Interaction of *Borrelia burgdorferi* Hbb with the p66 promoter

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ABSTRACT

*Borrelia burgdorferi*, an agent of Lyme disease, encodes the β3-chain integrin ligand P66. P66 is expressed by *B. burgdorferi* in the mammal, in laboratory media, and as the bacteria are acquired or transmitted by the tick, but is not expressed by the bacterium in unfed ticks. Attempts to reveal factors influencing expression revealed that P66 was expressed in all *in vitro* conditions investigated. Candidate regulators identified in a search of the *B. burgdorferi* genome for homologs to other bacterial transcription factors were cloned and introduced into *E. coli* carrying a p66 promoter-signal sequence-phoA (alkaline phosphatase, or AP) fusion. Three candidate transcription factors—two that decreased AP activity (Hbb and BB0527), and one that increased AP activity (BBA23)—were identified. BBA23 and BB0527 did not bind to the p66 promoter at physiologically relevant concentrations. In contrast, several promoter fragments, including p66, were bound by Hbb (BB0232), with slightly different affinities. Consistent with results from other laboratories, Hbb appears to recognize multiple DNA sequences. Changes in the expression of p66 and bb0232 in the tick at various points with respect to feeding on mice, along with the results of the reporter experiment in the surrogate host *E. coli*, are consistent with Hbb/BB0232 being involved in regulating p66 expression.

INTRODUCTION

Lyme disease is the most prevalent arthropod-borne disease in the USA (1,2). The causative agent, *Borrelia burgdorferi*, migrates between vertebrate hosts and the tick vector. The relatively small genome size of *B. burgdorferi* belies the complex regulation one would expect to be necessary for living in these different environments. Only three sigma (σ) factors have been identified in *B. burgdorferi*, relatively few when compared with the larger numbers found in other organisms. *Borrelia burgdorferi* encodes *rpoD*, the sigma 70 (σ70) subunit, in addition to *rpoS* (σ8) and *rpoN* (σ54) homologs. Typically, RpoD homologs are involved with housekeeping functions, whereas RpoS controls gene expression in stationary phase or the stress response, and RpoN is often involved in responses to nitrogen limitation or other stresses. *Borrelia burgdorferi* differs significantly from the classical patterns, in that RpoS, RpoN and the response regulator Rrp2 participate in a regulatory pathway that is critical to how *B. burgdorferi* responds to certain environmental changes (3–10).

A number of laboratories have investigated *in vitro* culture methods thought to model the differences between the unfed tick, the feeding tick and the mammal. The pH of the unfed tick midgut is slightly alkaline (11), and the temperature is that of the tick’s surroundings. However, as the tick begins to take its blood meal, the pH in the midgut drops to ~6.8 (11), and the temperature is near the surface temperature of the mammal, between 34°C and 37°C (11,12). Temperature, growth phase or cell density, pH, oxygen concentration, the addition of blood to the medium, migration between the vertebrate and tick environments, and time and tissue localization within a mammal have been shown to affect the expression of many genes in *B. burgdorferi* (11,13–26). Other genes affected by environmental cues were identified using dialysis membrane chambers (DMCs) implanted in rats (27,28), which at least partially allow for adaptation to the mammalian host by *B. burgdorferi*.

P66 is a *B. burgdorferi* surface-exposed outer membrane protein and a ligand for the β3-chain integrins (29–32). P66 is commonly recognized by Lyme disease patient sera (33,34), demonstrating that the protein is produced when the bacteria are in a mammalian host. Mapping of
the transcriptional start site revealed that p66 expression is apparently under the control of a $\sigma^{70}$-dependent promoter (29). This is corroborated by the fact that its expression was not changed in the rpoS or rpoN deletion mutants used for microarray experiments (6,8,35).

Indirect immunofluorescence studies showed that P66 protein levels do, however, vary at different stages in the tick life cycle of the tick (36). As compared with flagellin expression, P66 is not produced by B. burgdorferi in the midguts of unfed ticks, but as the ticks take their blood meal, P66 is expressed by a majority of B. burgdorferi (36). At 7 days post-repletion, P66 is expressed by only a fraction of the B. burgdorferi cells, indicating that the production of P66 is tied to the presence of the mammalian blood meal.

In similar studies, OspC undergoes a sharp peak of production coincident with transmission from the tick to the mammal (37). OspA is produced in the midgut of the uncloned B. burgdorferi strain B31 by artificial infection (38). Infected larvae and nymphs were inoculated from frozen stocks (passage 4 for N40 or passage 5 B31) into fresh medium, and grown to late exponential phase (unless otherwise specified). Bacteria were then subcultured 1:200 into fresh medium and grown again to late exponential phase (unless otherwise specified). Bacterial cell densities were determined by dark-field microscopy. Cultures were then divided so that 7 ml of culture was harvested for SDS-PAGE and immunoblot analysis, and 3 ml of culture was harvested for RNA analysis. The bacteria were then pelleted, washed in 1 ml PBS (phosphate-buffered saline) plus 0.2% BSA (bovine serum albumin), then again in 200 μl PBS. The pellets were resuspended in Trizol (Invitrogen, Carlsbad, CA) for subsequent RNA extraction, or in HEPES-buffered saline plus protease inhibitors [25 mM HEPES pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.1 TIU/ml aprotinin, 1 mM benzamidine, 10 μM pepstatin A and 1 mM PMSF (Sigma-Aldrich Co., St. Louis, MO)] for analysis of protein expression and stored at −70°C. In total, $5 \times 10^7$ B. burgdorferi were loaded per lane and SDS-PAGE and immunoblotting were performed according to standard protocols (42,43) (data not shown).

### MATERIALS AND METHODS

**Infection, feeding and collection of ticks**

*IXODES SCAPULARIS* larvae were infected with *B. burgdorferi* strain B31 by artificial infection (38). Infected larvae and control, uninfected larvae were fed separately on Rocky Mountain Laboratories (RML) mice, an outbred strain of Swiss–Webster mice, and allowed to molt to nymphs. Nymphs were held in the unfed state, or were fed on naïve mice and collected when replete. At unfed, replete and 9 days post-repletion time points, midguts of 15, 10 and 10 ticks, respectively, were dissected and stored in water at −80°C until RNA extraction. Animal experiments were performed under protocols approved by the RML Animal Care and Use Committee, prepared according to National Institutes of Health guidelines. The RML Animal Facility is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care.

**Bacterial strains and culture conditions**

*Escherichia coli* strains JM109 and BLR were used for cloning of genes; CC118 and CC118(DE3)pLysS were used for Alkaline Phosphatase (AP) assays, and BL21(DE3)pLysS and Rosetta-gami2(DE3)pLysS were used for protein expression and purification. Proteins expressed in and purified from the Rosetta-gami2(DE3)pLysS strain were used for electrophoretic mobility shift assays (EMSAs). Strain genotypes are provided in Table 1.

| Strain       | Genotype                                                                 | Source or reference          |
|--------------|---------------------------------------------------------------------------|------------------------------|
| JM109        | F' traD36 proA+ B+ lacq Δ(lacZ)M15/Δ (lac-proAB) ghtV44 c14- gyrA96 recA1 relA1 | Promega                      |
| BLR          | F' ompT gal dcm lon hsdS(r-g m-c) araB::T7RNAP-iptA recA-                  | Novagen                      |
| CC118        | araD139 (ara-leu)797 ΔlacX74 galE galK ΔphoA20 thi rpsE rpoB argE(Em) recA1 | A. Wright (45)               |
| CC118(DE3)pLysS | CC118 plus Δ(Δ3) pLysS(cm*)                                          | this study                    |
| BL21(DE3)pLysS | F' ompT gal dcm lon hsdS(r-g m-c) Δ(Δ3) pLysS(cm*)                          | Novagen                      |
| Rosetta-gami2(DE3)pLysS | Δ(ara-leu)7697 ΔlacX74 AphoA PruH phoR araD139 ahpC galE galK rpsL (DE3) | Novagen                      |

| Strain       | Genotype                                                                 | Source or reference          |
|--------------|---------------------------------------------------------------------------|------------------------------|
| JM109        | F' traD36 proA+ B+ lacq Δ(lacZ)M15/Δ (lac-proAB) ghtV44 c14- gyrA96 recA1 relA1 | Promega                      |
| BLR          | F' ompT gal dcm lon hsdS(r-g m-c) araB::T7RNAP-iptA recA-                  | Novagen                      |
| CC118        | araD139 (ara-leu)797 ΔlacX74 galE galK ΔphoA20 thi rpsE rpoB argE(Em) recA1 | A. Wright (45)               |
| CC118(DE3)pLysS | CC118 plus Δ(Δ3) pLysS(cm*)                                          | this study                    |
| BL21(DE3)pLysS | F' ompT gal dcm lon hsdS(r-g m-c) Δ(Δ3) pLysS(cm*)                          | Novagen                      |
| Rosetta-gami2(DE3)pLysS | Δ(ara-leu)7697 ΔlacX74 AphoA PruH phoR araD139 ahpC galE galK rpsL (DE3) | Novagen                      |

**Escherichia coli** strains JM109 and BLR were used for cloning of genes; CC118 and CC118(DE3)pLysS were used for Alkaline Phosphatase (AP) assays, and BL21(DE3)pLysS and Rosetta-gami2(DE3)pLysS were used for protein expression and purification. Proteins expressed in and purified from the Rosetta-gami2(DE3)pLysS strain were used for electrophoretic mobility shift assays (EMSAs). Strain genotypes are provided in Table 1.

**Borrelia burgdorferi** strains N40 (clone D10E9) and B31 (uncloned) were used for all pH, temperature, growth phase, mammalian components and tick cell culture medium experiments. *Borrelia burgdorferi* were grown in either BSK-II (39) or MKP (40) medium. For some in vitro tests of how the manipulations of culture conditions affect p66 expression, BSK-H (Sigma-Aldrich, St. Louis, MO) was also used. Tick cell culture medium (41) was used for some experiments.

*Borrelia* were inoculated from frozen stocks (passage 4 for N40 or passage 5 B31) into fresh medium, and grown to late exponential phase (~$1 \times 10^8$ bacteria/ml). Bacteria were then subcultured 1:200 into fresh medium and grown again to late exponential phase (unless otherwise specified). Bacterial cell densities were determined by dark-field microscopy. Cultures were then divided so that 7 ml of culture was harvested for SDS-PAGE and immunoblot analysis, and 3 ml of culture was harvested for RNA analysis. The bacteria were then pelleted, washed in 1 ml PBS (phosphate-buffered saline) plus 0.2% BSA (bovine serum albumin), then again in 200 μl PBS. The pellets were resuspended in Trizol (Invitrogen, Carlsbad, CA) for subsequent RNA extraction, or in HEPES-buffered saline plus protease inhibitors [25 mM HEPES pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.1 TIU/ml aprotinin, 1 mM benzamidine, 10 μM pepstatin A and 1 mM PMSF (Sigma-Aldrich Co., St. Louis, MO)] for analysis of protein expression and stored at −70°C. In total, $5 \times 10^7$ *B. burgdorferi* were loaded per lane and SDS-PAGE and immunoblotting were performed according to standard protocols (42,43) (data not shown).
Table 2. Primers used for qRT–PCR

| Target | Forward primer, 5’–3’ | Reverse primer, 5’–3’ | Limit of detection, copy number of genomes |
|--------|------------------------|------------------------|------------------------------------------|
| flaA (bb0668) | aaagttcaacaggttcaaaaagc | gatttcctagtttttcacgctc | <15 |
| flaB (bb0147) | aaggcactattcaggttcacagaggg | aagacggctagttttgcacgctc | 150 |
| ϕ66-MM (bb0603) | atttaaaagcacttactatggattcctcgttttgatcaagtagattttttattggg | tcgcctgtgtagttgctttgg | 150 |
| ϕ66-CC (bb0668) | tgaacaaagttcaacaagcatacagaaataa | tggcttgtgtagttgctttgg | 150 |
| ospA (bba15) | p66-MM (bb0603) | aagacggctagttttgcacgctc | 150 |
| ospC (bb019) | agaactctacagttcaaaagagcagattgactttcataagtagattttttattggg | tacaagagttgtagctgcaagctc | 150 |
| hbb (bb0232) | tgaagactctacagttcaaaagagcagattgactttcataagtagattttttattggg | atgattctgtagttttgcacgctc | 150 |

Primers were designed and tested by PCR, then by conventional RT–PCR prior to use in qRT–PCR.

Analysis of mRNA levels

RNA from ticks or cultured B. burgdorferi samples was purified using the Trizol fractionation method according to the manufacturer’s instructions, then treated with RNase-free DNase and repurified using the RNeasy kit (Qiagen, Valencia, CA). First-strand synthesis was performed using Superscript II RT (Gibco, Carlsbad, CA) and gene-specific reverse primers listed in Table 2. Each cDNA sample was then amplified by either conventional polymerase chain reaction (PCR) or quantitative PCR using gene-specific primers as listed in Table 2. All primers were checked against the NCBI database for potential homology to and amplification from the other genomes that had been sequenced, and were tested for sensitivity using known quantities of DNA purified from cultured B. burgdorferi. Conventional RT–PCR products were analyzed by electrophoresis through 10% acrylamide gels in Tris–borate–EDTA buffer. Quantitative RT–PCR (qRT–PCR) samples were amplified by use of the SYBR Green master mix (Qiagen, Valencia, CA) and either the ABI Prism 7700 sequence detection system (PE Biosystems/Applied Biosystems Inc., Foster City, CA) or the Stratagene MxPro 3000P system (Stratagene, La Jolla, CA). Melt point analysis was performed to assess homogeneity of the amplified product. All samples were amplified in parallel with standard curves using known quantities of purified genomic DNA from B. burgdorferi using the same primer sets in the same 96-well plates. Copy numbers of the target sequence cDNAs in the tick samples were determined from the Ct values of triplicate samples using the standard curves from the same reaction plate. Each mRNA preparation was analyzed by performing at least two cDNA synthesis reactions and subsequent qRT–PCR reactions. The data shown for the tick experiments are from one of two separate cohorts of ticks (biological replicates) that showed the same trends, although the absolute numbers of mRNA copies differed between the cohorts.

Production, purification, and analysis of recombinant candidate regulators

BL21(DE3)pLysS or Rosetta-gami2(DE3)pLysS E. coli strains containing the recombinant plasmids were grown in LB (Luria–Burtani) medium plus 30 μg/ml chloramphenicol and 50 μg/ml kanamycin overnight at 30°C. Five milliliters of overnight culture was added to 500 ml 2XYT (Yeast Extract Tryptone) medium containing 0.2% dextrose, chloramphenicol and kanamycin and grown to mid-exponential phase (OD<sub>600</sub> 0.4–0.6) at 30°C. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to cultures to a final concentration of 1 mM for induction of protein production, after which cultures were aerated for 1–2 h at 250 rpm and 30°C. Cells were harvested, chilled on ice for 30 min, and centrifuged at 4230×g for 10 min at 4°C. Pellets were resuspended in 20 ml HEPES-buffered saline, and centrifuged at 4230×g for 10 min at 4°C. The pellets were stored at −70°C. Pellets were lysed in a French pressure cell, in the presence of protease inhibitors (0.1 TIU/ml aprotinin, 1 mM benzamidine, 10 μM pepstatin A and 1 mM PMSF, 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, Gibbstown, NJ) were used to purify the recombinant His-6 tagged proteins from the supernatants. The purification was performed as per the manufacturer’s instructions, except that protease inhibitors (0.1 TIU/ml aprotinin, 1 mM benzamidine and 10 μM pepstatin A) were added to the binding and elution buffers, and 10% glycerol was added to the elution buffer. Protein purity was assessed by SDS–PAGE (42) (data not shown). Protein concentrations were determined for each sample using both the Bradford (BioRad, Hercules, CA) and BCA (Pierce, Rockford, IL) reagents. The tags were not cleaved from the recombinant proteins to allow detection throughout the experiments.
Table 3. Oligonucleotides used for cloning and sequencing

| Target, purpose | Genome coordinates | Forward primer, 5′–3′ | Reverse primer, 5′–3′ |
|-----------------|--------------------|------------------------|----------------------|
| bba23 cl,seq⁴   | 15303–15886        | tagggattaccaataatttgattgaataatttgaaatatatcaaacaggcaaaggtaatgttaggtaataagataggtggtggtggtggtggtggtcattgtaaacgctctctggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattct ggactcaggtctggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtctgtggagttgtaaattctggactcaggtc...
dried and exposed to Maximum Sensitivity (MS) X-ray film (Kodak, Rochester, NY) with an intensifier screen.

**RESULTS**

Expression of p66 in different laboratory culture conditions

We first attempted to mimic the conditions of the unfed tick midgut versus the fed tick midgut *in vitro* with regard to p66 expression by growing *B. burgdorferi* in BSK and MKP media under conditions in which we varied the temperature, starting pH and growth phase at which the cultures were harvested. Medium to which the mammalian-derived gelatin, serum and BSA had not been added was also tested. As previously reported, no significant differences in P66 were seen at the protein level, while at the mRNA level a maximum decrease of 10-fold was observed in level, while at the mRNA level a maximum decrease of significant differences in P66 were seen at the protein level. Medium to which the mammalian-derived components, and in fact do not reliably mimic the midgut of the unfed tick were identified with regard to P66 production. Moreover, the bacteria do not survive for more than a few days in medium devoid of the mammalian-derived components, and in fact do not replicate more than once, so changes in the mRNA level that are seen may not be reflected at the protein level if the protein turnover rate is slow. These results suggest, however, that P66 levels may be regulated at both the transcriptional and post-transcriptional levels. We therefore determined whether p66 mRNA levels vary in the tick with feeding status.

Assessment of p66 mRNA levels in the tick midgut

To determine whether changes at the protein level were reflected at the mRNA level, we examined six sets of tick samples: unfed, replete (‘drop-off’), or 9 days post-repletion samples from ticks that were either unfected or infected with *B. burgdorferi*. The time course differed slightly from the samples used in the immunofluorescence studies (36), but still included the conditions we were interested in, namely the absence or presence of the mammalian blood meal. qRT–PCR analysis of these samples examined expression of p66, flaA, ospA and ospC. Flagellar expression is used in *B. burgdorferi* as a control and a marker for spirochete presence, as flagellar operons are thought to be constitutively expressed in *B. burgdorferi*. Attempts to perform qRT–PCR using primers against the *B. burgdorferi* 16S rRNA proved unsuccessful due to high levels of background, even in the uninfected tick midgut samples, presumably due to the presence of other bacteria. OspA and OspC have long been considered hallmarks of differential expression during the tick-mammal cycle (47), and we examined their expression as controls.

For qRT–PCR, expression within the tick midguts was compared with standard curves of known concentrations of serial dilutions of *B. burgdorferi* genomic DNA. In this way, we were able to calculate limits of detection for each set of primers used (Table 1). We also used the BLAST program (http://www.ncbi.nlm.nih.gov/blast/) (48) to ensure that there were no obvious sequences related to the primers we designed in other organisms.

There is a clear increase in the expression of all genes, including flaA, in both the replete and the 9 days post-repletion samples. This likely reflects both an increase in the number of *B. burgdorferi* cells and transcriptional activity of the bacteria, so data for each gene are shown normalized to the flaA transcript (Figure 1). By immunofluorescence intensity, flagellar expression appears consistent in all infected tick samples, and was not seen in uninfected tick midguts (36). By qRT–PCR, p66 (bb0603) is not detectable above background in the infected, unfed ticks, indicating that P66 protein is not simply being degraded in the tick midgut (Figure 1). p66 is expressed to higher levels at repletion, and its expression is highest at 9 days. P66 protein expression was not detected in unfed tick midguts, was highest in the midguts of ticks at repletion, and was still highly expressed at 7 days post repletion (36). However, the expression of P66 at 16 days post-repletion was significantly decreased in the immunofluorescence studies. While the time points analyzed in the two experiments differ, p66 expression still increases dramatically in the presence of the mammalian blood meal by both immunofluorescence and qRT–PCR [this study, (36)]. At the mRNA level, the increase in p66 expression is greater than that of flaA expression following the tick blood meal.

The qRT–PCR data for ospA show that little transcript is detected in the unfed, infected tick, more is detected in the replete tick, and still more is found in the post-repletion tick midgut (Figure 1). These are the only results that are somewhat inconsistent with what was seen at the protein level (37,47). OspA by immunofluorescence is expressed to high levels in unfed tick midguts, and to slightly lower levels at repletion and
13 days post-repletion. However, the *ospA* qRT–PCR results may indicate transcriptional changes occurring that are not directly reflected at the protein level. The relatively quiescent bacteria in the unfed tick midgut may not turn over OspA protein rapidly, and again our results are consistent with complex regulation of gene expression versus protein production for at least some *B. burgdorferi* genes. For *ospC*, the qRT–PCR results are most directly consistent with the immunofluorescence data (Figure 1) (37,47). By qRT–PCR, there is a sharp peak in *ospC* expression in the infected, replete ticks. Expression is highest at repletion, lower at 9 days post-repletion, and lowest in unfed tick midguts. Based on the immunofluorescence studies, OspC is expressed to high levels briefly before repletion during tick feeding, but not at the other time points (37,47).

The qRT–PCR results are consistent with the immunofluorescence data in that transcription of *p66* increases dramatically when the blood meal is taken. The increases in transcript levels of all genes are likely to reflect increases in bacterial numbers and metabolic activity as well as gene-specific increases in expression levels, but the increases in bacterial numbers and metabolic activity are most likely reflected by the changes in the *flaA* mRNA levels. Although the expression of *flaA* and *p66* both increased in the fed versus unfed tick environments, the fold change for *p66* was much greater than that for *flaA* (Figure 1), suggesting the possibility that *p66* expression may be repressed in the unfed tick or activated in the fed tick. In addition, it is clear that *p66* production patterns at the protein level are distinct from those of *flaA*, *ospA* and *ospC*, despite the similarity of the *p66* and *ospA* mRNA expression patterns. These results suggest that there are additional specific factors encoded in the *B. burgdorferi* genome that may bind to and regulate the expression of the *p66* promoter, and possibly, expression of the gene at the post-transcriptional level.

**Identification of candidate transcriptional regulators**

The published *B. burgdorferi* strain B31 genome was surveyed for candidate regulators based on several parameters. First, we looked for orthologs of known transcriptional regulators in other bacteria, including the five main families of transcription factors (*araC/xylS, argR, lacI, lysR* and *ompR* families). We also looked for homologs of proteins outside the five major transcription factor families that have been identified as participating in transcriptional regulation. Finally, we looked for proteins predicted to contain DNA-binding domains such as helix-turn-helix (HTH) or helix-loop-helix motifs. We identified 14 potential transcription factors in the *B. burgdorferi* genome and further evaluated nine of these candidates (Table 5), only one of which, Hbb, had been characterized at the start of this investigation. Although we were able to amplify the remaining five genes by PCR, and were able to obtain clones of some of these candidates that were stable in *E. coli*, some are not in themselves likely to affect gene expression, but to cooperate with other proteins in a more complex network. Examples include proteins with GGDEF and EAL domains, which would potentially modulate cyclic digMP levels, and Rrp2, which is part of the RpoN-RpoS regulatory cascade that does not affect *p66* expression (49). We therefore focused on candidates that were predicted to have domains that might interact with...
DNA directly. Several of the candidate genes, \textit{bb0225}, \textit{bb0345}, \textit{bb0355} and \textit{bb0468}, have not previously been studied in \textit{B. burgdorferi}, although all are predicted to encode proteins with motifs of possible interest regarding regulation of gene expression, e.g. homology to known transcription factors in other bacteria, and HTH or other putative DNA-binding motifs (Table 4).

Three candidates that we identified, \textit{BB0232} (Hbb), \textit{BB0462} (EbfC) and \textit{BB0647} (Fur/BosR) have been identified by other laboratories as having site-specific DNA binding activity (13,46,50–54), and were examined by several groups, and although there is some controversy regarding its activities, they are consistent with its putative DNA-binding motifs (Table 4).

\textit{BB0232} (Hbb) was originally identified on the basis of complementation of \textit{E. coli} mutants defective in \textit{phoA} promoters (44). Hbb was later shown to bind specifically to a site in the \textit{dnaA-dnaN} intergenic region in the origin of replication of the \textit{B. burgdorferi} chromosome (46). Hbb also binds to the upstream regions of \textit{hbb}, \textit{ospC} and \textit{gacA} (46), but Hbb did not interact specifically with any \textit{oppA} promoter (44). EbfC (BB0462) was previously identified as a regulator of specific \textit{erm} loci (54) and interacts with the \textit{oppA} promoter (44). Fur/BosR (BB0647) (13,50,52,55) has been studied by several groups, and although there is some controversy regarding its activities, they are consistent with its similarity to Fur and Per proteins found in other bacteria. Fur/BosR interacts with the \textit{oppA} promoter as well as its own promoter and those of \textit{bb0153} (\textit{sodA}), \textit{bb0646} (a predicted hydrolase) and \textit{bb0690} (\textit{napA}) (44,50,52,56).

\textit{BB0527} is homologous to Baf (Bvg accessory factor), which helps to activate the Bvg two-component system of \textit{Bordetella pertussis} (57). In that system, BvgS is a transmembrane sensor kinase, and BvgA the positive regulator, of virulence gene expression in response to certain environmental signals in \textit{B. pertussis} (58). Baf augments BvgAS effects in \textit{E. coli}, and is essential for \textit{B. pertussis} viability (57), suggesting additional possible roles for the protein.

\textit{BB0232} in \textit{B. burgdorferi} is a \textit{MarR} homolog, and is the only one of the candidate transcription factors identified to date that is located on one of the plasmids in strain B31 (59,60). In \textit{E. coli}, \textit{MarR} encodes a repressor of the \textit{marRAB} operon, which controls the response of the bacterial cell to multiple antibiotics and to other environmental stresses (61). \textit{MarR} may bind as oligomers both at the −35 and −10 region of the \textit{mar} operator, and at the \textit{mar} ribosome-binding site (61). Binding of this protein to the operator appears to be influenced by a wide variety of signals, including antibiotics and reactive oxygen species.

\textbf{Functional characterization of candidate regulators}

Nine candidate regulators expressed from an \textit{E. coli} pET expression vector were introduced into \textit{phoA−} \textit{E. coli} carrying a second plasmid containing the \textit{p66} promoter plus secretion signal fused to the alkaline phosphatase (AP) reporter gene devoid of promoter and signal sequence. This fusion was generated to account for the possibility that some regulatory factors might bind to sites overlapping the translational start site, so the fragment cloned included the 5′ end of the structural gene. In addition, to account for the possibility that some factors might bind to sites considerably 5′ to the −35 element of the promoter, the fragment cloned was 556 bp in length. The AP assays were performed after growth of the dually transformed strains in liquid culture. At least three independent experiments were performed for each candidate regulator. Controls in all experiments included the \textit{p66} promoter fusion alone, with and without the pET vector with no \textit{B. burgdorferi} DNA; both of these control strains expressed similar low levels of AP activity that we termed ‘basal’. Three of the nine candidates tested reproducibly affected production of AP activity (\textit{P}-value of <0.01 by the Students’ two-tailed t-test in comparison to the control; Figure 2). These candidates were \textit{BB0232} (Hbb), which resulted in decreased AP expression from the \textit{p66} promoter, \textit{BB0527} (Baf homolog), which also resulted in decreased AP activity, and \textit{BB0232} (MarR homolog), which resulted in an increase in AP activity. The decrease in \textit{p66-phoA} expression is distinct from the effects of Hbb on the expression of the \textit{oppA1-oppA5} promoters in a similar reporter system, in which, if Hbb expression affected reporter activity at all, an increase was observed (44). The other candidates did not significantly affect the basal AP activity, although all nine candidate regulators were expressed after induction as analyzed by immunoblot (data not shown). Therefore, no further characterization of these six candidate regulators was performed as part of

\begin{table}[h]
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\begin{tabular}{|l|l|l|}
\hline
Genome locus & Description/possible roles & Putative functions \\
\hline
BB0225 & TIM-barrel protein & Putative transcriptional regulator, function unknown, conserved hypothetical \\
BB0232 & Hbb (HU-like protein) & Histone-like, potential roles in DNA replication, transcriptional regulation (46,53) \\
BB0345 & HTH-3 & Conserved hypothetical protein, family includes phage transcriptional control, bacterial methylase, plasmid copy control \\
BB0355 & Transcription Factor (CarD-like) & Transcriptional regulator homolog (92) \\
BB0462 & EbfC & Transcriptional regulator homolog (44,54) \\
BB0468 & Putative Transcription Factor & Homologous to Lac I family transcription factors in \textit{Brucella} spp. \\
BB0527 & Transcriptional Activator (Baf family) & Putative transcriptional activator, putative \textit{B. pertussis} toxin operon regulator \\
BB0647 & Fur family & Transcription factor activity, \textit{Borrelia} oxidative stress regulator, BosR (13,44,50,52) \\
\hline
\end{tabular}
\caption{Candidate regulators of \textit{p66} expression}
\end{table}
this study, although some were shown to affect expression of particular oppA promoters (44).

To determine whether Hbb, BB0527 and BBA23 interact directly with the p66 promoter region, we performed electrophoretic mobility shift assays (EMSAs, or gel shift assays) with purified, recombinant preparations of the three proteins identified in the reporter screen described above. Hbb bound to the p66 promoter at 1 nM, while BBA23 and showed no binding, and BB0527 only very inefficient binding, although this was reproducible (Figure 3). Therefore, within the E. coli cell, additional factors may contribute to the interaction of BB0527 and BBA23 with the p66 promoter, or their effects may be indirect. While BB0527 may interact with the p66 promoter, it appears that the interaction is of very low affinity and therefore may not be physiologically relevant. However, Hbb binding occurs at much higher affinity, and Hbb binding to the p66 promoter fragment may occur to multiple sites of different affinities, as several distinct species appear with increasing concentrations of Hbb. The shifted bands are due to Hbb-specific interaction with the DNA fragments, as ‘supershift’ assays in which antibody to the HA tag fused to the Hbb resulted in a further decrease in migration of the promoter fragment (Figure 3). The p66 promoter fragment was not shifted in the presence of BSA (Figure 3). Because Hbb does bind directly to the p66 promoter, this interaction was characterized further.

To assess the specificity of the interaction of Hbb with the p66 promoter region, we also examined the promoter regions of flaB, ospA and ospC. In addition, the origin of replication (oriC) of the linear chromosome of B. burgdorferi was tested because it is known to interact with Hbb (46). Hbb bound to all of these DNA fragments at nanomolar concentrations, although concentrations differed slightly between the different fragments (Figure 4A). In the presence of excess salmon sperm DNA, the binding by Hbb to each of the DNAs is disrupted at lower Hbb concentrations, but at high Hbb concentrations, only the p66 promoter and oriC fragments are still bound by Hbb (Figure 4B). These results suggest that the p66 promoter and oriC fragments bind Hbb with higher affinity than do the flaB, ospA, or ospC promoter fragments. Finally, Hbb binding to the p66 promoter was competed by unlabeled p66 promoter DNA, but not by flaB or oriC DNA (Figure 4C).

Figure 2. Activity of a p66 promoter-signal sequence-phoA fusion reporter in the presence of candidate regulators. PhoA+E. coli carrying the p66 promoter-signal sequence-phoA fusion, were transformed with plasmids encoding nine candidate B. burgdorferi regulatory proteins or the vector only control, and were tested for AP activity. Shown are the means ± standard deviations of three to five replicate experiments. To incorporate all data from independent experiments, activities in the presence of the candidate regulators were normalized to that of the vector only control. Statistically significant differences were observed for BBA23 (light bar, increased activity), and for BB0232, and BB0527 (black bars, decreased activity) with a P-value of less than 0.01 based on a Students’ two-tailed t-test.

Figure 3. Interaction of purified Hbb, BBA23 and BB0527 with the p66 promoter region. Various concentrations of purified recombinant proteins were incubated with 100 pM radiolabeled DNA for 30 min, and the reactions were run on non-denaturing 5% polyacrylamide gels. The gels were dried and exposed to film overnight at −70°C and were visualized by autoradiography. Hbb starts to shift the p66 promoter fragment at 1 nM (arrow), while BB0527 requires much higher concentrations, and BBA23 did not appear to interact with this DNA fragment. The p66 promoter fragment is homogeneous when analyzed by agarose gel electrophoresis but generally heterogeneous by acrylamide gel electrophoresis, even after gel purification.
Together, our results suggest that Hbb binds to the p66 promoter with an affinity similar to that reported for the oriC fragment (46). In their work with the oriC region, Kobryn et al. (46) demonstrated specificity sufficient to identify a binding site using DNase 1, KMnO$_4$ and hydroxyl radical footprinting. In the p66 promoter fragment used in this study, there is a site similar to that identified by Kobryn et al. and refined by Mow and Rice (46,62) (Figure 5). A 41 bp fragment (shown in blue in Figure 5A) containing this site was bound by Hbb, as was a shorter p66 promoter fragment in which this site was truncated, but both were bound by Hbb only at $\geq 2$ nM.

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**Figure 4.** Specificity of interaction of purified Hbb with *B. burgdorferi* DNA fragments. (A) Varying concentrations of Hbb were incubated with 100 pM radiolabeled DNA fragments, and the samples fractionated on non-denaturing gels, as described for Figure 3. Arrows indicate the first shifted band apparent for each DNA fragment, all of which appear at 1–2 nM Hbb. (B) One microgram salmon sperm DNA was added to each binding reaction as a competitor. (C) Hbb was held constant, and unlabeled DNA fragments were added at the concentrations shown.
(Figure 5B and data not shown), suggesting that binding with maximal affinity occurs only when an extended p66 upstream region is present.

Quantitative RT–PCR analysis of hbb mRNA levels in tick midguts

To determine whether the expression of hbb reflects the expression of p66 by B. burgdorferi in tick midguts, we performed qRT–PCR on the tick midgut samples used for the experiments shown in Figure 1. The pattern of hbb expression in infected ticks seen by qRT–PCR generally reflects that seen in the cases of p66, flaA and ospA in that the highest absolute transcript levels are seen post-repletion, and the lowest expression occurs when the ticks are unfed. The major difference between hbb and the other genes analyzed is that the hbb transcript levels are much lower, and that transcript is detected in the ticks that were not infected with B. burgdorferi at 9 days post repletion. This is most likely an artifact of the extremely low signals of both the p66 signals of both the

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DISCUSSION

Borrelia burgdorferi is intimately tied to both the tick vector and the vertebrate (usually mammalian) host. The bacteria reside in the tick midgut in apparent quiescence, then replicate and change gene and protein expression as the tick takes its blood meal. Several B. burgdorferi proteins and genes are produced or expressed very differently between the two environments. Changes in temperature, pH, cell density and other as-yet unidentified signals are thought to contribute to these changes. The paradigm for this change in gene expression is the induction of OspC in conjunction with decreased OspA expression while the tick is feeding (47). Changes in the expression of many other genes and gene products by B. burgdorferi in preparation for transit between tick and mammal have subsequently been observed by multiple investigators (6,12,35–37,47,63–73). Because digestion of the blood meal occurs intracellularly (74,75), and B. burgdorferi is extracellular, degradation of surface-exposed bacterial proteins by tick lytic enzymes is thought unlikely. qRT–PCR analysis of B. burgdorferi in tick midguts or salivary glands suggests transcriptional regulation of gene expression by B. burgdorferi during the tick life cycle (76–80). Post-transcriptional regulation, e.g. by small RNAs (81), or regulation of protein half-lives are other possible mechanisms by which B. burgdorferi could be altering its protein complement within the tick.

Transcriptional regulation of gene expression has been demonstrated for a number of B. burgdorferi genes. Primer extension was performed on p66 RNA, and a putative σ70-dependent promoter was identified (34). In accordance with this work, microarray studies of rpoN and rpoS mutants of B. burgdorferi do not indicate significant roles for these alternative sigma factors in p66 expression (6,8,35). We therefore hypothesized that σ70-dependent expression of p66 is modulated by accessory factors that result in the differences observed in fed versus unfed tick, especially since p66, ospA and the flagellar genes flaA and flaB are all thought to be σ70-dependent (29,82), but show different patterns of expression. In fact, the repression of ospA in the mammalian host is RpoS-dependent (3). These observations led us to search for potential factors in B. burgdorferi that affect p66 expression.

Of the candidate regulators identified, only Hbb (BB0232) appeared to interact with the p66 promoter in settings consistent with physiologic relevance, although additional factors may influence p66 mRNA and protein expression in B. burgdorferi during the tick-mammal infectious cycle. Hbb is a homolog of the architectural DNA-binding proteins, including the H-NS, IHF, FIS and HU-like proteins (46,53). Proteins in this family are known to play a variety of roles, including roles in DNA replication (46), promoting complex assembly by inducing severe bends in DNA (83), and transcriptional regulation by virtue of DNA bending or stabilizing sequence- and temperature-dependent secondary structures (84). HU- and IHF-like proteins, to which Hbb is more closely similar, tend to be involved in the activation of transcription due to formation of an open sequence- and temperature-dependent secondary structures (85,86). Of particular interest, H-NS in Yersinia pseudotuberculosis and Y. enterocolitica represses expression of the inv gene, which encodes the integrin ligand invasin. This repression is countered by competition for overlapping binding sites upstream of the −35 sequence by RovA, a marR family member. These results parallel those reported here, in which Hbb repressed the activity of a p66 promoter fusion to the reporter alkaline phosphatase, while BBA23, another MarR family member, increased reporter activity (87,88). It will be interesting to determine the effects of deletion of hbb (bb0232) and bba23 on p66 during the tick-mouse infectious cycle, but those experiments will require the generation of the mutants in B. burgdorferi.
Figure 5. The *p66* promoter region and Hbb-binding sites. (A) The *p66* upstream region is shown 5′–3′ with the −35 and −10 highlighted in red, the transcriptional start site highlighted in red (29), the putative ribosome binding site in green, the translational start site in green, and the candidate Hbb-binding site tested by EMSA highlighted in blue. Mismatches to a perfect palindrome within this site are highlighted in pink, the base at the axis of symmetry is in orange. The DNA sequences bound by Hbb and their affinities are from (46,62). Their binding site studies were refined with double stranded oligonucleotides (designated oligo 1, oligo 2 and oligo 3 in the alignment, shown without flanking 10 bp fragments) designed to test the roles of symmetry and IHF consensus sequences (62). The *p66* bases that are exact matches to the sequences bound by Hbb with affinities of <30 nM are indicated by black asterisks. (B) Hbb binds to the 41 bp region shown in blue in (A). The DNA fragment was formed by annealing two oligonucleotides, and tested for binding to Hbb by EMSA as described in Figures 3–5, with the exception that the samples were run on a higher percentage acrylamide gel. 

Our results are consistent with Hbb being involved in the reduction of *p66* expression. Because Hbb causes bends in the DNA to which it binds, full assembly of the RNA polymerase complex may not occur adjacent to a significant bend. Upstream (5′) of the −35 site of the *p66* promoter there is a candidate Hbb-binding site consistent with the requirements outlined by Mouw and Rice (62) (Figure 5A). Hbb does bind to this fragment (Figure 5B), but additional work is required to assess specificity and stoichiometry. One model consistent with our data is that Hbb is expressed at low levels, and that *hbb* transcription is not increased at a rate sufficient to keep up with bacterial replication as the tick feeds. Hbb would then be diluted as the bacteria divide, allowing access of RNA polymerase to promoters, such as that of *p66*, that were previously rendered inaccessible by Hbb. In fact, although *hbb* expression at the mRNA level does increase in the tick after the blood meal, this increase does not keep pace with the increases in either *p66* or *flaA* expression. The *p66*/*flaA* ratio is 0.033 in the unfed tick, increases to 2.36 in the fed tick and further increases to 7.18 in the tick 9 days post repletion. In contrast, the *hbb*/*flaA* ratio is 0.037 in the unfed tick, and decreases to 0.013 in the fed tick and remains at that level in the tick at 9 days. As the bacteria slow or stop replication, the intracellular Hbb concentration may then increase to levels to allow interaction with sites such as the *p66* promoter, and resumption of repression of transcription from this promoter. In preliminary studies using anti-Hbb generated in the laboratory of Dr Scott Samuels (90), Hbb in laboratory-cultivated *B. burgdorferi* is present at less than 750 copies of the protein molecule per cell (data not shown), which is low considering that Hbb forms a dimer (62) and that it is thought to have roles in DNA replication (46) and transcription. Our model, however, requires validation through additional experimentation. Hbb may also serve as a type of scaffold, upon which additional factors could build to influence gene expression. It is also possible that BBA23 competes with Hbb binding, and results in increased gene expression, but all of these possibilities require future experimentation. Future studies will also be required to define the Hbb binding site in the *p66* promoter. Ultimately, the most important experiment would be to mutate the gene in infectious *B. burgdorferi* and determine whether the bacteria are able to transcribe genes appropriately in the tick, in the mammal, and in transitions between these two hosts.

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