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Genetic analysis of petrobactin transport in *Bacillus anthracis*

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Summary

Iron acquisition mechanisms play an important role in the pathogenesis of many infectious microbes. In *Bacillus anthracis*, the siderophore petrobactin is required for both growth in iron depleted conditions and for full virulence of the bacterium. Here we demonstrate the roles of two putative petrobactin binding proteins FatB and FpuA (encoded by GBAA5330 and GBAA4766, respectively) in *Bacillus anthracis* iron acquisition and pathogenesis. Markerless deletion mutants were created using allelic exchange. The Δ*fatB* strain was capable of wild-type levels of growth in iron depleted conditions, indicating that FatB does not play an essential role in petrobactin uptake. In contrast, Δ*fpuA* bacteria exhibited a significant decrease in growth under low iron conditions when compared to wild-type bacteria. This mutant could not be rescued by the addition of exogenous purified petrobactin. Further examination of this strain demonstrated increased levels of petrobactin accumulation in the culture supernatants, suggesting no defect in siderophore synthesis or export but, instead, an inability of Δ*fpuA* to import this siderophore. Δ*fpuA* spores were also significantly attenuated in a murine model of inhalational anthrax. These results provide the first genetic evidence demonstrating the role of FpuA in petrobactin uptake.

Keywords

anthrax; iron transport; petrobactin

Introduction

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive, spore-forming bacterium that readily infects a variety of mammals. In the environment, *B. anthracis* exists primarily as dormant spores, which are the infectious form of this microorganism (Dixon *et al.*, 1999). Spores can enter a mammalian host through three distinct routes: cutaneous, gastrointestinal, or inhalational, each leading to distinct symptoms and disease course (Dixon *et al.*, 1999, Inglesby *et al.*, 1999, Inglesby *et al.*, 2002, Doganay & Welsby, 2006). Infection through the inhalational route leads to the most severe and potentially fatal form of the disease (Dixon *et al.*, 1999, Inglesby *et al.*, 1999, Inglesby *et al.*, 2002, Doganay & Welsby, 2006). Upon entry into the host, spores are phagocytosed by resident macrophages and transported to the regional lymph nodes (Dutz & Kohout, 1971, Dutz & Kohout-Dutz,

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1981, Guidi-Rontani *et al.*, 1999, Dixon *et al.*, 2000). While in the intracellular environment, *B. anthracis* spores germinate, transforming into rapidly dividing vegetative cells (Dixon *et al.*, 2000). It is thought that these bacilli begin to multiply within macrophages prior to the induction of cell death and escape from these immune cells (Dixon *et al.*, 2000). Once out of the macrophages, *B. anthracis* vegetative cells continue to grow rapidly in the bloodstream. Although symptoms during these early stages are relatively mild, the bacteria soon replicate to very high levels, approaching 10^8 organisms per milliliter of blood. Production of two binary toxins by these bacteria can lead to serious complications, including hypotension, shock, and eventually death (Dixon *et al.*, 1999).

Iron acquisition mechanisms are vital for the survival and pathogenesis of many infectious microbes, including *B. anthracis* (Ratledge & Dover, 2000, Faraldo-Gomez & Sansom, 2003, Wandersman & Delepelaire, 2004). In the mammalian host the concentration of free iron is limiting, with most of the iron sequestered into complexes such as transferrin and hemoglobin (Ratledge & Dover, 2000, Glanfield *et al.*, 2007). *B. anthracis*, and other pathogenic microbes, succeed in such environments by utilizing various iron acquisition methods including the production of siderophores and a variety of iron transport systems (Ratledge & Dover, 2000, Faraldo-Gomez & Sansom, 2003, Glanfield *et al.*, 2007). Siderophores are high-affinity iron binding molecules that are secreted by bacteria to sequester insoluble ferric iron from host complexes (Ratledge & Dover, 2000). *B. anthracis* produces two different siderophore molecules, bacillibactin and petrobactin (Cendrowski *et al.*, 2004, Koppisch *et al.*, 2005, Wilson *et al.*, 2006). Although the genes encoding bacillibactin biosynthesis are induced during iron starvation, this molecule is not required for growth of *B. anthracis* under iron limiting conditions (Cendrowski *et al.*, 2004). *B. anthracis* strains deficient in bacillibactin biosynthesis are fully virulent in a murine model of anthrax infection (Cendrowski *et al.*, 2004). In contrast, petrobactin biosynthesis has been clearly associated with anthrax pathogenesis. Strains unable to synthesize this siderophore exhibit attenuated growth under iron depleted conditions and are highly attenuated for murine virulence (Cendrowski *et al.*, 2004, Abergel *et al.*, 2006, Lee *et al.*, 2007, Pfleger *et al.*, 2007).

Once secreted siderophores bind extracellular iron they must be reacquired for the organism to take advantage of this system. Uptake of iron bound siderophores typically occurs through specific bacterial membrane receptor complexes (Faraldo-Gomez & Sansom, 2003). The specific transport system involved in petrobactin uptake has yet to be identified. Recently, Zawadzka *et al.* reported the identification of two *B. cereus* proteins, FatB and FpuA, capable of binding multiple forms of petrobactin *in vitro* (Zawadzka *et al.*, 2009a). The *B. anthracis* homologs of these proteins are encoded by genes designated GBAA5330 and GBAA4766, and have high homology to the *B. cereus* proteins (95% and 99% amino acid identity, respectively). Expression of these genes were also identified as significantly up-regulated by *B. anthracis* during iron starvation (Carlson *et al.*, 2009). In the current study, we sought to elucidate the role of these two petrobactin binding proteins in the pathogenesis of *B. anthracis*.

Results

B. anthracis Δ*fpuA* exhibits a growth defect in iron depleted media

We sought to determine the role of two genes encoding homologs of the *B. cereus* petrobactin binding proteins FatB and FpuA (encoded by GBAA5330 and GBAA4766, respectively) in *B. anthracis* iron acquisition. *B. cereus* homologs of these proteins are able to bind petrobactin *in vitro* (Zawadzka *et al.*, 2009a); however the function of these proteins in bacterial iron acquisition and pathogenesis remains to be elucidated. To accomplish this mutant *B. anthracis* strains lacking either GBAA4766 (Δ*fpuA*), GBAA5330 (Δ*fatB*), or both

genes (Δ*fpuA* Δ*fatB*) were created by markerless gene replacement (Fig. 1 and Table 1). PCR was used to screen all mutant strains for the expected gene deletions, as well as the presence of the virulence plasmid pXO1, prior to use in experiments (data not shown).

We hypothesized that if these proteins were involved in the uptake of petrobactin, mutants lacking the respective genes would exhibit significant growth defects in iron depleted media (IDM), similar to a mutant that cannot synthesize petrobactin. To test this, vegetative bacilli of wild-type *B. anthracis*, Δ*fpuA*, Δ*fatB*, and the Δ*fpuA* Δ*fatB* double mutant were inoculated into iron-depleted media (IDM) at an initial OD_{600} of 0.05 and growth was monitored hourly by measurement of optical density at 600nm. A mutant strain deficient for petrobactin biosynthesis (Δ*asbABCDEF*) was also included as a control (Cendrowski *et al.*, 2004, Lee *et al.*, 2007). Cultures of wild-type *B. anthracis* are capable of rapid growth under these conditions, reaching high density within four hours (Fig. 2A, solid diamonds). As previously reported, the petrobactin deficient mutant exhibited a growth defect in this medium, emphasizing the importance of this siderophore in the ability of *B. anthracis* to replicate in low iron conditions (Fig. 2A, open circles) (Cendrowski *et al.*, 2004). The Δ*fatB* mutant grew as well as wild-type *B. anthracis* in IDM, suggesting that this gene is not required for growth in low iron conditions (Fig. 2A, solid squares). In sharp contrast, both mutant strains lacking *fpuA* (Δ*fpuA* and Δ*fpuA* Δ*fatB*) exhibited severe growth defects in IDM, with each growing to levels nearly identical to the petrobactin-deficient strain (Fig. 2A). Introduction of the Δ*fpuA* mutant allele into the petrobactin biosynthesis deficient strain (Δ*asbABCDEF*) did not result in a stronger phenotype, further suggesting a role for FpuA in petrobactin dependent iron acquisition (data not shown). It should also be noted that none of the gene deletions studied here resulted in a general growth defect and all mutant strains grew equally well in brain heart infusion broth (Fig. 2B). Importantly, plasmid based expression of *fpuA* driven by its native promoter resulted in complementation of Δ*fpuA* as demonstrated by increased growth in IDM (Fig. 2C). The complementation studies were performed at room temperature as the pBKJ236 vector can not replicate at 37°C. These data indicate that FpuA, but not FatB, is necessary for *B. anthracis* growth in iron depleted conditions and Δ*fpuA* mutants are unable to efficiently utilize iron at the low levels available in this medium.

Δ*fpuA* mutants are able to produce and secrete petrobactin

It was possible that the growth defect observed for the Δ*fpuA* mutant was due to an unexpected loss of the ability to produce or secrete petrobactin, rather than an inability to import the siderophore. Since strains deficient in petrobactin production can be rescued by the addition of exogenous petrobactin to the media (Lee *et al.*, 2007), we sought to determine if the Δ*fpuA* mutants could also be complemented in the same manner. Wild-type, Δ*fpuA*, and Δ*asbABCDEF* spores were inoculated into IDM with or without supplemental petrobactin (2.5μM) and growth was monitored by increase in optical density (600nm) over a ten hour time course (Fig. 3). These experiments were performed in triplicate in 96-well plates due to the limited availability of purified petrobactin. All strains used in this study germinated with equal efficiencies (data not shown). As expected, wild-type spores exhibited significant growth in IDM with or without supplemental petrobactin. As previously observed, the Δ*asbABCDEF* strain, while unable to grow in IDM, grew at a rate similar to wild-type in cultures supplemented with petrobactin. Conversely, the Δ*fpuA* mutant was not rescued by the addition of supplemental petrobactin. This finding is consistent with the hypothesis that this protein is required for the uptake of iron-bound petrobactin molecules.

If FpuA is indeed the petrobactin receptor, we hypothesized that the Δ*fpuA* mutant would exhibit increased levels of petrobactin in culture supernatants due to their inability to import this molecule. The Arnow assay was used to measure the concentration of total catechols in

culture supernatants (Arnow, 1937). This colormetric assay measures the presence of both petrobactin and its immediate biosynthetic precursor, 3, 4-dihydroxybenzoic acid (DHB) (Cendrowski *et al.*, 2004, Garner *et al.*, 2004). Vegetative bacilli from wild-type, Δ*fpuA*, and Δ*asbABCDEF* were inoculated into IDM, as described previously. Supernatants were isolated from each of the cultures hourly for six hours post-inoculation and analyzed for catechol levels (Fig. 4A). No signal could be detected above background from supernatants isolated during the first two hours of growth. Importantly, supernatants from Δ*asbABCDEF* cultures, which should not contain any petrobactin, did not exhibit a reaction in this assay at any of the time points tested. The lack of detectable signal in supernatants from these mutants suggests that, at least at these timepoints, petrobactin and 3, 4-DHB are the only catechols in the media. Wild-type *B. anthracis* cultures did show an increase in catechol levels in supernatants over the time course studied. The Δ*fpuA* mutant exhibited significantly higher levels of extracellular catechols, presumably petrobactin, at all time points when compared to wild-type bacteria (Fig. 4A).

Since the Arnow assay is able to detect multiple molecules, we next sought to measure petrobactin levels directly from culture supernatants. Supernatants obtained from both wild-type and Δ*fpuA* mutant cultures grown for six hours were normalized to equivalent OD_{600} and examined using high performance liquid chromatography (HPLC). Peaks corresponding in retention time (37.5 min), UV absorbance spectra (peaks at 260 and 290 nm), and mass spectra (*m/z*: $[M+2H]^{2+}$= 360 and $[M+2H-H_2O]2+$= 351) to an authentic petrobactin standard were observed in both WT and Δ*fpuA* traces. HPLC peak integration revealed an approximate 65% increase of petrobactin accumulation per OD_{600} in Δ*fpuA* supernatants over that observed in wild type samples (Fig. 4B). Importantly, this peak was not observed in the Δ*asbABCDEF* strain which is unable to produce petrobactin (data not shown).

In order to examine the possibility that the altered petrobactin levels were due to increased production, we performed quantitative real-time PCR (Q-PCR) to determine levels of *asbA* expression after six hours of growth in IDM. Loss of *fpuA* expression in either single or double mutant strains did not lead to a significant increase in the transcription of this gene ($\log_2$ [fold change] = 0.6 ± 0.15). While it is more likely that the increase in petrobactin accumulation detected in Δ*fpuA* culture supernatants was due to the inability of this strain to import iron-bound petrobactin, we can not exclude the possibility that this slight increase in transcription is responsible for this observation.

Gallium associated growth inhibition requires FpuA

One test that has been used to show siderophore transport in other systems is gallium sensitivity. Gallium has been shown to bind to various siderophores, including petrobactin (Banin *et al.*, 2008, Zawadzka *et al.*, 2009b). Gallium is toxic to most bacterial cells and transport of siderophore-gallium complexes across the membrane often leads to cell death (Olakanmi *et al.*, 2000, Banin *et al.*, 2008). It is, therefore, possible to correlate defects in siderophore transport with increased resistance to gallium. This system was used here to more definitively show a decrease in petrobactin in mutant strains. Wild-type, Δ*asbABCDEF*, and Δ*fpuA* strains were grown in IDM with or without 20μM gallium sulfate. Growth was monitored by change in optical density (600nm) over time. The addition of gallium to cultures of wild-type *B. anthracis* led to a significant decrease in growth over time, with a peak inhibition of 40% observed at two hours (Table 2). As would be expected, growth of the petrobactin deficient strain Δ*asbABCDEF* was not significantly impaired by the addition of gallium to the culture media (Table 2). Finally, deletion of the putative petrobactin binding protein, FpuA, protects *B. anthracis* from gallium induced growth inhibition, suggesting that this strain is defective in transport of gallium across the membrane. The fact that strains lacking the putative receptor have the same phenotype as those deficient in petrobactin biosynthesis supports a decreased transport of petrobactin in

these mutants. These data provide further evidence that FpuA is required for transport of petrobactin into *B. anthracis*.

Role of FpuA in *B. anthracis* virulence

Since it is known that mutants unable to synthesize petrobactin exhibit significant attenuation in murine virulence (Cendrowski *et al.*, 2004, Pfleger *et al.*, 2008), we hypothesized that strains unable to import this siderophore would exhibit similar levels of attenuation. To test this hypothesis, we employed a murine model of inhalational anthrax. DBA/2J mice were used for these studies as these mice are known to be susceptible to *B. anthracis* Sterne strain (Welkos *et al.*, 1986). DBA/2J mice (n = 8) were inoculated intratracheally with wild-type, Δ*fpuA*, or Δ*asbABCDEF* spores and monitored over the course of fourteen days. When inoculated with 1.5×10^5 spores, mice receiving *B. anthracis* Δ*fpuA* exhibited increased survival compared to those infected with wild-type spores (Fig. 5). At this dose, all mice infected with wild-type spores succumbed to infection within five days post infection, while 87.5% of mice infected with Δ*fpuA* ($p = 0.0006$) survived the full two week experiment, with the only death occurring on the first day post-infection (Fig. 5A). In fact, even at a ten-fold higher dose (1.5×10^6 spores/mouse), the Δ*fpuA* mutant showed attenuated murine virulence compared to wild-type spores (Fig. 5B). At this dose, 75% of mice infected with Δ*fpuA* ($p = 0.01$) showed no sign of disease after fourteen days, while all of the mice infected with wild-type spores succumbed to infection by day four (Fig. 5B). These results are similar to what was observed for the mutant strain defective in petrobactin biosynthesis (data not shown and (Cendrowski *et al.*, 2004)). In order to calculate an accurate LD_{50} for both the Δ*fpuA* and Δ*asbABCDEF* strains, mice were infected with 1.5×10^7 spores of each of the mutants. Even at this dose four of eight mice infected with Δ*fpuA* failed to show any sign of disease during the course of the infection. The LD_{50} of Δ*fpuA* was determined to be 2.2×10^7 spores/mouse, approximately three logs higher than that of wild-type spores (Table 3). Interestingly, when mice were infected with a similar dose of the petrobactin biosynthesis deficient strain, Δ*asbABCDEF*, 100% of mice succumbed to disease by day four post-infection, indicating that Δ*fpuA* is slightly more attenuated than this strain ($p = 0.0002$). In fact, the Δ*asbABCDEF* mutant spores exhibited an LD_{50} nearly ten fold lower than that of the Δ*fpuA* mutants (Table 3).

Discussion

The ability of a microorganism to survive in low iron environments, such as a mammalian host, typically requires specialized systems of iron acquisition and uptake. Although *B. anthracis* encodes the biosynthesis machinery for two siderophores, only one of these, petrobactin, is required for efficient bacterial growth in low iron conditions and for full virulence in a murine model of anthrax infection (Cendrowski *et al.*, 2004, Lee *et al.*, 2007). Since this siderophore plays such an important role in *B. anthracis* pathogenesis, we sought to elucidate the role of two proteins recently implicated in petrobactin binding. We created deletion mutants lacking two putative siderophore binding proteins, FatB and FpuA, encoded by GBAA5330 and GBAA4766, respectively. *B. cereus* homologs of these proteins were recently shown to have the ability to bind to petrobactin, however their role in the pathogenesis of the bacterium remained untested. (Zawadzka *et al.*, 2009a). Here we have demonstrated the role of these genes during growth in low iron conditions as well as their effect on murine virulence.

Despite the ability of both FpuA and FatB to bind petrobactin *in vitro*, only FpuA appears important for growth of *B. anthracis* under iron limiting conditions. Mutants lacking *fpuA* exhibits a severe growth defect when either vegetative bacteria or spores were inoculated into IDM (Figs. 2, 3A). These cultures also exhibit growth kinetics nearly identical to those of *B. anthracis* strains unable to synthesize petrobactin (Fig. 2, 3A and (Cendrowski *et al.*,

2004,Lee *et al.*, 2007)). Importantly, although vegetative bacilli of these strains (Δ*asbABCDEF* and Δ*fpuA*) are capable of some growth in IDM (Fig. 2A), these strains are not capable of outgrowth from spores in this medium (Fig. 3). Although the growth kinetics were slightly slower than wild-type bacteria, replacement of this gene *in trans* did restore the ability of the bacteria to grow exponentially in IDM (Fig. 2C). It should be noted, a *B. anthracis* strain with deletions in both putative petrobactin binding proteins (Δ*fpuA* Δ*fatB*) grew to the same levels as Δ*fpuA* single mutants (Fig. 1 and data not shown). In contrast, mutants lacking FatB are not significantly impaired in their ability to grow in IDM (Fig. 2). In fact, growth curves for the Δ*fatB* mutant in IDM were nearly identical to those of wild-type bacilli (Fig. 2, filled squares). These data indicate that FatB does not play a role in uptake of petrobactin by *B. anthracis*. Curiously, the FatB homolog in *B. subtilis* has recently been shown to be required for uptake of petrobactin (Zawadzka *et al.*, 2009b). It should be noted that *B. subtilis* does not make petrobactin, nor does it have an ortholog of *fpuA*. A role for this protein in iron metabolism in *B. cereus* remains to be tested.

Importantly, we have shown that Δ*fpuA* bacteria are not deficient in petrobactin synthesis or secretion, but rather the growth defect of this strain appears to be caused by an inability to use this siderophore. The addition of supplemental petrobactin had no effect on the growth of the Δ*fpuA* strain (Fig. 3B). Addition of excess siderophore is able to restore the growth of *B. anthracis* strains deficient in petrobactin biosynthesis, indicating that the Δ*fpuA* is phenotypically distinct from these strains. Petrobactin production from Δ*fpuA* bacteria was conclusively shown using both the Arnow assay and HPLC (Fig. 4). At the time points tested in the Arnow assay, no signal could be detected in the supernatants of strains unable to synthesize petrobactin, raising the possibility that bacillibactin was not being secreted at these time points. This finding is not surprising given the fact that most bacillibactin secretion has been seen following 24 hour cultures and recently it was shown that bacillibactin secretion was undetectable until the bacteria had grown for 10 or more hours (Wilson *et al.*, 2006,Pfleger *et al.*, 2008,Wilson *et al.*, 2009). Although no catechols were detected using the Arnow assay on Δ*asbABCDEF* supernatants, these molecules were observed from both wild-type bacteria and the Δ*fpuA* mutant. Also, petrobactin was specifically detected in both wild-type and Δ*fpuA* supernatants using HPLC. Interestingly, petrobactin levels were significantly higher in supernatants of Δ*fpuA* cultures than in those of wild-type *B. anthracis*. It is likely that the Δ*fpuA* mutant strain is synthesizing petrobactin at levels comparable to wild-type bacteria, as only a minimal increase was observed in the transcription of the petrobactin biosynthetic operon during growth of this strain in IDM. These results suggest that the increased accumulation of petrobactin in the supernatants of Δ*fpuA* cultures is due to the inability of these strains to import petrobactin, thus trapping the iron-bound siderophore in the extracellular environment and depriving the bacterium of the necessary iron. Strains lacking the putative petrobactin receptor also exhibit a resistance to gallium induced growth inhibition. Since gallium can be bound by petrobactin, these results further support the idea that the Δ*fpuA* mutant is deficient in petrobactin uptake. Taken together, the inability of supplemental petrobactin to restore the growth of Δ*fpuA* mutants, the increased accumulation of petrobactin in the Δ*fpuA* culture supernatants, the increased resistance of Δ*fpuA* to gallium, and the fact that the homologous protein from *B. cereus* binds petrobactin, clearly implicate FpuA in the uptake of iron-bound petrobactin.

Although it was clear that Δ*fpuA* strains were deficient for growth under iron limiting conditions *in vitro*, it was also important to determine the role of this protein during infection. We have shown that the loss of a single putative petrobactin receptor in the Δ*fpuA* strain leads to severe attenuation in this infection model (Fig. 5). The LD_{50} of the Δ*fpuA* mutant was found to be nearly 3,000 times that of wild-type *B. anthracis* Sterne (Table 3). Similar to what was observed in the *in vitro* experiments, no significant difference was seen in the virulence of the double mutant (Δ*fpuA* Δ*fatB*) when compared to the mutant lacking

only *fpuA* (data not shown). The level of attenuation observed for these mutants further emphasizes the importance of iron acquisition through petrobactin and its receptor complex in *B. anthracis* pathogenesis.

The observed attenuation of strains unable to import petrobactin is consistent with published reports on the attenuation of petrobactin biosynthesis mutants (Cendrowski *et al.*, 2004, Pfleger *et al.*, 2008). Although the growth phenotypes of Δ*fpuA* and Δ*asbABCDEF* strains appear very similar in IDM, slight differences in murine virulence were observed between these two strains. The virulence of these two mutant strains appears similar at lower doses, with neither strain killing a significant number of the mice tested. A difference in virulence was observed at higher doses however, and the LD_{50} of Δ*asbABCDEF* mutants was found to be approximately eight fold lower than that of the Δ*fpuA* strain (Table 3). These results indicate that Δ*fpuA* mutants are slightly less virulent than even the petrobactin biosynthesis mutant, leading to the hypothesis that this receptor protein plays a secondary function during mammalian infection. It is possible that this receptor is able to scavenge siderophore molecules produced by other bacterial species, providing the bacterium an increased survival advantage within its host. Uptake of exogenous siderophores has been shown in other bacterial species including *B. cereus* and *Pseudomonas aeruginosa* (Ollinger *et al.*, 2006, Greenwald *et al.*, 2009), however the exact mechanism involved in *B. anthracis* remains to be elucidated.

The data presented here, combined with the fact that FpuA is able to bind petrobactin *in vitro* (Zawadzka *et al.*, 2009a), implicate this protein as a part of the petrobactin uptake system. FpuA has homology to substrate binding proteins from ABC family transporters (Miethke & Marahiel, 2007). In Gram-positive bacteria, these are membrane bound proteins which interact with a transmembrane permease to transport molecules into the bacterium (Miethke & Marahiel, 2007). The identity of the remaining components of this ABC transport system remains to be elucidated. Although some candidate proteins have been identified (Zawadzka *et al.*, 2009a), further study is required to show a role for these proteins in petrobactin uptake.

Iron uptake systems are thought to be strong candidate targets for vaccine and therapeutic treatments, since they are often implicated in pathogen virulence and their surface localization leaves them exposed to extracellular molecules and the immune system (Glanfield *et al.*, 2007). Studies in *E. coli*, *S. pneumoniae* and *S. aureus* have shown that vaccines targeting iron uptake systems can improve immune responses and disease survival in animal infection models (Jomaa *et al.*, 2006, Kuklin *et al.*, 2006, Alteri *et al.*, 2009). It has been postulated that iron uptake proteins from *B. anthracis*, including the two examined here, could serve as vaccine targets (Gat *et al.*, 2006). Antibodies to these proteins were present in the serum of infected guinea pigs, indicating that they are expressed by the bacterium during infection (Gat *et al.*, 2006). Since disruption of a single siderophore binding protein/iron uptake system in the Δ*fpuA* mutant leads to such dramatic phenotypes in *B. anthracis* growth and virulence it is likely that this protein could be a prime target for the development of new therapeutic measures. The data presented here indicate that a therapeutic blocking this receptor could greatly reduce the bacterium's ability to replicate within the low iron environment of the human host. Such a treatment could, hypothetically, allow the host immune system to eliminate the pathogen before bacterial replication and toxin production reach lethal levels.

Given that recombinant forms of *B. cereus* FpuA have been shown to bind petrobactin directly, that these proteins are nearly identical to their *B. anthracis* homologs, and the genetic evidence provided in this study, it is highly likely that FpuA serves as the receptor for petrobactin. In contrast, our data indicate that FatB does not play a role in petrobactin

uptake in *B. anthracis*. Finally, the severe attenuation of Δ*fpuA* strains in a murine model of inhalation anthrax raises the possibility that this protein might be a useful target for the development of new vaccines and therapeutics.

Experimental procedures

Bacterial strains and growth conditions

All of the work described in this manuscript was performed using the Sterne $34F_2$ ($pXO1^+$, $pXO2^-$) strain of *B. anthracis*. Initial spore stocks were prepared as follows. Strains were grown in modified G medium (Kim & Goepfert, 1974) for three days at 37°C with shaking and spores were prepared as previously described (Passalacqua *et al.*, 2006). Spores were stored at room temperature in sterile water and titered by hemacytometer (spores/ml) for animal studies. For all iron depletion studies, iron depleted media (IDM) was used (Cendrowski *et al.*, 2004). Experimental design for growth curves, Arnow assays, and RNA isolation experiments were done as follows. Spores were germinated in brain heart infusion (BHI) broth and incubated overnight with shaking, at room temperature. The following day, vegetative bacilli were diluted 1:100 in fresh BHI and grown 1–2 hours at 37°C. Actively growing bacilli were then washed 3x with phosphate buffered saline (PBS) (Invitrogen) and 3x with IDM to ensure removal of nutrients and potential iron sources carried over from the BHI. Washed bacteria were then used to inoculate cultures of IDM or iron replete media (IRM) [IDM + 20μM ferrous sulfate] at an OD_{600} = 0.05. Experiments performed with petrobactin were started with 1×10^5 spores per well in 96 well plates and performed in a Molecular Devices M2 plate reader. Growth was monitored by change in OD_{600} over ten hours. For gallium resistance experiments, strains were grown in IDM with or without 20μM gallium sulfate and growth was monitored over time. Percent growth inhibition was calculated as ([(OD_{600} IDM+gallium)/(OD_{600} IDM)]*100).

Mutant Construction

Mutant *B. anthracis* strains were constructed using allelic exchange as previously described (Janes & Stibitz, 2006). The Δ*fpuA* and Δ*fatB* single mutants were created in the wild-type Sterne $34F_2$ background by deletion of either GBAA4766 or GBAA 5330, respectively. The double mutant was created by deleting GBAA4766 in the Δ*fatB* single deletion strain. The Δ*asbABCDEF B. anthracis* strain used in these studies has been reported previously (Lee *et al.*, 2007). The sequence of all oligonucleotides used in creating and screening these mutant strains are available on request.

Complementation of gene deletions

fpuA along with the upstream promoter region (120 bp) was cloned into the temperature sensitive plasmid, pBKJ401 (a derivative of pBKJ236 (Janes & Stibitz, 2006)). The resulting vector, p*fpuA*+, was then introduced to Δ*fpuA* by conjugal transfer. Conjugates were selected by screening for erythromycin resistance. Because this vector can not replicate at 37°C, all growth of the plasmid containing strain was performed at 25°C.

Real time RT-PCR

cDNA synthesis was performed using Superscript III (Invitrogen) and 1 μg of total RNA. Real time reactions were performed with a 1:500 final dilution of template cDNA. Primer sets were designed for genes indicated using Primer 3 (Rozen & Skaletsky, 2000) and reactions were carried out on an BioRad ICycler real time machine using SYBR Green (BioRad). The *acoB* gene (GBAA2775) was used as the internal reference, as its expression was previously determined to remain unchanged during iron starvation (data not shown).

Measurement of petrobactin

Extracellular levels of catechols were measured using the Arnow assay (Arnow, 1937). Bacteria were grown in IDM and samples of cultures were removed at the indicated timepoints. Cultures were centrifuged and filtered using 0.2μm filters to remove bacteria. Samples were mixed with equal volumes of 0.5M HCl, nitrate-molybdate reagent (10% sodium nitrate and 10% sodium molybdate), and 1N NaOH. Positive reactions produce a red color and absorbance was determined at 515nm. Samples were normalized to OD_{600} of the original culture and data are presented as percent of wild-type petrobactin levels at the three hour timepoint.

For direct measurement of petrobactin, supernatants were obtained from three cultures of both wild type Sterne and Δ*fpuA* following six hours of growth in IDM. All cultures were diluted with IDM to equal OD_{600} and sterile-filtered. Samples of individual culture supernatants were mixed with nine volumes of LCMS grade methanol and incubated at −20° C for 20 minutes. Methanol crashes were centrifuged at 14,000 × g and 1600 μl of each supernatant was transferred to disposable 1.7 ml tubes for evaporation by vacuum centrifugation. Dried pellets were re-dissolved in 80 μl of ddH_2O and directly injected onto a Beckman Coulter System Gold for HPLC analysis. Peaks were separated using a C_{18} analytical reverse-phase column (Waters XBridge C_{18}, 5 μm, 6 × 250 mm) at a rate of 1 ml/min with the following solvent system: 5% methanol (MeOH) in water with 0.1% formic acid (FA) for 25 minutes then increased to 50% MeOH plus 0.1% FA over the next 28.5 minutes followed by a cleanup step with 100% MeOH plus 0.1% FA for the remaining 10 minutes. Mass spectrometric detection was performed by direct injection of a collected peak onto a Finnigan LTQ Linear Ion Trap (ThermoElectron) scanning in positive mode. An authentic standard of petrobactin was used for comparisons. The A_{290} peaks corresponding to petrobactin were integrated using the area annotation feature included in the 32 Karat Software package (Beckman Coulter). Peak areas corresponding to petrobactin were normalized with the mean of wild type sample values equaling 100% petrobactin production.

Murine infections

Intratracheal infections of DBA/2J mice (Jackson Laboratories) were performed as previously described (Heffernan *et al.*, 2007). Groups of eight mice were infected with either wild-type or mutant spores at a variety of doses ranging from 1×10^5 through 1×10^8 spores per mouse. Mice were monitored over a period of fourteen days. LD_{50} values were calculated using the Moving Average Interpolation program available at (http://falkow.stanford.edu/whatwedo/software/software.html) (Kim *et al.*, 2003).

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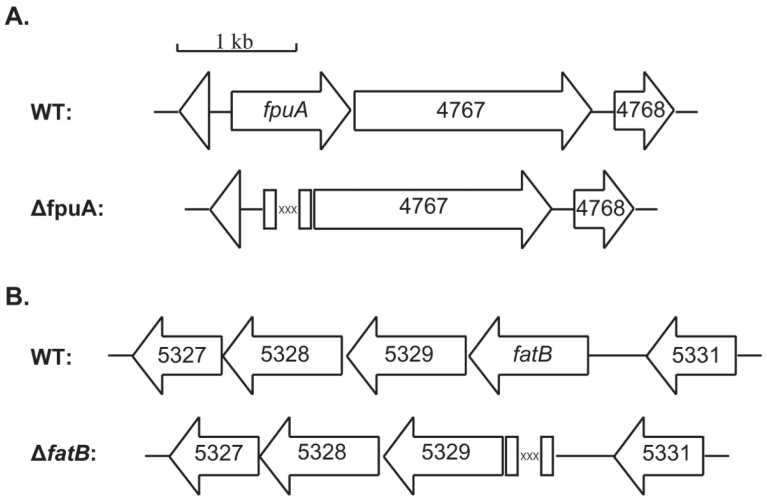

Figure 1. Construction of *B. anthracis* mutants

Gene deletion mutants were created by allelic exchange removing either (A) GBAA4766 (*fpuA*) or (B) GBAA5330 (*fatB*). For each deletion, the initial 30 and final 30 nucleotides of the gene were fused creating a markerless deletion. Deleted genetic material was replaced by three stop codons, represented by "X" above.

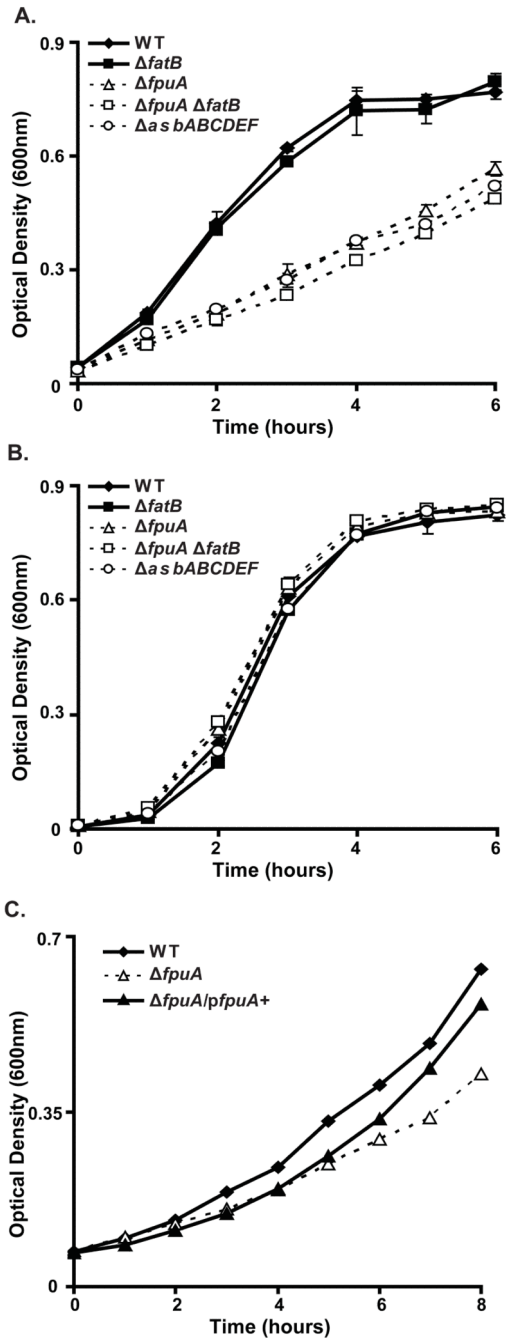

Figure 2. Growth of *B. anthracis* mutant strains in iron depleted media

(A and B) Wild-type (solid diamonds), Δ*fatB* (solid squares), Δ*fpuA* (open triangles), Δ*fpuA* Δ*fatB* (open squares), and Δ*asbABCDEF* (open circles) were grown in either iron depleted media (A) or BHI (B). (C) Wild-type (solid diamonds), Δ*fpuA* (open triangles), Δ*fpuA*/p*fpuA* + were grown in IDM at 26°C. All cultures were inoculated with vegetative bacilli at an initial OD_{600} = 0.05 and growth was monitored by measuring change in OD_{600} over time. Data presented are representative of least three individual experiments.

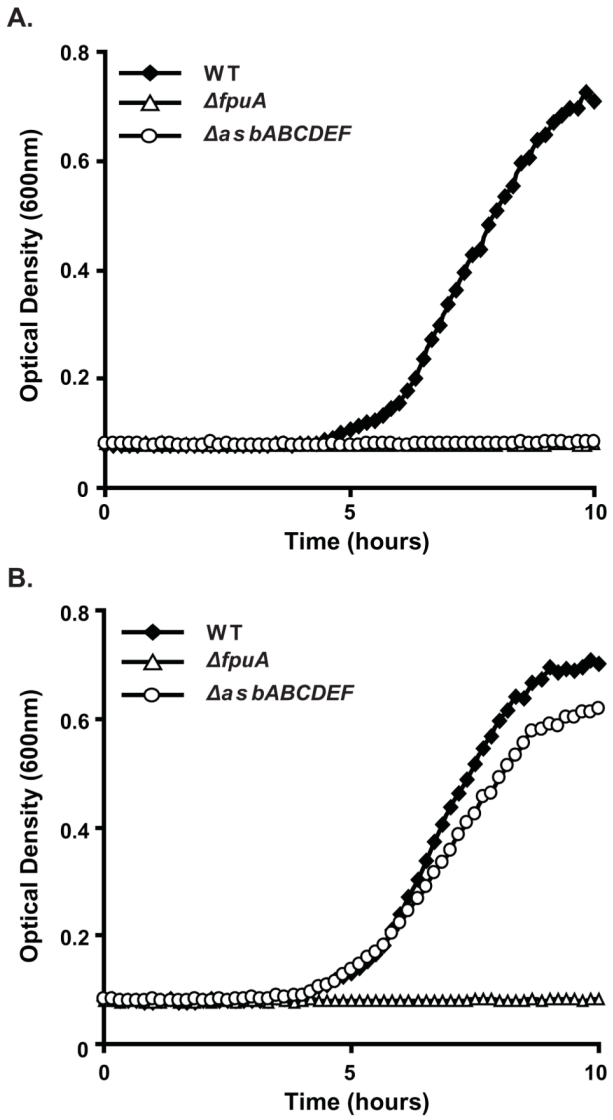

Figure 3. Addition of supplemental petrobactin does not rescue binding protein mutants

Wild-type (solid diamonds), Δ*fpuA* (open triangles), and Δ*asbABCDEF* (open circles) were grown in either (A) iron depleted media or(B) iron depleted media supplemented with 2.5μM petrobactin. Cultures were inoculated with spores and grown in microtiter plates. Data presented are representative of three individual experiments.

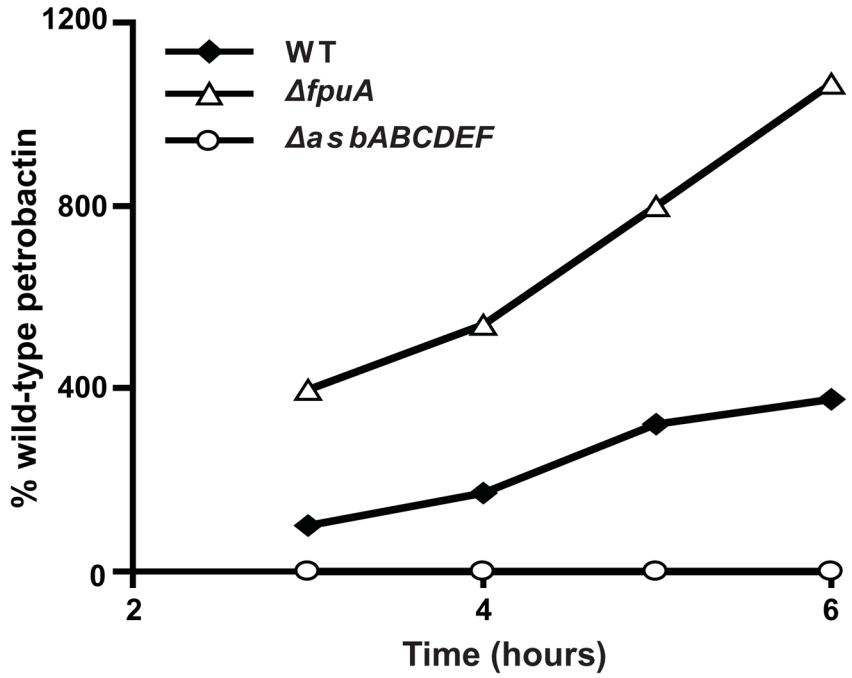

Figure 4. Increased petrobactin levels observed in Δ*fpuA* strains

(A) Extracellular catechol levels were measured over time in culture supernatants using the Arnow assay. Data were normalized to the OD_{600} of cultures at each timepoint and are presented as percent of wild-type petrobactin levels at three hours. No signal could be detected at timepoints prior to three hours. Error bars represent standard deviation of triplicate samples within one experiment. Data are representative of four individual experiments. (B) Comparison of petrobactin accumulation by HPLC. Supernatants from wild-type and Δ*fpuA* cultures that were grown for six hours and normalized to equivalent OD_{600} were observed by HPLC. A peak corresponding to an authentic petrobactin standard is observed in both WT and Δ*fpuA* traces (arrow).

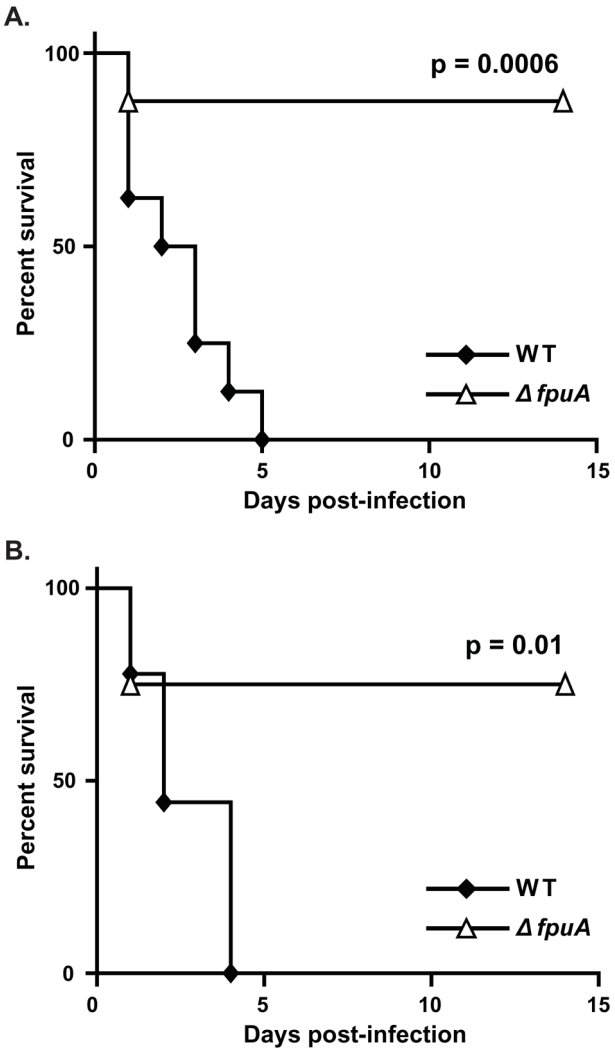

Figure 5. Attenuation of virulence of the Δ*fpuA* strain in a murine model of infection

DBA/2J mice were infected by intratracheal infection with WT (filled diamonds) or Δ*fpuA* (open triangles) spores at 1×10^5 (A), or 1×10^6 (B) spores per mouse. Mice were monitored for fourteen days. Survival curves for Δ*fpuA* were significantly different from wild-type at all doses tested by the log-rank test (p values indicated above).

Table 1

Bacillus anthracis strains used in this study

Strain	Relevant Characteristics	Reference
Sterne $34F_2$	Wild type ($pXO1^+$, $pXO2^-$)	(Sterne, 1939)
BA850	$34F_2$, Δ*asbABCDEF*	(Lee *et al.*, 2007)
PC101	$34F_2$, Δ*fatB*	This work
PC102	$34F_2$, Δ*fpuA*	This work
PC103	$34F_2$, Δ*fpuA* Δ*fatB*	This work

Table 2

Resistance of Δ*fpuA* to gallium treatment.

	hours			
	1	**2**	**3**	**4**
wild-type	25.0[a, b]	40.2	33.4	27.5
Δ*asbABCDEF*	4.6	6.0	8.7	5.0
Δ*fpuA*	4.6	4.6	4.6	8.0

[a] % growth inhibition

[b] results are representative of 4 independent experiments

Table 3

Attenuated virulence of Δ*fpuA* in DBA/2J mice.

Strain	LD_{50}
wild-type	7.5×10^3
Δ*fpuA*	2.2×10^7
Δ*asbABCDEF*	2.7×10^6