Articles

The Two-Component System *Bacillus* Respiratory Response A and B (BrrA−BrrB) Is a Virulence Factor Regulator in *Bacillus anthracis*†

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ABSTRACT: *Bacillus anthracis*, a bioterrorism threat as well as an agricultural concern, has complex mechanisms for regulation of its major virulence factors. Genome searches identified the putative two-component system that we designated *Bacillus anthracis* respiratory response (Brr)A−BrrB. A *brrA* deletion strain was constructed, and real-time reverse transcriptase polymerase chain reaction and Western blot analysis were used to assess the effect of BrrA−BrrB on levels of virulence factors, the regulator *atxA*, and growth characteristics. When *brrA* was deleted, the genes for anthrax toxins (lethal factor, protective antigen, and edema factor) where expressed 4−6-$\log_{10}$-fold less than in the parent Sterne strain. The global regulator *atxA* was downregulated when compared to *atxA* in the Sterne strain. Thus, the BrrA−BrrB two-component system positively regulates *B. anthracis* toxin genes as well as the *atxA* regulator. Aerobic growth was not affected by the Δ*brrA* mutation, but colonies showed differences in morphology, the mutant did not sporulate, and the strain lost the ability to synthesize cytochrome aa_3. Gel-shift mobility assays demonstrated that BrrA bound to the promoters of genes for both protective antigen and cytochrome aa_3, demonstrating that BrrA is a transcription factor. BrrA−BrrB has sequence similarity with the virulence regulator SrrA−SrrB in *Staphylococcus aureus* and the aerobic/anaerobic regulator, ResD−ResE, in *B. subtilis*, and appears to share regulatory mechanisms with ResD−ResE.

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive spore-forming rod, whose natural environment is the soil. However, when out of its natural environment, the organism causes serious disease in almost any mammal, especially grazing animals, such as sheep or cattle (*1*). Three types of anthrax disease have been described, depending upon the route of entry. The spores may enter via the skin, respiratory tract, or digestive tract (*2*, *3*). The cutaneous form of anthrax is most recognizable because it results in a black eschar on the skin. However, in humans, this is the least severe form of the disease and is easily treated with antibiotics. The other two forms of the disease are more severe and highly lethal.

B. anthracis carries two plasmids that are recognized as primarily responsible for virulence, pX01 and pX02. The plasmid pX01 carries the genes encoding the toxin proteins lethal factor (*lef*),[1] edema factor (*cya*), and protective antigen (*pagA*). Lethal factor is a zinc endoprotease, interfering with functions of the mitogen-activated protein (MAP) kinase pathway (*4*), leading to immense cytokine release, and resulting in shock-like symptoms. Edema factor is an adenylate cyclase, catalyzing cyclic AMP synthesis in the cytosol of cells (*5*). Both of these proteins are coupled with protective antigen to form functional toxins. Protective antigen interacts with one of the two identified host anthrax toxin receptors, tumor endothelial marker-8 (TEM8) or capillary morphogenesis gene-2 (CMG2), and forms pores through which edema factor and lethal factor pass (*6*−*8*) into the cytosol. The second plasmid pXO2 carries the genes for capsule synthesis, *capBCAD*, and is required for immune evasion in the course of infection (*9*).

Environmental conditions are important in *B. anthracis* virulence gene expression. *B. anthracis* requires both 37 °C temperatures and dissolved CO_2 or bicarbonate in growth media for toxins to be produced (*10*). The gene *atxA* (anthrax toxin activator) has been characterized as a global regulator of toxin expression because, when the gene is not expressed, toxin genes are not expressed (*11*). However, much remains unknown related to the regulation of toxin production, especially regulation by CO_2/bicarbonate. The mechanism

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[1] Abbreviations: *lef*, lethal factor gene; *cya*, edema factor gene; *pagA*, protective antigen gene; MAP, mitogen-activated protein; TEM8, tumor endothelial marker-8; CMG2, capillary morphogenesis gene-2; *capBCAD*, capsule genes; *atxA*, anthrax toxin activator gene; Srr, staphylococcal respiratory response; Brr, *Bacillus anthracis* respiratory response; LB, Luria broth; RPM, revolutions per minute; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; *gyrB*, gyrase B gene; *ctaA*, cytochrome aa_3 gene; TBE, Tris−borate−EDTA.

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by which *atxA* regulates toxin transcription is only just starting to be elucidated. The gene product has not been demonstrated to have DNA-binding activity but does have conserved histidine residues that serve as phosphorylation sites (*12*). Phosphorylation at site H199 activates toxin transcription, while phosphorylation at site H379 inhibits toxin transcription. The expression levels of *atxA* are constant across various CO_2/bicarbonate concentrations but are variable with temperature and various levels of glucose (*12*, *13*). A two-component system that senses the environment and then correspondingly regulates other genes is a potential additional mechanism by which toxin genes may be regulated.

Two-component systems are commonly associated with the regulation of virulence factors under strict environmental conditions (*14*, *15*). The two-component system in *S. aureus*, staphylococcal respiratory response (Srr)A−SrrB, is particularly interesting because it is similar in sequence to the ResD−ResE two-component system in *B. subtilis*, an organism in the same genus as *B. anthracis* (*16*). ResD−ResE is a regulator of anaerobic and aerobic metabolism in *B. subtilis*. Under anaerobic conditions, ResD−ResE regulates genes for nitrate reductases because *B. subtilis* uses nitrogen as the final electron receptor during anaerobic respiration. Under aerobic conditions, ResD−ResE controls genes that synthesize cytochrome aa_3, the most utilized cytochrome for *B. subtilis* respiration (*17*).

In the present studies, we identified a putative homologue of ResD−ResE in *B. anthracis* via sequence similarity. When the *B. anthracis* system, tentatively designated *B. anthracis* respiratory response (Brr), was deleted from the genome, aerobic growth was not affected but the organism lost the ability to grow anaerobically, sporulate, and produce cytochrome aa_3, properties in common with ResD−ResE. In addition to regulating anaerobic and aerobic metabolism, BrrA also positively regulated all three exotoxin virulence genes, *lef*, *pag*, and *cya*, as well as another global regulator of virulence, *atxA*. These studies provide a novel mechanism by which metabolism and toxins are regulated in *B. anthracis*.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. The Sterne strain 34F2 (Colorado Serum Co., Denver, CO) was used in all studies (Table 1 lists bacteria and plasmids used in these studies). For growth and toxin experiments, one colony from a plate was inoculated in Luria broth (LB) and grown to stationary phase over 18 h at 37 °C. Approximately 750 μL of the overnight culture was inoculated into fresh medium to give an absorbance at 600 nm wavelength of 0.1 in 25 mL of R medium (*18*, *19*) supplemented with 0.8% sodium bicarbonate. The culture was grown with mild shaking [120 revolutions per minute (RPM)] at 37 °C with 7% CO_2. To measure sporulation frequency, overnight cultures were added to Schaeffer's sporulation media and incubated at 37 °C for 12, 24, 36, or 48 h (*20*). Spores were selected by incubating a sample of the culture at 80 °C for 10 min. If an antibiotic was required in the medium, 5 μg/mL of erythromycin was used.

Construction of brrA Null Mutant and Complemented Strain. Genomic DNA was isolated from the Sterne strain using a DNA preparation kit (Qiagen, Valencia, CA). Single-overlap extension polymerase chain reaction (PCR), as described by Senanayake et al. (*21*), was used to create a DNA construct containing the flanking regions of *brrA* with the region of *brrA* deleted. Briefly, approximately 500 bp regions flanking *brrA* were amplified using PCR along with a 13 bp homologous region of the opposite flanking region. The upstream 5′ and downstream 3′ regions were amplified using primers shown in Table 2. The antisense primer for the upstream region and the sense primer for the downstream region were reverse complements of each other, being homologous to both the 3′ region of the upstream portion and the 5′ region of the downstream portion. The products were amplified over 30 cycles using Reddy mix PCR Mastermix (Abgene, Epsom, U.K.) with a melting temperature of 95 °C for 1 min, an annealing temperature of 45 °C for 1 min, and an extension temperature of 72 °C for 1 min. The resulting products were then used as templates in a second PCR reaction using the antisense primer for the upstream region and the sense primer for the downstream region to create a product where the two flanking segments were joined. These products were amplified using Reddy mix extensor PCR Mastermix (Abgene, Epsom, U.K.) with 30 cycles and a melting temperature of 95 °C for 1 min, an annealing temperature of 55 °C for 1 min, and an elongation temperature of 68 °C for 2 min. The products were purified using the PCR cleanup kit (Qiagen, Valencia, CA) before being digested with *Hin*d III and *Xba* I (New England Biolabs, Ipswich, MA) and then ligated with T4 ligase (Invitrogen, Carlsbad, CA) into the *Hin*d III and *Xba* I restriction sites into the temperature-sensitive vector pG-host$^+$5 to create pSMV17 (*22*). To introduce the plasmid

Table 1: Strains and Plasmids

strain or plasmid	relevant characteristics[a]	source or reference
strains		
E. coli XL-2 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q}*ZΔM15 Tn10* (Tet^r) Amy Cam^r]	Stratagene
B. anthracis		
34F2	pX01$^+$ pX02$^-$	Colorado Serum Co.
BA18	Δ*brrA*	this study
BA30	BA18 electroporated with pCE104, Em^r	this study
BA31	BA18 electroporated with pSMV9, Em^r	this study
plasmids		
pCE104	shuttle vector containing pE194 and pUC18, Em^r	*24*
pG+host5	shuttle vector with temperature-sensitive Gram-positive origin of replication	*22*
pSMV9	pCE104 with *brrAB* fragment cloned into the *Xma* I restriction site	this study
pSMV17	pGhost with 560 bp +2 region of *brrA* and +5 to +524 region of *brrA* cloned into the *Hin*d III and *Xba* I restriction sites	this study
pET28	Kan^r	Novagen
pSMV1	pET28 with *brrA* cloned into *Bam* HI and *Nde* I restriction sites	this study

[a] Tet^r, tetracycline resistance; Em^r, erythromycin resistance; Kan^r, kanamycin resistance.

Table 2: Primers Used in This Study

primer function	forward primer (+) and antisense primer (−)
clone upstream 500 bp region of *brrA*	(+)5′-TTTCATCTAGAGTGCATTATTTT-3′ *Xba* I[a]
	(−)5′-TATGGTCATCCACATATTATGTTGC-3′
clone downstream 500 bp region of *brrA*	(+)5′-GCAACATAATAGTGGATGACCATA-3′
	(−)5′-GCAATCAAGCTTGATAAGAATATAA-3′ *Hin*d III
clone *brrAB* into pCE104, *brrA* deletion confirmation	(+)5′-TATCCCGGGTTTACATTCATACGCATAG-3′ *Xma* I
	(−)5′-AACCCCGGGCTAAATTATACGATTCGG-3′ *Xma* I
clone *brrA* in pET28 for protein purification	(+)5′-CGCAACGGCATATCATGGAAAATGAATC-3′ *Bam* HI
	(−)5′-CTACTCCGGATCCTCAGTCGTTCACAACC-3′ *Nde* I
qRT-PCR primers	
gryB	(+)5′-GGTGTTGGGGCATCTGTACGT-3′
	(−)5′-TAAATCCGCAACCGGAATAC-3′
lef	(+)5′-CTTAAGGAACATCCCACAGAC-3′
	(−)5′-CTGTGGCTGCATATAATATCG-3′
pagA	(+)5′-AAGTGCATGCGTCGTTCTTT-3′
	(−)5′-TTTCAGCCCAAGTTCTTTCC-3′
cya	(+)5′-TGCGCTTTTCTTTAGCGTTTTC-3′
	(−)5′-TTTCTCAAATCCCCCTTTTTC-3′
atxA	(+)5′-GTGTTGTTCGCTATAACAATCTCC-3′
	(−)5′-GCTCTGAGGTTATAGACGCTACTG-3′
gel-shift probes	
ctaA	(+)5′- AAAACATGAACTTTTTGTGTCGAA-3′
	(−)5′-TGGATAAGTGACAATACGTTTT -3′
pagA	(+)5′-TTCAAAAACAGCTTCTGTGTCC -3′
	(−)5′- TTTTTGGCTTTTTAAACAGAACTT-3′
rpsC	(+)5′- GGTAAGCAAGTGGGTGAAGC-3′
	(−)5′- AATCGCAAATGCAGAGCACAA-3′

[a] Restriction enzyme recognition sites are underlined with the corresponding enzyme noted after the primer sequence.

into *B. anthracis*, pSMV17 was electroporated into competent cells using the method of Hoffmaster et al. (*23*). Transformants were selected on brain heart infusion agar plates (Difco, Franklin Lakes, NJ) containing erythromycin at the permissive temperature of 30 °C. To select for integration of the plasmid into the genome, liquid cultures were incubated at the nonpermissive temperature of 40 °C before being plated on brain heart infusion agar plates containing erythromycin and then incubated at 40 °C. To facilitate the loss of plasmids and, therefore, the second crossover event, colonies selected at the nonpermissive temperature were incubated and passaged for 3 days at the permissive temperature. The cultures were then plated on nonselective medium and then selective medium for the loss of antibiotic resistance.

For construction of a Brr-complemented Δ*brrA* strain, the entire *brrA*−*brrB* locus, including the predicted promoter region, was cloned into plasmid pCE104 to create pSMV9. This plasmid was introduced into the Δ*brrA* strain by electroporation to create strain BA31. pCE104 is a shuttle plasmid (*24*) that contains plasmids pE194 and pUC19, thus capable of replicating in both *B. anthracis* and *Escherichia coli*.

TMPD Plate Assay. *N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine (TMPD) (Sigma-Aldrich, St. Louis, MO) is an artificial terminal oxidase that can be oxidized by cytochrome aa_3. If oxidized, TMPD turns blue and can be seen when bacterial colonies to which TMPD was exposed turn blue. If not oxidized, the colonies remain their natural color. This is a convenient assay to determine if *B. anthracis* mutants produce cytochrome aa_3. The assay was adapted from the method of Mueller and Taber (*17*). Briefly, *B. anthracis* cells were plated on LB agar and grown for 16 h. The plates were incubated at −20 °C for 45 min to permeabilize cells and then sprayed with a thin layer of hairspray to fix the cells to the plate. An agar overlay was prepared consisting of 2.0 mL of molten 1.5% Bacto-agar (Difco, Franklin Lakes, NJ), 1.0 mL of 10% Triton X-100 in 0.1 M KPO_4 (pH 7.0), 0.2 mL of 10% sodium deoxycholate, 1.0 mL of 95% ethanol, and 1.0 mL of 1.0% TMPD. The solution was prepared immediately before use and poured over the cells. TMPD oxidase activity was detected within 5 min. KCN inhibits cytochrome aa_3 activity and was used as a control to show that oxidase activity was specific for the presence of cytochrome and not simply heme molecules.

Real-Time RT-PCR. Cultures were grown under conditions described above in R media supplemented with 0.8% sodium bicarbonate. A total of 3 mL of the culture was harvested at 4 h (0.7 absorbance at 600 nm wavelength), and the cells were pelleted (10000*g* at 15 min) and resuspended in TE buffer [(0.01 M Tris at pH 8.0 and 0.005 M ethylenediaminetetraacetic acid (EDTA)]. The bacteria were digested with 5 μg/mL lysozyme (Signal-Aldrich, St. Louis, MO) followed by total RNA isolation with the RNAqueous kit (Ambion, Austin, TX) according to the instructions of the manufacturer. Total RNA was then treated with DNA*free* (Ambion, Austin, TX) and LiCl precipitation to rid the RNA sample of contaminating DNA. Approximately 1 μg of total RNA was then reverse-transcribed with Superscript II (Invitrogen, Carlsbad, CA) using random hexamer primers. Approximately 0.5 μL of the 20 μL reverse-transcription reaction was used as a template for real-time PCR using SYBR-green PCR reagents from Qiagen (Valencia, CA), and the reactions were performed in an iCylcer machine (BioRad, Hercules, CA). Reactions were performed at 95 °C for 15 min for a hot start and then 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 20 s for a total of 40 cycles. Primers for use in the quantification of the mRNAs, gyrase B (*gyrB*), *pagA*, *lef*, *cya*, and *atxA*, are listed in Table 2. The *gyrB* gene was used as a normalizing gene because this gene has

been used successfully in other studies measuring mRNA in *B. anthracis* (*25*). Data were analyzed by the method described by Lival et al., but the method was altered slightly (*26*). The Ct value (the PCR cycle at which the amplified product reached a set threshold of detection) of the housekeeping gene (*gyrB*) was subtracted from the Ct value of each experimental gene. Then, the difference between the *gyrB* and the experimental gene was taken to the power of 2 to find the fold difference in the amounts of starting mRNA in the sample. The data reported represent three independent experiments.

Toxin Measurements. Cell-free culture media were harvested after 8 h and filtered through 0.22 μm filters. The culture media were then used in Western immunoblotting and enzyme-linked immunosorbent assay (ELISA) analysis. Primary antibodies were purchased from Chemicon International, Temecula, CA. LF antibody MAB8086 (used at 1/1000 dilution) and PA antibody MAB8081 (used at 1/1000 dilution) were used as primary antibodies in Western immunoblots. A secondary antibody, an anti-mouse IgG alkaline phosphate conjugate (Sigma-Aldrich, St. Louis, MO) was used at a 1/30 000 concentration. The Western immunoblots were developed colorimetrically by the addition of 50 μg/mL nitroblue tetrazolium and 1 μg/mL 5-bromo-4-chloro-3-indoyl phosphate. To detect BrrA, overnight cultures were harvested by centrifugation and digested with 20 μg/mL lysozyme. The cell membranes were then lysed by mechanical disruption using the Fast prep instrument (Qbiogene, Carlsbad, CA). The lysate was then centrifuged for 5 min at 20000*g* before Western analysis. The anti-BrrA antibody was prepared from rabbits immunized with purified BrrA. BrrA was purified from *E. coli* expressing *brrA* from the pET28 plasmid (Novagen, San Diego, CA) (Table 1) using the His-Bind kit (Novagen, San Diego, CA) according to the instructions of the manufacturer.

Gel-Shift Assays. To construct a probe for the *ctaA* promoter, ~50 bp of the sequence 180 bp 5′ of the translational start site for *ctaA* was PCR-amplified. The sequence selected was in the approximate region of the promoter sequence of the *B. subtilis ctaA* that has been shown to bind ResD (*27*). For the *pagA* promoter, ~100 bp of the sequence was amplified and selected ~35 bp 5′ of the translational start site of the P1 promoter region (*28*). To act as a negative control, 100 bp of the sequence from the internal coding sequence of *rpsC*, a ribosomal-binding sequence, was selected (for a list of primers, see Table 2). PCR products were labeled with digoxigenin using the digoxigenin gel-shift kit (Roche, Mannheim, Germany). BrrA was purified by the method described above, and either 0, 15, 30, 60, or 120 pM of BrrA was mixed with 1.2 pM of labeled probe with 0.1 μg of poly L-lysine and 1 μg of poly-[d(I-C)]. The reaction was allowed to incubate at room temperature for 20 min before electrophoresis in a 6% nondenaturing Tris−borate−EDTA (TBE) polyacrylamide gel (Invitrogen, Carlsbad, CA). DNA in the gel was electroblotted onto a positively charged membrane and developed with anti-digoxigenin antibody and chemiluminescent detection.

RESULTS

Identification of BrrA−BrrB. The two-component system SrrA−SrrB was previously identified in *S. aureus* as a global regulator of virulence. This system was identified on the basis of a similarity to a two-component system in *B. subtilis* ResD−ResE (*16*). ResD−ResE is a well-characterized global regulator of both anaerobic and aerobic metabolism. However, becasue *B. subtilis* is nonpathogenic, roles of ResD−ResE in virulence regulation could not be studied in this system. *B. subtilis* and *B. anthracis* share chromosomal similarity and many of the same physiology characteristics, including the ability to sporulate and grow aerobically as well as anaerobically using nitrogen as a final electron acceptor. This suggests that *B. anthracis* may have a ResD−ResE homologue because those are among the functions of this system. *B. anthracis* has almost 1 Mb more DNA in its genome than *B. subtilis*, including the two plasmids that carry recognized primary virulence factors (*29*). Therefore, we hypothesized that a ResD−ResE homologue in *B. anthracis* could have the same functions in *B. anthracis* as in *B. subtilis* but may also have an additional function to regulate exotoxin production.

The Ames strain (NC 003997, NCBI) genome sequence was searched for both a ResD−ResE two-component system and a SrrA−SrrB system, using TBLASTN (*30*). A putative two-component system (annotated in sequence as being the *B. anthracis* ResD−ResE proteins) was identified as having the highest similarity to both ResD−ResE and SrrA−SrrB (Figure 1). The identical sequence was identified in *B. anthracis* Sterne strain NC 005945. We tentatively named this system *B. anthracis* respiratory response A−B (BrrA−BrrB). The *brrAB* gene locus was 2486 bp, with *brrA* being 717 bp and *brrB* encompassing 1410 bp. *BrrA* and *brrB* overlapped by one nucleotide, suggesting that the genes were cotranscribed on an operon. BrrA was predicted to be 238 amino acids, encoding a 23 kDa protein, and BrrB was predicted to be 591 amino acids, encoding a 66.3 kDa protein. A domain search of the Conserved Domain Database predicted BrrA to be a response regulator and contain a DNA-binding region (*31*), similar to ResD. In addition, BrrB was predicted to have the same domains as ResE, such as HAMP, histidine kinase, and ATPase (*32*, *33*). BrrA, the putative response regulator in *B. anthracis*, at the amino acid level, was 75% identical (83% similar) to *B. subtilis* ResD, while BrrB was predicted to be 47% identical (67% similar) to ResE. BrrA was also 70% identical (77% similar) to *S. aureus* SrrA and BrrB and 31% identical (52% similar) to SrrB.

To characterize BrrA−BrrB function, *brrA* was deleted from the genome via allelic exchange. Deletion of the gene was confirmed using PCR (Figure 2A) and sequencing across the region. BrrA was detected in the parent Sterne and *brrA−brrB* complemented strain, BA31, but not in the Δ*brrA* mutant, BA18 (Figure 2B).

Growth of Strains and Phenotypic Effects. The Δ*brrA* mutant (BA18), parent Sterne strain, and BA31 (complemented strain) were grown under toxin, producing conditions (R medium, 0.8% bicarbonate with 7% CO_2, and 37 °C) over 10 h to compare aerobic growth rates (Figure 3). No difference was seen among the strains, demonstrating that *brrA* had no effect on aerobic growth rates under toxin-producing conditions. ResD−ResE in *B. subtilis* is responsible for regulating the nitrate reductase genes that allow for anaerobic growth (*34*). To determine if *brrA−brrB* was required for anaerobic growth, the parent strain, BA18

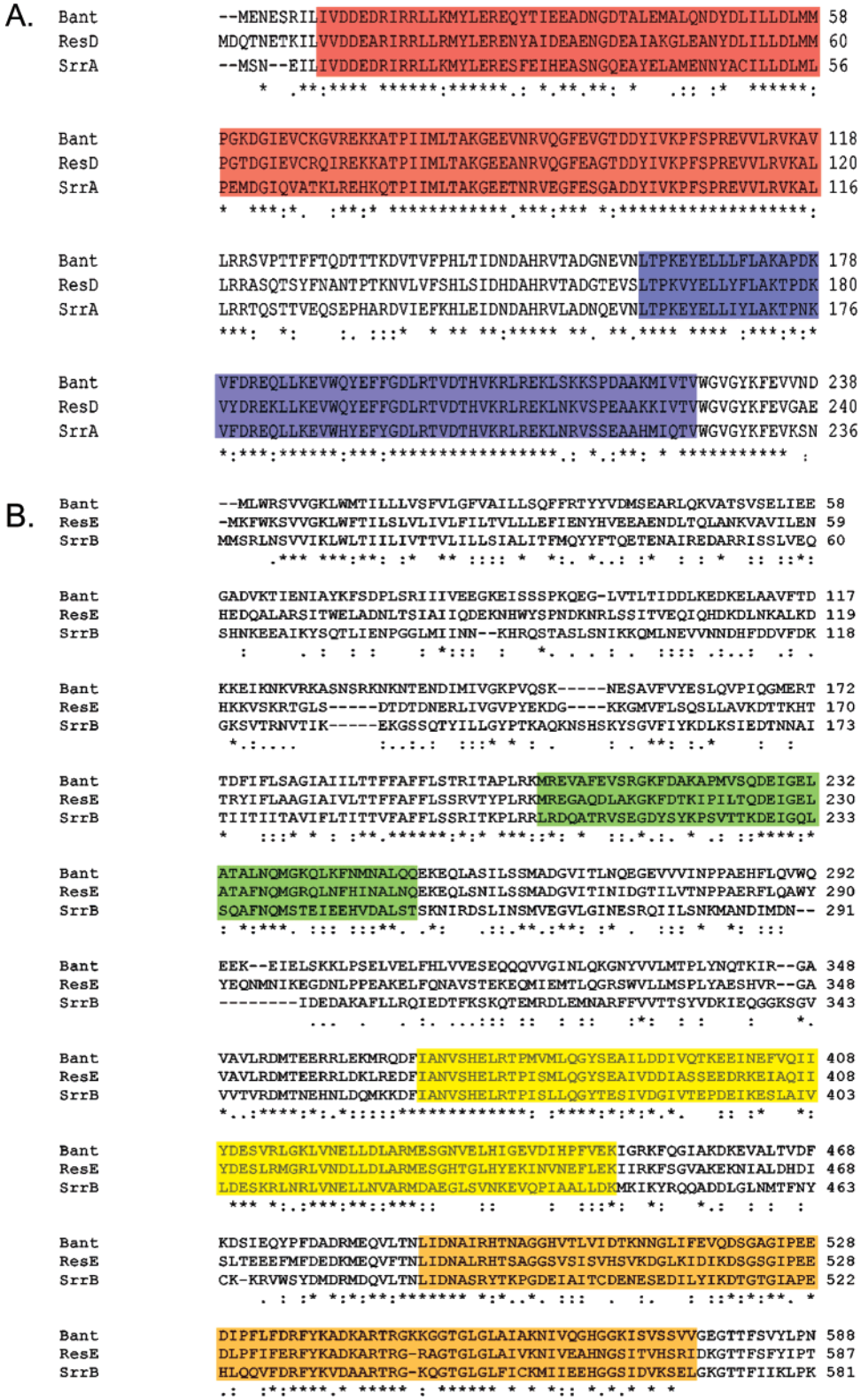

FIGURE 1: Clustal W global alignment of protein sequences (*39*). (A) SrrA, ResD, and the *B. anthracis* (Bant) putative protein, BrrA, are the predicted DNA-binding proteins (response regulators). The red region indicates the approximate region of the response receiver domain, and the blue region indicates the predicted DNA-binding domain. (B) SrrB, ResE, and the *B. anthracis* putative protein, BrrB, are the predicted histidine kinases. The green region indicates the predicted HAMP domain, and the yellow and orange domains indicate the histidine kinase and ATPase domains, respectively. The asterisks indicate amino acid residues with identity between the three proteins, and the dots indicate regions of amino acid similarity.

(Δ*brrA* mutant), or BA31 (complemented with *brrA*−*brrB*) were plated onto brain heart infusion agar plates, placed in an anaerobic chamber, and incubated for 72 h. The parent and complemented strains (BA31) grew, but the Δ*brrA* strain (BA18) failed to show demonstrable growth (data not shown). This suggests that *brrA* was required for anaerobic growth, similar to *resD*, and it was possible that *brrA* regulated nitrate reductase genes in *B. anthracis*. Although

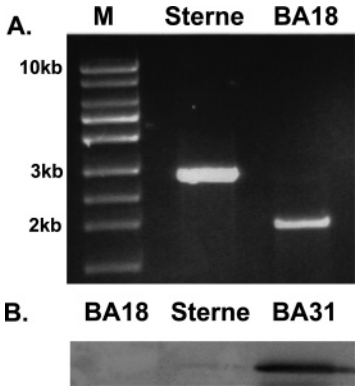

FIGURE 2: Confirmation of deletion of *brrA* via a double-crossover event between the *Xba* I−*Hin*d III regions (flanking regions of *brrA*) of pSMV17. (A) DNA agarose gel showing the PCR products using genomic DNA from either parent Sterne strain or the Δ*brrA* strain and primers flanking up- and downstream regions (Table 2). (B) Western blot analysis of cell lysates from parent Sterne, the Δ*brrA* strain (BA18), or the Δ*brrA* strain complemented with *brrA*−*brrB* expressed on a plasmid (BA31). BrrA was detected using rabbit anti-BrrA antibody.

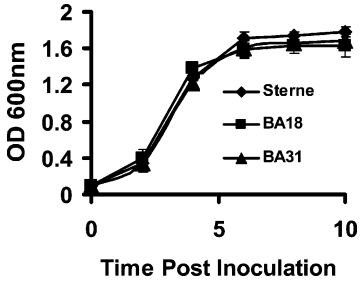

FIGURE 3: Samples of a stationary-phase culture (750 μL) from parent Sterne, Δ*brrA* (BA18), or Δ*brrA* complemented with *brrA*−*brrB* (BA31) strains were inoculated into 25 mL of R media supplemented with 0.8% sodium bicarbonate. Growth was monitored at 2, 6, 8, and 10 h post-inoculation.

there was no growth difference in aerobic liquid culture in 7% CO_2 conditions, colonies from aerobically grown BA18 (Δ*brrA* mutant) and parental strains were different. The BA18 (Δ*brrA* mutant) colonies were smaller with more irregular edges than the parent (Figure 4). The colony color was whiter for the mutant strain than the parent strain, and the colonies were less thick.

Because *resD* mutants in *B. subtilis* lack the ability to form mature spores, sporulation frequency was also measured between the *B. anthracis* Δ*brrA* mutant and parent strains. A total of 1 mL of stationary-phase cultures was inoculated into Schaeffer's sporulation medium and grown aerobically. At 12, 24, 36, and 48 h after inoculation, samples of each culture were used to assess both the numbers of viable cells and numbers of spores. No spores were formed in the BA18 (Δ*brrA* mutant) and BA30 (strain complemented with empty vector plasmid) strains over 48 h (Figure 5). However, after 48 h, nearly 85% of the parental Sterne culture was spores and approximately 50% of the BA31 culture (complemented with *brrA*−*brrB*) was spores. All cultures grew to the same vegetative cell density for the first 24 h, approximately 1.0×10^8 colony-forming units/mL. However, by 96 h after inoculation, no viable cells from either BA18 or BA30 mutant strain could be recovered.

ResD−ResE is not only required for anaerobic growth, but the two-component system also controls cytochrome aa_3

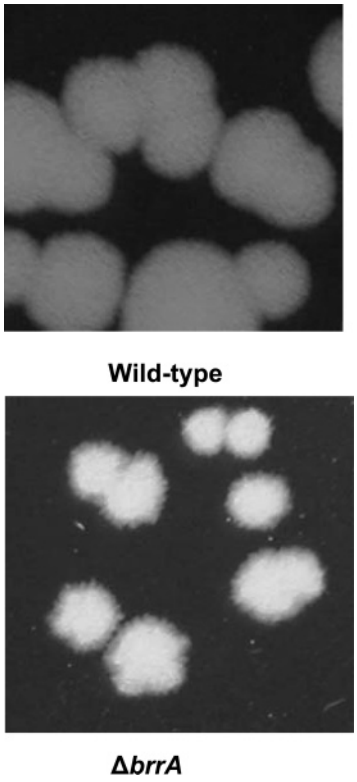

FIGURE 4: Colony morphology differences in the parent Sterne strain compared to the Δ*brrA* strain (BA18). Stationary-phase liquid cultures of parent Sterne and Δ*brrA* strains were plated onto brain heart infusion 1.5% agar plates and incubated at 37 °C for 18 h. Magnification was the same for both photographs.

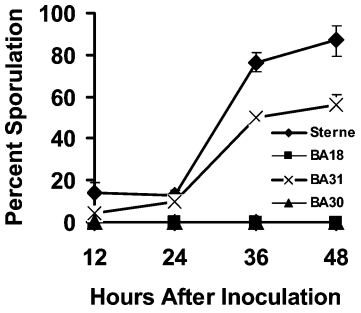

FIGURE 5: Sporulation frequency of Δ*brrA* mutants (Δ*brrA*, BA18) and Δ*brrA* complemented with pCE104, an empty plasmid (BA30) versus *brrA* expressing strains (parent Sterne and the Δ*brrA* mutant complemented with *brrA*−*brrB* on pSMV9, BA31). Approximately 1 mL of stationary-phase culture was inoculated into Schaeffer's sporulation media. Two samples of each culture were taken at 12, 24, 36, and 48 h after inoculation, and one sample was plated to count all viable cells. The second sample was heated at 80 °C for 10 min to kill vegetative cells and then plated to determine the number of spores in the sample.

synthesis through the gene *ctaA* (*35*). Therefore, *B. subtilis* mutants lacking *resD* do not produce cytochrome aa_3 (*34*). Cytochrome aa_3 activity can be tested using a TMPD plate assay. Cells of each *B. anthracis* strain were incubated on brain heart infusion agar for 24 h. They were fixed to the plates, and a TMPD agar overlay was then applied. When TMPD was added to the plates, the parent Sterne strain and BA31 (complemented with *brrA*−*brrB*) strain stained blue, while the BA18 (Δ*brrA* mutant) colonies remained white (Figure 6). When KCN (a cytochrome aa_3 inhibitor) was applied to the cells as a negative control, all colonies,

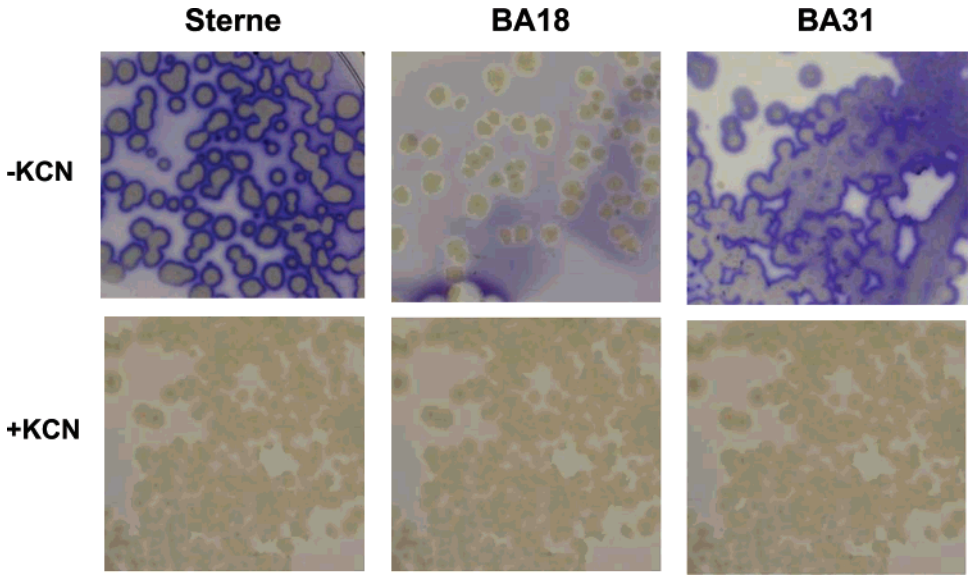

FIGURE 6: TMPD assay for cytochrome aa_3 activity. Stationary-phase cultures of parent Sterne, Δ*brrA* strain (BA18), and Δ*brrA* strain complemented with *brrA*−*brrB* (BA31) were plated on brain heart infusion agar plates and allowed to incubate for 16 h to allow the colonies to form. A TMPD agar overlay was then applied to the cells. TMPD was oxidized by cytochrome aa_3, if present, and turned the cells blue as seen with the wild-type and *brrA*−*brrB* complemented (BA31) strains. If no cytochrome aa_3 is present, the TMPD remains reduced and colorless and no staining occurs as seen with the Δ*brrA* mutant. KCN is an inhibitor of cytochrome aa_3 and is used as a negative control to demonstrate that the activity was due to the cytochrome.

regardless of strain tested, remained white, showing that the blue stain was from cytochrome aa_3 activity as opposed to the activity of cytochrome heme groups. These data suggest that *B. anthracis* strains lacking *brrA* do not make cytochrome aa_3. This result parallels ResD function in *B. subtilis*, where it has been shown that ResD, upon phosphorylation, binds the promoter of *ctaA*, directly regulating its expression (*27*).

Effects on Toxin. A carbon dioxide environment is essential for toxin production in *B. anthracis*. Because *brrA*−*brrB* may control genes expressed under various aerobic and anaerobic conditions and exotoxins are also controlled differentially by aerobic and anaerobic conditions, the effects of the *brrA* mutation on exotoxin production were examined. The parental Sterne strain, Δ*brrA* mutant (BA18), *brrA*−*brrB* complemented Δ*brrA* strain (BA31), and Δ*brrA* strain complemented with empty vector plasmid (BA30) were grown in R medium supplemented with 0.8% bicarbonate with 7% CO_2 at 37 °C. After 8 h, the culture media were harvested, and lethal factor, edema factor, and protective antigen were detected using Western blot analysis (Figure 7A). None of the three toxin components were detected in BA18 (Δ*brrA* mutant). However, the toxin was present in the parent Sterne strain and BA31 (*brrA*−*brrB* complemented). These data show that BrrA positively regulates exotoxin in *B. anthracis*. Quantitative real-time RT-PCR was conducted to determine if the effect on exotoxin production was transcriptional. Consistent with the Western analysis, mRNA transcript levels for *lef*, *pag*, and *cya* for both BA18 (Δ*brrA* mutant) as well as BA30 (Δ*brrA* strain complemented with empty vector plasmid) were 4−6 logs less than the levels for the parent Sterne strain (Figure 7B). The BA31 (*brrA*−*brrB* complemented) strain did not have toxin transcript levels quite at the level of the parent Sterne strain, but transcript levels were restored to a level at which protein was detected. These data suggest that BrrA was controlling toxin production at the level of transcription.

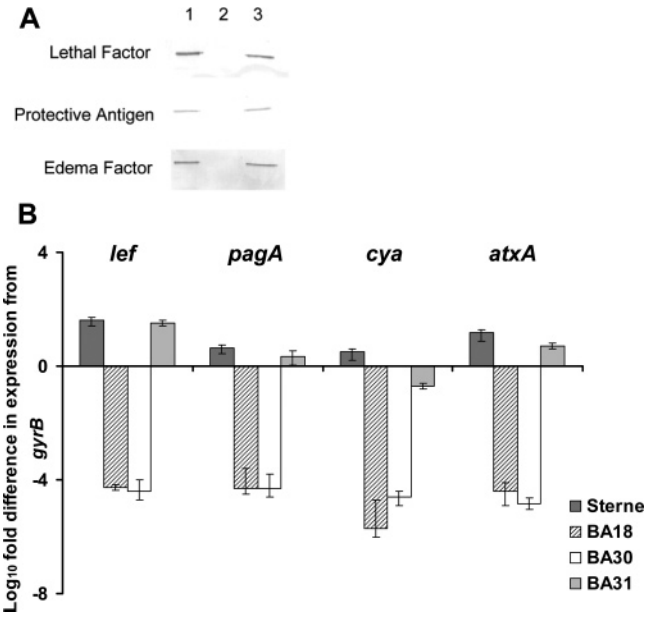

FIGURE 7: (A) Effect of *brrA* on virulence factors. Strains [lane 1, parent Sterne; lane 2, Δ*brrA* strain (BA18); lane 3, Δ*brrA* strain complemented with *brrA*−*brrB* on pSMV9 (BA31)] were grown for 8 h in R medium supplemented with 0.8% bicarbonate and 7% CO_2. Proteins in the cell supernates were separated using SDS−PAGE and subjected to Western analysis. (B) RNA was extracted from exponential-phase cultures growing in R medium with 0.8% bicarbonate and 7% CO_2. A total of 5 μg of total RNA was reverse-transcribed, and 0.5 μL of that reaction was used in a quantitative RT-PCR reaction to measure levels of the indicated gene. The gene, *gyrB*, was used to normalize the levels of RNA transcript in each sample. The transcript levels reported here represent the fold difference from the level of *gyrB* expression on a $\log_{10}$ scale. Strains used include parent Sterne strain, Δ*brrA* strain (BA18); Δ*brrA* strain complemented with empty plasmid (BA30); and Δ*brrA* strain complemented with *brrA*−*brrB* on pSMV9 (BA31).

AtxA is a protein that is characterized as a global regulator of *B. anthracis* virulence. However, AtxA has not been demonstrated to have DNA-binding activity, and it is unclear

how AtxA regulates exotoxin synthesis. Because BrrA controls virulence factor production, its affect on *atxA* was determined using real-time RT-PCR. In the absence of *brrA*, *atxA* was expressed at levels below the parent, suggesting that BrrA also positively regulated *atxA*.

Mobility gel-shift assays were carried out with representative promoters, those for *ctaA* and *pagA*, to determine if BrrA functioned as a transcription factor. Purified BrrA was incubated with promoter sequences of *ctaA*, *pagA*, or a negative control sequence, *rpsC*, that had been labeled with digoxigenin (Figure 8A). Analysis of the *ctaA* promoter revealed a band shift when 15 pM of BrrA was added to the binding reaction (Figure 8B). Analysis of the *pagA* promoter revealed a shift when 30 pM of BrrA was added to the binding reaction. Incubation of BrrA with *rpsC* did not reveal a shift, showing that BrrA does not bind DNA indiscriminately (Figure 8C). These results suggest that BrrA regulates *ctaA* and *pagA* expression by directly binding to their promoter regions and acting as a transcription factor.

DISCUSSION

Here, we begin characterization of a two-component system, BrrA−BrrB, as a critical global virulence regulator as well as system similar to the *B. subtilis* ResD−ResE two-component system. When *brrA* was deleted from the genome, all three exotoxin genes *lef*, *pag*, and *cya* were downregulated at the level of transcription. In addition, the Δ*brrA* mutant was deficient in sporulation, anaerobic growth, and cytochrome aa_3 synthesis, all similar characteristics of ResD mutants in *B. subtilis* (*34*). This suggests that *brrA* controls more than exotoxin production but also regulates key genes controlling many metabolic processes. This is the first report to connect exotoxin gene regulation in *B. anthracis* directly to metabolic regulation and suggests that these two regulation mechanisms are more interconnected than previously thought. On the basis of our prior observations that SrrA−SrrB in *S. aureus* regulates other global regulators of virulence factor production, in addition to regulating virulence factors directly, we hypothesize that BrrA−BrrB may likewise assume a central position in gene regulation in *B. anthracis*.

Similar to ResD, BrrA also binds to the *ctaA* promoter to regulate its expression. However, in addition, BrrA binds to the promoter of *pagA* also positively regulating its expression. More experiments will have to be completed to determine the extent of the direct promoter-binding regulation of BrrA. It is hypothesized that BrrA will also bind the promoters of *lef* and *cya*, because the toxin genes tend to be regulated analogously, and the nitrate reductase genes, because it has been shown that ResD binds these promoters in *B. subtilis* (*36*).

The question may then be asked: How does BrrA connect with the other two characterized virulence regulators, *atxA* and *abrB*? We propose that BrrA is a complement to the negative regulator *abrB*. The transition-state regulator *abrB* is highly expressed in the exponential phase and downregulates the expression of exotoxin genes (*37*). When *B. anthracis* enters the post-exponential phase of growth, *abrB* is downregulated and its protein product is not available to bind the toxin gene promoters, possibly allowing BrrA to bind the toxin gene promoters and upregulate their expression.

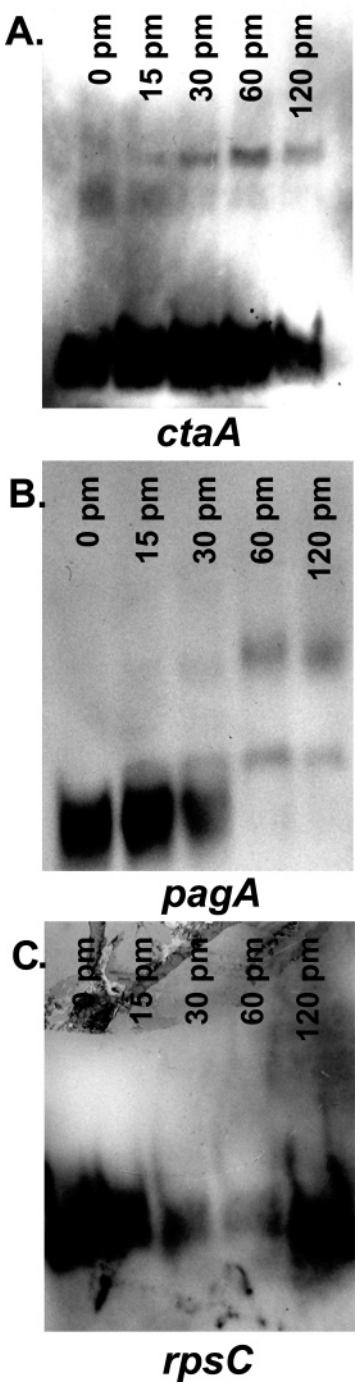

FIGURE 8: BrrA interacts with the promoters of *ctaA* and *pagA*. Increasing amounts (0, 15, 30, 60, or 120 pM) of BrrA were allowed to bind with the digoxigenin-labeled promoter segments of *ctaA* (A), *pagA* (B), or the negative control gene *rpsC* (C) before the complexes were electrophoresed in nondenaturing acrylamide gels and blotted onto a positively charged membrane. Samples were detected using an anti-digoxigenin antibody and chemiluminescent detection. Arrows indicate the positions of BrrA-bound probes.

The role of BrrB is also yet to be completely determined. The protein is predicted to be a histidine kinase; therefore, it is hypothesized that BrrB is the sensor that activates BrrA. The signal BrrB senses remain to be determined. Although BrrB is most likely anchored in the plasma membrane and is predicted to have an extracellular domain, the signal that BrrB is sensing could be located in the cytosol or plasma

membrane. Because BrrA−BrrB is associated with metabolic regulation, it is possible that BrrB is sensing the metabolic state of the cell, such as the level of cytochromes or redox potential near the membrane. The ArcAB system in *E. coli* is also a metabolic regulator in that organism, sensing the oxidative state of the cell. It was shown that this system directly senses the redox state of the quinone pool using cysteine residues located on the cytoplasmic domain of the ArcB histidine kinase protein (*38*). It is thus possible that BrrB is not sensing the CO_2 atmosphere directly but, rather, responding to the metabolic state that the cell is experiencing in the environment. Clearly, more needs to be done to characterize further the BrrA−BrrB system as a two-component regulator, but our work adds a critical piece to anthrax toxin regulation.

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