Borrelia burgdorferi Alters Its Gene Expression and Antigenic Profile in Response to CO₂ Levels

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The etiologic agent of Lyme disease, Borrelia burgdorferi, must adapt to the distinct environments of its arthropod vector and mammalian host during its complex life cycle. B. burgdorferi alters gene expression and protein synthesis in response to temperature, pH, and other uncharacterized environmental factors. The hypothesis tested in this study is that dissolved gases, including CO₂, serve as a signal for B. burgdorferi to alter protein production and gene expression. In this study we focused on characterization of in vitro anaerobic (5% CO₂, 3% H₂, 0.087 ppm O₂) and microaerophilic (1% CO₂, 3.48 ppm O₂) growth conditions and how they modulate protein synthesis and gene expression in B. burgdorferi. Higher levels of several immunoreactive proteins, including BosR, NapA, DbpA, OspC, BBK32, and RpoS, were synthesized under anaerobic conditions. Previous studies demonstrated that lower levels of NapA were produced when microaerophilic cultures were purged with nitrogen gas to displace oxygen and CO₂. In this study we identified CO₂ as a factor contributing to the observed change in NapA synthesis. Specifically, a reduction in the level of dissolved CO₂, independent of O₂ levels, resulted in reduced NapA synthesis. BosR, DbpA, OspC, and RpoS synthesis was also decreased with the displacement of CO₂. Quantitative reverse transcription-PCR indicated that the levels of the dbpA, ospC, and BBK32 transcripts are increased in the presence of CO₂, indicating that these putative borrelian virulence determinants are regulated at the transcriptional level. Thus, dissolved CO₂ may be an additional cue for borrelian host adaptation and gene regulation.

Lyme disease is a multisystemic, inflammatory disorder caused by the pathogenic spirochetal bacterium Borrelia burgdorferi (32, 44). In 2002, the Centers for Disease Control and Prevention reported 23,763 cases, indicating that Lyme disease is the leading tick-borne disease in the United States and, based on the 40% increase in reported cases, is a reemerging infectious disease (12). The reservoir for B. burgdorferi is the white-footed mouse (Peromyscus spp.), and ticks become infected when larvae feed on these mice. Humans can become infected when an Ixodes scapularis nymph takes a blood meal prior to molting into an adult, resulting in transmission of the spirochete from the tick midgut into mammalian tissue (32, 44). The disparate host milieus that B. burgdorferi occupies (i.e., the tick vector and a mammalian host) present a challenge for this spirochetal pathogen since it must quickly adapt to these different environments in order to establish an infection and avoid host clearance. Previous studies have shown that temperature and pH modulate gene expression in B. burgdorferi (7, 9, 10, 34, 36, 39, 45, 53). The best-characterized loci involved in differences in expression between the arthropod vector and mammalian host are the genes encoding the prominent surface-exposed lipoproteins OspA and OspC (3, 26, 34, 36, 40, 45, 55). OspA is expressed under conditions that model the tick environment (pH 7.5 and 23°C) (36, 40, 53, 56), and the influx of a blood meal into the tick midgut changes the temperature and pH to 35°C and 6.8, respectively, resulting in a switch to ospC expression (53). The expression of ospC (along with other genes) requires the RpoN-RpoS system in conjunction with the response regulator Rrp2 (26, 54, 55). This adaptive response enables the organism to traffic to the salivary glands prior to entering the dermal tissue of the mammalian host (21, 33, 35). Analyses of host-adapted B. burgdorferi have indicated that additional unidentified host factors may modulate gene expression (2, 7, 36).

Reactive oxygen species, oxygen, and CO₂/bicarbonate are known to alter gene expression in several distinct pathogenic bacteria through differential regulatory mechanisms (15, 24, 25, 41, 46). For example, several Bacillus anthracis toxin genes and a gene involved in capsule biosynthesis, capB, are coregulated by the anthrax toxin activator, AtxA, together with CO₂/bicarbonate levels (15, 17, 24, 25). Previous studies indicated that B. burgdorferi modulates gene expression in response to gas displacement, presumably via oxygen depletion (41). In this study we obtained data indicating that dissolved CO₂ also contributes to this process. The results presented here indicate that dissolved CO₂ levels affect expression of the genes examined previously (41), as well as several additional loci. Thus, the levels of dissolved CO₂ may serve as an additional cue used by B. burgdorferi to modulate gene expression in a manner that has potential importance for host adaptation.

MATERIALS AND METHODS

Strains and growth conditions. All strains of B. burgdorferi used in this study were grown in BSK-II medium supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR), which is referred to as complete BSK-II medium below. The CMRL-1066 medium (United States Biologicals, Swampscott, MA) used in BSK-II medium lacked any added bicarbonate. Low-passage,
infectious, clonal B31 derivative MSK5 and clonal 297 derivatives AH130 (parent), AH210 (ipsoN mutant), AH212 (ipsoN mutant), and AH123 (ipsoN mutant complemented by wild-type ipsoN gene) were used in this study (26, 29). Microaerophilic cultures were grown statically at 32°C and pH 7.8 with 1% atmospheric CO2, in complete BSK-II medium. The anaerobic culture conditions consisted of 5% CO2, 3% H2, pH 7.3, and 32°C in complete BSK-II medium. The level of oxygen was reduced 40-fold in the anaerobic complete BSK-II medium (0.087 ppm) compared to the microaerophilic medium (3.48 ppm), as determined using a DO-166 oxygen probe (Lazar Research Laboratories, Los Angeles, CA) (41).

All cultures were inoculated at a density of 1 × 10^8 cells per ml and grown to a density of 5 × 10^9 cells per ml to acquire RNA or protein samples. For the pH study, microaerophilic cultures were grown in complete BSK-II medium at pH 7.3, and the pH of anaerobic cultures with the same starting density were adjusted to 7.8. CO2 was displaced from anaerobically grown cultures by treatment with nitrogen gas for 20 min at a pressure of 10 lb/in^2, which reduced the CO2 level from 5,800 ppm to undetectable levels. Dissolved CO2 levels were measured using a CO-35 probe (Lazar Research Laboratories, Los Angeles, CA). RNA and protein samples were taken from anaerobic cultures and anaerobic cultures lacking CO2 at a density of 5 × 10^8 cells per ml. MSK5 was grown under anaerobic and microaerophilic conditions in modified BSK-II medium containing decreasing amounts of NaHCO3, so that the medium contained 25 mM, 15 mM, 5 mM, 1 mM, or no added NaHCO3; the highest concentration represented conventional BSK-II medium. The NaHCO3-modified medium was supplemented with NaCl to maintain a constant osmotic balance. To examine the role of different CO2 levels with a constant O2 content, AH130 and MSK5 were also grown statically in the presence of atmospheric O2 (3.48 ppm dissolved O2) and 5% atmospheric CO2 at 32°C or 37°C.

**SDS-PAGE and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using infection-derived and monospecific antiserum as primary antibodies were conducted as previously described (41).

**RNA isolation.** Three independent cultures of *B. burgdorferi* strains MSK5 and AH130 were grown to the exponential growth phase (i.e., 5 × 10^8 cells per ml), and total RNA was isolated from 1 × 10^7 cells using a Versagene kit (Genra Inc., Minneapolis, MN). RNA samples were treated with DNase I (Roche Inc., Indianapolis, IN) and Superase In (Ambion Inc., Austin, TX) to eliminate contaminating DNA and inhibit RNase activity, respectively. Three independent RNA samples of each strain tested were pooled, and DNA contamination and crude RNA yield were examined by PCR and reverse transcription (RT-PCR), respectively.

**Quantitative RT-PCR.** A defined set of genes was subjected to quantitative RT-PCR to ascertain whether the antigenic production observed was due to regulation at the transcriptional level. Oligonucleotide primers (Table 1) were designed with the Primer Express software (Perkin-Elmer Biosystems, Foster City, CA). Selected primer pairs were tested to confirm that they amplified a single product with a known size using genomic *B. burgdorferi* DNA as the template. Reverse transcription reactions were performed by combining TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) with purified *B. burgdorferi* total RNA. A control reaction with a mixture lacking reverse transcriptase was performed for each primer set using total RNA from each of the test strains to confirm that RNA was not present. Subsequently, the products from the reverse transcription reaction were subjected to real-time PCRs using an Applied Biosystems 7500 real-time PCR system. SYBR green PCRs were performed in triplicate, and each experiment was repeated in triplicate, resulting in nine data points for each gene of interest and for each *B. burgdorferi* strain tested. A constitutively expressed gene, flaB, which was not affected by any treatment tested in this study, was used for normalization as previously described (41). The levels of induction of genes induced during anaerobic-grown cultures with CO2 compared to the levels observed during anaerobiosis without CO2 were determined by the ΔΔCt method as previously described (7, 41).

**Statistical analyses.** The real-time RT-PCR data from three independent experiments were analyzed using a resampling bootstrap procedure and the permutation two-sample test. The bootstrap distribution provides an accurate estimate of the lower and upper limits, respectively, of a 95% confidence interval around the true mean. For the permutation two-sample test, the distribution of the data tested the null hypothesis that CO2 had no influence on gene expression. A P value of ≤0.01 was used. The data set for each gene was based on ΔΔCt values (relative to flaB for *B. burgdorferi* grown with or without CO2) to ensure that there were normal distributions for accurate probability estimates. All statistical tests and data resampling operations were performed with S-PLUS, version 7.02 (Insightful Corp., Seattle, WA).

**RESULTS**

Different antigenic compositions of *B. burgdorferi* under anaerobic and microaerophilic conditions. As *B. burgdorferi* moves through the disparate environments encountered in the arthropod vector and mammalian host, changes in temperature, pH, and other host factors modulate gene expression (2, 3, 7, 10, 16, 40, 45, 53, 55). Previous studies indicated that the redox environment of *B. burgdorferi* alters gene expression and protein synthesis in this spirochetal pathogen (4, 41). To address this question further, defined anaerobic and microaerophilic conditions were imposed to determine how dissolved gases, including CO2, affect borrelial gene regulation and protein synthesis. *B. burgdorferi* was grown statically in complete BSK-II medium at 32°C for all conditions tested unless indicated otherwise. Microaerophilic growth conditions were maintained with 1% atmospheric CO2, and the anaerobic environment was defined as 5% CO2 and 3% H2 atmospheric levels in a controlled anaerobic chamber. An oxygen electrode was used to measure dissolved oxygen levels in microaerophilic complete medium (3.48 ppm) and anaerobic complete medium (0.087 ppm), and the results indicated that the anaerobic culture conditions resulted in a 40-fold reduction in the dissolved oxygen level compared to the level in microaerophilic medium. All *B. burgdorferi* cells tested exhibited normal motility under both of the culture conditions mentioned above without a significant difference in growth (data not shown). The antigenic responses of *B. burgdorferi* to anaerobic and microaerophilic growth conditions were assessed by Western immunoblot analysis. AH130 and MSK5 samples from cultures grown under each condition were probed with serum from a patient with chronic Lyme disease or with infection-derived mouse serum (Fig. 1). Major antigenic differences for *B. burgdorferi* AH130 and MSK5 were observed throughout the immunoblot, and there was significantly greater synthesis of the antigens in the anaerobically grown cultures (Fig. 1).

### TABLE 1. Oligonucleotides used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Open reading frame</th>
<th>Designation</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
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<tr>
<td>BB0147</td>
<td>flaB</td>
<td>CAGCTAAATGTGCAAATCTTTTGCTT</td>
<td>TCCGTTGAAACCCCTTGGAGA</td>
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<tr>
<td>BB0153</td>
<td>sodA</td>
<td>GCTGCAGAAGACTTGGTGATTATG</td>
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<tr>
<td>BB0647</td>
<td>boxR</td>
<td>ACCCTATTCAACCTTTGACATTTAAGAT</td>
<td>GCTCTGATGGAAATTTTTTATTT</td>
</tr>
<tr>
<td>BBa24</td>
<td>dbpA</td>
<td>CAGATGACGCTGAAAGAATCTCTT</td>
<td>ACCGTGTTAATTTTTCCCTTTT</td>
</tr>
<tr>
<td>BBB19-MSK5</td>
<td>ospC (MSK5)</td>
<td>CGGATTCTTAATGGGGTTTACTTG</td>
<td>CAATAGCTTTAGCAGAAATTTTCT</td>
</tr>
<tr>
<td>BBB19-AH130</td>
<td>ospC (AH130)</td>
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</tr>
<tr>
<td>BBBK32</td>
<td></td>
<td>GAATAATAGGAGGATGACTCAAAGTG</td>
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</tr>
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**Primers (5'-3')**
The production of individual borrelial antigens was examined under anaerobic and microaerophilic conditions (Fig. 2). FlaB synthesis was unchanged under the experimental conditions employed and was used as a control to demonstrate equivalent protein levels in samples. Proteins associated with oxidative stress, including BosR, a borrelial oxidative stress regulatory protein, and NapA, a Dps/Dpr homolog, produced more of these specific antigens during anaerobic growth than during microaerophilic growth for both strains of *B. burgdorferi* analyzed, AH130 and MSK5 (Fig. 2). Previous studies demonstrated that NapA levels decreased when the culture medium was pretreated with nitrogen gas displacement and with Oxyrase to deplete all dissolved gases and O2, respectively (41). The results obtained here using an anaerobic chamber did not corroborate these findings, suggesting that the different culture conditions utilized accounted for the different NapA levels observed. The levels of the decorin binding adhesin, DbpA, and OspC increased under anaerobic conditions, similar to previously reported observations (41). In addition, the level of the borrelial RpoS sigma factor also increased when *B. burgdorferi* was grown under anaerobic conditions (Fig. 2).

**Effect of pH on borrelial protein production.** Increased levels of CO2/bicarbonate decreased the pH of complete BSK-II medium, and since pH is known to affect *B. burgdorferi* gene expression and protein production, the appropriate adjustments were made to the medium to compensate for the difference (9, 10). The pH values of anaerobic and microaerophilic complete BSK-II media were 7.3 and 7.8, respectively. To assess if the observed changes in specific borrelial protein production could be attributed to the difference in pH values, MSK5 was also grown anaerobically at pH 7.3 and 7.8 (lanes A) and under microaerophilic conditions at pH 7.3 and 7.8 (lanes M). Protein samples were probed with monospecific antisera to the borrelial proteins DbpA, NapA, and BosR. If the induction was due to differences in pH, then one would expect that the level of each antigen would be significantly induced when organisms were grown microaerophilically at pH 7.3. Although the level of DbpA increased somewhat in cells grown microaerophilically at pH 7.3, the level never approached what was observed under conventional anaerobic conditions (Fig. 2). Furthermore, the levels of NapA and BosR were not affected by lowering the pH under microaerophilic conditions (Fig. 3). Taken together, these results suggest that the change in protein production previously observed during anaerobic growth (Fig. 2) is independent of pH.

**Influence of CO2 and bicarbonate on *B. burgdorferi*.** The effect of CO2 and bicarbonate levels on borrelial protein synthesis was analyzed, as the atmospheric level of CO2 was 4% higher under the defined anaerobic growth conditions than...
under the microaerophilic growth conditions. Dissolved O₂ and CO₂ are present under both in vivo and in vitro growth conditions, and in this study nitrogen gas was used to indiscriminately displace both dissolved O₂ and CO₂ from the growth medium. A CO₂ probe was used to measure dissolved CO₂ levels in microaerophilic (1,700 ppm), anaerobic (5,800 ppm), and nitrogen gas-purged anaerobic (0 ppm) complete BSK-II media. To directly assess the effect of CO₂ on gene expression and protein production, anaerobic cultures were treated with nitrogen gas to completely displace the dissolved CO₂ (Fig. 4). Protein samples from the anaerobic, nitrogen gas-treated anaerobic, and microaerophilic cultures were probed with monospecific antisera to several borrelial proteins. As described above, constitutively synthesized FlaB was used as a control between samples and for the different treatments employed. The DpbA and OspC levels were greatly increased when CO₂ was present, suggesting that in addition to O₂, CO₂ levels modulate gene expression and protein production in B. burgdorferi (Fig. 4).

Dissolved CO₂ levels also affected the synthesis of borrelial RpoS and BosR, an alternate sigma factor and redox regulatory protein, respectively. Specifically, RpoS and BosR were induced greatly when CO₂ was present, followed by microaerophilic growth; anaerobic cultures produced by cells under these conditions than by cells grown anaerobically without CO₂ (Fig. 5). Furthermore, the reduction in the NapA level was attributed to a decrease in the dissolved oxygen level. In this study, the levels of NapA were evaluated when cells were grown in an anaerobic chamber containing 5% CO₂. Surprisingly, appreciably more NapA was produced by cells under these conditions than by cells grown anaerobically without CO₂ (Fig. 5). This hierarchy of regulation is consistent with the amounts of dissolved CO₂ available in the samples; that is, the larger the amount of CO₂ in the sample, the larger the amount of NapA produced. These results indicate that CO₂/bicarbonate levels dramatically influence NapA protein production in B. burgdorferi and suggest that dissolved oxygen plays a minor role in the regulation of napA.

Bacteria can sense CO₂/bicarbonate via adenylyl cyclase, which results in increased cAMP levels that, in turn, modulate gene expression (22, 52). Conventional microaerophilic growth conditions consist of atmospheric CO₂ and the 25 mM NaHCO₃ in BSK-II medium. To examine the effect of bicarbonate levels in anaerobically (5% CO₂) and microaerophilically (1% CO₂) grown B. burgdorferi, the concentration of sodium bicarbonate was decreased in the borrelial growth me-
of 25 mM, 15 mM, 5 mM, or 1 mM or with no additional NaHCO₃ under both anaerobic and microaerophilic conditions. Protein lysates from each strain grown under each condition were resolved by SDS-PAGE, immobilized on polyvinylidene difluoride membranes, and probed with antisera specific for the antigens indicated on the left. Constitutive synthesis of FlaB was used as a control to demonstrate that there were equivalent amounts of protein in the samples. Note that synthesis of the antigens tested in the microaerophilically grown samples generated protein species that were detected following longer exposure times.

**FIG. 6.** Antigen synthesis is influenced by CO₂ rather than by bicarbonate levels. A strain B31 derivative, MSK5, was grown with 25 mM (conventional concentration), 15 mM, 5 mM, 1 mM, and no added NaHCO₃ under both anaerobic and microaerophilic conditions. Protein lysates from each strain grown under each condition were resolved by SDS-PAGE, immobilized on polyvinylidene difluoride membranes, and probed with antisera specific for the antigens indicated on the left. Constitutive synthesis of FlaB was used as a control to demonstrate that there were equivalent amounts of protein in the samples. Note that synthesis of the antigens tested in the microaerophilically grown samples generated protein species that were detected following longer exposure times.

To confirm the finding that 5% CO₂ altered borrelial protein synthesis and to examine the role of oxygen in this process, AH130 and MSK5 were grown microaerobically in the presence of 5% CO₂ with atmospheric O₂ (3.48 ppm dissolved O₂) at 32°C and 37°C (Fig. 7). Then protein production was evaluated compared to the production under conventional microaerophilic growth conditions (1% CO₂, atmospheric O₂). A variable response was observed for OspC and BBK32, indicating that multiple factors affect certain strains differently to alter the synthesis of the protein species.

**FIG. 7.** Increased CO₂ levels in the presence of atmospheric O₂ result in levels of antigen synthesis comparable to levels observed for anaerobically grown *B. burgdorferi*. AH130 and MSK5 were grown under conventional microaerophilic growth conditions (1% CO₂ with atmospheric O₂) and with increased levels of CO₂ and atmospheric O₂ (either 5% CO₂ at 32°C or 5% CO₂ at 37°C). Protein lysates from each strain grown under each condition used were resolved by SDS-PAGE, immobilized on polyvinylidene difluoride membranes, and probed with antisera specific for the antigens indicated on the left. Constitutive synthesis of FlaB was used as a control to demonstrate that there were equivalent amounts of protein in the samples. Note that synthesis of BosR, NapA, DbpA, OspC, and BBK32 was observed in the samples grown with 1% CO₂ at 32°C following longer exposure times.
that there was a dramatic change at the transcriptional level for BBK32, *dbpA*, and *ospC* in the presence of CO₂. Specifically, the BBK32, *dbpA*, and *ospC* transcript levels were 17.4-fold, 18-fold, and 93.2-fold greater, respectively, when AH130 was grown anaerobically without detectable dissolved CO₂. The MSK5 transcript levels of BBK32, *dbpA*, and *ospC* were increased 3.82-fold, 4.41-fold, and 8.48-fold, respectively, in anaerobic samples containing CO₂. The increases in gene expression observed in strain AH130 (a strain 297 derivative) compared to strain MSK5 (a strain B31 derivative), particularly for *ospC*, are consistent with previously published observations for comparisons of expression in these strains (53) and suggest that while there are absolute expression differences between these strains, the trends observed are similar (Fig. 8).

The increased expression in the presence of CO₂ of lipoprotein genes (i.e., *ospC*, *dbpA*, and BBK32) involved in the adherence of *B. burgdorferi* to host structures suggests that CO₂ may serve as a cue for mammalian adaptation and that the regulation observed is at the transcriptional level. In addition to BBK32, *dbpA*, and *ospC*, two genes associated with the oxidative stress response (*bosR* and *sodA*) were also evaluated by RT-PCR, but the transcription of neither was enhanced greatly when cells were grown anaerobically with or without dissolved CO₂. Specifically, less-than-two-fold change was observed for *bosR* and *sodA*, although for *bosR* in strain MSK5 and for *sodA* in strain AH130 the increase in transcript production observed when CO₂ was present was statistically significant (Fig. 8). However, for *bosR*, for which there was great induction of protein production when CO₂ was present (Fig. 2, 4, and 7), the regulation observed appeared to be not linked to transcript production and instead may have been at the translational or posttranslational level.

**DISCUSSION**

Pathogenic bacteria modulate gene expression and protein synthesis in response to changing environmental conditions. *B. burgdorferi* adapts to the unique environments of the arthropod vector and mammalian hosts as infections are established during its complex enzootic life cycle (44). Previous studies have shown that changes in temperature and pH alter borrelial gene expression and protein synthesis (2, 3, 7, 10, 16, 34, 36, 40, 45, 53, 55). In addition, surgical implantation of dialysis membrane chambers containing *B. burgdorferi* into the peritoneal cavity of rats results in changes in gene expression and protein production that model a mammalian host-adapted state (2, 7, 36). Additional experimentation indicated that the changes observed could not be explained by altering the temperature and pH during in vitro cultivation, thus suggesting that there are other unidentified environmental signals that alter borrelial gene expression and protein synthesis during infection (2, 7, 36). The hypothesis presented here suggests that dissolved O₂ and CO₂ levels serve as a cue for *B. burgdorferi* to adapt to changing host environments, perhaps via the borrelial RpoS sigma factor and/or BosR regulator. The working hypothesis is that the midgut of *Ixodes* ticks is essentially anaerobic prior to a blood meal. Subsequently, in response to a blood meal, there are increases in temperature and tick respiration, resulting in higher levels of dissolved oxygen and potentially different levels of dissolved CO₂ as a result of cellular respiration. In addition, as *B. burgdorferi* disseminates in a mammalian host, the levels of dissolved O₂ and CO₂ in the various tissue locales are likely to vary. Thus, differences in dissolved O₂ and CO₂ levels may serve as potential signals perceived by *B. burgdorferi* to modulate gene expression in the mammalian host (43, 50). Consistent with this contention, several of the borrelial genes (*ospC*, *dbpA*, and BBK32) and products of these genes that were expressed or synthesized at higher levels when CO₂ and O₂ levels were altered in this study are also synthesized at higher levels in host-adapted spirochetes (2, 7, 36) and are antigenic following infection with *B. burgdorferi* (1, 11, 18, 23, 38, 51).

In addition to different levels of O₂, the dissolved CO₂ level fluctuates throughout the mammalian host, and the concentration is 1.5-fold higher in tissue than in arterial blood (49). Previous work demonstrated that there is a correlation between higher atmospheric CO₂ levels and the maintenance of infection-associated plasmids of *B. burgdorferi* during in vitro cultivation, suggesting that CO₂ imposes a selective pressure that preserves genome stability and thus infectivity (4). The ability to sense CO₂, either as CO₂ or as bicarbonate, influences the expression of virulence determinants in other pathogenic organisms, suggesting a potential role for CO₂ sensing in borrelial virulence gene expression (5, 28, 31, 52). Interestingly, *Bacillus anthracis* responds to CO₂/bicarbonate levels by inducing the toxin genes, *cya*, *lef*, and *pag*, as well as the capsule gene, *capB*, through the activity of the temperature-regulated anthrax toxin activator, AtxA (15, 17, 24, 25).

Another way in which living systems sense CO₂ is via the well-characterized enzyme adenyl cyclase (13, 28, 31, 52). Specifically, adenyl cyclase is able to directly sense bicarbonate or CO₂, which results in an increase in cAMP production by this enzyme (22, 47, 52). Accumulation of cAMP, a well-
known secondary messenger signal in both eukaryotes and prokaryotes, affects the expression of a number of genes throughout these systems (13, 28, 31). Whether the *B. burgdorferi* lone adenyl cyclase homolog functions in this capacity remains to be determined.

In previous work researchers assessed the effect of dissolved oxygen on microaerophilically grown *B. burgdorferi* by treating cultures with nitrogen gas or Oxyrase (41). In the current study, an anaerobic chamber was employed to grow cultures under oxygen-depleted conditions. Initially, the effect of anaerobiosis was assessed by looking at the synthesis of NapA inasmuch as previous studies had shown that NapA levels decreased when oxygen levels were reduced (41). In other organisms NapA has been shown to nonspecifically bind DNA in response to oxidative stress, and it is presumed to have a similar function in *B. burgdorferi* (14, 20, 30, 37). Surprisingly, in this study, higher levels of NapA were produced when *B. burgdorferi* was grown in the anaerobic chamber than when it was grown microaerophilically, suggesting that the regulatory cue for napA repression and induction observed previously was not limited to oxygen depletion alone. When nitrogen gas was used to indiscriminately displace all gases, including O2 and CO2, the levels of NapA were reduced compared to the levels both in microaerophilically grown *B. burgdorferi*, as described in a previous report (41), and in borrelial cells grown in an anaerobic chamber with CO2 (Fig. 2 and 5). Inasmuch as the level of oxygen (0.087 ppm) was the same when cells were grown in the anaerobic chamber, this result indicates that the absence of CO2 results in decreased synthesis of NapA (Fig. 5).

Previous studies demonstrated that pH alters gene expression in *B. burgdorferi* (9, 10, 36, 53). To examine the possibility that the differential synthesis of borrelial antigens was due to changes in pH as a result of alteration of the CO2 levels, the pHs of the anaerobic and microaerophilic media, determined to be 7.3 and 7.8, respectively, were adjusted so that the pH of the anaerobic medium was 7.8 and the pH of the microaerophilic medium was 7.3. Under these conditions, the synthesis of NapA, BosR, or DbpA was not altered compared to the synthesis in *B. burgdorferi* grown microaerophilically at pH 7.8 (Fig. 2), indicating that the regulation observed was not due to pH (Fig. 3). Subsequent comparisons indicated that the difference observed was due to CO2 levels (Fig. 4 and 5) and that, in addition to O2, CO2 serves an inducible signal that modulates a subset of genes in *B. burgdorferi*. Increasing CO2 levels in the presence of atmospheric oxygen confirmed the effect of CO2 on the synthesis of NapA and other borrelial antigens (Fig. 7).

The *B. burgdorferi* RpoN-RpoS two-component regulatory system responds to environmental stress by controlling the expression of lipoproteins associated with pathogenic mechanisms, specifically the decorin binding adhesin (encoded by dbpA), as well as the product of ospC (26, 53), which is involved in transmission of *B. burgdorferi* from the tick vector into the mammalian host (21, 35). To facilitate this response, a response regulatory protein, Rrp2, is activated by its cognate histidine kinase (encoded by BB0764), which contains a PAS domain. Together with the sigma factor RpoN, Rrp2 regulates the expression of rpoS (54). PAS domains detect numerous environmental signals, such as light, redox potential, oxygen, small ligands, and overall cell energy (48). Therefore, the borrelial PAS-containing histidine kinase may sense CO2 as well as the redox status of the cell to activate Rrp2 and, via RpoN/RpoS, dbpA and ospC. The observation that RpoS levels are enhanced most when CO2 is present supports this hypothesis (Fig. 4 and 7).

The borrelial oxidative stress regulator, BosR, is a member of the Fur family of regulatory proteins and has been associated with direct or indirect control of the expression of genes involved in combating oxidative stress and strategic host adaptation, including sodA, napA, dbpA, and BB0646, as well as having an autoregulatory effect (6, 27, 42). The fact that dbpA is regulated by RpoN/RpoS and apparently via BosR suggests that there is a possible cooperative effect between these distinct regulatory systems in response to various environmental cues, such as temperature, pH, and/or dissolved gases. Since in the study described here we focused on the effect of dissolved gases on borrelial gene expression, experiments were conducted to ascertain how bosR was regulated in response to dissolved O2 and CO2 levels. BosR synthesis increased under anaerobic conditions compared to the synthesis during microaerophilic growth independent of differences in pH or bicarbonate (Fig. 2, 3, and 6). When either the level of dissolved CO2 was reduced or dissolved CO2 was absent, BosR synthesis was dramatically decreased compared to the synthesis of BosR when the cells were grown either anaerobically or microaerophilically in 5% CO2 (Fig. 4 and 7). However, surprisingly, the quantitative RT-PCR analysis revealed no obvious transcriptional induction under the conditions employed (i.e., anaerobiosis with and without CO2) (Fig. 8). Thus, bosR is presumably regulated at the translational or posttranslational level rather than at the transcriptional level.

This study demonstrated that dissolved CO2 functions as an additional environmental signal that modulates gene expression and protein production by *B. burgdorferi*. Although all of the molecules involved in responding to dissolved CO2 have yet to be characterized, it is conceivable that the PAS domain of BB0764 senses CO2, as well as several other environmental cues, and initiates an Rrp2-dependent cascade that interfaces with the RpoN-RpoS regulatory pathway to increase the transcription of lipoprotein genes (i.e., ospC and dbpA). Interestingly, several adhesin genes purported to be important for mammalian infection (i.e., dbpA and BBK32) were also induced in the presence of CO2. There was also increased synthesis of BosR, the borrelial oxidative stress regulatory protein, and NapA in the presence of CO2, suggesting that in addition to the RpoN/RpoS inducible system, an additional multifactorial adaptive response by *B. burgdorferi* is employed. Further studies are necessary to elucidate the responses of these regulatory pathways to different dissolved oxygen and CO2 levels within the context of the pathogenic mechanisms operative during *B. burgdorferi* infection.

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