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Overexpression of CsrA (BB0184) Alters the Morphology and Antigen Profiles of *Borrelia burgdorferi*[▽]

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***Borrelia burgdorferi*, the agent of Lyme disease, alters its gene expression in response to highly disparate environmental signals encountered in its hosts. Among the relatively few regulators of adaptive gene expression present in the borrelial genome is an open reading frame (ORF), BB0184, annotated as CsrA (*c*arbon *s*torage *r*egulator *A*). CsrA, in several bacterial species, has been characterized as a small RNA binding protein that functions as a global regulator affecting mRNA stability or levels of translation of multiple ORFs. Consistent with known functions of CsrA, overexpression of CsrA from *B. burgdorferi* ($CsrA_{Bb}$) in *Escherichia coli* resulted in reduced accumulation of glycogen. We determined that $csrA_{Bb}$ is part of the *flgK* motility operon and that the synthesis of $CsrA_{Bb}$ was increased when *B. burgdorferi* was propagated under fed-tick conditions. Overexpression of $CsrA_{Bb}$ in *B. burgdorferi* strain B31 (ML23, lp25-negative clonal isolate) resulted in a clone, designated ES25, which exhibited alterations in colony morphology and a significant reduction in the levels of FlaB. Several lipoproteins previously characterized as playing a role in infectivity were also altered in ES25. Real-time reverse transcription-PCR analysis of RNA revealed significant differences in the transcriptional levels of *ospC* in ES25, while there were no such differences in the levels of other transcripts, suggesting posttranscriptional regulation of expression of these latter genes. These observations indicate that $CsrA_{Bb}$ plays a role in the regulation of expression of pathophysiological determinants of *B. burgdorferi*, and further characterization of $CsrA_{Bb}$ will help in better understanding of the regulators of gene expression in *B. burgdorferi*.**

Borrelia burgdorferi, the agent of Lyme disease, causes a multiphasic disorder affecting the cutaneous, musculoskeletal, cardiovascular, and nervous systems (77). This spirochetal pathogen is transmitted to vertebrate hosts through the bite of infected *Ixodes* species ticks (8). Lyme disease is a significant public health issue in certain areas of endemicity where there is an increased presence of the transmission vector that cohabitates with a variety of reservoir hosts (9, 59).

B. burgdorferi exhibits adaptive gene expression in response to highly disparate environmental signals such as temperature, pH, dissolved gases, and other undefined factors unique to its arthropod vector and mammalian hosts (1–5, 14, 21, 25, 38, 49, 58, 66, 67, 74). Differential gene expression plays a critical role in the transmission, colonization, and dissemination of *B. burgdorferi* in mammalian hosts. Several of these open reading frames (ORFs) include mediators that provide a selective advantage to *B. burgdorferi* by enhancing its ability to adhere to host matrices (24, 39, 64) and to evade the mediators of innate and adaptive immunity (16, 26, 48, 89), as well as other deleterious physiological processes encountered in its hosts (27). A number of studies have determined the significance of alterations of surface lipoproteins in response to these signals, and the molecular mechanisms responsible for these changes are beginning to be understood (41, 86).

The genome of *B. burgdorferi* includes a limited number of ORFs that have been annotated as regulators (22, 32). A central regulatory pathway comprising Rrp2-RpoN-RpoS has been characterized as regulating outer surface protein C (OspC), decorin binding protein A (DbpA), fibronectin binding protein BBK32, and several other borrelial determinants involved in various pathogenic processes (11, 17–20, 41, 88). Mutations in one or more of these regulators have also been characterized as having significant global effects on the transcriptional levels of a large number of ORFs in *B. burgdorferi* (12, 17, 20, 31, 61). Recently, it has also been shown that inactivation of *ospAB* constitutively activates the Rrp2-RpoN-RpoS pathway, indicating mechanisms that are unique to *B. burgdorferi* and to its survival strategies in the tick vector and mammalian hosts (40).

In addition to this regulatory pathway, the genome of *B. burgdorferi* codes for a homolog of CsrA (*c*arbon *s*torage *r*egulator *A*, BB0184), which has been shown to be an RNA binding protein in several eubacterial species (68, 70). CsrA plays a central role in the up- or downregulation of several metabolic processes not limited to glycolysis, glycogen biosynthesis, biofilm formation, and motility as well as playing a role in the regulation of virulence factors (69). CsrA has been shown to control gene expression posttranscriptionally via specific and high-affinity binding to 5′ untranslated regions of mRNA, by affecting translation or stability of RNA (6). Alternatively, the effects of CsrA can also be titrated by binding of the noncoding RNAs such as CsrB and CsrC, which have multiple binding sites to sequester CsrA (53). The *B. burgdorferi* CsrA ($CsrA_{Bb}$) has significant sequence similarity to CsrA proteins in other species, including the conserved domains that have been biochemically shown to be critical for the regulatory functions of CsrA in other eubacterial species (53). Hence, it is possible for $CsrA_{Bb}$ to have multiple functions in regulating

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

B. burgdorferi strain	Description[a]	Source or reference(s)
ML23	Noninfectious, lp25-negative clonal isolate of *B. burgdorferi* strain B31	45
ML23/pBBE22	ML23 carrying pBBE22 (pBSV2/E22$^+$) vector, Kan^r	51, 65
ML23/pBSV2	ML23 carrying empty pBSV2 vector, Kan^r	This study and reference 78
MSK5	Clonal isolate of *B. burgdorferi* strain B31 that contains all the plasmids and is highly virulent	45
ES25	ML23 with vector pES25, Kan^r	This study

[a] Kan^r, kanamycin resistance.

TABLE 2. Plasmids used in this study

Plasmid	Description[a]	Source or reference
pCR2.1	PCR cloning vector	Invitrogen
pET23a	Protein expression vector	Novagen
pML102	Customized donor plasmid for in vitro transposition	75
pBSV2	Borrelial shuttle vector conferring Kan^r	78
pES15	bb0184 (*csrA*$_{Bb}$) cloned into pCR2.1	This study
pES16	bb0184 (*csrA*$_{Bb}$) cloned into pET23a	This study
pES20	*csrA*$_{Bb}$-six-His tag cloned into pCR2.1	This study
pES23	P_{flgB}-*csrA*$_{Bb}$-six-His tag cloned into pML102	This study
pES25	P_{flgB}-*csrA*$_{Bb}$-six-His tag cloned into pBSV2	This study

[a] Kan^r, kanamycin resistance.

gene expression in *B. burgdorferi* even though there is no sequence information or identity of its cognate small RNA molecule.

Previous studies have shown the advantages of overexpression of select genes coding for lipoproteins in *B. burgdorferi*, facilitating evaluation of their contributions in terms of adherence and infectivity (81, 83). The methodology was to replace the native promoters of these genes with constitutive borrelial promoters to relieve the need for appropriate signals critical for their continued expression (82). This strategy is also useful in the analysis of potential regulators of gene expression either when the environmental conditions for their induction are yet to be identified or when it is difficult to biochemically or phenotypically characterize their role due to limitations of determining the levels of expression. Moreover, the regulators may exert their effects posttranscriptionally, and hence, their levels per se may not exhibit a change upon variation of environmental signals while their "state" (such as levels of phosphorylation, oxidation, and dimerization) under these various conditions could have significant bearing on gene expression (28).

We initially determined that there were minimal levels of $CsrA_{Bb}$ in various clonal isolates of *B. burgdorferi* propagated at pH 7.6 and 32°C with the use of anti-$CsrA_{Bb}$ serum. However, there was significant upregulation of $CsrA_{Bb}$ when the spirochetes were propagated under conditions mimicking the midgut of fed ticks (pH 6.8 and 37°C) compared to that of unfed ticks (pH 7.6 and 23°C). In this study, we describe the effect of overexpression of CsrA under the control of a borrelial promoter, P_{flgB}, by using the shuttle vector pBSV2 on morphology and expression of lipoproteins in *B. burgdorferi*. The analysis of the overexpression strain indicates that $CsrA_{Bb}$ has an effect on multiple determinants affecting morphology and motility and pathogenesis-related events of *B. burgdorferi.*

MATERIALS AND METHODS

Bacterial strains and growth conditions. A clonal, noninfectious isolate of *B. burgdorferi* strain B31 (ML23, Table 1), lacking linear plasmid 25 (lp25 negative), was used for the overexpression of $CsrA_{Bb}$ (44, 45, 65). All *B. burgdorferi* cultures used for transformations were grown in 1% CO_2 at 32°C in Barbour-Stoenner-Kelly II (BSK-II) liquid medium (pH 7.6) supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR) (51, 52). In order to determine the levels of synthesis of $CsrA_{Bb}$ under different host-specific conditions, we also 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 and 23°C) and after (pH 6.8 and 37°C) a blood meal to determine if these environmental signals altered levels of CsrA. *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 (27).

Expression of recombinant $CsrA_{Bb}$. Total genomic DNA obtained from *B. burgdorferi* clonal isolate MSK5 (Table 1) was used as a template to PCR amplify the full-length *csrA*$_{Bb}$ by using the primers (csrAF and csrAR) listed in Table 3 containing appropriate engineered restriction enzyme sites. The amplicon was cloned into pCR2.1-TOPO vector (Invitrogen), transformed into *E. coli* TOP10 cells, and subjected to blue/white colony screening in the presence of ampicillin (100 μg/ml) and kanamycin (50 μg/ml). The insert was excised with NdeI/XhoI and ligated into 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 junction of the plasmid containing the insert of the expected size was sequenced, and the plasmid, designated pES16 (Table 2), was used to transform the *E. coli* expression host (Rosetta; Novagen).

Detection of glycogen in *E. coli* overexpressing $CsrA_{Bb}$. *E. coli* (Rosetta) carrying the plasmid pES16 (Table 2) or the empty vector pET23a was propagated overnight in solid Kornberg's medium supplemented with 50 mM glucose, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and ampicillin (100 μg/ml) and stained for endogenous glycogen with iodine vapor as described previously (70).

Purification of $rCsrA_{Bb}$ and generation of anti-$CsrA_{Bb}$ serum. Recombinant CsrA (rCsrA$_{Bb}$) with a C-terminal six-histidine tag was overexpressed by inducing *E. coli* strains containing pES16 (pET23a-*csrA*$_{Bb}$) with 1 mM IPTG for 2 h (27). The bacterial pellets 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) per the manufacturer's instructions. The bound six-histidine-tagged proteins were eluted as 0.5-ml fractions with elution buffer (8 M urea, pH 4.5). The eluted proteins were analyzed on a sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel. Select fractions with the largest concentrations of eluted proteins were further purified using Amicon centrifugal filters (Millipore, Bedford, MA) to concentrate the proteins as well as to remove urea used in the purification process. An approximately 13-kDa protein was purified to homogeneity (data not shown), quantified by bicinchoninic acid assay (Pierce Thermo Fisher Scientific, Rockford, IL), and stored at −80°C until further use. The predicted molecular mass of CsrA is 9.53 kDa, but the rCsrA migrates as a 13-kDa protein, which could be attributed, in part, to the presence of the six-histidine tag. The purified rCsrA emulsified in equal volumes of Titermax (Sigma, St. Louis, MO) was used to immunize 6- to 8-week-old female BALB/c mice, and booster immunizations were given at days 14 and 21. Immunoblot analysis revealed the presence of specific antibodies to purified $rCsrA_{Bb}$ in the serum obtained on day 28 postimmunization (data not shown). Infection-derived serum was obtained from C3H/HeN mice at 8 weeks postinfection following intradermal inoculation with 1×10^3 spirochetes (MSK5, Table 1) via needle inoculation. 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.

TABLE 3. Oligonucleotides used in this study

Name[c]	Sequence (5′→3′)[a]
csrAF	ACGCCATATGCTAGTATTGTCAAGA (primer 3, 186949 to 186966)[b]
csrAR	ACGCCTCGAGATTTTCATTCTTGAAATA (primer 4, 187174 to 187191)[b]
csrAR2	ACGCGCGGCCGCGTGGTGGTGGTGGTGG TGATTTTCATTTTCATTCTTGAA
flgBaadAF	ACGCGAATTCGGAAGATTTCCTATTAAGG
regF	CCTGTTGAAGCAAGCATTGA (primer 1, 186034 to 186053)[b]
regR	AAAGGGACTTGCCCTTAACC (primer 5, 188093 to 188112)[b]
200bpF	GGTCGGACATTAAAGCAA (primer 2, 186753 to 186770)[b]
recAFq	ATGCTCTTGATCCTGTTTATGCAA
recARq	GGTATCAGGCTGACTAAGCCAAA
ospCFq	AATCAGTAGAGGTCTTGTCAAAAGCA
ospCRq	CCACAACAGGGCTTGTAAGCT
ospAFq	CCAAAGACAAGTCATCAACAGAAGAA
ospARq	GGTTCCGTCTGCTCTTGTTATTATTT
dbpAFq	GCAATTAAAAAAGACGCTGCTCTT
dbpARq	CCCCACTACCCGTTTTTTTATCT
p66Fq	GGAACAATTCAGCTGGGATACAA
p66Rq	CTGTTCCCCCTATTGCAAATG
BbA64Fq	TCAAAACTCAGCCAAGACTCAAAC
BbA64q	GCTAATTGAATGCTAAAGCCTCTGT
napAFq	TGCTGGTGATTATGGTACTGCTAATAT
napARq	AAGCAATGCCTTATGCATCCA
flaBFq	CAGCTAATGTTGCAAATCTTTTCTCT
flaBRq	TTCCTGTTGAACACCCTCTTGA
BbK32Fq	CAAGTAAGTGTAAAGACTGCAGCAAA
BbK32Rq	CTGCTTCAATGTTCTCTAATTTCCTTTT

[a] Restriction sites are underlined.

[b] Primer used for cDNA PCR (Fig. 2). Numbers indicate the coordinates in the chromosome as they appear in the TIGR database.

[c] Primers indicated with a letter q as a suffix were used for quantitative RT-PCR.

Overexpression of CsrA in *B. burgdorferi*. The insert from pES16 (Table 2) containing the full-length $CsrA_{Bb}$ with the six-histidine tag was used as a template with primers having engineered restriction enzyme sites NdeI and NotI (csrAF and csrAR2, Table 3). The amplicon was cloned into pCR2.1, digested with NdeI and NotI, and recloned into pML102 so as to facilitate expression of $CsrA_{Bb}$ under the control of a constitutive borrelial promoter, P_{flgB}. In order to overexpress $CsrA_{Bb}$ in *B. burgdorferi*, P_{flgB}-*csrA*$_{Bb}$-His tag was cloned into the borrelial shuttle vector pBSV2 using restriction enzymes that flank P_{flgB}-*csrA*-His tag in pCR2.1. The plasmid designated pES25 was used to transform a clonal isolate of *B. burgdorferi* strain B31 (ML23; lp25 negative) as described previously (51, 72). The transformants were selected on BSK-II agar overlay supplemented with kanamycin (200 μg/ml) and checked for the presence of pBSV2. The level of expression of $CsrA_{Bb}$ in *B. burgdorferi* transformants was also verified by immunoblot assay using monospecific anti-CsrA serum.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis. *B. burgdorferi* whole-cell lysates were prepared and separated on SDS-12.5% polyacrylamide gels as described previously (51, 52). The separated proteins were either visualized by Coomassie brilliant blue staining or transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond-P; GE Healthcare, Buckinghamshire, United Kingdom) and subjected to immunoblot analysis. The membranes were probed with anti-OspA monoclonal antibodies, mouse anti-OspC, anti-BBK32, anti-DbpA, anti-SodA, anti-P66, and rat anti-BBA64 (15) and rabbit anti-NapA serum. 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).

Morphology of *B. burgdorferi*. The control strain (ML23/pBSV2) and overexpression strain (ES25) were grown to a density of 5×10^7 spirochetes/ml and washed three times in Hanks balanced salt solution-5 mM $CaCl_2$-50 mM sucrose and evaluated using the Live/Dead *BacLight* bacterial viability kit (Molecular Probes, Invitrogen, Carlsbad, CA) in conjunction with confocal microscopy (27). The images were captured using a Zeiss LSM510 microscope and deconvolved using AutoQuantX (MediaCybernetics Inc., Bethesda, MD).

RNA extraction. RNA was extracted as previously described (73). Briefly, *B. burgdorferi* cultures were grown to a density of 2×10^7 to 3×10^7 spirochetes per ml and RNA was extracted by resuspending the bacterial pellets in RNA-Bee (Tel-Test, Inc., Friendswood, TX) at a ratio of 0.2 ml to 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* primers (recAFq and recARq) to detect contaminating DNA (55, 80). 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 3) specific to *ospC*, *ospA*, *dbpA*, *p66*, BBA64, *flaB*, and BBK32 at a final concentration of 100 nM, and quantitative real-time PCR was done using the ABI Prism 7300 system (Applied Biosystems). The induction of each gene in ES25 relative to that in MSK5 or ML23/pBSV2 was normalized to the levels of *recA* as previously described (55, 80). The threshold cycle (C_T) values of each of the genes from three independent experiments 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 $2^{-\Delta\Delta CT}$, where C_T is the cycle number of the detection threshold, as described previously (42, 73). To determine whether our real-time reverse transcription-PCR (RT-PCR) data were statistically significant, the differences between the normalized C_T values obtained for ES25 and those for the ML23/pBSV2 or MSK5 strain were subjected to a two-way analysis of variance followed by the Bonferroni post hoc test implemented in PRISM. Statistical significance was accepted when the P values were less than 0.05.

Analysis of genomic organization of *csrA*$_{Bb}$ in the borrelial chromosome. Total RNA was extracted from MSK5 as described above, and RNA samples devoid of contaminating DNA were reverse transcribed to cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). A primer specific to the 3′ end of *csrA*$_{Bb}$ (csrR, Table 3) was used in conjunction with primers specific to 200 bp (200bpF, Table 3) and 1,000 bp (regF, Table 3) upstream of *csrA*$_{Bb}$ that were predicted to generate 1,157-bp and 438-bp amplicons, respectively. In addition, a primer specific to the 5′ end of *csrA* (csrAF, Table 3) was used with a primer (regR, Table 3) corresponding to the downstream region of *csrA*$_{Bb}$ that would result in an amplicon of 1,163 bp. The total RNA (reverse-transcriptase-negative [−RT] control) and the cDNA generated from the total RNA from MSK5 were used as the template to determine if *csrA*$_{Bb}$ was cotranscribed with the members of a previously characterized *flgK* motility operon (34). Total genomic DNA from MSK5 served as the positive control for the expected size of the amplicons that would indicate if *csrA*$_{Bb}$ was organized within the *flgK* motility operon or was under the control of an independent promoter.

RESULTS

Overexpression of $CsrA_{Bb}$ in *E. coli* inhibits glycogen biosynthesis/accumulation. The ORF BB0184 present in the linear chromosome of *B. burgdorferi* has been annotated as a homolog of CsrA/RsmA (32). In several bacterial species, CsrA/RsmA has been characterized as a small RNA binding protein capable of regulating multiple metabolic and virulence mechanisms via precise interactions with its cognate small RNA molecules CsrB and CsrC (7, 53). Amino acid sequence analysis of borrelial CsrA indicated significant sequence conservation and the presence of two regions that have been biochemically shown to contain several critical residues (Fig. 1A) that mediate the interaction of CsrA with its cognate small RNA molecule in *E. coli* (53). An interesting feature of the borrelial CsrA in all three sequenced species (*B. burgdorferi* strain B31, *Borrelia afzelii* PKo, and *Borrelia garinii* PBi) is the presence of an additional 7 amino acids at the C terminus, even though the exact contribution of these residues to the function of $CsrA_{Bb}$ remains to be determined. Seminal studies of *E. coli* indicated that *csrA* negatively regulates expression of genes

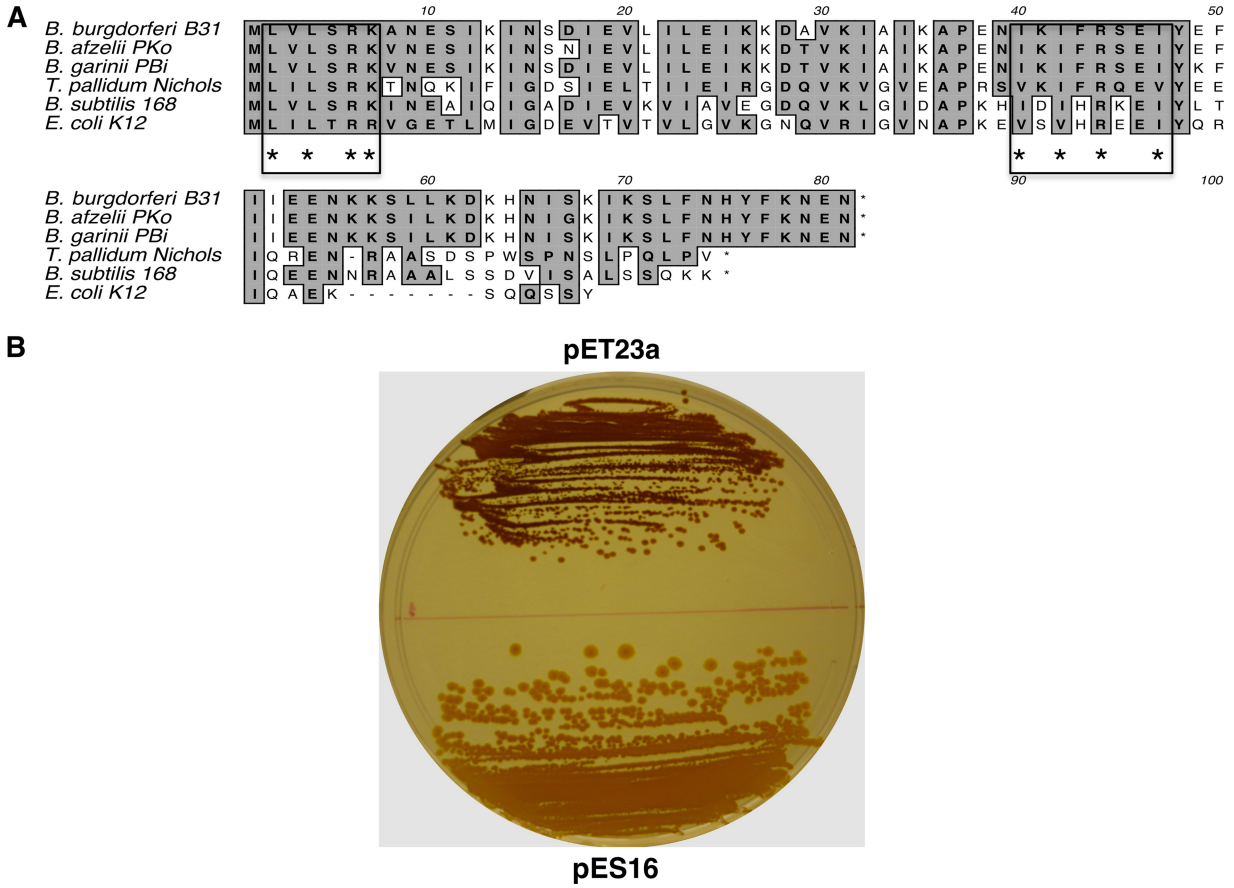


FIG. 1. Overexpression of $CsrA_{Bb}$ results in inhibition of glycogen synthesis in *E. coli*. (A) Sequence analysis of $CsrA_{Bb}$ revealed significant conservation of amino acid residues with the CsrA homologs present in various bacterial species. Several residues (*) within the two subdomains (indicated by boxes) that have been shown to be critical for binding of CsrA to small noncoding RNAs in other bacterial species are conserved in $CsrA_{Bb}$ (53). (B) Overexpression of $CsrA_{Bb}$ inhibits glycogen synthesis in *E. coli.* The *E. coli* Rosetta expression host containing either pES16 (pET23a/$csrA_{Bb}$) or pET23a alone was grown overnight on solid Kornberg's medium supplemented with 50 mM glucose and 1 mM IPTG at 37°C and exposed to iodine vapor, and the intense brown coloration of the colonies is indicative of the endogenous glycogen levels.

involved in biosynthesis of glycogen such as *glgB* (encoding glycogen branching enzyme), *glgC* (encoding ADP-glucose pyrophosphorylase), and *pckA* (encoding gluconeogenic enzyme phosphoenolpyruvate carboxy kinase), which results in reduced levels of glycogen synthesis and accumulation when levels of CsrA are elevated upon expression from a high-copy-number plasmid (70). Even though no typical homologs of the aforementioned genes involved in glycogen biosynthesis could be identified in *B. burgdorferi* based on sequence analysis, we determined if $CsrA_{Bb}$ would inhibit the synthesis and accumulation of glycogen when expressed in *E. coli*. When the *E. coli* Rosetta strain carrying pES16 (pET23a/$csrA_{Bb}$) was propagated in solid Kornberg's medium supplemented with 50 mM glucose and 1 mM IPTG, there was reduced accumulation of glycogen as determined by the exposure to iodine vapor compared to that in the *E. coli* Rosetta strain carrying the empty vector pET23a (Fig. 1B). This "complementation" analysis (in the presence of an intact copy of *csrA* in the *E. coli* Rosetta strain) demonstrated that $CsrA_{Bb}$ has several features similar to that of the *E. coli* CsrA to mediate the suppression of glycogen biosynthesis, even though this may not be its primary role in the pathophysiology of *B. burgdorferi*.

***$csrA_{Bb}$* is part of the *flgK* operon of *B. burgdorferi*.** Bioinformatic analysis of the genomic location of *csrA* in three different borrelial strains that have been sequenced revealed that it is downstream of several ORFs associated with motility. Ge et al. (34) demonstrated that the *flgK* motility operon consists of *flbF* (BB0180; unknown function), *flgK* (BB0181, encoding HAP1), *flgL* (BB0182, encoding HAP3), and *orfX* (BB0183, encoding FliW). The analysis of the whole-genome sequence of *B. burgdorferi* facilitated the annotation of the ORF immediately downstream of what was characterized as the *flgK* motility operon as *csrA* (BB0184). In order to determine if $csrA_{Bb}$ is indeed a part of the *flgK* operon, we performed RT-PCR using primers specific to $csrA_{Bb}$ and to regions up- and downstream of $csrA_{Bb}$ similar to procedures described previously (34, 62). As shown in Fig. 2A, when primer 2 (200bpF, within BB0183) and primer 4 (csrAR) were used with the total cDNA from MSK5, there was an amplification of the 431-bp fragment (Fig. 2B, lane 2) consistent with the size of the fragment generated using the total genomic DNA (Fig. 2B, lane 3). This observation indicated that $csrA_{Bb}$ was part of the *flgK* operon and is cotranscribed with its upstream neighbor, BB0183. Similarly, primer 1 (regF, within BB0182; Table 3) and primer 4 (csrAR,

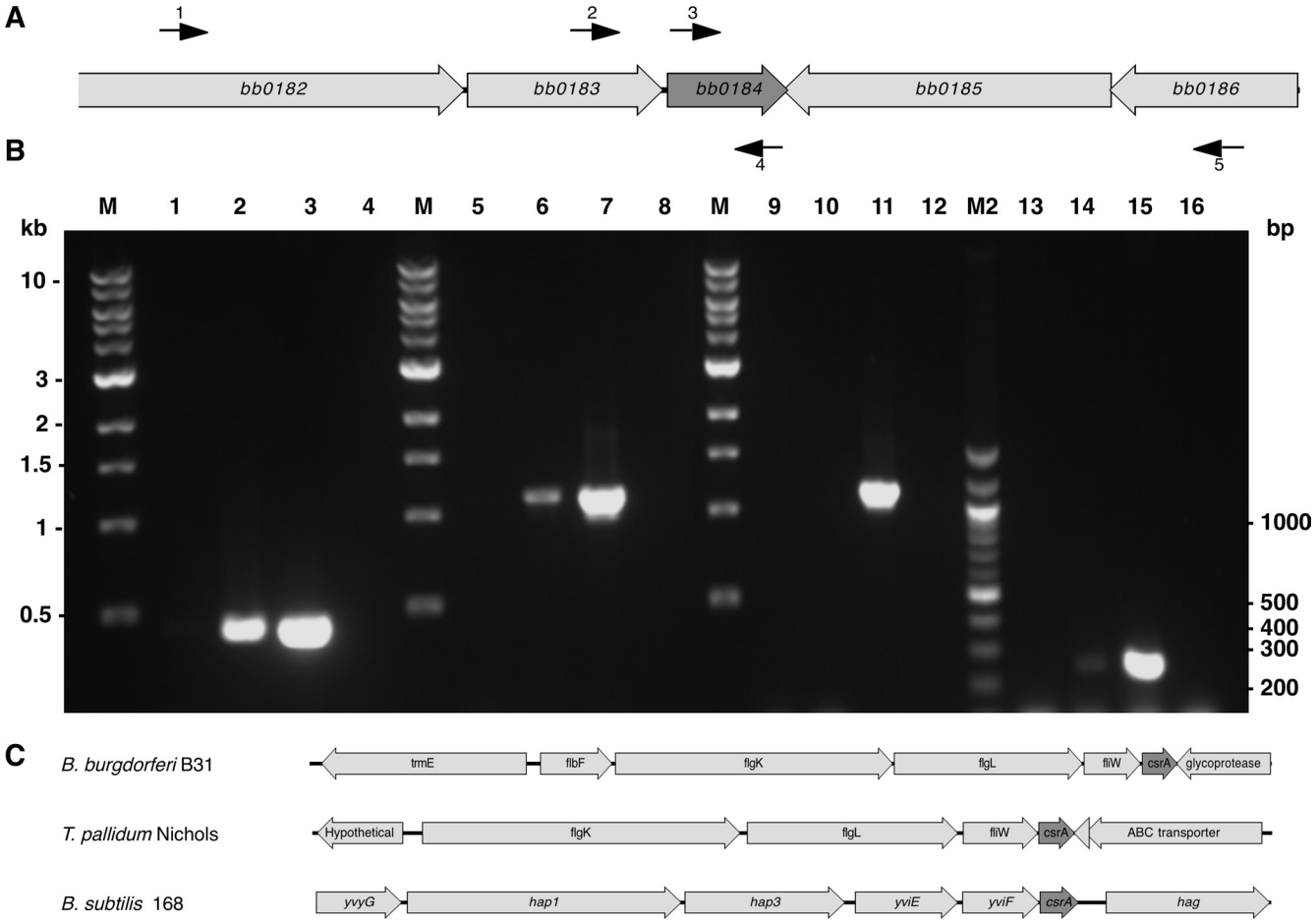


FIG. 2. $csrA_{Bb}$ (bb0184) is cotranscribed with bb0182-bb0183 and is the terminal ORF of the *flgK* motility operon. (A) Schematic representation of a region of the *flgK* motility operon that has been previously shown to extend from bb0180 to bb0183 (34). The arrows with numbers refer to the primers (Table 3) used in the RT-PCR depicted in panel B. (B) RT-PCR products separated on a 1% agarose gel stained with ethidium bromide. Lanes 1 to 4, PCR with primer sets 2 and 4; lanes 5 to 8, PCR with primer sets 1 and 4; lanes 9 to 12, PCR with primer sets 3 and 5; lanes 13 to 16, PCR with primer sets 3 and 4. The templates used in the PCRs are from MSK5 and are as follows: for lanes 1, 5, 9, and 13, total RNA (−RT control); for lanes 2, 6, 10, and 14, cDNA (+RT); for lanes 3, 7, 11, and 15, total genomic DNA; for lanes 4, 8, 12, and 16, PCR master mix with no template (double-distilled H_2O control). Lanes M and M2, molecular size markers in kilobases (M) or base pairs (M2) as indicated on left and right sides, respectively, of the image. The image was generated using the Versadoc imaging system (Bio-Rad Laboratories, Hercules, CA). (C) Genomic organization corresponding to the location of $csrA_{Bb}$ in the linear chromosome of *B. burgdorferi*. A similar organization of genes surrounding $csrA_{Bb}$ is also observed in the sequenced species *Borrelia afzelii* PKo and *Borrelia garinii* PBi (not shown). CsrA is also located downstream of homologs of flagellar biosynthesis in *Treponema pallidum* and upstream of *hag* in *B. subtilis*, mediating regulation of morphology by altering levels of Hag.

Table 3) amplified an 1,154-bp fragment when the total cDNA was used (Fig. 2B, lane 6) consistent with the size of the amplicon generated using total genomic DNA (Fig. 2B, lane 7). This observation further confirmed that $csrA_{Bb}$ is indeed a part of the *flgK* operon, as it is cotranscribed with BB0182 (*flgL*). There was no amplification when either of the primer sets (primers 1 and 4 or primers 2 and 4) was used with the total RNA (−RT control) as the template (Fig. 2B, lanes 1 and 5, respectively). Moreover, when primer 5 (regR, Table 3) was used with primer 3 (csrAF, Table 3), there was no amplification with the cDNA generated from total RNA of MSK5, as BB0186 is in the orientation opposite that of $csrA_{Bb}$ (Fig. 2B, lane 10). However, the same primer set generated an 1,163-bp amplicon when total genomic DNA was used as the template (Fig. 2B, lane 11). When primers 3 and 4 specific to $csrA_{Bb}$ were used, a 243-bp amplicon was generated with the cDNA as the template (Fig. 2B, lane 14), consistent in size with what was observed with total genomic DNA (Fig. 2B, lane 15). There was no amplification when total RNA (−RT) was used as the template with any of the primer sets used in this analysis (Fig. 2B, lanes 1, 5, 9, and 13) or with any of the primer sets used with PCR master mix without template (Fig. 2B, lanes 4, 8, 12, and 16). Taken together, these observations indicate that $csrA_{Bb}$ is cotranscribed with its upstream neighbors BB0183 and BB0182 and that the last ORF in the *flgK* operon is indeed $csrA_{Bb}$. The *csrA* homolog of another related spirochete, *Treponema pallidum*, is also organized similarly to that of $csrA_{Bb}$ (Fig. 2C). Moreover, in *Bacillus subtilis*, the *csrA* homolog is located upstream of *hag*, which in turn has been characterized as being involved in the motility of *B. subtilis* (84).

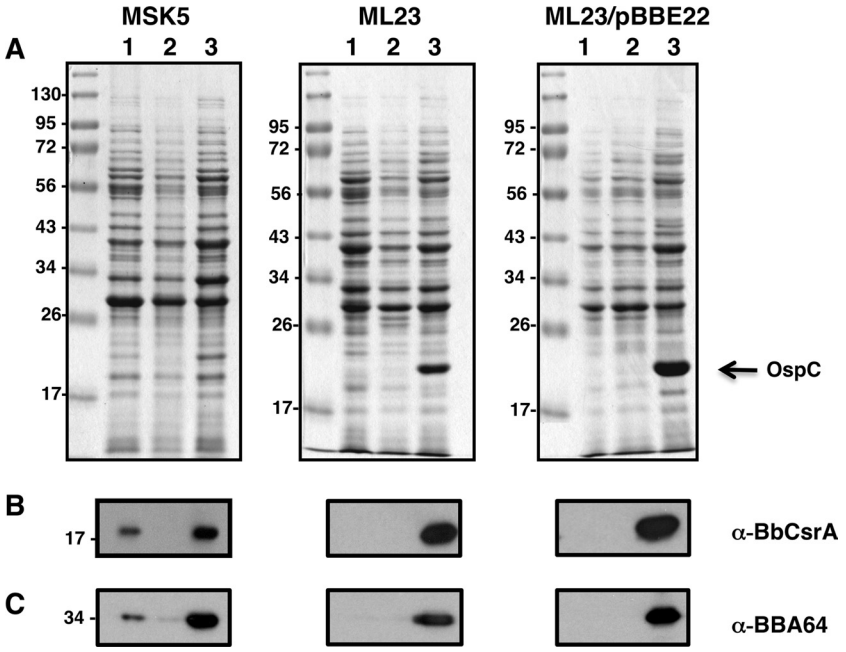


FIG. 3. Increased synthesis of $CsrA_{Bb}$ under fed-tick conditions. *B. burgdorferi* strain B31 clonal isolates MSK5, ML23, and ML23/pBBE22 were propagated under various conditions mimicking the unfed- or fed-tick midgut conditions such as pH 7.6 and 32°C (lanes 1), pH 7.6 and 23°C (lanes 2), and pH 6.8 and 37°C (lanes 3). (A) Total protein samples were separated on an SDS-12.5% polyacrylamide gel and stained with Coomassie brilliant blue. The arrow corresponds to OspC, and the markers on the left indicate the molecular masses of protein standards in kilodaltons. (B and C) Immunoblot analysis using monospecific serum against $CsrA_{Bb}$ (B) and BBA64 (C). The blots were developed by using the enhanced chemiluminescence system. The numbers to the left of each panel indicate the molecular masses in kilodaltons.

$CsrA_{Bb}$ synthesis is elevated under conditions that mimic the midgut of fed ticks. In order to determine if the level of $CsrA_{Bb}$ expression is dependent on the environmental signals present in the tick midgut before and after a blood meal, we analyzed protein lysates from MSK5, ML23, and ML23/pBBE22 propagated at pH 7.6 and 32°C and pH 7.6 and 23°C (unfed-tick midgut conditions) and pH 6.8 and 37°C (fed-tick midgut conditions), respectively, using monospecific anti-$CsrA_{Bb}$ antibodies. As shown in Fig. 3, the level of $CsrA_{Bb}$ was elevated under fed-tick conditions in all three strains (Fig. 3B, lanes 3, MSK5, ML23, and ML23/pBBE22) while $CsrA_{Bb}$ was absent under unfed-tick conditions (Fig. 3B, lanes 2, MSK5, ML23, and ML23/pBBE22). The level of $CsrA_{Bb}$ was reduced in MSK5 at pH 7.6 and 32°C compared to the level at pH 6.8 and 37°C, whereas it was absent in both ML23 and ML23/pBBE22 when the spirochetes were propagated at pH 7.6 and 32°C. There were increased levels of OspC in all three strains of *B. burgdorferi* (as indicated by the arrow in Fig. 3A) consistent with the previous studies that demonstrated significant increases in the levels of this lipoprotein under environmental conditions that mimicked the conditions of the midgut of fed ticks. In addition, the Coomassie blue-stained gel also served as a control for the relative loading of various samples, indicating that the increased level of $CsrA_{Bb}$ observed under fed-tick conditions was not an artifact of varied levels of protein separated on the SDS-polyacrylamide gel. We (51, 52) and others (38) have previously demonstrated that the levels of BBA64 encoded on linear plasmid 54 (lp54) increase when *B. burgdorferi* is propagated under fed-tick conditions (15, 36, 37, 66). Immunoblot analysis using rat anti-BBA64 serum demonstrated that the level of BBA64 is indeed elevated in *B. burgdorferi* under fed-tick conditions (Fig. 3C, lanes 3) and coincided with the increased levels of $CsrA_{Bb}$ under conditions mimicking the midgut of fed ticks (Fig. 3B, lanes 3) in MSK5, ML23, and ML23/pBBE22. There were no detectable levels of either $CsrA_{Bb}$ (Fig. 3B, lanes 2) or BBA64 (Fig. 3C, lanes 2) in any of these controls when they were propagated under conditions similar to those of the midgut of unfed ticks (pH 7.6 and 23°C). When these three control strains were propagated at pH 7.6 and 32°C, only MSK5 had detectable levels of $CsrA_{Bb}$ and BBA64 (Fig. 3B and C, lanes 1), whereas neither of these proteins was detected under the above conditions in both ML23 and ML23/pBBE22 (Fig. 3B and C, lanes 1). These observations suggested that the levels of $CsrA_{Bb}$ may, therefore, contribute to the alteration in the levels of determinants (such as OpsC and BBA64) of *B. burgdorferi* that facilitate its adaptation to the mammalian host following transmission from ticks. We decided to exploit the lack of synthesis of $CsrA_{Bb}$ in ML23 when propagated at pH 7.6 and 32°C to determine if constitutive overexpression of $CsrA_{Bb}$ (under the control of a heterologous promoter, P_{flgB}) will facilitate delineation of its role independently of the culture conditions that induced its synthesis. Moreover, we also intended to develop a strategy to isolate the homolog(s) of the cognate small RNA molecule interacting with $CsrA_{Bb}$ that has been reported to regulate gene expression in other bacterial systems.

Overexpression of $CsrA_{Bb}$ in *B. burgdorferi* alters its morphology. We overexpressed $CsrA_{Bb}$ (ES25) under the control of a constitutive promoter, P_{flgB} (35), with a C-terminal six-histidine tag in an lp25-deficient, clonal isolate of *B. burgdorferi*

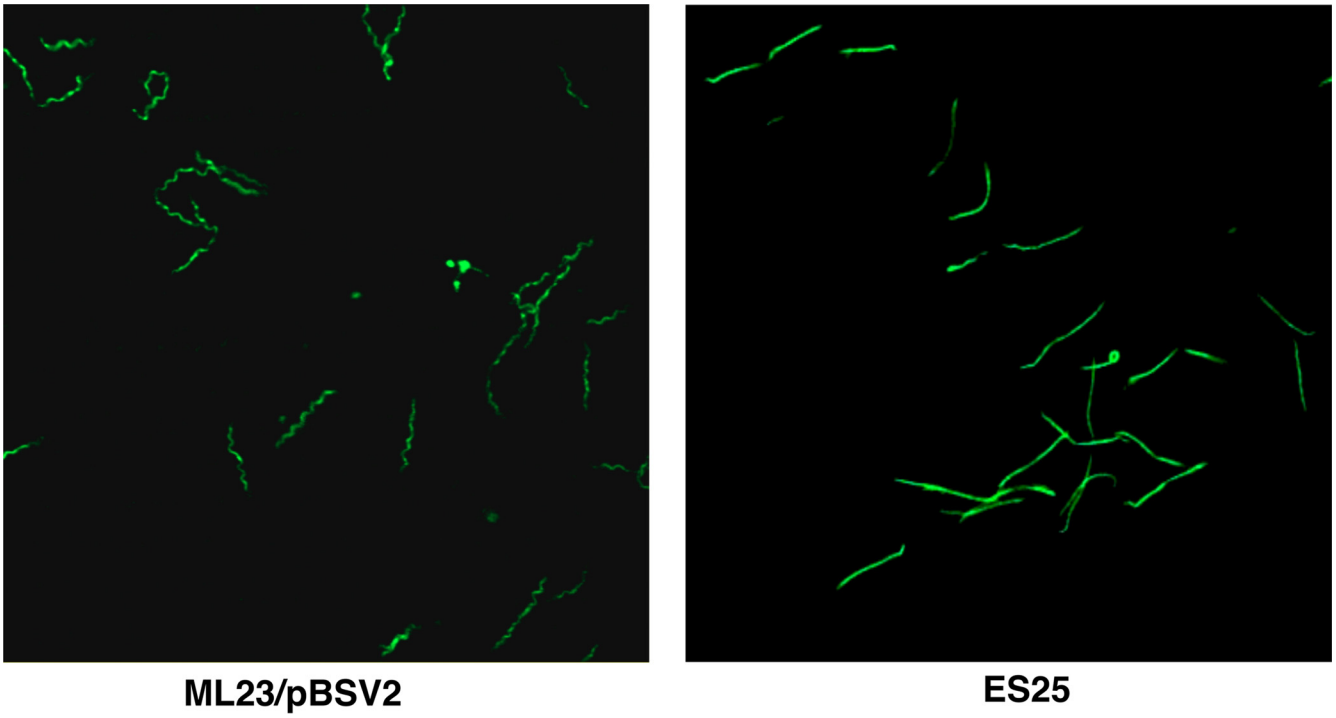
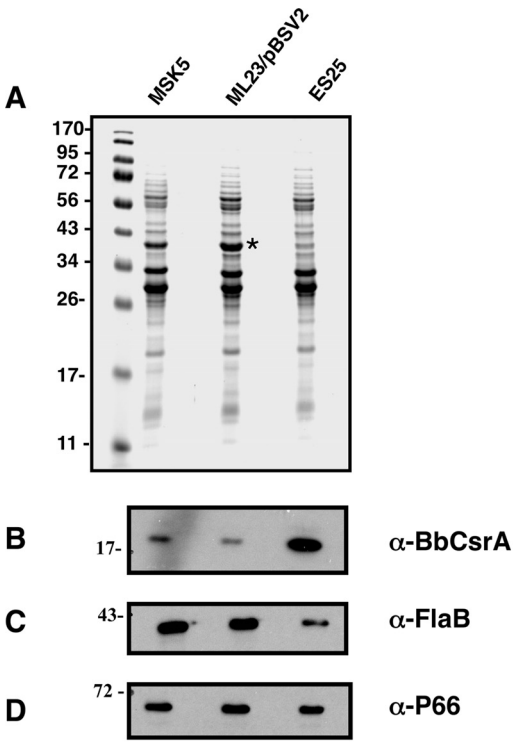


FIG. 4. Cell morphology of *B. burgdorferi* upon overexpression of CsrA. The spirochetes propagated in BSK-II medium supplemented with 6% normal rabbit serum at pH 7.6 and 32°C were washed, visualized using a Zeiss LSM510 microscope, and deconvolved using AutoQuant X following Live/Dead staining. Representative images are shown indicating the loss of "wave-form" morphology observed upon overexpression of $CsrA_{Bb}$ in ES25.

(ML23) by using the borrelial shuttle vector pBSV2 (78). Surprisingly, we found that the colonies on BSK-II agar overlays that grew in the presence of kanamycin (conferred by the shuttle vector) were compact compared to those of the parental strain (data not shown). Analysis of spirochetes following Live/Dead staining with confocal microscopy revealed significant differences between ES25 and the parental strain ML23/pBSV2 (Fig. 4). The cells from the parental strain had typical "wave-form" morphology, whereas those from ES25 appeared to be more "rod shaped" with reduced motility as visualized under dark-field microscopy (56). ES25 did not exhibit the typical corkscrew motility observed with the control strains but was motile as determined by dark-field microscopy. The altered morphology upon overexpression of $CsrA_{Bb}$ was observed at different culture densities ranging from 1×10^6 to 5×10^7 spirochetes/ml, indicating that it was independent of accumulation of various metabolites and/or changes in the conditions of the medium, and the morphology was persistent even after six in vitro passages of ES25. These observations indicated that the altered morphology is essentially due to overexpression of $CsrA_{Bb}$ under the control of the P_{flgB} promoter. Moreover, we have not observed such persistent changes in morphology when other borrelial determinants such as *sodA* or a variety of antibiotic resistance markers have been expressed under the control of P_{flgB} (27, 52, 74, 75).

Overexpression of $CsrA_{Bb}$ results in reduced expression of FlaB. In order to further characterize the effect of overexpression of CsrA, we analyzed the total proteins from ML23/pBSV2 and ES25 on a 12.5% SDS-polyacrylamide gel. Coomassie blue staining of the gels revealed a significant reduction in the levels of an approximately 41-kDa protein in ES25 compared to the levels of a similar-sized protein in the control strain (ML23/pBSV2) as well as in a clonal isolate of *B. burgdorferi* strain B31 (MSK5) carrying the full complement of plasmids (Fig. 5A). Mass spectrophotometric analysis of this protein isolated from ML23/pBSV2 (indicated by an asterisk in Fig. 5A) identified three different tryptic peptides with sequence identity with FlaB (BB0147) as shown in Fig. 5E. The N-terminal peptide (MIINHNTSAINASR) had 100% identity with the N-terminal sequence of FlaB, the endoflagellin of *B. burgdorferi*. Sequence analysis of additional peptides (ASDDA AGMGVSGKINAQIR and AINFIQTTEGNLNEVEK) had one amino acid difference from the deduced amino acid sequence of FlaB of *B. burgdorferi* (32). Moreover, immunoblot analysis of total protein lysates with anti-FlaB monoclonal antibodies indicated that there was less FlaB in ES25 than in ML23/pBSV2 and MSK5 (Fig. 5C). The reduced levels of FlaB coincided with increased levels of $CsrA_{Bb}$ observed in ES25 by immunoblot analysis using monospecific serum against $CsrA_{Bb}$ (Fig. 5B). The levels of $CsrA_{Bb}$ were significantly lower in both parental strain ML23/pBSV2 and MSK5 when they were propagated under conditions similar to those for ES25 at pH 7.6 and 32°C (Fig. 5B). $CsrA_{Bb}$ in the borrelial isolates, as determined by immunoblot analysis, migrates as an approximately 18-kDa protein, which is almost twice the predicted size. While this difference in the migration pattern could be due to dimerization, translational readthrough, or other unknown modifications, the specificity of the anti-CsrA serum to this band (18



E **MIINHNTSAINASR**NNGINAANLSKTQEKLSSGYRIN
R**ASDDAAGMGVSGKINAQIR**GLSQASRNTSK**AINF**
IQTTEGNLNEVEKVLVRMKELAVQSGNGTYSDADR
GSIQIEIEQLTDE

FIG. 5. Overexpression of CsrA in *B. burgdorferi* results in reduced expression of FlaB. A clonal isolate of *B. burgdorferi* strain B31 with the full complement of plasmids (MSK5), the parental strain ML23/pBSV2, and CsrA overexpression isolate ES25 were grown in BSK-II growth medium with 6% normal rabbit serum at pH 7.6 and 32°C to a density of 5×10^7 spirochetes/ml. (A) Total protein lysates were separated on an SDS-12.5% polyacrylamide gel and stained with Coomassie brilliant blue. The markers on the left indicate the molecular masses of protein standards in kilodaltons. An approximately 41-kDa protein that was present in ML23/pBSV2 and reduced in expression in ES25 is indicated with an asterisk. (B to D) Immunoblot analysis using monospecific anti-$CsrA_{Bb}$ serum (B), anti-FlaB monoclonal antibodies (C), or monospecific anti-P66 serum (D). (E) Sequences of peptides obtained on spectrophotometric analysis of the tryptic digests of the band indicated by an asterisk in ML23/pBSV2 are indicated in underlined boldface. The sequence of the first peptide (residues 1 to 14) had 100% identity to the deduced amino acid sequence of FlaB of *B. burgdorferi*. The other two peptides had one residue difference in each from the deduced sequence of FlaB from *B. burgdorferi*.

kDa) in the total lysates of *B. burgdorferi* clearly demonstrates that this protein is indeed $CsrA_{Bb}$. This conclusion is also supported by the increased detection of a similar-sized protein upon overexpression of $CsrA_{Bb}$ in ES25 (Fig. 5B). All three isolates had similar levels of P66, an integrin binding porin (76), providing evidence that increased expression of $CsrA_{Bb}$ has effects on only a select set of proteins. Taken together, these observations indicate that the levels of FlaB are reduced upon overexpression of $CsrA_{Bb}$ in *B. burgdorferi*. The effect of overexpression of CsrA on levels of FlaB of *B. burgdorferi* provides an added significance to its genetic location within the *flgK* operon, as shown in Fig. 2, even though the direct role of $CsrA_{Bb}$ in regulating FlaB expression is yet to be determined. It should be pointed out that ML23/pBVS2 at pH 7.6 and 32°C exhibits detectable levels of $CsrA_{Bb}$ (Fig. 5B, lane 2) compared to those in ML23 and ML23/pBBE22 (Fig. 3, lanes 1, ML23 and ML23/pBBE22). The samples depicted in Fig. 2 and 5 were from two separate experiments, and the effects of changes in the composition of the growth medium and cell density and the consequent changes in pH could have contributed to the increased levels of $CsrA_{Bb}$ in ML23/pBVS2, even though all three strains have essentially the same genetic background with or without the borrelial shuttle vector pBSV2. We, therefore, decided to determine if the increased level of $CsrA_{Bb}$ in ES25 observed under the unfed-tick conditions (Fig. 6J, lane 1), reflecting the levels from the heterologous promoter P_{flgB}, is sufficient to induce increased synthesis of proteins such as OspC and BBA64 which are not normally elevated in *B. burgdorferi* under unfed-tick conditions (51, 52).

Overexpression of CsrA alters expression of lipoproteins of *B. burgdorferi*. We further examined the levels of various lipoproteins in *B. burgdorferi* overexpressing $CsrA_{Bb}$. Antisera generated against low-dose infection of MSK5 in C3H/HeN mice recognized several antigens that were upregulated in ES25 compared to ML23/pBSV2 (Fig. 6A). The levels of expression of these lipoproteins were elevated in the clonal isolate MSK5, which correlates with increased expression of $CsrA_{Bb}$. We used monoclonal antibodies or monospecific sera to further identify the proteins differentially expressed in ES25. While the levels of OspA appears to be the same in ML23/pBSV2 and ES25 (Fig. 6B), there was a significant increase in the levels of OspC in ES25 over those in the control strain (Fig. 6C). This was also true with other lipoproteins such as BBK32 (Fig. 6D) and DbpA (Fig. 6E), which are well-characterized adhesins mediating adherence of *B. burgdorferi* to fibronectin (64, 75) and decorin (39), respectively. In addition, there was increased expression of BBA64 in ES25 while there was little or no expression of this lp54-encoded protein in both the parental control strain and MSK5 when these strains are propagated in BSK-II medium at pH 7.6 and 32°C. Previous studies have shown that increased expression of BBA64 was predominantly observed upon propagation of *B. burgdorferi* under in vitro growth conditions that mimic the tick midgut after the ingestion of a blood meal, namely, BSK-II growth medium at pH 6.8 and 37°C (21, 51, 52, 85). Therefore, the increased synthesis of BBA64 in ES25 can be attributed to the increased levels of $CsrA_{Bb}$. The levels of NapA and SodA (Fig. 6G and F) were found to be similar in both the parental control strain and ES25, suggesting that the overexpression of $CsrA_{Bb}$ does not seem to have a significant effect on proteins that have been characterized as mediating resistance to oxidative stressors in *B. burgdorferi* (13, 27, 47). Except for DbpA, the levels of OspC, BBK32, and BBA64 were higher in ES25 than in MSK5. The levels of NapA and SodA were also similar between MSK5, ML23/pBSV2, and ES25, suggesting a lack of significant correlation of levels of these proteins with the expression of $CsrA_{Bb}$.

Overexpression of $CsrA_{Bb}$ results in increased synthesis of OspC and BBA64 under unfed-tick midgut conditions (pH 7.6 and 23°C). In order to determine if $CsrA_{Bb}$ can exert regulatory effects on proteins that have been previously shown to be regulated by RpoS, we analyzed ES25 propagated under either

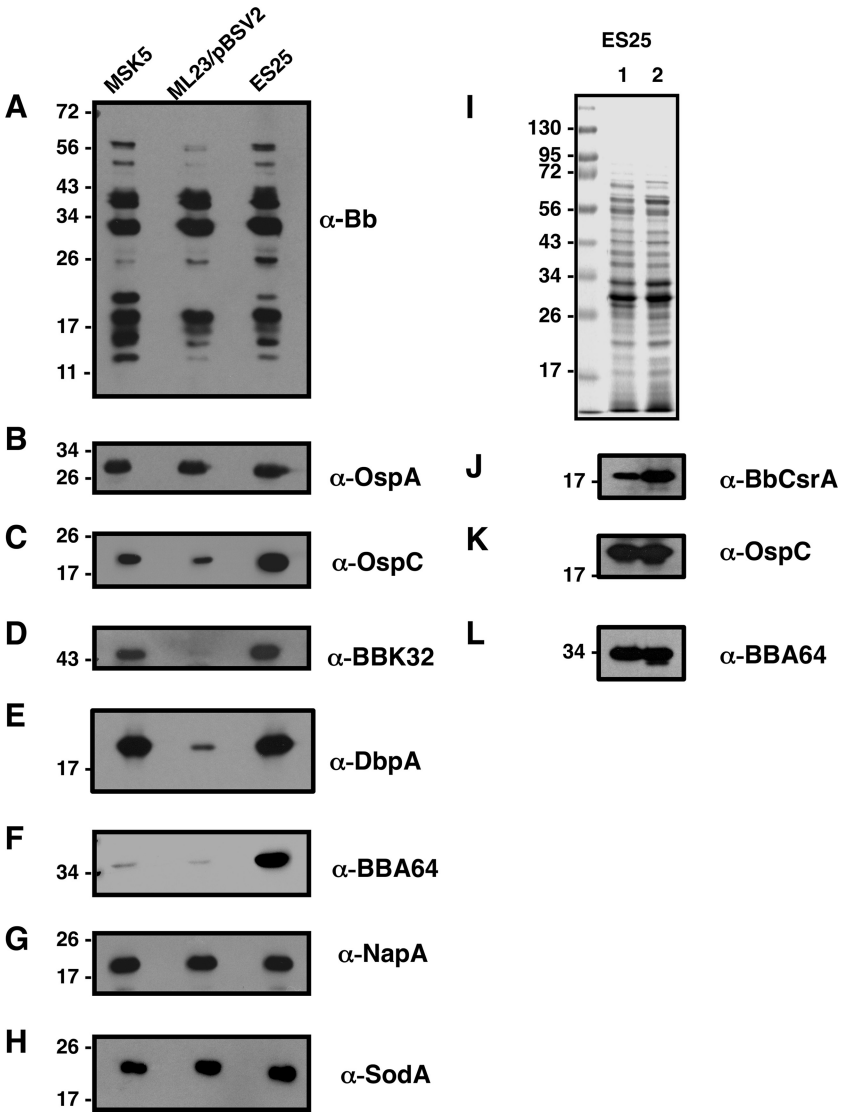


FIG. 6. Overexpression of CsrA results in increased expression of borrelial determinants associated with pathogenesis. Equivalent numbers of spirochetes from MSK5, ML23/pBSV2, and ES25 (as shown in Fig. 5A) propagated in BSK-II medium with 6% normal rabbit serum at pH 7.6 and 32°C to a density of 5×10^7 spirochetes/ml were resolved by SDS-PAGE, immobilized onto PVDF membranes, and probed with monoclonal antibodies or antisera as described in Materials and Methods. (A to H) Immunoblots were developed with *B. burgdorferi* infection-derived mouse serum (A), anti-OspA (B), anti-OspC (C), anti-BBK32 (D), anti-DbpA (E), anti-BBA64 (F), anti-NapA (G), or anti-SodA (H). (I to L) Overexpression of $CsrA_{Bb}$ results in increased synthesis of OspC and BBA64 under unfed-tick midgut conditions. The $CsrA_{Bb}$ overexpression strain, ES25, was propagated under conditions mimicking either the unfed-tick midgut (pH 7.6 and 23°C) (lanes 1) or the fed-tick midgut (pH 6.8 and 37°C) (lanes 2) to a density of 5×10^7 spirochetes/ml. Total protein lysates were resolved by SDS-PAGE and either stained with Coomassie blue (I) or immobilized onto PVDF membranes and probed with anti-$CsrA_{Bb}$ (J), anti-OspC (K), or anti-BBA64 (L). The numbers to the left of the panels indicate the molecular mass standards in kilodaltons proximate to each of the antigens.

unfed-tick conditions (pH 7.6 and 23°C) or fed-tick conditions (pH 6.8 and 37°C). Immunoblot analysis using anti-$CsrA_{Bb}$ serum demonstrated that increased levels of $CsrA_{Bb}$ in ES25 (in comparison to ML23 or ML23/pBBE22 [Fig. 3B]) grown under unfed-tick conditions (Fig. 6, ES25, lanes 1) are essentially due to the constitutive overexpression of $csrA_{Bb}$ under the control of a heterologous promoter, P_{flgB}. Moreover, there was a significant increase in the levels of $CsrA_{Bb}$ (Fig. 6J) when ES25 was propagated under fed-tick conditions (pH 6.8 and 37°C), which is due to the additive effects of increased expression from both the heterologous and native promoters of $csrA_{Bb}$. Interestingly, the levels of OspC (Fig. 6K) and BBA64 (Fig. 6L) in these samples were similar under the two types of culture conditions, suggesting that the overexpression of $CsrA_{Bb}$ from a constitutive promoter is sufficient to induce the levels of these lipoproteins that have been previously shown to be induced following the shift from pH 7.6 and 23°C to pH 6.8 and 37°C in an RpoS-dependent mechanism (41, 85, 86). The aforementioned observations demonstrate that $CsrA_{Bb}$ contributes to the induction of a select set of lipoproteins independently of the signal-dependent regulation seen when the spirochetes are cultivated at different temperatures or pHs. It

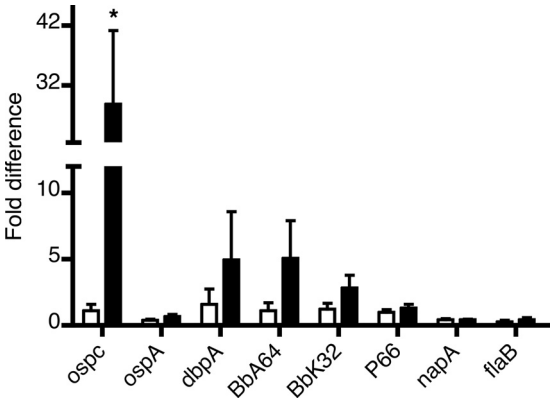


FIG. 7. Real-time RT-PCR analysis of select determinants upon overexpression of $CsrA_{Bb}$. RNA isolated from MSK5, ML23/pBSV2, and ES25 propagated in BSK-II growth medium at pH 7.6 and 32°C was subjected to RT-PCR as described in Materials and Methods. All samples were normalized relative to the *recA* value, and the ΔC_T values obtained from three independent experiments for each transcript were averaged. The $\Delta\Delta C_T$ for each transcript from ES25 relative to MSK5 (open bars) or from ES25 relative to ML23/pBSV2 (black bars) is shown as difference on the *y* axis. The asterisk indicates a sample whose C_T value was statistically significant (i.e., *P* value less than 0.05) in ES25 compared to ML23/pBSV2. The C_T values obtained for ES25 versus ML23/pBSV2 or MSK5 were subjected to a two-way analysis of variance followed by a Bonferroni post hoc test implemented in PRISM.

is not possible to ascertain if overexpression of $CsrA_{Bb}$ induces increased expression of RpoS without alterations in temperature or pH, which in turn mediates the upregulation of OspC or BBA64 from these studies. Taken together, overexpression of $CsrA_{Bb}$ appears to increase the levels of select lipoproteins, which may or may not be directly mediated by a concomitant increase in the levels of RpoS in ES25. It would be interesting to determine the levels of one or more genes, not under the control of the *rpoS* regulon, that could potentially be regulated only by $CsrA_{Bb}$.

Effect of overexpression of $CsrA_{Bb}$ on transcriptional levels of select ORFs. In order to confirm if the changes in the levels of proteins were due to transcriptional or posttranscriptional regulation by $CsrA_{Bb}$ (85), we used quantitative real-time RT-PCR of total RNA obtained from MSK5, ML23/pBSV2, and ES25. The transcript from *recA* was used to normalize the transcript levels of the three strains. As shown in Fig. 7, there was a statistically significant increase in the transcript levels of *ospC* (21.9-fold difference) in ES25 over those in the control strain ML23/pBSV2, which suggests that the increased level of this lipoprotein is due to transcriptional upregulation, even though it is not possible from this study to rule out the contribution of posttranscriptional events. There were no significant differences in the transcript levels of other genes between either ES25 and ML23/pBSV2 or ES25 and MSK5. Therefore, the increased levels of synthesis of multiple borrelial proteins observed with ES25 are probably due to posttranscriptional and translational events.

DISCUSSION

The ability of pathogenic bacteria to alter gene expression in response to environmental signals contributes significantly to their transmission, colonization, and dissemination capabilities within the host (30). The mechanisms responsible for regulating gene expression acquire greater significance for pathogens such as *B. burgdorferi* which not only are exposed to highly disparate environmental conditions in the arthropod and the mammalian hosts but also have limited metabolic capabilities requiring increased dependency on the acquisition of nutrients in these diverse microenvironments (63). In addition, the genome of *B. burgdorferi* codes for a limited number of regulators to control myriad metabolic processes, leading to overlapping or discrete networks responding to different environmental signals (32). Currently, much of the information on gene regulation in *B. burgdorferi* has been obtained from the analysis of a central regulatory pathway mediated by Rrp2-RpoN-RpoS (17, 20, 31, 41, 86). The activation of this pathway facilitates a concerted expression of a variety of borrelial determinants, such as OspC, DbpA/B, and BBK32, known to be involved in the pathogenic mechanisms of *B. burgdorferi* (41, 86). Other lipoproteins with less defined roles in the pathogenic process are also differentially expressed on activation of this pathway (33). Moreover, a few other regulatory proteins, such as BosR (13, 74) and a CarD-type domain-containing homolog, LtpA (87), have been characterized. BosR has been shown to regulate the levels of proteins involved in resistance to oxidative stress (13, 43).

In addition to the above regulators, the borrelial genome also codes for a homolog of CsrA (BB0184) with significant sequence identity at the amino acid level to similar homologs in other eubacterial species (Fig. 1A). This similarity was apparent functionally since the overexpression of $CsrA_{Bb}$ in *E. coli* was able to inhibit the synthesis/accumulation of glycogen presumably through the interactions with the promoter elements of genes involved in glycogen biosynthesis, such as *glgB*, *glgC*, and *pckA*. A search of the genome of *B. burgdorferi* did not reveal the presence of homologs of genes involved in glycogen biosynthesis, suggesting that $CsrA_{Bb}$ might regulate other processes unique to *B. burgdorferi*. However, it is interesting that the structural features of $CsrA_{Bb}$, mediated by its sequence similarity to the homolog present in *E. coli*, were able to functionally regulate inhibition of expression of glycogen biosynthesis genes. Therefore, it would be feasible to exploit this phenotype in delineating the regulatory functions of conserved domains or residues of $CsrA_{Bb}$ and to determine their contributions to the pathophysiology of *B. burgdorferi.*

There is significant conservation both in the amino acid sequences and in the locations of CsrA homologs in the three sequenced borrelial species, namely, *B. burgdorferi* strain B31, *B. afzelii* PKo, and *B. garinii* PBi (Fig. 1A). In addition, the location of $csrA_{Bb}$ was adjacent to the ORFs that were previously characterized as being part of the *flgK* (bb0180 to bb0183) motility operon (34). By employing an RT-PCR-based method (34, 62), we were able to show that bb0184 or $csrA_{Bb}$ is cotranscribed with its upstream neighbors bb0182 and bb0183. Based on these observations, it can be stated that $csrA_{Bb}$ is the terminal ORF of the *flgK* motility operon. When $CsrA_{Bb}$ was overexpressed in *B. burgdorferi*, there was a significant alteration in morphology with a concomitant decrease in the levels of FlaB (Fig. 4 and 5A and C). While a direct correlation between $csrA_{Bb}$ being a part of the *flgK* operon and its effects on FlaB levels upon overexpression cannot be made

at this juncture, it is interesting to speculate that the increased levels of $CsrA_{Bb}$ may downregulate the expression of genes of the *flgK* motility operon and thereby affect the stability of FlaB at the posttranscriptional levels. It has been previously demonstrated that there is a reduction in the levels of the flagellin proteins in the absence of the gene encoding the hook structural protein FlgE (71).

The role of CsrA in motility and morphology has been reported for several other bacterial species. In *B. subtilis*, CsrA was shown to repress *hag*, the gene encoding the flagellin protein, and overexpression of *csrA* reduced cell motility (84). This repression was mediated by CsrA blocking the binding of ribosomes with *hag* transcripts (7). Similarly, constitutive expression of CsrA in *Legionella pneumophila* resulted in elongated cell morphology, reduced motility, and significant reduction in the expression of *flaA* compared to that in the wild-type cells (54).

At this time, it is not known how the level of FlaB is regulated by borrelial CsrA, which could be either in conjunction with other mechanisms or through independent regulation of its levels (23, 57). It appears that the levels of CsrA are variable in different isolates, and it is interesting to speculate that the levels of the 41-kDa protein corresponding to FlaB as observed on the Coomassie blue-stained gel (Fig. 5A) reflect the levels of CsrA induced under similar growth conditions used in this study.

Since the effects of CsrA homologs in other eubacterial species have been shown to be antagonized via interactions with multiple binding sites present on noncoding small RNA molecules such as CsrB and CsrC, we hypothesized that the effects of CsrA in borrelial strains could be subjected to such regulatory effects by small RNA homologs (6, 46). Bioinformatic approaches to identify the small RNA molecule(s) that could titrate the effects of CsrA have not yielded sequences with significant homology to CsrB/CsrC, while a homolog of small RNA molecule $DsrA_{Bb}$ has been determined to mediate the temperature-induced regulation of RpoS (50). We, therefore, decided to initially determine the levels of CsrA in various borrelial isolates to establish if CsrA does indeed play a role in the pathophysiological response of *B. burgdorferi*. We generated monospecific serum against purified, recombinant borrelial CsrA following overexpression in *E. coli*. All three strains (Fig. 3B), propagated under fed-tick midgut conditions (pH 6.8 and 37°C), exhibited a significantly enhanced synthesis of $CsrA_{Bb}$, while there were no detectable levels of $CsrA_{Bb}$ when strains were propagated under unfed-tick midgut conditions (pH 7.6 and 23°C). The level of CsrA was detectable in a clonal isolate of *B. burgdorferi* strain B31 containing the full complement of plasmids and to a much lesser degree in ML23/pBSV2 when these strains were grown at pH 7.6 and 32°C even though these levels could partly reflect the constant changes in culture conditions such as density-dependent changes in pH, dissolved gases, or other nutrients (Fig. 5B, MSK5 and ML23/pBSV2). These levels, nevertheless, were much lower than those present in ES25 propagated under these conditions (Fig. 5B, ES25). Based on these observations and the apparent lack of identification of a homolog of a small RNA molecule that can titrate the effects of CsrA, we decided to overexpress CsrA under the control of borrelial promoter P_{flgB} by using a borrelial shuttle vector, pBSV2. A six-histidine tag was engineered at the C terminus of the overexpressed CsrA in order to facilitate methods for isolating the cognate noncoding small RNA molecule by using a variety of biochemical approaches.

We were surprised to find that the kanamycin-resistant borrelial colonies that grew on BSK-II agar overlay following transformation with the CsrA overexpression plasmid pES25 (Table 1) were compact or pinpoint sized compared to more diffuse colonies obtained following transformation with the shuttle vector alone as a control (data not shown). This observation led us to further characterize the overexpression clone ES25 to determine if the levels of CsrA could modulate expression of determinants critical to the pathophysiology of *B. burgdorferi.* When the spirochetes from these colonies were examined following *BacLight* Live/Dead staining in conjunction with confocal imaging, the control cells (ML23/pBSV2) had the typical "wave-form" morphology (Fig. 4) while ES25 had "rod-shaped" morphology essentially due to overexpression of CsrA (Fig. 4). The spirochetes with increased expression of $CsrA_{Bb}$ did not have the typical corkscrew motility observed with the control cells but were motile when observed by dark-field microscopy. When lysates of total proteins were separated on an SDS-polyacrylamide gel, ML23/pBSV2 had a protein of approximately 41 kDa that was significantly upregulated compared to that of ES25 (Fig. 5A). Mass spectrophotometric analysis of this protein yielded three N-terminal tryptic peptides with significant sequence identity with FlaB, the major endoflagellum of *B. burgdorferi*. Moreover, this observation was further confirmed by immunoblot analysis with anti-FlaB monoclonal antibodies. As shown in Fig. 5C, there was a significant reduction in levels of FlaB in ES25 and this decrease coincided with increased expression of $CsrA_{Bb}$ (Fig. 5B). P66, an integrin binding porin of *B. burgdorferi*, was found to be expressed at similar levels in different strains, indicating that the overexpression of CsrA does not have an effect on all the major borrelial determinants (Fig. 5D). Taken together, the aforementioned observations demonstrated that overexpression of CsrA results in significant reduction in the levels of FlaB in *B. burgdorferi*. In addition to its effect on the endoflagellum of *B. burgdorferi*, overexpression of CsrA significantly altered the levels of several lipoproteins. Infection-derived serum from mice infected with low doses of *B. burgdorferi* revealed increased immunoreactivity with several proteins in ES25 compared to the control strain ML23/pBSV2 (Fig. 6A). The levels of expression of these proteins were also similar to those seen with MSK5, which also exhibits increased levels of CsrA compared to ML23/pBSV2. While the levels of OspC (Fig. 6C), BBK32 (Fig. 6D), and DbpA (Fig. 6E) were elevated in ES25 over those in the control strain ML23/pBSV2, the levels of OspA remained unchanged, indicating that not all lipoproteins are affected by levels of $CsrA_{Bb}$ (Fig. 6B). While the levels of OspC and BBK32 were higher in ES25 than in MSK5, the levels of DbpA were similar between these two strains. The levels of both BBK32 and DbpA are detectable in ML23/pBVS22, but the levels are considerably lower than those in ES25. It is also interesting that this deviation is indicative of $CsrA_{Bb}$'s ability to differentially regulate the levels of various lipoproteins. We have previously shown that the levels of BBA64 can be upregulated in ML23/pBSV2 only upon propagation in BSK-II growth medium at pH 6.8 and 37°C, conditions which mimic the conditions in the midgut of ticks

following a blood meal (51, 52). The significant increase in the levels of BBA64 upon overexpression of $CsrA_{Bb}$ independent of the environmental signals like pH or temperature is indicative of a distinct role for $CsrA_{Bb}$, as BBA64 has been previously shown to be regulated by the RpoN/RpoS-dependent signaling network (20). It will be interesting to determine if the levels of BBA64 are coregulated by both CsrA and RpoN/RpoS in response to various environmental signals.

In order to correlate increased levels of $CsrA_{Bb}$ observed under fed-tick conditions with the increased level of select lipoproteins (OspC and BBA64) routinely observed under these conditions, we propagated ES25 under conditions that mimic the tick midgut before and after a blood meal. $CsrA_{Bb}$ was readily detectable when ES25 was propagated under unfed-tick conditions (Fig. 6J, lane 1), a finding which is essentially due to induction of $csrA_{Bb}$ under the control of P_{flgB} in the shuttle vector used to generate ES25. When ES25 was propagated under fed-tick conditions, there were considerably higher levels of CsrA (Fig. 6J, lane 2). This is presumably due to the additive effects of induction of $csrA_{Bb}$ from both the heterologous promoter (P_{flgB}) and its native promoter, as we have shown that there are increased levels of $CsrA_{Bb}$ under only fed-tick conditions in several clonal isolates of *B. burgdorferi* (Fig. 3A). Interestingly, the increased levels of $CsrA_{Bb}$ observed in ES25 under unfed-tick conditions correlate with increased levels of both OspC and BBA54, similar to those levels seen when ES25 is propagated under fed-tick conditions (Fig. 6K and L). This observation demonstrates that increased levels of OspC and BBA54 in ES25 at pH 7.6 and 23°C are essentially due to constitutive levels of $CsrA_{Bb}$ (from the heterologous promoter P_{flgB}) and are observed under environmental conditions where the levels of these lipoproteins are undetectable in the control strains (MSK5, ML23, ML23/pBBE22, and ML23/pBVS2). Since it has been shown elsewhere that the levels of both OspC and BBA64 are controlled by RpoS (33, 41), it appears either that $CsrA_{Bb}$ is able to regulate the levels of these lipoproteins independently of the levels of RpoS or that the increased levels of $CsrA_{Bb}$ may modulate the levels of RpoS under environmental conditions where it has minimal effects on the levels of OspC and BBA64.

The temperature-induced reciprocal regulation of OspC and OspA controlled by RpoS has been recently shown to be mediated by a small, noncoding RNA, $DsrA_{Bb}$, which has structural features that extensively base-pair with the upstream region of *rpoS* transcript (50). It was also elegantly demonstrated that *B. burgdorferi* mutants lacking $DsrA_{Bb}$ were unable to upregulate RpoS and OspC in response to temperature, while their regulation was unaffected in response to pH and cell density. Moreover, the regulation of RpoS was shown to be at the posttranscriptional level where the binding of $DsrA_{Bb}$ to the upstream region of the *rpoS* transcripts results in destabilization of a stable secondary structure, resulting in increased translational efficiency (50). While we have not delineated the differential roles of temperature, pH, or conditions of the medium associated with increased cell density in the levels of $CsrA_{Bb}$, there are several features of $DsrA_{Bb}$-RpoS interactions that are akin to the other small noncoding RNA-dependent gene regulation processes such as that observed with CsrA/CsrB/CsrC. Moreover, based on the observation that there are only a limited number of small noncoding RNA molecules in *B. burgdorferi* (60), it is interesting to speculate that signal-dependent gene expression may be regulated at multiple levels by a small set of regulators and their cognate small RNA molecules while the outcome of these mechanisms may result in predominantly differential regulation of members of the *rpoS* regulon. The increased level of $CsrA_{Bb}$ resulting in increased synthesis of OspC and BBA64 under conditions where the levels of RpoS would be minimal (pH 7.6 and 23°C or unfed-tick midgut conditions [Fig. 6J, K, and L]) suggests that either it might regulate these determinants independently of RpoS or it could potentially upregulate RpoS under unfed-tick conditions, leading to increased synthesis of OspC and BBA64. Analysis of borrelial strains with deletions in multiple regulators such as $csrA_{Bb}$, *rpoS*, and $dsrA_{Bb}$ will help in understanding the regulatory network of *B. burgdorferi*, which is responsive to a variety of environmental signals.

Several bacterial species exhibit altered levels of oxidative stress response proteins depending on the levels of regulation by CsrA (10, 29, 79). But in *B. burgdorferi*, overexpression of $CsrA_{Bb}$ does not seem to have an effect on the levels of two critical proteins involved in mediating resistance to oxidative stress, namely, NapA and SodA. Therefore, it appears that borrelial CsrA has regulatory functions that might facilitate adaptive gene expression unique to the pathophysiological needs of *B. burgdorferi* under different host-specific conditions.

Determination of levels of transcripts by quantitative real-time PCR analysis of cDNA obtained from MSK5, ML23/pBSV2, and ES25 indicated that only *ospC* transcripts were significantly increased in ES25 over those in the control strains. The increased transcript levels of *ospC* observed in ES25 may be due to transcriptional upregulation. The levels of transcripts of several other genes were not significantly higher in ES25 than in the control strains, even though the levels of their corresponding proteins were upregulated. This could possibly be due to posttranscriptional changes, destabilization of potential secondary structures in untranslated regions of mRNA, and/or increased translational capabilities mediated by $CsrA_{Bb}$. Although the levels of FlaB were significantly reduced in ES25, there was no significant corresponding decrease in levels of *flaB* transcripts in ES25, suggesting that the FlaB protein levels could be regulated posttranscriptionally as has been observed with several other CsrA-regulated genes. It is interesting to speculate that, based on transcript levels observed with *ospC* in ES25 and a lack of such increase in the levels of transcription of other genes, $CsrA_{Bb}$ may potentially target its regulation of gene expression, either directly or indirectly, at both the transcriptional and the posttranscriptional levels.

It is interesting to speculate that CsrA is a global regulator of gene expression in *B. burgdorferi*, where under certain conditions its upregulation such as in the fed-tick conditions results in altered morphology/motility with increased lipoprotein expression. This differential regulation could be significant in microenvironments such as the skin of a mammalian host where increased expression of adhesins such as DbpA or BBK32 may facilitate greater colonization capability in conjunction with reduced motility. The reduced levels of CsrA, favoring increased expression of FlaB, may provide an added advantage to the spirochetes under conditions requiring increased motility with a concomitant decrease in adherence (56, 57). This initial characterization of the role of CsrA in regu-

lating gene expression will help facilitate a better understanding of the mechanism and role of this potential global regulator in controlling the levels of pathophysiological determinants of *B. burgdorferi* that contribute to its survival and virulence in diverse hosts.

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