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Effects of Environmental Changes on Expression of the Oligopeptide Permease (*opp*) Genes of *Borrelia burgdorferi*

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We analyzed expression of a putative oligopeptide permease (Opp) of *Borrelia burgdorferi*. Unlike the *opp* operons of other bacteria for which there is a single substrate binding protein, *B. burgdorferi* codes for three substrate binding proteins (OppA-I to -III) in its *opp* operon and an additional two homologs on plasmids (OppA-IV and -V). Instead of a single promoter region regulating transcription of the entire operon, as seen in other bacterial *opp* operons, it appears that among *oppA-I*, *-II*, and *-III*, as well as *oppA-IV* and *-V*, each has a potential upstream promoter region. We tested the function of these putative promoter sequences by fusion to a promoterless β-galactosidase reporter gene in pCB182. Each of the promoter regions was found to be active. The level of activity in the reporter constructs closely paralleled the level of expression of each gene in in vitro-grown *B. burgdorferi*. Changes in carbon and nitrogen availability differentially affected individual promoters, but no changes in promoter activity were seen when *Escherichia coli* bacteria (with the promoter constructs) were grown in various concentrations of phosphate and leucine and changes in pH. Expression of specific *oppA* genes with *B. burgdorferi* varied significantly between its mouse and fed and unfed tick hosts. Differences in regulation of *opp* gene expression suggest a potential role in environmental response by the organism.

Borrelia burgdorferi is the causative organism of Lyme disease. One of the striking observations that has been made about *B. burgdorferi* in the postgenomic era is that of its apparent lack of synthetic machinery. *B. burgdorferi* does not appear to possess genes for the synthesis of amino acids, fatty acids, and other essential elements (5). As a result, the organism is dependent upon its environment to supply these essential nutrients. Other bacteria typically possess multiple peptide transport systems with different specificities to facilitate the utilization of peptides as a source for amino acids (17). The *B. burgdorferi* genome appears to code for a single peptide transport system only. This peptide transport system shows a high degree of similarity to both the oligopeptide permease (Opp) system and the dipeptide permease (Dpp) system of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. All of these systems encode a peptide binding protein (OppA or DppA), two transmembrane proteins (Opp or DppB and -C) and two ATP binding proteins (Opp or DppD and -F). The *B. burgdorferi opp* operon, however, differs in that it encodes two additional OppA-like proteins (OppA-II and OppA-III). While the *B. burgdorferi opp* operon is chromosomally encoded, at least two other OppA orthologues have been identified on borrelial plasmids (*oppA-IV* on cp26 and *oppA-V* on lp-54). In previous studies using a complementation system with the *E. coli* integral membrane proteins OppB and OppC, the ability of the borrelial OppA orthologues to bind and facilitate transport of peptides was confirmed (10).

The natural life cycle of *B. burgdorferi* requires it to adapt to wide variations in environmental conditions as it moves between its tick and mammalian hosts. Peptide transporters have been shown to play an important role in a diverse array of environmentally relevant functions, including nutrient acquisition, chemotaxis, quorum sensing, and conjugation (1, 8, 9, 11). The specificity in these functions typically derives from the specificity of the substrate binding protein. Whereas *E. coli* DppA appears to have fairly narrow substrate specificity, OppA in other bacteria has been shown to have very broad substrate specificity (6, 14–16, 20, 21). In comparison to *E. coli* OppA, the *B. burgdorferi* OppA proteins appear to have overlapping but narrower specificities, allowing for the potential of separate roles for each OppA protein (10). We were interested in the mechanisms of regulation and expression of the *B. burgdorferi* OppA proteins and whether they could be differentially expressed under changing environmental conditions. In this study, we examine the activities of the promoter regions of the *B. burgdorferi opp* genes and assess the impact of different environmental factors on gene expression.

MATERIALS AND METHODS

Bacterial strains and media. *Borrelia burgdorferi*, strain N40, was grown at 37°C or 25°C in Barbour-Stoenner-Kelly (BSK) H medium (Sigma, St. Louis, Mo.) as previously described (7). *E. coli* bacteria of strain Top10 (Invitrogen) were employed as host cells for the expression of β-galactosidase genes from reporter plasmids.

Plasmid constructions. Five *oppA* upstream regions were individually cloned from *B. burgdorferi* B31 DNA through PCR using specific primers with *Bam*HI and *Bgl*II restriction sites. The primers, URA1F (5′-TCGGATCCTAAAACCA GCACCCT-3′) and URA1R (5′-TCAGATCTTGGGATTTTCCTTTT-3′),

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TABLE 1. Sequences of primers used in this study

Primer	Sequence	Location on *oppA* gene
A1F	5′-ATGAAATATATAAAAA-3′	*oppAI* (1–16 bp)
T7A1R	5′-TAATACGACTCACTATAGGGTTGCTACCGTAAAGG-3′	*oppAI* (141–156 bp)
A2F	5′-ATGAAATTACAAAGGTC-3′	*oppAII* (1–17 bp)
T7A2R	5′-TAATACGACTCACTATAGGGCGACATTACCTCTGCT-3′	*oppAII* (135–152 bp)
A3F	5′-ATGAGCTTTAATAAAACTA-3′	*oppAIII* (1–19 bp)
T7A3R	5′-TAATACGACTCACTATAGGTATTGTCTCATCTACCA-3′	*oppAIII* (160–177 bp)
A4F	5′-GTATTATTTCTCAATTTAAT-3′	*oppAIV* (31–50 bp)
T7A4R	5′-TAATACGACTCACTATAGGGTCCTGGGATCTCCATCT-3′	*oppAIV* (197–215 bp)
A5F	5′-ATGATAATAAAAAAAAG-3′	*oppAV* (1–17 bp)
T7A5R	5′-TAATACGACTCACTATAGGGCCTATCGCTGCAAAA-3′	*oppAV* (136–151 bp)

URA2F (5′-TCGGATCCATAATAAAATTAAGT-3′) and URA2R (5′-TCAGATCTTAATTTTTTATACCT-3′), URA3F (5′-TCGGATCCATAATATAATAAACT-3′) and URA3R (5′-TCAGATCTTATTAACCTTTCCCC-3′), URA4F (5′-TCGGATCCTATTCCTCTCCTTTGA-3′) and URA4R (5′-TCAGATCTTACAAGCATCCTTACA-3′) and URA5F (5′-TCGGATCCTTTGGAGGCGATTTT-3′) and URA5R (5′-TCAGATCTATAGGAATACTGGAA-3′), were used for cloning the upstream regions of *oppA-I*, *-II*, *-III*, *IV*, and *-V*, respectively. The sequences underlined represent the restriction sites for *Bam*HI or *Bgl*II. PCR was performed with the following cycling parameters: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension cycle of 72°C for 7 min. The DNA fragments obtained from PCR were ligated into pCR2.1 vector (Invitrogen) for further amplification. After digestion by *Bam*HI and *Bgl*II, the DNA fragments were recovered using a Qiaquick gel extraction kit (Qiagen) and ligated to the promoter probe vector pCB182 or pCB192 (18) at *Bam*HI and *Bgl*II sites. The recombinant plasmids were transformed into Top10 by electroporation (Electroporator 2510; Eppendorf). A similar strategy was used for construction of deletions in *oppA* upstream regions to identify the DNA fragment with promoter function. All DNA sequences inserted into the promoter-probe vectors were confirmed by DNA sequencing. Plasmid DNA was purified using Qiaprep mini spin columns (Qiagen). All restriction enzymes and primers were purchased from Invitrogen unless otherwise stated.

β-Galactosidase assays. Bacterial cultures were grown to mid-logarithmic phase under various environmental and nutritional conditions. Growth curves for the bacteria were established experimentally prior to the experiments to ensure that bacteria were collected at similar stages of growth. Bacteria in logarithmic phase were centrifuged, washed and resuspended in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol [pH 7.0]), and incubated on ice (12). After absorbance was recorded at a wavelength of 600 nm, 10 μl of 1% sodium dodecyl sulfate and 20 μl of chloroform were added in each 0.8-ml sample. The samples were vortexed for 15 s and then placed at 30°C for 15 min. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) (160 μl of 4 mg/ml, dissolved in 0.1 M sodium phosphate buffer [pH 7.0]) was added and mixed vigorously for 10 s. The samples were incubated at 30°C for 15 to 30 min. 1 M Sodium carbonate (400 μl) was added to quench the reaction. The samples were clarified by centrifugation. β-Galactosidase activity was monitored by measuring the optical density at 420 nm (OD_{420}) and OD_{550}. Enzyme activity was calculated based on the following equation: $U = 1{,}000 \times [OD_{420} - (1.75 \times OD_{550})]/[\text{time} \times \text{vol} \times OD_{600}]$. One unit of enzyme activity was defined as the ability to hydrolyze 1 μmol of ONPG per min (12). Specific activity was expressed as units per absorbance unit of the culture at a wavelength of 600 nm.

RNase protection assay. An RNase protection assay (RPA) was employed as a tool to quantitatively detect the target RNA transcribed by each *oppA* gene in *B. burgdorferi*. To generate linear DNA templates with the core T7 promoter sequence for the preparation of RNA probes, the oligonucleotide primers listed in Table 1 were separately used to specifically amplify *oppA* gene fragments from each cloned *oppA* gene by PCR. Polymerase chain reactions were conducted under the same conditions as described above. The desired DNA fragments, after being amplified from each *oppA* gene, were separated by 1.5% agarose gel electrophoresis and extracted using a Qiaquick gel extraction kit according to the manufacturer's instructions.

To create a specific RNA probe, the linear PCR product containing the core T7 promoter sequence was used as the template DNA. Biotin-labeled RNA probes were individually synthesized with a MAXIscript T7 kit (Ambion), separated on urea–6% polyacrylamide gel, and then eluted into 350 μl of DEPC (diethylpyrocarbonate)-treated H_2O at 37°C overnight.

RNA was extracted from *B. burgdorferi*, using Trizol (Invitrogen) according to the manufacturer's instructions. The amount of RNA probe required to completely hybridize with the target RNA molecules in total RNA and the optimal hybridization temperatures were determined experimentally. Threefold molar excesses of each probe over target mRNA were used to ensure that every target RNA transcript was hybridized. Hybridized RNA was digested with RNase A-RNase T_1 at 37°C for 30 min and inactivated according to the manufacturer's instructions (MAXIscript; Ambion). The RNA samples were separated on a 6% polyacrylamide gel containing urea and transferred to a positively charged nylon membrane (Osmonics). Biotinylated RNA probes protected from RNase digestion by hybridizing to cRNA were detected chemiluminescently, using a BrightStar BioDetect nonisotopic detection kit (Ambion). Quantitation was performed by densitometric scanning (KS1D apparatus; Kodak) of exposed X-ray film.

Preparation of tick RNA. *Ixodes dammini* ticks were obtained from a laboratory colony derived from an Ipswich, Mass., population that has been determined to be free of inherited spirochetal infection. Outbred C3H mice are infected by nymphs infected by strain N40 (wild type), which is maintained in alternating tick-mouse-tick passages. Larvae are allowed to feed to repletion 3 weeks after the infected nymphs engorge. Upon repletion, engorged larvae are 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. RNA was purified according to the manufacturer's instructions.

Preparation of mouse RNA. C3H/HeN mice (Jackson Laboratories) were infected subcutaneously with 10^4 *B. burgdorferi* (cN40) bacteria. Mice were sacrificed at 2 weeks postinfection, and hearts were snap frozen in RNAlater (Ambion) until use. RNA was prepared using Trizol according to the manufacturer's instructions.

Reverse transcriptase PCR (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 (Ambion) at 37°C for 15 min. First-strand cDNA synthesis was performed, using SuperScript (Invitrogen) with random hexamer primers or gene-specific primers, according to the manufacturer's instructions. The generated cDNA was used as a template for real-time PCR amplification (ABI 7700; Applied Biosystems), using SYBR green fluorescent dye (SYBR Green Master Mix; Applied Biosystems) and specific primers for each *oppA* gene. Cycling parameters were 50°C for 5 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30s, and 55°C for 1 min. Specific primers RTA1F (5′-CTTTAACTAAAGTAGTTTTAAAGGGAAGTTCAGAT-3′) and RTA1R (5′-ACATTTGGTAATTTCCAGTTCTTCTGCTTCCTAGGAA-3′) for *oppA-I*, RTA2F (5′-GGCTCAAAGTACGTTGAAATGGTTAAATCGGTA-3′) and RTA2R (5′-TAATAGTCGCTTCTTAATTTTAGATTTTTGATTAG-3′) for *oppA-II*, RTA3F (5′-TGTTCTTACCAACAGCAGAAATACTGGCA-3′) and RTA3R (5′-TAATTCAGAAAGATAATAAACCTCTGATACA-3′) for *oppA-III*, RTA4F (5′-GTCACAGATAATACCATTACAGCTTA-3′) and RTA4R (5′-TAGAAGAAGTTATATTAAGAAATACA-3′) for *oppA-IV*, and RTA5F (5′-AAATATTTTAAAAGGTCAATATGAGATCTCAGTAAGGTC-3′) and RTA5R (5′-GTTTCCCGCAACACTGTATATTGGAATTATT-3′) for *oppA-V* were designed with the program Primer3, which was developed by Steve Rozen and Helen J. Skaletsky. All primer pairs were tested for specificity using templates for each of the other *oppA* genes. None of the primer pairs showed any cross-reactivity with other *opp* genes and all showed only a single band with *B. burgdorferi* DNA. Sequences of the products were confirmed to correspond with the predicted sequence by DNA sequencing. Calculations of relative expression of the gene of interest were normalized to *recA* gene expression with the $\Delta\Delta C_t$

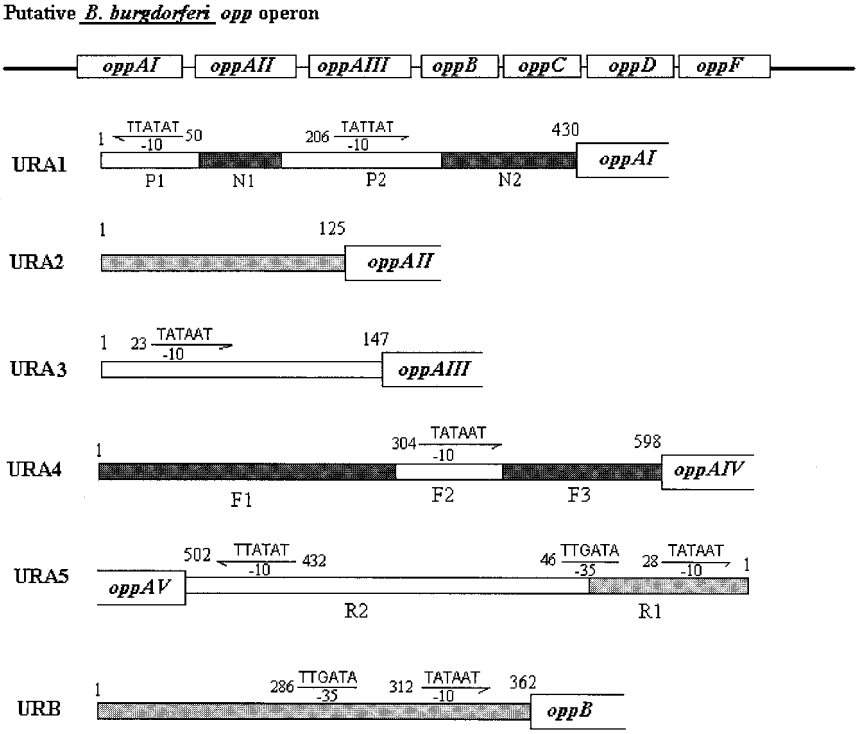

FIG. 1. Schematic representations of *opp* operon and upstream regions of five *oppA* genes and *oppB*. DNA sequences symbolize putative −10 and −35 regions of σ^{70} promoter; arrows indicate the orientations of promoters. Arrows facing to the right indicate sequences on the sense strand; arrows pointing to the left indicate sequences in the reverse orientation. The numbers represent the nucleotide positions in each upstream region. P1, N1, P2, and N2 represent DNA fragments of the *oppA-I* upstream region, F1, F2, and F3 represent those of *oppA-IV* upstream region, and R1 and R2 represent those of *oppA-V* upstream region.

method, in which the amount of target (normalized to an endogenous reference and relative to a calibrator) is given by $2^{-\Delta\Delta C_t}$, where C_t is the cycle number of the detection threshold. Calculations of absolute copy number per sample were calculated from standard curves generated with each individual primer set.

RESULTS

Analysis of *B. burgdorferi opp* promoters. The best-understood OPPs are those of *E. coli* and *S. enterica* serovar Typhimurium. Opp proteins in the *opp* operons of these bacteria appear to be under the regulation of a single promoter region which precedes the *oppA* gene, with little to no separation between the coding sequences. In contrast, the *B. burgdorferi opp* operon contains intergenic regions of sufficient size to potentially allow separate regulation of the three chromosomally encoded *oppA* genes and another potential promoter region upstream of the *oppB*, *oppC*, *oppD*, and *oppF* coding sequences. In addition, both plasmid-encoded OppA orthologues have upstream sequences that could potentially serve as promoter regions. In the chromosomally encoded *opp* operon of *B. burgdorferi*, three *oppA* genes (*oppA-I*, *oppA-II*, and *oppA-III*) are individually separated by substantial intergenic regions (*oppA-I* to *oppA-II*, 123 bp; *oppA-II* to *oppA-III*, 145 bp; and *oppA-III* to *oppB*, 361 bp), whereas the *oppB*, *oppC*, *oppD*, and *oppF* genes are separated by only a few base pairs (4 to 13 bp). A total of 430 bp separates *oppA-I* from the putative glycerol-3-P-*O*-acyltransferase gene that is transcribed in the reverse direction. There is a 598-bp region separating *oppA-IV* from an unknown reading frame on the plasmid cp26 and a 501-bp region separating *oppA-V* from a lipoprotein on the plasmid lp-54.

The *B. burgdorferi* genome encodes sequences for the classical, σ^{70}-type promoter as well as those for alternative sigma factors such as *rpoS* (σ^{38}) and *ntrA* (σ^{54}). An analysis of the upstream sequences of the *opp* genes for significant identity to the consensus σ^{70} binding sequences revealed classical −35 and −10 binding sequences upstream of *oppB*. However, we were unable to find both −35 and −10 σ^{70}-type sequences upstream of any *oppA* coding region. Putative −10 sequences without corresponding −35 sequences were found for several of the sequences upstream of individual *oppA* coding regions and are shown in Fig. 1. A search for binding sequences of alternative sigma factors did not reveal any sequences with >60% identity to a σ^{38}- or σ^{54}-dependent promoter region.

Cloning of *B. burgdorferi oppA* promoters into reporter plasmids. To confirm the promoter function of the upstream sequences of the *B. burgdorferi opp* genes, we cloned the sequences into the promoter-less β-galactosidase reporter plasmids pCB182 (*oppA-I*, *oppA-II*, *oppA-III*, *oppA-IV*, and *oppB*) and pCB192 (*oppA-V*). pCB182 and pCB192 differ only in the orientations of the multicloning sites upstream of the β-galactosidase gene. In addition, for *oppA-I*, *oppA-IV*, and *oppA-V*, which have large upstream intergenic regions of between 430 and 598 bp, we examined the effects of serial deletions of the upstream regions on promoter function to better localize the active sites. β-Galactosidase activity from strains of *E. coli* transformed with the various recombinant plasmids was

TABLE 2. β-Galactosidase activity for *B. burgdorferi opp* genes

Name of fragment (region)[a]	Specific activity (U)
URA1 (1–430 bp)	
P1 (1–87 bp)	331 ± 54
P1N1 (1–156 bp)	3.3 ± 0.6
P1N1P2 (1–299 bp)	249 ± 44
P1N1P2N2 (1–430 bp)	1.0 ± 0.2
N1P2N2 (71–430 bp)	87 ± 13
P2N2 (156–430 bp)	156 ± 19
N2 (275–430 bp)	11 ± 1.2
P2 (156–299 bp)	125 ± 19
URA2 (1–125 bp)	12 ± 1.2
URA3 (1–147 bp)	128 ± 7.1
URA4 (1–598 bp)	
F1F2F3 (1–598 bp)	1.6 ± 0.7
F1 (1–290 bp)	0.3 ± 0.5
F2 (290–400 bp)	8.7 ± 2.2
F2F3 (290–598 bp)	2.4 ± 0.5
URA5 (1–501 bp)	
R1R2 (1–501 bp)	29 ± 5.3
R1 (1–92 bp)	71 ± 2.6
R2 (93–501 bp)	22 ± 1.5
URB (1–362 bp)	56 ± 8.5

[a] URA1 to -5, upstream regions of *oppAI* to *-V;* URB, upstream region of *oppB.*

measured to establish the promoter activity for each upstream fragment. Results are shown in Table 2. There are marked differences in the promoter activities of the different upstream sequences, with the highest activity levels seen for *oppA-I* and *oppA-III* and minimal activity seen for *oppA-II* and *oppA-IV*.

Analysis of the serial deletions of the putative *oppA-I* promoter region revealed two separate sequences, designated P1 and P2, with high activity. Both sequences contain a putative −10 region *TATATT* for P1 and a *TATTAT* for P2, which are located at nucleotide positions 50 and 206, respectively (Fig. 1). The P1 fragment presumably functions as a promoter to control the transcription of a putative glycerol-3-P-*O*-acyltransferase gene upstream of *oppA-I* which is oriented in the reverse direction. Cloning of P1 into pCB192 confirmed that P1 possesses a strong biorientational promoter function. As P1N1 displays 100-fold-lower activity than that for P1 alone, it appears that P1 promoter function is unlikely to participate in regulating *oppA-I* gene transcription. This speculation is further supported by the finding that specific activity for N1P1 (bp 156 to 1) (data not shown) is sixfold higher than that for P1N1 (bp 1 to 156). Furthermore, P1N1P2 displays activity 75-fold higher than that for P1N1 but only twofold higher than that for P2, indicating that activity of P1N1P2 results primarily from the function of P2. The lack of significant difference (less than twofold) between the specific activities of N1P2N2 and P2N2 suggests that N1 fragments do not interfere with the function of P2.

Of note, the entire upstream region (P1N1P2N2) of *oppA-I* gene displayed markedly reduced activity compared with that of the truncated sequences. We believe this to be an artifact of the construction and not due to any inhibitory effects of the P1 region. As we will show in subsequent sections in which expression of *oppA-I* in *B. burgdorferi* is examined, the activities of the P2 sequences are more reflective of the promoter activity in *B. burgdorferi* than those revealed by our results using the entire intergenic region.

Although the sequence search in the nonsense chain of the *oppA-II* upstream region did not reveal any areas with identity to σ-binding promoter sequences, it does appear that the intergenic region between *oppA-I* and *oppA-II* has at least weak, independent promoter function (12 units), suggesting that there may be individual regulation of this gene. In contrast to the *oppA-II* promoter region, the *oppA-III* upstream region displayed a high specific activity level (128 units), suggesting that this fragment functions as a strong promoter. A perfect −10 σ^{70}-like promoter sequence (*TATAAT*) was found to be located at nucleotide position 23.

Specific activity for the *oppA-IV* upstream region located on plasmid cp26 showed the weakest activity (1.6 units). Serial deletions of the upstream sequence revealed negligible activity (0.3 unit) for the F1 fragment (1 to 299 bp), suggesting that the F1 fragment is not involved in promoter function for the *oppA-IV* gene. The F2F3 fragment (299 to 598 bp) had 2.4 units of specific activity, which is similar to the level of activity found for the intact *oppA-IV* upstream region (1.6 units). Sequence analysis revealed a putative −10 region sequence (*TATAAT*) in the F2 fragment (290 to 400 bp). Insertion of the F2 fragment into promoter-probe vector pCB182 gave F2 8.7 units of specific activity.

The promoter activity level of the *oppA-V* upstream region (1 to 501 bp) was intermediate compared with those of the other promoter regions (18-fold higher than that of *oppA-IV* and fivefold lower than that of *oppA-I*). Both the R1 fragment (1 to 92 bp) and the R2 fragment (93 to 501 bp) displayed promoter activity. The activity of the R2 fragment was comparable to the activity for the entire upstream sequence. Sequence analysis did reveal a putative −10 σ^{70}-like binding sequence (*TATATT*) located at position 432 (Fig. 1), which is close to the initiation codon of *oppA-V* gene.

Promoter activities for *oppA-1* (P1N2), *oppA-2* (URA2), *oppA-3* (URA3), *oppA-4* (URA4), and *oppA-5* (URA5) differed significantly from each other, with $P < 0.05$ by Kruskal-Wallis nonparametric analyses.

Expression of Opp genes in in vitro-grown *B. burgdorferi*. To confirm the results of our reporter assays, we examined expression of the *oppA* genes directly in *B. burgdorferi*. Quantitative RT PCR was used to determine the amount of mRNA for each *oppA* gene. The results are shown in Fig. 2A. Similar to what we found with the promoter activity in our reporter constructs, the amount of *oppA-I* transcripts in *B. burgdorferi* grown in BSK medium at 37°C was the largest, followed by those of *oppA-III* and *oppA-V* and, finally, *oppA-II*, in that order. It appeared that there was very little transcription of *oppA-IV* (over 4 logs fewer copies than that for *oppA-I*). This data was confirmed using RPAs, which showed similar trends (Fig. 2B).

To further establish that our reporter constructs reflect promoter activity in *B. burgdorferi*, we next sought to compare the effects of changes in temperature on promoter activity in the reporter constructs and on *oppA* gene expression in *B. burgdorferi*. Temperature change has been shown to be an important signal for expression of other *B. burgdorferi* genes and is an

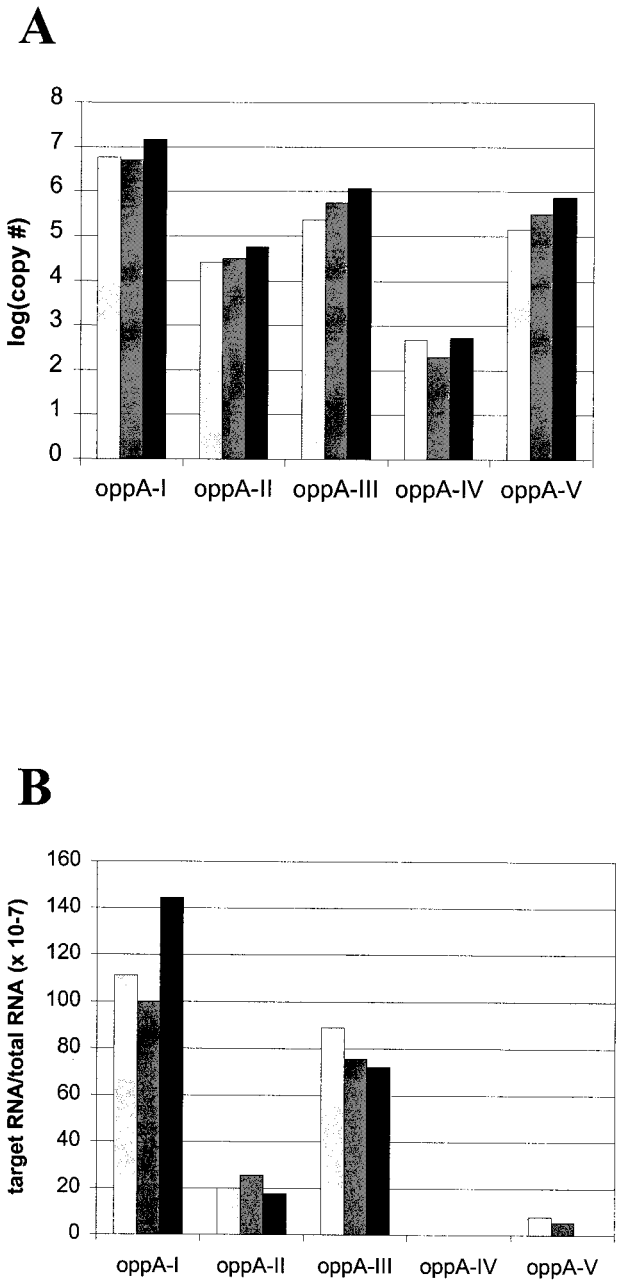

FIG. 2. Expression of *oppA* mRNA transcripts in cultured *B. burgdorferi*. Expression of the *oppA* genes was measured by RT PCR (A) and by RPA (B). For RT PCR, RNA was isolated from *B. burgdorferi* grown in BSK H medium at 37°C. cDNA was generated from total RNA, using gene-specific primers. For each sample, quantitative RT PCR for *oppA-I* to *-V* was performed. Determination of copy numbers was calculated by comparison to individual standard curves generated for each primer set. Panel A shows the results of three separate experiments. For RPA, total RNA was hybridized to biotinylated probes specific for each *oppA* gene and then digested with RNase A-RNase T_1. Samples were subjected to electrophoresis in a polyacrylamide gel and transferred to nylon membranes. Detection of biotinylated probes was done using chemiluminescence, and quantitation was performed by scanning densitometry. Data shown are individual results from three separate experiments.

important environmental variable in the life cycle of *B. burgdorferi* as it moves from its tick to mammalian hosts. Bono et al. (3) have previously shown that only *oppA-V* expression is significantly affected by temperature changes. Using real-time quantitative PCR technology, we confirmed these findings (Fig. 3). There were only small changes (less than fivefold) in the levels of mRNA transcripts for *oppA-I*, *oppA-II*, *oppA-III*, and *oppA-IV* between organisms grown at 25°C and those grown at 37°C. *oppA-V* showed a large increase (32-fold) in mRNA transcripts at 37°C versus 25°C.

We next examined the effects of temperature on our promoter constructs in *E. coli* (Table 3). Bacteria containing the reporter plasmids for the upstream regions of each of the five *oppA* genes and the region upstream of *oppB* were incubated at 25°C and 37°C and grown to mid-logarithmic phase. Prior to performing the experiments, bacterial growth curves for each strain at each temperature were established to ensure that samples were taken at the same growth stage. There was no significant difference in plasmid copy numbers in *E. coli* grown at the two temperatures, as measured by real-time PCR using total DNA as the template and primers for the plasmid and a host chromosomal gene. All of the reporter constructs showed statistically significant increases in activity at 37°C versus 25°C. For the *oppA-I*, *oppA-II*, *oppA-III*, and *oppA-IV* promoters, the increase was in the range of 3.1- to 3.6-fold. Of the *oppA* promoters, only the reporter plasmid for the *oppA-V* promoter region showed an increase in β-galactosidase activity greater than 5-fold (5.5-fold) when bacteria were grown at 37°C versus 25°C.

Effects of nutritional changes in the environment on activity of *oppA* promoters. Having confirmed that our reporter constructs in *E. coli* appear to reflect promoter activity in *B. burgdorferi*, we next sought to use these constructs to test the effects of various environmental changes on promoter activity for the *oppA* and *oppBCDF* genes. Because *B. burgdorferi* does not grow in defined media, many of these manipulations would be difficult or impossible to carry out directly in *B. burgdorferi*. Since the Opp systems of other bacteria play a major role in nutritional uptake of oligopeptides and may be regulated as part of a "feast or famine" response, we sought to determine whether specific changes in nutrient availability could change *opp* gene expression.

We first examined the effects of variations in the carbon and nitrogen availability in the culture medium on *oppA* promoter activity. The bacterial cells containing the various reporter plasmids were grown at 37°C in minimal medium, with a wide range of concentrations of D-glucose or NH_4Cl. Again, bacterial growth curves in each medium were monitored to ensure that measurements were taken at comparable growth phases. All bacterial cells used in β-galactosidase assays were grown to the mid-logarithm phase.

The promoter activity for the upstream regions of *oppA-II* and *oppA-IV* remained fairly stable over a wide range of glucose concentrations (0.05 to 1 mM). In contrast, promoter activity for *oppA-I*, *oppA-III*, and *oppA-V* showed steady increases with increasing glucose concentration; however, none of these increases were greater than twofold (Fig. 4A).

Promoter activities for the *oppA-I* and *oppB* upstream regions showed increases of approximately threefold, with increases in NH_4Cl concentration from 0.2 mM to 20 mM in the

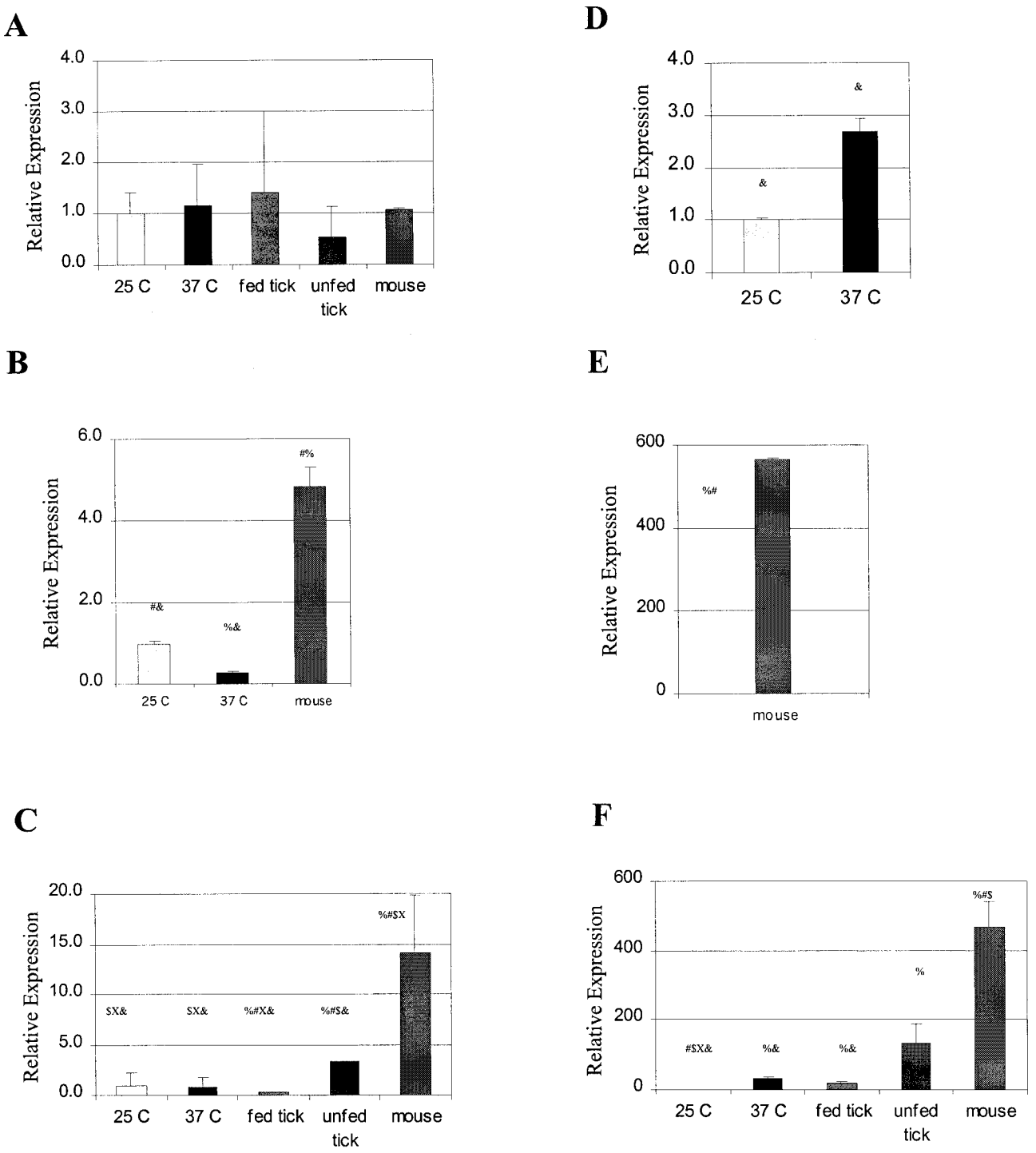

FIG. 3. Expression of *oppA* mRNA transcripts under various environmental conditions. Total RNA was isolated from in vitro-grown organisms at 25°C and 37°C or from fed and unfed *Ixodes* ticks. cDNA to total RNA was generated using random hexamers. Expression of mRNA for each individual *oppA* was examined by quantitative real-time RT PCR. Expression levels of *oppA-I* (A), *oppA-II* (B), *oppA-III* (C), *oppA-IV* (D), and *oppA-V* (E and F) are shown relative to that of expression of the gene in in vitro-grown organisms at 25°C, which was assigned a relative value of 1.0. Data shown are averages from two to three experiments performed in duplicate. Error bars represent standard errors of the means. Statistical significance was calculated using the Kruskal-Wallis method. %, significant ($P < 0.05$) differences in expression level compared with samples grown at 25°C; #, significant differences in expression level compared with samples grown at 37°C; $, significant differences in gene expression level in fed ticks; X, significant differences in gene expression level in unfed ticks; &, significant differences from expression levels in mouse tissue. Note that the scale changes for each graph. Data for *oppA-IV* are split into two graphs (panels E and F) due to the wide range of relative expression.

TABLE 3. Effect of temperature on *E. coli* promoter constructs

Region (DNA fragment)[a]	β-Galactosidase activity[b]		Ratio (37°C/25°C)	*P* value
	25°C	37°C		
URB	15 ± 1.4	66.4 ± 3.6	4.4	<0.05
URA1 (P1N1N2)	50.4 ± 4.1	155 ± 23.4	3.1	<0.05
URA2	3.9 ± 0.6	13.1 ± 0.6	3.4	<0.05
URA3	39 ± 3.2	133 ± 6.2	3.4	<0.05
URA4 (F1F2F3)	0.5 ± 0.2	1.8 ± 0.7	3.6	<0.05
URA5 (R1R2)	6.3 ± 0.3	34.4 ± 7.3	5.5	<0.05

[a] URB, upstream region of *oppB;* URA1 to -5, upstream regions of *oppAI* to *-V*.
[b] Miller units.

medium (Fig. 4B), suggesting a role for nitrogen availability in regulation of expression of these Opp proteins. Promoter activities for the other *oppA* genes showed minor increases in activity between 0.2 mM and 2 mM NH_4Cl that may be due to cell viability at the extremes of nitrogen limitation. Only *oppA-I* promoter activity continued to increase with increases in NH_4Cl concentration to 20 mM.

Regulation of Opp expression in other bacteria has been reported to be affected by phosphate concentration and leucine concentration (2, 19). However, we did not observe any changes in promoter activity in our constructs with changes in phosphate concentration (70 μM to 2 mM) or in leucine concentration (data not shown). Another environmental factor which has been shown to affect expression of other *B. burgdorferi* proteins is change in pH. Changes in pH are also physiologically relevant, as the bacteria must adapt to changes in pH as it moves from its tick to mammalian hosts. We did not observe any change in promoter activity for any of the *oppA* or *oppB* promoters with changes in pH over a range of 3.0 to 8.5 (data not shown).

Expression of *opp* genes in tick and mouse hosts. We next chose to examine expression of *oppA* mRNA transcripts by *B. burgdorferi* in its tick host. *B. burgdorferi* residing within a tick must adapt to vast changes in nutrient ability during periods between blood meals. We examined gene expression from *B. burgdorferi* in nymphal *Ixodes* ticks in the unfed state and after allowing them to feed on an uninfected mouse for 60 h (Fig. 3).

Expression of *oppA-II* and *oppA-IV* mRNA was below the limit of reliable detection in both fed and unfed ticks (less than 150 copies determined, using known quantities of spiked DNA). *oppA-I* expression showed a trend to increased expression in the fed tick, but this was not significant. Overall, expression of *oppA-I* in either fed or unfed ticks did not differ significantly from *oppA-I* expression in in vitro-grown organisms relative to expression of *recA*. In contrast, expression of both *oppA-III* and *oppA-V* was significantly increased in the unfed ticks compared to the fed ticks (9.5- and 7-fold, respectively). *oppA-V* expression in both the fed and unfed ticks was significantly higher than that in in vitro-grown organisms at 25°C (18- and 32-fold, respectively). Expression of *oppA-V* in the fed tick approximates expression of *oppA-V* from in vitro-grown organisms at 37°C (a less than twofold difference) but is fourfold less than the expression seen in unfed ticks.

We also examined expression of the *oppA* genes from organisms in infected mice. Once again, expression of *oppA-I* remained relatively unchanged between organisms isolated from in vitro cultures, unfed and fed ticks, and mouse tissue. Expression of *oppA-II* was moderately increased in mouse tissue (approximately fivefold) compared with in vitro-grown organisms and significantly higher than in either fed or unfed ticks, where expression was not detected. Both *oppA-III* and *oppA-V* expression levels were significantly increased in mouse tissue compared with that seen for either fed or unfed ticks. Expression of *oppA-V* relative to *recA* was almost 500-fold higher in mouse tissue than that seen for cultured organisms grown at 25°C and 25- and 3.5-fold higher than that seen for fed and unfed ticks, respectively.

However, the most significant change in expression was seen with *oppA-IV.* Expression of *oppA-IV*, which was undetectable in organisms isolated from ticks, was increased more than 500-fold compared to expression in cultured organisms.

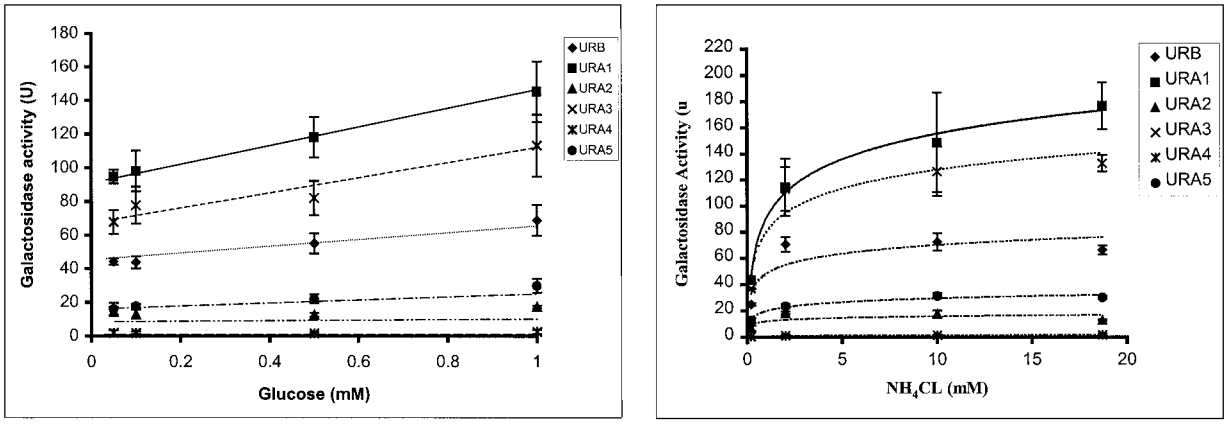

FIG. 4. Effects of glucose and NH_4Cl concentrations on *opp* promoter activity. Bacteria containing plasmid reporter constructs for each of the *oppA* promoter regions were grown at 37°C in minimal medium supplemented with 100 μg of ampicillin/ml, 0.2 mM L-leucine, and different glucose (A) and NH_4Cl (B) concentrations. Specific activity (U) was calculated from three experiments performed in duplicate. Error bars represent standard errors of the means. URA1, upstream region of *oppA-I*; URA2, upstream region of *oppA-II*; URA3, upstream region of *oppA-III*; URA4, upstream region of *oppA-IV*; URA5, upstream region of *oppA-V*; URAB, upstream region of *oppB*.

DISCUSSION

Sequence analysis of the upstream regions of *B. burgdorferi oppA-I*, *oppA-II*, *oppA-III*, *oppA-IV*, and *oppA-V* as well as *oppB* revealed little similarity, suggesting that expression of each gene is regulated by an independent mechanism(s). The data presented in this paper confirm that there are marked differences in the levels of expression of the various *opp* genes and that each may be subject to a different regulatory mechanism(s).

Our β-galactosidase reporter assays demonstrate that the upstream regions *of oppA-I*, *oppA-II*, and *oppA-III* all possess promoter activity. This represents further evidence to support the hypothesis that transcription can initiate at each *oppA* gene. Bono et al. have previously shown that the chromosomally encoded *oppA* genes, *oppA-I*, *oppA-II*, and *oppA-III*, may be transcribed as mono-, di,- or tricistronic messages (3). Based on our data, it appears that transcription of the chromosomally encoded *oppA* genes is initiated more frequently by the individual promoters rather than proceeding from the *oppA-I* promoter. If the majority of transcription of *oppA-I*, *oppA-II*, and *oppA-III* occurred through the activity of the *oppA-I* promoter, it would be expected that transcripts of the three genes would be equal or, if early termination occurred, that the amount of transcription would be largest for *oppA-I*, followed by those of *oppA-II* and, finally, *oppA-III*. On the other hand, if bicistronic messages originated from the *oppA-II* promoter, higher levels of *oppA-II* and *oppA-III* would be expected. However, in our studies of *oppA* transcription from in vitro-grown organisms, we have found that the amount of *oppA-II* transcription is almost 20-fold less than of that of *oppA-III* and over 200-fold less than that of *oppA-I*, as determined by RT PCR. This strongly suggests that transcription of *oppA-I* gene can be terminated before processing of *oppA-II* and that the majority of *oppA-III* transcription occurs through the activity of its own promoter.

After confirming that promoter activity measured in our *E. coli* reporter systems was consistent with the levels of transcription seen in *B. burgdorferi* under comparable conditions, we used this system to test the effects of various environmental changes. While to examine promoter activity in a heterologous system using a different host organism is certainly not optimal, the inability of *B. burgdorferi* to grow in defined media severely limits the ability to test the effects of these conditions on *B. burgdorferi* directly. Although we believe that the *E. coli* reporter system is reflecting activity of the *B. burgdorferi opp* promoters and have found this to be the case in some of the direct comparisons that are possible, it would be impossible to establish this for every condition. However, almost as important as the individual changes in promoter activity we described is the understanding that each of the promoter regions can respond differentially to the same environmental condition. For example, *oppA-V* expression is most affected by changes in temperature while *oppA-I* expression is the most affected by nitrogen concentration.

The ability to differentially regulate expression of its *oppA* genes could potentially provide *B. burgdorferi* with a mechanism for environmental adaptation. The organism is exposed to vast environmental changes as it moves from its tick and mammalian hosts. *B. burgdorferi* bacteria are acquired by larval ticks taking a blood meal from infected animals (4). The ticks do not take another meal until they molt into the nymphal stage months later. At the time of the next blood meal, when nutrients again become plentiful, the bacteria in the midgut of the organism begin to multiply rapidly and subsequently can infect the animal on which the tick is feeding. After the meal is complete, within days the number of organisms drops precipitously and remains low until the next blood meal. Narasimhan et al. have previously found that genes in the *opp* operon were differentially expressed in fed and unfed ticks but did not identify specific genes within the operon that accounted for the differences (13). We have found that expression of *oppA-II* and *oppA-IV* remains very low in the tick hosts and that *oppA-III* and *oppA-V* are up-regulated during periods of stable nutrient availability (unfed tick and mouse hosts). Expression of *oppA-II* appears to be specifically down-regulated in organisms growing in ticks compared with the activity of its promoter and expression in in vitro-grown organisms. In contrast, despite very weak promoter activity in vitro, *oppA-IV* undergoes tremendous up-regulation in organisms in the mouse host. The signals regulating these changes have not been identified and are likely to be complex. For example, it appears that factors other than temperature play a major role in determining *oppA-V* expression in fed and unfed ticks, since increases in temperature up-regulate *oppA-V* expression but expression in unfed ticks is higher than that in fed ticks. Increased nutrient availability appeared to play a role only for *oppA-I*, which was the only gene to show substantially increased promoter activity with increases in nitrogen availability.

Transport of specific peptides has been shown to mediate important bacterial adaptations such as chemotaxis, quorum sensing, and the feast or famine response (1, 8, 9, 11). It is tempting to speculate that the different OppA proteins are involved in separate adaptive functions. For example, could it be the case that OppA-I, which has the highest level of basal expression and which showed the greatest responsiveness to changes in nutrient availability, is involved in peptide transport for basic nutritional needs while OppA-4, which is expressed in high levels only in the mouse host, is involved in a more specific function such as peptide chemotaxis? Another possibility is that differences in expression and substrate specificity of the OppA proteins have evolved to allow the organism to adapt to different environmental niches, where the supplies of specific peptides can vary. Further studies of the conditions affecting expression of the individual OppA proteins as well as the substrate specificities of each protein will be necessary to achieve a better understanding of the role of each of the OppA proteins of this sole peptide transporter of *B. burgdorferi.*

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