Deletion of BBA64, BBA65, and BBA66 Loci Does Not Alter the Infectivity of *Borrelia burgdorferi* in the Murine Model of Lyme Disease[†]

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Borrelia burgdorferi, the causative agent of Lyme disease, alters its gene expression in response to highly disparate environmental signals encountered in its tick vector versus vertebrate hosts. Whole-genome transcriptional profile analysis of *B. burgdorferi*, propagated in vitro under mammalian-host-specific conditions, revealed significant upregulation of several linear plasmid 54 (lp54)-encoded open reading frames (ORFs). Among these ORFs, BBA64, BBA65, and BBA66 have been shown to be upregulated in response to multiple mammalian-host-specific signals. Recently, we determined that there was no significant difference in the ability of BBA64[−] mutant to infect C3H/HeN mice compared to its isogenic control strains, suggesting that *B. burgdorferi* might utilize multiple, functionally related determinants to establish infection. We further generated BBA65[−] and BBA66[−] single mutants in a noninfectious, lp25[−] clonal isolate of *B. burgdorferi* strain B31 (ML23) and complemented them with the minimal region of lp25 (BBE22) required for restoring the infectivity. In addition, we generated a BBA64[−] BBA65[−] BBA66[−] triple mutant using an infectious, clonal isolate of *B. burgdorferi* strain B31 (5A11) that has all of the infection-associated plasmids. There were no significant differences in the ability to isolate viable spirochetes from different tissues of C3H/HeN mice infected via intradermal needle inoculation with either the individual single mutants or the triple mutant compared to their respective isogenic parental strains at days 21 and 62 postinfection. These observations suggest that *B. burgdorferi* can establish infection in the absence of expression of BBA64, BBA65, and BBA66 in the murine model of Lyme disease.

Borrelia burgdorferi, the etiological agent of Lyme disease, causes a multiphasic disorder involving the cutaneous, musculoskeletal, cardiovascular, and nervous systems (58). This spirochetal pathogen is transmitted to many vertebrate hosts, including humans, through the bite of infected *Ixodes* spp. ticks and has a significant public health impact in certain areas of endemicity in the United States (6, 42). The ability of *B. burgdorferi* to successfully colonize a vertebrate host is dependent on its ability to undergo adaptive gene expression in response to the highly disparate environmental conditions that exist between the tick vector and the vertebrate host (1–4, 7, 11, 12, 17, 18, 35, 41, 43, 49). A plethora of determinants with or without characterized functions are expressed by *B. burgdorferi* that facilitates trafficking of the spirochetes from the tick vector to the mammalian host and also during the eventual dissemination and colonization of various host tissues (15, 16, 18, 20, 21, 37, 38, 53, 59, 67).

The analysis of the dynamics of gene expression in *B. burgdorferi*, propagated under in vitro growth conditions that mimic either the tick vector or mammalian host environment, has provided a large body of information with regard to the open reading frames (ORFs) that may play a significant role in the infectious process. Several whole-genome transcriptional analyses of *B. burgdorferi* grown under defined fed and unfed tick or mammalian host-specific conditions have revealed a preferen-

tial upregulation of ORFs present on the linear plasmid 54 (lp54) (3, 6, 10, 41, 49). In addition, lp54 encodes for borrelial adhesins such as the outer surface protein A (OspA) (44) and the decorin binding proteins A and B (DbpA/B) (25), as well as ORFs regulated by pH (12), temperature (52), and other undefined vertebrate host signals (1). Therefore, it is apparent that many ORFs present on lp54 without defined functions are critical for the transmission from tick and for the early stages of infection of mammalian hosts. In order to evaluate the direct significance of differentially expressed ORFs on infectivity, we generated mutants in ORFs that are significantly upregulated under fed-tick conditions and evaluated their abilities to infect C3H/HeN mice.

Sequence analysis of ORFs encoded on lp54 indicated that BBA64, BBA65, and BBA66 are related and originally classified as part of paralogous gene family 54 (pgf54) (13, 22). Even though some of the members have been reassigned to other functionally related families, the sequence similarity and distribution of these ORFs within the borrelial genome suggest that they may play a significant role in the pathogenic mechanisms of *B. burgdorferi*. Several members of this gene family exhibit preferential upregulation under fed-tick or mammalian host-specific conditions and hence are excellent targets for inactivation to determine their contribution to the colonization of mammalian hosts (14, 39, 40). Antibodies to BBA64, BBA65, and BBA66 were found to be present in sera from experimentally infected mice and in patients diagnosed with early, disseminated Lyme disease, indicating that some of the members of pgf54 are synthesized during the disease, implicating a role in the persistence of the spirochetes in infected hosts (14, 24). Taken together, these observations suggest that

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TABLE 1. Plasmids and *B. burgdorferi* strains used in this study

Plasmid or <i>B. burgdorferi</i> strain	Description ^a	Source or reference
Plasmids		
pCR2.1-TOPO	PCR cloning vector	Invitrogen
pML102	Customized donor plasmid for in vitro transposition	55
pMM2	4.5 kb of lp54 spanning BBA64 to BBA68 in pCR2.1-TOPO	36
pMM6	pMM2 with 1.6-kb insertion of P _{flgB} - <i>aadA</i> 138 bp from the 5' end of BBA65	This study
pMM7	pMM2 with 1.6-kb insertion of P _{flgB} - <i>aadA</i> 145 bp from the 5' end of BBA66	This study
pMM11	2.4 kb of lp54 downstream of BBA64 and upstream of BBA66 and deletion of lp54 coordinates 43015 to 45827 in pCR2.1	This study
pMM13	pMM11 with 1.6-kb insertion of P _{flgB} - <i>aadA</i> in lieu of lp54 coordinates 43015 to 45827	This study
<i>B. burgdorferi</i> strains		
5A11	B31 isolate with all infection-associated plasmids	46
ML23	B31, lp25 ⁻ , noninfectious	32
MSK5	B31 isolate with all infection-associated plasmids	32
MM6	B31, lp25 ⁻ BBA65 ⁻ , Str ^r	This study
MM7	B31, lp25 ⁻ BBA66 ⁻ , Str ^r	This study
MM13	B31, BBA64 ⁻ BBA65 ⁻ BBA66 ⁻ , Str ^r	This study
ML23/pBBE22 (wild type)	ML23 with pBBE22 (BBE22 ⁺), Kan ^r	36
MM6/pBBE22 (BBA65 ⁻ mutant)	MM6 with pBBE22 (BBA65 ⁻ BBE22 ⁺), Str ^r Kan ^r	This study
MM7/pBBE22 (BBA66 ⁻ mutant)	MM7 with pBBE22 (BBA66 ⁻ BBE22 ⁺), Str ^r Kan ^r	This study

^a All coordinates for DNA sequences listed here are based on lp54 sequences available at the National Center for Biotechnology Information (NC-001857). Str^r, streptomycin resistance; Kan^r, kanamycin resistance.

several members of the *pgf54* may play a role in *B. burgdorferi* infection either independently or in conjunction with other members of this gene family.

In a recent study, we described the generation of a BBA64 mutant and found no significant differences in the ability of this mutant to colonize various tissues compared to the isogenic control strains in the murine model of Lyme disease (36). Immunoblot analysis of the BBA64 mutant propagated under conditions that mimic the fed tick revealed increased synthesis of BBA65, suggesting that inactivation of one ORF may lead to compensatory upregulation of other related ORFs (36).

In order to directly test the significance of the BBA65 and BBA66 loci in the infectious process, we generated individual mutants by using a two-step process of inactivating these genes in a noninfectious, lp25-negative clonal isolate of *B. burgdorferi* strain B31 (ML23) and subsequently restored the minimal region of infectivity (BBE22) present on lp25 using the borrelial shuttle vector pBBE22 (31, 36, 45, 55). We also used an infectious, clonal isolate of *B. burgdorferi* strain B31 (5A11) that has all of the infection-associated plasmids to generate the BBA64/BBA65/BBA66 triple mutant (33, 34, 46). The purpose of the present study is to determine whether the inactivation of BBA65 or BBA66 individually or the deletion of three genetically linked *pgf54* members (BBA64, BBA65, and BBA66) leads to attenuation of infectivity in the murine model of Lyme disease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A clonal, noninfectious isolate of *B. burgdorferi* strain B31 (ML23), lacking linear plasmid 25 (lp25⁻) was used to generate BBA65- and BBA66-negative mutants (32) (Table 1). A clonal, infectious isolate of *B. burgdorferi* strain B31 designated 5A11 (B31/5A11) containing all infection-associated plasmids was used to generate the BBA64/BBA65/BBA66-negative triple mutant (46). All *B. burgdorferi* cultures used for transformations were grown in 1% CO₂ at 32°C in BSK-II liquid medium (pH 7.6) supplemented with 6% normal rabbit serum (Pel-Freez Biologicals). *Escherichia coli* TOP10 cells (Invitrogen) were used for all cloning steps and were cultured

in Luria-Bertani broth supplemented with appropriate concentrations of antibiotics.

Plasmid constructs for the generation of single and triple mutants. Plasmid pMM2 with 4.5-kb region of lp54 extending from the 3' end of BBA64 to the 5' end of BBA68 in pCR2.1-TOPO cloning vector was used as the target for in vitro mutagenesis reactions to insertionally inactivate BBA65 and BBA66 using the GPS mutagenesis system (New England Biolabs) as described previously (36, 54, 55). Plasmids with potential transposon "hits" resulting in insertional inactivation of the BBA65 or BBA66 loci were identified by PCR analysis, and the site of insertion was determined by DNA sequencing. A plasmid, designated pMM6, containing a transposon insertion at position 138 bp relative to the 5' end of BBA65 and pMM7 with insertion at 145 bp relative to the 5' end of BBA66 were chosen and used for the generation of the BBA65- and BBA66-negative mutants in *B. burgdorferi*. The orientation of the transposon insertion is in the same direction as that of BBA65 in pMM6, while it is in the opposite orientation of that of BBA66 in pMM7, respectively (Fig. 1E and F). In order to facilitate recombination via double-crossover events involving homologous borrelial sequences flanking the antibiotic resistance marker, both of the plasmids were linearized with PstI and precipitated with ethanol prior to transformation. The use of linearized plasmids facilitates allelic exchange via double-crossover events to insertionally inactivate genes of interest and excluding the potential for single-crossover events that could result in the incorporation of genetic elements from the PCR cloning vector pCR2.1-TOPO within the targeted regions of lp54.

A two-step overlap PCR strategy was used to generate a plasmid with deletion of three genes (BBA64, BBA65, and BBA66) of lp54. In the first step, a 1.2-kb fragment of lp54 downstream BBA64 gene was PCR amplified from *B. burgdorferi* B31 by using the primer pair A64down and delA64 (Table 2). Similarly, a 1.2-kb fragment of lp54 upstream of BBA66 was amplified using A66up and delA66 primers. Both of the primers sets had an engineered SalI site, as well as a 10-nucleotide overlap to facilitate the deletion process in the overlap PCR. In the second step, the 2.4-kp product was PCR amplified by using LATag polymerase (TaKaRa) with the primers A64down and A66up and PCR products from the first step as a DNA template with 31 cycles of 94°C for 1 min, 50°C for 1 min, and 68°C for 5 min, followed by a 10-min extension at 72°C. The resulting 2.4-kp product was then cloned in pCR2.1 vector (Invitrogen) and transformed into TOP10 *E. coli*. Individual colonies were subjected to blue-white screening, and plasmids were screened for the correct insert size by restriction enzyme digestion with EcoRI. A plasmid designated pMM11 with a SalI site in between the downstream region of BBA64 and the upstream region of BBA66 was used to insert the P_{flgB}-*aadA* (Str^r) with flanking SalI sites (PCR amplified from pML102 using the primers flgBaadAF and flgBaadAR) to insert a streptomycin resistance marker under the control of a borrelial promoter. A plasmid designated pMM13, with P_{flgB}-*aadA* inserted between flanking regions of BBA64 and

FIG. 1. Southern blotting confirms the mutation in the BBA65 and BBA66 genes of lp54 in ML23 (noninfectious, lp25⁻, *B. burgdorferi* strain B31). (A and B) Total genomic DNA from ML23 parental strain (lanes 1 and 2) and MM6 (BBA65⁻ mutant, lanes 3 and 4) was digested with HindIII (lanes 1 and 3) and BglII (lanes 2 and 4). Blots were probed with the *aadA* gene (Str^r marker, probe A) (A) and with probe B encompassing the BBA65 gene (B). (C and D) Total genomic DNA from ML23 parental strain (lanes 1 and 2) and MM7 (BBA66⁻ mutant, lanes 3 and 4) was digested with HindIII (lanes 1 and 3) and BglII (lanes 2 and 4). Blots were probed with the *aadA* gene (Str^r marker, probe A) (C) and with the probe C encompassing the BBA66 gene (D). The numbers on the left of each panel indicate the size of the markers in kilobases. (E and F) Schematic of the BBA64 to BBA68 region of lp54 with the transposon insertion (*P_{flgB}-aadA*) in BBA65 (E) and BBA66 (F). The black boxes indicate the location of the Tn7 repeats. The arrows indicate the orientations of BBA64R and BBA68F primers used to amplify the region of BBA64 to BBA68 on lp54. H3, HindIII.

BBA66 (resulting in the deletion of the sequence between the nucleotides 43015 and 45827 of lp54), was linearized with PstI and ethanol precipitated prior to being used for generation of the triple mutant using the clonal, infectious isolate of *B. burgdorferi* strain B31 (5A11). All of the nucleotide coordinates mentioned here were based on lp54 sequences available at the National Center for Biotechnology Information (NC-001857).

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5'→3') ^a
A64down	TTGCTGCTTTTGAATCTATGC
A66up	GCCAAAAAATCACCATCCG
delA64	ATTTCCTTGGCTCGACGAGAGAGAGAGATGCTTACAC
delA66	CTCTCTTCTGGTCGACGCAAGAAAATAAAAAACATCCC
flgBaadAF	ACGCGTCGACGGAAGATTTCCTATTAAGG
flgBaadAR	ACGCGTCGACTTATTTGCCGACTACCTTGGT
BBA65F	ACGCCATATGGATCTAAACAACAAAGAC
BBA65R	ACGCCTCGAGATTATTATTAATTTAAATAATGT
BBA66F	ACGCCATATGATTGATGCCAATCTAAAC
BBA66R	ACGCCTCGACATTATACTAATGTATGC

^a Restriction sites are underlined.

Generation of mutants in *B. burgdorferi*. A clonal derivative of *B. burgdorferi* strain B31 lacking linear plasmid 25, ML23, was electrotransformed either with pMM6 or with pMM7 as described previously (36, 51, 54, 55). An infectious, clonal derivative of *B. burgdorferi* strain B31 (5A11) was electrotransformed with pMM13 using a similar procedure. Transformants were selected on BSK-II agarose overlays containing 50 µg of streptomycin/ml as described previously. Individual colonies were screened by PCR using total genomic DNA as a template by forward and reverse primers specific to BBA65 (pMM6) or BBA66 (pMM7) or A64down with A66up (pMM13), respectively (Table 2). Transformants of ML23/pMM6 and ML23/pMM7 with an increase in the amplicon size by 1.6 kb due to the presence of the *P_{flgB}-aadA* marker compared to the wild-type parental control strain ML23 were considered positive and selected for further analysis. Transformants of 5A11/pMM13 with a 1.2-kb decrease in the size of amplicon compared to that of 5A11 parental strain were selected as positive. Even though the size of the deletion is 2.8 kb, the presence of the 1.6-kb Str^r marker (*P_{flgB}-aadA*) results in an amplicon with a size reduction of only 1.2 kb, a finding consistent with our deletion strategy described above. One of each representative mutant clone designated MM6 (BBA65⁻), MM7 (BBA66⁻), and MM13 (triple mutant), respectively, were further analyzed by Southern hybridization.

Southern blot analysis. Total genomic DNA was isolated from MM6, MM7, and MM13, as well as from the isogenic, parental control strains for single (ML23) and triple (5A11) mutants, respectively. The DNA was digested with

different restriction enzymes, separated on a 1% agarose gel, transferred onto a nylon membrane, and hybridized with enhanced chemiluminescence-labeled probes overnight at 42°C. The membranes were developed according to the manufacturer's instructions (GE Healthcare).

Restoring infectivity of MM6 and MM7 mutants with an ML23 (lp25⁻) background. The borrelial shuttle vector pBBE22 containing the minimal region of lp25 (BBE22 encoding PncA) required to restore the infectivity of ML23 in the murine model of Lyme disease was used to transform the BBA65⁻ (MM6) and BBA66⁻ (MM7) mutants (45, 50, 60). The transformants were selected on BSK-II agarose overlays supplemented with 200 µg of kanamycin/ml and 50 µg of streptomycin/ml. The presence of pBBE22 was verified by using primers specific to this borrelial shuttle vector as described previously (36, 45).

Plasmid profile of the mutants. The plasmid profiles of all clones and mutants were determined by PCR using primers sets as previously described (32).

SDS-polyacrylamide gel electrophoresis and immunoblot analysis. *B. burgdorferi* parental clones (5A11 and ML23) and mutants (MM6, MM7, and MM13) were cultivated in 45-ml cultures at pH 7.6 and 23°C to a density of $\sim 1 \times 10^7$ spirochetes/ml, at which time $\sim 5 \times 10^5$ spirochetes were transferred into 45 ml of BSK-II growth medium at pH 6.8 and 37°C. All cultures were then allowed to grow to late logarithmic phase (ca. 1×10^8 to 2×10^8 spirochetes/ml) in the above-described medium that mimicked tick midgut conditions before (pH 7.6 and 23°C) and after (pH 6.8 and 37°C) feeding. Whole-cell lysates of *B. burgdorferi* were prepared and separated on sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel electrophoresis as described previously (36). The separated proteins were either visualized by Coomassie brilliant blue staining or transferred to a polyvinylidene difluoride membrane and subjected to immunoblot analysis as described previously. The membranes were probed with rat anti-BBA64 serum, rat anti-BBA66 serum, or mouse anti-BBA65 serum (V. L. Sexton and J. Seshu, unpublished data). The blots were developed after incubation with appropriate dilutions of horseradish peroxidase-conjugated anti-rat or anti-mouse secondary antibodies using enhanced chemiluminescence-Western blotting detection reagents (GE Healthcare).

Infectivity studies. Groups ($n = 3$) of 6-week-old female C3H/HeN mice were inoculated intradermally at doses of 10^2 , 10^3 , 10^4 , and 10^5 spirochetes per mouse with the following borrelial strains: wild-type strains 5A11 and ML23/pBBE22 and mutant strains MM6/pBBE22, MM7/pBBE22, and MM13. At 21 days after inoculation, the spleen, left-tibiotarsal joint, left inguinal lymph node, heart, bladder, and a piece of abdominal skin were collected from each mouse and cultured in BSK-II growth medium to facilitate the isolation of spirochetes as previously described (31, 36, 55). The cultures were scored for growth of *B. burgdorferi* after 2 to 3 weeks using dark-field microscopy. All animal procedures were performed in accordance with the animal use protocol approved by the Institutional Animal Care and Use Committee at the University of Texas at San Antonio.

RESULTS

Construction of BBA65 and BBA66 single mutants. We intentionally inactivated the BBA65 and BBA66 genes using a customized transposon containing a streptomycin resistance marker under the control of a borrelial promoter ($P_{\text{flgB-aadA}}$) using an in vitro mutagenesis strategy as described previously (36, 54). Southern blot analysis of BBA65⁻ clone, designated MM6, showed that there was hybridization of the *aadA* probe to DNA fragments from MM6 (Fig. 1A, lanes 3 and 4) whereas there was no hybridization with the DNA from the parental strain (ML23) digested with HindIII or BglII (Fig. 1A, lanes 1 and 2). The sizes of these DNA fragments were consistent with the expected sizes based on the restriction enzyme profile of the region of BBA65 in lp54 and the presence of these sites on the customized transposon. We further confirmed that the sizes of DNA fragments from the isogenic parental control ML23 (Fig. 1B, lanes 1 and 2) and that of the MM6 (Fig. 1B, lanes 3 and 4) that hybridized to probe B were consistent with the expected sizes based on the presence of restriction enzyme sites, as well as on the site of the insertion of customized transposon (Fig. 1E). A similar analysis of the BBA66⁻ mutant, designated MM7, also revealed that the hybridization of

the labeled *aadA* was only with the DNA fragments from the mutant (Fig. 1C, lanes 3 and 4) and not with the DNA from ML23 (Fig. 1C, lanes 1 and 2). Moreover, the differences in the hybridization of the DNA from the parental and mutant strains with BBA66 gene as the labeled probe (probe C) indicated that the insertional inactivation occurred as expected in MM7 (Fig. 1D, lanes 3 and 4) and was consistent with the expected sizes based on the presence of restriction enzyme sites and the site of insertion of the customized transposon within BBA66 (Fig. 1F). These observations confirmed the inactivation of BBA65 and BBA66, respectively, as well as the clonality of these mutants. The size of the DNA fragments hybridizing with the respective probes indicate that the inactivation of BBA65 and BBA66 in ML23 was achieved by allelic exchange due to double-crossover events and not via a single-crossover which could result in insertion of DNA sequences from the nonhomologous regions of the PCR cloning vector pCR2.1 TOPO. The comparable growth characteristics of MM6/MM7 and the parental strain suggested that BBA65 and BBA66 are dispensable for in vitro growth. All of the strains (ML23, MM6, and MM7) were transformed with pBBE22 to provide the minimal region of lp25 required to restore infectivity.

Construction of the BBA64/BBA65/BBA66 triple mutant.

The gene organization of BBA64, BBA65, and BBA66 as adjacent ORFs with minimal intergenic regions on lp54 facilitated the deletion of these three ORFs using a single counterselectable borrelial marker (Str^r). In order to do this, we PCR amplified two regions of lp54, as indicated in Fig. 2A, where region 1 corresponds to the downstream sequence of BBA64 and the region 2 corresponds to the upstream sequence of BBA66 using the indicated primers. The two reverse primers (delA64 and delA66) had an engineered SalI site, as well as a 10-nucleotide overlapping sequence that facilitated the second overlap PCR to generate a deletion of the sequence corresponding to nucleotides 43015 to 45827 of lp54 (Fig. 2B). The presence of SalI in between regions 1 and 2 was used to insert $P_{\text{flgB-aadA}}$ (Str^r) with flanking SalI sites by conventional cloning steps, resulting in a construct schematically represented in Fig. 2C. This construct (pMM13) was used to generate the deletion of the BBA64, BBA65, and BBA66 loci in the infectious 5A11 clone of *B. burgdorferi* strain B31 using the transformation protocols described previously (36). The borrelial transformants were selected in the presence of 50 µg of streptomycin/ml, and the deletion was verified by using primers specific to the flanking regions of the deleted segment. One clone, designated MM13, was selected by using the PCR-based analysis described above. While the total length of the deleted fragment was 2.8 kb, the insertion of the 1.6-kb Str^r resistance cassette resulted in an amplicon that was only 1.2 kb smaller than the one from the parental strain (data not shown). Southern blot hybridization analysis of MM13 showed that there was hybridization of the *aadA* probe to DNA fragments from MM13 (Fig. 2E, lanes 3 and 4) whereas there was no hybridization with the DNA from the parental strain (5A11) digested with ScaI or HindIII (Fig. 2E, lanes 1 and 2). The sizes of these DNA fragments were consistent with the expected sizes based on the restriction enzyme profile of the region of lp54 and the region of lp54 deleted in the mutant. The same was true for DNA fragments from the isogenic parental control 5A11 (Fig. 2F, lanes 1 and 2) and that of the MM13 (Fig. 2F, lanes 3 and

FIG. 2. Southern blotting confirms the deletion of BBA64, BBA65, and BBA66 genes of lp54 in 5A11 (clonal, infectious *B. burgdorferi* strain B31). (A to D) Schematic representations of constructs generated to delete BBA64, BBA65, and BBA66 genes with the replacement of the intervening region with the P_{flgB} -*aadA* (Str^r marker) using a two-step overlap PCR-based method for deletion. (A) Schematic representation of the lp54 region spanning from BBA64 to BBA68. The positions of select restriction enzyme sites are indicated. H3, HindIII. (B) Schematic representation of lp54 region of pMM11. The arrows flanking the regions downstream of BBA64 and upstream of BBA66 represent the primers used to generate PCR products. An engineered SalI sites and 10-nucleotide overlapping sequence present on the primers (delA64 and delA66) facilitated the overlap PCR to delete the region lp54 from the nucleotide coordinates 43015 to 45827. Probe B used in the Southern blot analysis comprises of region 1 (downstream of BBA64) and region 2 (upstream of BBA66). (C) Schematic representation of lp54 region of pMM13. The cloning of P_{flgB} -*aadA* (Str^r) marker at the SalI site indicated in Fig. 2B resulted in the insertion of a selectable marker within the deleted region of lp54. The 2.8-kb deleted region of lp54 replaced with 1.6-kb Str^r marker resulted in fragments with a change in size of 1.2 kb in Southern blot analysis. (D) Schematic representation of the region of lp54 in the BBA64/BBA65/BBA66 triple mutant (MM13) generated using an infectious clonal isolate of *B. burgdorferi* strain B31 (5A11). (E and F) Total genomic DNA from 5A11 parental strain (lanes 1 and 2) and MM13 (BBA64/BBA65/BBA66 triple mutant, lanes 3 and 4) was digested with Scal (lanes 1 and 3) and HindIII (lanes 2 and 4). Blots were probed with the *aadA* (Str^r marker, probe A) (E) and with probe B comprising of the regions 1 and 2 (F). The probe B was designed to overlap with the sequence downstream of BBA64 and upstream of BBA66 to establish the deletion of much of the intervening region.

4) that hybridized to probe B (Fig. 2B, comprising the lp54 region that flanks the deleted sequence). The Southern hybridization analysis established not only the clonality of the mutant but also confirmed the PCR-based observations (data not shown) with regard to the nature of the deletion within lp54 present in the triple mutant and the parental strain. Moreover, the size of the hybridizing fragments with the respective probes confirmed allelic exchange via double-crossover events spanning homologous regions of borrelial DNA with no integration of nonhomologous regions of the plasmid used for transformation of *B. burgdorferi*. That the growth characteristics of MM13 were comparable to that of the parental strain suggested that the deletion of BBA64, BBA65, and BBA66 is dispensable for in vitro growth.

Expression of BBA64, BBA65, and BBA66 in the parental and mutant strains. To test the contribution of BBA65 and

BBA66 to the infectivity of *B. burgdorferi* in the mammalian host, it was necessary to confirm whether different environmental signals induced comparable levels of expression of pgf54 members in the strains to be used in the infectivity analysis. To determine the levels of expression of BBA64, BBA65, and BBA66, we propagated ML23/pBBE22, MM6/pBBE22, and MM7/pBBE22 at either pH 7.6 and 23°C or pH 6.8 and 37°C, mimicking the tick midgut conditions before or after feeding, respectively. Immunoblot analysis with mono-specific rat anti-BBA64, anti-BBA66 antibodies (8) or mono-specific mouse anti-BBA65 antibodies was used to detect the levels of these proteins (36). As shown in Fig. 3A, there were no overt differences in the protein profiles between the MM6 strain, the MM7 strain, and isogenic parental strain ML23 except between samples of strains propagated under different growth conditions. Previous studies have shown a significant

FIG. 3. Expression of BBA64, BBA65, and BBA66 proteins in response to mammalian host-specific conditions in single mutants. The ML23 (wild type), MM6 (BBA65⁻ mutant), and MM7 (BBA66⁻ mutant) were propagated at either pH 7.6 and 23°C (lane 1) or pH 6.8 and 37°C (lane 2), mimicking tick- and mammalian-host-specific conditions, respectively. (A) Total protein samples were separated on a SDS-12.5% polyacrylamide gel and stained with Coomassie brilliant blue. The arrow corresponds to OspC, and the markers on the left indicate the molecular masses of protein standards in kilodaltons. (B) Immunoblot analysis using monospecific serum against BBA64, BBA65, and BBA66 proteins. The blots were developed by using an enhanced chemiluminescence system. The numbers to the left of each panel indicate the molecular masses of protein markers in kilodaltons.

increase in the levels of outer surface protein C (OspC) when *B. burgdorferi* is exposed to conditions such as increased temperature, reduced pH, and other undefined signals that mimic the midgut environment of fed-tick or mammalian host (63). Hence, the levels of OspC serve as an indicator of the state of *B. burgdorferi* within fed ticks, as well as the functionality of critical regulatory pathways such as Rrp2-RpoN-RpoS (26, 64). This pathway has been shown to control the expression of lipoproteins such as OspC and DbpA that are important in the pathogenesis of *B. burgdorferi*. As expected, there was a significant increase in the levels of OspC in *B. burgdorferi* cultured at pH 6.8 and 37°C (Fig. 3A, lane 2, in all strains) compared to cultures grown at pH 7.6 and 23°C (Fig. 3A, lane 1, in all strains). This observation is consistent with previous reports demonstrating that a change in environmental conditions that mimic post-tick-feeding conditions (i.e., increased temperature and lowered pH) results in increased levels of OspC and that the *ospC* expression was similar between the mutant and the control strains (63).

Consistent with what has been observed previously (36), immunoblot analysis with monospecific anti-BBA64 serum revealed equivalent expression of BBA64 protein in all three strains propagated at pH 6.8 and 37°C (Fig. 3B, αA64, lane 2, all strains) with little or no expression at pH 7.6 and 23°C (Fig. 3B, αA64, lane 1, all strains). Immunoblot analysis of BBA65 and BBA66 mutants propagated at pH 6.8 and 37°C showed no reactivity to the respective monospecific antisera, confirming the loss of expression of the corresponding proteins in these mutants (Fig. 3B, αA65 and αA66). The parental strain when propagated at pH 6.8 and 37°C showed reactivity to both anti-

BBA65 and anti-BBA66 with little or no expression of these proteins when propagated at pH 7.6 and 23°C. Moreover, the BBA65 mutant showed reactivity to anti-BBA64 and anti-BBA66 (Fig. 3B, MM6, lane 2) when grown at pH 6.8 and 37°C, indicating that the loss of expression of BBA65 did not have an effect on either the upstream (BBA66) or the downstream (BBA64) ORFs. This was also true with the BBA66⁻ mutant (Fig. 3B, MM7, lane 2). This analysis also suggested that the loss of expression of BBA65 or BBA66 did not affect the expression of other critical determinants such as OspC, as well as the expression levels of neighboring ORFs. Although the orientation of the transposon was in the same direction as that of BBA65, the levels of BBA64, as determined by immunoblot analysis (Fig. 3B, MM6, lane 2) was similar to that of the parental control strain (Fig. 3B, ML23, lane 2). Hence, there was no effect of the orientation of the transposon (*P_{flgB-addA}*) on the levels of expression of the downstream ORF BBA64. Similarly, there was no effect on the levels of expression of BBA64 (Fig. 3B, MM7, lane 2, α-A64) and BBA65 (Fig. 3B, MM7, lane 2, α-A65) in MM7 even though the direction of the transposon was in the opposite orientation of that of BBA66. This immunoblot analysis also showed that the expression of BBA64, BBA65, and BBA66 was dependent on their respective promoters and that they are not a part of a single operon since inactivation of BBA66 did not have any effect on the levels of expression of downstream ORFs, namely, BBA65 and BBA64. In addition, this analysis also suggested that the signaling pathways involved in the expression of these proteins in response to various environmental signals was intact and that these mutants could be used to evaluate the significance of the role of BBA65 and BBA66 individually using the murine model of Lyme disease.

There were no overt differences between the parental strain 5A11 and the triple mutant MM13 in terms of the protein profile under the growth conditions tested (Fig. 4A). Immunoblot analysis also confirmed that the expression of all three proteins was completely abolished in the BBA64/BBA65/BBA66 triple mutant. As shown in Fig. 4B, there was no expression of these proteins in MM13 when the immunoblots were probed with anti-BBA64, anti-BBA65, and anti-BBA66 serum under the *in vitro* growth conditions used in the present study, whereas the parental control strain (5A11) showed reactivity with all three sera only when propagated at pH 6.8 and 37°C (Fig. 4B, 5A11, lane 2). Moreover, the level of induction of OspC was consistently lower in 5A11 (Fig. 4A) grown under conditions that mimic conditions within the midgut of fed ticks compared to ML23/pBBE22 (Fig. 3A, ML23). While this may be due to differences in the strains, the levels of OspC in the mutant (Fig. 4A, MM13, lane 2) and parental (Fig. 4A, 5A11, lane 2) strains appear to be similar, suggesting that it was not the process of generating the mutant that resulted in altered levels of expression of OspC. Immunoblot analysis of 5A11 and MM13 with anti-BBA65 serum showed cross-reactivity to a band independent of the culture conditions (Fig. 4B, all lanes, α-A65). There was no such cross-reactivity when lp25-negative ML23, MM6, or MM7 was probed with the same serum (Fig. 3B, all lanes, α-A65). Although the exact identity of this protein is not known, it is possible that this protein may be synthesized from lp25 present in 5A11 and MM13 or it could be due to differences in the expression of members of *pgf54*.

FIG. 4. Expression of BBA64, BBA65, and BBA66 proteins in response to mammalian host-specific conditions in the BBA64/BBA65/BBA66 triple mutant. The 5A11 (wild type) and MM13 (BBA64/BBA65/BBA66 triple mutant) strains were propagated at either pH 7.6 and 23°C (lane 1) or pH 6.8 and 37°C (lane 2), mimicking the tick- and mammalian-host-specific conditions, respectively. (A) Total protein samples were separated on a SDS–12.5% polyacrylamide gel and stained with Coomassie brilliant blue. The markers on the left indicate the molecular masses of protein standards in kilodaltons. (B) Immunoblot analysis using monospecific serum against BBA64, BBA65, and BBA66 proteins. The blots were developed by using the enhanced chemiluminescence system. The numbers to the left of each panel indicate the molecular masses of proteins markers in kilodaltons.

Plasmid profile of the triple mutant. Since we used a clonal, infectious isolate of *B. burgdorferi* strain B31 containing the infection-associated plasmids, we evaluated the plasmid profile of BBA64/BBA65/BBA66 triple mutant to ensure that plasmids such as lp25, lp28-1, and lp36 were present in the mutant. These linear plasmids have been shown to be essential for

infection in the murine model of Lyme disease. The plasmid profile of the MM13 strain was identical to the parental strain 5A11, and all of the critical infection-associated plasmids were present in both of the strains (data not shown). In addition to determining the presence of lp28-1, we also determined, by immunoblot analysis, that the levels of expression of VlsE in both the parental and the mutant strains were similar (data not shown) (31, 65–67). We also evaluated the plasmid profile of the MM6 and MM7 strains using the same set of primers and found that cp9 and lp25 are the only two plasmids that were not present (data not shown). The minimal region of lp25 required for infectivity of these single mutants and their isogenic parental strain ML23 was restored by the use of the borrelial shuttle vector pBBE22. By adopting this strategy of generating mutants in noninfectious and infectious clonal isolates and evaluating their plasmid profile for critical infection-associated plasmids (or their minimal regions), we have generated mutant strains with the requisite genetic background to test for infectivity in the murine model of Lyme disease.

Infectivity analysis of single and triple mutants in C3H/HeN mice. We recently reported the lack of a significant attenuation in the infectivity of a BBA64 mutant in the murine model of Lyme disease. The *in vitro* phenotypic analysis of the above mutant revealed an increase in the levels of BBA65 by immunoblot analysis, suggesting that the loss of one ORF may be compensated for by differential expression of other functionally related genes. We therefore evaluated the infectivity phenotype of BBA65- and BBA66-negative single mutants in the C3H/HeN mice after intradermal needle inoculation at doses ranging from 10^2 to 10^4 spirochetes per mouse. As shown in Table 3, there were no significant differences in the ability to isolate spirochetes from various tissues from mice infected with the parental, BBA65⁻ or BBA66⁻ strains at 21 days postinfection. Since it has been shown that BBA65 and BBA66 could play a role in the persistence of the spirochetes, we extended the infectivity analysis for 62 days using a single dose of infection (10^3 spirochetes per mouse) administered intradermally. Similar to the 21-day postinfection analysis, there were no significant differences in the ability of the BBA65⁻ and BBA66⁻ strains to colonize, disseminate, and survive for

TABLE 3. Infectivity analysis of BBA65⁻ and BBA66⁻ mutants in C3H/HeN mice

Time postinfection and strain	Dose	No. of positive cultures/no. tested obtained from:							No. of mice infected/no. tested
		Skin	Spleen	Joint	Lymph node	Heart	Bladder	All sites	
21 days									
Wild type (ML23/pBBE22)	10 ²	0/3	0/3	0/3	0/3	0/3	0/3	0/18	0/3
	10 ³	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
	10 ⁴	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
BBA65 [−] mutant (MM6/pBBE22)	10 ²	1/3	1/3	1/3	1/3	1/3	1/3	6/18	1/3
	10 ³	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
	10 ⁴	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
BBA66 [−] mutant (MM7/pBBE22)	10 ²	1/3	1/3	1/3	1/3	1/3	1/3	6/18	1/3
	10 ³	3/3	2/3	3/3	3/3	3/3	3/3	17/18	3/3
	10 ⁴	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
62 days									
Wild type (ML23/pBBE22)	10 ³	3/3	1/3	3/3	3/3	3/3	3/3	16/18	3/3
BBA65 [−] mutant (MM6/pBBE22)	10 ³	3/3	1/3	3/3	3/3	3/3	3/3	16/18	3/3
BBA66 [−] mutant (MM7/pBBE22)	10 ³	3/3	0/3	3/3	3/3	3/3	3/3	15/18	3/3

TABLE 4. Infectivity analysis of the BBA64/BBA65/BBA66-negative triple mutant in C3H/HeN mice

Time postinfection and strain	Dose	No. of cultures positive/no. tested obtained from:							No. of mice infected/no. tested
		Skin	Spleen	Joint	Lymph node	Heart	Bladder	All sites	
21 days									
Wild type (B31/5A11)	10 ²	1/3	1/3	1/3	1/3	1/3	1/3	6/18	1/3
	10 ³	2/5	2/5	1/4	2/5	1/4	2/5	10/28	2/5
	10 ⁴	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
	10 ⁵	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
Mutant (MM13)	10 ²	1/3	1/3	1/3	1/3	1/3	1/3	6/18	1/3
	10 ³	4/6	3/6	3/5	4/6	3/5	4/6	21/34	4/6
	10 ⁴	3/3	1/3	3/3	3/3	3/3	3/3	16/18	3/3
	10 ⁵	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
62 days									
Wild type (B31/5A11)	10 ³	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
Mutant (MM13)	10 ³	3/3	2/3	3/3	3/3	3/3	3/3	17/18	3/3

62 days in the C3H/HeN model of Lyme disease. Since both BBA65- and BBA66-negative single mutants did not exhibit any significant attenuation compared to their isogenic parental strain, we further evaluated the infectivity phenotype of the BBA64/BBA65/BBA66-negative triple mutant. As shown in Table 4, there was no significant difference in the ability of the triple mutant to colonize and disseminate to various tissues in C3H/HeN mice compared to its isogenic parental strain at day 21 postinfection. In addition, there was no significant difference in the ability to isolate spirochetes from various mouse tissues at day 62 postinfection, indicating that the triple mutant was competent to survive for longer periods of time in the mouse model. These aforementioned infectivity analyses using the single and triple mutants indicate that despite significant upregulation under mammalian host-specific conditions, BBA64, BBA65, and BBA66 are not required for the survival of *B. burgdorferi* in the C3H/HeN model of Lyme disease.

DISCUSSION

A number of recent studies have described a significant differential transcriptional regulation of plasmid-encoded ORFs when *B. burgdorferi* is propagated under conditions that mimic the mammalian host or conditions prevalent in the tick midgut following the ingestion of a blood meal (3, 7, 41, 49, 61). A similar upregulation also was observed when a wild-type strain of *B. burgdorferi* was propagated in dialysis membrane chambers implanted into peritoneal cavities of rats, notably in comparison to that of an *rpoS* mutant propagated under similar conditions (7, 10). These whole-genome transcriptional analyses revealed that BBA64, BBA65, and BBA66 were among the most significantly upregulated genes under mammalian-host-adapted conditions (10). While the transcriptional levels of various ORFs provide a means to identify a subset of genes compared to those that are downregulated or remain unchanged under mammalian-host-specific conditions, it is still imperative to determine the contributions of these ORFs either individually or, if feasible, in combination to the colonization and dissemination characteristics of *B. burgdorferi* in the experimental models of Lyme disease. Toward that end, we previously described the phenotypic analysis of BBA64⁻ mutant, which did not display a significant difference in infectivity

in the C3H/HeN model of Lyme disease (36). When the BBA64⁻ mutant was propagated at pH 6.8 and 37°C, there was increased expression of BBA65, suggesting that upregulation of closely related genes (based on sequence similarity) or proteins with related functions may compensate for the lack of expression of a single transcriptionally active ORF induced under different host-specific conditions. These observations led us to extend our studies to generate mutants in BBA65 and BBA66 individually, as well as to generate a triple mutant deleting BBA64, BBA65, and BBA66 together, and to characterize their in vitro and in vivo phenotypes.

The lack of reactivity of BBA65⁻ and BBA66⁻ mutants with monospecific sera against BBA65 and BBA66 clearly demonstrated the loss of synthesis of the corresponding proteins, while the regulatory pathways (such as RpoS/RpoN) that presumably play a significant role in the expression of these antigens were intact following generation of the mutants. Although there are distinct advantages such as an increased number of transformants and shorter duplication time using the lp25-negative strain of B31 (ML23), there is a drawback because these mutants are not competent for tick-borne transmission since they lack a critical determinant (BBE16) encoded in lp25 (30, 48). In order to overcome this limitation, we used an infectious clonal isolate (5A11) to generate the BBA64/BBA65/BBA66 triple knockout (46). We were successful in generating a triple mutant in lp54 in spite of the fact that 5A11 was more difficult to manipulate due to a slower growth rate and inherently lower transformation efficiency than ML23 (data not shown). The propagation of this strain at pH 6.8 and 37°C did not result in significant upregulation of OspC (compared to its growth at pH 7.6 and 23°C; Fig. 4), whereas the clonal, noninfectious lp25⁻ isolate of *B. burgdorferi* (ML23) exhibited a significant difference in OspC levels when propagated under mammalian-host-specific conditions (Fig. 3). The presence of lp25 in 5A11 and in the triple mutant (data not shown) would facilitate analysis of tick-borne transmission, since both the isolates contain lp25-encoded BBE16 needed for their competency to survive in ticks.

Consistent with several previous studies (14, 23, 28, 29), an important outcome of the aforementioned analysis is that BBA64, BBA65, and BBA66 are not part of an operon in the clonal isolates (ML23 and 5A11) of *B. burgdorferi* strain B31

used in the present study even though they are located adjacent to each other. This is based on the observation that insertional inactivation of BBA66 did not have any effect on the levels of expression of the downstream ORFs (BBA65 and BBA64) based on immunoblot analysis (Fig. 3B). The transposon used for inactivation was in the opposite orientation to that of BBA66, and hence there is no potential for expression of BBA65 and BBA64 due to the effects of constitutive borrelial promoter P_{flgB} driving the expression of the *aadA* gene conferring streptomycin resistance (Fig. 1E). It has been shown that distinct sequence-specific DNA-binding proteins recognize motifs in the upstream regions of BBA64 (23, 28) and BBA66 (14). The promoter/operator region of BBA64 has been shown to have a 43-nucleotide DNA-binding motif upstream of the -35 element (σ^{70} consensus sequence) with an inverted repeat sequence and a downstream poly(T) tract (23, 28). Similarly, the upstream promoter/operator region of BBA66 was shown to have both a σ^{70} consensus sequence similar to that of BBA64 and a σ^S -dependent promoter (14). Sequence analysis of the upstream region of BBA65 revealed motifs similar to -10 sequences of BBA64 or BBA66, but the lack of information on the transcriptional start site precludes a precise identification of these motifs. Moreover, there is an inverted repeat (nucleotides 44568 to 44594) and a poly(T) tract (nucleotides 44564 to 44570), suggesting that the upstream region of BBA65 may be regulated similarly to that of BBA64 and BBA66 (9, 57). It is apparent that BBA64, BBA65, and BBA66 are each regulated by their respective promoters and are not part of an operon based on functional promoter/operator analysis (14, 23), as well as the mutational analysis described here.

Infectivity analysis of C3H/HeN mice via intradermal needle inoculation and evaluation of the ability to isolate viable spirochetes at day 21 or 62 postinfection from different tissues did not exhibit any significant difference between the BBA65⁻ and BBA66⁻ single mutants and their parental control strains (Table 3). Moreover, there was no difference in the ability of BBA64/BBA65/BBA66 triple mutant to colonize and disseminate to various tissues in the C3H/HeN mice compared to its isogenic parental strain both after short-term (21 day) and long-term (62 day) infections, demonstrating that the lack of these three gene products does not alter the ability of *B. burgdorferi* to survive in the C3H/HeN mice after intradermal needle inoculation (Table 4). Since we did not observe a significant difference in the in vivo phenotype, the complementation of the inactivated or deleted genes *in trans* on the borrelial shuttle vector would not have provided any additional information and was therefore not carried out.

The lack of attenuation of infection with the mutants suggests that *B. burgdorferi* may utilize other proteins that are regulated similarly under fed-tick or mammalian-host-specific conditions. It is also possible that the requirement of BBA64, BBA65, and BBA66 is not critical for the initial stages of infection in the C3H/HeN model of Lyme disease since it is feasible that expression of major lipoproteins such as OspC, DbpA, and VlsE at wild-type levels in the mutants may be sufficient for the initial steps of the infectious process (47, 62). In order to determine whether there would be a difference in the in vivo phenotype between the mutants and their parental strains, we extended the infectivity analysis to 62 days and

found that there were no significant differences between these strains. It is also feasible that the requirement for BBA64, BBA65, and BBA66 either individually or in combination for *B. burgdorferi* is potentially at the tick-pathogen interface after the ingestion of a blood meal and warrants further detailed investigations.

Transcriptional analysis of *B. burgdorferi* propagated under conditions mimicking the vertebrate hosts using microarrays (in vitro) or by quantitative real-time PCR analysis of reverse-transcribed RNA from infected tissues (in vivo) provide significant information on the levels of expression of BBA64, BBA65, and BBA66 (10, 27, 41). The exact function of these proteins are yet to be defined; coupled with a lack of correlation of transcript levels to the extent of expression of these proteins on the surface, *B. burgdorferi* may partly limit the nature of the in vivo phenotypic analysis. Moreover, the temporospatial expression of proteins may not coincide with the infectivity analysis based on the ability to isolate viable spirochetes from different tissues. An isolate of cN40 obtained after 75 in vitro passages, N40-75, was able to infect mice but was deficient in its ability to cause inflammation in joints and heart (5). Moreover, the high-passage N40-75 isolate did not express BBA64, BBA65, BBA66, and DbpA/B, suggesting alterations in the regulatory pathways responsible for expression of these lipoproteins in this strain (5). While it is interesting to speculate that the triple mutant may have a similar infectious phenotype, it is important to emphasize that viable mutant spirochetes (MM13) were isolated in both the joint and cardiac tissues even at low doses of infection (10^2 spirochetes/mouse; Table 4). It is also possible that the lack of expression of DbpA/B in N40-75 may contribute to the attenuation of inflammation in joints and heart (56). Even though these differences have not been observed between the triple mutant and its parental strain, it will be interesting to evaluate quantitative differences in different tissues both in number of spirochetes and in the levels of tissue-specific pathology. It will also facilitate the identification of subtle differences that may exist between the mutants and their respective parental strains in their ability to colonize various tissues since such differences may be beyond the sensitivity levels of isolation of viable spirochetes from select tissues. This lack of sensitivity was apparent in our ability to detect viable spirochetes in only one of the three mice infected at low doses (10^2 spirochetes per mouse), even though we have had success in detecting viable spirochetes in multiple tissues in the past with this dose of infection in C3H/HeN mice (36).

The lack of an infectivity phenotype upon deletion of multiple ORFs that are transcriptionally upregulated on exposure of *B. burgdorferi* to fed-tick conditions that presumably facilitate adaptation to an environment encountered upon transmission to a mammalian host brings into focus the breadth of the adaptive processes that mediate establishment of *B. burgdorferi* infection in the murine host. Recently, it has been shown that the mutant spirochetes lacking *ospC* were rapidly cleared from the skin of SCID mice, suggesting that this major outer surface protein plays a role in providing resistance to innate immune mechanisms, whereas increased expression of any one of the other major borrelial lipoproteins such as OspA, OspE, VlsE, or Dbp protected the *ospC* mutant from clearance (62). Moreover, it was also shown that *B. burgdorferi* lacking lp28-1 (lack-

ing VlsE) was rapidly cleared upon infection due to lack of repression of OspC, which tended to be the target of adaptive immune response (19). Hence, it is interesting to speculate that transcriptional upregulation of select ORFs (or the lack of it) under fed- and unfed-tick or mammalian-host-specific conditions in *B. burgdorferi* may only provide suggestive information on the requirements of these ORFs for infection. Since the infectious processes involve the interaction of both the spirochetal determinants and multiple components of the hosts, analysis of mutants of upregulated ORFs using murine models of Lyme disease would provide a better understanding of the interactions at the host-pathogen interface. The concerted expression of these ORFs may also provide a selective advantage under various microenvironments of a mammalian host but at the same time may not have a critical bearing in the initial stages of infection or dissemination. Moreover, the plasticity of the borrelial genome with multiple paralogs distributed over its segmented genome may facilitate differential expression of related or even unrelated proteins that may provide the necessary advantage to establish infection in the murine model, and hence more global alterations such as the deletion of a regulator(s) of paralogous families or a related group of genes may result in a significantly attenuated set of borrelial mutants (13).

In summary, the deletion of three ORFs present on lp54 with significant transcriptional upregulation under fed-tick conditions did not have a significant bearing on the infectivity phenotype of *B. burgdorferi* in the C3H/HeN model of Lyme disease. A more global analysis of the effect of deletion of three ORFs from lp54 would further extend the observations of the present study and facilitate the understanding of host-pathogen interactions requiring the expression of these proteins.

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