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Siderophores of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*

Melissa K. Wilson [a], Rebecca J. Abergel [b], Kenneth N. Raymond [b], Jean E.L. Arceneaux [a], B. Rowe Byers [a,*]

[a] *Department of Microbiology, University of Mississippi Medical Center, Jackson, MS 39216-4505, USA*
[b] *Department of Chemistry, University of California, Berkeley, CA 94720-1460, USA*

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Abstract

Three *Bacillus anthracis* Sterne strains (USAMRIID, 7702, and 34F2) and *Bacillus cereus* ATCC 14579 excrete two catecholate siderophores, petrobactin (which contains 3,4-dihydroxybenzoyl moieties) and bacillibactin (which contains 2,3-dihydroxybenzoyl moieties). However, the insecticidal organism *Bacillus thuringiensis* ATCC 33679 makes only bacillibactin. Analyses of siderophore production by previously isolated [Cendrowski et al., Mol. Microbiol. 52 (2004) 407–417] *B. anthracis* mutant strains revealed that the *B. anthracis bacACEBF* operon codes for bacillibactin production and the *asbAB* gene region is required for petrobactin assembly. The two catecholate moieties also were synthesized by separate routes. PCR amplification identified both *asbA* and *asbB* genes in the petrobactin producing strains whereas *B. thuringiensis* ATCC 33679 retained only *asbA*. Petrobactin synthesis is not limited to the cluster of *B. anthracis* strains within the *B. cereus sensu lato* group (in which *B. cereus*, *B. anthracis*, and *B. thuringiensis* are classified), although petrobactin might be prevalent in strains with pathogenic potential for vertebrates.

Keywords: *Bacillus anthracis*; *Bacillus cereus*; *Bacillus thuringiensis*; Siderophore; Bacillibactin; Petrobactin; Iron; 2,3-Dihydroxybenzoic acid; 3,4-Dihydroxybenzoic acid; Protocatechuic acid; Anthrax

Despite the differences in the host range and pathogenic potentials of the zoonotic agent *Bacillus anthracis*, the soil and opportunistic pathogen *Bacillus cereus*, and the insect pathogen *Bacillus thuringiensis*, various studies suggest that the three species are a closely related collection of organisms within the *B. cereus sensu lato* group [1–4]. Independently isolated strains of *B. anthracis* display a high degree of genetic homogeneity, constituting a single cluster within the *B. cereus* group, while *B. cereus* and *B. thuringiensis* show greater genetic diversity. Nonetheless, strains of *B. cereus* and *B. thuringiensis* isolated from human or animal infections may share a set of virulence factors, forming a distinct sub-group of strains with pathogenic potential for vertebrates in the *B. cereus* group [4–6].

The ability to obtain sufficient iron for proliferation in the iron restricted environment of the vertebrate host is a nearly universal virulence trait of pathogenic microorganisms and production of iron acquisition cofactors called siderophores is prominent among the various iron uptake systems of pathogens [7]. Although production of more than one type of siderophore has been demonstrated in several microorganisms, *B. anthracis* uniquely excretes two catecholate siderophores with different catechol hydroxylation patterns. Garner et al. [8] showed that one of the siderophores incorporates 3,4-dihydroxybenzoyl (3,4-DHB) moieties at the iron chelation center. This siderophore later was identified by Koppisch et al. [9] as the previously isolated citrate siderophore called petrobactin (Fig. 1) which, together with a sulfonated petrobactin derivative, is the

* Corresponding author. Fax: +1 601 984 1708.
E-mail address: bbyers@microbio.umsmed.edu (B.R. Byers).

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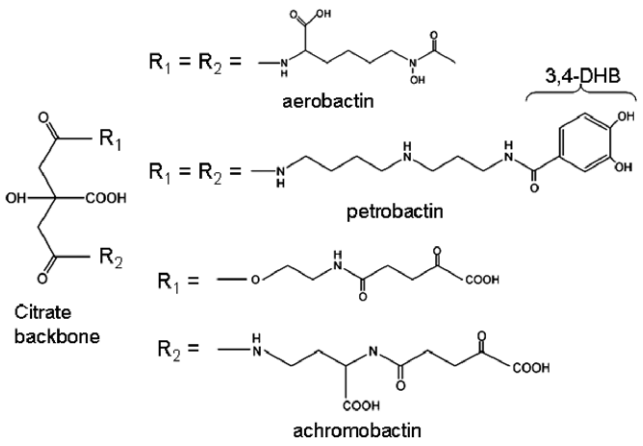

Fig. 1. Structures of representative citrate siderophores aerobactin [10], petrobactin [11], and achromobactin [12].

only known siderophore utilizing 3,4-DHB as an iron chelating component [11,14]. A second *B. anthracis* siderophore detected by Koppisch et al. [9] resembled the tris-catecholate bacillibactin first isolated from *Bacillus subtilis* (Fig. 2) which contains 2,3-dihydroxybenzoyl (2,3-DHB) units [13], the iron-liganding catechol occurring most often in siderophores of the catecholate family. Synthesis of some citrate siderophores is an important virulence trait [15] and production of the unusual 3,4-DHB siderophore petrobactin may be specific to the pathogenic *B. anthracis* cluster. However, evidence suggests that petrobactin may not be limited to *B. anthracis*; this evidence includes excretion of the petrobactin precursor 3,4-DHB by *B. cereus* [16], production of an unidentified catecholate siderophore(s) by *B. cereus* [17], the close relationship of *B. anthracis*, *B. cereus*, and *B. thuringiensis*, and results obtained by Garner [18] which tentatively indicate synthesis of petrobactin by *B. cereus*.

The *B. anthracis* genome includes at least two gene groupings for biosynthesis of siderophores, a five gene operon designated *bacACEBF* that is 79% similar to the *dhbACEBF* operon of *B. subtilis* that encodes the synthesis of bacillibactin and two adjacent genes (within another operon) named *asbA* and *asbB* that may be orthologs of the siderophore biosynthetic genes *iucA* and *iucC*, respectively [19]. The *iuc* gene family encodes assembly of several citrate based siderophores [15]. Using the *B. anthracis* Sterne 34F2 strain, Cendrowski et al. [19] constructed two strains, designated Δ*bacCEBF* and Δ*asbA*, with deletions in the respective gene groups. The deletion in each of the mutant strains reduced, but did not abolish, catechol excretion, suggesting that the operon *bacACEBF* and the two genes *asbAB* were involved in synthesis of different catecholate siderophores. The siderophores produced by each of the mutant strains were not identified. The Δ*asbA* strain was attenuated for growth in macrophages and for virulence in mice, leading to the conclusion that the siderophore encoded by the *asbAB* region was essential for growth in macrophages [19]. While this may be true, the deletion in the Δ*asbA* strain may have affected expression of genes (not necessarily related to siderophore production) downstream of the *asb* region resulting in impaired growth and lowered virulence of the *B. anthracis* Δ*asbA* strain.

Materials and methods

Organisms, siderophore production, and thin layer chromatography (TLC). *Bacillus anthracis* Sterne ($pXO1^+$ $pXO2^-$) strains USAMRIID and 7702 were obtained from P. Worsham, US Army Research Institute of Infectious Diseases, Frederick, Maryland, and T. Koehler, University of Texas-Houston Medical School, Houston. The Sterne strain *B. anthracis* 34F2 and the siderophore mutant strains *B. anthracis* Δ*bacCEBF* and *B. anthracis* Δ*asbA* derived from it were supplied by P. Hanna, University of Michigan School of Medicine, Ann Arbor. *B. cereus* ATCC 14579 and *B. thuringiensis* ATCC 33679 were purchased from the American Type Culture Collection, Manassas, Virginia, and *B. subtilis* W23 was a strain from this laboratory. For strains *B. anthracis* Δ*bacCEBF* and Δ*asbA*, 200 μg of spectinomycin per mL or 50 μg of kanamycin per mL, respectively, was usually added to all culture media.

For growth studies and excreted siderophore preparation, cells were harvested from brain–heart infusion (BHI) agar cultures and transferred to a controlled trace metal (CTM) medium [8] containing 0.01 μM Fe (low iron conditions) at a final inoculum of 10^4 colony forming units per mL. The CTM medium cultures were incubated in glass vessels (previously

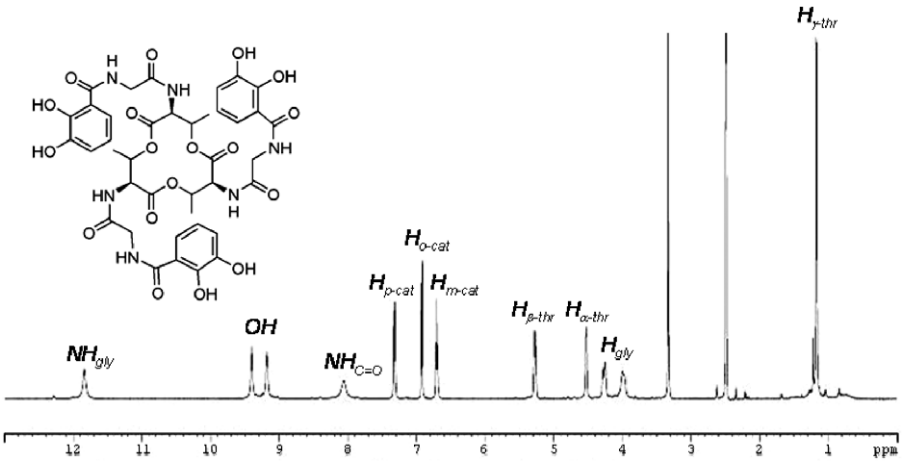

Fig. 2. ^{1}H NMR Spectra of bacillibactin purified from *B. cereus* in DMSO-d_6. Peaks are assigned directly on the spectra. Bacillibactin is composed of three types of fragments: the catechol iron binding moieties (cat), the threonine trilactone backbone (thr), and the glycine spacers (gly).

treated with EDTA to lower trace metal contamination) at the desired temperature with rotation at 300 rpm; growth was followed by turbidity measurements. After 24 h incubation, cultures were centrifuged and the supernatants filtered through 0.22 μm pore diameter filters. The cell free filtrates were processed immediately or stored frozen.

Bacillibactin was prepared for structural analyses from the low iron culture filtrates according to the method of Dertz et al. [20]. Fractions containing the excreted siderophores and siderophore precursors 3,4-DHB and 2,3-DHB were prepared from the filtrates by modifications of the methods used by Garner et al. [8]. The liquid CAS universal siderophore assay [21], which detects high affinity iron chelating agents, and the Arnow assay [22], which detects dihydroxy phenols or siderophores containing these components, as well as the color formed by addition of a 1% $FeCl_3$ solution to a siderophore solution were used to assess preparation of the siderophore-containing fractions. A fraction containing bacillibactin and the siderophore precursors 2,3-DHB and 3,4-DHB (if present) was prepared by extraction of acidified (pH 2–5) filtrates with ethyl acetate; after drying the extracts over desiccant, the ethyl acetate was evaporated and the residue dissolved in methanol. Petrobactin did not extract from the filtrates into ethyl acetate at these conditions. A fraction containing both petrobactin and bacillibactin was prepared from the filtrates (adjusted to pH 7 to prevent retention of 2,3-DHB and 3,4-DHB by Amberlite XAD-4) by binding to Amberlite XAD-4 and elution of the Arnow assay positive and iron reactive substances with methanol as previously described [8]. These eluates were concentrated by evaporation. Petrobactin was purified (described in Results and discussion) from this fraction for structural analyses.

The presence of siderophores and catechol siderophore precursors in the fractions was analyzed by cellulose TLC using Polygram CEL 400 precoated plastic sheets (Macherey-Nagel, Düren, Germany) developed with solvent 1, *n*-butanol/acetic acid/water (12:3:5), and solvent 2, *t*-butanol/methyl ethyl ketone/water/diethylamine (10:10:5:1). Authentic 2,3-DHB and 3,4-DHB (Sigma Chemical Co., St. Louis, MO) were used as standards. Iron reactive substances were detected with a freshly prepared 1% $FeCl_3$ spray.

HPLC, NMR, and mass spectroscopy. Chromatographic purification of products was accomplished using high pressure liquid chromatography on Waters Preparative C18 125 Å 55–105 μm silica gel (reverse phase). Gradient mixtures of acetonitrile, distilled water (purified through a Millipore Milli-Q reverse osmosis cartridge system), and trifluoroacetic acid were used for elution. ^{1}H NMR spectra were recorded at ambient temperature on a Brucker Avance AV-500 MHZ equipped with a TBI probe. Chemical shifts (δ) are given in ppm, downfield from tetramethylsilane (TMS), used as a standard. Mass spectra were recorded at the Mass Spectrometry Laboratory, College of Chemistry, University of California, Berkeley. Fast atom bombardment ionization was used with an *m*-nitrobenzyl alcohol (NBA) matrix.

Ethylenediamine-di(o-hydroxyphenylacetic acid) inhibition assay. The minimal inhibitory concentrations (MIC) of the chelating agent ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) for *B. anthracis* 34F2 and the siderophore mutant strains Δ*bacCEBF* and Δ*asbA* derived from *B. anthracis* 34F2 were determined by previously used methods [23]. BHI broth supplemented with EDDA from 0 to 2 mg/mL (that was aged one week to allow iron chelation) was inoculated at 10^3 colony forming units per mL and incubated at 37 °C for 48 h. Growth was determined visually and by turbidity measurements.

Screening for asb genes by PCR. The cultures were screened for *asb* gene homologs using boiled cell lysates, a primer concentration of 0.6 μM in all reactions, and PCR Mastermix (Promega, Madison, Wisconsin). Gel electrophoresis used 2% agarose gels containing ethidium bromide and a DNA Marker 100 bp ladder (Promega) to determine PCR product size. Primers were designed to amplify sequences within the *asbA* and *asbB* genes of *B. anthracis* Ames, *B. thuringiensis* 97-27 subsp. *konkukian*, and *B. cereus* ATCC 14579. The primers for the *asbA* amplicon were (forward) 5′-GGAGAATGGATTACAGAGG-3′ and (reverse) 5′-GTCGTCATA TAAATTTCCAG-3′. Those for the *asbB* amplicon were (forward) 5′-ACGATTGCACAATATGAAAGA-3′ and (reverse) 5′-CAATGG TTTGGAACTTCATG-3′. Primers amplifying the Ba813 sequence of *B. anthracis* served as a positive control [24].

Results and discussion

Siderophore production in B. anthracis, B. cereus, and B. thuringiensis

An objective of the present research was to determine if petrobactin was restricted to the *B. anthracis* cluster. To tentatively determine if synthesis of petrobactin was a trait exclusive to the *B. anthracis* subgroup, presumptive identification of the siderophores present in culture filtrates of strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis* was determined by cellulose thin layer chromatography (TLC) developed in two solvent systems (see Materials and methods). Although unfractionated culture filtrates could be tested, siderophore-containing fractions prepared from the filtrates by ethyl acetate extraction or by binding to and elution from Amberlite XAD-4 (see Materials and methods) gave more consistent R_f values on TLC. These analyses demonstrated two iron reactive siderophores in filtrates of *B. anthracis* Sterne strains USAMRIID, 34F2, and 7702 grown in CTM medium at low iron (Table 1). One of these iron reactive spots displayed R_f values of 0.55 and 0.16 in solvents 1 and 2, respectively, which corresponds to those previously reported for the *B. anthracis* 3,4-DHB siderophore [8] identified as petrobactin [9] and suggests that the three Sterne strains produced petrobactin. Moreover, *B. cereus* ATCC 14579 also appeared to synthesize petrobactin, indicating that the petrobactin siderophore was not restricted to the *B. anthracis* cluster in the *B. cereus* group of organisms, but the analyses failed to demonstrate production of petrobactin by *B. thuringiensis* ATCC 33679 (Table 1). All of the presumptive petrobactin producing strains also excreted the petrobactin catecholate precursor 3,4-DHB. To confirm synthesis of petrobactin by *B. anthracis* and *B. cereus*, the siderophore-containing methanol eluates of Amberlite XAD-4 were evaporated; the oily residues were dissolved in a minimal amount of methanol and slowly added to a stirred solution of diethyl ether (50 mL). The precipitate was filtered off to afford a brown powder. Purification of the crude material was achieved by reverse-phase HPLC on a semi-preparative C18 column. A gradient from 5% CH_3CN in ddH_2O/0.1% TFA to 30% CH_3CN in ddH_2O/0.1% TFA over 20 min at 22 mL/min was used to elute petrobactin as a single peak (detection by UV–vis absorption at 280 nm, $t_R = 11.2$–12.2 min). Subsequent lyophilization afforded ~3 mg of a white amorphous solid identified as petrobactin. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.42 (m, 4H), 1.55 (m, 4H), 1.80 (m, 4H), 2.53 (dd, $J = 14.5$ and 36 Hz, 4H), 2.89 (br, 8H), 3.04 (m, 4H), 3.27 (m, 4H), 5.54 (br, 1H), 6.76 (d, $J = 8.5$ Hz, 2H), 7.18 (d, $J = 8.5$ Hz, 2H), 7.27 (s, 2H), 8.01 (t, $J = 5.5$ Hz, 2H), 8.33 (t, $J = 5.0$ Hz, 2H), 8.40 (m, br, 2H), 9.13 (br, 2H), 9.56 (br, 2H) ppm. (+)-FABMS: m/z 719.4 (MH^+).

A second siderophore in the low iron culture filtrates of *B. anthracis* Sterne strains USAMRIID, 34F2, and 7702 displayed iron reactive R_f values of 0.97 and 0.94 in the

Table 1
Siderophores and siderophore precursors excreted by *B. anthracis*, *B. cereus*, and *B. thuringiensis*[a]

Siderophore or siderophore precursor	*B. anthracis* Sterne strains					*B. cereus* ATCC 14579	*B. thuringiensis* ATCC 33679
	USAMRIID	7702	34F2	Δ*bacCEBF*	Δ*asbA*		
Petrobactin	Yes	Yes	Yes	Yes	No	Yes	No
Bacillibactin	Yes	Yes	Yes	No	Yes	Yes	Yes
2,3-DHB[b]	No	No	No	No	No	Yes	Yes
3,4-DHB[b]	Yes	Yes	Yes	Yes	Yes	Yes	No

[a] Yes and no indicate excretion and lack of excretion, respectively, of the siderophore or siderophore precursor.
[b] 2,3-DHB, 2,3-dihydroxybenzoic acid; 3,4-DHB, 3,4-dihydroxybenzoic acid.

TLC solvents 1 and 2, respectively, identical to bacillibactin prepared by the method of Dertz et al. [20] from *B. subtilis* W23; an iron reactive component of similar R_f was excreted by *B. cereus* ATCC 14579 and *B. thuringiensis* ATCC 33679 (Table 1). Possible excretion of the free monomer 2,3-DHB also was detected in *B. cereus* and *B. thuringiensis* but this bacillibactin precursor was not seen in *B. anthracis* filtrates (Table 1). Koppisch et al. [9] raised the interesting possibility that the *B. anthracis* analog of bacillibactin might incorporate the 3,4-DHB moiety, given that this organism uses 3,4-DHB to form petrobactin. Because petrobactin does not extract into ethyl acetate from the acidified filtrates of iron restricted cultures, the ethyl acetate extraction-ether precipitation method of Dertz et al. [20] was used to recover and precipitate bacillibactin for structural analyses from filtrates of *B. anthracis* USAMRIID, *B. cereus* ATCC 14579, and *B. thuringiensis* ATCC 33679. Mass spectrometry and ^{1}H NMR analysis of the samples allowed identification of the siderophore. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.17 (d, $J = 6.5$ Hz, 9H), 3.99 (br d, $J = 12.0$ Hz, 3H), 4.27 (br d, $J = 12.0$ Hz, 3H), 4.53 (br s, 3H), 5.28 (m, $^2J = 6.5$ Hz, 3H), 6.70 (t, $J = 7.5$ Hz, 3H), 6.92 (d, $J = 6.5$ Hz, 3H), 7.32 (d, $J = 7.5$ Hz, 3H), 8.06 (br, 3H), 9.18 (br, 3H), 9.40 (br, 3H), 11.83 (br, 3H) ppm. (+)-FABMS: m/z 883.6 (MH^+). The presence of characteristic signals in the aromatic region of the ^{1}H NMR spectra of bacillibactin (one triplet and two doublets) confirmed a structure based on 2,3-DHB units rather than 3,4-DHB units, which would typically exhibit two doublets and one singlet in that same region. The spectral features observed here (Fig. 2) are consistent with those previously reported for bacillibactin when isolated from *B. subtilis*, showing that the three species *B. anthracis*, *B. cereus*, and *B. thuringiensis* produced authentic bacillibactin incorporating 2,3-DHB.

Petrobactin and bacillibactin genes: characterization of B. anthracis siderophore mutants

Using the *B. anthracis* Sterne 34F2 strain, Cendrowski et al. [19] constructed two strains, one (designated *B. anthracis* Δ*bacCEBF*) with a deletion in the operon homologous to the bacillibactin operon of *B. subtilis* and the other (designated *B. anthracis* Δ*asbA*) with a deletion in the *asb* region which may be orthologous to the siderophore synthetase superfamily that encodes assembly of the citrate siderophores [15]. Objectives of the present studies were to identify the putative siderophores encoded by each of these regions and to assess some of the growth characteristics of the mutant strains. We first examined the iron gathering efficiency of the Δ*asbA* and Δ*bacCEBF* strains by determining the capability of each mutant strain to grow in the presence of ethylenediamine-di-(*o*-hydroxy phenylacetic acid) (EDDA), an agent that chelates iron and tends to withhold it from an organism. Loss of siderophore production usually yields a mutant strain more sensitive than the parent to growth inhibition by EDDA. The minimal inhibitory concentrations (MICs) of EDDA for the Δ*asbA* and Δ*bacCEBF* mutant strains were identical to the parent strain *B. anthracis* 34F2 (at 1 mg/mL), suggesting that each of the mutant strains retained a high affinity iron uptake process, probably including a functional siderophore.

Growth of the mutant strain *B. anthracis* Δ*bacCEBF* in low iron (0.01 μM Fe) CTM medium was identical to the parent strain *B. anthracis* 34F2. Analyses by TLC of the filtrate fractions prepared from Δ*bacCEBF* cultures tentatively showed that neither bacillibactin nor 2,3-DHB was excreted, indicating that the *B. anthracis* operon *bacACEBF* encodes synthesis of bacillibactin. Iron reactive spots at the R_f values of petrobactin and 3,4-DHB were noted, suggesting production of only the 3,4-DHB siderophore petrobactin by the Δ*bacCEBF* mutant strain (Table 1). The data also show that the biosynthetic pathway for production of the petrobactin precursor 3,4-DHB is distinct from the 2,3-DHB biosynthetic pathway.

To confirm that the *B. anthracis* Δ*bacCEBF* strain synthesized the siderophore petrobactin, the fraction prepared by methanol elution of substances excreted by *B. anthracis* Δ*bacCEBF* that were bound by Amberlite XAD-4 (see Materials and methods) was evaporated to dryness. The residue was dissolved in a minimal amount of methanol and slowly added to a stirred solution of diethyl ether (50 mL). The precipitate was filtered off to afford a brown powder. Precipitation from methanol into diethyl ether was repeated three times. Purification of the crude material was achieved by reverse-phase HPLC on a semi-preparative C18 column. A gradient from 5% CH_3CN in ddH_2O/0.1% TFA to 30% CH_3CN in ddH_2O/0.1% TFA over 20 min at 22 mL/min was used to elute petrobactin as a single peak (detection by UV–vis absorption at 280 nm, $t_R = 11.2$–12.2 min). Subsequent lyophilization afforded

~6 mg of a white amorphous solid that was characterized by ^{1}H NMR spectroscopy and mass spectrometry, and identified as petrobactin.

Cendrowski et al. [19] reported that the other *B. anthracis* siderophore mutant, strain Δ*asbA*, showed reduced siderophore production and impaired growth when compared to the parent strain *B. anthracis* 34F2. In the present study, we found that while growth of *B. anthracis* Δ*asbA* in a rich medium (BHI) was similar to the parent strain, the mutant strain was unable to grow in the CTM medium at either low iron (0.01 μM) or high iron (1–10 μM) conditions. Therefore, to analyze the siderophore(s) made by *B. anthracis* Δ*asbA*, the organism first was grown in BHI broth containing EDDA at a concentration of 0.5 mg/mL (less than the MIC of 1 mg/mL) to yield iron starved cells. Growth was followed by turbidity measurements and at mid-log phase, cells were harvested by centrifugation and the cell pellet suspended in CTM medium (containing 0.005 μg Fe per mL) at the same turbidity. After 4, 12, and 24 h incubation at 37 °C, the filtrate from this suspension was analyzed by TLC for the presence of iron reactive siderophores. This method of analysis first was validated using the parent strain *B. anthracis* 34F2. When grown and transferred as described, production at 12 and 24 h of both petrobactin and bacillibactin by the parent strain was demonstrated by TLC. Analyses of similarly prepared supernatants using *B. anthracis* Δ*asbA* failed to show excretion of petrobactin, although the petrobactin precursor 3,4-DHB was produced by the mutant strain (Table 1). Analyses of the ethyl acetate extracts of supernatants obtained from the *B. anthracis* Δ*asbA* were done using standard NMR techniques, including nuclear overhauser enhancement spectroscopy (NOESY) and heteronuclear multiple bond correlation (HMBC). The ^{1}H NMR spectra of the residues dissolved in methanol exhibited the characteristic signals of both bacillibactin and 3,4-DHB: ^{1}H NMR (500 MHz, MeOD-d_4): δ 1.29 (d, $J = 6.5$ Hz, 9H), 4.02 (d, $J = 16.0$ Hz, 3H), 4.36 (d, $J = 16.0$ Hz, 3H), 4.51 (s, 3H), 5.49 (m, $^2J = 10.5$ Hz, 3H), 6.71 (t, $J = 8.0$ Hz, 3H), 6.92 (d, $J = 8.0$ Hz, 3H), 7.30 (d, $J = 8.0$ Hz, 3H) ppm, and δ 6.78 (d, $J = 8.0$ Hz, 1H), 7.26 (d, $J = 8.0$ Hz, 1H), 7.42 (s, 1H) ppm. To insure that the 3,4-DHB peaks corresponded to an entity separate from bacillibactin, standard ^{1}H–^{1}H NOESY and ^{1}H–^{13}C HMBC NMR experiments were performed on the same sample. In both cases, no correlation was observed between the peaks corresponding to bacillibactin and those belonging to 3,4-DHB. A 1:1 ratio was observed for the two compounds present in the sample. The siderophore mutant strain *B. anthracis* Δ*asbA* produced bacillibactin and excreted the petrobactin catecholate precursor 3,4-DHB.

These analyses show that the *asbA* gene is essential for assembly of petrobactin. The genes *asbA* and *asbB* may be orthologs of the aerobactin (Fig. 1) biosynthetic genes *iucA* and *iucC*, respectively, which encode enzymes that catalyze condensation of each of the aerobactin side chains with one of the carboxyl groups of the citric acid backbone [10]. Like aerobactin, petrobactin (Fig. 1) is a citrate based siderophore and it is possible that the *asbA* and *asbB* genes are required for addition of each of the two 3,4-DHB-spermidinyl moieties to assemble petrobactin.

PCR amplification of asb gene loci from B. anthracis and B. cereus; absence of asbB from B. thuringiensis ATCC 33679

Gene specific primers were designed and used to amplify the *asbA* and *asbB* loci and, as expected, amplicons corresponding to the target genes *asbA* and *asbB* were obtained from the petrobactin producing strains *B. anthracis* 34F2 and *B. cereus* ATCC 14579 (Table 2). The validity of the PCR amplification for these genes was shown by the absence from the *B. anthracis* Δ*asbA* strain of a target for the *asbA* specific primers, although the product representing the *asbB* gene was present, and by the absence of both genes from *B. subtilis* strain W23 (Table 2). Of interest was the absence of the *asbB* locus from *B. thuringiensis* ATCC 33679 whereas this organism had an apparent *asbA* gene (Table 2). These data confirm the involvement of the *asb* region in assembly of petrobactin but not in biosynthesis of the catechol 3,4-DHB. The possible presence of one of the two *asb* genes in the *B. thuringiensis* strain used here suggests the existence of petrobactin producing strains of this species, especially those pathogenic strains, for example, *B. thuringiensis* 97-27 subsp. *konkukian* that may have anthrax-like characteristics [4]. Blast searches show the presence of the *asb* region in this strain of *B. thuringiensis* [15].

In summary, three *B. anthracis* Sterne strains and *B. cereus* ATCC 14579 were found to produce two catecholate siderophores, the 3,4-DHB containing citrate siderophore petrobactin and the 2,3-DHB based siderophore bacillibactin, while the insecticidal organism *B. thuringiensis* ATCC 33679 makes only bacillibactin. Therefore, petrobactin synthesis is not limited to the *B. anthracis* cluster within the *B. cereus* group and excretion of bacillibactin may be a *B. cereus* group characteristic. Exclusive

Table 2
PCR amplification of *asbA* and *asbB* loci[a]

Gene loci amplified	*B. anthracis* strains		*B. cereus* ATCC 14579	*B. thuringiensis* ATCC 33679	*B. subtilis* W23
	34F2	ΔasbA			
asbA	Positive	Negative	Positive	Positive	Negative
asbB	Positive	Positive	Positive	Negative	Negative
Ba 813	Positive	Positive	Negative	Negative	Negative

[a] Positive and negative indicate amplification or lack of amplification, respectively, of the target locus.

production of bacillibactin or petrobactin by the mutant strains *B. anthracis* Δ*asbA* and *B. anthracis* Δ*bacCEBF*, respectively, confirmed that the *bacACEBF* operon codes for bacillibactin production and the *asb* region is required for petrobactin biosynthesis. Moreover, the siderophore precursors 2,3-DHB and 3,4-DHB are synthesized by different pathways but the metabolic source and the genes encoding biosynthesis of 3,4-DHB in these organisms are presently not defined. *B. thuringiensis* ATCC 33679 may retain at least one of the genes for assembly of petrobactin, suggesting the existence of *B. thuringiensis* strains that produce petrobactin. Petrobactin is a member of the citrate based siderophore family, a group of iron transport cofactors that enhance microbial virulence [15], and this siderophore may be prevalent in *B. anthracis* and other members of the *B. cereus* group with pathogenic potential for vertebrates.

Note on nomenclature

As structures are now available for the siderophores of *B. anthracis*, *B. cereus*, and *B. thuringiensis*, as well as the identities of some of the genes encoding production of the siderophores, it is appropriate that earlier names take precedence. Therefore, the 2,3-DHB siderophore originally designated anthrabactin [19] coded for by the *bacACEBF* operon should be called bacillibactin and the 3,4-DHB siderophore originally called anthrachelin [8,19] coded for in part by the *asb* region should be designated petrobactin.

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