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Temporal production of the two *Bacillus anthracis* siderophores, petrobactin and bacillibactin

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Abstract *Bacillus anthracis* secretes two siderophores, petrobactin (PB) and bacillibactin (BB). These siderophores were temporally produced during germination and outgrowth of spores (the usual infectious form of *B. anthracis*) in low-iron medium. The siderophore PB was made first while BB secretion began several hours later. Spore outgrowth early in an infection may require PB, whereas delayed BB production suggests a role for BB in the later stages of the infection. Incubation of cultures (inoculated as vegetative cells) at 37°C, as compared to 2°C, increased PB production and decreased secretion of BB, suggesting that the production of PB and BB responded to the host temperature signal. The dual siderophores of *B. anthracis* may fulfill independent roles in the life cycle of *B. anthracis*.

Keywords Siderophores · Petrobactin · Bacillibactin · Iron · Anthrax

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Introduction

To successfully establish an infection in the iron restricted environment of a normal mammalian host, a pathogenic microorganism must acquire iron from the host iron sources (Byers and Arceneaux 1998). Notable among the microbial iron uptake mechanisms, siderophores are low molecular mass iron chelating agents synthesized by microbes to gather iron and deliver it to the microbe. The concept that some siderophores are virulence factors was strengthened by the discovery that the mammalian protein siderocalin is a component of the host innate iron-depletion defenses that attacks siderophore-mediated iron uptake by binding siderophores (Goetz et al. 2002; Flo et al. 2004; Abergel et al. 2006). *Bacillus anthracis*, the etiologic agent of the disease anthrax, evades siderocalin by producing the virulence-associated siderophore petrobactin (PB) (Garner et al. 2004; Koppisch et al. 2005; Wilson et al. 2006). PB is constructed on a citrate bis-spermidine scaffold with two 3,4-dihydroxybenzoate (3,4-DHB) caps linked to the spermidinyl arms (Bergeron et al. 2004). The 3,4 hydroxylation pattern of the iron binding moieties is unusual in catecholate

siderophores and precludes insertion of PB within the binding pocket of siderocalin (Abergel et al. 2006)). *B. anthracis* Δ*asbA*, a mutant strain unable to produce PB, is both attenuated for growth in macrophages and significantly less virulent than its parent strain in a mouse infection assay (Cendrowski et al. 2004). *B. anthracis* also produces a second siderophore, bacillibactin (BB), that incorporates catecholate groups of the more commonly found 2,3 hydroxylation pattern (Koppisch et al. 2005) and which is found in several species of the genus *Bacillus*, including *Bacillus subtilis* (May et al. 2001). Siderocalin binds BB (Abergel et al. 2006), probably rendering BB unable to deliver iron to *B. anthracis* and loss of BB production does not alter the virulence of *B. anthracis* in a mouse model (Cendrowski et al. 2004). Although BB is a major iron deficiency rescue system in *B. subtilis* (Miethke et al. 2006), in iron depleted culture medium the capacity to produce BB does not support normal growth of *B. anthracis asb* mutant strains that fail to synthesize PB (Cendrowski et al. 2004; Lee et al. 2007) and outgrowth of spores of *B. anthracis* under iron restriction requires PB (Lee et al. 2007).

The usual infectious form of *B. anthracis* is the spore and spore germination in the herbivore host produces vegetative cells that multiply as the infection progresses (Mock and Fouet 2001). Both PB and BB are produced by pathogenic strains of *B. anthracis* (Koppisch et al. 2008b) but it is unknown if the two siderophores are produced simultaneously during spore germination and outgrowth in the iron restricted environment of the host (Byers and Arceneaux 1998) and if syntheses of the siderophores are responsive to host signals. Timed or temporal production of the siderophores might suggest independent roles for PB and BB during the *B. anthracis* life cycle from spore germination, vegetative cell replication, and final re-sporulation. A hierarchy of controls may adjust siderophore production to the life cycle of *B. anthracis*.

Materials and methods

Bacterial strains, culture media cultivation procedures, spore preparation, siderophore purification

The Sterne strain *B. anthracis* USAMRIID (Garner et al. 2004; Wilson et al. 2006) that lacks plasmid pXO2 of the two virulence plasmids pXO1 and pXO2 was obtained from P. Worsham. Bacteria were kept in long-term storage as spores. The Controlled Trace Metal (CTM) medium for growth and siderophore production studies was prepared as previously described (Garner et al. 2004), either with a high iron (Fe = 36 μM) or low iron (Fe = 0.1 μM) supplement. Spores used to inoculate cultures for determination of siderophore production during germination and outgrowth were prepared as follows. After incubation for 12–14 days at 30°C on sporulation agar (composed of, per l, 23 g nutrient agar, 0.5 g yeast extract, 0.006 g $MnCl_2$, and 0.078 g $CaCl_2$) the bacterial growth was removed from the agar and diluted in sterile water. The suspensions were incubated for 30 min in a 65°C water bath to kill vegetative cells (Turnbull et al. 2007). The spores then were washed four times by centrifugation and suspension of the pellets in sterile water. To remove the dead vegetative cells, the final spore suspension was filtered through a sterile glass microfiber 3.1 μm filter (Russell et al. 2007). Spores were enumerated as colony forming units (CFU) per ml by dilution plate counting on Brain–Heart Infusion (BHI) agar plates. For germination and outgrowth experiments, high- and low-iron CTM media (also supplemented with the germinant L-alanine at 50 mM concentration) were inoculated with spores at an initial A_{600} of 0.09. The cultures were incubated at 37°C or kept at 0°C (controls) and germination and outgrowth were followed by turbidity measurements (A_{600}). Culture samples were collected at timed intervals for analyses by reverse phase HPLC for amount and type of siderophore produced.

For cultures inoculated with vegetative cells, bacteria were transferred from BHI agar slants to 25 ml of high-iron CTM medium that was incubated at 37°C with shaking at 300 rpm for 16–17 h. This culture was centrifuged and the cell pellet washed once by suspension in CTM medium (without an iron supplement) and re-centrifugation. The final washed cell pellet was suspended and diluted appropriately in CTM medium (without an iron supplement) for inoculation of high- and low-iron CTM medium, usually at 10^4 CFU per ml. Cultures were incubated in air at the desired temperature with shaking at 300 rpm. Growth was followed by turbidity measurements (A_{600}).

Detection and quantification of siderophore secretion

The culture samples were filtered through 0.22 μm pore diameter filters to remove cells and the filtrates were then analyzed using high pressure liquid chromatography on Zorbax Rx-SIL C18 80 Å 5 μm porous silica support (reverse phase) with gradient mixtures of acetonitrile, water (purified through a Millipore Milli-Q cartridge system), and trifluoroacetic acid. Samples were injected as 20 μl aliquots onto an analytical Eclipse XDB-C18 column (Agilent). A gradient from 5% CH_3CN to 30% CH_3CN in ddH_2O/0.1% TFA over 20 min at 1 ml/min was used to elute 3,4-DHB (t_R = 5.3–5.4 min), PB (t_R = 6.6–6.7 min) and BB (t_R = 14.6–14.7 min) as discrete peaks (detection by UV–vis absorption at 254, 280 and 316 nm). Concentrations were determined against 0.1 mM standard ligand solutions using the liquid chromatography Agilent Chemstation. The siderophores PB and BB were purified from culture filtrates of *B. anthracis* USAMRIID (Wilson et al. 2006) and *Bacillus thuringiensis* ATCC 33679 (Dertz et al. 2006), respectively, following protocols described in the designated references; 3,4-DHB was obtained from Sigma Chemical Co. Due to the relative extinction coefficients of the siderophores at the detection wavelengths, the minimum detectable concentrations of 3,4-DHB, PB and BB in each 20 μl sample were 1.5, 1 and 1 μM, respectively.

Results and discussion

Temporal siderophore secretion; early production of PB during outgrowth from spores

Because the disease anthrax begins with spore germination in the host, we followed siderophore secretion during the early hours of germination and outgrowth in cultures inoculated with spores. When spores of *B. anthracis* were inoculated into either low- and high-iron CTM medium supplemented with the germinant L-alanine (50 mM) and incubated at 37°C, there was an initial decrease in culture turbidity due to germination of spores; subsequent spore outgrowth then was evidenced by an increase in turbidity (Fig. 1a). Similarly inoculated control cultures kept on ice did not show turbidity changes indicative of

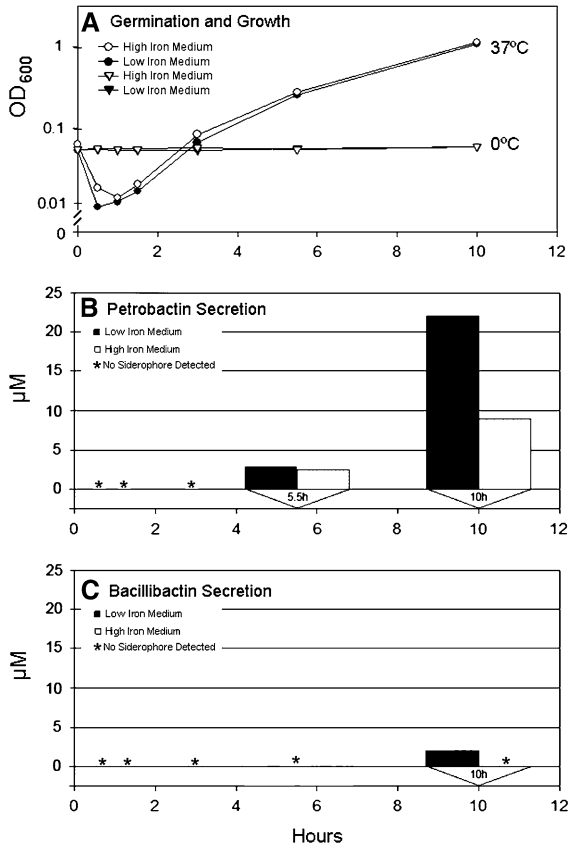

Fig. 1 Temporal secretion of PB and BB during germination and outgrowth of *B. anthracis* USAMRIID spores. Spores were inoculated in low- and high-iron CTM medium and germination and outgrowth of vegetative cells was followed by turbidity measurements (*Panel A*). Cell free culture samples were analyzed at the times indicated by reverse phase HPLC for PB (*Panel B*) and BB (*Panel C*). *Asterisks* indicate no siderophore was detected. Data presented is from a typical experiment

germination and outgrowth. The amounts of PB and BB present in the culture filtrates prepared at timed intervals were determined. Neither PB nor BB were detected at 0.5, 1, and 3 h, but at 5.5 h PB only was detected at concentrations of 3 and 2 μM in the filtrates from low-iron and high-iron cultures, respectively, (Fig. 1b). BB at a concentration of 2 μM was first detected later at 10 h and then only in the low-iron culture (Fig. 1c). At the time that BB was first detected, the PB concentrations had increased to 22 and 8 μM in low-iron and high-iron cultures, respectively. The initial and sole production of siderocalin resistant PB during outgrowth from spores may illustrate an early event in infection when PB is the

only useful siderophore in the presence of an intact host siderocalin defense that would inactivate BB. These data also reveal the stringent control over BB secretion exerted by the level of iron in the environment whereas PB is made at iron levels that abrogate BB production. These results confirm those of Lee et al. (2007) who found that spores of a *B. anthracis* mutant strain unable to produce PB did not outgrow on iron depleted agar unless supplied with PB or another utilizable iron source. These results also resemble the temporal production of the siderophores achromobactin and chrysobactin noted in the phytopathogen *Erwinia chrysanthemi*, a property that may allow the microorganism to cope with changing conditions in its plant host during an infection (Franza et al. 2004). In the pathogenic *Bordetella* species, multiple iron acquisition pathways also are differentially expressed, possibly to exploit early and late events of infection (Brickman and Armstrong 2009).

Host temperature and bicarbonate level increased PB secretion and decreased BB production

Host signals often trigger expression of bacterial virulence traits. Optimal expression of the toxins and capsule virulence factors of *B. anthracis* occurs during cultivation at 37°C in the presence of bicarbonate/CO_2 which are considered signals of the host environment (Sirard et al. 1994). Moreover, deletion of the genes for the bicarbonate transporter abolished toxin production, producing a strain that was avirulent the mouse model (Wilson et al. 2008). Syntheses of the two siderophores PB and BB in *B. anthracis* is regulated by iron level and by temperature (Garner et al. 2004; Koppisch et al. 2005). When cultures of *B. anthracis* (inoculated as vegetative cells into low-iron CTM medium) were incubated at 37°C, as compared to 23°C, secretion of the PB was increased from 40 μM to 60 μM, while total BB secretion was decreased from 20 μM to about 10 μM (Fig. 2). Growth at 37°C also increased secretion of the PB constituent 3,4-DHB. Koppisch et al. (2005) were unable to detect BB in culture filtrates of *B. anthracis* grown at 37°C in an atmosphere of 5% CO_2 and we did not observe BB on thin layer chromatograms of filtrates from *B. anthracis* USAMRIID cultures grown with added 0.4–0.8% bicarbonate in low-iron CTM medium at 37°C, although both PB and 3,4-DHB were identified on these chromatograms (data not shown).

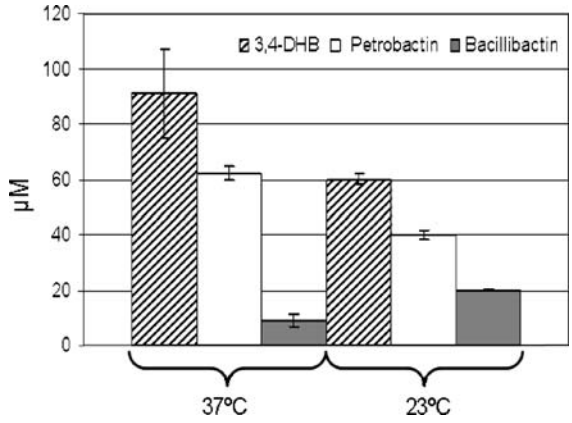

Fig. 2 Effect of incubation temperature on *B. anthracis* USAMRIID siderophore secretion determined at the maximum stationary phase of growth in low-iron CTM medium at 37°C. Concentrations of secreted products were normalized as μM per OD_{600} unit of culture turbidity

In addition to the host cue of iron restriction, the host temperature and the presence of CO_2/bicarbonate also altered syntheses of the dual siderophores of this microorganism. The combination of 37°C and CO_2/bicarbonate appeared to disable BB production, suggesting that BB synthesis might occur late in the infectious process when host conditions have been significantly changed.

Conclusions

B. anthracis physiology presents a complex pattern of temporal production of the two *B. anthracis* siderophores, PB and BB. In the herbivore host, siderocalin blocks utilization of BB; *B. anthracis* evades siderocalin with PB, yet BB production is preserved. PB was the first detectable siderophore secreted during outgrowth from spores while BB production began several hours later in the growth cycle. The initial outgrowth from spores in the infected host requires iron, which could be satisfied by PB mediated removal of iron from sources such as Fe-transferrin (Abergel et al. 2008). *B. anthracis* siderophore production also responded to cues of the host environment. A temperature of 37°C, compared to 23°C, and the presence of CO_2/bicarbonate increased production of the virulence siderophore PB and curtailed synthesis of BB. The role of BB in *B. anthracis* metabolism is

uncertain, although BB could be a participant at the last stages of the anthrax infection when the organism transitions from rapid vegetative growth to the sporulation phase. Production of PB may be essential for outgrowth from spores and onset of rapid cell division in the early stages of the infection. Preservation of BB production by *B. anthracis* suggests that the dual siderophores of *B. anthracis* fulfill unique and independent functions in the life cycle of *B. anthracis*.

The rare occurrence in catecholate siderophores of the 3,4-DHB unit that is found in PB may offer a point of vulnerability for rational anti-anthrax drug design. The 3,4 isomer of DHB is crucial to the capacity of PB to acquire iron in the presence of innate host defenses early in an infection. A specific block in *B. anthracis* 3,4-DHB synthesis (Koppisch et al. 2008a; Pfleger et al. 2008; Fox et al. 2008) might impede outgrowth of the vegetative cell stage from germinating spores and could, therefore, be an anthrax-preventive strategy. Administration of a drug that blocks PB production to livestock during an anthrax outbreak or to persons about to enter areas contaminated with *B. anthracis* spores, either accidentally or purposefully, could offer a significant adjunct to other means of protection from infection.

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