Regulators of Expression of the Oligopeptide Permease A Proteins of *Borrelia burgdorferi*\(^{\text{v}}\)

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*Borrelia burgdorferi* undergoes an infectious cycle that requires adaptation to different hosts and marked differences in environment. *B. burgdorferi* copes with its different environments by regulating the expression of proteins required for survival in specific settings. The *B. burgdorferi* oligopeptide permease (Opp) is one of only a few transporters encoded by the *B. burgdorferi* genome. Opp proteins in other bacteria serve multiple environmental adaptation functions. *B. burgdorferi* appears to broaden the usage of this transporter by utilizing five different substrate binding proteins (OppA proteins) that interact with the integral membrane components of the transporter. Expression of the OppA proteins is individually regulated and may play different roles in adaptation to host environments. Very little is known about the mechanisms used by *B. burgdorferi* to regulate the expression of different OppA proteins. Here we show that the alternative sigma factors, RpoS and RpoN, regulate the expression of *oppA* genes. Using a reporter assay with *Escherichia coli* gel shift binding assays, we also show that the *B. burgdorferi* BosR/Fur homologue interacts with the *oppA* promoter and that another candidate transcription factor, EbFC, interacts with the *oppA* promoter. Binding to the promoters was confirmed by gel shift assays. Expression of BosR/Fur in its different hosts does appear to parallel the expression of *oppA*. A better understanding of the factors involved in gene regulation in *B. burgdorferi* will help to identify coregulated proteins that may cooperate to allow the organism to survive in a specific environment.

*Borrelia burgdorferi*, the causative agent of Lyme disease, is able to adapt to a diverse range of hosts that pose very different challenges to its survival. *B. burgdorferi* accomplishes this, in part, by tight regulation of the expression of various proteins that are utilized for critical functions in specific hosts. A well-studied example of this is the regulation of outer surface protein A (OspA) of *B. burgdorferi*, a protein required for binding to the tick midgut but nonessential for survival in the mammalian host. *B. burgdorferi* minimizes the antigenic cost of expression of OspA by down-regulating its expression as the organism moves from its tick host to a mammalian host.

The *B. burgdorferi* genome appears to be profoundly deficient in genes devoted to the biosynthesis of fatty acids, nucleic acids, and amino acids. As a result, the organism is highly dependent on its external environment for acquisition of these essential nutrients. Peptides serve as a source of amino acids for many bacteria. *B. burgdorferi* carries a single peptide transport system that appears to be closely related to the oligopeptide permease (Opp) family of transporters. Opp transporters are ABC-type transporters with a peptide-binding protein that interacts with two inner membrane transmembrane proteins and two ATP binding proteins. The *B. burgdorferi* genome encodes five separate peptide-binding proteins that each appear to be capable of functioning with the integral membrane proteins of the transporter. Three of the genes (*oppA*1, -2, and -3) are located in the *opp* operon located on the chromosome, and two are on distinct plasmids (*oppA*4 is on lp26, and *oppA*5 is on lp54). It was previously shown that all five putative peptide-binding proteins are capable of facilitating the transport of small peptides and that many of the proteins have overlapping but distinct substrate preferences.

In addition to the transport of peptides for nutritional needs, peptide transport systems have been implicated in diverse non-nutritional bacterial functions. In *Bacillus subtilis*, the oligopeptide permease encoded by *spotK* (Opp) binds to an exported peptide, competence and sporulation stimulating factor (CSF), which acts intracellularly to signal cell density. CSF stimulates competence gene expression at low concentrations and inhibits competence gene expression and stimulates sporulation at high concentrations. Uptake of CSF by Opp from the extracellular environment allows *B. subtilis* to respond to changes in cell density. In *Enterococcus faecalis*, a plasmid-encoded peptide binding protein, PrgZ, with similarity to OppA, binds a peptide sex pheromone, cCF10, which signals conjugative transfer of a plasmid. PrgZ uses a chromosomal OppB/CD/F to process cCF10 and reach intracellular target molecules. *Escherichia coli* shows chemotaxis towards a variety of dipeptides (but not tripeptides) that is mediated by dipeptide permease A (DppA), a structurally related oligopeptide transport system.
to be mediated through Tap, which acts as a conventional signal transducer. Manson et al. have shown that Tap-mediated peptide chemotaxis requires the function of DppA but not the rest of the Dpp peptide transport system (14). More recently, for *Listeria monocytogenes*, OppA was shown to be critical to environmental adaptation, allowing the bacterium to grow at a low temperature and to survive intracellularly (4).

Expression of the *B. burgdorferi* oppA genes was previously shown to be differentially regulated under changing environmental conditions (26). In this study, we attempted to determine the mechanisms involved in the regulation of expression of *B. burgdorferi* oppA by examining the effects of alternative sigma factors and putative transcription factors on the activities of the oppA promoters.

**MATERIALS AND METHODS**

**Bacterial and mouse strains.** *B. burgdorferi* was grown in BSK-H medium (Sigma Co., St. Louis, MO). RpS0, RpS0N, and RpR2 mutants and complemented strains were shown to be differentially regulated under changing environments of the bacterium (18).

**Primer or strain**

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* From reference 18.

**Expression of the *B. burgdorferi* oppA genes**

The *B. burgdorferi* oppA genes was previously shown to be differentially regulated under changing environmental conditions (26). In this study, we attempted to determine the mechanisms involved in the regulation of expression of *B. burgdorferi* oppA by examining the effects of alternative sigma factors and putative transcription factors on the activities of the oppA promoters.
0.4 to 0.6. Protein production was induced by adding IPTG (isopropyl-β-d-thiogalactopyranoside) to a final concentration of 1 mM, and cells were shaken at 250 rpm for one to two additional hours. Cells were harvested, chilled on ice for 30 min, and centrifuged at 4,230 × g for 10 min at 4°C. Pellets were resuspended in 20 ml HEPES-buffered saline and centrifuged at 4,230 × g for 10 min at 4°C. The pellets were stored at −70°C until ready for lysis.

Pellets were lysed in a French pressure cell in the presence of protease inhibitors (0.1 trypsin inhibitor unit/ml aprotinin, 1 mM benzamidine, 10 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride [Sigma-Aldrich Co., St. Louis, MO]). Lysed cells were centrifuged at 26,890 × g for 30 min at 4°C. The supernatant was decanted and centrifuged at 38,720 × g for 20 min at 4°C.

His-bind kit columns (Novagen/EMD Biosciences, Darmstadt, Germany) were used to purify the recombinant His6-tagged proteins from the supernatants. The purification was performed per the manufacturer’s instructions, except that protease inhibitors (0.1 trypsin inhibitor unit/ml aprotinin, 1 mM benzamidine, and 10 μM pepstatin A) were added to the binding buffers. The expression and purity of the individual tagged proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie blue staining or by transfer to polyvinylidene difluoride membranes and immunoblotting using a mouse monoclonal anti-HA antibody (Cell Signaling, Beverly, MA).

Electrophoretic mobility shift assays (EMSAs). DNAs for the upstream regions of oppA4 and oppA5 containing promoter activity, as described previously (26), were prepared by PCRs utilizing the primers listed in Table 1. DNAs were labeled with digoxigenin by using a gel shift assay kit (Roche Biochemicals, Indianapolis, IN). Purified EthC or BosR/Fur was mixed with the appropriate labeled DNA at various concentrations in a buffer containing 20 mM HEPES, pH 7.6, 50 mM NaCl, 5 mM dithiothreitol, 5% glycerol, 50 μg/ml bovine serum albumin, 1 mM MgCl₂, and 0.1 μg poly(dI-dC) to reduce nonspecific interactions. For competition assays, unlabeled DNA with the same sequence as the labeled DNA was added in 125-fold excess to the mixture. The mixture was incubated at room temperature for 30 min before being loading into a 6% DNA retardation gel (Novex precast gel; Invitrogen, Carlsbad, CA). After electrophoresis, the gel was transferred to a nylon membrane (Bio-Rad, Hercules, CA). Blocking of the membrane, incubation with alkaline phosphatase-linked anti-digoxigenin antibody, and detection with the chemiluminescent substrate CDP-Star, and detection of alkaline phosphatase by exposure to film (Biomax; Kodak, Rochester, NY) were performed per the gel shift assay kit instructions.

Preparation of tick RNA. *Ixodes dammini* ticks were obtained from a laboratory colony derived from an Ipswich, MA, population that has been determined to be free of inherited spirochetal infection. Outbred CH3 mice were infected by nymphs infected with strain N40 (clone D10E9), which was maintained in alternating tick-mouse-tick passages. Larvae were allowed to feed to repletion 3 weeks after the infected nymphs engorged. Upon repletion, engorged larvae were collected and permitted to molt to the nymphal stage at 21°C and 95% relative humidity. Nymphal ticks were fed on uninfected mice for 60 h prior to removal.

Fed and unfed nymphal ticks were pooled into groups of 5 to 10 ticks, suspended in Trizol (Invitrogen), and homogenized for 30 s, using a rotor-stator homogenizer. RNAs were purified per the manufacturer’s instructions.

Preparation of mouse RNA. C57/HeN mice (Jackson Laboratory, Bar Harbor, ME) were infected subcutaneously with 10⁶ *B. burgdorferi* organisms (strain N40, clone D10E9). Mice were sacrificed at 2 weeks postinfection, and hearts were placed into RNA later (Applied Biosystems, Foster City, CA), snap frozen in liquid nitrogen, and stored at −70°C until use. RNAs were prepared using Trizol per the manufacturer’s instructions.

RT-PCR. Total RNA from *B. burgdorferi*, ticks, or mouse tissue was heated to 95°C for 10 min and then chilled. Samples were then treated with RNase-free DNase I (Applied Biosystems) at 37°C for 15 min. First-strand cDNA synthesis was performed using SuperScript (Invitrogen) with random hexamer primers or gene-specific primers per the manufacturer’s instructions. The generated cDNAs were used as a template for real-time PCR amplification (Cycler; Bio-Rad), using SYBR green fluorescent dye (SYBR green master mix; Qiagen) and specific primers for each oppA gene. Cycling parameters were 50°C for 5 min and 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 55°C for 1 min. The primers used for reverse transcriptase PCR (RT-PCR) are shown in Table 1 and were described previously (26). Calculations of relative expression of the gene of interest were normalized to recA gene expression by using the ΔΔCT method, where the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by the variable 2−ΔΔCT, where CT is the cycle number of the detection threshold.

Statistics. Comparisons of *B. burgdorferi* gene expression between control and test conditions were performed using the nonparametric Mann-Whitney U test for two-tailed tests.
roles of other identified or putative transcription factors of *B. burgdorferi*. Hbb (BB0232), EbfC (BB0462), and BosR/Fur (BB0647) have been annotated in the *B. burgdorferi* genome to have similarity to DNA binding proteins or transcription factors. EbfC and BosR/Fur have been shown to affect the expression of at least one *B. burgdorferi* gene (2, 5, 9, 22). To test their effects on the expression of *oppA* genes, we utilized a transcriptional reporter fusion system in *E. coli*. The genes for the transcription factors were cloned into an expression plasmid, and expression of the recombinant proteins was verified by Western blotting with an antibody raised against the HA epitope tag fused to each protein. A screen for the effects of these recombinant proteins on *oppA* expression was performed by cotransforming *E. coli* expressing the recombinant protein of interest with a reporter plasmid containing the promoter region of an *oppA* gene fused to *lacZ*. The effects of the recombinant protein were then measured by performing β-galactosidase assays as previously described (26).

Increases in β-galactosidase activity of 3-fold were seen for Hbb with the *oppA2* and *oppA4* promoters, for EbfC with the *oppA5* promoter, and for BosR/Fur with the *oppA4* promoter (Fig. 2). A decrease in β-galactosidase activity of 3-fold was seen only for EbfC with the *oppA4* promoter. As controls, multiple other *B. burgdorferi* proteins, including those with similarity to transcription factors of other organisms and those not predicted to have transcription factor properties, were tested, and none showed changes of 3-fold (data not shown).

**Binding of BosR/Fur and EbfC to oppA4 and oppA5 promoters.** We had previously shown that the expression of *oppA4* and *oppA5* was highly affected by environmental conditions, whereas the expression of other *oppA* genes, such as *oppA1*, was constitutive (26). Therefore, we chose to further study the interactions of EbfC with the *oppA5* promoter and of Hbb and BosR/Fur with the *oppA4* promoter. Purified recombinant

![FIG. 2. Effects of putative transcription factors on activities of OppA promoters. Plasmids expressing each putative transcription factor were cotransformed into *E. coli* BL21(DE3) with a *lacZ* reporter plasmid for each *oppA* promoter region. Bacteria were grown to an OD600 of 0.5. Cells were harvested, and β-galactosidase activity was measured. The control is pET30a plus the specific *oppA* reporter plasmid. Error bars represent standard errors of the means for three experiments performed in duplicate.](http://jb.asm.org/)

![FIG. 3. Purification of Hbb, EbfC, and BosR/Fur. Recombinant Hbb, EbfC, and BosR/Fur were expressed in *E. coli* as described in Materials and Methods. The recombinant proteins were expressed with a His6-HA-HA tag. Lysates of *E. coli* were clarified by centrifugation and then applied to Ni2+ columns. After being washed, recombinant proteins were eluted from the columns by using an imidazole gradient. Shown are Coomasie blue-stained gels of the purified proteins. The predicted sizes of the recombinant proteins with tags are as follows: BosR/Fur, ~25 kDa; EbfC, ~18 kDa; and Hbb, ~18 kDa. Note that there is a discrepancy between the predicted size of BosR/Fur and the actual migration. Identification of the protein was performed by sequencing and Western blotting. Lane 1, BosR/Fur; lane 2, EbfC; lane 3, Hbb; lane M, markers.](http://jb.asm.org/)
Hbb, Ebfc, and BosR/Fur proteins were prepared for use in EMSA. Purified proteins were added in increasing amounts to digoxigenin-labeled DNAs from promoter regions. Unlabeled DNA (125-fold excess) was added as a specific competitor (Comp) to wells containing the largest amount of protein. DNA from the recA coding region was used as a negative control. (A) BosR/Fur; (B) Ebfc. Arrows indicate shifted DNAs.

![Image](image1)

**FIG. 4.** Binding of BosR/Fur and Ebfc to OppA promoters. Binding of putative transcription factors to oppA promoters was determined by EMSA. Purified proteins were added in increasing amounts to digoxigenin-labeled DNAs from promoter regions. Unlabeled DNA (125-fold excess) was added as a specific competitor (Comp) to wells containing the largest amount of protein. DNA from the recA coding region was used as a negative control. (A) BosR/Fur; (B) Ebfc. Arrows indicate shifted DNAs.

The addition of Ebfc resulted in a shift of labeled oppA5 promoter DNA that was lost with the addition of a 125-fold excess of unlabeled oppA5 promoter DNA (Fig. 4). The addition of an equivalent amount of Ebfc did not result in a shift of labeled promoter regions from other oppA genes or of the recA promoter.

The addition of BosR/Fur resulted in a shift of labeled oppA4 promoter that was lost with the addition of a 125-fold excess of unlabeled oppA4 promoter. The addition of an equivalent amount of BosR/Fur did not result in a shift of labeled promoter regions from other oppA genes or of the recA promoter. One previous report suggested that the presence of Zn$^{2+}$ optimizes binding of BosR/Fur to its target sequences, although others have not found this to be true (5, 9). We did not see any significant changes in binding with the addition of various concentrations of Zn$^{2+}$ to the binding buffer (data not shown).

These data confirm the results of the reporter screen showing that Ebfc binds to the oppA5 promoter region and that BosR/Fur binds to the oppA4 promoter region. These interactions are consistent with the reporter assay data showing increases in the promoter activities of oppA4 by BosR/Fur and oppA5 by Ebfc.

**Comparison of expression of Ebfc and BosR/Fur with oppA expression under various environmental conditions.** If Ebfc and BosR/Fur regulate the expression of oppA5 and oppA4, respectively, then it is possible that their own regulation is affected by environmental conditions and parallels expression of the opp genes that they regulate. It was previously shown that oppA4 expression is greatly increased in *B. burgdorferi* organisms recovered from mouse tissue compared with that in organisms grown in vitro and is undetectable in organisms recovered from either unfed or feeding ticks (26). oppA5 expression was found to increase in *B. burgdorferi* organisms recovered from unfed ticks compared with either those from fed ticks or those from mouse tissue. We examined the expression of Ebfc and bosR/fur in fed and unfed ticks and in mouse heart tissue by qRT-PCR (Fig. 5).

The pattern of transcription of Ebfc did not match the expression of oppA5 under various environmental conditions. The relative expression of Ebfc decreased approximately five-fold (P = 0.014) in unfed nymphal ticks compared to that in organisms grown in vitro at 37°C. Expression in ticks that had taken their blood meal was essentially unchanged from that in organisms grown in vitro, and expression in organisms recovered from mouse heart tissue was only slightly elevated (1.5-fold). Deletion of RpoS, RpoN, or Rrp2 also did not significantly change the expression of Ebfc in vitro.

The relative expression of BosR/Fur more closely followed the pattern of oppA4 gene expression. bosR/fur transcripts were...
undetectable in unfed ticks. Transcripts in fed ticks were decreased compared with levels in organisms grown in vitro and increased in organisms recovered from mouse heart tissue \((P \leq 0.014\) for both).

**DISCUSSION**

In this study, we examined the roles of specific alternative sigma factors and candidate transcription factors in the expression of \(oppA\) genes of \(B. burgdorferi\). The five \(oppA\) genes of \(B. burgdorferi\) have previously been shown to be independently regulated and to respond differentially to changing environmental conditions (26). The \(B. burgdorferi\) genome contains genes encoding only three identifiable sigma factors, namely, RpoD, RpoS, and RpoN. The alternative sigma factors, RpoS and RpoN, have been found to regulate the expression of a cluster of proteins involved in environmental adaptation and infection of mice (6, 8, 27). Expression of many of these proteins appears to be influenced by temperature. Using knockout and complemented mutant bacteria, we found that of the \(oppA\) genes, RpoS and RpoN appear to be involved in the regulation of only \(oppA5\). This appears to be consistent with prior studies that have shown that among the \(B. burgdorferi\) \(oppA\) genes, only \(oppA5\) expression is affected by temperature (3, 26).

Non-sigma-factor-controlled expression of \(oppA\) genes is likely mediated by additional transcriptional activators or repressors. Compared with other bacteria, \(B. burgdorferi\) has only a limited number of putative genes with similarity to known transcription factor genes. Only a few functional transcription factors have been identified in \(B. burgdorferi\) to date, and their effects on the expression of \(OppA\) proteins have not previously been studied in detail (5, 9, 10, 22). Because of the difficulty in working directly to manipulate \(B. burgdorferi\) gene expression, we employed a reporter screen using the expression of \(B. burgdorferi\) putative transcriptional factors in \(E. coli\). While this is clearly suboptimal, use of the heterologous system allows for rapid screening of multiple protein-promoter pairs to identify promising interactions for more detailed examination. Here we show, using a reporter assay with \(E. coli\) and gel shift assays, that Ebfc (BB0462) binds to the \(oppA5\) promoter and BosR/Fur (BB0647) binds to the \(oppA4\) promoter region. Binding of both of these proteins to the respective \(OppA\) promoters results in increased promoter activity. Although another putative transcription factor, Hbb (BB0232), showed activity in our reporter assays, we were unable to confirm this activity due to nonspecificity of binding of our recombinant protein. Previous investigators have found specific binding by Hbb (10). The difference may be due to the tags that were used in our system or to subtle differences in the host strain or in the expression and purification procedures that resulted in changes in folding and activity of the protein. Even among our constructs, we did see differences in DNA affinity between Hbb proteins expressed from different host strains of \(E. coli\).

Ebfc has only recently been identified as a functional transcription factor. Babb et al. identified Ebfc by DNA affinity chromatography with the promoter region from the \(erpA\) gene of \(B. burgdorferi\) (2). Some of the Erp proteins (also known as OspE/OspF/Elp family proteins) bind to mammalian factor H proteins, which is hypothesized to protect the organism from complement-mediated killing (15, 23). Erp proteins are down-regulated in unfed ticks and up-regulated as ticks take their blood meals and in the mammalian host (16). We found that \(ebfc\) expression was greatly decreased in unfed ticks, which is consistent with its role in expression of the Erp proteins. Expression of \(ebfc\) did not match the expression of \(oppA5\) in the tick, which increases in unfed ticks compared with fed ticks (26). This may be due in part to the effects of other mechanisms that control \(oppA5\) expression, for example, the RpoS-RpoN system, which we found to have significant effects on \(oppA5\) expression. It should also be noted that because of the small numbers of organisms present in tick and mouse tissues, our studies, of necessity, measured transcription, not actual protein production. It is possible that Ebfc protein expression is not transcriptionally controlled and that actual protein levels differ significantly. Another possibility is that the activity of Ebfc on the \(oppA5\) promoter may not have been represented accurately by the heterologous \(E. coli\) reporter system. Babb et al. identified a putative Ebfc binding motif, TGT(A/T)ACA, which was not identified in the \(oppA5\) promoter region. It is unknown whether there are other sequences which bind Ebfc or whether the requirement for the TGT(A/T)ACA sequence is stringent.

BosR/Fur (BB0647) has previously been studied by three groups of investigators (5, 9, 22). It has variously been named BosR and reported as a homolog of PerR, a stress response repressor, and also Fur, for its similarity to ferric uptake regulation protein. Recombinant BosR/Fur can bind to both Fur and Per box sequences with similar affinities (9). There are data to suggest that it may act as both a repressor and an activator in \(B. burgdorferi\) (5, 9, 22). It has been shown to bind to the promoter of \(napA\), which is involved in the response by \(B. burgdorferi\) to oxidative stress, and to the superoxide dismutase (\(sodA\)) promoter. BosR also binds to its own promoter, indicating autoregulation, and binds to the BB0646 promoter as well, although the role for this is not yet clear (9). We found that BosR/Fur has an activator effect on the \(oppA4\) promoter region but not on other \(B. burgdorferi\) \(oppA\) promoter regions. An analysis of the \(oppA4\) promoter region did not reveal any segments that closely matched consensus Fur or Per binding sequences identified for \(E. coli\) or \(B. subtilis\), although multiple partial matches were seen. The expression of \(bosR/fur\) in vivo has not previously been described. Here we show that the expression of \(bosR/fur\) is greatly increased in the heart tissue of mice compared with expression in vitro or in either fed or unfed ticks. This expression pattern parallels that of \(oppA4\) and would be consistent with the need for increased expression of \(NapA\) under conditions of higher oxidative stress in the mammalian host.

In summary, we have now delineated some of the mechanisms by which \(B. burgdorferi\) is able to differentially regulate the expression of its \(oppA\) genes. There are undoubtedly additional mechanisms that are yet to be described that play roles in the control of these proteins. A better understanding of the mechanisms by which \(B. burgdorferi\) regulates gene expression and the proteins that are coregulated by different transcriptional factors will lead to new insights into how \(B. burgdorferi\) is able to adapt to the markedly different environments it encounters.
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