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Influence of the phosphoenolpyruvate:carbohydrate phosphotransferase system on toxin gene expression and virulence in *Bacillus anthracis*

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Summary

AtxA, the master virulence gene regulator of *Bacillus anthracis*, is a PRD-Containing Virulence Regulator (PCVR) as indicated by the crystal structure, posttranslational modifications and activity of the protein. PCVRs are transcriptional regulators, named for PTS Regulatory Domains (PRDs) subject to phosphorylation by the phosphoenolpyruvate phosphotransferase system (PEP-PTS) and for their impact on virulence gene expression. Here we present data from experiments employing physiological, genetic and biochemical approaches that support a model in which the PTS proteins HPr and Enzyme I (EI) are required for transcription of the *atxA* gene, rather than phosphorylation of AtxA. We show that *atxA* transcription is reduced 2.5-fold in a mutant lacking HPr and EI, and that this change is sufficient to affect anthrax toxin production. Mutants harboring HPr proteins altered for phosphotransfer activity were unable to restore *atxA* transcription to parent levels, suggesting that phosphotransfer activity of HPr and EI is important for regulation of *atxA*. In a mouse model for anthrax, a HPr⁻ EI⁻ mutant was attenuated for virulence. Virulence was restored by expressing *atxA* from an alternative, PTS-independent, promoter. Our data support a model in which HPr transfers a phosphate to an unidentified downstream transcriptional regulator to influence *atxA* gene transcription.

Introduction

For pathogens that encounter multiple host niches during infection, the ability to discern and respond to changing environmental signals, including nutrient availability, is paramount to survival in the context of the host. *Bacillus anthracis* is an example of a bacterial pathogen that occupies diverse host niches as it disseminates and proliferates. The bacterium causes anthrax disease in a wide variety of mammals. Following entry into the host via inhalation, gastrointestinal or cutaneous routes, *B. anthracis* can disseminate and thrive in a wide variety of tissues (Weiner and Glomski, 2012; Goossens and Tournier, 2015). As might be

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Author contributions

N.B., T.G.H., and T.M.K. contributed to the conception and design, acquisition, analysis and interpretation of the data. N.B. and T.M.K. wrote the article.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

expected for such a pathogen, control of classic virulence factor synthesis in *B. anthracis* is intertwined with regulation of key physiological processes. AtxA, a well-established transcription regulator of *B. anthracis*, positively controls the structural genes for the anthrax toxin proteins and the capsule biosynthesis operon, but also affects expression of genes associated with sporulation, branched chain amino acid biosynthesis and transport and other aspects of metabolism (Raynor et al., 2018). Moreover, while carbohydrate utilization has not been directly associated with AtxA, transcription of the *atxA* gene is influenced by glucose availability via carbon catabolite repression (Chiang et al., 2011), and toxin synthesis is high when the bacterium is cultured in defined medium containing glucose (Ristroph and Ivins, 1983).

A growing body of work on bacterial pathogens reveals relationships between aspects of carbohydrate metabolism and virulence gene expression (Poncet et al., 2009a). In *B. anthracis*, deletion of the gene encoding the catabolite control protein CcpA results in attenuated virulence in mice injected subcutaneously with the bacterium. This phenotype may be related to the observation that CcpA enhances transcription of *atxA* in response to glucose (Chiang et al., 2011). CcpA also enhances virulence factor production in *Enterococcus faecalis*, *Listeria monocytogenes* and *Streptococcus pneumoniae* (Poncet et al., 2009a). In *Streptococcus gordonii* and *Streptococcus sanguis*, CcpA reduces production of H₂O₂ and organic acids which are important for competition with other oral microbes and colonization (Redanz et al., 2018).

In Gram-positive bacteria, CcpA activity is influenced by the histidine-containing phosphocarrier protein HPr. In the presence of a preferred carbon and energy source, glycolytic intermediates such as glucose-6-phosphate and fructose-bis-phosphate, as well as energy in the form of ATP, stimulate the kinase activity of HPr Kinase/Phosphorylase (HPrK/P). HPrK/P phosphorylates HPr at a conserved serine residue (Ser46), changing the conformation of HPr to facilitate interaction with CcpA. CcpA associated with HPrSer46-P can bind target DNA sequences known as catabolite responsive elements (*cre* sites). Binding of CcpA to *cre* sites is generally inhibitory to transcription and controls the expression of genes encoding catabolic enzymes necessary for the use of secondary carbon sources (Deutscher et al., 2014).

An overarching control mechanism for selective carbon source utilization, and in some cases virulence, is the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). The PTS is an energy-dependent sugar import system comprised of general proteins, Enzyme I (EI) and HPr (which also serves as a co-effector of CcpA) and sugar-specific Enzyme II (EII) proteins. In this system, EI autophosphorylates, using a phosphate from phosphoenol pyruvate (PEP), is an end product of glycolysis. The high energy phosphate is transferred to HPr on a conserved histidine residue (His15 in *B. subtilis*), and subsequently relayed to an EIIB protein that transfers the phosphate to the EIIB domain. EIIB phosphorylates the incoming sugar transported by the transmembrane protein EIIC. This phosphorylation event serves to charge the sugar to enter glycolysis and prevent sugar efflux from the cell, as EII has low affinity for phosphorylated sugars (Deutscher et al., 2014).

In a model based primarily on studies of the non-pathogen *Bacillus subtilis*, HPr can also transfer the phosphate from the specific histidine residue to certain transcriptional regulators. The phosphorylated domains of these regulators are known as PTS regulation domains (PRDs). In *B. subtilis*, HPr-mediated phosphorylation of PRD-containing regulators affects transcription of genes involved in sugar transport and utilization (Stulke et al., 1998). The presence of PTS substrates influences the phosphorylation state of the various components of the system, and therefore influences the ability of the PTS to phosphorylate and control PRD-containing regulators. When HPr is phosphorylated at His15 it can transfer the phosphate to PRD-containing regulators thereby activating their activity. In the absence of a cognate sugar, the EII proteins of a specific system are found in their phosphorylated form and may phosphorylate the PRD-containing regulator to inactivate the regulator activity. Alternatively, in the presence of specific substrate, the EII proteins transfer phosphate to the substrate rather than the PRD-containing regulator, thereby relieving the repressive phosphorylation on regulator activity (Deutscher et al., 2014).

An emerging class of PRD-containing proteins has been reported in pathogens. The regulons of these transcription regulators include genes encoding virulence factors. PRD-containing virulence regulators, termed 'PCVRs', include AtxA and its paralogues AcpA and AcpB in *B. anthracis*, Mga in *Streptococcus pyogenes*, MgaSpn in *S. pneumoniae*, and most recently, PafR in uropathogenic *Escherichia coli* (Hondorp and McIver, 2007; Solano-Collado et al., 2013; Baum et al., 2014). AtxA is the only full-length PRD-containing protein for which the high-resolution crystal structure has been solved. The AtxA protein has two helix-turn-helix domains at the amino-terminus, followed by two PRDs and a region resembling EIIB of the PTS at the carboxy-terminus (Hammerstrom et al., 2015). As is true for other well-studied PRD-containing regulators, AtxA is subject to phosphorylation at histidine residues within its PRDs. Phosphorylation at His199 allows AtxA activity as a transcriptional regulator, while phosphorylation at His379 inhibits AtxA activity by preventing dimerization (Tsvetanova et al., 2007; Hammerstrom et al., 2011). In a recombinant *B. subtilis* mutant containing the *B. anthracis* toxin gene *pagA* and an IPTG-inducible *atxA* gene, AtxA-dependent transcription of *pagA* was increased in a HPr⁻ EI⁻ (PTS⁻) mutant relative to the PTS⁺ parent, suggesting that the PTS influences AtxA activity (Tsvetanova et al., 2007). Based on the AtxA structure, the phosphorylation events that affect AtxA dimerization and function, and the reported PTS effect on AtxA in *B. subtilis*, we hypothesized that AtxA is phosphorylated by HPr to control regulator activity in its native host, *B. anthracis*. In this report, we provide data demonstrating that in *B. anthracis*, the PTS is not responsible for phosphorylation of AtxA but rather transcription of the *atxA* gene.

Results

Toxin synthesis in a PTS-deficient *B. anthracis* mutant

We sought to determine if the PTS affects AtxA-mediated toxin gene expression in *B. anthracis*. We generated a *ptsHI*-null mutant, UT439, from our parent *B. anthracis* strain, ANR-1. Supernates from cultures grown in CA medium with 0.8% of dissolved bicarbonate in 5% of atmospheric CO₂ (CACO₃), conditions reported to allow production of the toxin proteins, edema factor (EF), lethal factor (LF) and protective antigen (PA), at high levels

(Haines et al., 1965), were probed with anti-LF, anti-EF and anti-PA sera using western blotting. As shown in Fig. 1, the toxin proteins were readily detected in culture supernates of the parent strain but were not detected in culture supernates of the *ptsHI*-null mutant harboring an empty vector. Complementation of the mutant, with *ptsH* (encoding HPr) and *ptsI* (encoding EI) individually, was not sufficient to restore toxin synthesis. However, when both *ptsH* and *ptsI* were present on the complementation vector, toxin production was restored to a level similar to that of the parent strain. These data, together with the previous demonstration of a PTS effect on AtxA-dependent expression of the PA gene *pagA* in recombinant *B. subtilis* (Tsvetanova et al., 2007), support the hypothesis that the PTS is required for AtxA activity.

AtxA activity during growth in PTS and non-PTS sugars

The influence of the PTS on PRD-containing regulators differs in response to specific sugars (Stulke et al., 1998). When a PTS substrate is present in the medium, the cognate EII PTS components are stimulated to relay phosphate to the incoming sugar, thus affecting the flux of phosphate through the system. To ascertain whether AtxA activity is affected by the presence of PTS substrates (PTS sugars) versus sugars transported by alternative means (non-PTS sugars), we assessed AtxA activity in cultures grown in CACO₃ supplemented with 0.1% of glycerol and 0.1% of the specified added sugar. Our *in vivo* assay for AtxA activity employs strain UT376 (pUTE991). In this strain, *atxA* is deleted from its native locus and expressed from an IPTG-inducible promoter, and the *lef* promoter (of the lethal factor gene) at the native locus is fused to a promoter-less *lacZ* gene (Hammerstrom et al., 2011). Induction of *atxA*, uncouples transcriptional control of *atxA* from AtxA function, allowing quantitative assessment of AtxA activity. β -galactosidase activity was measured in cell lysates from cultures grown in the presence of the PTS substrates (glucose, fructose or mannitol) and in the presence of non-PTS sugars (glycerol or melibiose). As shown in Fig. 2, there was no significant difference in AtxA activity in samples taken from late log phase cultures containing PTS and non-PTS sugars. These data indicate that engagement of the PTS components of the fructose-, glucose- and mannitol-specific EII systems does not influence AtxA activity.

PTS-mediated phosphotransfer of ³²P

In vitro transfer of phosphate from PTS proteins to PRD-containing regulators has been demonstrated for many PRD-containing regulators (Stulke et al., 1997; Martin-Verstraete et al., 1998; Schmalisch et al., 2003). We sought to detect EI- and HPr-mediated phosphorylation of AtxA in conditions similar to those reported for other PRD-containing proteins. His₆-tagged proteins were purified from *B. anthracis* and incubated in various combinations with ³²P-labeled PEP using conditions previously reported (Tortosa et al., 2001; Schmalisch et al., 2003; Joyet et al., 2010). Reactions were subjected to SDS-PAGE followed by phosphor-imaging to assess protein phosphorylation (Fig. 3). The *B. anthracis* homologue of GlcT, a well-studied PRD-containing regulator from *B. subtilis*, was used as a positive control. As seen in Fig. 3, EI was successfully labeled with the ³²P-PO₄ when incubated alone or with other proteins (lanes 1 and 5–11). A radiolabeled band corresponding to phosphorylated HPr was detected when HPr and EI were incubated together (lanes 5 and 8–11), but not when HPr was incubated without EI (lanes 2, 12

and 13), indicating transfer from phosphorylated EI to HPr, as expected. Phosphorylated GlcT was detected when incubated with EI and HPr (lane 8), indicating that the purified PTS proteins were functional in transferring $^{32}\text{P-PO}_4$ to a PRD-containing regulator in our system. We did not detect phosphorylated AtxA when the protein was incubated with $^{32}\text{P-PEP}$ in any combination of the PTS proteins, including a reaction containing excess AtxA (lane 10). To control for the possibility that AtxA was purified in its fully phosphorylated form, and therefore unable to accept $^{32}\text{P-PO}_4$, purified AtxA was treated with agarose-bound alkaline phosphatase prior to incubation with HPr and EI. Again, phosphorylated AtxA was not detected (lane 11).

AtxA activity in a PTS-null mutant

To further investigate the potential role of the PTS in AtxA function, we used the *Plef-lacZ* reporter system to compare AtxA activity in the PTS⁺ strain UT376 (pUTE991) and PTS-null mutant, UT417 (pUTE991). Cells were cultured in CACO₃, and activity at the *Plef-lacZ* reporter was determined following induction of *atxA* expression with IPTG. β -galactosidase activity of the PTS⁺ and PTS⁻ mutants was comparable in cultures containing similar levels of AtxA (Fig. 4). However, *Plef-lacZ* expression by UT408, a PTS-null mutant expressing *atxA* from its native locus, was severely diminished.

Together, the absence of detectable differences in AtxA activity in cultures grown in PTS- and non-PTS sugars (Fig. 2), the lack of PTS-mediated AtxA phosphorylation *in vitro* (Fig. 3), and no detectable difference in AtxA activity between PTS⁺ and PTS⁻ strains when *atxA* transcription was controlled by an IPTG-inducible promoter (Fig. 4), indicate that the PTS does not affect AtxA function and phosphorylation in *B. anthracis*. On the contrary, data showing reduced expression of *Plef-lacZ* in a PTS-null mutant expressing *atxA* from its native promoter combined with our observation that LF production decreased in the absence of the PTS (Fig. 1) suggested a role of the PTS in *atxA* transcription.

PTS effects on transcription of *atxA*

Our data revealed a relationship between PTS-controlled toxin synthesis and transcription of *atxA*. A previous study reported that *atxA* transcription increases in the presence of glucose in a CcpA-dependent manner (Chiang et al., 2011). In *B. subtilis* and other Gram-positive organisms, CcpA is activated in the presence of rapidly metabolized carbon sources; ATP stimulates HPr kinase to phosphorylate HPr at a conserved serine residue and HPr-SerP interacts with CcpA to positively allow for binding of CcpA to target *cre* sites. To explore the possibility that the decrease in toxin production in the absence of the PTS was due to CcpA control of *atxA* transcription, we assessed the *PatxA-lacZ* reporter in parent, PTS-null and *ccpA*-null mutants. Transcription of the *PatxA-lacZ* reporter was decreased in the *ptsHI*-null the *ccpA*-null mutant (Fig. 5A). Reporter activity in the *ptsHI*-null was restored when both HPr and EI were expressed, but not when either HPr or EI were expressed individually. In agreement with decreased levels of *atxA* transcript in the PTS-null mutant, lethal factor was undetectable in the supernates of this strain, as well (Fig. 5B). According to the model developed in *B. subtilis* and the observation made by Chiang et al. (2011) in *B. anthracis*, enhanced transcription of *atxA* in the presence of glucose is attributed to HPr-mediated control of CcpA activity. However, our experiments revealing the requirement

for the presence of both HPr and EI to restore *atxA* transcription to the parent level, suggested a mechanism independent of CcpA. Generally, HPr affects transcription of genes through two pathways; by interacting with CcpA in its HPrSer46 phosphorylated form, and by phosphorylating PRD-containing transcriptional regulators. A third mechanism exists in *B. subtilis*, in which His15-phosphorylated HPr directly activates the transcription factor YesS similar to how HPrSer46P interacts with CcpA (Poncet et al., 2009a; 2009b). The need for both EI and HPr to restore *atxA* transcription in the PTS mutant suggests HPr affects *atxA* transcription in an EI-dependent manner, either through phosphor-transfer to a downstream, PRD-containing regulator, or by direct interaction with a downstream regulator in its His14-phosphorylated form. To discern between these possibilities, we generated phosphovariants of HPr and assessed their ability to complement the *ptsHI*-null mutant when co-expressed with EI. We reasoned that if phosphotransfer activity is necessary, any substitutions to HPr-His14 would not functionally complement the *ptsHI* mutant, however, if HPr directly interacts with a downstream regulator in its His14-phosphorylated form, mimicking phosphorylation using a His to Asp substitution should allow for a functional complementation of the PTS mutant.

The phosphovariants with substitutions at His14 (H14A and H14D) were unable to complement the PTS-null phenotype (Fig. 5), suggesting phosphotransferase activity at this residue is necessary for *atxA* transcription. A His to Ala substitution at HPrS46 did not affect *atxA* transcription, in agreement with no change in *atxA* observed in the *ccpA* mutant. The change measured by β -galactosidase assays was small, but significant, and enough to strongly affect toxin production. As shown in Fig. 5, the decrease in *atxA* transcription observed in the PTS mutant and non-functional HPr complementation strains, correlated with undetectable toxin levels in the culture supernates. Overall, these data support a model in which the PTS functions to phosphorylate a downstream regulator that directly or indirectly affects *atxA* transcription, and that this regulation is important for virulence factor production.

***atxA* promoter expression during growth in different sugars**

According to the model for phosphate flow in the PTS, transfer of phosphate from His15 of HPr to the EI_{IIA} component of the active sugar-specific PTS complex is modified by the presence or absence of PTS sugars. Therefore, PTS sugars also affect HPr-mediated phosphate transfer to PRD-containing regulators (Stulke et al., 1998). Our data suggest a PRD-containing regulator is phosphorylated by HPr to activate transcription of *atxA*. To better understand the PTS-mediated control of *atxA*, we assessed expression of the *atxA-lacZ* promoter fusion in the presence of various PTS and non-PTS sugars. As shown in Fig. 6, we did not observe significant differences in *atxA* transcription in cultures grown in the presence of PTS sugars glucose, fructose and mannitol, and in the non-PTS sugars glycerol and melibiose. Thus, we are unable to attribute PTS-mediated control of *atxA* transcription to a specific sugar in these growth conditions.

The PTS affects *atxA* transcripts originating from the primary promoter P1

Transcription of *atxA* is initiated from two promoters; P1 is proximal to the translation start site and is the most predominant start site, while P2 is located approximately 600

bp upstream from P1 and is expressed weakly (Dai et al., 1995; Bongiorno et al., 2008). To further discern the PTS effect on *atxA* transcription, we questioned if differences in PTS control of *atxA* transcription could be associated with a specific transcriptional start site. We assessed relative expression of a *PatxA-lacZ* reporter containing the full length (P1+P2) promoter region, or solely the proximal (P1) promoter region in the parent strain and PTS-null mutant. As shown in Fig. 7, expression of *PatxA-lacZ* was decreased in the PTS-null mutant for both reporter constructs, indicating that P1 is sufficient for PTS control of *atxA* transcript levels.

Altered AtxA expression affects virulence

We sought to determine if the PTS and PTS-controlled expression of *atxA* influenced *B. anthracis* virulence in a murine model of late stage anthrax infection in which toxin synthesis is critical. A/J mice are complement-deficient and are susceptible to toxin-mediated infection with the non-capsulated ANR-1 strain (Welkos et al., 1986). Our previous studies have demonstrated that an *atxA*-null mutant is avirulent in this model when mice are injected via the tail vein with up to 10^9 spores (Dale et al., 2012).

To determine if the PTS plays an important role in the toxin-dependent A/J model, we compared the virulence of the parent strain to that of the *ptsHI*-null mutant UT439 (Fig. 8). All mice infected with the parent strain were moribund within 96 h and *B. anthracis* was recovered from the liver, lung and spleen. All mice infected with UT439 survived the seven-day experiment with no symptoms of disease (Fig. 8A) and *B. anthracis* was not recovered from tissue of the collected organs (Fig. 8B).

To uncouple *atxA* transcription from the PTS, we created UT447, a mutant in which *atxA* is constitutively expressed from a non-PTS-controlled promoter (*atxA**). Mice infected with UT447 succumbed to disease (Fig. 8A) and the bacterium disseminated to all tissues tested (Fig. 8B). Virulence and dissemination of UT449, which contains *atxA** but is *ptsHI*-null, was not statistically different from UT447, indicating that the avirulent phenotype of UT439, a *ptsHI*-null mutant that carries *atxA* under control of its native promoter, is due to the effect of the PTS on *atxA* expression. We also note that the UT447 (*atxA**)-infected mice exhibited significantly prolonged survival compared to mice infected with the parent strain (Fig. 8A), suggesting that native transcriptional control of *atxA* affects virulence.

With the exception of the avirulent UT439 mutant, *B. anthracis* CFU were recovered from liver, spleen and lung tissues. Interestingly, tissues from moribund mice infected with strains harboring *atxA**, contained approximately 10-fold higher numbers of CFU than mice infected with the parent strain, suggesting that deregulation of *atxA* transcription affects bacterial load.

Discussion

The high-resolution structure of AtxA combined with previous biochemical studies demonstrating relationships between AtxA phosphorylation, dimerization and activity (Tsvetanova et al., 2007; Hammerstrom et al., 2011; 2015), have placed AtxA in a class of transcription regulators whose activity is controlled by components of the PTS. For

many of these regulators, direct phosphorylation by HPr has been demonstrated (Martin-Verstraete et al., 1998; Tortosa et al., 2001; Schmalisch et al., 2003; Joyet et al., 2010). In the current model for PTS control of these regulators, phosphorylation by HPr positively affects regulator activity during growth in PTS substrates. In the absence of PTS substrates, phosphorylation by substrate-specific EII proteins inactivates regulator activity (Deutscher et al., 2014).

In this work, we tested for relationships between AtxA and the PTS. Our three-pronged approach to examine PTS control of AtxA activity revealed (1) no differences in AtxA activity during growth of *B. anthracis* in PTS versus non-PTS substrates, (2) no direct phosphorylation of AtxA by HPr or EI *in vitro* and (3) no change in AtxA activity in the absence of HPr and EI. Surprisingly, and in contrast to the generally accepted mechanism for control of PRD-containing regulators, results of our experiments support a model in which the PTS proteins HPr and EI control *atxA* gene transcription rather than AtxA protein activity. Deletion of the PTS renders *B. anthracis* avirulent and virulence is restored in a PTS⁻ mutant expressing a recombinant PTS-independent *atxA* gene.

The overall model by which the PTS affects transcription in bacteria includes many aspects of HPr function. These activities include (1) HPr-mediated phosphor-transfer to PRD-containing transcriptional regulators, (2) interaction of HPrSer46P with CcpA, and, in the case of *B. subtilis* and (3) interaction of HPrHis15P with YesS, a transcriptional activator of pectin/rhamnogalacturonan utilization genes (Poncet et al., 2009b). Our results suggest that regulation of *atxA* transcription is achieved through control of an unidentified upstream regulator that is controlled by HPr and EI to affect transcription from the primary promoter of *atxA*. The *B. anthracis* genome does not indicate the presence of a YesS homologue and we have not been successful in identifying another PRD-containing regulator that affects *atxA* transcription. Interestingly, Chiang and coworkers (2011) showed CcpA elevated *atxA* transcript levels two-fold in response to the presence of glucose. Our data did not reveal a significant difference in expression of the AtxA reporter *PatxA-lacZ* between the parent and *ccpA*-null strain. It is possible that the conflicting data are due to strain differences or assay sensitivity. We note that CcpA control of the close *atxA* homologue *mga* in group A streptococcus has been reported, but there are many differences in regulation of *mga* and *atxA* transcription. For example, *mga* is autogenously regulated (Almengor et al., 2007), while *atxA* is not subject to such control, as indicated by evidence of transcriptional activity from the *PatxA-lacZ* reporter in the *atxA*-null mutant (Fig. 5).

Multiple reports have indicated that AtxA activity is affected by the phosphorylation state of His199 in PRD1 and His379 in PRD2 of the protein. Our results support a model in which HPr does not affect phosphorylation of AtxA directly, so the protein(s) responsible for AtxA phosphorylation remain(s) unknown. Comparison of the amino acid sequences of AtxA and other PCVRs relative to well-studied PRD-containing regulators, reveals a key difference in histidine placement. It is possible that location of these specific residues within the PRDs affects substrate susceptibility. As previously shown by Stulke and coworkers (Stulke et al., 1998) and modified here (Fig. 9), multiple PRDs have a histidine residue at position 7 of the alignment presented and a conserved arginine at position 14. For most of these proteins, phosphorylation of the histidine at position seven has been demonstrated.

PRD-containing regulators of the PCVR subgroup (Group 1 in Fig. 9) appear to differ in histidine placement, as well as phosphorylation sites for those proteins in which the sites have been identified. In addition, the arginine in PRDII is not as well conserved in the PCVRs as it is in the *B. subtilis* PRD-regulators. Four out of the five PCVRs presented do not have the conserved arginine in PRDII at position 14 of the alignment. Finally, PRDI seems to be highly conserved with regard to the glycine and tyrosine residues at positions 28 and 54 respectively, whereas PRDII has a highly conserved lysine at position 47. These differences in the PRD sequences may be due to placement of the PRDs proximal to other functional domains of the proteins. For instance, PRDI is proximal to the nucleotide binding domains of the proteins whereas PRDII is more distal, or closer to the C-terminus, and for the transcriptional activators PRDII is adjacent to the EIIA and/or EIIB domains.

A few PRD-containing regulators have been identified for which phosphorylation by HPr is not necessary for activation. MtlR, a PRD-containing regulator found in many Gram-positive organisms, is responsible for regulating transcription of mannitol import utilization genes. The MtlR homologue in *Lactobacillus casei* is not phosphorylated by HPr and EI, and functions independently of these proteins. MtlR homologues in other bacteria are phosphorylated by HPr at a histidine residue within PRDII to activate the protein, however in *L. casei* the histidine normally found in PRDII has been substituted with a tyrosine (Joyet et al., 2015). *L. casei* may have undergone a number of changes from the conserved MtlR sequence to establish HPr-independence and subsequently changed the no longer needed His to Tyr. MtlR in *L. casei*, like other organisms, is subject to phosphorylation by EIIB^{Mtl} at His590 in the EIIA^{Mtl} domain of the protein, and this phosphorylation acts to inhibit MtlR activity. Other examples of HPr-independent function of PRD-containing regulators exist in *B. subtilis*. GlcT and SacY, the transcription antiterminators responsible for controlling glucose and sucrose import machinery respectively, are phosphorylated by HPr and EI, however, phosphorylation does not affect regulator activity (Crutz et al., 1990; Stulke et al., 1997). Our data suggest that AtxA is another example of a PRD-containing protein that has evolved to be active independent of the PTS.

Our results, examining PTS-AtxA relationships in *B. anthracis*, are not in agreement with results of experiments that tested AtxA activity in the non-native host *B. subtilis*. Tsvetanova and coworkers (Tsvetanova et al., 2007) employed recombinant *B. subtilis* strains harboring an AtxA reporter (toxin gene *pagA* promoter-*lacZ* fusion) and expressing *atxA*. They showed that AtxA activity at the *pagA* promoter was increased 2.5-fold in a *ptsHI*-null mutant relative to a PTS⁺ strain. Taken together, the findings suggest differences in PTS – AtxA relationships in *B. anthracis*, the native host for AtxA and *B. subtilis*, which does not carry the *atxA* gene. MtlR is a good example of host-specific control of PRD-containing regulators. MtlR, unlike AtxA, is native to multiple Gram-positive species. Despite the high degree of MtlR amino acid sequence conservation between *L. casei*, *B. subtilis* and *Geobacillus stearothermophilus*, the mechanisms for MtlR control in these organisms differ. In *B. subtilis* and *G. stearothermophilus*, MtlR is phosphorylated by HPr and EI at a conserved histidine in PRD2 to activate protein activity, whereas in *L. casei* it is not. In *B. subtilis*, MtlR must also be sequestered to the membrane via interaction with unphosphorylated EIIB^{Mtl} in order to be active (Joyet et al., 2015). The observation that

AtxA is controlled differently in *B. anthracis* and *B. subtilis* raises the possibility that the systems function differently in two organisms.

Control of PCVRs by the PTS extends the influence of the sugar import system such that in addition to regulating bacterial physiology, the PTS can also affect virulence gene expression in infected hosts. In our study, we found that the PTS-null mutant is attenuated for virulence and virulence was restored when *atxA* transcription is under control of a non-PTS responsive promoter.

Interestingly, our data showed that placement of *atxA* under control of a non-regulated promoter results in attenuation for virulence relative to the parent ANR-1. These results are not entirely unexpected. In *Listeria monocytogenes* and *Yersinia pestis* altering virulence determinants such that they are constitutively active, attenuates virulence to different degrees (Mitchell et al., 2017; Kryptou et al., 2019). For *B. anthracis*, strains expressing *atxA* constitutively were attenuated yet showed an increase in CFU per gram of infected organs relative to parent (ANR-1)-infected mice. These results intimate a more complex process leading to death of the host rather than simply a change in dissemination and/or proliferation.

In this work, we found that a PRD-containing regulator hypothesized to be controlled post-translationally by the PTS, is in fact controlled by the PTS at the level of transcription. While many studies of PRD-containing proteins have focused on protein function and regulation of activity, relatively little is known regarding potential influence of carbohydrate metabolism and signaling on transcription of the genes encoding these regulators. Our data support a model in which HPr transfers phosphate to a transcriptional regulator that regulates the transcription of a PRD-containing regulator, *atxA*. Future studies will aim to identify the target of HPr phosphorylation and to determine if regulation of *atxA* occurs via a direct or indirect mechanism.

Experimental procedures

Strains and growth conditions

Strains and plasmids are shown in Table 1. *B. anthracis* strains were derived from the Ames non-reverting, pXO1⁺ pXO2⁻ strain, ANR-1. *Escherichia coli* strains TG1 and GM2163 were used for cloning. Unless otherwise indicated, *E. coli* cultures were grown in Luria Burtani (LB) broth (Bertani, 1951) with shaking at 37°C.

Inocula for *B. anthracis* cultures were obtained from overnight cultures grown in brain-heart infusion (BHI) broth (Becton, Dickson and Company, Franklin Lakes, NJ) with shaking at 30°C. Stationary phase cultures were transferred to fresh medium at a starting OD₆₀₀ of 0.08 and incubated with shaking at 37°C. For preparation of cells for electroporation, subcultures were grown in BHI to an OD₆₀₀ between 0.6–0.8. Cell lysates used for β -galactosidase assays and western blotting were prepared from subcultures grown in CACO₃ (Casamino Acids medium Thorne and Belton, 1957; Hadjifrangiskou et al., 2007) buffered with 100 mM HEPES [pH 8.0], containing 0.1% of glycerol and supplemented with 0.1% of w/v glucose (or other sugars as specified), as well as 0.8% of w/v sodium bicarbonate) and

incubated in 5% of atmospheric CO₂. For strains harboring *atxA* under control of the *hyperspank* promoter (*Phyperspank*) (Britton et al., 2002), expression was induced with isopropyl β-d-thiogalactoside (IPTG) during early exponential phase at 2 h and harvested at early stationary phase at 4 h. Strains harboring *ptsH* and *ptsI* under a xylose-inducible promoter were induced with 0.1–2% of xylose 1 h post-inoculation and harvested at early stationary phase at 4 h. Cell pellets were used immediately or stored at –80°C.

Antibiotics were added to media when appropriate, at the following concentrations; carbenicillin (100 µg ml⁻¹), spectinomycin (100 µg ml⁻¹), erythromycin (300 µg ml⁻¹) for *E. coli* and (5 µg ml⁻¹) for *B. anthracis*.

DNA isolation and manipulation

Standard procedures were used for cloning in *E. coli* TG1. Primers used for PCR are shown in Table S1. Plasmid isolation from *E. coli* was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI). *E. coli* GM2163 was used to isolate non-methylated plasmid DNA for electroporation into *B. anthracis* (Marrero and Welkos, 1995). *B. anthracis* DNA was obtained using UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Restriction enzymes, T4 DNA ligase and Phusion DNA polymerase were purchased from New England Biolabs (Ipswich, MA). Bullseye Taq DNA Polymerase was purchased from MidSci (St. Louis, MO).

Generation of marker less deletions

Markerless deletions in *B. anthracis* were constructed using the temperature-sensitive integration vector pHY304 as described previously (Raynor et al., 2018). The *ptsHI* operon was removed from the ANR-1 chromosome via homologous recombination. Primers TH235 and TH245 were used to amplify the left flanking region surrounding the *ptsHI* operon and primers TH246 and TH244 were used to amplify the right flanking region. The two amplicons were fused together by splicing by overlapping extension PCR (SOE-PCR) (Horton et al., 1989), subcloned into pHY304 and used to transform *B. anthracis*. The *ptsHI*, *atxA* double mutant UT448 was constructed by removing the *atxA* gene from the *ptsHI* mutant UT439 as described using the plasmid pUTE937. Details regarding the construction of pUTE937 can be found in Hammerstrom et al. (2011). The *ccpA*-null mutant, UT433 was generated following the same protocol using the primers NB063 and NB064 to generate the LFR and NB065 and NB066 to generate the RFR.

Generation of *plcR*:GBAA_2500promoter-*atxA*-His₆

The promoter of GBAA_2500 was chosen to control *atxA* transcription because GBAA_2500 transcript levels are comparable to *atxA* transcript levels in cultures grown in toxin-inducing conditions (Raynor et al., 2018). The *atxA*-His₆ construct was fused to the promoter region and cloned into the *plcR* locus, a nonfunctional gene in *B. anthracis* that has been used to express cloned genes (Raynor et al., 2018). To confirm the promoter was not controlled by the PTS, expression of the promoter-*atxA* fusion in a parent strain and a *ptsHI*-null mutant was compared using western blotting (data now shown).

The *plcR*::GBAA_2500promoter-*atxA*-His₆ construct was generated by fusing two fragments together via SOE-PCR. The fragments generated contained *atxA* with a C-terminal His₆-tag and *Bam*HI RE, and the region upstream of GBAA_2500 predicted to contain the transcriptional start site and a *Bam*HI RE site.

The *plcR* flanking regions were amplified with *Bam*HI cut site in the middle of the two regions in order to promote incorporation of the *plcR*::GBAA_2500promoter-*atxA*-His₆ construct into the *plcR* locus via homologous recombination. Primers ES40 and ES41 were used to amplify the right flanking region upstream of the *plcR* locus. Primers ES42 and ES43 were used to amplify the left flanking region downstream of the *plcR* locus. The two flanking regions were fused via SOE-PCR.

Generation of the HPr and EI expression vectors

To create the His₆-EI (pUTE1141) and His₆-HPr (pUTE1142) constructs, the *ptsHI* operon was amplified from the *Bacillus anthracis* genome using primers NB074 and NB075. The *atxA* RBS was added to the 5' end of the product using primer walking with primers NB074 and NB076. The PCR product generated by NB074 and NB076 was used as template for all the HPr and EI-expressing plasmids. pUTE1141 the pUTE657-based plasmid expressing His₆-EI was constructed using primers NB074 and NB145 and sub-cloned into pUTE657 using *Sa*II and *Sph*I. pUTE1142 the pUTE657-based His₆-HPr expressing vector was constructed using primers NB144 and NB076.

Site directed mutagenesis was employed to create the HPr phosphovariants, primers listed in the primer table.

To use the plasmid-based *PatxA-lacZ* reporter and the HPrEI expression vectors simultaneously, we employed the pAW285 vector (Han and Wilson, 2013) with a different origin of replication from the pHT304-*lacZ* based *PatxA-lacZ* reporter. To move His₆-HPr and His₆-EI from pUTE657 based plasmids the following primers were used. Primers NB146 and NB148 were used to amplify the *ptsHI* operon with His₆-tagged EI and His₆-tagged HPr from pUTE1140 and the phosphovariants created by site-directed mutagenesis. Primers NB148 and NB149 were used to amplify His₆-HPr, and NB146 and NB147 were used to amplify His₆-EI. The constructs were subcloned into pAW285 using the restriction enzymes *Bam*HI and *Hind*III.

Overproduction of *B. anthracis* proteins

For the *in vitro* phosphorylation assay, proteins were purified from *B. anthracis* cultures grown in 1 liter of BHI at 37 C. After two h of growth 0.5 M IPTG was added to the medium and cultures continued to incubate for four more h. Cells were collected on 0.22 µm pore-size filter unit and resuspended in Buffer A-PIC (20 mM Tris-Cl pH 8.0, 100 mM NaCl, 5% of glycerol, 30 mM imidazole pH 7.9, 1X EDTA-free Complete proteinase inhibitor cocktail), followed by centrifugation at 5000 × g and freezing of the cell pellets at -80°C.

Native levels of HPr were determined using western blot with an antibody raised against specific peptides within the HPr sequence (a gift from Bethyl Laboratories Inc.,

Montgomery, TX). A gradient of xylose was used to determine the proper induction levels of HPr and EI from the pAW285 constructs. EI is expressed from its native RBS within the *ptsH* coding sequence. We therefore determined the level of EI-His₆ expressed from the *ptsHI* locus with both *ptsH* and *ptsI* His-tagged, to determine the native levels of EI.

Purification of proteins from *B. anthracis*

Frozen pellets were thawed on ice and resuspended in 10 mL Buffer A (10 mM Tris-HCl pH 7.5, 200 mM NaCl, 1mM imidazole) w/v Complete EDTA-free proteinase inhibitor cocktail (Buf A+PIC), supplemented with 1 mM MgCl₂ and 10 µl DNase 1. Cells were lysed via French press (3 passages through small cell set on 1,000 psi). Cell debris were collected by centrifugation at 16,000 × *g* for 20 min at 4°C. Soluble cell lysate was transferred to pre-washed NTA-Ni resin. 1 ml NTA-Ni resin slurry (Qiagen, 50% of resin solution) was washed twice with 4 volumes Buf A+PIC. The lysate was incubated with the resin in batch for 2 h at 4°C. Beads were washed in batch two times using four bead volumes of BufA+PIC. Beads were then resuspended in 1.25 ml BufA, transferred to a gravity column and washed with 15 ml BufA. Proteins were eluted using 1 ml of Buffer E (Buffer A with imidazole) with a gradient of imidazole concentrations. Bradford reagent was used to detect protein in fractions. The fractions with protein were combined and dialyzed to maintain proteins in 50 mM HEPES pH 7.5, 5 mM MgCl₂, 5% glycerol and stored at 4°C.

Purification of PEP carboxykinase

PEP carboxykinase (His-PEPCK) was expressed and purified from *E. coli* C41(DE3) harboring plasmid pKSM879 (gift from K. McIver, University of Maryland). A colony obtained from LB agar was used to inoculate three 2 liter baffled flasks each containing 500 ml ZYP5052 autoinduction medium (Studier, 2005). The cells were cultured at 37°C for 24 h. After harvesting cells via centrifugation (10 min, 6,200 × *g*, 4°C), the cell pellet was washed twice with 50 ml 100 mM NaH₂PO₄ pH 8.0 and stored at -80°C. The cell pellet was resuspended in 35 ml Buffer 1 (20 mM imidazole, 20 mM NaH₂PO₄, 500 mM NaCl, pH 7.4) containing Complete EDTA-free proteinase inhibitor and cell membranes were ruptured during five passages through a French press (large cell, set on 1,000 psi). The sample was centrifuged at 6,200 × *g* at 4°C for 10 min. The soluble material was transferred to a new tube and centrifuged for 20 min at 20,000 × *g* and 4°C before applying to 2 ml NTA-Ni resin prewashed with Buffer 1. After binding for 1 h at 4°C, the resin was washed in batch using 8 ml Buffer 1. The resin was transferred to a gravity-flow column and subsequently washed with 25 ml Buffer 1. PEPCK was eluted from the resin with 10 ml Buffer 2 (500 mM imidazole, 20 mM NaH₂PO₄, 500 mM NaCl, pH 7.4). 20 µl Ni chelation buffer (250 mM EDTA, 50 mM NaH₂PO₄) was added per ml fraction and fractions containing HisPEPCK were dialyzed four times against 1 L Buffer 3 (20 mM Tris-Cl pH 7.4, 1 mM EDTA, 100 mM KCl). The induction and purification yielded 100 mg His-PEPCK.

Synthesis of ³²P-PEP

³²P-PEP was synthesized and isolated according to methods established previously (Mattoo and Waygood, 1983) using γ-³²P-ATP (PerkinElmer, Waltham, Massachusetts) and PEPCK. 0.5 ml reactions containing 50 mM HEPES pH 7.5, 12.5 mM KF, 5 mM MgCl₂, 1 mM oxaloacetate, 500 µg PEPCK, 50 µM γ-³²P-ATP (2 mCi ml⁻¹, 10 Ci

mmol⁻¹) were incubated for 5 min at room temperature (21°C). The reaction was diluted 10-fold with ddH₂O, and the newly synthesized ³²P-PEP was isolated via anion exchange chromatography using AG-1-X8 Bicarbonate resin. The hydroxide form of the AG-1-X8 resin (BioRad) was converted to the bicarbonate form according to the product manual. The PEP reaction was added to 2 ml of resin and ³²P-PEP was eluted using a step-wise gradient consisting of 5 ml 0.3M, 0.4 M, 0.6 M, and 0.7 M triethylammonium bicarbonate. ³²P-PEP was present in fractions containing 0.6 M and 0.7 M triethylammonium. The fractions were concentrated five-fold via vacuum centrifugation in a vacufuge (Eppendorf, Hamburg, Germany).

In vitro phosphotransfer assay

Phosphotransfer assays were conducted using purified His₆-EI, His₆-HPr, GlcT-His₆ and AtxA-His₆. Reactions contained 3 µg His₆-EI, 1.6 µg His₆-HPr, 2 µg AtxA-His₆ and 2 µg GlcT-His₆ in a total volume of 30 µl buffer (50 mM Tris-Cl pH 7.4, 5 mM MgCl₂). ³²P-PEP (0.5 µCi, 10 Ci mmol⁻¹) was added to a final concentration of 10 µM and the solutions were incubated for 30 min at 37°C. Reactions were quenched by adding SDS loading buffer (final concentration of loading buffer was 5% glycerol, 100 mM DTT, 2% SDS, 40 mM Tris-Cl pH 7.4). Samples were subjected to SDS-PAGE (12% of poly-acrylamide SDS gel). The gels were dried and exposed to a phosphorimaging screen to detect ³²P-labeled protein. The screens were scanned using a STORM 840 scanner and analyzed with ImageJ software.

β-galactosidase assay

One-ml samples were collected from cultures grown to late exponential phase. Cell pellets were resuspended in 1 ml Z-buffer (60 mM NaH₂PO₄ · 7H₂O, 40 mM NaH₂PO₄ · H₂O, 10 mM KCl, 1 mM MgSO₄ · 7H₂O, 50 mM β-mercaptoethanol [added just prior to use]), transferred to tubes containing 400 µl 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK), and beaten for 1 min using a Mini BeadBeater (BioSpec Products) to lyse the cells. Lysates were centrifuged at 10,000 × *g* to pellet debris, and supernatant fractions were used to assay β-galactosidase activity. Liquid β-galactosidase assays were performed as previously described by Miller et al. (1972). To determine relative activity of the experimental samples, the activity obtained for each experimental strain was divided by the activity obtained for the parent, or control strain, setting the parent/control to one. Figures show data averaged from at least three independent cultures. Error bars indicate one standard deviation.

Western blot analysis

Four milliliter culture samples were collected via centrifugation at 21,130 × *g*. Culture supernates and cell pellets were separated and processed immediately or stored at -80°C. Cell pellets were resuspended in 550 µl KTE-PIC (10 mM Tris-HCl pH 8.0, 100 mM KCl, 10% ethylene glycol and EDTA-free complete proteinase inhibitor) and transferred to tubes containing 400 µl 0.1 mm zirconia/silica beads (BioSpec Products) and beaten for two min using a Mini BeadBeater (BioSpec Products) to lyse the cells. Lysates were centrifuged at 10,000 × *g* and the soluble fraction collected. SDS loading buffer (final concentration of loading buffer was 0.05% of bromophenol blue, 0.1M DTT, 10% of glycerol, 2% of SDS and 5 mM Tris-Cl pH 6.8) was added to the soluble fractions.

Suspensions were boiled and subjected to SDS-PAGE. Proteins were transferred to PVDF membranes, blocked with 5% of milk in TBS-T (20mM Tris base, 137 mM NaCl, 0.1% of Tween 20; pH 7.6) and probed with the appropriate primary antibodies. 1:1,000 for anti-EF and anti-LF sera, and anti-PA antibody (List Labs, Campbell, California). His₆-tagged proteins were detected using THETMHis antibody (Genscript, Piscataway, NJ, USA) diluted 1:5,000 in TBST. RNA polymerase β subunit was used as a load control and was probed with an antibody generated by ThermoFisher (Rockford, IL). Membranes were washed three times for five min each with TBST and probed with an HRP-conjugated secondary antibody. Membranes were washed again and developed using the SuperSignal West Dura chemiluminescent substrate (Thermo Scientific).

Preparation of vegetative *B. anthracis* cells for I.V. injection

B. anthracis spores ($\sim 10^7$) were incubated in 1 ml BHI at 37°C shaking for 15 min. The entire spore outgrowth was transferred to 30 ml CACO₃ for 4 h to an OD₆₀₀ of 0.4–0.6. Cells were collected using a 0.22 μ m pore-size filter unit and rinsed twice with 25 ml Dubelco's phosphate buffered saline (DPBS) without calcium or magnesium. Cells were resuspended in DPBS to an OD₆₀₀ of ~ 0.4 . Prior to infection, an aliquot of the resuspension was diluted and plated on LB agar to determine the final inoculation dose (in CFU ml⁻¹). One milliliter syringes with 27 gauge needles were loaded with 100 μ l vegetative cell suspension.

Mouse infections

All mouse protocols were approved by The University of Texas Health Science Center Institutional Animal Care and Use Committee and performed using accepted veterinary standards. About 7–8-week-old female A/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen-free vivarium at The University of Texas Health Science Center. Food and water were supplied to the mice *ad libitum*. The mice were housed 3–5 per cage and were allowed to acclimate to their surroundings for 72 h prior to being used in experiments. Mice were sedated with 3–6 mg kg⁻¹ acepromazine 5–10 min prior to injection. Mice were infected intravenously using 27 gauge needles (Becton, Dickinson and Company, Franklin Lakes, NJ). The tail vein was injected with 100 μ l containing approximately 1,000 heat sensitive colony forming units. Mice were monitored two to six times per day for seven days according to the approved protocol. When mice presented with multiple signs of disease and were determined moribund, they were sacrificed and the liver, lungs and spleen were collected to determine CFU per organ.

CFU determination and statistics

Organs from deceased mice were collected, weighed and resuspended in sterile PBS and 400 μ L 2.3 mm diameter zirconia/silica beads (BioSpec Products, Barlettsville, OK). Tissues were homogenized by bead beating for 1 min, followed by 1 min on ice and again bead beating for 1 min. Homogenates were diluted and plated on LB agar and incubated at 30°C overnight. Plates containing 30–300 colonies were counted and used to calculate the CFU g⁻¹ tissue. A two-sample permutation test (Berry et al., 2019) was performed to determine the significance of the differences in CFU per g and survival curves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Toxin production by parent and mutant strains. Culture lysates and supernates were obtained at late exponential phase (4 h) of growth from cultures grown in CACO₃. Samples of culture supernatants were concentrated 10X and subjected to SDS-PAGE followed by western blot with α -LF serum, and α -THETMHis mAb to detect HPr and EI. *ptsHI* (UT439) was complemented with EV (empty vector pAW285) or genes *ptsH* (pUTE1143), *ptsI* (pUTE1144) or *ptsHI* (pUTE1145) as indicated.

Fig. 2.

AtxA activity at the *lef* promoter in the presence of PTS sugars. Cultures of the *B. anthracis* reporter strain UT376(pUTE991) were grown in CACO₃ containing 0.1% of glycerol and 0.1% of added sugar as indicated. Cells were collected from cultures at late exponential phase (4 h growth) for AtxA activity assays and western blotting with α -THETMHis antibody. AtxA activity was assessed as β -galactosidase activity from a *Plef-lacZ* transcriptional fusion. Data represent the average of three independent experiments. Error bars represent standard deviation.

Fig. 3.

In vitro phosphorylation assay with the PTS proteins HPr and EI. Proteins present in each lane are indicated by '+'. Hexa-His EI, HPr, GlcT, and AtxA were induced in *B. anthracis* and purified via nickel-affinity purification. ³²P-PEP was mixed with the proteins and incubated at 37°C for 30 min.

A. Samples were separated on 10% of poly-acrylamide-SDS gels and stained with Coumassie blue.

B. The proteins were subjected to SDS-PAGE, dried and exposed to a phosphor-imaging screen. Proteins present in each lane are indicated by the tables. ¹Ten-fold more AtxA was added to the reaction. ²Purified AtxA was treated with calf-intestine alkaline phosphatase followed by removal of the phosphatase prior to incubation.

Fig. 4.

AtxA activity at the *lef* promoter in the absence of the PTS. *B. anthracis lef-lacZ* reporter strains UT376(pUTE991)(PTS⁺), UT417(pUTE991)(PTS⁻) and UT408 (PTS⁻) were grown in CACO₃. Strains harboring pUTE991 express *atxA* from an IPTG-inducible promoter. Strain UT408 expresses *atxA* from its native promoter. Cells were collected at late exponential phase for β-galactosidase assays (AtxA activity) and for western blotting of cell lysates with α-THETMHis antibody (AtxA level). β-galactosidase activity represents the average of three experiments. Error bars represent standard deviation. A representative western is shown.

Fig. 5.

Transcription of *PatxA-lacZ*. Cultures of parent, PTS⁻ mutant, and PTS complementation strains carrying the *PatxA-lacZ* reporter pUTE843 were grown in CACO₃ to late exponential phase and collected for β -galactosidase assays and western blotting.

A. β -gal activity from the *PatxA-lacZ* transcriptional fusion. **p* value < 0.05, ***p* value < 0.01.

B. Western blot of concentrated culture supernatants with anti-LF antibody.

Fig. 6.

Transcription of *PatxA-lacZ* in the presence of PTS sugars. Cultures of the parent strain carrying the *PatxA-lacZ* reporter pUTE843 were grown in CACO₃ containing 0.1% of glycerol and 0.1% of added sugar as indicated. Cells were collected from cultures at late exponential phase (4 h growth) for β -galactosidase assays. Data represent the average of three independent experiments. Error bars indicate one standard deviation.

Fig. 7.

Relative expression of *lacZ* driven by *atxA* promoter regions P1+P2 (–770 bp to translational start) and P1 alone (–72 bp to translational start).

A. Schematic representation of *atxA* promoter region.

B. Cultures of *B. anthracis* strains harboring *PatxA-lacZ* constructs were grown in casamino acids medium containing 0.2% of glucose and 0.9% of bicarbonate in 5% of atmospheric CO₂. Cells were collected from cultures at late exponential phase (4 h growth) for analysis of β-galactosidase activity representative of activity from the *atxA* promoter regions.

Data represent the average of three independent experiments. Error bars indicate standard deviation.

Fig. 8.

Virulence of parent and *ptsHI* mutants.

A. Survival curves of mice infected intravenously with vegetative *B. anthracis* are shown. A/J mice were injected i.v. with 2.75×10^3 CFU of the parent (black solid line; $n = 6$), 4.5×10^3 CFU of parent constitutively expressing *atxA* (ANR-1 *atxA**) (black dashed line; $n = 6$), 3×10^3 CFU of *ptsHI*-null (gray solid line; $n = 6$), or 3×10^3 CFU of *ptsHI atxA** (gray dashed line, $n = 6$) vegetative cells.

B. CFU g⁻¹ of tissue collected. Two sample permutation analysis was performed to compare each strain to the other. ** $p < 0.05$.

Fig. 9. Alignment of PTS regulation domains. Modified from Stulke et al. (1998). Amino acid sequences denoted 'I' correspond to PRDs proximal to the amino-terminus. Sequences denoted 'II' correspond to PRDs, proximal to the carboxy-terminus. Bracketed groups 1, 2 and 3 indicate: PCVRs in Gram-positive organisms; PRD-containing transcription activators; and PRD-containing transcription antiterminators respectively. Highly conserved

amino acids are highlighted in black. Residues with similarity are highlighted in gray. Red boxes indicate published data suggesting phosphorylation of the indicated histidine.

Table 1

B. anthracis strains and plasmids used in this study.

Name	Description	Source
<i>Strains</i>		
ANR-1	<i>B. anthracis</i> , Parent strains, pXO1 ⁺ , pXO2 ⁻	Welkos et al. (2001)
UT374	ANR-1-derivative <i>atxA</i> -null	Dale et al. (2012)
UT376	ANR-1-derivative, <i>lef</i> promoter - <i>lacZ</i> fusion (<i>P</i> <i>lef-lacZ</i>) at native <i>lef</i> locus, <i>atxA</i> -null	Hammerstrom et al. (2011)
UT408	ANR-1 derivative, <i>lef</i> promoter - <i>lacZ</i> fusion (<i>P</i> <i>lef-lacZ</i>) at native <i>lef</i> locus, <i>ptsHI</i> -null	This work
UT417	UT376-derivative, <i>lef</i> promoter - <i>lacZ</i> fusion (<i>P</i> <i>lef-lacZ</i>) at native <i>lef</i> locus, <i>atxA</i> -null, <i>ptsHI</i> -null	This work
UT433	ANR-1-derivative <i>acpA</i> -null	This work
UT439	ANR-1 derivative, <i>ptsHI</i> -null	This work
UT447	UT374-derivative, <i>atxA</i> -null, GBAA_2500 promoter region fused to <i>atxA-His₆</i> and incorporated in to the <i>plcR</i> locus	This work
UT448	UT439-derivative, <i>ptsHI</i> -null, <i>atxA</i> -null	This work
UT449	UT448-derivative, <i>ptsHI</i> -null, <i>atxA</i> -null, GBAA_2500 promoter region fused to <i>atxA-His₆</i> and incorporated in to the <i>plcR</i> locus	This work
<i>Plasmids</i>		
pAW285	Xylose-inducible expression vector; Cm ^r	Han and Wilson (2013)
pUTE657	Expression vector derived from pDR111 and pBC16 with IPTG-inducible <i>Phyper-spank</i> , Spec ^r Amp ^r	Hammerstrom et al. (2011)
pUTE839	pHT304-18z-derived <i>atxA</i> promoter- <i>lacZ</i> fusion vector; contains sequence from -770 to +99	Dale et al. (2012)
pUTE843	pHT304-18z-derived <i>atxA</i> promoter- <i>lacZ</i> fusion vector; contains sequence from -72 to +99	Dale et al. (2012)
pUTE991	pUTE657-derived expression vector for AtxA-His ₆ (6xHis-epitope on the C-terminus of AtxA) the <i>atxA</i> ribosome binding site and coding region controlled by <i>Phyper-spank</i>	Hammerstrom et al. (2011)
pUTE1034	pUTE657 - derived expression vector for HPr and EI; the <i>ptsH</i> ribosome binding site, <i>ptsH</i> coding region, <i>ptsI</i> ribosome binding site, and <i>ptsI</i> coding region controlled by <i>Phyper-spank</i>	This work
pUTE1098	pUTE657-derivative expression vector for GlcT-His ₆ (C-terminally tagged GlcT); the AtxA ribosome binding site, <i>glcT</i> coding region controlled by <i>Phyper-spank</i>	This work
pUTE1141	pUTE657-derivative expression vector for His ₆ -EI (N-terminally tagged EI); the AtxA ribosome binding site, <i>ptsI</i> coding region controlled by <i>Phyper-spank</i>	This work
pUTE1142	pUTE657-derivative expression vector for His ₆ -HPr (N-terminally tagged HPr); the AtxA ribosome binding site, <i>ptsH</i> coding region controlled by <i>Phyper-spank</i>	This work
pUTE1143	pAW285-derived, His ₆ -HPr (N-terminally tagged HPr)	This work
pUTE1144	pAW285-derived, His ₆ -EI (N-terminally tagged EI)	This work
pUTE1145	pAW285-derived, His ₆ -HPr His ₆ -EI (N-terminally tagged HPr and EI)	This work
pUTE1151	pAW285-derived, His ₆ -HPr His ₆ -EI (N-terminally tagged HPr and EI) HPr H14A	This work

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Name	Description	Source
pUTE1152	pAW285-derived, His ₆ -HPt His ₆ -EI (N-terminally tagged HPt and EI) HPt H14D	This work
pUTE1153	pAW285-derived, His ₆ -HPt His ₆ -EI (N-terminally tagged HPt and EI) HPt S46A	This work
pUTE1154	pAW285-derived, His ₆ -HPt His ₆ -EI (N-terminally tagged HPt and EI) HPt H14A S46A	This work
pUTE1155	pAW285-derived, His ₆ -HPt His ₆ -EI (N-terminally tagged HPt and EI) HPt H14D S46A	This work

Ap^r, ampicillin resistant; Cm^r, chloramphenicol; Km^r, kanamycin; Sp^r, spectinomycin.