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# Analysis of the *ospC* Regulatory Element Controlled by the RpoN-RpoS Regulatory Pathway in *Borrelia burgdorferi*

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**Outer surface lipoprotein C (OspC) is a key virulence factor of *Borrelia burgdorferi*. *ospC* is differentially regulated during borrelial transmission from ticks to rodents, and such regulation is essential for maintaining the spirochete in its natural enzootic cycle. Recently, we showed that the expression of *ospC* in *B. burgdorferi* is governed by a novel alternative sigma factor regulatory network, the RpoN-RpoS pathway. However, the precise mechanism by which the RpoN-RpoS pathway controls *ospC* expression has been unclear. In particular, there has been uncertainty regarding whether *ospC* is controlled directly by RpoS ($\sigma^s$) or indirectly through a transactivator (induced by RpoS). Using deletion analyses and genetic complementation in an OspC-deficient mutant of *B. burgdorferi*, we analyzed the *cis* element(s) required for the expression of *ospC* in its native borrelial background. Two highly conserved upstream inverted repeat elements, previously implicated in *ospC* regulation, were not required for *ospC* expression in *B. burgdorferi*. Using similar approaches, a minimal promoter that contained a canonical −35/−10 sequence necessary and sufficient for $\sigma^s$-dependent regulation of *ospC* was identified. Further, targeted mutagenesis of a C at position −15 within the extended −10 region of *ospC*, which is postulated to function like the strategic C residue important for E$\sigma^s$ binding in *Escherichia coli*, abolished *ospC* expression. The minimal *ospC* promoter also was responsive to coumermycin $A_1$, further supporting its $\sigma^s$ character. The combined data constitute a body of evidence that the RpoN-RpoS regulatory network controls *ospC* expression by direct binding of $\sigma^s$ to a $\sigma^s$-dependent promoter of *ospC*. The implication of our findings to understanding how *B. burgdorferi* differentially regulates *ospC* and other *ospC*-like genes via the RpoN-RpoS regulatory pathway is discussed.**

*Borrelia burgdorferi*, the spirochetal agent of Lyme disease, is maintained in nature via a complex enzootic cycle involving *Ixodes scapularis* ticks and small rodents (50, 51). During transmission, the spirochete differentially expresses many of its constituent proteins for adaptation to its diverse host environments. Among those differentially regulated in this manner are outer surface lipoproteins A (OspA) and C (OspC) (11, 31, 32, 47, 48). OspA is expressed principally by spirochetes harbored in unfed, flat ticks and functions as an essential adhesion molecule for colonization and survival within the tick midgut (34–36, 62). OspC, which is upregulated in *B. burgdorferi* at the time of tick engorgement, is essential for the *B. burgdorferi* infection of mice (21) and for the migration of *B. burgdorferi* from tick midguts to salivary glands (15, 20, 37).

Given their importance in the life cycle of *B. burgdorferi* and/or the pathogenesis of Lyme disease, the elucidation of the regulatory networks that govern the differential expression of OspA and OspC has become a central focus for understanding the molecular mechanisms by which *B. burgdorferi* adapts to its disparate host environments. However, the discernment of the molecular basis of gene regulation in *B. burgdorferi* generally has been hampered by a lack of systems for genetically manipulating the spirochete, particularly for virulent strains (7, 56). Nonetheless, recent advances in borrelial genetics have led to the development of selectable markers and shuttle vectors (5, 12, 14, 16, 44, 45, 53), targeted gene inactivations (for a review, see reference 41), and identification of *B. burgdorferi* virulence factors (21, 37, 39, 62). Similar advances also have culminated in the discovery of the first *B. burgdorferi* genetic regulatory network, the RpoN-RpoS pathway (25, 61). In this pathway, a two-component response regulator, Rrp2, functions as an enhancer-binding protein (EBP), along with the alternative sigma factor RpoN ($\sigma^N$), to control the expression of another alternative sigma factor, RpoS ($\sigma^s$). RpoS, in turn, regulates the expression of OspC, other "group I" lipoproteins (e.g., DbpA and the Mlp family) (58, 59), and additional infection-associated immunogens (61).

The discovery of the RpoN-RpoS regulatory network prompts an important question concerning how $\sigma^s$, in particular, induces the expression of *ospC* and other virulence-associated genes. One possibility is that $\sigma^s$ controls *ospC* expression via an unidentified transactivator, which could bind to the regulatory region for the activation of *ospC*. Relative to this hypothesis, two sets of conserved inverted repeats (IRs) located upstream of the *ospC* promoter (Fig. 1) have been proposed to be candidate binding sites for a potential transactivator(s) (29, 55). An alternative possibility is that *ospC* contains a $\sigma^s$-dependent promoter; in this case, $\sigma^s$ would directly control the transcriptional activation of *ospC* by binding

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FIG. 1. Upstream regions of the *ospC* genes of *B. burgdorferi* strains 297 and B31. Pairs of divergent arrows denote the two putative inverted repeat elements ($IR_1$ and $IR_2$). The −35 and −10 promoter elements, ribosomal-binding site (RBS), and the ATG start codon are shown in boldface type. Filled arrowheads indicate the starting positions of each deletion (Δ) described in the legend to Fig. 3A. The −15 C residue (boxed) within the extended −10 region was targeted for mutagenesis. The asterisks mark two previously identified transcriptional initiation sites (28, 29, 33). min, start of deletion made to yield the minimal promoter construct diagrammed in Fig. 3A.

to the *ospC* promoter. Along these lines, predicated on determinations of transcriptional initiation, *ospC* has been predicted to possess a typical −35/−10 $\sigma^{70}$ promoter (18, 28, 29, 33). However, sequence information alone is likely insufficient for distinguishing between $\sigma^s$ and $\sigma^{70}$ promoters, inasmuch as $\sigma^s$ and $\sigma^{70}$ are highly related and recognize the same core promoter elements (19, 24). Recent studies have shown that $\sigma^s$ promoter selectivity is attained by several promoter-specific sequence elements, architectural DNA-binding proteins, or DNA topology (24). For example, in *Escherichia coli*, $\sigma^s$ preferentially recognizes promoters on a relaxed template (26) and DNA relaxation is required for transcription by the holoenzyme containing $\sigma^s$ ($E\sigma^s$) during the cellular response to osmotic stress (6). Interestingly, in *B. burgdorferi*, the regulation of *ospC* gene expression involves not only the RpoN-RpoS signaling pathway (25) but also DNA supercoiling (1), raising the possibility that *ospC* utilizes a $\sigma^s$-dependent promoter. Additional experiments are therefore warranted to define whether the *ospC* gene utilizes a $\sigma^{70}$ or a $\sigma^s$ promoter.

Regarding initial efforts to investigate *ospC* promoter activity, Sohaskey et al. (49) first showed that when transiently expressed in *B. burgdorferi*, a 551-bp promoter region of *ospC* was capable of driving the expression of a chloramphenicol acetyltransferase (CAT) reporter gene. Carroll et al. (8) later constructed a stable shuttle vector for *B. burgdorferi* in which the 179-bp region upstream of *ospC* (containing the IRs) was fused to a green fluorescent protein (*gfp*) reporter gene. When subjected to different environmental conditions, the construct in *B. burgdorferi* regulated the expression of GFP akin to OspC expression (8). More recently, Eggers et al. (13) further analyzed the activity of the *ospC* promoter in a surrogate *E. coli* background using another *gfp* reporter system. Despite these efforts, a direct examination of the *ospC* promoter element and its influence on OspC expression in the relevant *B. burgdorferi* background has been lacking. To this end, we previously constructed an *ospC* mutant complemented with a shuttle vector harboring a complete wild-type *ospC* gene (37). Herein, we used this construct to generate nested deletions within the upstream regulatory region of *ospC*; this approach has allowed us to identify the *cis* element involved in *ospC* regulation in the native background of *B. burgdorferi*. The implications of our findings relative to the control of *ospC* expression by the RpoN-RpoS regulatory pathway in *B. burgdorferi* are discussed.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Low-passage, wild-type clone BbAH130 of *B. burgdorferi* strain 297, the OspC-deficient mutant, the OspC-deficient mutant complemented with a shuttle vector carrying a wild-type *ospC* gene, and the RpoS-deficient mutant were described previously (25, 37). High-passage *B. burgdorferi* strain 297 was obtained by continuously passaging it more than 230 times in vitro, and a high-passage *B. burgdorferi* strain B31 (B31-A) was provided by Patricia Rosa (5). Spirochetes stored at −70°C were inoculated into Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma Chemical Co., St. Louis, MO) (38) and were cultivated under various environmental conditions of temperature (23° or 37°C) and pH (7.5 or 8.0) (58). Cultures were harvested for analysis at the late logarithmic phase of growth ($5 \times 10^7$ cells/ml), as determined by enumerating spirochetes via dark-field microscopy. For coumermycin $A_1$ experiments, *E. coli* strain DH5α was grown at 23°C and then was diluted 1:100 in Luria-Bertani broth (with or without 1 μg/ml of coumermycin $A_1$). Cultures were allowed to grow at 23°C for about 2 h (optical density at 600 nm of about 0.4). *B. burgdorferi* was cultivated in BSK-H medium at 23°C until the mid-logarithmic phase of growth, at which time the culture was diluted 1:100 in BSK-H medium containing or lacking 20 ng/ml of coumermycin $A_1$; the cultures were then allowed to grow at 23°C (for about 2 weeks) to the mid-logarithmic phase. *E. coli* TOP10 (Invitrogen, Carlsbad, CA) was used as a host for cloning experiments.

**Promoter mutagenesis.** Construction of the shuttle vector pOspC-wt (pBSV2-OspC) was described previously (37). pOspC-wt contains the complete coding region of *ospC* as well as the 141-bp sequence upstream of the ATG initiation codon (see Fig. 3A). To construct shuttle vectors with various deletions in the region of the *ospC* promoter, a series of PCRs using the Expand High Fidelity PCR system (Roche Diagnostics, Indianapolis, IN) were performed. The template was pOspC-wt, and the primer pairs for each construct are listed in Table 1. The resulting PCR fragments were cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA). The resulting plasmids and pOspC-wt were then digested with HindIII and XbaI and ligated together to generate pOspC-Δ$IR_1$, pOspC-Δ$IR_{1+2}$, pOspC-min, and pOspC-Δ-35. To construct a pOspC-C/A point mutation, a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used with the corresponding primer pairs (Table 1). The template used was pOspC-wt. Sequence analysis was performed to verify the desired mutation. A resultant HindIII-XbaI fragment was excised and then subcloned back into pOspC-wt to ensure that only the desired mutation was present in pOspC-C/A.

***B. burgdorferi* transformations.** The OspC-deficient (streptomycin-resistant) mutant (37) or the RpoS-deficient (erythromycin-resistant) mutant (25) was made electrocompetent and transformed with various shuttle vector constructs. Transformants were selected by kanamycin treatment. The general procedure for transforming *B. burgdorferi* has been described previously (42, 62). Twenty to 50 μg of plasmid DNA was used in each transformation experiment. After electroporation, the mixture (0.05 ml) was diluted into 100 ml of BSK-H medium and was incubated at 32°C overnight to allow for recovery. Relevant antibiotics were added to the cultures in the following final concentrations: 50 μg/ml for streptomycin, 50 ng/ml for erythromycin, and 200 μg/ml for kanamycin. The cultures were then aliquoted into multiple 96-well tissue culture plates (230 μl/well). Two to 3 weeks after plating, the wells that contained positive cultures were identified by color change of the medium; the presence of viable spirochetes was verified by dark-field microscopy. In general, 10% or less of the wells were positive for growth and therefore were considered to be clonal as a result of limiting dilution

TABLE 1. Oligonucleotide primers used in this study

| Primer name | Sequence | Purpose |
|---|---|---|
| ospC-wt-5′ | CAAATTAAGACAATATTTTTCAAATTC | 5′ primer; PCR of the *ospC* gene for constructing pOspC-wt |
| ospC-$IR_1$ | ATAAATATTGAAGAATTTG | 5′ primer; PCR of the *ospC* gene for constructing pOspC-Δ$IR_1$ |
| ospC-no-IR | AAATATTTTTTCAAATAAA | 5′ primer; PCR of the *ospC* gene for constructing pOspC-Δ$IR_{1+2}$ |
| ospC-min | AATTGAAAAACAAAATTGTTGGACTA | 5′ primer; PCR of the *ospC* gene for constructing pOspC-min |
| ospC-no-35 | GTTGGACTAATAATTCATAAATAA | 5′ primer; PCR of the *ospC* gene for constructing pOspC-Δ-35 |
| ospC-wt-3′ | GA**TCTAGA**CAAGAAATCTTTCTTGACTTATATTGA | 3′ primer; PCR of the *ospC* gene for constructing all of the plasmids listed above. An XbaI site (boldface letters) is attached for the purpose of cloning. |
| ospC-C/A-5′ | GAGATCTAAATATTTTTTCAAATAAAAAATTGAAAAACAAAATTG TTGGAATAATAATTCATAAATAAAAAG | 5′ primer; generation of a C/A mutation at the −15 position within the *ospC* promoter |
| ospC-C/A-3′ | CTTTTTATTTATGAATTATTATTCCAACAATTTTGTTTTTCAATTTT TTATTTGAAAAAATATTTAGATCTC | 3′ primer; generation of a C/A mutation at the −15 position within the *ospC* promoter |

(10). To confirm that these clones harbored the desired shuttle vector, whole-cell lysates were used to transform *E. coli* TOP10. Plasmid DNA was rescued from *E. coli* transformants, and restriction digestions were performed to verify recovery of the pertinent plasmid.

**RT-PCR.** Total RNAs from *B. burgdorferi* were isolated using a NucleoSpin RNA II purification kit (BD Biosciences, Palo Alto, CA) according to instructions provided by the manufacturer. To remove potential DNA contamination, RNA was further treated with DNA-*free* DNase Treatment and Removal Reagents (Ambion, Austin, TX). The concentration of RNA was determined by UV spectrophotometry using an ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). RT-PCRs were performed using a Titan One Tube RT-PCR system (Roche Diagnostics, Indianapolis, IN). Conditions for reverse transcriptase (RT)-PCRs were as recommended by the manufacturer; a 50-μl buffered reaction mixture contained 40 ng of bacterial RNA, 0.4 μM concentrations of each of the oligonucleotide primers, 5 mM dithiothreitol, 0.2 mM concentrations of each deoxynucleoside triphosphate, 5 U of RNase inhibitor, and 1 μl of enzyme mixture. Primers used for amplification of *ospC* and *rpoS* transcripts were described previously (25). Five microliters of the RT-PCR mixture was used for agarose gel electrophoresis.

**SDS-PAGE and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were carried out as previously described (60). Cells were loaded in gel lanes at $5 \times 10^7$ cells per lane. Rat polyclonal antisera against OspC and $\sigma^s$ and monoclonal antibody 8H3-33 against FlaB were described previously (58). For coumermycin $A_1$ experiments, OspC was detected by immunoblotting with monoclonal antibody 4B8F4 (for detection in *E. coli*) (33) or with rabbit polyclonal antiserum (for detection in *B. burgdorferi*). Most immunoblots were developed colorimetrically; for some of these, densitometry was used to assess the relative amounts of protein per gel lane using a Kodak Gel Logic 200 instrument with 1D image analysis software (version 3.6; Kodak, Rochester, NY). For other selected immunoblots (e.g., coumermycin $A_1$ experiments and certain experiments involving the detection of $\sigma^s$), blotted membranes were developed by chemiluminescence using either ECL Plus Western Blotting Detection system (Amersham Biosciences, Piscataway, NJ) or Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA).

## RESULTS

***trans*-Complementation for studying the *ospC* promoter.** As an initial approach for assessing how *ospC* is controlled by RpoS, we exploited a previously constructed *ospC* mutant of *B. burgdorferi* ($OspC^-$) complemented with a wild-type version of *ospC* that was cloned into the shuttle vector pBSV2 (pOspC-wt) (37, 53) (Fig. 2A and B). The advantage of our approach was that *ospC* expression could be assessed directly via the detection of OspC in *B. burgdorferi*. To first examine whether regulation of the cloned *ospC* gene in pOspC-wt was similar to that of a native, endogenous *ospC* gene, wild-type *B. burgdorferi* and the complemented OspC mutant ($OspC^-$/pOspC-wt) were cultivated in BSK-H medium under various temperature and pH conditions. In both wild-type *B. burgdorferi* and the $OspC^-$/pOspC-wt complemented strain, *ospC* expression was induced at an elevated temperature (Fig. 2C, lanes 2 and 8) and was inhibited by increased culture pH (Fig. 2C, lanes 3 and 9). These results confirmed that the *ospC* gene located on a shuttle vector responded to environmental stimuli in a manner similar to that of an endogenous *ospC* gene, indicating the suitability of *trans*-complementation for assessing *ospC* gene regulation.

**IR elements are not required for the expression of *ospC*.** Previously, two partially overlapping IRs were identified upstream of the *ospC* gene of *B. burgdorferi* (29) (Fig. 1). These IRs are highly conserved among *ospC* homologs of many *B. burgdorferi* strains (29, 55) and have been hypothesized to be involved in *ospC* regulation (1, 55). To assess the potential role(s) of the IRs in *ospC* expression, we first generated complementation constructs lacking either $IR_1$ or both $IR_1$ and $IR_2$ (Fig. 3A). The resulting mutated constructs were then transformed into the $OspC^-$ mutant, and OspC expression was monitored by immunoblot analysis. Densitometry revealed that plasmids harboring either deletion construct in the $OspC^-$ mutant still promoted the expression of OspC (lanes 4 and 5) at levels close to that for the wild type (lane 3), indicating that neither of the two IRs upstream of *ospC* is required for *ospC* expression.

**Minimum promoter for *ospC* expression.** Deletion of the two IRs removed the majority of the sequence upstream of the −35/−10 promoter of *ospC* that was previously noted by others (18, 28, 29, 33) (Fig. 3A). To further examine whether an additional 18 bp remaining immediately upstream of the −35 consensus sequence played a role in *ospC* regulation, the 18-bp

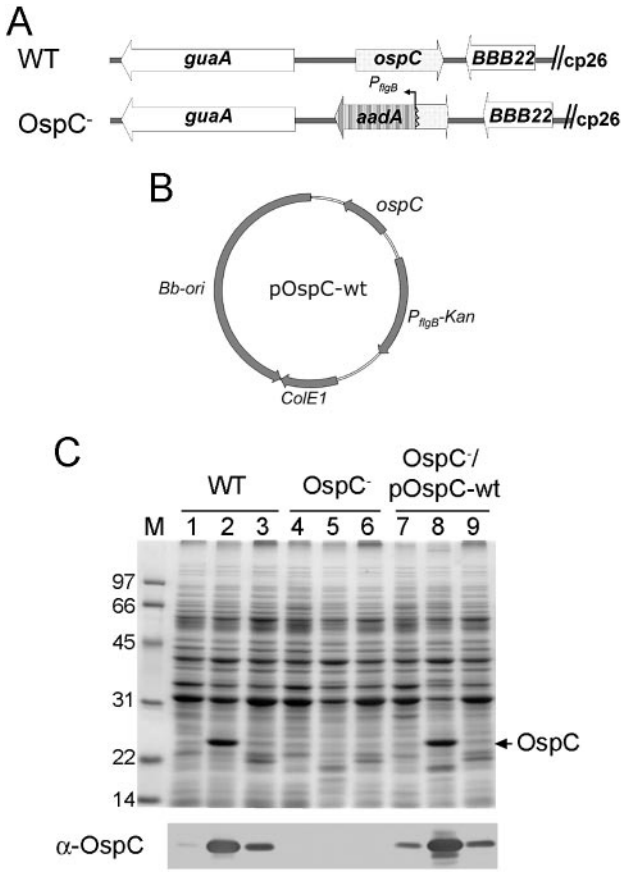


FIG. 2. Approach for studying the regulation of *ospC*. (A) Structure of the *ospC* locus in cp26 (top) and *ospC* disruption by the *aadA* (streptomycin resistance) marker (16) (bottom). Only the relevant portions of cp26 are shown. WT, wild type. (B) The shuttle vector pBSV2 (53) harboring a wild-type copy of *ospC* (pOspC-wt) used for genetic complementation of the *ospC* mutant of *B. burgdorferi*. In this construct, the kanamycin resistance gene is driven by the constitutive promoter of the borrelial *flgB* gene ($P_{flgB}$-*Kan*). (C) SDS-PAGE (Coomassie blue staining) (top) and immunoblotting (bottom) of whole-cell lysates of *B. burgdorferi* strains cultivated under various conditions of temperature and pH. M, molecular mass marker (in kilodaltons). WT, parental low-passage *B. burgdorferi* 297. OspC$^-$, the *ospC* mutant. OspC$^-$/pOspC-wt, the *ospC* mutant complemented with the shuttle vector pOspC-wt. Lanes 1, 4, and 7, spirochetes cultivated in BSK-H medium (pH 7.5) incubated at 23°C. Lanes 2, 5, and 8, spirochetes cultivated in BSK-H medium (pH 7.5) at 37°C. Lanes 3, 6 and 9, spirochetes cultivated in BSK-H medium (37°C) adjusted to pH 8.0. The migration of OspC in the Coomassie blue-stained gel (top) is indicated by the arrow. In the immunoblot (bottom), only the relevant portion of the gel is shown.

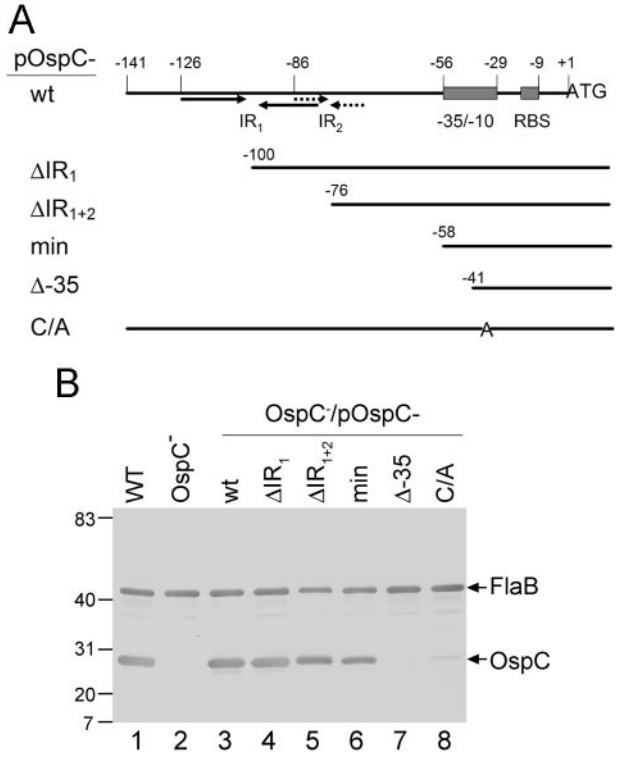


FIG. 3. Influence of upstream *cis* elements on *ospC* expression. (A) Diagram of a series of shuttle vector constructs containing various versions of the upstream region of *ospC*. Nucleotide positions are relative to the ATG start codon, where A is position +1. All six constructs are denoted by the prefix pOspC- followed by the pertinent deletion or mutation (indicated at the left). wt, wild-type *aspC* gene; $\Delta IR_1$, deletion of $IR_1$; $\Delta IR_{1+2}$, deletion of $IR_1$ and $IR_2$; min, minimal promoter for *ospC*; Δ-35, deletion of $IR_1$, $IR_2$, and the −35 sequence; C/A, targeted point mutation of C (within the extended −10 region) to A. (B) Immunoblot of the *ospC* mutant (OspC$^-$) transformed with the various pOspC shuttle vectors shown in panel A. All cultures were grown at 37°C (pH 7.5) and were harvested at the late logarithmic phase of growth. Antibodies directed against OspC and FlaB were pooled. Numbers at the left denote protein molecular mass markers (in kilodaltons). Densitometry results for OspC are as follows: lane 1, 2.0; lane 2, 0.008; lane 3, 2.3; lane 4, 2.2; lane 5, 2.0; lane 6, 1.7; lane 7, 0.05; and lane 8, 0.2. WT, wild type.

region was deleted. The resulting construct contained only the minimal −35/−10 sequence (pOspC-min) (Fig. 3A). The OspC$^-$ mutant of *B. burgdorferi* complemented with pOspC-min readily expressed OspC (Fig. 3B, lane 6). However, a complementation construct lacking an additional 17 nucleotides extending into the putative −35 consensus sequence had greatly diminished OspC expression in the OspC$^-$ mutant (Fig. 3B, lane 7). These results indicate that a minimal −35/−10 *ospC* promoter sequence is necessary and sufficient for OspC expression in *B. burgdorferi*.

Recent data demonstrate that although the −35/−10 consensus sequences for $\sigma^s$ and $\sigma^{70}$ promoters tend to be indistinguishable (19, 24), some minor sequence differences may exist, especially within the extended −10 element (4). In this regard, a −13 C residue in this extended −10 region is strategic for interacting with E$\sigma^s$ in *E. coli* (4). We therefore mutagenized a candidate C residue conserved at position −15 within the *ospC* minimal promoter (Fig. 1) and assessed the influence of this point mutation on *ospC* expression. As shown in Fig. 3B (lane 8), the OspC$^-$ mutant complemented with this construct expressed significantly lower levels of OspC than the mutant transformed with the wild-type *ospC* gene.

To examine whether the *ospC* gene driven only by a minimal promoter remained responsive to environmental stimuli, *B. burgdorferi* strain OspC$^-$/pOspC-min was cultivated under various conditions of temperature and pH. As in the case of either wild-type *B. burgdorferi* or OspC$^-$/pOspC-wt (Fig. 2C), *ospC* expression in the OspC$^-$/pOspC-min strain was inhibited by

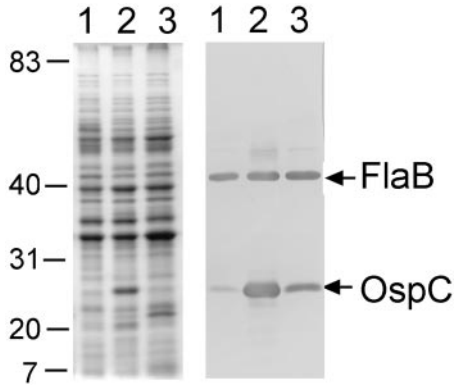


FIG. 4. Influence of culture temperature and pH on *ospC* expression driven by a minimal promoter. Spirochetes were cultivated in BSK-H medium (pH 7.5) at 23°C (lanes 1) or 37°C (lanes 2) or adjusted to pH 8.0 in medium at 37°C (lanes 3). Whole-cell lysates were either stained with Coomassie blue (left) or immunoblotted with pooled antibodies directed against FlaB and OspC (right). Numbers at the left denote protein molecular mass markers (in kilodaltons).

decreased temperature and increased culture pH (Fig. 4, lanes 1 and 3), indicating normal *ospC* regulation.

**$\sigma^s$ controls *ospC* expression via the minimal promoter.** Wild-type *ospC* expression is controlled via the RpoN-RpoS regulatory network, which culminates in $\sigma^s$ regulating *ospC* expression (25, 61). To garner evidence that $\sigma^s$ directly controls *ospC* expression via interaction with the minimal promoter, the complementation construct pOspC-min was transformed into an *rpoS*-deficient mutant of *B. burgdorferi* (25). The resulting strain, RpoS$^-$/pOspC-min, no longer expressed *ospC* at either the RNA or protein level (Fig. 5), demonstrating

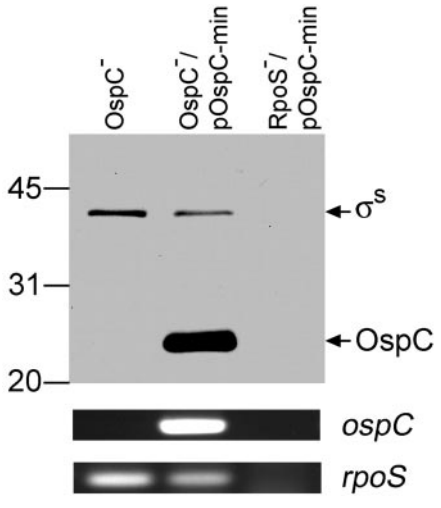


FIG. 5. *ospC* expression driven by the minimal promoter is $\sigma^s$ dependent. Top panel, immunoblot of whole-cell lysates of the *ospC* mutant (OspC$^-$), *ospC* mutant transformed with pOspC-min (OspC$^-$/pOspC-min), or an *rpoS* mutant (RpoS$^-$) transformed with pOspC-min. All cultures were grown at 37°C and harvested at the late logarithmic phase of growth. Antisera directed against $\sigma^s$ and OspC were pooled for immunoblotting. Numbers at the left denote protein molecular mass markers (in kilodaltons). Bottom two panels, RT-PCR and agarose gel electrophoresis for the detection of *ospC* and *rpoS* transcripts, respectively. Only the relevant portions of the agarose gel are shown.

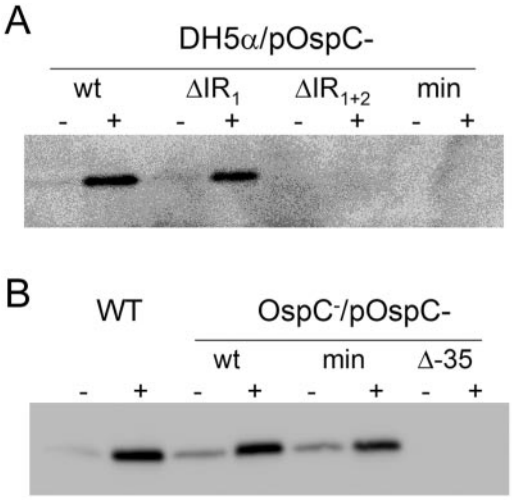


FIG. 6. Influence of coumermycin $A_1$ on *ospC* expression driven by various promoter constructs. (A) *E. coli* strain DH5α carrying various mutant *ospC* promoters (top of panel A; abbreviations as defined for Fig. 3A) was treated (+) or not treated (−) at 23°C with 1 μg/ml of coumermycin $A_1$. (B) Wild-type (WT) *B. burgdorferi* or various complemented derivatives of the *ospC* mutant (OspC$^-$) (top of panel B; abbreviations as in Fig. 3A) were either treated (+) or not treated (−) at 23°C with 20 ng/ml of coumermycin $A_1$. Whole-cell lysates were then immunoblotted with either monoclonal antibody (panel A) or antiserum (panel B) directed against OspC and developed using chemiluminescence.

that $\sigma^s$ controls *ospC* expression via the minimal promoter.

**Influence of coumermycin $A_1$ on activity of the *ospC* promoter.** Elevated culture temperature reduces DNA supercoiling in *B. burgdorferi* that, in turn, induces the expression of *ospC* (1). This same OspC induction was mimicked by treatment of *B. burgdorferi* with coumermycin $A_1$, an inhibitor of DNA gyrase that also culminates in decreased DNA supercoiling (43). Relaxation of supercoiling is one of the factors that enhances promoter selectivity by E$\sigma^s$ (24). In addition, the IRs were postulated to play a role in the regulation of *ospC* by supercoiling (1). To examine which *ospC* promoter element(s) responds to DNA supercoiling, *E. coli* (Fig. 6A) and the *B. burgdorferi* OspC$^-$ mutant (Fig. 6B) carrying the complementing plasmids were cultivated at room temperature and treated with coumermycin $A_1$. In the heterologous *E. coli* strain transformed with a wild-type *ospC* gene, OspC is not expressed by *E. coli* cultivated at room temperature, whereas coumermycin $A_1$ treatment induced the expression of OspC in *E. coli* cultivated under the same conditions (Fig. 6A). This same coumermycin $A_1$ induction effect was obtained in *E. coli* when $IR_1$ of *ospC* was absent (Fig. 6A). However, plasmid constructs that lacked both $IR_1$ and $IR_2$ (pOspC-$\Delta IR_{1+2}$) or that contained only the minimal promoter (pOspC-min) failed to respond to coumermycin $A_1$ treatment (Fig. 6A). In wild-type *B. burgdorferi* cultivated at room temperature for 2 weeks, 20 ng/ml of coumermycin $A_1$ also induced the expression of *ospC* (Fig. 6B). The same coumermycin $A_1$ induction effect was observed with the OspC$^-$ strain of *B. burgdorferi* complemented with either a wild-type copy of *ospC* (pOspC-wt) or the minimal promoter of *ospC* (pOspC-min) (Fig. 6B). Treatment of the same complemented strains of *B. burgdorferi* with 100 ng/ml of coumermycin $A_1$ for 24 h yielded the same results (data not shown). Of note, the

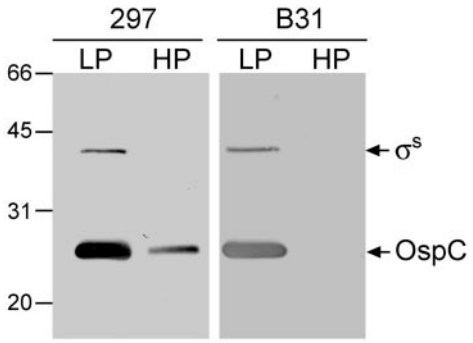


FIG. 7. $\sigma^s$ expression correlates with OspC expression in low- and high-passage strains of *B. burgdorferi* 297 and B31. Low-passage (LP) and high-passage (HP) *B. burgdorferi* strains 297 and B31 were cultivated in BSK-H medium at 37°C and were harvested at the late logarithmic stage of growth. Antisera directed against $\sigma^s$ and OspC were pooled for immunoblotting. Numbers at the left denote protein molecular mass markers (in kilodaltons).

minimal promoter of *ospC* remained responsive to coumermycin $A_1$ treatment and, thus, to a DNA supercoiling effect in *B. burgdorferi*, but not in *E. coli* transformed with the same plasmid.

**Loss of *ospC* expression correlates with the loss of $\sigma^s$ in high-passage populations of *B. burgdorferi*.** Continuous serial passage of *B. burgdorferi* in vitro results in a reduction or loss of OspC expression (30, 47, 57). Inasmuch as our data indicate that the expression of *ospC* is under the direct control of $\sigma^s$, we investigated whether the loss of OspC expression in high-passage populations of *B. burgdorferi* correlated with a concomitant loss of $\sigma^s$. To examine this, low- and high-passage populations of *B. burgdorferi* strains 297 and B31 were subjected to SDS-PAGE and immunoblotting for colorimetric detection of OspC and $\sigma^s$ (Fig. 7). OspC was absent in high-passage strain B31, a finding that correlated with an undetectable level of $\sigma^s$ (Fig. 7). A minimal quantity of OspC was detected in high-passage strain 297 even though $\sigma^s$ also was not detectable in the same strain (Fig. 7). However, when similar immunoblots were developed by a more sensitive chemiluminescence method, a small amount of $\sigma^s$ was detected (data not shown). These combined findings suggest that the continuous in vitro passage of *B. burgdorferi* results in a loss of $\sigma^s$ that, in turn, leads to the loss of *ospC* expression.

## DISCUSSION

The *B. burgdorferi* genome encodes about 175 known or putative lipoprotein genes comprising about 10% of the total *B. burgdorferi* genomic coding capacity (9, 17). This remarkable feature distinguishes *B. burgdorferi* from virtually all other pathogenic bacteria (17) and has engendered the contention that the membrane lipoproteins likely play an important role in the adaptation of *B. burgdorferi* to both its arthropod and mammalian hosts (22, 54). In this regard, it is increasingly well documented that a number of these lipoproteins indeed are differentially expressed during the transmission of *B. burgdorferi* between ticks and mammals (2); recent demonstration of the essential roles for the OspA and OspC in spirochete transmission and mammalian infection further underscores the importance of lipoproteins in the complex life cycle of *B. burgdorferi* (21, 37, 62). Continuing efforts to elucidate the molecular mechanisms that modulate the expression of borrelial lipoproteins thus likely will hold the key for understanding how *B. burgdorferi* survives in nature via its complex adaptive responses (22, 41, 46).

Previously, we identified a novel genetic regulatory network, the RpoN-RpoS pathway, that governs the expression of several borrelial lipoproteins, including OspC, DbpA, and the Mlp family (i.e., "group I" lipoproteins) (58). In the present study, we exploited *ospC* as a model system for further understanding the mechanism(s) governing the expression of lipoprotein genes regulated by the RpoN-RpoS pathway. Regulation by the RpoN-RpoS pathway, however, is predicated on the requirement for a $\sigma^s$-dependent promoter to drive expression of the downstream target gene. Prior reports, however, have suggested that the *ospC* promoter was the $\sigma^{70}$ type (based on sequence analysis and primer extension studies) (18, 28, 29, 33), and the additional presence of two conserved IR elements upstream of the *ospC* gene potentially also constituted a binding site(s) for a putative transactivator of *ospC* regulation. As such, one attractive hypothesis has been that *ospC* actually is regulated indirectly by the RpoN-RpoS pathway via the induction of a requisite transactivator, followed by expression of *ospC* via a $\sigma^{70}$-like promoter.

By exploiting an *ospC* mutant of *B. burgdorferi* and a shuttle vector carrying a wild-type copy of *ospC*, herein we now have provided several lines of evidence that the *ospC* gene likely is regulated directly by the binding of $\sigma^s$ to its $\sigma^s$-dependent promoter. First, the two IRs (potential transactivator-binding site[s]) were dispensable; a minimal $-35/-10$ promoter sequence was both necessary and sufficient for *ospC* expression. Second, the minimal *ospC* promoter defined in our study remained responsive to key environmental stimuli typically associated with the regulation of *ospC* (e.g., temperature and pH). Third, as shown in complementation studies with a $\sigma^s$-deficient mutant of *B. burgdorferi*, the *ospC* gene containing the minimal promoter remained $\sigma^s$ dependent. Fourth, consistent with the fact that $E\sigma^s$ polymerase binds preferentially to relaxed DNA (26), *ospC* expression controlled by the minimal promoter increased in response to coumermycin $A_1$.

The alternative sigma factor $\sigma^s$ is a general stress factor that controls the expression of many genes essential for bacterial stationary-phase adaptation (23). Although $\sigma^s$ modulates the expression of a distinct group of genes, its structure and molecular function are very similar to those of the housekeeping sigma factor, $\sigma^{70}$ (RpoD), and the consensus promoter sequence for $E\sigma^s$ is similar to that used by $E\sigma^{70}$ (19, 24). In fact, a typical $\sigma^s$-dependent promoter binds to both $E\sigma^s$ and $E\sigma^{70}$ in vitro, thereby precluding the use of electrophoretic gel shift assays for distinguishing between $\sigma^s$ and $\sigma^{70}$ promoters. As such, there has been a great interest in elucidating the mechanism by which $E\sigma^s$ recognizes and discriminates its cognate promoter in vivo. In *E. coli*, several factors, such as a high-salt condition, presence of an additional *trans* regulator, or the local relaxation of target DNA, have been shown to contribute to promoter selectivity for $E\sigma^s$ (24). In addition, certain promoter sequence elements, especially a C nucleotide within the extended $-10$ region, can play an important role in $E\sigma^s$ selectivity (4). This C nucleotide was suggested to interact with a key Lys173 residue of $\sigma^s$ in *E. coli* (4). In the present study, we showed that mutation of a candidate $-15$ C nucleotide within the extended $-10$ region of the minimal promoter for *ospC*

greatly diminished OspC expression, further suggesting that a C residue within this extended −10 region also is important for $\sigma^s$-dependent activation in *B. burgdorferi*. On the other hand, $\sigma^s$ of *B. burgdorferi* does not possess an obvious Lys residue that corresponds to Lys173 of *E coli* $\sigma^s$. Further experiments are therefore warranted to identify the residue(s) in *B. burgdorferi* $\sigma^s$ involved in interacting with the −15 C nucleotide.

Eggers et al. (13) recently performed an analysis of promoter elements involved in the expression of *ospC* and other *B. burgdorferi* genes using a GFP reporter system in *E. coli*. Although our results regarding *ospC* expression are largely in agreement with those of Eggers et al. (13), some differences are noteworthy. Eggers et al. (13) showed that deletion of the IR elements upstream of the *ospC* promoter significantly reduced the level of *ospC* promoter activity in *B. burgdorferi*; it therefore was concluded that the upstream (IR) region likely functions as an enhancer-binding site for maximal expression of *ospC*. In contrast, densitometry performed on immunoblots indicated that deletion of both IRs did not dramatically affect *ospC* expression in *B. burgdorferi*. The reason for this discrepancy is unclear but may be grounded in the fact that we assayed for native OspC expression, whereas Eggers et al. (13) used a GFP-based reporter assay.

Much of the work reported by Eggers et al. (13) exploited *E. coli* as a surrogate system for assessing *ospC* promoter activity. Whereas using surrogate systems can be valuable, in our studies we noted differences in the regulation of *ospC* when present in *E. coli* or its native *B. burgdorferi* background. For example, $IR_2$ was required for *ospC* induction by coumermycin $A_1$-induced relaxation of DNA supercoiling in *E. coli*, but not in *B. burgdorferi*. This may be due to differences in $\sigma^s$ function, plasmid topology, or other *trans*-acting factors that exist between the two species. Thus, with continuing advances in borrelial genetics, particularly the applications of targeted mutagenesis, gene inactivation, and improved shuttle vectors, studying *B. burgdorferi* gene regulation in the relevant native background should be the preferred experimental approach.

A distinguishing feature of *B. burgdorferi* as a prokaryote is its remarkable plasmid complexity (52). While the extremely large complement of circular and linear plasmids in *B. burgdorferi* likely offers a genetic plasticity that allows it to adapt readily to its diverse arthropod and mammalian hosts, it also may engender the genetic instability that typifies the population biology of *B. burgdorferi* (3, 52). The spontaneous loss by *B. burgdorferi* in culture of lp25, encoding the *pncA* gene essential for *B. burgdorferi* infectivity (39), is a prime example of this kind of genetic instability (27, 40, 57). Another genetic phenomenon, yet to be elucidated, is that continuous passage of *B. burgdorferi* in vitro also results in greatly diminished *ospC* expression (47, 57). Our data appear to provide the first insights into the loss of *ospC* expression. Namely, the loss of *ospC* expression correlated with the apparent loss of (or reduction in) $\sigma^S$ within the *B. burgdorferi* population; such loss in OspC expression was not attributed to the loss of cp26 or the loss of *ospC* or *rpoS* (data not shown). Although the precise mechanism accounting for this phenomenon thus remains unclear, we hypothesize that continuous in vitro passage of *B. burgdorferi* may adversely influence the ability of Rrp2 to become activated by its cognate histidine kinase, thereby blocking *rpoS* expression. Alternatively, $\sigma^S$ in *B. burgdorferi* under continuous in vitro passage may be susceptible to a form of posttranscriptional regulation, such as proteolysis, that has been observed for other bacteria (23).

OspC has been classified with other borrelial lipoproteins, denoted "group I" lipoproteins, such as DbpA, OspF, and the Mlp family, which appear to be regulated by similar environmental cues. Therefore, the fact that *ospC* is regulated directly by $\sigma^s$ may be applicable to the regulation of other group I lipoprotein genes. On the other hand, such extrapolation to other group I lipoprotein genes will require further experimental corroboration, as presented herein for *ospC*. This is particularly important given the fact that although *dbpA* is induced by elevated temperature, its responsiveness to culture pH differs from that of *ospC* (58). In the case of the *mlp* genes, they are coordinately regulated in a pattern very similar, but not identical, to that of *ospC* (59). Thus, it is premature to conclude that *dbpA* or the *mlp* lipoprotein genes have $E\sigma^s$ promoters. It is therefore not inconceivable that another layer of gene regulation, yet to be elucidated, remains for the regulation of other group I lipoprotein genes.

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