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atxA Controls *Bacillus anthracis* Capsule Synthesis via *acpA* and a Newly Discovered Regulator, *acpB*

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Two regulatory genes, *acpA* and *atxA*, have been reported to control expression of the *Bacillus anthracis* capsule biosynthesis operon *capBCAD*. The *atxA* gene is located on the virulence plasmid pXO1, while pXO2 carries *acpA* and the *cap* genes. *acpA* has been viewed as the major regulator of the *cap* operon because it is essential for capsule gene expression in a pXO1$^-$ pXO2$^+$ strain. *atxA* is essential for toxin gene transcription but has also been implicated in control of the *cap* genes. The molecular functions of the regulatory proteins are unknown. We examined *cap* gene expression in a genetically complete pXO1$^+$ pXO2$^+$ strain. Our results indicate that another pXO2 gene, *acpB* (previously called pXO2-53; accession no. NC002146.1:49418-50866), has a role in *cap* expression. The predicted amino acid sequence of AcpB is 62% similar to that of AcpA and 50% similar to that of AtxA. Assessment of *cap* gene transcription revealed that *cap* expression was not affected in a pXO1$^+$ pXO2$^+$ *acpB*-null mutant and was slightly reduced in an isogenic *acpA* mutant. However, *cap* gene expression was abolished in an *acpA acpB* double mutant. Microscopic examination of capsule synthesis by the mutants corroborated these findings. *acpA* and *acpB* expression is controlled by *atxA*; capsule synthesis and transcription of *acpA* and *acpB* were markedly reduced in an *atxA* mutant. The data suggest that, in a strain containing both virulence plasmids, *atxA* is the major regulator of capsule synthesis and controls *capBCAD* expression indirectly, via positive regulation of *acpA* and *acpB*.

Many bacterial pathogens require a cell-associated capsule for virulence. During infection, capsulated organisms have a selective advantage over noncapsulated organisms for numerous reasons. In some species, capsules allow the bacteria to avoid the host's nonspecific immune response by hindering complement binding and subsequent phagocytosis (7, 11, 12). Other capsules specifically bind complement components that prevent the membrane attack complex from forming (18, 26). In some cases, capsules are poorly immunogenic or nonimmunogenic and the lack of a strong specific immune response against the outer surface of bacterial cells facilitates long-term survival in the host (26). Finally, capsules of some bacteria have been reported to play roles in attachment to host cell surfaces (6, 8).

The unique protein capsule of *Bacillus anthracis* is considered to be essential for establishment of infection leading to anthrax disease. The *B. anthracis* capsule is composed of γ-linked D-glutamic acid residue polymers of up to 216 kDa in size (30). The highly negatively charged capsule protects bacterial cells inside the host in a number of ways. The capsule hinders complement binding and decreases the ability of immune cells to phagocytose the bacteria. Treatments that disrupt or remove the capsule allow *B. anthracis* to be more easily engulfed by phagocytes (19, 24). In addition, due to the simplistic chemical composition of the capsule, there is little if any specific immune response generated toward the capsule (22, 34). In this manner, the bacteria remain relatively "invisible" to immune cells of the host.

Fully virulent *B. anthracis* strains possess two large plasmids, pXO1 (182 kb; accession no. NC001496.1) and pXO2 (96 kb; accession no. NC002146.1). The biosynthetic genes for capsule synthesis are located on pXO2 (accession no. M24150 [*capBCA*] and D14037 [*capD*]) while the three structural genes for the anthrax toxin proteins (*pagA* [accession no. NC001496.1:133161-135455], *lef* [accession no. NC001496.1:127442-129871], and *cya* [accession no. NC001496.1:154224-156626]) are located on pXO1. The absence of either plasmid leads to attenuation in most animal models (17, 42). For reasons of safety and easy manipulation in the laboratory, the majority of virulence gene regulation studies have employed attenuated strains harboring only one of the two plasmids (20).

Examination of capsule gene regulation and function in attenuated pXO1$^-$ pXO2$^+$ *B. anthracis* strains leads to the identification of the capsule biosynthesis genes, *capBCAD* (23, 24), and the capsule gene activator, *acpA* (40). The *cap* genes are transcribed as a single operon and are predicted to encode proteins responsible for the biosynthesis, transport, and attachment of the D-glutamic acid residues on the bacterial surface (24, 25). In a recent publication by Urushibata and coworkers (39), the CapB homolog in *Bacillus subtilis* natto, YwsC, was shown to catalyze the polymerization of L-glutamic acid residues to form poly-D-glutamic acid polymers. The YwsC protein is similar to enzymes belonging to the ADP-forming amide bond ligase family. For enzymes of this type, ADP and P_i are detected as end products of the polymerization reaction (13). The *B. anthracis* CapB protein is predicted to have four domains found in ADP-forming amide bond ligases and has significant amino acid homology to proteins of this class (13). The

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TABLE 1. Strains used in this study

Strain name[a]	Plasmid content	Genotype	Relevant characteristics	Source or reference
6602	pXO1$^-$ pXO2$^+$			ATCC[b]
7702	pXO1$^+$ pXO2$^-$			5
UM23C11td10	pXO1$^-$ pXO2$^+$		pXO2 from 6602 transduced into cured strain UM23C1-1	14
UT500	pXO1$^+$ pXO2$^+$		pXO2 from 6602 transduced into 7702	2
UT501	pXO1$^+$ pXO2$^+$	*atxA*	Km^r	2
UT502	pXO1$^+$ pXO2$^+$	*acpA*	Sp^r	2
UT503	pXO1$^+$ pXO2$^+$	*atxA acpA*	Sp^r Km^r	2
UT229	pXO1$^-$ pXO2$^+$	*acpB*	Km^r	This work
UT525	pXO1$^+$ pXO2$^+$	*acpB*	Km^r	This work
UT526	pXO1$^+$ pXO2$^+$	*acpA acpB*	Sp^r Km^r	This work
UT537	pXO1$^+$ pXO2$^+$	*atxA acpB*	Sp^r Km^r	This work
UT252	pXO1$^+$ pXO2$^-$	*atxA*	Sp^r	This work
UT536	pXO1$^+$ pXO2$^+$	*atxA*	Sp^r	This work

[a] UT229 was derived from UM23C11td10. All other UT strains are isogenic, excluding genotypes indicated.
[b] ATCC, American Type Culture Collection.

CapC homolog YwtA was found to be essential for polyglutamic acid polymer formation in *B. subtilis* natto since deletion of *ywtA* eliminates the ability of this strain to secrete the polymers. Secondary and tertiary structure predictions indicate that CapC may be an integral membrane protein, perhaps functioning to transport the polymers to the outside of the cell (24, 39). It is interesting that, although many *Bacillus* species produce and secrete poly-D-glutamic acid, only *B. licheniformis* and *B. anthracis* produce a cell-associated glutamic acid capsule. The *capA* homolog *ywtB*, although not essential for polyglutamic acid production, is required for optimal production of glutamic acid polymers in *B. subtilis* natto (39). The fourth gene of the *cap* operon, *capD*, is unique to *B. anthracis* and encodes an enzyme that depolymerizes large capsule polymers, releasing lower-molecular-weight D-glutamic acid polymers into the environment (37). Makino et al. (25) demonstrated that a *capD*-null mutant is avirulent in a mouse model for anthrax. Virulence was restored by the addition of lower-molecular-weight capsule polymers at the time of injection, thus indicating a direct role for the exogenous lower-molecular-weight polymers in virulence.

Akin to the capsule gene regulation studies, researchers examining toxin gene regulation and toxin function have generally employed strains carrying only pXO1 (20). The structural genes for the toxin proteins, *pagA*, *lef*, and *cya*, encoding protective antigen, lethal factor, and edema factor, respectively, and the regulatory gene *atxA*, which is required for expression of the toxin genes, were identified in experiments with pXO1$^+$ pXO2$^-$ strains (3, 10, 21, 28, 31, 32, 35, 36, 41, 43). The toxin genes and *atxA* are all located on pXO1. *atxA* has been shown to regulate toxin synthesis in culture and in a mouse model for anthrax (10, 21, 36).

Recent transcriptional profiling studies employing a genetically complete pXO1$^+$ pXO2$^+$ strain and isogenic *atxA* and *acpA* regulatory mutants have revealed a significant role for *atxA* and a minor role of *acpA* in *cap* gene regulation (2). We showed that *acpA* is not essential for capsule gene expression in a pXO1$^+$ pXO2$^+$ strain background; an *acpA*-null mutant produces capsule similarly to the parent strain. These findings are in contrast to a report showing that *acpA* is essential for capsule gene expression and capsule synthesis in a pXO1$^-$ pXO2$^+$ strain (40). We also determined that capsule gene expression is significantly reduced in an *atxA*-null pXO1$^+$ pXO2$^+$ strain (2). Previously, it was shown that *atxA* was able to complement an *acpA*-null mutant and positively regulate capsule gene transcription and capsule synthesis in a pXO1$^-$ pXO2$^+$ strain (38). However, the significance of *atxA* in capsule gene regulation in genetically complete strains was not recognized in this genetic background. The cross talk between pXO1 and pXO2 appears to be unidirectional; *acpA* does not have any effect on the expression of the toxin genes (2, 15, 38). The molecular mechanism(s) by which *atxA* and *acpA* regulate the expression of the toxin and capsule genes has not been elucidated.

Here we further explore capsule gene expression in a genetically complete *B. anthracis* strain and investigate the effects of *atxA*, *acpA*, and a newly discovered regulator, *acpB*, on capsule gene expression and capsule synthesis. Based on our findings, we propose a new model whereby *atxA* controls capsule gene transcription and synthesis via positive regulation of *acpA* and/or *acpB* expression.

MATERIALS AND METHODS

Strains. Table 1 is a complete list of strains, including plasmid content and relevant genotypes. Construction of UT500 (pXO1$^+$ pXO2$^+$ parent strain), UT501 (*atxA*), UT502 (*acpA*), and UT503 (*atxA acpA*) was described previously (2). An *acpB*-null mutation in strain UM23C1-1td10 (pXO1$^-$ pXO2$^+$) was created by replacement of coding sequences of *acpB* (open reading frame pXO2-53; accession no. NC002146.1:49418-50866; 109 nucleotides [nt] downstream of the translational start codon to 100 nt upstream of the translational stop codon) with an Ω kanamycin resistance cassette (29), using a protocol described previously (10). The mutation was confirmed using the PCR with oligonucleotide primers corresponding to sequences upstream and downstream of *acpB* in combination with primers internal to the Ω cassette. This *acpB*-null mutant was named UT229.

A similar gene replacement method was used for construction of a spectinomycin-resistant *atxA*-null mutant of strain 7702 (pXO1$^+$ pXO2$^-$). The *atxA* coding sequences 132 nt downstream from the translational start to 51 nt upstream from the translational stop were replaced with an Ω spectinomycin resistance cassette (33). The mutation was confirmed using the PCR as described above with primers corresponding to sequences adjacent to the *atxA* gene and within the Ω cassette. This *atxA*-null mutant was named UT252.

CP51-mediated transduction was used to transfer mutations between isogenic

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

Gene	Accession no.	Forward primer (+) and anti sense primer (−)	Probe (5′ FAM[a])
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
16S rRNA	NC003997.3:9335–10841	(+) TTCGGGAGCAGAGTG (−) AACATCTCACGACACGAG	CAGGTGGTGCATGGTTGTC

[a] FAM, 6-carboxyfluorescein.

strains (14). The *acpB*- and *atxA*-null mutations of UT229 and UT252 were transduced to recipient strains UT500 and UT501 to yield UT525 (pXO1$^+$ pXO2$^+$ *acpB*) and UT536 (pXO1$^+$ pXO2$^+$ *atxA*), respectively. The *acpB*-null mutation of UT229 was transduced into recipient strains UT502 and UT536 to yield UT526 (pXO1$^+$ pXO2$^+$ *acpA acpB*) and UT537 (pXO1$^+$ pXO2$^+$ *atxA acpB*), respectively. Null mutations in all transductants were confirmed using the PCR.

Media and growth conditions. Strains were grown in conditions shown previously to promote capsule synthesis (14). $NBYCO_3$ medium was nutrient broth yeast agar (14) supplemented with 0.8% (wt/vol) sodium bicarbonate. LBgoh was Luria-Bertani (1) broth containing 0.5% glycerol to suppress sporulation. When indicated, media contained kanamycin (50 μg/ml; Fisher Scientific, Pittsburgh, Pa.) and/or spectinomycin (100 μg/ml; Sigma-Aldrich, St. Louis, Mo.). Thirty milliliters of LBgoh 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 of approximately 0.1. Cultures were grown in 5% CO_2 at 37°C with stirring (200 rpm). Under these growth conditions the isogenic parent and mutant strains had similar growth rates.

Microscopy. *B. anthracis* cells were visualized using a Labophot Nikon microscope, and pictures were obtained using a COOLPIX995 digital camera. For India ink preparations, vegetative cells from $NBYCO_3$ broth cultures were added directly to microscope slides and coverslips were placed directly on top of the cells. India ink (Becton Dickinson Microbiology Systems, Sparks, Md.; undiluted or diluted to 70% in nutrient broth yeast agar) was added to the edges of the coverslip. Capsule was visualized as the exclusion of ink particles around cells. Pictures were taken using the 100× objective with oil immersion. Capsule was later measured using Metavue software version 5.0r3 (Universal Imaging Corporation, Downingtown, Pa.).

Real-time (quantitative) reverse transcription-PCR (Q-RT-PCR). RNA was extracted from cultures grown in $NBYCO_3$ broth as described above until mid-exponential phase (optical density at 600 nm of ~0.7), using the protocol and reagents of the Ribopure Bacteria kit (Ambion, Austin, Tex.). Typically, 10 to 30 μg of RNA was obtained from 1 ml of culture. RNA preparations were treated with DNase-Free solution (Ambion) according to the protocol of the supplier.

TaqMan Q-RT-PCR was performed using a 7700 Sequence Detector (Applied Biosystems, Foster City, Calif.) (4, 16). Specific quantitative assays for *atxA*, *acpA*, *acpB*, *capB*, 16S rRNA, *gyrB*, and *capB* genes were developed using sequences from GenBank (Table 2) and Primer Express software (Applied Biosystems). cDNA was synthesized in a 10-μl total volume by adding 6 μl of an RT master mix (400 nM assay-specific reverse primer, 500 μM deoxynucleotides, Superscript II buffer, dithiothreitol, and 10 U of Superscript II reverse transcriptase [Invitrogen, Carlsbad, Calif.]) followed by 4 μl of sample (25 ng of RNA/μl) per well of a 7700 96-well plate. Each sample was measured in triplicate, in addition to a control without reverse transcriptase. Each plate also contained an assay-specific sDNA (synthetic amplicon oligonucleotide) standard spanning a 5-log range and a no-template control. Plates were covered with Biofilm A (MJ Research, Waltham, Mass.) and incubated in a thermocycler (MJ Research) for 30 min at 50°C followed by 10 min at 72°C. Subsequently, 40 μl of a PCR master mix (400 nM forward and reverse primers, 100 nM fluorogenic probe [except that *atxA*, *acpA*, and *acpB* assay mixtures contained 50 nM fluorogenic probe], 5 mM $MgCl_2$, 200 μM deoxynucleotides, PCR buffer, and 1.25 U of *Taq* polymerase [Invitrogen]) was added directly to each well of the cDNA plate. RT master mixes and all RNA samples were pipetted by a Tecan Genesis RSP 100 robotic workstation (Tecan US, Research Triangle Park, N.C.); PCR master mixes were pipetted utilizing a Biomek 2000 robotic workstation (Beckman, Fullerton, Calif.). Each assembled plate was then capped and run in the 7700 sequence detector under the following cycling conditions: 95°C for 1 min and 40 cycles of 95°C for 12 s and 60°C for 1 min. The resulting data were analyzed using SDS software (Applied Biosystems) with carboxy-X-rhodamine (ROX) as the reference dye.

Synthetic DNA oligonucleotides used as standards (sDNA) encompassed exactly the entire 5′-to-3′ amplicon for the assay (Biosource International, Camarillo, Calif.). It has been shown for several assays that in vitro-transcribed RNA amplicon standards (sRNA) and sDNA standards have the same PCR efficiency when the reactions are performed as described above (G. L. Shipley, personal communication).

Due to the inherent inaccuracies in quantitating total RNA by absorbance, the amount of RNA added to an RT-PCR mixture from each sample was more accurately determined by measuring the *gyrB* and 16S rRNA transcript levels in each sample. Final data reported here were normalized to *gyrB*, although similar results were obtained when data were normalized to the 16S rRNA gene. Due to the high level of expression of the 16S rRNA gene, RNA employed in this assay was diluted 1/500.

Nonquantitative RT-PCR. RNA was isolated and DNase treated as described above for Q-RT-PCR. RT-PCR was performed using the protocol and reagents supplied in the RETROscript kit by Ambion. Briefly, 1 μg of RNA was mixed with 2 μl of random decamers (50 μM) for a final volume of 12 μl in water. The mixture was heated to 68°C for 4 min and then immediately placed on ice. A master mix comprised of 2 μl of 10× RT buffer, 4 μl of deoxynucleoside triphosphate mix (2.5 mM each), 1 μl of RNase inhibitor (10 U/μl), and 1 μl of Moloney murine leukemia virus (MMLV) reverse transcriptase enzyme (100 U/μl) was added to the RNA solution (final volume, 20 μl), and the mixture was incubated at 42°C for 1 h. The mixture was then heated to 92°C for 2 min to inactivate the MMLV reverse transcriptase enzyme. A control sample that contained RNA and all of the RT components except MMLV reverse transcriptase was prepared simultaneously. The PCRs were then performed using the RNA control sample, the cDNA sample, and a DNA template control.

Statistical analysis. For microscopic studies, a minimum of 200 cells from at least five independent growth curves of each strain were counted for every time point tested. For each strain and growth phase (mid-exponential, late exponential, and late stationary), capsule thickness and percentage of encapsulated cells were displayed graphically and summarized as means and 95% confidence intervals.

For quantitative RT-PCR assays, at least five independent RNA extractions were performed for each strain. Transcript levels of *atxA*, *acpA*, *acpB*, and *capB* were normalized to corresponding values for the *gyrB* gene. Data were then transformed by taking logarithms in order to stabilize within-group variances. Differences in expression of each gene as a function of the mutation status of the other genes were evaluated using two-way analysis of variance. For example, *acpA* was analyzed using data from non-*acpA*-mutated strains in a two-way analysis of variance with terms for ± *atxA* and *acpB*. There was no triple mutant strain, and therefore *capB* data were analyzed using three two-way analyses of variance, the first with terms for ± *atxA* crossed with ± *acpA* in wild-type *acpB* strains, the second with terms for ± *atxA* crossed with ± *acpB* in wild-type *acpA* strains, and the third with terms for ± *acpA* crossed with ± *acpB* in wild-type

atxA strains, respectively. Fold differences in expression due to mutation of one or more regulators were estimated by back-transformation (exponentiating).

RESULTS

Capsule synthesis by a pXO1$^+$ pXO2$^+$ *B. anthracis* strain and isogenic regulatory mutants. In recent work, we constructed a genetically complete pXO1$^+$ pXO2$^+$ *B. anthracis* strain, UT500, and examined the effects of previously identified virulence gene regulators *atxA* and *acpA* on the *B. anthracis* transcriptome (2). In addition to revealing several previously unidentified *atxA*-regulated genes, the transcriptional profiles of the parent strain and isogenic *acpA*- and *atxA*-null mutants indicated that, while *acpA* had a twofold effect on transcription of the capsule operon, *capBCAD*, *atxA* had an eight- to ninefold effect on capsule gene expression. Capsule phenotypes of the *acpA* and *atxA* mutants corroborated the transcriptional analysis. A mutant with a deletion of *atxA* was severely impaired for capsule synthesis, while an *acpA*-null mutant produced capsule at levels comparable to those for the parent strain, UT500. A mutant with deletions of *acpA* and *atxA* was noncapsulated. These results were surprising in light of previous data suggesting that *acpA* is the major regulator of capsule gene expression and capsule synthesis (38, 40).

A detailed analysis of the *atxA*-regulated genes revealed that *atxA* positively regulates transcription of *acpA* and an *acpA* homolog, pXO2-53 (accession no. NC002146.1:49418-50866), also located on pXO2, 12-fold and 9-fold, respectively. We have renamed pXO2-53 "*acpB*." This gene is predicted to encode a protein with 62% amino acid similarity to AcpA and 50% similarity to AtxA. The similarity among the three proteins is throughout the length of the entire protein (Fig. 1). We hypothesized that *acpB* may play a role in capsule gene expression and capsule synthesis.

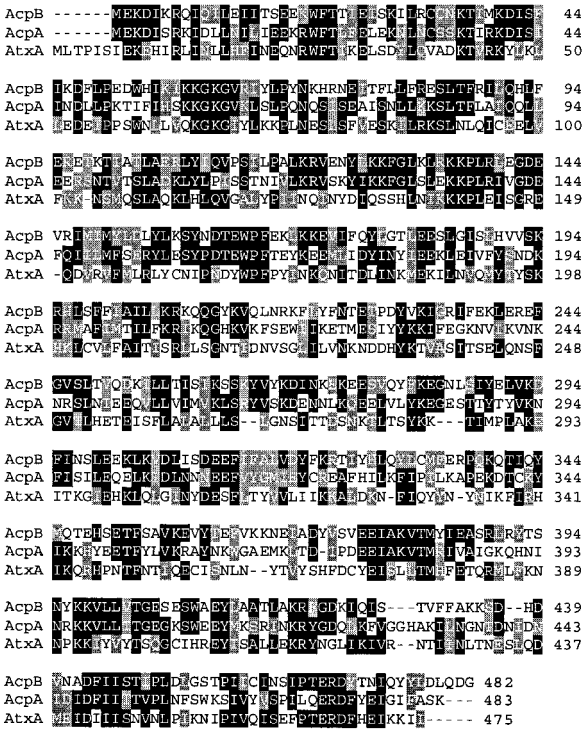

FIG. 1. Comparison of the predicted amino acid sequences of AcpB (pXO2-53; protein identification no. NP_053208.1), AcpA (pXO2-64; protein identification no. NP_053219.1), and AtxA (pXO1-119; protein identification no. NP_052815.1). Black and gray backgrounds indicate identity and similarity, respectively, between residues. The sequences were aligned using CLUSTAL W (1.74) multiple sequence alignment.

To determine if *acpB* contributes to capsule synthesis in *B. anthracis*, we constructed an *acpB*-null mutation and examined the capsule phenotypes of mutants with deletions of *acpB* in the presence and absence of the other regulators. As shown in Fig. 2, at early stationary phase, the *acpB*-null mutant cells (UT525) were indistinguishable from the fully capsulated cells of the parent strain (UT500). Likewise, capsule synthesis by the *acpA* mutant (UT502) was similar to that observed for the parent strain. As reported previously (2), a thin capsule was observed on some cells of the *atxA* mutant (UT501). The same phenotype was observed for the *atxA acpB* double mutant (UT537). No capsule was evident for the *atxA acpA* mutant (UT503), and surprisingly, cells of a mutant with deletions of both *acpA* and *acpB* (UT526) appeared completely devoid of capsule. This finding was unexpected given that the single *acpA* (UT502) and *acpB* (UT525) mutants appeared to produce capsule in amounts similar to those of the parent strain.

To more quantitatively assess capsule production by the mutants, we determined the percentages of capsulated cells and the average capsule diameter on cells of UT500 and the mutants following growth in batch culture. Cells were examined at mid-exponential, late exponential, and early stationary phase. As shown in Fig. 3, UT500 was capsulated at all three time points measured and the average diameter of the capsule increased from 1.18 to 2.55 μm as the culture transitioned from mid-exponential to early stationary phase. In contrast, only 13% of UT501 (*atxA*) cells produced a visible capsule at early stationary phase and the average diameter of the capsule was 0.91 μm, significantly less than that found on UT500. The capsule of the *atxA acpB* mutant (UT537) appeared very similar to that of the *atxA* mutant (UT501), with 13% of the cells making a thin capsule at early stationary phase. As reported previously (2), UT503 (*atxA acpA*) did not produce capsule at any time point measured. The small amount of capsule produced by UT501 and UT537 and the complete lack of capsule synthesis by UT503 suggest that, in the absence of *atxA*, *acpA* has a larger effect on capsule synthesis than does *acpB*.

Deletion of *acpA* (UT502) or *acpB* (UT525) resulted in cells that were capsulated throughout growth. However, the average diameter of the capsule on UT502 cells was smaller than that on UT500 and UT525 at all time points tested, again indicating that *acpA* has a greater effect on capsule synthesis than does *acpB*. Unlike the individual *acpA* and *acpB* mutants, the double mutant UT526 was profoundly affected for capsule synthesis, since less than 0.1% of the cells were capsulated. These data suggest that the positive effect of *atxA* on capsule synthesis can be attributed to *atxA* control of *acpA* and *acpB*.

Correlation of *capB* transcript levels with capsule synthesis. Q-RT-PCR was used to assess the effect of each regulator on expression of *capB*, the first gene of the *cap* operon. *capB* transcript levels were measured in each of the single and dou-

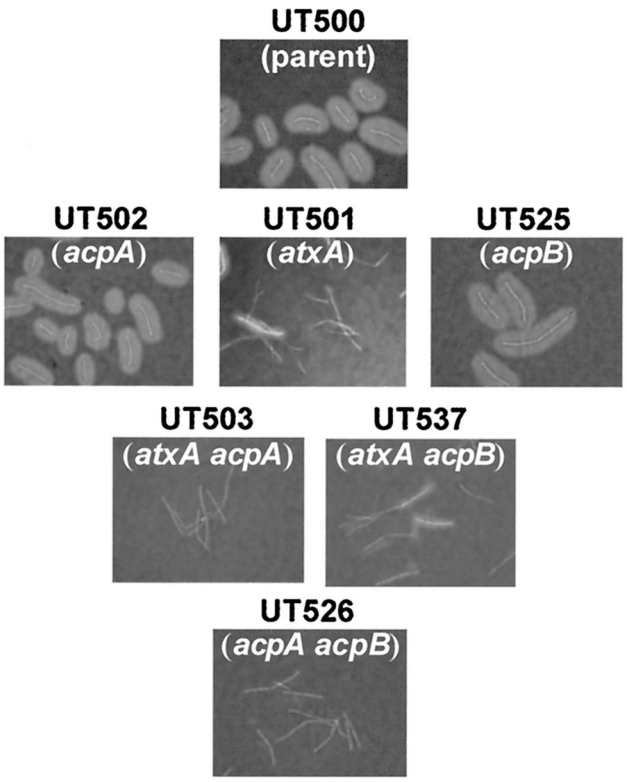

FIG. 2. Capsule production by UT500 and mutants. Cultures were grown to early stationary phase, and capsule was visualized in India ink preparations. Genotypes are as indicated.

ble mutant strains following growth to late exponential phase. All three regulators, *atxA*, *acpA*, and *acpB*, affected capsule synthesis at the level of transcription (Fig. 4A). *capB* transcript levels were 32-fold lower in UT501 (*atxA*) than in UT500, indicating that *atxA* has a significant effect on *capB* gene expression ($P < 0.01$). *capB* transcript levels in UT525 (*acpB*) did not differ significantly from those in UT500, while UT502 (*acpA*) showed a twofold decrease in *capB* transcript compared to UT500 ($P < 0.01$). The noncapsulated strains UT503 (*atxA acpA*) and UT526 (*acpA acpB*) showed a dramatic decrease in *capB* transcript levels of more than 2 orders of magnitude ($P < 0.01$). Overall, the *capB* transcript levels in each of the mutant strains correlated with the amount of capsule produced by each strain.

Epistatic relationships among *atxA*, *acpA*, and *acpB*. Epistatic relationships among the three regulators were determined by measuring the transcript levels of each regulator in UT500 and the mutant strains. The *atxA* transcript levels did not differ in UT500, UT502 (*acpA*), UT525 (*acpB*), and UT526 (*acpA acpB*) (Fig. 4B). However, assessment of transcript levels in UT500 and UT501 by Q-RT-PCR showed that *atxA* had a large effect on the expression of *acpA* (40-fold) (Fig. 4C) and *acpB* (15-fold) (Fig. 4D). These data suggest that *atxA* acts upstream of both *acpA* and *acpB*. Nonetheless, it is notable that *acpA* and *acpB* were transcribed at a low level even in the absence of *atxA*. Finally, *acpA* had a small effect on the expression of *acpB* (1.5-fold, $P = 0.03$), and there was an additive effect of *atxA* and *acpA* on *acpB* gene expression, since deletion of both genes resulted in a 22.5-fold reduction in the *acpB* expression level (Fig. 4D). No effect of *acpB* on *acpA* expression was observed (Fig. 4C).

Taken together, the data indicate that the positive effect of *atxA* on capsule gene expression and capsule synthesis can be attributed to *atxA* control of *acpA* and *acpB*. *acpA* and *acpB* appear to be functionally similar. Individually, neither gene has a large effect on *capB* gene expression and capsule synthesis. However, deletion of both *acpA* and *acpB* results in a dramatic decrease in *capB* gene expression and capsule synthesis. Thus, in a pXO1$^+$ pXO2$^+$ strain, *atxA* does not influence *cap* gene expression directly but requires one of the downstream regulators. Furthermore, the results demonstrate that *acpA* has a greater effect on capsule gene expression than does *acpB*. Expression of *capB* was twofold lower in UT502 (*acpA*) than in UT500, while *capB* gene expression by UT525 (*acpB*) did not differ from that by UT500. Also, *capB* transcript levels were higher in UT537 (*atxA acpB*) than in UT503 (*atxA acpA*).

CO_2-enhanced expression of *acpB*. Expression of *capB* and the toxin genes *pagA*, *cya*, and *lef* is enhanced when *B. anthracis* is cultured in media containing bicarbonate and grown in elevated atmospheric CO_2. The bicarbonate-CO_2 signal is considered to be of physiological significance for a mammalian pathogen (20, 27). Previous studies employing primer extension and immunoblotting experiments have shown that steady-state levels of *atxA* mRNA and AtxA protein do not differ in cells grown in the presence and absence of CO_2-bicarbonate (9). In contrast, results of RNA-DNA hybridization (slot blotting) experiments indicate that CO_2-bicarbonate has a strong positive effect on *acpA* expression. *acpA* mRNA was detected in cells cultured in elevated bicarbonate-CO_2 but not detected when cells were grown aerobically (40).

Considering the functional similarity between *acpA* and *acpB*, we tested for *acpB* expression during aerobic growth of *B. anthracis*. Figure 5 shows the results of RT-PCR with RNA from cells cultured in elevated CO_2-bicarbonate and cells cultured aerobically. Very low levels of *acpB* mRNA were detected in RNA isolated from aerobically grown cells. Significantly higher levels of *acpB* mRNA were detected in comparable amounts of RNA isolated from cells grown in elevated CO_2-bicarbonate. Control experiments testing expression of *atxA*, *acpA*, and *gyrB* confirm that, among these genes, only *acpA* expression is induced by the CO_2-bicarbonate signal. Thus, as is true for *acpA*, *acpB* expression is increased during growth in elevated CO_2-bicarbonate.

DISCUSSION

Previous studies of capsule gene regulation in attenuated *B. anthracis* strains led to the identification of two positive regulators, *acpA* and *atxA*. *acpA*, a gene located on pXO2, was shown to be essential for capsule synthesis in a pXO1$^-$ pXO2$^+$ strain (40). *atxA*, located on pXO1 and originally identified as the toxin gene regulator (10, 21, 36), was also shown to positively regulate the expression of the capsule biosynthetic gene operon (15, 38). We recently demonstrated that in a genetically complete (pXO1$^+$ pXO2$^+$) *B. anthracis* strain, *atxA* is the master regulator of capsule gene expression while *acpA* plays a minor role in capsule gene regulation (2). Our findings were in contrast to those of previous studies employing attenuated

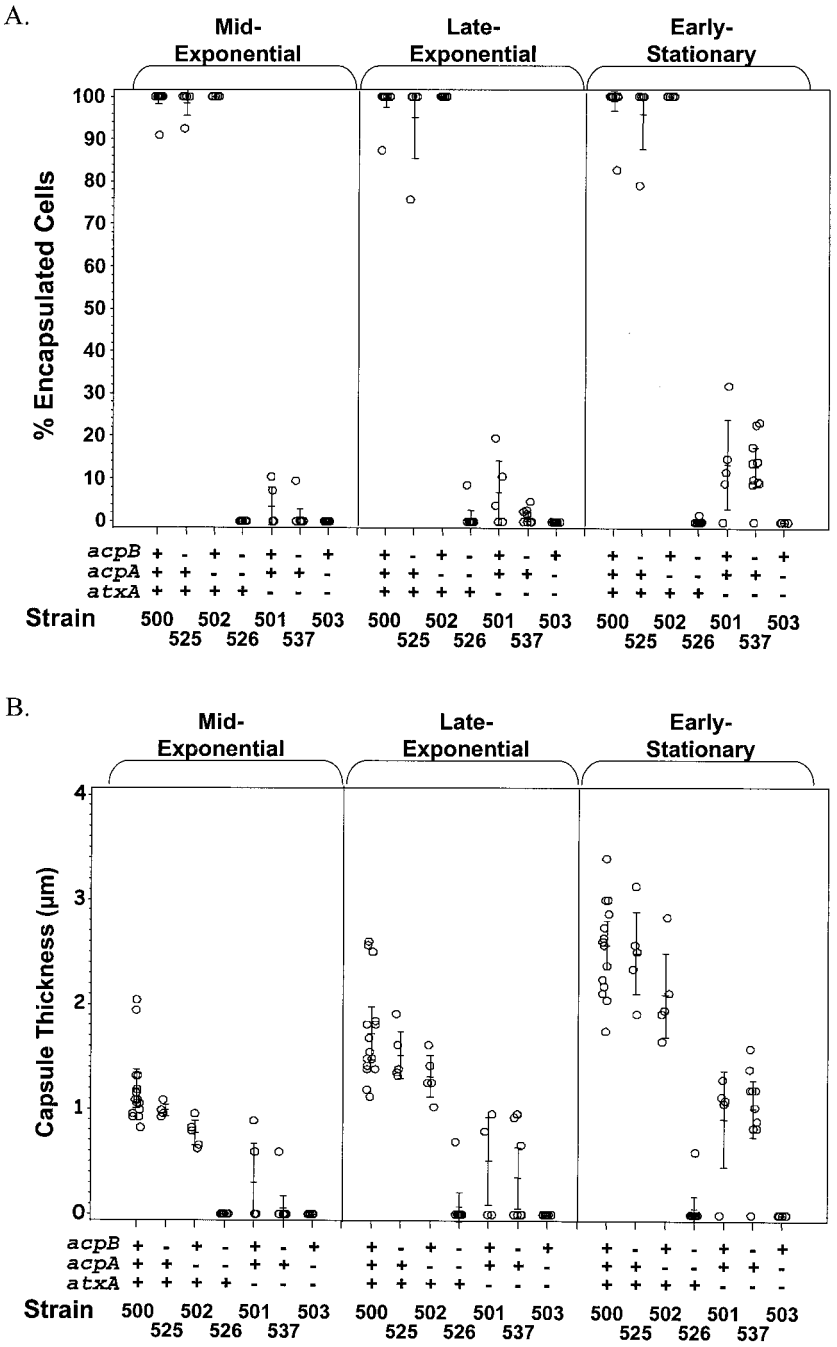

FIG. 3. Comparison of capsule production by UT500 and mutant strains at mid-exponential, late exponential, and early stationary growth phases. (A) Percentages of capsulated cells. (B) Mean diameters of capsules. Each circle represents data obtained from a single culture. Vertical lines with horizontal hash marks show means and 95% confidence intervals. Strain names are listed at the bottom, and the corresponding genotypes are indicated above (+ or − for the *acpB*, *acpA*, and *atxA* genes).

($pXO1^-$ $pXO2^+$) strains (38, 40). In the same investigation, we also demonstrated that *atxA* positively regulates expression of *acpA* (2). Studies reported here reveal that a third gene, *acpB*, is involved in capsule gene regulation in a genetically complete ($pXO1^+$ $pXO2^+$) *B. anthracis* strain. In a $pXO1^+$ $pXO2^+$ strain with deletion of both *acpA* and *acpB*, no capsule synthesis is evident even though *atxA* is expressed in this strain. Based on our findings, we propose a new model for capsule gene regulation whereby *atxA* regulates the expression of the capsule biosynthetic gene operon via a positive effect on transcription of *acpA* and *acpB*. *acpA* and *acpB* appear to have partial overlapping function. Deletion of either gene in a $pXO1^+$ $pXO2^+$ strain has a small impact on capsule gene transcription and capsule synthesis. In contrast, *atxA* mutant

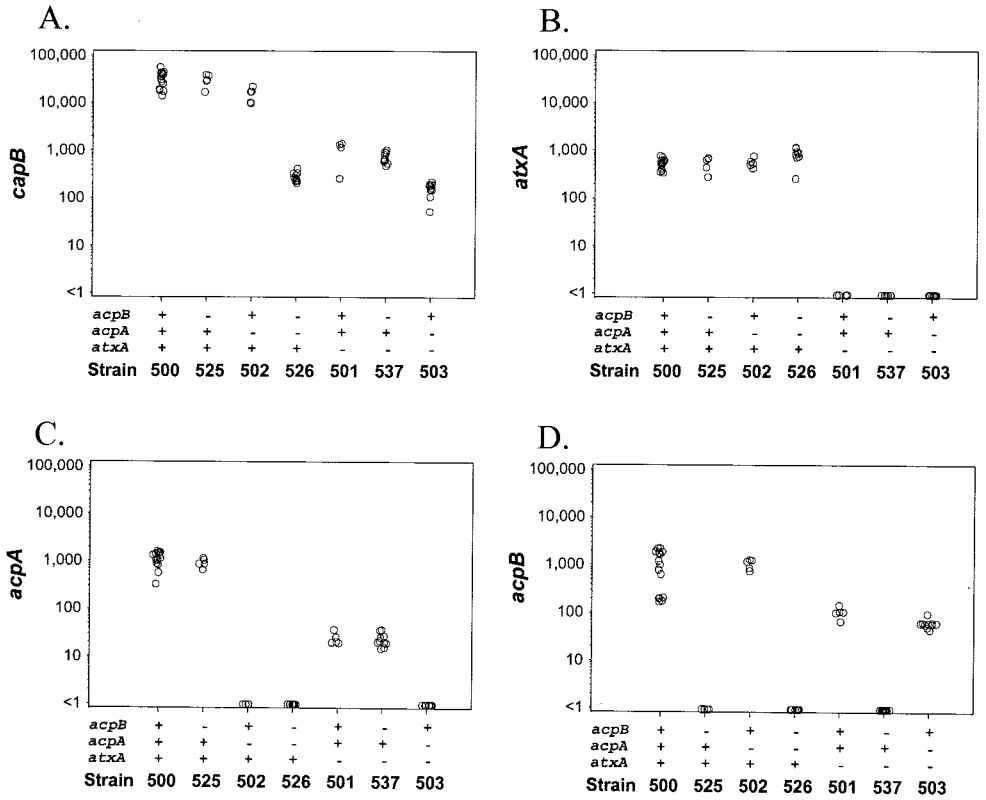

FIG. 4. Real-time transcript levels of *capB*, *atxA*, *acpA*, and *acpB* in UT500 and mutants. Data were normalized to *gyrB*. Each circle represents a result obtained from a single experiment. Circles less than 1 indicate that no transcript was detected.

cells are drastically impaired for *capB* gene expression and capsule synthesis.

The poorly capsulated phenotype of the *atxA* mutant cells may be due to limited expression of *acpA* and *acpB*. Expression of both genes is significantly decreased in the *atxA*-null strain. Results of experiments reported previously have indicated that *acpA* expression is limiting for capsule production (38, 40). Typically, pXO1$^-$ pXO2$^+$ strains are noncapsulated during growth in air or in physiologically relevant levels of bicarbonate-CO_2 (0.8% bicarbonate–5% CO_2). However, growth of such strains in buffered media in high atmospheric CO_2 (20%), conditions reported to increase expression of *acpA* (40), results in capsulated cells (14). Moreover, overexpression of *acpA* in a pXO1$^-$ pXO2$^+$ strain leads to capsule production during growth in air (38). The CO_2-induced increase in *acpA* expression (40) and *acpB* expression, as shown here, may lead to increased *cap* gene expression and capsule synthesis during growth in 20% CO_2, allowing for capsule synthesis even in the absence of *atxA*.

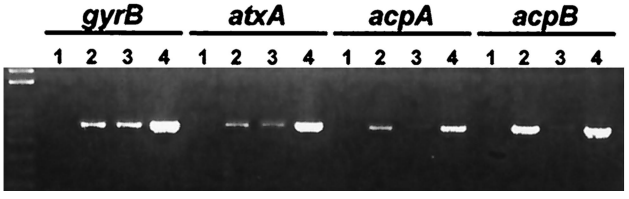

FIG. 5. Regulator expression during growth in air and elevated CO_2. Transcripts of genes indicated were detected using RT-PCR. Data shown are representative of three experiments using RNA prepared from different cultures. Lanes 1, RNA template control (CO_2) (similar results were obtained using RNA from cultures grown in air); lanes 2, cDNA template (CO_2); lanes 3, cDNA template (air); lanes 4, DNA template PCR control.

Although expression of both *acpA* and *acpB* is controlled by *atxA* and bicarbonate-CO_2, there are some important functional differences between the two regulators. In a pXO1$^-$ pXO2$^+$ strain, an *acpA* mutant is noncapsulated following growth in 20% CO_2 (40), while an *acpB*-null mutant is capsulated and indistinguishable from the pXO1$^-$ pXO2$^+$ parent strain (data not shown). The phenotypes of the *acpA* and *acpB* null mutants in a pXO1$^-$ strain background strengthen data indicating that *acpA* has a larger effect on *cap* gene expression than does *acpB*.

acpA and *acpB* are unique in another aspect. We showed previously that *atxA* and *acpA* have a synergistic effect on capsule gene expression (2). Results of Q-RT-PCR experiments reported here also reveal that deletion of both *atxA* and *acpA* has a much larger effect on *capB* gene expression than does deletion of either gene individually. *capB* transcript levels were decreased 32-fold in the *atxA* mutant and 2-fold in the *acpA* mutant. Yet, the *atxA acpA* double mutant showed a 200-fold decrease in the *capB* transcript level. In contrast, *capB* transcript levels in the *atxA* mutant and the *atxA acpB* double mutant were comparable, with 32-fold and 43-fold decreases,

respectively. Thus, unlike *acpA*, *acpB* does not appear to act synergistically with *atxA* for *capB* gene activation.

The functional dissimilarities observed between *acpA* and *acpB* may be due to differences in the primary sequence and/or steady-state levels of the gene products. The predicted amino acid sequences of the two proteins are 62% similar throughout. Slightly different secondary and tertiary structures may result in proteins with disparate affinities for their target(s). Future experiments will address the potential roles of protein concentration and stoichiometry in regulator function. It is important to note that specific DNA binding by AtxA, AcpA, and/or AcpB has not been demonstrated and the mechanism(s) of regulation has not yet been established.

Our results indicate that, in a genetically complete parent strain, *atxA* positively influences *acpA* and *acpB* expression but is not absolutely required for expression of these genes. Our data and findings reported by others suggest that *acpA* and *acpB* are limiting for *cap* gene transcription. Unlike *atxA*, the steady-state levels of *acpA*, *acpB*, and *capB* transcripts are positively controlled by bicarbonate-CO_2, and elevated levels of this signal can increase capsule synthesis even in the absence of *atxA* (14, 40). Uchida et al. (38) reported a positive effect of *atxA* on *capB* transcription when the two genes were cloned on separate multicopy plasmids in a pXO1$^-$ pXO2$^-$ strain (in the absence of *acpA* and *acpB*). Taken together, the data indicate some functional overlap among *acpA*, *acpB*, and *atxA*. Indeed, the predicted amino acid sequence of AtxA is 47 and 50% similar to those of AcpA and AcpB, respectively. We hypothesize that the mechanism for *cap* gene expression is highly dependent upon the stoichiometry of the three regulators. The physiological significance of the three regulators with regard to capsule synthesis during infection has not been assessed. Future research will address the role of each capsule gene regulator with regard to capsule synthesis and virulence in vivo.

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