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## Gene Regulation Pathways Affect Toxin Gene Expression, Sporulation and Pigment Generation in Bacillus Anthracis and Bacillus Cereus

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GENE REGULATION PATHWAYS AFFECT TOXIN GENE EXPRESSION,  
SPORULATION AND PIGMENT GENERATION IN *BACILLUS ANTHRACIS* AND  
*BACILLUS CEREUS*

by

HESONG HAN

Under the Direction of Eric Gilbert, PhD

ABSTRACT

*B. anthracis* alters its virulence gene expression profile in response to a number of environmental signals, including levels of bicarbonate and CO<sub>2</sub>. Virulence plasmid pXO1 is important to *Bacillus anthracis* pathogenicity as it carries the genes encoding the anthrax toxin and virulence regulatory factors. Induction of toxin and other virulence genes requires the pXO1-encoded AtxA regulatory protein. The cytochrome *c* maturation system influences the expression of virulence factors in *Bacillus anthracis*. *B. anthracis* carries two copies of the *ccdA* gene, encoding predicted thiol-disulfide oxidoreductases that contribute to cytochrome *c* maturation. Loss of both *ccdA* genes results in a reduction of cytochrome *c* production, an increase in virulence factor expression, and a reduction in sporulation efficiency. pXO1 also carries a gene encoding an Hfq-like protein, *pXO1-137*. Loss of *pXO1-137* results in significant growth defects and reductions in toxin gene expression only when grown under toxin inducing conditions. Similarly, loss of a small RNA on pXO1, *sRNA-1*, results in similar growth defects and reductions in toxin gene production. Both increased and decreased expression of *pXO1-137* and *sRNA-1* result in growth defects suggesting narrow functional set points for Hfq and sRNA levels.

INDEX WORDS: toxin gene, cytochrome *c*, sporulation, sRNA, Hfq

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by

HESONG HAN

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2017

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## **DEDICATION**

This paper is dedicated to my family and friends who supported me through this meaningful yet hard journey.

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# 1 CHAPTER ONE INTRODUCTION AND PURPOSE

## 1.1 Virulence and sporulation regulation in *Bacillus anthracis* and *Bacillus cereus*

*Bacillus anthracis* is a Gram-positive, endospore-forming bacterium that is the etiological agent of anthrax (1). Several virulence factors, including the anthrax toxin and capsule, contribute to virulence in the mammalian host. Expression of both the toxin and capsule genes is dependent upon the master virulence regulator AtxA. Loss of *atxA* strongly decreases transcription of toxin and capsule genes and renders *B. anthracis* avirulent in animal models of infection (2). The direct interaction between AtxA and the promoters of its target genes has not been demonstrated (Fig 1), and the mechanism by which AtxA regulates its target genes remains unclear (3).

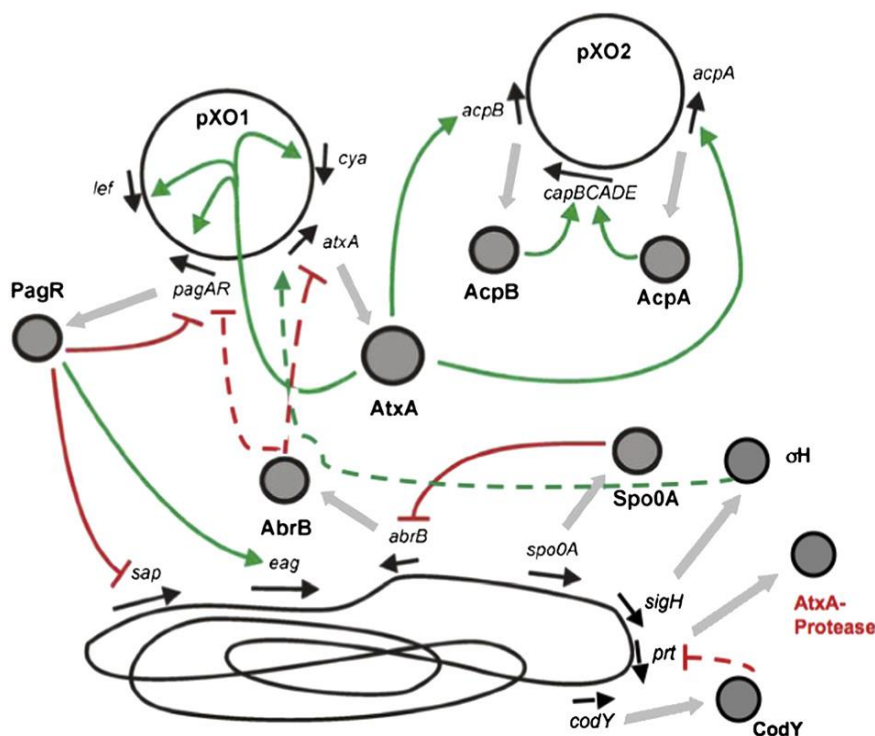


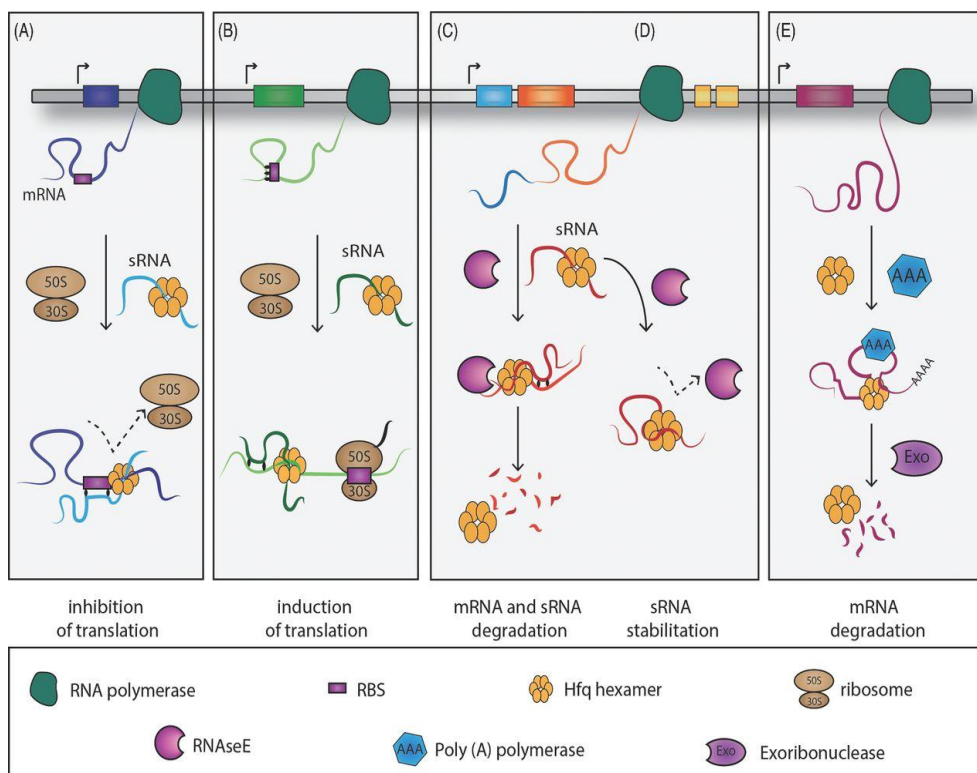
Figure 1.1 *B. anthracis* regulatory networks involving AtxA.

Schematic representation of *B. anthracis* regulatory networks involving AtxA. 0923-2508see front matter © 2010 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved. doi:10.1016/j.resmic.2010.09.006

We recently reported that loss of the two small *c*-type cytochromes of *B. anthracis*, *c<sub>550</sub>* and *c<sub>551</sub>*, leads to increased transcription of *atxA* and increased production of AtxA- regulated virulence factors(4). These observations led us to further investigate the roles of *c*-type cytochromes and cytochrome *c* maturation pathways in *B. anthracis* physiology and virulence. While little is known about cytochrome production and activity in *B. anthracis*, our understanding is informed by findings in the closely related organism, *Bacillus subtilis*. The thiol-disulfide oxidoreductase protein CcdA is required for cytochrome *c* maturation in *B. subtilis* (5). In the *B. anthracis* genome, there are two genes predicted to encode CcdA-like proteins. It is unclear why *B. anthracis* carries two copies of *ccdA* when *B. subtilis* can carry out both efficient sporulation and *c*-type cytochrome production with a single copy of *ccdA*.

*Bacillus cereus* is a Gram-positive, spore-forming, soil-dwelling bacterium that can be pathogenic in humans (6). *B. cereus* is most often observed as a causative agent of food poisoning but is also responsible for a wide range of opportunistic and nosocomial infections (7). Despite high degree of genetic similarity between *B. cereus* and *B. anthracis*, the molecular mechanisms of pathogenesis are dissimilar. Pathogenesis of *B. cereus* relies on a number of hemolytic and non-hemolytic enterotoxins controlled by the quorum-sensing regulatory system PlcR-PapR (Fig 2), all encoded by genes on the main bacterial chromosome (8). It is unclear that whether *c*-type cytochromes and cytochrome *c* maturation pathways have any effect on toxin gene expression and sporulation in *B. cereus*. Virulence plasmid pXO1 is important to *Bacillus anthracis* pathogenicity as it carries the genes encoding the anthrax toxin and virulence regulatory factors. *B. anthracis* alters its virulence gene expression profile in response to a number of environmental signals, including levels of bicarbonate and CO<sub>2</sub> (12). Induction of toxin and other virulence genes requires the pXO1-encoded AtxA regulatory protein. pXO1 also

carries a gene encoding an Hfq-like protein, pXO1-137, and a small RNA, sRNA1. Loss either of these two genes results in significant growth defects and reduction in toxin gene expression under toxin inducing conditions. While the Hfq protein-sRNA system is well established in *E. coli*, it has never been reported how this system affects toxin gene expression in *B. anthracis* (Fig 2) (13).



*Figure 1.2 Mechanisms of RNA-binding protein-Hfq.*

Schematic representation of the mechanisms of action employed by the RNA-binding protein, Hfq. ©C FEMS 2015. All rights reserved DOI: 10.1093/femsre/fuv022

## 1.2 Pigment generation in *Bacillus anthracis*

Production of melanin pigments contributes to microbial pathogenesis, as it is associated with virulence in many microorganisms (9). Pyomelanin is a melanin-like pigment produced by the spontaneous oxidation of homogentisic acid. Homogentisic acid is an intermediate in the pathway catabolizing L-tyrosine and L-phenylalanine to acetoacetate and fumarate in many

species. Homogentisic acid normally does not accumulate in significant amounts as it is converted to maleylacetoacetate by the activity of homogentisate 1, 2-dioxygenase. In the absence of homogentisate 1, 2-dioxygenase, homogentisic acid accumulates in the cell and spontaneously oxidizes to pyomelanin, a water-soluble brown pigment (10). Pyomelanin is also known as alkaptomelanin in the human genetic disorder alkaptonuria, in which L-tyrosine catabolism is disrupted by the loss of human homogentisate 1, 2-dioxygenase, resulting in the accumulation of damaging brown pigment in tissues (11). In *B. anthracis*, loss of *hmgA*, encoding homogentisate 1, 2-dioxygenase, results in accumulation of a melanin-like pigment.

### **1.3 Specific aims and hypotheses**

#### **Specific Aim 1: What are the functions of CcdA1 and CcdA2?**

#### **Hypothesis: CcdA1 and CcdA2 would alter toxin gene expression and sporulation.**

In *B. subtilis*, CcdA is required for *c*-type cytochrome maturation (Fig 3). CcdA accepts electrons from thioredoxin in the cytoplasm and transfers the electrons to ResA at the cell surface. Reduced ResA can then reduce a disulfide linkage on apo-cytochrome *c*, facilitating covalent attachment of heme and production of functional *c*-type cytochromes (14). It also is involved in spore generation by targeting StoA, another thiol-disulfide oxidoreductase which plays an important role in forespore formation (15). Examination of the *B. anthracis* genome reveals the presence of two *ccdA*-like genes. BAS1647 (GenBank: AAT53964.1) encodes a protein with 65% amino acid similarity relative to *B. subtilis ccdA* (GenBank: CAB13677.1). BAS3455 (GenBank: AAT55761.1) encodes a protein with 68% amino acid similarity relative to *B. subtilis ccdA*. BAS1647 and BAS3455 share 63% amino acid similarity. We will refer to BAS1647 as *ccdA1* and BAS3455 as *ccdA2*. The function of CcdA1 and CcdA2 is unknown, but

giving the similarity of *B. anthracis* and *B. subtilis*, it is safe to assume that two of them involved in cytochrome *c* maturation and sporulation pathway in *B. anthracis*. Since matured *c*-type cytochromes are associated with virulence expression in *B. anthracis*, CcdA1 and CcdA2 likely affect virulence gene expression. More interesting question would be why there are two copies of CcdAs in *B. anthracis*? Is it simply due to gene duplication? Or do they evolved to different pathways? Here, I will characterize CcdA1 and CcdA2, and investigate the functional similarity and difference between CcdA1 and CcdA2.

## c-type cytochrome maturation pathway

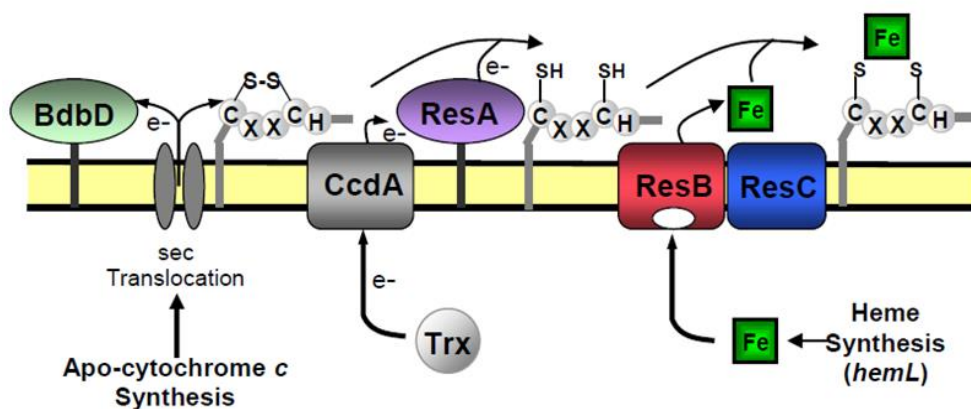
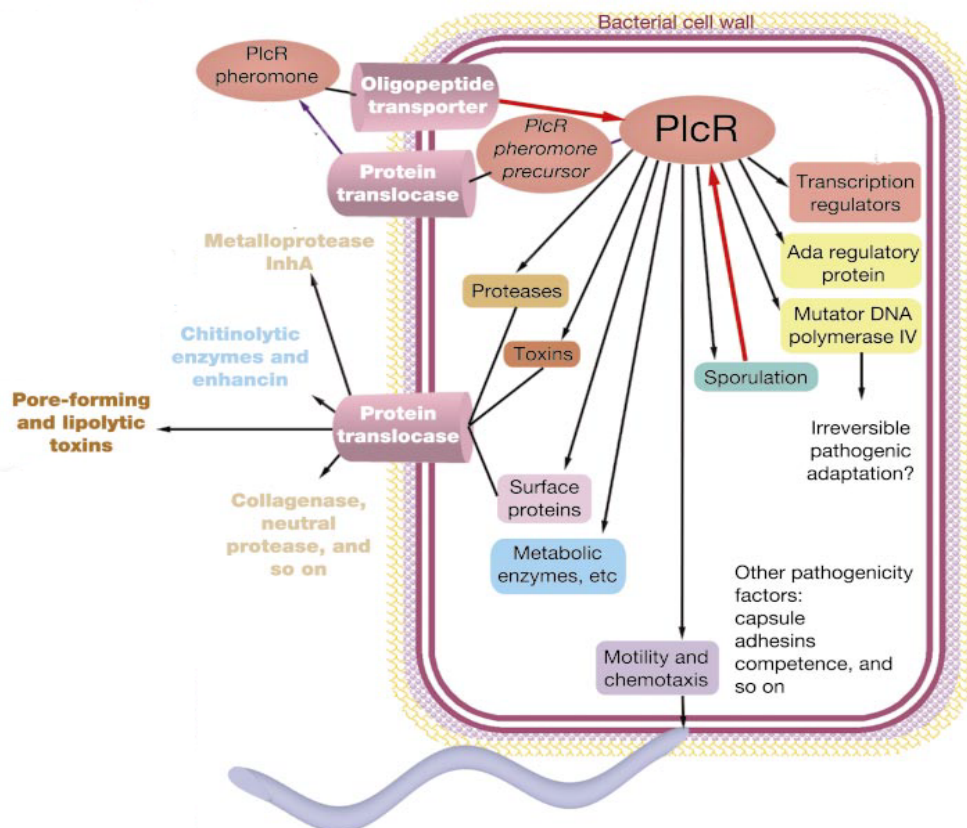


Figure 1.3 c-type cytochrome maturation pathway in *B. subtilis*.

**Specific Aim 2: Do *c* type cytochrome and its maturation pathway affect virulence expression and spore formation in *B cereus*, like they do in *B anthracis*?**

**Hypothesis: cytochrome *c* may not be involved in virulence expression due to lack of AtxA, but maturation pathway should play a role in spore formation.**

*B. cereus* produces a number of secreted proteins, including hemolysins, phospholipases, and enterotoxins, that cause host cellular damage. The three most important enterotoxins are the non-hemolytic enterotoxin (Nhe), hemolysin BL (Hbl), and cytotoxin K (CytK) (16). Unlike *B. anthracis* with master regulator AtxA, *B. cereus* does not contain virulence plasmids pXO1 and pXO2. Majority of pathology metabolic pathways are controlled by the quorum-sensing regulatory system PlcR (Fig4) (17). The roles of *c*-type cytochromes and their maturation pathway on virulence gene expression and sporulation have never been studied. With absence of AtxA, it will be anticipated the virulence gene expression would not alter. However, the spore formation would have significant decreased without proper pathway. I will test the hypothesis that the major effect of process is on spore generation but not virulence gene expression.



*Figure 1.4 PlcR regulon networks.*

Schematic representation of the potential PlcR regulon. © 2003 Nature Publishing Group doi:10.1038/nature01582

**Specific Aim 3: Does pigment accumulation affect physiology and pathology state of *B. anthracis*?**

**Hypothesis: Loss of *hmgA* would result in response changes of oxidase and UV stress, as well as the virulence profile.**

Melanin can protect cells from oxidative damage, alter phagocytosis and phagocytotic killing, modify virulence factor activity, and change responses to antimicrobial compounds (18). Melanin production can also alter susceptibility to UV damage, protecting microorganisms from environmental damage (19). It raises the question whether the pigment accumulated by lack of *hmgA* would have the same protective effect under oxidase and UV damage like melanin in most Gram-negative microorganism. It would also be beneficial to have better understanding the role of *hmgA* in spore assembling process. Brown pigment coat outside spore is essential for survival under harsh condition. However, in *B. subtilis*, there is no predicted homogentisate 1, 2-dioxygenase production due to lack of *hmgA*-like gene. The melanin-like pigment is produced by the copper-dependent laccase, CotA (20).

**Specific Aim 4: How do *pXO137* and *sRNA1* alter the toxin gene expression?**

**Hypothesis: Hfq-like *pXO137* and *sRNA1* would directly target *atxA* in post-transcriptional level.**

Regulation through small RNAs (sRNAs) has emerged as a major mechanism of post-transcriptional control of bacterial gene expression. sRNAs control virulence gene expression in many pathogens (21). A recent study identified two sRNA carried on the *B. anthracis* virulence plasmid pXO1 that are induced in response to bicarbonate/CO<sub>2</sub> through an AtxA-dependent mechanism (22). Efficient binding of sRNAs to their targets often requires RNA-binding chaperone proteins called Hfq. In many bacteria, loss of Hfq can result extensive alteration alter the pattern of global gene expression and alterations in virulence (23). *B. anthracis* carries at least three Hfq-like proteins, two encoded on the main chromosome and one encoded on pXO1. Here I will exam whether *pXO137* and *sRNA1* are indeed working as Hfq-sRNA system to regulate virulence gene expression.

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## 2 CHAPTER TWO: THE TWO CCDA OF *BACILLUS ANTHRACIS* DIFFERENTIALLY AFFECT VIRULENCE GENE EXPRESSION AND SPORULATION

### 2.1 Introduction

*Bacillus anthracis* is a Gram-positive, endospore-forming bacterium that is the etiological agent of anthrax. Several virulence factors, including the anthrax toxin and capsule, contribute to virulence in the mammalian host (1). Expression of both the toxin and capsule genes is dependent upon the master virulence regulator AtxA. Loss of *atxA* strongly decreases transcription of toxin and capsule genes (2, 3) and renders *B. anthracis* avirulent in animal models of infection (4). The direct interaction between AtxA and the promoters of its target genes has not been demonstrated, and the mechanism by which AtxA regulates its target genes remains unclear. AtxA activity is regulated posttranscriptionally by alternate phosphorylation and dephosphorylation (5), the global regulator CodY (6), and multimerization in response to growth conditions (7). *atxA* transcription is directly regulated by the transition state regulator AbrB, which connects virulence regulation to the sporulation pathway (8). *atxA* transcription is also indirectly regulated in response to glucose through the catabolite repressor protein CcpA (9).

We recently reported that loss of the two small *c*-type cytochromes of *B. anthracis*, *c*<sub>550</sub> and *c*<sub>551</sub>, leads to increased transcription of *atxA* and increased production of AtxA-regulated virulence factors (10). These observations led us to further investigate the roles of *c*-type cytochromes and cytochrome *c* maturation pathways in *B. anthracis* physiology and virulence. The *c*-type cytochromes are distinct from other cytochromes due to their covalently attached heme (11). Maturation *c*-type cytochromes is a complex, multistep process (12). While little is

known about cytochrome production and activity in *B. anthracis*, our understanding is informed by findings in the closely related organism, *Bacillus subtilis*. *B. subtilis* carries a system II cytochrome *c* maturation system that is similar to those found in Gram-positive bacteria, cyanobacteria, the beta-, delta-, and epsilonproteo bacteria, and chloroplasts (12, 13). The *c*-type cytochrome is initially produced as an apoprotein in the cytoplasm, missing its required cofactor, heme. The apocytochrome *c* is secreted across the membrane by the Sec translocation system. At the membrane, the cytochrome *c* maturation protein ResA reduces cysteine residues in the heme binding pocket of apocytochrome (14), while ResB and ResC transport and covalently attach heme to the reduced apocytochrome to generate the final, active form of cytochrome *c* (15, 16).

The thiol-disulfide oxidoreductase protein CcdA is also required for cytochrome *c* maturation in *B. subtilis*. CcdA is an integral membrane protein with six transmembrane (TM) domains with conserved cysteine residues in TM1 and TM4 (17, 18). The protein is homologous to a portion of *Escherichia coli* DsbD (18, 19). CcdA was first identified in *B. subtilis* during a screen for mutations that result in decreased production of *c*-type cytochromes (18). Loss of CcdA also results in disruption of late events in spore formation, indicating multiple extracellular functions of CcdA (20, 21). Subsequent work has shown that CcdA accepts electrons from thioredoxin in the cytoplasm and transfers the electrons to ResA at the cell surface (22). Reduced ResA can then reduce a disulfide linkage on apocytochrome *c*, facilitating covalent attachment of heme and production of functional *c*-type cytochromes (14, 23). The phenotypes associated with loss of CcdA can be suppressed by mutations in BdbC and BdbD, two other protein thiol-disulfide oxidoreductases that are involved in an oxidative pathway, because the disulfide bond in apocytochrome *c* that is reduced by ResA is no longer formed (14, 24). In addition to its interactions with ResA, CcdA reduces StoA, which contributes to spore formation and may

reduce YneN, which has no known function (12, 25). In the *B. anthracis* genome, there are two genes predicted to encode CcdA-like proteins (26), a feature found in members of the pathogenic *Bacillus cereus* group but not found in many other groups of bacilli (27). It is unclear why *B. anthracis* carries two copies of *ccdA* when *B. subtilis* can carry out both efficient sporulation and *c*-type cytochrome production with a single copy of *ccdA*. In this report, we show that the duplicate CcdA proteins have dissimilar activities in the cytochrome *c* maturation and spore formation pathways and that these differences are reflected in their gene expression profiles. Further, the activities of the two *B. anthracis* CcdA proteins are altered when expressed in *B. subtilis* and are distinct from the activity of endogenous *B. subtilis* CcdA.

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## 2.2 Materials and Methods

**Bacterial strains and growth conditions.** *B. anthracis* strain 34F2 (pXO1+ pXO2-) and its derivatives were routinely grown in LB or brain heart infusion (BHI) broth supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol,

7.5 µg/ml erythromycin, 5 µg/ml lincomycin, 25 µg/ml, and kanamycin, 7.5 µg/ml. 5-bromo-4-chloro-3-indolyl-β-D galactopyranoside (X-Gal) (40µg/ml) was added to LB agar to monitor β-galactosidase activity as necessary. Competent cells of *B. anthracis* were prepared following the method of Koehler et al (24), and electroporation was performed using the Bio-Rad-Gene Pulser according to the instructions of the supplier.

*B. subtilis* strain JH642 and its derivatives were routinely grown in LB or NSMP (nutrient sporulation medium phosphate) (17) supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol, 5 µg/ml, erythromycin, 5 µg/ml, lincomycin, 25 µg/ml, and kanamycin, 5 µg/ml. *B. subtilis* transformation was performed as previously described (51).

*Escherichia coli* TG1, C600 and DH5α competent cells were used for the propagation and isolation of all plasmid constructs. *E. coli* transformation was performed in chemically competent cells as previously described (39). Transformants were selected on LB agar supplemented with ampicillin, 100 µg/ml, chloramphenicol, 7.5 µg/ml, or kanamycin, 30 µg/ml.

**Plasmid and strain construction.** Strains and plasmids are listed in Table 1. Oligonucleotide primers are listed in Table S1. Markerless gene deletions in *B. anthracis* and *B. subtilis* were generated through a modification of the technique of Janes and Stibitz (22), as previously described (49) with the exception that plasmid pSS4332 (9) was used in place of plasmid pBKJ223 (22). The retention of plasmid pXO1 in *B. anthracis* strains was confirmed by PCR using primers atxAU5'Bam and atxAD3'Pst

The expression plasmid pAW285 was created by PCR amplification of JH642 genomic DNA to produce a DNA fragment containing the xylose repressor, xylR, and the XylR-regulated

xylA promoter of *B. subtilis* using primers xyl5'Eco and xyl3'Bam. The fragment was then cloned into plasmid pHP13 (19) at the EcoRI and BamHI sites. The resulting plasmid allows subcloning of genes downstream of the XylR-repressed xylA promoter, thereby placing their expression under a strong constitutive promoter in *B. anthracis* that can be further induced by xylose.

**$\beta$ -Galactosidase assays.** *B. anthracis* strains harboring gene promoter fusions on the replicative vector pTCV-lac (34) were grown at 37°C in LB supplemented with the appropriate antibiotics.  $\beta$ -galactosidase activity was assayed as described previously and specific activity was expressed in Miller units (28, 50).

**Quantitative RT-PCR.** RNA was extracted from *B. anthracis* using the UltraClean Microbial RNA Isolation Kit (MoBio, Carlsbad, CA). RNA was treated with Turbo DNase (Life Technologies, Carlsbad, CA) and quantified using an Eppendorf Bio- spectrophotometer. Quantitative, real time RT-PCR was performed using the Power SYBR RNA-to-Ct 1-step kit (Life Technologies) on a Prism 7500 Fast Real Time PCR System (Life Technologies). Data is presented as ( $\Delta\Delta C_T$ ) value calculated from the results of at least three independent experiments.

**TMPD Oxidase Staining.** The presence of active c-type cytochrome was verified by a colorimetric assay using N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) as an artificial electron donor that can be oxidized by cytochrome *caa3*. TMPD oxidase staining was performed on *B. anthracis* or *B. subtilis* grown on NSMP as previously described (26, 49).

**Sporulation analysis.** Sporulation assays were performed using strains grown in NSMP or SM (Schaeffer's sporulation medium) (40), containing chloramphenicol if necessary, at 37°C for 48 hours. Sporulation was initially monitored by visualization of cells on a Zeiss Axio Imager microscope and enumeration of spores and vegetative cells from multiple

independent fields. Sporulation efficiency was assayed by chloroform treatment and enumeration of spores and vegetative cells as previously described (5). Sporulation efficiency is presented as percentage of spores relative to total viable cells.

**Operon and transcription analysis.** Operon organization was determined by conventional RT-PCR using 34F2 RNA extracted using the UltraClean Microbial RNA Isolation Kit. Following extraction, RNA was treated with Turbo DNase. cDNA was synthesized using SuperScript III reverse transcriptase and random hexamers (Life Technologies). Sets of primers flanking the intergenic regions for each gene pair were used to amplify cDNA. Amplicons were separated by electrophoresis on 1% agarose gels, stained by ethidium bromide, and visualized on a UVP gel documentation system.

For gene expression analysis, the intergenic region upstream of the first linked gene of each of operon were amplified using primers indicated in Table S1 in the supplemental material. The PCR fragments were then cloned into pTCV-*lac* (35), and sequences were confirmed by DNA sequencing. Transcriptional activities of the promoter fragments were then tested under a variety of conditions using  $\beta$ -galactosidase analysis.

## 2.3 Results

**Identification and description of *ccdA1* and *ccdA2* loci.** Disruption of the cytochrome *c* maturation pathway in *B. anthracis* causes increased transcription of virulence genes, the result of loss of the two small c-type cytochromes *c*<sub>550</sub> and *c*<sub>551</sub> (49). In previous transposon mutagenesis screens for mutants that cause increased transcription of virulence genes, the *B. anthracis* orthologs of most non-essential genes known to be required for cytochrome *c* maturation in the model organism *B. subtilis* were disrupted (26, 49). A gene

important to cytochrome *c* production in *B. subtilis* is *ccdA*, which encodes a protein thiol-disulfide oxidoreductase that contributes to covalent attachment of heme to apocytochrome *c* at the cell surface (14, 42, 43). *ccdA* is also involved in spore formation in *B. subtilis* (41). A *ccdA* disruption was not isolated in a screen for mutants with increased transcription of virulence genes that isolated mutants in most other cytochrome *c* maturation genes (49). Examination of the *B. anthracis* genome reveals the presence of two *ccdA*-like genes. BAS1647 (GenBank: AAT53964.1) encodes a protein with 65% amino acid similarity relative to *B. subtilis ccdA* (GenBank: CAB13677.1). BAS3455 (GenBank: AAT55761.1) encodes a protein with 68% amino acid similarity relative to *B. subtilis ccdA*. BAS1647 and BAS3455 share 63% amino acid similarity. We will refer to BAS1647 as *ccdA1* and BAS3455 as *ccdA2*. The lack of *ccdA* disruption mutants our previous screen may be due to the existence of two complementary copies of *ccdA*.

The genomic context of both *ccdA1* and *ccdA2* is different than that of *B. subtilis ccdA*. *B. subtilis ccdA* is co-transcribed with two downstream genes, *yneI* and *yneJ*, but neither of the downstream genes contributes to sporulation or cytochrome *c* maturation (41). *ccdA1* appears to be part of a large gene cluster containing genes predicted to be involved in multiple cellular processes. Of note is the presence immediately downstream of *ccdA1* of an ortholog of *yneN*, a membrane-bound protein with a thioredoxin-like domain that may interact with *ccdA* in *B. subtilis* (15). To determine the structure of putative *ccdA1* operon, linkage analysis was performed by RT-PCR using 34F2 RNA and sets of primers flanking the intergenic regions of each potential gene pair. As shown in Figure S1, amplicons were obtained for each intergenic region between BAS1644 and BAS1649, indicating that transcription of these genes is linked and constitute an operon. The genes flanking either end of the BAS1644-9

operon are transcribed in the opposite direction and are not part of the operon. These findings are consistent with a recent global transcriptome analysis that identified *ccdA1* as part of a six-gene operon (32). Linkage analysis of *ccdA2* indicates that it is unlinked to either of the surrounding genes transcribed in the same orientation. The lack of upstream linkage is unsurprising due to the large intergenic region and predicted transcriptional terminator between BAS3456 and *ccdA2*. A linkage between *ccdA2* and BAS3454, a *yneJ*-like gene, might have been expected given the similarities to the operon organization in *B. subtilis* (*ccdA-yneI-yneJ*) (29, 41), but the lack of an *yneI*-like gene between *ccdA2* and the *yneJ*-like BAS3454 indicates genomic dissimilarities between the two species.

**Characterization of *ccdA* deletion strains.** To investigate the role of *ccdA1* and *ccdA2* in virulence gene expression, cytochrome c maturation, and sporulation, we created markerless deletion strains in the *B. anthracis* 34F2 background. Strain AW-A027 is missing *ccdA1*, strain AW-A028 is missing *ccdA2*, and strain AW-A029 is missing both *ccdA1* and *ccdA2*. A growth curve in LB of the double deletion strain is shown in Figure 1A. Loss of *ccdA1* and *ccdA2* singly (data not shown) or loss of both in the double deletion did not result in significant growth defects.

*B. anthracis* virulence gene expression was measured by  $\beta$ -galactosidase assays using transcriptional fusions of either the *atxA* (4) or *pagA* (45) promoter to a *lacZ* reporter. As shown in Figure 1B loss of both *ccdA1* and *ccdA2* resulted in increased transcription of *atxA* during exponential phase and increased transcription of *pagA* during early stationary phase, similar to the phenotype of a cytochrome c maturation or a *cccA/cccB* knock-out mutant as observed previously (49). As *atxA* over-expression in exponential phase was shown to be the cause of increased *pagA* transcription, we focused more carefully on early *atxA* expression. Figure 2

shows the early *atxA* expression profile of multiple strains. As expected of the parental 34F2 strain, *atxA* expression was low at 3 hours and remains unchanged at 4 hours. In contrast, the *resB* deletion strain, in which cytochrome *c* production is completely abolished, showed a more than five-fold increase in *atxA* expression at 3 hours that decreased significantly at 4 hours, consistent with previous findings (49). The *atxA* transcription profile in the *ccdA1/ccdA2* double deletion strain was similar to the *resB* deletion strain, though the expression level was slightly lower. The *ccdA1* deletion strain resembled the parental 34F2 strain with no increased *atxA* expression. The *ccdA2* deletion strain had increased *atxA* expression relative to 34F2 and the *ccdA1* deletion strain but lower than the double deletion. These results suggested that loss of both *ccdA1* and *ccdA2* is required for strongly increased virulence gene expression and that there is some degree of complementarity between CcdA1 and CcdA2.

Next, the cytochrome *c* oxidase activity of the mutants was assayed by TMPD staining. TMPD is an artificial electron donor that interacts specifically with cytochrome *c* oxidase (cytochrome *caa3*) and is oxidized to a blue colored product that stains colonies in the presence of active cytochrome *c* in the membrane. Parental and mutant strains were grown on NSMP agar plates, stained with TMPD, and scored for the appearance of blue color. As shown in Table 2, the parental 34F2 and the *ccdA1* deletion strains were stained using TMPD, indicating active cytochrome *c* oxidase. In contrast, the *ccdA2* and *ccdA1 ccdA2* deletion strains were not stained using TMPD, indicating lack of active cytochrome *c* oxidase. These findings are consistent with the *atxA* transcription results, suggesting that loss of *ccdA2* results in loss of cytochrome *c* while loss of *ccdA1* has little effect.

*ccdA* is also involved in spore formation in *B. subtilis* in an activity distinct from its role in cytochrome *c* maturation (41). To test the effect of loss of *ccdA1* and *ccdA2* in sporulation of

*B. anthracis*, parental and mutant strains were grown on SM media. Sporulation efficiency was monitored by both microscopic examination and liquid sporulation assays. As shown in Table 2, the sporulation efficiency of the *ccdA1* deletion strain was similar to the parental 34F2 while sporulation of the *ccdA2* and *ccdA1/ccdA2* deletion strains were significantly reduced.

Sporulation was also tested using a different sporulation-inducing medium, NSMP, and sporulation efficiency was similar to the results obtained using SM (data not shown). These results suggest that loss of *ccdA2* results in decreased sporulation while loss of *ccdA1* has no observable effect on sporulation.

***ccdA2* is expressed at higher levels than *ccdA1*.** Our initial characterization of the *ccda* deletion strains suggests that *ccdA2* is responsible for all observed phenotypes while loss of *ccdA1* has no measurable effect. This may be due to differential activities of the two protein products, i.e. CcdA1 does not function in cytochrome *c* maturation and sporulation pathways. Alternatively, the results could be due to different levels of gene expression, i.e. *ccdA1* is expressed at much lower levels than *ccdA2* such that loss of CcdA1 can easily be compensated for by the presence of abundant CcdA2. We performed quantitative RT-PCR to measure *ccdA1* and *ccdA2* mRNA levels in the parental 34F2 strain. Assays were performed in triplicate and analyzed by the comparative Ct ( $2^{-\Delta\Delta C_t}$ ) method relative to the *gyrB* control. Under conditions used in the previous phenotypic characterization assays, *ccdA2* was expressed 14.2 ( $\pm 0.91$ ) fold higher than *ccdA1*. The higher levels of *ccdA2* expression can explain the lack of effect in the *ccdA1* single deletion as the loss of the smaller amount of CcdA1 can easily be compensated by the much more abundant CcdA2, assuming that protein concentrations are consistent with mRNA amounts. This may also explain the increase in *atxA* expression in the *ccdA1/ccdA2*

deletion relative to the *ccdA2* deletion as the small amount of CcdA1 production lost in the double mutant further reduces *c*<sub>550</sub> and *c*<sub>551</sub> levels and increases *atxA* transcription.

**CcdA2 is active in cytochrome *c* maturation and sporulation while CcdA1 is only active in cytochrome *c* maturation.** Our earlier analysis of the *ccdA1* and *ccdA2* single deletion strains was hampered by substantial differences in expression. To eliminate the differences in complementation approach, *ccdA1*, *ccdA2*, and *B. subtilis ccdA* were cloned into the expression plasmid pAW285. This plasmid places the gene of interest under the control of the *B. subtilis xylA* promoter, which is constitutively active in the absence of added xylose in *B. anthracis* (data not shown), thereby eliminating differences in transcription from the endogenous promoters.

*atxA* gene expression was monitored by  $\beta$ -galactosidase analysis in the parental 34F2 and *ccdA1ccdA2* double deletion strain carrying various plasmids (Figure 4). Addition of the empty pAW285 or any of the CcdA-expressing plasmids did not carrying empty plasmid overexpressed *atxA* in a pattern similar to our previous data, suggesting the empty plasmid does not affect expression. Addition of plasmids carrying *ccdA1*, *ccdA2*, or *ccdA* of *B. subtilis* reduced *atxA* expression levels to that of the parental strain. These data suggest that any of *ccdA1*, *ccdA2* or *B. subtilis ccdA*, when expressed at similar levels, can complement the *atxA* overexpression phenotype associated with loss of CcdA activity.

Cytochrome *c* maturation in the parental 34F2 and *ccdA* deletion strains carrying various plasmids was assayed by TMPD oxidase staining (Table 2). Addition of empty or *ccdA*-expressing plasmids did not alter cytochrome *c* oxidase activity in the parental 34F2 strain. The *ccdA2* and *ccdA1ccdA2* strains transformed with empty plasmid were deficient in TMPD staining, indicating that the empty plasmid does not alter cytochrome *c* maturation. Consistent

with the *atxA* expression data, addition of plasmids carrying *ccdA1*, *ccdA2*, or *ccdA* of *B. subtilis* restored TMPD staining in all deficient strains.

Sporulation efficiency assays were also performed in the complementation. Sporulation efficiency assays were also performed in the complementation to parental strain levels in the *ccdA2* and *ccdA1ccdA2* deletion strains. Surprisingly, addition of *ccdA1* was unable to complement the sporulation defect in the *ccdA2* and *ccdA1ccdA2* deletion strains. Addition of xylose to the cultures, which increases expression levels almost 5-fold, did not alter sporulation in the presence of the *ccdA1* complementation plasmid (data not shown), demonstrating that inability to complement is not the result of expression levels. While *ccdA2* and *B. subtilis ccdA* can complement all phenotypes associated with loss of CcdA activity, *ccdA1* was incapable of complementing sporulation deficiency.

**Neither *ccdA1* nor *ccdA2* can fully complement the loss of *ccdA* in *B. subtilis*** Previous observations indicate that *ccdA* of *B. subtilis* provided in trans can fully complement the phenotypes associated with loss of *ccdA1* and *ccdA2* in *B. anthracis*. We investigated the converse: whether *ccdA1* and *ccdA2* can complement the loss of *ccdA* in *B. subtilis*. A markerless deletion strain missing *ccdA* was constructed in the JH642 strain of *B. subtilis*. This strain, AW-S002, was defective in cytochrome *c* oxidase activity, as measured by loss of TMPD oxidase activity, and had reduced sporulation efficiency relative to parental JH642. AW-S002 was transformed with the same complementation plasmids previously used in *B. anthracis*. Unlike in *B. anthracis*, neither *ccdA1* nor *ccdA2* could restore TMPD oxidase activity in the *ccdA* deletion strain (Table 3). In contrast, *ccdA* of *B. subtilis* could restore TMPD oxidase activity in the *ccdA* deletion strain. When sporulation efficiency was measured in the same strains, *ccdA2* and *B. subtilis ccdA* could restore sporulation to parental strain levels while

addition of *ccdA1* did not affect sporulation, observations similar to what was seen in *B. anthracis*. The same complementation plasmid stocks used for the *B. subtilis* experiments were again independently transformed into both *B. anthracis* and *B. subtilis*, and these strains showed the same phenotypes previously reported, excluding plasmid irregularities as an explanation our observations. Neither *ccdA1* nor *ccdA2* could complement cytochrome c oxidase deficiency while only *ccdA2* could complement sporulation deficiency, indicating that the activities of *B. anthracis* CcdA1 and CcdA2 are dissimilar when expressed in *B. subtilis* relative to *B. anthracis*.

Table 2.1 Strains and plasmids used in Chapter 2.

Strain or plasmid	Relevant characteristic(s)	Source
<i>B. anthracis</i> strains		
34F2	pXO1+ pXO2-	Laboratory stock
AW-A027	Markerless deletion of <i>ccdA1</i>	This study
AW-A028	Markerless deletion of <i>ccdA2</i>	This study
AW-A029	Markerless deletion of <i>ccdA1</i> and <i>ccdA2</i>	This study
34F2 $\Delta$ <i>resB</i>	Markerless deletion of <i>resB</i>	10
<i>B. subtilis</i> strains		
JH642	<i>pheA1 trpC2</i>	Laboratory stock
AW-S002	Markerless deletion of <i>ccdA</i>	This study
Plasmids		
pORI-Cm-I-Sce1	pORI-Cm vector with I-Sce1 recognition site, Cm <sup>R</sup>	33
pSS4332	I-Sce1 expression plasmid, Kan <sup>R</sup>	33
pTCVlac-pagA	pagA-lacZ fusion, Kan <sup>R</sup>	5
pTCVlac-atxA12	atxA-lacZ fusion, Kan <sup>R</sup>	41
pAW184	Regions flanking BAS1647 Cm <sup>R</sup>	This study
pAW184	Regions flanking BAS3455 Cm <sup>R</sup>	This study
pHP13	Cloning/shuttle plasmid Cm <sup>R</sup>	34
pAW285	Xylose-inducible expression plasmid Cm <sup>R</sup>	This study
pAW304	<i>ccdA1</i> expression plasmid Cm <sup>R</sup>	This study
pAW305	<i>ccdA2</i> expression plasmid Cm <sup>R</sup>	This study
pAW305	<i>B.subtilis ccdA</i> expression plasmid Cm <sup>R</sup>	This study
pAW350	<i>ccdA2-lacZ</i> fusion Kan <sup>R</sup>	This study
pAW355	<i>ccdA1-lacZ</i> fusion Kan <sup>R</sup>	This study
pAW360	Regions flanking <i>B. subtilis ccdA</i>	This study

Table 2.2 Cytochrom c oxidase activity and sporulation efficiency in *B. anthracis*.Table 2: Cytochrome c oxidase activity and sporulation efficiency in strains of *B. anthracis*.

	TMPD Staining				Sporulation Efficiency			
	34F2	<i>ccdA1</i>	<i>ccdA2</i>	<i>ccdA1ccdA2</i>	34F2	<i>ccdA1</i>	<i>ccdA2</i>	<i>ccdA1ccdA2</i>
pAW285 (Empty)	+	+	-	-	53	55	21	23
pAW304 ( <i>ccdA1</i> )	+	+	+	+	52	51	19	20
pAW305 ( <i>ccdA2</i> )	+	+	+	+	50	53	56	53
pAW306 ( <i>B. subtilis ccdA</i> )	+	+	+	+	ND	55	57	53

<sup>a</sup> +, TMPD staining detected. -, no TMPD staining detected.

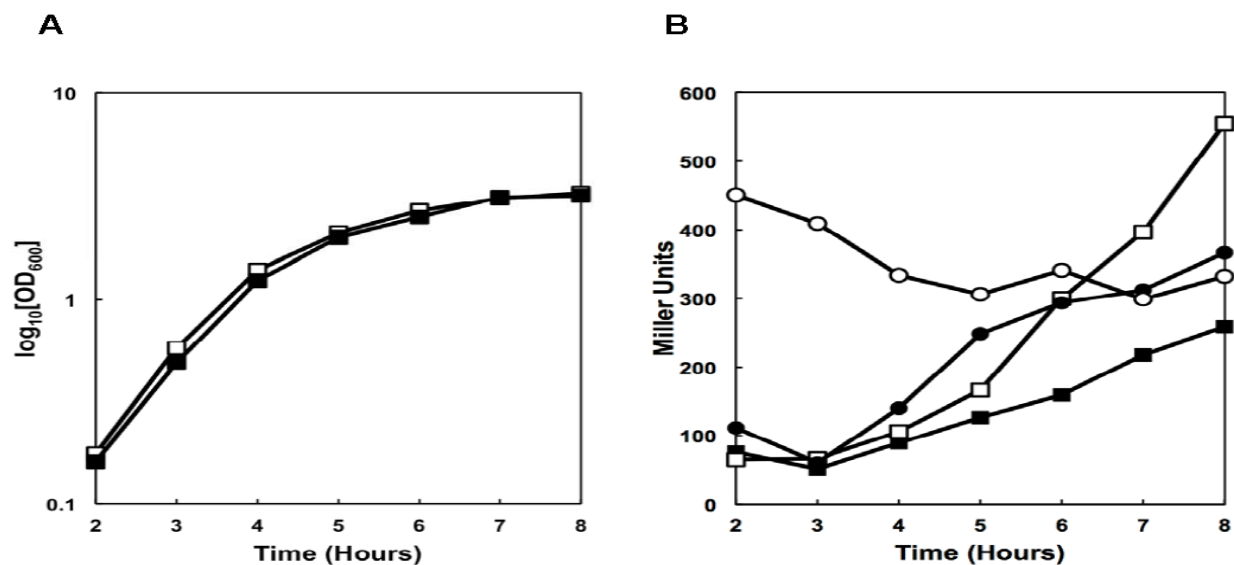
<sup>b</sup> Sporulation values represent percentage of spores relative to the total number of viable cells. Results are the average of three independent assays. Standard error of the mean was less than 10% of the mean in all experiments. ND, not determined

Table 2.3 Cytochrom c oxidase activity and sporulation efficiency in *B. subtilis*.Table 3: Cytochrome c oxidase activity and sporulation efficiency in strains of *B. subtilis*.

	TMPD Staining		Sporulation Efficiency	
	JH642	<i>ccdA</i>	JH642	<i>ccdA</i>
pAW285 (Empty)	+	-	34	2
pAW304 ( <i>ccdA1</i> )	+	-	37	3
pAW305 ( <i>ccdA2</i> )	+	-	34	34
pAW306 ( <i>B. subtilis ccdA</i> )	+	+	35	34

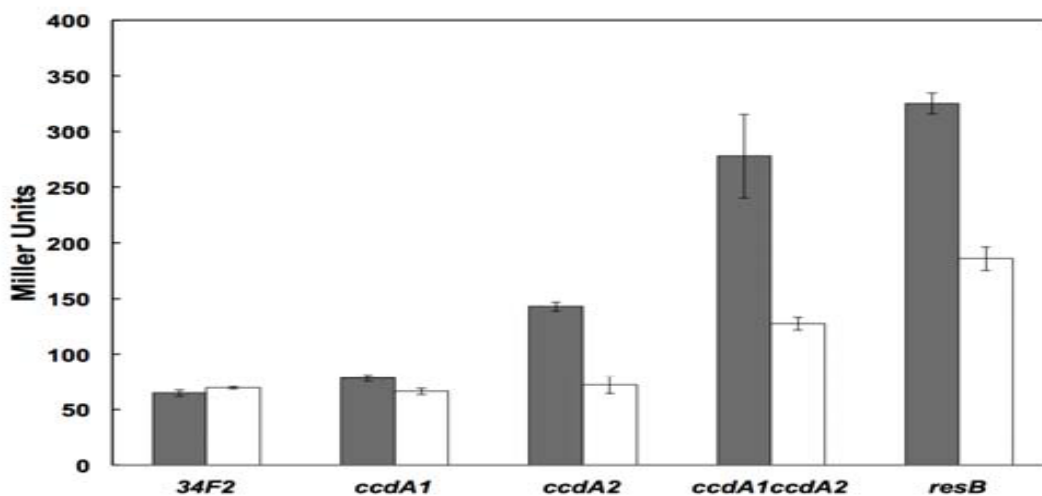
<sup>a</sup> +, TMPD staining detected. -, no TMPD staining detected.

<sup>b</sup> Sporulation values represent percentage of spores relative to the total number of viable cells. Results are the average of three independent assays. Standard error of the mean was less than 10% of the mean in all experiments. ND, not determined



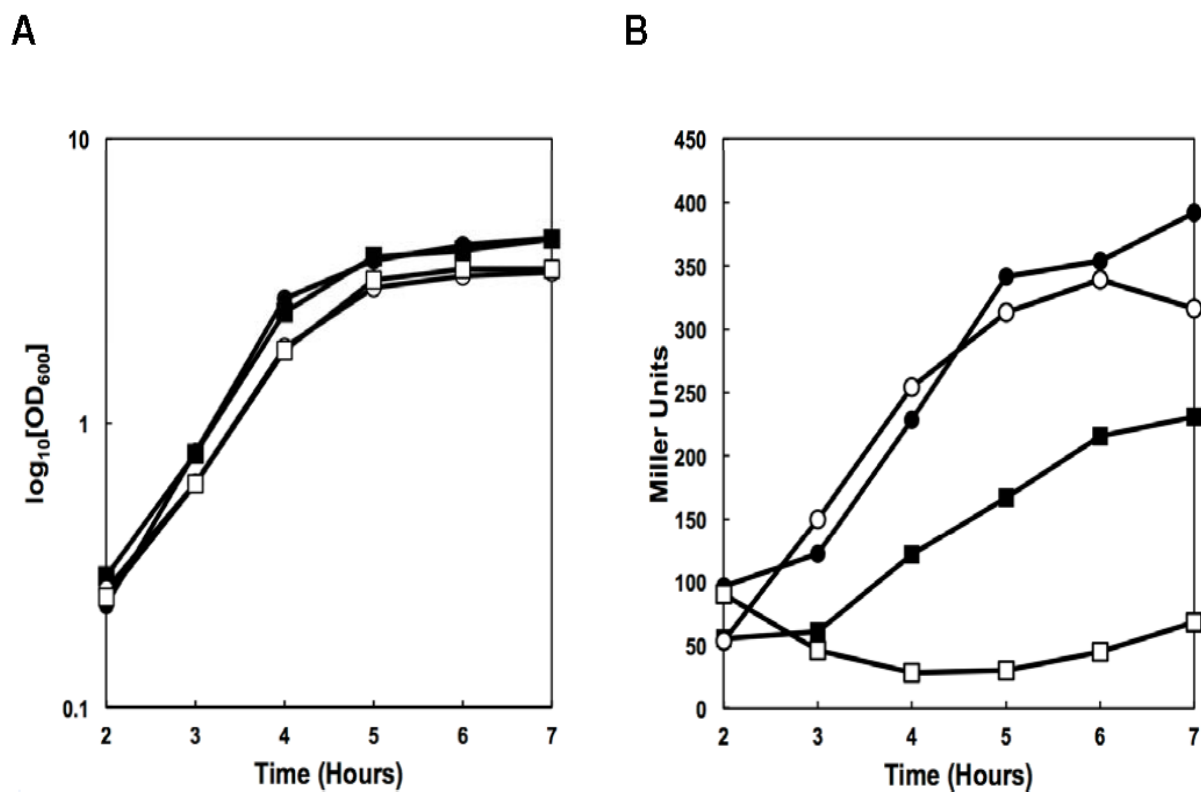
*Figure 2.1 Growth and virulence gene expression in ccdA1/ccdA2 mutant strain.*

**A.** Cell growth of parental and mutant strains grown in LB at 37 °C. ■, 34F2; □, AW-A029(ccdA1/ccdA2). **B.** β-galactosidase activity in pagA and atxA reporter strains grown in LB supplemented with Kanamycin at 37 °C. ■ 34F2 pagA-lacZ; ● 34F2 atxA-lacZ; □, AW-A029 pagA-lacZ; ○, AW-A029 atxA-lacZ.



*Figure 2.2 Early atxA expression in ccdA mutant strain.*

β-galactosidase activity of parental and mutant strains carrying atxA-lacZ reporter grown in LB supplemented with Kanamycin at 37 °C. Filled bars represent atxA expression at 3 hours post-inoculation and empty bars represent atxA expression at 4 hours post-inoculation



*Figure 2.3 Gene expression profile of ccdA1 and ccdA2.*

**A.** Cell growth of parental 34F2 strain carrying *ccdA1* (pAW355) and *ccdA2* (pAW350) reporter plasmids grown in the medium indicated supplemented with Kanamycin at 37°C. **B.**  $\beta$ -galactosidase activity of parental 34F2 strain carrying *ccdA1* (pAW355) and *ccdA2* (pAW350) reporter plasmids grown in the medium indicated supplemented with Kanamycin at 37°C. For both panels: ■, *ccdA1*-lacZ in LB; ●, *ccdA2*-lacZ in LB; □, *ccdA1*-lacZ in NSMP; ○, *ccdA2*-lacZ in NSMP.

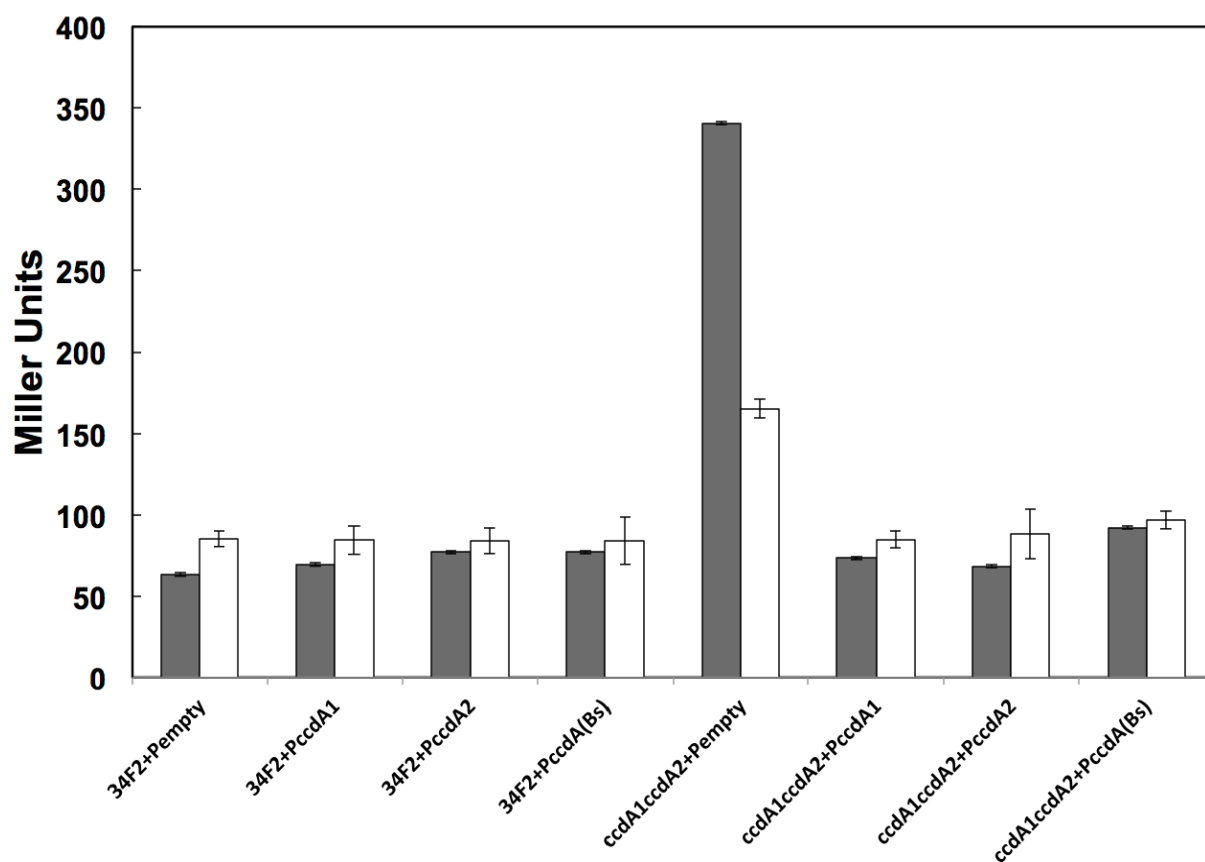


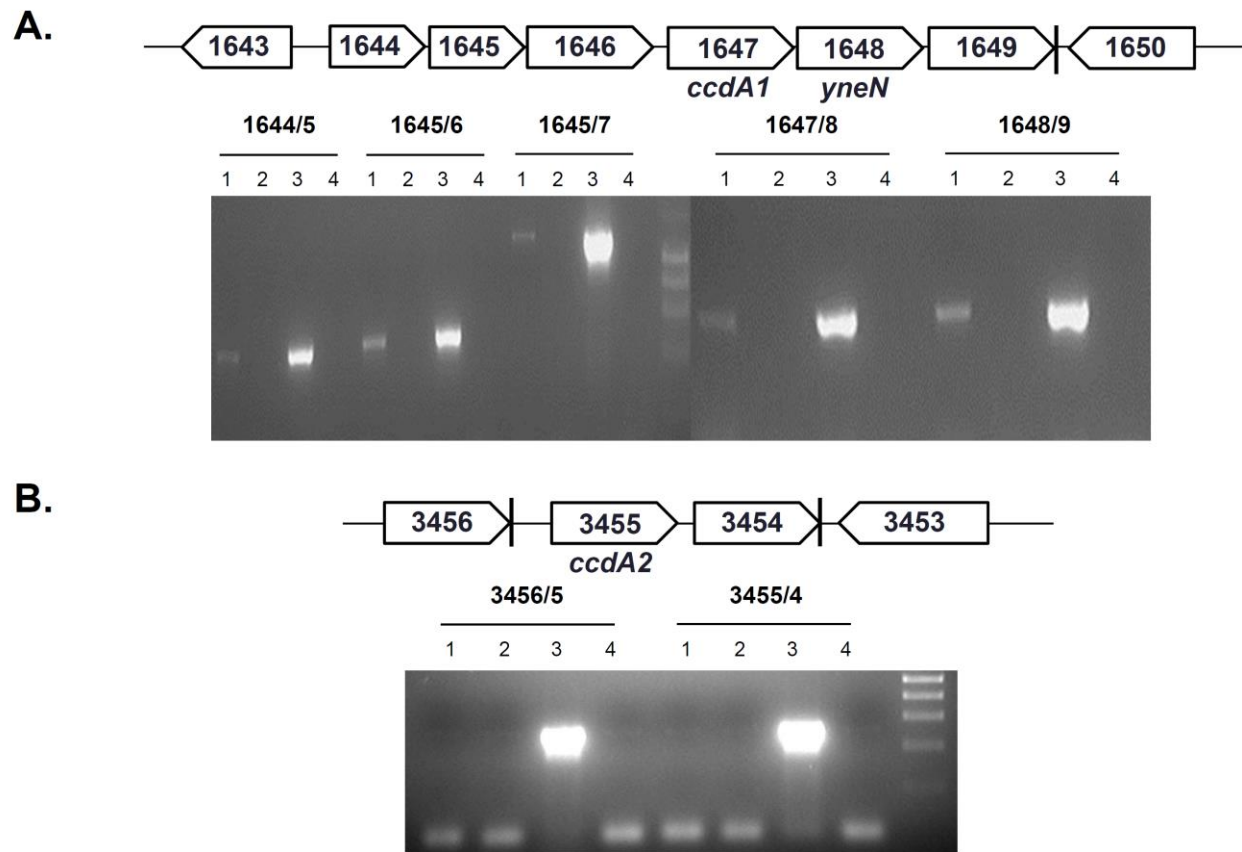
Figure 2.4 Early *atxA* expression in parental and *ccdA1/ccdA2* mutant strains carrying *ccdA* expression plasmids. .

$\beta$ -galactosidase activity of parental and mutant strains carrying *atxA-lacZ* reporter grown in LB supplemented with Kanamycin and Chloramphenicol at 37°C. Filled bars represent *atxA* expression at 3 hours post-inoculation an empty bars represent *atxA* expression at 4 hours post-inoculation

Table 2.4 Oligonucleotide primers used in Chapter 2.

Table S1: Oligonucleotide primers used in this study

Name	Function	Sequence
ccdA1U5'Bam	Region upstream of BAS1647 for deletion of <i>ccdA1</i>	5'-CACCGGATCCTGTAAAAGGATATGGCGACAA
ccdA1U3'Sal	Region upstream of BAS1647 for deletion of <i>ccdA1</i>	5'-TGCCTCGACCCCTGAAACACTACAACAA
ccdA1D5'Sal	Region downstream of BAS1647 for deletion of <i>ccdA1</i>	5'-CACCGTCGACCTATTCGGCGGATTACAGG
ccdA1D3'Pst	Region downstream of BAS1647 for deletion of <i>ccdA1</i>	5'-TGCCTGCAGACCTCACGATCCACTCCATC
ccdA1USeq	Check primer upstream of BAS1647	5'-TGAAATGGCAAAATGCGTAAA
ccdA1E5'Bam	Amplification of <i>ccdA1</i> ORF	5'-CACCGGATCCTGTTTCAGGGGAATAAAATG
ccdA1E3'Sal	Amplification of <i>ccdA1</i> ORF	5'-TGCCTCGACTTAAAAACCTGTAATCCGCCG
1644F	<i>ccdA1</i> operon mapping	5'-GATCGGGAATTGTTTGCACCT
1645R	<i>ccdA1</i> operon mapping	5'-TCATTTTCTTCGTTTGATTGGA
1645F	<i>ccdA1</i> operon mapping	5'-TCCAATCAAACGAAGAAAATGA
1646R	<i>ccdA1</i> operon mapping	5'-TCGGACGAAATAGCCCAAT
1646F	<i>ccdA1</i> operon mapping	5'-CATTTGCAAAAACAGCAATCA
1647R	<i>ccdA1</i> operon mapping	5'-TGATTCCAATCCAGCTTACTGA
1647F	<i>ccdA1</i> operon mapping	5'-ATCCTGAAGGGGCACTTCTT
1648R	<i>ccdA1</i> operon mapping	5'-ACACCATGATGCCAAAAAT
1648F	<i>ccdA1</i> operon mapping	5'-TCGTCACGGATTTAAGTTTCC
1649R	<i>ccdA1</i> operon mapping	5'-TGCCCTTTTTACTTTTCCA
ccdA1RTF	<i>ccdA1</i> qRT-PCR	5'-GGCAGAGAAAAAGGTGCAGT
ccdA1RTR	<i>ccdA1</i> qRT-PCR	5'-AGAAGTGCCCTTCAGGATT
ccdA1P15'Eco	Region upstream of <i>ccdA1</i> operon for <i>lacZ</i> fusion	5' CACCGAATTCGCCACCAATGACAGCTAAT
ccdA1P13'Bam	Region upstream of <i>ccdA1</i> operon for <i>lacZ</i> fusion	5'- TCGCGATCCTCGAATCGAAAAATCCACTTTTT
ccdA2U5'Bam	Region upstream of BAS3455 for deletion of <i>ccdA2</i>	5'-CACCGGATCCTTGTGGGGAATTAGGGACT
ccdA2U3'Sal	Region upstream of BAS3455 for deletion of <i>ccdA2</i>	5'-TGCCTCGACGATTGTTCCACTCCTCTCC
ccdA2D5'Sal	Region downstream of BAS3455 for deletion of <i>ccdA2</i>	5'-CACCGTCGACTTTGGTGGATTACTGGTTTTT
ccdAD3'Pst	Region downstream of BAS3455 for deletion of <i>ccdA2</i>	5'-TGCCTGCAGATACGTCGTGGCTGGGATAG
ccdA2USeq	Check primer upstream of BAS3455	5'-TCCGATTGTAGTGCAGTGT
ccdA2E5'Bam	Amplification of <i>ccdA2</i> ORF	5'-CACCGGATCCGGAGAGGAGTGGAAACAATGC
ccdA2E3'Sal	Amplification of <i>ccdA2</i> ORF	5'-TGCCTCGACTAAAAACAGTAAATCCACCAA
3455F	<i>ccdA2</i> operon mapping	5'-TTGGATTCCGCTATTCCGTTT
3456F	<i>ccdA2</i> operon mapping	5'-GCAGCGTTTGTGTTTGGATT
3455R	<i>ccdA2</i> operon mapping	5'-GATAAAGCGGCAAGCAACAT
ccdA2RTF	<i>ccdA2</i> qRT-PCR	5'-TAATTGGGTTGGCATTGCT
ccdA2RTR	<i>ccdA2</i> qRT-PCR	5'-AGCACGAAGAACGGAATAGC
ccdA2P15'Eco	Region upstream of <i>ccdA2</i> operon for <i>lacZ</i> fusion	5'- CACCGAATTCGATGGTGAATGTATTCCG
ccdA2P13'Bam	Region upstream of <i>ccdA2</i> operon for <i>lacZ</i> fusion	5'- TCGCGATCCGCGCCAAACGCTAAAAA
BsccdAE5'Bam	Amplification of <i>B. subtilis ccdA</i> ORF	5'-CACCGGATCCTGATTTTGTCAACTTGAAGGAA
BsccdAE3'Sal	Amplification of <i>B. subtilis ccdA</i> ORF	5'-TGCCTCGACTCAAAAACAGTAAAGCCTCC
BsccdAU5'Bam	Region upstream of <i>ccdA</i> for deletion of <i>ccdA</i>	5' CACCGGATCCTGATGCAATGGGAATGAAA
BsccdAU3'Sal	Region upstream of <i>ccdA</i> for deletion of <i>ccdA</i>	5'- TGCCTCGACTCCCATGTATCATCCCTTC
BsccdAD5'Sal	Region downstream of <i>ccdA</i> for deletion of <i>ccdA</i>	5'- CACCGTCGACGCTTACTGGTTTTGACGGTAA
BsccdAD3'Pst	Region downstream of <i>ccdA</i> for deletion of <i>ccdA</i>	5'- TGCCTGCAGGCTTACAGCTTCCAGCACT
BsccdA2USeq	Check primer upstream of <i>ccdA</i>	5'- GCTCATAGGTGTTGCACCTCG
atxAU5'Bam	Check primer upstream of <i>atxA</i>	5'- CACCGGATCCTGCATTCTTGACCAGTTGGA
atxAD3'Pst	Check primer downstream of <i>atxA</i>	5'- TGCCTGCAGCAGCTCATCAGATGATTT



*Figure 2.5 Transcriptional linkage analysis of ccdA1 and ccdA2 loci.*

RT-PCR was performed on RNA extracted from 34F2 using primer sets flanking intergenic regions. **A.** *ccdA1* loci. **B.** *ccdA2* loci. For each primer set, four reactions were performed: Lanes 1, RNA with reverse transcriptase; Lanes 2, RNA without reverse transcriptase; Lanes 3, Genomic DNA; Lanes 4, RNA/DNA negative. Diagram above each reaction set depicts gene orientation of mapped loci with vertical bars representing predicted transcriptional stop sites.

## 2.4 Discussion

Membrane bound thiol-disulfide oxidoreductases contribute to several processes important to metabolism and virulence in *B. anthracis*. There is conservation among components between *B. anthracis* and the well-characterized model organism *B. subtilis*. One significant difference lies in the fact that, unlike *B. subtilis*, which carries one *ccdA*, *B. anthracis* carries two copies of *ccdA*. In *B. anthracis*, CcdA2 functions in cytochrome *c* maturation, *atxA* regulation, and sporulation. In contrast, CcdA1 functions in cytochrome *c* maturation and *atxA*

regulation but has no activity in efficient sporulation. The actual activities of the two proteins are partially masked by the significant differences in expression levels.

The presence of two distinct *ccdA* genes is a feature found throughout the pathogenic *B. cereus* group. Comparison of genomic sequences deposited to GenBank reveals a *ccdA1/ccdA2* pair in all screened genomes of the pathogenic *B. cereus* group, including *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis*. In contrast, most other non-pathogenic environmental bacilli, including *B. subtilis*, *B. pumilus*, and *B. halodurans*, carry only one *ccdA*, which is more similar to *ccdA1*. Interestingly, some other bacilli, including *Geobacillus spp.* and *B. licheniformis*, carry multiple *ccdA1*-like genes. The presence of a distinct *ccdA2*-like gene appears to be common in the pathogenic bacilli group, suggesting the genetic events that generated two distinct *ccdA* genes occurred following separation of the *B. cereus* group from the other bacilli. The potential contribution of *ccdA2*-like genes to pathogenicity of these organisms may merit further investigation.

The operon organization of *ccdA1* and *ccdA2* is distinct from that of *B. subtilis ccdA*. *ccdA1* is the fourth gene in a six-gene operon. The purpose of the linkage of these genes is unclear as most of the genes have no known function and predictions of activity based on similarity of conserved domains reveals little commonality. A gene of interest in the operon is the fifth gene, BAS1648, just downstream of *ccdA1*, which is predicted to encode a protein with 59.4% amino acid similarity to *B. subtilis* YneN. YneN is one of the 8 membrane-bound thiol-disulfide oxidoreductase proteins in *B. subtilis*, a group which also includes CcdA, ResA, StoA, BdbA, BdbB, BdbC, and BdbD (3, 13, 15, 27). Unlike CcdA, which is a polytopic membrane protein, YneN possesses a single transmembrane segment (14). YneN is unique among this group in that it has no known function in *B. subtilis*, and clearly is not involved in either

cytochrome *c* maturation or sporulation (14, 15). The proximity of the genes suggests that CcdA1 and the YneN-like protein might be a functional pair with CcdA1 serving as the electron donor to the YneN-like protein, as has been previously suggested (29).

The gene expression profiles of *ccdA1* and *ccdA2* are consistent with their observed activities. When grown on rich medium, *ccdA1* and *ccdA2* are expressed robustly. The relevance of significantly higher expression of *ccdA2* relative to *ccdA* to reduced need for *ccdA1* given its reduced spectrum of cellular activity. When grown in the parental 34F2 strain grown in rich medium is unclear, but could perhaps be related to sporulation inducing media, *ccdA2* expression was unchanged, but *ccdA1* expression dropped to near background levels. As CcdA1 is not needed in sporulation, lack of expression under sporulation-inducing conditions could be expected. Experimental induction of artificially high transcription of either *ccdA1* or *ccdA2* using our complementation plasmids does not alter the phenotypes of the parental strain or the complementation strains without induction, suggesting that increased production of either CcdA does not strongly affect *B. anthracis* physiology.

When the *B. anthracis ccdA* complementation plasmids were placed in a *B. subtilis ccdA* deletion strain, a different pattern of activity emerged than that seen in *B. anthracis*. We found that neither CcdA1 nor CcdA2 was active in cytochrome *c* maturation, odd given the similarity in the target thiol-disulfide oxidoreductase in cytochrome *c* maturation, ResA, in both organisms. CcdA2 could restore sporulation while CcdA1 could not, identical to the situation in *B. anthracis*. This, too, is unexpected in light of our previous findings given the low degree of similarity between the CcdA sporulation target thiol-disulfide oxidoreductase, StoA, and any other predicted thiol-disulfide oxidoreductase in the *B. anthracis* genome. We cannot rule out the possibility that our in trans complementation approach may not accurately reflect the

activities of native CcdA, but the internal consistency of these experiments does highlight functional differences. These observations suggest that specificity of thiol-disulfide oxidoreductases cannot easily be predicted and is dependent upon factors beyond the conservation of the active cysteine residues.

Thiol-disulfide oxidoreductases have been implicated in the pathogenesis of several organisms, including *E. coli* (16, 52), *Vibrio cholera* (33), and *Haemophilus influenzae* (36), among many others (reviewed in 21)). The effect the loss of *ccdA1/2* on virulence of *B. anthracis* is unclear. The virulence gene dysregulation upon loss of cytochrome *c* activity is unique: increased *atxA* only during exponential phase that leads to increased toxin expression in stationary phase though a mechanism still under investigation. This effect, however, is seen only under in vitro conditions that do not induce strong toxin production (49). It was recently reported that manipulation of the *atxA* promoter to overexpress AtxA did not alter virulence in a murine model of infection (11). While this experiment did not directly replicate the unique *atxA* expression profile found in loss of *ccdA1/2*, it does raise questions about the relevance of AtxA overexpression. It is also unclear as to whether loss of *c*-type cytochromes at the cell surface has any effect of virulence of *B. anthracis*. Beyond the connection between cytochrome *c* maturation and virulence regulation through AtxA, the association between CcdA and spore formation provides a link to *B. anthracis* pathogenicity. Spores of *B. anthracis* are the infectious particle and are capable of persisting outside the host under adverse environmental conditions. Germination of spores to vegetative cells allows production of virulence factors, but the vegetative cells are incapable of initiating infection (18, 23), thus the process of sporulation is essential to perpetuate the infectious cycle of *B. anthracis*. Factors that alter or reduce

sporulation, such as loss of CcdA1/2, disrupt the pathogenic lifecycle of *B. anthracis* and can significantly reduce the infectious success of *B. anthracis*.

While these observations provide a unique view of the function and specificity of these duplicate *ccdA* genes, they also highlight the gaps in our knowledge of thiol- disulfide oxidoreductases. Thiol-disulfide oxidoreductases are essential to correct assembly of surface-exposed proteins that are important to many processes in *B subtilis*, including sporulation (41, 42), competence (27), cytochrome maturation (43), and lantibiotic production (12). It is certainly possible that thiol-disulfide oxidoreductases could be involved in other as yet undefined processes in Gram-positive bacteria. Further, thiol-disulfide oxidoreductases are an interesting drug target, given their roles in the pathogenesis of many organisms and evidence suggesting that inhibitors can block viral infection (37). Additional work is required to define the relationships of CcdA1/2 and other thiol-disulfide oxidoreductases to their cognate targets in order to define their activities in bacterial physiology and virulence.

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### 3 CHAPTER THREE: CYTOCHROME *C*<sub>551</sub> AND THE CYTOCHROME *C* MATURATION PATHWAY AFFECT VIRULENCE GENE EXPRESSION IN *BACILLUS CEREUS* ATCC14579

#### 3.1 Introduction

*Bacillus cereus* is a Gram-positive, spore-forming, soil-dwelling bacterium that can be pathogenic in humans. *B. cereus* is most often observed as a causative agent of food poisoning but is also responsible for a wide range of opportunistic and nosocomial infections (reviewed in 1). This species belongs to the *B. cereus* group that includes the closely related species *Bacillus anthracis* and *Bacillus thuringiensis* (2). Despite high degree of genetic similarity between *B. cereus* and *B. anthracis*, the molecular mechanisms of pathogenesis are dissimilar. Pathogenesis of *B. cereus* relies on a number of hemolytic and non-hemolytic enterotoxins controlled by the quorum-sensing regulatory system PlcR-PapR, all encoded by genes on the main bacterial chromosome (3-5). Pathogenesis of *B. anthracis* relies on production of anthrax toxin and capsule, expression of which are controlled by the virulence regulatory protein AtxA, and these virulence factors are encoded by genes on extra- chromosomal virulence plasmids (6-8). While *B. anthracis* carries the same chromosomally-encoded enterotoxin genes as *B. cereus*, they are not expressed due to a truncation mutation in *plcR* (3, 9).

*B. cereus* produces a number of secreted proteins, including hemolysins, phospholipases, and enterotoxins, that cause host cellular damage. The three most important enterotoxins are the non-hemolytic enterotoxin (Nhe), hemolysin BL (Hbl), and cytotoxin K (CytK) (10, 11). Nhe is a tripartite toxin encoded by three contiguous genes that contributes to diarrheal disease through pore formation in host cell membranes (10, 12-14). Hbl is a similar tripartite toxin that also

causes pore formation in host cell membranes (10, 15, 16). The similarity in structure and activity of Hbl and Nhe enterotoxin complexes has led to the hypothesis that the *nhe* and *hbl* operons are the result of a gene duplication event in an ancestral species (14). CytK is a  $\beta$ -barrel channel-forming toxin that damages host cells by membrane pore formation and contributes to necrotic enteritis, a potentially fatal outcome of *B. cereus* food poisoning (11). Production of virulence factors in *B. cereus* is tied to the metabolic state of the cell, and several metabolic regulators have been shown to alter the expression profiles of enterotoxin genes. The catabolite control protein CcpA represses transcription of the Nhe and Hbl operons by binding to putative CRE sites (17). Expression of the enterotoxin genes is also influenced by the pleiotropic regulator CodY, tying virulence expression to intracellular GTP and branched-chain amino acid levels (18). Enterotoxin production is sensitive to the oxidation-reduction potential through complex interactions between multiple regulators. In *B. cereus* strain F4430/73, Nhe and Hbl expression increases when is grown under anaerobic conditions (19, 20) due to changes in the activity of the redox regulator Fnr and the two-component system ResDE in response to changes in oxidoreduction potential (ORP). ResDE and Fnr directly regulate expression of Hbl and Nhe and indirectly regulate all enterotoxin expression through changes to PlcR expression (20-22). An additional two-component system, OhrRA, is also involved in regulating enterotoxin production in response to changes in ORP (23).

It was recently shown in *B. anthracis* that the *c*-type cytochromes affect virulence gene expression of the anthrax toxin genes (24). *c*-type cytochromes contribute, though are not essential, to aerobic respiration and are distinct from other cytochromes due to their covalently attached heme (25). In *B. subtilis*, five membrane-bound proteins with covalently bound heme have been identified: the 39-kDa subunit II of cytochrome *caa*<sub>3</sub> (CtaC), the 28-kDa cytochrome *c*

subunit of the *bc* complex (QcrC), the 25-kDa cytochrome b subunit of the cytochrome *bc* complex (QcrB), the 13-kDa cytochrome *c*<sub>550</sub> (CccA), and the 10-kDa cytochrome *c*<sub>551</sub> (CccB) (26, 27). The functions of the two small *c*-type cytochromes, CccA and CccB, remain unknown. Both the *B. cereus* and *B. anthracis* genomes contain *c*-type cytochrome genes similar to those of *B. subtilis*. In *B. anthracis*, loss of either the *c*-type cytochrome maturation system or simultaneous loss of CccA and CccB results in increased toxin gene production through altered transcription of the *B. anthracis*-specific virulence regulator AtxA. Neither CtaC nor QcrBC contribute to virulence regulation (24). Production of *c*-type cytochromes requires a number of proteins that constitute the cytochrome *c* maturation system. *c*-type cytochromes are initially produced as apo-proteins in the cytoplasm, missing their required co-factor, heme. The apo-cytochrome *c* is secreted across the membrane by the Sec translocation system. At the membrane, the oxidoreductase ResA reduces cysteine residues in the heme binding pocket of apo-cytochrome (28), while ResB and ResC transport and covalently attach heme to the reduced apo-cytochrome to generate the final, active form of cytochrome *c* (29, 30). Loss of ResB and ResC in *B. anthracis* also results in increased toxin gene production through AtxA. Toxin and AtxA gene expression is identical in the  $\Delta cccAB$  strain and the  $\Delta resBC$  strain, indicating that the role of ResBC in altered virulence gene expression is the production of active CccA and CccB (24).

Here we report that virulence gene expression in *B. cereus* is also influenced by *c*-type cytochromes and their maturation system. Loss of only the small *c*-type cytochrome CccB increases enterotoxin and *plcR* expression, but deletion of the cytochrome maturation system increases virulence gene expression more strongly than the loss of CccB alone. The increased virulence gene expression is *plcR*-dependent. Hemolytic activity in the cytochrome and

cytochrome maturation system mutants is also increased. These results reveal a newly identified regulatory network controlling *B. cereus* virulence gene expression and highlight differences in virulence regulation between *B. cereus* and *B. anthracis*.

### 3.2 Materials and Methods

**Bacterial strains and growth conditions** *B. cereus* strain ATCC14579 and its derivatives were routinely grown in LB, NSMP (nutrient sporulation medium phosphate) (31), or brain heart infusion (BHI) broth supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol (7.5 µg/ml), erythromycin (25 µg/ml), lincomycin (25 µg/ml), and kanamycin (150 µg/ml). 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) (40 µg/ml) was added to LB agar to monitor-galactosidase activity as necessary. Competent cells of *B. cereus* were prepared following the method of Koehler et al (32), and electroporation was performed using the Bio-Rad-Gene Pulser according to the instructions of the supplier.

*E. coli* TG1, C600 and DH5 $\alpha$  competent cells were used for the propagation and isolation of all plasmid constructs. *E. coli* transformation was performed in chemically competent cells as previously described (33). Transformants were selected on LB agar supplemented with ampicillin (100 µg/ml), chloramphenicol (7.5 µg/ml), or kanamycin (30µg/ml).

**Plasmid and strain construction** Strains and plasmids are listed in Table 1. Oligonucleotide primers are listed in Table 2. Marker-less gene deletions in *B. cereus* were generated through a modification of the technique of Janes and Stibitz (34), as previously described (35). The  $\beta$ - galactosidase reporter plasmid pAW325 was created by digesting pTCV-*lac* (36) with SnaBI and PstI, blunt-ending with T4 DNA polymerase, and re-ligating, resulting

in a promoter-less *lacZ* transcriptional fusion plasmid carrying only a kanamycin-resistance cassette.

**$\beta$ -Galactosidase assays** *B. cereus* strains harboring gene promoter fusions on the replicative vector pTCV-lac or pAW325 were grown at 37°C, as indicated, in LB supplemented with the appropriate antibiotics.  $\beta$ -galactosidase activity was assayed as described previously and specific activity was expressed in Miller units (37, 38).

**TMPD Oxidase Staining** The presence of active c-type cytochrome oxidase was verified by a colorimetric assay using N,N,N,N-tetramethyl-p-phenylenediamine (TMPD) as an artificial electron donor that can be oxidized by cytochrome *caa3*. TMPD oxidase staining was performed on *B. cereus* grown on NSMP as previously described (24, 29).

**Sporulation analysis** Sporulation assays were performed using strains grown in NSMP or SM(Schaeffer's sporulation medium) (39) at 37°C for the time indicated in each experiment. Sporulation was initially monitored by visualization of cells on a Zeiss Axio Imager microscope and enumeration of spores and vegetative cells from multiple independent fields. Sporulation efficiency was assayed by chloroform treatment and enumeration of spores and vegetative cells as previously described for *B. anthracis* (40). Sporulation efficiency is presented as percentage of spores relative to total viable cells.

**Quantitative hemolysis assay** Overnight cultures of *B. cereus* were diluted to an OD<sub>600</sub> of 0.01 in LB and grown for 7 hours at 37°C. Samples were centrifuged for 5 minutes at 10,000xg, and supernatants were transferred to a new tube. Supernatant samples were diluted as indicated in PBS. Supernatant samples were then added to 3% washed sheep red blood cells in PBS and loaded onto 96-well microtiter plates. Only PBS was added as 0% hemolysis control, and 1% Triton X100 was added as 100% hemolysis control. Plates were incubated for 30 minutes

at 37°C and then read at OD<sub>540</sub>. Percentage hemolysis was calculated as  $\{1 - (\text{OD}_{\text{Sample}} - \text{OD}_{100\% \text{ control}}) / (\text{OD}_{0\% \text{ control}} - \text{OD}_{100\% \text{ control}})\} \times 100$ . Results were plotted and a 50% hemolysis value was calculated.

### 3.3 Results

***B. cereus* ATCC14579 carries a single *resB* gene and produces active cytochrome *c* oxidase.** In *B. subtilis* and *B. anthracis*, a single *resB* gene encodes a protein required for covalent attachment of heme to apo-cytochrome *c* to produce active *c*-type cytochromes. According to the published genome sequence of *B. cereus* type strain ATCC14579, ResB is encoded by two genes, BC1474-5, predicted to encode the n- terminal and c-terminal portions of ResB, respectively (41). BC1474 (GenBank: AAP08454.1) and BC1475 (GenBank: AAP08455.1) have 98% amino acid identity relative to the ResB of *B. anthracis* (GenBank: AAT53704.1). To confirm this observation, the region encoding BC1474 and BC1475 was amplified by PCR and sequenced. Complete sequencing of *resBC* region demonstrated that the frame shift mutation indicated in the ATCC14579 genome sequence is not present in our amplified sequence (data not shown), indicating that ResB is encoded by a single, full-length gene highly similar to *B. anthracis resB*. *B. cereus* ATCC14579 is positive for TMPD- oxidase activity, confirming cytochrome *c*-maturation activity of the strain (Table 3).

**Loss of *resBC* results in increased expression of virulence genes.** In *B. anthracis*, loss of the cytochrome *c* maturation pathway results in increased transcription of the anthrax toxin genes (24, 35). In order to investigate the role of cytochrome *c* maturation pathways in *B. cereus* virulence gene regulation, a strain containing a marker-less deletion of *resBC* was created (AW-C006). Loss of *resBC* resulted in complete abolition of cytochrome *c* oxidase activity as

indicated by TMPD oxidase staining (Table 3). Loss of *resBC* did not significantly affect growth in LB (Figure 1A) or BHI (data not shown). To assay the role of ResBC in virulence gene expression, the previously mapped promoter regions of the major enterotoxin genes *cytK* (42), *hbl* (43), and *nhe* (44), as well as the virulence regulator *plcR* (3, 5), were fused to a promoter-less *lacZ* gene and promoter activity was monitored by  $\beta$ -galactosidase analysis. As shown in figure 1B, promoter activity of all three enterotoxin genes was increased relative to the parental strain as the cultures enter stationary phase. In *B. anthracis*, increased toxin gene expression was associated with an increase in transcription of the virulence regulatory gene *atxA* during exponential growth (24). In *B. cereus*, an increase in *plcR* promoter activity was observed during stationary phase relative to the parental strain, similar to the profiles of the enterotoxin genes (figure 1B inset). A second, independent *resBC* deletion strain was generated (AW- C019), and the phenotypes associated with this strain are the same as the first  $\Delta$ *resBC* strain (data not shown).

To confirm of the role of *resBC* in virulence gene expression, a region containing *resBC* was cloned into plasmid pAW285 (35), placing *resBC* transcription under the control of an exogenous promoter. Figure 2 represents virulence gene expression at 7 hours post inoculation, during stationary phase when maximum differences in promoter activity were seen in the  $\Delta$ *resBC* strain. Addition of *resBC* to the parental ATCC14579 strain had no effect on *cytK* promoter activity relative to the parental strain transformed with empty vector. Complementation of *resBC* in trans expressed from *B. subtilis xylA* promoter reduced *cytK* expression to parental levels in the  $\Delta$ *resBC* strain, confirming that the increased virulence gene expression in these mutants is due to the loss of *resBC*.

**Loss of only *cccB* results in increased expression of virulence genes.** In *B. anthracis*, the increased virulence gene expression associated with loss of cytochrome *c* maturation is the result of deficiency in both the two small *c*-type cytochromes encoded by *cccA* and *cccB*. Loss of other *c*-type cytochromes has no effect on virulence gene expression (24). Deletions were made of all four *c*-type cytochromes (*cccA*, *cccB*, *ctaC*, and *qcrBC*) singly and in combinations of multiple genes. Cytochrome *c* activity was assayed in the mutants by TMPD oxidase analysis (Table 3). As expected, strains lacking *ctaC*, encoding a subunit of cytochrome *c*-oxidase, were TMPD-negative. All other cytochrome mutants were TMPD-positive, showing that loss of these genes has no effect on the activity of cytochrome *c* oxidase. None of the cytochrome *c* mutants displayed a growth defect in LB when grown under aerobic conditions. To analyze gene expression,  $\beta$ -galactosidase analysis was performed using the four virulence gene reporters. Figure 3 represents promoter activity at 7 hours post inoculation, when maximum differences in promoter activity were seen in the  $\Delta resBC$  strain. The relative gene expression profiles of all four virulence reporters are similar across tested strains. Loss of *cccA* does not alter promoter activity, while loss of *cccB* increases promoter activity, though not as strongly as the  $\Delta resBC$  strain. A strain missing both *cccA* and *cccB* was similar to the  $\Delta cccB$  strain. This is different than what was seen in *B. anthracis*, where loss of either small *c*-type cytochrome had no effect individually and loss of both *cccA* and *cccB* completely replicated the virulence gene over-expression phenotype of the  $\Delta resBC$  mutant (24). Single deletions of *ctaC* and *qcrBC* had no effect on promoter activity (data not shown). Promoter activity in a strain carrying deletion of all *c*-type cytochrome genes was similar to the  $\Delta cccB$  single deletion mutant but still below the levels of the  $\Delta resBC$  mutant.

The *cccB* coding region was cloned into plasmid pHT315S (45), placing *cccB* transcription under the control of a constitutively active, exogenous promoter. Addition of *cccB* to the parental ATCC14579 strain had no effect on *cytK* promoter activity relative to the parental strain transformed with empty vector (Figure 4). Complementation of *cccB* in trans expressed from a constitutively active, artificial SPAC promoter reduced *cytK* promoter activity to parental levels in the  $\Delta cccB$  and  $\Delta cccAB$  strains. Similar reductions in promoter activity were seen in the  $\Delta cccB$  and  $\Delta cccAB$  strains carrying other virulence gene reporters (data not shown), further confirming that the increased promoter activity in these mutants is due to the loss of *cccB*.

**Hemolytic activity is increased in cytochrome mutants.** *B. cereus* is a  $\beta$ -hemolytic bacterium due to the production of hemolysins, including hemolysin BL, encoded by the *hbl* gene used in our promoter analysis. In support of the gene expression analysis, quantitative hemolysis assays were performed on the cytochrome mutants. As shown in figure 5, hemolysis in the  $\Delta cccA$  mutant was similar to the parental strain. Strains that lacked *cccB*, *cccAB* or all four *c*-type cytochrome genes increased hemolysis to similarly high levels relative to the parental strain. Deletion of *resBC* further increased hemolysis beyond the levels measured in the  $\Delta cccB$  mutant. These results are consistent with the  $\beta$ -galactosidase data and provide functional confirmation of gene expression analysis.

**Sporulation is unaffected in cytochrome mutants.** A previous report suggests that loss of the small *c*-type cytochrome *c550*, encoded by *cccA*, alters sporulation initiation in *Bacillus subtilis* (46). The effect of loss of *c*-type cytochromes or *c*-type cytochrome maturation on sporulation in *B. cereus* was measured by a liquid sporulation assay. As shown in Table 3, sporulation efficiency was similar to the parental stain in all mutants tested, including mutants

missing *cccA*, indicating that loss of neither the *c*-type cytochromes nor the *c*-type cytochrome maturation system affects sporulation in *B. cereus*.

**Toxin expression in *resBC* mutant is PlcR-dependent.** In *B. anthracis*, increased toxin gene expression was the result of increased *atxA* transcription (24). *plcR* transcription was elevated in the  $\Delta resBC$  mutant and in the mutants containing a deletion of *cccB*. To further investigate the role of *plcR*, marker-less deletion strains were made missing *plcR-papR* and both *resBC* and *plcR-papR*. As shown in Figure 6, toxin gene and *plcR* expression levels were very low in the  $\Delta plcR-papR$  mutant, as expected based on previous findings (3, 5), but expression levels were above those of promoter-less pTCV-lac. Promoter activities in the  $\Delta resBC \Delta plcR-papR$  strain was similar to the  $\Delta plcR-papR$  deletion strain, suggesting that loss of *resBC* has no effect on virulence gene expression in the absence of *plcR-papR*.

Table 3.1 Strains and plasmids used in Chapter 3.

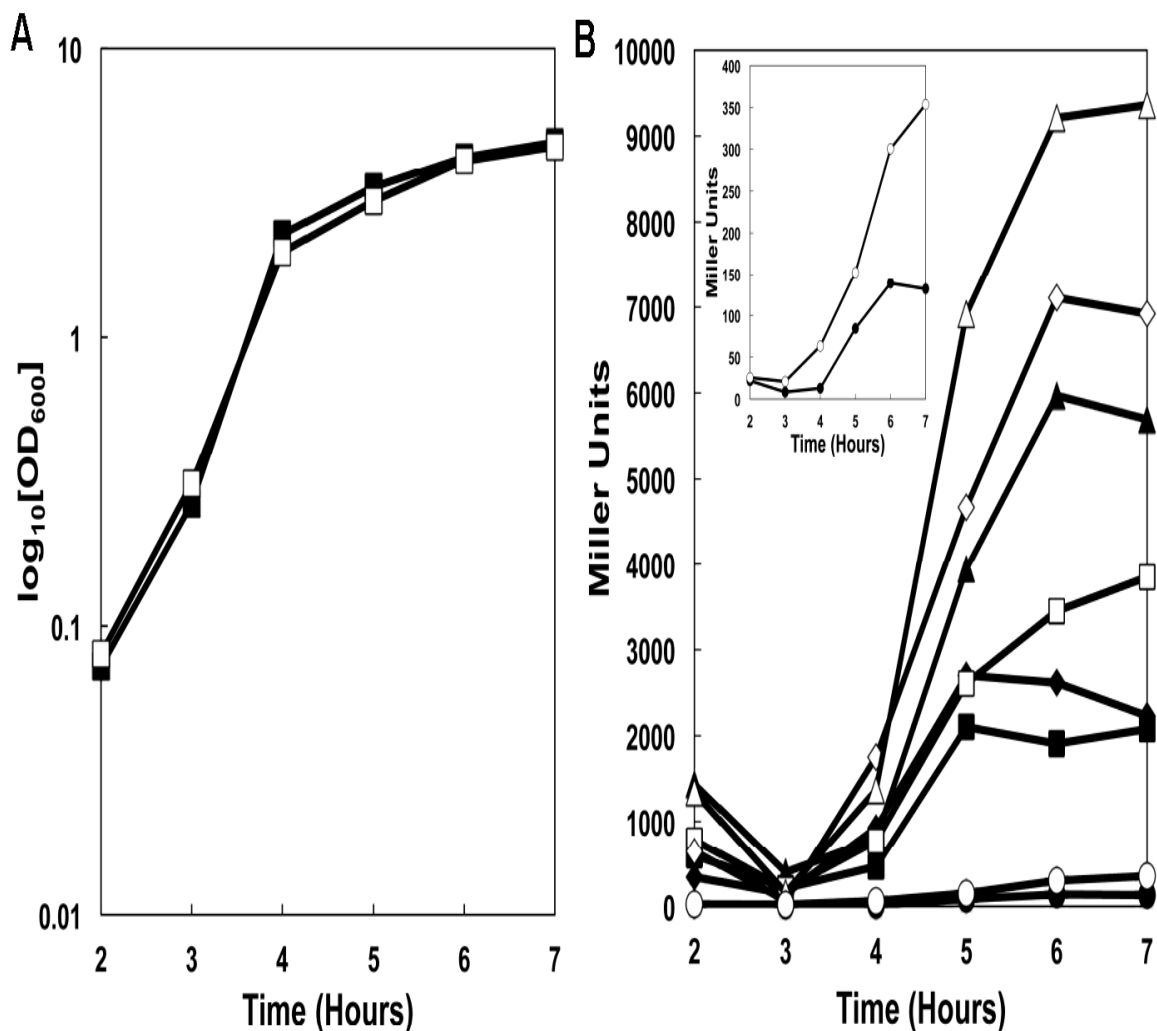
Table 1: Strains and plasmids used in this study.		
Strain or plasmid	Relevant Characteristics	Source
<b><i>B. cereus</i></b>		
ATCC14579	Type strain of <i>B. cereus</i>	Laboratory stock
AW-C006	Markerless deletion of <i>resBC</i>	This study
AW-C007	Markerless deletion of <i>plcR-papR</i>	This study
AW-C008	Markerless deletion of <i>cccA</i>	This study
AW-C009	Markerless deletion of <i>cccB</i>	This study
AW-C011	Markerless deletions of <i>cccA</i> and <i>cccB</i>	This study
AW-C012	Markerless deletions of <i>resBC</i> and <i>plcR-papR</i>	This study
AW-C013	Markerless deletion of <i>ctaC</i>	This study
AW-C017	Markerless deletion of <i>qcrBC</i>	This study
AW-C016	Markerless deletions of <i>cccA</i> , <i>cccB</i> , <i>ctaC</i> , and <i>qcrBC</i>	This study
AW-C019	Markerless deletion of <i>resBC</i>	This study
<b>Plasmids</b>		
pHT315s	Shuttle vector with P <sub>SPAC</sub> promoter, Amp <sup>R</sup> , Erm <sup>R</sup>	45
pORI-Cm-I-SceI	pORI-Cm vector with I-SceI recognition site, Cm <sup>R</sup>	40
pSS4332	I-SceI expression plasmid, Kan <sup>R</sup>	35
pTCV-lac	Promoterless vector for <i>lacZ</i> fusion, Erm <sup>R</sup> Kan <sup>R</sup>	36
pAW250	<i>nheA-lacZ</i> transcriptional fusion in pTCV-lac, Erm <sup>R</sup> Kan <sup>R</sup>	This study
pAW270	<i>cytK-lacZ</i> transcriptional fusion in pTCV-lac, Erm <sup>R</sup> Kan <sup>R</sup>	This study
pAW285	Xylose-inducible expression plasmid, Cm <sup>R</sup>	35
pAW287	<i>hblL2-lacZ</i> transcriptional fusion in pTCV-lac, Erm <sup>R</sup> Kan <sup>R</sup>	This study
pAW288	<i>plcR-lacZ</i> transcriptional fusion in pTCV-lac, Erm <sup>R</sup> Kan <sup>R</sup>	This study
pAW325	Promoterless vector for transcriptional <i>lacZ</i> fusion, Kan <sup>R</sup>	This study
pAW330	Regions flanking BC1474-6 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW340	Regions flanking BC5349-50 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW349	Regions flanking BC4288 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW352	Regions flanking BC5187 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW353	<i>cccB</i> expression vector, Cm <sup>R</sup>	This study
pAW368	Regions flanking BC3943 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW369	Regions flanking BC1523-4 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW395	<i>cytK-lacZ</i> transcriptional fusion in pAW325, Kan <sup>R</sup>	This study
pAW396	pHT315S-based <i>cccB</i> expression vector, Amp <sup>R</sup> , Erm <sup>R</sup>	This study
pAW426	pAW285-based <i>resBC</i> expression vector, Cm <sup>R</sup>	This study

<sup>a</sup> Cm<sup>r</sup>, chloramphenicol resistance, Kan<sup>r</sup>, Kanamycin resistance

Table 3.2 Oligonucleotide primers used in Chapter 3.

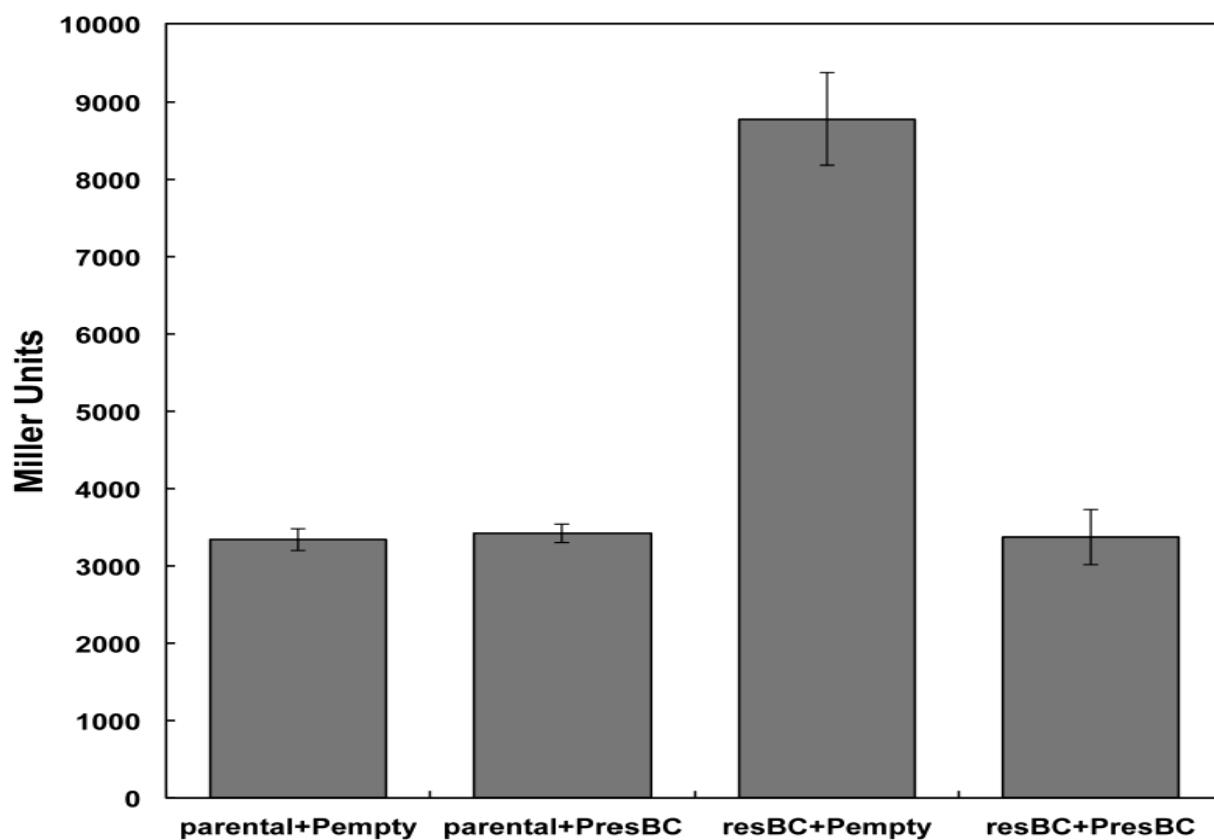
Table 2: Oligonucleotide primers used in this study

Name	Function	Sequence
BC14745'Bam	Region upstream of BC1474 for amplification of <i>resB</i>	5'-CACCGGATCCTTGC GGTAACCTTGAGGTTAGAA
BC14753'Sal	Region downstream of BC1475 for amplification of <i>resB</i>	5'-TGCCTCGACATCGTTTCCCCACCTTAAC
BcresBU5'Acc	Region upstream of BC1474 for deletion of <i>resBC</i>	5'-CACCGGTACCTGGTGTGTAGGATTTACCC
BcresBU3'Sal	Region upstream of BC1474 for deletion of <i>resBC</i>	5'-TGCCTCGACTTCTAACCTCAAGTTACCGCAAT
BcresCD5'Sal	Region downstream of BC1476 for deletion of <i>resBC</i>	5'-CACCGTCGACAATTATTGCCGGTTTACATTCA
BcresCD3'Pst	Region downstream of BC1476 for deletion of <i>resBC</i>	5'-TGCCTGCAGTGTGCGCTTTCTTCTCACGAA
BcresBUSeq	Check primer upstream of BC1474	5'-GCGAGAGAATTGACACCACA
BC4288U5'Bam	Region upstream of BC4288 for deletion of <i>cccA</i>	5'-CACCGGATCCGGACCATGCAGCCTATGAAT
BC4288U3'Sal	Region upstream of BC4288 for deletion of <i>cccA</i>	5'-TGCCTCGACTCCCCCTTCTATACCCTGA
BC4288D5'Sal	Region downstream of BC4288 for deletion of <i>cccA</i>	5'-CACCGTCGACCGTATGACAAGCCTTTTGC
BC4288D3'Pst	Region downstream of BC4288 for deletion of <i>cccA</i>	5'-TGCCTGCAGTCATGGATAAGCTCCCAACC
BC4288USeq	Check primer upstream of BC4288	5'-TAGGCCGTGGTATGCTTTTC
BC4288DSeq	Check primer downstream of BC4288	5'-AGAGTAAGGCGCTGCAACAT
BC5187U5'Bam	Region upstream of BC5187 for deletion of <i>cccB</i>	5'-CACCGGATCCGGAGAGCAAAAAGAAATCG
BC5187U3'Sal	Region upstream of BC5187 for deletion of <i>cccB</i>	5'-TGCCTCGACACTTCTATGCTCCCCATT
BC5187D5'Sal	Region downstream of BC5187 for deletion of <i>cccB</i>	5'-CACCGTCGACAAGGTGAAATGGACGAGTTGA
BC5187D3'Pst	Region downstream of BC5187 for deletion of <i>cccB</i>	5'-TGCCTGCAGTACACGCTCAGCAATTCGAT
BC5187E5'Bam	Amplification of <i>cccB</i> ORF	5'-CACCGGATCCGGGGAGCGGATAGAAGTGAAA
BC5187E3'Sal	Amplification of <i>cccB</i> ORF	5'-TGCCTCGACTCAACTCGTCCATTTACCTT
plcRU5'Bam	Region upstream of <i>plcR</i> for deletion of <i>plcR-papR</i>	5'-CACCGGATCCACTAACAGTTGTGATTACTCCATT
plcRU3'Sal	Region upstream of <i>plcR</i> for deletion of <i>plcR-papR</i>	5'-TGCCTCGACGTGCATACTTACTACCCATCC
papRD5'Sal	Region downstream of <i>papR</i> for deletion of <i>plcR-papR</i>	5'-CACCGTCGACCGTCTTACTTAGCGGGTTG
papRD3'Pst	Region downstream of <i>papR</i> for deletion of <i>plcR-papR</i>	5'-TGCCTGCAGCCTGCCCAATTTATCCCTCT
plcR3'promBam	Region upstream of <i>plcR</i> for <i>lacZ</i> fusion	5'-TGC GGATCCTAATTTTTCTGCGTGCAT
plcR5'promXma2	Region upstream of <i>plcR</i> for <i>lacZ</i> fusion	5'-CACCCCCGGGTTTGCAAATTCATTTGATACGG
nhe5'promXma	Region upstream of <i>nheA</i> for <i>lacZ</i> fusion	5'-CACCCCCGGGCCGGTTACGGTCTGTTATAC
nhe3'promBam	Region upstream of <i>nheA</i> for <i>lacZ</i> fusion	5'-TGC GGATCCGCTTACAGGAATGAAGCAA
cytK5'promEco	Region upstream of <i>cytK</i> for <i>lacZ</i> fusion	5'-CACCGAATTCACAGCCAGCCCTCCTTATTGA
cytK3'promBam	Region upstream of <i>cytK</i> for <i>lacZ</i> fusion	5'-TGC GGATCCGAACGTTTCA TACAATCACTTCC
hbl5'promEco2	Region upstream of <i>hbl</i> for <i>lacZ</i> fusion	5'-CACCGAATTCGTTAATAATGATATTAGGATGTTTTGTG
hbl3'promBam2	Region upstream of <i>hbl</i> for <i>lacZ</i> fusion	5'-TGC GGATCCACTCCTTTACCATTGTTTTATAACT
BcctaCU5'Bam	Region upstream of BC3943 for deletion of <i>ctaC</i>	5'-CACCGGATCCTGGGATTTATGGTGCCTTCA
BcctaCU3'Sal	Region upstream of BC3943 for deletion of <i>ctaC</i>	5'-TGCCTCGACAGTCGCCACTGTTTCTTCA
BcctacD5'Sal	Region downstream of BC3943 for deletion of <i>ctaC</i>	5'-CACCGTCGACTGACTGGCAAATATGGCAAC
BcctaCD3'Pst	Region downstream of BC3943 for deletion of <i>ctaC</i>	5'-TGCCTGCAGTGAGATTTGCAAGCCGAGTA
BcctaCUSeq	Check primer upstream of BC3943	5'-AGAACAAGACCCGTCCTCAA
BcqcrBU5'Bam	Region upstream of BC1523 for deletion of <i>qcrB</i>	5'-CACCGGATCCTGACCTGTGATGAGCGTAT
BcqcrBU3'Sal	Region upstream of BC1523 for deletion of <i>qcrB</i>	5'-TGCCTCGACAATCTTTTATCCACCCAAATCA
BcqcrCD5'Sal	Region downstream of BC1524 for deletion of <i>qcrC</i>	5'-CACCGTCGACGAAGCTTTCCGAATTCGTTG
BcqcrCD3'Pst	Region downstream of BC1524 for deletion of <i>qcrC</i>	5'-TGCCTGCAGCATCGTTATGCAACGTCAC
BcqcrBUSeq	Check primer upstream of BC1523	5'-TTGGAAGAGAAATGGGGAAA
BcresBCE5'Sal	Amplification of <i>resB</i> and <i>resC</i> ORF	5'-CACCGTCGACTAGATACAGGGCGGTGAGA
BcresBC53'Pst	Amplification of <i>resB</i> and <i>resC</i> ORF	5'-TGCCTGCAGTGACATGCACCTCCCTATTTT



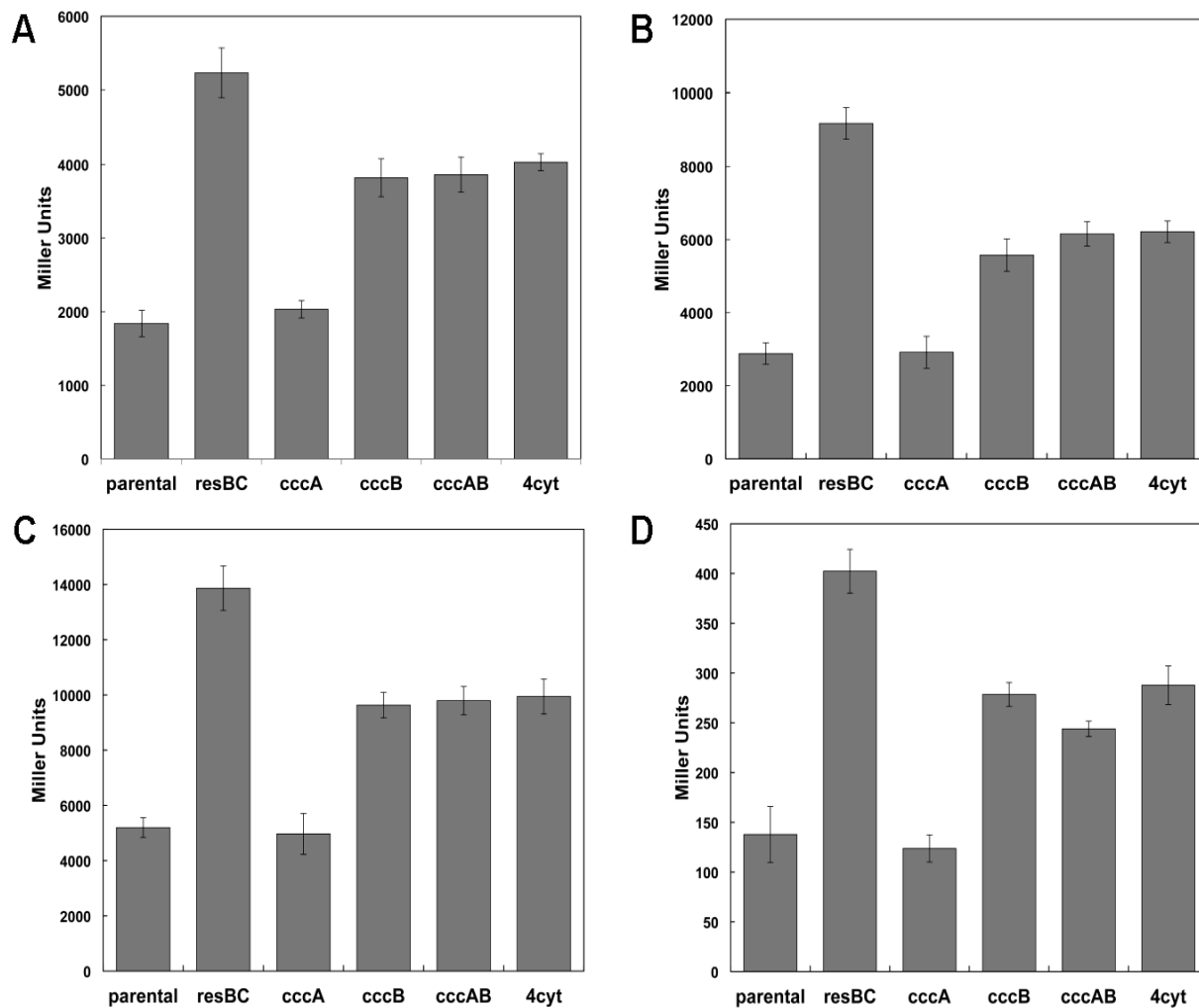
*Figure 3.1 Growth and virulence gene expression in parental and  $\Delta resBC$  mutant strain.*

**A.** Cell growth of parental and mutant strains grown in LB at 37°C. ■, ATCC 14579; □, AW-C006 ( $\Delta resBC$ ). **B.**  $\beta$ -galactosidase activity in virulence gene reporter strains grown in LB supplemented with kanamycin at 37°C. ■, ATCC 14579 *nhe-lacZ*; ◆, ATCC 14579 *cytK-lacZ*; ▲, ATCC 14579 *hbl-lacZ*; ●, ATCC 14579 *plcR-lacZ*; □, AW-C006 *nhe-lacZ*; ◇, AW-C006 *cytK-lacZ*; Δ, AW-C006 *hbl-lacZ*; ○, AW-C006 *plcR-lacZ*. Inset graph magnifies results from *plcR-lacZ* reporters



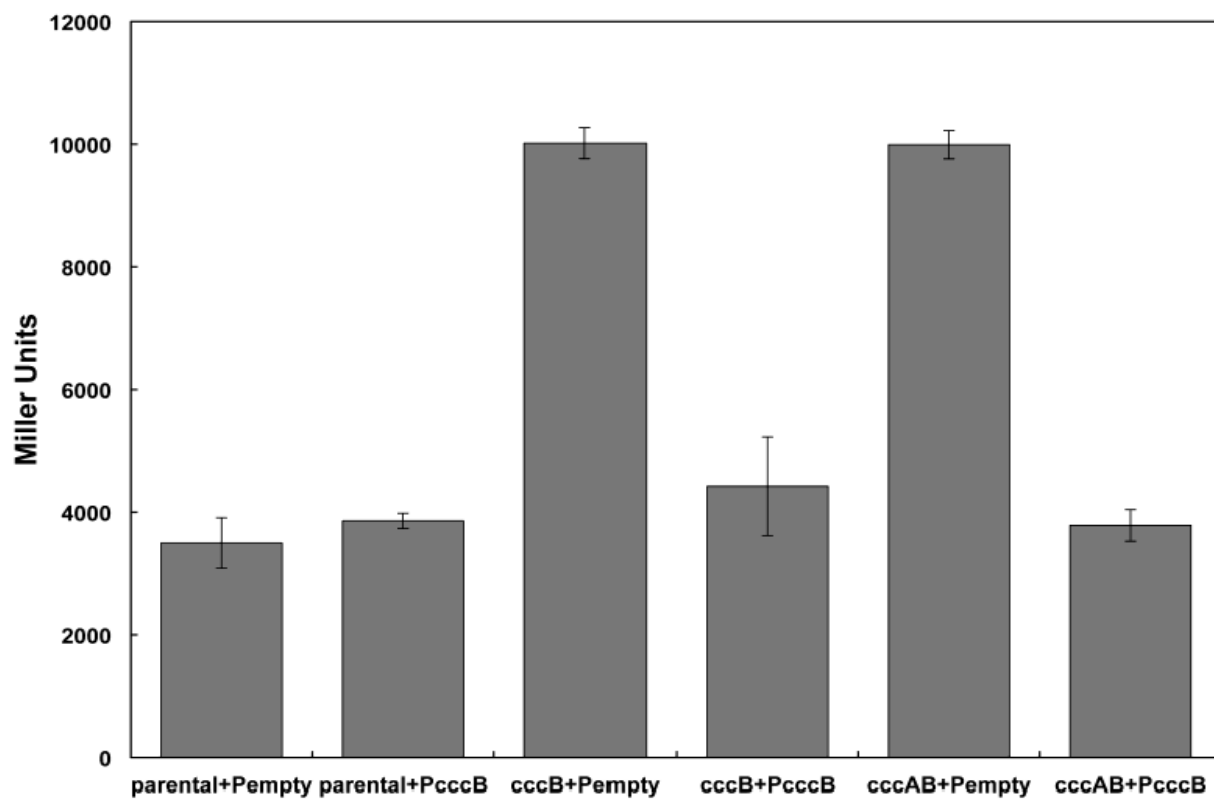
*Figure 3.2 Complementation analysis in  $\Delta resBC$  mutant.*

$\beta$ -galactosidase activity of ATCC 14579 and AW-C006 ( $\Delta resBC$ ) strains carrying the *cytK-lacZ* reporter plasmid and either empty complementation plasmid (pAW285) or *resBC* complementation plasmid (pAW426), as indicated, grown in LB supplemented with kanamycin and chloramphenicol at 37°C for 7 hours post inoculation. Data was obtained from 3 independent cultures and error bars represent standard deviation from the mean. Two tailed unpaired *t* test indicate P value of  $P < 0.0001$  between empty vector and complementation plasmid in  $\Delta resBC$  strain.



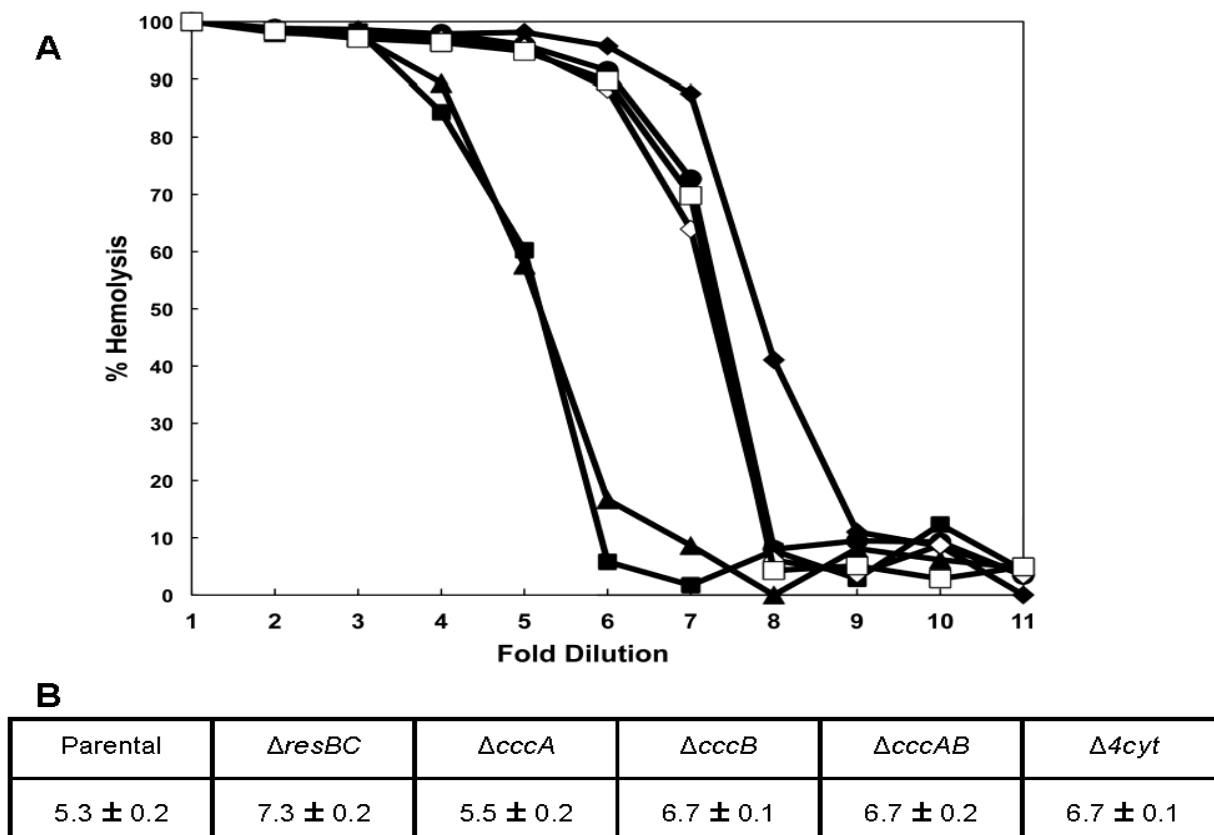
**Figure 3.3** Virulence gene expression in parental and mutant strains.

$\beta$ -galactosidase activity of parental and mutant strains carrying reporters, as indicated, grown in LB supplemented with kanamycin at 37°C for 7 hours post inoculation. Data was obtained from 3 independent cultures and error bars represent standard deviation from the mean. **A.** *nhe-lacZ*; **B.** *cytK-lacZ*; **C.** *hbl-lacZ*; **D.** *plrC-lacZ*. Two tailed unpaired *t* tests indicate P values of  $P < 0.0001$  between  $\Delta resBC$  and parental strain and  $P < 0.005$  between  $\Delta resBC$  and  $\Delta cccB$  mutant for all reporters.



*Figure 4.4 Complementation analysis in  $\Delta cccB$  mutant strains.*

$\beta$ -galactosidase activity of ATCC 14579, AW-C009 ( $\Delta cccB$ ), and AW-C011 ( $\Delta cccAB$ ) mutant strains carrying the *cytK-lacZ* reporter plasmid and either empty complementation plasmid (pHT315S) or *cccB* complementation plasmid (pAW396), as indicated, grown in LB supplemented with kanamycin, erythromycin, and lincomycin at 37°C for 7 hours post inoculation. Data was obtained from 3 independent cultures and error bars represent standard deviation from the mean. Two tailed unpaired *t* tests indicate P values of  $P < 0.0001$  between empty vector and complementation plasmid in both  $\Delta cccB$  and  $\Delta cccAB$  strains.



**Figure 4.5 Hemolytic activity of parental and mutant strains.**

Cell supernatants of indicated strains grown in LB at 37°C for 7 hours diluted as indicated. **A.** Hemolytic activity presented as percentage of red blood cell lysis relative to 0% hemolysis and 100% hemolysis controls. Data presented are average of three independent cultures. ■, ATCC 14579; ◆, AW-C006 ( $\Delta resBC$ ); ▲, AW-C008 ( $\Delta cccA$ ); ●, AW-C009 ( $\Delta cccB$ ); □, AW-C011 ( $\Delta cccAB$ ); ◇, AW-C016 (4cyt). **B.** Mean dilution value at which 50% hemolysis occurs is three independent cultures  $\pm$  standard deviation of the mean.

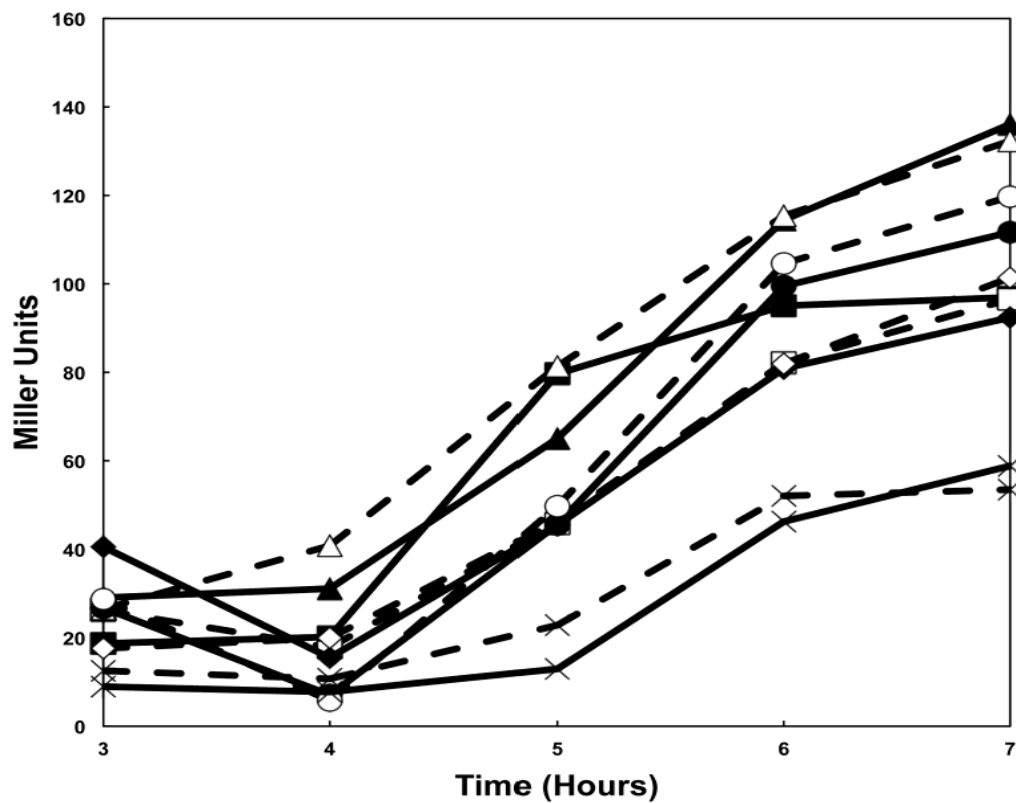


Figure 3.6 Virulence gene expression in  $\Delta plcR-papR$  and  $\Delta plcR-papR \Delta resBC$  mutant strains.

$\beta$ -galactosidase activity in virulence gene reporter strains grown in LB supplemented with kanamycin at 37°C. Filled symbols and solid lines represent AW-C007 ( $\Delta plcR-papR$ ) while empty symbols and dashed lines represent AW-C012 ( $\Delta plcR-papR \Delta resBC$ ). ■,  $nhe-lacZ$ ; ▲,  $cytK-lacZ$ ; ●,  $hbl-lacZ$ ; ○,  $plcR-lacZ$ ; x- promoter-less  $lacZ$

Table 3.3 TMPD activity and sporulation efficiency in wild-type and mutant *B. cereus* strains.

TABLE 3 TMPD activity and sporulation efficiency in wild-type and mutant *B. cereus* strains

Strain	Genotype	TMPD activity <sup>a</sup>	% sporulation <sup>b</sup>
ATCC 14579	Parental	+	27.3 ± 2.8
AW-C006	$\Delta resBC$	–	26.7 ± 1.6
AW-C008	$\Delta cccA$	+	ND
AW-C009	$\Delta cccB$	+	27.8 ± 3.3
AW-C011	$\Delta cccAB$	+	27.5 ± 2.7
AW-C013	$\Delta ctaC$	–	ND
AW-C017	$\Delta qcrBC$	+	ND
AW-C016	4 cyt	–	26.2 ± 2.0

<sup>a</sup> +, TMPD staining detected; –, no TMPD staining detected.

<sup>b</sup> Percentage of spores relative to the total number of viable cells. Values are means and standard deviations from the means for three independent assays. ND, not determined.

### 3.4 Discussion

This study reveals a role for the *c*-type cytochromes and the *c*-type cytochrome maturation pathway in the regulation of virulence gene expression in *B. cereus*. Loss of the cytochrome *c* maturation genes *resB* and *resC* results in increased expression of the major enterotoxins and the virulence regulator *plcR*. This effect can be partially, but not completely, attributed to loss of *cccB*, encoding cytochrome CccB (*c551*). These findings are similar to a

previous report that the two small *c*-type cytochromes, CccA and CccB, control virulence gene expression in *B. anthracis*; however, there appear to be significant differences in the molecular mechanisms of regulation. Unlike *B. anthracis*, where loss of both *cccA* and *cccB* is required for increased anthrax toxin expression, loss of only *cccB* in *B. cereus* results in increased virulence gene expression. In *B. anthracis*, the presence of CccA can complement the loss of CccB, but in *B. cereus*, CccA does not play a role in this regulatory pathway. CccA and CccB are similar in the amino acid sequence of their extracellular, heme-binding domains but differ in their membrane-anchoring systems: CccA has a predicted  $\alpha$ -helical transmembrane polypeptide membrane anchor while CccB is membrane attached by a diacylglycerol membrane anchor (27). Although CccA and CccB in *B. cereus* may have distinct activities, these functions remain unclear. As in *B. subtilis* and *B. anthracis*, the functions of CccA and CccB are most likely redox-related and associated with electron transfer (26), but they are not required for aerobic respiration as growth is not affected. None of the remaining *c*-type cytochromes (CtaC and QcrBC) are involved in the regulation of virulence gene expression indicating a specific participation of CccB in enterotoxin gene expression.

Previous studies have examined the role of ORP on enterotoxin production in *B. cereus* F4430/73. Anaerobic conditions lead to increased production of *nhe* and *hbl* (19, 47), while *cytK* and *plcR* transcription is largely unchanged (48). In this report, using *B. cereus* strain ATCC14579, we demonstrate increased *plcR*, *nhe*, *hbl*, and *cytK* transcription under aerobic conditions in response to the loss of cytochrome *c* maturation or CccB. As control of enterotoxin production occurs at both the transcriptional and post-transcriptional level (20, 47), increased hemolysis in the  $\Delta resBC$  and  $\Delta cccB$  functionally demonstrates increased enterotoxin production. These findings may reflect the previously noted differences in response to redox conditions

between strains of *B. cereus* (49). The function of CccB remains unclear as loss of CccB has no effect on growth or sporulation in *B. anthracis*, *B. subtilis*, or *B. cereus* (24, 27). In some thermophilic Bacilli, *c<sub>551</sub>* plays a significant role in oxidation of the terminal oxidase in the respiratory chain and is synthesized under air-limited conditions (50, 51), but the oxidase genes in this pathway are not present in *B. cereus*. It is possible that CccB may play an unappreciated role in respiratory control through an uncharacterized pathway.

These findings are unexpected in light of the differences in virulence gene regulation between *B. cereus* and *B. anthracis*. In *B. anthracis*, loss of cytochrome maturation activity results in increased toxin gene expression through increased *atxA* transcription (24). An *atxA*-like transcriptional regulator is not present in the *B. cereus* genome; instead, transcription of the gene encoding the major virulence regulator in *B. cereus*, *plcR*, is increased in the absence of *c*-type cytochrome maturation. Presence of PlcR is required for the cytochrome-dependent increase in virulence gene expression, arguing against a regulatory mechanism independent of PlcR. There is little similarity in the structure, regulation, or known activities of AtxA and PlcR, which makes a common mechanism acting upon both regulatory proteins unusual. The similarity in phenotypes with loss of cytochrome *c* maturation despite differences in regulatory networks raises the possibility of a conserved, previously unidentified regulatory pathway at work in both organisms.

The increased virulence gene expression and hemolysis in the  $\Delta resBC$  strain relative to the strain missing all known *c*-type cytochromes was surprising given that the only known functions of ResBC are related to heme delivery to *c*-type cytochromes (29, 30). Several possibilities may explain this discrepancy. The ResDE two-component system, known to regulate some enterotoxin gene expression, is encoded by genes downstream of *resBC*. In *B.*

*cereus* and *B. subtilis*, *resDE* is transcribed both from a promoter upstream of *resA*, producing a *resABCDE* transcript, and from a promoter upstream of *resD*, producing a *resDE* transcript (20, 52). A polar effect on expression of *resDE* is unlikely due to the marker-less nature of the *resBC* deletion and that addition of *resBC* in trans to the  $\Delta$ *resBC* mutant can complement the over-expression phenotype. Alternatively, ResBC could be transferring heme to a *c*-type cytochrome not found in *B. anthracis* or *B. subtilis*. This is unlikely given the highly similar gene content relative to *B. anthracis*, the lack of additional predicted *c*-type cytochromes in the *B. cereus* ATCC14579 genome, and the lack of additional predicted transmembrane proteins containing the characteristic CXXCH heme binding motif found in *c*-type cytochromes. Finally, ResBC could have additional activities in *B. cereus* that are not present in or have not been observed in either *B. anthracis* or *B. subtilis*. The role of ResBC in *B. cereus* and related organisms requires further investigation.

Despite the high degree of chromosomal synteny between *B. cereus* and *B. anthracis*, the chromosomally-encoded virulence regulatory networks are significantly different. The specificity of virulence control is of interest in light of fatal diseases caused by strains of *B. cereus* that carry virulence genes of both *B. cereus* and *B. anthracis* (53, 54). Insight into the mechanistic differences in the control of virulence factor expression would be helpful in understanding and tracking these dangerous pathogenic strains.

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## 4 CHAPTER FOUR: LOSS OF HOMOGENTISATE 1, 2-DIOXYGENASE ACTIVITY IN *BACILLUS ANTHRACIS* RESULTS IN ACCUMULATION OF PROTECTIVE PIGMENT

### 4.1 Introduction

*Bacillus anthracis* is a Gram-positive, endospore-forming bacterium that is the etiological agent of anthrax. Virulence in the mammalian host depends on production of anthrax toxin and capsule, carried on virulence plasmids pXO1 and pXO2, respectively (1). Expression of both the toxin and capsule genes is regulated by the master virulence regulator AtxA. The activity of AtxA and the production of virulence factors are tied through several regulatory networks to the physiological state of the cell (2–6).

Production of melanin pigments contributes to microbial pathogenesis, as it is associated with virulence in many microorganisms (reviewed in 7). Melanin can protect cells from oxidative damage, alter phagocytosis and phagocytotic killing, modify virulence factor activity, and change responses to antimicrobial compounds (7–9). Melanin production can also alter susceptibility to UV damage, protecting microorganisms from environmental damage (10). Pyomelanin is a melanin-like pigment produced by the spontaneous oxidation of homogentisic acid (11). Homogentisic acid is an intermediate in the pathway catabolizing L-tyrosine and L-phenylalanine to acetoacetate and fumarate in many species (12–14). Homogentisic acid normally does not accumulate in significant amounts as it is converted to maleylacetoacetate by the activity of homogentisate 1, 2-dioxygenase. In the absence of homogentisate 1, 2-dioxygenase, homogentisic acid accumulates in the cell and spontaneously

oxidizes to pyomelanin, a water-soluble brown pigment. Pyomelanin is also known as alkaptomelanin in the human genetic disorder alkaptonuria, in which L-tyrosine catabolism is disrupted by the loss of human homogentisate 1,2-dioxygenase, resulting in the accumulation of damaging brown pigment in tissues (14). In this report, we have identified and characterized the gene encoding homogentisate 1, 2-dioxygenase (*hmgA*) of *B. anthracis*. Loss of *hmgA* results in accumulation of pyomelanin in the presence of L-tyrosine and L-phenylalanine. Pyomelanin protects *B. anthracis* cells from UV damage but loss of *hmgA* does not alter other metabolic and virulence characteristics.

## 4.2 Materials and Methods

**Bacterial strains and growth conditions.** *B. anthracis* strain 34F2 (pXO1+ pXO2-) and its derivatives were routinely grown in LB or brain heart infusion (BHI) broth supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol (7.5 µg/ml), spectinomycin (100 µg/ml), and kanamycin (7.5 µg/ml). As indicated, *B. anthracis* was grown in a modified formulation of defined R-Media (15) buffered to pH 7.0 without 0.8% NaHCO<sub>3</sub> under 5% CO<sub>2</sub>. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 µg/ml) was added to LB agar to monitor β-galactosidase activity as necessary. Competent cells of *B. anthracis* were prepared following the method of Koehler et al (16), and electroporation was performed using the Bio-Rad-Gene Pulser according to the instructions of the supplier.

*E. coli* TG1, C600 and DH5α competent cells were used for the propagation and isolation of all plasmid constructs. *E. coli* transformation was performed in chemically competent cells as previously described (17). Transformants were selected on LB agar supplemented with

ampicillin (100 µg/ml), chloramphenicol (7.5 µg/ml), spectinomycin (100 µg/ml), or kanamycin (30 µg/ml).

**Transposon mutagenesis and analysis.** Transposition was performed in the 34F2 strain using the Mariner-based transposon delivery system pAW068 (18) and the protocols previously described (19). The mutants were screened for altered morphology when grown on LB-agar plates containing spectinomycin. To identify the site of insertions, mutant strains were grown overnight in BHI containing 0.5% glycerol and spectinomycin at 28°C. Genomic DNA was extracted from the overnight cultures using UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA). The site of insertion was identified by restriction digestion of genomic DNA using NsiI. Digested genomic DNA was then re-ligated and used to transform *E. coli*. The presence of the pUC origin of replication allows re-ligated DNA containing the transposed sequence to replicate in *E. coli* as a spectinomycin-resistant plasmid. Following isolation of plasmid from *E. coli*, sequencing of transposon-flanking DNA was performed using transposon-specific primers oMarSeq1 (5'- GCTTGTCATCGTCATCCTTGT) and oMarSeq2 (5'- GGCCGCGAAGTTCCTATT).

**Plasmid and strain construction.** Strains and plasmids are listed in Table 1. A 723 bp region upstream of BAS0228 was PCR amplified from 34F2 genomic DNA using primers BAS0228U5'Bam (5'-CACCGGATCC GCGTTTGAGCAGCATGTAAA) and BAS0228U3'Sal (5'-TGCGTCGACGGAATTGTACA TGTCGTTTATG) while a 638 bp region downstream of BAS0228 was amplified using primers BAS0228D5'Sal (5'-CACCG TCGACAAAGAGGCATGCTCAATAACG) and BAS0228D3'Pst (5'- TGCTGCAGCGTTACATCCGCAACCATC) using OneTaq DNA polymerase (NEB). The upstream PCR fragment was cloned into the BamHI and SalI restriction sites of pORI-I-SceI (20)

to generate plasmid pAW406. The downstream region was then cloned into the SalI and PstI sites of pAW406 to generate pAW407. A 590 bp region upstream of BAS0227 was PCR amplified using primers BAS0227U5'Bam (5' CACC GTCGACGAAAAGGAAGCAGG TGATGAG) and BAS0227U3'Sal (5' TCGTCTGACTCATAGGGAGGAATC ATGATGA) while a 610 bp region downstream of BAS0227 was amplified using primers BAS0227D5'Sal (5'-CACCGTCGACGAAAAGGAAGCAGGTGATGAG) and BAS0227D3'Pst (5'-TGCCTGCAGCTCACAAAACGG GCTATGCT) using OneTaq DNA polymerase. The upstream PCR fragment was cloned into the BamHI and SalI restriction sites of pORI-Cm-I-SceI to generate plasmid pAW416. The downstream region was then cloned into the SalI and PstI sites of pAW416 to generate pAW417. A region containing the coding region of BAS0028 was amplified using primers BAS0228U5'Bam and BAS0228U3'Sal. The PCR product was digested with SnaBI and BamHI and blunt-ended with T4 DNA polymerase. The PCR product was then cloned into pAW285, digested with EcoRI and blunt-ended with T4 DNA polymerase, to generate complementation plasmid pAW444. The sequence of all plasmids was verified by DNA sequencing. Marker-less gene deletions in *B. anthracis* were generated through a modification of the technique of Janes and Stibitz (21), as previously described (22).

**Vis-NIR Absorption Spectroscopy.** Cultures of parental and mutant strains were grown for 48 hours at 37°C. Cell-free supernatants were prepared by centrifugation of cultures at 10,000xg followed by transfer of supernatant to new tubes. The optical spectra of the supernatants were recorded on a Cary 5000 UV-Vis-NIR spectrophotometer at room temperature in 50 mM potassium phosphate buffer pH 7.5 as previously described (23).

**Cell-free extract analysis.** Overnight cultures of *B. anthracis* strains grown in LB were diluted to OD<sub>600</sub> of 0.01 in fresh LB and grown for 6 hours at 37°C. Cells were pelleted by

centrifugation and resuspended in phosphate-buffered saline. Cells were then disrupted by sonication, cell debris removed by filtration, and cell-free extracts transferred to new tubes. Amino acids were added as indicated to cell-free extracts at the following concentrations based on composition of R-medium: L-phenylalanine 125mg/L; L-tryptophan 35mg/L; L-tyrosine 144mg/L. After overnight incubation at 37°C, samples were assayed visually for color change and spectroscopically at OD<sub>400</sub>.

**UV and H<sub>2</sub>O<sub>2</sub> sensitivity tests.** For UV protection assays, *B. anthracis* strains were grown in LB at 37°C for 28 hours. The cultures were transferred into a petri dish and irradiated with UV at 302 nm as indicated in figure. Serial dilutions were performed and plated on LB-agar. After 18 hours at 37°C, colony forming units (CFU) were counted.

For H<sub>2</sub>O<sub>2</sub> sensitivity by disk diffusion analysis, *B. anthracis* strains were grown in LB at 37°C to an OD<sub>600</sub> of 0.6. Aliquots of bacterial cultures were then added to LB-agar cooled to 50°C and poured into petri dishes. Cooled and solidified plates were overlaid with H<sub>2</sub>O<sub>2</sub>-saturated paper disks at concentrations indicated. Plates were incubated overnight at 37°C and the diameters of the zones of clearance were measured.

H<sub>2</sub>O<sub>2</sub> protection was also assayed in liquid cultures similar to assays described previously (24). *B. anthracis* strains were grown in LB at 37°C for 24 or 48 hours. Bacteria were then pelleted by centrifugation and resuspended in cell-free supernatants. H<sub>2</sub>O<sub>2</sub> was added at concentrations indicated and cultures incubated for 15 minutes at 37°C. Serial dilutions were performed and plated on LB-agar. After 18 hours at 37°C, CFU of pre- and post-treatment cultures were counted.

**Sporulation and germination analysis.** Sporulation assays were performed using strains grown in SM (Schaeffer's sporulation medium) (25) at 37°C for 48 hours. Sporulation was

initially monitored by visualization of cells on a Zeiss Axio Imager microscope. Sporulation efficiency was assayed by chloroform treatment and enumeration of spores and vegetative cells as previously described for *B. anthracis* (22). Sporulation efficiency is presented as percentage of spores relative to total viable cells. For germination experiments, strains were grown in SM media for 72 hours at 37°C. Spores were harvested by heating samples to 80°C for 30 minutes followed by 3 washes in sterile water. Spores were enumerated by microscopy using a hemocytometer.  $5 \times 10^3$  spores were resuspended in 1 ml LB broth, incubated for 60 minutes at 37°C, serially diluted, and plated on LB-agar. After 18 hours at 37°C, CFU were counted.

**$\beta$ -Galactosidase assays.** *B. anthracis* strains harboring gene promoter fusions on the replicative vector pTCV-lac were grown at 37°C in LB supplemented with kanamycin.  $\beta$ -galactosidase activity was assayed as described previously and specific activity was expressed in Miller units (26,27).

### 4.3 Results

**Transposon mutagenesis generates a pigment-producing mutant.** The 34F2 (pXO1+ pXO2-) strain of *B. anthracis* was randomly mutagenized by Mariner transposition using plasmid pAW068. pAW068 carries a Mariner-based transposon system which delivers a transposon containing a spectinomycin-resistance cassette for selection of transposon mutants and a Gram-negative origin of replication for plasmid rescue and identification of mutants (18). Spectinomycin-resistant insertional mutants were generated as previously described (18, 28), plated on LB-agar containing spectinomycin, and incubated for 24 hours at 37°C. Colonies derived from one transposon mutant, annotated 34F2-tM104, became a light brown color after 24 hours. After continued incubation for an additional 24 hours at 37°C, the colonies became dark

brown and a brown pigment was observed in the solid media surrounding the mutant colony. Sequencing of the transposon insertion site of the pigmented mutant identified an insertional site 127 nt downstream of the predicted translation start site of BAS0228. BAS0228 is predicted to encode a homogentisate 1, 2-dioxygenase. Homogentisate 1, 2-dioxygenase, also known as HmgA, has been shown to be involved in the catabolism of L- tyrosine and L-phenylalanine in several species, converting homogentisate to maleylacetoacetate (12–14). Loss of HmgA activity results in accumulation of homogentisate, which oxidizes to form a brown pigment known as pyomelanin. *hmgA*-like genes are found among members of the *Bacillus cereus* group, such as *B. cereus*, *B. anthracis*, and *B. thuringiensis*, but are not found in the related model organism *Bacillus subtilis* or in other pathogenic firmicutes. Genetic organization of the BAS0228 region is shown in Fig 1. There is a 34 nt overlap between the 5' end of *hmgA* and the 3' end of BAS0227. BAS0227 is predicted to encode a fumarylacetoacetate hydrolase (HmgB), the final enzyme in a tyrosine and phenylalanine catabolism pathway that converts fumarylacetoacetate to fumarate and acetoacetate in many species.

**Characterization of directed *hmgA* and *hmgB* deletion mutants.** To confirm the phenotype of the transposon mutant, a strain containing a marker-less deletion of *hmgA*, named AW-A127, was created. As with the transposon mutant, directed deletion of *hmgA* resulted in colonies that produce a brown pigment when grown on LB-agar (Fig 2A). When grown in liquid media, brown color is observed in the  $\Delta hmgA$  strain beginning as the cells enter stationary phase, after approximately 5 hours post inoculation, and pigment continues to accumulate in the cultures as incubation continues. Fig 2B demonstrates the extent of pigment accumulation in culture supernatants at 72 hours post inoculation. The brown pigment was analyzed by spectroscopy. Fig 2C shows a spectroscopic scan of cell-free supernatants of the parental strain

subtracted from the  $\Delta hmgA$  strain. The  $\Delta hmgA$  supernatants generate a strong absorbance peak near OD<sub>400</sub> that was not found in the parental strain, consistent with observations of pyomelanin in other species (10, 14). Loss of *hmgA* did not affect growth in LB at 37°C relative to the parental strain (Fig 2D).

To demonstrate that loss of *hmgA* is responsible for the observed phenotypes, in trans complementation analysis was performed. The coding region of *hmgA* was cloned into vector pAW285, producing complementation plasmid pAW444, placing *hmgA* expression under the control of a highly active promoter. The  $\Delta hmgA$  strain carrying the empty plasmid still produces pigment while the  $\Delta hmgA$  strain carrying the *hmgA* complementation plasmid no longer produces pigment (Fig 3). Neither the *hmgA* complementation plasmid nor the empty plasmid affect the parental 34F2 strain.

As the genes predicted to encode *hmgA* and *hmgB* partially overlap, a marker-less deletion of *hmgB*, named AW-A130, was created. As shown in Fig 2A and 2B, loss of *hmgB* does not result in pigment accumulation. Further, loss of *hmgB* does not alter growth characteristics of the strain relative to parental 34F2 (data not shown). The  $\Delta hmgB$  strain was not further analyzed.

**Pyomelanin is produced in presence of L-tyrosine and L-phenylalanine.** *B. anthracis* strains were grown in a derivative of a commonly used defined medium, R-medium, without added bicarbonate (R-bic). Using a defined medium allows removal and addition of specific amino acids to determine contributions to pyomelanin production. Growth of 34F2 and the  $\Delta hmgA$  strain were similar when grown in R-bic or R-bic without L-phenylalanine, L-tyrosine, and L-tryptophan (Fig 4A). Addition or removal of any of these individual amino acids did not alter the growth characteristics of either strain (data not shown). Pigment production did not

occur under any conditions tested with parental 34F2 strain (Fig 4B). In the  $\Delta hmgA$  mutant, pigment production occurred in the presence of L-tyrosine and L-phenylalanine but not the presence of L-tryptophan (Fig 4C).

To confirm these findings, pigment production was tested in cell-free extracts of the parental and mutant strains. 34F2 and the  $\Delta hmgA$  strains were grown to early-stationary phase at 37°C in LB, cells disrupted by sonication, and cell debris removed by filtration. Amino acids were then added to the cell-free extracts and incubated for 48 hours at 37°C. With extracts of the parental strain, pigment was not produced under conditions tested (Fig 5). In extracts of the  $\Delta hmgA$  strain, pigment was observed with the addition of exogenous L-tyrosine or L-phenylalanine but not with the addition of exogenous L-tryptophan. Combined, these findings indicate that, in the  $\Delta hmgA$  strain, pyomelanin is produced in the presence of L-tyrosine and L-phenylalanine.

**Pyomelanin protects *B. anthracis* from UV but not oxidative damage.** Reports from other organisms indicate that production of pyomelanin protects cells from UV and/or H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress (9, 10, 14, 24). UV sensitivity was tested as previously described (10). Significantly more cells of the pyomelanin-producing  $\Delta hmgA$  strain survived exposure to UV as compared to the parental 34F2 (Fig 6A). Complementation of *hmgA* in trans increases sensitivity of the  $\Delta hmgA$  strain to UV radiation to parental 34F2 levels (Fig 6B), indicating that pyomelanin production confers protection from UV radiation.

Initially, H<sub>2</sub>O<sub>2</sub>-sensitivity was tested by a simple disk diffusion assay using paper discs saturated with several different concentrations of H<sub>2</sub>O<sub>2</sub>. No difference in the sizes of zones of inhibition were observed between 34F2 and the  $\Delta hmgA$  strain in a range between 1 mM and 200 mM of H<sub>2</sub>O<sub>2</sub> (data not shown). The protective effects of pyomelanin-containing supernatants on

the 34F2 strains were assayed similar previous studies (9, 24). As shown in Fig 6 C, no significant difference was seen in the ability of 34F2 or  $\Delta hmgA$  supernatants to protect 34F2 against  $H_2O_2$  damage, suggesting that pyomelanin does not protect *B. anthracis* against  $H_2O_2$ -induced oxidative damage.

**Loss of *hmgA* does not alter virulence gene expression.** As production of anthrax toxin is important to virulence of *B. anthracis*, the effect of loss of *hmgA* on virulence gene expression was assayed by  $\beta$ -galactosidase analysis. Transcriptional fusions of the promoters of a toxin subunit, *pagA*, and the master virulence regulator, *atxA*, to a *lacZ* reporter were introduced into the 34F2 and  $\Delta hmgA$  strains. No difference in  $\beta$ -galactosidase activity was observed with either reporter between the 34F2 and  $\Delta hmgA$  strains in exponential or stationary phase growth (Fig 7). No difference in  $\beta$ -galactosidase activity was seen in samples allowed to grow to 24 and 48 hours, when pigment accumulation was high (data not shown). These data suggest that neither loss of *hmgA* nor production of pyomelanin significantly affect virulence gene expression.

**Loss of *hmgA* does not alter sporulation or germination.** The processes of sporulation and germination are essential to the life cycle of *B. anthracis*. As this is the first exploration of HmgA in a sporulating bacterium, the effects of loss of *hmgA* on sporulation and germination were investigated. Sporulation was assayed by a liquid sporulation assay. Sporulation efficiency, as measured by percentage of spores relative to the total number of viable cells from three independent cultures, for the 34F2 strain was  $48.9\% \pm 7.9\%$  while the  $\Delta hmgA$  strain was  $49.3\% \pm 1.2\%$ . Germination analysis was performed by calculating CFU from a fixed number of purified spores from three independent spore preparations. For 34F2,  $7.7 \times 10^6 \pm 4.1 \times 10^4$  CFU/ml were recovered while for the  $\Delta hmgA$  strain  $8.0 \times 10^6 \pm 3.6 \times 10^4$  CFU/ml were recovered. These

data suggest that loss of *hmgA* and pigment production affect neither sporulation nor germination in *B. anthracis*.

Table 4.1 Strains and plasmids used in Chapter 4

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Source
<b><i>B. anthracis</i> strains</b>		
34F2	pXO1 <sup>+</sup> pXO2 <sup>-</sup>	Laboratory stock
34F2-tM104	Mariner transposon insertion into BAS0028	This study
AW-A127	Markerless deletion of BAS0228 ( $\Delta hmgA$ )	This study
AW-A130	Markerless deletion of BAS0227 ( $\Delta hmgB$ )	This study
<b>Plasmids</b>		
pORI-Cm-I-SceI	pORI-Cm vector with I-SceI recognition site, Cm <sup>R</sup>	[20]
pSS4332	I-SceI expression plasmid, Kan <sup>R</sup>	[34]
pTCVlac- <i>pagA</i>	<i>pagA-lacZ</i> transcriptional fusion in pTCV-lac, Kan <sup>R</sup>	[3]
pTCVlac- <i>atxA12</i>	<i>atxA-lacZ</i> transcriptional fusion in pTCV-lac, Kan <sup>R</sup>	[20]
pAW068	Mariner transposon delivery plasmid, Cm <sup>R</sup> Spc <sup>R</sup>	[18]
pAW285	Xylose-inducible expression plasmid, Cm <sup>R</sup>	[22]
pAW406	Region upstream of BAS0228 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW407	Regions flanking BAS0228 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW416	Region upstream of BAS0227 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW417	Regions flanking BAS0227 in pORI-Cm-I-SceI, Cm <sup>R</sup>	This study
pAW444	BAS0228 coding region in pAW285, Cm <sup>R</sup>	This study

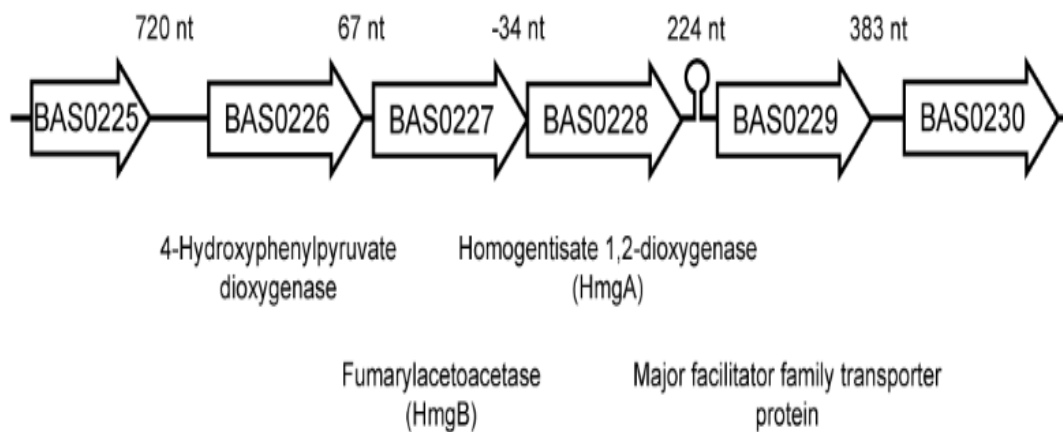
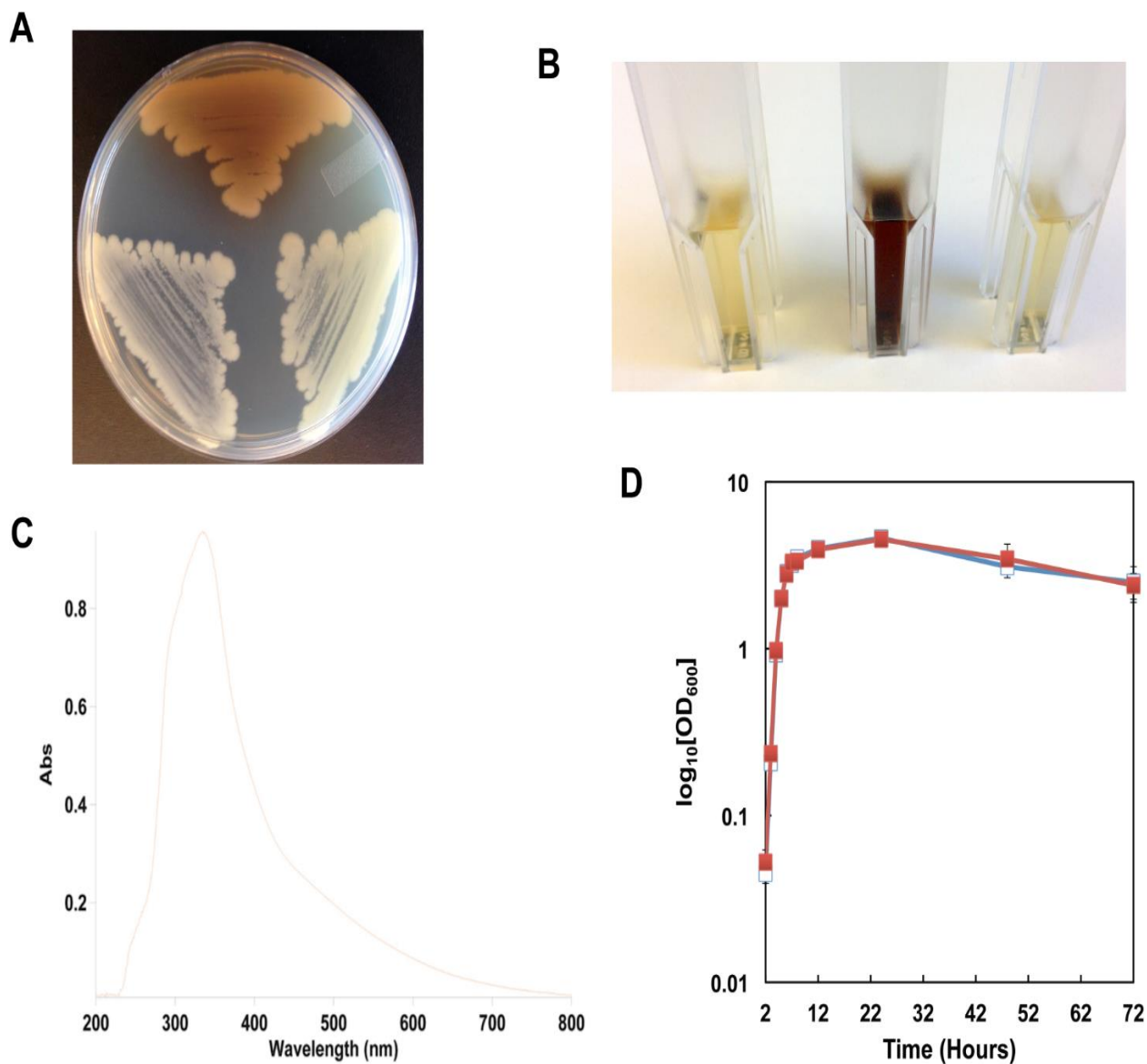


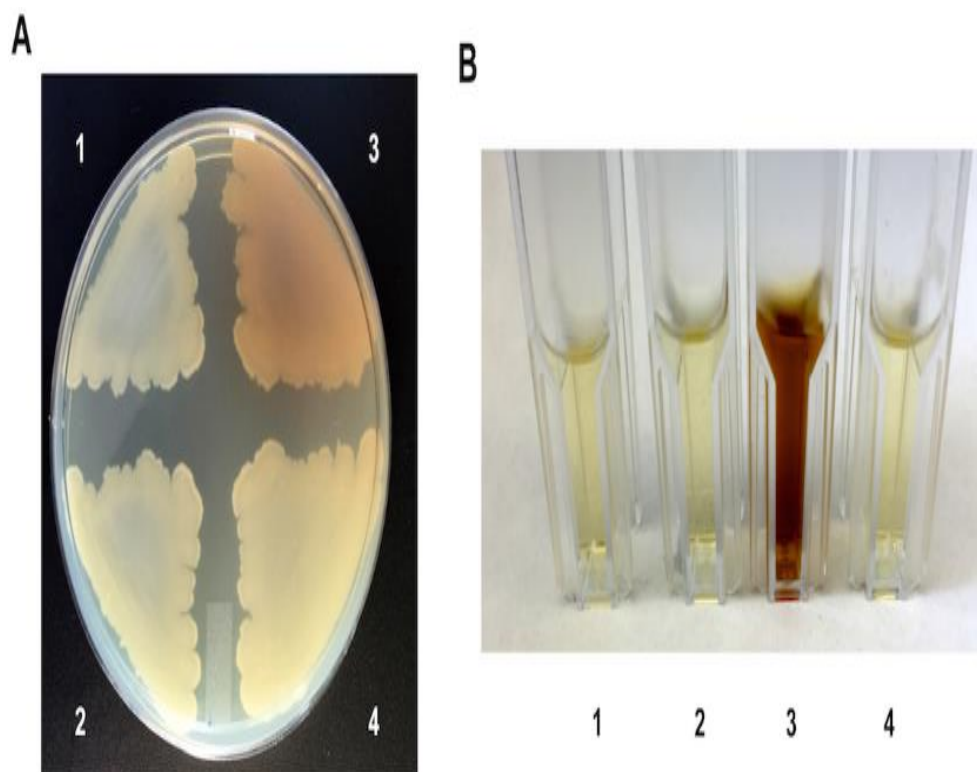
Figure 4.1 Genetic organization of BAS0228 (*hmgA*) region

Numbers above specify size of intergenic regions. Predicted transcriptional terminator indicated downstream of *hmgA*



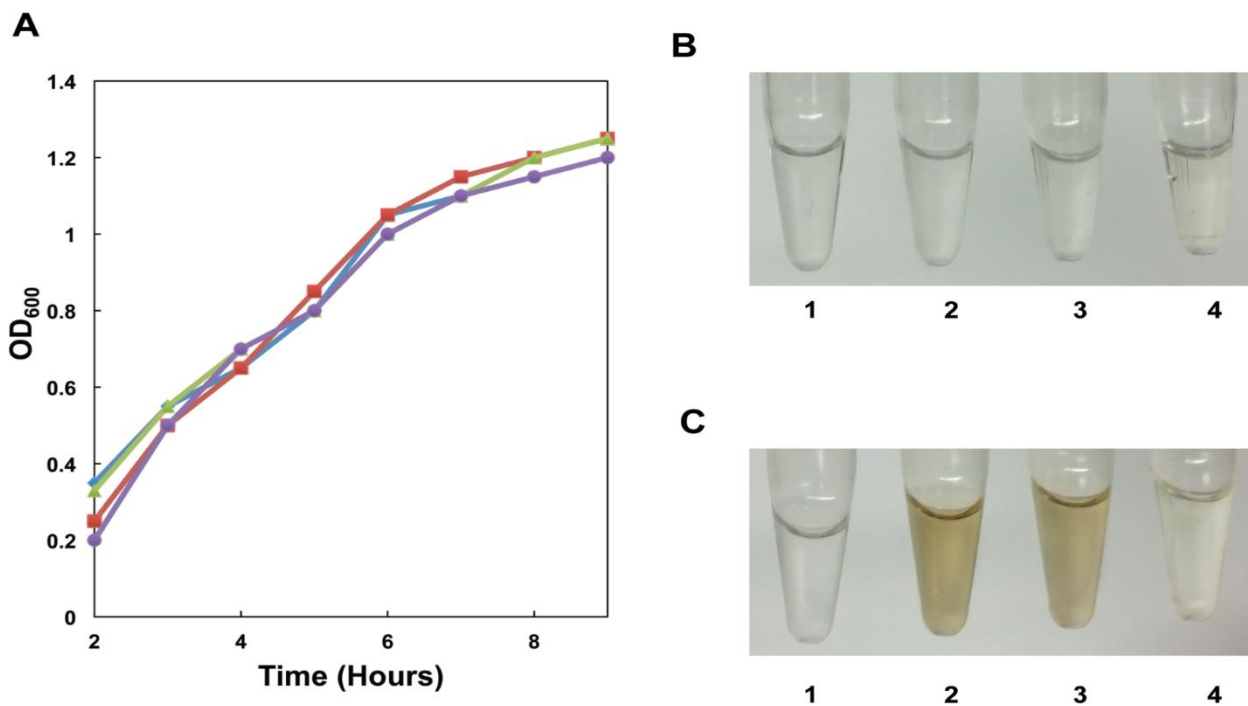
**Figure 4.2 Characteristics of  $\Delta hmgA$  mutant strain**

**A.** Pigment production in strains grown on LB-agar for 48 hours at 37°C. Top, AW-A127 ( $\Delta hmgA$ ); Bottom left, parental 34F2; Bottom right, AW-A130 ( $\Delta hmgB$ ). **B.** Pigment production in supernatants of strains grown in LB for 72 hours at 37°C. Left, 34F2; Center, AW-A127 ( $\Delta hmgA$ ); Right, AW-A130 ( $\Delta hmgB$ ). **C.** Optical spectra of AW-A127 ( $\Delta hmgA$ ) supernatant following subtraction of 34F2 supernatant spectra. **D.** Cell growth of parental and mutant strains grown in LB at 37°C. -■- 34F2; -□- AW-A127 ( $\Delta hmgA$ ).



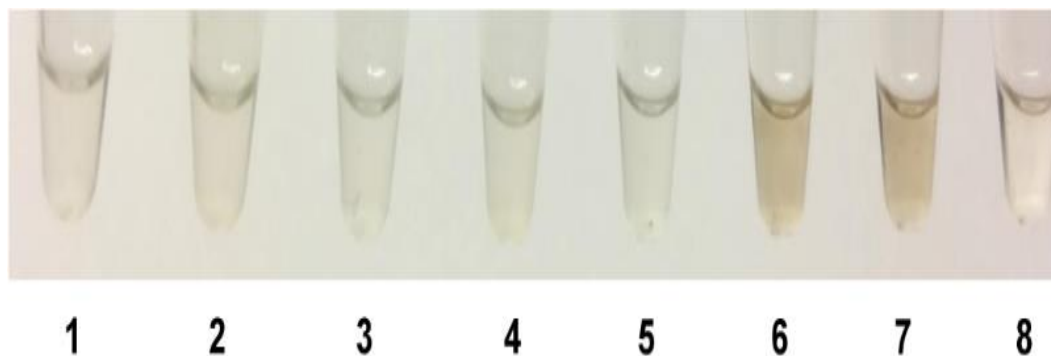
*Figure 4.3 Complementation analysis of  $\Delta hmgA$  mutant strain*

**A.** Pigment production in strains grown on LB-agar for 48 hours at 37°C. **B.** Pigment production in supernatants of strains grown in LB for 72 hours at 37°C. For both: 1. 34F2 + pAW285 (empty vector); 2. 34F2 + pAW444 (*hmgA*); 3. AW-A127 ( $\Delta hmgA$ ) + pAW285 (empty vector); 4. AW-A127 ( $\Delta hmgA$ ) + pAW444 (*hmgA*)



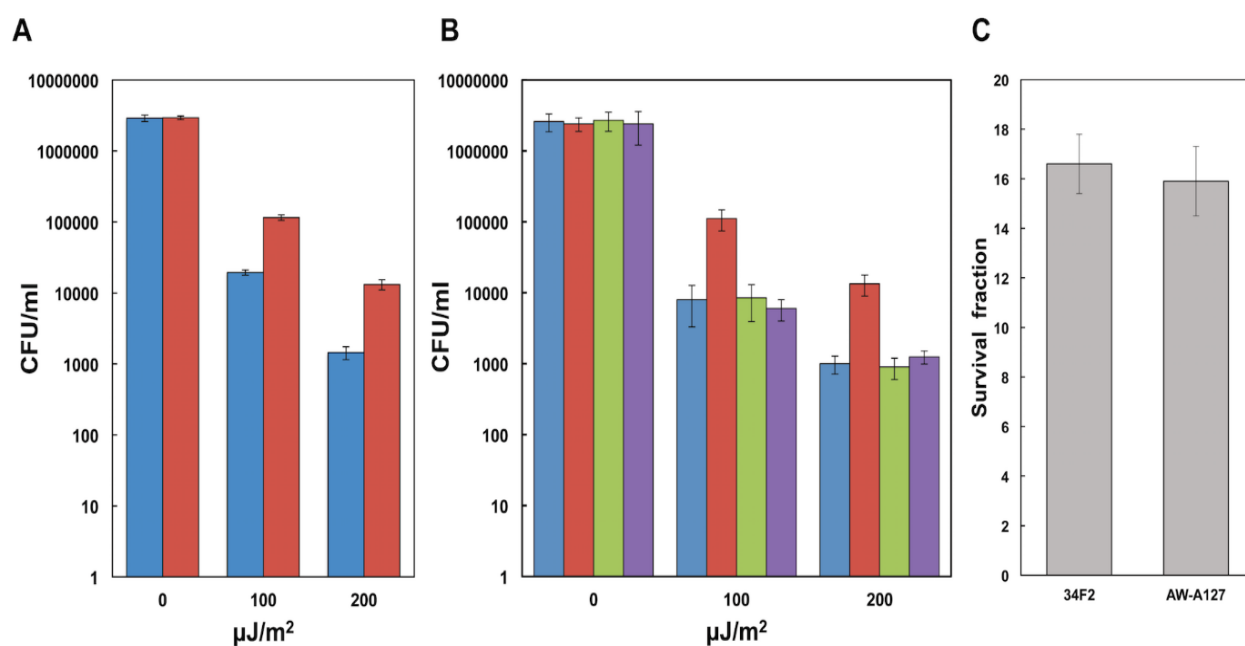
*Figure 4.4 Growth and pigment production in defined medium*

- A.** Cell growth of parental and mutant strains grown in R-Bic medium at 37°C. -■- 34F2 in R-bic; -◆- AW-A127 ( $\Delta$ hmgA) in R-bic; -▲- 34F2 in R-bic without L-tryptophan, L-phenylalanine, or L-tyrosine; -●- AW-A127 in R-bic without L-tryptophan, L-phenylalanine, or L-tyrosine. **B.** Pigment production in 34F2 supernatants. **C.** Pigment production in AW-A127 supernatants. 1, R-bic without L-tryptophan, L-phenylalanine, or L-tyrosine; 2, R-bic without L-tryptophan or L-phenylalanine; 3, R-bic without L-tryptophan or L-tyrosine; 4, R-bic without L-phenylalanine or L-tyrosine.



**Figure 4.5 Pigment production in cell-free extracts**

Cell-free extracts from cells grown in LB at 37°C for 6 hours. Amino acids then added to cell-free extracts, as indicated, and incubated for an additional 24 hours at 37°C. 1, 34F2, no added amino acids; 2, 34F2 with L-tyrosine; 3, 34F2 with L-phenylalanine; 4, 34F2 with L-tryptophan; 5, AW-A127 ( $\Delta hmgA$ ), no added amino acids; 6, AW-A127 with L-tyrosine; 7, AW-A127 with L-phenylalanine; 8, AW-A127 with L-tryptophan.



**Figure 4.6 UV and  $\text{H}_2\text{O}_2$  protection**

**A.** UV protection. Survival of 34F2 (blue) or AW-A127 ( $\Delta hmgA$ ) (red) following irradiation with UV at 302 nm as indicated. Data was obtained from 3 independent cultures and error bars represent standard deviation from the mean. Two tailed unpaired  $t$  test indicate P value of  $P < 0.0001$  between 34F2 and AW-A127 at both levels of UV exposure. **B.** UV protection of complemented strains. Survival of 34F2+pAW285 (blue), AW-A127+pAW285 (red), 34F2+pAW444 (green), and AW-A127+pAW444 (purple) following irradiation with UV at 302 nm as indicated. Data was obtained from 3 independent cultures and error bars represent standard deviation from the mean. **C.**  $\text{H}_2\text{O}_2$  survival. Survival of 34F2 cells in 50mM  $\text{H}_2\text{O}_2$  when resuspended in supernatants from 34F2 or AW-A127 ( $\Delta hmgA$ ) cultures. Data was obtained from 3 independent cultures and error bars represent standard deviation from the mean.

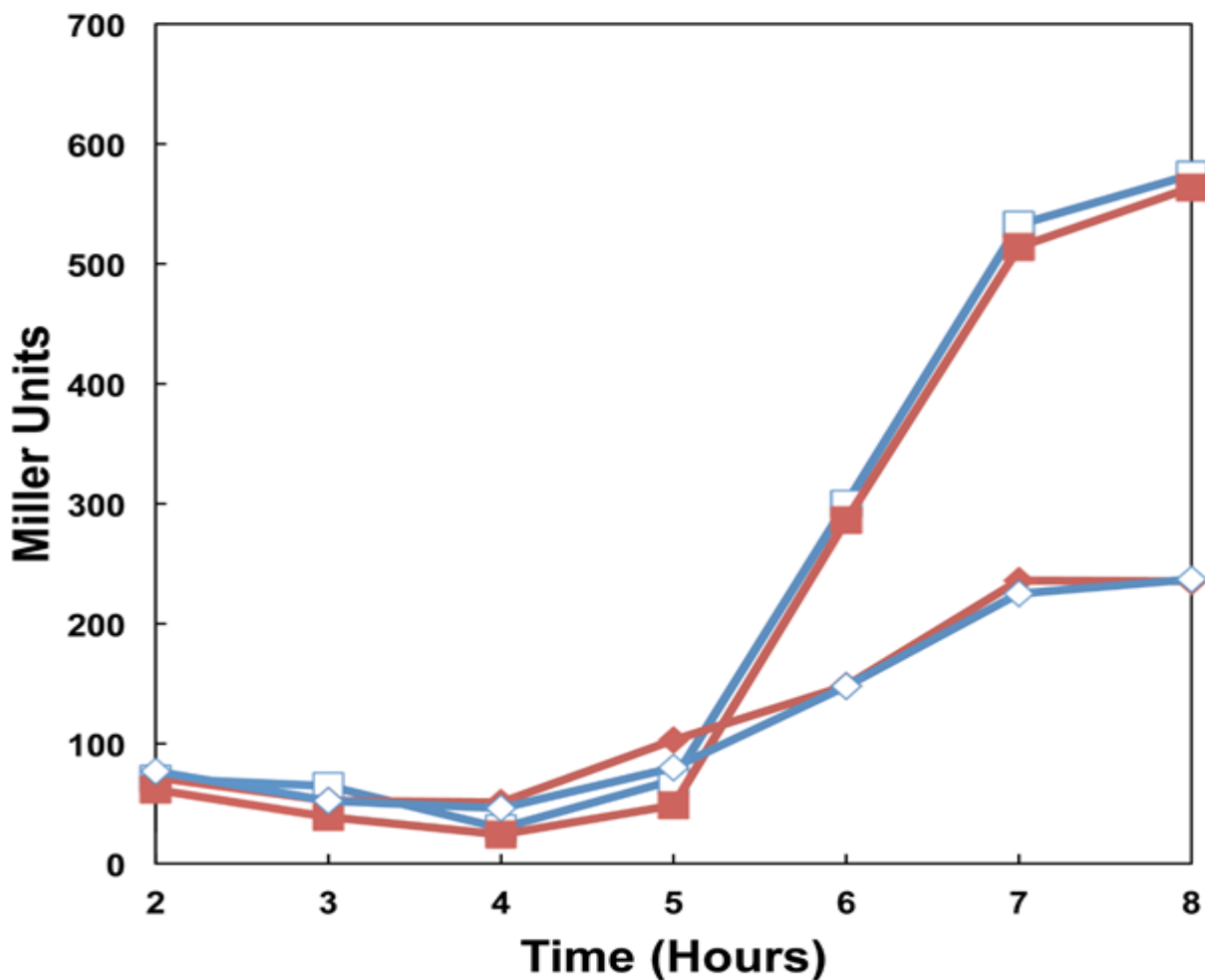


Figure 4.7 Virulence gene expression in 34F2 and  $\Delta hmgA$  strains

#### 4.4 Discussion

Loss of *hmgA* in *B. anthracis* results in accumulation of pyomelanin in the presence of L-tyrosine and L-phenylalanine. Neither the loss of *hmgA* nor the accumulation of pyomelanin affects the growth characteristics of *B. anthracis*, indicating that this aromatic amino acid catabolism pathway is not essential. It is unclear if the complete L-tyrosine and L-phenylalanine catabolism pathway with homogentisate as an intermediate is present in *B. anthracis*. *B. anthracis* carries a putative protein-dependent phenylalanine hydroxylase (BAS4253) which

could function to convert L-phenylalanine to L-tyrosine, allowing L-phenylalanine to be degraded in the homogentisate pathway. A number of genes are predicted to encode aminotransferases (BAS1428, BAS1454, and BAS2746) potentially capable of converting L-tyrosine to 4-hydroxy-phenylpyruvate. A predicted 4-hydroxyphenylpyruvate dioxygenase, which converts 4-hydroxyphenylpyruvate to homogentisate, is encoded by BAS0226. HmgA (BAS0228) then converts homogentisate to maleylacetoacetate. The next steps in maleylacetoacetate degradation are unclear. BAS0227 encodes a predicted fumarylacetoacetate hydrolase that converts fumarylacetoacetate to fumarate and acetoacetate, but a gene encoding maleylacetoacetate isomerase, converting maleylacetoacetate to fumarylacetoacetate, cannot be identified in the *B. anthracis* genome. A previous report indicates that in some *Bacillus* species, maleylacetoacetate can be degraded by a maleylacetoacetate hydrolase to acetoacetate and maleic acid (29). A gene encoding a maleylacetoacetate hydrolase also cannot be directly identified in the *B. anthracis* genome. Total transcriptome analysis indicates that BAS0226, BAS0227 (*hmgB*), and BAS0228 (*hmgA*) are coordinately transcribed as part of an operon (30), suggesting a connection between the activities of the HmgA and HmgB. The fate of maleylacetoacetate in the homogentisate pathway requires additional analysis.

The production of pigment in cell free extracts of cells in the early stationary phase of growth indicates that the enzymes required to convert L-phenylalanine and L-tyrosine to homogentisic acid are produced at this time; however, pigmentation in batch culture is just becoming detectable in early stationary phase. The appearance of pigment relies on conversion of 4-hydroxyphenylpyruvate to homogentisic acid followed by spontaneous oxidation of homogentisic acid to pyomelanin (11). When batch cultures of the  $\Delta hmgA$  strain are grown without agitation, the development of pigment is delayed compared to a culture grown with

agitation controlled for differences in cell density (data not shown), likely due to decreased oxidation of homogentisic acid. The rate of homogentisic acid production and the oxidation of homogentisic acid to pyomelanin cannot be established from these experiments. Further analysis of homogentisic acid production and oxidation under specific conditions would be needed to optimize pyomelanin production. *hmgA*-like genes are not found widely in Gram-positive bacteria. In fact, *hmgA*-like genes in the firmicutes are largely restricted to the *Bacillus cereus* group, which includes *B. anthracis*, *B. cereus*, and *B. thuringiensis*. The genome of *Bacillus subtilis*, the model organism for Bacillus physiology and genetics, does not encode a predicted homogentisate 1, 2-dioxygenase. *B. subtilis* does produce a protein coat that contains a brown pigment around the endospore. This melanin-like pigment is produced by the copper-dependent laccase, CotA (31). CotA confers resistance to UV light and hydrogen peroxide damage (31, 32). *B. anthracis* does possess a CotA-like protein (33), but this protein would produce pigment in the spore, not in vegetative cells as with the loss of HmgA. The relevance of pyomelanin accumulation to pathogenesis of *B. anthracis* is unclear. Neither loss of *hmgA* nor pyomelanin accumulation affect expression of key virulence factors. Pyomelanin production does not appear to protect *B. anthracis* cells from oxidative damage, a feature important to anthrax pathogenesis (34). While pyomelanin protects vegetative cells from UV damage, *B. anthracis* would mostly likely be exposed to UV radiation outside the host when in the form of UV-resistant spores. It may be possible that pyomelanin protects cells from other stresses found during the course of infection that were not assayed. Further analysis of pyomelanin-producing mutants in cellular and animal models of infection may be of value.

#### 4.5 References

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## 5 CHAPTER FIVE: HFQ-MEDIATED SMALL RNA REGULATION OF METABOLISM AND VIRULENCE GENE EXPRESSION IN *BACILLUS ANTHRACIS*

### 5.1 Introduction

*Bacillus anthracis*, the causative agent of anthrax, is a Gram-positive, non-motile, endospore forming bacterium. *B. anthracis* carries two virulence plasmids, pXO1 and pXO2, which carry virulence genes that are required for pathogenic damage in the mammalian host. pXO1 carries more than 200 open reading frames, including the three anthrax toxin subunits; *pagA* encodes Protective Antigen (PA), *lef* encodes Lethal Factor (LF), and *cya* encodes Edema Factor (EF) (1, 2). pXO1 also carries the virulence regulatory protein, AtxA, which coordinately controls expression of all three toxin subunit genes (3, 4). The smaller pXO2 plasmid, carrying just over 100 open reading frames, encodes the proteins responsible for production of the poly-D-glutamic acid capsule (5, 6). Regulatory cross talk occurs as the pXO1-encoded AtxA regulates expression of genes on pXO1, pXO2, and the bacterial chromosome (7-9). Expression of the major plasmid-encoded virulence factors is influenced by a number of environmental signals and the growth state of the cell (4, 10-18). The mechanisms by which various environmental signals are perceived by the cell and converted into changes in gene expression often appear to involve altered production and/or activity of AtxA (3, 4, 11, 16,17). AtxA activity is regulated post-transcriptionally by phosphorylation and dephosphorylation (12, 19), and AtxA accumulation is influenced by the pleiotropic regulator CodY (14, 16). The only transcriptional regulator directly shown to bind the *atxA* promoter and control *atxA* transcription

is the transition state regulator AbrB, which ties toxin gene expression into the developmentally regulated sporulation phosphorelay (11, 20). Other as yet unidentified proteins may also bind to the *atxA* promoter and regulate transcription (21). *atxA* transcription has also been shown to be indirectly regulated by two small *c*-type cytochromes (13) and the catabolite repression protein CcpA (14). One of the most potent environmental inducers of virulence gene expression is bicarbonate/CO<sub>2</sub>; when grown in media containing bicarbonate under 5% atmospheric CO<sub>2</sub>, toxin gene expression increases 10-20 fold (10, 22-24). Bicarbonate/ CO<sub>2</sub> induction of virulence gene expression appears to be AtxA-dependent (4, 25), but *atxA* transcription and AtxA protein levels are not strongly altered in response to bicarbonate/ CO<sub>2</sub> (23, 25). Bicarbonate/ CO<sub>2</sub> levels affect AtxA post-transcriptionally by altering the dimerization status of AtxA (17). Additional factors that directly alter AtxA post-transcriptionally function have not been identified.

Regulation through small RNAs (sRNAs) has emerged as a major mechanism of post-transcriptional control of bacterial gene expression. sRNAs control virulence gene expression in many pathogens, and sRNAs have been studied extensively in model organisms (26, 27). sRNAs are 50 to 300 nucleotide RNAs that commonly bind to complementary sequences in mRNA targets to positively or negatively regulate gene expression. A recent study identified two sRNA carried on the *B. anthracis* virulence plasmid pXO1 that are induced in response to bicarbonate/ CO<sub>2</sub> through an AtxA-dependent mechanism (28). Efficient binding of sRNAs to their targets often requires Sm-like, RNA-binding chaperone proteins called Hfq (29). In many bacteria, loss of Hfq can result extensive alteration alter the pattern of global gene expression and alterations in virulence (27). Loss of the single Hfq in the Gram-positive model organism *Bacillus subtilis* results in reduced survival in long-term, stationary-phase cultures (30), and the pXO1- encoded Hfq can bind to small RNA in vitro (31). Further analysis

suggests that the pXO1-encoded Hfq can complement an *E. coli hfq* deletion strain but that overexpression of this Hfq is toxic to *E. coli* (32). The function of the three Hfq in *B. anthracis* was not further analyzed. In this report, we analyze the function of pXO1-137, the pXO1-encoded Hfq, and a pXO1-encoded sRNA. We demonstrate that loss of either pXO1-137 or sRNA-1 results in significant growth defects and reductions in toxin gene expression only in toxin induction medium. Cells appear to be sensitive to both under- and over-production of Hfq and sRNA. *pXO1-137* and *sRNA-1* control virulence gene expression by promoting translational initiation of AtxA. This report demonstrates a phenotype associated with Hfq and sRNA in *B. anthracis* and introduces a new mechanism of control of the long mysterious AtxA master virulence regulator.

## 5.2 Materials and Methods

**Bacterial strains and growth condition.** *B. anthracis* strain 34F2 (pXO1+ pXO2-) and its derivatives were routinely grown in LB or brain heart infusion (BHI) broth supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol (7.5 µg/ml), erythromycin (5 µg/ml), lincomycin (25 µg/ml), and kanamycin (7.5 µg/ml). 5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside (X-Gal) (40 µg/ml) was added to LB agar to monitor β-galactosidase activity as necessary. As indicated, *B. anthracis* was grown in R-Media (22) under 5% CO<sub>2</sub>. Competent cells of *B. anthracis* were prepared as previously described (19).

*E. coli* TG1, C600, and DH5α competent cells were used for the propagation and isolation of all plasmid constructs. *E. coli* transformation was performed in chemically competent cells (46). Transformants were selected on LB agar supplemented with ampicillin (100 µg/ml), chloramphenicol (7.5 µg/ml), or kanamycin (30 µg/ml) Plasmid and strain construction

**Plasmid and strain construction.** Strains and plasmids are listed in Table 1.

Oligonucleotide primers are listed in Table S1. Markerless gene replacements in *B. anthracis* were generated by allelic exchange as previously described (33, 34). The pXO1-137 complementation plasmids pAW377 and pAW427 were created through PCR amplification of the pXO1-137 ORF using primers pXO1-137E5'Bam and pXO1-137E3'Sal cloned into pAW285 and pHT315S, respectively. The pXO1 sRNA-1 complementation plasmids pAW437 and pAW435 were created through PCR amplification of pXO1 sRNA-1 using primers sRNA1U and sRNA1D cloned into pAW285 and pHT315spac, respectively. The *atxA-lacZ* translational fusion plasmid pAW457 was created by first amplifying a fragment of the *atxA* promoter and ORF from nucleotide position -945 to +60 relative to the translation start site using primers *atxA* fus5'Eco and *atxA* fus3'2. A second fragment containing the *lacZ* coding region, missing the initiator codon, was amplified from plasmid pTCV-*lac* using primers *lacZ* fus5'2 and *lacZ* fus3'Bam. The two fragments were joined by splicing by overlap extension PCR (35) and the assembled product cloned into plasmid pHP13.

**$\beta$ -Galactosidase assays.** *B. anthracis* strains harboring promoter or translational fusions were grown at 37°C in LB or R-Media supplemented with the appropriate antibiotics.  $\beta$ -galactosidase activity was assayed as described previously and specific activity was expressed in Miller units (36, 37)

**Quantitative RT-PCR.** RNA was extracted from *B. anthracis* using the UltraClean Microbial RNA Isolation Kit (MoBio, Carlsbad, CA). RNA was treated with Turbo DNase (Life Technologies, Carlsbad, CA) and quantified using an Eppendorf Bio-spectrophotometer.

Quantitative, real time RT-PCR was performed using the Power SYBR RNA-to-Ct 1- step kit

(Life Technologies) on a Prism 7500 Fast Real Time PCR System (Life Technologies). Data is presented as  $\Delta C_t$  calculated from the results of at least three independent experiments.

**Purification of recombinant AtxA.** Plasmid pAW237 contains the coding sequence of *B. anthracis* AtxA (excluding the initial start codon) in the *E. coli* expression vector pET15b. The plasmid was constructed so as to add an N-terminal 6-His tag to the expressed AtxA. pAW410 was transformed into *E. coli* strain BL21(DEProtein was expressed and purified as previously described (18).

**Immunoblotting.** *B. anthracis* strains were grown in R-Media at 37° under 5% CO<sub>2</sub>, and cell pellets were isolated by micro centrifugation of cell suspensions. Cell pellets were lysed following resuspension in buffer (10mM Tris-HCl [pH8.0], 300 mM NaCl, 10 nM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, and 1x protease inhibitor cocktail) by sonication on ice for 20 seconds for 3 cycles. SDS sample buffer (50 mM Tris-HCl [pH6.8], 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.1% Bromphenol blue) was added to each supernatant. Samples were boiled for 5 minutes and loaded on 10% SDS-PAGE gels. The amount loaded was normalized relative to cell growth and total amount of protein as confirmed by Coomassie staining of gels. The gels were run at 30 mA for approximately 2 hr. The gels were transferred to a PVDF membrane (BioRad) in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) at 20V overnight. The membranes were incubated for 30 minutes at room temperature in blocking buffer (5% dried milk in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20)) followed by addition of a rabbit polyclonal  $\alpha$ -AtxA antibody diluted 1:15,000. The  $\alpha$ -AtxA polyclonal serum (17) was a generous gift of Theresa M. Koehler, The University of Texas – Houston Health Science Center. The blots were washed 5 times and then incubated for 1hr at RT with horseradish peroxidase-conjugated goat anti-rabbit antibody (BioRad) diluted 1:10,000 in

blocking buffer. Following washing of the membrane, binding of the antibodies was probed using the ECL Plus kit (GE), and the protein bands were visualized by exposure to film.

### 5.3 Results

**Loss of *pXO1-137* results in decreased growth and toxin gene expression under toxin-inducing conditions.** Virulence plasmid pXO1 carries an open reading frame, *pXO1-137*, with significant similarity to bacterial Hfq-like genes. *pXO1-137* is surrounded by more than 300 base pair intergenic regions upstream and downstream of its coding region (2), suggesting that *pXO1-137* is not part of a larger operon. To investigate the role of *pXO1-137* in the physiology of *B. anthracis*, a strain, AW-A049, was constructed containing a markerless gene deletion of *pXO1-137* in the 34F2 (pXO1+ pXO2-) background. When grown under non-toxin inducing conditions in LB at 37°C, growth of the  $\Delta pXO1-137$  strain was unchanged relative to the parental strain (Figure 1A). Similarly, no difference in activity of the *pagA* promoter (directing transcription of the protective antigen subunit of anthrax toxin) or the *atxA* promoter (directing transcription of the AtxA master virulence regulator) was observed under non-inducing conditions as assayed using promoter-*lacZ* fusion constructs (Figure 1B). In contrast, when grown under toxin inducing conditions, in R-Media at 37°C under 5% CO<sub>2</sub>, growth of the  $\Delta pXO1-137$  strain was significantly reduced relative to 34F2 (Figure 1C). *pagA* promoter activity was significantly reduced in the  $\Delta pXO1-137$  strain while *atxA* promoter activity was similar to the 34F2 strain (Figure 1D). To expand our gene expression analysis, the two additional toxin promoters were included, and samples collected at 8 hours from three independent cultures for each. As shown in Figure 1E, promoter activities of *pagA*, *cya* (directing transcription of the edema factor subunit of anthrax toxin), and *lef* (directing

transcription of the lethal factor subunit of anthrax toxin) in the  $\Delta pXO1-137$  strain were significantly reduced relative to 34F2, but *atxA* promoter activity was unchanged. To confirm these findings, a second, independent  $\Delta pXO1-137$  strain was created in the 34F2 background, AW-A115, and the phenotype of this strain was identical to that of our first  $\Delta pXO1-137$  strain (AW-A049) (data not shown). These observations indicate that cell growth and promoter activities of the toxin subunit genes are strongly reduced but only under toxin inducing conditions. As AtxA is the only regulator known to directly control transcription of all three toxin subunits, it was noteworthy that *atxA* promoter activity was unchanged.

**Growth and virulence gene expression is sensitive to altered levels of pXO1-137 expression.** In an attempt to complement the  $\Delta pXO1-137$  strain in trans, the pXO1-137 coding region was cloned into expression plasmid pHT315s, placing pXO1-137 under a constitutively active SPAC promoter. Unexpectedly, the 34F2 strain carrying the *pXO1-137* expression plasmid had a significant growth defect similar to the  $\Delta pXO1-137$  when grown in R-Media (Figure 2A). Addition of either the complementation plasmid or empty vector had no effect on the growth defect in the  $\Delta pXO1-137$  strain. Similarly, *pagA* promoter activity in the 34F2 strain carrying the pXO1-137 expression plasmid was strongly decreased to levels similar to the  $\Delta pXO1-137$  strain (Figure 2B), while addition of the *pXO1-137* expression plasmid did not alter *pagA* expression in the  $\Delta pXO1-137$  strain relative to the empty vector. As an alternative approach, we cloned pXO1-137 coding region into plasmid pAW285, which places *pXO1-137* under the control of a xylose-inducible promoter. Whether *pXO1-137* expression was repressed or induced by xylose, growth and *pagA-lacZ* expression of the parental strain carrying the pXO1-137 expression plasmid were reduced to levels similar to the  $\Delta pXO1-137$  strain (Figure S1). Growth of 34F2 and the  $\Delta pXO1-137$  strain were unaltered in the presence of the *pXO1-137*

expression plasmid when grown under non-toxin inducing conditions (data not shown). These observations suggest that *B. anthracis* is sensitive to both loss of *pXO1-137* and overproduction of *pXO1-137*.

**Loss of a pXO1-encoded small RNA results in decreased growth and toxin gene expression under toxin-inducing conditions.** Hfq-like proteins modulate gene expression by modulating binding of sRNA to their mRNA targets (29). McKenzie et al. recently identified two small RNAs on pXO1 through transcriptome analysis of pXO1 genes regulated by AtxA and the presence of CO<sub>2</sub>/bicarbonate. One of these sRNAs, *sRNA-1* is predicted to interact with a region in the 5'UTR of *atxA* (28). Another strain, AW-A133, was created containing a markerless deletion of pXO1 *sRNA-1*. Like with the  $\Delta pXO1-137$  strain, growth, *atxA* promoter activity, and *pagA* promoter activity of the  $\Delta sRNA-1$  strain was unchanged relative to the parental strain when grown under non-toxin inducing conditions in LB at 37°C (Figure 3A and B). When grown in R-Media at 37°C under 5% CO<sub>2</sub>, growth of both the  $\Delta sRNA-1$  and  $\Delta pXO1-137$  strains were significantly reduced relative to 34F2 (Figure 3C). *pagA* promoter activity was significantly reduced in the  $\Delta sRNA-1$  and  $\Delta pXO1-137$  strains while *atxA* promoter activity was similar to the 34F2 strain (Figure 3D). When three independent replicate samples were collected at 8 hours activity of *pagA*, *cya*, and *lef* in the  $\Delta sRNA-1$  and  $\Delta pXO1-137$  strains were significantly reduced relative to 34F2, but *atxA* promoter activity was unchanged. Further, toxin gene promoter activities were lower in the  $\Delta sRNA-1$  relative to the  $\Delta pXO1-137$  strain (Figure 3E), suggesting the possibility of *pXO1-137*-independent regulatory activities of *sRNA-1*. As loss of *sRNA-1* and *pXO1-137* result in very similar phenotypes, *pXO1-137* likely interacts with *sRNA-1* to facilitate binding to target mRNA transcripts for further regulation.

**Growth and virulence gene expression is sensitive to altered levels of *sRNA-1*.** When *pXOI-137* was added in trans to the parental strain, growth and toxin promoter activity was reduced to a level similar to the  $\Delta pXOI-137$  strain. We also constructed a *sRNA-1* expression plasmid using pAW285, placing *sRNA-1* under the control of a xylose-inducible promoter. Just as with *pXOI-137*, the 34F2 strain carrying the *sRNA-1* expression plasmid had a significant growth defect similar to the  $\Delta pXOI-137$  and  $\Delta sRNA-1$  strains when grown in R-Media (Figure 4A). *pagA* promoter activity was also strongly reduced in the 34F2 strain in the presence of the *sRNA-1* expression plasmid (Figure 4B). Like with the  $\Delta pXOI-137$  strain, reduced growth and *pagA* promoter activity in the  $\Delta sRNA-1$  strain was unaltered by addition of the *sRNA-1* expression plasmid. The data presented were from strains grown in the absence of xylose where *sRNA-1* expression is reduced. Addition of xylose had no effect of the observed phenotypes of the 34F2 or  $\Delta sRNA-1$  strains (data not shown). *B. anthracis* is sensitive to both loss and overproduction of either *pXOI-137* or *sRNA-1*.

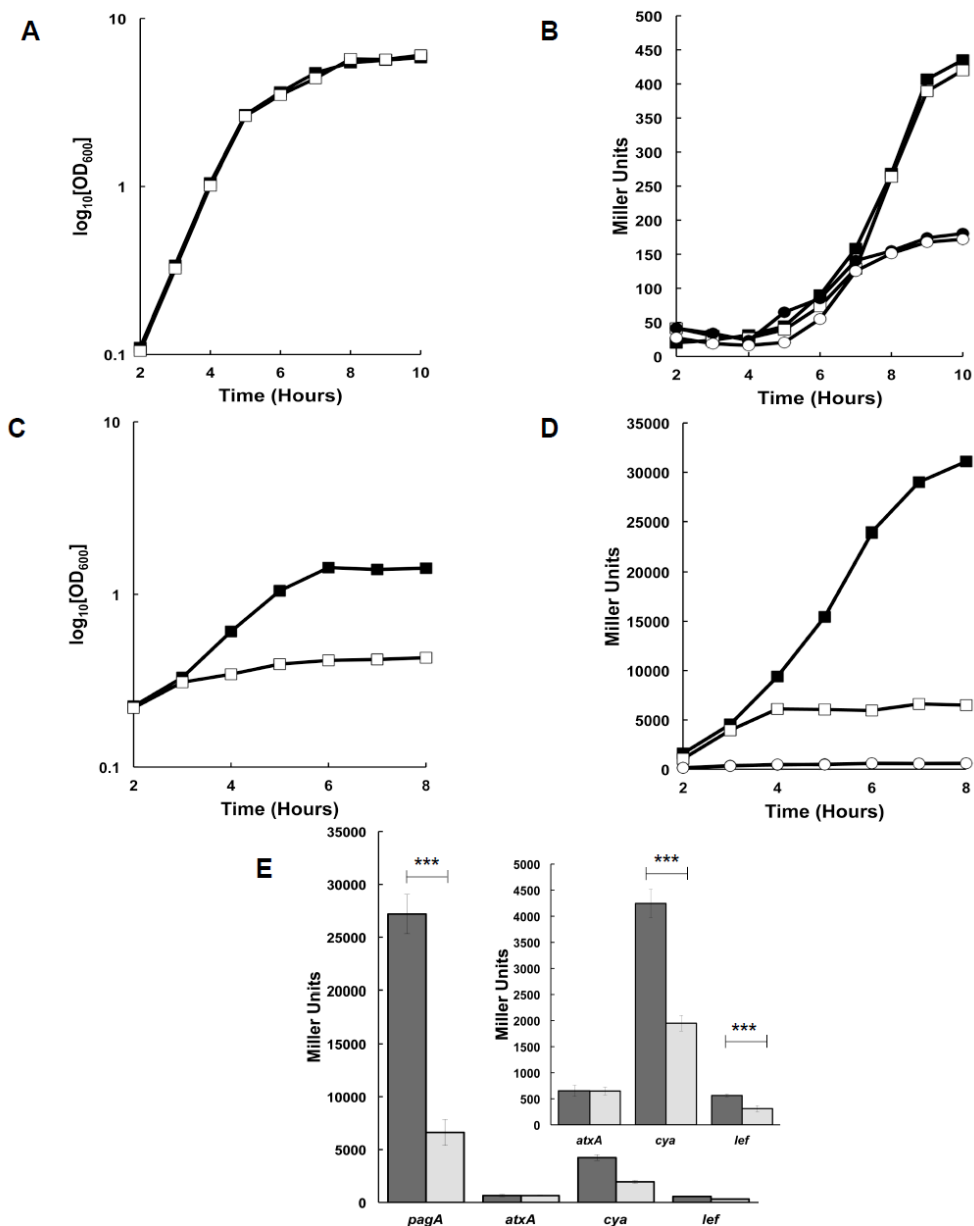
***atxA* mRNA transcript levels are unaltered in  $\Delta pXOI-137$  strain.** Our data demonstrate that loss of either *pXOI-137* or *sRNA-1* results in a coordinate decrease in promoter activities of all the toxin subunit genes, suggestive of an AtxA-mediated process, but *atxA* promoter activity is unaltered in the mutants. To address this discrepancy, steady state levels of *atxA* mRNA were assayed by quantitative reverse transcriptase PCR. Equal amounts of total RNA were loaded in each reaction, and data presented as *atxA* levels relative to the *gyrB* control from three independent reactions. As shown in Figure 5A, *atxA* transcript levels in the  $\Delta pXOI-137$  strain were unchanged relative to the parental 34F2 strain. These findings are consistent with measurements *atxA* promoter activity, indicating that *atxA* transcription is unchanged with loss of *pXOI-137*.

**AtxA protein levels are reduced in the  $\Delta pXOI-137$  and  $\Delta sRNA-1$  strains.** Thus far, we have shown that *atxA* promoter activity and *atxA* mRNA transcript levels are unaltered in the  $\Delta pXOI-137$  and  $\Delta sRNA-1$  strains despite the coordinate decrease in toxin gene expression indicative of an AtxA-mediated effect. We next assayed AtxA protein levels by immunoblotting with  $\alpha$ -AtxA polyclonal serum (17). As show in Figure 5B, AtxA protein was detected in the parental 34F2 strain, but AtxA was undetectable in the  $\Delta pXOI-137$  and  $\Delta sRNA-1$  strains. As additional controls, AtxA was also undetectable in a strain containing a markerless deletion of *atxA*, and the  $\alpha$ -AtxA polyclonal serum detected purified His6- tagged recombinant AtxA protein. While *atxA* transcription is unchanged in the mutant strains, AtxA protein levels are significantly reduced, suggesting that *pXOI-137* and *sRNA-1* contribute to post-transcriptional regulation of AtxA expression.

**Loss of *pXOI-137* or *sRNA-1* reduces translational initiation of *atxA*.** Our data suggesting post-transcriptional regulation involving *pXOI-137* and *sRNA-1*, together with computational RNA alignment analysis indicating that sRNA-1 binds to the *atxA* 5'-UTR (28), point to potential regulation of *atxA* translation. We developed a translational fusion of *atxA*, including the mapped promoters of *atxA*, the putative *sRNA-1* binding site, and the first 30 amino acids of AtxA, fused in-frame to the *lacZ* coding region missing its translational initiation codon. When the translational fusion was assayed in the 34F2 background,  $\beta$ -galactosidase activity was low during exponential phase growth and began to increase as the cells entered stationary phase (Figure 6), similar to the expression profile of the *atxA* promoter fusion. When grown in LB, no difference in growth or  $\beta$ -galactosidase activity between the parental and mutant strains was observed (Figure 6B). When grown in toxin- inducing R-Media, the expected growth defect was seen in the  $\Delta pXOI-137$  and  $\Delta sRNA-1$  strains (Figure 6C).  $\beta$ -galactosidase activity in the  $\Delta pXOI-$

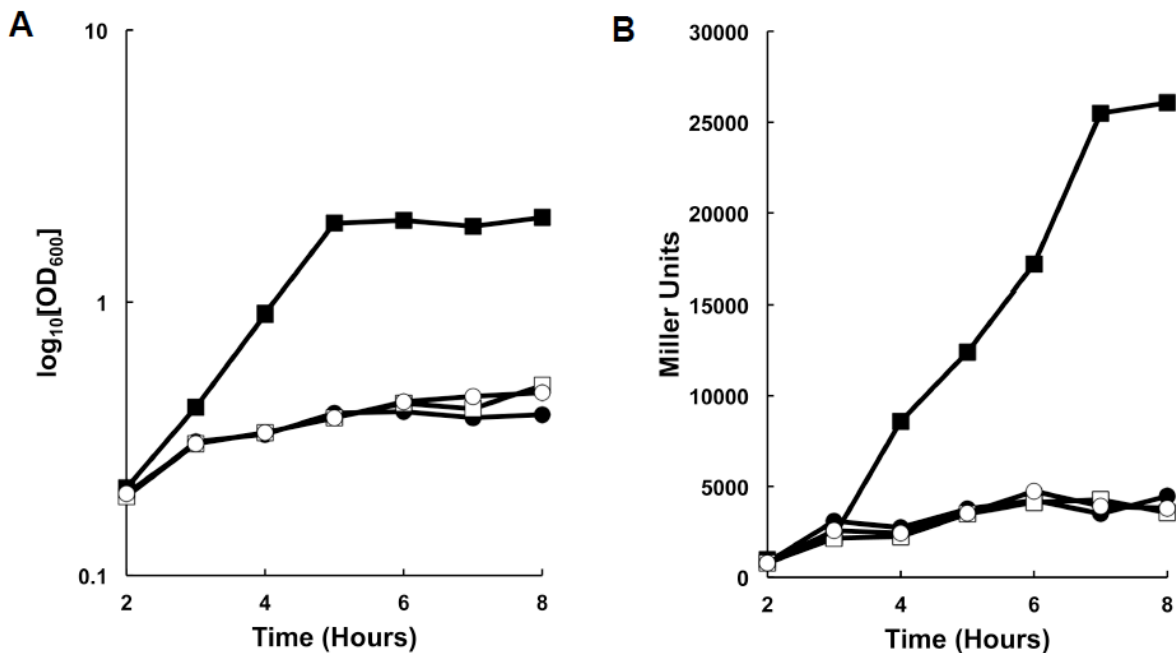
*137* and  $\Delta sRNA-1$  strains carrying the *atxA* translation fusion reporter was no longer induced during entry to stationary phase, remaining much lower than the expression levels in the parental 34F2 strain (Figure 6D). These observations indicate that *atxA* translation initiation is strongly reduced in the mutant strains and provide a mechanism for control of toxin gene expression by *pXO1-137* and *sRNA-1*.

**Loss of pXO1, but not *atxA*, results in an early growth defect in toxin-inducing media.** An unexpected finding in the  $\Delta pXO1-137$  and  $\Delta sRNA-1$  strains was a significant growth defect as loss of AtxA has not been shown to affect growth (4). To investigate the possibility that other factors on pXO1 contribute to the phenotype, the growth of a 34F2 strain cured of pXO1 was compared to  $\Delta atxA$ ,  $\Delta pXO1-137$ , and  $\Delta sRNA-1$  strains cultured in R-Media under 5% CO<sub>2</sub>. As expected, deletion of *atxA* had no effect on growth (Figure 7A). Deletion of pXO1-137 and sRNA-1 resulted in a significant defect beginning shortly after inoculation in fresh media, and growth was lower throughout the remainder of the experiment. The pXO1- strain showed an intermediate phenotype: growth was reduced through 4 hours, similar to the  $\Delta pXO1-137$  and  $\Delta sRNA-1$  strains, but, beginning at 5 hours, growth rate increased until total OD<sub>600</sub> matched 34F2 by 8 hours. Samples were collected at several time points to emphasize low growth early and recovery to parental levels late (Figure 7B). When grown in rich media in the absence of bicarbonate/CO<sub>2</sub>, no difference in growth was observed between the strains (Figure S2).



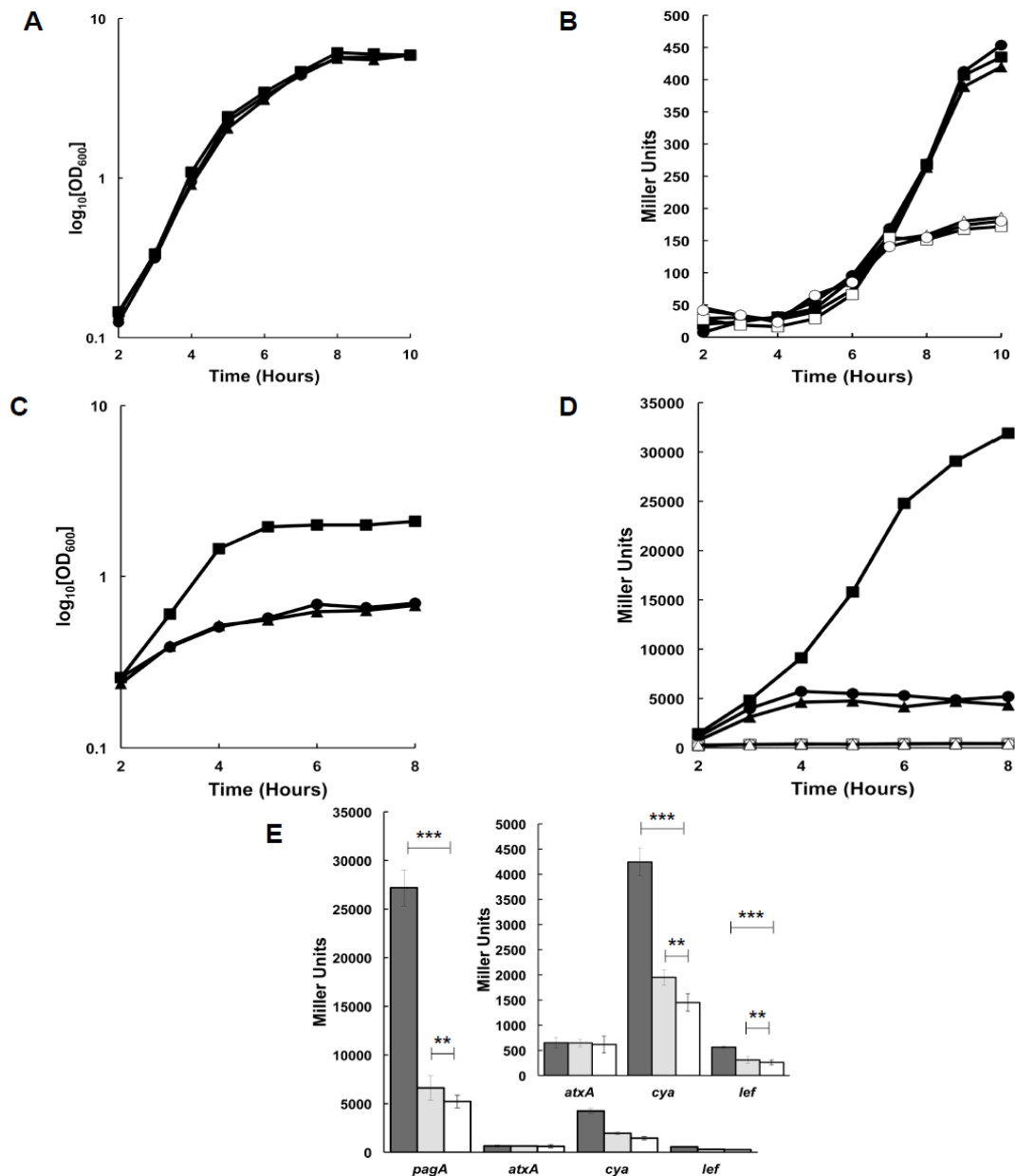
**Figure 5.1** Growth and virulence gene expression in *pXO1-137* mutant strains.

**A.** Cell growth of parental and mutant strains grown in LB at 37°C. ■ 34F2; □ Δ*pXO1-137*. **B.** β-galactosidase activity in *pagA* and *atxA* reporter strains grown in LB supplemented with kanamycin at 37°C. ■ 34F2 *pagA-lacZ*; ● 34F2 *atxA-lacZ*; □ Δ*pXO1-137 pagA-lacZ*; ○ Δ*pXO1-137 atxA-lacZ*. **C.** Cell growth of parental and mutant strains grown in R-Media at 37°C under 5% CO<sub>2</sub>. ■ 34F2; □ Δ*pXO1-137*. **D.** β-galactosidase activity in *pagA* and *atxA* reporter strains grown in R-Media at 37°C under 5% CO<sub>2</sub> supplemented with kanamycin. ■ 34F2 *pagA-lacZ*; ● 34F2 *atxA-lacZ*; □ Δ*pXO1-137 pagA-lacZ*; ○ Δ*pXO1-137 atxA-lacZ*. **E.** β-galactosidase activity of parental and mutant strains carrying reporters, as indicated, grown in R-Media supplemented with kanamycin at 37°C under 5% CO<sub>2</sub> for 8 hours post inoculation. Dark grey bars represent 34F2 and light grey bars represent Δ*pXO1-137*. Data was obtained from 3 independent cultures and error bars represent standard deviation from the mean. \*\*\* indicates two tailed unpaired *t* tests with P values of P<0.0001.



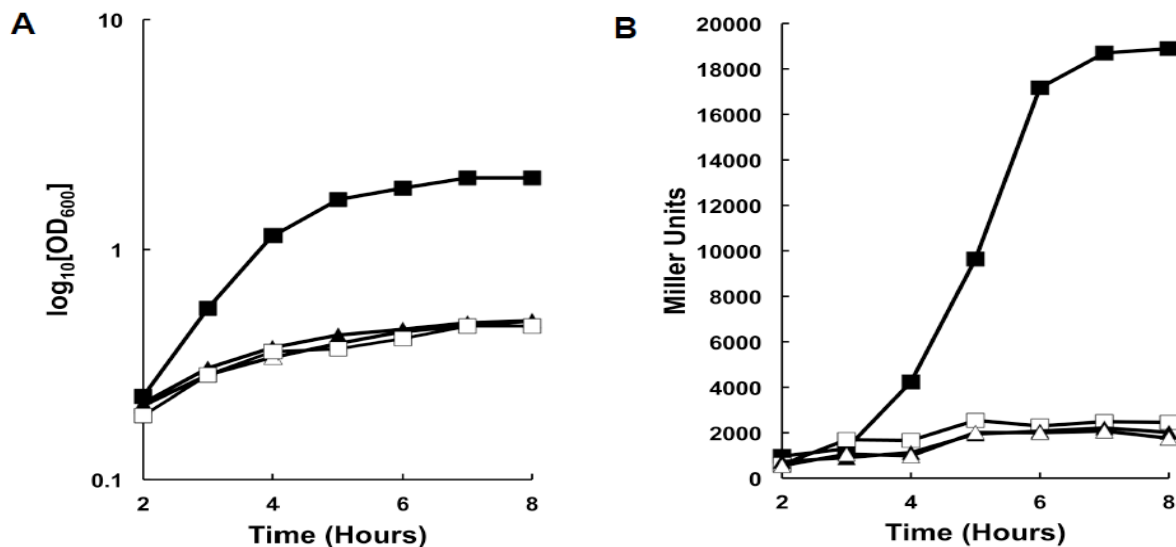
*Figure 5.2 Growth and virulence gene expression in pXO1-137 mutant strain with pXO1-137 complementation plasmid.*

**A.** Cell growth in R-Media at 37°C under 5% CO<sub>2</sub> supplemented with erythromycin and lincomycin. **B.** β-galactosidase activity of parental and mutant strains carrying the pAW324 pagA-lacZ reporter plasmid grown in in R-Media at 37°C under 5% CO<sub>2</sub> supplemented with kanamycin, erythromycin, and lincomycin. For both panels, ■ 34F2 with pHT315S (empty vector); □ 34F2 with pAW427 (pXO1-137 expression); ● ΔpXO1-137 with pHT315S (empty vector); ○ ΔpXO1-137 with pAW427 (pXO1-137 expression).



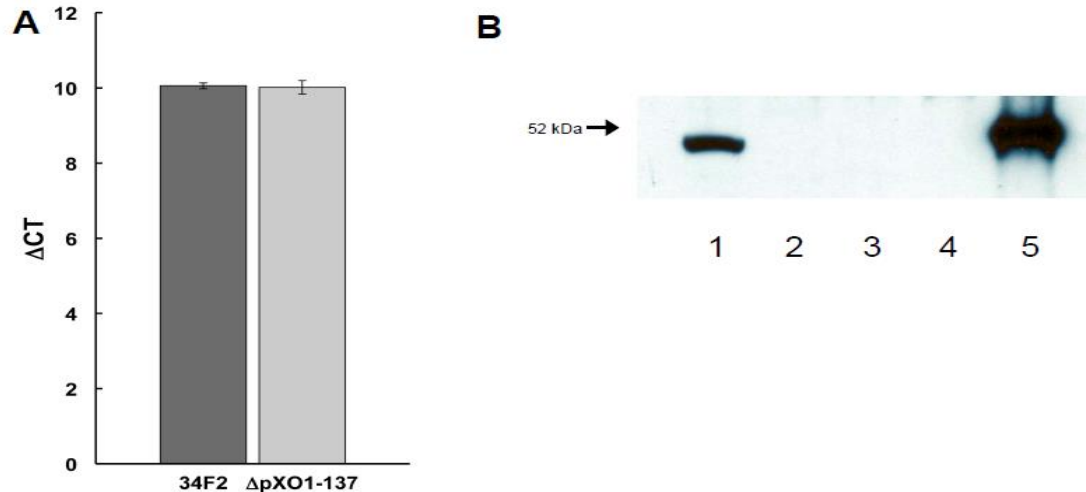
**Figure 5.3** Growth and virulence gene expression in *pXO1-sRNA1* mutant.

**A.** Cell growth of parental and mutant strains grown in LB at 37°C. ■ 34F2; ●  $\Delta pXO1-137$ ; ▲  $\Delta sRNA-1$ . **B.**  $\beta$ -galactosidase activity in *pagA* and *atxA* reporter strains grown in LB supplemented with kanamycin at 37°C. ■ 34F2 *pagA-lacZ*; ●  $\Delta pXO1-137$  *pagA-lacZ*; ▲  $\Delta sRNA-1$  *pagA-lacZ*; □ 34F2 *atxA-lacZ*; ○  $\Delta pXO1-137$  *atxA-lacZ*; △  $\Delta sRNA-1$  *atxA-lacZ*. **C.** Cell growth of parental and mutant strains grown in R-Media at 37°C under 5% CO<sub>2</sub>. ■ 34F2; ●  $\Delta pXO1-137$ ; ▲  $\Delta sRNA-1$ . **D.**  $\beta$ -galactosidase activity in *pagA* and *atxA* reporter strains grown in R-Media at 37°C under 5% CO<sub>2</sub> supplemented with kanamycin. ■ 34F2 *pagA-lacZ*; ●  $\Delta pXO1-137$  *pagA-lacZ*; ▲  $\Delta sRNA-1$  *pagA-lacZ*; □ 34F2 *atxA-lacZ*; ○  $\Delta pXO1-137$  *atxA-lacZ*; △  $\Delta sRNA-1$  *atxA-lacZ*. **E.**  $\beta$ -galactosidase activity of parental and mutant strains carrying reporters, as indicated, grown in R-Media supplemented with kanamycin at 37°C under 5% CO<sub>2</sub> for 8 hours post inoculation. Dark grey bars represent 34F2, light grey bars represent  $\Delta pXO1-137$ , and white bars represent  $\Delta sRNA-1$ . Data was obtained from 3 independent cultures and error bars represent standard deviation from the mean. \*\*\* indicates two tailed unpaired *t* tests with P values of P<0.0001. \*\* indicates two tailed unpaired *t* tests with P values of P<0.005.



**Figure 5.4** Growth and virulence gene expression in *sRNA-1* mutant strain with *sRNA-1* complementation plasmid.

**A.** Cell growth in R-Media at 37°C under 5% CO<sub>2</sub> supplemented with chloramphenicol. **B.**  $\beta$ -galactosidase activity of parental and mutant strains carrying the pAW324 *pagA-lacZ* reporter plasmid grown in in R-Media at 37°C M under 5% CO<sub>2</sub> supplemented with kanamycin, and chloramphenicol. For both panels, ■ 34F2 with pAW285 (empty vector); □ 34F2 with pAW437 (*sRNA-1* expression); ▲  $\Delta sRNA-1$  with pAW285 (empty vector); △  $\Delta sRNA-1$  with mpAW437 m (*sRNA-1* expression).



**Figure 5.5** *atxA* transcript and AtxA protein levels.

**A.** RNA extracted from 34F2 and  $\Delta pXO1-137$  strains grown in R-Media at 37°C under 5% CO<sub>2</sub> at 8 hours post-inoculation normalized between samples for total RNA was subjected to quantitative, real time RT-PCR. Relative expression levels were calculated by the  $\Delta\text{Ct}$  method for *atxA* relative to *gyrB* transcript levels. Data was obtained from 3 independent experiments and error bars represent standard deviation from the mean. **B.** Immunoblotting of total protein extracted from strains grown in R-Media at 37°C under 5% CO<sub>2</sub> to 8 hours post-inoculation. Amount of extracted sample loaded was normalized relative to total protein. AtxA protein was detected by probing with polyclonal polyclonal  $\alpha$ -AtxA serum. Lane 1, 34F2; Lane 2, AW-A002 ( $\Delta atxA$ ); Lane 3, AW-A049 ( $\Delta pXO1-137$ ); Lane 4, AW-A133 ( $\Delta sRNA-1$ ); Lane 5, purified His6-AtxA.

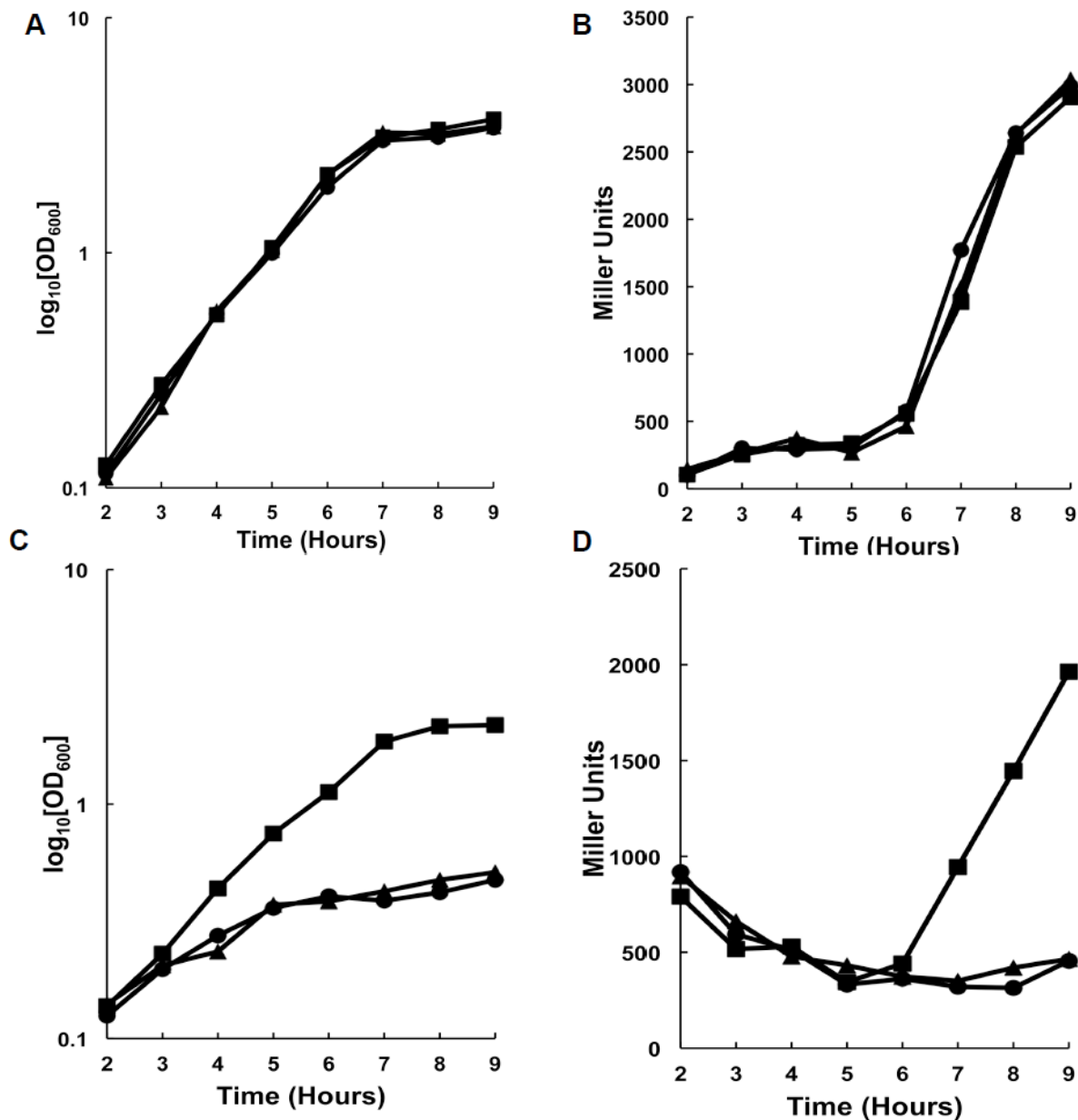


Figure 5.6 Growth and *atxA* translational initiation in *pXO1-137* and *sRNA1* mutant strains.

**A.** Cell growth of strains carrying plasmid pAW457 (*atxA* translational fusion to *lacZ*) grown in LB supplemented with chloramphenicol at 37°C. **B.** β-galactosidase activity strains carrying plasmid pAW457 grown in LB supplemented with chloramphenicol at 37°C. **C.** Cell growth of strains carrying plasmid pAW457 grown in R-Media at 37°C under 5% CO<sub>2</sub> supplemented with chloramphenicol. **D.** β-galactosidase activity strains carrying plasmid pAW457 grown in R-Media at 37°C under 5% CO<sub>2</sub> supplemented with chloramphenicol at 37°C. For all panels: ■ 34F2; ● Δ*pXO1-137*; ▲ Δ*sRNA-1*.

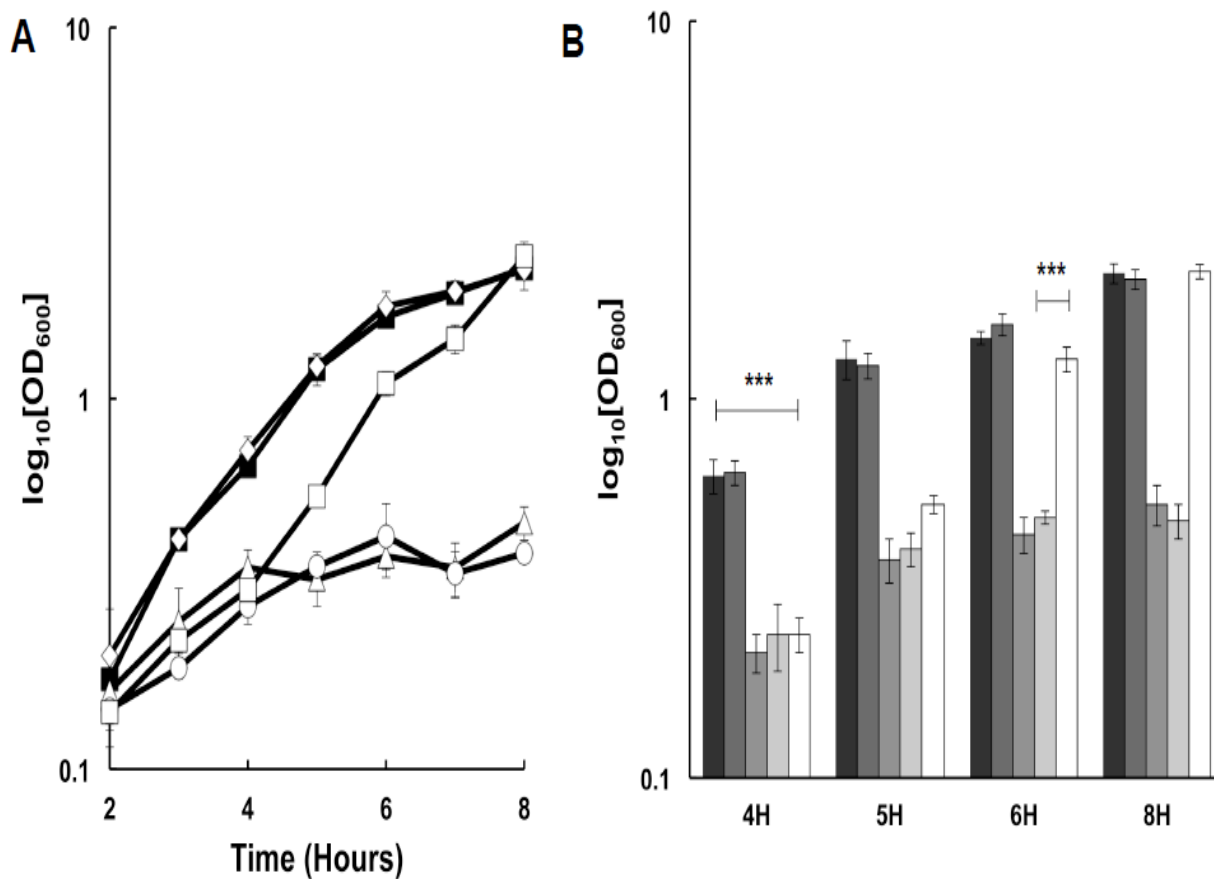
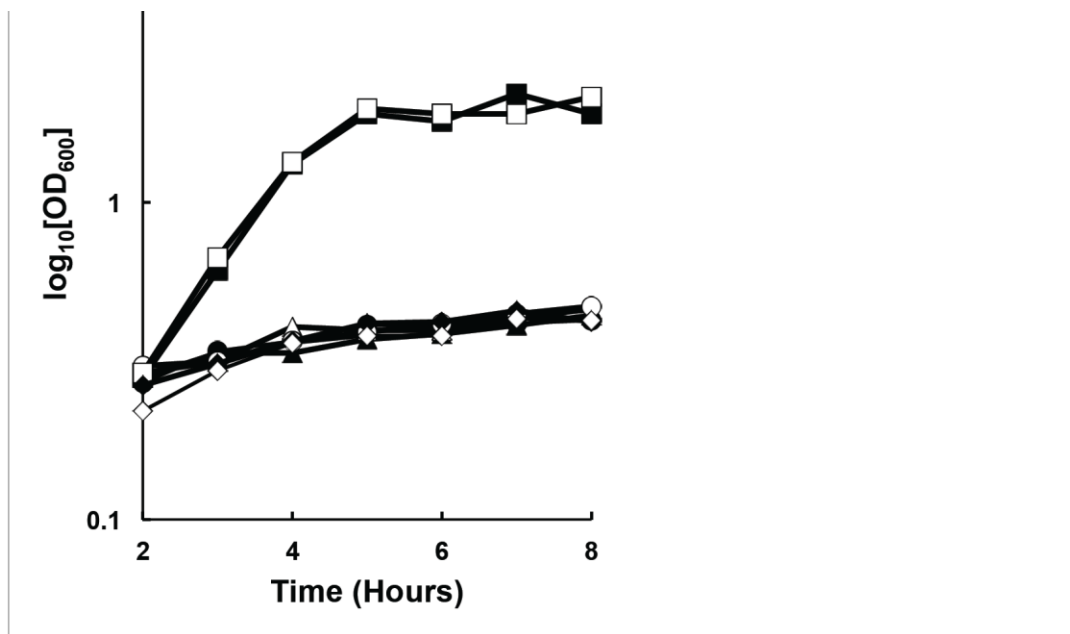


Figure 5.7 Growth comparisons of parental,  $\Delta atxA$ ,  $\Delta pXOI-137$ ,  $\Delta sRNA-1$  and  $pXOI-1$  strains.

A. Cell growth in R-Media at 37°C under 5% CO<sub>2</sub>. ■ 34F2; ◇  $\Delta atxA$ ; ○  $\Delta pXOI-137$ ; △  $\Delta sRNA-1$ ; □  $pXOI-1$ . B. Cell growth in R-Media at 37°C under 5% CO<sub>2</sub> collected at time points as indicated. Strains as indicated from darkest to lightest shade: 34F2;  $\Delta atxA$ ;  $\Delta pXOI-137$ ;  $\Delta sRNA-1$ ;  $pXOI-1$ . \*\*\* indicates two tailed unpaired *t* tests with P values of P<0.0001.



*Figure 5.8 Growth and virulence gene expression in pXO1-137 mutant strain with xylose-inducible pXO1-137 complementation plasmid*

**A.** Cell growth in R-Media at 37°C under 5% CO<sub>2</sub> supplemented with chloramphenicol and kanamycin. **B.** β-galactosidase activity of parental and mutant strains carrying the pTCVlac-pagA reporter plasmid grown in R-Media at 37°C under 5% CO<sub>2</sub> supplemented with chloramphenicol and kanamycin. For both panels, ■ 34F2 with pAW285 (empty vector); □ 34F2 with AW285 with xylose; ◆ 34F2 with pAW377 (pXO1-137 expression); ◇ 34F2 with pAW377 with xylose; ▲ ΔpXO1-137 with pAW285; △ ΔpXO1-137 with pAW285 with xylose; ● ΔpXO1-137 with pAW377; ○ ΔpXO1-137 with pAW377 with xylose.

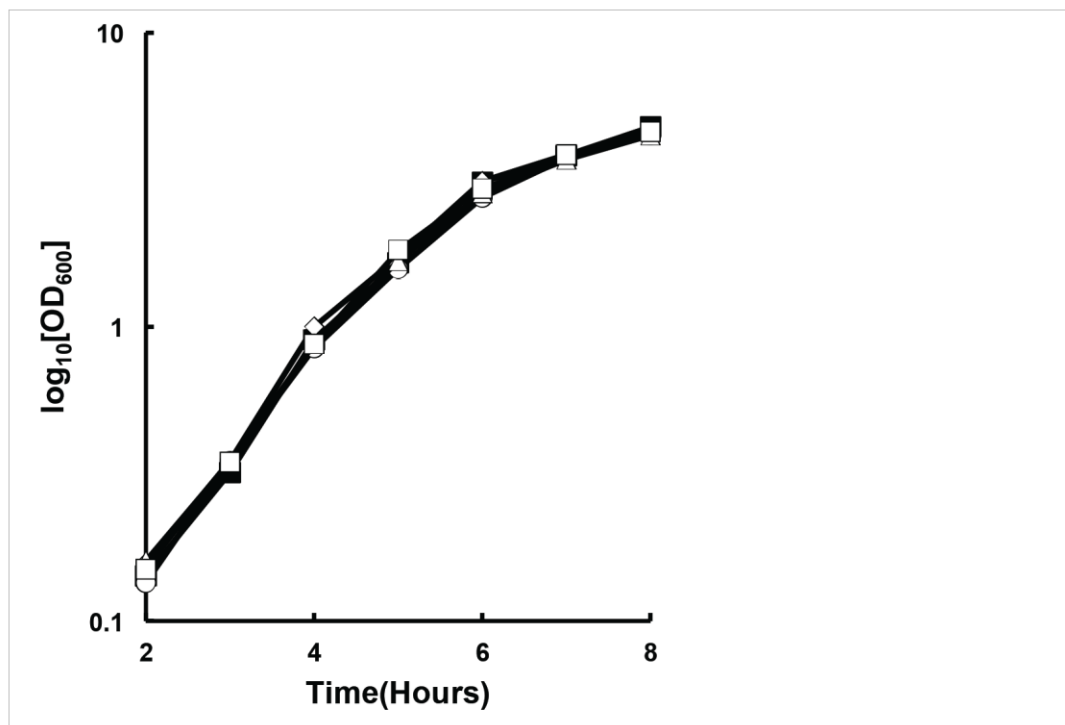


Figure 5.9 Growth comparisons of parental  $\Delta atxA$ ,  $\Delta pXO1-137$ ,  $\Delta sRNA-1$  and pXO1-1 strains.

A. Cell growth in LB at 37°C. ■ 34F2; ◇  $\Delta atxA$ ; ○  $\Delta pXO1-137$ ; △  $\Delta sRNA-1$ ; □ pXO1.

Table 5.1 Oligonucleotide primers used in Chapter 5

Table S1: Oligonucleotide primers used in this study.

Primers	Sequence
pXO1-137U5'Bam	5'-CACCGGATCCTATGAAAATCGGGGAGTCCA
pXO1-137U3'Sal	5'-GCGTCGACTCTTCCTCTCTTTAAACGTTCTAATCT
pXO1-137D5'Sal	5'-CACCGTTCGACAAGGATTCACCCCAGGAAAT
pXO1-137D3'Pst	5'-TGCCTGCAGTGACAACACTATTCAAGCAGGTAATGA
pXO1-sRNA-1U5'Bam	5'-CACCGGATCCAATCACTAGGTAGGCTCA
pXO1-sRNA-1U3'Sal	5'-TGCCTGCAGAGTTTAAATCCCTACGTTTCC
pXO1-sRNA-1D5'Sal	5'-CACCGTTCGACAGAGGTAACAAAGTATCCATCTAAA
pXO1-sRNA-1D3'Pst	5'-TGCCTGCAGTCAATTCTTTTTATCGAATCGTG
pXO1-137E5'Bam	5'-CACCGGATCCAAAGAGAGGAAGAACTATTCATGC
pXO1-137E3'Sal	5'-TGCCTGCAGCGGTATATATTTATAAAGATACTGTTGAAATAGCAT
sRNA1U	5'-TGTTAAGTGATGCATGTTTAATAATTCATTAG
sRNA1D	5'-CTTTGTTTACCTCTGTTAAGTCCAG
atxAfus5'Eco	5'-CACCGAATTCCTTTTTAATAATAATCAAAAATTAATTATTG
atxAfus3'2	5'-CCCGTAATCTTACGTCAGTAACTTC AAAGTGATGATAATTAATTAATCTTATATGTTTCT
lacZfus5'2	5'-GAAGTTACTGACGTAAGATTACGGG
lacZfus3'Bam	5'-TGC GGATCCGGTTCCTTATTTTTGACACCA
atxART5'5'	5'-ACGAAATTCCTCCATCTTGG
atxART5'3'	5'-TCAACAAAGGAAAGGGATTCA
AtxAE5'Xho	5'-CACCTCGAGCTAACACCGATATCCATCGAAAA
AtxAE3'Bam	5'-TGC GGATCCTTATATTATCTTTTTGATTCATGAAAATCTCT

## 5.4 Discussion

Hfq-mediated small RNA regulation of AtxA activity provides new insight into virulence regulation in *B. anthracis*. While other modes of post-transcriptional regulation of AtxA have been demonstrated or suggested, such as AtxA dimerization status (17), alternative phosphorylation/dephosphorylation in PRD-like domains (12, 37), and CodY-mediated AtxA alteration in steady state AtxA levels (14, 16), this report is the first demonstration of regulation at the level of translational initiation. sRNA-mediated positive regulation of translational

initiation has been observed in many other bacterial systems. A common mechanism involves binding of the sRNA to the 5'UTR of the target mRNA, releasing RNA secondary structure blocking the Shine Dalgarno sequence, thereby promoting translation. Classic examples include RNAIII regulation of *hla* in *Staphylococcus aureus* (38) and DsrA regulation of *rpoS* in *E. coli* (39). Our phenotypic findings and the bio-informatic predictions of McKenzie et al. (28) are consistent with such a translational regulation mechanism controlling AtxA production in *B. anthracis*. The phenotypes associated with loss of *pXOI-137* and *sRNA-1* are observed only when strains are grown in toxin-inducing medium in the presence of bicarbonate/CO<sub>2</sub>. The precise contribution of growth conditions and nutrient availability to sRNA regulation of AtxA may warrant further investigation. Several transcriptomics studies have shown that *pXOI-137* and/or *sRNA-1* are up-regulated in response to bicarbonate/CO<sub>2</sub> (28, 40). Induction of *pXOI-137* and *sRNA-1* expression provides a mechanism by which AtxA activity is induced by bicarbonate/CO<sub>2</sub> without altering *atxA* transcription (23). Post-transcriptional regulation by altered translational initiation, as demonstrated here, and AtxA dimerization (17) provide two mechanisms that control AtxA activity in response to bicarbonate/CO<sub>2</sub>. Additionally, transcription of both *pXOI-137* and *sRNA-1* is regulated by AtxA (28). This suggests that production of active AtxA further amplifies production of AtxA, thus providing positive feedback to AtxA and increasing virulence gene expression. Several studies have pointed to the importance of physiologic set points for proper activity of both Hfq and sRNA. In *E. coli*, increased Hfq concentrations can result in a decrease, rather than the predicted increase, of some sRNA activities. This appears to be the result of Hfq binding singly to either mRNA or sRNA to create non-functional complexes rather than forming functional Hfq-sRNA-mRNA complexes (41). Similarly, when sRNA expression is induced, limiting amounts of Hfq available for

functional complex formation can result in reduced sRNA regulation (42). That overexpression of either *pXO1-137* or *sRNA-1* alters growth even in the parental 34F2 strain of *B. anthracis* would appear to be consistent with findings in *E. coli* and point to the importance of fine tuning in the expression of both Hfq and sRNA.

Altered levels of *pXO1-137* or *sRNA-1* result in a significant growth defect when grown under toxin-inducing conditions. As loss of AtxA does not alter growth, these observations would suggest that *pXO1-137* and *sRNA-1* are regulating other factors that do affect growth. A 34F2 strain cured of virulence plasmid pXO1 has an intermediate growth defect: a defect during exponential phase that was resolved as the cells entered stationary phase. This would suggest multiple phases of *sRNA-1* growth control. One interpretation of these data is that perhaps *pXO1-137* and *sRNA-1* regulate factors on the chromosome that are important to early growth, as loss of pXO1 causes slow growth, while they regulate factors on pXO1 reducing late growth that would be absent in the pXO1- strain. McKenzie et al. predicted additional targets of *sRNA-1* on pXO1, but their analysis did not extend to sequences on the main bacterial chromosome (28). Of particular interest to our phenotype is a predicted sRNA-1-regulated *relA*-like gene. RelA-SpoT family proteins are involved in inducing the stringent response in response to nutritional stress through production of the alarmone (p)ppGpp (24), and *B. subtilis* carries multiple, small (p)ppGpp synthetases that appear to respond to different signals (43). A chromosomally-encoded RelA was characterized in *B. anthracis* (44), but the pXO1-encoded *relA*-like gene has not been studied. Perhaps this sRNA-1-regulated *relA*-like gene contributes to late growth regulation under toxin inducing conditions, and, in its absence in the pXO1- strain, this growth control is lost. Clearly, pXO1-137 and sRNA-1 regulation is complex and additional work is required to identify their targets and modes of regulation. AtxA directly and indirectly controls a number of

other genes on pXO1, pXO2, and the main bacterial chromosome. Of special significance are the indirect effects of AtxA on capsule production through the pXO2-encoded AcpA and AcpB (7-9) and the S-layer surface proteins through pXO1-encoded PagR (45). AtxA is essential to virulence in animal models of anthrax infection (46). Further investigation is needed to understand the complexity of sRNA-1-mediated regulation of AtxA in global gene regulation and virulence control. pXO1 carries an additional bicarbonate CO<sub>2</sub>/ induced sRNA, sRNA-2 (28), and the role of pXO1-137 on sRNA-2 activity is unknown. Two additional Hfq are encoded on the main bacterial chromosome (our observations) (32, 40), and investigations into their function and cross-talk are currently

## 5.5 References

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## 6 CHAPTER SIX: DISCUSSION

### 6.1 Role of membrane bound thiol-disulfide oxidoreductases in toxin expression and sporulation

First, we demonstrate that membrane bound thiol-disulfide oxidoreductases contribute to several processes important to metabolism and virulence in *B. anthracis*. There is conservation among components between *B. anthracis* and the well-characterized model organism *B. subtilis*. One significant difference lies in the fact that, unlike *B. subtilis*, which carries one *ccdA*, *B. anthracis* carries two copies of *ccdA*. In *B. anthracis*, CcdA2 functions in cytochrome *c* maturation, *atxA* regulation, and sporulation. In contrast, CcdA1 functions in cytochrome *c* maturation and *atxA* regulation but has no activity in efficient sporulation. The actual activities of the two proteins are partially masked by the significant differences in expression levels. Interestingly, the presence of two distinct *ccdA* genes is a feature found throughout the pathogenic *B. cereus* group, including *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis*. In contrast, most other non-pathogenic environmental bacilli, including *B. subtilis*, *B. pumilus*, and *B. halodurans*, carry only one *ccdA*, which is more similar to *ccdA1*. Thiol-disulfide oxidoreductases have been implicated in the pathogenesis of several organisms, including *E. coli*, *Vibrio cholera*, and *Haemophilus influenzae*, among many others (1-4). The effect the loss of *ccdA1/2* on virulence of *B. anthracis* is unclear. The virulence gene dysregulation upon loss of cytochrome *c* activity is unique: increased *atxA* only during exponential phase that leads to increased toxin expression in stationary phase though a mechanism still under investigation. While our observations provide a unique view of the function and specificity of these duplicate *ccdA* genes, they also highlight the gaps in our

knowledge of thiol- disulfide oxidoreductases. Thiol-disulfide oxidoreductases are essential to correct assembly of surface-exposed proteins that are important to many processes in *B subtilis*, including sporulation, competence, cytochrome maturation, and lantibiotic production (5-8). It is certainly possible that thiol-disulfide oxidoreductases could be involved in other as yet undefined processes in Gram-positive bacteria. Further, thiol-disulfide oxidoreductases are interesting drug targets, given their roles in the pathogenesis of many organisms and evidence suggesting that inhibitors can block viral infection (9). Additional work is required to define the relationships of CcdA1/2 and other thiol-disulfide oxidoreductases to their cognate targets in order to define their activities in bacterial physiology and virulence.

## **6.2 New element in *B. cereus* virulence gene expression regulation pathway**

We revealed a role for the *c*-type cytochromes and the *c*-type cytochrome maturation pathway in the regulation of virulence gene expression in *B. cereus*. Loss of the cytochrome *c* maturation genes *resB* and *resC* results in increased expression of the major enterotoxins and the virulence regulator *plcR*. This effect can be partially, but not completely, attributed to loss of *cccB*, encoding cytochrome CccB (*c551*). We demonstrate increased *plcR*, *nhe*, *hbl*, and *cytK* transcription under aerobic conditions in response to the loss of cytochrome *c* maturation or CccB. These findings are unexpected in light of the differences in virulence gene regulation between *B. cereus* and *B. anthracis*. In *B. anthracis*, loss of cytochrome maturation activity results in increased toxin gene expression through increased *atxA* transcription (10). An *atxA*-like transcriptional regulator is not present in the *B. cereus* genome; instead, transcription of the gene encoding the major virulence regulator in *B. cereus*, *plcR*, is increased in the absence of *c*-type cytochrome maturation. Presence of PlcR is required for the cytochrome-dependent increase in virulence gene expression, arguing against a regulatory mechanism independent of PlcR. There

is little similarity in the structure, regulation, or known activities of AtxA and PlcR, which makes a common mechanism acting upon both regulatory proteins unusual. The similarity in phenotypes with loss of cytochrome *c* maturation despite differences in regulatory networks raises the possibility of a conserved, previously unidentified regulatory pathway at work in both organisms. Despite the high degree of chromosomal similarity between *B. cereus* and *B. anthracis*, the chromosomally-encoded virulence regulatory networks are significantly different. The specificity of virulence control is of interest in light of fatal diseases caused by strains of *B. cereus* that carry virulence genes of both *B. cereus* and *B. anthracis* (11.12). Insight into the mechanistic differences in the control of virulence factor expression would be helpful in understanding and tracking these dangerous pathogenic strains.

### **6.3 Pyomelanin pigment function in *B. anthracis***

We investigated the impact of pigment generation on cell physiology and pathology. Loss of *hmgA* in *B. anthracis* results in accumulation of pyomelanin in the presence of L-tyrosine and L-phenylalanine. Neither the loss of *hmgA* nor the accumulation of pyomelanin affects the growth characteristics of *B. anthracis*, indicating that this aromatic amino acid catabolism pathway is not essential. The loss of *hmgA* does not affect virulence gene expression nor sporulation and germination, suggesting HmgA has no significant effect on *B. anthracis* cell lifecycle. In contrast, the accumulation of pyomelanin production confers protection from UV radiation, indicating that HmgA may involve in cell survival pathway. While pyomelanin protects vegetative cells from UV damage, *B. anthracis* would mostly likely be exposed to UV radiation outside the host when in the form of UV-resistant spores. However, the protein coat that contains a brown pigment around the endospore is produced by the copper-dependent laccase, CotA (13). This melanin-like pigment protein would only be present in the spore, not in

vegetative cells as with the loss of HmgA. The relevance of pyomelanin accumulation to pathogenesis of *B. anthracis* is unclear. It may be possible that pyomelanin protects cells from other stresses found during the course of infection that were not assayed. Further analysis of pyomelanin-producing mutants in cellular and animal models of infection may be of value.

#### **6.4 New AtxA translational regulation pathway**

We later demonstrated that Hfq-mediated small RNA regulation of AtxA activity in *B. anthracis*. While other modes of post-transcriptional regulation of AtxA have been demonstrated or suggested, such as AtxA dimerization status, alternative phosphorylation/dephosphorylation in PRD-like domains, and CodY-mediated AtxA alteration in steady state AtxA levels (14-16), this is the first demonstration of regulation at the level of translational initiation. Several transcriptomics studies have shown that pXO1-137 and/or sRNA-1 are up-regulated in response to bicarbonate/CO<sub>2</sub> (17). Induction of pXO1-137 and sRNA-1 expression provides a mechanism by which AtxA activity is induced by bicarbonate/CO<sub>2</sub> without altering *atxA* transcription. Along with dimerization, these are two mechanisms that control AtxA activity in response to bicarbonate/CO<sub>2</sub>. Additionally, transcription of both pXO1-137 and sRNA-1 is regulated by AtxA (18). This suggests that production of active AtxA further amplifies production of AtxA, thus providing positive feedback to AtxA and increasing virulence gene expression. Altered levels of pXO1-137 or sRNA-1 result in a significant growth defect when grown under toxin-inducing conditions. As loss of AtxA does not alter growth, these observations would suggest that pXO1-137 and sRNA-1 are regulating other factors that do affect growth. Further investigation is needed to understand the complexity of sRNA-1-mediated regulation of AtxA in global gene regulation and virulence control. pXO1 carries an additional bicarbonate/CO<sub>2</sub> induced 2sRNA, sRNA-2 (19), and the role of pXO1-137 on sRNA-2 activity is unknown. Two

additional Hfq are encoded on the main bacterial chromosome, and their function and cross-talk should be addressed.

Overall bacteria gene regulation pathway is a complicated yet efficient process which plays crucial role in majority of bacteria metabolism. This dissertation is dedicated to have a better understanding how the regulation pathway affects both physiology and pathology of *B. anthracis* and *B. cereus* from several different angles.

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