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Borrelia burgdorferi Alters Its Gene Expression and Antigenic Profile in Response to CO_2 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 CO_2, 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% CO_2, 3% H_2, 0.087 ppm O_2) and microaerophilic (1% CO_2, 3.48 ppm O_2) 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 CO_2. In this study we identified CO_2 as a factor contributing to the observed change in NapA synthesis. Specifically, a reduction in the level of dissolved CO_2, independent of O_2 levels, resulted in reduced NapA synthesis. BosR, DbpA, OspC, and RpoS synthesis was also decreased with the displacement of CO_2. Quantitative reverse transcription-PCR indicated that the levels of the *dbpA*, *ospC*, and BBK32 transcripts are increased in the presence of CO_2, indicating that these putative borrelial virulence determinants are regulated at the transcriptional level. Thus, dissolved CO_2 may be an additional cue for borrelial 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 CO_2/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 CO_2/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 CO_2 also contributes to this process. The results presented here indicate that dissolved CO_2 levels affect expression of the genes examined previously (41), as well as several additional loci. Thus, the levels of dissolved CO_2 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,

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TABLE 1. Oligonucleotides used for quantitative RT-PCR

Open reading frame	Designation	Primers (5′-3′)	
		Forward	Reverse
BB0147	*flaB*	CAGCTAATGTTGCAAATCTTTTCTCT	TTCCTGTTGAACACCCTCTTGA
BB0153	*sodA*	GCTGCCAGAACTTGGTTATGATTAT	AAAACCATTATGATGCTTGCTATGAT
BB0647	*bosR*	ACCCTATTCAACTTGACGATATTAAAGAT	GCCCTGAGTAAATGATTTCAATAGATT
BBa24	*dbpA*	CAGATGCAGCTGAAGAGAATCCT	ACCCTTTGTAATTTTTCTCTCATTTTT
BBB19-MSK5	*ospC* (MSK5)	CGGATTCTAATGCGGTTTTACTTG	CAATAGCTTTAGCAGCAATTTCATCT
BBB19-AH130	*ospC* (AH130)	TGAACTTGCTACTAAAGCTATTGGTAAAA	CCTGCTAACAATGTTCCATTATGC
BBK32		GAATATAAAGGGATGACTCAAAGTT	TTTGGCCTTAAATCAGAATCTATAGTAAGA

infectious, clonal B31 derivative MSK5 and clonal 297 derivatives AH130 (parent), AH210 (*rpoS* mutant), AH212 (*rpoN* mutant), and AH123 (*rpoN* mutant complemented by wild-type *rpoN*) were used in this study (26, 29). Microaerophilic cultures were grown statically at 32°C and pH 7.8 with 1% atmospheric CO_2 in complete BSK-II medium. The anaerobic culture conditions consisted of 5% CO_2, 3% H_2, 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 1×10^4 cells per ml and grown to a density of 5×10^7 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 pHs of anaerobic cultures with the same starting density were adjusted to 7.8. CO_2 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 CO_2 level from 5,800 ppm to undetectable levels. Dissolved CO_2 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 CO_2 at a density of 5×10^7 cells per ml. MSK5 was grown under anaerobic and microaerophilic conditions in modified BSK-II medium containing decreasing amounts of $NaHCO_3$, so that the medium contained 25 mM, 15 mM, 5 mM, 1 mM, or no added $NaHCO_3$; the highest concentration represented conventional BSK-II medium. The $NaHCO_3$-modified medium was supplemented with NaCl to maintain a constant osmotic balance. To examine the role of different CO_2 levels with a constant O_2 content, AH130 and MSK5 were also grown statically in the presence of atmospheric O_2 (3.48 ppm dissolved O_2) and 5% atmospheric CO_2 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 antisera 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., 5×10^7 cells per ml), and total RNA was isolated from 1×10^9 cells using a Versagene kit (Gentra 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 *B. burgdorferi* strain to confirm that DNA 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 anaerobiosis with CO_2 compared to the levels observed during anaerobiosis without CO_2 were determined by the $\Delta\Delta C_t$ 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 CO_2 had no influence on gene expression. A P value of ≤ 0.01 was used. The data set for each gene was based on $\Delta\Delta C_t$ values (relative to *flaB* for *B. burgdorferi* grown with or without CO_2) 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 CO_2, 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 CO_2, and the anaerobic environment was defined as 5% CO_2 and 3% H_2 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).

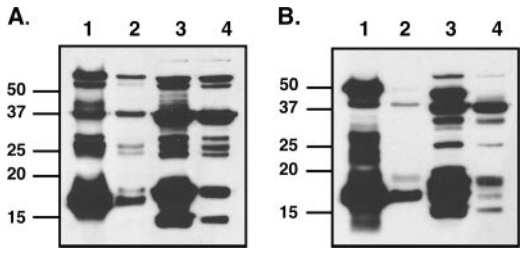

FIG. 1. Immunoreactive proteins induced when *B. burgdorferi* AH130 and MSK5 are grown under anaerobic or microaerophilic conditions with constant CO_2 levels. Protein samples were probed with serum from a patient with chronic Lyme borreliosis (A) or with infection-derived mouse serum (B). Lanes 1 and 2 contained protein from the strain 297 clonal derivative AH130 grown anaerobically and microaerophilically, respectively. Lanes 3 and 4 contained protein from the strain B31 clonal derivative MSK5 grown anaerobically and microaerophilically, respectively. The numbers on the left indicate the molecular masses (in kilodaltons) of protein markers.

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 O_2, respectively (41). The results obtained here using an anaerobic chamber

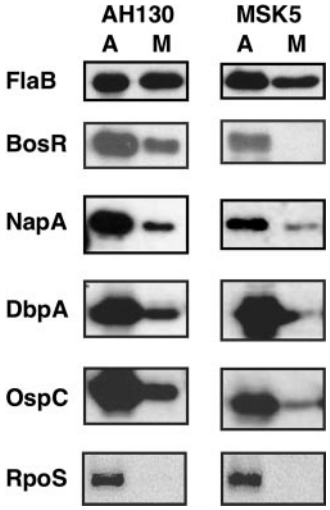

FIG. 2. Antigens from *B. burgdorferi* strains AH130 and MSK5 are synthesized differentially when the organisms are cultivated under anaerobic (lanes A) and microaerophilic (lanes M) growth conditions. Protein lysates from each strain grown under each condition were resolved by SDS-PAGE, immobilized on polyvinylidene difluoride membranes, and probed with antiserum specific for the antigens indicated on the left. Constitutively synthesized FlaB was used as a control to demonstrate that there were equivalent amounts of protein in the samples.

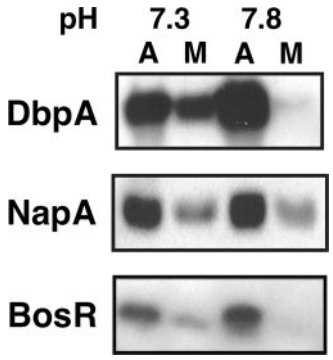

FIG. 3. Modulation of the protein produced under the experimental conditions used is independent of the pH. A *B. burgdorferi* B31 derivative, MSK5, was grown under anaerobic conditions at pH 7.3 and 7.8 (lanes A) and under microaerophilic conditions at pH 7.3 and 7.8 (lanes M). Protein samples were probed with monospecific antisera to the borrelial proteins DbpA, NapA, and BosR.

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 CO_2/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 in medium whose pH was adjusted to 7.8 and concurrently, the pH of microaerophilic medium was adjust to 7.3 to reflect the hydrogen ion concentration of the alternate growth conditions (Fig. 3). Under these conditions, DbpA, NapA, and BosR protein production did not change in response to pH compared to the protein profiles observed for microaerophilically or anaerobically grown *B. burgdorferi*, as shown in Fig. 2. 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 CO_2 and bicarbonate on *B. burgdorferi*. The effect of CO_2 and bicarbonate levels on borrelial protein synthesis was analyzed, as the atmospheric level of CO_2 was 4% higher under the defined anaerobic growth conditions than

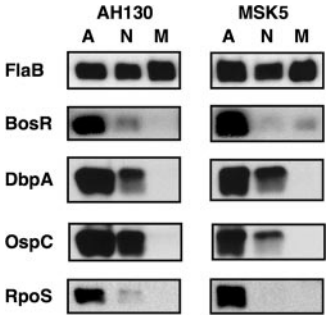

FIG. 4. Displacement of dissolved CO_2 modulates antigen synthesis in *B. burgdorferi*. AH130 and MSK5 cultures were grown anaerobically (0.087 ppm O_2, 5% CO_2) (lanes A), anaerobically with CO_2 removed by N_2 gas displacement (0.087 ppm O_2, no CO_2) (lanes N), and microaerophilically (3.48 ppm O_2, 1% CO_2) (lanes M). 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.

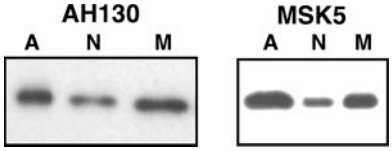

FIG. 5. Synthesis of NapA is affected by CO_2 levels. NapA production was assessed by immunoblot analysis of protein lysates from strains AH130 and MSK5 grown either anaerobically (0.087 ppm O_2, 5% CO_2) (lanes A), anaerobically with CO_2 removed by N_2 gas displacement (0.087 ppm O_2, no CO_2) (lanes N), or microaerophilically (3.48 ppm O_2, 1% CO_2) (lanes M).

under the microaerophilic growth conditions. Dissolved O_2 and CO_2 are present under both in vivo and in vitro growth conditions, and in this study nitrogen gas was used to indiscriminately displace both dissolved O_2 and CO_2 from the growth medium. A CO_2 probe was used to measure dissolved CO_2 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 CO_2 on gene expression and protein production, anaerobic cultures were treated with nitrogen gas to completely displace the dissolved CO_2 (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 DbpA and OspC levels were greatly increased when CO_2 was present, suggesting that in addition to O_2, CO_2 levels modulate gene expression and protein production in *B. burgdorferi* (Fig. 4).

Dissolved CO_2 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 *B. burgdorferi* was grown anaerobically in the presence of 5% CO_2 compared to the synthesis in cells grown anaerobically without CO_2 or in microaerophilically grown *B. burgdorferi* (Fig. 4). Note that BosR, DbpA, and OspC, although not readily detectable (Fig. 4), were synthesized in microaerophilically grown *B. burgdorferi* if the exposure time of the blot was increased (data not shown). This suggests that the production of some borrelial proteins is influenced by dissolved CO_2 levels, as well as dissolved oxygen levels. The enhanced production of DbpA and OspC is consistent with the increased synthesis of RpoS since previous studies have demonstrated that borrelial RpoS is required for expression of *dbpA* and *ospC* (26, 53). Taken together, these results suggest that CO_2 is an additional signal that is integrated by *B. burgdorferi* to modulate gene expression.

Since RpoS, OspC, and DbpA levels were increased by anaerobiosis, we examined whether the increases were dependent on the well-characterized borrelial RpoN-RpoS regulatory system (8, 19, 26, 53, 55). To assess this, a *B. burgdorferi rpoN* mutant, an *rpoS* mutant, and the complement of the *rpoN* mutant were grown under microaerophilic, anaerobic, and nitrogen-treated anaerobic conditions. In the presence of CO_2, the synthesis of RpoS, DbpA, and OspC was significantly reduced in the absence of RpoN, but the levels reverted to wild-type levels in the *rpoN* complemented strain (data not shown), indicating that increased production of RpoS, OspC, and DbpA is dependent on a functional RpoN and apparently does not involve other regulatory loci, at least under these experimental conditions.

Previous results demonstrated that NapA levels were reduced when *B. burgdorferi* was grown in BSK-II medium purged with nitrogen gas compared to the levels in microaerophilically grown samples (41). In the previous study, the decrease 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% CO_2. Surprisingly, appreciably more NapA was produced by cells under these conditions than by cells grown anaerobically without CO_2 (Fig. 5). Furthermore, the reduction in the NapA level was apparently dependent on a decrease in the CO_2 level independent of oxygen since the levels of NapA produced anaerobically with CO_2 were slightly greater than the levels found in microaerophilically grown *B. burgdorferi* (Fig. 2 and 5). In fact, the level of NapA production was maximal when O_2 was missing and CO_2 was present, followed by microaerophilic growth; anaerobic growth without CO_2 resulted in the smallest amount of NapA for both strain MSK5 and strain AH130 (Fig. 5). This hierarchy of regulation is consistent with the amounts of dissolved CO_2 available in the samples; that is, the larger the amount of CO_2 in the sample, the larger the amount of NapA produced. These results indicate that CO_2/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 CO_2/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 CO_2 and the 25 mM $NaHCO_3$ in BSK-II medium. To examine the effect of bicarbonate levels in anaerobically (5% CO_2) and microaerophilically (1% CO_2) grown *B. burgdorferi*, the concentration of sodium bicarbonate was decreased in the borrelial growth me-

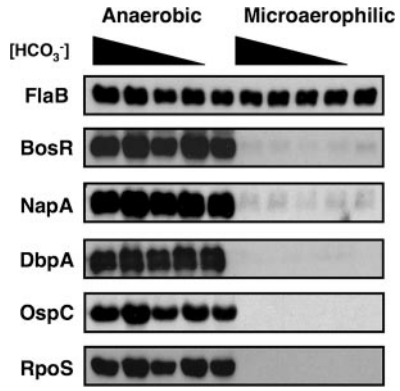

FIG. 6. Antigen synthesis is influenced by CO_2 rather than by bicarbonate levels. A strain B31 derivative, MSK5, was grown with 25 mM (conventional concentration), 15 mM, 5 mM, 1 mM, and no added $NaHCO_3$ under both anaerobic and microaerophilic conditions. Protein lysates from each strain grown under 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.

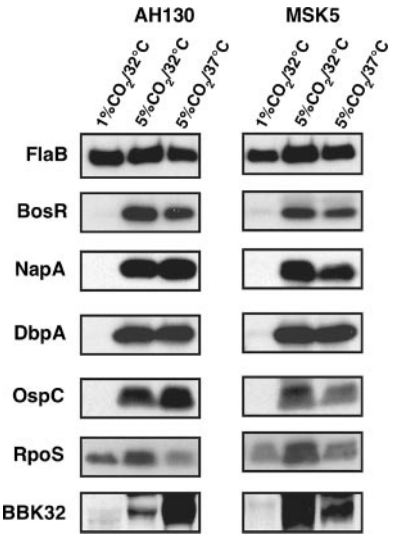

FIG. 7. Increased CO_2 levels in the presence of atmospheric O_2 result in levels of antigen synthesis comparable to levels observed for anaerobically grown *B. burgdorferi*. AH130 and MSK5 were grown under conventional microaerophilic growth conditions (1% CO_2 with atmospheric O_2) and with increased levels of CO_2 and atmospheric O_2 (either 5% CO_2 at 32°C or 5% CO_2 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% CO_2 at 32°C following longer exposure times.

dium while the overall salt content was controlled (Fig. 6). MSK5 was grown anaerobically or microaerophilically with additional $NaHCO_3$ added to the medium at a concentration of 25 mM, 15 mM, 5 mM, or 1 mM or with no additional $NaHCO_3$ in addition to the equilibrium that was already present between bicarbonate and dissolved CO_2. Under these conditions, changes in antigen production were evaluated (Fig. 6). FlaB was not affected by bicarbonate levels and served as a control for equal protein loading (Fig. 6). Decreasing the concentration of sodium bicarbonate did not influence the synthesis of BosR, DbpA, OspC, RpoS, or NapA (Fig. 6). The synthesis of these antigens was consistent with the synthesis observed for anaerobic and microaerophilic growth conditions (Fig. 2, 4, and 5), and the results indicated that the level of production observed is not appreciably affected by the addition of bicarbonate (Fig. 6).

To confirm the finding that 5% CO_2 altered borrelial protein synthesis and to examine the role of oxygen in this process, AH130 and MSK5 were grown microaerophilically in the presence of 5% CO_2 with atmospheric O_2 (3.48 ppm dissolved O_2) at 32°C and 37°C (Fig. 7). Then protein production was evaluated compared to the production under conventional microaerophilic growth conditions (1% CO_2, atmospheric O_2, 32°C), and FlaB antibody was used to control for equivalent protein loading. As in previous experiments, there was increased synthesis of BosR, NapA, DbpA, OspC, RpoS, and BBK32 in the presence of 5% CO_2 compared to the synthesis in the presence of 1% CO_2 when the oxygen levels remained constant. It is important to note that BosR, NapA, DbpA, OspC, and BBK32 were detectable in the samples grown in the presence of 1% CO_2 at 32°C when the blot was exposed for a longer time, indicating that there was a great difference in protein production when CO_2 was limiting (Fig. 7). For RpoS, there was apparently more synthesis under microaerophilic conditions than observed previously (Fig. 4) due to the longer exposure times required to see antigen in any of the samples tested. Regardless, the overall trend (i.e., more RpoS as the level of CO_2 increased) was consistent. These results suggest that CO_2 levels modulate the production of borrelial proteins that are believed to be important for adaptation in the mammalian host environment.

To examine the effect of temperature on the response to CO_2, cultures were grown at 32°C with 1% CO_2 or 5% CO_2 and compared with borrelial cells grown at 37°C with 5% CO_2. In nearly all cases, an increase in the CO_2 level resulted in enhanced synthesis of borrelial proteins independent of the temperature (Fig. 7). For strain MSK5 (a B31 derivative), an increase in the temperature did not enhance synthesis of the antigens tested (Fig. 7). A variable response was observed for strain AH130 (a 297 derivative) compared to MSK5, particularly for OspC and BBK32, indicating that multiple factors affect certain strains differently to alter the synthesis of the protein species.

CO_2 regulated gene expression. To determine the method of CO_2 regulation, the results of quantitative real-time RT-PCR of total RNA were analyzed for select genes of *B. burgdorferi* strains AH130 and MSK5. Total RNA from these strains was isolated from three independent anaerobic and nitrogen-treated anaerobic cultures to allow a comparison of the effect of dissolved CO_2 on gene expression independent of dissolved O_2 (Fig. 8). An endogenous, constitutively expressed gene, *flaB*, was used as a normalization control. The results showed

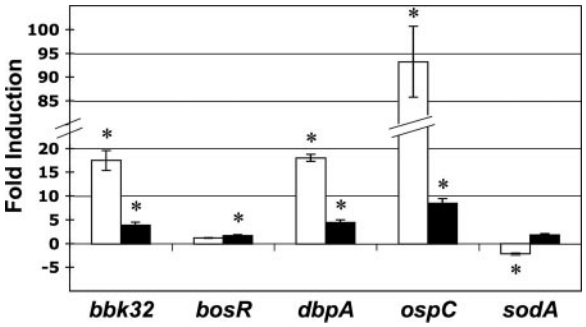

FIG. 8. Quantitative RT-PCR indicates that *B. burgdorferi* modulates gene expression at the transcriptional level when it is grown anaerobically in the presence of CO_2. The data show the ratio of the level of transcripts produced under anaerobic conditions with CO_2 present to the level of transcripts produced under anaerobic conditions without CO_2 for the genes indicated at the bottom and are the averages for three experiments, each done in triplicate. The error bars indicate standard errors. The results for strains AH130 (a 297 derivative) and MSK5 (a B31 derivative) are indicated by open bars and solid bars, respectively. The data for all samples were normalized to the data for an endogenous control, *flaB*, whose transcription was not affected by either of the conditions used in this experiment. The asterisks indicate that differences are statistically significant at a P value of <0.01.

that there was a dramatic change at the transcriptional level for BBK32, *dbpA*, and *ospC* in the presence of CO_2. 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 in the presence of dissolved CO_2 than when cells were grown anaerobically without detectable dissolved CO_2. 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 CO_2. 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 CO_2 of lipoprotein genes (i.e., *ospC*, *dbpA*, and BBK32) involved in the adherence of *B. burgdorferi* to host structures suggests that CO_2 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 CO_2. Specifically, a less-than-twofold 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 CO_2 was present was statistically significant (Fig. 8). However, for *bosR*, for which there was great induction of protein production when CO_2 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 O_2 and CO_2 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 CO_2 as a result of cellular respiration. In addition, as *B. burgdorferi* disseminates in a mammalian host, the levels of dissolved O_2 and CO_2 in the various tissue locales are likely to vary. Thus, differences in dissolved O_2 and CO_2 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 CO_2 and O_2 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 O_2, the dissolved CO_2 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 CO_2 levels and the maintenance of infection-associated plasmids of *B. burgdorferi* during in vitro cultivation, suggesting that CO_2 imposes a selective pressure that preserves genome stability and thus infectivity (4). The ability to sense CO_2, either as CO_2 or as bicarbonate, influences the expression of virulence determinants in other pathogenic organisms, suggesting a potential role for CO_2 sensing in borrelial virulence gene expression (5, 28, 31, 52). Interestingly, *Bacillus anthracis* responds to CO_2/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 CO_2 is via the well-characterized enzyme adenylyl cyclase (13, 28, 31, 52). Specifically, adenylyl cyclase is able to directly sense bicarbonate or CO_2, 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 adenylyl 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 O_2 and CO_2, 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 CO_2 (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 CO_2 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 CO_2 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 CO_2 levels (Fig. 4 and 5) and that, in addition to O_2, CO_2 serves an inducible signal that modulates a subset of genes in *B. burgdorferi*. Increasing CO_2 levels in the presence of atmospheric oxygen confirmed the effect of CO_2 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 CO_2 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 CO_2 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 O_2 and CO_2 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 CO_2 was reduced or dissolved CO_2 was absent, BosR synthesis was dramatically decreased compared to the synthesis of BosR when the cells were grown either anaerobically or microaerophilically in 5% CO_2 (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 CO_2) (Fig. 8). Thus, *bosR* is presumably regulated at the translational or posttranslational level rather than at the transcriptional level.

This study demonstrated that dissolved CO_2 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 CO_2 have yet to be characterized, it is conceivable that the PAS domain of BB0764 senses CO_2, 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 CO_2. There was also increased synthesis of BosR, the borrelial oxidative stress regulatory protein, and NapA in the presence of CO_2, 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 CO_2 levels within the context of the pathogenic mechanisms operative during *B. burgdorferi* infection.

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