Regulation of Anthrax Toxin Activator Gene (atxA) Expression in Bacillus anthracis: Temperature, Not CO2/Bicarbonate, Affects AtxA Synthesis

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Anthrax toxin gene expression in Bacillus anthracis is dependent on the presence of atxA, a trans-acting regulatory gene located on the resident 185-kb plasmid pXO1. In atxA+ strains, expression of the toxin genes (pag, lef, and cya) is enhanced by two physiologically significant signals: elevated CO2/bicarbonate and temperature. To determine whether increased toxin gene expression in response to these signals is associated with increased atxA expression, we monitored steady-state levels of atxA mRNA and AtxA protein in cells cultured in different conditions. We purified histidine-tagged AtxA (AtxA(His)) from Escherichia coli and used anti-AtxA(His) serum to detect AtxA in protein preparations from B. anthracis cells. AtxA was identified as a protein with an apparent size of 56 kDa in cytoplasmic fractions of B. anthracis cells. Our data indicate that atxA expression is not influenced by CO2/bicarbonate levels. However, the steady-state level of atxA mRNA in cells grown in elevated CO2/bicarbonate at 37°C is five- to sixfold higher than that observed in cells grown in the same conditions at 28°C. A corresponding difference in AtxA protein was also seen at the different growth temperatures. When atxA was cloned on a multicopy plasmid in B. anthracis, AtxA levels corresponding to the atxA gene copy number were observed. However, this strain produced significantly less pag mRNA and protective antigen protein than the parental strain harboring atxA in single copy on pXO1. These results indicate that increased AtxA expression does not lead to a corresponding increase in pag expression. Our data strongly suggest that an additional factor(s) is involved in regulation of pag and that the relative amounts of such a factor(s) and AtxA are important for optimal toxin gene expression.

Bacillus anthracis causes anthrax in animals, including humans. The major virulence factors produced by this gram-positive, sporulating bacillus are a poly-α-glutamic acid capsule and the anthrax toxins, edema toxin and lethal toxin. Edema toxin is composed of protective antigen (PA) and edema factor (EF), while lethal toxin is a combination of PA and lethal factor (LF) (for a review, see reference 14). The structural genes for the toxin proteins and the genes required for capsule synthesis are plasmid encoded. The toxin genes, cya, lef, and pag, encoding EF, LF, and PA, respectively, are located non-contiguously within a 30-kb region of pXO1 (185 kb) (16). The capsule genes, capB, capC, and capA, which are essential for encapsulation, and a gene, dep, which is associated with depolymerization of the capsule, are located contiguously and in the same direction of transcription on pXO2 (95 kb) (23).

Expression of the toxin and capsule genes by B. anthracis during growth in vitro has been shown to be influenced by culture conditions. Capsule and toxin expression is enhanced during growth in certain minimal media in the presence of bicarbonate or under elevated (5% or greater) atmospheric CO2 (15). In addition, toxin synthesis is increased during growth at 37°C compared to 25°C. In all culture conditions, toxin gene promoter activity and toxin protein yield are highest during the late log phase of growth. CO2/bicarbonate and temperature effects on virulence gene expression are thought to be significant for a pathogen which infects mammalian host tissues. Control of the capsule and toxin genes by these environmental signals is at the level of transcription (2, 3, 13, 18, 24). In the case of the toxin genes, mutants harboring transcriptional lacZ fusions have been used to monitor relative promoter activity. At late log phase, expression of a pag-lacZ transcriptional fusion on pXO1 is induced five- to eightfold over that observed in air (0.03% CO2). Growth in 20% CO2 increases the transcription up to 19-fold (13). When cells are grown in bicarbonate medium, promoter activity of all three toxin genes is four- to sixfold greater in cultures incubated at 37°C than in cultures grown at 28°C (18).

Two genes have been identified as trans-acting regulators of toxin and capsule gene transcription. The pXO1-encoded gene atxA (anthrax toxin activator) activates transcription of all three toxin genes (4, 22). The pXO2-encoded gene acpA activates transcription of capB (24). Using the Tox+ Cap+ Weybridge strain of B. anthracis (pXO1, pXO2), we have focused on the role of atxA in CO2/bicarbonate-enhanced transcription of cya and lef, and pag. The 1.5-kb atxA gene maps between cya and pag and is predicted to encode a 476-amino-acid polypeptide with a molecular weight of 55,673 (22). This gene is essential for expression of the toxin genes during culture in vitro and during infection in a mouse model (4). atxA is required for transcription from the unique start sites of cya and lef and for transcription from the major start site, P1, of the pag gene (4, 13). Moreover, CO2/bicarbonate-enhanced transcription of the toxin genes is dependent on atxA. Low levels of toxin gene transcripts can be detected in atxA+ cells grown in air. However, the steady-state levels of transcripts increase dramatically when the same strain is grown in elevated atmospheric CO2. An atxA-null mutant does not produce detectable levels of cya,
ence of CO₂/bicarbonate. We present evidence that increased
in vitro are affected by growth temperature but not by the pres-

report that
atxA
B. anthracis
gene in

mRNA and AtxA protein in
atxA
B. anthracis
dition.

mRNA and AtxA protein in
atxA
B. anthracis

Bifunctional

pMK4
Ap" in E. coli, Cm" in B. anthracis

pUTE29
Ap" in E. coli, Te" in B. anthracis

pUTE34
2.7-kb SnaBI-EcoRI PX01 fragment containing atxA cloned in pUTE29

pUTE142
2.7-kb SnaBI-EcoRI PX01 fragment containing atxA cloned in pMK4

Strains

B. anthracis

UM44
pXO1 transcribing atxA

UM44(pUTE29)
UM44 electroporated with pUTE29; Ind- Te"

UM44(pUTE34)
UM44 electroporated with pUTE34; Ind- Te"

UM44-1C9
pXO1 derivative of UM44; Ind- Str, C. Thorne

UM44-1C9(pUTE34)
UM44-1C9 electroporated with pUTE34; Ind- Str, Te"

UT53
atxA-null derivative of UM44; atxA is replaced by ßlon-2; Ind- Km"

UT53(pUTE34)
UT53 electroporated with pUTE34; Ind- Km" Te"

E. coli

BL21(DE3)
F- ompT raf-1 mB- lacIq (lac-proAB) (dam-4 (lac-pro) X111 thi-1 glnV44 relA1)

GM1684
F' lacPΔM15 proph + (dam-4 (lac-pro) X111 thi-1 glnV44 relA1)

HMS174(DE3) pLYS
F' recA lacIq (lac-pro) lacZAM15 recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 (Δ(lac-proAB)) mcrA

JM109
F' traD36 proA+ proB+ lacIq lacZAM15 recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 (Δ(lac-proAB)) mcrA

a Abbreviations: Ap", ampicillin resistance; Cm", chloramphenicol resistance; Ind, indole: Km", kanamycin resistance; Te", tetracycline resistance; Tox, anthrax toxin proteins.

b BGSC, Bacillus Genetic Stock Center.

c Derived from the Weybridge strain.

lelf, or pag P1 mRNA transcripts during growth in either condition.

In work reported here, we examined expression of the atxA
gene in B. anthracis grown in different culture conditions. We
report that atxA encodes a cytoplasmic protein with an appar-
ent size of 56 kDa in B. anthracis cells. Steady-state levels of
atxA mRNA and AtxA protein in B. anthracis cells cultured in
vitro are affected by growth temperature but not by the pres-
ence of CO₂/bicarbonate. We present evidence that increased
PA synthesis during growth in elevated CO₂/bicarbonate and
at 37°C is not due to increased expression of atxA. A strain
harboring an atxA deletion on pX01 can be complemented by the
addition of atxA in trans on a multicopy plasmid. Such strains
overproduce AtxA, yet PA synthesis is not restored to the
wild-type level.

MATERIALS AND METHODS

Strains, media, and growth conditions. Strains and their relevant character-
istics are listed in Table 1. All B. anthracis strains are derivatives of the Wey-
bridge strain, a capsule-negative toxigenic isolate originally obtained from the

For preparation of cellular protein and RNA, B. anthracis cultures were grown in
CA broth (20) containing tryptophan (20 μg/ml) and buffered with 100 mM
HEPES (pH 8.0). For cultures incubated in 5% or greater atmospheric CO₂,
sodium bicarbonate was added to a final concentration of 0.8%. Generally, 30 ml
of CA broth in a 250-ml flask was inoculated with cells from a selective Luria-
Bertani (LB) (5) agar plate. The initial optical density (A600) was approximately
0.1. Cultures were incubated with vigorous stirring in air, 5% CO₂, or 20% CO₂.
The growth temperature was 37°C unless noted otherwise.

For plasmid DNA extraction, B. anthracis strains were grown in brain heart
infusion medium (Difco, Detroit, Mich.) containing 50% horse serum (Gibco,
Grand Island, N.Y.), and Escherichia coli strains were grown in LB medium.
Isopropyl-β-D-thiogalactoside (IPTG; 0.5 mM) and 5-bromo-4-chloro-3-indolyl-
β-D-galactopyranoside (X-Gal; 40 μg/ml) were used in LB agar to monitor
β-galactosidase activity for the construction of plasmids. All antibiotics were
purchased from Sigma (St. Louis, Mo.) and were added to media at the following
concentrations when appropriate: ampicillin, 100 μg/ml; carbenicillin, 50 μg/ml;
chloramphenicol, 34 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 10 μg/ml for
E. coli cultures and 5 μg/ml for B. anthracis cultures.

DNA isolation, manipulation, and plasmid constructions. Plasmid DNA was
extracted from B. anthracis strains by the method of Green et al. (8). Miniprepa-
rations of plasmid DNA from E. coli and recombinant DNA techniques were
conducted and maintained in E. coli JM109. Plas-
mid pUTE142 was constructed by ligating a 2.7-kb SnaBI-EcoRI PX01 fragment
containing atxA into SnaBI-EcoRI-digested pMK4.

Plasmid pUTE179 is a derivative of pET-15b (Novagen, Madison, Wis.) in
which the atxA gene is translationally fused to a region encoding a 20-amino-acid
polypeptide containing six consecutive histidine residues and a thrombin cleav-
ge site. Transcription of the recombinant atxA gene is under the control of a
promoter recognized by T7 RNA polymerase. Expression of such gene fusions is
IPTG inducible in E. coli strains harboring a chromosomal T7 RNA polymerase
gene driven by a lacUV5 promoter (Novagen). The plasmid was constructed as
follows. First, an NdeI restriction site overlapping the atxA translational start

TABLE 1. Strains and plasmids used in this study

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<th>Plasmid or strain</th>
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<td>Stratagene</td>
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<td>This work</td>
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<td>UM44 electroporated with pUTE29; Ind- Te'</td>
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<tr>
<td>BL21(DE3)</td>
<td>F- ompT raf-1 mB- lacIq (lac-proAB) (dam-4 (lac-pro) X111 thi-1 glnV44 relA1)</td>
<td>Novagen</td>
</tr>
<tr>
<td>GM1684</td>
<td>F' lacPΔM15 proph + (dam-4 (lac-pro) X111 thi-1 glnV44 relA1)</td>
<td>R. Kolter</td>
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<td>HMS174(DE3)pLYS</td>
<td>F' recA lacIq (lac-proAB) lacZAM15 recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 (Δ(lac-proAB)) mcrA</td>
<td>Novagen</td>
</tr>
<tr>
<td>JM109</td>
<td>F' traD36 proA+ proB+ lacIq lacZAM15 recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 (Δ(lac-proAB)) mcrA</td>
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Expression and purification of AtxA(His). Recombinant AtxA protein carrying a 20-amino-acid-residue amino-terminal extension including the affinity tag, AtxA(His), was purified from *E. coli* HMS714(DE3)pLYsS(pUTE179) by using the protocol supplied by Novagen, with some modifications. Briefly, *E. coli* HMS714(DE3)pLYsS was transformed with pUTE179. A transformant colony from a selective LB plate was inoculated into 50 ml of LB broth containing carbenicillin. The culture was incubated with shaking at 37°C to an optical density (a600) of approximately 0.7 and then held overnight at 4°C. Cells were collected by centrifugation and inoculated into 900 ml of LB broth containing carbenicillin to an a600 of approximately 0.03. The culture was incubated with shaking to an a600 of 0.7. IPTG was added to a final concentration of 1 mM, and the culture was further incubated for 2 h. The culture was chilled on ice before centrifugation at 4°C. Pelleted cells were resuspended in 10 ml of binding buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 10% (v/v) glycerol, 2 μM pepstatin A, 0.6 μM leupeptin, and 0.4 mM phenylmethylsulfonyl fluoride (PMSF). The cell suspension was passed through a French press minicell (SLM Aminco, Urbana, Ill.) three times at 20,000 lb/in². The lysate was centrifuged at 16,000 × g for 60 min, and the supernatant was collected for purification of AtxA(His) protein.

The recombinant protein was purified from the cell lysate by affinity chromatography as follows. A 1.6 mM NiSO₄-charged His.Bind resin column (Novagen) was washed with washing buffer (50 mM sodium chloride [pH 6.0], 0.5 M NaCl, 20 mM Tris-HCl [pH 8.0]) containing 10% (v/v) glycerol, 0.4 mM PMSF, and 0.1% Triton X-100. The column was equilibrated with binding buffer. Approximately 10 ml of cell lysate was applied to the column. Binding buffer (16 ml) was applied to the column followed by 9.6 ml of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 10% (v/v) glycerol and 0.4 mM PMSF. The protein was eluted from the column with 9.6 ml of eluate buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 10% (v/v) glycerol, 0.4 mM PMSF, and 0.1% Triton X-100. The eluate was concentrated by centrifugation in a Centricon 30 concentrator (Amicon, Beverly, Mass.).

**Production of anti-AtxA(His) antisera.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel slices containing the 60-kDa protein purified from *E. coli* HMS714(DE3)pLYsS(pUTE179) were solubilized and used to immunize a female New Zealand rabbit by Cocalico Biologicals (Reamstown, Pa.). The animal received approximately 900 μg of protein via intradermal injections over a 15-week period. Eighteen weeks following the first injection, the rabbit was sacrificed and serum was collected. To reduce nonspecific reactions with non-AtxA proteins, the serum was incubated with protein extracts made from the pXO1 *B. anthracis* strain (ATCC 391). The mixture was centrifuged, and the supernatant was used for Western blotting experiments.

**Immunoblotting.** *B. anthracis* cultures were grown in CA medium to an a600 of 0.8. Cells were separated from culture supernatants by filtration through cellulose (0.45-μm pore size; Nalgene, Rochester, N.Y.). Cells collected on filters were washed with buffer A (40 mM HEPEs [pH 8.0], 60 mM KCl, 1 mM EDTA, 10% [v/v] glycerol) and resuspended in buffer A containing 0.1 mM dithiothreitol, 0.4 mM PMSF, 2 μM pepstatin A, and 0.6 μM leupeptin. Cells were disrupted by passing through a French press minicell three times at 20,000 lb/in². Soluble and insoluble fractions were separated by centrifugation at 100,000 × g for 1 h. Pellets were solubilized by boiling in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. Protein concentrations in soluble fractions were determined by the Bio-Rad (Hercules, Calif.) protein assay reagent, with bovine serum albumin as the standard.

Culture supernatant and cellular protein samples were applied directly to nitrocellulose membranes by vacuum blotting or subjected to SDS-PAGE (7.5% polyacrylamide gel) and subsequently transferred to nitrocellulose membranes. PA secreted into culture media is subject to various degrees of proteolysis. Therefore, a slot-blotting apparatus (Hoefer Scientific, San Francisco, Calif.) was used to assay PA. Protein blots were blocked in TBS-T buffer (20 mM Tris base, 137 mM sodium chloride [pH 7.6], 0.1% Tween 20) with 10% nonfat milk for 2 h at room temperature. The membranes were reacted with rabbit anti-PA serum (1:10,000 diluted in TBS-T with 5% nonfat milk) or rabbit anti-AtxA(His) serum (1:2,000) overnight at 4°C. Membranes were washed in TBS-T and finally reacted with a 1:1000 dilution of anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (1:4,000 diluted in TBS-T with 5% nonfat milk) for 1 h at room temperature. Antiserum-reactive material was visualized on autoradiographs by using an enhanced chemiluminescence immunoblotting kit purchased from Amersham (Buckinghamshire, England). All experiments were run in duplicate, using samples from at least two different cultures. Relative band intensities resulting from application of serially diluted culture supernatant samples were determined by using a ScanJet 4C scanner (Hewlett-Packard, Camas, Wash.).

**RESULTS**

**Expression and purification of AtxA(His).** We purified AtxA(His), a fusion protein in which a 20-amino-acid-lead peptide containing six consecutive histidine residues is fused to the amino terminus of AtxA, from *E. coli* HMS714(DE3)pLYsS containing pUTE179. Figure 1 shows a Coomassie blue-stained gel in which samples of cellular protein from HMS714(DE3)pLYsS were examined. A faint band representing an apparent 60-kDa protein was observed in samples from IPTG-induced cultures of a strain carrying pUTE179 (lane 3). This faint band was not seen in samples from a strain carrying pUTE179 (lane 3). A faint band representing an apparent 60-kDa protein was observed in samples from IPTG-induced cultures of a strain carrying pUTE179 (lane 3). This faint band was not seen in samples from a strain carrying pUTE179 (lane 3). A faint band representing an apparent 60-kDa protein was observed in samples from IPTG-induced cultures of a strain carrying pUTE179 (lane 3). This faint band was not seen in samples from a strain carrying pUTE179 (lane 3).

**RNA analysis.** Methods for RNA extraction and primer extension experiments have been described previously (21). RNA was extracted from *B. anthracis* cultures grown to late log phase (a600 of 0.8) in CA medium. RNA was quantitated spectrophotometrically and also visualized using 1.2% formaldehyde gels. The oligonucleotide primers 5'-CCCTATITATTTTGCATCTCTAGAA-3' and 5'-CCCTGTTGGCTGAAACTATATATCGAG-3' were labeled with [γ-32P]ATP (6,000 Ci/mmol; Amersham), hybridized to 50 ng of RNA, and extended by using avian myeloblastosis virus reverse transcriptase (Promega). The 5' ends of the atxA and pag genes were sequenced by the dye-deoxy chain-termination method (1), using the appropriate primers and a Sequenase version 2.0 DNA sequencing kit purchased from United States Biochemical Corp. (Cleveland, Ohio). The [γ-32P]ATP (>1,000 Ci/mmol) for sequencing was purchased from Amersham Corp. (Arlington Heights, Ill.). Primer extension and sequencing reaction mixtures were subjected to electrophoresis on 6% polyacrylamide and 42% urea gels.

**FIG. 1.** Coomassie blue-stained SDS-polyacrylamide gel showing AtxA(His) in *E. coli* HMS714(DE3)pLYsS cells and in eluates from affinity chromatography of cell extracts. Lanes: 1, protein standards (sizes are indicated in kilodaltons); 2, HMS714(DE3)pLYsS(pET-15b) cells; 3, HMS714(DE3)pLYsS(pUTE179) cells; 4, eluate from HMS714(DE3)pLYsS(pET-15b); 5, eluate from HMS714(DE3)pLYsS(pUTE179) eluate.
containing the 60-kDa protein were solubilized and used as antigen to generate anti-AtxA(His) serum in a rabbit.

Approximately 500 μg of purified AtxA(His) protein was obtained from a 900-ml culture of *E. coli* HMS174 (DE3)pLysS(pUTE179). This level of expression is far below those reported for other affinity-tagged proteins cloned and expressed using the pET system (Novagen). It is unclear why AtxA(His) was not expressed at higher levels. We also transformed pUTE179 into *E. coli* BL21(DE3) to test for AtxA-(His) expression. This strain lacks the Lon and OmpT proteases (9). Low-level synthesis of the recombinant protein by this strain was also observed (data not shown). Considering the regulatory role of AtxA in *B. anthracis* gene expression, it is possible that the fusion protein interferes with gene expression in *E. coli*.

**Detection of AtxA in *B. anthracis* cell extracts.** We used antiserum generated against purified AtxA(His) to monitor synthesis of AtxA by *B. anthracis*. Cultures of *B. anthracis* UM44(pXO1<sup>-</sup>), UM44-1C9(pXO1<sup>-</sup>), and UT53(pXO1<sup>-</sup>, atxA) were grown to late log phase in 5% CO<sub>2</sub> at 37°C. Soluble and insoluble fractions from disrupted cells were tested for the presence of AtxA by immunoblotting with anti-AtxA(His) antiserum. Results are shown in Fig. 2. A band indicating an approximately 56-kDa protein was detected in the soluble fraction from UM44 (lane 2). After prolonged exposure of the autoradiograph film, this band was not present in the soluble fractions from the atxA strains UM44-1C9 and UT53 (lanes 3 and 4, respectively). The band was also not detected in the insoluble fractions from UM44 (lane 1), UM44-1C9, and UT53 (data not shown). These results indicate that the atxA gene encodes a soluble protein in *B. anthracis* cells with an apparent size of 56 kDa, consistent with the predicted molecular weight of 55,673.

**Temperature effect on atxA expression.** Transcription of the toxin genes in cells growing under elevated CO<sub>2</sub>/bicarbonate conditions is increased when cultures are incubated at 37°C compared to 28°C (18). To determine whether increased temperature enhances *atxA* expression, we examined the relative amounts of *atxA* mRNA and AtxA protein in UM44 cells grown to late log phase under 5% CO<sub>2</sub> at 37 and at 28°C. The results of primer extension reactions are shown in Fig. 3A. As reported previously (4), one primer extension product was observed, indicating a single putative transcriptional start site. The relative intensities of bands representing primer extension products indicate that the steady-state level of *atxA* mRNA in cells grown at 37°C is five- to sixfold higher than the level in cells grown at 28°C.

Steady-state levels of AtxA protein were also increased in UM44 cells grown at high temperature. Soluble protein fractions of the cells grown at different temperatures were analyzed by immunoblotting with anti-AtxA(His) antiserum. Prolonged exposure of the autoradiograph film resulted in a faint band representing a low level of AtxA protein in fractions from cells grown at 28°C. The relative band intensities (Fig. 3B) indicate that approximately 10-fold more AtxA protein was obtained from cells grown at 37°C than from those grown at 28°C.

To determine whether the difference in *atxA* expression at the different growth temperatures could be related to a change in growth rate, we examined the doubling time of *B. anthracis* UM44 cultures grown at the different temperatures. As shown in Fig. 3C, the lag phase was somewhat longer for the culture grown at 28°C than for the culture grown at 37°C. However, the log-phase growth rates were similar. The doubling time for the 28°C culture was 68 min, while the doubling time for the 37°C culture was 61 min. These data indicate that increased *atxA* expression at 37°C is most likely not a reflection of an altered growth rate.

**CO<sub>2</sub> effect on *atx* expression.** The *atx* gene is required for CO<sub>2</sub>/bicarbonate-enhanced transcription of the toxin genes (4, 13, 22). Results of experiments reported previously indicated that steady-state levels of *atxA* mRNA are not increased when UM44 cells are grown in 5% CO<sub>2</sub> compared to growth in air (4). To further examine whether growth in high atmospheric CO<sub>2</sub> affects *atxA* expression, we compared the relative amounts of *atxA* mRNA and AtxA protein in UM44 cells grown under varying CO<sub>2</sub> concentrations. As shown in Fig. 3D, *atxA* expression was not enhanced in cells grown in 5% CO<sub>2</sub> compared to cells grown in 28% CO<sub>2</sub>.
of ata mRNA and AtxA protein in cells grown to late log phase in air, 5% CO2, and 20% CO2. Primer extension reactions were performed with total RNA extracted from cells grown in the atmospheric CO2 concentrations indicated. The end-labeled primer was complementary to a 23-nucleotide sequence located 55 nucleotides downstream of the first nucleotide of the ata translational start site. Lanes G, A, T, and C correspond to the dideoxy sequencing reactions carried out with the same oligonucleotide primer. (B) Equivalent amounts of soluble proteins from cells grown in different atmospheric CO2 concentrations were tested for AtxA protein by Western blot analysis. Purified AtxA(His) was used as a control.

To determine whether steady-state levels of AtxA protein differed in the cultures, soluble protein fractions were analyzed by immunoblotting with anti-AtxA(His) antiserum. Bands indicative of AtxA were detected in all protein samples, and no significant differences in band intensity were observed when the primer extension products were compared (Fig. 4A), confirming that steady-state levels of ata mRNA do not differ in cells grown under different atmospheric CO2 levels.

Influence of ata copy number on AtxA and protective antigen synthesis. CO2/bicarbonate- and temperature-induced expression of the toxin genes is observed only in the presence of the ata gene. Mutants harboring ata mutations are Tox− even when grown in optimal conditions for toxin gene expression (CO2/bicarbonate and 37°C) (4, 13, 22). Such mutants become Tox+ when ata is introduced in trans in multicopy. However, the amount of toxin produced is less than that synthesized by the ata− parent strain (13).

We examined AtxA protein levels in strains harboring the ata gene in multicopy. Relative steady-state levels of AtxA were compared in cell extracts from UM44, UT53, UT53 (pUTE34), and UM44-1C9(pUTE34) cultures incubated in 5% CO2 at 37°C. Plasmid pUTE34 carries the ata gene. Results are shown in Fig. 5A. Strains harboring the cloned ata gene in multicopy produced over 10-fold more AtxA protein than UM44, which harbors a single copy of ata on pXO1. No significant differences in steady-state AtxA levels were observed in the presence of pXO1.

Supernatants from the same cultures were tested for relative amounts of PA. The PA levels detected in supernatants of UT53(pUTE34) cultures, which overproduced AtxA, were reduced to less than one-half of that detected in supernatants from the parent strain UM44. Reduced PA synthesis by UT53(pUTE34) was also observed in cultures grown at 28°C (data not shown). Similar results were obtained when ata was cloned in multicopy in UM44, which also carries ata on pXO1 in its normal genetic locus. Results are shown in Fig. 5B. The relative amount of PA in the culture supernate of UM44(pUTE34) was approximately one-third of that detected in supernatants from the parent strain UM44. Reduced PA synthesis by UT53(pUTE34) was also observed in cultures grown at 28°C (data not shown). Similar results were obtained when ata was cloned in multicopy in UM44, which also carries ata on pXO1 in its normal genetic locus. Results are shown in Fig. 5B. The relative amount of PA in the culture supernate of UM44(pUTE34) was approximately one-third of that detected in culture supernatants of the strain harboring the vector only, UM44(pUTE29).

To determine if the observed decrease in PA synthesis was a reflection of decreased transcription of the pag gene, we examined steady-state pag mRNA levels in UM44(pUTE34) and UM44(pUTE29). Results of primer extension experiments are shown in Fig. 5C. The relative amounts of primer extension products corresponding to transcripts mapping to the CO2/ata-regulated start site P1 indicated that pag P1 transcript levels were significantly lower in RNA from UM44(pUTE34).
than in RNA from UM44(pUTURE29). Overproduction of AtxA did not appear to be toxic, since similar growth rates were noted when the strains were grown at 28 or 37°C (data not shown). These results indicate that pag expression is decreased in strains which carry atxA in multicopy and overproduce AtxA.

To rule out the possibility that the cloned atxA gene on pUTURE34 differed from the wild-type gene, the DNA sequence of the cloned atxA gene was determined and compared to the sequence of the pXO1-encoded atxA gene, generated by PCR. Sequence analysis revealed that the nucleotide sequences were identical. It should be noted that the atxA gene sequence from our laboratory strains, which were derived from the Weybridge strain of B. anthracis, differs from the sequence first reported by Uchida et al. (22). Our sequence does not have the leucine codon, TTA, which is present at positions 1171 to 1173 in the sequence reported by Uchida et al. Also, our sequence contains a phenylalanine codon, TTT, at positions 2107 to 2109, rather than the tyrosine codon, TAT, reported previously.

**DISCUSSION**

To date, only one gene has been characterized as a regulator of anthrax toxin gene expression in B. anthracis. The atxA gene is located on the 185-kb plasmid pXO1, which carries the three structural genes for the toxin proteins, PA, LF, and EF. Transcription of the unlinked toxin genes, pag, lef, and cya, is dependent on atxA; mutants harboring transposon insertions in atxA or deleted for the atxA gene are toxic (4, 13, 22). The atxA gene also appears to function in vivo. An atxA-null mutant has been demonstrated to be avirulent in mice, and the antibody response to all three toxin proteins is decreased significantly in atxA-null mutant-infected mice (4).

Transcription of the anthrax toxin genes is enhanced when B. anthracis is grown in certain minimal media in the presence of elevated CO₂/bicarbonate. Expression of the B. anthracis is grown in certain minimal media in the presence or 5% atmospheric CO₂. Expression of the B. anthracis atxA gene is located on the 185-kb plasmid pXO1, which carries the three structural genes for the toxin proteins, PA, LF, and EF. Transcription of the unlinked toxin genes, pag, lef, and cya, is dependent on atxA; mutants harboring transposon insertions in atxA or deleted for the atxA gene are toxic (4, 13, 22). The atxA gene also appears to function in vivo. An atxA-null mutant has been demonstrated to be avirulent in mice, and the antibody response to all three toxin proteins is decreased significantly in atxA-null mutant-infected mice (4).

Transcription of the anthrax toxin genes is enhanced when B. anthracis is grown in certain minimal media in the presence of elevated CO₂/bicarbonate (2, 13, 18). The CO₂/bicarbonate effect is specific and not related to changes in growth rate or the buffering capacity of dissolved bicarbonate in the culture medium. Other than toxin and capsule synthesis by B. anthracis, there are a few examples of CO₂/bicarbonate-regulated virulence gene expression. Cholera toxin synthesis by Vibrio cholerae is enhanced during growth in media containing 0.2 to 0.4% sodium bicarbonate, which corresponds to the bicarbonate concentration in cholera stool (10). Toxic shock syndrome toxin 1 synthesis by Staphylococcus aureus is stimulated at CO₂ concentrations present in the vaginal environment (11). Cryptococcus neoformans produces capsule in response to growth in elevated CO₂ (7). The molecular mechanism for CO₂/bicarbonate-enhanced virulence gene expression has not been elucidated in any of these examples.

Toxin gene expression by B. anthracis is also influenced by growth temperature. Transcription of the pag gene, for example, increases four- to sixfold when cells are grown in CO₂ bicarbonate medium at 37°C compared to growth at 28°C (18). Numerous microbial pathogens which infect mammals exhibit temperature-regulated virulence gene expression. In some cases, theremo regulation of gene expression has been shown to be associated with changes in local DNA structure (17) and can be dependent on histone-like proteins that are involved in determining DNA topology (6).

Considering the signals which influence expression of pag and the other atxA-regulated toxin genes, we investigated expression of the atxA gene itself. Our data indicate that steady-state levels of atxA mRNA and AtxA protein are not influenced by growth in the presence of elevated CO₂/bicarbonate. However, atxA RNA transcript and protein levels are increased in cultures grown at 37°C relative to 28°C. Interestingly, overproduction of AtxA by strains harboring atxA in multicopy does not result in elevated PA synthesis when cells are grown at either temperature. Taken together, these results indicate that increased pag expression at 37°C cannot be attributed directly to increased atxA expression at the high temperature.

We were surprised to find that strains which carry atxA in multicopy and overproduce AtxA actually synthesize decreased levels of pag mRNA and PA relative to the parent strains. This result indicates that either increased copy number of the atxA gene or increased AtxA protein level adversely affects pag activation. These data provide compelling evidence that an additional regulatory factor(s) is involved in pag activation. If pag activation is mediated by AtxA alone, then one would predict that increased AtxA would either (i) increase pag expression, if AtXA is limiting, or (ii) have no effect on pag expression, if AtxA is not limiting.

We propose that an additional regulatory factor(s) affects AtxA function. AtxA may be conformationally altered, covalently modified, or associated with some cofactor, in response to the modulating affect of some other protein. In this model, the additional regulatory factor would be limiting in cells which overproduce AtxA. If unmodified (nonfunctional) AtxA interferes with the ability of modified AtxA to activate pag expression, then pag expression would be reduced, relative to wild-type gene expression, when unmodified AtxA is abundant.

Other observations support the idea that a factor(s) other than AtxA is important for control of the toxin genes. There is currently no evidence that AtxA itself interacts with the control regions of the toxin genes. The predicted amino acid sequence of AtxA does not indicate that it is a DNA-binding protein, and sequence-specific interactions of AtxA and the promoter regions of the toxin genes have not been demonstrated. Also, atxA-mediated activation of pag has been demonstrated in a Bacillus subtilis strain harboring atxA and a pag-lacZ transcriptional fusion on separate multicopy plasmids (22). However, preliminary results in our laboratory indicate that CO₂/bicarbonate-enhanced activation is not observed in B. subtilis (12). The atxA-mediated activation of pag also occurs in aerobically grown B. anthracis, but the level of pag expression is significantly enhanced in response to CO₂/bicarbonate (13). This may indicate that an additional B. anthracis gene is required for CO₂/bicarbonate-enhanced pag activation. If such an additional regulatory factor exists, it may be present on the B. anthracis chromosome. We have demonstrated CO₂/bicarbonate-enhanced pag expression in a pXO1¹ pXO2² strain of B. anthracis which carries pag and atxA on separate multicopy plasmids (13). It has also been proposed that B. anthracis harbors a gene for a protein which represses toxin gene expression in the absence of the CO₂/bicarbonate signal. Uchida et al. (22) isolated a B. anthracis mutant which produces toxin at high levels during aerobic growth. However, a specific gene associated with the mutation has not been reported.

Studies are under way to identify additional regulatory genes in B. anthracis and to examine the effects of the gene products on AtxA activity. Characterization of such genes will lead to increased understanding of the mechanism by which B. anthracis senses and responds to environmental cues which trigger virulence gene expression.

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