, Valencia, CA), ligated into the pCR2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA), and transformed into One Shot TOP10 *Escherichia coli* (Invitrogen, Carlsbad, CA). A clone from each transformation was verified to contain the plasmid insert and was sequenced for the *flaB* promoter and *ospA* gene.

Analysis of mRNA. Total RNA from each sample was purified as described previously (15). Oligonucleotide PCR primers and TaqMan internal probes of genes that encode flagellin and OspA were based on sequences of *B. burgdorferi* strain N40 (14) (Table 1), while sequences from this study were based on strain B31. Primers OSPC-146F and OSPC-235R (Table 1) were synthesized to amplify a 90-bp fragment of the *ospC* gene. The internal oligonucleotide probe OSPC-205p (Table 1) was labeled at the 5′ end with the reporter dye 6-carboxyfluorescein and at the 3′ end with the quencher dye 6-carboxytetramethylrhodamine. To distinguish between transcriptional activity of *ospA* that is constitutively expressed in the mutant from native *ospA* present normally, primers and a probe were designed based on the *flaB-ospAB* open reading frame that would distinguish between *ospA* produced from our inserted construct from natively produced *ospA*. Primers FLAB+OSPAB-F and FLAB+OSPAB-R were synthesized to amplify a 231-bp fragment, and the internal oligonucleotide probe FLAB+OSPAB-p was labeled as the other probes (Table 1). For absolute quantification of RNA, standard curves were created with in vitro-transcribed RNA using the RiboProbe in vitro transcription system (Promega, Madison, WI).

ELISA. Blood samples were obtained from all C3H mice by tail bleed at 4, 15, 30, and 60 days postinoculation, and serum was separated for serology (enzyme-linked immunosorbent assay [ELISA]). Microtiter plates (96-well; Nunc Immuno-Max Maxi-Sorp, Wiesbaden, Germany) were coated with either 1 μg/ml of *B. burgdorferi* lysate or recombinant OspA in carbonate buffer (pH 9.6) and were incubated overnight at 4°C. Coated plates were washed with phosphate-buffered saline-Tween 20 and blocked for 1 h with 1% bovine serum albumin, and then serial threefold dilutions of serum samples (starting at 1:100) were added to each well. The plates were incubated overnight at 4°C, washed again, and then incubated for 2 h with alkaline phosphate-conjugated rat anti-mouse immunoglobulin G (heavy and light chains) diluted 1:5,000. Alkaline phosphatase substrate (Sigma, St. Louis, MO) at 1 mg/ml was added to each well for color development. The optical density was measured at a test wavelength of 405 nm with an ELISA reader (Molecular Devices Corporation, Sunnyvale, CA). Each assay included positive and negative controls for serum. Cut-off points for each dilution were established by testing the absorbance of normal mouse sera to determine the means and then adding three standard deviations above the mean.

Arthritis. Rear legs (tibiotarsus and knee) and hearts were processed for histology by fixation in neutral buffered formalin at pH 7.2. After fixation, bones were demineralized, and then all tissues were embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin. The prevalence of arthritis in each mouse was determined by examination of one knee and tibiotarsus from each mouse and scored as 0, 1, or 2 joints positive. Arthritis severity in the tibiotarsus was scored on a scale of 0 (negative) to 3 (severe). Sections through the heart, including the great vessels at the heart base, were examined for active inflammation, and carditis for each mouse was scored as either present or absent. All tissue sections were examined blindly.

TABLE 2. Spirochete strains used in this study

Strain	Introduced DNA	Plasmid(s) missing	Source or reference
MI-A3	None	cp9	10
A3-pB22	pBSV2+22	cp9, lp25, lp21	This study
A3-pB22-FA	pBSV2+22+FA	cp9, lp25	This study

Statistical comparisons. For the incidence of arthritis, comparisons were made between C3H and SCID mice infected with the three *B. burgdorferi* strains as well as between strains among the C3H and SCID mice. For quantitative PCR (qPCR) and RT-PCR, evaluations were made between strains among the C3H and SCID mice. Comparisons were made by using Student's *t* test, and calculated *P* values of <0.05 were considered significant.

RESULTS

Creation of a *B. burgdorferi* strain that constitutively produces OspA and B. A DNA construct containing the *ospAB* open reading frame was fused to the *flaB* promoter to create a spirochete strain that constitutively produces OspA and B. This DNA construct was cloned into the *B. burgdorferi* shuttle vector pBSV2+22 that contains a kanamycin resistance cassette and the BBE22 gene from *B. burgdorferi* lp25 (Fig. 1). lp25, which encodes genes required for survival of spirochetes in the tick and the mouse, is a barrier to transformation of spirochetes, and the plasmid is commonly lost during genetic manipulation of spirochetes. The gene on lp25 that is principally required for mouse infection is BBE22 (21). By providing this gene on the shuttle vector, it is possible to create strains missing lp25 that are still infectious to mice. Moreover, the presence of this gene on the shuttle vector serves as a selection marker in the mouse and prevents plasmid loss.

Two strains of spirochetes were created by introduction of shuttle vectors. Strain A3-pB22 was created by introduction of the pBSV2+22 shuttle vector alone, and strain A3-pB22-FA was created by introduction of the same shuttle vector with the addition of the *ospAB* operon under the control of the *flaB* promoter. A plasmid profile of both strains revealed that lp25 was missing from both as expected, while A3-pB22 was additionally missing lp21 (Table 2). lp21 has been shown previously not to be necessary for mouse infection (22, 25). Otherwise, all other plasmids were present. It was not possibly to directly assess the level of OspA produced from the shuttle vector because the native copy of OspA is produced at very high levels in culture, and even at very high dilutions, differences are difficult to see. However, *B. burgdorferi* strain MI-A3 has a premature stop codon in the native *ospB* gene which leads to the production of a truncated version of OspB (10). As the *ospAB* operon on the shuttle vector construct did not have the premature stop codon, we could assay for the presence of full-length OspB as a measure of protein produced off the *flaB* promoter on the shuttle vector as indirect evidence of OspA production. Although Liang et al. (19) showed that OspB might have regulation independent of OspA in vivo, they and others have shown that in vitro, both OspA and B are regulated from the same promoter (6, 17, 19). In fact, full-length OspB production was seen in the A3-pB22-FA mutant by Western blots (Fig. 2). The protein profile of the three strains was also

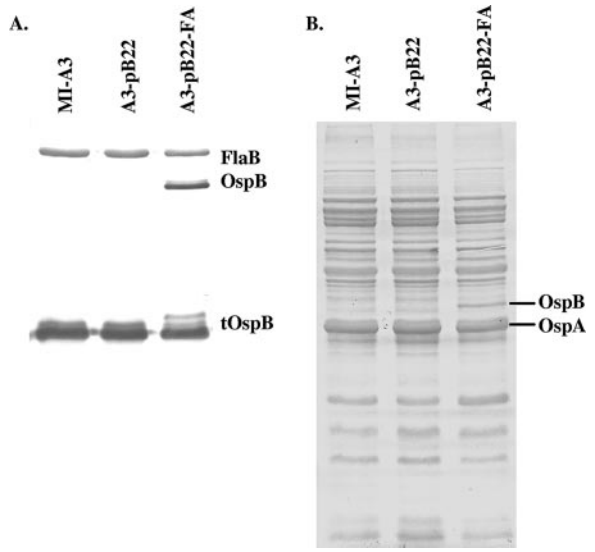

FIG. 2. Western blot (A) of the spirochete strains showing production of full-length OspB for the A3-pB22-FA mutant as well as the truncated version of OspB (tOspB) produced in all three strains and total protein (B) showing locations of OspA and full-length OspB.

examined, and no obvious differences among levels of proteins produced among the three strains were obvious (Fig. 2). In addition, RT-PCR performed on RNA from in vitro-grown MI-A3 and A3-pB22-FA strains showed that A3-pB22-FA spirochetes produced transcripts from the inserted DNA construct (Fig. 3).

Infection of mice with spirochetes constitutively producing OspA and B. C3H and SCID mice were injected with the B31 MI-A3, A3-pB22, or A3-pB22-FA spirochete strain and necropsied 4, 15, 30, and 60 days after infection. By day 15 and through 60 days postinfection, nearly all mice were infected, with the exception of the C3H mice given the A3-pB22-FA strain, which had no infection by organ culture at any time point tested (Table 3). SCID mice given the A3-pB22-FA strain, on the other hand, were positive at 15 to 60 days postinoculation (Table 3).

A qPCR analysis of spirochete numbers in different tissues confirmed that A3-pB22-FA was less infectious than MI-A3 and A3-pB22 in C3H mice. Most of the mice did not have detectable spirochete numbers until 15 days postinoculation (Fig. 4). In SCID mice, all three strains of spirochetes grew to similar numbers, although SCID mice generally had higher bacterial loads than the C3H mice in different tissues and at all time points (Fig. 4). In C3H mice, *B. burgdorferi* strains MI-A3 and A3-pB22 were recovered in similar numbers from several tissues, whereas the A3-pB22-FA strain was detected only from the heart tissues at 60 days postinoculation and from the tibiotarsus joints at 15 days postinoculation.

TABLE 3. Infection of mice as seen by organ culture at different time points postinoculation

Strain	Mouse type	No. of mice positive/total no. of mice tested on day:			
		4	15	30	60
MI-A3	C3H	0/5	5/5	4/5	5/5
	SCID	1/5	5/5	5/5	5/5
A3-pB22	C3H	0/5	4/5	4/5	4/5
	SCID	0/5	5/5	5/5	5/5
A3-pB22-FA	C3H	0/5	0/5	0/5	0/5
	SCID	0/5	5/5	5/5	5/5

OspA and B expression in mice. We assumed that placement of the *ospAB* operon under the regulation of the *flaB* promoter would lead to the continuous expression of this operon in infected animals. Serological studies confirmed that strain A3-pB22-FA produced more OspA than MI-A3 or A3-pB22. When sera from C3H mice infected with the B31 MI-A3, A3-pB22, or A3-pB22-FA spirochete strain were tested by ELISA using cell lysates from MI-A3 spirochete as an antigen, all three of the spirochetes induced specific antibody by 15 days after infection (Fig. 5). No difference in serum reactivity was seen from days 4 to 30 postinoculation (Fig. 5). A noticeable rise in reactivity to MI-A3 spirochetes from mice injected with the MI-A3 strain was seen at 60 days postinoculation. On the other hand, when sera from mice injected with the three strains were reacted against recombinant OspA protein, mice injected with MI-A3 and A3-pB22 spirochetes had little or no reactivity to OspA. Sera from mice given the A3-pB22-FA strain had strong reactivity to OspA (Fig. 5). These results confirm that

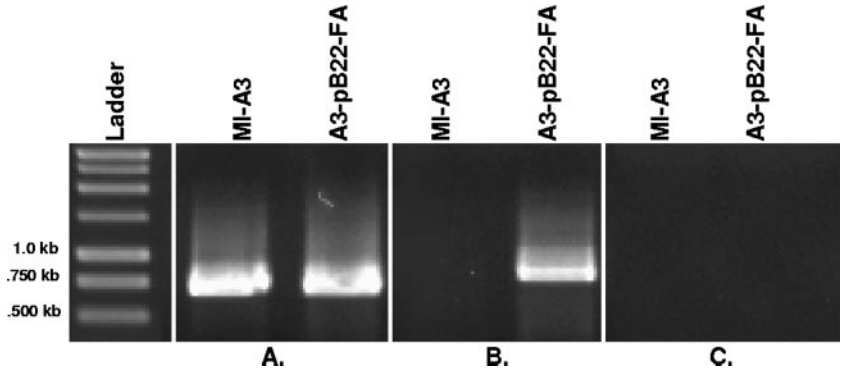

FIG. 3. PCR of RT-PCR showing *ospA* transcripts in the MI-A3 and A3-pB22-FA strains (A) and *flaB* promoter/*ospA* gene construct transcripts in the A3-pB22-FA strain (B). (C) PCR of RT-PCRs without reverse transcriptase, showing that the samples contained no DNA carryover.

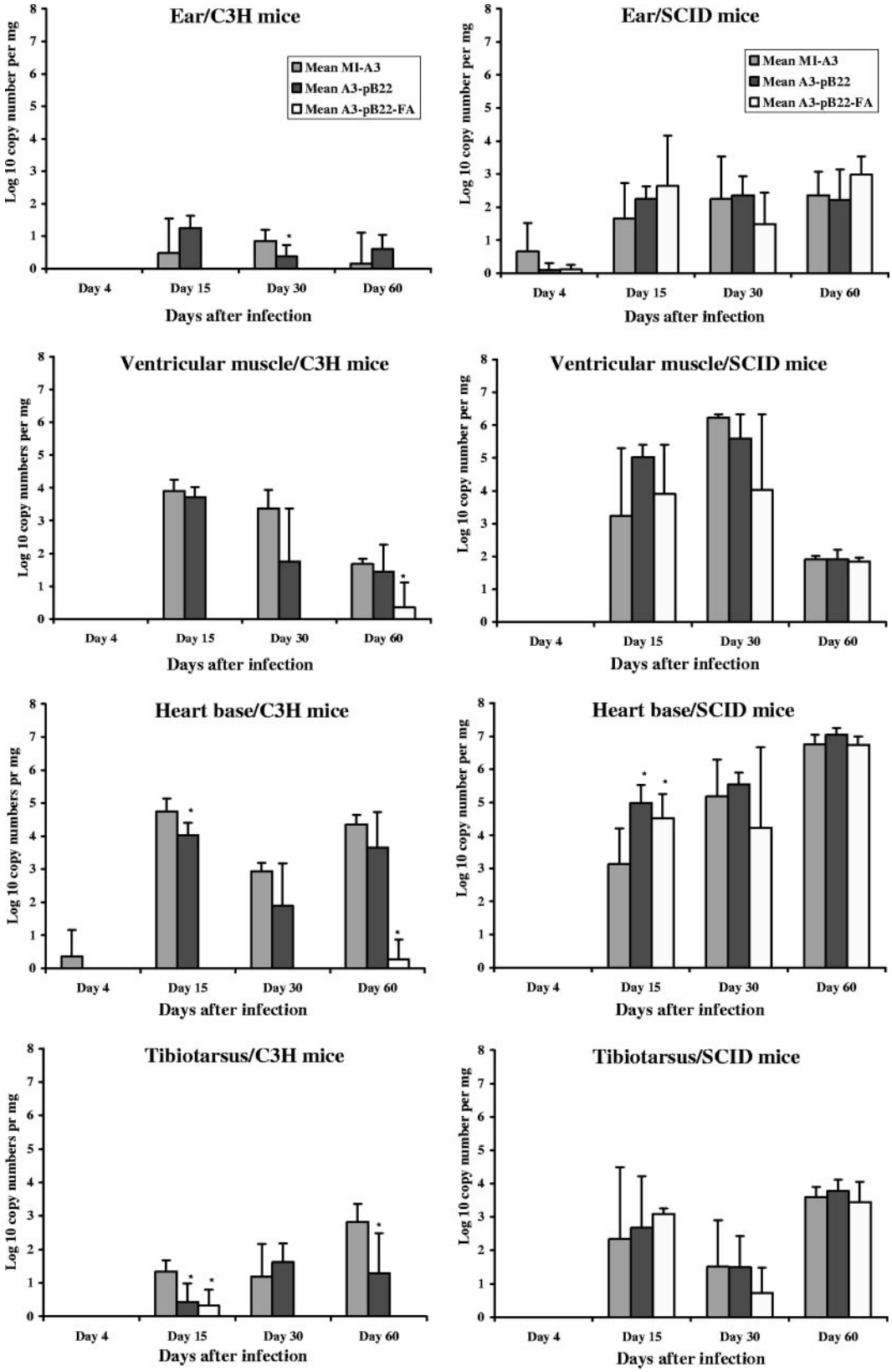

FIG. 4. Estimation of spirochete numbers by qPCR in different tissues of C3H and SCID mice 4, 15, 30, and 60 days postinoculation. Cycle threshold (C_T)values of 40 were considered negative (no amplification), and C_T values < 40 were considered positive. The threshold of detection was estimated to be 10 spirochetes (14). ∗, mean significantly different than that of the MI-A3 strain ($P \leq 0.05$).

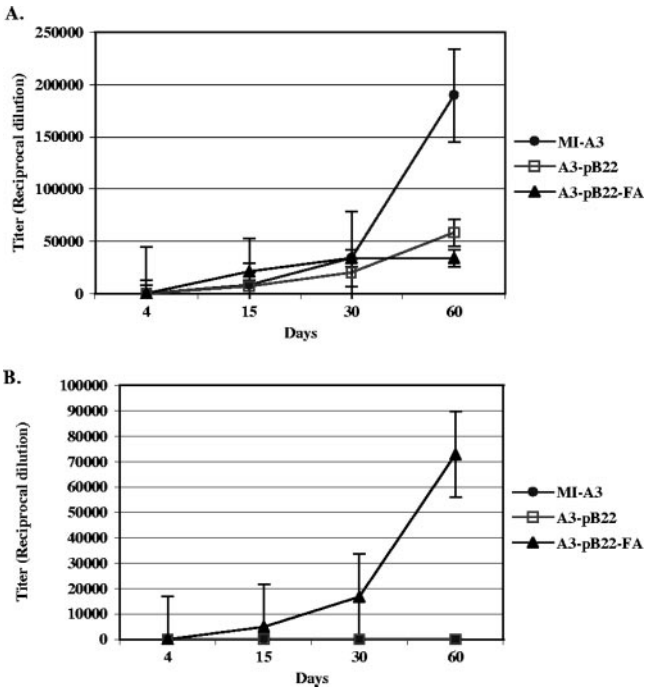

FIG. 5. ELISA results for serum from mice injected with three strains of spirochetes reacted against MI-A3 spirochetes (A) and OspA protein (B).

strain A3-pB22-FA produced OspA in C3H mice unlike the control strains.

We also used RT-PCR to monitor the expression of o*spA*, *ospC*, and *flaB* in mice at 4, 15, 30 and 60 days postinoculation. Transcript levels of the three genes were the same or higher in SCID mice than in C3H mice (Fig. 6). Transcript levels of the *ospC* and *flaB* genes from the A3-pB22-FA strain were comparable to the levels produced by the control strains in SCID mice (Fig. 6). Surprisingly, the A3-pB22-FA strain produced low levels of *ospA* transcript in SCID mice that were similar to the low levels observed for the control *Borrelia* strains (Fig. 6).

Disease severity in mice infected with spirochetes constitutively producing OspA and B. Although OspA is primarily produced in the vector, the protein is occasionally produced in the vertebrate host, and some investigators have proposed that OspA production is linked to more severe disease. To determine if OspAB production in the context of a *Borrelia* infection was associated with more severe disease, mice infected with A3-pB22-FA and the control strains were examined for arthritis and carditis at days 15, 30, and 60 after infection. In most cases, the arthritis and carditis scores were higher for SCID mice than in C3H mice, as seen by others (4) (Table 4). Of interest is the lack of carditis in C3H mice for the two mutant strains compared to the wild type MI-A3 strain, although all three strains produced carditis in SCID mice. The lack of carditis for the two mutants, which were both missing lp25, suggests a role for lp25 in the causation of carditis in C3H mice. However, the *B. burgdorferi* strain constitutively producing OspAB (A3-pB22-FA) did not produce more severe disease compared to the control strains MI-A3 and A3-pB22 in SCID mice or C3H mice (Table 4) ($P \leq 0.5$).

Sequence of A3-pB22-FA mutants from SCID mice. DNA from 4 SCID mice infected with the A3-pB22-FA strain from 15, 30, and 60 days postinfection was sequenced to test for possible mutations in the plasmid that might lead to impairment of OspA production. Two of the samples from mice infected for 15 and 30 days showed no deviations at all from the known sequence. The other two samples had 2 and 3 single base pair substitutions, respectively, located in the *flaB* promoter and *ospA* gene (Fig. 7).

DISCUSSION

OspA is a *Borrelia* protein that is primarily produced in the tick. OspA is down regulated as the spirochetes move from the tick to a host during a blood meal. In the needle inoculation model of murine Lyme disease, spirochetes injected into rodents do express *ospA* for up to 5 days before down regulation occurs (8). Mutants missing the *ospAB* operon are infectious to mice but not to ticks, which further supports a role for these proteins in the vector and not the host (28). Although OspA is not a dominant antigen produced by spirochetes in the mammalian host, production has been detected under some conditions. In people with Lyme disease, OspA is produced in small amounts or not at all during the early stages of infection (1, 11, 18, 26). Most people with Lyme disease develop a single or multiple erythema migrans skin lesions at early stages of infection, biopsies from these sites have shown no *ospA* mRNA (11), and sera from patients with erythema migrans have had little or no reactivity to OspA proteins (18, 26). However, with the onset of Lyme arthritis 5 months to 7 years after the initial infection, an antibody response to OspA occurs in some people in which levels of anti-OspA antibody correlate with severity of arthritis (1). The possibility that OspA acts as an autoantigen with similarities to proteins produced in human neural tissue as well as to a peptide from human leukocyte function-associated antigen 1 has been proposed (2, 12). In fact, the gene encoding OspA was among the first *B. burgdorferi* genes to be cloned, and a recombinant OspA vaccine was approved for use in people and animals. However, the vaccine is no longer available, in part because of fears that the protein or an immune response against the protein could induce arthritis.

Our results also support the view that the OspAB operon is not of importance in the murine model of Lyme disease, as very low levels of *ospA* transcript and no OspA antibody were detected in C3H mice infected with the control strains MI-A3 and A3-pB22. C3H mice infected with *Borrelia* strain A3-pB22-FA, however, did develop an anti-OspA response. This observation confirms that the *flaB-ospAB* construct was expressed to higher levels in C3H mice than the native *ospA* which is down regulated in mice. Spirochetes producing OspA from the *flaB* promoter were almost totally cleared from C3H mice, most likely because of the immune response against OspA.

The conclusion that OspA antibodies in C3H mice cleared infection was also supported by the fact that strain A3-pB22-FA was able to persist in SCID mice that cannot mount a specific immune response. Surprisingly, the overall *ospA* transcript level in SCID mice was low even in mice infected with strain A3-pB22-FA, where *ospA* is controlled by the constitutive *flaB* promoter. One possible explanation for this result is that posttranscriptional mechanisms regulate *ospAB* mes-

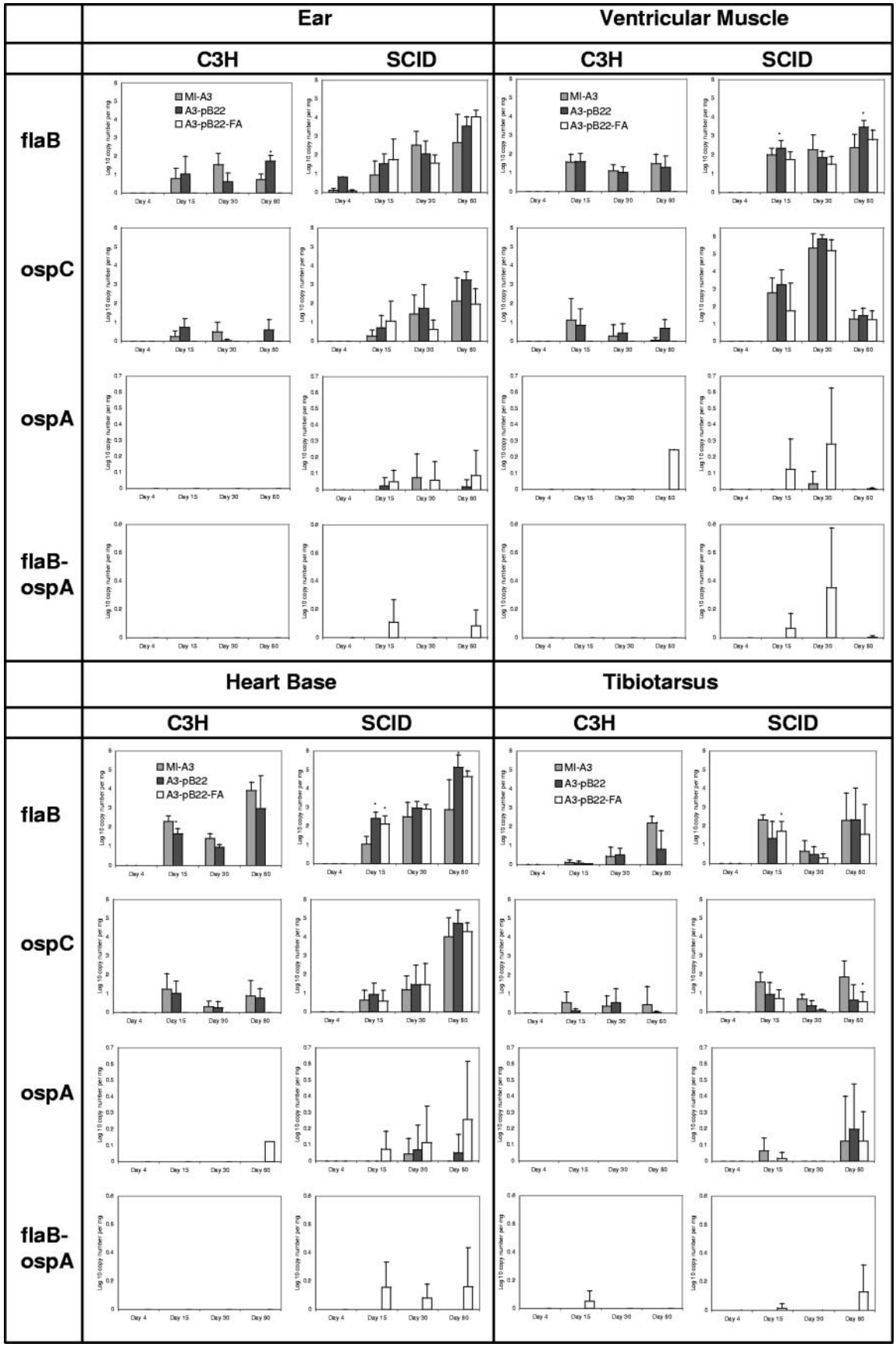

FIG. 6. RT-PCR of three different strains of spirochetes in C3H and SCID mice at 4, 15, 30, and 60 days postinoculation, where "ospA" represents native *ospA* transcripts and "flaB-ospA" represents *ospA* transcripts produced from the inserted plasmid vector in the A3-pB22-FA mutant. Results for both *ospA* transcripts have been scaled differently to show small differences. *, mean significantly different than that of the MI-A3 strain ($P \leq 0.05$).

TABLE 4. Arthritis and carditis scores for mice infected with three strains of spirochetes at varying time points postinoculation

Strain	Mouse type	Mean tibiotarsal arthritis severity (range, 0 to 3) ± SD on day:			Mean arthritis prevalence (range, 0 to 2) ± SD on day:			Carditis prevalence (no. positive/no. examined) on day:		
		15	30	60	15	30	60	15	30	60
MI-A3	C3H	0	1.0 ± 0	0.8 ± 0.8	0	1.0 ± 0	0.6 ± 0.5	5/5	5/5	1/5
	SCID	1.3 ± 0.4[a]	2.6 ± 0.5[a]	3.0 ± 0[a]	2.0 ± 0[a]	2.0 ± 0[a]	2.0 ± 0[a]	2/5	5/5	5/5
A3-pB22	C3H	0	0	0.4 ± 0.5	0	0	0.4 ± 0.5	0/5	0/5	0/5
	SCID	0[b]	0.8 ± 0.4[a,b]	2.3 ± 0.4[a,b]	0.8 ± 0.4[a,b]	1.4 ± 0.5[a]	1.8 ± 0.4[a]	5/5	5/5	5/5
A3-pB22-FA	C3H	0	0	0	0	0	0	0/5	0/5	0/5
	SCID	0.4 ± 0.5	0.9 ± 0.6[b]	2.2 ± 0.4[a,b]	1.4 ± 0.5[a]	1.3 ± 0.96	1.8 ± 0.4[a]	4/5	4/4	5/5

[a] The mean for SCID mice was significantly different from that for C3H mice for a given strain ($P \leq 0.05$).
[b] The mean for SCID mice given the A3-pB22 or A3-pB22-FA strain was significantly different from that for SCID mice given the MI-A3 strain ($P \leq 0.05$).

sage levels in the host. Alternatively, it is possible that OspA-producing spirochetes are under strong negative selection even in immunodeficient mice. In fact, when the *flaB-ospAB* construct was sequenced from four mice, two of the mice had point mutations in the *flaB* promoter region. However, it is not known whether the specific point mutations observed had an affect on *ospA* transcription. Further studies are needed to understand why the A3-pB22-FA spirochete mutant produced low levels of *ospA* in SCID mice.

Although C3H mice given the A3-pB22-FA strain of spirochetes were not positive by organ culture at all time points tested, some organs were positive for infection by qPCR and by RT-PCR. These results suggest that, despite the fact that the vast majority of mutant spirochetes were cleared from the mice, some spirochetes were able to persist. These spirochetes were possibly hidden in immune-privileged areas of the heart and joints or they were escape mutants, as seen in Xu et al. (27) for similarly constructed OspC-overproducing spirochetes in immunocompetent mice.

Some people with Lyme disease develop arthritis that does not resolve even after successful treatment of the bacterial infection. Although the exact etiology of treatment-resistant Lyme arthritis is not known, some investigators believe that an OspA-induced autoimmune reaction is responsible for this condition (1, 2, 12, 18). An arthritic reaction to OspA has also been shown in rats (5), suggesting that constitutive OspA production might lead to higher levels of arthritis. A study by Yang et al. (28) showed that an OspAB-negative mutant actually caused a more pronounced arthritis than the wild-type strain, suggesting that OspA is not linked to arthritis in the murine model of Lyme disease. Strain A3-pB22-FA constitutively producing OspA did not cause any symptoms of acute or lingering disease in C3H mice. Instead the strain stimulated a robust OspA antibody response in mice that lead to the clearance of infection.

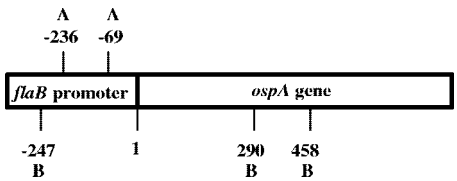

FIG. 7. Location of point mutations within the vector insert of the A3-pB22-FA strain isolated from SCID mice at 30 days (A) and 60 days (B) postinfection. Locations within the insert are based on the start codon of the *ospA* gene, where the "A" in the ATG codon represents position 1. All mutations consisted of an "A" in the parental sequence that was replaced by a "G" in the recovered mutant, except for the mutation at location 290, which had a "C" in the parental sequence that was replaced by a "T."

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