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Transcriptional Analysis of the *Bacillus anthracis* Capsule Regulators

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The poly-D-glutamic acid capsule of *Bacillus anthracis* is essential for virulence. Control of capsule synthesis occurs at the level of transcription and involves positive regulation of the capsule biosynthetic operon *capBCAD* by a CO_2/bicarbonate signal and three plasmid-borne regulators: *atxA*, *acpA*, and *acpB*. Although the molecular mechanism for control of *cap* transcription is unknown, *atxA* affects *cap* expression via positive control of *acpA* and *acpB*, two genes with partial functional similarity. Transcriptional analyses of a genetically complete strain indicate that *capB* expression is several hundred-fold higher during growth in 5% CO_2 compared to growth in air. *atxA* was expressed appreciably during growth in air and induced only 2.5-fold by CO_2. In contrast, expression of *acpA* and *acpB* was induced up to 23-fold and 59-fold, respectively, by CO_2. The 5′-end mapping of gene transcripts revealed *atxA*-regulated and *atxA*-independent apparent transcription start sites for *capB*, *acpA*, and *acpB*. Transcripts mapping to all *atxA*-regulated start sites were increased during growth in elevated CO_2. The *acpA* gene has one *atxA*-regulated and one *atxA*-independent start site. *acpB* lies downstream of *capBCAD*. A single *atxA*-independent start site maps immediately upstream of *acpB*. *atxA*-mediated control of *acpB* appears to occur via transcriptional read-through from *atxA*-dependent start sites 5′ of *capB*. One *atxA*-independent and two *atxA*-regulated start sites map upstream of *capB*. Transcription from the *atxA*-regulated start sites of *capBCAD* was reduced significantly in an *acpA acpB* double mutant but unaffected in mutants with deletion of only *acpA* or *acpB*, in agreement with the current model for epistatic relationships between the regulators.

Control of virulence gene expression in *Bacillus anthracis* is highly dependent upon *atxA*, a regulatory gene located on virulence plasmid pXO1. In genetically complete strains harboring pXO1 and the second virulence plasmid, pXO2, *atxA* acts as a global regulator controlling expression of the pXO2-encoded capsule biosynthetic gene operon, *capBCAD* (3, 6, 9, 22); the toxin structural genes, *pagA*, *lef*, and *cya* on pXO1 (4, 13, 20); and a number of other genes located on the plasmids and chromosome (3). The mechanism by which *atxA* exerts its effect on target gene transcription is unknown. A direct effect of *atxA* on transcription has not been demonstrated for any *atxA*-controlled gene.

The *capBCAD* genes are required for virulence in a mouse model for inhalation anthrax (7). The capsule biosynthetic genes *capBCA* are predicted to encode the proteins responsible for the synthesis, transport and attachment of the poly-D-glutamic acid capsule polymers to the outside of the bacterial cells (14, 15). Enzymatic or structural functions for CapB, CapC, and CapA have not been demonstrated. CapD (formerly Dep) is an enzyme that depolymerizes the large capsule polymers into smaller D-glutamic acid peptide fragments that are released from the surface of the bacterial cells (21). Given the significance of the capsule biosynthetic gene operon in virulence, determining the mode of regulation of these genes is of interest.

In our current model for capsule gene regulation, *atxA* controls *cap* gene transcription and capsule synthesis via the positive regulation of two pXO2-encoded regulators, *acpA* and *acpB*. The model arose from studies employing a genetically complete ($pXO1^+$ $pXO2^+$) parent strain and isogenic mutants with deletion of *atxA*, *acpA*, and/or *acpB* (6). In $pXO1^+$ $pXO2^+$ strains, while deletion of *acpA* or *acpB* alone does not appreciably affect *capB* transcription or capsule synthesis, an *acpA acpB* double mutant exhibits drastically reduced *capB* transcription and is noncapsulated. Thus, *acpA* and *acpB* have some functional similarity. The amino acid sequences of the predicted products of these genes are approximately 62% homologous. Moreover, the proteins also share significant amino acid sequence similarity with the predicted product of *atxA*.

For many *atxA*-controlled genes, including *acpA* and *acpB*, expression is induced during growth in 5% atmospheric CO_2 or in media containing bicarbonate (2, 6, 11, 13, 18, 22, 23). CO_2-induced transcription of all three toxin genes has been demonstrated in experiments employing promoter-reporter gene fusions (2, 13, 18). RNA slot blot analysis of *capB*, the first gene of the capsule biosynthetic operon, and *acpA* transcripts demonstrated an increase in both transcripts during culture in elevated CO_2 (22, 23). We recently demonstrated elevated *acpB* expression during growth in 5% CO_2 using reverse transcription-PCR (RT-PCR) (6).

CO_2/bicarbonate is likely to be a physiologically significant signal encountered by the bacterium in the host environment. Concentrations of bicarbonate/CO_2 (15 to 40 mM) in the bloodstream of the host (5) are comparable to the concentration of bicarbonate/CO_2 present in the bicarbonate-supplemented growth media during culture in vitro (48 mM). Although induction of *cap* gene expression in vivo has not been assessed quantitatively, our recent experiments employing a mouse model for inhalation anthrax demonstrate the impor-

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TABLE 1. Strains used in this study

Strain name	Plasmid content	Genotype	Relevant characteristic(s)[a]	Reference
UT500	pXO1$^+$ pXO2$^+$		pXO2 from 6602 transduced into 7702	3
UT501	pXO1$^+$ pXO2$^+$	*atxA*	Km^r	3
UT502	pXO1$^+$ pXO2$^+$	*acpA*	Sp^r	3
UT525	pXO1$^+$ pXO2$^+$	*acpB*	Km^r	6
UT526	pXO1$^+$ pXO2$^+$	*acpA acpB*	Sp^r Km^r	6

[a] Abbreviations: Km^r, kanamycin resistance; Sp^r, spectinomycin resistance.

tance of the capsule biosynthetic operon and its regulators during infection (7). The noncapsulated *acpA acpB* mutant is completely attenuated in the mouse model. The 50% lethal dose and mean time to death for the mutant were comparable to those of a mutant with deletion of the entire capsule biosynthetic gene operon, *capBCAD*, suggesting that the regulators function similarly during in vivo and in vitro growth.

Here we further investigate the expression patterns of *capB* and the *cap* gene regulators, *acpA* and *acpB*, with respect to the CO_2/bicarbonate signal during culture in vitro. We also identify *atxA*- and CO_2-controlled transcripts of *capB*, *acpA*, and *acpB* to further elucidate the relationships between these regulators and this important cue.

MATERIALS AND METHODS

Strains. Table 1 contains a complete list of strains, including plasmid content and relevant genotypes. Construction of the strains was described previously (3, 6).

Media and growth conditions. For analysis of gene expression during growth in elevated CO_2, strains were grown under conditions known to promote capsule synthesis as described previously (6, 8). $NBYCO_3$ medium was nutrient broth yeast medium (8) supplemented with 0.8% sodium bicarbonate (wt/vol). LBgoh was Luria-Bertani (1) broth containing 0.5% glycerol to suppress sporulation. When indicated, media contained kanamycin (50 μg/ml) (Fisher Scientific, Pittsburg, PA) and/or spectinomycin (100 μg/ml) (Sigma-Aldrich, St. Louis, MO). Briefly, 30 ml of LBgoh (plus antibiotics when required) in a 250-ml flask was inoculated with vegetative cells from an $NBYCO_3$ plate. Cultures were incubated at 30°C with agitation (200 rpm) for 12 to 14 h. Cultures were diluted into 50 ml of $NBYCO_3$ broth (without antibiotics) to obtain an initial optical density at 600 nm (OD_{600}) of approximately 0.1. Cultures were grown in 5% CO_2 at 37°C with stirring (200 rpm) and sampled at early, mid-exponential, late-exponential, and early-stationary phases. Under these growth conditions, the parent and isogenic mutant strains had similar growth rates. For analysis of gene expression during growth in air, duplicate cultures were grown in unsupplemented NBY broth and incubated in air at 37°C with agitation (200 rpm).

Real-time Q-RT-PCR. RNA was extracted from cultures using the protocol and reagents of the Ribopure Bacteria kit (Ambion, Austin, TX). Typically, 10 to 30 μg of RNA was obtained from 1 ml of culture. RNA preparations were treated with DNase-Free (Ambion, Austin, TX) according to the protocol of the supplier. The protocol and equipment used for quantitative RT-PCR (Q-RT-PCR) assays were described previously (6). The primer and probe sequences for the assays are shown in Table 2.

TABLE 3. Primer sequences used in primer extension and nonquantitative PCR assays[a]

Gene	Primer name	Primer sequence (5′→3′)
acpA	MD28	CTGTTTGTCATGTAAATAATTCTT
	MD33	AGAAATGGCTTCAGAAATTG
	MD34	TCGGCTAATATCTTTTTCCAT
acpB	MD62	TGTAAACCATTTTTCTTCGC
	MD64	TCCCTTGCTTTTAAGAATGT
	MD65	GTCAGAATCCCTGGTTTGTA
	MD108	GATTCAGCAGTGTTTCCAAT
	MD129	AGGCCTTAATTAAAGACGAGA
capB	PE2	CCATTTCTATACTAGATGTTGCATG

[a] DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa). PCRs were performed using a PE-Applied Biosystems (Norwalk, Conn.) PCR system 9700.

Nonquantitative reverse transcription-PCR. RNA was isolated and DNase treated as described above for Q-RT-PCR. Nonquantitative RT-PCR was performed using the protocol and reagents supplied in the RETROscript kit by Ambion. The protocol has been described previously (6). Primers MD62 and MD129 were employed. Primer sequences are shown in Table 3.

Primer extension. For primer extension analysis, RNA was extracted from cultures using RNAwiz (Ambion, Austin, TX) according to the protocol provided by the supplier. Typically between 20 and 100 μg of RNA was obtained per ml of culture. The method for primer extension analysis has been described previously (13, 19). Briefly, 30 to 50 μg of RNA was incubated in the presence of end-labeled primer [γ-^{32}P]ATP (10mCi/ml). Reverse transcription reactions were carried out with Superscript (Invitrogen, Carlsbad, CA). Primer sequences are listed in Table 3. Primers MD28, MD33, and MD34 were used for analysis of *acpA* transcripts. Primers MD62, MD64, MD65, and MD108 were used for analysis of *acpB* transcripts. For analysis of the *capB* gene, primer PE2 developed by Uchida et al. (22) was employed.

The 5′ ends of the *acpA*, *acpB*, and *capB* genes were sequenced using the *fmol* Sequencing kit (Promega, Madison, WI) according to the protocol of the supplier. Primers employed in the sequencing reactions were the same primers used for the corresponding primer extension reactions (listed above).

RESULTS

Quantitative and temporal assessment of CO_2-enhanced *capB*, *acpA*, and *acpB* expression during culture. Although CO_2-enhanced expression of *capB*, *acpA*, and *acpB* has been

TABLE 2. Primer and probe sequences used in Q-RT-PCR assays

Gene	Accession no.[a]	Forward primer (+) and antisense primer (−)	Probe (5′FAM)[b]
gyrB	NC003997.3:4584-6506	(+) ACTTGAAGGACTAGAAGCAG (−) TCCTTTTCCACTTGTAGATC	CGAAAACGCCCTGGTATGTATA
atxA	NC001496.1:150042-151469	(+) ATTTTTAGCCCTTGCAC (−) AAGTTAATGTTTTATTGCTGTC	CTTTTATCTCTTGGAAATTCTATTACCACA
acpA	NC002146.1:68909-70360	(+) ATTATCTTTACCTCAGAATCAG (−) AACGTTAATGATTTCTTCAG	CAATTTCTGAAGCCATTTCTAATCTT
acpB	NC002146.1:49418-50866	(+) TTTTTCAATACCTTGGAACT (−) AATGCCTTTTAGAAACCAC	CTTGAAGAATCATTAGGAATCTCATTACA
capB	NC002146.1:56089-57483	(+) TTTGATTACATGGTCTTCC (−) CCAAGAGCCTCTGCTAC	ATAATGCATCGCTTGCTTTAGC

[a] Each accession number is followed by the version number and the nucleotide region for that sequencing project.
[b] FAM, 6-carboxyfluorescein.

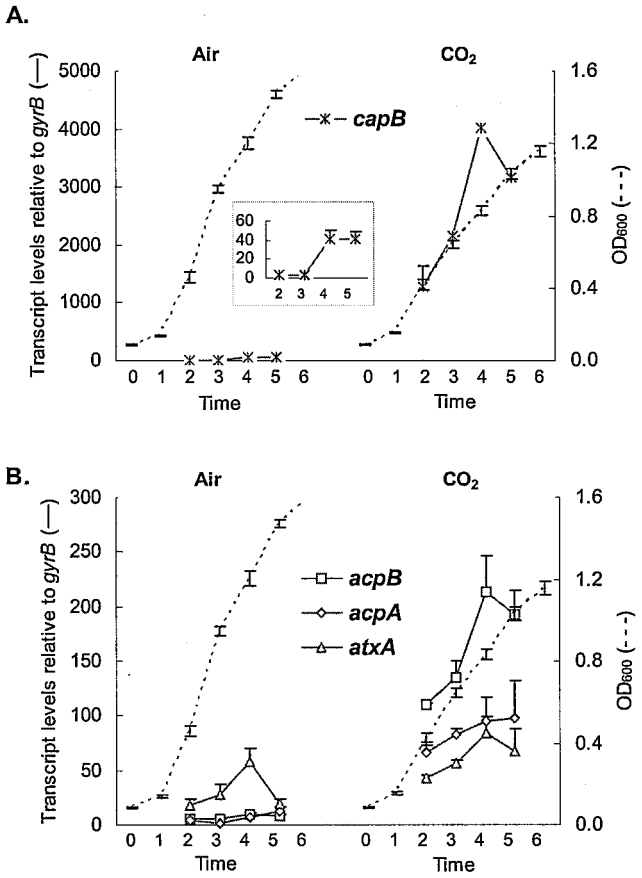

FIG. 1. Real-time transcript levels detected during growth in elevated CO_2 and during growth in air for (A) *capB* and (B) *atxA*, *acpA*, and *acpB*. The transcript levels shown represent four different data sets that were normalized to *gyrB* transcript levels. A representative growth curve is shown for each experiment. The inset in panel A shows *capB* transcript levels using a different scale.

reported, the expression patterns of the regulators and the capsule biosynthesis gene during culture were not known. We used Q-RT-PCR (Taqman) to accurately measure *capB* transcript levels during growth in air and in 5% atmospheric CO_2 (Fig. 1A). The *capB* gene is the first gene in the capsule biosynthetic gene operon. Throughout growth, *capB* transcript levels were 57- to 448-fold higher when cells were cultured in the presence of 5% CO_2, compared to cells grown in air. *capB* transcription was extremely low during growth in air, but increased 12- to 15-fold as the culture entered the late-exponential growth phase (Fig. 1A, insert). The highest *capB* transcript levels observed during growth in air were still remarkably less than levels observed at any time throughout growth in elevated CO_2.

We used the same method to assess the relative transcript levels of *atxA*, *acpA*, and *acpB* throughout growth in the presence of elevated CO_2 and during growth in air (Fig. 1B). Q-RT-PCR results revealed that during growth in air, *acpA* and *acpB* expression was relatively unchanged and very low. *atxA* was expressed at a higher level than either *acpA* or *acpB* (two- to fivefold), and expression levels were highest at late-exponential phase under this growth condition. The expression of *acpA* and *acpB* was markedly higher in 5% CO_2 than during culture in air. Expression of the *acpA* and *acpB* genes was induced 16- to 23-fold and 7- to 59-fold, respectively, depending on the growth phase. In contrast, the presence of CO_2 only affected *atxA* gene transcription approximately 2.5-fold.

The *capB* gene has *atxA*-regulated and *atxA*-independent transcriptional start sites. A previous report indicated two major transcriptional start sites for *capB*, P1 and P2, located 731 and 625 bp upstream of the translational start, respectively (Fig. 2) (22). These results were obtained from experiments employing strains lacking both virulence plasmids and carrying *capBCA* and *acpA* or *atxA* on multicopy vectors. The data indicated that P1 and P2 were *atxA* and *acpA* regulated, but *acpA* had a much larger effect on transcription than *atxA* (22). We performed *capB* primer extension reactions using the same primer as Uchida et al. (PE2) (22), and RNA was obtained from the $pXO1^+$ $pXO2^+$ strain UT500 and isogenic mutants deleted for specific regulatory genes. All cultures were grown to mid-exponential phase in air or in elevated CO_2.

In contrast to the previous report, we found that transcripts mapping to P1 and P2 were reduced in the *atxA* mutant but unaffected in the *acpA* mutant (Fig. 3A). Transcription from P1 and P2 was also unaffected in the *acpB* mutant. Nonetheless, steady-state levels of transcripts originating at P1 and P2 decreased appreciably in the *acpA acpB* double mutant. These results are in accord with our previously established model for *capB* gene regulation (6) in which one of the two regulators, *acpA* or *acpB*, is required for *atxA*-mediated *trans*-activation of *capB* transcription. Deletion of either *acpA* or *acpB* does not significantly affect *capB* transcripts because the two regulators have some partial functional similarity.

Our data also revealed a previously unidentified transcriptional start site, P3, that is located 796 bp upstream from the translational start site of *capB* (Fig. 2). P3 transcription is not affected by *acpA* or *acpB* (Fig. 3). We observed a slight but reproducible increase in P3 transcription in the *atxA*-null mutant, suggesting that other factors may be involved in regulation of transcription from this start site. It is unlikely that P1 and P2 transcripts are the result of *atxA*-dependent processing of the P3 transcript since P3 transcription remained unchanged in the *acpA acpB* mutant as P1 and P2 transcripts decreased significantly. Other bands (unlabeled in Fig. 3) were visualized consistently but did not appear to be affected by the capsule gene regulators. It is likely that these minor bands correspond to regions of RNA with secondary structure that lead to pausing of reverse transcriptase. The *B. anthracis* genome is A-T rich (65% for the chromosome, 67.5% for pXO1, and 67% for pXO2) (17). Alternatively, the bands may represent processed P3 transcripts.

atxA has been linked to CO_2-enhanced gene expression in *B. anthracis* (11, 13). Previous reports of toxin gene expression have indicated *atxA*/CO_2-regulated transcriptional start sites for *lef*, *cya*, and *pagA* (4, 13). The *lef* and *cya* genes each have one apparent start site that is *atxA* dependent and CO_2 induced (4). The *pagA* gene has two apparent start sites; one is constitutively expressed at a low level, while the other is *atxA* dependent and CO_2 induced (13). To determine whether the *atxA*-regulated transcriptional start sites observed for the *capB* gene were also influenced by CO_2, we compared the primer extension results obtained using RNA from the parent strain

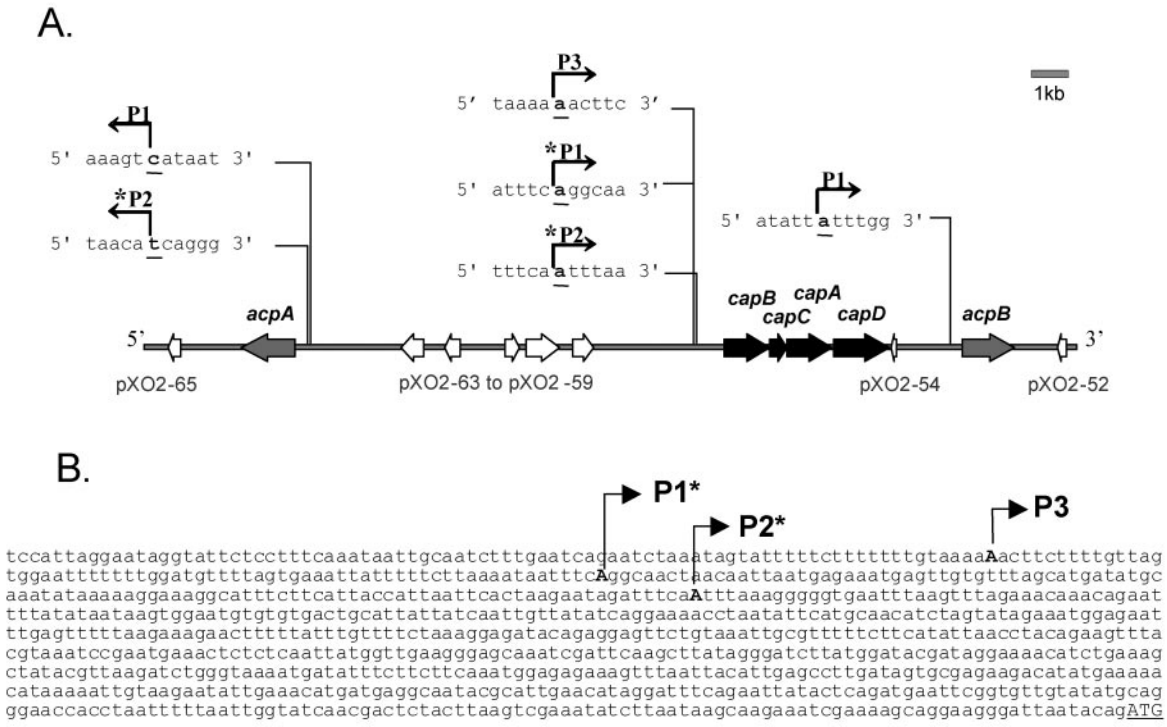

FIG. 2. (A) Model showing transcriptional start sites for the capsule biosynthetic gene operon (*capBCAD*) and the *cap* gene regulators, *acpA* and *acpB*. (B) DNA sequence of the region upstream of *capB*. The predicted translational start site is underlined and capitalized. Nucleotides corresponding to the transcriptional start sites are in bold and capitalized. ∗ denotes *atxA*/CO_2-regulated start site. P1 and P2 upstream of *capB* were reported previously by Uchida and coworkers (17).

cultured in air and RNA isolated from the parent strain grown in elevated CO_2 (Fig. 3B). Levels of transcripts corresponding to both *atxA*-regulated start sites, P1 and P2, were elevated during growth in 5% CO_2. Thus, the low level of *capB* transcript detected during aerobic growth (Fig. 1A) most likely correlates with transcripts initiating at P3.

We searched for consensus sequences upstream of the *capB* transcriptional start sites to reveal putative promoter regions. Canonical −10 and −35 sequences typical of *B. subtilis* σ^A promoters (10) were not apparent upstream of the P3 start site (Fig. 2B), suggesting that additional regulatory mechanisms may be involved in controlling transcription at this start site. We noted a potential −10 but not a −35 consensus sequence upstream of P1. Prototypical −10 and −35 sequences were not readily identifiable for the P2 start site, indicating that P2 may be a breakdown product of P1, as suggested previously by Uchida (22).

***acpB* is cotranscribed with *capD*.** *acpB* transcript levels decrease significantly (15-fold) in the absence of *atxA* (6). We performed 5′-end mapping experiments using RNA isolated from the parent strain and the *atxA*-null mutant to map *atxA*-regulated and *atxA*-independent transcriptional start sites for *acpB* and revealed potential promoter sequences. Using various primers (Table 3), we identified a single transcriptional start site, P1, located 310 bp upstream from the translational start codon (Fig. 2A and Fig. 4). A weak −10 promoter sequence (GATAAT) was identified upstream of this start site, but a canonical −35 sequence was not readily discernible.

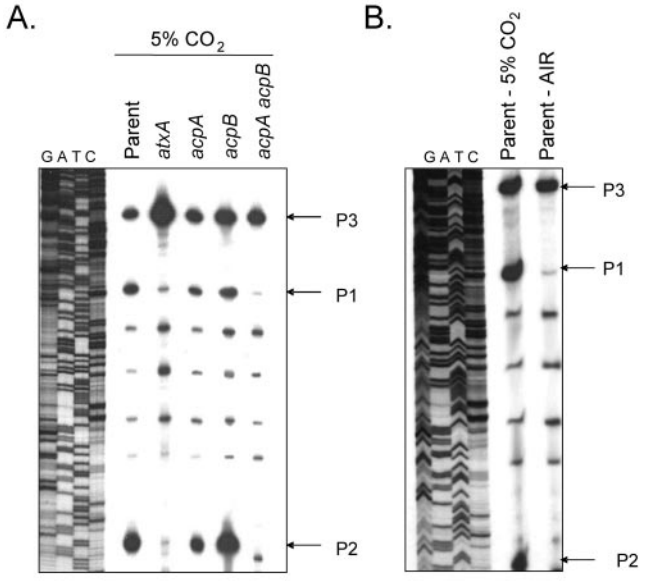

FIG. 3. Primer extension analysis of *capB* transcripts. PE2 primer was employed (22). (A) RNA was extracted from cells grown in 5% CO_2. Lane 1, UT500 (Parent); lane 2, UT501 (*atxA*); lane 3, UT502 (*acpA*); lane 4, UT525 (*acpB*); lane 5, UT526 (*acpA acpB*). (B) RNA was extracted from cells as shown. Lane 1, UT500 (Parent) grown in 5% CO_2; lane 2, UT500 grown in air.

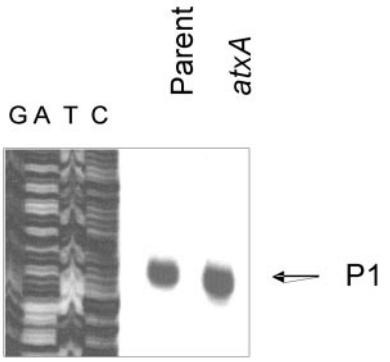

FIG. 4. Primer extension analysis of *acpB* transcripts. MD65 primer was employed (See Materials and Methods). RNA was extracted from cells grown in 5% CO_2. Lane 1, UT500; lane 2, UT501 (*atxA*).

Surprisingly, the steady-state level of this transcript was unchanged in the *atxA* mutant.

The *acpB* gene is located approximately 2 kb downstream from and in the same orientation as the *cap* gene operon (17) (Fig. 2A). The inability to identify an *atxA*-regulated transcriptional start site in the region between *capD*, the last gene of the capsule biosynthetic gene operon and *acpB* suggested that *acpB* may be cotranscribed with *capD* and that *atxA*-regulation of *acpB* results from transcription associated with the *capBCAD* operon. We employed RT-PCR to test for transcripts extending from *capD* to *acpB*. Using primers corresponding to sequences of the *capD* and *acpB* genes we obtained the amplicon shown in Fig. 5, indicating cotranscription of the two

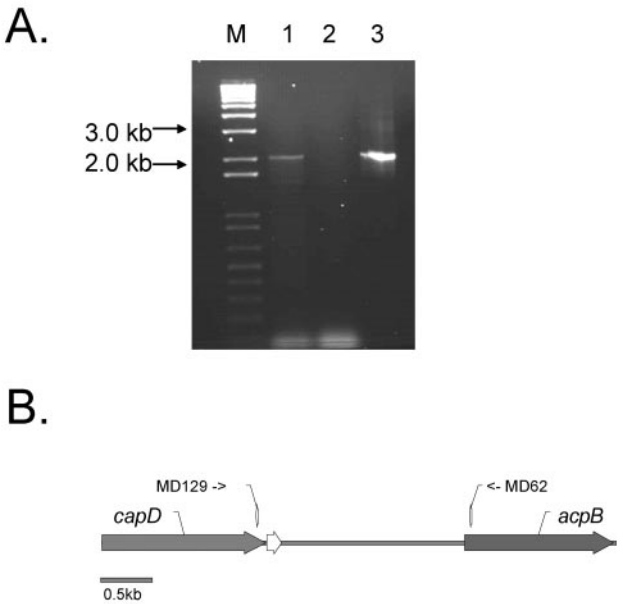

FIG. 5. RT-PCR of the *capD-acpB* region. (A) *capD-acpB* transcript was detected. Lane M, DNA markers with sizes as indicated; lane 1, cDNA template plus reverse transcriptase; lane 2, cDNA template minus reverse transcriptase; lane 3, DNA template, PCR control. (B) Illustration showing location of primers MD62 and MD129 employed in the PCRs used in panel A. The open arrow indicates the position of pXO2-54.

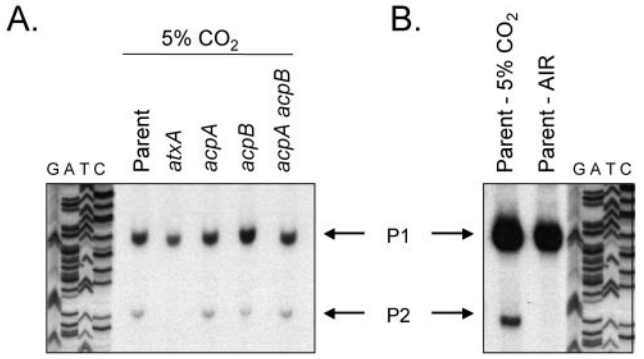

FIG. 6. Primer extension analysis of *acpA* transcripts. MD28 primer was employed. (A) RNA was extracted from cells grown in 5% CO_2. Lane 1, UT500 (Parent); lane 2, UT501 (*atxA*); lane 3, UT502 (*acpA*); lane 4, UT525 (*acpB*); lane 5, UT526 (*acpA acpB*). (B) RNA was extracted from cells as shown. Lane 1, UT500 (Parent) grown in 5% CO_2; lane 2, UT500 grown in air.

genes. Primer extension experiments employing various primers corresponding to the *capBCAD* coding sequence did not reveal transcripts with 5′ ends mapping within the region (data not shown). Taken together, the data indicate that *acpB* is cotranscribed with *capD* and suggest that *atxA*-mediated regulation of *acpB* occurs via the *atxA*-dependent P1 and P2 start sites of *capB* (Fig. 2).

The *acpA* gene has *atxA*-regulated and *atxA*-independent transcriptional start sites . We performed a similar analysis of the other *atxA*-controlled *cap* regulator, *acpA*. Primer extension analysis of the *acpA* gene revealed two major transcriptional start sites, P1 and P2, located 379 and 353 bp, respectively, upstream from the translational start (Fig. 2A and 2B and Fig. 6A). P1 expression was unaffected by *atxA*, yet P2 expression was *atxA*-regulated since P2 transcripts were undetectable in the *atxA*-null mutant (Fig. 6A). There was no evidence of *acpA* autoregulation as P1 and P2 transcription did not vary in the *acpA* mutant samples. Similarly, in an *acpB* mutant and an *acpA acpB* double mutant, the *acpA* P1 and P2 transcript levels did not change, indicating that *atxA*-mediated regulation of *acpA* does not appear to require any of these factors. Similar to the results obtained for the *capB* gene, transcripts from the *atxA*-regulated start site of *acpA*, P2, but not the *atxA*-independent start site, P1, were increased during growth in elevated CO_2 (Fig. 6B). Canonical −10 and −35 promoter sequences could not be readily identified upstream of either the P1 or P2 site, indicating that the expression of *acpA* is most likely sigma A independent.

DISCUSSION

In the work described here, we characterized transcription of *capB*, the first gene of the *B. anthracis* capsule biosynthetic operon; *acpA* and *acpB*, functionally similar *cap* regulators; and *atxA*, a master regulator controlling *acpA* and *acpB* expression. In light of the complex regulation of capsule synthesis, we wanted to (i) assess temporal expression of *capB* and the regulators in batch culture, (ii) examine expression of the genes in response to elevated CO_2, a signal considered to be important in the host; and (iii) establish the apparent tran-

scriptional start sites for these genes, distinguishing regulated and nonregulated transcripts.

Capsule synthesis and *capB* gene expression correlate directly. When *B. anthracis* is grown in nutrient broth yeast medium (NBY) supplemented with 0.8% bicarbonate in an atmosphere of 5% CO_2, capsule is first observed during the mid-exponential phase and cells with the largest capsule diameter are visualized at the early stationary phase (6). The quantitiative RT-PCR analyses performed here indicate that *capB* gene expression was several hundred-fold higher during growth in elevated CO_2 than that observed during growth in air. The highest level of *capB* transcript observed during growth in 5% CO_2 was detected at mid- to late-exponential phase, just before cells become fully capsulated. *capB* transcription is detected throughout growth in air, yet microscopic examination of India ink preparations of the parent strain do not show capsule on the surface of cells grown in this environment. This suggests that a minimal level of *cap* transcript must be attained before capsule can be readily identified on the bacterial cell surface. Temporal expression patterns of *acpA* and *acpB* indicated that the transcript levels for both regulators in cultures grown in 5% CO_2 were relatively high at early exponential phase and increased further as the culture grew. These data suggest that the CO_2 signal is sensed quickly by the bacterium, leading to a rapid increase in expression of both *acpA* and *acpB* that subsequently leads to induction of *capB*.

Two apparent transcriptional start sites for *capB* were identified previously (22). However, the regulation of these start sites did not completely agree with the current model for capsule gene regulation in a genetically complete strain. In the work described here, we have established the apparent transcriptional start sites for *capB*, distinguishing regulated and nonregulated transcripts, in a pXO1$^+$ pXO2$^+$ strain. Results of primer extension of *capB* transcripts confirmed two *atxA*-regulated transcriptional start sites, P1 and P2, as reported previously by Uchida (22). However, in contrast to the previous report, P1 and P2 transcript levels were unaffected in the *acpA* mutant. It is likely that our findings differ from previously published data because the steady-state levels of *atxA* and *acpA* are extremely important for regulation of *capB* expression. In the previous study (22), elevated P1 and P2 transcription was observed in a pXO1$^-$ pXO2$^-$ strain containing the *capBCA* genes and *atxA* or *acpA* cloned on high-copy vectors. Taken together, the data suggest that overexpression of *acpA* can lead to an increase in P1 and P2 transcription in the absence of *atxA*. In addition, overexpression of *atxA* may bypass the requirement for *acpA* or *acpB* for *cap* gene activation. We determined that in the pXO1$^+$ pXO2$^+$ *acpA acpB* strain, *cap* gene expression is significantly reduced and the strain is noncapsulated, indicating that the levels of *atxA* normally present in a genetically complete strain are not sufficient for positive regulation of P1 and P2 transcription in the absence of *acpA* and *acpB*. The low levels of P1 and P2 transcripts detected in the *atxA* mutant are likely the result of positive regulation by the low levels of *acpA* and *acpB* transcripts present in an *atxA* mutant. Finally, the nonregulated *capB* transcription start site P3, was not noted previously by Uchida and coworkers (22). The basal level of *capB* transcription observed in the noncapsulated *acpA acpB* mutant and during growth in air most likely results from transcription at this site. A slight increase in P3 transcript was observed in the *atxA* mutant suggesting an additional level of control.

Primer extension of *acpA* transcripts revealed an *atxA*/CO_2-regulated transcript (P2) and an *atxA*-independent transcript (P1). P1 transcription is evident during growth in air and in the absence of *atxA*. *atxA*-regulated P2 transcription is induced in the presence of elevated CO_2. *atxA*-independent expression of *acpB* results from transcription initiating at a start site (P1) immediately upstream of *acpB*, whereas *atxA*-mediated regulation of *acpB* expression occurs via read-through transcription from *capD*. Using primer extension analysis, we were unable to demonstrate mRNAs with 5′ ends mapping within the *capBCAD* region (data not shown), indicating that *atxA*-regulated *acpB* expression may be attributed to the *atxA*-regulated promoters P1 and P2 of *capB*. A *capBCAD-acpB* transcript would result in a positive feedback loop for *acpB* expression. Nevertheless, data supporting the existence of a 9-kb *capBCAD-acpB* mRNA molecule are lacking. Northern hybridization experiments reported by Makino et al. (15) reveal a 6-kb transcript associated with *capBCAD*. Our quantitative reverse transcription-PCR analyses of UT500 show that *capB* expression is 20- to 50-fold higher than expression of *acpB* (Fig. 1) (6), but analysis of sequences in the *capBCADacpB* region does not reveal potential transcription terminators. Further work will address the stability of a possible mRNA corresponding to *capBCAD-acpB*.

The data described here and work published previously suggest that *acpA* and *acpB* levels are limiting for *capB* transcription in the absence of *atxA* and/or elevated CO_2 (6, 22, 23). *B. anthracis* strains cultured in air are normally noncapsulated, but overexpression of *acpA* in a pXO1$^-$ pXO2$^+$ strain leads to capsule production during growth in air (22). Additionally, *capB* primer extension experiments employing RNA from a pXO1$^-$ pXO2$^-$ strain with the *capBCA* genes and *acpA* cloned on high-copy vectors revealed high levels of transcripts mapping to P1 and P2 in the absence of *atxA* (22).

Transcriptional regulation of *atxA* is unlike that observed for *acpA* and *acpB*. We only observed a small increase (2.5-fold) in *atxA* transcript levels during growth in elevated CO_2 compared to that observed during growth in air. *atxA* has a single transcriptional start site, and a previous study employing primer extension reactions and Western hybridizations indicated no difference in *atxA* expression during growth in elevated CO_2 versus growth in air (4). Consensus sequences for promoter recognition by sigma A RNA polymerase (−10 and −35 sequences) have been noted upstream of *atxA*. Such sequences have not been found upstream of *acpA*, *acpB*, or *capBCAD*. Finally, some data suggest that, in contrast to *acpA* and *acpB*, *atxA* levels are not limiting for target gene expression. Dai et al. (4) demonstrated that overexpression of *atxA* actually leads to a decrease in expression of *pagA* and decreased toxin synthesis, and Sirard et al. (18) showed that a second copy of the *pagA* promoter cloned on pXO1did not affect expression of the *pagA* gene at the normal pXO1 locus.

The complex regulation of capsule gene expression in *B. anthracis*, which includes the CO_2 signal, a regulatory cascade with functionally similar regulators, multiple regulated and nonregulated transcription start sites, and a potential positive feedback loop, is made even more intriguing by the amino acid sequence similarity of the three regulatory proteins. The mas-

ter regulator, AtxA, is approximately 50% similar to the *cap* regulators AcpA and AcpB. AcpA and AcpB are 62% similar to each other. In all cases, the amino acid sequence similarity is throughout the proteins and not limited to a specific region or predicted domain. Secondary and tertiary protein structure predictions suggest that the proteins are soluble, basic proteins. They do not contain strong motifs indicative of nucleic acid binding; however, BLAST results indicate some amino acid homology to the transcriptional regulator Mga (40 to 45% similar) of *Streptococcus pneumoniae* and BglG (40 to 45% similar), an antiterminator protein in *Escherichia coli*. Mga has been shown to bind directly to the promoter sequences of the genes that it activates (16), while BglG is an antiterminator protein that binds to the leader sequence of mRNA to allow read-through of its target genes (12). Neither DNA nor RNA binding activities have been ascribed to the *B. anthracis* proteins. Further investigations will be directed toward discovery of the molecular functions of these unique regulators and the mechanism by which their expression and/or function is linked to the CO_2/bicarbonate signal.

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