INFECTION AND IMMUNITY, Aug. 1997, p. 3091–3099 Vol. 65, No. 8
0019-9567/97/$04.00+0

The Anthrax Toxin Activator Gene *atxA* Is Associated with CO_2-Enhanced Non-Toxin Gene Expression in *Bacillus anthracis*

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Received 10 February 1997/Returned for modification 15 April 1997/Accepted 15 May 1997

The *Bacillus anthracis* toxin genes, *cya*, *lef*, and *pag*, can be viewed as a regulon, in which transcription of all three genes is activated in *trans* by the same regulatory gene, *atxA*, in response to the same signal, CO_2. In *atxA*$^+$ strains, toxin gene expression is increased 5- to 20-fold in cells grown in 5% CO_2 relative to cells grown in air. CO_2-enhanced toxin gene transcription is not observed in *atxA*-null mutants. Here, we used two independent techniques to obtain evidence for additional CO_2-induced *atxA*-regulated genes. First, total protein preparations from *atxA*$^+$ and *atxA* isolates grown in 5% CO_2 and in air were examined by two-dimensional electrophoresis. Comparison of the resulting protein patterns indicated that synthesis of non-toxin proteins is influenced by growth in elevated CO_2 and the toxin gene regulator, *atxA*. Second, we generated random transcriptional *lacZ* fusions in *B. anthracis* with transposon Tn*917*-LTV3. Transposon-insertion libraries were screened for mutants expressing CO_2-enhanced *atxA*-dependent β-galactosidase activity. DNA sequence analysis of transposon insertion sites in 17 mutants carrying CO_2- and *atxA*-regulated fusions revealed 10 mutants carrying independent insertions on the 185-kb toxin plasmid pXO1 which did not map to the toxin genes. The *tcr-lacZ* fusion mutants (*tcr* for toxin coregulated) were Tox$^+$, indicating that these genes may not be involved in anthrax toxin gene activation. Our data indicate a clear association of *atxA* with CO_2-enhanced gene expression in *B. anthracis* and provide evidence that *atxA* regulates genes other than the structural genes for the anthrax toxin proteins.

Bacillus anthracis, the etiological agent of anthrax, produces two known virulence factors: a tripartite toxin and a poly-D-glutamic acid capsule. Synthesis of the toxin proteins (edema factor, lethal factor, and protective antigen) and capsule is induced during growth of the organism in certain minimal media in the presence of bicarbonate or under elevated (5% or greater) atmospheric CO_2 levels (15). The CO_2-bicarbonate effect is at the level of transcription for the three toxin genes, *cya*, *lef*, and *pag*, all located on pXO1 (185 kb), and the capsule gene, *capB*, located on pXO2 (95 kb) (2, 12, 25, 32). Induction of virulence gene expression by the CO_2-bicarbonate signal may be physiologically significant for an organism which invades mammalian host tissues.

The pXO1 gene *atxA* (anthrax toxin activator) is required for increased levels of toxin gene transcripts during growth in elevated CO_2 levels (7, 12, 30). AtxA also appears to activate toxin expression in vivo; *atxA*-null mutants are avirulent in mice, and mice infected with an *atxA*-null mutant show a decreased immunological response to the toxin proteins (7). In vitro transcription and translation of the 1.4-kb *atxA* gene results in synthesis of a 53-kDa protein (30), and *atxA*$^+$ *B. anthracis* strains contain a 56-kDa cytosolic protein which reacts with antibody generated against recombinant AtxA (6). The predicted amino acid sequence of AtxA shares some homology with two other regulators. AtxA is 51% similar and 28% identical to AcpA, which activates the *B. anthracis* capsule genes in response to elevated CO_2-bicarbonate levels (32). AtxA is 47% similar and 22% identical to Mga, a transcriptional activator of virulence genes in *Streptococcus pyogenes* (20). In each case, the homology is relatively weak and distributed throughout the proteins.

atxA is the only *B. anthracis* gene known to play a role in toxin gene expression. This regulatory gene can activate all three of the toxin genes in trans (7, 12, 30). However, the mechanism for *atxA*-mediated gene activation has not been elucidated. Sequence analysis of the toxin gene promoter regions has revealed no obvious similarities, and there is no evidence that AtxA is a DNA-binding protein. The purpose of this study was to determine whether *atxA* regulates genes other than *cya*, *lef*, and *pag* in response to the CO_2-bicarbonate signal. Here we report evidence for pXO1-encoded non-toxin genes which are controlled by a common signal and/or mechanism.

MATERIALS AND METHODS

Strains, media, and growth conditions. *Escherichia coli* JM109 and LE392 were used as hosts for cloning. Isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 40 μg/ml) were used in Luria-Bertani (LB) agar (8) to monitor β-galactosidase activity in the construction of plasmids.

B. anthracis strains and their relevant characteristics are listed in Table 1. All *B. anthracis* strains are derivatives of the Weybridge strain, a capsule-negative toxigenic isolate originally obtained from the Microbiological Research Establishment, Porton Down, England. For DNA extractions, the *B. anthracis* strains were grown brain heart infusion medium (BHI) (Difco, Detroit, Mich.) containing 10% horse serum. For transduction and electroporation experiments, *B. anthracis* strains were grown in BHI containing 0.5% glycerol (BHIG). XO minimal medium contained glucose (0.5%), ferric chloride (40 μg/ml), thiamine hydrochloride (10 μg/ml), glycine (200 μg/ml), L-methionine (40 μg/ml), L-proline (40 μg/ml), L-serine (40 μg/ml), L-threonine (40 μg/ml), $(NH_4)_2SO_4$ (2 mg/ml), KH_2PO_4 (6 mg/ml), K_2HPO_4 (14 mg/ml), sodium citrate (1 mg/ml), $MgSO_4 \cdot 7H_2O$ (0.005 mg/ml), $MnSO_4 \cdot H_2O$ (0.00025 mg/ml), L-glutamic acid (2 mg/ml), and agar (1.5%).

Culture supernatant samples for immunoblotting experiments were obtained

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Relevant characteristic(s)[a]	Source and/or reference[b]
Plasmids		
B. anthracis pXO1	Tox^+	27
E. coli		
pBR322	Ap^r Tc^r	NEB
pBSIIKS$^+$	Ap^r	Stratagene
Bifunctional		
pLTV3	Tc^r Cm^r Em^r; carries Tn*917*-LTV3	4
pMK3	Ap^r in *E. coli*; Cm^r in *B. anthracis*	BGSC
pUTE29	Ap^r in *E. coli*; Tc^r in *B. anthracis*	12
pUTE172	pUTE29 derivative, ΔLacZ α peptide sequences	This work
pUTE178	3.6-kb *Bam*HI-*Eco*RI fragment of pXO1 containing *atxA* cloned into pUTE172	This work
Strains		
B. anthracis		
UM23[c]	Tox^+ Ura^-	3
UM23C1-2	pXO1$^-$ derivative of UM23, Ura^-, Rif^r	C. Thorne
UM44[c]	Tox^+ Ind^-	27
UM44-1C9	pXO1$^-$ derivative of UM44; Ind^- Str^r	C. Thorne
UT36	Isolated from Tn*917*-LTV3 insertion library of UM44 (pLTV3); carries *lef*::*lacZ* fusion on pXO1; Ind^- Em^r	
UT53	*atxA*-null derivative of UM44; *atxA* is replaced by Ω*km-2*; Ind^- Km^r	7
UT61	*atxA*-null derivative of UM23; *atxA* is replaced by Ω*km-2*; Ura^- Km^r	This work
E. coli		
GM1684	F′ F-*lacI*^{q} DM15 *pro*$^+$/*dam-4* Δ(*lac-pro*)*X111 thi-1 glnV44* (*relA1*)	R. Kolter
JM109	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15/*recA1 endA1gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *mcrA*	34
LE392	F′ *hsdR514 supE44 supF58 lacY1 galT22 metB1 trpR55*	16

[a] Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Ind, indole; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Tox, anthrax toxin proteins.

[b] Abbreviations: NEB, New England Biolabs; BGSC, Bacillus Genetic Stock Center.

[c] UM23 and UM44 were derived from the Weybridge strain.

from *B. anthracis* cultures grown in CA broth (29) buffered with 100 mM HEPES (pH 8.0). Cell-associated protein preparations to be analyzed by two-dimensional electrophoresis and immunoblotting were derived from *B. anthracis* cultures grown in R medium (23). CA and R media contained 0.8% sodium bicarbonate when incubated in 5% CO_2. CA plates containing X-Gal (40 μg/ml) (CAX) were used to monitor the β-galactosidase activity of *B. anthracis* mutants. All synthetic media were supplemented with L-tryptophan (40 μg/ml) or uracil (40 μg/ml), as required for the auxotrophy of UM44- and UM23-derived mutants, respectively.

All antibiotics were purchased from Sigma (St. Louis, Mo.) or Fisher Scientific (Pittsburgh, Pa.) and were added to media at the following concentrations when appropriate: ampicillin, 100 μg/ml; erythromycin, 1 μg/ml; lincomycin, 25 μg/ml; kanamycin, 20 μg/ml for *E. coli* and 50 μg/ml for *B. anthracis*; and tetracycline, 10 μg/ml for *E. coli* and 5 μg/ml for *B. anthracis.*

Protein preparation for two-dimensional electrophoresis. Cells from selective LB agar plates were inoculated into 250-ml flasks each containing 15 ml of R medium such that the initial optical density at 600 nm (OD_{600}) was 0.1 to 0.2. Cultures were incubated in air or in 5% CO_2 with vigorous stirring at 37°C to a final OD_{600} of 0.8 to 1.0. The cells were collected from 2-ml culture samples by centrifugation, washed twice with 0.05 M Tris (pH 7.5) containing 0.002 M phenylmethylsulfonyl fluoride, resuspended in 2 ml of sample buffer I (1% sodium dodecyl sulfate [SDS], 200 mM dithiothreitol, 28 mM Tris HCl, 22 mM Tris OH), and passed through a French press three times at 20,000 lb/in^2. The lysates were boiled for 10 min and then cooled on ice for 5 min. A 100-μl volume of sample buffer II (24 mM Tris base, 476 mM Tris HCl, 50 mM $MgCl_2$, 1 mg of DNase I per ml, 0.25 mg of RNase A per ml) was added to each lysate, and the lysates were incubated at room temperature for 5 min to digest DNA and RNA. The lysates were centrifuged at 16,000 × *g* for 10 min. Trichloroacetic acid was added to the supernatants to a final concentration of 10%, and the samples were left on ice for 10 min. Following centrifugation at 16,000 × *g* for 5 min, protein pellets were washed twice with 500 μl of cold acetone. The pellets were air dried for 5 min and resuspended in 200 μl of sample buffer III (9.9 M urea, 4% Nonidet NP-40, 2.2% ampholytes, 100 mM dithiothreitol) containing ampholytes in the pH 4 to 8 range. Finally, the samples were centrifuged in a Beckman Airfuge at 100,000 × *g* for 1 h. The protein concentrations of the supernatants were determined with the Bio-Rad protein assay reagent as specified by the manufacturer, with bovine serum albumin (Sigma) as the standard. The protein concentrations ranged from 1.5 to 6.0 μg/μl. For each strain and growth condition, protein was prepared from three separate cultures. Each protein preparation was then subjected to two-dimensional electrophoresis three times.

Two-dimensional electrophoresis. The Investigator two-dimensional electrophoresis system and all reagents were purchased from Oxford Glycosystems (Bedford, Mass.). Isoelectric focusing gels contained ampholytes in the 4 to 8 pH range. Samples containing 50 to 100 μg of protein were focused to equilibrium for 17.5 h under the following conditions: maximum voltage, 2,000 V; holding voltage, 125 V; current, 110 mA/gel; and 18,000 V-h. The protein was run in the second dimension on a 10% duracryl gel at a maximum voltage of 500 V and a maximum power of 20,000 mW/gel until the bromophenol blue dye front was 1 cm from the bottom (about 4.5 h). The gels were silver stained by a modification of the protocol of Morissey (19) described by Oxford Glycosystems.

Immunoblotting. For detection of lethal factor and protective antigen in culture supernatants and AtxA in cell extracts, *B. anthracis* cultures were grown in CA medium to an OD_{600} of 0.8. The cells were separated from culture supernatants by filtration through cellulose acetate membranes (pore size, 0.45 μm; Nalge, Rochester, N.Y.). To prepare crude cell extracts, the cells collected on filters were resuspended in sample buffer I containing 5 mM phenylmethylsulfonyl fluoride and disrupted by passage through a French press minicell three times at 20,000 lb/in^2. The soluble fraction was obtained following centrifugation at 16,000 × *g* for 5 min. Protein concentrations in soluble fractions were determined by using the Bio-Rad protein assay reagent with bovine serum albumin as the standard. Comparable amounts of protein from the cell extracts were subjected to SDS-polyacrylamide gel electrophoresis (7% polyacrylamide).

Supernatants from cultures grown in CA medium were applied to nitrocellulose membranes by vacuum blotting with a slot blot apparatus (Hoefer Scientific, San Francisco, Calif.). Proteins from one- and two-dimensional gels were transferred to nitrocellulose membranes by electroblotting. The membranes were reacted with rabbit anti-protective-antigen (PA) serum (diluted 1:6,000 in 20 mM Tris OH, 137 mM NaCl, 0.1% Tween 20, 5% milk [pH 7.6]), rabbit anti-lethal-factor (LF) serum (1:5,000), or rabbit anti-AtxA serum (1:2,000) for 2 h at room temperature. They were then washed in TBS-T with 5% milk and finally reacted with donkey anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (diluted 1:4,000 in TBS-T with 5% milk) for 1 h at room temperature. Immunoreactive material was visualized on autoradiographs with an enhanced

chemiluminescence immunoblotting (Amersham, Little Chalfont, England). Rabbit anti-LF serum was kindly provided by P. Hanna.

DNA isolation and manipulation and plasmid constructions. Plasmid DNA was extracted from *B. anthracis* by the method of Green et al. (11). Minipreparations of plasmid DNA from *E. coli*, transformation of *E. coli*, and recombinant DNA techniques were carried out by standard procedures (1). *B. anthracis* was electroporated with plasmid DNA from *E. coli* GM1684, as described previously (12). Restriction enzymes, reverse transcriptase, T4 DNA ligase, and Klenow fragment were purchased from Promega (Madison, Wis.), Boehringer Mannheim (Indianapolis, Ind.), or New England Biolabs (Beverly, Mass.). The DNA sequence encoding the α-peptide of β-galactosidase was removed from the shuttle vector pUTE29 (12) to create pUTE172. Briefly, pUTE29 was cleaved with *Nar*I and *Hin*dIII and the DNA ends were blunted with Klenow fragment prior to ligation with T4 DNA ligase.

Transposon-mediated mutagenesis and sequence analysis of 5′ junction fragments. The temperature-sensitive delivery vector pLTV3 (4) was used to deliver Tn*917*-LTV3 insertions to the genome of *B. anthracis* UM44(pXO1), as described previously (12). The 5′ junction fragments of Tn*917*-LTV3 insertion mutants were cloned in *E. coli* LE392 with *Xba*I or *Kpn*I, as described by Camilli et al. (4). *B. anthracis* DNA sequences (400 to 800 bases) upstream of transposon insertion sites were determined with the Perkin-Elmer Applied Biosystems Prism 377 DNA sequencer. The Tn*917*-LTV3 primer was kindly provided by D. Portnoy. Sequence data were analyzed with the FASTA or BLAST program of the University of Wisconsin Genetics Computer Group software package.

β-Galactosidase assays. It has been reported that the growth and specific enzyme activity of *B. anthracis* cells producing high levels of β-galactosidase can be highly variable when cells are grown in liquid media. Autolysis of the cells leads to the release of inactive β-galactosidase into the culture medium (24). Therefore, we measured the specific β-galactosidase activity of cells grown on solid medium. *B. anthracis* cells were streaked onto CA plates and incubated at 37°C for 24 h. The cells were subsequently removed from the plate with a toothpick and solubilized in 1% toluene in Z buffer. β-Galactosidase activity was measured by the *o*-nitrophenylgalactoside hydrolysis procedure of Miller (18). Protein concentrations of nonpermeabilized cell suspensions were determined with the Bio-Rad protein assay reagent as specified by the manufacturer, with bovine serum albumin as the standard. The results shown are from representative experiments. There was some day-to-day variation in specific activity, but absolute differences due to altered growth conditions and between strains remained the same.

CP51-mediated transductions. A temperature-sensitive mutant of the generalized transducing phage CP51, kindly provided by C. B. Thorne, was used to transduce Ω*km-2* insertion mutations and plasmids between *B. anthracis* isolates. The phage was propagated and assayed as described previously (7, 28). Recipient strains were grown overnight in BHIG containing antibiotics when appropriate. Transductions were performed on HA membranes (pore size, 0.45 μm; diameter, 47 mm; Millipore, Bedford, Mass.) placed on LB agar plates. Membranes were spread with 0.1 ml of a CP51 lysate containing approximately 10^9 PFU. Recipient cultures were applied to the phage-coated membranes in 10-μl drops containing approximately 7×10^6 CFU. Plates were incubated at 40°C for 2 h. The membranes were subsequently transferred to LB agar containing kanamycin (for Ω*km-2* transduction) or tetracycline (for plasmid transduction) and incubated overnight at 40°C.

Nucleotide sequence accession number. The nucleotide sequence of *tcr-2* has been deposited in the GenBank database under accession no. AF003936.

RESULTS

Analysis of CO_2- and *atxA*-regulated protein synthesis by two-dimensional protein electrophoresis. Transcription of the known *atxA*-regulated genes (*pag*, *lef*, and *cya*) is enhanced when *B. anthracis* strains are grown in elevated (5% or greater) levels of CO_2. To determine whether *atxA* affects the synthesis of proteins other than the toxin components and to observe global changes in the protein composition of *B. anthracis* cells following growth in air in comparison with 5% CO_2, we examined protein samples from cultures of UM44(pXO1) and the *atxA*-null derivative UT53 by two-dimensional electrophoresis. UM44 cells were cultured in air and 5% CO_2 so that changes in protein composition due to growth in different atmospheres could be compared to *atxA*-dependent changes.

Figure 1a shows a representative gel containing a protein sample from UM44 grown in a 5% CO_2 atmosphere. Boxed regions A to D represent areas containing silver-stained spots which differed consistently from those observed in protein preparations from UM44 grown in air and from UT53 grown in 5% CO_2. Figure 1b shows regions A to D from gels containing protein from UM44 grown in 5% CO_2 (Fig. 1a), UM44 grown in air, and UT53 (*atxA*) grown in 5% CO_2, as indicated. The arrows indicate consistent differences in the intensity of silver-stained spots.

Seven silver-stained spots inside box A represent proteins of approximately 85,000 Da, which are clearly *atxA* and CO_2 dependent. We predicted that these spots were likely to include, and may be exclusively, the toxin proteins. Edema factor (EF), PA, and LF have apparent sizes of 89, 85, and 83 kDa, respectively, when purified proteins from *B. anthracis* culture supernatants are analyzed on one-dimensional SDS-polyacrylamide gels. Furthermore, PA is known to migrate as different isoforms when subjected to isoelectric focusing (14, 15). The results of immunoblotting experiments with anti-toxin antisera revealed that the four spots representing proteins with higher molecular masses reacted with anti-PA antiserum while the three spots representing proteins with lower molecular masses reacted with anti-LF antiserum (data not shown). Spots representing PA and LF are indicated in Fig. 1b box A. Antibody to EF was not available.

Boxes B, C, and D contain spots representing proteins which were CO_2 and/or *atxA* dependent yet did not react with anti-toxin antisera. Boxes B and C show four spots which appear to be weakly *atxA* and CO_2 dependent. Box D shows two spots which are *atxA* dependent but not CO_2 dependent. These data indicate that synthesis of nontoxin proteins is influenced by growth in elevated CO_2 and by the toxin gene regulator, *atxA*.

Isolation of mutants harboring CO_2-enhanced *atxA*-dependent transcriptional *lacZ* fusions. To search for genes subject to CO_2 and *atxA* control, we generated random promoter-*lacZ* fusions in UM44(pXO1) by using transposon Tn*917*-LTV3 and screened for regulated β-galactosidase synthesis. Tn*917*-LTV3 generates transcriptional *lacZ* fusions when it inserts into a gene in the proper orientation. This transposon has a high frequency of transposition to pXO1. Mutants carrying transposon insertion mutations on the *B. anthracis* chromosome have been obtained only when pXO1$^-$ strains were used (11a). Therefore, our screens were biased toward finding pXO1-encoded CO_2- and *atxA*-regulated genes.

Spores from Tn*917*-LTV3 insertion libraries were plated on CAX agar and incubated in 5% CO_2. Mutants which produced blue colonies were considered to harbor active transcriptional *lacZ* fusions. These isolates were then tested for apparent CO_2-enhanced β-galactosidase activity by being transferred to fresh CAX plates and screened for mutants that produced blue colonies when incubated in 5% CO_2 and white to light blue colonies when incubated in air. To determine whether the mutants harboring apparent CO_2-enhanced fusions were also *atxA* regulated, we deleted the *atxA* gene from these isolates and screened them for decreased β-galactosidase activity following growth in 5% CO_2. *B. anthracis* UT53 is an *atxA*-null mutant in which the *atxA* coding sequence is replaced with the Ω*km-2* element. Mutants harboring CO_2-enhanced fusions were transduced to kanamycin resistance with bacteriophage CP51 propagated on UT53. Km^r transductants were Tox$^-$ and carried the *atxA*-null mutation of UT53, as indicated from the results of immunoassays and Southern hybridization experiments (data not shown). The β-galactosidase activity of the *atxA* mutants was compared to that of the parent transposon insertion mutants when incubated in 5% CO_2 on CAX plates. *atxA*-null mutants which produced white colonies were considered to carry *atxA*-dependent transcriptional *lacZ* fusions.

To map the Tn*917*-LTV3 insertion sites in CO_2- and *atxA*-regulated mutants, we cloned the *B. anthracis* DNA flanking the 5′ ends of Tn*917*-LTV3 insertions in 17 CO_2- and *atxA*-regulated mutants obtained from five different transposon insertion libraries. Restriction fragment analysis of multiple

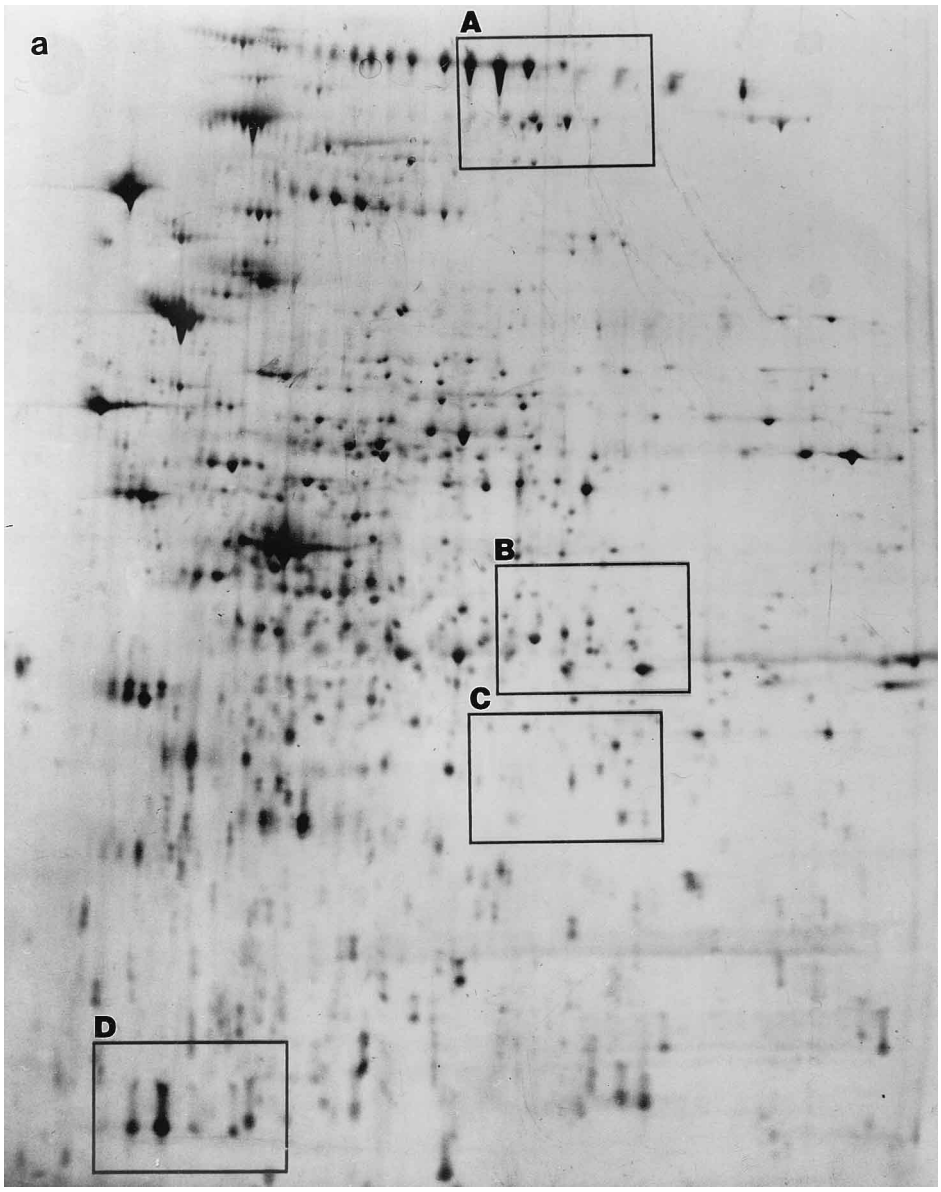

FIG. 1. Two-dimensional electrophoresis of a protein sample from *B. anthracis*. Isoelectric focusing gels contained ampholytes in the pH 4 to 8 range. The samples were electrophoresed in the second dimension in gels containing 10% duracryl. Boxed regions (A, B, C, and D) contain spots representing *atxA*- and/or CO_2-enhanced proteins. (a) Representative gel showing the protein profile of UM44 grown in 5% CO_2. (b) Comparison of regions A to D in gels containing protein from UM44 grown in air, UM44 grown in 5% CO_2, and UT53 grown in 5% CO_2, as indicated. Region A contains seven CO_2- and *atxA*-enhanced protein spots. The four spots to the right of the arrow marked P represent protective antigen. The three spots to the right of the arrow marked L represent lethal factor (see Results). Other arrows indicate spots representing unidentified *atxA*- and/or CO_2-enhanced proteins.

clones indicated that all but two mutants harbored single transposon insertions. Two different clones were obtained from each of two mutants, UT20 and UT82, indicating that each of these isolates carries two different transposon insertions. Results of Southern hybridization experiments (data not shown) and analysis of 400- to 800-nucleotide (nt) DNA sequences adjacent to the transposon insertion sites revealed that 10 of the 17 mutants examined harbored Tn*917*-LTV3 insertions on pXO1 at independent sites which did not correspond to the toxin genes. The remaining seven mutants carried transposon insertions in known CO_2-enhanced *atxA*-dependent genes, thus confirming that the strategy can be used to locate CO_2- and *atxA*-regulated promoters. Two mutants carried insertions in *pag*, and five carried insertions in *lef*. The *pag* and *lef* insertion mutants have Tn*917*-LTV3 insertions in different locations, indicating that these isolates are not siblings (Fig. 2). None of the mutants tested carried insertions in the other known CO_2- and *atxA*-regulated gene, *cya*. This may be due to the low level of *cya* expression, relative to *pag* and *lef*, observed in *B. anthracis* (15, 25).

As was noted in the analysis of CO_2- and *atxA*-regulated protein synthesis, a strong correlation between CO_2-enhanced and *atxA*-dependent regulation was observed when transcriptional *lacZ* fusion mutants were isolated. Table 2 shows the results of three representative experiments, each with an independently generated Tn*917*-LTV3 insertion library. An average of 39% of the transposon mutants appeared to synthesize β-galactosidase when incubated in 5% CO_2. Of these, 12%

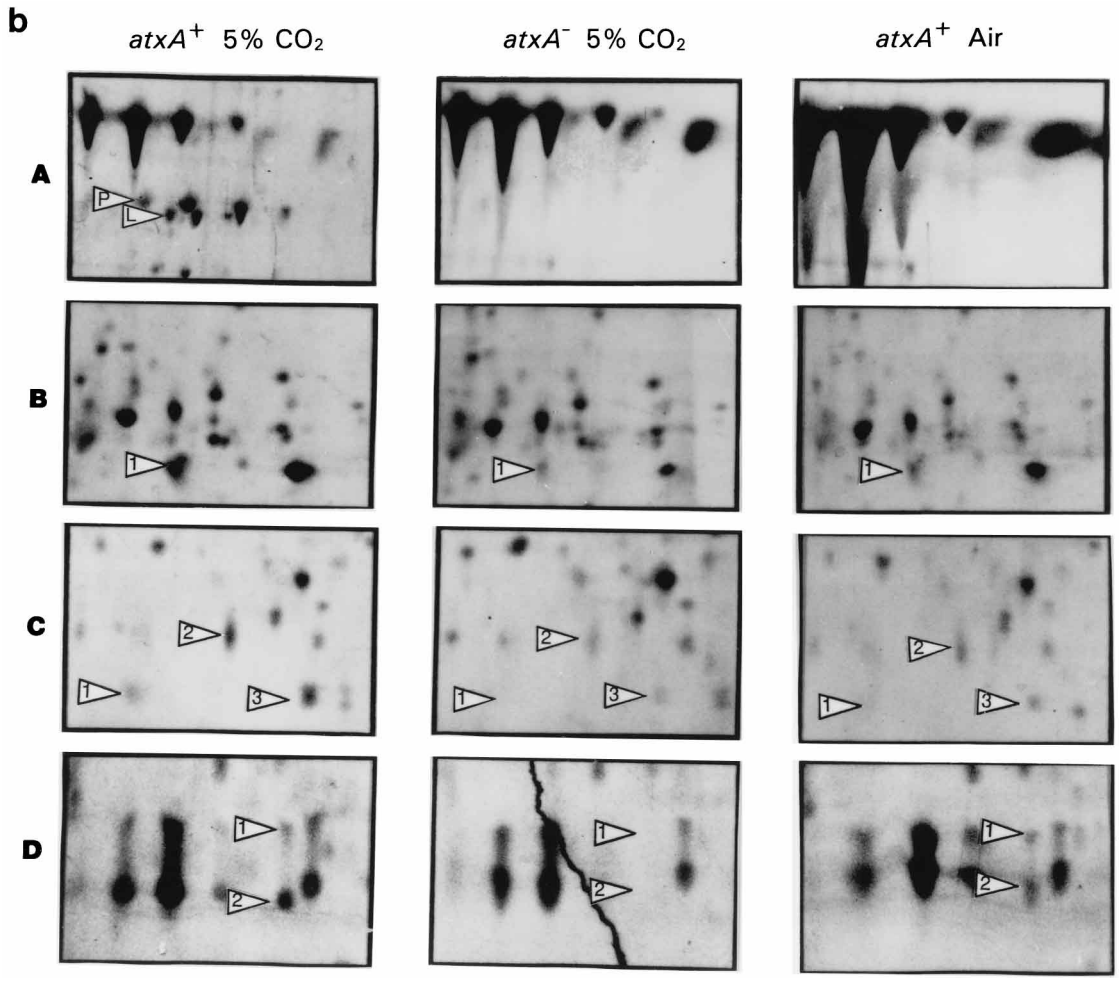

FIG. 1—*Continued.*

appeared to synthesize greater amounts of β-galactosidase when grown in 5% CO_2 than in air. The majority of these CO_2-enhanced fusions (79%) were also *atxA* dependent. In the experiments shown, 8 of the 11 CO_2- and *atxA*-regulated mutants obtained carried transposon insertions in independent sites outside of the toxin genes.

Quantitation of *tcr-lacZ* fusion expression in *atxA*$^+$ and *atxA* mutants. We designated the fusions in CO_2- and *atxA*-regulated mutants *tcr-lacZ* (for toxin coregulated). The specific β-galactosidase activity of the *atxA*$^+$ *tcr-lacZ* fusion mutants was determined following growth in air and in 5% CO_2. UT36, a *lef-lacZ* fusion mutant, served as a control. As shown in Fig. 3, the magnitude of the effect of CO_2 on *tcr-lacZ* expression varied in the different mutants; induction levels ranged from 2.4-fold (for UT25) to 40-fold (for UT82). β-Galactosidase activity of UT36 was enhanced 33-fold in cells grown at elevated CO_2 levels. When the *atxA*-null mutation was transduced into the *tcr-lacZ* mutants and UT36, the specific enzyme activity of cells grown at elevated CO_2 concentrations was reduced to levels comparable to or below that of *atxA*$^+$ isolates grown in air.

Since *atxA* has been shown to activate toxin gene expression in *trans* (12, 30), we tested for complementation of the *atxA*-null mutants by the addition of pUTE178, a multicopy plasmid carrying the *atxA* gene. The specific enzyme activity of the mutants was determined following growth in 5% CO_2. As shown in Table 3, when pUTE178 was transduced into the *atxA*-null derivative of UT36, which carries a *lef-lacZ* fusion,

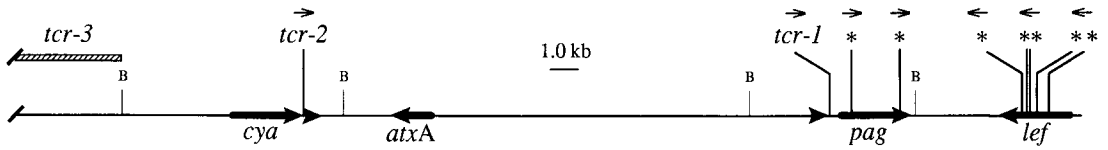

FIG. 2. Tn*917*-LTV3 insertion sites in mutants carrying *atxA*- and CO_2-activated promoter-*lacZ* fusions which map to known regions on pXO1. A 30-kb region of the plasmid, which contains all known genes, is shown. *, insertion sites in *pag* or *lef* genes. *tcr-1* and *tcr-2*, insertion sites in mutants UT34 and UT76, respectively. The hatched bar indicates a 14.3-kb *Bam*HI fragment containing the *tcr-3* insertion site of UT20. The small arrows indicate the transcriptional orientation of the transposon-associated *lacZ* genes. B, *Bam*HI.

TABLE 2. Screen for Tn*917*-LTV3 insertion mutants harboring CO_2-enhanced *atxA*-dependent transcriptional *lacZ* fusions

Expt[a]	No. of fusions/ no. of insertions (%)[b]	No. of CO_2-enhanced fusions/total no. of fusions (%)[c]	No. of *atxA*-dependent fusions/no. of CO_2-regulated fusions (%)[d]
1	31/120 (26)	8/31 (26)	7/8 (87)
2	79/120 (66)	3/61 (5)	3/3 (100)
3	29/120 (24)	3/25 (12)	1/3 (33)
Combined	139/360 (39)	14/117 (12)	11/14 (79)

[a] Results of three representative experiments are shown. For each experiment, an independent Tn*917*-LTV3 insertion library was tested.

[b] Insertion mutants (Em^r Tc^s isolates) were screened on CAX plates for *lacZ* expression (Lac^+) during growth in 5% CO_2. Mutants which produced blue colonies were considered to harbor active transcriptional *lacZ* fusions.

[c] Mutants which appeared Lac^+ in 5% CO_2 were screened for decreased *lacZ* expression on CAX plates incubated in air. Mutants which produced colonies appearing more blue following growth in 5% CO_2 than following growth in air were considered to harbor CO_2-enhanced transcriptional *lacZ* fusions.

[d] The *atxA* gene was deleted in mutants harboring CO_2-regulated fusions. *atxA* isolates were screened for decreased *lacZ* expression, relative to *atxA*$^+$ parental strains, when incubated in 5% CO_2.

β-galactosidase activity was fully restored to the level detected in the *atxA*$^+$ parent strain. Interestingly, only a subset of the CO_2- and *atxA*-regulated *tcr-lacZ* mutants harboring the *atxA*-null mutation were complemented by pUTE178. Expression of the *tcr-lacZ* fusions in *atxA*-null derivatives of UT79, UT25, UT34, and UT33 was activated when pUTE178 was transduced into these isolates (Table 3). Transduction of pUTE178 into *atxA*-null mutants of UT79 and UT25 restored the specific β-galactosidase activity of these isolates to 91 and 72% of the activity detected in the parent strains. However, the enzyme activity of *atxA*-null mutants of UT34 and UT33 carrying pUTE178 was only 21 and 20% of the levels detected in the parent strains. The *atxA*-null derivatives of the remaining six *tcr-lacZ* mutants were not complemented by pUTE178 (data not shown).

To confirm that AtxA synthesis was restored in mutants carrying the *atxA*-null mutation and harboring pUTE178, we tested cell extracts for the presence of AtxA. The results of

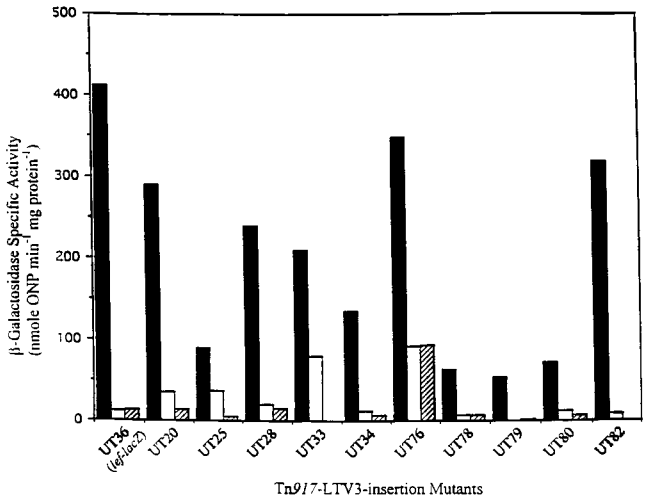

FIG. 3. CO_2- and *atxA*-induced expression of β-galactosidase by *tcr-lacZ* isolates. Specific enzyme activity was determined for cells grown in 5% CO_2 (solid bars) or air (open bars) and for *atxA*-null mutants grown in 5% CO_2 (hatched bars). UT36 harbors a *lef-lacZ* transcriptional fusion. The data shown were obtained with cells grown for 24 h on solid CA medium as described in Materials and Methods.

TABLE 3. Complementation of the *atxA tcr-lacZ* isolates[a]

Isolate	β-Galactosidase activity (nmol of ONP min^{-1} mg of protein^{-1}) of:		
	atxA$^+$	*atxA*	*atxA* (pUTE178)[b]
UT36 (*lef-lacZ*)	412	14	430 (104%)
UT79	53	2	48 (91%)
UT25	90	5	65 (72%)
UT34	135	6	28 (21%)
UT33	210	0	42 (20%)

[a] The data shown were obtained with cells grown for 24 h on solid CA medium, as described in Materials and Methods. The β-Galactosidase activity of mutants following growth in CA broth to late log phase indicated similar induction ratios.

[b] Plasmid pUTE178 carries the *atxA* gene. Numbers in parentheses represent β-galactosidase activity relative to the *atxA*$^+$ parent strain.

immunoblotting experiments indicated that the steady-state level of AtxA in strains carrying pUTE178 was approximately threefold greater than that of the parental strain, UM44, which harbors a single copy of *atxA* on pXO1 (data not shown). Thus, lack of complementation of some of the *atxA*-null mutants by pUTE178 was not due to the absence of AtxA protein.

Sequence analysis of *tcr*–Tn*917*-LTV3 junction regions. DNA sequences upstream of the transposon insertion sites in the *tcr-lacZ* mutants, and the corresponding predicted protein sequences, were examined for possible clues to the function of putative *tcr* genes by testing for similarities to sequences in the database(s) with the BLAST and FASTA algorithms. For the majority of the mutants, these sequences did not show significant similarity to known genes or proteins.

Sequence analysis revealed that mutant UT34 (*tcr-1*) carried a transposon insertion 161 bp downstream of a 576-nt open reading frame designated *orf-1* (Fig. 2). This open reading frame, which is located adjacent to the 5′ end of the *pag* gene, was identified previously by Welkos et al. (33). *orf-1* (sequence accession no. M22589) has significant homology (61% similar and 37% identical) to the carboxy terminus of *pag*. Potential −10 and −35 RNA polymerase recognition sites were located upstream of the predicted translational start codon. However, sequence analysis did not reveal a consensus ribosome-binding site (33). The 5′ ends of *pag* mRNA transcripts have been mapped to positions downstream of *orf-1*, indicating that these genes are not cotranscribed (12).

The transposon insertion site in UT76 (*tcr-2*), was located 217 bp downstream of the *cya* gene and 35 bp upstream of a previously unidentified 332-nt open reading frame (Fig. 2). This open reading frame has significant sequence similarity to *apt* (adenine phosphoribosyltransferase) genes of many organisms (unpublished data). The transcriptional orientation of the transposon-associated *lacZ* gene in UT76 is the same as that of *cya* and the downstream ORF. The precise 3′ end of *cya* mRNA has not been determined. It is possible that transcription of the *lacZ* gene in this mutant is controlled by the *cya* promoter. However, the β-galactosidase activity expressed by UT76 during growth at elevated CO_2 levels is comparable to that expressed by a *lef-lacZ* fusion (Fig. 3). This relatively high activity would be unexpected for a *cya-lacZ* fusion, considering the relatively low strength of the *cya* promoter (25).

UT20 carried two Tn*917*-LTV3 insertions. The sequence of the DNA 5′ of one of the insertion sites did not show significant similarity to known genes or proteins. However, the DNA sequence 5′ of the other insertion site (*tcr-3*) has the potential to encode a protein with several transmembrane domains, as indicated from the results of hydropathy analysis. This insertion site mapped to a 14.3-kb *Bam*HI fragment of pXO1 (Fig. 2).

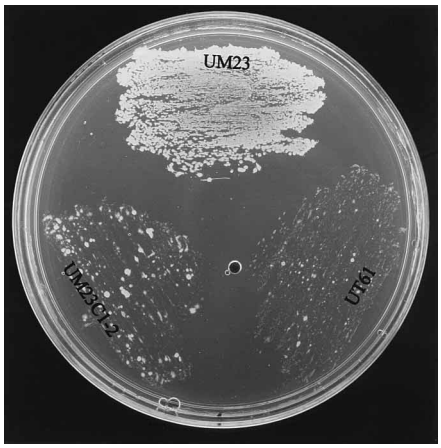

FIG. 4. Growth of UM23(pXO1$^+$), UM23C1-2(pXO1$^-$), and UT61(pXO1$^+$ *atxA*) on XO medium. Spores were streaked to plates and incubated at 37°C in air for 2 days.

Toxin synthesis by *tcr-lacZ* mutants. To determine whether the *tcr* genes may play a role in toxin gene expression, the *tcr-lacZ* mutants were tested for PA and LF synthesis. Culture supernatants from cells grown in 5% CO_2 were examined for PA and LF by immunoblotting (data not shown). None of the mutants exhibited decreased toxin protein synthesis, indicating that the transposon insertion mutations in these isolates did not insertionally inactivate any genes required for toxin production.

Growth of *atxA*-null and pXO1$^-$ strains on minimal medium. *B. anthracis* strains cured of pXO1 grow poorly on certain minimal media compared to pXO1$^+$ strains. Poor growth of cured isolates on minimal medium is not due to a simple auxotrophic requirement, and specific genes associated with this cured-strain phenotype have not been identified (26). Considering our data indicating *atxA*-dependent expression of numerous pXO1-encoded genes, we questioned whether the growth of a pXO1$^+$ *atxA* mutant may resemble that of a pXO1$^-$ strain on this medium. Strain UM23(pXO1$^+$), which was isolated from the Weybridge strain and grows well on minimal XO medium, was used in previous growth studies by Thorne (26). We transduced the *atxA*-null mutation of UT53 into UM23 to generate the *atxA* strain UT61. Spores of UM23 (pXO1$^+$), UM23C1-2(pXO1$^-$), and UT61(pXO1$^+$, *atxA*) were streaked on XO minimal medium plates and incubated at 37°C in air. As shown in Fig. 4, UM23 grew well on this medium, producing white, rough colonies characteristic of nonencapsulated *B. anthracis* strains. UM23C1-2 and UT61 grew poorly, producing small, wispy colonies with occasional large-colony mutants.

This observation indicates that the altered growth of cured strains on minimal XO medium is related to the absence of *atxA* and provides further evidence that *atxA* has a profound effect on gene expression. Previously, the only known phenotype of *atxA* mutants was reduced toxin synthesis. It is possible that the cured phenotype of the *atxA*-null mutant is due to lack of expression of *atxA*-regulated chromosome- or plasmid-encoded genes which are important for growth on XO medium.

DISCUSSION

Microbial pathogens adapt to host environments during infection by altering gene expression in response to host-specific cues. In many cases, specific regulators of gene expression have been identified as proteins which play roles in sensing a specific signal, processing the change, and ultimately activating or repressing the expression of genes. In *Bacillus anthracis*, the *atxA* gene, located on the 185-kb plasmid pXO1, is a key player in the response of the organism to increased CO_2 or bicarbonate levels in mammalian hosts. Transcription of the three structural genes for the anthrax toxin proteins, *pag*, *lef*, and *cya*, all located on pXO1, is enhanced during growth in 5% or greater atmospheric CO_2 (2, 12, 25). CO_2-induced transcription of each toxin gene is dependent upon *atxA* (7, 12, 30).

In this paper, we have presented evidence that the expression of additional *B. anthracis* genes is subject to control by the same signal and regulatory factor as is expression of the anthrax toxin genes. The protein profiles of *atxA*$^+$ and *atxA B. anthracis* strains, as examined by two-dimensional SDS-polyacrylamide gel electrophoresis, revealed six proteins, other than the toxin proteins, which appeared to be *atxA* induced. The levels of four of these also appeared to be enhanced during growth in elevated CO_2. Genetic studies, in which transposon-generated promoter-*lacZ* fusion libraries were screened for regulated fusions, also revealed a strong correlation between CO_2-enhanced and *atxA*-dependent gene expression, indicating that the *atxA* gene is an important regulator of multiple changes in gene expression in response to the CO_2 signal.

Our regulated fusions may represent as many as 10 toxin-coregulated (*tcr*) promoters. The transcriptional start sites for the putative *tcr* genes have not yet been determined. However, the relative effect of CO_2 on expression of the promoter-*lacZ* fusions in the different isolates varies significantly, and sequence analysis indicates that the *B. anthracis* DNA-*lacZ* junction sites do not overlap within 400 to 800 bp in the different mutants. All of the putative *tcr* genes map to pXO1, which also carries the toxin genes. Nevertheless, we cannot rule out *atxA*-mediated regulation of chromosomal genes. Our screen was biased toward the isolation of pXO1-encoded *tcr-lacZ* fusions because the promoter-*lacZ*-generating transposon Tn*917*-LTV3 transposes preferentially to the toxin plasmid, rather than the chromosome, in strains harboring pXO1 (11a). Since our strain did not carry the other *B. anthracis* virulence plasmid, pXO2 (95 kb), our experiments did not test for *tcr* genes on this plasmid. Plasmid pXO2 carries genes required for capsule synthesis, *capB*, *capC*, *capA*, and *dep* (31). Capsule synthesis by pXO2$^+$ strains is enhanced in the presence of pXO1 (10, 11). It will be interesting to determine whether *atxA* plays a role in increased expression of these genes and others on pXO2.

The mechanism by which the *atxA* gene product activates gene expression and mediates CO_2-enhanced transcription is not known. Virulence gene regulons have been described for numerous pathogenic bacteria. In many cases, regulatory proteins have been demonstrated to recognize and bind specific DNA sequences in the control regions of different genes. Examples include the PrfA-regulated operons of *Listeria monocytogenes*, which are also controlled by temperature (5, 9, 13, 22), and the Mga-regulated virulence genes of *Streptococcus pyogenes*, which respond to a number of physiologically significant signals (17). It is possible that AtxA activates gene expression in an analogous manner. However, AtxA has not been shown to specifically bind DNA sequences in the vicinity of the *pag*, *lef*, and *cya* gene promoters, and no sequence similarity has been found in the toxin gene promoter regions which might specify a *cis*-acting element mediating the response. Moreover, the predicted amino acid sequence of AtxA does not suggest that it has characteristics common to DNA-binding proteins. Nevertheless, a model could be proposed in which the function

or expression of some other DNA-binding protein(s) is controlled by AtxA.

The promoter activity of the different *tcr-lacZ* mutants isolated here may be regulated by more than one *atxA*-dependent mechanism. We were surprised to find that only 4 of 10 *atxA tcr-lacZ* fusion mutants were complemented by pUTE178 harboring the *atxA* gene. We have determined that the steady-state level of AtxA in strains carrying pUTE178 is approximately threefold greater than that in strains carrying *atxA* in single copy on pXO1. For some of the *tcr-lacZ* fusions, the relative level of AtxA protein may be critical for optimal *tcr* gene expression. The promoter activity of the *lef-lacZ* fusion did not appear to be negatively affected by increased AtxA levels. However, in other work, we have demonstrated that overproduction of AtxA can negatively affect *pag* gene expression (6).

So what are the functions of the putative *tcr* genes? The *tcr-lacZ* fusion mutants do not exhibit any obvious phenotype. We initiated this study with the intent of searching for *B. anthracis* genes which play a role in *atxA*-mediated toxin gene expression. We postulated that if AtxA does not directly activate toxin gene expression, *atxA* may be required for activation of some other gene(s) necessary for toxin gene transcription. We were surprised to find that all *tcr-lacZ* mutants tested produced toxin proteins. Either the putative *tcr* genes are not required for toxin gene expression or, in some cases, the transposon insertion did not result in inactivation of the putative *tcr* gene. It is possible that these genes play a role in virulence, independent of toxin synthesis. Mice infected with pXO1$^+$ strains harboring toxin gene mutations show an immunological response to *B. anthracis* extracellular antigens, while mice infected with a pXO1$^-$ strains do not show an immunological response to these antigens (21). This suggests that genes on pXO1 other than the toxin genes may be important for survival in the host.

Future studies will identify open reading frames which correspond to the transcriptional fusions. Insertional mutagenesis of these genes will allow us to test for function of the *tcr* gene products. In addition, once the transcriptional start sites of the *tcr* genes are identified, the promoter regions can be examined for similarities. Studies of these newly discovered *atxA*-regulated genes will facilitate the investigation of the molecular basis for *atxA*-mediated activation of gene expression and possibly reveal non-toxin gene products associated with *B. anthracis* virulence.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI33537 from the National Institutes of Health.

We thank Kathleen Borkovich for helpful discussions during the course of this work. We thank Bonnie Dunbar for advice about two-dimensional gel electrophoresis and Malcolm Winkler for use of equipment. We acknowledge Zhihao Dai for sequencing *tcr-2.*

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Editor: P. E. Orndorff