Role of the BBA64 Locus of *Borrelia burgdorferi* in Early Stages of Infectivity in a Murine Model of Lyme Disease

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*Borrelia burgdorferi*, the causative agent of Lyme disease, undergoes rapid adaptive gene expression in response to environmental signals encountered during different stages of its life cycle in the arthropod vector or the mammalian host. Among all the plasmid-encoded genes of *B. burgdorferi*, several linear plasmid 54 (lp54)-encoded open reading frames (ORFs) exhibit the greatest differential expression in response to mammalian host-specific temperature, pH, and other uncharacterized signals. These ORFs include members of the paralogous gene family 54 (pgf 54), such as BBA64, BBA65, and BBA66, present on lp54. In an attempt to correlate transcriptional up-regulation of these pgf 54 members to their role in infectivity, we inactivated BBA64 and characterized the phenotype of this mutant both in vitro and in vivo. There were no major differences in the protein profiles between the BBA64 mutant and the control strains, while immunoblot analysis indicated that inactivation of BBA64 resulted in increased levels of BBA65. Moreover, there was no significant difference in the ability of the BBA64 mutant to infect C3H/HeN mice compared to that of its parental or complemented control strains as determined by culturing of viable spirochetes from infected tissues. However, enumeration of spirochetes using quantitative real-time PCR revealed tissue-specific differences, suggesting a minimal role for BBA64 in the survival of *B. burgdorferi* in select tissues. Infectivity analysis of the BBA64 mutant suggests that *B. burgdorferi* may utilize multiple determinants to establish infection in mammalian hosts.

Lyme disease is the most prevalent arthropod-borne infection in the United States and remains a significant public health issue in certain geographic loci (3, 46). It is a multiphasic disorder with clinical symptoms involving the cutaneous, musculoskeletal, cardiovascular, and nervous systems (62). *Borrelia burgdorferi*, the causative agent of Lyme disease, is transmitted to several vertebrate hosts, including humans, by the bite of infected *Ixodes* ticks. When ticks consume a blood meal from mammalian hosts, there is a rapid alteration of gene expression in *B. burgdorferi*, facilitating adaptation of the spirochetes to the highly disparate environmental conditions that exist between the tick vector and the vertebrate host (4, 9, 10, 15, 29, 30–32, 44, 53, 63, 67). This adaptive gene expression may aid in the efficient trafficking of the spirochetes from the tick vector to the mammalian host and subsequently facilitate dissemination and colonization of various host tissues (16, 22).

Whole-genome transcriptional analyses using *B. burgdorferi*, propagated under in vitro growth conditions that mimic either the tick vector or mammalian host environment, have revealed preferential expression of plasmid-encoded genes (4, 44, 53, 66). Among the several linear and circular plasmids present in *B. burgdorferi*, linear plasmid 54 (lp54) encodes the largest number of open reading frames (ORFs) that exhibit differential gene expression in response to these changing environmental signals. Moreover, lp54 encodes adhesins, such as outer surface protein A (OspA; BBA15) (15, 17, 18, 48, 70) and decorin binding proteins A and B (DbpA and DbpB; BBA24/ BBA25) (23, 24), as well as pH-regulated (9, 10) and temperature-regulated proteins, such as the members of the paralogous gene family 54 (pgf 54) (22). Members of pgf 54 that are clustered on the right terminal region of lp54 are a part of the lp54-specific “transcriptome” observed in response to exposure of *B. burgdorferi* to multiple mammalian host-specific signals (2, 11, 21, 44, 45, 53, 66). Among these pgf 54 members, BBA64, BBA65, BBA66, and BBA73 have been shown to be transcriptionally elevated in response to mammalian host-specific pH (7.0) or temperature (35°C) present in fed ticks (12, 44, 53). This is in contrast to a lack of expression of BBA64 and BBA66 at pH 8.0 and 23°C, mimicking the conditions associated with unfed ticks (66). A recent study demonstrated the presence of antibodies against BBA64 and BBA66 in the serum of mice experimentally infected with *B. burgdorferi* and in serum samples of patients diagnosed with early, disseminated Lyme disease (42). Moreover, the temporal expression of various members of the pgf 54 in an infected mammalian host was also attributed to the involvement of multiple regulatory pathways (12, 22). Taken together, these observations suggest that BBA64 may play a role in *B. burgdorferi* infection either independently or in association with other members of the pgf 54.

Transcriptional up-regulation of an ORF in *B. burgdorferi* grown under mammalian host-specific conditions and/or the presence of serum antibodies against an antigenic determinant in infected hosts are two commonly used correlates for establishing the significance of an individual ORF in the infectious process (4, 5, 16, 44, 53). However, a more direct approach...
would be to generate strains with mutations in one or more differentially expressed ORFs and determine their infectivities with the appropriate animal models. This would help establish a correlation between signal-dependent transcriptional regulation of an ORF and its role in the infectious process. The purpose of the present study was to determine whether the inactivation of BBA64 leads to attenuation of infectivity with the murine model of Lyme disease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A clonal derivative of *B. burgdorferi* sensu stricto strain B31, ML23, lacking linear plasmid 25 (lp25), was exclusively used for generation of mutants (mts) (36). Another clonal derivative, MSK5, which contains all plasmids, was also used in infectivity experiments (35, 36). All *B. burgdorferi* cultures used for transformation were grown in 1% CO2 at 32°C in Barbour-Stoener-Kelly II (BSK-II) liquid medium supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR). We propagated various *B. burgdorferi* strains to a density of 1×10^{6} to 2×10^{9} ml of spirochetes in BSK-II growth medium (40 ml cultures) that mimicked tick microbiota conditions before (pH 7.6 and 23°C) and after (pH 6.8 and 37°C) a blood meal or shifted from pH 7.6/23°C to pH 6.8/37°C (67). A combination of the above conditions was also employed to dissect the roles of pH and temperature as either discrete or overlapping signals in the regulation of expression of the pgf members.

In vitro mutagenesis. A 4.5-kb region of lp54 extending from the 3′ end of BBA64 to the 5′ end of BBA68 was amplified using primers BBA64R and BBA68F (see Table 2). The amplicon was ligated into the pCR2.1-TOPO cloning vector, and a clone, designated pMM2, containing a region of lp54 between BBA64 and BBA68 was isolated either with the *aadA* marker compared to that for the wild-type (wt) parental control strains (data not shown). One of the mt clones, designated MM4, was further characterized by Southern blot analysis. To further confirm the inactivation of BBA64, total genomic DNA was extracted from MM4 and from the isogenic parental control strain, ML23 (36, 61). The DNA was digested with different restriction enzymes, separated on a 1% agarose gel, transferred onto a nylon membrane (Amersham Biosciences), and hybridized with the labeled probes overnight at 42°C and developed as per the manufacturer’s instructions.

### Generation of BBA64 mt in ML23.

A clonal derivative of *B. burgdorferi* strain B31 lacking lp25, ML23, was electrotransformed with pMM4 using a procedure described previously (35, 36, 35, 56, 60, 61). After electroporation, the transformants were incubated for 24 h at 32°C in BSK-II growth medium without antibiotics and plated on BSK-II agarose overlays containing 50 μg/ml streptomycin. The plates were then incubated at 32°C in 1% CO2 for 14 to 18 days or until individual colonies were visible in the overlays. Colonies were isolated aseptically into BSK-II growth medium with 50 μg/ml streptomycin and grown at 32°C until the cultures reached a density of 5×10^{7} spirochetes per ml. One milliliter of these cultures was used to extract total genomic DNA, and the presence of the P<sub>Tn7</sub>-aad<sub>A</sub> cassette within BBA64 was confirmed using primers specific to the ends of Tn7 (Tn7N and Tn7S), as well as forward and reverse primers specific to BBA64 (Table 2). Two recombinants were identified in which the BBA64 ampiclon size was increased by 1.6 kb due to the presence of a P<sub>Tn7</sub>-aad<sub>A</sub> marker compared to that for the wild-type (wt) parental control strains (data not shown). One of the mt clones, designated MM4, was further characterized by Southern blot analysis.

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR2.1-TOPO</td>
<td>PCR cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBBE22</td>
<td>Borreial shuttle vector confers Kan'&lt;br&gt;50</td>
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</tr>
<tr>
<td>pML102</td>
<td>Customized donor plasmid for in vitro transposition</td>
<td>61</td>
</tr>
<tr>
<td>pMM2</td>
<td>pMM2 with 1.6-kb insertion of P&lt;sub&gt;Tn7&lt;/sub&gt;-aad&lt;sub&gt;A&lt;/sub&gt; at 154 bp from 5′ end of BBA64</td>
<td>This study</td>
</tr>
<tr>
<td>pBBE22/bba64&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Borreial shuttle vector with BBA64-BBA65</td>
<td>This study</td>
</tr>
<tr>
<td>β-Actin/pCR2.1</td>
<td>347 bp of β-actin in pCR2.1-TOPO</td>
<td>This study; 47</td>
</tr>
</tbody>
</table>

**Strains**<br>ML23: B31, lp25<sup>−</sup>, noninfectious 36<br>MSK5: B31 isolate with all infection-associated plasmids 36<br>MM4: B31, lp25<sup>−</sup>, BBA64<sup>−</sup>, Str<sup>+</sup> 36<br>ML23/pBBE22 (wt): ML23 with pBBE22 (BBA64<sup>−</sup>), Kan'<br>MM4/pBBE22 (mt): MM4 with pBBE22 (BBA64<sup>−</sup> BBA68<sup>−</sup>), Str<sup>+</sup> Kan'<br>MM4/pBBE22-bba64<sup>−</sup> (ct): MM4 with pBBE22/BBA64<sup>−</sup>, BBA68<sup>−</sup>, Str<sup>+</sup> Kan'<br>

**TABLE 2.** Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Sequence (5′→3′)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
<td>BBA64F</td>
<td>ACGCCATATGGGACGCAATTGAAGACAAA</td>
</tr>
<tr>
<td>BBA64R</td>
<td>ACGCCATGGCAGCTTATGGGACGAAAT</td>
</tr>
<tr>
<td>BBA65F</td>
<td>ACGCCATATGGGACTTAAACACAAAAAGAC</td>
</tr>
<tr>
<td>BBA65F</td>
<td>ACGCCATATGGGACTTAAACACAAAAAGAC</td>
</tr>
<tr>
<td>BBA68F</td>
<td>ACGCCATATGGGACGCAATTGAAGACAAA</td>
</tr>
<tr>
<td>Tn7N</td>
<td>ACTTTATTGTCATAGTTTAGATCTATTTG</td>
</tr>
<tr>
<td>Tn7S</td>
<td>TCCCCCACTTATCTCAGAAATTCCTAAAA</td>
</tr>
<tr>
<td>acF</td>
<td>TACCCCCACTGTCGCCCATC (Ref 47)</td>
</tr>
<tr>
<td>actR</td>
<td>GGGATGCGACGAGTATGCATCC (Ref 47)</td>
</tr>
<tr>
<td>flagellinF</td>
<td>TCTTTTCTTCGTGGATGGAAGAGGAC</td>
</tr>
<tr>
<td>flagellinR</td>
<td>CCAAGAAGGAGCCCTGCGAAA</td>
</tr>
<tr>
<td>LE+actF</td>
<td>CAAGCTCTCATCTTGGCAACAGG</td>
</tr>
<tr>
<td>LE+actR</td>
<td>CCAAGAAGGAGCCCTGCGAAA</td>
</tr>
<tr>
<td>E22F</td>
<td>ACGCGGATACCTTTTATATTGATGCCGTTT</td>
</tr>
<tr>
<td>E22R</td>
<td>ACGCGGATACCTTTTATATTGATGCCGTTT</td>
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*a* Restriction sites underlined.
Complementation of BBA64 mt. In order to restore a functional copy of BBA64 in the mt, a region of lp54 corresponding to the DNA coordinates 42453 and 44409 with engineered restriction enzyme sites was amplified using primers BBA64R and BBA65F (Table 2) and cloned into the pCR2.1-TOPO cloning vector. The insert was excised using BamHI and PstI and ligated into the borrelial shuttle vector pBBE22, containing the minimal region of lp52 needed to restore the infectivity of ML23 in the murine model of Lyme disease (50, 61). The BBA64 mt (MM4) was transformed by electroporation with either pBBE22/ bbba64 or pBBE22 alone to generate both a complemented (ct) strain and a BBA64-negative mt containing the minimal region of infectivity (Table 1). The transformants were selected on BSK-II agar overlays supplemented with 50 μg/ml of streptomycin and 200 μg/ml of kanamycin. The presence of pBBE22/ bbba64 and pBBE22 was verified using primers specific to the BBE22 region (Table 2; see Fig. 3), and levels of the BBA64 protein in the parental, mt, and ct strains were verified by immunoblot analysis (data not shown).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analysis. B. burgdorferi whole-cell lysates were prepared and separated by SDS-12.5% polyacrylamide gel electrophoresis as described previously (59). The separated proteins were either visualized by Coomassie brilliant blue staining or transferred onto a polyvinylidene difluoride membrane (Amersham Hybond-P; GE Healthcare, Buckinghamshire, United Kingdom) and subjected to immuno blot analysis. The membranes were probed with rat anti-BBA64, anti-BBA66 serum (5), or with mouse anti-BBA65 (V. L. Sexton and J. Seshu, unpublished data). Immunoblots developed with mouse anti-P66 serum served as a control for levels of loading of proteins from different strains (M. D. Esteve-Gassent and J. Seshu, unpublished data). The blots were developed following incubation with appropriate dilutions of horseradish peroxidase-conjugated antirat or antimouse secondary antibodies. The membranes were then developed with ECL Western blotting reagents (GE Healthcare, Buckinghamshire, United Kingdom).

PCR analysis of plasmid profiles of strains used in infectivity. Total DNA was extracted from liquid cultures (10 ml) of each strain when the density of the spirochetes reached 1 × 10^8 cells/ml. The spirochetes were pelleted at 4,000 × g for 20 min at 4°C and washed twice with phosphate-buffered saline (pH 7.4), and total genomic DNA was extracted and used as a template as described previously (61). Primers used to amplify the targeted regions in different plasmid linear and circular plasmids have been described previously (36). The amplicons were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide. A primer set (E22F/E22R) (Table 2) was also used to amplify the BBE22 region of lp52 present in the borrelial shuttle vector pBBE22.

Infectivity studies. All animal procedures were done in accordance with the approved animal use protocol from the Institutional Animal Care and Use Committee of The University of Texas at San Antonio. Groups (n = 3) of 6-week-old female C3H/HeN mice were infected with 10^3 spirochetes per mouse intradermally with the wt (ML23/pBBE22) and BBA64 mt (MM4/pBBE22) strains of B. burgdorferi. At 62 days postinfection, the spleen, left tibiotarsal joint, left inguinal lymph node, heart, and bladder and a piece of abdominal skin were removed aseptically from infected mice and the tissues were processed to isolate spirochetes in BSK-II growth medium as previously described. The right tibiotarsal joints from each of the mice were examined for histopathological changes. The joints were formalin fixed, decalcified, sectioned, and stained with hematoxylin and eosin using standard procedures. The specimens were qualitatively examined to determine if histopathological lesions were present in the joints after long-term infection. Due to the limitation on the number of specimens after the long-term infection, we did not evaluate the histopathology using the scoring system as a measure of inflammation.

RESULTS

Construction of the BBA64 mt. We inactivated the BBA64 gene using a customized transposon containing a streptomycin resistance marker under the control of a borrelial promoter (P_sflg), employing an in vitro mutagenesis strategy as described previously (60, 61). A plasmid obtained using this approach, designated pMM4, with insertional inactivation of BBA64 at position 154 relative to its 5′ end, was used to transform a noninfectious, lp52-negative (lp52- ) clonal isolate of B. burgdorferi strain B31 (ML23). We employed a two-step approach, where we first used ML23 to generate the BBA64::Str mt, exploiting the lack of a restriction/modification system (BBE02) carried on lp25 to obtain higher transformation frequencies (33) and overcoming the lack of infectivity in the second step by the use of a borrelial shuttle vector (pBBE22) that encoded the minimal region of lp25, thereby restoring infectivity (50).

Following the transformation of ML23 with pMM4, streptomycin-resistant colonies were screened using a PCR-based approach with primers specific to BBA64. Two positive clones were identified based on the observation that the BBA64 am plicon size was increased by 1.6 kb compared to that of the wt parental control strain due to the presence of the P_sflg/aadA marker (data not shown). Southern blot analysis of one of the positive clones, designated MM4, showed that there was hybridization of the aadA probe to DNA fragments of 2.8 kb and 5.1 kb from MM4 (Fig. 1A, lanes 3 and 4, respectively) while there was no hybridization with the DNA from the parental strain (ML23) digested with EcoRV or HindIII (Fig. 1A, lanes 1 and 2, respectively). The sizes of these DNA fragments were consistent with expected sizes based on the restriction enzyme profile of the region of BBA64 in lp54 and the presence of these sites on the customized transposon. We further con-
firmed that the DNA fragments of 2.9 kb (EcoRV fragment) and 3.5 kb (HinDIII fragment) from the isogenic parental control (ML23) hybridized to labeled BBA64 used as a probe (Fig. 1B, lanes 1 and 2, respectively) and that there was a 1.6-kb increase in the corresponding size of the HinDIII fragment in MM4 (Fig. 1B, lane 4). This increase in the size of the HinDIII fragment is due to the presence of the aadA gene (Str'). A similar increase in the EcoRV fragment was not observed, since the additional EcoRV site (Fig. 1B, lane 3) within the transposon renders the corresponding fragment from MM4 smaller than that in the parental strain (Fig. 1C). These observations confirmed the inactivation of BBA64 and the clonality of the mt MM4. The comparable growth characteristics of MM4 and the parental strain suggested that BBA64 is dispensable for in vitro growth.

Complementation of MM4. Since MM4 (BBA64::Str' mt) was generated in a noninfectious, lp25- strain of B. burgdorferi, it was essential to restore the minimal region of lp25 required for infectivity. This was achieved by transformation of both ML23 and MM4 with pBBE22 carrying the region of lp25 that would restore a functional copy of BBE22 or pncA in trans (50, 54, 61). In addition, MM4 was also transformed with pBBE22 carrying a functional copy of BBA64 (a region of lp54 corresponding to the DNA coordinates 42563 and 44409) to obtain a genetically complemented strain. Transformants selected in the presence of kanamycin (conferring by pBBE22) were screened by PCR using primers specific to BBE22 or BBA64 and were found to carry pBBE22 (Table 2). At this stage, it was critical to determine if all the transformed strains expressed VlsE, an lp28-1-encoded lipoprotein that exhibits antigenic variation facilitating persistence of B. burgdorferi in the murine model of Lyme disease (55, 71–73). Immunoblot analysis of wt (ML23/pBBE22), BBA64 mt (MM4/pBBE22), and ct (MM4/pBBE22-bba64') strains using anti-VlsE serum revealed similar levels of expression of VlsE (data not shown), indicating that all these strains carried the two critical determinants of infectivity, namely, pncA and vlsE. Thus, by adopting a two-step transformation strategy, we successfully generated a BBA64 mt along with its parental and ct strains that have the requisite genetic background to facilitate infectivity analysis using the murine model of Lyme disease.

Plasmid profile of BBA64 mt. In addition to evaluating the expression of VlsE present on lp28-1, we also determined the plasmid profile of the BBA64 mt and the control strains. As shown in Fig. 3, the plasmid profile of wt and mt strains was identical whereas the ct strain does not contain lp5 and cp9. All three strains retain lp28-1 (which encodes VlsE), needed for persistence. While all three strains do not show an amplicon specific to lp25 (using primers specific for BBE16), primers specific to the E22 region of lp25 (Table 2) did amplify a 2-kb fragment in all three strains. This is due to the transformation of all three strains with the borrelial shuttle vector pBBE22, which restores the minimal region of lp25 required for infectivity. Previous studies (35, 51) have demonstrated little or no significant loss of infectivity with isolates of B. burgdorferi lacking lp5 or cp9, and hence we proceeded with the infectivity analysis using the ct strain lacking the aforementioned plasmids.

Expression of BBA64, BBA65, and BBA66 proteins in the wt, mt, and ct strains. To test the contribution of the BBA64 gene to the infectivity of B. burgdorferi for the mammalian host, it was necessary to confirm if different environmental signals induced comparable levels of expression of pgf 54 members in the strains to be used in the infectivity analysis (12, 22, 43). Immunoblot analysis using monospecific rat anti-BBA64 or anti-BBA66 (5) or monospecific mouse anti-BBA65 antibodies was used to detect the levels of these proteins. As shown in Fig. 2A, there were no overt differences in the protein profiles between the BBA64 mt and the parental or ct strain. Previous studies have shown a significant 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 mammalian host (10, 67). Hence, the levels of OspC serve as an indicator of the mammalian host-adapted state of B. burgdorferi as well as the functionality of critical regulatory pathways, such as the Rrp2-RpoN-RpoS pathway. This pathway has been shown to control expression of lipoproteins important for pathogenesis, such as OspC and DbpA (6–8, 20, 28, 68, 69). As expected, there was a significant increase in the levels of OspC in B. burgdorferi cultured at pH 6.8/37°C (Fig. 2A, lane 2, for all strains) or when shifted from pH 7.6/23°C to pH 6.8/37°C (Fig. 2A, lane 3, for all strains) compared to levels in cultures grown at pH 7.6/37°C (Fig. 2A, lane 1, for all strains). This observation is consistent with previous reports demonstrating that a change
in environmental conditions that mimic tick feeding (increased temperature and lowered pH) results in increased levels of OspC (9, 10, 67) and that ospC expression was similar between the mt and the control strains.

To delineate the role of temperature and pH as signals for the induction of the BBA64, BBA65, and BBA66 proteins, we performed immunoblot analysis using the different borrelial strains that were propagated initially at pH 7.6/23°C and then shifted at low densities to growth media at pH 7.6/23°C (Fig. 2, lane 1) or pH 6.8/37°C (Fig. 2, lane 3). Samples propagated initially at pH 6.8/23°C with a shift to pH 6.8/37°C (Fig. 2, lane 2) were also analyzed. There was no detectable expression of the BBA64, BBA65 and BBA66 proteins in any of the strains with propagation at pH 7.6/37°C (Fig. 2B, α-A64, α-A65, and α-A66, lane 1; all strains). When the wt strain was shifted from pH 6.8/23°C to pH 6.8/37°C, there was a significant increase in the levels of the BBA64, BBA65, and BBA66 proteins (Fig. 2B, lane 2). A similar increase was also noted for the ct strain under identical growth conditions (Fig. 2B, lane 2). When the pH of the growth media was reduced to 6.8 from 7.6 with a concomitant increase in temperature from 23°C to 37°C, there was increased expression of each of the analyzed lipoproteins (Fig. 2B, lane 3, wt and ct). There was no expression of the BBA64 protein when the BBA64 mt was cultivated under conditions described for its parental control, confirming the complete loss of expression of the BBA64 protein in the mt (Fig. 2B, α-A64, lanes 1, 2 and 3; mt). We noted an increased expression of the BBA65 protein in the BBA64 mt compared to results for the wt strain when the spirochetes were grown at pH 6.8/37°C (Fig. 2B, α-A65, lane 2; mt) or shifted from pH 7.6/23°C to 6.8/37°C (Fig. 2B, α-A65, lane 3; mt). The BBA64 mt, however, did not exhibit an increase in the levels of BBA66 (Fig. 2B, α-A66, lanes 2 and 3; mt) compared to those for the parental control under similar growth conditions. There were no significant differences in the levels of expression of BBA66 between the parental control and the ct samples (Fig. 2B, α-A66, lanes 2 and 3). Immunoblot analysis using mouse anti-P66 serum established that the levels of P66 were independent of pH and temperature and that there were comparable levels of P66 between the BBA64 mt and its parental and ct strains (Fig. 2B, α-P66, lanes 1, 2, and 3; all strains). These observations were consistent with those of a previous study where it was demonstrated that the levels of P66 were not affected by either temperature, pH, or a combination of these factors (14).

The above analysis also suggests that the loss of expression of BBA64 did not affect other critical determinants which are known to play a role in the infectious process, albeit this analysis was limited to OspC and P66.

We further analyzed if pH, independently of temperature, has an overriding effect on the expression of the BBA64, BBA65, and BBA66 proteins. All three strains (wt, mt, and ct) were first grown at pH 7.6/23°C and then inoculated into growth media at pH 6.8/37°C, pH 7.6/23°C, or pH 6.8/23°C at low densities to facilitate adaptation to the latter environmental conditions. As previously described, the levels of OspC were found to be enhanced only in borrelial samples from cultures at pH 6.8/37°C for all three strains (Fig. 2, lane 3). There was no expression of BBA65 and BBA66 at pH 7.6/23°C or at pH 6.8/23°C, indicating that temperature and pH have a combined effect on the expression of these members of pgf 54 (data not shown).

**Infectivity analysis of BBA64 mt in C3H/HeN mice.** Recent studies have demonstrated that several members of the pgf 54 present on lp54 are upregulated under mammalian host-specific conditions (4, 44, 53, 66). However, the individual or collective contribution of these genes to infectivity has not been studied. We have shown that all three strains have the requisite genetic background and regulatory pathways (Fig. 2 and 3) to allow direct analysis of the role of BBA64 in the initial colonization and dissemination of *B. burgdorferi* using the murine model of Lyme disease (61). As shown in Table 3, there were no significant differences when the spirochetes were propagated in BSK-II medium from different tissues in mice infected with either the BBA64 mt strain or its parental or ct strain, indicating that loss of the BBA64 gene alone does not result in the attenuation of *B. burgdorferi* infectivity in the murine host.

Even though there is no significant difference among the wt, mt, and ct strains in their ability to disseminate and colonize different tissues, we further enumerated the spirochetes by real-time PCR analysis to see if there are any subtle differences in the numbers of spirochetes in different tissues (61). As shown in Fig. 4 (representative data from mice infected with 10⁸ spirochetes/strain/mouse), there were no significant differences (*P > 0.05*) in the levels of spirochetal burden in the skin...
from mice infected with either the wt, mt, or ct strain. This was true for mice infected with different doses of spirochetes (10^2 to 10^5; P < 0.05; data not shown). However, there were significantly higher numbers of spirochetes in lymph nodes (P < 0.01), spleen (P < 0.05), and joints (P < 0.01) from mice infected with the BBA64 mt strain than from mice infected with the wt or ct strain (10^3 spirochetes/mouse) (Fig. 4). Mice infected with higher doses (10^4 and 10^5 spirochetes/mouse) of the BBA64 mt did not exhibit significant differences in the number of spirochetes in the spleen and joints (P > 0.05) from those for the control strains, while the opposite was true with lower doses (10^2 and 10^3 spirochetes/mouse; P < 0.05). The lymph nodes, however, had a significantly higher spirochetal burden for mice infected with the BBA64 mt at all the tested doses (10^2 to 10^5 spirochetes/mouse) than for the mice infected with the wt or ct strain at the same levels. Quantification of spirochetes in target tissues indicated that while the loss of BBA64 did not have an effect on the total numbers of spirochetes in the skin (independent of the size of the inocula), there were significantly higher numbers of BBA64-negative spirochetes in the lymph nodes (at all inocula) than was the case for the control strains. These results indicate that there are subtle tissue-specific differences in the distribution of the BBA64 mt even though these differences were not apparent in terms of propagation of spirochetes from infected tissues in BSK-II growth medium.

There was no growth of spirochetes in tissues isolated from mice infected with ML23 and cultured in BSK-II growth medium (Table 3), but we were able to detect spirochete-specific DNA in all the tissues tested. This may be due to the presence of residual DNA from spirochetes that were not completely cleared even though they were not capable of growth and replication in the BSK-II growth medium (35). It has previously been shown that ML23 (lp25^-^) is capable of dissemination to various tissues in C3H/HeN mice but cannot be propagated in BSK-II growth medium from these tissues after 2 to

![FIG. 3. Plasmid profiles of the parental (wt; ML23/pBBE22), BBA64 mt (MM4/pBBE22), and ct (MM4/pBBE22-bba64^-^-) strains. PCR analysis was performed as described in Materials and Methods using primers pairs listed in reference 36. The amplicons were resolved by electrophoresis in a 1.5% agarose gel. The primer pair used to generate the amplicon in the lane designated bbe22 is listed in Table 2. Molecular size markers (in base pairs) are indicated on the left.](http://iai.asm.org/)

### Table 3. The BBA64 locus is dispensable for infection of C3H/HeN mice

<table>
<thead>
<tr>
<th>Strain and dose</th>
<th>No. of cultures positive/no. tested</th>
<th>No. of mice infected/no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skin</td>
<td>Spleen</td>
</tr>
<tr>
<td>MSK5 10^3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>ML23 10^3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>ML23 10^5</td>
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<tr>
<td></td>
<td>10^5</td>
<td>3/3</td>
</tr>
<tr>
<td>mt (MM4/pBBE22)</td>
<td>10^2</td>
<td>0/3</td>
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<td></td>
<td>10^3</td>
<td>3/3</td>
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<td>10^5</td>
<td>3/3</td>
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4 days postinfection (35). Borrelial flaB was not present in tissues isolated from uninfected mice (data not shown).

We extended the infectivity analysis to 62 days postinfection to determine if long-term infections would facilitate determination of differences between wt and mt strains using a limited number of mice (n ≥ 3) and a single inoculum dose (10^3 spirochetes/animal). As shown in Table 4, there were no significant differences in the ability to propagate wt or mt spirochetes from different tissues at 62 days postinfection. However, histological examination of the joint tissues indicated a greater degree of inflammatory cellular infiltration in the joints of mice infected with the mt strain than for those infected with the wt (Fig. 5). Consistent with results of previous studies, the degree of inflammatory cellular infiltration was greater in mice infected with the wt strain than for those infected with the mt strain (i.e., the case for joints from uninfected control mice; mock; Fig. 5). The inflammatory infiltrates consisted predominantly of mononuclear cells. Since the long-term infection was carried out with a limited set of mice and a single dose of infection, we were unable to conduct quantitative scoring to measure inflammation induced in a dose-dependent manner. Nonetheless, there were increased levels of tissue inflammation in joints from mice infected with the BBA64 mt strain compared to results with the wt strain at 62 days postinfection, which may be related to an increased number of spirochetes present in the joint tissues of mice infected with the mt compared to that for the wt controls at 21 days postinfection as determined by quantitative real-time PCR (Fig. 4).

In summary, in vivo analyses of the BBA64 mt indicated that there were no significant differences in the ability to propagate spirochetes in the growth medium from various tissues of mice infected with the BBA64::Strr mt or the control strains. Quantitative real-time PCR revealed that there were differences with various levels of significance in the numbers of spirochetes in different tissues. The foregoing analysis demonstrates that...

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**FIG. 4.** Quantitative real-time PCR analysis of the spirochetal burden in mice infected with the BBA64 mt. Groups (n = 3) of 6-week-old C3H/HeN female mice were infected intradermally with B. burgdorferi strains—wt (ML23/pBBE22), mt (MM4/pBBE22), or ct (MM4/pBBE22-bba64+)—at doses ranging logarithmically from 10^2 to 10^5 spirochetes per mouse. A similar group (n = 3) of mice was infected with MSK (10^3) or ML23 (10^4 and 10^5) to serve as the positive or negative control, respectively, for infection. Total genomic DNA was isolated from tissues (skin, spleen, lymph node, and joint) using the High Pure PCR template preparation kit, and quantitative real-time PCR was performed. Results for the infection dose of 10^3/mouse are shown. Numbers of borrelial flaB copies were normalized against total mouse β-actin copies. Only two out of three samples of lymph nodes from mice infected with the ct strain had detectable levels of spirochetal DNA. The asterisks indicate levels of significance as follows: **, P < 0.01; *, P < 0.05.

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**TABLE 4.** The BBA64 locus is dispensable for long-term infection of C3H/HeN mice

<table>
<thead>
<tr>
<th>Strain, dose</th>
<th>No. of cultures positive/no. tested</th>
<th>No. of mice infected/no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skin</td>
<td>Spleen</td>
</tr>
<tr>
<td>wt (ML23/pBBE22), 10^3</td>
<td>3/3</td>
<td>1/3</td>
</tr>
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</table>
loss of BBA64 does not result in any significant changes in the protein profile of *B. burgdorferi*, nor does it alter infectivity in the murine model of Lyme disease.

**DISCUSSION**

*B. burgdorferi* alters its gene expression to adapt to the highly disparate environmental conditions that it encounters in the tick vector and the mammalian host. Several whole-genome transcriptional profile analyses revealed that much of the above differential gene expression was associated with the plasmid-encoded genes present in lp54 (4, 44, 53, 66). Lp54 encodes several determinants with known functions, such as OspA/B, DbpA/B, and CRASP-1 (23–27, 34, 40), as well as several members of pgf 54 which are transcriptionally upregulated under mammalian host-specific conditions albeit with no defined functions (4, 5, 11, 21, 22, 53, 66). We therefore hypothesized that the loss of expression of genes that may facilitate the initial stages of adaptation of the spirochetes to the mammalian-host environment may result in the attenuation of the virulence of *B. burgdorferi*.

Contrary to our expectation, inactivation of BBA64 did not exhibit any significant difference either in the protein profile (Fig. 2) or in infectivity with the murine model of Lyme disease (Table 3). Subtle differences were observed in the expression of BBA65, another member of pgf 54, along with differences in the number of mt spirochetes in select tissues (Fig. 4). There was also no significant difference in infectivity between wt and mt strains following a long-term infection (62 days postinfection), while there was an increased inflammatory response in joints of mice infected with the BBA64 mt (Fig. 5). This analysis has helped to establish that even though BBA64 is transcriptionally upregulated, its inactivation does not have a significant bearing on infectivity of *B. burgdorferi* in the murine model of Lyme disease.

Due to the difficulties associated with genetic manipulation of infectious strains of *B. burgdorferi* (54), we employed a two-step transformation strategy to inactivate BBA64 and confirmed that all the strains retained plasmids critical for infectivity. The first step was to inactivate BBA64 in a noninfectious *lp25*/H11002 strain of *B. burgdorferi* (ML23) (35). The second step was to restore the minimal region of infectivity using a borrelial shuttle vector (50, 61). By this approach, we limited the effects of restriction/modification systems present on select linear plasmids (like *lp25*-encoded BBE02), which resulted in reduced transformation efficiencies (37). We were able to successfully generate a BBA64 mt (Fig. 1) and subsequently restore the minimal region of infectivity to facilitate analysis of the role of BBA64 in mammalian host infectivity (Table 3). This strategy has been used previously to determine the effect of loss of the fibronectin binding protein BBK32 in the murine model of Lyme disease and hence is a viable strategy to obtain mts that can be tested for infectivity (61). A limitation inherent in this strategy is the inability to analyze the phenotype of the mts in terms of acquisition, survivability, or transmission by ticks because the complementation step restores only the minimal region of *lp25* (BBE22) required for infectivity of the mammalian host but not the region required for long-term survival in ticks and for completion of the life cycle (BBE16).
(52, 64, 65). Nonetheless, generation of mts using this approach facilitated analysis of the effect of loss of one or more genes in the infectivity of *B. burgdorferi* for the mammalian host.

Since the differential expression of pgf 54 family members in response to mammalian host-specific signals is crucial to the initial stages of infectivity, it was important to determine if the regulation of expression of a subset of these members is similar in the wt parental strain, the BBA64 mt, and the ct strain. As shown in Fig. 2, the expression of the BBA64 protein in the wt parental strain (ML23/1pBBE22) was consistent with results in previous studies (9). There was increased expression of the BBA64 protein when the wt strain was grown at a low pH (6.8) and higher temperature (37°C), while there was no detectable expression of the BBA64 protein at a higher pH (7.6) and lower temperature (23°C). These studies clearly indicated the significance of the coordinate effects of pH and temperature in the up-regulation of BBA64 and that the gene regulatory circuits are intact and functional in wt, mt, and ct strains.

There was no evidence of expression of BBA64 in immunoblot analysis of the BBA64 mt, confirming the loss of expression of this determinant, while complementation of the mt with a functional copy of BBA64 resulted in the restoration of expression when it was propagated under mammalian host-specific conditions (Fig. 2). There were no overt differences in the protein profile of the mt and control strains. However, the levels of expression of BBA65 were found to be higher by immunoblot analysis for the mt than for the parental wt strain, suggesting that the loss of BBA64 may result in altered expression of other pgf 54 members. Contrary to our expectation, we also observed increased levels of expression of BBA65 in the ct strain. This may be due to a lack of effect of providing BBA64 in *trans* to restore the BBA65 protein to wt levels. Alternatively, it is also possible that the lack of cp9 and lp5 in the ct strain may have yet-to-be-determined effects, albeit subtle, on the expression of these members of pgf 54. The loss of BBA64 did not have an effect on the levels of expression of another member of pgf 54, namely, BBA66.

The process of generating mts may inadvertently result in selection of mutations in distal genes or loss of plasmids with significant effects on the infectivity of *B. burgdorferi* (36, 41, 51, 64). There were no overt differences in the protein profile and the level of OspC was significantly higher under mammalian host-specific conditions in all three strains, indicating that both the regulatory mechanisms and the expression of other critical determinants of virulence were similar and intact in the mt and control strains (1, 58, 63, 67). Moreover, we also analyzed the plasmid profile of the parental, mt, and ct strains and found that all the plasmids critical for infectivity (lp25 and lp28-1) were present in all three strains. The two plasmids that were missing in the ct strain, namely, cp9 and lp5, have been shown to be dispensable for infectivity of *B. burgdorferi* in the murine model of Lyme disease (36, 51). Moreover, the ct strain did not show any significant difference in levels of infectivity from the wt strain, indicating that the lack of cp9 or lp5 did not have a significant bearing on the outcome of infectivity.

Recently, Gilmore et al. described the kinetics of expression of the BBA64, BBA65, and BBA66 genes during persistent infection in mice up to 100 days and found that the expression of BBA64 was reduced at 20 days postinfection (22). While the expression of BBA64 remained low, the transcriptional levels of both BBA65 and BBA66 were found to be elevated during the entire course of infection, suggesting a role for multiple mechanisms or regulators being involved in their expression. Analysis of the upstream region of BBA64 revealed a σ^70 consensus sequence very similar in organization to that of BBA64 (12, 30, 31), suggesting a direct or indirect role of the RpoN-RpoS regulatory pathway in the expression of BBA64, BBA65, and BBA66. These observation were further corroborated by the loss of expression of BBA66 in the absence of either σ^32 (RpoN) or σ^3 (RpoS) (12). The RpoN-RpoS regulatory pathway has been characterized as influencing the expression of several lipoproteins, such as OspC and DpBpA (20, 28, 68, 69). More recently, BBA64 was found to be upregulated by greater than 18-fold in the wt strain compared to results for the rpoS mt when these strains were propagated under in vitro growth conditions (8). Furthermore, there was greater than 1,600-fold differential expression of BBA64 in the wt strain compared to results for the rpoS mt when the strains were grown under host-adapted conditions (8). A highly significant, concomitant transcriptional up-regulation was also observed with BBA65, BBA66, and BBA73, indicating that these pgf 54 members are regulated in an rpoS-dependent manner and in response to mammalian host-adapted conditions (8). It is conceivable from the above analysis that up-regulation of multiple pgf 54 members would facilitate the full spectrum of infectivity.

While whole-genome transcriptional profiles facilitate identification of a subset of genes that may play a role in a defined environmental niche, phenotypic analysis of mts in one or more of these genes facilitates a more direct evaluation of their roles in mediating adaptation to those conditions. For example, if these upregulated ORFs encode lipoproteins, it is important that the surface expression of these proteins coincide temporally in microenvironments that facilitate migration of spirochetes from the midgut to the salivary glands or participate in other stages of infection, such as mediating attachment to host matrices or evasion of innate immunity (13, 39). Even though BBA64 has been shown to be significantly upregulated under mammalian host-adapted conditions, its inactivation did not alter the dissemination and colonization of the mammalian host (Tables 3 and 4). Therefore, it is critical to evaluate the link between transcriptional levels of borrelial genes and their role in infectivity using the murine model of Lyme disease (8).

Even though BBA64 is dispensable for infectivity, enumeration of spirochetes in infected tissues, on the other hand, revealed differences, which indicates that the expression of BBA64 temporally or in specific microenvironments may play a role in facilitating the full spectrum of infectivity in the murine model of Lyme disease. There were no significant differences in the numbers of spirochetes in the skin of mice infected with the mt or the control strain (Fig. 4). However, there were increased numbers of spirochetes present in the spleen, joints, and lymph nodes of mice infected with the BBA64 mt compared to results for the control strains (Fig. 4). While the exact mechanisms responsible for these observations are not known, it is interesting to speculate that there is a minimal role for BBA64 for survival of *B. burgdorferi* in certain select tissues. But at the same time, these differences in the spirochetal burden in different tissues as determined by quan-
tative real-time PCR may not translate into a significant difference in infectivity, since this assay does not distinguish between live and dead spirochetes. It is also plausible that the lack of antibodies directed against the BBA64 protein, as would be expected for mice infected with the BBA64-negative mt, may also lead to reduced neutralization of the spirochetes and thereby facilitate increased colonization in these tissues. However, long-term infections (62 days) using the murine model of Lyme disease also did not reveal any significant differences between the mt and wt even though this analysis was done using a single infectious dose. This is consistent with the observation that there are no apparent mechanisms of antigenic variation associated with the expression of the members of pgf 54 (12). There was an increased level of inflammation in joint tissues associated with the mt strain, and this correlates with the ability to detect increased numbers of mt spirochetes in the joints.

The lack of attenuation of infectivity of the BBA64 mt can be explained by compensatory mechanisms that are responsible for the colonization and dissemination of *B. burgdorferi* (19). Since BBA64 is one of 12 members of the pgf 54, it is possible that one or more paralogs of this gene family are upregulated to compensate for the loss of BBA64 (22). This scenario is consistent with the observation that BBA65 is elevated in the BBA64 mt at pH 6.8/37°C. While it is not possible to directly attribute the lack of attenuation of the BBA64 mt to the up-regulation of BBA65, it is plausible that compensatory gene expression over a broad array of pgf 54 members may alleviate the effect of inactivation or loss of expression of an individual paralog. Alternatively, the sequence similarity of these members could result in determinants that functionally compensate for the loss of one or more members of pgf 54. In a recent study, temporal expression of BBA64, BBA65, and BBA66 monitored by quantitative real-time PCR indicated that while BBA64 expression was considerably reduced in mouse tissues from that observed during in vitro growth, there was increased expression of BBA65 and BBA66 throughout the course of infection and up to 100 days postinfection (22). This suggested a preferential role for BBA64 in the vector-to-mammalian-host transmission and/or in the early stages of infection.

Our study indicates that there is no significant reduction in the infectivity of the BBA64 mt from that of its isogenic parental or ct strain following short-term (21 days) or long-term (62 days) infection based on cultivation of spirochetes from infected tissues. However, this does not preclude the possibility of a contribution of BBA64 to the trafficking of spirochetes from the tick midgut to the salivary glands or subsequent transmission to mammalian hosts. The transcriptional down-regulation of BBA64 in the mammalian host (22) may indicate the lack of a role for this protein in the course of infection. This may therefore translate into a lack of attenuation of infectivity following short- or long-term infection. Inactivation of proteins in *B. burgdorferi* that serve as adhesins, such as BBK32 (38, 61) and Bgp (49), has resulted in either partial or no attenuation of infectivity, suggesting the role of multiple borrelial determinants in initiation and maintenance of infection. Hence, it is interesting to speculate that infectivity analysis of a BBA64/BBA65/BBA66-negative triple mt will help further characterize the role of a subset of pgf 54 family members in the colonization and dissemination of *B. burgdorferi* within the mammalian host. Analysis of the in vivo phenotype of strains with mutations in ORFs that are significantly upregulated under mammalian host-specific conditions will contribute to understanding their role in infectivity and possibly in determining their function(s).

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