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CsrA Modulates Levels of Lipoproteins and Key Regulators of Gene Expression Critical for Pathogenic Mechanisms of *Borrelia burgdorferi*[▽]

S. L. Rajasekhar Karna,† Eva Sanjuan,†‡ Maria D. Esteve-Gassent,§ Christine L. Miller, Mahulena Maruskova, and J. Seshu*

South Texas Center for Emerging Infectious Diseases and Department of Biology, The University of Texas at San Antonio, San Antonio, Texas 78249

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Carbon storage regulator A (CsrA) is an RNA binding protein that has been characterized in many bacterial species to play a central regulatory role by modulating several metabolic processes. We recently showed that a homolog of CsrA in *Borrelia burgdorferi* (CsrA$_{Bb}$, BB0184) was upregulated in response to propagation of *B. burgdorferi* under mammalian host-specific conditions. In order to further delineate the role of CsrA$_{Bb}$, we generated a deletion mutant designated ES10 in a linear plasmid 25-negative isolate of *B. burgdorferi* strain B31 (ML23). The deletion mutant was screened by PCR and Southern blot hybridization, and a lack of synthesis of CsrA$_{Bb}$ in ES10 was confirmed by immunoblot analysis. Analysis of ES10 propagated at pH 6.8/37°C revealed a significant reduction in the levels of OspC, DbpA, BBK32, and BBA64 compared to those for the parental wild-type strain propagated under these conditions, while there were no significant changes in the levels of either OspA or P66. Moreover, the levels of two regulatory proteins, RpoS and BosR, were also found to be lower in ES10 than in the control strain. Quantitative real-time reverse transcription-PCR analysis of total RNA extracted from the parental strain and *csrA$_{Bb}$* mutant revealed significant differences in gene expression consistent with the changes at the protein level. Neither the *csrA$_{Bb}$* mutant nor the *trans*-complemented strain was capable of infection following intradermal needle inoculation in C3H/HeN mice at either 10^3 or 10^5 spirochetes per mouse. The further characterization of molecular basis of regulation mediated by CsrA$_{Bb}$ will provide significant insights into the pathophysiology of *B. burgdorferi*.

Lyme disease is the most prevalent arthropod-borne illness in the United States, with symptoms affecting the cutaneous, musculoskeletal, cardiovascular, and nervous systems (88). The causative agent of Lyme disease is the spirochetal pathogen *Borrelia burgdorferi*, which is transmitted to vertebrate hosts via the bite of infected *Ixodes* species ticks (8). The ability of *B. burgdorferi* to undergo adaptive gene expression in response to vertebrate host-specific signals upon the ingestion of a blood meal by the infected ticks contributes significantly to its transmission capabilities to vertebrate hosts (1–5, 13, 21, 35, 53, 66, 73, 74, 82, 83, 98, 100). While a number of studies have focused on the levels of expression of open reading frames (ORFs) encoding proteins with or without a known function, the regulatory networks that define the transcriptional regulation are beginning to be understood in greater detail (15, 16, 20, 41, 99). The genome of *B. burgdorferi* encodes a limited set of known regulators of gene expression (33). Therefore, it is conceivable that a network of multiple regulators may contribute to the fine-tuning of the signal-dependent adaptive gene expression in *B. burgdorferi* that facilitates transmission and colonization between hosts with highly disparate microenvironments (10, 11, 18, 28, 33, 44, 55, 68, 80).

A number of borrelial determinants that play a crucial role in the pathogenic processes have been shown to be regulated by a central regulatory pathway comprising Rrp2-RpoN-RpoS (16, 20, 32, 41, 61, 99). These include outer surface protein C (OspC), decorin binding protein A (DbpA), fibronectin binding protein (BBK32), other linear plasmid 54 (lp54)-encoded ORFs such as BBA64 and BBA34, and a growing list of ORFs known to be responsive to the shift in environmental signals that are different between the arthropod and vertebrate hosts, such as temperature, pH, dissolved gases, and other undefined factors (21, 26, 37–40, 45, 70, 82, 83, 89). Recently, it has been shown that a small RNA molecule, *B. burgdorferi* DsrA (DsrA$_{Bb}$), regulates the levels of RpoS in response to a temperature shift from 23°C to 37°C and thereby serves as a molecular thermometer modulating temperature-induced, RpoS-regulated genes (56). Moreover, regulatory functions mediated by BosR (Borrelia oxidative stress regulator), cyclic-di-GMP levels, and RNA chaperone Hfq in oxidative stress response, motility, and pathogenic mechanisms have added to our understanding of regulation of gene expression in *B. burgdorferi* (29, 44, 55, 68, 74, 84, 91).

In addition to the above regulators, the borrelial genome encodes a homolog present in many bacterial species termed carbon storage regulator A (CsrA). This small RNA binding protein has been characterized to be a global regulator affecting mRNA stability or levels of translation of multiple ORFs (46, 75, 76, 92, 93). We recently showed that there was increased expression of *Borrelia burgdorferi* CsrA (CsrA$_{Bb}$) when

* Corresponding author. Mailing address: BSE3.230, Department of Biology, The University of Texas at San Antonio, One UTSA Circle, San Antonio, TX 78249. Phone: (210) 458-6578. Fax: (210) 458-5658. E-mail: j.seshu@utsa.edu.

† Contributed equally to the work and should be considered first authors.

‡ Present address: Department of Microbiology and Ecology, University of Valencia, Valencia, Spain.

§ Present address: Department of Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843.

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description[a]	Source or reference
Plasmids		
pCR2.1-TOPO	PCR cloning vector	Invitrogen
pML102	Customized donor plasmid for *in vitro* mutagenesis	85
pBBE22	Borrelial shuttle vector confers Kan^r derived from pBSV2 with BBE22 region of lp25	71
pES1	1 kb of chromosome upstream of $csrA_{Bb}$ in pCR2.1	This study
pES4	1 kb of chromosome downstream of $csrA_{Bb}$ in pCR2.1 (with intact *bb0185*)	This study
pES8	2 kb of chromosome upstream of $csrA_{Bb}$ and downstream of $csrA_{Bb}$ and deletion of chromosome coordinates in pCR2.1	This study
pES25	P*flgB*-$csrA_{Bb}$ six-His tag cloned into pBSV2	80
pES10	pES8 with 1.2-kb insertion of P_{flgB}-*aadA*	This study
pES50	P*flgB*-$csrA_{Bb}$ in pCR2.1	This study
pES51	P*flgB*-$csrA_{Bb}$ cloned into pBSV2	This study
pES52	pES51 with BBE22	This study
B. burgdorferi strains		
ML23	B31, lp25-negative, noninfectious clonal isolate	48, 49
MSK5	B31 isolate with all infection-associated plasmids	48, 49
ES10	ML23, $csrA_{Bb}$ negative, Str^r	This study
ML23/pBBE22 (wt)	ML23 with pBBE22 (BBE22$^+$), Kan^r	57, 58
ES10/pBBE22 ($csrA_{Bb}$-negative mutant)	ES10 complemented with pBBE22/Str^r Kan^r	This study
ES10/ES52	ES10 with ES52/Str^r Kan^r	This study

[a] Str^r, streptomycin resistance; Kan^r, kanamycin resistance.

B. burgdorferi was propagated under fed-tick conditions (80). Moreover, the overexpression of $csrA_{Bb}$ under the control of a constitutive borrelial promoter, P_{flgB}, in *B. burgdorferi* strain B31 (ML23, lp25-deficient clonal isolate) that had an intact chromosomal copy of $csrA_{Bb}$ resulted in increased levels of several lipoproteins that have been extensively characterized to play a role in infectivity to various degrees, such as OspC (27, 36, 69), DbpA (9, 86, 87), and BBK32 (15, 17, 70, 85).

In this study, we describe the effect of deletion of $csrA_{Bb}$ in a noninfectious, lp25-deficient clonal isolate of *B. burgdorferi* strain B31 (ML23) which was complemented with the minimal regions of lp25 using the shuttle vector pBBE22 alone or along with a functional copy of $csrA_{Bb}$ (pES52). We evaluated the ability of the $csrA_{Bb}$ mutant (mt) to modulate levels of borrelial lipoproteins and two regulators of gene expression (RpoS and Borrelia oxidative stress regulator [BosR]) in *B. burgdorferi* in response to key environmental signals that mimic the post-fed-tick conditions. Consistent with the findings made in the previous study, the deletion of $csrA_{Bb}$ reduced the levels of several lipoproteins and key regulators involved in the adaptation of *B. burgdorferi* for colonization of vertebrate hosts. Moreover, the $csrA_{Bb}$ mutant was incapable of colonization of C3H/HeN mice following intradermal needle inoculation at a dose of either 10^3 or 10^5 spirochetes per mouse. However, complementation of the mutant with an intact copy of $csrA_{Bb}$ in *trans* on a borrelial shuttle vector did not restore the parental infectivity phenotype. These observations indicate that $CsrA_{Bb}$ plays a key role in the regulation of expression of pathophysiological determinants of *B. burgdorferi* and may be required for the colonization of various tissues in the C3H/HeN mouse model of Lyme disease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A clonal, noninfectious isolate of *B. burgdorferi* strain B31 (ML23; Table 1) lacking linear plasmid 25 was used for the deletion of $csrA_{Bb}$ (48, 49, 72). All *B. burgdorferi* cultures used for transformations were grown in 1% CO_2 at 32°C in BSK-II liquid medium (pH 7.6) supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR) unless otherwise stated (57, 58). We had previously shown that the level of $CsrA_{Bb}$ synthesis is increased under fed-tick conditions. We therefore propagated various *B. burgdorferi* strains (Table 1) to a density of 5×10^7 spirochetes/ml in BSK-II growth medium that mimicked the tick midgut before (pH 7.6/23°C) and after (pH 6.8/37°C) a blood meal to determine if these environmental signals altered the levels of a subset of proteins modulated by $CsrA_{Bb}$ (80). *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) and Rosetta(DE3)(pLysS) (Novagen, Madison, MI) strains were used for all procedures involving cloning and overexpression of recombinant proteins, respectively. The *E. coli* strains were cultured in Luria-Bertani (LB) broth supplemented with appropriate concentrations of antibiotics (29).

Expression and purification of recombinant proteins for generation of monospecific sera. Total genomic DNA obtained from *B. burgdorferi* clonal isolate MSK5 (Table 1) was used as the template to PCR amplify *dbpA*, *bbk32*, *bba34*, *p66*, *rpoS*, and *pncA* using forward and reverse primers containing appropriate engineered restriction enzyme sites (Table 2). The leader sequences of lipoproteins were not included to generate the expression constructs to facilitate induction of increased levels of recombinant proteins in *E. coli*. The amplicons were cloned into the pCR2.1-TOPO vector (Invitrogen) and transformed into *E. coli* TOP10 cells, and the cells were subjected to blue/white colony screen in the presence of ampicillin (100 μg/ml) and kanamycin (50 μg/ml). The inserts were excised with NdeI/XhoI and ligated into the pET23a expression vector. The ligated products were electrotransformed into *E. coli* TOP10 cells and screened by restriction enzyme digestion for the presence of the insert of the appropriate size. The junctions of plasmids containing inserts of the expected size were sequenced, and the plasmids were used to transform the *E. coli* expression host (Rosetta; Novagen). Recombinant OspC, BBK32, DbpA, BBA34, P66, RpoS, and PncA with a C-terminal 6× histidine tag were overexpressed by inducing *E. coli* strains containing appropriate plasmids with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h (29). The bacterial cells were disrupted using a French press in denaturing lysis buffer (8 M urea, pH 8), and the supernatants were collected, clarified by centrifugation, and subjected to affinity purification using nickel-nitrilotriacetic acid beads (Qiagen, Valencia, CA), according to the manufacturer's instructions. The bound 6× histidine proteins were eluted as 0.5-ml fractions with elution buffer (8 M urea, pH 4.5). The eluted proteins were analyzed by sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel electrophoresis (PAGE). Select fractions with the highest concentrations of eluted proteins were further purified using Amicon centrifugal filters (Millipore, Bedford, MA) to concentrate the proteins as well as to remove the urea used in the purification process. In some cases the proteins were purified to homogeneity

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′→3′)[a]
BBA34F	ACGCCATATGTCTAAACCAAAAGATA
BBA34R	ACGCCTCGAGGTCAAGAAAATATCTTGCTG
OspC_F/NdeI	ACGCCATATGAATAATTCAGGGAAAGATGGG
OspC_R/XhoI	ACGCCTCGAGAGGTTTTTTTGGACTTTCTGC
BBK32F/NdeI	ACGCCATATGATAAGATATGAAATGAAAGAG
BBK32R/XhoI	ACGCCTCGAGGTACCAAACGCCATTCTT
DbpA_F/NdeI	ACGCCATATGGGACTACCAGGAGCAACA
DbpA_F/XhoI	ACCCTCGAGGTTATTTTTGCATTTTTCATC
RpoS/F	ACGCCATATGAACATATTTAGTAATGAG
RpoS/R	ACGCCTCGAGATTTATTTCTTCTTTTAA
PncAF/NdeI	ACGCCATATGTTGCCAGTATCTAACAGT
PncAR/XhoI	ACGCCTCGAGTATATTAAGCTTACTTTG
csrAF	ACGCCATATGCTAGTATTGTCAAGAAAAGCA
csrAR	ACGCCTCGAGATTTTCATTCTTGAAATA
csrAR	ACGCGCGGCCGCGTGGTGGTGGTGGTGGTGAT TTTCATTTTCATTCTTGAA
csrRegF (185949–185966)[b]	AATTTATATTGACAATAT
csrAF/Sal (186949–186963)	CGCACGCACGCGTCGACTGACAATACTAGCAT
csrRegR (188178–188194)	CCAAATCTTTTTTTTAT
csrAR/Sal (187177–187191)	GCGTGCGTGCGGTCGACTTCAAGAATGAAAAT
S1 (CsrA200upF)	GGTCGGACATTAAAGCAA
S2(CsrA200downR)	CAGTGCTTATTGGGAACA
FlgBF/ERI	ACGCGAATTCGGAAGATTTCCTATTAAGG
csrAR/PstI/NotI	ACGCCGCCGGCGGACGTCAATTTTCATTCTTGA AATA
aadAR	CATTATTTGCCGACTACCTTGG
aadAF	ATGAGGGAAGCGGTGATC
aadaR/salI	ACGCGTCGACTTATTTGCCGACTACCTTGG
FlgBF/salI	ACGCGTCGACGGAAGATTTCCTATTAAGC
rpoSFRq[c]	AGATATGCGGGTAAAGGGTTAAAA
rpoSRRq	CAGCAGCTCTTATTAATCCCAAGTT
bb0185FRq	ACCCCAGTGCAGTTGTATAACTTT
bb0185RRq	AAGCACTGAATTTGGATCAATCTG
bb0183FRq	GCAATCCATCAATAAAGACGTAAGTTT
bb0183RRq	CGACCAATCTTTTTCCTGGATATTT
bosRFRq	GCATTGGAAAAAGTCGGCATT
bosRRRq	GATGCTTTCATTCCTAATTCTGATGTT

[a] Restriction enzyme sites are underlined.

[b] The numbers within parentheses refer to the nucleotide coordinates of the chromosome of *B. burgdorferi* (available at NCBI under accession number NC_001318) that correspond to the sequence of the primers used for generating the $csrA_{Bb}$ deletion mutant.

[c] Rq, quantitative real-time primers.

using an S&S Elutrap electroseparation system as described previously (47). Recombinant proteins purified to homogeneity (data not shown) were quantified by bicinchoninic acid (Pierce, Thermo Fisher Scientific, Rockford, IL) assay and stored at −80°C until further use. The purified recombinant proteins were emulsified in equal volumes of Titermax adjuvant (Sigma, St. Louis MO) and used to immunize 6- to 8-week-old female BALB/c mice. Booster immunizations were given at days 14 and 21. Immunoblot analysis was carried out to determine the specificities of the antibodies against the recombinant proteins and total borrelial lysates with the serum obtained on day 28 postimmunization (data not shown). 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.

Plasmid constructs for generation of $csrA_{Bb}$ mutant. A two-step PCR process was used to generate the $csrA_{Bb}$ deletion mutant as described previously (58). Briefly, the region of the chromosome corresponding to 1 kb up- and downstream of $csrA_{Bb}$ (*bb0184*) was amplified using primer sets (CsrRegF/CsrAF/Sal and CsrRegR/CsrAR/Sal), and each amplicon was cloned into the PCR cloning vector pCR2.1 to derive the plasmids, designated pES1 and pES4, respectively. The DNA coordinates of the chromosome of *B. burgdorferi* corresponding to the sequence for each of the primers used in the generation of these fragments are indicated in parentheses in Table 2. The insert from pES1 was excised using BamHI/SalI and cloned into pES4 digested with the same enzymes to generate pES8, resulting in a construct where $csrA_{Bb}$ was deleted by juxtaposing the up- and downstream regions of $csrA_{Bb}$ with the concomitant generation of a single SalI site. The DNA coordinates for $csrA_{Bb}$ in the borrelial chromosome are between nucleotide (nt) 186949 and nt 187194, and the two-step PCR described above resulted in the deletion of $csrA_{Bb}$ sequence from nt 186964 to nt 187177. The construct therefore retained 15 nucleotides of $csrA_{Bb}$ at the 5′ end and 18 nucleotides at the 3′ end, resulting in the deletion of 213 out of 246 nucleotides of $csrA_{Bb}$, and hence, the construct was not expected to alter the up- and downstream sequences flanking $csrA_{Bb}$. The streptomycin resistance gene *aadA* under the control of P_{flgB} with flanking SalI sites was digested and cloned into pES8 with compatible restriction enzyme sites, resulting in pES10. The junctions of plasmid pES10 were verified by sequencing and were consistent with the restriction enzyme sites used in its construction. This plasmid was used to delete $csrA_{Bb}$ in *B. burgdorferi* by homologous recombination, facilitating counterselection of mutants in the presence of streptomycin.

Generation of $csrA_{Bb}$ deletion mutant in *B. burgdorferi*. A clonal derivative of *B. burgdorferi* strain B31 lacking linear plasmid 25, ML23, was electrotransformed with pES10 (43, 57, 58, 79, 83–85). Transformants were selected on BSK-II agarose overlays containing 50 μg/ml of streptomycin as described previously (85). Individual colonies were screened by PCR using total genomic DNA as the template and forward and reverse primers S1 and S2, respectively (Table 2). Transformants of ML23/pES10 which exhibited an increase in the amplicon size by 1.5 kb due to the presence of the P_{flgB}-*aadA* marker compared to the size of wild-type (wt) parental control strain ML23 (610-bp amplicon) were considered positive and selected for further analysis. One mutant, designated ES10, was further analyzed by Southern hybridization.

Southern blot analysis. Total genomic DNA isolated from ML23 and ES10 was digested with different restriction enzymes, separated on a 1% agarose gel, transferred onto a nylon membrane, and hybridized with enhanced chemiluminescence (ECL)-labeled probes overnight at 42°C. The membranes were developed according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, United Kingdom) and as described previously (29, 57, 58).

Complementation and restoration of infectivity in ES10. The borrelial shuttle vector pBBE22 containing the minimal region of lp25 (*bbe22* encoding PncA) required to restore the infectivity of ML23 in the murine model of Lyme disease was used to transform $csrA_{Bb}$ mutant ES10 (32, 71, 90). The transformants were selected on BSK-II agarose overlays supplemented with 200 μg/ml of kanamycin and 50 μg/ml of streptomycin. The presence of pBBE22 was verified using primers specific to this borrelial shuttle vector as described previously (57, 58, 71). We also transformed ES10 with pES52 that had P_{flgB}-$csrA_{Bb}$ cloned into pBBE22 in order to restore a constitutively expressed $csrA_{Bb}$ along with the minimal region of lp25 required for infectivity (see Fig. 5G). Since we showed previously that $csrA_{Bb}$ is the terminal ORF of the *flgK* motility operon, we decided to complement $csrA_{Bb}$ in *trans* under the control of a constitutive promoter due to the limitation of cloning the entire *flgK* motility operon and due to the lack of information on the minimal upstream region of $csrA_{Bb}$ needed for its expression (34, 80).

Plasmid profiles of mutants. *B. burgdorferi* has 21 linear and circular plasmids, in addition to its ~960-kb linear chromosome, and many of them are required for virulence. Therefore, it is critical to determine the plasmid profiles of isolated clones if they are to be used in infectivity analysis. The plasmid profiles of all clones and mutants were determined by PCR using the primer sets previously described (49).

SDS-PAGE and immunoblot analysis. *B. burgdorferi* whole-cell lysates were prepared and separated on SDS–12.5% polyacrylamide gels as described previously (57, 58) The separated proteins were either visualized by Coomassie brilliant blue staining or transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond-P; GE Healthcare) and subjected to immunoblot analysis as described previously (29, 57, 58, 80). The membranes were probed with monoclonal antibodies against OspA and FlaB or mouse, rat, or rabbit antibodies against $CsrA_{Bb}$, RpoS, OspC, BBK32, DbpA, P66, BBA34, PncA, BBA64 (14), NapA, and BosR. The blots were developed following incubation with appropriate dilutions of horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-rat secondary antibodies using ECL Western blotting reagents (GE Healthcare).

RNA extraction and quantitative real-time PCR analysis. In order to determine if the differences in the levels of select determinants observed in the parental and mutant strains correlated with their transcript levels, total RNA was extracted as previously described (83). Briefly, *B. burgdorferi* cultures were propagated under conditions (either pH 7.6/32°C or pH 6.8/37°C) that resulted in differential expression of $CsrA_{Bb}$ to a density of 2×10^7 to 3×10^7 spirochetes per ml. The samples were washed in Hanks balanced salt solution, and the bacterial pellets were resuspended in RNA-Bee RNA extraction reagent (Tel-Test, Inc. Friendswood, TX) at a ratio of 0.2 ml for every 10^6 cells. Following extraction with chloroform, the RNA was precipitated with isopropanol, washed with 75% ethanol, air dried, and resuspended in RNase-free water. The RNA was treated twice at 37°C for 45 min with DNase I to remove any contaminating DNA, and the total RNA was quantified spectrophotometrically. In order to evaluate the purity of the RNA sample, real-time PCR was done using *recA*-

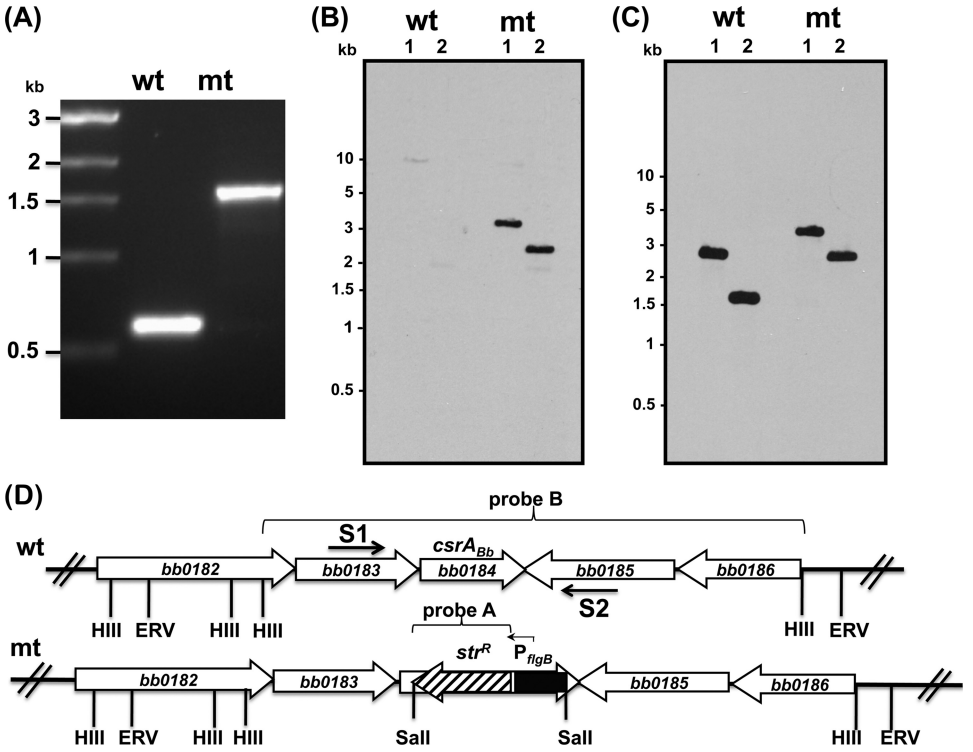

FIG. 1. PCR and Southern blotting confirm the deletion of $csrA_{Bb}$ in the chromosome of ML23 (noninfectious, lp25-deficient, clonal isolate of *B. burgdorferi* strain B31). (A) Total genomic DNA from parental strain ML23 (wt) and $csrA_{Bb}$ deletion strain ES10 (mt) was subjected to PCR analysis using primers specific to a region up- and downstream of $csrA_{Bb}$ on the linear chromosome of *B. burgdorferi* (primers S1 and S2; Table 2). An amplicon of 610 bp was amplified in the wild-type strain, whereas the size of the amplicon was 1.5 kb in the mutant, consistent with the replacement of the $csrA_{Bb}$ gene with a 1.2-kb P_{flgB}-*aadA* insert conferring streptomycin resistance. The amplicons were separated on a 1% agarose gel and visualized by staining with ethidium bromide. Molecular size markers are indicated to the left. (B and C) Total genomic DNA from ML23 parental strain (wt, lanes 1 and 2) and $csrA_{Bb}$ mutant strain ES10 (mt, lanes 1 and 2) was digested with HindIII (lane 1) or EcoRV (lanes 2). The digested DNA was separated on 1% agarose gels and transferred to nylon membranes. Blots were probed with the *aadA* gene (Str^r marker, probe A; mt in panel D) (B) and the region encompassing the chromosome around $csrA_{Bb}$ ($csrA_{Bb}$, probe B; wt in panel D) (C). The numbers on the left of each panel indicate the sizes of the markers. (D) Schematic of the $csrA_{Bb}$ region of chromosome of *B. burgdorferi* strain B31 (wt) showing the site of insertion of the streptomycin resistance marker (P_{flgB}-*aadA*) replacing $csrA_{Bb}$ (mt). The arrows indicate the orientations of primers S1 and S2, used to amplify the region of $csrA_{Bb}$ shown in panel D. HIII, HinDIII, ERV-EcoRV.

specific primers (primers recAFq and recARq) to detect contaminating DNA (94). The RNA samples devoid of contaminating DNA were reverse transcribed to cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Real-time PCRs were set up with SYBR green PCR master mix with various oligonucleotide primers (Table 2), as described previously (80), specific to *rpoS*, *ospC*, *ospA*, *flaB*, *bba64*, *dbpA*, *bbk32*, *napA*, *p66*, bosR, *bb0183*, and *bb0185* at a final concentration of 100 nM. Quantitative real-time PCR was done using an ABI Prism 7300 system (Applied Biosystems). The level of induction of each gene in ES10 relative to that in ML23 was normalized to the level of induction of *recA* as previously described (65, 94). The cycle number of the detection threshold (C_T) values of each of the genes were averaged following normalization, and the levels of induction were determined with the $\Delta\Delta C_T$ method, where the quantity of each transcript was determined by the equation $2^{-\Delta\Delta CT}$ as described previously (42, 83). To determine whether our real-time reverse transcription-PCR data were statistically significant, the fold differences in the normalized C_T values obtained for ES10 versus those obtained for ML23 were subjected to unpaired Student's *t* test implemented in Prism software. Statistical significance was accepted when the *P* values were less than 0.05.

Infectivity studies. Groups of 6-week old female C3H/HeN mice ($n = 3$) were each inoculated intradermally with either 10^3 or 10^5 spirochetes per mouse with the following borrelial strains: the wild-type strain (ML23/pBBE22) (57), the mutant strain (ES10/pBBE22), and the complemented strain (ES10/pES25). Twenty-one days after inoculation, the spleen, left tibiotarsal joint, left inguinal lymph node, heart, bladder, and a piece of abdominal skin were collected and cultured in BSK-II growth medium to facilitate isolation of spirochetes as previously described (43, 48, 57, 58, 85). The cultures were scored for growth of *B. burgdorferi* after 2 to 3 weeks using dark-field microscopy.

RESULTS

Construction of $csrA_{Bb}$ mutant. We generated a plasmid construct (pES10) where $csrA_{Bb}$ was replaced with the streptomycin resistance marker (P_{flgB}-*aadA*) flanked by up- and downstream regions of $csrA_{Bb}$. The deletion construct was generated such that it retained 15 nucleotides of $csrA_{Bb}$ at the 5′ end and 18 nucleotides at the 3′ end and, hence, was not expected to alter the up- and downstream sequences flanking $csrA_{Bb}$. This plasmid, linearized with ScaI (inactivating the ampicillin gene), was used to transform a clonal isolate of *B. burgdorferi* strain B31 lacking lp25. The transformants were selected in the presence of 50 μg/ml of streptomycin and screened by PCR using primers S1 and S2 (Table 2). As shown in Fig. 1A, when total genomic DNA from the parental strain was used, there was amplification of a 610-bp fragment, while the size of the amplicon in the mutant was approximately 1.5 kb. This was due to the replacement of the $csrA_{Bb}$ gene with a

1.2-kb P_{flgB}-*aadA* fragment, resulting in an amplicon of 1.5 kb. We further confirmed the deletion by Southern blot analysis, where the total genomic DNA from the mutant strain digested with EcoRV or HindIII hybridized to the labeled *aadA* gene, whereas there was no such hybridization with the DNA from the parental strain (Fig. 1B). Moreover, when a region of the chromosome comprising $csrA_{Bb}$ was used as a probe, there was hybridization with 2.73-kb and 1.76-kb fragments in the parental strain, whereas the sizes of these corresponding fragments in the mutant strain were 3.75 kb and 2.73 kb, respectively (Fig. 1C). This hybridization pattern is consistent with the deletion of $csrA_{Bb}$ (except for 15 nucleotides at its 5′ end and 18 nucleotides at the 3′ end) and the presence of the P_{flgB}-*aadA* conferring streptomycin resistance in the mutant strain (Fig. 1D). We then verified the plasmid profile of the mutant using the primer pairs described above, and it was identical to that of the parental strain, in that lp25 and cp9 were absent (data not shown). While the absence of cp9 has been shown to have no major limitations in the pathogenic mechanisms of *B. burgdorferi*, the absence of lp25 would render both strains noninfectious, which was overcome by complementation with pBBE22 to restore the minimal regions of lp25 required for infectivity (49, 71, 72, 77).

Deletion of $csrA_{Bb}$ results in reduced levels of RpoS and BosR. We had previously shown that clonal isolates of *B. burgdorferi* strain B31 exhibited increased levels of $CsrA_{Bb}$ when they were propagated under conditions that mimic those in the fed tick, namely, pH 6.8/37°C, than when they were propagated under regular laboratory growth conditions, which are pH 7.6/32°C (80). In order to determine the effect of deletion of $csrA_{Bb}$, we propagated ML23 and ES10 at the unfed-tick midgut (pH 7.6/23°C) and fed-tick midgut (pH 6.8/37°C) conditions and analyzed the levels of key regulators of gene expression that have been shown to play a role in the pathogenic processes. As shown in Fig. 2A, propagation of ES10 (Fig. 2A, lane 2, mt) under fed-tick conditions did not result in an increase in the levels of OspC, while there was a dramatic increase in OspC in ML23 (Fig. 2A, lane 2, wt) propagated under the same conditions. We also determined that the levels of RpoS and BosR were lower in the mutant propagated under fed-tick conditions (Fig. 2B, lanes 2, mt, α-RpoS and α-BosR) than in the parental strain grown under the same conditions. As expected, we were unable to detect $CsrA_{Bb}$ in the mutant strain grown under unfed-tick or fed-tick conditions (Fig. 2B, lanes 1 and 2, mt, α-$CsrA_{Bb}$). We also observed that the levels of $CsrA_{Bb}$, RpoS, and BosR were undetectable in the parental strains when they were propagated under unfed-tick conditions (Fig. 2B, lane 1, wt). There were no detectable variations in the levels of P66 between the parental and mutant strains grown *in vitro* under either unfed- or fed-tick conditions (22–24, 62). Since these regulators are known to affect the levels of a variety of lipoproteins that play a role in the pathogenesis of *B. burgdorferi*, we employed a panel of antisera to determine the variations in the levels of several of these determinants between the parental and mutant strains.

Deletion of $csrA_{Bb}$ results in reduced levels of select lipoproteins. Consistent with what we have observed in the previous study, where overexpression of $CsrA_{Bb}$ resulted in increased levels of select lipoproteins, deletion of $csrA_{Bb}$ resulted in reduction of the levels of these lipoproteins when *B. burgdorferi*

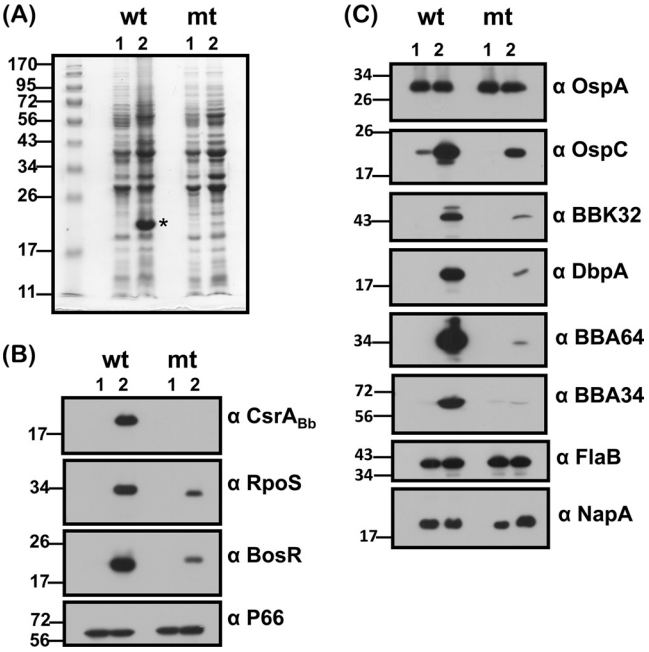

FIG. 2. Deletion of $csrA_{Bb}$ results in decreased expression of borrelial determinants associated with pathogenesis. Equivalent numbers of spirochetes from ML23 (wt) and ES10 (mt) propagated in BSK-II medium with 6% normal rabbit serum to a density of 5×10^7 spirochetes/ml under conditions that mimicked either the unfed tick (pH 7.6/23°C; lane 1) or fed tick (pH 6.8/37°C; lane 2) were resolved by SDS–12.5% PAGE. The gels were stained with Coomassie blue (A), or the separated proteins were electrotransfered onto PVDF membranes. Immunoblots were developed with antibodies consisting of mouse or rabbit serum against regulators of gene expression indicated to the right (B) or with antibodies consisting of mouse, rat, or rabbit antisera against the proteins indicated to the right (C). The blots were developed using an enhanced chemiluminescence system. The asterisk in panel A indicates increased levels of OspC in the wt strain under the fed-tick condition. The numbers to the left of the panels indicate the molecular mass standards (in kilodaltons) proximate to each of the antigens.

was propagated under fed-tick conditions. We found that the levels of OspC, BBK32, DbpA, BBA64 (14), and BBA34 (95) were reduced in the $csrA_{Bb}$ mutant (Fig. 2C, lane 2, mt) when it was propagated under fed-tick conditions, while the levels of these proteins were much higher in the parental strain, ML23 (Fig. 2C, lane 2, wt). The levels of these proteins, as expected, were lower or undetectable when either ML23 or ES10 was propagated under unfed-tick conditions (Fig. 2C, lanes 1, wt and mt). An exception to these observations are the levels of OspA, which did not exhibit differences between parental and mutant strains grown under conditions mimicking either the unfed tick or the fed tick (Fig. 2C, wt and mt, α-OspA). We found that there were no major differences in the levels of FlaB and NapA between the parental and mutant strains, even though the transcriptional level of *flaB* was less in the mutant by real-time PCR analysis (Fig. 3), arguing for probable transcriptional and posttranscriptional mechanisms of regulation of some of these borrelial determinants by $CsrA_{Bb}$. Taken together, these findings demonstrate that $CsrA_{Bb}$ is able to modulate several pathogenic determinants and hence could

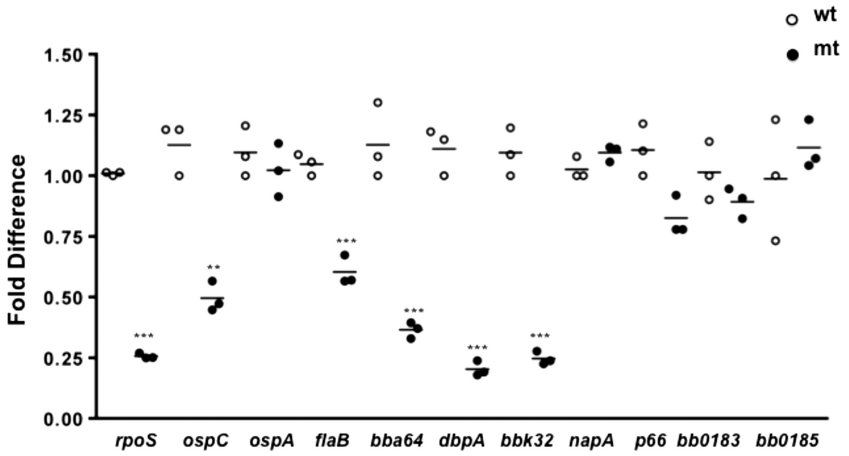

FIG. 3. Real-time reverse transcription-PCR analysis of select determinants in the $csrA_{Bb}$ mutant. RNA was isolated from ML23 (wt) and ES10 (mt) propagated in BSK-II growth medium following a shift from conditions mimicking the unfed-tick midgut (pH 7.6/23°C) to conditions mimicking the fed-tick midgut (pH 6.8/37°C) and was subjected to reverse transcription-PCR, as described in Materials and Methods. The values for all samples were normalized relative to the value for *recA*, and the change in C_T value for each transcript was obtained as an average of each sample analyzed in triplicate. The $\Delta\Delta C_T$ values for each transcript from ES10 (filled circles) relative to that for each transcript from ML23 (open circles) are shown as the fold difference on the *y* axis. Mean values are indicated by horizontal bars. The ΔC_T values obtained for ES10 and ML23 were subjected to unpaired Student's *t* test implemented in Prism software. The asterisks indicate samples whose C_T values are statistically significantly different between ES10 and ML23 (**, $P < 0.01$; ***, $P < 0.001$).

play a major role in the adaptation of *B. burgdorferi* for transmission/colonization of vertebrate hosts.

Effect of deletion of $csrA_{Bb}$ on transcriptional levels of select ORFs. In order to determine if the changes in the levels of proteins observed were due to transcriptional or posttranscriptional regulation by $CsrA_{Bb}$, we used quantitative real-time reverse transcription-PCR of total RNA obtained from the parental and mutant strains propagated at pH 6.8/37°C, where we anticipated the effect of $CsrA_{Bb}$ to be most dramatic due to increased levels of this regulator. The transcript level from *recA* was used to normalize the transcript levels of both strains. As shown in Fig. 3, there were statistically significant decreases in the levels of *rpoS*, *ospC*, *flaB*, *bba64*, *dbpA*, and *bbk32* in the mutant compared to the levels in the wild-type parental strain. Except for *flaB*, the transcript levels of all other genes were consistent with the increased levels of the respective proteins in the parental strain compared to those in the $csrA_{Bb}$ deletion strain (Fig. 2C). While we observed a significant decrease in the levels of *flaB* transcripts in the $csrA_{Bb}$ deletion strain, we did not observe a corresponding decrease in the levels of its translated products by immunoblot analysis. There were no significant differences in the transcript levels of *ospA*, *napA*, and *p66* in the mutant strain compared to those in the parental strain, and this finding correlates with the similar levels of these proteins observed on immunoblot analysis (Fig. 2C). We did not observe a significant difference in the transcript levels of *bb0183* and *bb0185*, which are located up- and downstream of $csrA_{Bb}$ in the parental or mutant strains, suggesting that the deletion of $csrA_{Bb}$ did not result in polar effects. Since several of the genes whose levels were elevated are transcriptionally regulated by RpoS, we determined the levels of *rpoS* that are induced when parental and mutant strains were propagated at pH 7.6/32°C (where the protein levels of $CsrA_{Bb}$ were minimal in the wild-type strain) or shifted from pH 7.6/23°C to pH 6.8/37°C (where the protein levels of $CsrA_{Bb}$ were substantial) so as to evaluate the contributions of $csrA_{Bb}$ to the *rpoS* transcript levels (Fig. 4). As shown in Fig. 4, there was a significant increase in *rpoS* levels in the parental strain compared to those in the $csrA_{Bb}$ mutant propagated under fed-tick conditions. We also determined the levels of *bosR* transcripts and found that there was a significant reduction in the $csrA_{Bb}$ mutant compared to the levels in the parental strain ($P < 0.001$) under fed-tick conditions. While the total levels of induction of *bosR* transcripts upon shifting of the growth conditions from unfed- to fed-tick conditions were lower than those of *rpoS*, there was still a significant difference in the level of *bosR* transcripts both in the parental ($P < 0.05$) and in the $csrA_{Bb}$ mutant ($P < 0.01$) strains between these two conditions. Taken together, these observations demonstrate that $CsrA_{Bb}$ plays a major role in regulating the levels of lipoproteins or their key regulators and thereby could affect the infectivity potential of *B. burgdorferi*.

Complementation of $csrA_{Bb}$ mutant. Since we generated the mutant in an lp25-negative clone of *B. burgdorferi* strain B31, we complemented the mutant strain with pBBE22 in order to restore the minimal region of infectivity (*pncA*) present on lp25. We also cloned $csrA_{Bb}$ under the control of a constitutive borrelial promoter, P_{flgB}, into pBBE22 and isolated borrelial colonies on BSK-II agar overlays supplemented with streptomycin and kanamycin. The rationale for this approach is (i) to take the best first approach to generate a plasmid that will facilitate complementation of $csrA_{Bb}$ in *trans* on a shuttle vector that will also provide the minimum region of lp25 required for restoring infectivity in the borrelial strain used for generating the mutant; (ii) to overcome the physical limitation of the entire DNA segment comprising the *flgK* operon, including its promoter regions, since $csrA_{Bb}$ is the terminal ORF of this motility operon (34, 80); (iii) a lack of evidence of whether $csrA_{Bb}$ transcript levels are driven only by the promoter present upstream of the first ORF of this operon (*bb0180*, *flbF*) or whether there are additional internal promoters immediately

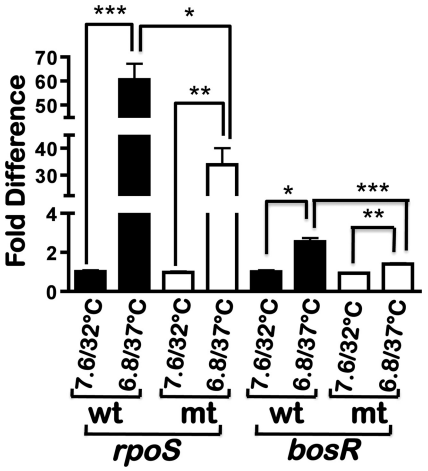

FIG. 4. Real-time reverse transcription-PCR analysis of levels of *rpoS* and *bosR* in *csrA*$_{Bb}$ mutant propagated at pH 7.6/32°C (undetectable levels of $CsrA_{Bb}$) or pH 6.8/37°C (increased levels of $CsrA_{Bb}$). RNA was isolated from ML23 (wt) and ES10 (mt) propagated in BSK-II growth medium following a shift from conditions mimicking the unfed-tick midgut (pH 7.6/23°C) to conditions mimicking the fed-tick midgut (pH 6.8/37°C) or propagated under laboratory growth conditions (pH 7.6/32°C) and was subjected to reverse transcription-PCR using primers specific to *rpoS*, *bosR*, or *recA*, as described in Materials and Methods. The values for all samples were normalized relative to the value for *recA*, and the change in C_T values for *rpoS* and *bosR* was obtained as an average of each sample analyzed in triplicate. The $\Delta\Delta C_T$ values for each transcript from ES10 (open bars) relative to each transcript from ML23 (filled bars) are shown as fold difference on the *y* axis, and error bars are indicated. The ΔC_T values obtained for ES10 and ML23 were subjected to unpaired Student's *t* test implemented in Prism software. The asterisks indicate samples whose C_T values are statistically significantly different between ES10 and ML23 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

upstream of *csrA*$_{Bb}$; and (iv) the fact that transcript stability of a large mRNA species of a multigene operon of which *csrA*$_{Bb}$ is a component could by itself alter the protein levels of $CsrA_{Bb}$. It is also feasible, though not proven, that the expression of *csrA*$_{Bb}$ under the control of the *flbF* promoter may not be regulated similarly to its expression under the control of a constitutive *flgB* promoter. Hence, we derived plasmid pES52 from pBBE22 by cloning *csrA*$_{Bb}$ in *trans* and the minimal region of lp25 required for infectivity in the C3H/HeN mouse model of Lyme disease. The transformed colonies were readily identified on the basis of the appearance of a pinpoint colony morphology suggestive of the expression levels of $CsrA_{Bb}$ from a constitutive promoter, P_{flgB}, similar to what was observed in the overexpression strain (ES25) (80). The colonies were screened for the presence of pBBE22 by PCR, and a clone designated ES52 was verified for the expression of a select set of proteins, such as OspC, OspA, DbpA, and BBA64, as well as for colony morphology. As shown in Fig. 5B, there was increased expression of OspC, DbpA, and BBA64 in the complemented strain compared to that in the mutant strain, and the levels were comparable to what were observed in the parental strain for all proteins except DbpA. There were no overt differences, other than the levels of FlaB, in the total protein profile in the complemented strains and the parental or mutant strain (Fig. 5A and B, α-FlaB). The above observation was consistent with what was observed in strain ES25, where there was a decrease in the levels of FlaB upon increased synthesis of $CsrA_{Bb}$ from a shuttle vector expressing *csrA*$_{Bb}$, in *trans*, under the control of P_{flgB} (80). We also examined the spirochetes labeled with BacLight Live/Dead stain using confocal microscopy and observed that the complemented strain had a rod-shaped morphology, whereas the parental and mutant strains had a waveform morphology (Fig. 5C). This phenotype is consistent with the synthesis of $CsrA_{Bb}$, as we have observed this phenotype in the borrelial strain (ES25) expressing both the native copy of *csrA*$_{Bb}$ and a copy from the shuttle vector driven by a constitutive promoter, P_{flgB} (80). It needs to be emphasized that even though the levels of several determinants (OspC, OspA, NapA, and P66) were similar in the complemented and parental strains, the levels of BosR were much lower in the complemented strain (Fig. 5B). Since the levels of key determinants that contribute to the colonization capabilities are reduced rather than completely absent in the complemented strain, we decided to determine the infectivity potential of the *csrA*$_{Bb}$ mutant and its partially complemented strain in the C3H/HeN mouse model of Lyme disease.

$CsrA_{Bb}$ is required for the infection in the C3H/HeN mouse model of Lyme disease following intradermal needle inoculation. As shown in Table 3, we were unable to isolate the *csrA*$_{Bb}$ mutant from any of the tissues from the C3H/HeN mice infected with either 10^3 or 10^5 spirochetes per mouse, while the parental strain was able to infect and colonize C3H/HeN mice at both doses (two of three and three of three mice infected with 10^3 or 10^5 spirochetes per mouse, respectively). This demonstrated that $CsrA_{Bb}$ is critical for infection following intradermal needle inoculation in the mouse model of Lyme disease and that this *in vivo* phenotype is presumably due to the decrease in the levels of a variety of determinants involved in the pathogenic processes of *B. burgdorferi*.

We were surprised to note that our complemented strain was incapable of establishing infection in the C3H/HeN mice, even at a dose of 10^5 spirochetes per mouse, following intradermal needle inoculation, even though the levels of OspC were similar to those of the parental strain by immunoblot analysis. In order to further determine the reasons for the lack of infectivity associated with the complemented strain, we expanded our *in vitro* phenotypic analysis of all three strains. Transcriptional analysis of the levels of expression of *bb0183* and *bb0185* did not reveal any significant differences between the parental and mutant strains, suggesting that the deletion of *csrA*$_{Bb}$, *per se*, did not have any polar effects that could have altered the expression levels of the up- or downstream ORFs contributing to the lack of infectivity of the mutant and complemented strains. We analyzed the levels of select determinants in borrelial lysates prepared from parental, mutant, or complemented strains propagated after the growth conditions were shifted from 23°C/pH 7.6 (unfed-tick midgut condition) to 37°C/pH 6.8 (fed-tick midgut condition) (Fig. 5D). Immunoblot analysis revealed reduced levels of $CsrA_{Bb}$, RpoS, BosR, and OspC in the complemented strain compared to those in the wild-type parental strain (Fig. 5D), even though the changes in the levels of FlaB were consistent with the in *trans* expression of *csrA*$_{Bb}$. In addition, we also examined the levels of expression of PncA synthesized from either pBBE22 or pES52 (pBBE22 with an intact copy of *csrA*$_{Bb}$), used to provide the minimal regions of lp25 required for infectivity for the

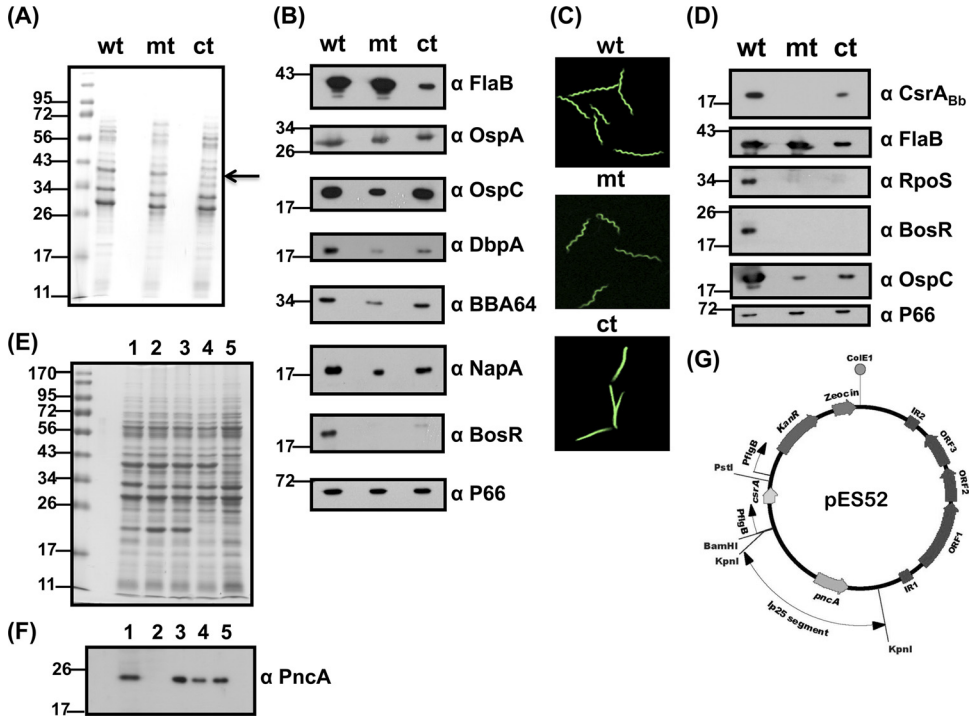

FIG. 5. Complementation analysis of *csrA*$_{Bb}$ deletion mutant. (A) Equivalent number of spirochetes from ML23/pBBE22 (wt), ES10/pBBE22 (mt), and ES10/pES52 (complemented [ct]) propagated in BSK-II medium with 6% normal rabbit serum at pH 7.6/32°C to a density of 5×10^7 spirochetes/ml were resolved by SDS–12.5% PAGE. The gels were stained with Coomassie blue or the separated proteins were electrotransfered onto PVDF membranes. The arrow in panel A corresponds to the FlaB protein in *B. burgdorferi.* (B) Immunoblots were developed with mouse, rat, or rabbit serum against the borrelial antigens indicated to the right side of each blot. (C) The spirochetes were stained with BacLight Live/Dead stain, visualized using a Zeiss LSM510 microscope, and deconvolved using AutoQuant X image deconvolution software. Representative images are shown indicating the loss of the waveform morphology observed in the complemented strain indicating constitutive expression of CsrA$_{Bb}$ in *trans* from the shuttle vector pBBE22/P$_{flgB}$-*csrA*$_{Bb}$. (D) Immunoblot analysis of borrelial strains propagated by shifting the growth conditions from those for an unfed tick (pH 7.6/23°C) to those for a fed tick (pH 6.8/37°C) with the antibody reagent indicated to the right of each of the blots. The blots were developed using an enhanced chemiluminescence system, and the molecular mass markers (in kilodaltons) are indicated to the left of each blot. (E) Equivalent numbers of spirochetes from MSK5 (lane 1), ML23 (lane 2), ML23/pBBE22 (lane 3, wt), ES10/pBBE22 (lane 4, mt), and ES10/pES52 (lane 5, ct) propagated in BSK-II medium with 6% normal rabbit serum at pH 7.6/32°C to a density of 5×10^7 spirochetes per ml were resolved by SDS–12.5% PAGE. The gels were either stained with Coomassie blue as a control for equivalent loading of proteins or (F) transferred to PVDF membranes and probed with anti-PncA antibodies. The blots were developed using an enhanced chemiluminescence system, and the molecular mass markers (in kilodaltons) are indicated to the left of each blot. (G) Schematic representation of pES52 on the basis of the sequences of pBSV2 (90) and pBBE22 (71). The construction of this plasmid is described in Materials and Methods. P$_{flgB}$, promoter sequence from the *flgB* gene of *B. burgdorferi*; kanR, kanamycin resistance gene; *csrA*, carbon storage regulator A of *B. burgdorferi.*

parental and mutant strains and the complemented strains, respectively. There were no differences in the levels of PncA between these strains (Fig. 5F, lanes 3, 4, and 5). The levels of PncA detected in these strains were similar to the levels present in a clonal isolate of *B. burgdorferi* strain B31 containing the full complement of plasmids (strain MSK5; Fig. 5F, lane 1), while there was no reactivity in the clonal isolate lacking lp25 (strain ML23; Fig. 5F, lane 2). The lack of infec-

TABLE 3. Infectivity analysis of *csrA*$_{Bb}$ mutant in C3H/HeN mice at 21 days postinfection

Strain and dose (no. of spirochetes/mouse)	No. of cultures positive/no. tested							No. of mice infected/no. tested
	Skin	Spleen	Joint	Lymph node	Heart	Bladder	All sites	
wt (ML23/E22)								
10^3	1/3	0/3	2/3	2/3	2/3	2/3	9/18	2/3
10^5	3/3	1/3	1/3	3/3	3/3	3/3	14/18	3/3
mt (ES10/pBBE22)								
10^3	0/3	0/3	0/3	0/3	0/3	0/3	0/18	0/3
10^5	0/3	0/3	0/3	0/3	0/3	0/3	0/18	0/3
Complemented (ES10/pES52)								
10^3	0/3	0/3	0/3	0/3	0/3	0/3	0/18	0/3
10^5	0/3	0/3	0/3	0/3	0/3	0/3	0/18	0/3

tivity with the complemented strain therefore appears to be unrelated to the levels of other factors essential for infection, such as PncA, but rather appears to be due to the reduced levels of $CsrA_{Bb}$ generated in *trans* from the shuttle vector, pES52.

In spite of the limitation on the lack of complementation of the $csrA_{Bb}$ mutation, the observation that deletion of $csrA_{Bb}$ resulted in a strain that was incapable of establishing infection in the C3H/HeN mouse model demonstrates that $CsrA_{Bb}$ plays a central role in regulating expression of determinants critical for infection. This *in vivo* phenotype of the $csrA_{Bb}$ mutant, as the data suggest, could be due to the direct or indirect effects of $CsrA_{Bb}$ on the levels of RpoS and BosR, which in turn drastically altered the levels of key determinants of pathogenesis. It is also feasible that lack of expression of $CsrA_{Bb}$ could have a direct effect on the levels of expression of other unknown factors contributing to infectivity of *B. burgdorferi* in the C3H/HeN mice. Even though we were unable to restore the wild-type infectivity in the complemented strain, it did give insights into the significance of the role of $CsrA_{Bb}$, notably, from the standpoint of the need for optimal levels of $CsrA_{Bb}$ and its effect on other key regulators to recapitulate the infectivity phenotype of the parental strain in the C3H/HeN mouse model of Lyme disease.

DISCUSSION

We have recently shown that the homolog of CsrA present in *B. burgdorferi* has certain unique properties that are relevant to the pathophysiology of the agent of Lyme disease (80). We observed that (i) when *B. burgdorferi* was propagated under conditions that mimic the fed-tick midgut (pH 6.8/37°C), the levels of $CsrA_{Bb}$ were elevated compared to those obtained under conditions that mimic the unfed-tick midgut (pH 7.6/23°C); (ii) increased levels of $CsrA_{Bb}$ due to expression from a strong constitutive promoter resulted in increased levels of several lipoproteins; (iii) the levels of some of the borrelial determinants, such as OspC and BBA64, were increased upon overexpression of $CsrA_{Bb}$ independent of the shifting of growth conditions from unfed-tick to fed-tick conditions; and (iv) $csrA_{Bb}$ was the terminal ORF of the *flgK* motility operon and affects the levels of the major flagellin, FlaB. A caveat in this study was the expression of $CsrA_{Bb}$ from a borrelial shuttle vector in *B. burgdorferi* under the control of a constitutive promoter (P_{flgB}) in the presence of a native copy of $csrA_{Bb}$ in the chromosome. As a follow-up to this study, we isolated a $csrA_{Bb}$ null mutant and found that it did not exhibit altered morphology or any apparent growth defect (data not shown). There was no synthesis of $CsrA_{Bb}$ in the mutant, as expected, under both the unfed- and fed-tick conditions (Fig. 2B, lanes 1 and 2, mt), while there were increased levels when the parental strain was grown under conditions that mimic those in the fed tick compared to those obtained under conditions that mimic the unfed tick (Fig. 2B, lanes 1 and 2, wt). Consistent with the observations reported in the previous study, the deletion of $csrA_{Bb}$ resulted in reduced levels of several key determinants involved in the pathogenic mechanisms of *B. burgdorferi*, such as OspC, BBK32, DbpA, BBA64, and BBA34, in the mutant (Fig. 2C, lane 2, mt) compared to the parental strain (Fig. 2C, lane 2, wt) propagated under fed-tick conditions. The levels of these proteins were minimal, as expected when both the wild-type and mutant strains were propagated under unfed-tick conditions (Fig. 2C, lanes 1, wt and mt). At the same time, several other proteins, such as OspA, FlaB, and NapA, did not show major differences in the levels of expression between the parental and mutant strains, nor did the levels change under different growth conditions (Fig. 2C, lanes 1 and 2, wt and mt). Moreover, contrary to a recent observation, under the growth conditions employed in our analysis (with 1% CO_2), we did not see a change in the level of NapA in response to reduced levels of BosR observed with the $csrA_{Bb}$ mutant or when the parental strain was propagated under unfed-tick midgut conditions, where the levels of this oxidative stress regulator were minimal (44). Collectively, these findings suggest that the regulatory functions of $CsrA_{Bb}$ are specific to select borrelial determinants and that this regulation occurs either directly or indirectly through the modulation of other key regulators, such as RpoS and BosR (30). The exact molecular interaction between these regulators or if there is a hierarchy of regulators controlled by yet to be identified intermediates remains to be determined.

While it has been known for some time that several key pathogenesis-related proteins are under the control of a central regulatory pathway comprising Rrp2-RpoN-RpoS, recently it has been shown that BosR, a regulator of oxidative stress response proteins such as SodA, also regulates the levels of major lipoproteins involved in the pathogenic processes (29, 44, 68). Since we observed a drastic reduction in the levels of the same lipoproteins regulated by RpoS and BosR in the $csrA_{Bb}$ mutant, we determined the levels of the above regulators by immunoblot assay. To our surprise, we found that the $csrA_{Bb}$ mutant had drastically lower levels of both RpoS and BosR than the wild-type control strain when they were propagated under fed-tick condition (Fig. 2B, lanes 2, wt and mt). The levels of these regulators were not detectable in either strain when they were propagated under unfed-tick conditions (Fig. 2B, lane 1, wt and mt), and this trend is similar to what has been observed with expression levels of $CsrA_{Bb}$. Taken together, these observations clearly demonstrate that $CsrA_{Bb}$ plays a key role in regulating select proteins that are known to regulate the mediators of the pathogenic mechanisms of *B. burgdorferi* (19, 32, 41). Since both RpoS and BosR are reduced in levels in the $csrA_{Bb}$ mutant propagated under the fed-tick conditions, it is interesting to speculate that the increased levels of $CsrA_{Bb}$ may either regulate the transcriptional levels of these regulators or stabilize the corresponding protein products, which in turn could lead to increased synthesis of select lipoproteins (10). We therefore determined the transcriptional levels of select proteins that are differentially synthesized in the $csrA_{Bb}$ mutant and the parental control strain under conditions where there were maximal levels of synthesis of $CsrA_{Bb}$.

The CsrA homologs in several other pathogenic bacteria are known to alter gene expression by interacting with small RNA molecules (CsrB/CsrC), which in turn titrate the levels of CsrA to limit its effects on levels of expression of proteins (6, 7, 75, 81). While we are yet to identify the homologs of CsrB/CsrC, if they exist, in *B. burgdorferi*, we isolated total RNA following propagation of the spirochetes under fed-tick conditions to capture the effects of increased levels of $CsrA_{Bb}$ observed under these conditions on transcript levels of other regulators/

lipoproteins. We determined that there was a significant decrease in the levels of transcripts specific to *rpoS*, *bosR*, *ospC*, *flaB*, *bba64*, *dbpA*, and *bbk32* in the mutant compared to the parental strain by quantitative real-time PCR analysis (Fig. 3). The transcript levels of *ospA*, *napA*, and *p66* in the $csrA_{Bb}$ mutant, on the other hand, were not different from those of the parental strain, consistent with what was observed at the protein level. These observations suggest that borrelial determinants that are differentially expressed between the parental and mutant strains were due to transcriptional differences rather than posttranscriptional regulation, which is known to be mediated by CsrA homologs in other bacterial species (63, 64, 81). It is also possible that $CsrA_{Bb}$ may regulate levels of *rpoS* and *bosR* posttranscriptionally, which in turn could alter the transcriptional levels of members of their respective regulons. We further determined if the levels of *rpoS* transcripts in the parental and mutants strains were significantly different following the change in their growth conditions from where the levels of $CsrA_{Bb}$ were minimal (pH 7.6/32°C) or from where the levels of $CsrA_{Bb}$ were maximal (pH 6.8/37°C). As expected, there was a significant increase in the levels of *rpoS* following the shift from pH 7.6/32°C to pH 6.8/37°C in both the parental and mutant strains. At the same time, the levels of increase in the *rpoS* transcripts were much higher in the parental strain (60-fold increase) than in the $csrA_{Bb}$ mutant (30-fold increase). While it is not possible to unequivocally state that the increase in the levels of *rpoS* in the parental strain is due to either increased transcription from the *rpoS* gene or increased stability of the *rpoS* transcripts, it is clear that one possible mechanism for differences in the levels of members of the *rpoS* regulon between the parental and mutant strains is the level of *rpoS* transcripts. This observation is unique to the *csrA* homolog of *B. burgdorferi*, in that there is a reduction in the levels of *rpoS* transcripts in the $csrA_{Bb}$ mutant compared to those in the parental strain under environmental conditions that are known to induce increased levels of both the *rpoS* transcripts and the members of its regulon (20, 25).

The connection between CsrA and RpoS in other bacterial systems, notably, in *E. coli*, has been evaluated, where CsrA represses stationary-phase genes involved in gluconeogenesis and glycogen biosynthesis, while RpoS has the opposite effect (50–52, 96). It is therefore interesting to speculate that the connection between borrelial *rpoS* and $csrA_{Bb}$ could be mediated by the ratio of accumulation of certain metabolites such as acetate or acetyl phosphate in the $csrA_{Bb}$ mutant that may play a role in increasing the level of *rpoS* transcription or act as a signal for proteolysis of RpoS, respectively, via certain proteases (12, 31). Indeed, *B. burgdorferi* has homologs of both phosphotransacetylase (*pta*, BB0589) and acetate kinase (*ackA*, BB0622) that could contribute to accumulation of acetyl phosphate from acetyl coenzyme A and P_i or acetate and ATP, respectively, depending on the availability of select host-specific nutrients (12, 54, 59, 60), and a recent report has established the relevance of these metabolites in altering the Rrp2-RpoN-RpoS pathway (97). A link between *rpoS* and *csrA* has also been established in *Legionella pneumophila* through the regulation of *csrB* homologs *rsmY* and *rsmZ*, which are regulated by a two-component system, LetAS (78). Hence, it is possible that the regulation of levels of RpoS in *B. burgdorferi* could be due to the accumulation of key metabolites of carbohydrate metabolism in the $csrA_{Bb}$ mutant or through the functions of other undetermined intermediates.

There are significant implications to the reduction in the levels/stability of the *rpoS*/*bosR* transcripts in the $csrA_{Bb}$ mutant in terms of the levels of key determinants associated with the pathogenic mechanisms of *B. burgdorferi*. The $csrA_{Bb}$ mutant was incapable of infection at doses of both 10^3 and 10^5 spirochetes per mouse, while the parental strain was capable of colonization in two of three mice at the lower dose and all three mice at the higher dose in the C3H/HeN mouse model of Lyme disease following intradermal needle inoculation. This demonstrates that the combined effect of reduction in the levels of a variety of borrelial antigens, most notably, OspC, DbpA, and BBK32, and the levels of other unknown determinants on colonization and dissemination of *B. burgdorferi* in the vertebrate host is profound. This is consistent with what has previously been shown, that is, that there is an increase in the level of $CsrA_{Bb}$ under fed-tick conditions, which in turns facilitates increased synthesis of key determinants needed for successful colonization of the vertebrate host (80). Taken together, these observations demonstrate that $CsrA_{Bb}$ is required for the infectious process of *B. burgdorferi* in the C3H/HeN model of Lyme disease.

We were surprised to observe that the $csrA_{Bb}$ mutant complemented in *trans* with a functional copy of $csrA_{Bb}$ on a shuttle vector was incapable of colonization in C3H/HeN mice at a dose of either 10^3 or 10^5 spirochetes per mouse. We initially screened for the levels of a select set of proteins known to play a role in the infectious process and found that the levels of OspA, OspC, BBA64, NapA, and P66 were similar to those in the parental strain (Fig. 5C). This analysis was carried out with parental, mutant, and complemented strains propagated at pH 7.6/32°C. Since $csrA_{Bb}$ was under the control of a constitutive promoter in the complemented strain, we expected that the levels of $CsrA_{Bb}$ should be independent of environmental signals, such as temperature and pH. Moreover, other than the levels of FlaB (lower in the complemented strain), several immunogenic antigens were comparable between the parental and complemented strain (Fig. 5A and B). The levels of DbpA were slightly higher in the complemented strain than in the mutant but were lower than those in the parental strain. We also observed that the levels of BosR were not restored to wild-type levels upon complementation and that the levels of FlaB were reduced, consistent with in *trans* expression of $CsrA_{Bb}$. Based on these observations, however, we decided to test the complemented strain along with parental and mutant strains with the expectation that there would be some level of infection at least at higher doses (10^5 spirochetes per mouse) in the C3H/HeN mice. On the contrary, we observed that the complemented strain was incapable of establishing infection even at the high dose, suggesting that the restoration of a select set of determinants alone to wild-type levels was insufficient for infectivity. We then examined the cells following BacLight Live/Dead staining and found that the complemented strain had an altered morphology (rod shaped), whereas the parental and mutant strains had the typical waveform morphology (Fig. 5C). While this phenotype was indicative of complementation of $csrA_{Bb}$ in *trans*, the reduction in the levels of FlaB could also be a factor contributing to the lack of infection associated with the complemented strain. We shifted the growth conditions of

all three strains from unfed-tick (pH 7.6/23°C) to fed-tick (pH 6.8/37°C) conditions and found that the complemented strain was indeed deficient in levels of $CsrA_{Bb}$, RpoS, BosR, and other members of their respective regulons, such as OspC (Fig. 5D), collectively contributing to the lack of infectivity of the complemented strain. Since *csrA*$_{Bb}$ (*bb0184*) is the terminal ORF of the *flgK* motility operon and is expressed as a part of a polycistronic message, neither the upstream (*bb0183*) nor the downstream (*bb0185*) ORF was transcriptionally different between the parent and mutant strains, indicating that their levels are not a factor contributing to the lack of infectivity associated with the mutant or the complemented strain (Fig. 3). In addition, we also verified the levels of PncA in all three strains since we used a noninfectious lp25-deficient strain (ML23) to generate the mutant. As shown in Fig. 5F, we did not see any difference in the levels of this enzyme, which is essential for infectivity in all three strains, and therefore, the lack of infectivity associated with the mutant and complemented strains is not due to a lack of PncA or due to polar effects of deletion of *csrA*$_{Bb}$. Even though the lack of complementation of the wild-type infectious phenotype was a limitation in this study, it appears that the regulatory role of $CsrA_{Bb}$ is required for infectivity of *B. burgdorferi* in the mouse model of Lyme disease. These observations, collectively, underscore the importance of the regulated levels of synthesis of $CsrA_{Bb}$ at sufficient levels during the transition of the spirochetes from unfed-tick to fed-tick conditions in regulating the pathogenic mechanisms needed for establishment of infection in vertebrate hosts.

The observations presented in this report indicate that the $CsrA_{Bb}$ may interact with RpoS or BosR directly to either stabilize the transcripts or regulate the protein levels of RpoS via the expression of an unknown protease. Alternatively, if $CsrA_{Bb}$ was directly responsible for the transcriptional levels of *rpoS*, it could be expected that the transcript levels are nonexistent in the mutant strain. The latter scenario is unlikely, based on the levels of *rpoS* observed in the mutant strain (Fig. 4). There is precedence for this type of regulation of the *rpoS* transcripts, where a small, noncoding RNA, $DsrA_{Bb}$, extensively base pairs with the upstream region of *rpoS*, resulting in destabilization of a stable secondary structure, leading to increased translational efficiency (56). A borrelial mutant lacking *dsrA*$_{Bb}$ was incapable of upregulating the levels of RpoS and OspC in response to temperature, while their regulation was unaffected in response to pH and cell density (56). While we have not delineated the role of individual environmental signals, such as temperature, pH, and the conditions of the media, associated with increased cell density on the levels of $CsrA_{Bb}$, the alterations in the levels of other key regulators, such as RpoS and BosR, suggest that a small set of regulators with a complex network of interactions leads to a fine-tuned gene regulatory mechanism facilitating the adaptation of *B. burgdorferi* to hosts with vastly different environmental conditions. Moreover, the lack of a large number of small noncoding RNA molecules in *B. burgdorferi* (67) adds to the possibility that signal-dependent gene expression may be regulated at multiple levels by a small set of regulators and their cognate small molecules, which in turn may converge these signals for direct or indirect modulation of the levels of RpoS/BosR for regulating the levels of the virulence determinants of *B. burgdorferi*. It will be interesting to determine the molecular basis of the interaction of $CsrA_{Bb}$ with RpoS and BosR, which will further our understanding of the regulatory networks operative in *B. burgdorferi*. Taken together, these observations indicate that $CsrA_{Bb}$ is a global regulator of gene expression affecting levels of expression of several pathogenic determinants via RpoS and BosR and is required for infection of *B. burgdorferi* in the mouse model of Lyme disease. The mechanisms and the hierarchy of interactions between these key regulators, which play a complex, yet unique, role in regulating gene expression in *B. burgdorferi* remain to determined.

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