CsrA (BB0184) Is Not Involved in Activation of the RpoN-RpoS Regulatory Pathway in *Borrelia burgdorferi*

Zhiming Ouyang, Jianli Zhou, Michael V. Norgard

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

Borrelia burgdorferi encodes a homologue of the bacterial carbon storage regulator A (CsrA). Recently, it was reported that CsrA contributes to *B. burgdorferi* infectivity and is required for the activation of the central RpoN-RpoS regulatory pathway. However, many questions concerning the function of CsrA in *B. burgdorferi* gene regulation remain unanswered. In particular, there are conflicting reports concerning the molecular details of how CsrA may modulate *rpoS* expression and, thus, how CsrA may influence the RpoN-RpoS pathway in *B. burgdorferi*. To address these key discrepancies, we examined the role of CsrA in differential gene expression in the Lyme disease spirochete. Upon engineering an inducible *csrA* expression system in *B. burgdorferi*, controlled hyperexpression of CsrA in a merodiploid strain did not significantly alter the protein and transcript levels of *bosR*, *rpoS*, and RpoS-dependent genes (such as *ospC* and *dbpA*). In addition, we constructed isogenic *csrA* mutants in two widely used infectious *B. burgdorferi* strains. When expression of *bosR*, *rpoS*, *ospC*, and *dbpA* was compared between the *csrA* mutants and their wild-type counterparts, no detectable differences were observed. Finally, animal studies indicated that the *csrA* mutants remained infectious for and virulent in mice. Analyses of *B. burgdorferi* gene expression in mouse tissues showed comparable levels of *rpoS* transcripts by the *csrA* mutants and the parental strains. Taken together, these results constitute compelling evidence that CsrA is not involved in activation of the RpoN-RpoS pathway and is dispensable for mammalian infectious processes carried out by *B. burgdorferi*.

Borrelia burgdorferi, the etiological agent of Lyme disease, survives in nature via a complex enzootic life cycle involving *Ixodes* tick vectors and various mammalian hosts (1, 2). As it cycles between these two diverse niches, *B. burgdorferi* undergoes dramatic adaptive changes. This host adaptation is primarily mediated through differential gene expression in response to specific tick or host environmental signals (3–10). To date, a variety of factors with putative regulatory functions have been identified in *B. burgdorferi* (10–12). Among these is a novel regulatory cascade, the RpoN-RpoS pathway, which is central for *B. burgdorferi* virulence and host adaptation. This pathway is composed of two alternative sigma factors, σ^S (RpoS) and σ^{54} (RpoN), in which σ^{54} directly binds to a canonical $-24/-12$ promoter and initiates *rpoS* transcription (13, 14). When RpoS is produced, it modulates the expression of key virulence-associated outer membrane lipoproteins, such as outer surface protein C (OspC), OspA, decorin binding protein B (DbpB), and DbpA (13, 15–28). As in other bacterial σ^{54} -dependent systems, a putative AAA+ activator ATPase, also known as the bacterial enhancer binding protein (bEBP) Rrp2, is required for the transcriptional activation of *rpoS* in *B. burgdorferi* (21, 29–33). Uniquely, *rpoS* transcription also requires another key factor, the Fur homologue BosR (34–39). Although many molecular details behind the activation of *rpoS* by BosR remain unknown, our recent studies revealed that BosR directly activates *rpoS* transcription through binding to a novel DNA sequence (38). In addition, expression of *rpoS* has also been reported to be influenced by a small noncoding RNA, *B. burgdorferi* DsrA (DsrA_{Bb}); its chaperone, Hfq (40, 41); and a putative RNA-binding protein, BB0184 (42–47).

The *B. burgdorferi* protein designated BB0184 was originally annotated as a homologue of carbon storage regulator A (CsrA) (11). In other bacteria, such as *Escherichia coli*, CsrA, together with three other components (CsrB, CsrC, and CsrD), constitutes a global Csr system (48–52). As a key factor determining the func-

tion of the entire system, CsrA primarily modulates gene expression at the posttranscriptional level. Specifically, CsrA binds as a homodimer to a consensus sequence (5'-RUACARGGAUGU-3') on its target mRNA or its small regulator RNAs. When CsrA binds to a site near the Shine-Dalgarno sequence, it typically blocks the binding of ribosomes and facilitates mRNA decay, thus preventing the translation of the target mRNA. CsrA has also been reported to function as a positive regulator to enhance gene expression (49, 51, 53). In this positive regulation, the binding of CsrA stabilizes mRNA, which in turn leads to the increase of target transcripts and subsequently enhances gene translation. The activity of CsrA is tightly regulated by CsrB and CsrC, two small noncoding regulatory RNAs containing 18 or 9 CsrA binding sites, respectively (48, 49). Through these CsrA binding sites, CsrB and CsrC sequester CsrA and thus impede the binding of CsrA to its target mRNA. In addition, the CsrA system contains a membrane-bound protein, CsrD (48, 49, 51, 54). As a negative regulatory protein, CsrD binds with high affinity to CsrB and CsrC, stimulates the degradation of these two small RNAs, and antagonizes their activities. In a wide variety of bacterial pathogens, the Csr system is involved in numerous cellular functions such as central

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Address correspondence to Michael V. Norgard, michael.norgard@utsouthwestern.edu.

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carbon metabolism, quorum sensing, biofilm formation, motility, stress responses, and the production of virulence factors (48–51). As such, the Csr system, in particular, its key determinant, CsrA, plays important roles in bacterial physiology and pathogenesis.

The potential role of CsrA in *B. burgdorferi* gene regulation was first reported in a CsrA hyperexpression study (44). By employing a CsrA expression shuttle vector, Sanjuan et al. reported that the syntheses of several virulence-associated lipoproteins, including OspC, DbpA, BBK32, and BBA64, were highly increased when CsrA was hyperexpressed in a noninfectious *B. burgdorferi* B31 strain, isolate ML23 (44). The compelling evidence that the expression of these proteins is controlled by RpoS in *B. burgdorferi* (13, 16, 19, 21, 55–58) engendered the notion that CsrA may activate expression of these genes through RpoS. Subsequently, Karna et al. (43), from the same lab, reported that, relative to gene expression in the noninfectious parental strain ML23, production of RpoS and RpoS-dependent OspC, DbpA, BBK32, and BBA64 was reduced in an isogenic *csrA* mutant, ES10, suggesting that CsrA is involved in the expression of the RpoS regulon in *B. burgdorferi*. Recently, by analyzing gene expression in a *csrA* deletion mutant in a low-passage virulent strain, B31A3, Sze and Li (45) also reported that transcription of *rpoS*, *ospC*, and *dbpA* was repressed when *csrA* was inactivated.

Despite these seemingly concordant findings, some significant anomalies exist among those previous studies. First, in the study of Sanjuan et al. (44), although the synthesis of OspC, DbpA, BBK32, and BBA64 was highly increased in the strain hyperexpressing CsrA, the effects of CsrA hyperexpression on the transcription of these genes were different. Notably, when CsrA was hyperexpressed, transcription of *ospC* was markedly induced, whereas the transcription of *dbpA*, *bbk32*, or *bba64* was not affected (44). These findings suggested that CsrA may modulate *ospC* at the transcriptional level but modulates DbpA, BBK32, and BBA64 at the posttranscriptional level. However, by analyzing the mutant deficient in *csrA*, researchers from the same lab reported that the transcription of all of the targets, including *ospC*, *dbpA*, *bbk32*, and *bba64*, was significantly repressed in the mutant, suggesting that CsrA modulates the expression of these genes at the transcriptional level, probably through RpoS (43). Second, Karna et al. (43) reported that the production of BosR, the key activator essential for *rpoS* transcription, was abolished at both the protein and mRNA levels in the *csrA* mutant, suggesting that the lack of RpoS in their mutant was due to the lack of BosR. In contrast, in the study of Sze and Li (45), the levels of BosR mRNA and protein were not altered in the *csrA* mutant. Rather, they described that CsrA indirectly modulated *rpoS* transcription via its influence on the level of acetyl phosphate, a potential small phosphate donor for the bEBP Rrp2 (59). Moreover, when analyzing gene expression in the *csrA* mutant, Karna et al. (43) reported that the transcription of *flaB* was decreased in the mutant but the protein level of FlaB remained unchanged. To the contrary, when analyzing the *csrA* mutant, Sze et al. (46) found that the transcription of *flaB* was not affected by *csrA* inactivation. Rather, the synthesis of FlaB was significantly increased in the *csrA* mutant, implying that CsrA represses *flaB* expression at the posttranscriptional level (46). Finally, in the study by Karna et al. (43), despite the fact that the expression of *bosR*, *rpoS*, *ospC*, *dbpA*, *bbk32*, and *bba64* was abolished or highly repressed in the *csrA* mutant, gene expression was not restored when *csrA* was complemented. In the complemented strain, when *csrA* was highly expressed, expression of *bosR* and

rpoS was still lacking and the expression of *ospC*, *dbpA*, *bbk32*, and *bba64* was still repressed (43). These incongruous results have confounded our understanding of the possible role of CsrA in modulating gene expression in *B. burgdorferi* and imply that other unknown changes, rather than the inactivation of *csrA*, caused the gene expression defects in the previously reported *csrA* mutant (43).

In an attempt to garner further clarity on the role of CsrA in *B. burgdorferi* gene regulation and Lyme disease pathogenesis, we created mutants deficient in *csrA* in two widely used infectious *B. burgdorferi* strains (B31 and 297). Strikingly, our data indicated that the expression of *bosR*, *rpoS*, and RpoS-dependent *ospC* and *dbpA* was not altered in the *csrA* mutants, suggesting that CsrA is not involved in the regulation of *rpoS* expression in *B. burgdorferi*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains and plasmids used in this study are described in Table 1. Low-passage-number infectious *B. burgdorferi* strains B31 (60) and 297 (61) were used as the wild-type (WT) strains throughout this study. Isogenic *csrA* mutants were created as described below. The virulent *B. burgdorferi* strain B31 clone B31A3 and its isogenic *csrA* mutant (the *bb0184::kan* mutant) (45) were provided by Chunhao Li (The State University of New York at Buffalo, Buffalo, NY). *B. burgdorferi* was routinely cultured at 37°C and 5% CO₂ in either Barbour-Stoenner-Kelly II (BSK-II) medium (62) or BSK-H medium (Sigma) supplemented with 6% rabbit serum (Pel-Freez). The pH of the media was typically 7.6, except as otherwise stated (e.g., pH 6.8). When appropriate, supplements were added to the media at the following concentrations: erythromycin (Ery), 60 ng/ml; gentamicin, 50 µg/ml; kanamycin (Kan), 150 µg/ml; or streptomycin (Str), 150 µg/ml. Spirochetes were enumerated by dark-field microscopy. *E. coli* strains were cultured in Luria-Bertani (LB) medium supplemented with appropriate antibiotics at the following concentrations: ampicillin (Amp), 100 µg/ml; gentamicin, 50 µg/ml; kanamycin, 50 µg/ml; or spectinomycin (Spt), 100 µg/ml.

Generation of *csrA* mutants in *B. burgdorferi*. To disrupt *csrA* in the infectious *B. burgdorferi* clonal isolate 297 (13), a suicide plasmid, pALH384, was constructed. Briefly, a DNA fragment comprising *csrA* and its upstream and downstream flanking regions was PCR amplified using primers priAH33 and priAH34 (see Table S1 in the supplemental material). The resulting PCR product was cloned into the pGEM-T vector (Promega), resulting in pALH356. One EcoRI site was then introduced into *csrA* in pALH356 through site-directed mutagenesis using primers priAH46 and priAH47 (see Table S1 in the supplemental material). The resulting plasmid was designated pALH360. The erythromycin resistance (*Ery*^r) gene (*ermC*) (13) was then cloned into pALH360 at the EcoRI site, resulting in the plasmid pALH384. In this construct, *ermC* was inserted in *csrA* in the opposite direction of *csrA* transcription. All constructs were confirmed using PCR amplification, restriction digestion, and sequence analysis.

To inactivate *csrA* in the virulent *B. burgdorferi* strain B31 through allelic exchange, a suicide vector (pOY236) was created. The 821-bp 5' arm for creating pOY236 was PCR amplified using primers ZM279F and ZM279R, whereas the 829-bp 3' arm was amplified using primers ZM280F and ZM280R (see Table S1 in the supplemental material). After digestion with *Asc*I, these two fragments were fused together. By using this ligated DNA as the template, PCR was employed to amplify a fragment comprising the upstream and downstream regions of *csrA* by using primers ZM279F and ZM280R. The obtained fragment was cloned into the pGEM-T Easy vector (Promega), yielding pOY227. The *PflgB*-*kan* cassette excised from pJD55 (15) using *Asc*I was cloned into pOY227 at the *Asc*I site. In the resulting construct, pOY236, the *PflgB*-*kan* cassette was inserted in *csrA* in the opposite direction as *csrA* transcription.

Plasmid pALH384 or pOY236 was then electroporated into *B. burgdorferi* 297 or B31, respectively, to create the *csrA* mutants. Transformation of *B. burgdorferi* was performed as previously described (33, 63, 64).

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>B. burgdorferi</i> strains		
B31	Infectious <i>B. burgdorferi</i> , tick isolate	60
OY153/B7	B31 <i>csrA::kan</i>	This study
OY153/B12	B31 <i>csrA::kan</i>	This study
297	Infectious <i>B. burgdorferi</i> , human spinal fluid isolate	61
AH227	297 <i>csrA::ermC</i> Ery ^r	This study
B31A3	Infectious clonal isolate of strain B31	45
<i>bb0184::kan</i> mutant	<i>csrA</i> mutant of strain B31A3 Kan ^r	45
<i>E. coli</i> TOP10F'	F' [<i>lacI</i> ^q Tn10 (Tet ^r)] <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>ara</i> Δ 139 Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
Plasmids		
pGEM-T Easy	TA cloning vector, Amp ^r	Promega
pGEM-T	TA cloning vector, Amp ^r	Promega
pJSB275	<i>E. coli</i> and <i>B. burgdorferi</i> shuttle vector, Spt ^r Str ^r	31
pOY340	<i>csrA</i> -FLAG cloned into pJSB275, Spt ^r Str ^r	This study
pJD55	Shuttle vector, Spt ^r Str ^r	15
pOY227	PCR product of 279F/280R cloned into pGEM-T Easy, Amp ^r	This study
pOY236	<i>PflgB</i> - <i>kan</i> cloned into pOY227, Amp ^r Kan ^r	This study
pALH356	PCR product of priAH33 and priAH34 cloned into pGEM-T, Amp ^r	This study
pALH360	An EcoRI site introduced into pALH356, Amp ^r	This study
pALH384	<i>ermC</i> cloned into pALH360, Amp ^r Ery ^r	This study

Transformants were selected by using kanamycin or erythromycin and confirmed by PCR amplification and reverse transcriptase (RT) PCR.

Generation of an IPTG-inducible *csrA* expression construct. To experimentally control *csrA* expression in *B. burgdorferi*, a *csrA* expression construct was generated using the *lac*-based gene-inducible expression system (65). Briefly, *csrA* was amplified from *B. burgdorferi* B31 using primers ZM373F and 373R2 (see Table S1 in the supplemental material). In the resulting PCR product, a DNA fragment encoding the FLAG tag (DYKDDDDK) was added to the 3' end of *csrA* to ultimately assist in the detection of CsrA expression in *B. burgdorferi*. This fragment was digested with NdeI and HindIII and then cloned into pJSB275 (31) that had been digested with the same restriction enzymes. In the resulting plasmid, pOY340 (Fig. 1A), *csrA* transcription is directly controlled by the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible T5 promoter (also known as the promoter PpQE30) from plasmid pQE30 (Qiagen). The plasmid pOY340 was then transformed into *B. burgdorferi* virulent strain B31, yielding the merodiploid strain OY212.

Infection of mice by *B. burgdorferi*. The murine needle-challenge model of Lyme borreliosis (66, 67) was employed to assess the infectivity of the *csrA* mutants. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at UT Southwestern Medical Center. Prior to infection, bacteria were carefully enumerated by dark-field microscopy. Each C3H/HeN mouse (Charles River Laboratories) was infected with 10⁴ spirochetes via intradermal injection. At 6 weeks postinoculation, mice were sacrificed and tissue biopsy specimens were collected. Spirochetal infection was confirmed by PCR and culture methods as described previously (22). Briefly, for recovering spirochetes from mouse tissue samples, biopsy specimens of skin, heart, and joints were cultured in BSK-II medium supplemented with 1 $\times$ *Borrelia* antibiotic mixture (BAM; Sigma). The outgrowth of spirochetes in these cultures was assessed using dark-field microscopy. For RNA isolation, ~30-mg portions of mouse back skin were harvested from each injected mouse. Samples were immediately immersed into 1 ml of the TRIzol reagent (Invitrogen) and frozen at -80°C.

RNA isolation and cDNA synthesis. RNA isolation was performed as previously described (21, 22, 63). Briefly, total RNA was isolated using the TRIzol reagent according to the manufacturer's instructions. After genomic DNA was digested with RNase-free DNase I (GenHunter Corporation), RNA was further purified using an RNeasy minikit (Qiagen)

and quantified using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies). For RT-PCR, cDNA was generated from 1 μ g of RNA using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

qRT-PCR analysis. Real-time quantitative RT-PCR (qRT-PCR) was employed to compare gene expression in various *B. burgdorferi* strains. Primers (see Table S2 in the supplemental material) specific for *B. burgdorferi* genes *csrA*, *rpoS*, *ospC*, *flaB*, and *eno* were designed by using Primer Express software (Applied Biosystems) and validated using 10-fold dilutions (10 to 0.00001 ng) of *B. burgdorferi* genomic DNA in an absolute quantification test on an ABI 7500 qRT-PCR system (Applied Biosystems). Standard curves created for all primers had a slope of -3.3 ± 0.3 (data not shown). For measuring gene expression, qRT-PCR using Platinum SYBR green quantitative PCR SuperMix-UDG (Invitrogen) was performed as previously described (21). The threshold cycle (C_T) relative quantification method ($\Delta\Delta C_T$) was used to calculate the variation of gene expression between *B. burgdorferi* parental strains and the corresponding mutants. As described before (45), the *B. burgdorferi* *enolase* gene (*eno*) was used as an endogenous control to normalize all qRT-PCR data.

SDS-PAGE and immunoblot analyses. SDS-PAGE and immunoblot analyses were carried out as previously described (21, 38). Briefly, a volume of whole-cell lysate equivalent to 4×10^7 bacteria was loaded per lane on a 12.5% polyacrylamide gel. Resolved proteins were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane for immunoblot analysis. FLAG, FlaB, RpoS, BosR, OspC, and DbpA were detected using the monoclonal anti-FLAG M2 antibody (Sigma), the chicken IgY anti-FlaB, anti-RpoS monoclonal antibody 6A7-101, anti-BosR rat polyclonal antibody α -BosR, anti-OspC monoclonal antibody 1B2-105A, or anti-DbpA monoclonal antibody 6B3, respectively. Immunoblots were developed colorimetrically using 4-chloro-1-naphthol as the substrate or by chemiluminescence using an ECL Plus Western blotting detection system (Amersham Biosciences).

RESULTS

Expression of *bosR*, *rpoS*, and RpoS-dependent *ospC* and *dbpA* is not altered when *csrA* expression is upregulated in *B. burgdorferi*. The possible involvement of CsrA in *B. burgdorferi* gene regulation was first reported in a CsrA hyperexpression study.

FIG 1 Controlled hyperexpression of CsrA does not alter the expression of *bosR*, *rpoS*, *ospC*, or *dbpA* in *B. burgdorferi*. (A) Construction of an IPTG-inducible *csrA* expression shuttle plasmid. To create pOY340, *csrA* was amplified from *B. burgdorferi* B31 and cloned into pJSB275. ORF, open reading frame. (B) PCR analysis of genomic DNA isolated from *B. burgdorferi* B31 and OY212. SDS-PAGE (C) and immunoblotting (D) were performed to analyze gene expression in OY212/A7. Bacteria were grown at 37°C in BSK-II medium (pH 7.6) with various concentrations of IPTG. When bacterial growth reached $\sim 10^8$ cells per ml, spirochetes were harvested. Approximately 4×10^7 spirochetes were loaded onto each lane of a 12.5% SDS-polyacrylamide gel. In panel C, the concentrations of IPTG are indicated above the image, and the arrows indicate OspC and FlaB in the SDS-polyacrylamide gels. The specific antibodies (denoted with α) used in the immunoblot (D) are indicated on the right. In the immunoblot (D), 1×10^7 spirochetes were used for the detection of FlaB, whereas 4×10^7 spirochetes were used for the detection of FLAG, RpoS, OspC, DbpA, and BosR. (E) Quantitative RT-PCR analyses of gene expression in OY212/A7 grown with various concentrations of IPTG. Data were collected from three independent experiments, and the bars represent the mean measurements $\pm$ standard deviations. For data normalization, the *B. burgdorferi* enolase gene (*eno*) was used as an internal control.

When constitutively hyperexpressing CsrA from the *B. burgdorferi* *flgB* promoter (P_{flgB}) in noninfectious *B. burgdorferi* B31 (strain ML23), Sanjuan et al. reported that the syntheses of RpoS-dependent OspC, DbpA, BBK32, and BBA64 were highly enhanced, implying that CsrA modulates *rpoS* expression (44). In an attempt to provide further experimental evidence for this conclusion, we generated a tightly controllable CsrA expression construct, pOY340 (Fig. 1A). From this construct, *B. burgdorferi* LacI (BbLacI), the repressor for the IPTG-inducible promoter PpQE30, was constitutively expressed, allowing CsrA expression to be solely dependent on IPTG added exogenously into the medium. This construct was then transformed into *B. burgdorferi* virulent strain B31, yielding two streptomycin-resistant clones, OY212/A7 and OY212/B1. The presence of the *csrA*-FLAG DNA fragment in these two clones was confirmed by PCR. As shown in Fig. 1B, a DNA fragment comprising *csrA*-FLAG was detected in both OY212/A7 and OY212/B1, but not in strain B31. PCR amplification also confirmed that these clones contained the *aadA* gene conferring streptomycin resistance (Fig. 1B).

To induce CsrA incrementally in *B. burgdorferi*, various concentrations of IPTG were added to the BSK-II medium. Cells were collected when bacterial growth reached early stationary phase ($\sim 10^8$ cells/ml). As shown in Fig. 1D, when various amounts of IPTG were added to the medium, the synthesis of CsrA-FLAG (assessed by anti-FLAG antibody) in *B. burgdorferi* OY212/A7 was increased in a dose-dependent manner. qRT-PCR analyses re-

vealed that *csrA* transcription was enhanced by 1.3-, 2.6-, 20.9-, 20.2-, and 19.2-fold, respectively, when 10, 20, 30, 50, or 100 μ M IPTG was added to the medium (Fig. 1E). When CsrA was modestly induced (in BSK-II medium containing 10 μ M or 20 μ M IPTG), *B. burgdorferi* exhibited normal morphology (Fig. 2). However, when CsrA was hyperexpressed (in BSK-II medium containing 30 μ M or higher concentrations of IPTG), the cell morphology of spirochetes was substantially affected, resulting in elongated rod-shaped organisms (Fig. 2).

To discern whether CsrA influences expression of *bosR*, *rpoS*, and RpoS-dependent *ospC* and *dbpA*, gene expression was measured in strain OY212/A7 by SDS-PAGE and immunoblot analyses. As shown in Fig. 1C and D, despite the production of CsrA-FLAG in bacteria cultivated in BSK-II medium containing IPTG, the protein levels of BosR, RpoS, OspC, and DbpA were not altered (compared to the levels of gene expression in spirochetes grown in BSK-II medium containing 0 μ M IPTG). Gene expression was also measured by using qRT-PCR analyses. As shown in Fig. 1E, relative to gene transcription in *B. burgdorferi* cultivated in BSK-II medium without exogenous IPTG, the transcription of *bosR*, *rpoS*, *ospC*, or *dbpA* in this strain was altered less than 2-fold. Specifically, when gene transcription in spirochetes grown in BSK-II medium with 10, 20, 30, 50, or 100 μ M IPTG was compared with gene expression in bacteria grown without extraneous IPTG, *rpoS* transcription was changed only 1.1-, 1.1-, 0.7-, 0.9-, or 0.7-fold, respectively; *ospC* expression was altered at 1.1-, 1.4-,

FIG 2 Hyperexpression of CsrA in *B. burgdorferi* alters bacterial morphology. Bacteria grown in BSK-II medium with various IPTG concentrations were subjected to dark-field microscopic analyses. The concentrations of IPTG are indicated below the images.

1.1-, 0.7-, or 1.0-fold, respectively; *dbpA* transcription was altered at 1.0-, 1.3-, 1.4-, 0.9-, or 1.4-fold, respectively; and *bosR* transcription was altered at 1.2-, 1.2-, 0.6-, 0.8-, or 0.6-fold, respectively. Similar results were also obtained when gene expression was examined in strain OY212/B1 (data not shown).

Disruption of *csrA* in *B. burgdorferi* strain 297. The *csrA*-deficient mutant of *B. burgdorferi* strain 297 (designated AH227) was generated by Anette Hübner in 2001 (Fig. 3A). The mutant AH227 exhibited spirochetal morphology and movement identical to that of 297 under dark-field microscopy. The inactivation of *csrA* in AH227 was confirmed by PCR amplification. Using primers ZM307F2 and ZM373R, a 606-bp fragment comprising *bb0183* and *csrA* was amplified in WT 297 (Fig. 3B, lane 1), while a 1,664-bp fragment was amplified in the *csrA* insertion mutant AH227 (Fig. 3B, lane 2), which is in agreement with the insertion of the 1,058-bp *ermC* in *csrA*. PCR employing *csrA*-specific primers also confirmed the inactivation of *csrA* in this mutant; as shown in Fig. 3B, in the WT strain 297 (lane 1), a 246-bp fragment of *csrA* was amplified by using the primer pair ZM373F and ZM373R, whereas no fragment was amplified from the *csrA* mutant AH227 (lane 2). Because several plasmids, such as lp25, lp28-1, lp54, lp36, and cp26 (68–71), are required by *B. burgdorferi* to establish mammalian infection, the plasmid profile of the *csrA* mutant was examined by PCR, as previously described (63, 70). The *csrA* mutant AH227 had lost plasmids lp25 and lp28-1 during its construction but retained all other plasmids contained in its parental strain, 297 (data not shown). The absence of *csrA* expression in the mutant was confirmed by RT-PCR. Contrary to *csrA* transcription in the WT strain 297 (Fig. 3C, lane 1), the *csrA* transcript was not detected in the mutant AH227 (Fig. 3C, lane 2).

Because it has been proposed that *csrA* is cotranscribed with *bb0181* (*flgK*), *bb0182*, and *bb0183* in *B. burgdorferi* (43–45), RT-

PCR using gene-specific primers was also performed to assess whether genes flanking *csrA* were expressed in the *csrA* mutant. Consistent with previous studies (43, 45), transcription of *bb0181*, *bb0182*, and *bb0183* was readily detected in the *csrA* mutant as well as in the WT 297 strain (Fig. 3C).

FIG 3 Inactivation of *csrA* in *B. burgdorferi* strain 297. (A) Schematic representation of the *bb0181* to *bb0185* genes in the *B. burgdorferi* 297 chromosome and the insertion of *ermC* by homologous recombination. Arrows indicate the approximate positions of the oligonucleotide primers used for subsequent analyses. (B and C) Analyses of the wild-type strain 297 and the *csrA* mutant AH227 by PCR (B) and RT-PCR (C). The specific primer pairs are indicated on the right. Lanes 1, 297; lanes 2, the *csrA* mutant AH227.

FIG 4 Gene expression in WT strain 297 and the *csrA* mutant AH227. SDS-PAGE (A) and immunoblotting (B) were employed to analyze gene expression. Bacteria grown in BSK-II medium at pH 7.6 or pH 6.8 were collected when growth reached the stationary phase. Lanes WT, the wild-type strain 297; lanes M, the *csrA* mutant AH227. In panel A, approximate molecular masses are indicated on the left; the arrows indicate OspC and FlaB. In panel B, $\sim 4 \times 10^7$ spirochetes were used for the detection of RpoS, OspC, DbpA, and BosR; $\sim 3 \times 10^6$ spirochetes were used for the detection of FlaB. Specific antibodies (denoted with α) are indicated on the right. (C) Gene expression was examined by qRT-PCR. Bacteria grown in BSK-II medium at pH 7.6 or pH 6.8 were harvested when growth reached the stationary phase. Data were collected from three independent experiments, and the bars represent the mean measurements $\pm$ standard deviations. All qRT-PCR data were normalized relative to the *eno* value.

***csrA* inactivation in *B. burgdorferi* 297 does not alter the expression of *bosR*, *rpoS*, *ospC*, or *dbpA*.** Gene expression was compared between WT strain 297 and the *csrA* mutant AH227. In some experiments here and subsequently, culture media at times were adjusted to pH 6.8 (from pH 7.6), which is one of the environmental conditions particularly conducive to activating the RpoN-RpoS pathway (8–10, 13, 22, 24, 72). As shown in Fig. 4A and B, when bacteria were grown in BSK-II medium at pH 7.6 or pH 6.8, similar levels of BosR, RpoS, OspC, and DbpA were detected between the WT (lanes WT) and the mutant (lanes M). To examine whether gene transcription was altered in the *csrA* mutant, qRT-PCR analyses were performed. As shown in Fig. 4C, transcription of *bosR*, *rpoS*, *ospC*, and *dbpA* appeared not to be significantly altered in the *csrA* mutant. Relative to gene expression in WT strain 297, a change in *rpoS* expression in the mutant AH227 was only 0.78-fold or 1.15-fold when spirochetes were grown under pH 7.6 or pH 6.8, respectively. Similar results were obtained when *B. burgdorferi* strains were cultured in BSK-H medium (data not shown).

Disruption of *csrA* in *B. burgdorferi* strain B31. Because all previous *csrA* mutants (42–45) were created in *B. burgdorferi* strain B31, we also created two mutants (OY153/B7 and OY153/B12) deficient in *csrA* in this strain. By introducing the suicide plasmid pOY236 into the low-passage virulent strain B31, we obtained two kanamycin-resistant *csrA* mutants (Fig. 5A). The inactivation of *csrA* in these strains was confirmed by PCR amplification. Using primers ZM307F2 and ZM373R, a 606-bp fragment

FIG 5 Inactivation of *csrA* in *B. burgdorferi* strain B31. (A) Schematic representation of the *bb0181* to *bb0185* genes in the *B. burgdorferi* B31 chromosome and the insertion of the *PflgB-kan* cassette by homologous recombination. Arrows indicate the approximate positions of the oligonucleotide primers used for subsequent analyses. (B and C) Analyses of the wild-type strain B31 and the *csrA* mutants by PCR (B) and RT-PCR (C). The specific primer pairs are indicated on the right. Lanes 1, B31; lanes 2, the *csrA* mutant OY153/B7; lanes 3, mutant OY153/B12.

comprising *bb0183* and *csrA* was amplified in the WT strain B31 (Fig. 5B, lane 1), whereas a 1,578-bp fragment was amplified in the *csrA* deletion mutants OY153/B7 (Fig. 5B, lane 2) and OY153/B12 (Fig. 5B, lane 3). This is consistent with the replacement of a 171-bp internal coding region of *csrA* by the 1,143-bp *PflgB-kan* cassette. PCR employing *csrA*-specific primers was also performed to confirm the inactivation of *csrA* in these mutants. As shown in Fig. 5B, in the WT strain B31 (lane 1), a 164-bp or 246-bp fragment of *csrA* was amplified using the primer pair ZM406F and ZM406R or the primer pair ZM373F and ZM373R, respectively, whereas no fragment was amplified from the *csrA* mutant OY153/B7 (lane 2) or OY153/B12 (lane 3). Plasmid profiling using PCR amplification indicated that the mutants OY153/B7 and OY153/B12 retained all plasmids contained in the parental strain B31 (data not shown).

To confirm the deletion of *csrA* in the mutants, RT-PCR was performed. As expected, *csrA* transcripts were detected in the WT strain B31 (Fig. 5C, lane 1), but not in the mutants OY153/B7 (Fig. 5C, lane 2) and OY153/B12 (Fig. 5C, lane 3). Additionally, genes flanking *csrA* (*bb0181*, *bb0182*, and *bb0183*) were transcribed in the *csrA* mutant as well as in WT B31 (Fig. 5C).

***csrA* inactivation in *B. burgdorferi* B31 does not alter the expression of *bosR*, *rpoS*, *ospC*, and *dbpA*.** To further investigate the potential role of CsrA in *B. burgdorferi* gene regulation, the synthesis of BosR, RpoS, OspC, and DbpA was compared between the parental strain and the *csrA* mutant using immunoblotting. As shown in Fig. 6A and B, when spirochetes were cultivated in BSK-II medium at pH 7.6, the levels of BosR, RpoS, OspC, and DbpA were not significantly altered when *csrA* was inactivated. Comparable levels of BosR, RpoS, OspC, and DbpA were produced in WT and the *csrA* mutants (lanes M1, OY153/B7; lanes

FIG 6 Gene expression in WT strain B31 and two B31-derived *csrA* mutants. SDS-PAGE (A) and immunoblotting (B) were performed to examine gene expression. Bacteria grown in BSK-II medium at pH 7.6 or pH 6.8 were collected when growth reached the stationary phase. Lanes WT, the WT strain B31; lanes M1, the *csrA* mutant OY153/B7; lanes M2, the mutant OY153/B12. In panel A, approximate molecular masses are indicated on the left; the arrows indicate OspC and FlaB. In panel B, $\sim 4 \times 10^7$ spirochetes were used for the detection of RpoS, OspC, DbpA, and BosR; $\sim 1 \times 10^7$ spirochetes were used for the detection of FlaB. Specific antibodies (denoted with α) are indicated on the right. (C, D) Gene expression was examined by qRT-PCR. Bacteria grown in BSK-II medium at pH 7.6 (C) or pH 6.8 (D) were harvested when growth reached the stationary phase. Data were collected from three independent experiments, and the bars represent the mean measurements $\pm$ standard deviations. In the experiments, *eno* was used as an internal control to normalize all qRT-PCR data.

M2, OY153/B12). To determine whether transcription of these genes was changed in the mutants, qRT-PCR was carried out; *bosR*, *rpoS*, *ospC*, and *dbpA* were transcribed at similar levels among WT and the *csrA* mutants cultivated in BSK-II medium at pH 7.6 (expression changes for all of these genes were less than 2-fold) (Fig. 6C). Spirochetes were also cultivated in BSK-II medium at pH 6.8. Under this condition, no significant difference of *bosR*, *rpoS*, *ospC*, and *dbpA* expression was observed between the WT and the *csrA* mutants when either protein or mRNA levels were examined (Fig. 6A, B, and D). Similar comparative results were obtained when spirochetes were cultivated in BSK-H medium at either pH 7.6 or pH 6.8 (data not shown).

Previously, another *csrA* mutant was generated in the low-passage virulent strain B31 (clone B31A3) (45). By examining gene expression using immunoblotting and RT-PCR, Sze and Li reported that expression of *ospC*, *dbpA*, and *rpoS* was decreased at both the protein and mRNA levels when *csrA* was deleted (45). Here, we also compared gene expression in the *bb0184::kan csrA* mutant and its parental strain, B31A3. To this end, bacteria were grown in BSK-II medium at either pH 7.6 or pH 6.8. As shown in Fig. 7A and B, in our hands, the mutant (lanes M) produced levels of BosR, RpoS, OspC, and DbpA comparable to those for the WT strain B31A3 (lanes WT). Moreover, as assessed by qRT-PCR, comparable levels of *bosR*, *rpoS*, *ospC*, and *dbpA* transcripts were detected in the mutant and the parental strains (Fig. 7C). When spirochetes were cultivated in BSK-II medium at pH 7.6 or 6.8, the *rpoS* expression change in the *csrA* mutant (relative to WT B31A3) was 0.74-fold or 1.17-fold, respectively. Comparable results were

also obtained when *B. burgdorferi* was grown in BSK-H medium (data not shown).

CsrA is not essential for *B. burgdorferi* to infect mice via needle inoculation. The fact that no substantial differences in the expression of BosR and RpoS or their downstream virulence-associated gene targets were found between *B. burgdorferi* *csrA* mutants and their counterpart WT strains suggested that the *csrA* mutants likely retained their ability to infect mammalian hosts. To test this hypothesis, the murine intradermal inoculation model was employed. Of note, the strain 297-derived *csrA* mutant AH227 could not be used for these studies, inasmuch as this strain lacks two virulence-associated plasmids (lp25 and lp28-1). However, the B31 *csrA* mutants (OY153/B7 and OY153/B12) retained all virulence-associated plasmids, and thus, they were amenable to mouse infectivity studies. C3H/HeN mice were thus infected via needle inoculation with WT B31 and the two *csrA* mutants. Specifically, each mouse was injected with 10^4 spirochetes. After 6 weeks, mice were sacrificed and assessed for *B. burgdorferi* infection by culturing mouse skin, heart, and joint tissue specimens in BSK-II medium. Cultures were monitored continually for 4 weeks for spirochete growth. As expected, motile spirochetes were recovered from all tissue specimens of mice infected with WT B31 (Table 2). Moreover, bacterial growth was also observed in cultures from mice infected with the *csrA* mutants (OY153/B7 and OY153/B12) (Table 2). These data were further corroborated by RT-PCR analyses of the tissue biopsy specimens isolated from injected mice. As shown in Fig. 8A, *B. burgdorferi* *flaB* transcripts were readily detected in all skin specimens isolated from mice injected

FIG 7 Gene expression in the *B. burgdorferi* *bb0184::kan csrA* mutant and its parental strain, B31A3. SDS-PAGE (A) and immunoblotting (B) were performed to analyze gene expression. Bacteria grown in BSK-II medium at pH 7.6 or pH 6.8 were collected when growth reached the stationary phase. Lanes WT, the WT strain B31A3; lanes M, the *bb0184::kan csrA* mutant. In panel A, approximate molecular masses are indicated on the left; the arrows indicate OspC and FlaB. In panel B, $\sim 4 \times 10^7$ spirochetes were used for the detection of RpoS, OspC, DbpA, and BosR; $\sim 3 \times 10^6$ spirochetes were used for the detection of FlaB. Specific antibodies (denoted with α) are indicated on the right. (C) Gene expression was examined by qRT-PCR. Bacteria grown in BSK-II medium at pH 7.6 or pH 6.8 were harvested when growth reached the stationary phase. Data were collected from three independent experiments, and the bars represent the mean measurements $\pm$ standard deviations. All qRT-PCR data were normalized relative to the *eno* value.

with B31 or the *csrA* mutants OY153/B7 and OY153/B12, implying the existence of live spirochetes in these samples. These results suggest that *csrA* is not required by *B. burgdorferi* to establish mammalian infection.

Inactivation of *csrA* does not alter the expression of *rpoS* during mouse infection by *B. burgdorferi*. To investigate whether *rpoS* expression was affected in the *csrA* mutants during mouse infection, qRT-PCR was performed to compare *rpoS* transcription in mice infected with the WT strain B31 or the *csrA* mutants. Borreliar *rpoS* was expressed in all mice infected with B31 or the *csrA* mutants at comparable levels (Fig. 8B). More specifically, relative to gene expression in skin samples isolated from B31-infected mice, *rpoS* transcription was changed only 0.81-fold or 0.74-fold, respectively, in tissue samples isolated from mice infected with OY153/B7 or OY153/B12 (Fig. 8B). These data suggest

that CsrA does not impact *rpoS* expression during mammalian infection.

Effect of CsrA on *B. burgdorferi* *flaB* expression. To investigate whether CsrA influences *flaB* expression in *B. burgdorferi*, we examined the expression of *flaB* when *csrA* was upregulated in a controllable fashion; as shown in Fig. 1C and D, when IPTG was added to BSK-II medium to induce *csrA* expression, the synthesis of FlaB was significantly repressed in a dose-dependent manner. However, when gene transcription was assessed by qRT-PCR, transcription of *flaB* was not significantly altered (Fig. 1E). These results prompted us to assess the expression of *flaB* in the *csrA* mutants. As shown in Fig. 6A and B, compared with the protein levels in strain B31 (lane WT), synthesis of FlaB was not significantly altered in the *csrA* mutants OY153/B7 (lane M1) and OY153/B12 (lane M2). Moreover, qRT-PCR analyses revealed

TABLE 2 Infectivity of *B. burgdorferi* in mice^a

Strain, clone	Description	No. of cultures positive/total no. of specimens examined				No. of mice infected/ total no. of mice
		Heart	Joint	Skin	All sites	
B31	Wild-type <i>B. burgdorferi</i>	3/3	3/3	6/6	12/12	6/6
OY153/B7	B31 <i>csrA::kan</i>	3/3	3/3	6/6	12/12	6/6
OY153/B12	B31 <i>csrA::kan</i>	4/4	4/4	7/7	15/15	7/7
<i>bb0184::kan</i> mutant	B31A3 <i>csrA::kan</i>	ND ^b	ND	5/5	5/5	5/5

^a All mice were treated with 10^4 spirochetes. Data were collected from two independent experiments.

^b ND, not determined.

FIG 8 Analyses of gene expression in tissue specimens isolated from mice infected with *B. burgdorferi*. (A) RT-PCR was performed to assess *B. burgdorferi flaB* transcription in back skin biopsy specimens isolated from mice injected with B31, OY153/B7, or OY153/B12. (B) *rpoS* expression in skin samples isolated from mice infected with B31 or the *csrA* mutants was compared by using qRT-PCR. Data were collected from three independent experiments, and the bars represent the mean measurements $\pm$ standard deviations. All data were normalized with those for the internal control, *eno*.

that *flaB* transcription was only slightly changed in the *csrA* mutants (Fig. 9A). More specifically, when bacteria were grown at pH 7.6 or pH 6.8, *flaB* transcription in OY153/B12 (relative to gene transcription in B31) was altered only 0.73-fold or 1.3-fold, respectively. Similar data were obtained when gene expression was compared between WT B31A3 and the *bb0184::kan* mutant (Fig. 7A and B and 9A) or between WT 297 and the mutant AH227 (Fig. 4A and B and 9A). Finally, *flaB* expression was also compared between B31 and the *csrA* mutants during mouse infection. As shown in Fig. 9B, compared with gene transcription in skin biopsy specimens isolated from B31-infected mice, *flaB* transcription was only slightly changed at 0.82-fold or 1.5-fold, respectively, in skin samples isolated from mice infected by the mutant OY153/B7 or OY153/B12.

DISCUSSION

Our laboratory has had a long-standing interest in elucidating genetic regulatory mechanisms in virulent *B. burgdorferi*. This

prompted the creation in 2001 of an initial *csrA* mutant in *B. burgdorferi* strain 297 by Anette Hübner. However, at that time, borreliac genetics was in its very early stages, and as such, there were many obstacles to creating targeted mutants and corresponding complemented strains. Spontaneous plasmid loss was, and still is, an obstacle to the genetic manipulation of *B. burgdorferi* (8, 10, 73, 74). Consequently, our original *csrA* mutant (AH227) lacked the virulence-associated plasmids lp25 and lp28-1, which precluded us at that time from assessing a role for CsrA in *B. burgdorferi* mouse infectivity and pathogenesis. However, recent reports implicating CsrA in a potential layer of control over the RpoN-RpoS regulatory pathway (42–45) prompted us to reassess the role(s) of CsrA in *B. burgdorferi* gene expression and pathogenesis, with emphasis on attempting to reconcile a number of paradoxes and contradictions. In contrast to recent findings (42–45), our data from this study clearly demonstrate that CsrA does not impact the expression of *rpoS* or RpoS-dependent *ospC* and *dbpA* in *B. burgdorferi*. The first line of evidence supporting our conclusion emanates from studying the effects of CsrA up-regulation on *B. burgdorferi* gene expression. Through an IPTG-inducible promoter, the hyperexpression of *csrA* was controlled in the low-passage virulent *B. burgdorferi* strain B31 (Fig. 1). When CsrA was upregulated in *B. burgdorferi*, the protein or mRNA levels of BosR, RpoS, OspC, and DbpA were not significantly altered. This result differs from what was reported in a previous study (44); constitutive hyperexpression of CsrA from PflgB resulted in the enhanced synthesis of RpoS-dependent OspC, DbpA, BBK32, and BBA64 in a noninfectious *B. burgdorferi* strain (44). The reason for this discrepancy currently remains unknown. It may result from the different strains used in these studies. It may also emanate from the different strategies used to induce *csrA* expression. In the current study, *csrA* hyperexpression, controlled by the IPTG-inducible promoter PpQE30, was induced gradually in virulent strain B31 by increasing the amount of IPTG. In the study by Sanjuan et al. (44), CsrA was constitutively hyperexpressed in a noninfectious strain of B31. Nevertheless, it is noteworthy that caution should be given when interpreting gene expression changes in studies employing gene overexpression. Although overexpression is conducive to analyzing the function of genes that cannot be inactivated, it has been widely reported that the abnormal expression of a regulatory protein in bacteria may cause pleiotropic effects on cell physiology, resulting in biologically irrelevant phenotypes (75–79). Gene expression changes observed under this aberrant condition may be due to inadvertent

FIG 9 Expression of *flaB* in *B. burgdorferi* assessed by qRT-PCR. (A) Bacteria grown in BSK-II medium at pH 7.6 or pH 6.8 were harvested when growth reached the stationary phase. (B) RNA was isolated from back skin samples isolated from infected mice. The bars represent the mean measurements $\pm$ standard deviations. All data were normalized with those for the internal control, *eno*.

indirect effects, rather than direct regulation from the regulatory protein. In fact, as revealed in the current study, as well as in two previous studies (44, 46), when CsrA was hyperexpressed in *B. burgdorferi*, an abnormal elongated rod-shape morphology and a delayed *in vitro* growth phenotype were observed, implying dramatic changes in bacterial physiology and metabolism.

Genetic mutation, as a parallel approach to the aforementioned *csrA* upregulation study, was also employed to investigate the role of CsrA in *rpoS* expression. In this regard, we generated isogenic *csrA* mutants in two commonly used low-passage infectious *B. burgdorferi* strains, including B31 and 297. Surprisingly, when comparing gene expression at the protein and mRNA levels, the expression of *rpoS*, *ospC*, *dbpA*, and *bosR* was not significantly altered in the *csrA* mutants (Fig. 4 and 6). Similar results were also obtained when gene expression was compared between another low-passage virulent strain, B31 (clone B31A3), and its isogenic *csrA* mutant, the *bb0184::kan* mutant, generated by another independent research group (Fig. 7) (45). All of these combined observations suggest that CsrA does not modulate the expression of *bosR*, *rpoS*, *ospC*, or *dbpA* in *B. burgdorferi*. Different results have been reported in a previous study (43). Specifically, a *csrA* mutant (ES10) was created in noninfectious *B. burgdorferi* strain ML23 (43). By assessing gene expression in the mutant, Karna et al. reported that transcription of *bosR*, *rpoS*, and RpoS-dependent *ospC*, *dbpA*, *bbk32*, and *bba64* was considerably repressed when *csrA* was inactivated, suggesting that CsrA may act as an activator to induce *rpoS* transcription (43). Accordingly, the *csrA* mutant was incapable of infecting mice when 10^5 mutant spirochetes were injected into mice, implying that CsrA is essential for *B. burgdorferi* infection of mice (43). However, it is noteworthy that these phenotypes could not be restored when the *csrA* mutation was complemented (43). Moreover, the production of RpoS or BosR was not restored in the complemented strain, despite the fact that CsrA was highly expressed (43). These data suggest that the phenotypes and gene expression changes observed in that *csrA* mutant (43) did not result from the inactivation of *csrA*.

Further evidence supporting our conclusion emerged upon examining gene expression in *B. burgdorferi* during infection. When *B. burgdorferi* gene expression was compared in tissue samples isolated from animals infected with the WT strain B31 or the *csrA* mutants, similar levels of *rpoS* transcripts were detected among these samples (Fig. 8), indicating that the expression of *rpoS* was not significantly altered in the *csrA* mutants during mouse infection. Given that BB0184 is the only protein predicted to be a CsrA homologue in *B. burgdorferi* (11), it is unlikely that another factor(s) carrying out the CsrA function compensated for the loss of CsrA in our mutants. Our combined data provided herein strongly suggest that CsrA does not impact the expression of *rpoS*, *ospC*, *dbpA*, or *bosR* in *B. burgdorferi*.

Previously, by analyzing gene expression in the *csrA* mutant derived from a noninfectious strain, Karna et al. (43) reported that the mRNA levels of *flaB* were reduced in the mutant, but the protein levels of FlaB were not changed. In contrast, Sze et al. (46) reported that the protein levels of FlaB were highly elevated when *csrA* was inactivated, but *flaB* transcription remained unaltered. Surprisingly, our data from the current study revealed that both the mRNA and protein levels of *flaB* were not affected by *csrA* inactivation, implying that CsrA likely does not impact *flaB* expression in *B. burgdorferi*. Nonetheless, consistent with previous studies (44, 46), our data also revealed that FlaB synthesis was

highly repressed when *csrA* was upregulated in *B. burgdorferi*, despite the fact that *flaB* transcription was not significantly altered. Thus, it still remains an open question as to whether CsrA modulates *flaB* expression in *B. burgdorferi*.

Another striking finding of our study is that our *csrA* mutants were still capable of infecting mice. Previously, CsrA was reported to be essential for the ability of *B. burgdorferi* to infect mice, because mice were not infected even by high needle inoculation doses (e.g., 10^5 spirochetes) (43, 45). However, in the current study, when 10^4 mutants were intradermally injected into mice, our *csrA* mutants OY153/B7 and OY153/B12 were able to establish infection in distant organs, such as skin, heart, and joints, suggesting that CsrA is not required by *B. burgdorferi* to infect mice, at least via the direct intradermal route. This conclusion was supported by two other observations. First, we also examined the infectivity of the previous *bb0184::kan csrA* mutant created by another group (45). Our data showed that, when 10^4 *bb0184::kan* mutants were injected into mice, all mice became infected. Second, in an independent study, the *bb0184::kan csrA* mutant was found to be able to infect mice via tick transmission (X. Frank Yang, unpublished data).

Among the potential transcriptional regulators encoded by *B. burgdorferi*, the RpoN-RpoS regulatory pathway plays a central role in governing *B. burgdorferi* virulence expression and host adaptation (8–10, 13). Hence, there has been an impetus to elucidate other potential layers of gene regulation that might impact the activation of the RpoN-RpoS pathway or that may fine-tune its ultimate downstream regulatory action. Although CsrA does not function as a master regulator in *B. burgdorferi* to modulate expression of σ^{54} -dependent *rpoS* and RpoS-dependent *ospC* and *dbpA*, this protein may be involved in regulating other unknown cellular processes in *B. burgdorferi*.

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