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## Control of the master virulence regulatory gene atxA in *Bacillus anthracis*

Jennifer L. Dale

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CONTROL OF THE MASTER VIRULENCE REGULATORY  
GENE *ATXA* IN *BACILLUS ANTHRACIS*

by

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CONTROL OF THE MASTER VIRULENCE REGULATORY  
GENE *ATXA* IN *BACILLUS ANTHRACIS*

A

Dissertation

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
Graduate School of Biomedical Sciences

and

The University of Texas  
M.D. Anderson Cancer Center  
Graduate School of Biomedical Sciences

in Partial Fulfillment  
of the Requirements  
for the Degree of  
DOCTOR OF PHILOSOPHY

By

Jennifer Lynn Dale, B.S.

Houston, TX

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# CONTROL OF THE MASTER VIRULENCE REGULATORY

## GENE *ATXA* IN *BACILLUS ANTHRACIS*

Publication No. \_\_\_\_\_

Jennifer Lynn Dale, B.S.

Supervisory Professor: Theresa M. Koehler, Ph.D.

Transcription of the *Bacillus anthracis* structural genes for the anthrax toxin proteins and biosynthetic operon for capsule are positively regulated by AtxA, a transcription regulator with unique properties. Consistent with the role of *atxA* in virulence factor expression, a *B. anthracis atxA*-null mutant is avirulent in a murine model for anthrax. In batch culture, multiple signals impact *atxA* transcript levels, and the timing and steady state level of *atxA* expression is critical for optimal toxin and capsule synthesis. Despite the apparent complex control of *atxA* transcription, only one *trans*-acting protein, the transition state regulator AbrB, has been demonstrated to directly interact with the *atxA* promoter. The AbrB-binding site has been described, but additional *cis*-acting control sequences have not been defined. Using transcriptional *lacZ* fusions, electrophoretic mobility shift assays, and Western blot analysis, the *cis*-acting elements and *trans*-acting factors involved in regulation of *atxA* in *B. anthracis* strains containing either both virulence plasmids, pXO1 and pXO2, or only one plasmid, pXO1, were studied. This work demonstrates that *atxA* transcription from the major start site P1 is dependent upon a consensus sequence for the housekeeping sigma factor SigA, and an A+T-rich upstream element (UP-element) for RNA polymerase (RNAP). In addition, the data show that a *trans*-acting protein(s) other than AbrB negatively impacts *atxA* transcription when it binds specifically to a 9-bp palindrome within *atxA* promoter sequences located downstream of P1. Mutation of the palindrome

prevents binding of the *trans*-acting protein(s) and results in a corresponding increase in AtxA and anthrax toxin production in a strain- and culture-dependent manner.

The identity of the *trans*-acting repressor protein(s) remains elusive; however, phenotypes associated with mutation of the repressor binding site have revealed that the *trans*-acting repressor protein(s) indirectly controls *B. anthracis* development. Mutation of the repressor binding site results in misregulation and overexpression of AtxA in conditions conducive for development, leading to a marked sporulation defect that is both *atxA*- and *pXO2-61*-dependent. *pXO2-61* is homologous to the sensor domain of sporulation sensor histidine kinases and is proposed to titrate an activating signal away from the sporulation phosphorelay when overexpressed by AtxA. These results indicate that AtxA is not only a master virulence regulator, but also a modulator of proper *B. anthracis* development. Also demonstrated in this work is the impact of the developmental regulators AbrB, Spo0A, and SigH on *atxA* expression and anthrax toxin production in a genetically incomplete (*pXO1*<sup>+</sup>, *pXO2*<sup>-</sup>) and genetically complete (*pXO1*<sup>+</sup>, *pXO2*<sup>+</sup>) strain background. AtxA and anthrax toxin production resulting from deletion of the developmental regulators are strain-dependent suggesting that factors on *pXO2* are involved in control of *atxA*. The only developmental deletion mutant that resulted in a prominent and consistent strain-independent increase in AtxA protein levels was an *abrB*-null mutant. As a result of increased AtxA levels, there is early and increased production of anthrax toxins in an *abrB*-null mutant. In addition, the *abrB*-null mutant exhibited an increase in virulence in a murine model for anthrax. In contrast, virulence of the *atxA* promoter mutant was unaffected in a murine model for anthrax despite the production of 5-fold more AtxA than the *abrB*-null mutant. These results imply that AtxA is not the only factor impacting pathogenesis in an *abrB*-null mutant. Overall, this work highlights the complex regulatory network that governs expression of *atxA* and provides an additional role for AtxA in *B. anthracis* development.

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# **Chapter I**

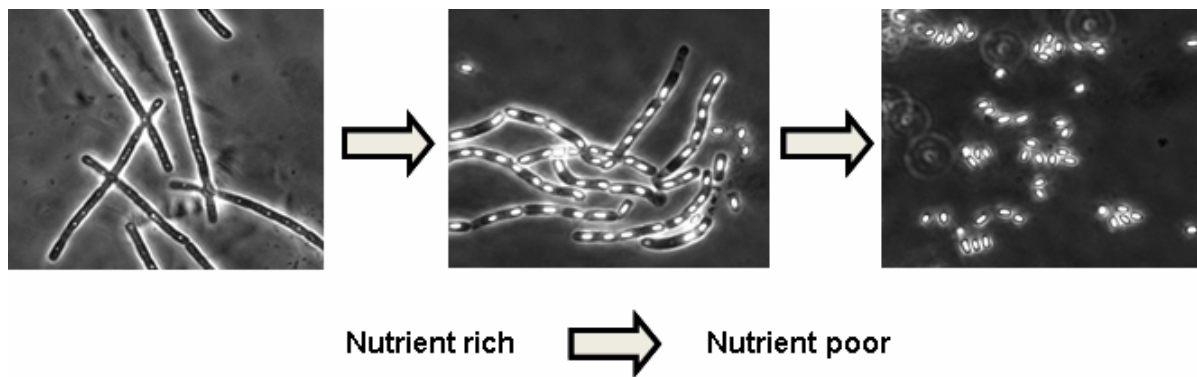
## **Introduction**

### 1.1. Physiology of the *Bacillus cereus* group members

The *Bacillus cereus* group, also referred to as “group 1 bacilli” or “*Bacillus cereus sensu lato*”, contains six related *Bacillus* species: *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus cereus sensu stricto*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, and *Bacillus pseudomycoides*. These species are rod-shaped, Gram-positive aerobic or facultatively anaerobic developmental bacteria that can form endospores in response to nutrient deprivation (Fig. 1-1). *Bacillus* spores are resistant to environmental stresses such as desiccation, heat, UV light, and chemicals enabling persistence of the organism (88). The *Bacillus* species are saprophytic organisms growing under nutrient rich conditions including some soil environments, and are common inhabitants of the gut of invertebrates (81).

The most well studied members of the *B. cereus* group, *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis*, are pathogens with common and unique properties that facilitate disease. *B. anthracis* is the etiological agent of anthrax disease while *B. cereus sensu stricto* and *B. thuringiensis* are opportunistic human pathogens causing mild food poisoning. *B. cereus sensu stricto* and *B. thuringiensis* can also cause local and systemic hospital-acquired infections; however, these diseases are less common. Even though *B. thuringiensis* can cause opportunistic human infections, it is considered primarily to be a pathogen of insects.

The *B. cereus* group members are closely related in chromosomal gene content and synteny (73, 123). Distinguishing characteristics between the species are often attributed to the presence or absence of virulence-associated plasmids. *B. anthracis* contains two extrachromosomal virulence plasmids, pXO1 and pXO2. The structural genes for anthrax toxin, *pagA* (PA), *cya* (EF), and *lef* (LF) are located on pXO1 (84) while the capsule biosynthetic operon, *capBCADE*, is located on pXO2 (27, 97, 112). The *B. cereus sensu stricto* cereulide (emetic toxin) synthesis genes are located on a pXO1-like plasmid



**Figure 1-1. Phase micrograph of a sporulating *B. anthracis* culture.** Vegetative cells (left panel) replicate and divide until nutrients are scarce at which point they form endospores (middle panel) and finally release fully developed spores (right panel) from the mother cell.

(46, 47, 88, 96, 143), and the *B. thuringiensis* insecticidal toxin genes are located on large, transmissible plasmids (123). Lack of virulence plasmids typically attenuates *B. cereus* group members with the exception of *B. cereus sensu stricto* isolates that produce chromosome-encoded enterotoxins (143).

The growth temperature and local environment of each *B. cereus* group member species correlates with the conditions required for optimal virulence factor production. Psychrotolerant species such as *B. cereus sensu stricto* synthesizes maximal amounts of cereulide (emetic toxin) when cultured between 12-22°C (49). Growth of *B. cereus sensu stricto*, and likely select isolates of *B. thuringiensis*, at 37°C in low oxydoreduction anaerobic environments such as those in the small intestines, enhances production of enterotoxins (45). *B. anthracis* produces its virulence factors, anthrax toxin and capsule, when cultured at 37°C in a minimal medium containing dissolved bicarbonate and elevated atmospheric CO<sub>2</sub>, conditions relevant for pathogenesis (9, 10, 29, 145, 161). *B. thuringiensis* produces its insecticidal pro-toxin crystalline aggregates in sporulating mother cells that are released at the completion of sporulation.

## **1.2. Sporulation of the *Bacillus* genus**

With most infections caused by *B. cereus* group members, the spore constitutes the infectious form of the organism. Members of the *Bacillus* genus undergo a developmental process that results in vegetative cells differentiating into dormant spores. This process of development, referred to as sporulation, has been best-characterized in *B. subtilis*, the archetype *Bacillus* species. *B. subtilis*, like the *B. cereus* group members, is a soil bacterium that senses and responds to environmental stimuli. In nutrient deprived conditions, *B. subtilis* senses the lack of nutrients and initiates sporulation. Sporulation is considered the last resort for *Bacillus* species survival since the developmental process is energy exhaustive. When cultured in the laboratory, sporogenous strains will initiate

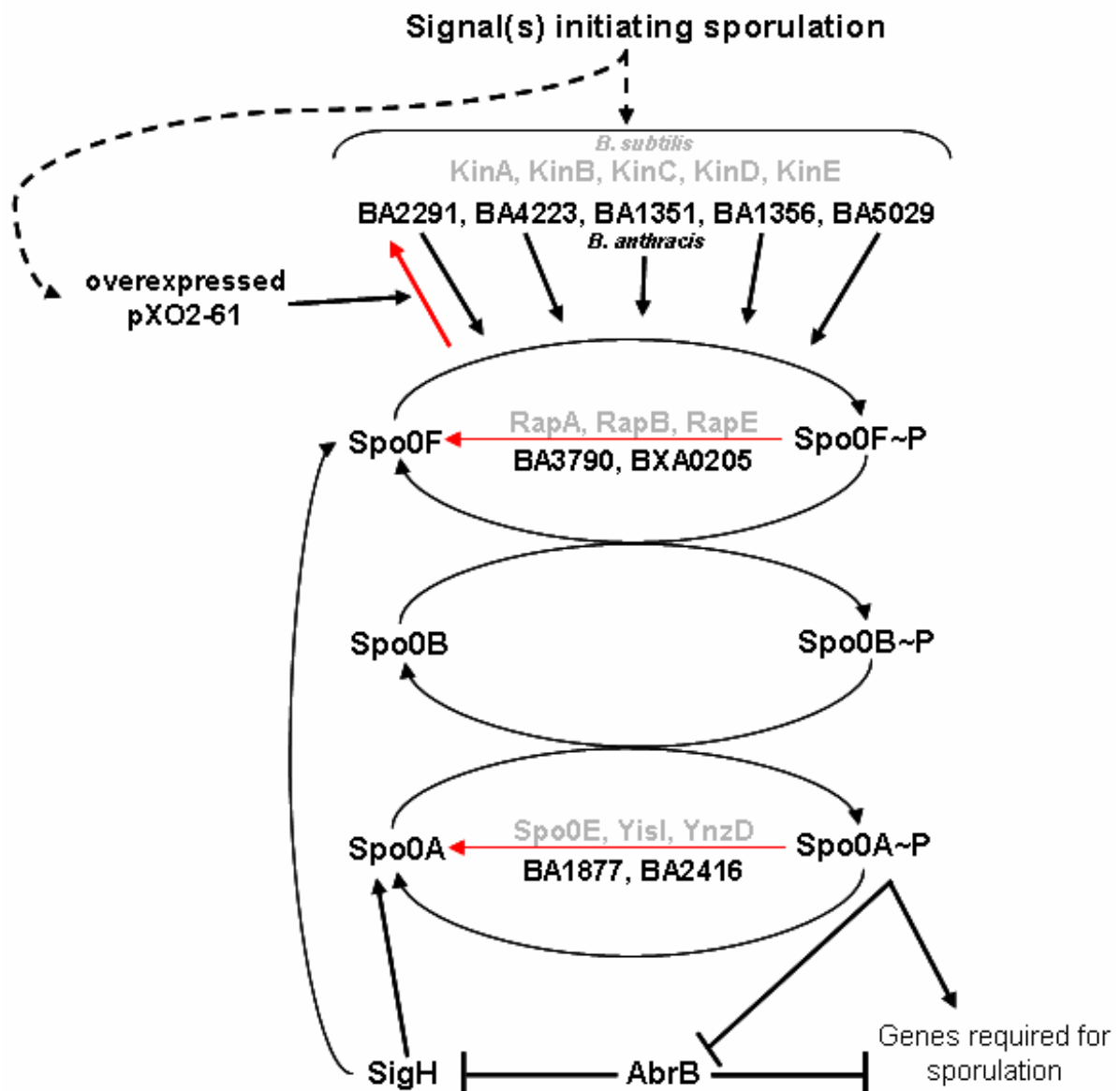
sporulation during the transition from exponential to stationary phase of growth. Multiple pleiotropic regulators are part of the developmental process involved in spore formation and have been termed transition-state regulators. The most important of these transition-state regulators is AbrB.

The primary role of transition-state regulators such as AbrB is to prevent the inappropriate expression of genes whose functions are only needed during stationary phase of growth. Several of these stationary phase-specific genes are required for sporulation initiation and are repressed during exponential growth by AbrB to prevent premature sporulation initiation. AbrB is a DNA-binding protein that directly binds to the promoter region of over 40 different *B. subtilis* genes. Examination of AbrB-controlled promoter regions has not revealed a consensus sequence, which suggests AbrB binds its target promoters based on DNA structure (148-150, 154, 178, 179). The threshold AbrB concentration required for repressing sporulation genes has a narrow range. AbrB autoregulates its own expression during exponential growth. As cells transition into stationary phase *abrB* is repressed leading to a drop in AbrB concentrations below the effective range. This repression of *abrB* transcription at the transition into stationary phase is attributed to the master response regulator Spo0A (121, 147, 153).

Upon nutrient limitation, a multi-component signal transduction phosphorelay is initiated which leads to the phosphorylation and activation of Spo0A (22). In *B. subtilis*, the phosphorelay contains a number of sensor histidine kinases, phosphorylated response regulators, and phosphatases (Fig. 1-2). Spo0A is the master response regulator that is capable of both activating and repressing genes required for sporulation initiation by binding to its cognate '0A box' (107, 144, 147, 152). One of the crucial roles of Spo0A~P is to repress *abrB* transcription, relieving repression of post-exponential sporulation genes.

In order for Spo0A to become phosphorylated, cells must sense the depletion of nutrients and trigger activation of the phosphorelay by enabling autophosphorylation of





**Figure 1-2. Multi-component signal transduction sporulation phosphorelay.** See text for details. *B. subtilis* specific proteins are depicted by gray lettering. *B. anthracis* proteins are depicted in black below the gray lettered *B. subtilis* protein orthologues. The response regulators Spo0F, Spo0B, and Spo0A, and the developmental regulators SigH and AbrB are present in both *B. anthracis* and *B. subtilis*.

sensor histidine kinases (KinA, KinB, KinC, KinD, and/or KinE). The exact signal for induction is unknown. The activating phosphoryl group is transferred from the sensor histidine kinase to the intermediate response regulator Spo0F. Using the phosphotransferase protein Spo0B, the phosphoryl group is transferred from Spo0F to Spo0A. The use of Spo0F and Spo0B as response regulator intermediates provides an additional level of regulation of the phosphorelay.

Other controls of the phosphorelay include phosphatases and positive feedback loops. Members of the Rap family of phosphatases (RapA, RapB, and RapE) dephosphorylate Spo0A~P indirectly by removing the phosphoryl group from Spo0F, whereas Spo0A~P is directly dephosphorylated by the Spo0E family of phosphatases (Spo0E, YisI, and YnzD) (82, 111, 117, 118). Spo0A~P levels continue to increase as a result of a feedback loop including AbrB and the alternative sigma factor SigH (150, 155). Transcription of *sigH* is repressed by AbrB; therefore, Spo0A~P repression of *abrB* leads to increased levels of SigH (148, 171). The *spo0A* and *spo0F* genes contain SigH-controlled promoters that are activated to increase expression of these two phosphorelay response regulators. Spo0A~P further activates, directly and indirectly, all the necessary genes required for the subsequent cascade of events which ultimately leads to complete development of *B. subtilis* and release of the endospore from the mother cell (reviewed in (121)).

Components of the sporulation phosphorelay are conserved among *B. cereus* group members most likely resulting in similar control of sporulation initiation among species. Of particular interest to researchers investigating sporulation in *B. cereus* group members is the etiological agent of anthrax disease, *B. anthracis*. Orthologues of Spo0F, Spo0B and Spo0A were identified in *B. anthracis* (144). In addition, five sensor histidine kinases (BA4223, BA2291, BA1351, BA1356 and BA5029) were identified in *B. anthracis* based on the amino acid conservation surrounding the active-site histidine of the major *B. subtilis*

sensor histidine kinase, KinA. The *B. anthracis* sensor histidine kinases were determined to be functional using a series of genetic deletion and complementation assays (21). Negative regulation of the phosphorelay involves the production of Spo0F- and Spo0A-specific phosphatases, Raps and Spo0E-like proteins, respectively. Two Rap phosphatases, one chromosome-encoded (BA3790) and the other pXO1-encoded (BXA0205), identified in *B. anthracis* were capable of directly dephosphorylating Spo0F. When overexpressed, the Rap phosphatases negatively impacted sporulation (16). Spo0A is directly dephosphorylated by the Spo0E-family of proteins. *B. anthracis* contains four genes that are homologues to the *B. subtilis* Spo0E, two (BA1877 and BA2416) of which were readily expressed in *B. anthracis* and shown to actively inhibit sporulation (15).

A unique attribute of *B. anthracis* is the expression of two plasmid-encoded proteins (pXO1-118 and pXO2-61) that also affect sporulation. When overexpressed, pXO2-61, and to a lesser extent pXO1-118, decrease sporulation efficiency. These proteins bear homology to the signal sensor domain of the *B. anthracis* sensor histidine kinase BA2291. It has been proposed that overexpression of *pXO2-61* titrates a signal away from BA2291 which results in BA2291 conversion to a phosphatase specific for Spo0F (174). The presence of these plasmid-associated sporulation inhibitors suggest that *B. anthracis* has developed additional control mechanisms of the phosphorelay enabling adaptation to growth within a host where sporulation does not occur.

### **1.3. *Bacillus anthracis* and anthrax disease**

As the etiological agent of anthrax disease, *B. anthracis* is the most renowned member of the *B. cereus* group. *B. anthracis* infection results in one of three forms of anthrax disease: cutaneous, gastrointestinal, or inhalation. The specific type of anthrax disease depends on the route of entry of the *B. anthracis* spore. Cutaneous anthrax is the most commonly reported of the three diseases and results from entry of spores into cuts or

abrasion in the skin. If recognized and treated properly, cutaneous anthrax is rarely fatal. The rarest form of anthrax, gastrointestinal anthrax, results from ingestion of spores. It is difficult to diagnose gastrointestinal anthrax; therefore, the lack of early recognition and treatment often leads to a lethal outcome. Inhalation anthrax is the most well-known and well-studied form of disease. Spores enter the lungs where they are phagocytosed by resident macrophages and dendritic cells which transfer the spores to regional lymph nodes enabling the spores to germinate and disseminate (8, 34, 39, 106, 128). Initial nonspecific flu-like symptoms of inhalation anthrax result in difficult diagnosis of disease. If untreated, inhalation anthrax progresses to full respiratory distress, septicemia, shock and eventually death. Inhalation anthrax is nearly always fatal.

*B. anthracis* can evade and escape the host immune response primarily by production of anthrax toxin and a poly-γ-D-glutamic acid capsule. Anthrax toxin is arguably the most important virulence factor produced by *B. anthracis*. The toxin is comprised of three pXO1-encoded proteins: protective antigen (PA), lethal factor (LF) and edema factor (EF). Binary combination of PA and LF is termed “lethal toxin” (LT) and combination of PA and EF is termed “edema toxin” (ET). Anthrax toxin entry is initiated when PA (85 kDa) binds to host cells via specific receptors (ANTXR1 and ANTXR2), is cleaved by a furin-like protease, and forms a multimeric prepore that is capable of binding LF (83 kDa) and/or EF (89 kDa). Upon endocytosis of the protein-receptor complex and endosomal acidification, the PA prepore undergoes a conformational change enabling insertion into the endosomal membrane and translocation of LT and ET into the host cell cytosol (13, 51, 89, 104, 157, 180). LF is a zinc-dependent metalloprotease that inhibits the MAPK signal transduction pathway ultimately resulting in host cell death (44, 86, 163). EF is a calmodulin-dependent adenylyl cyclase that elevates cellular levels of cAMP causing host cell edema (90, 163).

As is true for most bacteria that produce a capsule, the *B. anthracis* capsule is anti-phagocytic and associated with dissemination during infection (42, 57, 83, 97, 116). The *B.*

*anthracis* capsule composition is unique from other bacteria. Instead of producing a polysaccharide capsule like most bacteria, the *B. anthracis* capsule is composed solely of D-glutamic acid residues that are gamma-linked to form homopolymers (125). Encapsulated *B. anthracis* is likely protected from the destructive response of host cells because of its anti-immunogenic properties.

#### **1.4. Major pleiotropic regulators in the *Bacillus cereus* group members**

Virulence factor production is coordinately controlled in *B. cereus* group members in response to specific signals and regulators. In *B. anthracis*, the master virulence regulator AtxA (anthrax toxin activator) controls expression of the anthrax toxins and poly-γ-D-glutamic acid capsule (18). *B. cereus sensu stricto* and *B. thuringiensis* rely on the pleiotropic transcriptional regulator PlcR to control expression of their virulence factors (1, 59, 91). Differential gene expression among the *B. cereus* group species can be attributed in part to PlcR- and AtxA-controlled activities in these species.

The *atxA* gene is located on the *B. anthracis* virulence plasmid pXO1 (87, 165). The AtxA regulon includes *B. anthracis*-specific structural genes for anthrax toxin, *pagA*, *cya*, and *lef*, located on pXO1, and the capsule biosynthetic operon, *capBCADE*, located on pXO2. In addition, AtxA controls some chromosomal genes common to *B. cereus sensu stricto* and *B. thuringiensis* (18). Typical *B. cereus sensu stricto* and *B. thuringiensis* strains do not carry the plasmid harboring *atxA* and therefore exhibit differential expression of AtxA-controlled chromosomal genes.

The global regulator in *B. cereus sensu stricto* and *B. thuringiensis* species, PlcR, is encoded by the chromosome and controls expression of several genes, many associated with pathogenesis (1, 59, 91). The *plcR* gene in *B. anthracis* contains a strain-specific nonsense mutation that results in a truncated, non-functional protein (102). *B. anthracis*

carries multiple PlcR targets, but the lack of a functional PlcR results in minimal or no expression of these genes (127).

Interestingly, expression of a *B. thuringiensis plcR* gene in a *B. anthracis* strain containing *atxA* resulted in a significant decrease in sporulation, a phenotype that was rescued by deletion of *atxA*. It has been proposed that the *plcR* and *atxA* regulons in *B. anthracis* are not compatible and that the nonsense mutation within the *B. anthracis plcR* gene provided a selective advantage for evolution of the species (102). Nevertheless, recent reports of unusual *B. cereus sensu stricto* strains suggest that in certain strain backgrounds PlcR and AtxA can coexist. *B. cereus* G9241, which causes an anthrax-like disease, carries *plcR* and *atxA* genes and expresses factors attributed to both regulons (78).

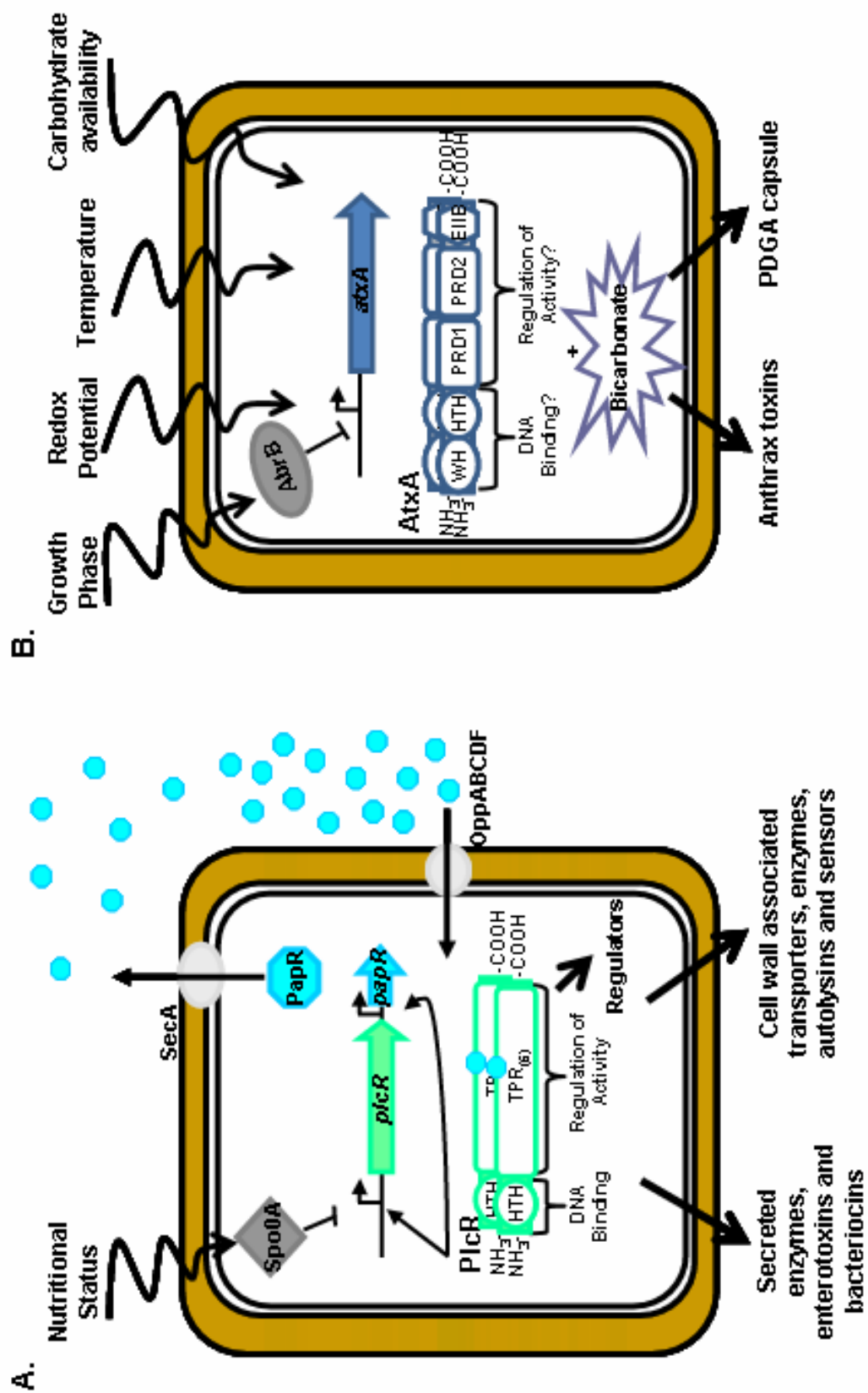
### **1.5. PlcR: A pleiotropic regulator in *Bacillus cereus sensu stricto* and *Bacillus thuringiensis***

The pleiotropic transcriptional regulator PlcR, initially discovered as a positive regulator of the phospholipase *C* gene in *B. thuringiensis* (91), controls multiple genes encoding secreted toxins and degradative enzymes, cell wall associated proteins, and cytoplasmic regulatory proteins in *B. thuringiensis* and *B. cereus sensu stricto* (1, 59, 91). PlcR regulation is not apparent in *B. anthracis* because the *plcR* locus contains a nonsense mutation resulting in a truncated nonfunctional protein (1, 102, 140). Proteomic studies, transcriptional profiling, and *in silico* analyses have been employed to determine PlcR regulons in several *B. thuringiensis* and *B. cereus sensu stricto* strains (1, 59, 60, 114). Established PlcR-controlled virulence genes include enterotoxins, hemolysins, proteases and phospholipases. These genes are spread throughout the genome and do not form pathogenic islands on the chromosome (1).

The 34-kDa PlcR protein contains an amino-terminal helix-turn-helix DNA-binding domain and a carboxy-terminal regulatory domain consisting of 11 helices that form five tetratricopeptide repeats (TPR) (37). PlcR activity is dependent upon interaction with the quorum-sensing peptide PapR (peptide activating PlcR) (138). In the current model for PlcR/PapR function (Fig. 1-3A), PapR is synthesized as a 48-amino acid peptide and secreted by the SecA machinery. Once outside of the cell, PapR is proteolytically processed to a heptapeptide that is imported into the cell via the OppABCD transport system (17, 61). Inside the cell, the PapR heptapeptide associates with PlcR to activate target genes. The crystal structure of PlcR:PapR indicates that PapR binds to the concave side of PlcR TPR domain helices 5 and 7 triggering dimerization of two PlcR:PapR complexes via the TPR domains (37).

The *plcR/papR* genes form a bicistronic cluster that is autogenously controlled (1, 91) and *B. cereus* group members can be classified into four distinct groups based on the sequence and specificity of the PlcR:PapR pair. PlcR groups I, II, III and IV are associated with PapR heptapeptides including the carboxy-terminal sequences LPFE(F/Y), VP(F/Y)E(F/Y), MPFEF, and LPFEH, respectively (17, 139). The first and last amino acids of these peptide sequences determine specificity of PlcR:PapR for its target genes (17, 138, 139). The PlcR:PapR complex binds to a consensus DNA sequence, the palindromic 'PlcR box' (TATGNAN<sub>4</sub>TNCATA), located up to 200 nucleotides upstream of the -10 box of a promoter region (1, 114). *In silico* and genetic analyses have revealed variability in PlcR box sequences. The A+T-content is higher in the vicinity of PlcR boxes that are active when grown in rich conditions [Luria-Bertani broth (LB), 30°C] (59). PlcR-target genes typically contain promoter regions that resemble the canonical -10 region of the housekeeping sigma factor, SigA, and a -35 recognition region that is slightly different than the typical SigA consensus sequence (1, 59).

*plcR* transcription is controlled by the developmental regulator Spo0A. Two Spo0A





**Figure 1-3. Models for AtxA and PlcR control of virulence gene expression.** (A) *plcR* gene activation and PlcR:PapR function in *B. cereus* group members. Signals that impact *plcR/papR* transcription include nutritional status and cell density. The master response regulator Spo0A binds directly to the *plcR* promoter to repress transcription. PlcR contains a DNA-binding domain, HTH, and tetratricopeptide repeats, TPRs, that regulate activity. PapR is exported by the SecA machinery, proteolytically processed to a heptapeptide, and imported into the cell by the OppABCD transport system. Mature processed PapR associates with PlcR enabling dimerization and regulation of activity. The PlcR:PapR complex autogenously controls the *plcR/papR* bicistronic gene cluster in addition to multiple genes encoding secreted toxins and degradative enzymes, cell wall associated proteins, and cytoplasmic regulatory proteins. (B) *atxA* gene activation and AtxA function in *B. anthracis*. Multiple signals including growth phase, redox potential, temperature and carbohydrate availability impact the transcription of *atxA*. The growth phase transition state regulator AbrB binds directly to the *atxA* promoter region to repress transcription. Predicted functional domains of AtxA are: winged helix (WH) and helix-turn-helix (HTH) for DNA binding; PEP:sugar dependent phosphotransferase system (PTS) domains (PRD1 and PRD2), for regulation of activity; and enzyme IIB component of the PTS (EIIB), for multimerization. In the presence of elevated CO<sub>2</sub>/bicarbonate, AtxA positively affects transcription of the anthrax toxin genes and the biosynthetic operon for synthesis of poly-D-glutamic acid (PDGA) capsule.

boxes flank the 'PlcR box' upstream of *plcR*. Active phosphorylated Spo0A is thought to repress *plcR* transcription by competing with PlcR: PapR for binding to the *plcR* promoter region (92). During batch culture in rich media, transcription of *plcR* and PlcR-regulated genes increases at the transition from exponential to stationary phase of growth. When cells are cultured in sporulation media, phosphorylated Spo0A prevents *plcR* activation (92). Thus, elevated *plcR* transcription occurs when the nutrient status keeps Spo0A~P levels low, and when cells are at a high density due to quorum-sensing (59, 92).

Deletion of *plcR* in pathogenic *B. cereus sensu stricto* and *B. thuringiensis* strains decreases virulence in insect larvae, mice, and rabbit eye models (25, 132, 138). A majority of strains synthesize a functional PlcR protein, but a small proportion (1%) contain *plcR* or *papR* genes with mutations rendering non-functional proteins. *B. anthracis* harbors multiple orthologues of *plcR*-regulated genes, but does not contain a functional PlcR due to a point mutation in *plcR* that results in a truncated protein (1, 140). Introduction of a functional *B. thuringiensis*-derived PlcR into *B. anthracis* facilitates expression of genes with PlcR boxes in their promoter regions, including genes encoding proteases, hemolysins and phospholipases. However, increased expression of these PlcR-regulated virulence factors did not influence the virulence of *B. anthracis* in a murine model of anthrax infection (102).

### **1.6. AtxA: A unique regulator in *Bacillus anthracis***

The *atxA* gene, initially named for its involvement in anthrax toxin gene activation, encodes a master virulence regulator of the anthrax toxin genes *pagA*, *lef*, and *cya*. *atxA* is located on the virulence plasmid pXO1 within a 45-kb pathogenicity island that includes the structural genes for anthrax toxin (103, 112). The capsule biosynthetic operon, *capBCADE*, and the capsule gene regulator *acpA*, both located on pXO2, are also positively controlled at the transcriptional level by AtxA (36, 40, 50, 67, 87, 101, 136, 165, 166). Transcriptional profiling and other experiments have revealed over 100 additional *atxA*-regulated genes

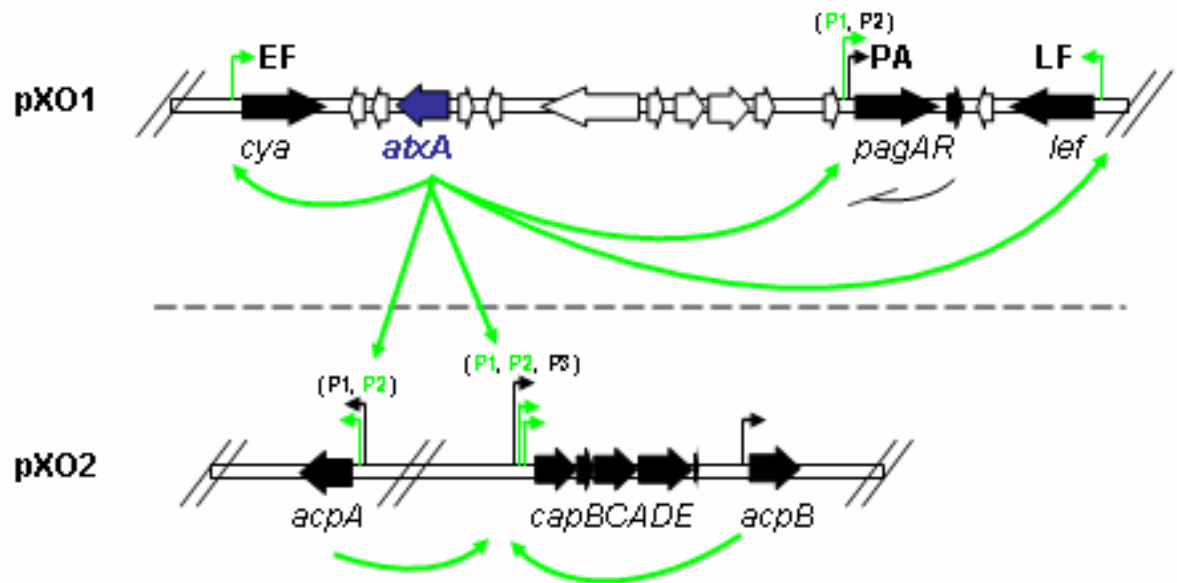
located on pXO1, pXO2, and the chromosome (18, 75, 77). A *B. anthracis atxA*-null strain is avirulent in a murine anthrax model demonstrating the necessity of AtxA for virulence (36).

The molecular mechanism by which AtxA regulates its target genes is unknown. AtxA has an apparent molecular weight of 55.6 kDa and motifs suggestive of DNA-binding and regulation of activity. Analysis of the AtxA amino acid sequence reveals two putative DNA-binding motifs, a winged-helix (WH) and helix-turn-helix (HTH), near the amino-terminus. However, no specific DNA-binding activity has been shown. Located near the center and carboxy-terminus are regions similar to proteins involved in the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) (70, 164). These regions are predicted to regulate AtxA activity. Two putative PTS regulation domains (PRD1 and PRD2) are located near the center of AtxA. PRDs are common to transcriptional regulators that control genes associated with carbohydrate metabolism. Phosphorylation of specific histidine residues within PRDs affect protein oligomerization and function (38). Two sites of phosphorylation were identified within the putative PRDs of AtxA. Phosphorylation of H199 in PRD1 increased AtxA activity whereas phosphorylation of H379 in PRD2 decreased the activity of AtxA (164). The carboxy-terminus contains a motif similar to the enzyme IIB (EIIB) component of the PTS. EIIB proteins function in the PTS to phosphorylate incoming carbohydrates as they pass through their cognate EIIC permeases (38). The EIIB domain of AtxA was shown to facilitate AtxA multimerization. Elevated AtxA activity has been attributed to multimerization of the protein (70).

Transcription of the toxin genes, capsule biosynthetic operon, and many other AtxA-controlled genes is enhanced when cultured in the presence of elevated atmospheric CO<sub>2</sub> and 0.8% dissolved bicarbonate, conditions considered physiologically relevant for pathogenesis (Fig. 1-4) (9, 18, 28, 33, 36, 50, 75, 77, 87, 101, 137). The monocistronic transcripts of the *cya* and *lef* genes map to single start sites and transcription of both genes

is *atxA*-dependent. The *pagA* gene is part of a bicistronic operon, *pagA**pagR*, which contains two transcription start sites (76). The major start site (P1) is *atxA*-dependent while the minor start site (P2) is expressed constitutively at a relatively low level (36, 87). AtxA positively affects transcription of the *cap* operon via control of the pXO2-encoded capsule regulators *acpA* and *acpB* (40, 67, 166, 169). The monocistronic transcripts of *acpA* map to two apparent transcription start sites; one start site (P1) is constitutively activate at a low level while the other (P2) is *atxA*-dependent. The *acpB* gene can be transcribed from its own promoter as a monocistronic transcript, or as part of a multi-cistronic transcript with the *cap* operon via transcriptional read-through. The *cap* operon contains three apparent transcription start sites; one start site (P3) is constitutively active at a relatively low level while the two other start sites (P1 and P2) are *atxA*-dependent. Co-transcription of the *cap* operon and *acpB* creates a positive feedback loop for *capBCADE* transcription (41, 166). Nucleotide sequence similarities in promoter regions of *atxA*-regulated genes are not apparent. It has been suggested that DNA curvature plays a role in AtxA regulation of its target toxin and capsule genes (69).

The steady state level of AtxA does not appear to be significantly affected by CO<sub>2</sub>/bicarbonate; however, multiple signals impact the transcription of *atxA* (Fig. 1-3B). In batch culture, *atxA* is expressed at relatively low levels during early exponential growth and expression peaks as the cells transition into stationary phase of growth (131). This growth phase dependent control of *atxA* expression is attributed to the transition state regulator AbrB. *atxA* expression is increased in an *abrB*-null mutant (131). AbrB is a DNA-binding pleiotropic regulator, often a repressor, which controls a plethora of post-exponential phase genes during logarithmic growth. Repression of genes by AbrB is relieved as cells transition into stationary phase (121, 151). AbrB is the only *trans*-acting factor which has been shown to directly and specifically bind the *atxA* promoter region (151). In the archetype *Bacillus* species, *B. subtilis*, AbrB is part of a feedback loop of regulators which



**Figure 1-4. Anthrax toxin and capsule gene regulation.** See text for details. The anthrax toxin genes, *cya*, *pagAR*, and *lef*, are located on the virulence plasmid pXO1. The *cap* operon, *capBCADE*, and capsule gene regulators *acpA* and *acpB* are located on the virulence plasmid pXO2. *atxA*-dependent promoters are shown in green. Constitutively active promoters are depicted in black.

include the master response regulator Spo0A and the alternative sigma factor SigH. In *B. anthracis*, *atxA* expression is positively controlled by another component of the feedback loop, SigH, in a strain-dependent and AbrB-independent manner (14, 68). Transcription of *atxA* is also increased during early exponential growth in small c-type cytochrome mutants. The small c-type cytochromes were shown to indirectly repress *atxA* when cultured in the absence of elevated CO<sub>2</sub>/bicarbonate; however, addition of CO<sub>2</sub>/bicarbonate eliminated the increased *atxA* expression phenotype making the importance of c-type cytochromes in pathogenesis unlikely (175).

Other signals impacting *atxA* transcription and AtxA protein levels are temperature and carbohydrate availability. In agreement with the requirement of AtxA for *B. anthracis* pathogenesis, optimal expression of *atxA* occurs at 37°C (35). Glucose is an additional signal impacting *atxA* transcription. Regulation of transcription in response to carbohydrate availability is often controlled by the carbon catabolite protein CcpA (170). Deletion of *ccpA* indirectly decreased transcription of *atxA* and also resulted in attenuation in a murine model for anthrax (32). Finally, the pleiotropic DNA binding protein CodY which senses and responds to cell energy and nutrient status (71, 124, 135) was shown to affect AtxA stability. AtxA protein levels decreased in a *codY*-null strain; however, *atxA* transcription, mRNA stability, and *atxA* translation were unaffected. It has been suggested that the deletion of *codY* results in the synthesis of a protease that directly influences AtxA stability (168).

In addition to control of transcription initiation, *atxA* transcript stability is regulated. Transcription of *atxA* initiates from two start sites, P1 (36) located 99-nts upstream of the translational start and P2 (14) located 650-nts further upstream of P1. Both P1 and P2 appear to contain putative consensus sequences for the housekeeping sigma factor SigA and are therefore presumably controlled by SigA containing RNA polymerase (RNAP). A positive retroregulation stem-loop structure starting 497-nts downstream from the *atxA*

translational stop codon has been shown to stabilize the mRNA (11). The exact mechanism by which the long 3' UTR regulates *atxA* expression is not completely understood; however, similar retroregulation systems are present in the control of *B. thuringiensis* cryotoxin genes (2, 176).

### 1.7. Gaps in knowledge and significance of research

Several signals impact *atxA* expression; however, the *cis*-acting elements and *trans*-acting factors that directly control transcription of *atxA* are largely unknown. Multiple studies suggest that precise control of the timing and steady state level of *atxA* transcription are required for optimal expression of AtxA target genes in batch culture (18, 35, 68, 166). The timing and transcript level of *atxA* is impacted by growth phase, redox potential, temperature, and carbohydrate availability (32, 35, 131, 175). Little is known regarding the direct mechanisms by which redox potential, temperature, or carbohydrate availability affect *atxA* transcription. However, the *B. anthracis* growth phase/transition state regulator AbrB was shown to directly repress *atxA* transcription (131). The AbrB binding site in the *atxA* promoter region overlaps the housekeeping sigma factor, SigA, putative -35 consensus sequence (151). This suggests that AbrB represses *atxA* transcription by competing with SigA containing RNA polymerase for binding to the *atxA* promoter region. Additional investigation of the *cis*-acting elements required for *atxA* transcription could impact our understanding of the molecular mechanisms controlling *atxA*. The results of such investigations could also reveal additional *trans*-acting factor binding sites, other than the AbrB-binding site, required for *atxA* transcription.

In addition to affecting *atxA* transcription, AbrB regulates development of *B. anthracis* from the vegetative cell state to a dormant spore. The spore constitutes the infectious form of *B. anthracis*; therefore, factors that affect *B. anthracis* sporulation may also impact virulence and pathogenesis. The developmental regulators Spo0A, AbrB, and

SigH have been well-characterized in *B. subtilis* and were shown to possess similar functions in *B. anthracis* (see section 1.2). Spo0A, AbrB and SigH are key components in the sporulation phosphorelay. Deletion of *spo0A* or *sigH* abrogates sporulation in *B. anthracis* (68, 177). In addition to their role in *Bacillus* species development, Spo0A, AbrB, and SigH also control *atxA* transcription. AbrB binds to specific sequences in the *atxA* promoter region to repress transcription of *atxA*. Spo0A positively affects *atxA* via control of *abrB* expression. SigH control of *atxA* occurs via its positive effect on *spo0A*, and in one strain, *sigH* positively regulates *atxA* transcription in a *spo0A*- and *abrB*-independent manner (68, 151). An *atxA*-null strain is avirulent in a murine model of anthrax disease demonstrating the necessity of *atxA* expression for virulence (36). Experiments directed toward assessing the impact developmental regulators have on AtxA expression and virulence could provide a better understanding of the role the established regulatory network (Spo0A/AbrB/SigH) has on *B. anthracis* pathogenesis.

Other factors impacting *B. anthracis* sporulation are plasmid-encoded and positively influenced by AtxA. Deletion of *atxA* in a Sterne-like strain (pXO1+, pXO2-) resulted in more efficient sporulation than parent when grown in a rich medium (75). Expression of a *B. thuringiensis plcR* gene in a *B. anthracis* strain containing *atxA* resulted in a significant decrease in sporulation, a phenotype that was rescued by deletion of *atxA*. *B. anthracis* contains a species-specific mutation within the *plcR* gene resulting in a truncated, non-functional protein. It has been proposed that coexpression of the AtxA- and PlcR-regulons in *B. anthracis* is not compatible; therefore, a mutation within the *B. anthracis plcR* occurred as a result of selective evolution (102). Overexpression of the highly *atxA*-controlled gene *pXO2-61* led to a marked decrease in *B. anthracis* sporulation resulting from a potential titration of signal from the sporulation sensor histidine kinase BA2291 (174). These results suggest that AtxA not only controls *B. anthracis* virulence gene expression, but also regulates spore development. Investigations into what role, if any, AtxA has on spore



development could provide evidence for a link between *B. anthracis* sporulation and toxin production. Finally, by determining the molecular mechanisms controlling *atxA* expression, it can be determined what physiological relevance altered *atxA* expression has on *B. anthracis* disease progression.

## **Chapter II**

### **Materials and Methods**

## 2.1. Growth conditions

*B. anthracis* was grown in Luria-Bertani (LB) (6) medium for electroporations and DNA extractions. Cell lysates and culture supernatants for Western blot analysis were obtained from cells cultured in toxin-inducing (Casamino acids [CA] medium (161) buffered with 100 mM HEPES [pH 8.0] and 0.8% [wt/vol] sodium bicarbonate at an atmosphere of 5% CO<sub>2</sub>) and/or sporulation (Phage assay [PA] medium (159) in atmospheric air) conditions. Samples obtained for  $\beta$ -galactosidase assays were cultured in toxin-inducing conditions. Briefly, an overnight culture of *B. anthracis* grown in LB medium supplemented with appropriate antibiotics and incubated with agitation at 30°C was used to inoculate CACO<sub>3</sub> (toxin-inducing) or PA (sporulation) medium comprising 10% of the volume of an Erlenmeyer flask. Cultures were incubated at 37°C with agitation for sporulation conditions and 37°C with agitation and an atmosphere of 5% CO<sub>2</sub> for toxin-inducing conditions. Antibiotics were added to media when necessary: spectinomycin (100µg/ml), erythromycin (300µg/ml for *E. coli*; 5µg/ml for *B. anthracis*), carbenicillin (100µg/ml). All chemicals were purchased from Fisher unless otherwise stated.

## 2.2. Strain construction

Strains and plasmids are shown in Table 2-1. *B. anthracis* strains were derived from the Sterne-like strains (pXO1+, pXO2-) ANR-1 (Ames non-reverting) and UM44, and the genetically complete Ames strain (pXO1+, pXO2+). *E. coli* TG1 and GM2163 strains were used for cloning purposes.

Isogenic  $\Delta atxA$ ,  $\Delta sigH$ , and  $\Delta spo0A$  mutants were created in the ANR-1 strain (UT374, UT399, and UT400, respectively) and in the Ames background (UTA22, UTA16, and UTA28, respectively), using the markerless temperature-sensitive integration system described previously (120). Oligonucleotide primers are shown in Table 2-2. DNA sequences upstream of the *atxA* gene (-1009 to +99 relative to the P1 transcription start

site) were amplified using primers JR170 and JR171, and regions downstream of the *atxA* translational stop (+1528 to +2517) were amplified using primers JR172 and JR173. Regions surrounding *sigH* were amplified using primers JR119 and JR120 which amplified sequences from -1026 to -1 (relative to the *sigH* translational start site), and primers JR121 and JR122 which amplified sequences from +658 to +1664. DNA sequences surrounding *spo0A* were amplified using primers JD191 and JD205 to amplify sequences from -1053 to +3 (relative to the *spo0A* translational start site) and primers JD206 and JD194 to amplify sequences from +793 to +1887. Splicing by overlap extension PCR (PCR-SOE) (79) was used to fuse the upstream and downstream fragments of each respective gene. The PCR-SOE product was cloned into the temperature-sensitive integration vector pHY304. According to established protocols, strains were cultured until the desired mutation was discovered (120).

Similar methods were used to create the isogenic *atxA*-up mutants in the ANR-1 and Ames backgrounds, UT398 and UTA26, respectively. Briefly, sequences surrounding the *atxA* ORF (-866 to +1527, relative to the P1 transcription start site) were amplified using primers TH134 and TH49 and cloned into pGEM-T easy (Promega, Madison, WI). Quickchange PCR (146) methods were used in combination with primers JD195 and JD196 to mutate sequences from +14 to +22 relative to the *atxA* P1 transcription start. The mutated sequence was cloned into pHY304. *atxA* was deleted from UTA26 to create strain UTA31 using DNA obtained from UTA26 for amplification purposes and the same methods described to create the single  $\Delta atxA$  mutant. Double deletion mutants containing  $\Delta atxA$ ,  $\Delta sigH$ ,  $\Delta spo0A$ , and *atxA*-up were created using the methods described above.

Creation of the ANR-1  $\Delta abrB$  (UT384), Ames  $\Delta abrB$  (UTA27), Ames  $\Delta pX02-61$  (UTA9), and any double mutants containing deletion of these genes utilized an  $\Omega$ -spec cassette and methods described previously (131). Deletion of *abrB* from the ANR-1 strain background was performed using pUTE416 and methods described previously (131). To

delete *abrB* from the Ames strain background, regions surrounding *abrB* were amplified using primers JD176 and JD177 to amplify sequences from -839 to +3 (relative to the *abrB* translational start site) and primers JD178 and JD179 to amplify regions from +283 to +1123. 870-nts upstream (-838 to +13 relative to the translational start site) and 859-nts downstream (+441 to +1299) of the *pXO2-61* ORF were amplified using primer pairs KT3/KT4 and KT1/KT2, respectively. PCR products for the *abrB* mutation were cloned into pUTE583 while the *pXO2-61* ORF deletion was cloned into pUTE568. The upstream and downstream fragments of the deletion construct flanked an  $\Omega$ -spec cassette. *pXO2-61* was initially deleted from the Pasteur-like strain 9131(pXO2) to create UT287 and further transduced into Ames using the CP51 phage (160).

*B. anthracis* strains harboring *atxA* promoter – *lacZ* (*PatxA-lacZ*) fusion vectors were constructed to monitor *atxA* promoter activity over time using  $\beta$ -galactosidase assays. The *atxA* promoter region was amplified to generate several 5' and 3' truncated fragments which were subsequently cloned upstream of a promoterless *lacZ* gene on pHT304-18z (3) using HindIII and XbaI restriction enzyme sites. Mutation of the putative SigA consensus sequence within the *atxA* promoter was performed using Quickchange PCR (146) and appropriate oligonucleotide primers (Table 2-2).

### **2.3. DNA isolation and manipulation**

Plasmid isolation from *E. coli*, transformations into *E. coli*, and recombinant techniques were performed using standard methods (6). Nonmethylated plasmid DNA for electroporation into *B. anthracis* (87, 98) was obtained from *E. coli* GM2163 cells. *B. anthracis* DNA extractions for verification purposes were performed using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc.). Restriction enzymes, T4 DNA lygase, and Taq polymerase were purchased from NEB.

## 2.4. Preparation of crude cellular extract

*B. anthracis* crude cellular extract was prepared as described previously (162) using some modifications. *B. anthracis* was cultured in 500-ml of CACO<sub>3</sub> medium and an atmosphere of 5% CO<sub>2</sub> to an OD<sub>600</sub> of 0.6 – 0.8. Cells were collected by passing cultures through a 0.2 µm polyethersulfone (PES) membrane filter (Corning Incorporated, Corning, NY). Cells were washed with 50 ml of TDE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], 100 mM KCl, 1 mM dithiothreitol, 10% ethylene glycol) followed by 30 ml of TEG (25 mM Tris-HCl [pH 8.0], 5 mM EGTA [pH 8.0]). Finally, cells were rinsed from the filter using 10 ml of TEG. Suspensions were centrifuged for 5 min at 2,260 x g at 4°C in a Rotanta 460 R Centrifuge. Supernates were discarded and cell pellets were stored at -80°C. The pellets were resuspended in a mixture of approximately 4 ml of TDE and 1.5 ml of TEG. To lyse the cells, each suspension was passed three times through a French press at 20,000 lb/in<sup>2</sup>. KCl was added to a final concentration of 100 mM and the lysates were centrifuged at 58,820 x g for 1 h at 4°C using a Beckman TL-100 ultracentrifuge. To remove contaminating nucleic acids, 1/10<sup>th</sup> the volume of a 30% streptomycin sulfate solution was added in a dropwise manner to the lysate at 4°C and stirred for 30 min. The lysate was then centrifuged at 15,700 x g for 10 min at 4°C in an Eppendorf Centrifuge 5415 R. Proteins were precipitated with ammonium sulfate to 70% saturation at 4°C for 30 min and then centrifuged at 15,700 x g for 15 min at 4°C using an Eppendorf Centrifuge 5415 R. The precipitated protein pellet was resuspended in 1 ml of TDE and dialyzed in the same buffer.

## 2.5. Electrophoretic mobility shift assays (EMSAs)

Primers used for probe construction are listed in Table 2-3. Probes were radioactively labeled using direct PCR incorporation of α-<sup>32</sup>P dATP (Perkin Elmer, Boston, MA). Approximately 1 ng of radiolabeled probe was added to binding reactions consisting of 500 ng synthetic DNA (PolydI-dC·PolydI-dC [Thermo Scientific, Milwaukee, WI]), 1-35 µg of

crude cellular extract, 10 µg bovine serum albumin [BSA], and TDE to a final volume of 10-15 µl. When appropriate, 2.5- to 100-fold excess unlabeled competitor was added to each binding reaction. The reactions were incubated for 15 min at room temperature (RT). DNA-protein complexes were resolved using electrophoresis on a 5% native polyacrylamide gel at 4°C. The gels were dried and visualized using a STORM phosphorImager (GE Healthcare, Piscataway, NJ).

## **2.6. $\beta$ -galactosidase assays**

One-ml samples were obtained from cultures at early exponential (2h), transition (4h), and stationary (7h) phases of growth.  $\beta$ -galactosidase assays were performed as described by Miller *et al.* (105). Briefly, cell pellets were resuspended in Z-buffer (60mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 40mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 10mM KCl, 1mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 50mM  $\beta$ -mercaptoethanol [added just prior to use]), transferred to tubes containing 400 µl of 0.1 mm Zirconia/Silica Beads (BioSpec Products, Bartlesville, OK) and bead beat for 1 min using a Mini BeadBeater (BioSpec Products) to lyse the cells. Debris was pelleted using centrifugation and the supernate was used to assay  $\beta$ -galactosidase activity. Figures show data averaged from three independent cultures.

## **2.7. Western blot analysis**

Cell lysates and culture supernates were obtained from *B. anthracis* cultures grown in toxin-inducing and sporulation conditions during early exponential (2h), transition (4h), and stationary (7h) phases of growth. Four-milliliter samples were obtained per time point and centrifuged at 10,000 x *g* for 10 min. To assess lethal factor (LF), protective antigen (PA), and edema factor (EF) protein levels, 1 ml of corresponding supernatant was passed through a 0.2 µm filter (Thermo Scientific) and applied to a nitrocellulose membrane using a slot blot apparatus (Hoefer Scientific, San Francisco, CA). Protein loads were normalized to

OD<sub>600</sub>. Membranes were blocked overnight at 4°C in 1xTBS-T (20mM Tris base, 137mM NaCl, 0.1% tween 20, pH 7.6) containing 2.5% BSA. Primary antibody ( $\alpha$ -LF,  $\alpha$ -PA, or  $\alpha$ -EF) was resuspended in 1xTBS-T and allowed to react with the membrane for 1h at room temperature (RT). Membranes were washed with 1xTBS-T and further incubated with corresponding secondary antibody (goat  $\alpha$ -rabbit-HRP [Bio-rad]) for 1h at RT. Membranes were washed as described above and developed using the SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific).

To assess AtxA protein levels, 4-ml cultures were centrifuged as described above. Cell pellets were washed twice with KTE-PIC (10 mM Tris-HCl pH 8, 100 mM KCl, 10% ethylene glycol, and EDTA-free Complete proteinase inhibitor [Roche]) and resuspended in KTE-PIC to a final volume of 450  $\mu$ l. The cell resuspension was transferred to 1.5 ml screw-cap tubes containing 400  $\mu$ l of 0.1 mm Zirconia/Silica Beads. Samples were lysed mechanically for 2.5 min using a Mini BeadBeater, placed on ice for 5 min, and subjected to mechanical lyses for an additional 2.5 min. After centrifugation, cell lysate was mixed with SDS sample buffer (5% glycerol, 100 mM DTT, 2% SDS, 40 mM Tris-Cl pH 6.8), boiled, and subjected to SDS-PAGE. Protein loads were determined based on OD<sub>600</sub> values and normalized to RNA polymerase  $\beta$  (ANR-1-derivatives) or Ponceau S (0.1% [w/v] Ponceau S in 5% [v/v] acetic acid) stained membranes (Ames-derivatives). SDS-PAGE gels were equilibrated in 1 x CAPS Buffer (10 mM CAPS pH 11, 10% methanol) for 30 min prior to protein transfer. Proteins were transferred to a PVDF membrane at 4°C using a Hoefer transfer unit (Hoefer, Holliston, MA, USA) containing 1 x CAPS Buffer at 50 V for 2h. Membranes were blocked in 1xTBS-T containing 5% non-fat dry milk overnight at 4°C. Primary antibodies ( $\alpha$ -AtxA [(70)] and  $\alpha$ -RNA polymerase  $\beta$  [Santa Cruz Biotechnology, Santa Cruz, CA, USA]) were resuspended in 1xTBS-T and allowed to react with the membrane for 1h at RT. The membranes were washed in 1xTBS-T and further incubated with corresponding secondary antibody (goat  $\alpha$ -rabbit-HRP for AtxA Westerns, or goat  $\alpha$ -



mouse-HRP for RNA polymerase  $\beta$  Westerns) for 1h at RT. Blots were washed in 1xTBS-T and further developed using the SuperSignal West Dura Chemiluminescent Substrate. For re-probing, membranes were stripped using the Restore Western Blot Stripping Buffer (Thermo Fisher) for 30 min at 37°C.

## **2.8. Heat-resistant CFU determination**

Growth curves were performed using toxin-inducing and sporulation conditions. One-milliliter samples were obtained to determine the percentage of heat-resistant colony forming units (CFU) during transition (4h) and stationary (7h and 10h) phases of growth. Using LB, the *B. anthracis* cultures were serially diluted and 100  $\mu$ l of the final dilution suspension was plated on LB agar using spread plating methods. The remaining dilution suspension was heat-shocked at 65°C for 45 min and plated as described above. All plates were incubated overnight at 37°C to determine numbers of heat-sensitive and heat-resistant CFU. The percentage of heat-resistant CFU was calculated by dividing the number of heat-resistant CFU by the number of heat-sensitive CFU.

Samples used to determine total heat-resistant CFU/ml were obtained from cells cultured in sporulation conditions for 24 hours. Using H<sub>2</sub>O, the *B. anthracis* cultures were serially diluted, heat-shocked at 65°C for 45 min, and 100  $\mu$ l of the final dilution was plated on LB agar using spread plating methods. All plates were incubated overnight at 37°C to determine the number of total heat-resistant CFU/ml.

## **2.9. Preparation of *B. anthracis* vegetative cells for intravenous (i.v.) infection**

*B. anthracis* spores ( $\sim 10^7$ ) were incubated in 1 ml of brain-heart infusion (BHI) medium for 10 min at 37°C. The entire spore outgrowth suspension was transferred to 25 ml of CACO<sub>3</sub> and incubated in 5% CO<sub>2</sub> to an OD<sub>600</sub> of approximately 0.6. At this OD<sub>600</sub>, all cultures contained approximately  $10^7$  CFU/ml devoid of refractile spores as observed using

phase contrast microscopy. Cultures were centrifuged at 5,708 x g for 10 min in a Sorval RC-5B Superspeed Centrifuge, the medium was removed, and the cells were washed with 25 ml of 1 x Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium (Mediatech Inc., Manassas, VA). Cells were washed a total of two times and then resuspended in 25 ml of 1 x DPBS. Prior to infection, an aliquot of the resuspension was diluted and plated on LB to determine the final CFU/ml inoculation dose.

## **2.10. Mouse infections**

All mouse protocols were approved by The University of Texas Health Science Center Institutional Animal Care and Use Committee and performed using accepted veterinary standards. Female 6- to 8-week-old A/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen-free vivarium at The University of Texas Health Science Center. Food and water were supplied to the mice *ad libitum*. The mice were housed 3 per cage and were allowed to acclimate to their surroundings for 7 days prior to being used in the experiments. Mice were infected intravenously using a 30-gauge needle. The tail vein was injected with 50 µl containing approximately  $10^2$  or  $10^3$  heat sensitive CFU.

## **2.11. Microscopy**

*B. anthracis* cells were visualized using a Nikon Eclipse TE2000-U microscope and images were captured using MetaMorph version 6.2r6 (Universal Imaging Corporation). Phase contrast microscopy was used to visualize sporulating cells. India ink (Becton Dickinson Microbiology Systems, Sparks, MD) exclusion methods and DIC imaging were used to visualize capsule.

## **2.12. RNA purification**

Four-milliliter samples were obtained from *B. anthracis* cultures during the transition (4h) phase of growth. Samples were centrifuged at 10,000 x g for 10 min at 4°C, the supernatant was decanted, and 500 µl of culture medium (CACO<sub>3</sub> or PA) was added to each pellet. Cell pellets were stored at -80°C. RNA was extracted using a hot acid-phenol method. An equal volume, 500 µl, of 65°C saturated acid phenol (pH 4.3 [Fisher]) was added to each sample and transferred to screw-cap tubes containing 400 µl of 0.1 mm Zirconia/Silica Beads. The samples were homogenized for 1 min using a Mini BeadBeater, incubated at 65°C for 5 min, and bead-beat for an additional 1 min. Homogenized samples were centrifuged at 16,000 x g for 3 min at 4°C. Following centrifugation, the aqueous phase was transferred to a new 2 ml Eppendorf tube and 500 µl of 65°C saturated acid phenol was added to remove any remaining organic material. Samples containing saturated acid phenol were vortexed, incubated at room temperature (RT) for 5 min, and centrifuged at 16,000 x g for 3 min at 4°C. Following centrifugation, 0.3 volumes of chloroform was added to the aqueous phase and incubated at RT for 10 min with agitation. The mixture was centrifuged for 15 min at 16,000 x g at 4°C and the aqueous phase was transferred to a sterile tube. To precipitate the RNA, ½ starting volume of DEPC-treated H<sub>2</sub>O and 1 volume isopropanol was added to the aqueous phase and incubated at RT for 10 min. RNA was pelleted at 4°C for 15 min at 16,000 x g. The supernatant was removed and RNA pellets were washed with 75% ice-cold EtOH, dried in an Eppendorf Vacufuge (Brinkmann Instruments, Inc., Westbury, NY), and resuspended in DEPC-treated H<sub>2</sub>O. RNA concentrations were quantified using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific).

### **2.13. Real-time quantitative PCR (RT-qPCR)**

Purified RNA samples (2.5 – 5 µg) were DNase treated using 5U of RQ1 DNase enzyme (Promega) for 30 min at 37°C. DNase reactions were stopped using 0.1 volume or

5  $\mu$ l (whichever was greater) RQ1 stop buffer (Promega) and incubated at room temperature for 2 min. DNase-treated RNA was precipitated with 1/10<sup>th</sup> volume of 3 M Na-acetate pH 5.2 (Ambion, Grand Island, NY) and 2 volumes of ice-cold 100% EtOH for a minimum of 30 min on ice. The mixture was centrifuged at 16,000 x g for 30 min at 4°C. RNA pellets were washed with 1 ml of ice-cold 75% EtOH, dried in an Eppendorf Vacufuge, and resuspended in DEPC-treated water. RNA concentrations were quantified using a NanoDrop Spectrophotometer ND-1000. RT-qPCR assay information is provided in Table 2-4. RT-qPCR assays were performed in the Quantitative Genomics Core Laboratory at The University of Texas Health Sciences Center in Houston, Texas. All real-time qPCR assays used in this publication were designed and validated by QGCL staff to ensure they pass the minimum requirements for efficiency, sensitivity and template specificity.

cDNA was synthesized in 5  $\mu$ l (384-well plate) total volume by the addition of 3  $\mu$ l/well RT master mix consisting of: 400 nM assay-specific reverse primer, 500  $\mu$ M deoxynucleotides, Superscript II (or Affinityscript) buffer and 1 U/ $\mu$ l Superscript II (or Affinityscript) reverse transcriptase (Invitrogen, Carlsbad, CA), to a 384-well plate (Applied Biosystems, Foster City, CA) and followed by a 2  $\mu$ l volume of sample (25-50 ng/ $\mu$ l). For 96-well plates, 6  $\mu$ l RT master mix was added to each well followed by 4  $\mu$ l of RNA sample (25 ng/ $\mu$ l). Each sample was assayed in triplicate plus a control without reverse transcriptase to assess DNA contamination levels. Each plate also contained an assay-specific sDNA (synthetic amplicon oligo) standard spanning a 5-log template concentration range and a no template PCR control. Both were added into RT master mix with reverse transcriptase. Each plate was covered with Biofilm A (Bio-Rad, Hercules, CA) and incubated in a PTC-100 or DYAD thermocycler (Bio-Rad, Hercules, CA) for 30 min at 50°C followed by 72°C for 10 min. PCR master mix, 15  $\mu$ l/well, was added directly to the 5  $\mu$ l RT volume. Final concentrations for the PCR were 400 nM forward and reverse primers (IDT, Coralville, IA), 100 nM fluorogenic probe (Biosearch Technologies, Novato, CA), 5 mM MgCl<sub>2</sub>, and 200  $\mu$ M deoxynucleotides, PCR buffer, 150 nM SuperROX dye (Biosearch Technologies, Novato, CA) and 0.25 U JumpStart Taq polymerase per reaction (Invitrogen, Carlsbad, CA), final concentrations. RT master mixes and all RNA samples and DNA oligo standards were pipetted by a Tecan Genesis RSP 100 robotic workstation (Tecan US, Research Triangle Park, NC); PCR master mixes were pipetted utilizing a Biomek 2000 robotic workstation (Beckman, Fullerton, CA). Each assembled plate was then covered with optically clear film (Applied Biosystems, Foster City, CA) and run in a 7900 real-time instrument using the following cycling conditions: 95°C, 2 min; followed by 40 cycles of 95°C, 12 sec and 60°C, 30 sec. The resulting data were analyzed using SDS 2.3 (7900) software (Applied Biosystems, Foster City, CA) with

FAM reporter and ROX as the reference dye. Synthetic, PAGE purified DNA oligos used as standards (sDNA) encompassed at least the entire 5' – 3' PCR amplicon for the assay (Sigma-Genosys, The Woodlands, TX). Each oligo standard was diluted in 100 ng/μl *E. coli* tRNA-H<sub>2</sub>O (Roche Diagnostics, Indianapolis, IN) and spanned a 5-log range in 10-fold decrements starting at 0.8 pg/reaction (24, 56, 72, 109). 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 with PCR amplicons of less than 100 bases in length (G.L. Shipley, personal communication).

**Table 2-1.** Strains and plasmids used in this study.

Name	Description <sup>a</sup>	Source or reference
<u>Strains</u>		
UM44	Webridge strain, pXO1+, pXO2-, Ind-	C. Thorne
UT291	UM44-derived $\Delta abrB/sigH$ mutant, Spo-, SpcR, Kan <sup>R</sup>	Hadjifrangiskou <i>et al.</i> 2007
ANR-1	Ames strain, pXO1+, pXO2-	Welkos <i>et al.</i> 2001
UT374	ANR-1-derived $\Delta atxA$ mutant	This work
	UT374 containing the <i>atxA</i> complementation vector pUTE926	This work
UT374(pUTE926)		
UT375	ANR-1-derived $\Delta lef$ mutant	Hammerstrom <i>et al.</i> 2011
UT384	ANR-1-derived $\Delta abrB$ mutant	This work
UT399	ANR-1-derived $\Delta sigH$ mutant	This work
UT398	ANR-1-derived <i>atxA</i> -up mutant; mutated at positions +14 to +22	This work
UT400	ANR-1-derived $\Delta spo0A$ mutant	This work
UT401	ANR-1-derived $\Delta spo0A/abrB$ mutant	This work
UT402	ANR-1-derived $\Delta spo0A/sigH$ mutant	This work
Ames	pXO1+, pXO2+	Ravel <i>et al.</i> 2009
UTA9	Ames-derived $\Delta pXO2-61$ mutant	This work
UTA16	Ames-derived $\Delta sigH$ mutant	This work
UTA22	Ames-derived $\Delta atxA$ mutant	This work
UTA26	Ames-derived <i>atxA</i> -up mutant; mutated at positions +14 to +22	This work
UTA27	Ames-derived $\Delta abrB$ mutant	This work
UTA28	Ames-derived $\Delta spo0A$ mutant	This work
UTA29	Ames-derived $\Delta spo0A/sigH$ mutant	This work
UTA30	Ames-derived $\Delta spo0A/abrB$ mutant	This work
UTA31	Ames-derived $\Delta pXO2-61/atxA$ -up mutant	This work
UTA32	Ames-derived $\Delta atxA/atxA$ -up mutant	This work
<u>Plasmids</u>		
pHT304-18z	Promoterless <i>lacZ</i> vector, Amp <sup>R</sup> in <i>E.coli</i> , Erm <sup>R</sup> in <i>B. anthracis</i>	D. Lereclus
pUTE839	pHT304-18z-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; contains sequences from -770 to +99	This work
pUTE843	pHT304-18z-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; contains sequences from -72 to +99	This work
pUTE890	pHT304-18z-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; contains sequences from -56 to +99	This work
pUTE891	pHT304-18z-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; contains sequences from -36 to +99	This work
pUTE901	pUTE843-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; mutated at position -8 of the SigA consensus	This work
pUTE902	pUTE843-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; mutated at position -35 of the SigA consensus	This work

pUTE904	pUTE843-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; mutated at position -11 of the SigA consensus	This work
pUTE905	pUTE843-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; mutated at position -9 of the SigA consensus	This work
pUTE906	pUTE843-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; mutated at position -34 of the SigA consensus	This work
pUTE907	pUTE843-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; mutated at position -30 of the SigA consensus	This work
pUTE914	pHT304-18z-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; contains sequences from -72 to +13	This work
pUTE915	pHT304-18z-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; contains sequences from -72 to +61	This work
pUTE918	pHT304-18z-derived <i>atxA</i> promoter - <i>lacZ</i> fusion vector; contains sequences from -72 to +31	This work
pUTE926	<i>atxA</i> complementation vector; contains sequences from -72 to +1527	This work
pUTE971	pHT304-18z-derived <i>atxA</i> promoter – <i>lacZ</i> fusion vector; contains mutated sequences from +14 to +22	This work

<sup>a</sup>numeric values relative to *atxA* P1 transcriptional start

**Table 2-2.** Primers used in this study.

Name	Sequence (5' to 3') <sup>a</sup>	Brief description <sup>b</sup>
JR170	GGCCGCGGAGAGCCGCATTAACT	<i>atxA</i> markerless mutation (SacII)
JR171	GGGCATGTCTATAATTGATTCTCCTTTCCTG	<i>atxA</i> markerless mutation
JR172	GAGAATCAATTATAGACATGCCCTTTAAATA TTTGTTTAATGACAC	<i>atxA</i> markerless mutation
JR173	GGCTCGAGCGCTTGTCTCACAATCTCATC	<i>atxA</i> markerless mutation (XhoI)
JR119	GGGCTCGAGATGAAATTGAAGACCCGCAT	<i>sigH</i> markerless mutation (XhoI)
JR120	GTAGCTCTTGTTACTTGATCCCTCCGACCGC TA	<i>sigH</i> markerless mutation
JR121	GTCGGAGGGATCAAGTAACAAGAGCTACAG GTGTAAAAATCACCTG	<i>sigH</i> markerless mutation
JR122	GGGCTCGAGTCTCATGAAGTTCAAAGTCGA AATC	<i>sigH</i> markerless mutation (XhoI)
JD176	TCTAGACTCCTATTGGAAATTTAGAAGATAT GAC	<i>abrB</i> allelic exchange (XbaI)
JD177	TATGGGATCCTAAGCTTCTCAATTATGAGAA GC	<i>abrB</i> allelic exchange (BamHI)
JD178	CTTAGGATCCCATAATTCTTTTCCTCCTAAA GAAATAG	<i>abrB</i> allelic exchange (BamHI)
JD179	GTCGACGATCTACTAGTTGCTTGAAGATTTT TTC	<i>abrB</i> allelic exchange (Sall)
JD191	CCCTCTAGACACGGCTAATGCTGTCGGTC	<i>spo0A</i> markerless mutation (XbaI)
JD205	CTTTTCGACACTGAGAATAGAAGAAGTAAGA GATTTAAG	<i>spo0A</i> markerless mutation
JD206	AGATAAGAGTCACAGCTTTTCCTCCCTTACC G	<i>spo0A</i> markerless mutation
JD194	CCCGTCGACGGAAACAGGCGAACCCTTA	<i>spo0A</i> markerless mutation (Sall)
KT1	GAATTCCATCACCGTTAGTGAATCCT	<i>pXO2-61</i> allelic exchange (EcoRI)
KT2	GGATCCTCGGTAAAGACAGAGAAAGC	<i>pXO2-61</i> allelic exchange (BamHI)
KT3	GGATCCTATCGACAAAGAAGGCATTT	<i>pXO2-61</i> allelic exchange (BamHI)
KT4	GAGCTCAGTATGCTTTGCATTTTGGT	<i>pXO2-61</i> allelic exchange (SacI)
YC9-1	AAGCTTAAAAAACTAATAACCCCCCT	<i>atxA</i> locus -770 (HindIII)
JD50	AAGCTTAGAAACAAAAACCAATTTTCC	<i>atxA</i> locus -72 (HindIII)
JD89	AAGCTTTTTCCCTTAAAAAATCATTCCC	<i>atxA</i> locus -56 (HindIII)
JD90	AAGCTTCCCAAATATGTGTTTTGAAATATAA	<i>atxA</i> locus -36 (HindIII)
JD111	AAGCTTTATAATAGCATTTGTCAGGTCATCT G	<i>atxA</i> locus -13 (HindIII)
JD228	TATAATAGCATTTGTCAGGTCATCTG	<i>atxA</i> locus -13
JD37	TCTAGAGTCTATAATTGATTCTCCTTT	<i>atxA</i> locus +99 (XbaI)
JD107	CAGAATATTAGAATTAACGGACATTTAAC	<i>atxA</i> locus +61
JD112	TCTAGACAGAATATTAGAATTAACGGACATT TAAC	<i>atxA</i> locus +61 (XbaI)
JD108	GTATTTTAACCATGTCATCAGATGA	<i>atxA</i> locus +31
JD113	TCTAGAGTATTTTAACCATGTCATCAGATGA	<i>atxA</i> locus +31 (XbaI)
JD109	CAGATGACCTGACAAATGCTATTATA	<i>atxA</i> locus +14
JD114	TCTAGACAGATGACCTGACAAATGCTATTAT A	<i>atxA</i> locus +14 (XbaI)
JD110	AAGCTTTCAGGTCATCTGATGACATGGTTAA	<i>atxA</i> locus +2 (HindIII)
JD229	TTAACGGTATTTTAACCATGTCATC	<i>atxA</i> locus +36



JD95	CCCAAAATATGTGTTTTGAAAT <u>G</u> TAATAGCA TTTGTCAAGGTCATC	<i>atxA</i> promoter SigA -11 SDM
JD96	GATGACCTGACAAATGCTATT <u>A</u> CATTTCAAA ACACATATTTTGGG	<i>atxA</i> promoter SigA -11 SDM
JD97	CAAAATATGTGTTTTGAAATAT <u>I</u> ATAGCATTT GTCAGGTCATCTG	<i>atxA</i> promoter SigA -9 SDM
JD98	CAGATGACCTGACAAATGCTAT <u>A</u> ATATTTCA AAACACATATTTTG	<i>atxA</i> promoter SigA -9 SDM
JD99	AAAATATGTGTTTTGAAATATA <u>I</u> TAGCATTTG TCAGGTCATCTGA	<i>atxA</i> promoter SigA -8 SDM
JD100	TCAGATGACCTGACAAATGCTA <u>A</u> TATATTTT AAAACACATATTTT	<i>atxA</i> promoter SigA -8 SDM
JD101	CCAATTTTTCCCTTAAAAAAATCAGTCCCAA AATATGTGTTTTGAAATA	<i>atxA</i> promoter SigA -35 SDM
JD102	TATTTCAAAACACATATTTTGGGACTGATTTT TTTAAGGGAAAAATTGG	<i>atxA</i> promoter SigA -35 SDM
JD103	CAATTTTTCCCTTAAAAAAATCAT <u>G</u> CCCCAAA TATGTGTTTTGAAATAT	<i>atxA</i> promoter SigA -34 SDM
JD104	ATATTTCAAAACACATATTTTGGG <u>C</u> ATGATTT TTTAAGGGAAAAATTG	<i>atxA</i> promoter SigA -34 SDM
JD105	TTCCCTTAAAAAAATCATTCCCGAAATATGT GTTTTGAAATATAA	<i>atxA</i> promoter SigA -30 SDM
JD106	TTATATTTCAAAACACATATTT <u>C</u> GGAATGAT TTTTTTAAGGGAA	<i>atxA</i> promoter SigA -30 SDM
JD195	CATTTGTCAGGTCATCTG <u>CGTCACGTTTT</u> AA AATACCGTTAAATG	<i>atxA</i> promoter SDM +14 to +22
JD196	CATTTAACGGTATTTTAA <u>ACGTGACG</u> CAGA TGACCTGACAAATG	<i>atxA</i> promoter SDM +14 to +22
TH49	GCGAAAGCTTATATTATCTTTTTGATTTCATG	<i>atxA</i> -up markerless mutation (+1527, HindIII)
TH134	GACAAAAATAAAATAGAATTGAATTCTTTTTA ATATAATC	<i>atxA</i> -up markerless mutation (-866, EcoRI)
JD139	TCGAAGTAATTGCCCTAGTGAAG	<i>PspoVG</i> probe
JD124	CTTGTTTCACCACTTTTC	<i>PspoVG</i> probe
JD230	AGTTTATTTTGTAATAGTGTATCAGG	<i>PcodY</i> probe
JD231	ACTTGGAAGCTAGGGCGAG	<i>PcodY</i> probe

<sup>a</sup>underline denotes restriction site

<sup>b</sup>numeric values relative to *atxA* P1 transcriptional start

**Table 2-3.** *atxA* promoter probes used in this study.

<b>Probe size (bp)</b>	<b>Sequence (relative to <i>atxA</i> P1 transcription start site)</b>	<b>Primers used</b>
171	-72 to +99	JD50, JD37
133	-72 to +61	JD50, JD107
103	-72 to +31	JD50, JD108
85	-72 to +14	JD50, JD109
97	-36 to +61	JD90, JD107
74	-13 to +61	JD111, JD107
60	+2 to +61	JD110, JD107
49	-13 to +36	JD228, JD229

**Table 2-4.** RT-qPCR primers and probes used in this study.

Name	Sequence (and nucleotide in relation to start codon (and strand))	Accession number	PCR efficiency (%)	Length of product (bases)
<i>gyrB</i> F	ACTTGAAGGACTAGAAGCAG (54(+))	NC_007530	99	68
<i>gyrB</i> R	GTCCTTTTCCACTTGTAGATC (121(-))	NC_007531	99	68
<i>gyrB</i> probe	FAM-CGAAAACGCCCTGGTATGTATA-BHQ1 (76(+))	NC_007532	99	68
<i>atxA</i> F	ATTTTATAGCCCTTGCAC (774(+))	NC_003980	93	71
<i>atxA</i> R	AAGTTAATGTTTATTGCTGTC (884(-))	NC_003981	93	71
<i>atxA</i> probe	FAM-CTTTTATCTCTTGGAAATTCTATTACCACA-BHQ1 (795(+))	NC_003982	93	71
<i>pXO2-61</i> F	GTGAATAAATTCAGCAATA (154(+))	AE17335	95	75
<i>pXO2-61</i> R	GAGATAGAAATAACATCCA (228(-))	AE17336	95	75
<i>pXO2-61</i> probe	FAM-TTTCATCAATACGCTCTCTTG-BHQ1 (179(+))	AE17337	95	75

### **Chapter III**

**An unidentified *trans*-acting repressor protein(s) directly regulates  
expression of *atxA***

### 3.1. Introduction

Regulation of gene expression at the level of transcription is often associated with *trans*-acting proteins and *cis*-acting promoter sequences that work in concert to affect the function of RNA polymerase (RNAP). In response to environmental cues, regulatory proteins can interact directly with RNAP to alter its activity or interact with specific sequences or structures in the promoter region to impact RNAP binding or processivity. Certain genes are subject to complex control in which multiple *trans*-acting factors and sequences in the promoter region function coordinately or independently to affect transcription (reviewed in (94)). The major virulence gene regulator of *Bacillus anthracis*, AtxA, positively affects transcription of the anthrax toxin and capsule biosynthetic genes (36, 40, 50, 67, 87, 101, 136, 165, 166). AtxA has motifs associated with DNA-binding, PTS-dependent phosphorylation, and multimerization (70, 85, 164, 166), but the precise molecular mechanism by which AtxA impacts transcription is not clear. Nevertheless, steady state levels of AtxA are critical for optimal transcription of the anthrax toxin and capsule genes (18, 35, 40, 68, 166).

Multiple signals have been shown to impact *atxA* transcript and protein levels. These signals include temperature, carbohydrate availability, redox potential, metabolic state, and growth phase. In agreement with the significance of AtxA in pathogenesis, *atxA* transcript levels are 5- to 6-fold greater in cultures grown at 37°C compared to cultures incubated at 30°C (35). In the presence of glucose, the catabolite control protein CcpA stimulates transcription of *atxA* indirectly by an unknown mechanism (32). The redox state of the cell also appears to control *atxA* transcription. Wilson *et al.* (175) showed early and increased expression of *atxA* when small c-type cytochromes were deleted. However, the enhanced *atxA* expression was only apparent when cells were cultured in medium not conducive for toxin production, so relevance for the small c-type cytochromes in virulence may be minimal. Another *trans*-acting factor, CodY, post-translationally controls AtxA

protein levels. A *codY*-null mutant produces less AtxA than parent due to an unknown mechanism (168).

Only one *trans*-acting factor has been reported to bind directly to the *atxA* promoter region. The transition state regulator AbrB represses *atxA* transcription by binding to a 43-bp region located 25 to 67 bp upstream of the P1 transcription start site (151). *atxA* expression is increased in an *abrB*-null mutant (68, 131). AbrB has been well studied in the archetype *Bacillus* species *B. subtilis* as a transcriptional regulator associated with cell development. The AbrB regulon includes several post-exponential phase genes associated with metabolic and physiological processes. Transition state regulators such as AbrB function to prevent the inappropriate expression of genes whose products have growth phase-specific functions (121, 150). AbrB is part of a feedback loop of regulators including the master response regulator Spo0A and the alternative sigma factor SigH. In *B. subtilis*, SigH positively affects *spo0A*, phosphorylated Spo0A represses *abrB*, and AbrB represses *sigH* (54, 66, 122, 147, 148). *B. anthracis* homologues of these proteins appear to perform similar roles, but in addition, they impact transcription of *atxA*. AbrB directly binds the *atxA* promoter region repressing transcription, and Spo0A positively controls *atxA* expression by repressing *abrB*. The *B. anthracis* *spo0A* promoter has a SigH recognition sequence comparable to that of the *B. subtilis* gene (68). SigH control of *atxA* occurs via its positive affect on *spo0A*, and in one strain, *sigH* positively regulates *atxA* expression in a *spo0A*- and *abrB*-independent manner (14, 68, 131, 151).

Transcription of *atxA* initiates from two start sites, P1 (36) located 99-nts upstream of the translational start site, and P2 (14) located an additional 650-nts upstream of P1. A modest decrease in *atxA* expression was observed upon deletion of P2 demonstrating P1 is the dominant transcription start site (14). There is a putative consensus sequence for the housekeeping sigma factor SigA, but not SigH, upstream of each transcription start site. However, direct transcription of *atxA* by SigA-RNAP has not been established. In addition

to control of transcription initiation, *atxA* mRNA stability is regulated. An extended 3' UTR contains a positive retroregulation stem-loop structure thought to protect *atxA* mRNA from exonucleolytic degradation (11, 176).

In this study, I sought to delineate *cis*-acting elements associated with regulation of *atxA* transcription and to test for the presence of additional *trans*-acting factors controlling transcription of *atxA*. Here I show that a region with similarity to the consensus sequence for recognition by SigA, and an UP element 5' of P1, the major transcription start site, are required for *atxA* transcription from P1. I also provide evidence for binding of a *trans*-acting repressor(s) other than AbrB to a palindromic sequence located downstream of the *atxA* P1 transcription start site. Elevated *atxA* transcription in a mutant altered for the repressor-binding site resulted in increased AtxA and anthrax toxin production. Nevertheless, virulence was unaffected by overexpression of AtxA.

## 3.2. Results

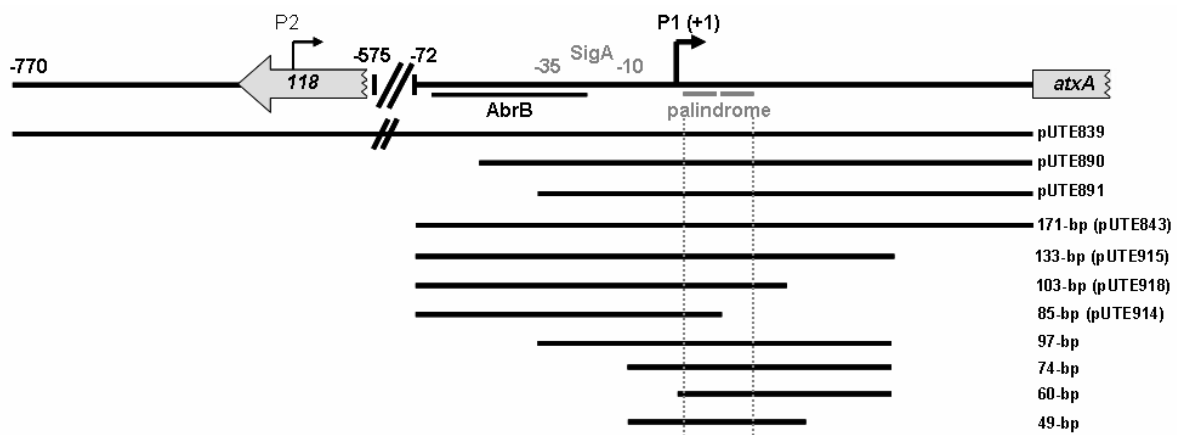
### 3.2.1. The SigA consensus sequence is required for transcription from P1.

Dai *et al.* (36) reported previously the presence of a putative consensus sequence for the housekeeping sigma factor SigA upstream of the *atxA* P1 transcription start site from sequences -36 to -8, relative to P1. This region of the *atxA* promoter differs from the established *B. subtilis* SigA consensus by two nucleotides within the putative -35 region (TTccCA). To determine if the putative SigA consensus is required for *atxA* transcription, I mutated single nucleotides within the -35 and -10 consensus regions and monitored *atxA* promoter activity using  $\beta$ -galactosidase assays. DNA sequences (171-bp) containing mutated and non-mutated *atxA* promoter regions (Fig. 3-1) were transcriptionally fused to a promoterless *lacZ* gene and introduced into *B. anthracis*. During culture in conditions conducive for toxin gene expression (CACO<sub>3</sub> + 5% CO<sub>2</sub>), the parent strain harboring the native *atxA* promoter-*lacZ* fusion exhibited a maximum  $\beta$ -galactosidase activity of 60 Miller Units (MU) at the transition to stationary phase (Fig. 3-2), consistent with previous reports of *atxA* expression (68, 131). Mutation of any single nucleotide within either the predicted SigA -35 or -10 region resulted in little to no  $\beta$ -galactosidase activity (Fig. 3-2). These results indicate that the putative SigA consensus located upstream of P1 is required for *atxA* promoter activity and suggest that SigA-RNAP transcribes *atxA* from the P1 initiation site.

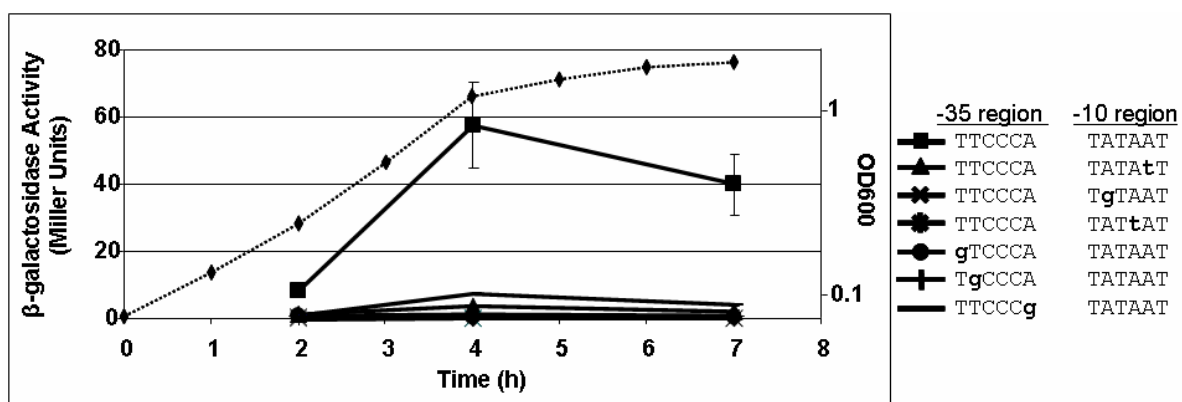
### 3.2.2. A *trans*-acting protein(s) other than AbrB binds specifically to the *atxA* promoter region.

The only *trans*-acting factor that has been shown to directly bind to DNA within the *atxA* promoter region is the transition state regulator AbrB (131, 151). To determine if *trans*-acting factors other than AbrB bind to *atxA* promoter DNA, I performed electrophoretic





**Figure 3-1. Schematic representation of the *atxA* promoter region.** The DNA fragments used for electrophoretic mobility shift assays (denoted by x-bp) and 5' or 3' deletion fragments cloned into a promoterless *lacZ* construct (denoted by a pUTE#) are depicted below the *atxA* promoter. The putative 9-bp palindrome sequence is located between positions +3 to +21 (grey bars).

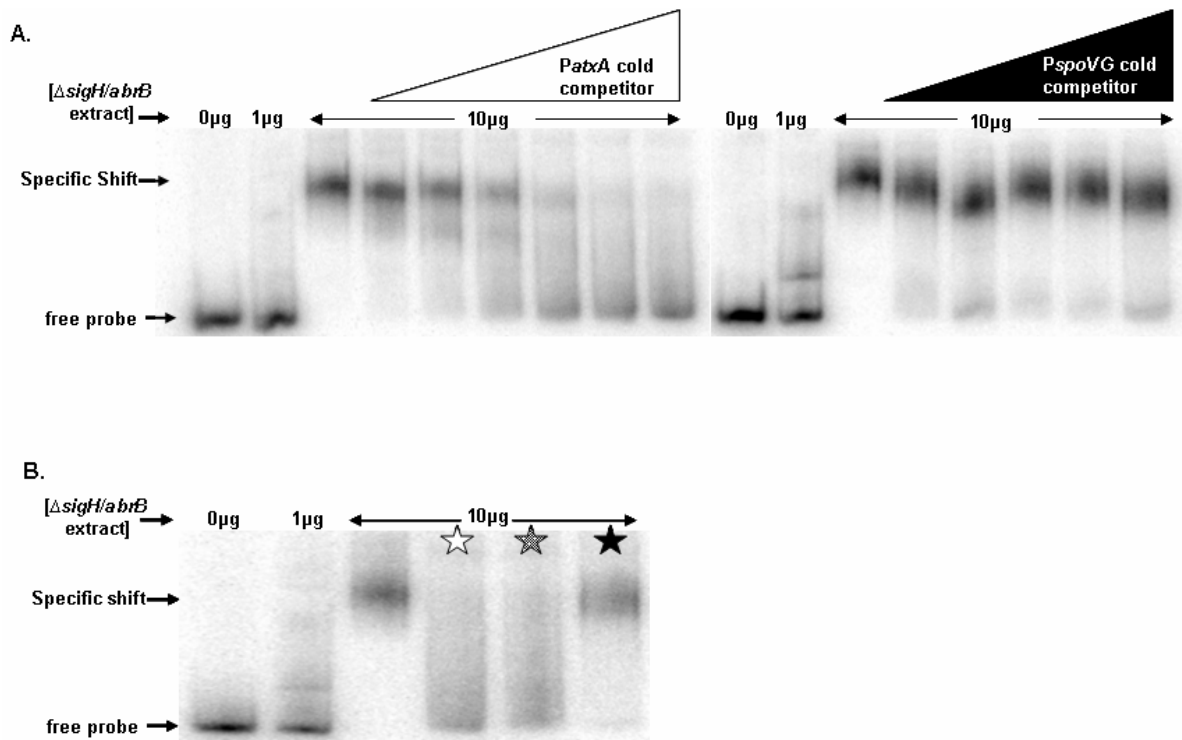


**Figure 3-2. Evidence for SigA-dependent *atxA* transcription.** *B. anthracis* mutants containing transcriptional *PatxA-lacZ* fusions were cultured in  $\text{CACO}_3 + 5\% \text{CO}_2$ .  $\beta$ -galactosidase activity was assessed at early exponential (2h), transition (4h), and stationary (7h) phases of growth. Specific mutations within the putative SigA -35 and -10 consensus sequences are denoted by lowercase, bold lettering. A representative growth curve is depicted by the hashed line with diamond symbols. These data were averaged from three independent cultures.

mobility shift assays (EMSAs) using *B. anthracis* crude cellular extracts from cultures grown in toxin-inducing conditions and a radiolabeled *atxA* promoter probe. Soluble cell extract was obtained from an *abrB/sigH*-null strain to eliminate transcription factors previously reported to influence *atxA* transcription (68, 131, 151). A 171-bp *atxA* promoter (*PatxA*) region containing the *AbrB* binding site, *SigA* consensus, and sequences from P1 to the translational start of *atxA* (+1 to +99) was used as the radiolabeled *atxA* probe (Fig. 3-1). Addition of 10 µg cellular extract resulted in a DNA-shift of the 171-bp *PatxA* probe. To show specificity of the shift, increasing concentrations of unlabeled *PatxA* were added to the binding reactions as cold competitor. The addition of unlabeled *PatxA* competitor resulted in a diminished DNA-shift and an increased abundance of free probe (Fig. 3-3A). The *spoVG* promoter (*PspoVG*) was used as a nonspecific unlabeled competitor. *spoVG* is controlled by both *SigH*-RNAP and *AbrB* (52, 126), two transcription factors also shown to control *atxA* transcription. Addition of increasing concentrations of unlabeled *PspoVG* competitor did not result in a diminished DNA-shift (Fig. 3-3A). These results indicate that a *trans*-acting factor(s) other than *AbrB* and *SigH* binds specifically to the *atxA* promoter region and not the *spoVG* promoter.

I considered that the factor responsible for the DNA-shift might be *SigA*-RNAP because the 171-bp *atxA* promoter probe contained the *SigA* consensus required for *atxA* transcription. To determine if *SigA*-RNAP was the *trans*-acting factor(s) that led to a specific DNA mobility shift, I used an unlabeled competitor DNA that contained a mutation within the *SigA* -35 consensus (gTCCCA). This mutation abolished *atxA* promoter activity in β-galactosidase assays (Fig. 3-2). Similar to competition with the unlabeled non-mutated *PatxA* DNA, excess *PatxA* DNA harboring the mutated *SigA* -35 consensus sequence resulted in a diminished DNA-shift (Fig. 3-3B). These results suggest that the observed DNA mobility shift is not attributed to *SigA*-RNAP binding the *atxA* promoter region.

Treatment of *B. anthracis* cellular extract with proteinase K resulted in protein

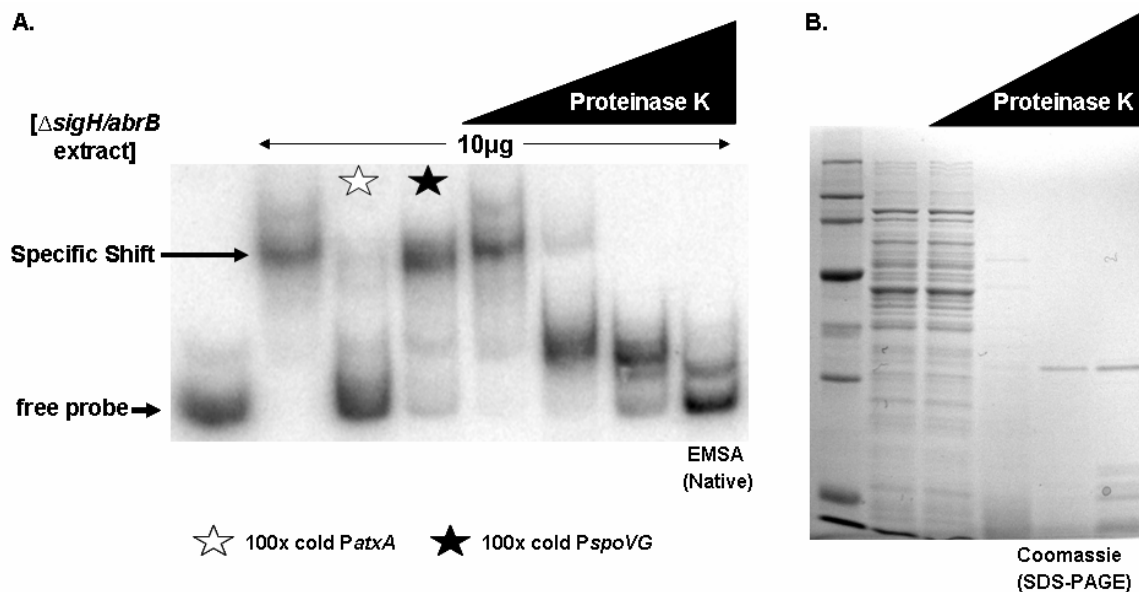


**Figure 3-3. A *trans*-acting factor(s) binds specifically to the *atxA* promoter region. B.** *anthracis* *sigH/abrB*-null soluble cellular extract mixed with radiolabeled 171-bp *PatxA*. Soluble cellular extract was obtained from cells cultured in  $\text{CACO}_3 + 5\% \text{CO}_2$ . (A) EMSAs using increasing concentrations of *PatxA* (2.5- to 80-fold excess) and *PspoVG* (10- to 50-fold excess) unlabeled competitors. (B) EMSAs using 50-fold more specific and non-specific unlabeled competitors than radiolabeled *PatxA*. Unlabeled competitors include 171-bp *PatxA* (white star), 171-bp *PatxA* with mutated SigA -35 sequence (gTCCCA; dotted star), and non-specific 187-bp *PspoVG* (black star). These data are representative of three separate experiments.

degradation and a diminished DNA-shift (Fig. 3-4), indicating that the *trans*-acting factor(s) is a protein(s). In addition, cellular extract obtained from a plasmid-cured *B. anthracis* strain added to the *PatxA* probe resulted in a DNA-shift indicating the *trans*-acting protein(s) is encoded by the chromosome (data not shown). Taken together, these results indicate that a *trans*-acting protein(s) other than AbrB, SigH, or SigA-RNAP specifically binds the *atxA* promoter region.

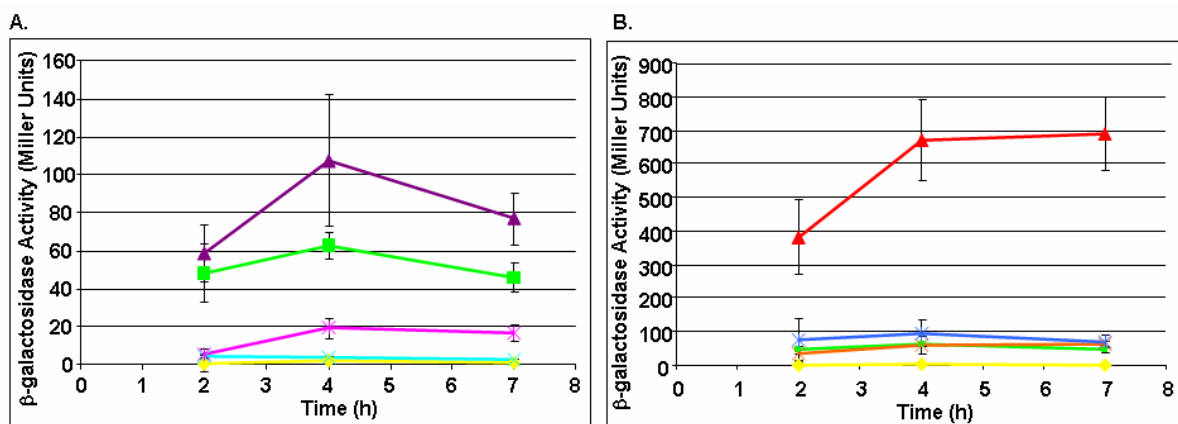
### **3.2.3. Additional *cis*-acting regulatory elements located within the *atxA* promoter region.**

In order to identify a putative binding site for the *PatxA* specific *trans*-acting protein(s) within the *atxA* promoter region and reveal any additional *cis*-acting elements controlling *atxA*, I performed 5' and 3' deletion analysis of the *atxA* promoter region. Truncated *atxA* promoter regions transcriptionally fused to the promoterless *lacZ* gene were introduced into *B. anthracis* and  $\beta$ -galactosidase assays were used to monitor *atxA* promoter activity (Fig. 3-1 and Fig. 3-5). Comparison of strains carrying pUTE839 and pUTE843 showed that deletion of P2 resulted in a 1.7-fold decrease in *atxA* promoter activity (Fig. 3-5A). Similar results showing a modest change in *atxA* promoter activity upon P2 deletion were reported by Bongiornoi *et al.* (14). Activity of a clone harboring only the P1 start site (pUTE843) decreased 3.2-fold when sequences from -72 to -54 relative to the P1 transcription start site (+1) were deleted (pUTE890). An additional 16.9-fold decrease in *atxA* promoter activity was observed when sequences from -72 to -36 were deleted (pUTE891). These results indicate the presence of a positive *cis*-acting element located within the sequences from -72 to -35. This region is A+T-rich and sequences from -65 to -44 resemble an UP-element recognized by the  $\alpha$ -subunits of RNAP (62). The data suggest that SigA-RNAP requires all or some of the sequences from -72 to -35 for maximal *atxA* transcription.



**Figure 3-4. A *trans*-acting protein(s) binds specifically to the *atxA* promoter region.**

Proteinase K treated *B. anthracis* soluble cellular extract incubated with radiolabeled 171-bp PatxA. Soluble cellular extract was obtained from cells cultured in CACO<sub>3</sub> + 5% CO<sub>2</sub>. Cellular extract was treated with 0, 10, 100, and 1000 μg/ml of proteinase K for 30 min at room temperature. (A) EMSAs using soluble cellular extract that was incubated with increasing concentrations of proteinase K. Unlabeled competitors include 171-bp PatxA (white star) and non-specific 187-bp PspoVG (black star). (B) Coomassie stained SDS-PAGE of proteinase K treated soluble cellular extract. These data are representative of three separate experiments.



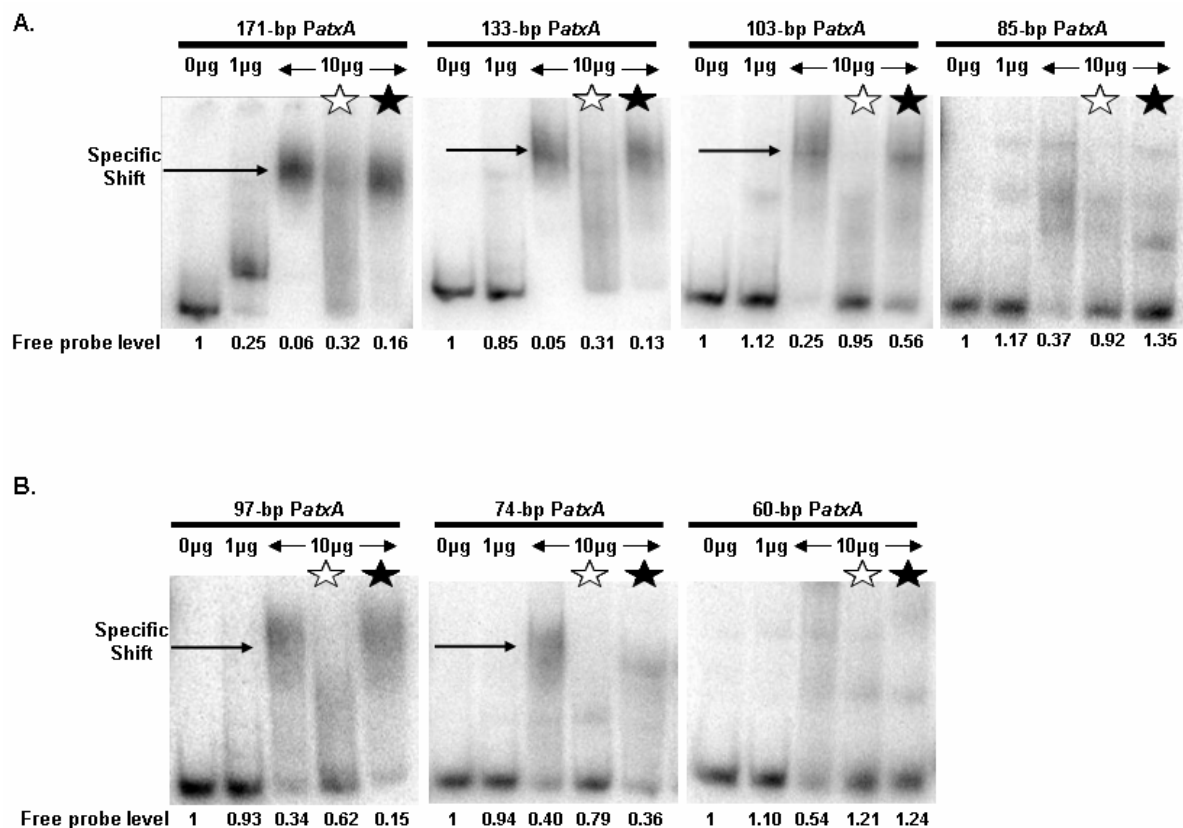
**Figure 3-5. 5' and 3' *atxA* promoter deletion analysis.** (A) 5' *PatxA* deletion constructs and (B) 3' *PatxA* deletion constructs transcriptionally fused to a promoterless *lacZ* gene, and corresponding  $\beta$ -galactosidase assays. *B. anthracis* ANR-1 harboring the *PatxA-lacZ* constructs were cultured in toxin-inducing conditions and samples were obtained during early exponential (2h), transition (4h) and stationary (7h) phases of growth. Symbols: pUTE839 / 904-bp *PatxA* (triangles – purple), pUTE843 / 171-bp *PatxA* (squares – green), pUTE890/153-bp *PatxA* (star - pink), pUTE891 / 135-bp *PatxA* (X – teal), pUTE915 / 133-bp *PatxA* (X – orange), pUTE918 / 103-bp *PatxA* (star – blue) pUTE914 / 85-bp *PatxA* (triangle – red), empty vector (diamond – yellow). These data were averaged from three independent cultures.

The 3' deletion analysis showed a 14.9-fold increase in *atxA* promoter activity when sequences from +14 to +31 (pUTE914), relative to the P1 transcription start site (+1), were deleted (Fig. 3-5B). Deletion of sequences from +31 to +99 (pUTE915 and pUTE918) resulted in no measurable difference in *atxA* promoter activity compared to the full-length *atxA* promoter control (pUTE843). These results suggest that a repressor protein(s) binds to the *atxA* promoter region and that sequences downstream of the P1 initiation site (+1) are required for binding. Together, the 5' and 3' *atxA* promoter deletion analyses indicate that sequences upstream and downstream of the P1 transcription initiation site (+1) are important for regulation of *atxA* promoter activity.

#### **3.2.4. A putative repressor protein(s) binding site is located near the P1 transcription start site.**

To define the *cis*-acting region required for repressor protein(s) binding, I performed EMSAs using truncated *atxA* promoter regions as radiolabeled probes and cellular extract from *B. anthracis* ANR-1. Similar DNA-shifts were observed using cellular extracts prepared from an *abrB/sigH*-null strain (data not shown). As seen previously, a DNA-shift was observed when cellular extract was added to the 171-bp *PatxA* probe containing the AbrB binding site, SigA consensus, and sequences from P1 to the translational start of *atxA* (+1 to +99). The DNA-shift was specific for the *atxA* and not *spoVG* promoter since excess unlabeled *PatxA* but not unlabeled *PspoVG* resulted in a diminished DNA-shift (Fig. 3-6A). Specific DNA-shifts were also observed when the *atxA* promoter probe was truncated from the 3' end to produce 133- and 103-bp probes indicating that *atxA* promoter sequences from +32 to +99 are not required for repressor protein binding (Fig. 3-6A). These results are in agreement with the comparable  $\beta$ -galactosidase activity of transcriptional fusions carrying the 171-, 133-, and 103-bp *atxA* promoter regions (Fig. 3-5B). 3' deletion of an additional 18-bp containing sequences from +14 to +31 (85-bp *PatxA* probe) resulted in a





**Figure 3-6. Defining the *cis*-acting region required for *trans*-acting protein(s) binding.**

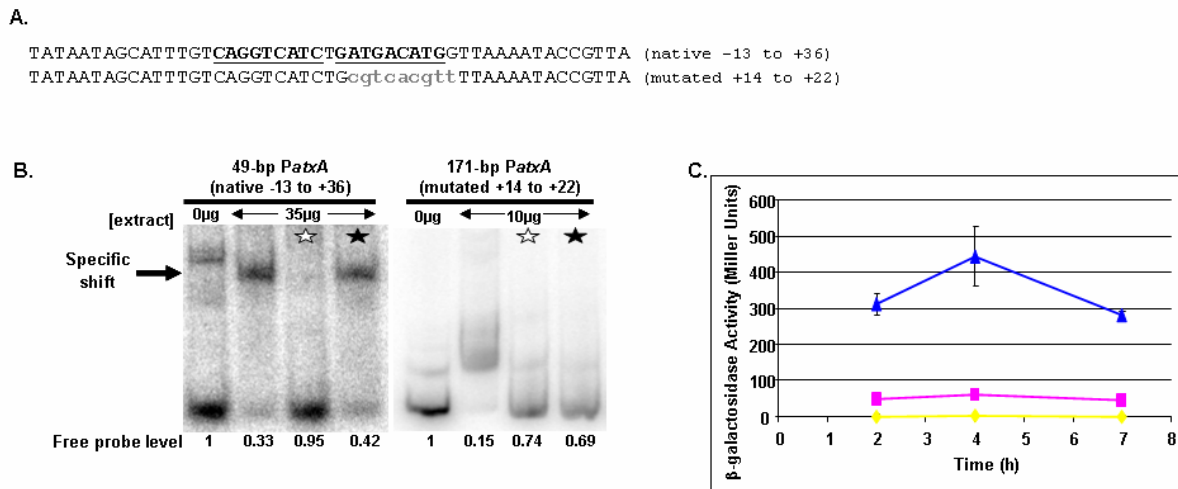
*B. anthracis* cellular extract obtained from cells cultured in CACO<sub>3</sub> + 5% CO<sub>2</sub> was incubated with radiolabeled (A) 3' and (B) 5' *Patx*A deletion constructs. Refer to Figure 1 for schematics of the 3' and 5' deletion constructs. Quantitative values of free probe are depicted below each lane. Symbols: 100 ng specific *Patx*A unlabeled competitor (white star), 100 ng non-specific *PspoVG* unlabeled competitor (black star). These data are representative of three separate experiments.

non-specific DNA-shift (Fig. 3-6A). These results and the  $\beta$ -galactosidase activity results which showed a 14.9-fold increase in *atxA* promoter activity using the 85-bp *atxA* promoter-*lacZ* fusion (Fig. 3-5B) suggest that sequences downstream of the P1 transcription start site are required for repressor protein(s) binding to the *atxA* promoter.

I further defined the repressor protein(s) binding site by testing 5' deletions of the 133-bp *PatxA* probe in EMSA experiments. Specific DNA-shifts were observed using 5' *PatxA* deletion probes of 97- and 74-bp (Fig. 3-6B). These probes are partially or fully deleted for the AbrB binding site confirming that AbrB is not the protein causing the observed DNA-shifts (Fig. 3-1). When the entire region upstream of P1 was deleted (60-bp *PatxA* probe), no specific DNA-shift was observed (Fig. 3-6B). Take together, the deletion analyses suggest that the repressor protein(s) binding site is located between sequences from -13 to +31, relative to the P1 transcription start site (+1).

### **3.2.5. A palindromic sequence required for repressor binding.**

*In silico* analysis of the *atxA* promoter region using a program established by Technion Israel Institute of Technology (<http://bioinfo.cs.technion.ac.il/>) revealed an imperfect 9-bp palindrome separated by 1-bp (CAxGTCATC) within the sequences from -13 to +31 (Fig. 3-7A). Since palindromic sequences in DNA often represent regulatory protein binding regions, I questioned whether this region of DNA was necessary and sufficient for repressor protein(s) binding. A radiolabeled 49-bp *PatxA* probe containing the 9-bp palindrome with an additional 15-bp on either side (native -13 to +36 [Fig. 3-7A]) was constructed to determine if this region of the *atxA* promoter was sufficient for binding of the *atxA* repressor protein(s). EMSA experiments revealed that addition of soluble cellular extract to the 49-bp *PatxA* probe resulted in a specific DNA-shift. Addition of excess unlabeled *PatxA* DNA, but not unlabeled *PspoVG* DNA, resulted in a diminished DNA-shift (Fig. 3-7B). These results indicate that only sequences from -13 to +36 are required for



**Figure 3-7. The *atxA* promoter region contains a 9-bp palindromic sequence required for repressor binding.** (A) *atxA* promoter sequences from -13 to +36 relative to the P1 transcription start site. The palindromic sequence is denoted by bold, underlined letters. Nucleotides mutated using site-directed mutagenesis are denoted by lowercase, grey lettering. (B) EMSAs using cellular extract obtained from *B. anthracis* cultured in  $\text{CaCO}_3$  + 5%  $\text{CO}_2$  incubated with radiolabeled PatxA probes of sizes 49-bp or 171-bp. Quantitative values of free probe are depicted below each lane. Symbols: PatxA unlabeled competitor (white star), PspovG unlabeled competitor (black star). These data are representative of three separate experiments. (C)  $\beta$ -galactosidase activity assays comparing *atxA* promoter activity in a parent strain (pink squares; pUTE843) versus a strain in which the native *atxA* promoter sequences from +14 to +22 were mutated (blue triangles; pUTE971). Empty vector control (pHT304-18z) is denoted by yellow diamonds. These data were averaged from three independent cultures.

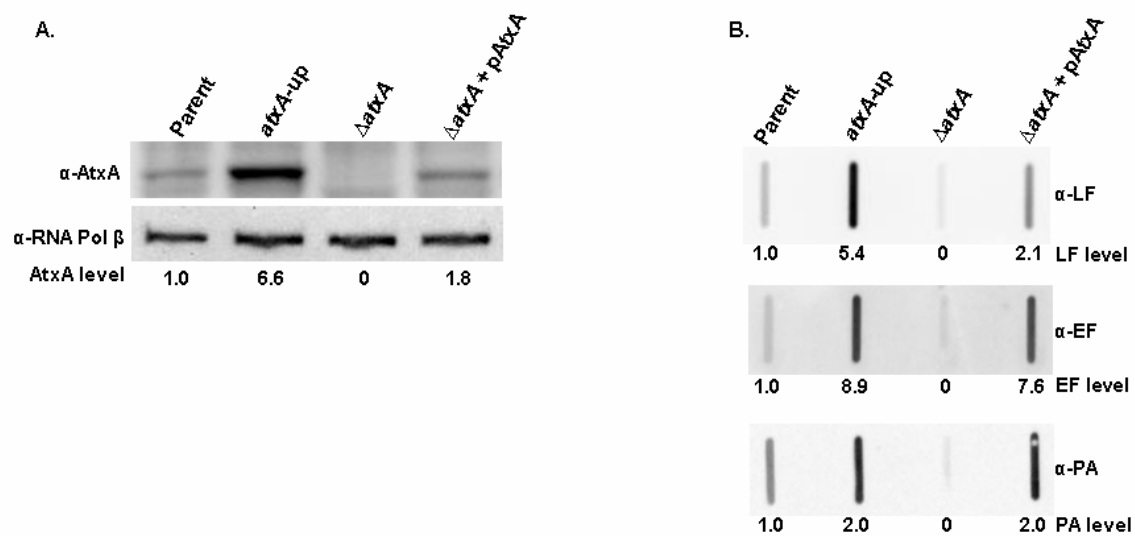
repressor protein(s) binding to the *atxA* promoter region.

To confirm the specific requirement of the palindromic sequence for repressor binding, I tested a mutant containing nucleotide mutations within the palindrome in EMSAs. Transversion mutations were created from sequences +14 to +22 that contain 8- of the 9-bp within the downstream portion of the palindrome (Fig. 3-7A). A specific DNA-shift was no longer apparent when the +14 to +22 mutated *atxA* promoter probe was used in EMSAs with soluble cellular extract (Fig. 3-7B) further confirming the importance of the palindrome for binding of the repressor protein(s). I also observed a 7-fold increase in *atxA* promoter activity when the +14 to +22 mutated *atxA* promoter was transcriptionally fused to a promoterless *lacZ* gene and introduced into *B. anthracis* (Fig. 3-7C). Together, these data defined a 49-bp region of the *atxA* promoter located near the P1 transcription start that contains a 9-bp palindrome required for binding of the *atxA* repressor protein(s).

### **3.2.6. Anthrax toxin expression is increased when the putative *atxA* repressor binding site is mutated.**

Saile *et al.* (131) reported previously that deletion of *abrB* resulted in early and increased toxin gene expression. The increased toxin gene transcription is associated with elevated *atxA* promoter activity (68, 131). Since *atxA* promoter activity increased 7-fold upon mutation of the palindrome (Fig. 3-7C), I mutated the native *atxA* promoter sequences from +14 to +22 (renamed *atxA*-up) and tested for a comparable increase in AtxA protein levels. The steady state level of AtxA protein produced by the *atxA*-up mutant was 6.6-fold higher than that of the parent. AtxA was not produced in an *atxA*-null strain and complementation of *atxA* resulted in 1.8-fold more AtxA than parent (Fig. 3-8A). Western blot analysis of culture supernates revealed increased levels of the anthrax toxin proteins. The *atxA*-up mutant produced 5.4-, 8.9-, and 2-fold more LF, EF, and PA, respectively, than the parent strain (Fig. 3-8B). An *atxA*-null mutant showed little to no anthrax toxin

production compared to parent, and complementation of the *atxA*-null strain showed restoration of the anthrax toxin proteins to levels above that of parent (Fig. 3-8B). In total, these results showed increased expression of AtxA, LF, EF, and PA when a portion of the putative *atxA* repressor binding site was mutated suggesting derepression of *atxA* transcription.



**Figure 3-8. Increased AtxA expression results in elevated anthrax toxin (LF, EF, and PA) production.** Production of (A) AtxA and (B) LF, EF, and PA by parent and mutant *B. anthracis* strains. Culture samples were obtained during transition phase (4h) of growth. (A) Samples were subjected to SDS-PAGE and Western blot analysis using rabbit  $\alpha$ -AtxA antibody raised against *B. anthracis* AtxA or mouse  $\alpha$ -RNA Pol  $\beta$  antibody raised against *E. coli* RNA Pol  $\beta$ . (B) Samples were subjected to Slot blot Western analysis using rabbit  $\alpha$ -LF, rabbit  $\alpha$ -EF, and goat  $\alpha$ -PA antibodies raised against *B. anthracis* proteins. These data are representative of three separate experiments.

### 3.3. Discussion

An *atxA*-null strain produces little to no anthrax toxin and is avirulent in a murine model for anthrax disease demonstrating the necessity of *atxA* for *B. anthracis* virulence (36). Transcription factors that impact *atxA* expression both at the transcriptional and post-translational level have been shown to affect *B. anthracis* virulence. The carbon catabolite protein CcpA, which senses and responds to carbon availability in Gram-positive bacteria, positively affects *atxA* transcription indirectly by an unknown mechanism. Deletion of the *ccpA* gene results in decreased *atxA* transcription and attenuated virulence in a subcutaneous murine model of anthrax disease (32, 170). The pleiotropic transcription regulator CodY, well characterized in *B. subtilis* as a regulator that responds to cell energy and nutrient status (124, 141), modulates AtxA stability in batch culture. A *codY*-null mutant exhibits decreased AtxA protein levels and is avirulent in a subcutaneous model of anthrax disease (168). The mechanism for CodY regulation of AtxA is unknown; however, it has been proposed that deletion of *codY* results in the production of a protease, chaperone, or adaptor protein that directly influences AtxA stability.

In this chapter, I expanded on previous investigations of transcriptional control of *atxA* by examining *cis*-acting elements and *trans*-acting factors that directly influence *atxA* expression. Transcription initiation relies on promoter recognition by RNA polymerase (RNAP). The sigma subunit of RNAP confers promoter specificity and directs RNAP to its target gene(s) while the  $\alpha$ -subunits of RNAP contribute to promoter recognition by interacting with a specific A+T-rich sequence, referred to as the UP-element, located upstream of the -35 sigma factor consensus. The binding of RNAP  $\alpha$ -subunits to the UP-element stabilizes and strengthens RNAP binding (12, 62, 129). Here, I showed that *atxA* transcription requires sequences resembling the SigA consensus and an UP-element for RNAP.

There are conflicting reports regarding regulation of *atxA* by the alternative sigma factor SigH. When an *atxA* promoter–*lacZ* reporter construct was cloned into the heterologous host *B. subtilis*, *atxA* promoter activity was controlled by SigH in an AbrB-dependent manner (151). Bongiorni *et al.* (14) examined *atxA* promoter activity in the *B. anthracis* Sterne-like strain 34F2 and showed that *atxA* transcription did not require SigH. Hadjifrangiskou *et al.* (68) showed previously that SigH positively controls *atxA* transcription in an AbrB-independent manner in the Sterne-like strain UM44. In addition, Hadjifrangiskou *et al.* (68) reported that SigH-RNAP transcribes an *atxA* promoter template *in vitro*. However, based on subsequent work and data presented here, I believe the *in vitro* transcript generated using SigH-RNAP resulted from end-to-end transcription of the DNA template, followed by degradation to a shorter RNA transcript. Taken together, these data indicate that SigH controls *atxA* by an unknown mechanism that varies among strains.

In this work, I have demonstrated that in addition to AbrB, another *trans*-acting protein(s) specifically binds the *atxA* promoter and represses transcription. My data indicate that the *atxA* *trans*-acting repressor protein(s) specifically binds to a 9-bp palindrome located immediately downstream of P1 from sequences +3 to +21 (CAGGTCATC – 1-nt spacer – GATGACaTG). Using the *in silico* database PRODORIC (108), I searched for known transcription factors that could potentially interact with the 9-bp palindrome. The search did not reveal any candidate transcription factors so I broadened the search parameters. Instead of using the 9-bp palindrome sequence alone, the entire 49-bp *atxA* promoter region required for binding of the *trans*-acting repressor protein(s) was used in an *in silico* search. The search revealed potential interaction of the *Pseudomonas aeruginosa* -10 sigma factor AlgU (99) and the *Escherichia coli* aerobic respiration control protein ArcA (80).

The *P. aeruginosa* AlgU protein is a sigma factor with predicted sequence similarity to the alternative sigma factor SigH of *Bacillus* species (99). A search for AlgU homologues

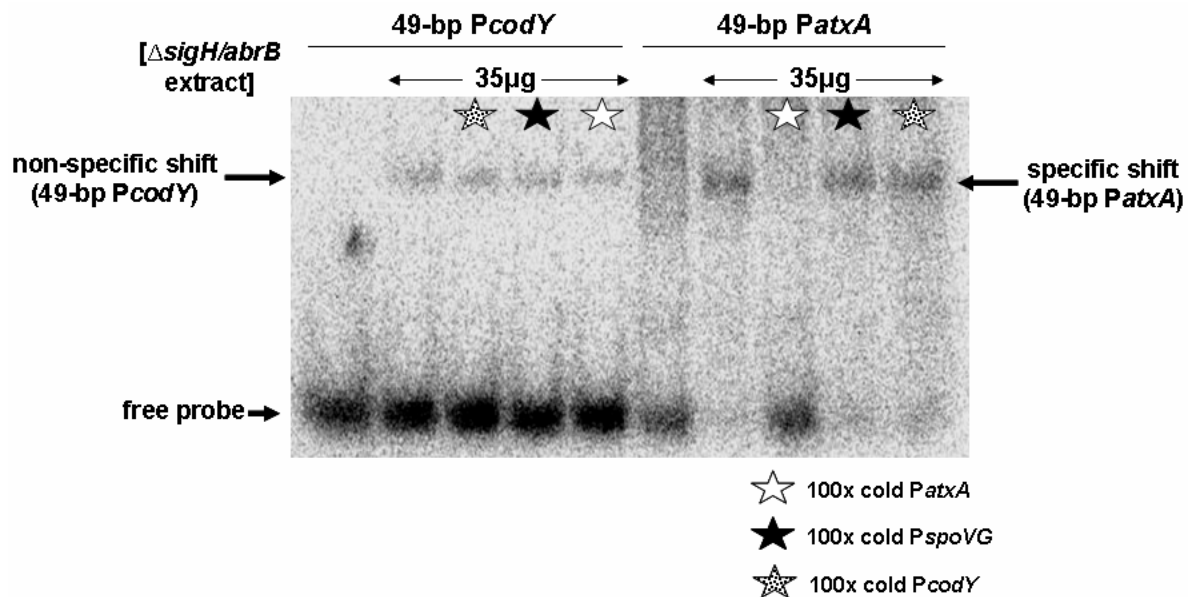


in *B. anthracis* revealed several sigma factors. Since I have already determined that the putative SigA consensus was required for *atxA* promoter activity, and the potential binding site for AlgU is downstream of this region, I ruled out AlgU homologues as the potential *atxA* *trans*-acting repressor binding protein(s). The *E. coli* ArcA protein is a DNA-binding response regulator that is part of the ArcAB two-component system. ArcA is activated by ArcB when oxygen availability is low, and represses a variety of aerobic enzymes (65). A search for *B. anthracis* protein homologues of *E. coli* ArcA revealed more than 30 proteins annotated as DNA-binding response regulators that contained anywhere from approximately 50-60% similarity. Without any additional information, I am unable to prioritize these genes as potential candidates for regulators of *atxA*. Therefore, the *in silico* analysis revealed that no known *trans*-acting factors in the database were likely candidates for specific interaction with the *atxA* promoter region.

To determine if other *B. anthracis* promoter sequences contained the 9-bp palindrome and were therefore regulated in a manner similar to *atxA*, I employed an *in silico* search using the PRODORIC database (108). The *in silico* analysis revealed 14 potential regions throughout the *B. anthracis* chromosome that contain an imperfect version of the 9-bp palindrome. Other than the *atxA* promoter on pXO1, regions containing the 9-bp palindrome were not identified on plasmid sequences. A majority of the genes in which the imperfect 9-bp palindrome was found upstream of the start codon do not have gene annotations. One of the few annotated genes, *codY*, was of particular interest considering that CodY post-transcriptionally modulates AtxA protein levels (168). I performed EMSAs using the *codY* promoter region and cellular extract from *B. anthracis*; however, no specific DNA-shift was observed (Fig. 3-9). These results suggest that the *atxA*-associated palindrome is a unique binding site for the repressor protein(s), but do not rule out the possibility of interactions with the other 13 palindromic sequences identified in the *in silico* search.

Mechanisms by which *trans*-acting factors control transcription are in part associated with the location of the binding site in the promoter region. Typically, the location of a *trans*-acting binding site determines whether the given transcription factor will activate or repress gene expression. The master response regulator Spo0A can activate or repress transcription of its target genes depending on the location of its cognate '0A' box (107, 144, 147, 152). Typically, Spo0A stimulates transcription when '0A' boxes are located upstream of the transcription start site and represses target genes when '0A' boxes are located downstream of the start site. Active Spo0A binds to sequences upstream of the *spoII*G operon transcriptional start site enabling RNAP to efficiently contact the -10 consensus sequence and initiate steps subsequent to closed-complex formation (130). In contrast, Spo0A represses *abrB* transcription by binding downstream of the *abrB* P1 and P2 transcription start sites likely preventing the progression of RNAP from P1 and blocking binding of RNAP to P2 (119, 147). Another example of a transcription factor that uses the location of its cognate binding site to activate or repress its target genes is the carbon catabolite protein CcpA. CcpA, with its accessory protein HPr, activates transcription of the group A streptococcus master virulence regulator Mga by binding to a *cre*-element located upstream of the P1 transcription start site (5). In contrast, CcpA-HPr represses the alternative carbon metabolism genes *acsA*, *araB*, and *amyE* by binding to *cre*-elements located downstream of the each transcription start site (55, 170, 181).

Here I showed that the *atxA* repressor protein(s) requires sequences within a 9-bp palindrome located immediately downstream of the *atxA* P1 transcription start site. Mutation of the 9-bp palindrome in the native *atxA* promoter region resulted in derepression of *atxA* and elevated AtxA and anthrax toxin production. My working model is that the *atxA* repressor protein(s) likely prevents the progression of RNAP from P1 and/or interferes with RNAP binding to the *atxA* promoter region.



**Figure 3-9. The *trans*-acting repressor protein(s) does not bind the *codY* promoter palindrome.** *B. anthracis sigH/abrB*-null soluble cellular extract mixed with radiolabeled 49-bp *PcodY* and 49-bp *PatxA* probes. Soluble cellular extract was obtained from cells cultured in  $\text{CACO}_3 + 5\% \text{CO}_2$ . EMSAs using radiolabeled *PcodY* resulted in a non-specific mobility shift (left side of gel) whereas radiolabeled *PatxA* resulted in a specific mobility shift (right side of gel). Unlabeled competitors include 49-bp *PatxA* (white star), 187-bp *Pspo0VG* (black stars), and 49-bp *PcodY* (dotted star). These data are representative of two separate experiments using either  $\Delta\text{sigH/abrB}$  (UT291) or parent (ANR-1) cellular extract.

## **Chapter IV**

### **AtxA modulates *B. anthracis* development**

NOTE: Maureen Ty helped perform experiments related to sample preparation for AtxA and LF Western blot analysis. Gregory L. Shipley, Ph.D., and the Quantitative Genomics Core Laboratory at The University of Texas Health Science Center Houston performed the RT-qPCR assays and data normalization.

#### 4.1. Introduction

Sporulation is a developmental process that members of the *Bacillus* genus undergo in response to unfavorable, nutrient deplete growth conditions. The spore is metabolically inactive, resistant to environmental stresses, and can survive until conditions are favorable for germination into a vegetative cell. The process of sporulation is energy exhaustive; therefore, is considered a last resort of survival for the bacterium. In the archetype *Bacillus* species, *B. subtilis*, the developmental process of sporulation has been well-studied. Nutrient deprivation is sensed by a multi-component signal transduction phosphorelay resulting ultimately in phosphorylation of the master response regulator Spo0A, and a commitment to sporulation (reviewed in (121)). *B. anthracis*, the etiological agent of anthrax disease, possesses orthologues of the signal transduction phosphorelay that enable the bacterium to sporulate in a manner similar to that of *B. subtilis* (15, 16, 21, 144).

The *B. anthracis* spore constitutes the infectious form of the bacterium. *B. anthracis* infection results in one of three forms of anthrax disease, cutaneous, gastrointestinal, or inhalation, depending on the route of infection. The most well-studied form of anthrax disease is inhalation anthrax. Upon entry into the lungs, *B. anthracis* spores are phagocytosed by resident alveolar macrophages and dendritic cells, which serve as vehicles for transit to the regional lymph nodes (8, 34, 39, 106, 128). Spores that survive the initial immune response are capable of germinating into vegetative bacilli and disseminating throughout the body. The bacilli do not initiate sporulation inside the host.

The master virulence regulator of *B. anthracis*, AtxA, is required for optimal expression of the anthrax toxins and capsule which promote *in vivo* survival of the bacterium. An *atxA*-null *B. anthracis* strain is avirulent in a murine model of anthrax disease (36). The structural genes for anthrax toxin, *pagA*, *lef*, and *cya*, located on pXO1, and the capsule biosynthetic operon, *capBCADE*, located on pXO2, are positively controlled by AtxA (26, 36, 40, 50, 67, 84, 87, 97, 101, 112, 113, 136, 165, 166).

Transcription of the anthrax toxin and capsule genes is enhanced by host related cues such as elevated CO<sub>2</sub>. In the absence of elevated CO<sub>2</sub> and/or *atxA*, little to no anthrax toxin or capsule is produced by *B. anthracis* (9, 18, 28, 33, 36, 50, 70, 75, 77, 87, 101, 137, 165). *B. anthracis* evades the host immune response primarily by the production of anthrax toxin and a poly-γ-D-glutamic acid capsule.

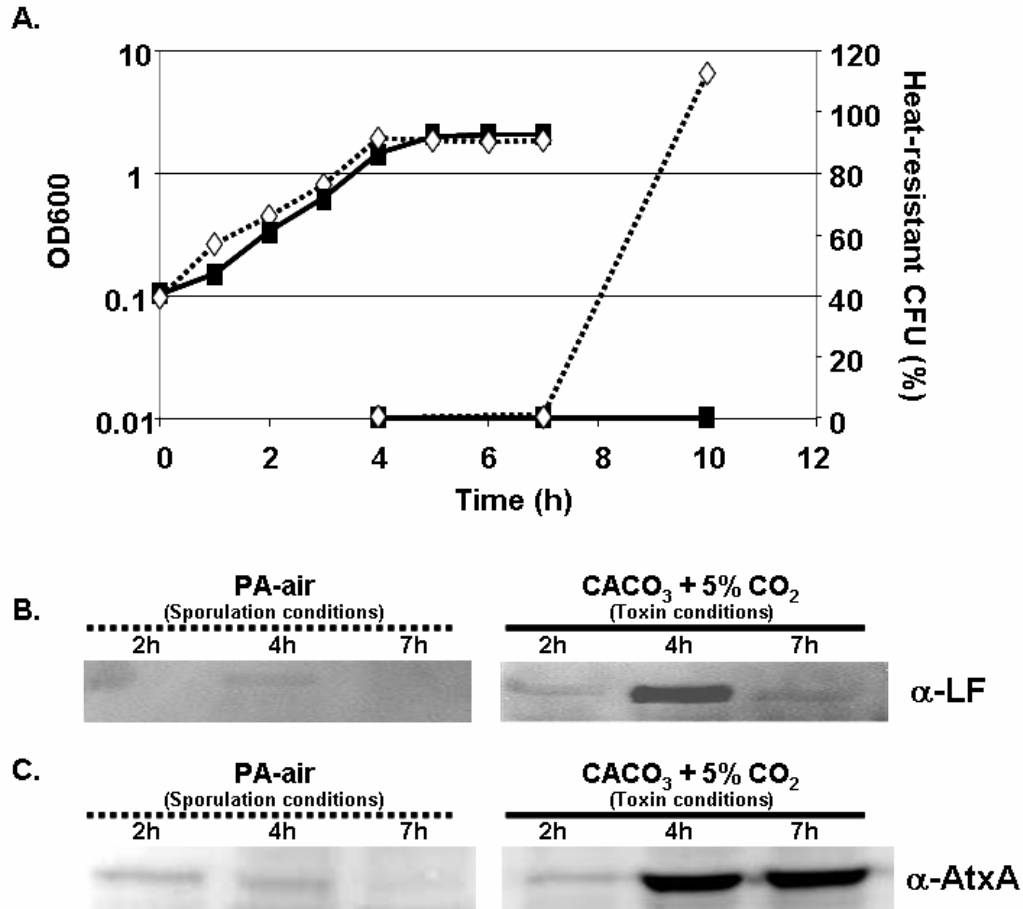
In this chapter, I show that anthrax toxin production and *B. anthracis* development are inversely related. I also demonstrate that there is a condition-dependent expression of AtxA that inversely corresponds with the production of anthrax toxin. To assess phenotypes attributed to altered expression of AtxA in different culture conditions, I mutated a regulatory region within the *atxA* promoter of the genetically complete Ames strain. Upon mutation of the *atxA* promoter region, I found that AtxA expression and sporulation are not significantly affected in toxin-inducing conditions. Conversely, AtxA is overexpressed in sporulation conditions and a marked decrease in spore formation is observed. The AtxA-associated sporulation defect is dependent upon the pXO2 gene *pXO2-61*, predicted to encode a protein homologous to the sensor domain of sporulation sensor histidine kinases.

## 4.2. Results

### 4.2.1. *B. anthracis* anthrax toxin production and sporulation are inversely related

*B. anthracis*, like all other *Bacillus* species, develops into environmentally resistant spores in response to nutrient deprivation. Limitation of nutrients can be modeled in batch culture by incubating cells in media for extended periods of time without nutrient supplementation. I sought to examine sporulation of *B. anthracis* using different culture conditions: a rich medium incubated in air (PA-air), or a semi-defined minimal medium containing dissolved bicarbonate and incubated in 5% CO<sub>2</sub> (CACO<sub>3</sub>). The latter growth condition is thought to model physiologically relevant conditions encountered by the bacterium during infection whereas the former is believed to model environmental conditions outside the host. *B. anthracis* growth rates were similar when cultured in PA-air or CACO<sub>3</sub> (Fig. 4-1A). When the cells were cultured in PA-air, there was an increase in the percentage of heat-resistant CFU over time, indicative of sporulation. In contrast, there were little to no heat-resistant CFU obtained for cells cultured in CACO<sub>3</sub> indicating that this growth condition is not conducive for sporulation (Fig. 4-1A).

In addition to assessing sporulation, I performed Westerns to examine both AtxA and Lethal Factor (LF) protein levels when cultured in the two growth conditions. LF expression peaked during the transition into stationary phase and protein levels decreased within stationary phase when cultured in CACO<sub>3</sub> (Fig. 4-1B). Transition phase occurs within four hours after inoculation of cells from an overnight culture using our culture methods. Pflughoeft *et al.* (120) showed previously that degradation of LF during stationary phase is due to the presence of *B. anthracis* extracellular proteases that target the anthrax toxin proteins. Conversely, LF synthesis was not observed during any growth phase when *B. anthracis* was cultured in PA-air (Fig. 4-1B). In concordance with the absence of LF production in PA-air, AtxA levels were minimal and decreased over time when *B. anthracis*



**Figure 4-1. Toxin production and sporulation are inversely related.** (A) Growth curve and heat-resistance CFU determination of Ames cultured in toxin-inducing ( $\text{CACO}_3 + 5\% \text{CO}_2$ ; solid line/squares) and sporulation (PA-air; hashed line/diamonds) conditions. (B) Production of LF in sporulation and toxin-inducing conditions. Cell-free supernatants were obtained from early exponential (2h), transition (4h), and stationary (7h) phases of growth and subjected to Western blot analysis using rabbit  $\alpha$ -LF antibody. Protein loads were normalized to  $\text{OD}_{600}$ . (C) Production of AtxA in sporulation and toxin-inducing conditions. Samples were obtained from early exponential (2h), transition (4h), and stationary (7h) phases of growth and subjected to Western blot analysis using rabbit  $\alpha$ -AtxA antibody. Protein loads were normalized to  $\text{OD}_{600}$  values per sample time point. These data are representative of three separate experiments.

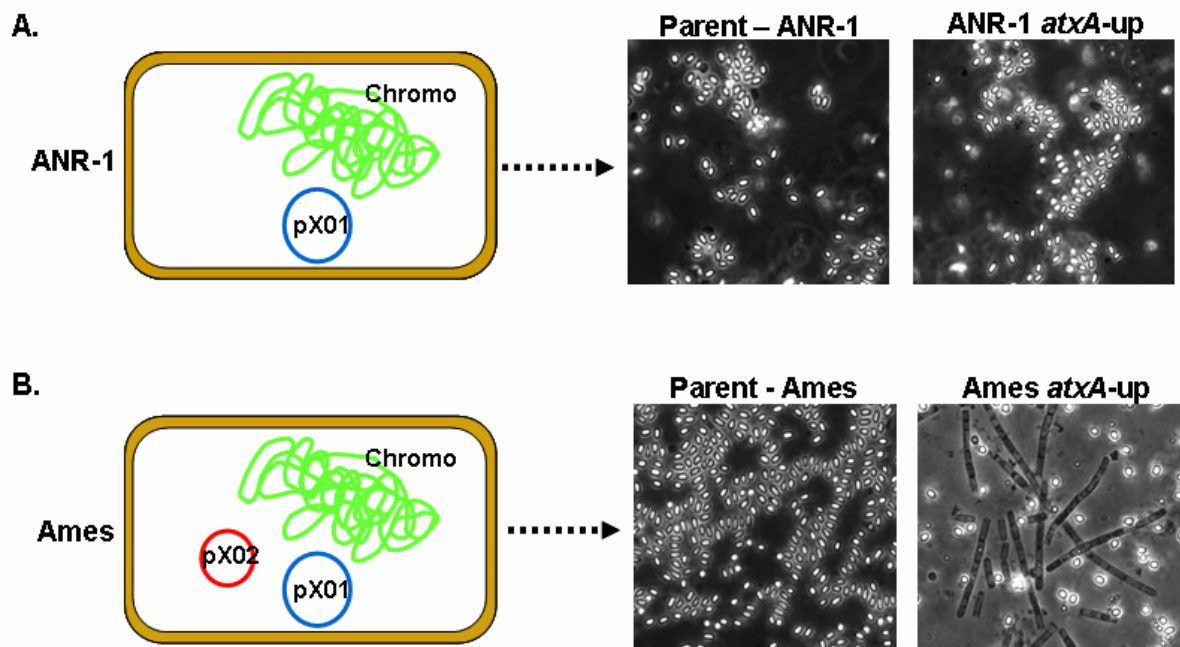


was cultured in PA-air (Fig. 4-1C). The opposite was true when *B. anthracis* was cultured in CACO<sub>3</sub>, AtxA protein levels increased as the cells transitioned into stationary phase (Fig. 4-1C). Together, these results demonstrate an inverse relationship between sporulation and anthrax toxin production. Furthermore, the results indicate that there is a condition-dependent inverse expression of AtxA. *B. anthracis* sporulates but produces little to no AtxA and LF in sporulation conditions (PA-air), whereas when cultured in toxin-inducing conditions (CACO<sub>3</sub> + 5% CO<sub>2</sub>), *B. anthracis* does not sporulate but produces AtxA and LF.

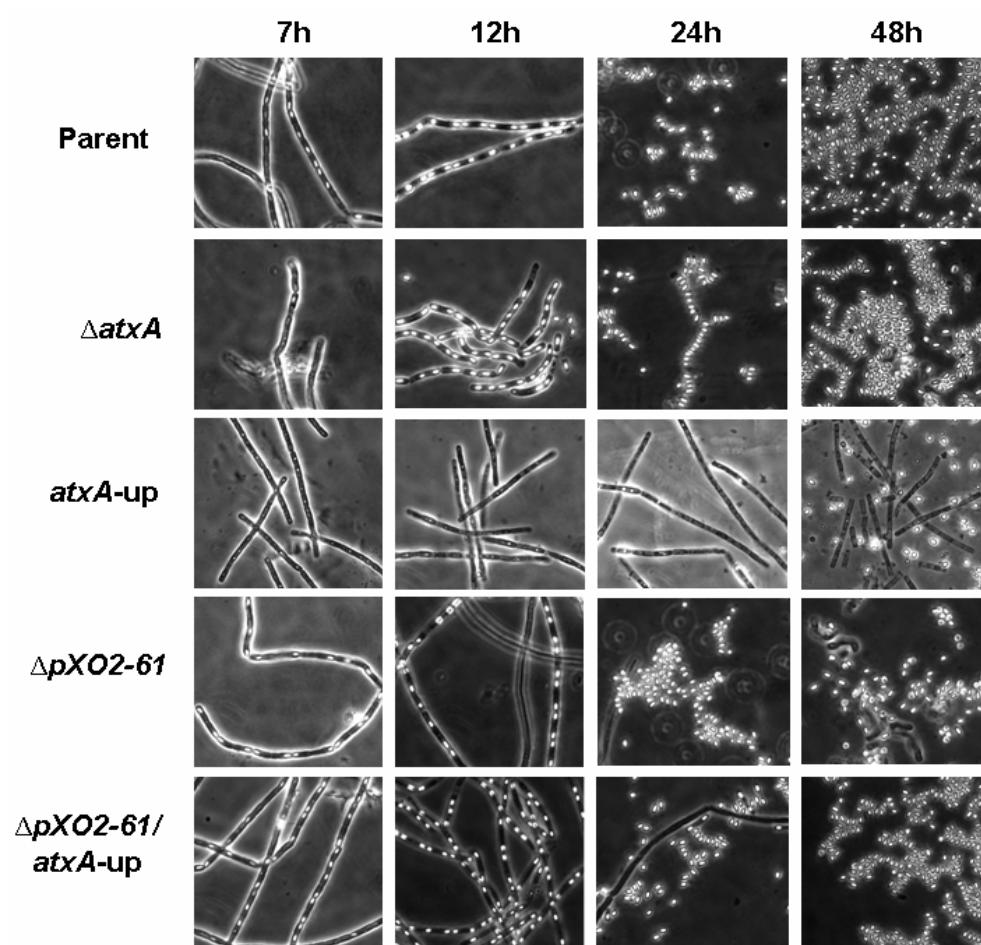
#### **4.2.2. Misregulation of *atxA* results in a pXO2-dependent sporulation defect**

I reported in Chapter 3 the creation of a *B. anthracis* Sterne-like ANR-1 (pXO1+, pXO2-) mutant (ANR-1 *atxA*-up) that overexpresses AtxA in toxin-inducing conditions. The ANR-1 *atxA*-up mutant contains specific nucleotide mutations within the native *atxA* promoter region putative *trans*-acting repressor binding site (see section 3.2.6). In order to assess the impact of altered *atxA* regulation in the genetically complete *B. anthracis* Ames (pXO1+, pXO2+) strain, I created an Ames *atxA*-up mutant in the same manner as the ANR-1 *atxA*-up mutant (see section 2.2). Sporulation of the Ames *atxA*-up mutant was significantly impaired whereas no sporulation defect was observed in the ANR-1 *atxA*-up mutant (Fig. 4-2) indicating that the sporulation defect was pXO2-dependent.

In an attempt to determine the extent of the pXO2-dependent sporulation defect in the Ames *atxA*-up mutant, I performed a time course experiment where *B. anthracis* sporulation was examined using phase-contrast microscopy. *B. anthracis* Ames and Ames-derivatives were cultured in sporulation conditions (PA-air) over a 48 hour time period (Fig. 4-3). The *B. anthracis* parent and *atxA*-null strains contained visible endospores by 7 hours of growth. The cells were in stationary phase of growth at this time point (Fig. 4-1A). Within 24 hours after initiation of the time course, several free floating spores were apparent in both the parent and *atxA*-null strain backgrounds. The abundance of free



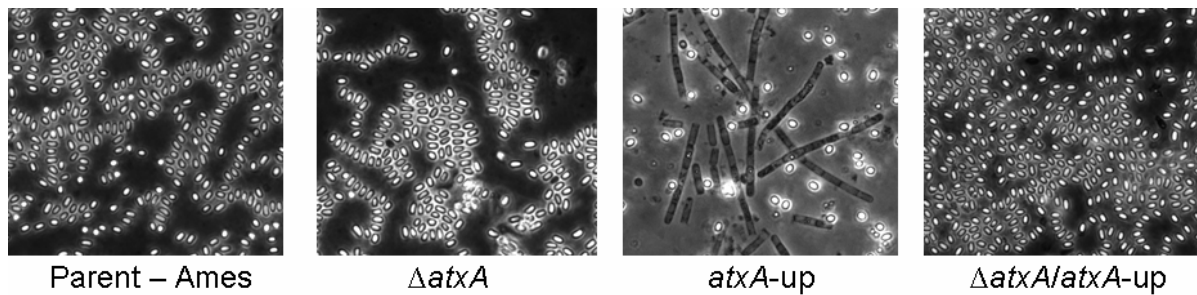
**Figure 4-2. An Ames *atxA*-up mutant is impaired for spore formation.** Phase contrast microscopy showing representative *B. anthracis* (A) ANR-1 and (B) Ames cultures incubated in sporulation conditions (PA – air) after 48 hours.



**Figure 4-3. Sporulation of *B. anthracis* when cultured in sporulation conditions (PA-air).** Phase contrast microscopy showing *B. anthracis* sporulation patterns of parent and mutant strains in batch culture over a 48 hour time course. These data are representative of three separate experiments.

floating spores increased by 48 hours. In comparison, the Ames *atxA*-up mutant displayed a visible delay and decreased efficiency in sporulation. Several vegetative bacilli were present throughout the time course and free floating spores were only visible after 48 hours of growth (Fig. 4-3). This appearance of free spores was delayed by 24-hours compared to the parent and *atxA*-null strains. To ensure that the sporulation defect was attributed to the deregulation of *atxA* and was not associated with misregulation of other genes controlled by the putative *trans*-acting repressor protein, I deleted *atxA* in the Ames *atxA*-up mutant background. This strain contained the mutated *atxA* promoter region but lacked the *atxA* gene (referred to as  $\Delta atxA/atxA$ -up). The sporulation defect observed in the Ames *atxA*-up mutant was relieved upon deletion of *atxA* in the  $\Delta atxA/atxA$ -up mutant (Fig. 4-4). These results indicate that the sporulation defect seen in the Ames *atxA*-up mutant was *atxA*-dependent.

Next, I sought to investigate the mechanism behind the *atxA*- and pXO2-dependent sporulation defect. Previously reported transcriptional profiling results from Bourgogne *et al.* (18) showed that a pXO2 gene, *pXO2-61*, was positively regulated 54-fold by *atxA*. *pXO2-61* exhibits high sequence similarity to the signal sensor domain of the BA2291 sporulation sensor histidine kinase which is a key component of the sporulation phosphorelay (174). Overexpression of *pXO2-61* in a Sterne-like strain resulted in a marked decrease in sporulation that was suppressed by deletion of the sensor histidine kinase BA2291 (174). Therefore, I questioned whether the decreased sporulation observed in the Ames *atxA*-up mutant was a result of *pXO2-61* overexpression due to derepression of *atxA*. To test the affect of *pXO2-61* on sporulation, I deleted *pXO2-61* in the Ames-derived strains and monitored sporulation over a 48 hour time course using phase-contrast microscopy. A  $\Delta pXO2-61$  strain displayed a sporulation pattern similar to both the parent and *atxA*-null strain, and the double  $\Delta pXO2-61/atxA$ -up mutant exhibited an identical sporulation profile (Fig. 4-3). These results indicate that the Ames *atxA*-up sporulation



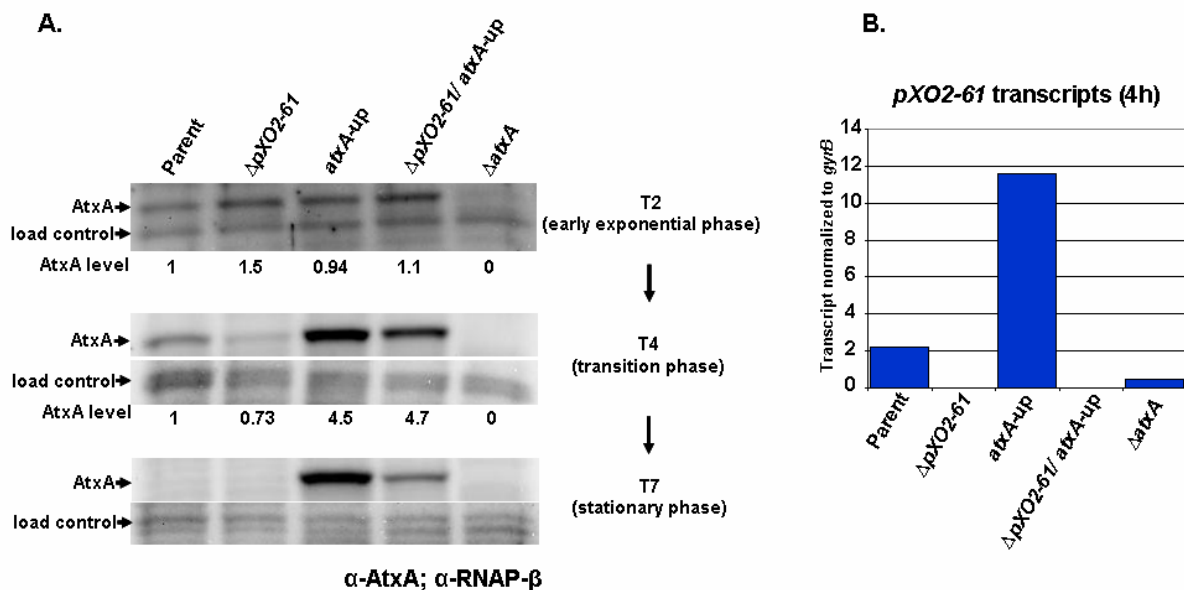
**Figure 4-4. The *atxA*-up sporulation defect is *atxA*-dependent.** Phase contrast microscopy showing representative cultures of parent and mutant *B. anthracis* strains incubated in sporulation conditions (PA – air) after 48 hours.

defect was both *atxA*- and *pXO2-61*-dependent.

Phase-contrast microscopy provides a qualitative assessment of sporulation. I wanted to determine if the observed spores were fully developed, so the number of total heat-resistant CFU was quantified. To this end, aliquots obtained from the *B. anthracis* 24-hour cultures were incubated at 65°C for 45 min and plated to determine the total number of heat-resistant CFU of parent and mutant strains when cultured in sporulation conditions. The parent,  $\Delta atxA$ ,  $\Delta pXO2-61$ , and  $\Delta pXO2-61/atxA$ -up strains all contained approximately  $10^8$  heat-resistant CFU whereas the Ames *atxA*-up mutant produced 1000-fold fewer heat-resistant CFU ( $\sim 10^5$ ) (Table 4-1). These results are in agreement with my interpretation of the phase-contrast microscopy data. Taken together, the results indicate that the Ames *atxA*-up mutant sporulation delay is *pXO2-61*-dependent and suggest that *pXO2-61* is overexpressed in the Ames *atxA*-up strain background.

#### **4.2.3. Increased expression of AtxA leads to elevated *pXO2-61* transcription**

AtxA protein levels diminished when cultures of the Ames parent strain grown in sporulation conditions transitioned from exponential to stationary phase (Fig. 4-1C). I wanted to determine if the steady state level of AtxA changed similarly in cultures of the Ames *atxA*-up and *pXO2-61* mutants. As was true for the parent strain, the  $\Delta pXO2-61$  mutant showed a decrease in AtxA levels from early exponential (T2) to stationary phase (T7) of growth (Fig. 4-5A). In contrast, AtxA protein levels were elevated 4.5-fold in the Ames *atxA*-up and 4.7-fold in the  $\Delta pXO2-61/atxA$ -up mutants compared to parent. AtxA was not expressed in the *atxA*-null strain. The increased expression of AtxA was most evident at transition (T4) and stationary (T7) phases of growth (Fig. 4-5A). A decrease in AtxA levels was apparent in the  $\Delta pXO2-61/atxA$ -up mutant compared to the Ames *atxA*-up mutant at stationary phase (T7) when the  $\Delta pXO2-61/atxA$ -up mutant had begun to sporulation (Fig. 4-3 and Fig. 4-5A).



**Figure 4-5. AtxA protein abundance and *pXO2-61* transcript levels in sporulation conditions (PA-air).** (A) AtxA protein levels in parent and mutant strain backgrounds. Culture samples were obtained during early exponential (T2), transition (T4), and stationary (T7) phases of growth. Samples were subjected to Western blot analysis using rabbit  $\alpha$ -AtxA antibody. Protein loads were determined based on OD<sub>600</sub> values and normalized to cross-reactive products from  $\alpha$ -AtxA or  $\alpha$ -RNAP- $\beta$  blots. These data are representative of three separate experiments. (B) RT-qPCR of *pXO2-61* transcripts normalized to *gyrB* in parent and mutant strains. These data represent average values of detectable transcripts from three independent cultures.

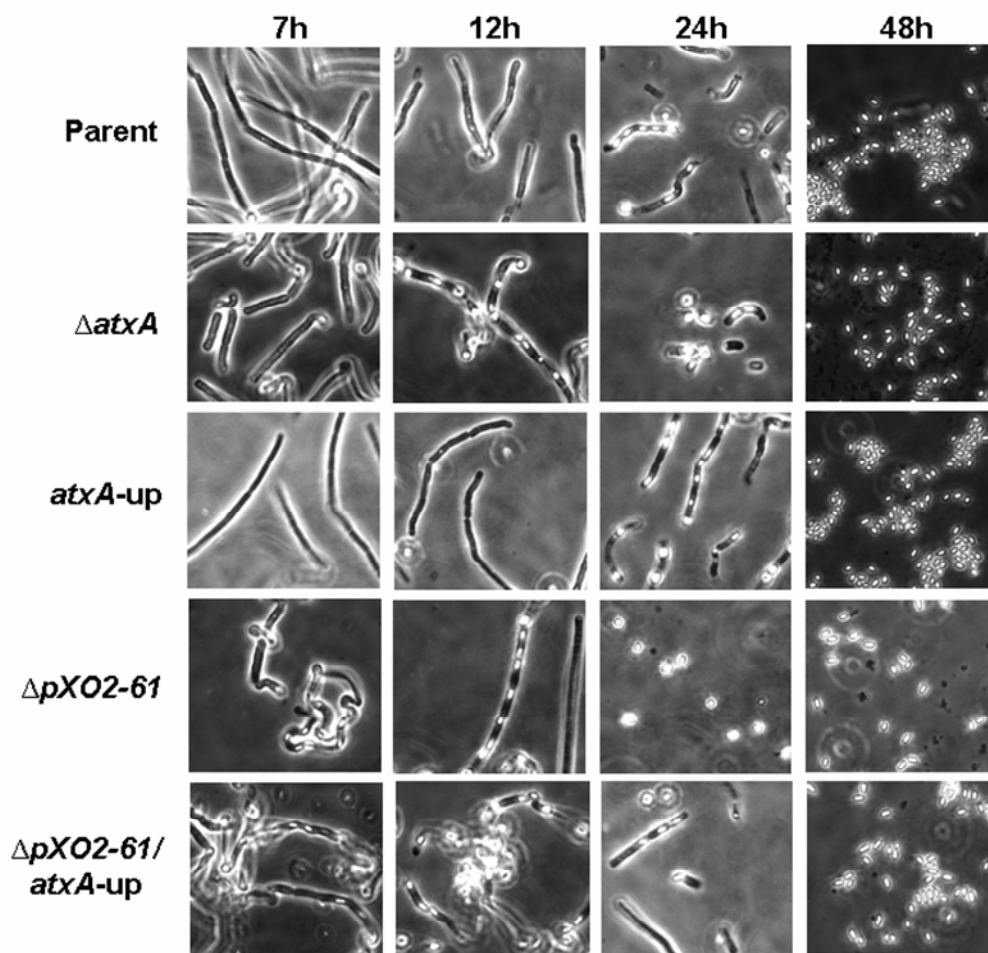
Since antibodies for *pXO2-61* are not available, I performed RT-qPCR on *pXO2-61* transcripts in the various Ames-derivatives at the transition phase of growth (T4). *pXO2-61* transcripts were elevated approximately 5-fold in the Ames *atxA*-up mutant compared to parent and 26-fold compared to the *atxA*-null strain (Fig. 4-5B). There was no detectable *pXO2-61* transcript in the single or double  $\Delta pXO2-61$  strain backgrounds. These results indicate that the overexpression of AtxA resulted in elevated *pXO2-61* transcription and a corresponding *pXO2-61*-dependent sporulation defect.

#### **4.2.4. *B. anthracis* sporulates when cultured in toxin-inducing conditions**

*B. anthracis* cultured in toxin-inducing conditions (CACO<sub>3</sub> + 5% CO<sub>2</sub>) for 10 hours resulted in few heat-resistant CFU, suggesting that the cells were not developing into spores (Fig. 4-1A). However, following prolonged incubation, phase-contrast microscopy revealed cells harboring spores (Fig. 4-6). There were visible endospores forming in the parent strain by 24 hours with several free floating spores by 48 hours. Sporulation of the Ames *atxA*-up mutant was virtually identical to that of the parent strain background. The *atxA*-null strain produced phase-bright endospores earlier than parent and the Ames *atxA*-up mutant at the 12 hour time point. Both the single  $\Delta pXO2-61$  and double  $\Delta pXO2-61/atxA$ -up mutants were the first to develop endospores at the 7-hour time point.

Quantitative determination of sporulation showed that there were 1- to 2-log fewer heat-resistant CFU when cells were cultured in toxin-inducing versus sporulation conditions. There were 10<sup>6</sup>-10<sup>7</sup> heat-resistant CFU in toxin-inducing conditions compared to 10<sup>8</sup> heat-resistant CFU when cells were cultured in sporulation conditions (Table 4-1). Nonetheless, parent and mutant strains were capable of sporulating in toxin-inducing conditions. In contrast to cells cultured in sporulation conditions, the *atxA*-up mutant did not have a drastic sporulation defect compared to parent in toxin-inducing conditions (Fig. 4-3 and Fig 4-5). Overall, these results suggest that the impact of altered AtxA expression is greater in





**Figure 4-6. Sporulation of *B. anthracis* when cultured in toxin-inducing conditions ( $\text{CACO}_3 + 5\% \text{CO}_2$ ).** Phase contrast microscopy showing *B. anthracis* sporulation patterns of parent and mutant strains in batch culture over a 48 hour time course. These data represent three separate experiments.

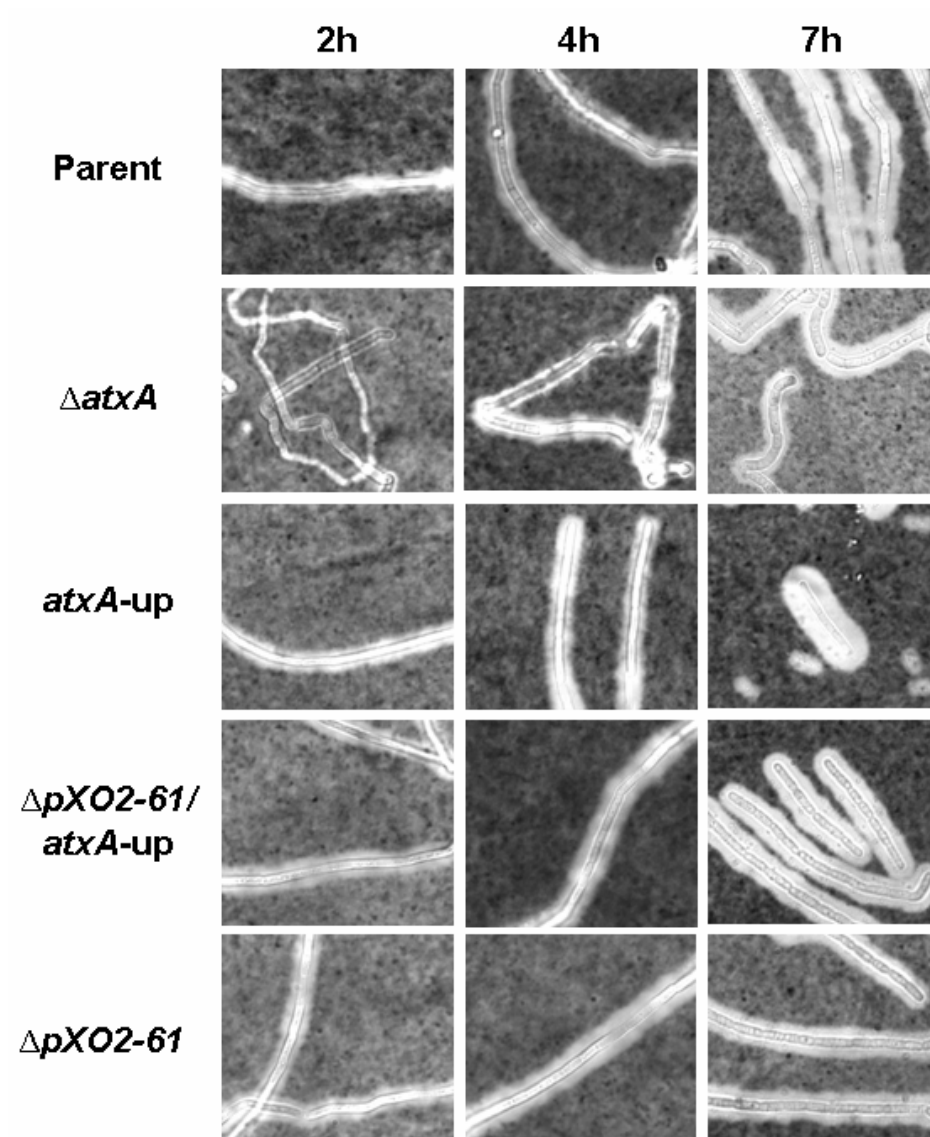
sporulation conditions compared to toxin-inducing conditions.

Toxin-inducing conditions are optimal for *B. anthracis* capsule production (40, 42, 64, 97). I wanted to determine if altered expression of *atxA* and/or deletion of *pXO2-61* had any impact on capsule production, and whether the formation of capsule affected sporulation. Using India Ink exclusion assays, I examined capsule production in the various Ames-derivatives. Capsule production was unaffected in the Ames *atxA*-up,  $\Delta pXO2-61$ , and  $\Delta pXO2-61/atxA$ -up mutant strains indicating that misregulation of *atxA*, and deletion of *pXO2-61*, does not impact capsule formation. These results also indicate that capsule production does not inhibit spore development (Fig. 4-7).

#### **4.2.5. AtxA and *pXO2-61* levels are unaffected by *atxA* misregulation in toxin-inducing conditions**

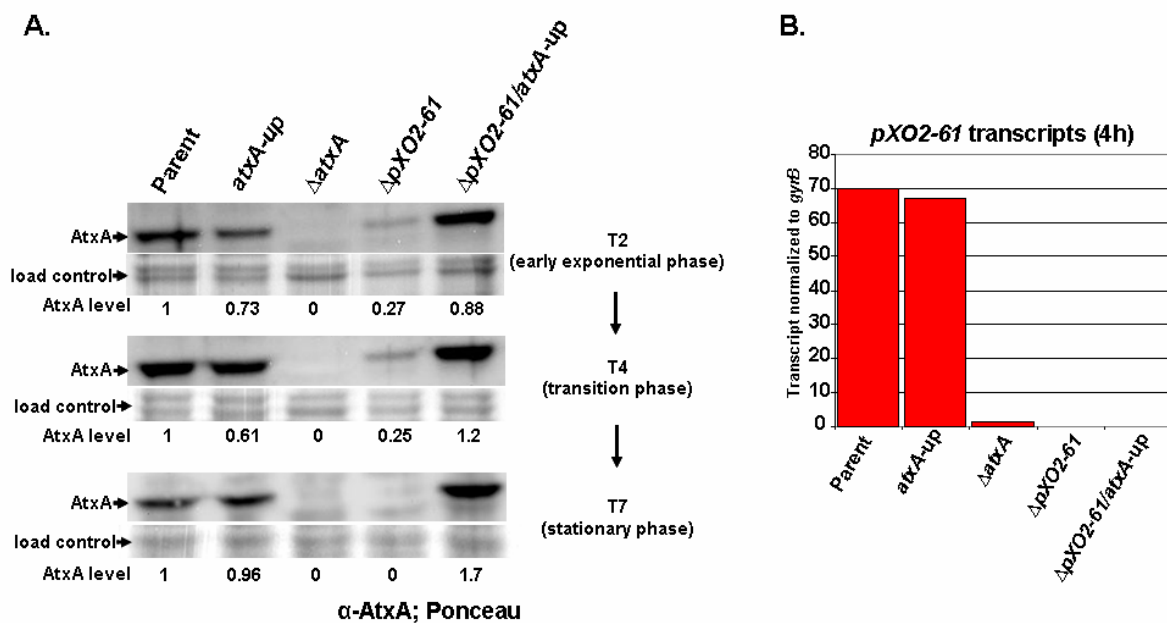
The steady state level of AtxA in the parent background increased from early exponential (T2) to stationary phase (T7) of growth in toxin-inducing conditions (Fig. 4-1C). I sought to determine if AtxA protein levels would vary in the *atxA*-up and *pXO2-61* mutant derivatives when cultured in toxin-inducing conditions. There was a minimal difference in AtxA protein levels between the parent, *atxA*-up mutant, and the double  $\Delta pXO2-61/atxA$ -up strains (Fig. 4-8A). These results do not correlate with the increased expression of AtxA in the ANR-1 *atxA*-up mutant when cultured in toxin-inducing conditions suggesting that factors on pXO2 also impact AtxA (Fig. 3-8). Additional evidence suggestive of pXO2 regulation of AtxA is apparent in the  $\Delta pXO2-61$  strain background. AtxA protein levels diminished over time when *pXO2-61* was deleted (Fig. 4-8A). The observed decrease in AtxA levels in the  $\Delta pXO2-61$  strain is similar to the pattern of AtxA expression when cultured in sporulation conditions (Fig. 4-1C). An *atxA*-null mutant did not express AtxA (Fig. 4-8A).

*pXO2-61* transcript levels were also determined in the various Ames-derivatives at



**Figure 4-7. Capsule production of *B. anthracis* parent and mutant strains in toxin-inducing conditions ( $\text{CACO}_3 + 5\% \text{CO}_2$ ).** Qualitative analysis of capsule production using India ink exclusion assays. These data represent three separate experiments.

the transition phase of growth (T4) when cultured in toxin-inducing conditions. *pXO2-61* transcripts were comparable in the parent and *atxA*-up mutant with less than a 2-fold change in relative transcript levels (Fig. 4-8B). There was an approximate 52-fold decrease in *pXO2-61* transcript levels in the *atxA*-null mutant compared to parent and the *atxA*-up mutant. No *pXO2-61* transcripts were detected in the single or double  $\Delta pXO2-61$  strains. Strains with decreased *pXO2-61* transcripts ( $\Delta pXO2-61$ ,  $\Delta pXO2-61/atxA$ -up, *atxA*-null) sporulated earlier than the *B. anthracis* strains expressing *pXO2-61* (parent, Ames *atxA*-up). In total, these results suggest differential control of AtxA when cultured in toxin-inducing versus sporulation conditions and also implicate *pXO2-61* in control of *atxA* expression.



**Figure 4-8. AtxA protein abundance and *pXO2-61* transcript levels in toxin-inducing conditions ( $\text{CACO}_3 + 5\% \text{CO}_2$ ).** (A) AtxA protein levels in parent and mutant strain backgrounds. Culture samples were obtained during early exponential (T2), transition (T4), and stationary (T7) phases of growth. Samples were subjected to Western blot analysis using rabbit  $\alpha$ -AtxA antibody. Protein loads were determined based on  $\text{OD}_{600}$  values and normalized to Ponceau S stained membranes. These data are representative of three separate experiments. (B) RT-qPCR of *pXO2-61* transcripts normalized to *gyrB* in parent and mutant strains. These data represent an average of three independent experiments.

**Table 4-1.** Total heat-resistant CFU/ml of parent and mutant strains cultured in sporulation and toxin-inducing conditions.

	Parent	$\Delta atxA$	<i>atxA</i> -up	$\Delta pXO2-61$	$\Delta pXO2-61/atxA$ -up
PA - air	$2.4 \times 10^8$	$2.2 \times 10^8$	$6.3 \times 10^5$	$2.4 \times 10^8$	$1.5 \times 10^8$
CACO <sub>3</sub> - 5% CO <sub>2</sub>	$4.7 \times 10^6$	$4.9 \times 10^6$	$1.1 \times 10^7$	$9.3 \times 10^6$	$1.1 \times 10^7$

### 4.3. Discussion

In this chapter, I showed that *B. anthracis* sporulation and anthrax toxin production were inversely related in a condition-dependent manner. The inverse relationship between *B. anthracis* sporulation and anthrax toxin production is physiologically significant for anthrax disease. During infection, *B. anthracis* remains in the vegetative cell state and synthesizes anthrax toxin proteins and other factors that facilitate pathogenesis; sporulation does not occur. Collection of cerebrospinal fluid and blood from *B. anthracis* infected mammals shows the presence of infiltrating vegetative cells, not spores. Conversely, when vegetative cells are exposed to environments outside of the host, toxins are not produced and *B. anthracis* sporulates efficiently (23, 106, 128, 133). Toxin synthesis and sporulation in *B. anthracis* are linked by common transcriptional regulators: the master response regulator Spo0A, the transition state regulator AbrB, and the alternative sigma factor SigH. Spo0A, AbrB, and SigH have been well-characterized in the archetype *Bacillus* species, *B. subtilis*, and are crucial for the appropriate regulation of development/sporulation (reviewed in (121)). The *B. anthracis* orthologues perform similar roles, but in addition, control *atxA* expression (15, 16, 21, 68, 131, 144, 151).

AtxA positively controls transcription of the structural genes for the anthrax toxin proteins, located on pXO1, the capsule biosynthetic operon, carried on pXO2, and multiple other pXO1, pXO2, and chromosomal genes (18, 36, 40, 50, 67, 75, 77, 84, 87, 97, 101, 112, 113, 136, 165, 166). Here I demonstrated that AtxA protein levels not only impacted anthrax toxin production but also affected *B. anthracis* development in a growth condition-dependent manner. My results showed an inverse expression of AtxA in toxin-inducing versus sporulation conditions. The steady state level of AtxA increased from early exponential to stationary phase of growth in toxin-inducing conditions whereas AtxA protein levels were minimal and decreased over time in sporulation conditions. Altering the regulation of *atxA* by mutating the *atxA* promoter region resulted in overexpression of AtxA

and a marked decrease in *B. anthracis* sporulation when cultured in sporulation conditions. Taken together, these results suggest that AtxA plays a role in *B. anthracis* spore development.

Previous reports have implicated *atxA* in proper spore development, but never directly shown AtxA involvement. Deletion of *pXO1* or *atxA* itself results in more efficient spore formation when cultured in rich media (75, 158). In addition, Mignot *et al.* (102) reported that expression of a *B. thuringiensis plcR* gene in a *B. anthracis* strain containing *atxA* resulted in a significant decrease in sporulation, a phenotype that was rescued by deletion of *atxA*. PlcR is a pleiotropic transcriptional regulator in the *B. cereus* group members that controls multiple genes, several of which are associated with pathogenesis (1, 59, 91). Most *B. cereus* group member species, except *B. anthracis*, contain a functional *plcR* gene. The *B. anthracis plcR* gene contains a species-specific nonsense mutation rendering it inactive (102). Typical *B. cereus* group members do not possess and/or express both *atxA* and *plcR* suggesting that mutation of *plcR* resulted in a selective advantage for *B. anthracis*. The final line of evidence suggesting *atxA* is involved in spore development is that overexpression of a highly *atxA*-regulated gene, *pXO2-61*, in a Sterne-like strain of *B. anthracis* led to a marked decrease in sporulation (174).

*pXO2-61* bears homology to the signal sensor domain of one of the major *B. anthracis* sporulation sensor histidine kinases, BA2291. *B. anthracis* sporulation initiates when a signal is sensed by sensor histidine kinases (i.e. BA2291) which further transduce the signal through a multi-component signal transduction phosphorelay (reviewed in (121)). Using phosphotransfer experiments, White *et al.* (174) demonstrated that BA2291 possesses phosphatase activity and can remove phosphate from Spo0F, the initial response regulator of the phosphorelay. Therefore, it has been proposed that when BA2291 is not bound by an activating signal it converts from a kinase to a phosphatase that negatively impacts sporulation at the Spo0F level. The sporulation defect observed as a



result of *pXO2-61* overexpression, in a Sterne-like strain of *B. anthracis*, was suppressed when BA2291 was deleted indicating that the phenotype was BA2291-dependent (174). It was proposed that overexpression of *pXO2-61* titrates signal away from BA2291 resulting in conversion of BA2291 from a kinase to a phosphatase inhibiting the sporulation phosphorelay. Bourgogne *et al.* (18) demonstrated that *pXO2-61* is strongly regulated by AtxA. Transcriptional profiling showed a 54-fold decrease in *pXO2-61* expression when *atxA* was deleted. My results indicated that increased expression of AtxA led to elevated *pXO2-61* transcription which resulted in delayed and decreased sporulation. These data provide evidence for coordinate regulation of toxin gene expression and sporulation by AtxA.

I also demonstrated that *B. anthracis* sporulation during culture in toxin-inducing conditions is delayed and less efficient than the sporulation that occurs during culture in traditional sporulation conditions. These results would suggest that *B. anthracis* has the capability of sporulating *in vivo*. Nevertheless, sporulation has not been observed *in vivo*. This is likely due to AtxA-dependent increased transcription of *pXO2-61*. AtxA is required for the expression of anthrax toxin and capsule enabling *in vivo* survival of *B. anthracis*. Here I have demonstrated an additional function of AtxA is to modulate sporulation of *B. anthracis* by controlling *pXO2-61* transcript levels. Comparison of *pXO2-61* transcript levels in conditions conducive (sporulation conditions) and not conducive (toxin-inducing) for sporulation, I saw that *pXO2-61* transcripts were elevated approximately 35-fold in non-sporulation conditions (toxin-inducing). I propose that elevated expression of a known AtxA-regulated sporulation inhibitor, *pXO2-61*, is a mechanism developed by *B. anthracis* to prevent premature sporulation during anthrax disease.

I only observed sporulation of *B. anthracis* in toxin-inducing conditions following prolonged incubation. Unlike conditions encountered in host tissues, nutrients become deplete over extended periods of time in batch culture. In addition, frequent removal of the

*B. anthracis* cultures from the CO<sub>2</sub> environment to obtain samples may have decreased CO<sub>2</sub> signaling that is required for optimal AtxA activity (70). Examining *B. anthracis* sporulation in closed vessels during growth in toxin-inducing conditions may be more representative of the host environment.

Environment-appropriate control of the key regulator of *Listeria monocytogenes* pathogenesis, PrfA, was shown to be critical for optimal survival of the bacterium inside and outside of the host (20). Similar to *B. anthracis*, *L. monocytogenes* is a saprophytic soil bacterium that has adapted to life within mammalian host cells (63, 81). Constitutive activation of PrfA resulted in a hyper-virulent phenotype in mice; however, as a consequence, *L. monocytogenes* was no longer suited for *ex vivo* growth. Improper regulation of PrfA tipped the balance toward survival of *L. monocytogenes* in the host versus the environment. My work provides evidence for an AtxA-dependent mechanism of *B. anthracis* survival inside and outside the host. AtxA is required for anthrax toxin and capsule production enabling survival within the host whereas AtxA is not required for spore formation. Instead, the expression of AtxA dampens sporulation of *B. anthracis*. The inappropriate timing of *B. anthracis* sporulation during infection could be detrimental to the bacterium and result in increased vulnerability to the host immune response. In addition, inhibition of sporulation outside the host could make the bacterium more susceptible to environmental stresses such as heat, desiccation, and antimicrobials produced by other soil bacteria. To my knowledge, this is the first report showing a direct relationship between AtxA production and *B. anthracis* development.

## **Chapter V**

### **The developmental regulators AbrB, Spo0A, and SigH differentially regulate *atxA* expression in a strain-dependent manner**

NOTE: Malik Raynor and Prabhat Dwivedi, Ph.D., performed the animal injections. Jason Rall, Ph.D., performed experiments related to quantification of *atxA* transcript levels. Gregory L. Shipley, Ph.D., and the Quantitative Genomics Core Laboratory at The University of Texas Health Science Center Houston performed the RT-qPCR assays and data normalization.

## 5.1. Introduction

*B. anthracis* produces two critical virulence factors, anthrax toxin and capsule, to escape and evade the host immune response during infection. The structural genes for anthrax toxin include, *pagA*, *lef*, and *cya*, which encode PA (protective antigen), LF (lethal factor), and EF (edema factor), respectively. The toxin genes are located non-contiguously within a pathogenicity island on the 182-kb virulence plasmid pXO1 (84, 103, 113). Transcription of the toxin genes is strongly affected by the pXO1-encoded *trans*-acting regulator AtxA. Each toxin gene contains, at minimum, an *atxA*-dependent transcription start site (36, 76, 87). The capsule biosynthetic operon, *capBCADE*, located on the 96-kb virulence plasmid pXO2 is also positively affected by AtxA (36, 40, 50, 67, 87, 101, 136, 165, 166). Transcription of the *cap* operon involves crosstalk between the two virulence plasmids. In a genetically complete strain containing pXO1 and pXO2, AtxA directs transcription of the *cap* operon via two pXO2-encoded regulators AcpA and AcpB. The *acpA* gene and *cap* operon contain *atxA*-dependent transcription start sites while *acpB* can be transcribed from its own promoter or via transcriptional read-through of the *cap* operon. Co-transcription of *acpB* with *capBCADE* results in a positive feedback loop for *cap* operon transcription (41).

Expression of the anthrax toxin and capsule genes, and many other *atxA*-regulated genes, is enhanced when cells are cultured in 5% CO<sub>2</sub> with media containing 0.8% dissolved bicarbonate (9, 18, 28, 33, 36, 50, 75, 77, 87, 101, 137). The CO<sub>2</sub>/bicarbonate signal is considered physiologically significant for pathogenesis. However, the molecular mechanism by which AtxA and CO<sub>2</sub>/bicarbonate control the toxin and capsule genes is unknown. AtxA is a 55.6-kDa basic protein with putative helix-turn-helix and winged-helix motifs near the amino-terminus, but no specific DNA binding has been demonstrated for this protein. Common *cis*-acting regions of *atxA*-dependent promoters have not been identified. Nucleotide sequence similarities in promoter regions are not apparent; therefore,

it has been suggested that DNA curvature plays a role in AtxA regulation of its target toxin and capsule regulator genes (69).

In addition to the plasmid-encoded regulators, the chromosome-encoded transcription factors AbrB, CcpA, and CodY indirectly affect toxin gene expression by controlling *atxA* at the transcriptional and post-translational level. The effect of AbrB and CcpA on capsule production has not been assessed, but deletion of *codY* has been reported to have no effect on capsule production (31). The carbon catabolite protein CcpA and the pleiotropic regulator CodY both sense the nutritional status within a cell to activate or repress their target genes (71, 124, 135, 170). A *ccpA*-null mutant exhibits a decrease in *atxA* transcription and produces little to no anthrax toxin (32). Deletion of *codY* results in decreased toxin production due to decreased AtxA protein levels (168). The mechanisms by which CcpA controls *atxA* transcription and CodY controls AtxA protein levels are unknown. The transition state regulator AbrB is the only identified *trans*-acting factor that specifically binds the *atxA* promoter (151). An *abrB*-null mutant shows early and increased transcription of the anthrax toxin genes (131).

AbrB is a pleiotropic DNA-binding regulator that represses post-exponential phase genes during logarithmic growth (121, 152, 154, 156). One of the direct targets of AbrB is *sigH*, a gene encoding an alternative sigma factor that directs the transcription of genes associated with the transition into stationary phase of growth (148). SigH is required during the early stages of sporulation to directly promote transcription of several crucial sporulation genes (19, 43). Most importantly, SigH (with core RNA polymerase [RNAP]) activates transcription of *spo0A*, the master response regulator of sporulation (122). AbrB, Spo0A, and SigH are all part of a feedback mechanism that ultimately controls expression of each other in addition to multiple other genes. In *B. subtilis*, SigH-RNAP directs transcription of *spo0A*, phosphorylated Spo0A represses *abrB*, and AbrB represses *sigH* (54, 66, 122, 147, 148, 151).

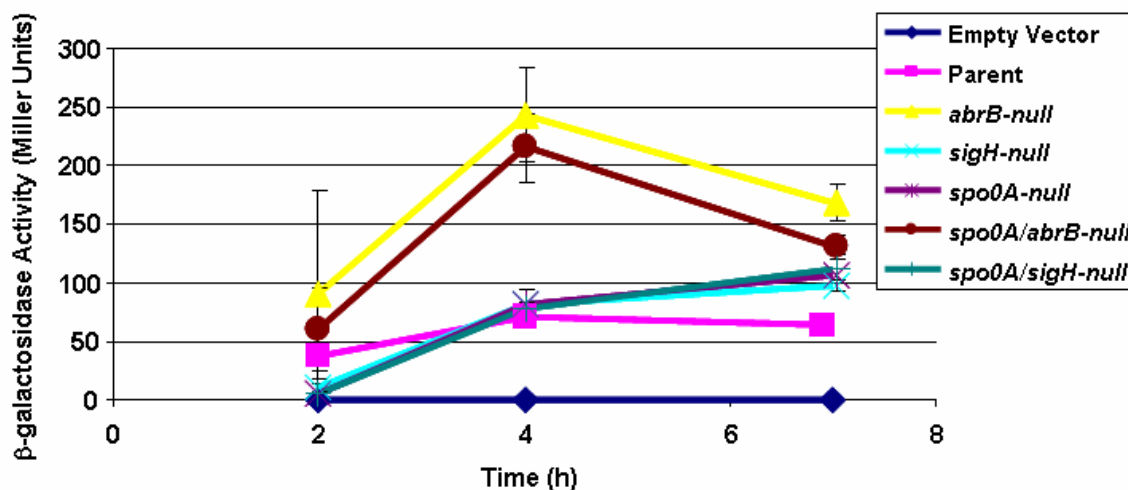
In *B. anthracis*, AbrB, Spo0A, and SigH appear to be part of a similar feedback loop regulating the expression of each other, but in addition, control *atxA* expression. Multiple studies have revealed effects of AbrB, Spo0A, and SigH on *atxA* transcription using different *B. anthracis* strains and the heterologous host *B. subtilis* (14, 68, 131, 151). The results of these studies indicate that AbrB binds to specific sequences in the *atxA* promoter region to repress *atxA* transcription. Spo0A positively affects *atxA* via control of *abrB* expression. SigH control of *atxA* occurs via its positive effect on *spo0A*, and in one strain, *sigH* positively regulates *atxA* expression in a *spo0A*- and *abrB*-independent manner (14, 68, 151).

In this study, I sought to investigate the physiological relevance of *atxA* regulators in *B. anthracis* disease progression by examining the impact AbrB, Spo0A, and SigH have on AtxA, anthrax toxin, and capsule production. Using a genetically incomplete strain of *B. anthracis* (ANR-1 [pXO1+, pXO2-]), I demonstrate that AtxA and anthrax toxin production supports the previously established AbrB/Spo0A/SigH model of *atxA* regulation. However, AtxA and anthrax toxin production are largely different in a genetically complete *B. anthracis* strain (Ames [pXO1+, pXO2+]) mutated for the developmental regulators because of factors on pXO2. Capsule production is modestly affected by deletion of the developmental regulators. Finally, I show that an *abrB*-null mutant, in the genetically incomplete ANR-1 strain background, is more virulent than the parent and a strain overexpressing AtxA (ANR-1 *atxA*-up mutant).

## 5.2. Results

### 5.2.1. The developmental regulator AbrB negatively controls *atxA* transcription

There is conflicting evidence regarding the impact of the developmental regulators AbrB, Spo0A, and SigH on *atxA* transcription. Different *B. anthracis* Sterne-like (pXO1+, pXO2-) strain backgrounds in addition to the heterologous host *B. subtilis* were used in previous investigations assessing *atxA* expression in developmental regulator mutants. In an attempt to clarify the effects of AbrB, SigH, and Spo0A on *atxA* transcription, I examined *atxA* promoter activity in the ANR-1 (pXO1+, pXO2-) strain of *B. anthracis*. ANR-1 is a pXO2-cured toxigenic, noncapsulated variant of the genetically complete, clinical isolate Ames (pXO1+, pXO2+) strain and is therefore considered physiologically relevant (172). *atxA* promoter activity was monitored during early exponential (2h), transition (4h), and stationary (7h) phases of growth when cultured in toxin-inducing conditions using  $\beta$ -galactosidase assays (Fig. 5-1). The minimal *atxA* promoter (171-bp, see Fig. 3-1) was transcriptionally fused to a promoterless *lacZ* gene and introduced into the *B. anthracis* ANR-1 parent and isogenic developmental regulator mutants. The ANR-1 parent strain containing the *atxA* promoter-*lacZ* fusion (*PatxA-lacZ*) exhibited a maximum  $\beta$ -galactosidase activity of 72 Miller Units (MU). Single deletion of either *sigH* or *spo0A* and a double *sigH/spo0A* mutant resulted in parent levels of  $\beta$ -galactosidase activity at the transition phase of growth (4h; 82 MU, 81 MU, and 78 MU, respectively) suggesting SigH and Spo0A have no effect on *atxA* transcription. Deletion of *abrB* resulted in an increase in  $\beta$ -galactosidase activity to 244 MU, consistent with AbrB repression of *atxA*. Similarly, a double *abrB/spo0A*-null mutant exhibited 216 MU of  $\beta$ -galactosidase activity. Taken together, these data suggest that AbrB represses *atxA* transcription while SigH and Spo0A do not affect *atxA* expression.



**Figure 5-1.  $\beta$ -galactosidase activities of *PatxA-lacZ* fusions in parent and isogenic developmental regulator mutant strains.** Samples were obtained from cultures grown in toxin-inducing conditions during early exponential (2h), transition (4h), and stationary (7h) phase of growth. The *PatxA-lacZ* low copy number plasmid was introduced into parent (ANR-1; pink),  $\Delta abrB$  (UT384; yellow),  $\Delta sigH$  (UT399; teal),  $\Delta spo0A$  (UT400, purple),  $\Delta spo0A/abrB$  (UT401; maroon),  $\Delta spo0A/sigH$  (UT402; green), and an empty vector control strain (ANR-1 (pHT304-18z); blue). These data are averaged from three independent cultures.

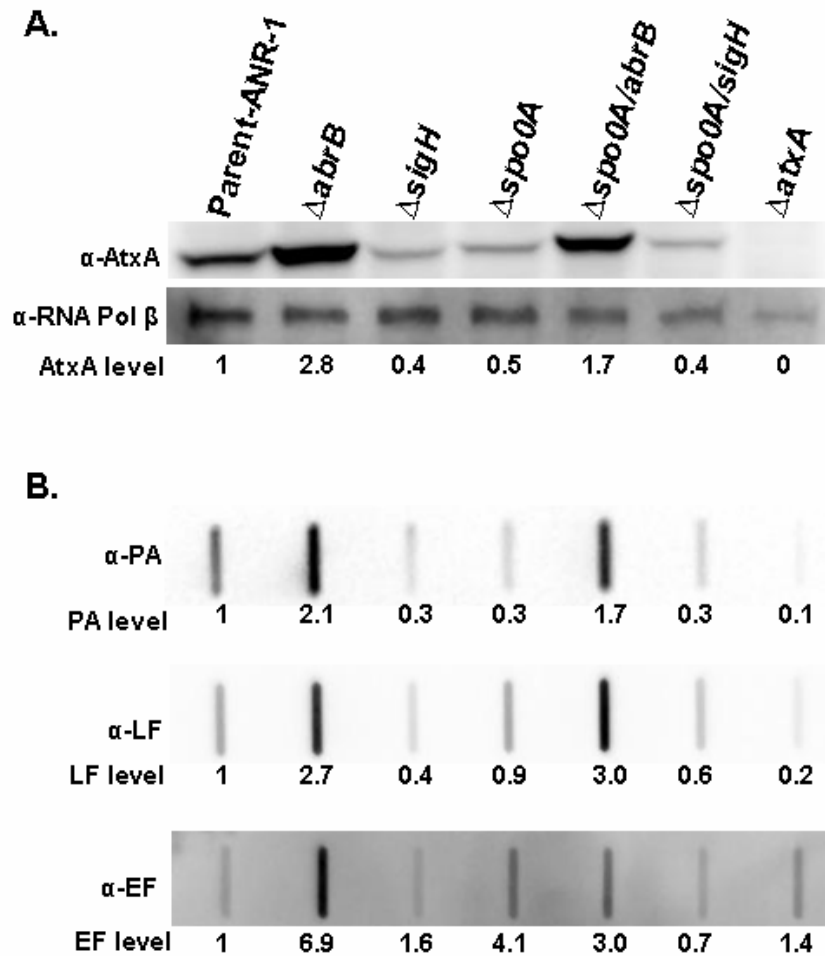


### 5.2.2. Factors on pXO2 impact AtxA expression

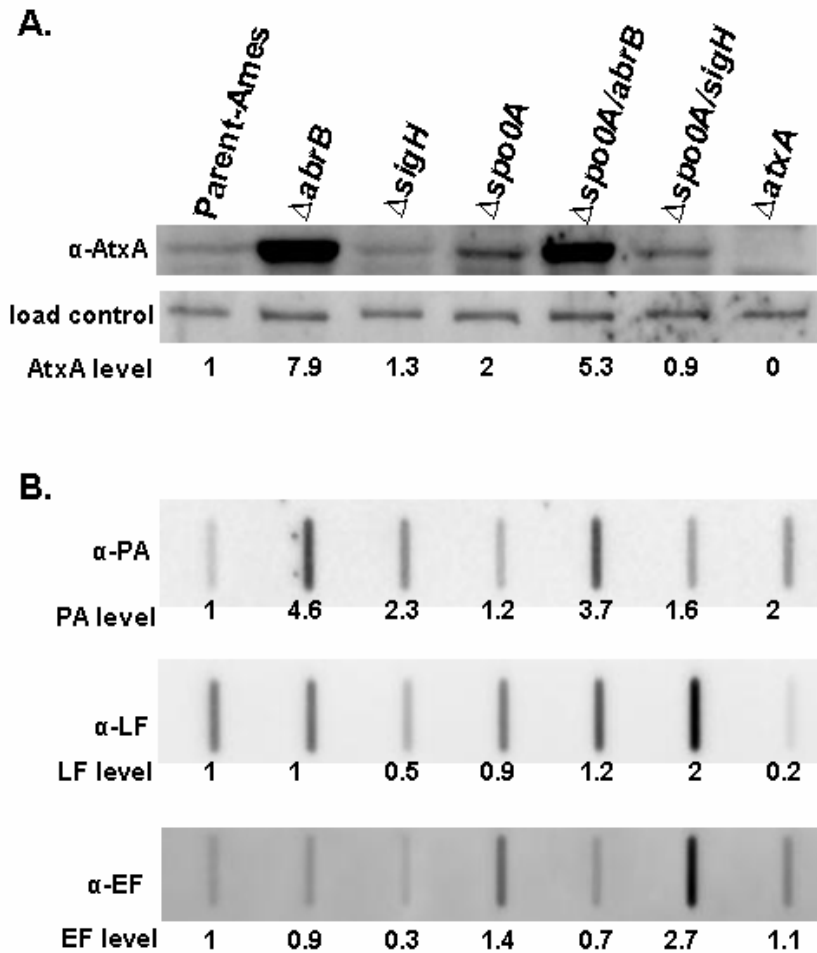
AtxA protein levels were examined in the ANR-1 parent and isogenic developmental regulator mutants to determine if the protein levels reflected *atxA* promoter activity (Fig. 5-2A). I also determined the impact deletion of the developmental regulators had on the anthrax toxin proteins PA, LF, and EF (Fig. 5-2B). There was a 2.8-fold increase in AtxA protein levels in the *abrB*-null mutant with a corresponding 2.1- to 6.9-fold increase in all three anthrax toxin proteins. These results support the model in which AbrB represses *atxA* transcription. The *spo0A/abrB*-null strain exhibited a subtle increase in AtxA protein levels with a corresponding 1.7- to 3-fold increase in the anthrax toxin proteins. These results support the AbrB/Spo0A/SigH model for regulation of *atxA* transcription.

Single or double deletion of *sigH* and *spo0A* in ANR-1 resulted in decreased AtxA, PA, and LF production. These results do not correlate with the parent level of  $\beta$ -galactosidase activity each *PatxA-lacZ* fusion exhibited in the *sigH* and *spo0A* mutant derivatives (Fig. 5-1). The results suggest that *lacZ* transcripts were stabilized or AtxA was regulated post-translationally in the *sigH*-, *spo0A*-, and *sigH/spo0A*-null strain backgrounds. EF production was at parent levels or elevated in the *sigH*- and *spo0A*-null mutants suggesting factors other than AtxA are impacting EF protein levels in the mutant backgrounds. An ANR-1 *atxA*-null mutant did not express AtxA and produced little to no anthrax toxin. Taken together, these data indicate that AbrB represses *atxA* transcription while SigH and Spo0A positively influence AtxA expression in the ANR-1 strain background.

I previously determined that factors on pXO2 influence AtxA expression (see section 4.2.5). Therefore, I wanted to determine the impact AbrB, Spo0A, and SigH had on AtxA and anthrax toxin production in the genetically complete *B. anthracis* Ames strain and isogenic developmental regulator mutants (Fig. 5-3). There was a 2.8-fold increase in AtxA expression in the Ames *abrB*-null compared to the ANR-1 *abrB*-null mutant (Fig. 5-3 and



**Figure 5-2. Influence of ANR-1 isogenic developmental regulators on AtxA and anthrax toxin expression.** Production of (A) AtxA and (B) LF, EF, and PA by ANR-1 parent and mutant strains. Culture samples were obtained from *B. anthracis* strains grown in toxin-inducing conditions during transition phase (4h) of growth. (A) Samples were subjected to SDS-PAGE and Western blot analysis using rabbit  $\alpha$ -AtxA antibody raised against *B. anthracis* AtxA or mouse  $\alpha$ -RNA Pol  $\beta$  antibody raised against *E. coli* RNA Pol  $\beta$ . (B) Samples were subjected to Slot blot Western analysis using rabbit  $\alpha$ -LF, rabbit  $\alpha$ -EF, and goat  $\alpha$ -PA antibodies raised against *B. anthracis* proteins. Quantified protein levels normalized to load control and parent strain. These data are representative of three separate experiments.

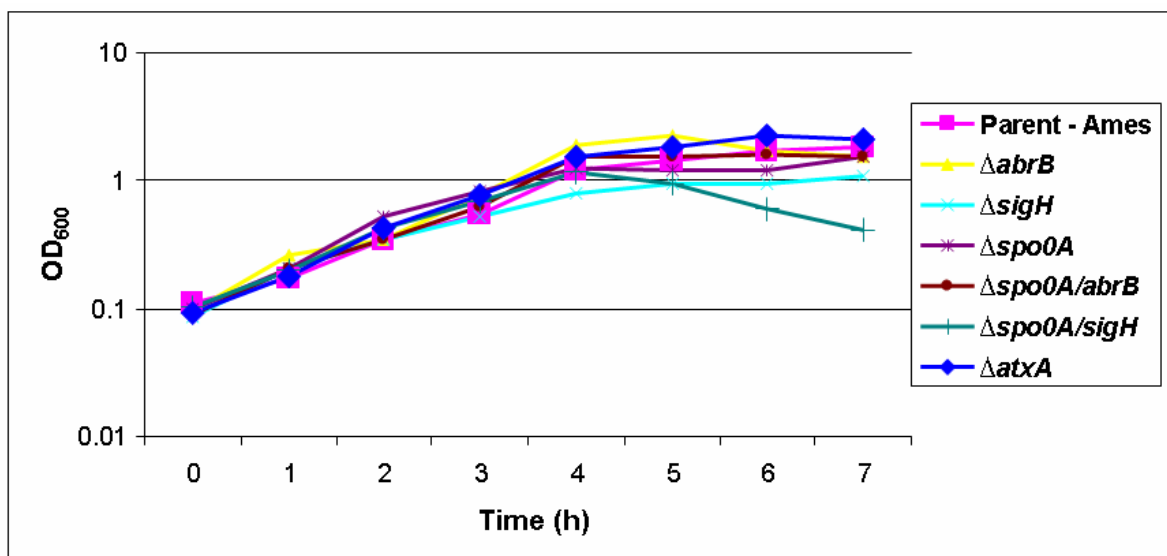


**Figure 5-3. Influence of Ames isogenic developmental regulators on AtxA and anthrax toxin expression.** Production of (A) AtxA and (B) LF, EF, and PA by Ames parent and mutant strains. Culture samples were obtained from *B. anthracis* strains grown in toxin-inducing conditions during transition phase (4h) of growth. (A) Samples were subjected to SDS-PAGE and Western blot analysis using rabbit  $\alpha$ -AtxA antibody raised against *B. anthracis* AtxA or mouse  $\alpha$ -RNA Pol  $\beta$  antibody raised against *E. coli* RNA Pol  $\beta$ . (B) Samples were subjected to Slot blot Western analysis using rabbit  $\alpha$ -LF, rabbit  $\alpha$ -EF, and goat  $\alpha$ -PA antibodies raised against *B. anthracis* proteins. Quantified protein levels normalized to load control and parent strain. These data are representative of three separate experiments.

Fig. 5-2). AtxA expression increased approximately 8-fold in the Ames *abrB*-null mutant versus parent which resulted in a 4.6-fold increase in PA expression. Similarly, the Ames *spo0A/abrB*-null mutant exhibited an approximate 5-fold increase in AtxA compared to parent. This resulted in a 3.7-fold increase in PA production. LF and EF production were unaffected in the *abrB*- and *spo0A/abrB*-null mutants possibly due to misregulation of secreted proteases that affect anthrax toxin stability (120). These secreted proteases specifically target the anthrax toxin proteins for degradation and are indirectly controlled by AbrB and Spo0A (7, 120, 121, 134).

Single or double deletion of *sigH* and *spo0A* in Ames resulted in AtxA protein levels similar to, or modestly increased, compared to parent. In agreement with the small changes in AtxA levels, there was little to no change in anthrax toxin production in the Ames *sigH*- and *spo0A*-null mutants. Anthrax toxin production did increase 1.6- to 2.7-fold in the *spo0A/sigH*-null mutant; however, this strain exhibited a growth defect that resulted in cell lysis over time (Fig. 5-4), impacting accurate determination of intracellular versus secreted protein levels. In total, these results are opposite of the decreased AtxA protein levels observed in the ANR-1 *sigH* and *spo0A* mutant derivatives suggesting interplay between factors on pXO2. Most surprisingly, an Ames *atxA*-null mutant did not express AtxA, but produced PA, LF, and EF at levels comparable to the parent strain, suggesting factors on pXO2 also control the anthrax toxins independent of *atxA*. Taken together, AtxA and anthrax toxin production in the Ames developmental regulator mutants was largely different than AtxA protein levels in the ANR-1 developmental regulator mutant derivatives indicating that factors on pXO2 impact both AtxA and anthrax toxin expression.

Multiple techniques were used to create a double *sigH/abrB*-null mutant in the ANR-1 and Ames strain backgrounds; however, the double deletion mutant could not be created due to unknown reasons. To this end, I could not clear up confusion regarding whether SigH controls AtxA expression in an AbrB-dependent or -independent manner. Variable

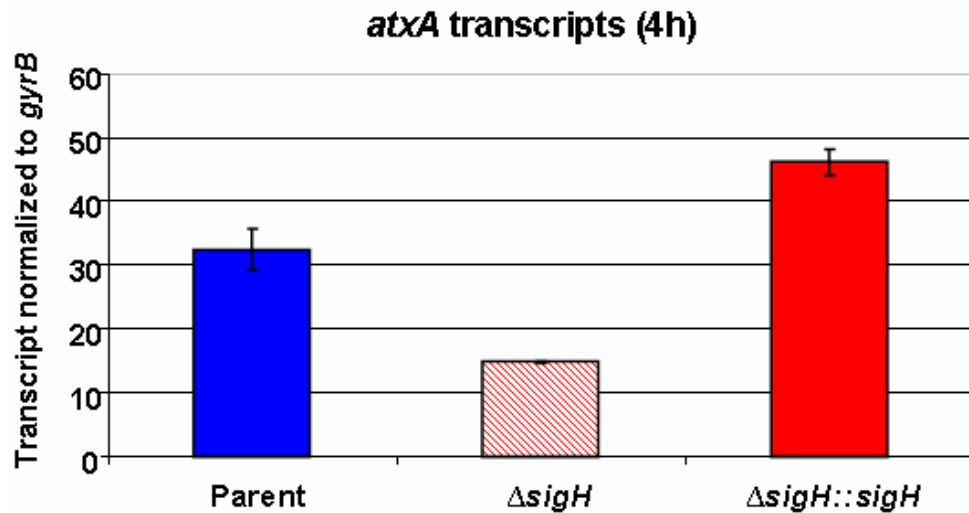


**Figure 5-4. Growth curve of Ames parent and developmental regulator mutants.** *B. anthracis* strains were cultured in toxin conditions (CACO<sub>3</sub> + 5% CO<sub>2</sub>) and the optical density at 600 nm (OD<sub>600</sub>) was measured hourly. These data are representative of three independent cultures.

phenotypes were often observed in the Ames *sigH*-null mutant concerning AtxA and anthrax toxin protein levels. In addition, creation of the Ames and ANR-1 *sigH*-null mutants was difficult and the ANR-1 *sigH*-null mutant sporulation defect could not be complemented (data not shown). In combination, these results suggest that SigH is a critical sigma factor and pleiotropic regulator in *B. anthracis*.

### 5.2.3. SigH positively controls *atxA* transcription

The impact of the alternative sigma factor SigH on *atxA* transcription is highly controversial. Hadjifrangiskou *et al.* (68) demonstrated SigH positively controlled *atxA* transcription in an AbrB-independent manner whereas other groups reported that SigH control of *atxA* transcription was AbrB-dependent (14, 151). In all previous reports, different *Bacillus* species strains were used including genetically incomplete Sterne-like (pXO1+, pXO2-) *B. anthracis* strains and a *B. subtilis* heterologous host strain. Therefore, I wanted to determine if SigH controlled *atxA* transcription in the genetically complete *B. anthracis* Ames strain. *atxA* transcript levels were determined in the Ames parent, *sigH*-null, and *sigH* complemented strains using RT-qPCR. The relative transcript levels were normalized to the housekeeping gene *gyrB*. A *sigH*-null strain exhibited a 2.2-fold decrease in *atxA* transcripts compared to parent. When *sigH* was reintroduced into the *sigH*-null mutant, *atxA* transcript levels increased to levels slightly higher than parent indicating that SigH positively controls *atxA* transcription (Fig. 5-5). However, since a double *sigH/abrB*-null mutant could not be created, it is unclear if the observed phenotype is AbrB-dependent or – independent. The 2.2-fold decrease in *atxA* transcripts in the Ames *sigH*-null mutant did not translate to a measurable decrease in AtxA protein levels (Fig. 5-3A). Nevertheless, these results demonstrate that SigH positively controls *atxA* transcription in a genetically complete *B. anthracis* strain background.



**Figure 5-5. *atxA* transcript levels in parent and *sigH* mutant strains.** Sample for RT-qPCR were obtained from *B. anthracis* parent (Ames, blue),  $\Delta sigH$  (UTA16; red partial fill), and  $\Delta sigH::sigH$  (UTA17; red) cultured in toxin-inducing conditions during transition (4h) phase of growth. Transcript levels normalized to the housekeeping gene *gyrB*. Data are averaged from three independent cultures. Strain creation and RNA manipulation performed by Jason Rall, Ph.D.

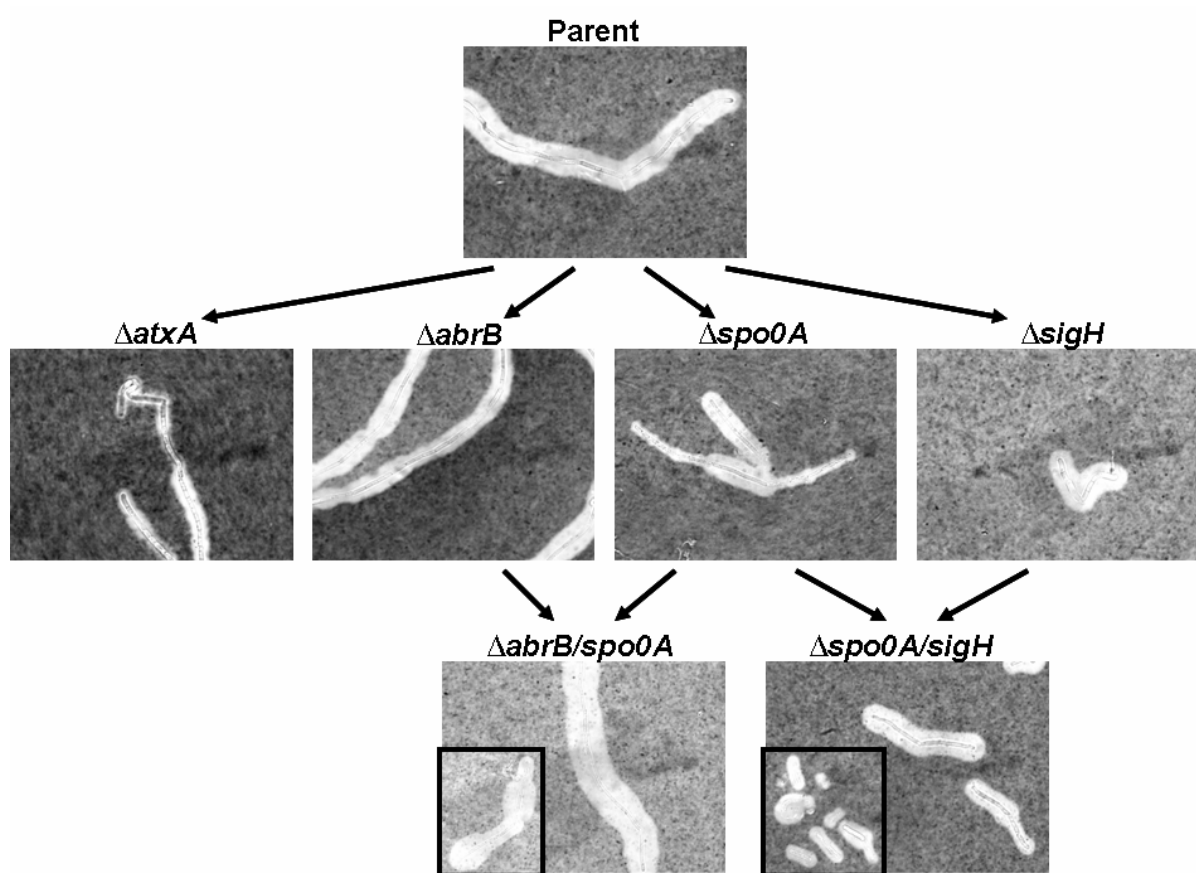
#### 5.2.4. Altered AtxA expression affects capsule production

AtxA positively affects transcription of the capsule biosynthetic operon via control of the capsule gene regulators *acpA* and *acpB* (40, 67, 166, 169). Since the developmental regulators control *atxA* transcription, I decided to determine if altered AtxA expression would impact capsule production. The production of capsule was assessed using India ink exclusion assays in which the capsule appears as a halo surrounding the bacilli. All strains produced capsule; however, the amount and uniformity of the capsule varied among the strain backgrounds (Fig. 5-6). Capsule synthesis by the *atxA*-null strain was delayed and reduced compared to the parent strain. The single *abrB*- and *sigH*-null mutants produced capsule similar to parent while mutation of *spo0A* resulted in irregular production of capsule. Some bacilli within a chain of a *spo0A*-null mutant produced parent levels of capsule while other bacilli within the same chain had reduced capsule production. The *abrB/spo0A*- and *spo0A/sigH*-null mutants exhibited mixed phenotypes where some chains produced parent levels of capsule and other chains were more variable for capsule production. In addition, a single or double *sigH*-null mutant produced shorter and smaller cells that were always capsulated. Together, these results indicate that altered AtxA expression impacted the uniform production of capsule.

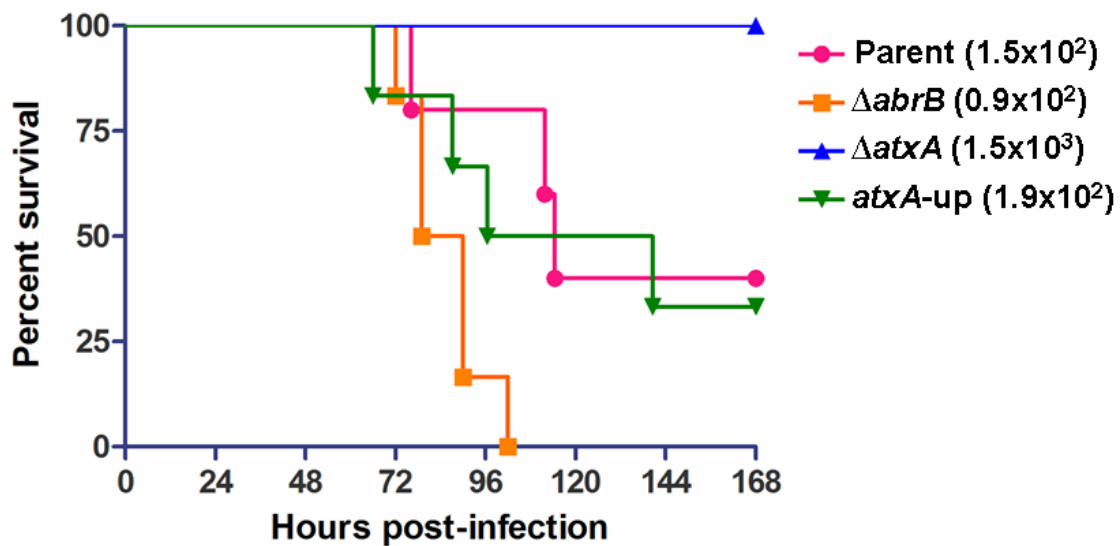
#### 5.2.5. An *abrB*-null mutant is more virulent than parent

Given the increased toxin production by the *abrB*-null and *atxA*-up mutants, I considered whether the mutants would display increased virulence in a murine model in which toxin plays an important role in pathogenesis. Vegetative cells obtained from *B. anthracis* ANR-1 parent and isogenic *atxA*-null, *abrB*-null, and *atxA*-up (see section 3.2.6) strains were injected via the tail vein into 6- to 8-week-old female A/J mice. Mice were monitored for signs of disease for 7 days post-infection. A/J mice are complement deficient therefore more susceptible to anthrax toxin and are often used when studying the affects of





**Figure 5-6. Capsule production is modestly affected by mutation of the developmental regulators AbrB, Spo0A, and SigH.** *B. anthracis* Ames parent and mutant strains were cultured in toxin-inducing conditions and capsule was visualized during stationary (7h) phase of growth using India ink exclusion assays. Micrograph inserts depict representative altered capsule phenotypes of each respective mutant strain. These data are representative of three separate experiments.



**Figure 5-7. Elevated AtxA expression is not sufficient for increased virulence.**

Survival curves of mice infected intravenously (i.v.) with vegetative ANR-1-derived *B. anthracis* strains. A/J mice were injected i.v. with  $1.5 \times 10^2$  CFU of parent (n=6),  $1.9 \times 10^2$  CFU of *atxA*-up (n=6),  $0.9 \times 10^2$  CFU of  $\Delta abrB$  (n=6), and  $1.5 \times 10^3$  CFU of  $\Delta atxA$  (n=3) vegetative cells. An  $\Delta atxA$  mutant is avirulent. Both the parent and *atxA*-up mutant had similar virulence with no significant difference in MTD. The  $\Delta abrB$  mutant had a MTD of 84.5 h. Injections performed by Malik Raynor and Prabhat Dwivedi, Ph.D.

*B. anthracis* toxin on virulence (173). There was no significant difference in the mean time to death (MTD) of mice infected with  $10^2$  CFU of the parent or an *atxA*-up mutant. Infection with the parent and *atxA*-up mutant strains resulted in a MTD of approximately 113 and 119 h, respectively. The ANR-1 *abrB*-null mutant was more virulent than parent and the *atxA*-up strain with a MTD of 84.5 h. An *atxA*-null mutant was avirulent (Fig. 5-7).

The *atxA*-up mutant produced more AtxA, PA, LF, and EF than both parent and the *abrB*-null mutant (Fig 3-8 and Fig. 5-2A); however, the *atxA*-up mutant was not more virulent. It is possible that the phenotype of the *atxA*-up mutant in batch culture is not mirrored during infection. These results indicate that increased production of AtxA and anthrax toxin expression in batch culture does not necessarily translate to an increase in virulence in a murine model for anthrax disease. The results also suggest that the increased virulence of an *abrB*-null mutant is not attributed to increased AtxA and anthrax toxin production.

### 5.3. Discussion

The AbrB/Spo0A/SigH model for regulation of *atxA* transcription was initially established using the heterologous host *B. subtilis* parent and isogenic developmental regulator mutants harboring a *B. anthracis atxA* promoter – *lacZ* fusion (*PatxA-lacZ*) (151). *B. subtilis* is the most extensively studied *Bacillus* species and has been used for several years as the model Gram-positive bacterium. *B. anthracis* is similar to *B. subtilis* and contains several homologous proteins with similar functions. *B. anthracis* is a member of the *Bacillus cereus* group that contains six closely related species, which does not include *B. subtilis*. It has become apparent based on similar studies regarding the role of AbrB, Spo0A, and SigH on *atxA* expression performed in *B. anthracis* that *B. subtilis* is not the best model system to examine *B. anthracis* gene regulation.

In *B. subtilis*, *abrB* negatively controls *atxA* expression while *spo0A* and *sigH* both positively affect *atxA* expression in an *abrB*-dependent manner (151). There are conflicting reports as to whether SigH controls *atxA* transcription independent of AbrB in *B. anthracis*. Hadjifrangiskou *et al.* (68) demonstrated that *atxA* expression requires *sigH* and that SigH positively controls *atxA* transcription in an *abrB*-independent manner. The *atxA* promoter does not contain a consensus sequence for SigH; therefore, it is suggested that SigH indirectly activates *atxA* transcription. Alternatively, it has been proposed that the *B. anthracis* and *B. subtilis* SigH proteins are functionally different and that the *B. anthracis* SigH recognizes a non-canonical consensus sequence. In contrast, Bongiorno *et al.* (14) showed that *atxA* transcription does not require SigH and that *atxA* promoter activity is unaffected by deletion of *sigH*. The lack of agreement between results obtained in *B. anthracis* and the use of different *B. anthracis* strain backgrounds suggests that the SigH phenotype is strain-dependent.

The activity of AbrB, Spo0A, and SigH is growth phase dependent; therefore, growth condition impacts their function. Spo0A is the master response regulator for the initiation of

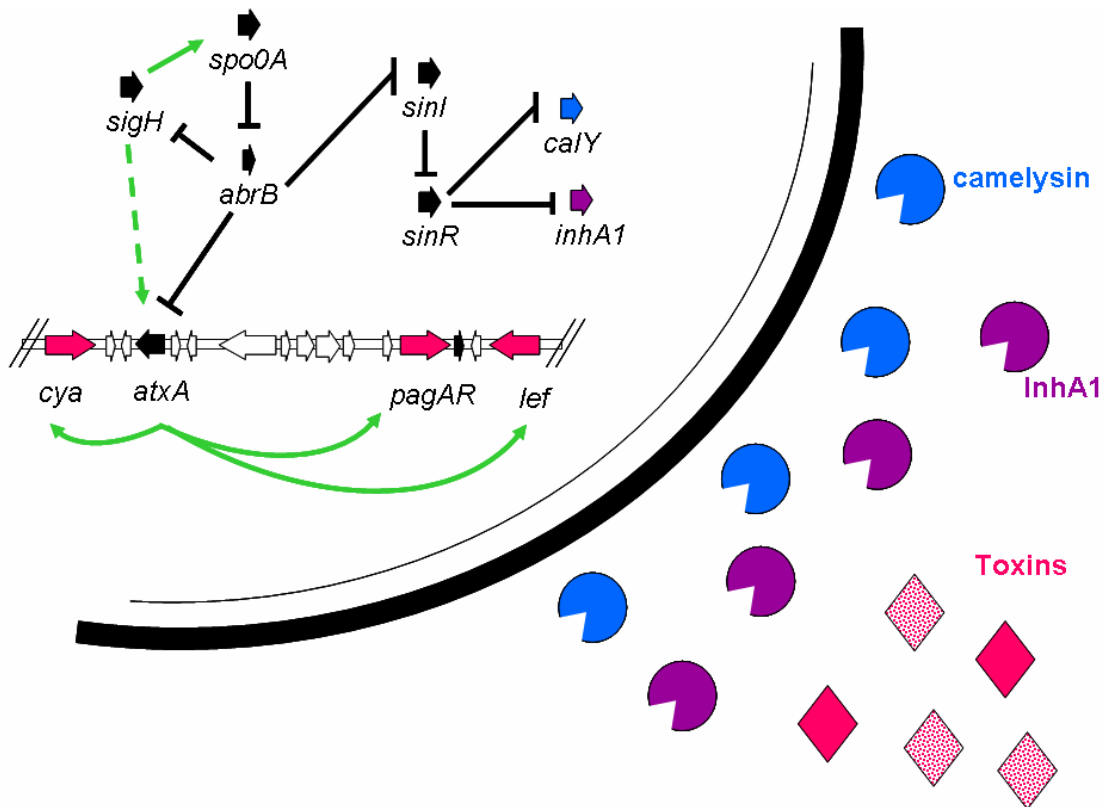
sporulation and is activated when conditions are conducive for sporulation (142). Strauch *et al.* (151) examined *PatxA-lacZ* expression in *B. subtilis* cells cultured in conditions conducive for sporulation (Schaeffer's sporulation medium) when Spo0A can actively repress *abrB*. Therefore, deletion of *B. subtilis sigH* reduced transcription of *spo0A* enabling increased repression of *PatxA-lacZ* by AbrB. This *sigH*-null phenotype was not apparent when Bongiorno *et al.* (14) determined *PatxA-lacZ* expression levels in *B. anthracis* likely because the culture conditions used were not conducive for sporulation. Instead, Bongiorno *et al.* (14) cultured *B. anthracis* in either LB/air or R medium containing 0.8% dissolved bicarbonate and an atmosphere of 5% CO<sub>2</sub>. The later growth condition is physiologically significant for a bacterial pathogen and is thought to model the host environment. Hadjifrangiskou *et al.* (68) also used growth conditions thought to model growth within a host (CACO<sub>3</sub> + 5% CO<sub>2</sub>); however, *atxA* expression required *sigH*. The slight variation in media composition and/or the difference in *B. anthracis* strain could explain the discrepancy with the Bongiorno *et al.* (14) results regarding SigH control of *atxA* transcription.

Here I showed that the established AbrB/Spo0A/SigH model for *atxA* regulation did not take into account factors on pXO2 that could control AtxA expression. Single and double deletions of *abrB*, *spo0A*, and *sigH* resulted in different AtxA expression levels depending on *B. anthracis* strain background. *abrB* had a more pronounced negative impact on AtxA production in the genetically complete Ames versus genetically incomplete ANR-1 strain background. *sigH* and *spo0A* appeared to be strong positive regulators of AtxA production in ANR-1 whereas they had little to no effect on AtxA expression in Ames. The only difference between ANR-1 and Ames is the presence of pXO2. I previously showed that deletion of *pXO2-61* resulted in decreased AtxA expression suggesting that *pXO2-61* positively impacts *atxA* by an unknown mechanism (see section 4.2.5). The data I have presented herein and the results I showed in Chapter 4 indicate that factors on pXO2

impact AtxA expression. It is becoming increasingly apparent that future studies determining the molecular mechanisms controlling *atxA* expression must be employed in genetically complete *B. anthracis* strain backgrounds.

In this chapter, I have also demonstrated that anthrax toxin production does not directly correlate with AtxA expression levels in the genetically complete Ames isogenic developmental regulator mutants. Pflughoeft *et al.* (120) reported previously that one of the most abundant proteins in the secretome of *B. anthracis* is the zinc metalloprotease InhA1 (immune inhibitor A1) which targets the anthrax toxin proteins (Fig. 5-8). An *inhA1*-null mutant displays increased and extended production of PA, LF, and EF (120). InhA1 levels are controlled by the transition state regulator SinR, and the SinR antagonist SinI. SinR represses *inhA1* transcription during exponential growth. As a culture transitions into stationary phase, SinI protein levels accumulate due to relieved repression by AbrB (7, 121, 134). This results in SinI interaction with SinR and relieved repression of *inhA1*. Once expressed, InhA1 is post-translationally regulated by the protease camelysin. In a genetically incomplete strain of *B. anthracis*, there is an inverse relationship between camelysin and InhA1 production, as camelysin levels increase InhA1 levels decrease (120). The regulation of *calY* and *inhA1* has not been extensively studied in a genetically complete *B. anthracis* strain. Therefore, the impact deletion of the developmental regulators *abrB*, *spo0A*, and *sigH* ultimately has on camelysin and InhA1 expression levels is unknown. Furthermore, the unexpected increases and decreases in secreted PA, LF, and EF could be a result of indirect camelysin and InhA1 misregulation by AbrB.

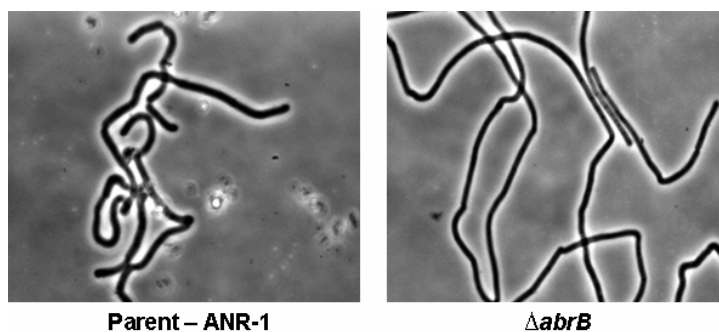
Finally, I showed that an ANR-1 *abrB*-null mutant was more virulent than parent and an ANR-1 *atxA*-up mutant. Both the *abrB*-null and *atxA*-up mutants produced elevated levels of AtxA and anthrax toxin when cultured *in vitro*. The impact of altered AtxA levels on virulence has not been examined previously. Rather, previous investigations have focused



**Figure 5-8. Model for post-translational regulation of the anthrax toxins proteins by secreted proteases when cultured in toxin-inducing conditions.** The structural genes for anthrax toxin, *cya*, *pagA*, and *lef*, are positively controlled by AtxA. The expression level of AtxA is controlled by the developmental regulators AbrB, Spo0A, and SigH which in turn regulate each others expression in addition to multiple other genes. AbrB controls SinI/R which regulate *calY* (camelysin) and *inhA1* (InhA1) production. In the culture supernatant, camelysin and InhA1 are capable of degrading the anthrax toxins. Solid shapes represent active proteins while spotted shapes signify cleaved (inactive) proteins.

on the virulence of *B. anthracis* strains defective for AtxA or anthrax toxin production (30-32, 36, 74, 93, 95, 168). Overexpression of AtxA and anthrax toxins did not correlate with the severity of anthrax disease. The increased virulence exhibited by the *abrB*-null mutant and lack of enhanced virulence in the *atxA*-up mutant indicates that AbrB is affecting other factors that are influencing pathogenesis. In batch culture, an *abrB*-null mutant produces longer chains of bacilli than parent or an *atxA*-up mutant (Fig. 5-9). Glomski *et al.* (58) have reported previously that tail vein injections of *B. anthracis* 'long bacteria' (approximately 20µm in length) were detected solely in the lungs of mice and resulted in rapid mortality. We did not examine the dissemination pattern or histopathology of *B. anthracis* infected mice. Therefore, it is possible that an *abrB*-null mutant leads to obstructive morphologies within the host. Future studies examining the histopathology of an *abrB*-null infected mouse would be valuable for understanding the mechanism by which an *abrB*-null mutant enhances *B. anthracis* virulence.





**Figure 5-9. Extended chaining phenotype of an  $\Delta abrB$  mutant.** Phase contrast microscopy showing *B. anthracis* parent and  $\Delta abrB$  strains in toxin-inducing batch culture conditions ( $\text{CACO}_3 + 5\% \text{CO}_2$ ). These data are representative micrographs. Microscopy performed by Prabhat Dwivedi, Ph.D.

## **Chapter VI**

### **Discussion**

*B. anthracis* is an endemic soil bacterium that has adapted to two different lifestyles: a nonpathogenic lifestyle of survival and saprophytic growth in the soil, and a pathogenic lifestyle of inhabiting mammalian hosts. The developmental characteristics of *B. anthracis* enable the bacterium to exist as an environmentally resistant spore in the soil, and a vegetative cell that produces essential virulence factors within the host. *B. anthracis* senses and responds to environment-specific signals to facilitate adaptation and growth in different conditions. These signals elicit responses such as production of the virulence factors, anthrax toxin and capsule, and development into dormant spores. My work is the first to demonstrate that in batch culture conditions there is an inverse relationship between *B. anthracis* toxin production and sporulation. In addition, my studies are the first to reveal that cellular development, like toxin production, is affected by the regulatory protein AtxA.

Using different batch culture conditions, I modeled physiologically relevant conditions encountered by *B. anthracis* during infection (toxin-inducing conditions), and environmental conditions in the soil (sporulation conditions). My data indicate that in specific culture conditions, there is an AtxA-associated inverse relationship between toxin production and sporulation. In conditions that favor sporulation, *B. anthracis* sporulates but produces little to no AtxA and LF. In conditions that are not conducive for sporulation, *B. anthracis* does not sporulate but produces AtxA and LF (Fig. 4-1). Furthermore, overexpression of AtxA results in a marked sporulation defect (Fig. 4-2) which in combination with the above data implicates the master virulence regulator AtxA in *B. anthracis* spore development. Moreover, the inverse expression of AtxA in toxin-inducing versus sporulation conditions strongly suggests that *atxA* is differentially regulated by *trans*-acting factors in each culture condition.

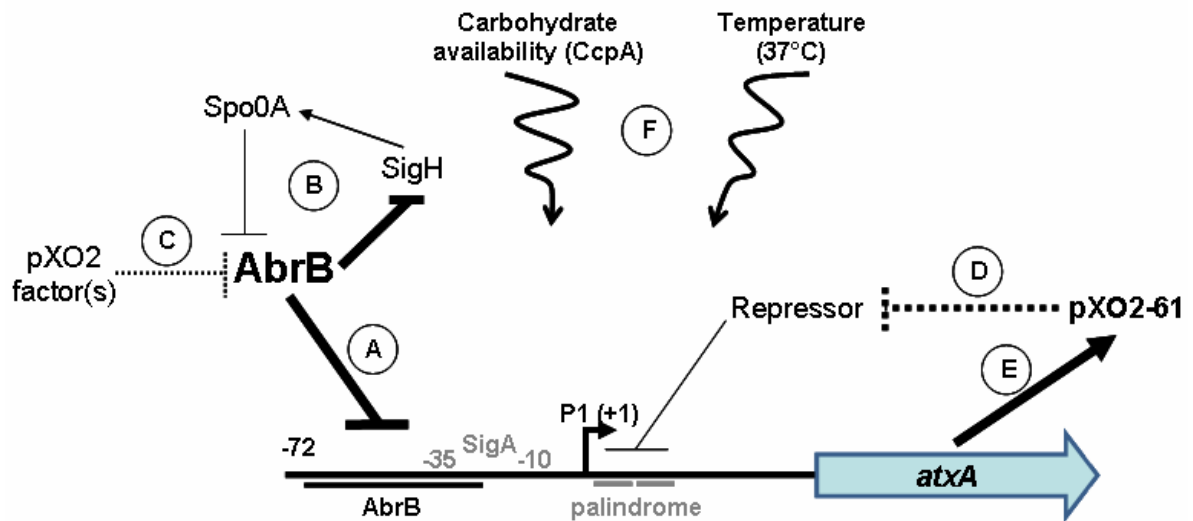
The only *trans*-acting factor shown to directly bind to the *atxA* promoter repressing transcription is the transition state regulator AbrB. Here I demonstrate that an additional *trans*-acting factor(s) other than AbrB binds specifically to the *atxA* promoter region to

negatively impact *atxA* expression (Fig. 3-3 and Fig. 3-4B). In this discussion, I refer to the unidentified *trans*-acting factor(s) that controls *atxA* transcription as the “*atxA* repressor protein(s)”. Based on my work, I consider a model in which *B. anthracis* requires AbrB and the *atxA* repressor protein(s) to differentially regulate *atxA* transcription in two distinct environments. I propose that AbrB controls *atxA* in toxin-inducing conditions whereas the *atxA* repressor protein(s) predominately regulates *atxA* transcription in sporulation conditions. Since the transcriptional regulation of *atxA* is complex and involves multiple signals and *trans*-acting factors that function in growth condition- and strain-dependent manners, the discussion is organized in different sections. These sections explain *atxA* regulation in each culture condition, the potential impact of *atxA* repressor protein(s) binding on AbrB interaction with the *atxA* promoter region, and the affect of *AtxA* overexpression on virulence. There is partial overlap between regulators in each culture condition; therefore, some repetition occurs in each section.

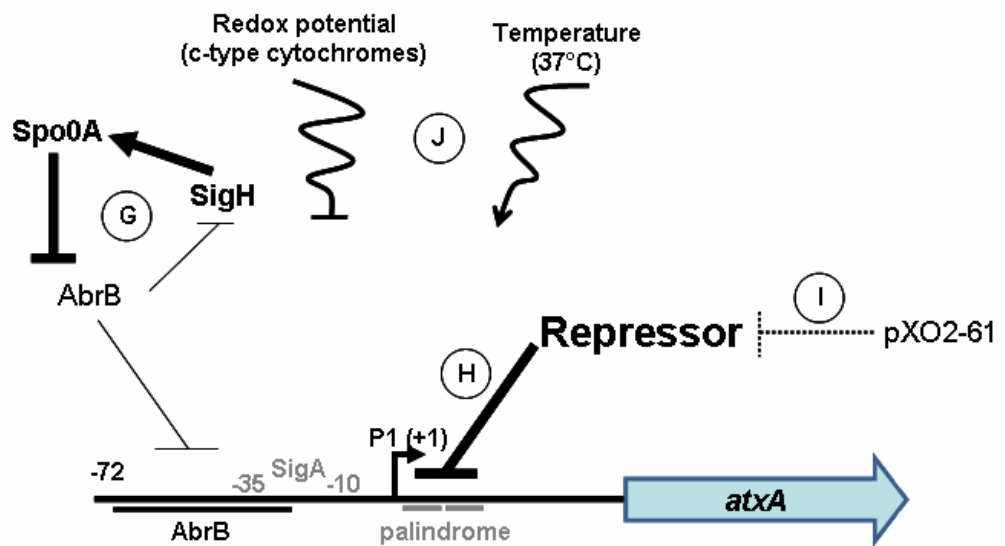
### **Regulation of *atxA* in toxin-inducing conditions (CACO<sub>3</sub> + 5% CO<sub>2</sub>)**

The most critical factor controlling *atxA* expression in toxin-inducing conditions is the transition-state regulator AbrB. AbrB represses *atxA* transcription by binding to a 43-bp region located upstream of the P1 transcription start site (151) (Fig. 6-1A). *atxA* transcripts are relatively low during exponential phase indicating that AbrB does not completely repress transcription. As the culture approaches stationary phase of growth, AbrB protein levels decrease (110) and *atxA* transcripts increase (131). Extensive studies in the archetype *Bacillus* species, *B. subtilis*, have revealed that AbrB is a DNA binding protein that controls several post-exponential phase genes associated with metabolic and physiological processes (121, 150). One of the direct targets of AbrB is the alternative sigma factor gene *sigH*. AbrB, SigH, and the master response regulator Spo0A are all part of a feedback loop, as depicted in Fig. 6-1B. Transcription of *sigH* is repressed by AbrB,

## Toxin-inducing conditions



Sporulation conditions
------------------------



**Figure 6-1. Model for regulation of *atxA* gene expression in toxin-inducing and sporulation conditions.** The developmental regulators AbrB, Spo0A, and SigH regulate *atxA* transcription in a condition-dependent manner. In toxin-inducing conditions, AbrB binds to a region upstream of P1 to actively represses *atxA* expression (A), whereas in sporulation conditions, AbrB plays a minor role in control of *atxA* (G). Data suggest that factors on pXO2 control AbrB protein levels (C). In toxin-inducing conditions, *atxA* positively controls *pXO2-61* transcription (E). In sporulation conditions, the *atxA* repressor protein (referred to as ‘Repressor’ in the model) interacts with a palindromic sequence located downstream of P1 (H). Activity of the repressor is down-regulated by pXO2-61 (D and I). Additional signals impacting *atxA* expression are carbohydrate availability, temperature, and redox potential (F and J). Thick lines denote important *trans*-acting factors or signals controlling *atxA* expression in the given culture condition. Thin lines denote minimal impact. Hashed lines indicate suggested function. Curved lines/arrows represent indirect affects on *atxA* transcription.

SigH (with core RNAP) transcribes *spo0A*, and phosphorylated Spo0A represses *abrB*. *B. anthracis* contains orthologues of the developmental regulators. Therefore, it is inferred that the developmental regulators perform similar functions in *B. anthracis*. In large part, the expression of AbrB and SigH is controlled by the threshold level of active/phosphorylated Spo0A. Spo0A is phosphorylated by components of the sporulation phosphorelay that are activated in response to nutrient limitation. I propose that toxin-inducing conditions are not optimal for Spo0A phosphorylation; however, as nutrients are depleted during the transition into stationary phase of growth low levels of Spo0A~P repress *abrB*. Fujita *et al.* (53) showed that in *B. subtilis* Spo0A~P binds to the *abrB* promoter with relatively high affinity. Therefore, *abrB* repression by Spo0A~P requires a low threshold level of protein. In *B. anthracis*, this repression of *abrB* by Spo0A~P results in increased *atxA* transcription.

My work demonstrates that the AbrB/Spo0A/SigH model for regulation of *atxA* is strain-dependent. This model was initially determined based on results obtained from *B. subtilis* and *B. anthracis* Sterne-like (pXO1+, pXO2-) strains harboring transcriptional *atxA* promoter–*lacZ* fusions (14, 68, 131, 151). I examined *atxA* transcription in the Sterne-like strain ANR-1, which is more physiologically relevant than the previously used Sterne-like strains. ANR-1 is a pXO2-cured toxigenic, noncapsulated variant of the clinical isolate Ames that has a documented genomic background and is less of a lab strain. Using the ANR-1 parent and developmental regulator mutants, I determined variations exist in the AbrB/Spo0A/SigH model for regulation of *atxA* transcription. First, I observed a greater increase (3.4-fold) in *atxA* promoter activity than AtxA protein levels (2.8-fold) in the *abrB*-null mutant. Previously, AtxA protein levels were not quantified in an  $\Delta$ *abrB* mutant. Instead, it was assumed that AtxA protein levels reflected *atxA* promoter activity which increased approximately 4-fold among the different strain backgrounds (68, 131, 151). Nonetheless, anthrax toxin protein levels were elevated 2.1- to 6.9-fold in the  $\Delta$ *abrB* mutant

compared to parent. Second, the  $\Delta spo0A/abrB$  mutant exhibited a 3-fold increase in *atxA* promoter activity; however, there was a subtle 1.7-fold increase in AtxA protein levels which translated to a 1.7- to 3-fold increase in the anthrax toxin proteins.

After examination of *B. anthracis* mutant phenotypes in a genetically incomplete (pXO1+, pXO2-) strain background, it is common laboratory practice to determine if the given phenotypes 'hold true' in a genetically complete (pXO1+, pXO2+) strain background. Using the Ames strain that harbors pXO1 and pXO2, my results demonstrate that AbrB is the only developmental regulator to significantly affect *atxA* expression. Deletion of *abrB* resulted in a 7.9-fold increase in AtxA protein levels, and the  $\Delta spo0A/abrB$  mutant exhibited a 5.3-fold increase in expression of AtxA. The level of AtxA modestly increased or was unchanged in the single and double *sigH* and *spo0A* mutants. This result does not support the AbrB/Spo0A/SigH model of *atxA* regulation since deletion of *sigH* or *spo0A* should result in elevated AbrB and constant repression of *atxA*. In total, these results would suggest that additional factors other than SigH and Spo0A negatively control AbrB in a genetically complete strain background. I propose a model by which factors present on pXO2 negatively control AbrB in a *sigH*- and *spo0A*-independent manner (Fig. 6-1C). To test this model, a library of pXO2 genes could be introduced into a pXO2-cured *B. anthracis* strain harboring an *abrB* reporter construct to screen for *abrB* phenotypes. Transcriptional and translational *abrB* reporter constructs could be created to identify the level at which *abrB* is regulated by pXO2 factors. In addition, pXO2 could be analyzed for annotated regulatory genes that could serve as targets for directed gene deletion and examination into what role, if any, there is on AbrB expression. Interestingly, anthrax toxin production in the various Ames isogenic developmental mutants did not directly correlate with the level of AtxA which also suggests that factors on pXO2 are indirectly controlling *B. anthracis* anthrax toxin-specific proteases via control of AbrB (for details see section 5.3 and Fig. 5-



7). In total, these results would imply that factors on pXO2 are indirectly controlling both *atxA* expression and anthrax toxin levels.

Another line of evidence suggesting that factors on pXO2 regulate *atxA* expression is that mutation of the *atxA* repressor protein(s) binding site results in overexpression of AtxA in a genetically incomplete (ANR-1) and not genetically complete (Ames) strain background. Furthermore, AtxA protein levels decreased 4-fold in a  $\Delta pXO2-61$  mutant and were restored to parent levels in the double  $\Delta pXO2-61/atxA$ -up mutant. Transcriptional profiling experiments showed that *atxA* positively affects *pXO2-61* transcription (54-fold), and overexpression of *pXO2-61* was shown to negatively impact sporulation (18, 174). The affect of *pXO2-61* overexpression on sporulation will be detailed in the next section. Here I will focus on how the lack of *pXO2-61* negatively affects AtxA protein levels. I propose that pXO2-61 somehow represses or inhibits the activity of the *atxA* repressor protein(s) (Fig. 6-1D). Future experiments assessing the mechanism by which pXO2-61 controls AtxA expression might reveal a direct affect on the *atxA* repressor protein(s). Crude cellular extract obtained from an ANR-1 *B. anthracis* strain overexpressing *pXO2-61* could be used in EMSAs with a radioactive *PatxA* probe. A diminished DNA mobility shift would infer that pXO2-61 is necessary and sufficient for indirectly regulating *atxA* expression via the *atxA* repressor protein(s). No change in the DNA mobility shift would suggest that other factors on pXO2 are involved in negatively controlling the *atxA* repressor protein(s). Overall, these results underscore the importance of using a genetically complete strain of *B. anthracis* to investigate *atxA* regulation.

Other factors shown to influence *atxA* transcription in toxin-inducing conditions are temperature and the presence of glucose (carbohydrate availability) (Fig. 6-1F). In concordance with the significance of AtxA for *B. anthracis* pathogenesis, the optimal temperature for *atxA* transcription is 37°C (35). In the presence of glucose, *atxA* transcription is stimulated due to indirect activation by the carbon catabolite protein CcpA

(32). The mechanism by which temperature and CcpA positively control *atxA* transcription is not clear.

### **Regulation of *atxA* in sporulation conditions (PA-air)**

The most critical regulator of *atxA* expression in sporulation conditions is the *atxA* repressor protein(s). Sequences within a 9-bp palindrome located immediately downstream of the *atxA* P1 transcription start site are critical for *atxA* repressor protein(s) binding and negative regulation of *atxA* transcription (Fig. 6-1H). Unlike in toxin-inducing conditions, AbrB plays little to no role in the regulation of *atxA* transcription in sporulation conditions. *atxA* transcript levels decrease approximately 3- to 4-fold in sporulation conditions and are unaffected by the deletion of *abrB* (Hadjifrangiskou, M. and J.L. Dale unpublished). This correlates with the fact that *abrB* transcripts are low in sporulation conditions as a result of increased threshold levels of Spo0A~P binding to the *abrB* promoter region repressing transcription (Fig. 6-1G).

One of the most intriguing phenotypes associated with mutation of sequences within the 9-bp palindrome repressor binding site, was the pXO2-dependent marked decrease in sporulation that resulted due to overexpression of AtxA. The sporulation defect was suppressed by deletion of *pXO2-61*. The pXO2-61 protein is homologous to the sensor domain of sporulation sensor histidine kinases which are key components of the sporulation phosphorelay. White *et al.* (174) demonstrated that overexpression of this highly AtxA-regulated gene led to a sporulation defect. I took those results one step further and showed that misregulation of *atxA* resulted in the physiologically significant developmental defect due to elevated *pXO2-61* transcription. Since the typical transcript level of *pXO2-61* is low in sporulation conditions, I did not observe any differences in AtxA protein levels in a  $\Delta pXO2-61$  mutant compared to parent. This suggests that *pXO2-61* has a minimal impact on the *atxA* repressor protein(s) in sporulation conditions unlike the observed impact *pXO2-*

61 had on AtxA levels in toxin-inducing conditions (Fig. 6-1I). Previous publications have alluded to AtxA or factors on pXO1 having an impact on *B. anthracis* development based on indirect evidence (75, 102, 158). To my knowledge, my data is the first work directly demonstrating that AtxA modulates spore development.

The mechanism by which overexpression of *pXO2-61* negatively impacts sporulation is not clear. White *et al.* (174) determined that the marked sporulation defect observed in a *pXO2-61* overexpression mutant was BA2291-dependent. BA2291 is one of the major sporulation sensor histidine kinases that initiates the sporulation phosphorelay in *B. anthracis* (21). It has been proposed that pXO2-61 is capable of titrating away an activating signal from BA2291 that results in BA2291 conversion to a phosphatase of Spo0F, a key sporulation phosphorelay transducer protein. Recently, Eswaramoorthy *et al.* (48) demonstrated that the major sporulation sensor histidine kinase in *B. subtilis*, KinA, is activated by multimerization and not detection of a sporulation-specific signal. There are no orthologues of BA2291 in *B. subtilis*; however, it would be interesting to determine if one of the *B. anthracis* major sporulation sensor histidine kinases (i.e. BA2291) is capable of forming homo- or hetero-complexes. Furthermore, affinity purification pull-down experiments using pXO2-61 could determine if and/or what factors associate with the protein to inhibit sporulation when overproduced.

Other factors affecting *atxA* transcription in sporulation conditions include redox potential and temperature. A relationship between redox potential and *atxA* expression was demonstrated when genes involved in cytochrome c maturation were deleted. Deletion of two small c-type cytochromes resulted in early and increased transcription of *atxA* that was transient and indirect (175). Similar to toxin-inducing conditions, *atxA* transcription is optimal at 37°C. The mechanisms by which the small c-type cytochromes and temperature affect *atxA* transcription remain elusive.

### **The *atxA* repressor protein(s) and AbrB bind the *atxA* promoter region independently**

My data indicate that nucleotides within the palindromic sequence (+14 to +22), located downstream of the P1 transcription start site, are required for the *atxA* repressor protein(s) to interact with the *atxA* promoter region. The presence of a *trans*-acting factor binding site downstream of a transcription start site is not uncommon. In both *B. subtilis* and *B. anthracis*, the *abrB* promoter contains two tandem 'OA' boxes for Spo0A recognition located downstream of the transcription start site from sequences +7 to +34, relative to the *abrB* P2 start site (147, 151). Binding of Spo0A to the *abrB* promoter negatively regulates gene transcription (119, 147, 154). Another similarity between the *atxA* and *abrB* promoter regions is the presence of AbrB binding sites upstream of the transcription start sites. AbrB represses *atxA* transcription by binding to a 43-bp region located 25 to 67 bp upstream of the P1 transcription start site (151). The *abrB* promoter autoregulates its own expression and contains both a high-affinity (-14 to -43) interaction region and contiguous low-affinity (-44 to approximately -120) binding region for AbrB (154). Strauch *et al.* (147) demonstrated that the separation between the AbrB and Spo0A binding sites within the *abrB* promoter region resulted in independent binding of each *trans*-acting factor to its cognate DNA sequence. Even though AbrB binds to its target DNA sequence based on DNA structure and curvature, the conformational change caused by Spo0A binding did not alter the binding of AbrB. Based on these results, I propose a model whereby the *atxA* repressor protein(s) binds to the *atxA* promoter in an *abrB*-independent manner. Future investigations establishing the epistatic and biochemical relationship between AbrB and the *atxA* repressor protein(s) could determine if the proteins function in a cooperative or competitive manner to control *atxA* expression.

The *atxA* repressor protein(s) must first be identified in order to determine epistatic and biochemical relationships with AbrB. In the course of my studies, I was unable to determine the identity of the *atxA* repressor protein(s). However, in my attempts to purify

and identify the *atxA* repressor protein(s) from *B. anthracis* crude cellular extract I obtained knowledge regarding the general characteristics of the *atxA* repressor protein(s). Using biochemical methods of purification, I determined that the *atxA* repressor protein(s) could stably bind a cation exchange column and elute off the column using 496-696 mM NaCl. The methods used for partial purification of the *atxA* repressor protein(s) revealed that the pI of the protein(s) is  $\geq 5.6$ . In addition, the *atxA* repressor protein(s) was shown to be unstable; freeze/thaw cycles resulted in loss of DNA-binding activity. Based on these preliminary results, future work should focus on using biochemical methods of purification including the use of a high-resolution cation exchange column and elution gradients within the range of 496-696 mM NaCl. The semi-enriched cellular extract can further be used with affinity purification methods and an *atxA* promoter concatamer containing the minimal repressor binding site to “pull-down” and identify the *atxA* repressor protein(s). Once identified, purified *atxA* repressor protein(s) could be used in DNase I footprinting experiments with AbrB to determine if the binding of one protein affects interaction of the other protein for binding to the *atxA* promoter region.

### **Impact of increased AtxA expression on virulence**

An  $\Delta atxA$  mutant is severely attenuated for virulence in a murine model for anthrax disease demonstrating the necessity of *atxA* for pathogenesis (36) (Fig. 5-6). Several researchers have investigated the impact of decreased *atxA* and anthrax toxin production on virulence (30-32, 36, 74, 93, 95, 168). However, no one has investigated the consequence of increased AtxA and anthrax toxin production on virulence. For the first time, I had the opportunity to determine if elevated AtxA and anthrax toxin production affected virulence of *B. anthracis* in a murine model of anthrax disease. Surprisingly, my results showed that an *atxA*-up mutant which produces 6.6-fold more AtxA than the parent strain did not exhibit enhanced virulence whereas the  $\Delta abrB$  mutant which produces a

modest 1.4-fold increase in AtxA was more virulent. It is possible that a specific and critical level of *atxA* is required for optimal expression of the toxin genes, or other *atxA*-regulated genes, *in vivo*. Overexpression of anthrax toxins does not correlate with the severity of disease; therefore, it is also possible that too much toxin production is problematic for virulence. Alternatively, it is possible that there is another *abrB*-controlled phenotype such as cell length that is important for virulence. An  $\Delta$ *abrB* mutant produces extremely long chains of bacilli in batch culture (Fig. 5-9). Glomski *et al.* (58) have reported previously that the size of bacteria in the inoculating culture influences the dissemination of bacteria to the lungs and other organs. 'Long bacteria' (approximately 20 $\mu$ m in length) were detected solely in the lungs of mice and resulted in rapid mortality when injected via the tail vein. Future histopathology experiments on mice infected with the  $\Delta$ *abrB* mutant could help determine if the mutant strain produces 'long bacteria' *in vivo*. Furthermore, assessing the dissemination of an  $\Delta$ *abrB* mutant could reveal if an altered dissemination pattern is attributed to the virulence phenotype.

### **Relationships between bacterial development and toxin production in other pathogens**

Links between signals and regulators controlling sporulation and virulence gene expression have also been found in other bacterial pathogens. Another member of the Firmicute phylum, *Clostridium difficile*, is a spore-forming, pathogenic bacterium that causes infections ranging from mild diarrhea to fatal pseudomembranous colitis. The spore constitutes the etiological form of *C. difficile* infections and once ingested can germinate and colonize the gut where it produces toxins (4, 100). Hypervirulent strains of *C. difficile* have become an emerging problem in hospitals and the mechanism(s) leading to hypervirulence is being investigated by several laboratories. There are conflicting reports regarding a potential relationship between sporulation and virulence in this species.

Akerlund *et al.* (4) reported an inverse correlation between *C. difficile* toxin yield and sporulation. These results support the author's hypothesis that toxin production and sporulation represent alternative survival strategies for *C. difficile* in nutrient depleted conditions. In contrast, a recent report by Merrigan *et al.* (100) demonstrates that hypervirulent clinical isolates of *C. difficile* sporulate earlier and produce more spores than non-hypervirulent strains. These results suggest a relationship exists between sporulation and toxin production. Discrepancies between the two reports could be a result of different culturing methods and media. Nonetheless, further research is required to determine if there is a relationship between *C. difficile* toxin production and development.

A more closely related species to *B. anthracis*, *B. thuringiensis*, is also a developmental pathogen that produces secreted virulence factors. The pathogenic properties of *B. thuringiensis* are in large part attributed to expression of the pleiotropic virulence regulator PlcR (see sections 1.4 and 1.5). The *plcR* gene is repressed by Spo0A, the master response regulator for sporulation initiation, suggesting a relationship between development and virulence factor synthesis. Nevertheless, overexpression of *plcR* in *B. thuringiensis* has no negative effect on sporulation (102). Interestingly, *B. anthracis* does not possess a functional *plcR* gene, but introduction and overexpression of the *B. thuringiensis plcR* gene results in a marked sporulation defect. The *B. anthracis* sporulation defect is rescued upon deletion of *atxA* (102). These results indicate that *plcR* is negatively controlling sporulation in an *atxA*-dependent manner and that *atxA* is the important factor contributing to the developmental defect. The exact mechanism by which simultaneous expression of *plcR* and *atxA* is inhibitory for *B. anthracis* sporulation is not understood.

Clearly, potential links between development and virulence gene expression in other bacteria are worthy of exploration. With the advent of methods such as RNA-seq and Tn-seq, facile identification of genes required for bacterial growth and adaptation to different environments is possible (115, 167). These methods could help elucidate common

regulators within a species of pathogenic bacteria involved in the control of survival within and outside the host. In addition, using methods like RNA-seq and Tn-seq on *B. anthracis* grown in toxin-inducing versus sporulation conditions could help define additional factors that are part of the regulatory network governing *atxA* expression in each culture condition.

### **Concluding remarks**

Regulation of *atxA* is the subject of investigation by several laboratories with the main focus of identifying signals and *trans*-acting factors responsible for *atxA*-regulated anthrax toxin and capsule production. My work is the first to demonstrate that AtxA is not only a master virulence regulator of the anthrax toxins and capsule, but also modulates spore development. This work demonstrates that *B. anthracis* senses and responds to its local environment by controlling the expression level of *atxA* using different culture condition-dependent regulators (AbrB and *atxA* repressor protein(s); Fig. 6-1). I showed that a *trans*-acting factor(s) other than AbrB, the *atxA* repressor protein(s), binds specifically to the *atxA* promoter region and negatively controls transcription. Furthermore, mutation of the repressor binding site results in a significant sporulation defect that is *atxA*- and *pXO2-61*-dependent. These results suggest that *B. anthracis* has developed a control mechanism that involves AtxA regulation of *pXO2-61* as an adaptive measure for continued growth within or outside the host environment. Finally, my data demonstrate that pXO2 negatively impacts AtxA protein levels in specific growth conditions and affects anthrax toxin production independent of *atxA*. These results demonstrate the importance of using a genetically complete *B. anthracis* strain to determine all the regulatory factors controlling *atxA* and toxin gene expression. Overall, this work expands on the complex regulatory networks governing control of *atxA* and demonstrates the impact of AtxA expression on *B. anthracis* pathogenesis and development.



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