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Two small *c*-type cytochromes affect virulence gene expression in *Bacillus anthracis*

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Abstract

Regulated expression of the genes for anthrax toxin proteins is essential for the virulence of the pathogenic bacterium *Bacillus anthracis*. Induction of toxin gene expression depends on several factors, including temperature, bicarbonate levels, and metabolic state of the cell. To identify factors that regulate toxin expression, transposon mutagenesis was performed under non-inducing conditions and mutants were isolated that untimely expressed high levels of toxin. A number of these mutations clustered in the heme biosynthetic and cytochrome *c* maturation pathways. Genetic analysis revealed that two heme-dependent, small *c*-type cytochromes, CccA and CccB, located on the extracellular surface of the cytoplasmic membrane, regulate toxin gene expression by affecting the expression of the master virulence regulator AtxA. Deregulated AtxA expression in early exponential phase resulted in increased expression of toxin genes in response to loss of the CccA-CccB signaling pathway. This is the first function identified for these two small *c*-type cytochromes of *Bacillus* species. Extension of the transposon screen identified a previously uncharacterized protein, BAS3568, highly conserved across many bacterial and archeal species, as involved in cytochrome *c* activity and virulence regulation. These findings are significant not only to virulence regulation in *B. anthracis*, but also to analysis of virulence regulation in many pathogenic bacteria and to the study of cytochrome *c* activity in Gram-positive bacteria.

Keywords

Bacillus anthracis; cytochrome; AtxA; *pagA*; resB; heme; CccB; CccA

Introduction

Bacteria are able to sense external signals and integrate these signals into regulatory circuits in order to adapt to and survive changes in environmental conditions. Environmental regulation in pathogenic bacteria is particularly important as they must recognize both metabolic and host-derived signals in order to promote bacterial growth in the host conditions. *Bacillus anthracis* presents an especially interesting system of environmental regulation as the organism leads a complex life cycle that includes periods of quiescence as spores in the external environment and periods of growth and pathogenesis once the bacteria finds itself in a suitable host. But even in the host, the bacteria must carefully balance growth, development, and virulence in order to propagate and disseminate. The complex regulatory networks controlling *B. anthracis* virulence and development are slowly being

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revealed (Perego and Hoch, 2008), but far too little is known of the mechanisms underlying the detection and propagation of host signals.

Both the anthrax toxin and the antiphagocytic capsule are essential to *B. anthracis* virulence. The tripartite anthrax toxin is encoded by three non-contiguous genes, *lef*, *cya* and *pagA*, carried on the virulence plasmid pXO1 (Okinaka *et al*., 1999). *lef* encodes Lethal Factor (LF), a zinc metalloprotease targeting host MAP-kinase signaling (Duesbery *et al*., 1998), *cya* encodes dema Factor (EF), an adenylate cyclase that increases cellular cAMP levels (Leppla, 1982), and *pagA* encodes Protective Antigen (PA), which forms a pore allowing entry of toxin components (Milne *et al*., 1994). The antiphagocytic poly-D-glutamic acid capsule, which is essential for bacterial dissemination in the host (Drysdale *et al*., 2005), is encoded by genes in the *cap* operon carried on virulence plasmid pXO2 (Makino *et al*., 1989). Transcription of the toxin and capsule genes is regulated by AtxA, encoded by the *atxA* gene on pXO1 (Dai *et al*., 1995;Uchida *et al*., 1997). Activity of AtxA is regulated by multiple regulatory circuits. Transcription of *atxA* is dependent on a dual promoter and repressed by the transition state regulator AbrB, which, in turn, is regulated by components of the sporulation phosphorelay (Saile and Koehler, 2002;Bongiorni *et al*., 2008). Post-translationally, the activity of AtxA protein is regulated through phosphorylation and dephosphorylation of two histidine residues (Tsvetanova *et al*., 2007).

Expression of *B. anthracis* virulence factors is stimulated by conditions suggestive of the host environment. Optimal toxin expression levels occur at 37°C in media supplemented with bicarbonate, *in vitro* conditions thought to mimic those of the mammalian host (Sirard *et al*., 1994;Ristroph and Ivins, 1983). The bicarbonate transporter is a required component of the regulatory pathway that controls the induction of toxin gene expression by elevated bicarbonate levels (Wilson *et al*., 2008b). However, toxin gene expression is regulated in the absence of bicarbonate by other, unknown factors.

In order to identify other regulatory pathways that regulate toxin expression, a transposon mutagenesis screen was performed that identified mutants overexpressing the protective antigen gene, *pagA*, under non-toxin inducing conditions. Several toxin-overexpressing mutations clustered in genes of the heme synthesis and cytochrome *c* maturation pathways. By extending the results of the mutagenesis screen through a combination of biochemical and genetic approaches, a novel regulatory pathway was identified that requires extracellular presentation of two small *c*-type cytochromes. These two cytochromes are required to repress transcription of the AtxA regulatory protein which, in turn, results in down regulation of toxin genes expression. Additionally, the transposon mutagenesis identified a highly conserved but previously uncharacterized protein that affects both cytochrome *c* activity and toxin gene expression.

Results

Identification of transposon mutants affecting toxin gene expression

The regulatory circuit required for control of virulence gene expression in *B. anthracis* is not fully understood as several regulatory inputs known to control toxin and capsule gene expression currently have no mechanistic basis. In order to probe for regulators of toxin gene expression in *B. anthracis*, the Sterne 3F42 strain ($pXO1^+$ $pXO2^-$) carrying a fusion of the protective antigen gene promoter (*pagA*) to the *lacZ* reporter on the replicative vector pTCV*lac* (Poyart and Trieu-Cuot, 1997) was mutagenized using the transposon delivery vector pAW016 (Wilson *et al*., 2007). pAW016 carries a mini-Tn10 transposon with a spectinomycin-resistance cassette on a temperature-sensitive replicative plasmid. Spectinomycin-resistant transposon insertion clones were screened for altered *pagA*

expression by scoring for blue color on X-Gal plates in growth conditions non-inductive of toxin production (i.e. 37°C, LB in air without bicarbonate) (Leppla, 1988).

Among the pool of mutants that overexpressed *pagA* relative to the parental strain were a number of disruptions of genes predicted to be involved directly or indirectly in heme production or cytochrome *c* activity and one insertion in a gene encoding a hypothetical protein of unknown function (Table 2 and Fig. S1).

Insertions were found in:

BAS4358 (GenBank: AAT56656.1) encoding a protein that shares 80% amino acid identity with the product of the *B. subtilis hemL* gene (GenBank: AAA22515.1). In *B. subtilis*, HemL converts glutamate-1-semialdehyde to 5-aminolevulinic acid (5-ALA) required for heme biosynthesis. In the absence of *hemL*, a small amount of 5-ALA is produced spontaneously from glutamate-1-semialdehyde resulting in reduced heme production (Hansson *et al*., 1991). As in *B. subtilis*, BAS4358 is the last gene in the heme biosynthetic cluster. Two different insertions into BAS4358 were isolated and both insertions resulted in increased *pagA* expression.

BAS1384 (GenBank: AAT53704.1) encoding a protein with 62% amino acid identity with the product of the *B. subtilis resB* gene (GenBank: AAA67495.1). ResB is an integral membrane protein that is required for cytochrome *c* assembly and maturation (Sun *et al*., 1996;Le Brun *et al*., 2000). ResB is similar to other System II cytochrome *c* maturation proteins, such as the Ccs1 protein of *Chlamydomonas reinhardtii* and CcsB of *Bordetella pertussis*, and likely functions in a complex with ResC to export and process heme for extracellular attachment to cytochrome *c* (Beckett *et al*., 2000;Hamel *et al*., 2003). Two independent insertions into BAS1384 were isolated occurring at two unique locations, and both insertions resulted in increased *pagA* expression. As in *B. subtilis*, BAS1384 is located between the genes *resA* and *resC*.

BAS1385 (GenBank: AAT53705.1) encoding a protein with 71% amino acid identity to the product of the *B. subtilis resC* gene (GenBank: CAB14245.1). ResC, like ResB, is an integral membrane protein similar to other System II cytochrome *c* maturation proteins (Le Brun *et al*., 2000). As in *B. subtilis*, BAS1385 is located downstream of the genes annotated as *resA* and *resB*.

BAS3568 (GenBank: AAT55872.1) encoding a predicted membrane protein with four transmembrane helices containing the PFam DUF420 domain. This gene is well conserved among many Gram-positive bacteria, and its closest ortholog in *B. subtilis* is the uncharacterized *yozB* gene (amino acid identity 52.4%, similarity 69.8%).

Quantitation of expression in these transposon mutant strains by means of β-galactosidase assays showed that the rate of induction of *pagA* was 4 fold higher in the *resB*, *resC*, and BAS3568 mutants and 2.5 fold higher in the *hemL* mutant compared to the parental strain while the growth rate was unaffected (Fig. 1A and B).

The clustering of transposon mutations deregulating *pagA* expression in genes involved in heme synthesis and cytochrome *c* maturation strongly suggested a role for respiration in environmental sensing and toxin production.

Heme-defective mutants overexpress *atxA* during exponential growth

Experiments were undertaken to distinguish whether the transposon mutants affected *pagA* expression directly or were influencing the global virulence regulator AtxA required for toxin gene expression in *B. anthracis* (Koehler *et al*., 1994;Uchida *et al*., 1993). To

investigate the effect of each transposon mutation on *atxA* transcription, the mutant strains were cured of the pTCV*lac-pagA* plasmid and re-transformed with the pTCV*lac-atxA* reporter plasmid, resulting in strains expressing β-galactosidase from the *atxA* promoter. Results of a time course experiment monitoring *atxA* expression in the transposon mutants and parental strain grown in LB broth containing kanamycin are shown in Figure 1C. The parental strain expressed *atxA* at low level in early exponential phase and an increase in expression occurred at mid exponential phase (Dai *et al*., 1995). The *resB* (34F2tB21), *resC* (34F2tB18), *hemL* (34F2tB3) and BAS3568 (34F2tB23) transposon mutants expressed *atxA* at very high levels during early exponential growth but expression dropped to near parental levels once the cells reached stationary phase. To the best of our knowledge, this pattern of exponential phase deregulation of *atxA* transcription followed by a return to normal expression levels in stationary phase has not previously been observed and is unique to these mutants.

Characterization of markerless, in-frame deletion mutants

The genetic characterization of transposon-generated mutants could be affected by stability or polarity issues that may interfere with the interpretation of the results. Thus, strains containing markerless deletions of transposon-identified genes were constructed to confirm the *pagA*- and *atxA*-overexpression phenotypes identified with the transposon mutants. All deletion strains were produced using a modification of the method of Janes and Stibitz (Janes and Stibitz, 2006). This method results in a markerless, in-frame deletion of the gene of interest, thereby reducing the possibility of polar effects on the expression of downstream genes that can be associated with transposon insertions.

A markerless deletion of *B. anthracis* BAS1384, resulting in strain 34F2Δ*resB*, was transformed with the pTCV*lac-pagA* or pTCV*lac-atxA* plasmids to test the effect of *resB* deletion on *pagA* and *atxA* expression. Unlike the transposon mutant 34F2 tB21(Figure 1A), the markerless deletion mutant 34F2Δ*resB* showed a modest growth defect relative to the parental strain (Figure 2A). 34F2Δ*resB* also expressed *pagA* at a 4 fold higher rate than the parental strain after induction at the transition phase (Figure 2B) and approximately 10-fold more *atxA* than the parental strain during early exponential phase (Figure 2C). Consistently, the AtxA protein was overexpressed in the 34F2Δ*resB* strain during exponential phase as shown by Western Blot analysis (Fig. 2C and Fig. S2). These data confirmed the contribution of *resB* to the *atxA* and *pagA* overexpression phenotypes observed in the transposon mutants.

Transposon insertions obtained in both *resB* and *resC* resulted in similar levels of *pagA* and *atxA* overexpression. By analogy to the similar cytochrome *c* maturation proteins in *B. subtilis*, expression of both ResB and ResC in *B. anthracis* is most likely required to form an active heme delivery complex and ResC expressed in the absence of ResB should be inactive (Hamel *et al*., 2003). To test for the effect of *resC* deletion in *B. anthracis*, a markerless *resBC* deletion strain was constructed that completely deleted the coding regions of both BAS1384 and BAS1385. 34F2Δ*resBC* grew similarly to the 34F2Δ*resB* strain, both growing to a final density less than the parental strain (Figure 2A). The expression of *pagA* (Fig. 2B) and *atxA* (Fig. 2C) was identical to the 34F2Δ*resB* single deletion strain, demonstrating that deletion of *resBC* had no additional effect over deletion of *resB* alone.

A strain containing a deletion of *resA*, a gene in the *resABC* operon encoding a thiol-disulfide oxidoreductase involved in cytochrome *c* maturation (Erlendsson *et al*., 2003), was also constructed (34F2Δ*resA*). When compared to 34F2Δ*resB*, this strain showed a similar pattern of *atxA*- and *pagA*-overexpression (data not shown), further indicating that disruption of cytochrome *c* maturation contributes to the observed virulence expression phenotypes.

The markerless deletion of *hemL* (BAS4358) resulted in increased *pagA* expression during stationary phase (Figure 2B) and increased *atxA* expression during early exponential growth (Figure 2C), similar to the transposon insertion mutants isolated in *hemL*. Though the pattern of overexpression in 34F2Δ*hemL* was similar to 34F2Δ*resB*, the level of β-galactosidase expression was lower in the 34F2Δ*hemL*, consistent with a reduction, but not elimination, of heme production (Hansson *et al*., 1991).

The analysis of markerless, in-frame deleted strains confirmed the original phenotypes of the *resB, resC,* and *hemL* transposon-generated mutants and indicated they were indeed the result of loss-of-function mutations.

Activity of cytochrome *c* oxidase in the transposon mutants affected in toxin gene expression

The *B. anthracis* orthologs of *B. subtilis* heme synthesis and cytochrome *c* maturation proteins identified in our transposon mutagenesis screen had not been studied previously, so it was essential to demonstrate that these gene products contribute to cytochrome *c* activity in this organism. A simple test for cytochrome *c* oxidase activity involves N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD) staining of *B. anthracis* colonies grown on solid media plates. 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* oxidase in the membrane (Le Brun *et al*., 2000).

B. anthracis parental and mutant strains were grown on NSMP and visually scored for TMPD oxidase staining (Table 2 and Fig. 3). A mutant in the CtaC subunit of cytochrome *caa3* was also constructed as a negative control (see below). As expected for a subunit of cytochrome *c* oxidase, disruption of *ctaC* (BAS3856) resulted in no TMPD oxidase staining, demonstrating a lack of cytochrome *c* oxidase activity. The *resB* (BAS1384) and *resC* (BAS1385) mutants also did not produce active cytochrome *c* oxidase, consistent with observations of orthologous gene disruptions in *B. subtilis* (Le Brun *et al*., 2000) (Table 2). The *hemL* (BAS4358) mutant showed a reduction in TMPD oxidase staining indicative of reduced, rather than abolished, cytochrome *c* oxidase activity, consistent with *B. subtilis* observations that heme production occurs at reduced levels in the absence of *hemL* (Hansson *et al*., 1991) (Table 2 and Fig. 3). As heme is required for cytochrome *c* oxidase activity, a reduction in cellular levels of the heme precursor 5-aminolevulinic acid (5-ALA) (Hansson *et al*., 1991) in the absence of *hemL* would result in reduced TMPD oxidase staining. Consequently, complementation of the oxidase defect and *pagA* overexpression phenotype in the *hemL* mutant was obtained by the addition of 5-ALA to the medium, while no complementation was observed for the 34F2Δ*ctaC* mutant (Fig. 3 and Fig. S1). These results confirmed that the *hemL*, *resB*, and *resC* genes of *B. anthracis* are required for cytochrome c maturation and their deletion results in toxin gene overexpression.

Increased *pagA* expression is AtxA-dependent

Inactivation of *hemL*, *resB*, or *resC* resulted in increased transcription of both *pagA* and *atxA* (Figs. 1 and 2). Because AtxA is required to activate transcription of *pagA* (Koehler *et al*., 1994;Uchida *et al*., 1993), increased *atxA* transcription in these mutant strains may directly lead to increased *pagA* transcription. However, it was reported that when AtxA production was increased by expressing the gene from a multi-copy plasmid, *pagA* expression actually decreased (Dai and Koehler, 1997), indicating that, under some conditions, increased AtxA expression did not lead directly to increased *pagA* expression.

To evaluate the role of AtxA in *pagA* overexpression in these mutants, a markerless deletion of *resB* was made in a strain containing an insertional inactivation of the *atxA* gene (Tsvetanova *et al*., 2007). This strain was transformed with the *pagA-lacZ* and *atxA-lacZ* reporter plasmids, and *pagA* and *atxA* expression was compared to the parental and single deletion strains. Deletion of *atxA* alone resulted in decreased *pagA* expression relative to the parental strain (Figure 4A) but did not significantly effect *atxA* expression (Figure 4B). The *resB-atxA* double deletion strain expressed *pagA* at reduced levels similar to the *atxA* deletion alone (Figure 4A), suggesting that AtxA is required for *pagA* overexpression in the *resB* mutant. Moreover, the *resB-atxA* double deletion strain expressed *atxA* at increased levels during exponential growth similar to the *resB* deletion alone (Figure 4B), indicating that AtxA is not required for its own overexpression in the *resB* mutant.

resB mutant effect on AtxA

It was important to determine whether the *resB*-dependent induction of *pagA* expression was dependent on *atxA* induced expression or whether it depended on an effect on AtxA activity. To test these possibilities, the *atxA* coding gene was placed under control of a non-inducible (constitutively expressed) *spac* promoter in a modified pTCV-lac plasmid (see Experimental Procedures). This construct was then transformed in a strain carrying a *pagA-lacZ* reporter fusion integrated isotopically in the pX01 plasmid of strains 34F2 Δ*atxA* and 34F2Δ*atxA*Δ*resB* (see Experimental Procedures). Transcription analysis by β-galactosidase assays (Fig. 4C) indicated that expression of AtxA from the *spac* promoter complemented the *atxA* deletion on pX01, but did not result in *pagA* overexpression in the absence of *resB* compared to the parental strain. These results indicated that the *resB*-dependent induction of *pagA* appears solely due to the induction of *atxA* transcription and not to a modification of its activity.

Because *atxA* is transcribed from two independent promoters, we determined which promoter(s) was affected in the *resB* mutant. Transcriptional analysis was carried out using *atxA-lacZ* reporter constructs carrying only the proximal P1 promoter (pAtxA10), both the proximal and distal P1 and P2 promoters (pAtxA12) or only the distal P2 promoter (pAtxA20) (Bongiorni *et al*., 2008). The results shown in Fig. 4C indicated that induction of *atxA* transcription in early exponential phase in the *resB* mutant occurred only at the proximal P1 promoter.

These results showed that the induction of *pagA* expression resulting from a deletion of *resB* was solely the consequence of deregulated *atxA* expression occurring at the P1 promoter and did not involve regulation of AtxA activity.

Inactivation of two *B. anthracis* small *c*-type cytochromes is required for *atxA*-overexpression

In *B. subtilis*, 5 membrane-bound proteins with covalently bound heme have been identified: the 39 kDa subunit II of cytochrome *caa*3 (CtaC), the 28 kDa cytochrome *c* of the *bc* complex (QcrC), the 25 kDa cytochrome *b* subunit of the cytochrome *bc* complex (QcrB), the 13 kDa cytochrome *c*550 (CccA), and the 10 kDa cytochrome *c*551 (CccB) (von Wachenfeldt and Hederstedt, 1990;Bengtsson *et al*., 1999). ResBC is required for synthesis of all of these cytochromes except the 25 kDa cytochrome *b* subunit of the cytochrome *bc* complex (QcrB) (von Wachenfeldt and Hederstedt, 2002). By BlastP analysis we have identified *B. anthracis* orthologs of all 5 *B. subtilis* membrane-bound proteins with covalently bound heme. BAS3856 (AAT56157) shares 53% amino acid identity with CtaC (NP_389372), BAS1433 (AAT53753) shares 94% amino acid identity with QcrB (NP_390136), BAS1434 (AAT53754) shares 71% amino acid identity with QcrC (NP_390135), BAS4193 (AAT56492) shares 46% amino acid identity with CccA

(NP_390398), and BAS5035 (AAT57324) shares 41% amino acid identity with CccB (NP_390135).

To investigate the roles of these proteins in virulence gene regulation, *B. anthracis* 34F2 derivative strains were generated containing markerless deletions of each gene (henceforth, each *B. anthracis* deletion strain will be annotated after the orthologous gene in *B. subtilis*). Heme attachment to QcrB does not appear to be ResBC-dependent, but given the paucity of information of QcrB and QcrC function and their operon organization on the chromosome, both coding genes were deleted in a single strain (34F2Δ*qcrBC*). Single deletion strains showed no significant growth defect and only the 34F2Δ*ctaC* strain was deficient in TMPD-oxidase staining (Fig 3 and data not shown). When transformed with the *lacZ* reporter plasmids, none of the single deletion strains overexpressed *pagA* or *atxA* relative to the parental strain (Figure 5).

Exploiting the advantages of the markerless deletion method, a single strain containing deletions of all 5 cytochrome genes (34F2Δ5*cyt*) was generated. This strain, unlike the single deletion strains, overexpressed *atxA* and *pagA* in a pattern identical to the 34F2Δ*resB* strain, suggesting that loss of multiple cytochromes is required for deregulation of *atxA* and *pagA* expression (Fig. 5).

The small *c*-type cytochromes CccA and CccB share 58% amino acid similarity overall, with the extracellular heme-attachment domains being the most conserved region, (Fig. S3), hinting that these two proteins may functionally overlap. A double deletion strain, *cccA cccB* (34F2Δ*cccA-B*) fully mimicked the *pagA*- and *atxA*-overexpression phenotype of both the 34F2Δ*resB* and 34F2Δ5*cyt* strains. While loss of either *cccA* or *cccB* did not affect toxin gene expression, the deletion of both genes simultaneously was necessary to deregulate *pagA* and *atxA*, suggesting that CccA and CccB can functionally complement one another.

A previously uncharacterized transmembrane protein contributes to regulation of *atxA* and *pagA* expression

One additional strain, 34F2tB23, was identified which overexpressed *pagA* in stationary phase and overexpressed *atxA* in exponential phase (Table 2 and Figure 1). This strain was partially deficient in TMPD-oxidase staining, suggesting a deficiency in cytochrome *c* oxidase function (data not shown). The transposon insertion in strain 34F2tB23 disrupted the BAS3568 gene. A markerless deletion strain (34F2ΔBAS3568) retained the *atxA*- and *pagA*-overexpression phenotype and reduction of TMPD-oxidase staining of the transposon mutant strain (Fig. 6 and data not shown), demonstrating that disruption of BAS3568 is responsible for the observed phenotypes. As with a *resB-atxA* double mutant strain, a BAS3568-*atxA* double disruption strain expressed *pagA* at levels identical to the *atxA* disruption strain (data not shown), which indicated that *pagA* overexpression in the BAS3568 strain is also *atxA*-dependent. Deletion of both BAS3568 and *resB* resulted in overexpression of *pagA* and *atxA* at levels similar to either of the single deletion strains alone (Fig. 6). The lack of an additive effect of *resB* and BAS3568 deletions suggests that both protein products function in the same pathway. Thus, the product of the BAS3568 gene is a newly identified component required for full cytochrome *c* activity whose function remains to be determined.

Bicarbonate induction of toxin expression eliminates the cytochrome-deficient phenotype

The initial transposon mutagenesis screen and subsequent analyses were performed on strains grown in the absence of added bicarbonate or CO_2, conditions which do not induce high level of toxin gene expression.

To investigate the role of this CccA-CccB newly identified heme-dependent regulatory pathway during bicarbonate-induced toxin induction, *pagA* and *atxA* expression levels were monitored in 34F2 and 34F2Δ*resB* strains grown in R-Media (Ristroph and Ivins, 1983) both with and without added bicarbonate. As shown in Figure 7, when grown in R-Media without added bicarbonate at 37° under 5% atmospheric CO_2, the 34F2Δ*resB* strain overexpressed *pagA* relative to the parental strain. The timing and extent of *pagA* overexpression was different from what was observed in strains grown in LB broth under air as the overexpression began much earlier and the extent of overexpression was reduced (approximately 2-fold in R-Media without $NaHCO_3$ versus approximately 4-fold in LB-broth). In the parental strain, expression of *pagA* was increased and expression was induced earlier in R-Media without bicarbonate, compared to growth in LB. When these strains were grown in R-Media with added 0.8% $NaHCO_3$, *pagA* expression was strongly induced, as expected, increasing almost 15-fold over the parental strain grown in the absence of added $NaHCO_3$. With bicarbonate induction, however, there was no difference in expression of *pagA* between the parental and *resB* mutant strains.

The expression of *atxA* in 34F2ΔresB was elevated early in R-Media without bicarbonate, similar to the expression pattern seen in LB broth in air. However, early overexpression of *atxA* in the 34F2Δ*resB* strain was lost when cells were grown in the presence of bicarbonate. Expression of *atxA* is not induced by bicarbonate (Dai and Koehler, 1997;Bongiorni *et al*., 2008), but these expression data clearly demonstrated that addition of bicarbonate to growth media eliminates the *atxA* overexpression phenotype of a *resB* mutant.

Discussion

B. anthracis regulates virulence gene expression by recognizing signals from the host environment. The mechanistic basis of *in vivo* and *ex vivo* toxin induction by elevated temperature (Dai and Koehler, 1997) and bicarbonate (Wilson *et al*., 2008b;Bartkus and Leppla, 1989) have become clearer in recent years, though much remains unknown about the connections between signals and regulatory pathways. While induction of virulence gene expression is vital to pathogenesis, repression of virulence gene expression may also be important for survival and replication of the organism in other conditions. In this work, we investigated a novel regulatory pathway that represses virulence gene expression under *ex vivo* non-host conditions.

This study has revealed a specific function in virulence regulation for two small highly conserved *c*-type cytochromes, CccA and CccB, in the *Bacillus* genus. Deletion of the genes for CccA and CccB of *B. anthracis* resulted in a transient deregulation of expression of the gene for the AtxA virulence regulator in the early exponential phase of growth. In turn, this deregulation gave rise to an increased rate of toxin gene expression (*pagA*). Increased expression was not limited to toxin genes but extended to other AtxA-regulated genes such as pX02-61 (Bourgogne *et al*., 2003;White *et al*., 2006) and was also detected when cells were grown at 25°C (Dai and Koehler, 1997) (data not shown). Moreover, the study identified a new putative component of the cytochrome *c* maturation pathway in the product of the BAS3568 gene which is well conserved among Gram-positive bacteria but whose function has remained unknown until now (Fig. 8).

Little is known about cytochromes and their maturation in *B. anthracis but* cytochrome *c* maturation systems (ccm) have been studied in other organisms. Production of cytochrome *c* has recently been shown to contribute to a variety of other processes in specific bacteria, for example iron acquisition in *Rhizobium leguminosarum* (Yeoman *et al*., 1997) and virulence in *Legionella pneumophila* (Naylor and Cianciotto, 2004). Ccm systems in Gram-positive

bacteria are distinct from those found in Gram-negative bacteria and more closely resemble the ccm systems in chloroplasts and cyanobacteria (Kranz *et al*., 1998). In *B. subtilis*, apo-cytochrome *c* is produced inside the cell and exported to the extracellular face of the cytoplasmic membrane (Fig. S4) (Crow *et al*., 2005). Once at the extracellular surface, the ResA, CcdA, and BdbD proteins cooperate to maintain the two cysteine residues in the CXXCH heme-attachment motif in the reduced state (Schiött *et al*., 1997;Erlendsson and Hederstedt, 2002). ResB and ResC likely cooperate to transport heme across the cytoplasmic membrane and attach the heme via two thioester bonds to reduced apo-cytochrome *c* to generate the mature cytochrome *c* (Le Brun *et al*., 2000).

CccA and CccB are similar in the amino acid sequence of their cytochrome c domains (Fig. S3) but differ in their membrane anchoring systems: CccA has a predicted α-helical transmembrane polypeptide membrane anchor while CccB is membrane attached by a diacylglycerol membrane anchor (Bengtsson *et al*., 1999). The reason for conservation of two highly similar small *c*-type cytochromes with no known function in *Bacillus* species when such arrangements are uncommon in other bacteria is still a mystery. In *B. anthracis*, the two cytochromes are clearly involved in a signaling pathway to virulence gene expression but their precise function is still unknown (Fig. 8). As is typical for *c*-type cytochromes, the function of CccA and CccB is most likely redox-related and associated with electron transfer (von Wachenfeldt and Hederstedt, 2002), but they are not required for aerobic respiration as growth is not affected. Deletion of both genes is required for *atxA* overexpression, suggesting one cytochrome can compensate for the loss of the other for this pathway. None of the remaining *c*-type cytochromes (CtaC and QcrBC) are involved, on an individual basis, in the regulation of *atxA* expression indicating a specific participation of CccA and CccB in virulence gene expression, at least, in *B. anthracis*.

The activity of CccA and CccB on virulence gene expression requires heme and its covalent attachment to these two cytochromes as indicated by the requirement for HemL and ResABC to regulated *atxA* expression in the early exponential phase of growth. A deletion of the *hemL* gene only partially affected cytochrome *c* oxidase activity but deregulated *atxA* expression suggesting that in conditions of low heme availability (as in the case of an *hemL* mutation) the CccA and CccB cytochromes may not have the highest affinity for the enzymes, presumably ResBC, carrying out the heme binding reaction to the apo-cytochromes. Complementation of the *hemL* deficiency by the 5-ALA intermediate in heme biosynthesis fully complemented the mutation, confirming the requirement of heme for the cytochrome activity on *atxA* transcription. The transient deregulation of *atxA* expression in the *resBC* mutants also confirmed that not only heme but also its presentation and covalent attachment to the cytochromes are necessary to regulate virulence gene transcription.

Our results also revealed that the protein encoded by BAS3568 is involved in cytochrome *c* maturation and, consequently, virulence regulation. BAS3568 is required for full cytochrome *c* oxidase function as its deletion resulted in partial loss of TMPD-oxidase activity. The BAS3568 product likely functions more generally in cytochrome *c* maturation as both cytochrome *c* oxidase and virulence expression were disrupted when the gene was deleted, indicative of loss of caa_3, c_{550} and c_{551} activity. As *atxA*- and *pagA*-overexpression levels were lower than in the *resB* mutant and a very low level of TMPD-oxidase staining was maintained, loss of BAS3568 strongly reduced but did not completely eliminate cytochrome *c* maturation. The role of the BAS3568 ortholog *yozB* in *B. subtilis* is unknown but our results may reveal much more about the function of this previously uncharacterized protein.

In *B. subtilis*, the ResDE two-component signal transduction system regulates a number of genes involved in aerobic and anaerobic respiration, including the *resABC* operon (Sun *et*

al., 1996). Deletion of *resDE*, in our hands, had no effect on either *pagA* or *atxA* transcription, demonstrating that this two-component regulatory system plays no role in the phenotype observed for *resB* deletion or in regulation of toxin expression (Wilson *et al*., 2008a;Vetter and Schlievert, 2007). Either the ResDE regulatory pathway in *B. anthracis* differs from *B. subtilis* or *resABC* expression in the absence of the two-component system is sufficient for maturation of the CccA and/or CccB in the growth conditions tested.

The question arising from this work is the mechanism by which elevated early exponential phase transcription of *atxA* leads to elevated stationary phase transcription of *pagA*. When *atxA* was expressed in its normal context from a multi-copy plasmid, overexpression of AtxA led to decreased *pagA* expression (Koehler *et al*., 1994;Dai and Koehler, 1997). In contrast, overexpression of *atxA* early in exponential phase induced by cytochrome *c* deficiency led to increased *pagA* expression but not changes in the timing of *pagA* expression. Also, increased *atxA* expression in an *abrB* mutant resulted in increased *pagA* expression throughout the growth cycle although the transcription patterns are significantly different from the ones observed in the cytochrome pathway deletion mutants (Fig. S5) (Saile and Koehler, 2002). These observations suggest that the timing or context of AtxA expression, not necessarily the total amount of AtxA produced, influences the regulatory activity of AtxA. AtxA produced at different stages of growth may become differentially modified in a way that influences activity, perhaps via phosphorylation/dephosphorylation of the histidine residues we have previously investigated (Tsvetanova *et al*., 2007). Notably, a *resB* mutation results in overexpression of *pagA* even in the presence of the AtxA H199D and H379A mutant proteins confirming that the cytochrome *c* deficiency only affects *atxA* gene expression (data not shown).

The response to bicarbonate is clearly essential to *B. anthracis* virulence regulation and the present results indicate that bicarbonate signals both activation and repression of toxin expression. The bicarbonate activation pathway, which relies on bicarbonate import through an ABC transporter (Wilson *et al*., 2008b), eliminated the *atxA* and *pagA* overexpression phenotype of the *resB* mutant, suggesting that the bicarbonate activation pathway completely overrides the regulatory pathway exerted by the CccA-B cytochromes. The transient nature of the overexpression phenotype resulting from the absence of the CccA and CccB cytochromes potentially suggests the involvement of an inducer of transcription which the bicarbonate response would neutralize. Alternatively, increased AtxA expression may induce a gene that feeds back to repress AtxA. However, the involvement of multiple negative regulators cannot be ruled out at this time. Further, activation requires the intracellular presence of bicarbonate (Wilson *et al*., 2008b) while these data suggest the involvement of extracellular signal(s) propagated by the two cytochromes, indicating *B. anthracis* can regulate the response to bicarbonate from both intra- and extra-cellular signals.

The pathway of virulence gene expression regulation identified by this study adds another level of control to the already complex AtxA regulatory circuit. AtxA plays an important role in the induction of *pagA* expression, but transcription of *atxA* is not induced by bicarbonate (Dai and Koehler, 1997;Bongiorni *et al*., 2008;Wilson *et al*., 2008b). Conversely, repression of *pagA* in the cytochrome *c*-dependent pathway and non-host conditions does operate through regulation of *atxA* transcription.

Our findings highlight the role of integrated metabolic systems in directing the expression of virulence genes. Rather than commit to a dedicated and metabolically expensive virulence regulatory system, many bacteria use or enhance existing metabolic pathways that already sense changes in the environment to regulate expression of genes required for growth in the host environment. There are already several examples of metabolic integration of virulence

regulation in *B. anthracis*, beyond the new system presented here, such as the connection of toxin expression to the sporulation phosphorelay through AbrB (Saile and Koehler, 2002), regulation of toxin in response to bicarbonate import (Wilson *et al*., 2008b), and the influence of the histidine phosphorylation on AtxA activity (Tsvetanova *et al*., 2007). The mechanism connecting cytochrome *c* activity to virulence regulation is an on-going topic of investigation, but these studies could prove valuable in the study of other Gram-positive pathogenic bacteria that share conserved regulatory circuits.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

The *B. anthracis* strains used in this study are listed in Table 1. *B. anthracis* Sterne 34F2 ($pXO1^+$ $pXO2^-$) and its derivatives were routinely grown in LB broth supplemented with the appropriate antibiotics at the following concentrations: spectinomycin (100 μg/ml), chloramphenicol (7.5 μg/ml), tetracycline (5 μg/ml), or 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, 5 μg/ml 5-aminolevulinic acid (5-ALA) (Sigma) was added to growth media. To induce high-level toxin expression, LB-agar plates or LB liquid media containing 0.8% sodium bicarbonate and 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.0 were incubated in a 5% CO_2 atmosphere. As indicated, *B. anthracis* was grown in R-Media with or without 0.8% $NaHCO_3$ (Ristroph and Ivins, 1983) under 5% CO_2. Competent cells of *B. anthracis* were prepared following the method of Koehler *et al* (Koehler *et al*., 1994).

E. coli K-12 TG1, SCS110 and DH5α competent cells were used for the propagation and isolation of all plasmid constructs. *E. coli* transformation was performed by electroporation using the Bio-Rad-Gene Pulser according to the supplier. Transformants were selected on LB broth supplemented with ampicillin (100 μg/ml), spectinomycin (100 μg/ml), chloramphenicol (10 μg/ml), or kanamycin (30 μg/ml).

Plasmid pTCVlac-*pagA* is a derivative of the *pagA-lacZ* reporter plasmid described in Tsvetanova et al (Tsvetanova *et al*., 2007) obtained by cloning the 580 bp EcoRI-BamHI fragment from pJM115 into pTCVlac (Poyart and Trieu-Cuot, 1997). Plasmid pTCVSpac-AtxA is a derivative of pTCV-lac (Poyart and Trieu-Cuot, 1997) carrying the *spac* promoter from pMutin2 (Vagner *et al*., 1998), the *atxA* coding region and a deletion of the *lacZ* gene. The lack of the *lacI* gene results in constitutive expression of the *spac* promoter once this plasmid is introduced in *B. anthracis. The* details of its construction will be described elsewhere. The construction of plasmids pAtxA10, pAtxA12, and pAtxA20 was described in Bongiorni et al (Bongiorni *et al*., 2008). Plasmid pORI-pagA-lacZ is a derivative of pORI-Cm (Brunsing *et al*., 2005) carrying the *pagA-lacZ* fragment from pTCV-lac-*pagA*. The details of its construction will be described elsewhere.

Transposon mutagenesis

The temperature sensitive mini-Tn10 transposon delivery plasmid pAW016 (Wilson *et al*., 2007) was electroporated into the *B. anthracis* 34F2 strain containing the replicative vector pTCV*lac-pagA* (Table S1) and plated on LB with kanamycin, spectinomycin, and chloramphenicol. Resulting transformants were screened for lack of premature transposition and for plasmid loss at non-permissive temperature as previously described (Wilson *et al*., 2007). Plasmid-containing strains were used to inoculate LB broth containing kanamycin and spectinomycin, and cultures were incubated overnight at the permissive temperature of 28°C. The following morning, the overnight cultures were diluted 1:1000 in fresh LB broth with kanamycin and spectinomycin and incubated 6 hours at the non-permissive temperature

of 37°C. Cultures were then serially diluted, the dilutions plated on LB-agar containing kanamycin, spectinomycin, and X-Gal, and the plates incubated overnight at 37°C. The following day, the spectinomycin- and kanamycin-resistant colonies were screened for alterations in *pagA-lacZ* expression. Mutants that showed a significant alteration in color were re-streaked on LB-agar with kanamycin, spectinomycin, and X-Gal to confirm phenotype, and on LB-agar with chloramphenicol to confirm loss of the transposon delivery plasmid. Mutants that retained their *pagA-lacZ* expression phenotype and were unable to grow on chloramphenicol were retained for further analysis.

Transposon analysis and sequencing

Spectinomycin-resistant and chloramphenicol-sensitive 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 a panel of restriction enzymes, including SalI, NsiI, EcoRI, and SacI. Digested genomic DNA was then re-ligated and used to transform *E. coli*. The presence of the pUC origin of replication allowed 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 the transposon-specific primer TSP3E (Table S2). Genomic DNA was screened for retention of virulence plasmid pXO1 by PCR amplification using the *atxA*-specific primer set AtxA5′promEco and AtxA3′Bam (Table S2).

β-Galactosidase assays

B. anthracis strains harboring the *pagA-lacZ* (Tsvetanova *et al*., 2007) or *atxA-lacZ* (pAtxA12) (Bongiorni *et al*., 2008) fusions on the replicative vector pTCV-lac (Poyart and Trieu-Cuot, 1997) were grown at 37°C in LB or R medium supplemented with the appropriate antibiotics. β-galactosidase activity was assayed as described previously and specific activity was expressed in Miller units (Miller, 1972;Wilson *et al*., 2008a).

TMPD Oxidase Staining

N,N,N’,N’-tetramethyl-*p*-phenylenediamine (TMPD) is an artificial electron donor that can be oxidized by cytochrome caa_3, resulting in a colorimetric change indicative of the presence of functional cytochrome *c* oxidase. TMPD oxidase staining was performed with *B. anthracis* colonies grown on NSMP agar plates (nutrient sporulation medium phosphate) (Fortnagel and Freese, 1968) as previously described (Le Brun *et al*., 2000).

Markerless Gene Deletion

Gene deletions in *B. anthracis* were generated through a modification of the technique of Janes and Stibitz (Janes and Stibitz, 2006). Regions upstream and downstream of the gene to be deleted were cloned in the temperature sensitive plasmid pORI-I-SceI (Bongiorni *et al*., 2007) using the PCR primers listed in Table S2. The resulting plasmids (listed in Table S1) were electroporated into *B. anthracis* 34F2 and grown at the permissive temperature of 28°C in the presence of chloramphenicol. Bacteria were then shifted to the non-permissive temperature of 37°C in the presence of chloramphenicol to achieve targeted plasmid integration by homologous recombination. Following plasmid integration, the protocol of Janes and Stibitz (Janes and Stibitz, 2006) was followed to generate the markerless deletion. Diagnostic PCR was carried out to ensure that the entire coding sequence had been correctly deleted and that plasmid pXO1 was retained.

SDS-PAGE and Western blotting

B. anthracis strains were grown in LB Broth at 37°, and cell pellets were isolated at time points indicated by microcentrifugation of cell suspensions. Cell pellets were lysed following resuspension in buffer (10 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 300 mM NaCl, and 10 mM 2-mercaptoethanol) by sonication for 20 seconds for 3 cycles and then centrifuged. SDS sample buffer was added to each supernatant, and samples were boiled for 5 minutes and loaded on 10% SDS-PAGE gels. The amount loaded was normalized relative to cell growth. The gels were run at 30mA for approximately 2 hr. The proteins in the gel were transferred to a PVDF membrane (BioRad) in transfer buffer (25mM Tris base, 192mM 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 α-AtxA antibody diluted 1:5,000. The blots were washed 5 times in TBST 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 PhosphorImager analysis (Molecular Dynamics).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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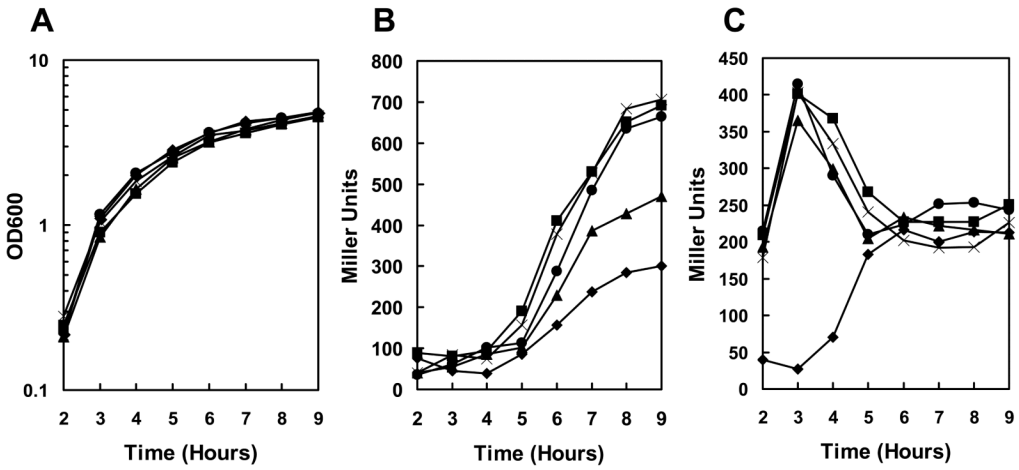

Figure 1.

Transcription analysis of *pagA* and *atxA* expression in transposon mutant strains. Strains carrying a *pagA-lacZ* or *atxA-lacZ* fusion on the replicative vector pTCV-*lac* were grown in LB broth supplemented with kanamycin at 37°C. β-galactosidase assays were carried out on samples taken at hourly intervals as indicated. **A.** Cell growth of *pagA-lacZ* reporter strains (cell growth of *atxA-lacZ* reporter strains was similar) **B.** β-galactosidase activity of *pagA-lacZ* reporter strains. **C.** β-galactosidase activity of *atxA-lacZ* reporter strains. Symbols in all three panels: -◆- 34F2; -▪- 34F2tB18 (*resC*); -X- 34F2tB21 (*resB*); -●- 34F2tB23 (BAS3568); -▲- 34F2tB24 (*hemL*).

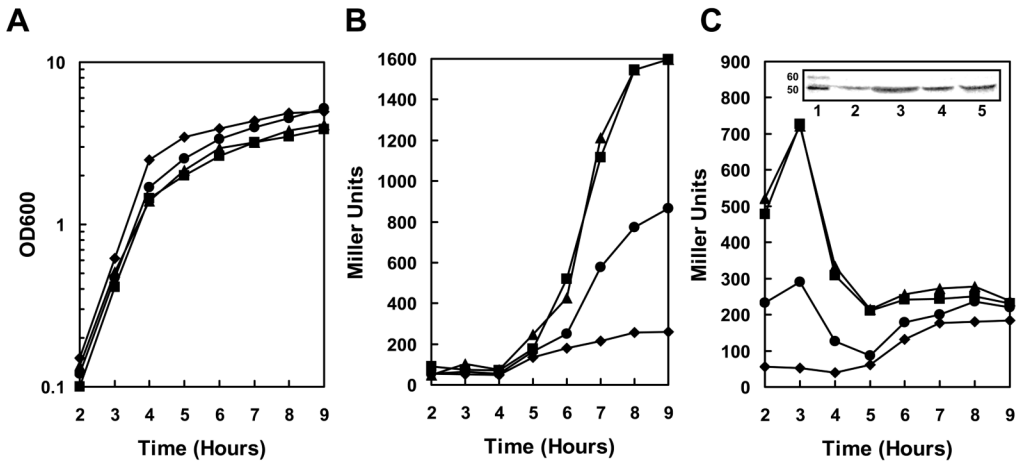

Figure 2.

Transcription analysis of *pagA* and *atxA* expression in *resB, resBC* and *hemL* mutant strains. Strains carrying a *pagA-lacZ* or *atxA-lacZ* fusion on the replicative vector pTCV-*lac* were grown in LB broth supplemented with kanamycin at 37°C. β-galactosidase assays were carried out on samples taken at hourly intervals as indicated. **A.** Cell growth of *pagA-lacZ* reporter strains (cell growth of *atxA-lacZ* reporter strains were similar) **B.** β-galactosidase activity of *pagA-lacZ* reporter strains. **C.** β-galactosidase activity of *atxA-lacZ* reporter strains. Symbols in all three panels: -◆- 34F2; -▪- 34F2△*resB*; -▲- 34F2△ *resBC*; -●- 34F2△*hemL*. The inset in panel C represents the Western blot analysis of AtxA on *B. anthracis* cell lysates collected after 3 and 8 hr of growth in LB Broth at 37°C. The amount of sample loaded on a 10% SDS-PAGE was normalized relative to cell growth. Lane 1: Magic Mark XP (Invitrogen); Lane 2: 34F2 after 3 hr of growth, Lane 3: 34F2△*resB* after 3 hr of growth; Lane 4: 34F2 after 8 hr of growth; Lane 5: 34F2△*resB* after 8 hr of growth. A full size of this Western blot is shown in Fig. S1.

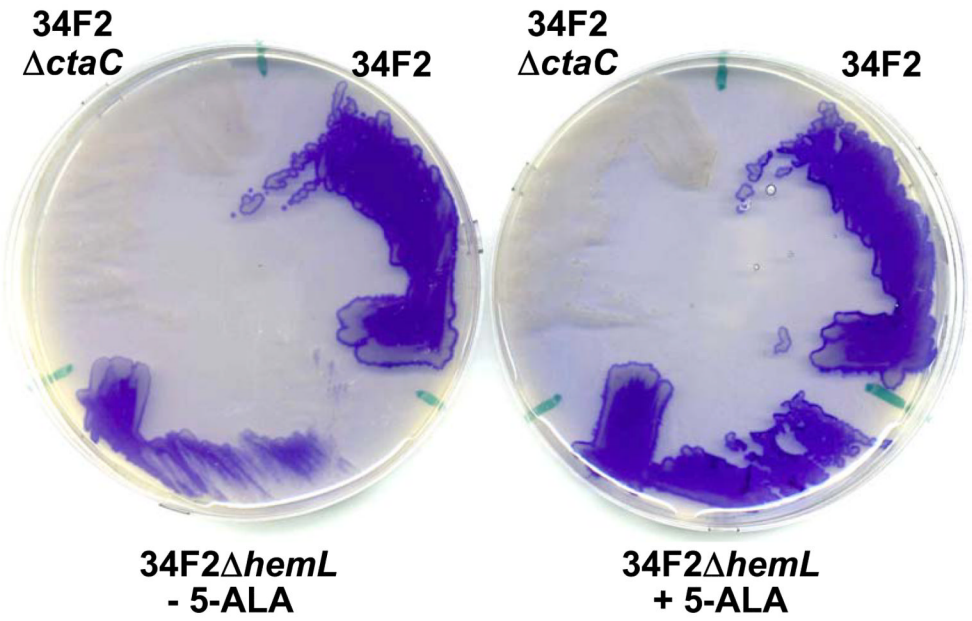

Figure 3.

Analysis of cytochrome *c* oxidase activity in *B. anthracis* parental and *hemL* mutant strains using TMPD oxidase staining. Strains were isolated on NSMP-agar plates in the absence (−) or presence (+) of 5 mg/ml 5-ALA. Upon exposure to TMPD, the staining for oxidase activity of strain 34F2 (full activity) or 34F2Δ*ctaC* (lack of activity) was not affected by the addition of 5-ALA. The partial staining of strain 34F2ΔhemL in the absence of 5-ALA was increased to the level of the parental strain when 5-ALA was present in the medium.

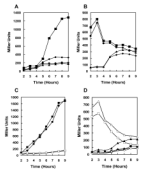

Figure 4.

Transcription analysis of *pagA* and *atxA* expression in *resB* and *atxA-resB* mutant strains. β-galactosidase assays were carried out on samples taken at hourly intervals as indicated. **A. and B.** Strains carrying a *pagA-lacZ* or *atxA-lacZ* fusion on the replicative vector pTCV-*lac* were grown in LB broth supplemented with kanamycin at 37°C. **A.** *pagA-lacZ* reporter strains. **B.** *atxA-lacZ* reporter strains. Symbols in both panels: -◆- 34F2; -▪- 34F2Δ*resB*; -▲- 34F2Δ*atxA*; -●- 34F2Δ*atxA*Δ*resB*. **C.** The 34F2Δ*atxA* strain carrying an isotopically integrated *pagA-lacZ* reporter was transformed with plasmids pTCV-lac-spac or the pTCV-spac-AtxA and β-galactosidase activity was determined. Symbols: -▪- 34F2Δ*atxA*::*pagA-lacZ*/pTCV-spac-AtxA; -□- 34F2Δ*atxA*::*pagA-lacZ*/pTCV-spac; -●- 34F2Δ*atxA*Δ*resB*::*pagA-lacZ*/pTCV-spac-AtxA; -□- 34F2Δ*atxA*Δ*resB*::*pagA-lacZ*/pTCV-spac. **D.** The *resB* deletion affects only the P1 promoter of *atxA*. The 34F2 and 34F2Δ*resB* strains were transformed with different *atxA-lacZ* fusion constructs in pTCV-lac (Bongiorni *et al*., 2008) and β-galactosidase activity was measured. Symbols: -▪- 34F2/pAtxA10; -□- 34F2Δ*resB*/pAtxA10; -▲- 34F2/pAtxA12; -△- 34F2Δ*resB*/pAtxA12; -◆- 34F2/pAtxA20; -◇- 34F2Δ*resB*/pAtxA20.

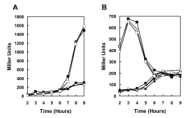

Figure 5.

Transcription analysis of *pagA* and *atxA* expression in cytochrome *c* deletion strains. Strains carrying a *pagA-lacZ* or *atxA-lacZ* fusion on the replicative vector pTCV-*lac* were grown in LB broth supplemented with kanamycin at 37°C. β-galactosidase assays were carried out on samples taken at hourly intervals as indicated. **A.** *pagA-lacZ* reporter strains. **B.** *atxA-lacZ* reporter strains. Symbols in both panels: -◆- 34F2; -▪- 34F2Δ*resB*; -▲- 34F2Δ*ctaC*; -X- 34F2Δ*qcrBC*; -○- 34F2Δ*cccA*; -●- 34F2Δ*cccB*; -△- 34F2Δ*cccA-B*; -□- 34F2Δ5*cyt*

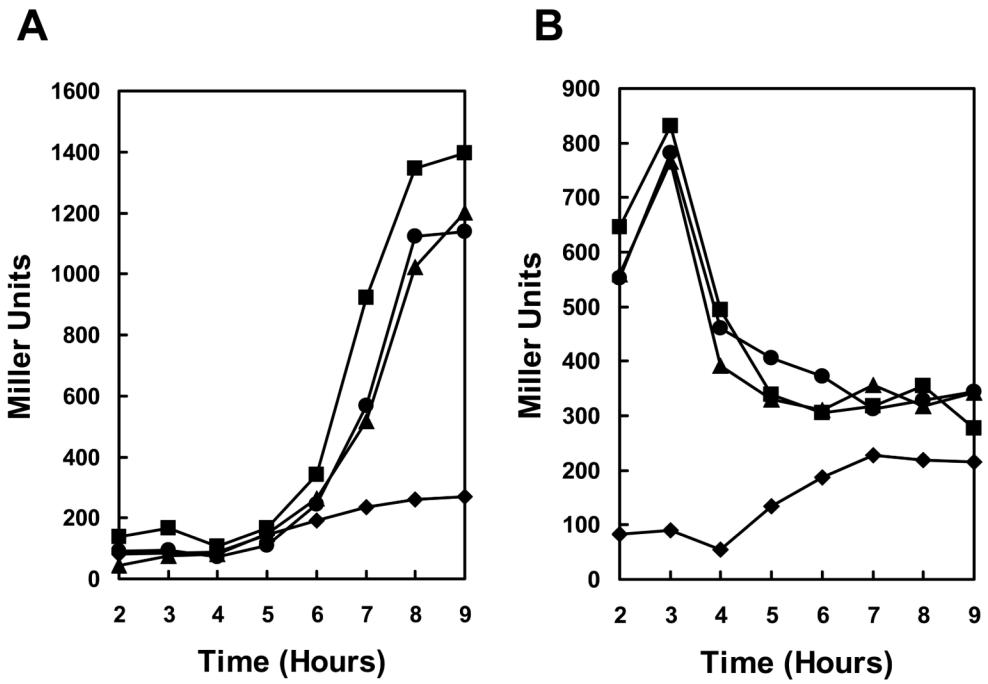

Figure 6.

Transcription of *pagA* and *atxA* in BAS3568 and *resB* mutant strains. Strains carrying a *pagA-lacZ* or *atxA-lacZ* fusion on the replicative vector pTCV-*lac* were grown in LB broth supplemented with kanamycin at 37°C. β-galactosidase assays were carried out on samples taken at hourly intervals as indicated. **A.** *pagA-lacZ* reporter strains. **B.** *atxA-lacZ* reporter strains. Symbols in both panels: -▲- 34F2; -▪- 34F2△*resB*; -▲- 34F2△BAS3568; -●- 34F2△*resB*△BAS3568

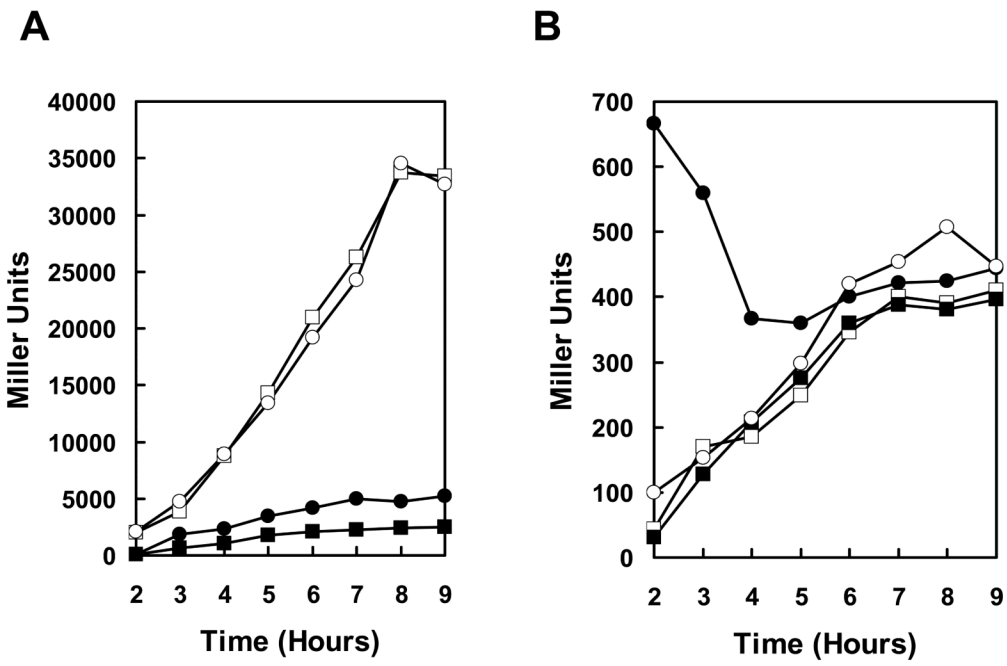

Figure 7.

Transcription analysis of *pagA* and *atxA* expression under toxin-inducing growth conditions. Strains carrying a *pagA-lacZ* or *atxA-lacZ* fusion on the replicative vector pTCV-*lac* were grown in R-Media with or without added $NaHCO_3$ supplemented with kanamycin at 37°C under 5% atmospheric CO_2. β-galactosidase assays were carried out on samples taken at hourly intervals as indicated. **A.** *pagA-lacZ* reporter strains. **B.** *atxA-lacZ* reporter strains. Symbols in both panels: -▪- 34F2 without added $NaHCO_3$; -□- 34F2 with 0.8% $NaHCO_3$; -○- 34F2△*resB* without added $NaHCO_3$; -●- 34F2△*resB* with 0.8% $NaHCO_3$.

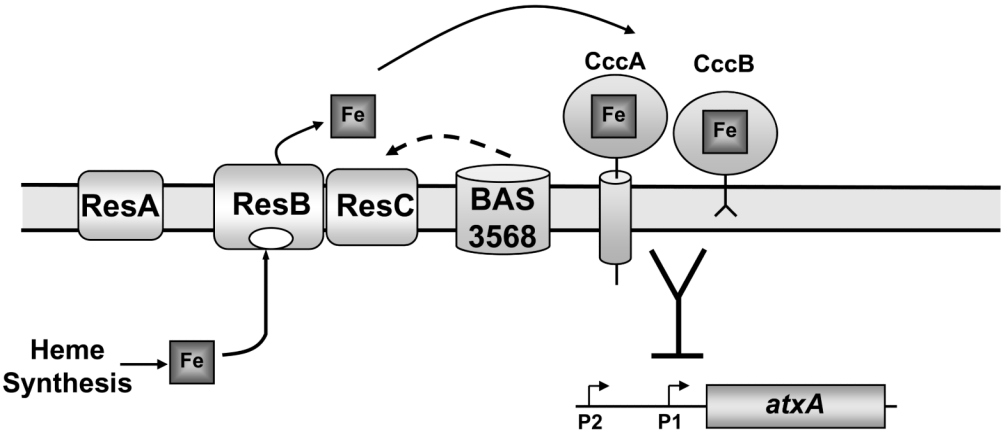

Figure 8.

Schematic representation of the small *c*-type cytochrome pathway regulating virulence gene expression in *B. anthracis*. Intracellularly synthesized heme, through a pathway that requires the Hem proteins including HemL, is transported across the membrane by the ResBC proteins (Ahuja *et al*., 2007) and is covalently attached to CccA and CccB with the involvement of ResA (Le Brun *et al*., 2000). Either CccA or CccB can then act to indirectly repress *atxA* transcription at the P1 promoter and their function is redundant. The BAS3568 protein, which is required for full cytochrome oxidase activity, is also involved in repression of *atxA* transcription in early exponential phase by an unknown mechanism (indicated by the broken arrow) likely to act on the cytochrome *c* maturation pathway. The BAS3568 protein does not share amino acid similarity with the Res proteins. The sidedness of the membrane is indicated by “in” and “out”.

Table 1

B. anthracis strains used in this study

Strain	Relevant Characteristics	Source
34F2	pXO1^{+} pXO2^{-}	Laboratory stock
34F2 *pagA-lacZ*	*pagA-lacZ* on pTCV-lac, Kan^{R}	(Tsvetanova *et al*, 2007)
34F2 *atxA-lacZ*	pATXA12 on pTCV-lac, Kan^{R}	(Bongiorni *et al*, 2008)
34F2 tB3	BAS4358::mini-Tn10, Spc^{R}	pAW016→34F2
34F2 tB17	BAS4358::mini-Tn10, Spc^{R}	pAW016→34F2
34F2 tB18	BAS1385::mini-Tn10, Spc^{R}	pAW016→34F2
34F2 tB21	BAS1384::mini-Tn10, Spc^{R}	pAW016→34F2
34F2 tB23	BAS3568::mini-Tn10, Spc^{R}	pAW016→34F2
34F2 tB28	BAS1384::mini-Tn10, Spc^{R}	pAW016→34F2
34F2 Δ*atxA*	*atxA*::*spc*	(Tsvetanova *et al*, 2007)
34F2 Δ*resB*	GGATCC in place of BAS1384	pAW050→34F2
34F2 Δ*resBC*	GGATCC in place of BAS1384-5	pAW073→34F2
34F2 Δ*hemL*	GTCGAC in place of BAS4358	pAW057→34F2
34F2 ΔBAS3568	GTCGAC in place of BAS3568	pAW066→34F2
34F2 Δ*abrB*	ACGCGT in place of BAS0036	pORISceI-abrB→34F2
34F2 Δ*atxA*Δ*resB*	*atxA*::*spc*, GGATCC in place of BAS1384	pAW050→34F2Δ*atxA*
34F2 Δ*atxA*ΔBAS3568	*atxA*::*spc*, GGATCC in place of BAS3568	pAW066→34F2Δ*atxA*
34F2 Δ*resB*ΔBAS3568	GGATCC in place of BAS3568 and BAS1384	pAW066→34F2Δ*resB*
34F2 Δ*ctaC*	GTCGAC in place of BAS3856	pAW121→34F2
34F2 Δ*qcrBC*	GTCGAC in place of BAS1433-4	pAW125→34F2
34F2 Δ*cccA*	GTCGAC in place of BAS4193	pAW131→34F2
34F2 Δ*cccB*	GTCGAC in place of BAS5035	pAW135→34F2
34F2 Δ*resA*	GTCGAC in place of BAS1383	pAW148→34F2
34F2 Δ3*cyt*	GTCGAC in place of BAS3856 and BAS1433-4	pAW125→34F2Δ*ctaC*
34F2 Δ4*cyt*	GTCGAC in place of BAS3856, BAS1433-4, and BAS4193	pAW131→34F2Δ3*cyt*
34F2 Δ5*cyt*	GTCGAC in place of BAS3856, BAS1433-4, BAS4193, and BAS5035	pAW131→34F2Δ4*cyt*
34F2 Δ*cccA-B*	GTCGAC in place of BAS4193 and BAS5035	pAW135→34F2Δ*cccA*

Table 2

mini-Tn10 transposon mutants identified in the cytochrome *c*/heme pathways

Strain Number	Locus	Name/Annotation	Function	*pagA-lacZ* Increase [a]	*atxA-lacZ* Increase *b*	TMPD staining [c]
tB3	BAS4358*	glutamate-1-semialdehyde-2,1-aminomutase (HemL)	heme synthesis	+	E	+/−
tB17	BAS4358*	glutamate-1-semialdehyde-2,1-aminomutase (HemL)	heme synthesis	+	E	+/−
tB18	BAS1385	ResC	cytochrome *c* maturation	++	E	−
tB21	BAS1384*	ResB	cytochrome *c* maturation	++	E	−
tB23	BAS3568	conserved hypothetical protein	unknown	++	E	+/−
tB28	BAS1384*	ResB	cytochrome *c* maturation	++	E	−

* multiple strains with unique transposon insertions into the same gene

[a]: + = *pagA-lacZ* expression higher than parental strain; ++ = *pagA-lacZ* expression much higher than parental strain

[b]: E = induced *atxA-lacZ* expression in exponential phase

[c]: +/− =staining reduced relative to parental strain; − = no staining