

BB0844, an RpoS-Regulated Protein, Is Dispensable for *Borrelia burgdorferi* Infectivity and Maintenance in the Mouse-Tick Infectious Cycle[∇]

Sukalyani Banik,^{1†} Darya Terekhova,^{1‡} Radha Iyer,¹ Christopher J. Pappas,¹
Melissa J. Caimano,² Justin D. Radolf,^{2,3,4} and Ira Schwartz^{1*}

Department of Microbiology and Immunology, New York Medical College, Valhalla, New York 10595,¹ and
Departments of Medicine,² Pediatrics,³ and Genetics and Developmental Biology,⁴ University of
Connecticut Health Center, Farmington, Connecticut 06030

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The genome of *Borrelia burgdorferi*, the causative agent of Lyme disease, is comprised of a large linear chromosome and numerous smaller linear and circular plasmids. *B. burgdorferi* exhibits substantial genomic variation, and previous studies revealed genotype-specific variation at the right chromosomal telomere. A correlation has also been established between genotype and invasiveness. The correlation between chromosome length and genotype and between genotype and invasiveness suggested that a gene(s) at the right chromosome telomere may be required for virulence. Of particular interest was *bb0844*, an RpoS-regulated gene at the right telomere, the expression of which is induced when the spirochete undergoes adaptation to the mammalian host. The structure of the right chromosomal telomere was examined in 53 *B. burgdorferi* clinical isolates of various genotypes. Four distinct patterns were observed for *bb0844*: (i) chromosomal localization, (ii) plasmid localization, (iii) presence on both chromosome and plasmid, and (iv) complete absence. These patterns correlated with the *B. burgdorferi* genotype. On the basis of available sequence data, we propose a mechanism for the genomic rearrangements that accounts for the variability in *bb0844* genomic localization. To further explore the role of BB0844 in the spirochete life cycle, a *bb0844* deletion mutant was constructed by allelic exchange, and the viability of wild-type and *bb0844* deletion mutants was examined in an experimental mouse-tick infection model. The *bb0844* mutant was fully infectious in C3H/HeJ mice by either needle inoculation or tick transmission with *B. burgdorferi*-infected *Ixodes scapularis* larvae. Naïve larval ticks acquired both wild-type and mutant spirochetes with equal efficiency from *B. burgdorferi*-infected mice. The results demonstrate that BB0844 is not required for spirochete viability, pathogenicity, or maintenance in the tick vector or the mammalian host. At present, a defined role for BB0844 in *B. burgdorferi* cannot be ascertained.

Borrelia burgdorferi, the Lyme disease pathogen, has a complex life cycle in nature which involves an arthropod vector and diverse mammalian hosts (6, 46). The type strain, B31, has a complex genome comprised of a linear chromosome and at least 21 linear and circular plasmids (12, 19). The linear replicons have covalently closed hairpin telomeric ends (11, 24). The chromosome of *B. burgdorferi* strain B31 is 910,725 bp long and includes 853 putative open reading frames (ORFs) (19). A majority of the chromosomal sequence and gene order is conserved among *B. burgdorferi* strains; however, the chromosomes vary in length. These differences were shown to be the result of variability in the right telomeric region (10, 11) and have been confirmed by sequencing of additional *B. burgdorferi* genomes (40). It has been proposed that linear plasmid fragments were added to the right chromosomal telomere by means of nonhomologous recombination, after which portions

of the transposed plasmid sequences underwent mutational decay (12, 13, 25).

Numerous approaches have been employed for typing of *B. burgdorferi* isolates (56). For example, North American *B. burgdorferi* isolates can be classified into three rRNA gene spacer types (RST) based on the rRNA gene spacer sequence (32) and 17 types based on the *ospC* sequence (41). Importantly, a correlation between genotype and pathogenicity has been observed. Isolates with an RST1 genotype were more likely than RST3 isolates to produce disseminated infection in humans or mice (55, 58, 59). Similarly, *OspC* type A, B, I, H, and K isolates were most likely to disseminate (41, 58). Comparative genome hybridization analysis of 16 *B. burgdorferi* isolates revealed that ORFs *bb0843.1* to *bb0853.1* were absent from the chromosomes of most of the RST3 isolates analyzed (11, 49). Overall, the length of the right chromosomal telomere correlated with the RST; RST1 and RST2 isolates contained longer right chromosomal telomeric regions than most RST3 strains, whose chromosomes end with ORF *bb0843* (11, 25, 49). The right telomere region of strain B31MI contains only two intact ORFs, *bb0844* and *bb0852* (19). *bb0844* encodes a hypothetical protein of 323 amino acid residues with a predicted molecular mass of 37.5 kDa. The encoded sequence contains a signal peptidase II recognition signal and is predicted to be a lipoprotein. It should be noted that in the recent reannotation of the

* Corresponding author. Mailing address: Department of Microbiology and Immunology, New York Medical College, Valhalla, NY 10595. Phone: (914) 594-4658. Fax: (914) 594-4176. E-mail: schwartz@nymc.edu.

† S.B. and D.T. contributed equally to this research.

‡ Present address. Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, TX 75390.

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B. burgdorferi B31MI genome sequence on the Institute for Genomic Research (TIGR) comprehensive microbial resource (<http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi>), *bb0844* has been renamed *bb0860*; the gene name remains as *bb0844* in GenBank. In this report, *bb0844* is used throughout.

Regulation of gene expression is a key strategy that *B. burgdorferi* employs in response to diverse environmental signals encountered in arthropod and mammalian hosts. Central to the process of differential gene expression is the alternative sigma factor RpoS, which governs differential expression of various *B. burgdorferi* genes during its enzootic life cycle (8, 27, 60). RpoS appears to serve a “gatekeeper” function; its induction during nymphal feeding (i.e., the RpoS-on state) results in repression of tick phase genes and the expression of genes whose products are required for transmission to the mammal or establishment and maintenance of mammalian infection. Expression of genes repressed by RpoS during mammalian infection resumes when spirochetes are acquired by naïve larvae (i.e., the RpoS-off state) (9, 36). *bb0844* was reported to be an RpoS-regulated gene with an expression pattern identical to that of *ospC* and *dbpA*, two well-characterized *B. burgdorferi* virulence factors (3, 9, 21, 43, 48, 51, 57). *bb0844* is induced under mammalian-host-like conditions (5, 9, 52). A proteome-wide array analysis revealed that BB0844 elicits an antibody response in humans and laboratory-infected mice, indicating that BB0844 is expressed during mammalian infection (2).

Given the evidence for expression of *bb0844* by spirochetes infecting mammalian hosts and the variable presence of this gene among clinical isolates, which appears to correlate with virulence, we hypothesized that *bb0844* might contribute to *B. burgdorferi* infectivity. To explore this possibility, we analyzed the right chromosomal telomeric regions of 53 *B. burgdorferi* clinical isolates and studied the effect of *bb0844* deletion on the ability of *B. burgdorferi* to infect mice and to survive throughout an experimental mouse-tick-mouse cycle.

MATERIALS AND METHODS

Bacterial strains and growth. The *B. burgdorferi* isolates employed in this study are listed in Table 1. Most clinical isolates were obtained from patients with early Lyme disease presenting with erythema migrans at the Lyme Disease Practice of Westchester Medical Center. A number of these isolates have been described previously (49, 55). Wild-type (c162) and *rpoS* mutant (c174) strain 297 isolates (7) harboring constitutively expressed PflaB-green fluorescent protein (GFP) reporters stably inserted into their endogenous cp26 plasmids (16) were used to compare expression of *bb0844* within *Ixodes scapularis* nymphs. All isolates were cultivated in modified Barbour-Stoenner-Kelly (BSK-S) medium (54).

PCR amplification and DNA sequence analysis. The presence of the right chromosomal telomeric ORFs was determined by PCR using primers listed in Table 2. *B. burgdorferi* DNA was isolated from 1×10^9 cells using a Puregene DNA purification kit (Gentra Systems Inc., Minneapolis, MN). PCR conditions were optimized for each primer set, and the products were analyzed by electrophoresis in 1.5% agarose gels. For sequencing, PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). DNA sequencing was performed by GeneWiz Inc., South Plainfield, NJ. Sequence alignments were performed using ClustalW (<http://www.ebi.ac.uk/clustalw/>).

Analysis of *bb0844* expression in ticks by real-time reverse transcription (RT)-PCR. Total RNA was isolated from infected ticks (~150 fed larvae or flat nymphs; ~75 engorged nymphs) using TRIzol (Invitrogen) according to the manufacturer's instructions. Contaminating genomic DNA was removed using Turbo DNase (Ambion, Inc. Austin, TX); the absence of contaminating DNA was confirmed by testing each treated RNA in *Taq* polymerase amplification reaction mixtures containing primers specific for *flaB* as previously described (8). DNase-treated RNAs (~4 µg per sample) were converted to cDNA using SuperScript First-Strand Synthesis for RT-PCR (Invitrogen). A control reaction

TABLE 1. *B. burgdorferi* isolates used in this study

RST type	Isolate(s) ^a	OspC type	Biological source ^d
RST1	B55, B88, B253, B360, B404, B470, B479, B489, B515, B160	A	Human EM skin biopsy specimen
	BL206, BL564		Lyme disease patient blood
	B242, B250, B310, B350, B134, MR623, MR660, BL214, BL325	B	Human EM skin biopsy specimen Lyme disease patient blood
RST2	297 ^b	K	Lyme disease patient CSF
	B379		Human EM skin biopsy specimen
RST3	BL515, BL538	C	Lyme disease patient blood
	JD1 ^c		<i>I. scapularis</i>
	B283, B361, B408, B460, B500, B331	I	Human EM skin biopsy specimen
	BL333, BL280, BL624, BL638		Lyme disease patient blood
	B240, B453, B477	D	Human EM skin biopsy specimen
	B143, B399, B485	U	Human EM skin biopsy specimen
	B268, B369, B385, MR616	G	Human EM skin biopsy specimen
	B348, B418, B146	E	Human EM skin biopsy specimen
	B236	J	Human EM skin biopsy specimen
	BL285		Lyme disease patient blood
	B356	M	Human EM skin biopsy specimen
BL522		Lyme disease patient blood	

^a Unless otherwise noted, isolates are from patients with early Lyme disease. B, skin biopsy isolate; BL, blood isolate.

^b Reference 46.

^c Reference 39.

^d EM, erythema migrans; CSF, cerebrospinal fluid.

lacking reverse transcriptase was carried out for each sample using total RNA to ensure that no DNA contamination was present. The cDNA thus generated was used as a template for quantitative PCR (qPCR) using primers and probes specific for *flaB* and *bb0844* (Table 2). Reaction mixtures containing cDNA generated without reverse transcriptase or containing primers alone were included as controls. For quantification, *flaB* and *bb0844* amplicons were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and recombinant plasmid DNAs were prepared to generate standard curves. Transcript copy numbers were calculated using the iCycler postrun analysis software based on internal standard curves and then normalized against copies of *flaB* present in the same cDNA. The statistical significance of observed differences in expression was evaluated by an unpaired *t* test with two-tailed *P* values and a 95% confidence interval using GraphPad (La Jolla, CA) Prism software (v. 5.0).

Construction of a *bb0844* deletion mutant. The strategy for construction of a *bb0844* mutant is presented schematically in Fig. 1. This resulted in a 397-bp deletion in the central portion of *bb0844* (positions 363 to 760 in the *bb0844* coding region) that was replaced with a 1,222-bp sequence containing a streptomycin/spectinomycin resistance cassette driven by the *B. burgdorferi* *flgB* promoter. Specifically, a 638-bp fragment encompassing the region immediately upstream (including 363 bp of *bb0844* coding sequence) and a 588-bp fragment encompassing the region immediately downstream (including 212 bp of *bb0844* coding sequence) of *bb0844* were PCR amplified using primer pairs BB0844R/BB0844EcoRIR and BB0844F/BB0844EcoRIL, respectively. Each of the PCR products was ligated separately into pGEM-T (Promega, Madison, WI) and transformed into *Escherichia coli* DH5α, and transformants were selected by

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5'→3') ^a	Coordinates in B31 chromosome ^b	Purpose
BB0843F	TTATGGTATCAACTTTGGGATAACAG	901760–901785	Right-telomere ORF detection
BB0843R	ACAATAGAAAAGAAATAACCCAAAGC	903285–903260	
BB0843-4F	TCAGTTGTTATTGCCATGCAG	903064–903084	Mutant construction or confirmation
BB0843-4R	TTAAAATCAAAGTCTTCTGTAAAGG	903902–903877	
BB0844F	TTGCAATCTGTAATGCCTTTT	903553–903573	
BB0844R	GCAACAAGATGGTTTTGTTTCT	905174–905153	
BB0852F	AAAAGGATATGAAAATTATTCGCTAAA	908198–908224	
BB0852R	ATAGGGGCTTTTACTTTTCTTGA	909782–909759	
BB0844outF	TTGTTTGATAGGATTAGAACAGAGG	903496–903520	
BB0844outR	TGAATCGTTTTAAAGGGGGTA	905304–905283	
BB0844EcoRIL	GGGAATTCACATAGTAATTTAGCAATACAGGC	904140–904116	
BB0844EcoRIR	GGGAATTCATCAACTAATTTAGCAAATTC AAG ACTCC	904537–904567	
flgB _p F	TAATACCCGAGCTTCAAGGAAG	304150–304129	Real-time RT-PCR
aadAR	GACGTCATTATTTGCCGACTACC	NA	
156a lp28-1F	GAGGCAGAGGATGCTTTAAACCAAT	NA	
156a lp28-1R	AACTCTTAAGGCGTTGTAATTCGTAATTT	NA	
WI91-23 lp28-1R	CCTAATTTCCCGATGCTCTTCAAAAC	NA	
BB0844seqR	ACAAGCAGAGGTGCATGTTG	904448–904429	
FlaB-F	CTTTTCTCTGGTGAGGGGAGCTC	148259–148238	
FlaB-R	GCTCCTTCTGTTGAACACCC	148288–148308	
BB0844-574F	TTGCAGATGCTCTCTCGCTAAG	904237–904258	
BB0844-674R	GGCCGATTTTTACGTTTGTATTT	904158–904181	
FL-Probe	FAM-CTTGAACCGGTGCAGCCTGAGCA-BHQ1	148264–148286	

^a Underlining indicates an incorporated restriction site. FAM, 6-carboxyfluorescein.

^b NA, not applicable.

growth on LB agar containing 100 µg/ml ampicillin. Recombinant pGEM-T plasmids were digested with EcoRI and SphI (New England BioLabs, Ipswich, MA) or EcoRI and SpeI in order to identify clones carrying inserts in the desired orientations. These plasmids were digested with EcoRI and SphI, and the digestion products were ligated and transformed into *E. coli* DH5α as described above. The presence of both portions of *bb0844* in the resultant plasmids was confirmed by digestion with SphI and SpeI, which yielded the expected 1.25-kb insert; this plasmid was designated pGEM-TΔ*bb0844*. A 1,222-bp fragment containing a streptomycin/spectinomycin resistance gene from *Shigella flexneri* under the *B. burgdorferi* *flgB* promoter (*flgBp-aadA*) was amplified from plasmid pKFSS1 (18) using primers flgB_pF and aadAR and converted to blunt ends with T4 DNA polymerase. To introduce a selective marker between the upstream and the downstream fragments, pGEMTΔ*bb0844* was digested with EcoRI, converted to blunt ends with T4 DNA polymerase, and ligated with the *flgBp-aadA* blunt-ended PCR product. The ligation mixture was transformed into *E. coli* DH5α, colonies were selected on LB agar plates containing 100 µg/ml of spectinomycin, and plasmids from selected colonies were analyzed by SpeI and SphI digestion. The ampicillin resistance gene was inactivated in the resulting plasmids by digestion with Psp1406I (New England BioLabs), which introduces a 300-bp deletion into *bla*, and ampicillin-sensitive, spectinomycin-resistant clones were selected after transformation into *E. coli* DH5α. The desired plasmids were isolated, electroporated into competent *B. burgdorferi* B31A3 cells, selected in BSK-S medium containing streptomycin (100 µg/ml), and analyzed by PCR to confirm that a double-crossover event had occurred at the *bb0844* locus. The total plasmid content of the *bb0844* mutant clones (Δ*bb0844*) was determined as described previously (28), and those clones that retained all the plasmids present in the parental strain were selected for further study.

Syringe inoculation of mice. Four- to six-week-old pathogen-free C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were divided into groups of four and inoculated subcutaneously with 0.1 ml of either wild-type B31A3 or Δ*bb0844* mutant spirochetes (1×10^4 spirochetes/mouse) or an equal volume of fresh BSK-S medium. Ear punch samples were collected at 14 and 21 days postinoculation, washed several times in 70% ethanol or phosphate-buffered saline (PBS), and cultured in BSK-S medium supplemented with *Borrelia* antibiotic mixture (Sigma-Aldrich, St. Louis, MO). Twenty-one days after inoculation, skin biopsy samples from the site of inoculation were collected from each mouse and cultivated as described above. Blood samples (200 µl) were collected on day 14 postinoculation and assessed for serum IgG response to *B. burgdorferi* antigens using a Marblot Strip Test System (MarDx Diagnostics, Inc., Carlsbad, CA). The

mice were sacrificed 35 days after inoculation, following which the skin, joints, and urinary bladders were collected aseptically for culture. All cultures were maintained at 37°C for at least 4 weeks and examined weekly for the presence of live spirochetes by dark-field microscopy.

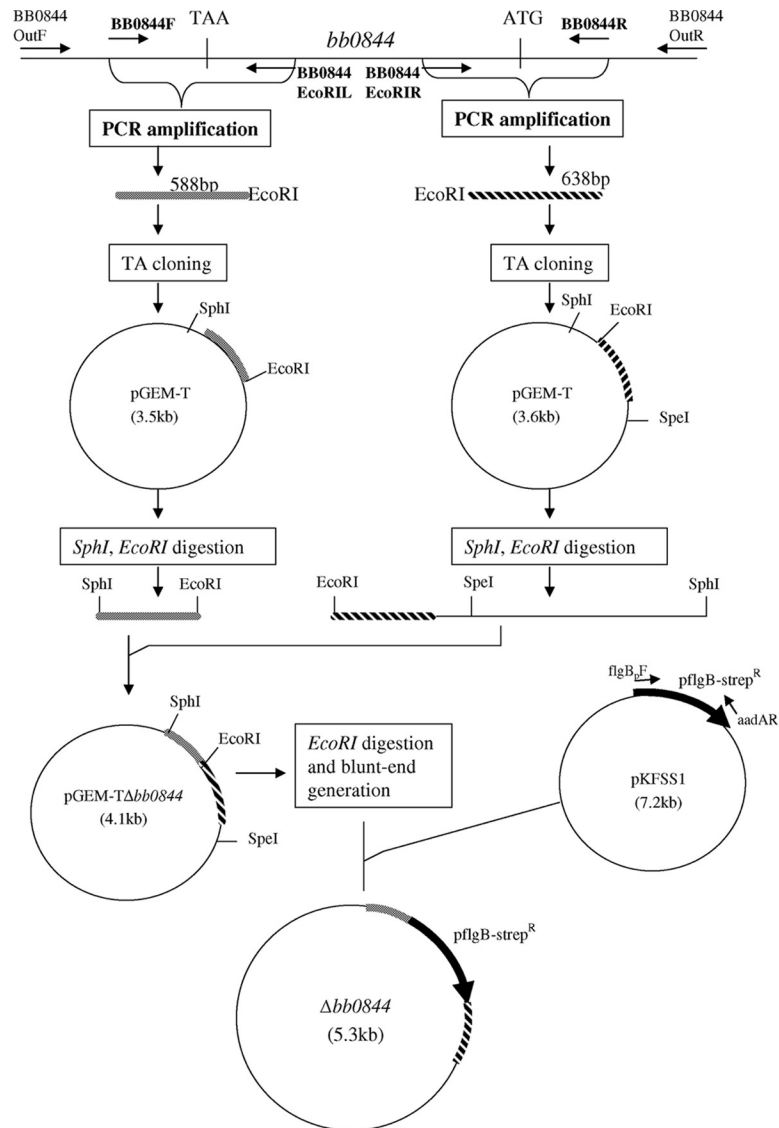
Spirochete acquisition and transmission by ticks. The pathogen-free *I. scapularis* larvae used in this study were obtained from a colony maintained at Yale University and kindly provided by Durland Fish. For spirochete acquisition, approximately 400 naïve larvae were placed on mice infected with either wild-type or Δ*bb0844* mutant spirochetes (two mice/isolate) and allowed to feed to repletion. Engorged (fed) larvae were collected at drop-off over a 5-day period and allowed to molt to the nymphal stage. Immersion-fed larvae used for qRT-PCR studies were infected with *B. burgdorferi* wild-type and Δ*rhoS* isolates as previously described (36).

For transmission studies, nymphs infected with either wild-type or Δ*bb0844* mutant spirochetes were placed on naïve C3H/HeJ mice (10 nymphs/mouse) and allowed to feed to repletion. Three weeks after infestation, the mice were sacrificed, and joints, urinary bladder, and ear punch biopsy specimens were cultivated and examined for spirochete infection as described above.

RESULTS

Structure of the right chromosomal ends of *B. burgdorferi* clinical isolates. Previous comparative genomic studies revealed substantial genotype-dependent variation at the right chromosomal telomere beginning with *bb0843* (12, 49). Of the ORFs in this region, only two, *bb0844* and *bb0852*, appear to encode potentially functional genes (25, 49). The structure of the right chromosomal telomeric region was explored in 53 *B. burgdorferi* clinical isolates representing all three RST and 11 *ospC* genotypes. The presence of four chromosomal loci, *bb0843*, *bb0843.1*, *bb0844*, and *bb0852*, was determined by PCR using primers listed in Table 2. The results are summarized in Table 3. All strains contained *bb0843*, in agreement with previous reports that this is the last ORF in *B. burgdorferi* isolates with short chromosomes (10). All 21 RST1 and 2 RST2

A



B

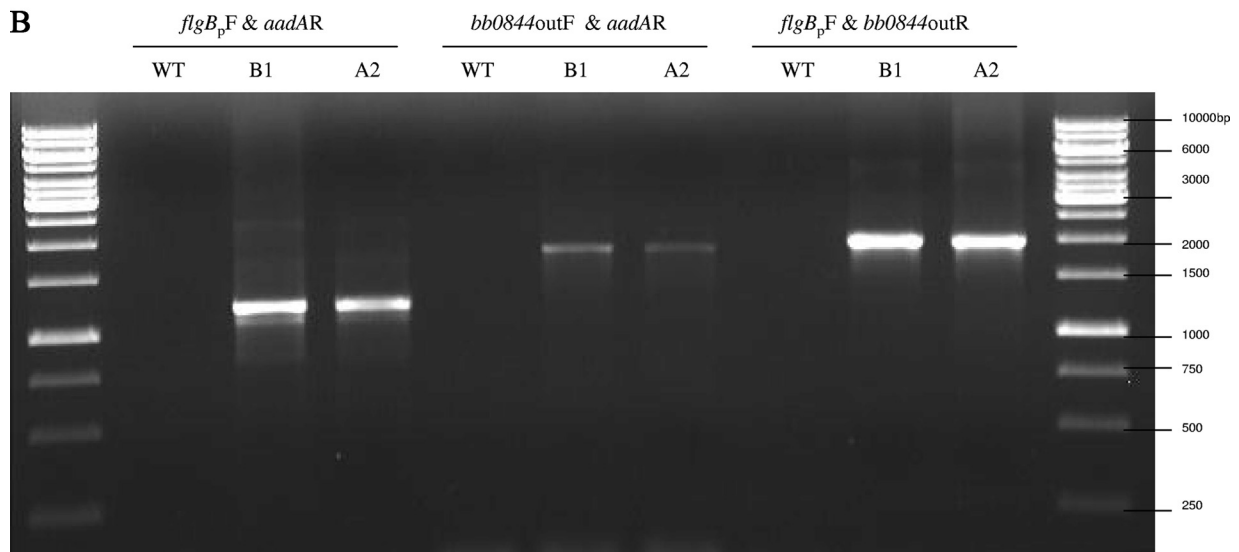


FIG. 1. Construction and characterization of a *bb0844* deletion mutant. (A) Strategy for partial deletion of *bb0844*. (B) PCR analysis of the wild type and two independent deletion mutant clones (B1 and A2). The combination of primers used for each PCR is shown at the top, and the positions of the primers are shown in panel A. The migration positions of a 1-kb DNA ladder are shown on the right.

TABLE 3. Presence of right chromosomal loci in RST1, RST2, and RST3 isolates

RST	OspC type	No. tested	Presence of ^a :			
			<i>bb0843</i>	<i>bb0843.1</i>	<i>bb0844</i>	<i>bb0852</i>
1	A	12	+	+	+	+
	B	9	+	+	+	+
2	K	2	+	+	+	+
3	C	3	+	+	+	+
	D	3	+	+	+	-
	G	4	+	+	+	-
	U	3	+	+	+	-
	I	10	+	-	+	-
	E	3	+	-	-	-
	J	2	+	-	-	-
M	2	+	-	-	-	

^a +, present; -, absent.

isolates analyzed carried all four loci, indicative of the presence of longer chromosomes. In contrast, RST3 strains were heterogeneous with respect to the presence of the intergenic spacer between *bb0843* and *bb0844* (*bb0843.1*), *bb0844*, and *bb0852*; *bb0843.1* and *bb0844* were detected in 14 of 30 (47%) and 23 of 30 (77%) RST3 isolates, respectively, whereas *bb0852* was present in only 3 (Table 3). Particularly noteworthy, 10 isolates contained *bb0844* but not *bb0843.1* or *bb0852*, suggesting that *bb0844* may be present on a genomic element other than the chromosome in some strains.

Linkage between right chromosomal telomere structure and *ospC* genotype. Previous work has shown that the RST and *ospC* genotypes are linked (22), and further, that disseminated infection is correlated with the genotype (41, 58, 59). It was, therefore, of interest to investigate whether a correlation exists between the structure of the right telomeric region, the presence of *bb0844*, and the *ospC* genotype. Four different patterns were observed (Table 3). As noted, RST1 (OspC types A and B), RST2 (OspC type K), and some RST3 (OspC type C) isolates scored positive for all four loci, indicating the presence of a long B31-like chromosome. Isolates harboring OspC types D, G, and U contained *bb0844*, but not *bb0852*, suggesting that their chromosomes are shortened by deletion of sequences distal to *bb0844*; the precise termini of these deletions were not explored further. Isolates containing OspC types E, J, and M yielded a PCR product only for *bb0843*, consistent with a shorter chromosome. Interestingly, all 10 OspC type I isolates tested positive for *bb0843* and *bb0844* but not for the chromosomal region between these loci (*bb0843.1*), suggesting an alternative genomic location for *bb0844*.

To further explore this possibility, total genomic DNA from several OspC type I isolates was separated by pulsed-field gel electrophoresis and probed for *bb0844* by Southern blotting. The results indicated that *bb0844* is located on a smaller replicon, most likely on a linear plasmid of 25 to 28 kbp, rather than on the chromosome (data not shown). Genomic sequences of several *B. burgdorferi* strains have recently been reported (40). BLAST analysis revealed a sequence with >99% identity to *bb0844* on plasmid lp28-1 of strain WI91-23. Based on *ospC* sequence, WI91-23 is OspC type I and has a shorter chromosome of 896 to 935 kbp in length (10, 40).

Primers were designed based on the WI91-23 lp28-1 sequence (accession no. CP001456). Primer WI91-23 lp28-1R is located outside the *bb0844* coding region and was used in combination with primer BB0844seqR, which is within the coding region, to perform PCR amplification with genomic DNAs from B500 and B408, two previously described OspC type I isolates (55). The resulting 1.5-kb amplicons were purified and sequenced; these sequences aligned perfectly with that from WI91-23 lp28-1. Thus, OspC type I isolates have a shorter chromosome terminating at *bb0843* but include a complete *bb0844* on a linear plasmid, most likely lp28-1.

BLAST analysis also revealed the presence of *bb0844* on lp28-1 of isolate 156a, an RST2, OspC type H strain (1). Primers 156a lp28-1F and 156a lp28-1R (Table 2) were designed based on the 156a lp28-1 sequence (accession no. CP001273) and used for PCR amplification with genomic DNA of isolates 297 and B379 (both RST2, OspC type K strains). These strains contain a complete copy of *bb0844* based on PCR analysis (Table 3). A 1.2-kb region that spans the junction of *bb0844* and lp28-1 was PCR amplified, and sequence analysis indicated that all three isolates contained identical sequence in this region. Unlike OspC type I isolates, strains 156a, 297, and B379 contain a complete copy of *bb0844* on the chromosome, whereas the 752 nucleotides at the 5' end of the *bb0844* coding region are deleted from the copy on lp28-1. Thus, these strains contain only one complete copy of *bb0844* located on the chromosome.

Strain 64b, an OspC type B isolate, also contained *bb0844* sequences in two different genomic locations. Both the chromosomal copy (Bbu64b_0872; accession no. ABKA02000005) and the plasmid copy on lp28-1 (Bbu64b_F0009 of lp28-1; accession no. CP001423.1) are 99% identical to the B31 *bb0844* sequence. Thus, this strain contains two complete copies of *bb0844*.

Sequence analysis of *bb0844* in selected RST 3 isolates. The DNA sequence of *bb0844*, including 223-bp upstream and 325-bp downstream flanking regions, was determined for four OspC type I isolates (B361, B460, B500, and BL333) and one OspC type C isolate (BL515) following PCR amplification using primers BB0844F and BB0844R (Table 2). The *bb0844* coding region was highly conserved, containing only six nucleotide polymorphisms among the isolates that would result in mostly conservative amino acid changes in the deduced protein sequences. The upstream region of *bb0844* was identical in all isolates, including the B31MI type strain, and encompasses a sequence with significant homology to the *B. burgdorferi* consensus RpoS promoter (see Table 4 in reference 9). Greater variability was observed in the 3' flanking region, particularly between 94 and 150 bp downstream of the *bb0844* termination codon, where several nucleotide changes were observed in RST3 isolates compared to B31MI (data not shown). The results suggest that expression of *bb0844* is regulated similarly regardless of the genomic location.

Differential expression of *bb0844* in *B. burgdorferi*. Previous microarray analyses of temperature-shifted *B. burgdorferi* or spirochetes cultivated under mammalian-host-like conditions indicated that, similar to *ospC*, expression of *bb0844* is absolutely dependent on RpoS (5, 9, 52). To extend these findings to *in vivo* conditions, *bb0844* expression was measured in *B. burgdorferi*-infected fed larvae and unfed and fed nymphal

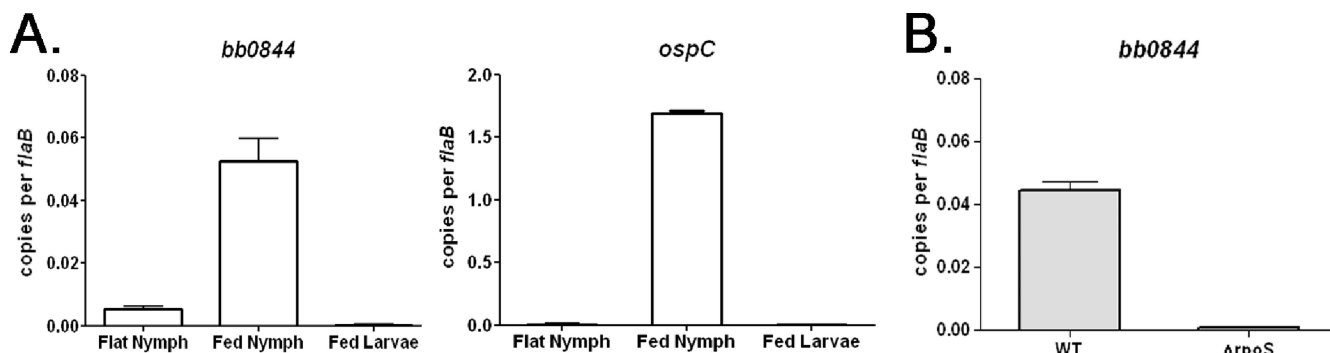


FIG. 2. (A) Real-time RT-PCR analysis of *bb0844* and *ospC* expression in flat (unfed) nymphs, fed nymphs, and fed larvae. Ticks were infected as larvae by infestation of mice that had been syringe inoculated with *B. burgdorferi* strain 297. Expression of *bb0844* was significantly induced upon nymphal feeding ($P = 0.0005$). (B) Real-time RT-PCR analysis of *bb0844* in fed nymphal ticks infected as larvae by immersion with either wild-type (WT) or *rpoS* mutant ($\Delta rpoS$) *B. burgdorferi* strain 297. Expression of *bb0844* was significantly reduced in the mutant ($P < 0.0001$). The values represent the average *flaB*-normalized copy number and standard error of the mean (SEM) for each gene.

ticks. *bb0844* transcript was detected only in fed nymphs, and expression was absolutely dependent on RpoS (Fig. 2). It should be noted that even in fed nymphs, *bb0844* was poorly expressed; transcript levels were more than 30-fold lower than that for *ospC*, a well-characterized RpoS-dependent gene. Despite numerous attempts, *bb0844* transcript was not detected in infected mouse tissue (data not shown).

Construction and characterization of a *bb0844* deletion mutant. To determine the requirement for *bb0844* during the borrelial enzootic cycle, a deletion mutant was constructed in strain B31A3 by deleting a portion of *bb0844* and replacing it with a spectinomycin resistance cassette by allelic exchange (Fig. 1A). Verification of the presence of the spectinomycin resistance cassette in mutant clones and confirmation that a double-crossover event took place were accomplished by PCR using the primer sets shown in Fig. 1A. The PCR results were consistent with replacement of 397 bp of *bb0844* with *flgBp-aadA* by a double-crossover event (Fig. 1B). The absence of any detectable *bb0844* transcript in the mutant clones further confirmed functional inactivation of the gene (data not shown). There were no differences in the growth characteristics of wild-type and *bb0844* mutant strains cultivated in BSK-S medium at 37°C. Furthermore, no obvious differences in spiro-

chete morphology were observed between wild-type and *bb0844* mutant strains when examined by dark-field microscopy (data not shown).

BB0844 is not required for infection of mice. To assess the role of BB0844 in the establishment of mammalian infection, mice were needle inoculated subcutaneously with 1×10^4 spirochetes of either wild-type B31A3 or one of the two independent mutant clones ($\Delta bb0844A2$ and $\Delta bb0844B1$). Infectivity was assessed by *in vitro* cultivation of mouse tissues at several time points postinfection and by serology. All infected mice were culture positive for spirochetes in ear and skin tissues at 21 days postinfection (Table 4). All mice infected with wild-type and $\Delta bb0844B1$ mutant strains were culture positive for *B. burgdorferi* at day 35 postinoculation, and all mice seroconverted (Table 4). These findings demonstrate that *bb0844* is not required for *B. burgdorferi* infectivity or persistence in mice when introduced by needle inoculation.

Acquisition and transmission of BB0844 deletion mutant strains by ticks. To determine whether the absence of BB0844 influences the ability of *B. burgdorferi* to be acquired and transmitted by ticks, naïve larvae were allowed to feed on mice infected with either wild-type or *bb0844* mutant strains ($\Delta bb0844A2$ and $\Delta bb0844B1$). Fed larvae were collected at

TABLE 4. Recovery of *B. burgdorferi* from mice infected with wild type, $\Delta bb0844A2$ and $\Delta bb0844B1$ strains by needle inoculation

<i>B. burgdorferi</i> strain	Time postinfection	No. of positive cultures/no. tested (%)					Serology
		Ear	Skin	Joint	Bladder		
B31A3 WT	Day 14	6/6 (100.0)	NT	NT	NT	6/6 (100.0)	
	Day 21	6/6 (100.0)	6/6 (100.0)	NT	NT		
	Day 35 ^a	NT ^b	4/4 (100.0)	4/4 (100.0)	4/4 (100.0)		
$\Delta bb0844$ A2	Day 14	4/4 (100.0)	NT	NT	NT	4/4 (100.0)	
	Day 21	4/4 (100.0)	4/4 (100.0)	NT	NT		
	Day 35 ^a	NT	1/2 (50.0)	2/2 (100.0)	1/2 (50.0)		
$\Delta bb0844$ B1	Day 14	4/4 (100.0)	NT	NT	NT	4/4 (100.0)	
	Day 21	4/4 (100.0)	4/4 (100.0)	NT	NT		
	Day 35 ^a	NT	2/2 (100.0)	2/2 (100.0)	2/2 (100.0)		

^a Only two of four animals from the mutant-infected groups were sacrificed. The remaining two mice from each group were used for larval tick feeding as described in Materials and Methods.

^b NT, not tested.

TABLE 5. Infectivity of *Δbb0844* in mice via tick bite after 3 weeks of infection

<i>B. burgdorferi</i> strain	Number of mice infected/no. of mice tested (%)		
	Ear	Bladder	Joint
B31A3 WT	2/2 (100.0)	2/2 (100.0)	2/2 (100.0)
<i>Δbb0844</i> A2	3/3 (100.0)	2/3 (66.6)	3/3 (100.0)
<i>Δbb0844</i> B1	3/3 (100.0)	2/3 (66.6)	3/3 (100.0)

drop-off, allowed to molt into nymphs, and used to examine the requirement for *bb0844* during spirochetal transmission to a mammalian host. There was no difference between the spirochete loads in larvae fed on either wild-type- or mutant-infected mice ($2,512 \pm 1,265$ versus $2,215 \pm 815$, respectively). Naïve C3H/HeJ mice were infested with 10 nymphs infected with either wild-type or *bb0844* mutant spirochetes. All mice fed upon by nymphs infected with either wild-type or mutant strains were culture positive for spirochetes in ear and joint tissues (Table 5). Taken together, these results demonstrate that *bb0844* is not required for spirochete acquisition, maintenance, or transmission by *I. scapularis*. Furthermore, *bb0844* is dispensable for infection of mice by either needle inoculation or tick transmission.

DISCUSSION

With the exception of the right telomere, the linear chromosome of *B. burgdorferi* is highly conserved with respect to sequence and gene order (11, 49). The variation of the right telomere is the result of a 6- to 14-kbp extension of the chromosomal end in certain strains (10, 11). Among the ORFs on the chromosomal extension is *bb0844*, an RpoS-dependent gene (9). Given the proposed role of RpoS as a regulator of genes required for establishment of mammalian infection (9, 36), it was of interest to explore the presence of *bb0844* among a collection of *B. burgdorferi* clinical isolates with different pathogenic properties representing most *ospC* genotypes. The results indicate four patterns of *bb0844* genomic localization: (i) complete absence, (ii) presence only on the chromosome, (iii) presence only on a plasmid, or (iv) presence of a complete gene copy on the chromosome and either a complete copy or a 5'-truncated copy on a plasmid. The genomic location of *bb0844* correlates with the *B. burgdorferi* genotype in a manner similar to that described previously for chromosome length variation (10, 49). *OspC* types A, B, C, and K have the largest chromosomes, as indicated in the current study by the presence of *bb0852*. B31, a typical *OspC* type A strain, contains an extra 8.5 kbp at the right chromosomal end; Sh-2-82 and JD1, *OspC* types K and C, respectively, have chromosomes which are approximately 12 kbp longer than that of B31(40). The chromosomes of Sh-2-82 and JD1 have not been sequenced, and thus, the precise nature of the right chromosomal ends in these strains beyond that of B31 is not currently known. *OspC* types D, G, and U contain *bb0844* on the chromosome but lack *bb0852*, suggesting that these genotypes possess a right chromosomal end extension that is shorter than that of B31. Casjens et al. (11) analyzed the right chromosomal telomere for 31 *B. burgdorferi* isolates, mostly of tick or wildlife origin, by Southern blotting using probes derived from conserved and

extended regions of the chromosome. They noted a pattern essentially identical to that observed in the current study. The present data extend the previous findings to human clinical isolates and provide a correlation between the genotype and the structure of the right chromosomal end.

Previous studies suggested that the *B. burgdorferi* genotype predicts the capacity of a strain for hematogenous dissemination. RST1 strains have a significantly greater tendency to produce disseminated infection than do RST3 isolates (29, 55, 58, 59). All 21 RST1 isolates (12 *OspC* type A and 9 *OspC* type B) analyzed in the current study had a pattern identical to that of strain B31. In contrast, RST3 isolates were heterogeneous in terms of the presence or absence of the four right chromosomal loci tested. Of particular note are strains of *OspC* types E, J, M, and I. None of the 17 isolates with these *OspC* types had *bb0844* on their chromosomes, and presumably all have the shortest chromosomal right end. *OspC* types E, J, and M isolates completely lack the gene. Despite this, BL285 (*OspC* type J) and BL522 (*OspC* type M) were recovered from the blood of Lyme disease patients, while B348 (*OspC* type E) disseminates in mice (albeit with kinetics that differ from those of *OspC* type A strains) (15, 23, 55), indicating that *bb0844* is not required for hematogenous dissemination. In contrast, *OspC* type I isolates lack *bb0844* in the chromosome and instead carry it on a plasmid of approximately 28 kb. The recently completed sequences of *OspC* type I strain WI91-23 plasmids allowed a more careful analysis of the genomic localization of *bb0844* in these strains. Beginning at nucleotide 394 of plasmid lp28-1, WI91-23 contains 6,654 nucleotides that are highly similar to the right chromosomal end of B31 and include ORFs *bb0844* to *bb0853*. The coding region in WI91-23 lp28-1 designated F0011 (BBUWI91-23_F0011) and the 80 bp upstream and downstream are >99% identical to the sequence of B31 *bb0844*; the deduced amino acid sequence of F0011 is 98.8% identical (319/323 residues) to that of BB0844. We conclude that a complete copy of *bb0844* is carried on lp28-1 in *OspC* type I strains and that *bb0844* expression would be dependent on RpoS regardless of the genomic location of the gene.

The results also indicate that *OspC* type K isolates carry *bb0844* on the chromosome, as they were PCR positive for all tested B31 ORFs, including *bb0852*. This is consistent with the results of Casjens et al. and Huang et al. (11, 25) showing that strains of this genotype have right chromosome extensions that are longer than that of B31. A complete genome sequence for an *OspC* type K isolate is not available; however, strain 156a, an *OspC* type H isolate (closely related phylogenetically to *OspC* type K isolates [33]), has been sequenced. Analysis revealed that this strain contains a complete copy of *bb0844* on the chromosome and a partial copy on lp28-1. Interestingly, the *bb0844* copy on lp28-1 is missing the 5' 752 bp of the *bb0844* coding region. Using primers based on the strain 156a sequence, we amplified identical sequences for two *OspC* type K strains. We conclude that *OspC* type K strains do not contain an intact *bb0844* on lp28-1; rather, these strains carry a complete, functional copy of *bb0844* only on the chromosome.

What is the mechanism underlying the variation in *bb0844* content and location? Kobryn and Chaconas (30) demonstrated that hairpin telomeres could be fused by reversal of the telomere resolution reaction catalyzed by ResT. On this basis,

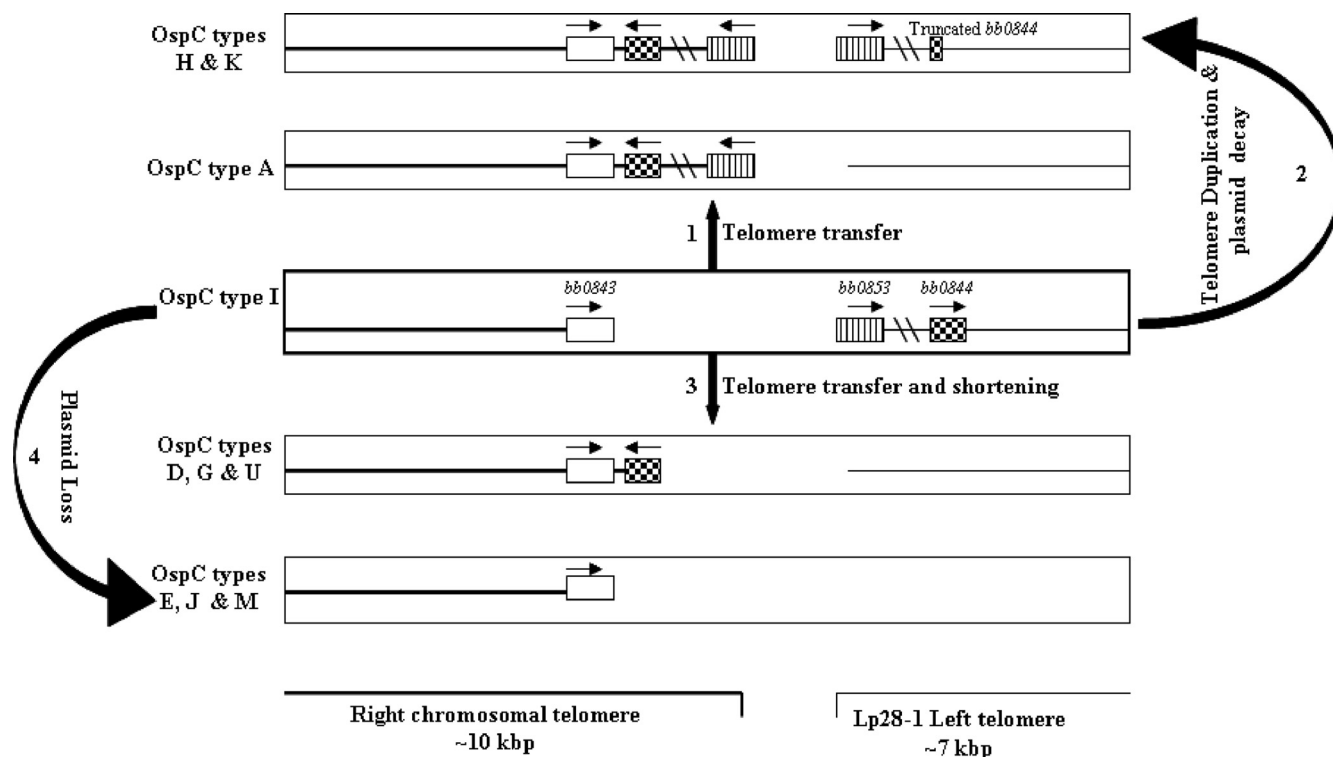


FIG. 3. Proposed evolution of the right chromosomal end of *B. burgdorferi* and the *bb0844* genomic location. The schematic diagram shows the final product following ResT-mediated reversal of telomere resolution based on the mechanisms proposed by Chaconas and coworkers (13, 14, 30). The rectangles denote the presence of the chromosome and plasmid in a single cell. The numbers refer to alternative outcomes discussed in the text.

they suggested that the most likely mechanism for variation at the chromosomal right end is extension via addition of linear plasmid sequences in a process that may be dependent on ResT, a telomere resolvase (13, 30). Telomere fusion would require base pairing between the six nucleotide sticky ends generated during the first step (substrate cleavage) (13, 30). Sequences for most of the *B. burgdorferi* chromosomal and plasmid telomeres have recently been reported and can be characterized into three distinct types based on the presence, sequence, and position of the so-called box 1; only telomeres with complementary sticky ends between the cleavage sites can undergo fusion by reversal of the telomere resolution (14, 53). Interestingly, the chromosome right end and the left end of Lp28-1 are both type 1 telomeres with compatible sticky ends (53), and thus, fusion mediated by ResT would be permitted. Based on the data from the current study and available sequence information, we propose the following evolution of right chromosomal ends and *bb0844* localization (Fig. 3). The ancestral strain is likely to have been similar to OspC type I, which has the shortest chromosome and carries *bb0844* on a linear plasmid. Telomere fusion between the *bb0844*-containing plasmid and the right chromosomal end, followed by deletion or mutation, could result in several outcomes: (i) telomere transfer, resulting in chromosome extension and the presence of *bb0844* only on the chromosome (e.g., *ospC* type A); (ii) telomere duplication and either maintenance of both gene copies (e.g., strain 64b) or decay of the plasmid copy of *bb0844* (*ospC* types K and H); (iii) telomere transfer followed

by shortening of the chromosomal extension, resulting in removal of regions distal to *bb0844*, including *bb0852* (*ospC* types D, G, and U); alternatively, this arrangement could be derived by telomere transfer from a currently unidentified plasmid containing *bb0844* but not the sequence distal to it); and finally, (iv) loss of the *bb0844*-containing plasmid from the OspC type I ancestral strain would yield isolates completely lacking *bb0844* (OspC types E, J, and M).

RpoS induces expression of genes whose products are required for either transmission to the mammalian host or establishment and maintenance of mammalian infection (8). Transcription of *bb0844* is induced in the mammalian-host-adapted state (5, 9, 52), and its expression is abrogated in Rrp2 and BosR mutants, two regulatory proteins required for expression of *rpoS* (4, 37, 38). In this study, *bb0844* was found not to be expressed in fed larvae or unfed nymphs, and its transcription was induced in fed nymphs. This is the pattern expected for an RpoS-dependent gene, as *rpoS* is induced on nymphal feeding and is not expressed at other tick stages (9). The *bb0844* transcript level is quite low in fed nymphs (0.05 copy/copy of *flaB*), as is the case *in vitro* and in dialysis membrane chambers (9). *bb0844* transcripts could not be detected in infected mice (data not shown), presumably due to relatively low transcript levels and the low spirochete loads in the infected animals. Nevertheless, BB0844 induces an antibody response in both humans and mice (2), confirming not only that it is expressed *in vivo*, but also that the protein is highly immunogenic. Eggers et al. (17) proposed that *B. burgdorferi*

recognizes an extended -10 region to distinguish between RpoS and σ^{70} promoters. The putative *bb0844* promoter sequence has a 10/11 match to the *B. burgdorferi* consensus RpoS promoter (9). Thus, the molecular basis for the low levels of *bb0844* transcript is unclear at present. It is possible that *bb0844* transcript levels are subject to posttranscriptional regulation (e.g., mRNA stability).

We hypothesized that BB0844 would play a role in mammalian infection based on two considerations: comparative genomic analysis indicated the absence of *bb0844* in isolates that disseminate less readily in mammals (49), and *bb0844* is coordinately expressed with other genes required for infectivity (e.g., *ospC*) due to its RpoS dependence (9). However, absence of *bb0844* had no discernible effect at any step in an experimental tick-mouse infection model. *bb0844* mutant strains were fully capable of establishing infection in mice, whether by needle or tick inoculation. Mutant spirochetes were acquired by ticks and persisted through the entire tick life cycle. Therefore, *bb0844* is not essential to *B. burgdorferi* survival *in vivo*, and its function is dispensable for spirochete pathogenicity and maintenance in the tick-mouse infectious cycle. This is perhaps not surprising, given that certain spirochete lineages (e.g., OspC types E, J, and M) lack *bb0844* yet are maintained in the tick-mammal enzootic cycle in nature and can infect humans (23).

RpoS is a key factor that governs differential gene expression in *B. burgdorferi* as it transitions from growth in the tick vector to growth in the mammal. It is therefore reasonable to predict that genes that are part of the RpoS regulon are important for mammalian infection. Caimano et al. (9) reported that the RpoS regulon is comprised of 137 genes, including *bb0844*. The current results demonstrate that not all genes whose expression is coordinately regulated with those required for virulence are themselves necessary for that process. It should be noted that there are numerous contradictory reports about the roles of other RpoS-dependent genes (e.g., *dbpA*, *bbk32*, and *bba64*) in mammalian infection, although these discrepancies may depend on the experimental mode of infection (3, 20, 31, 34, 35, 42–45). Our findings underscore the necessity to demonstrate required protein function by mutagenesis experiments, rather than reliance on correlative data alone.

It is assumed that BB0844 had a required function at some point in the evolutionary history of *B. burgdorferi*, although the biological function of BB0844 is not known. It is possible that other proteins of unknown function can provide redundant activities and compensate for the absence of BB0844. In such a case, it might be expected that the *bb0844* coding region would decay, but sequence analysis of multiple isolates revealed only a small number of nucleotide polymorphisms that would result in only conservative amino acid changes in the encoded protein. Alternatively, it is possible that BB0844 does indeed have a required function that cannot be discerned using currently available experimental models. For example, BB0844 may be required for survival in an alternative reservoir host (other than mice). At present, *bb0844* can be considered an addition to the growing list of *B. burgdorferi* genes (e.g., *ospD*, *luxS*, and *chbC*) that, based upon experimental models currently available, do not appear to be required during the enzootic cycle (26, 47, 50).

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