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

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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

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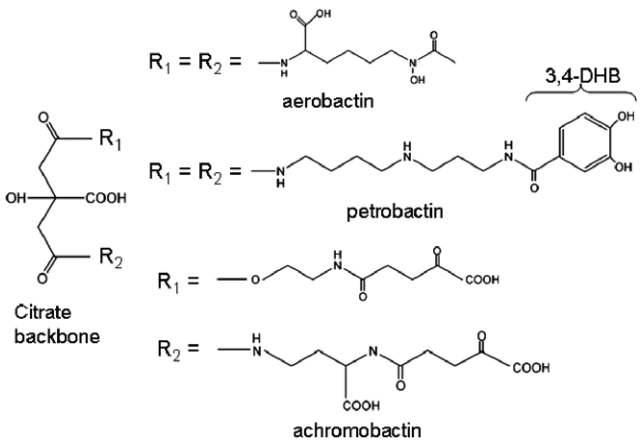

Fig. 1. Structures of representative citrate siderophores aerobactin [10], petrobactin [11], and achromobactin [12].

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

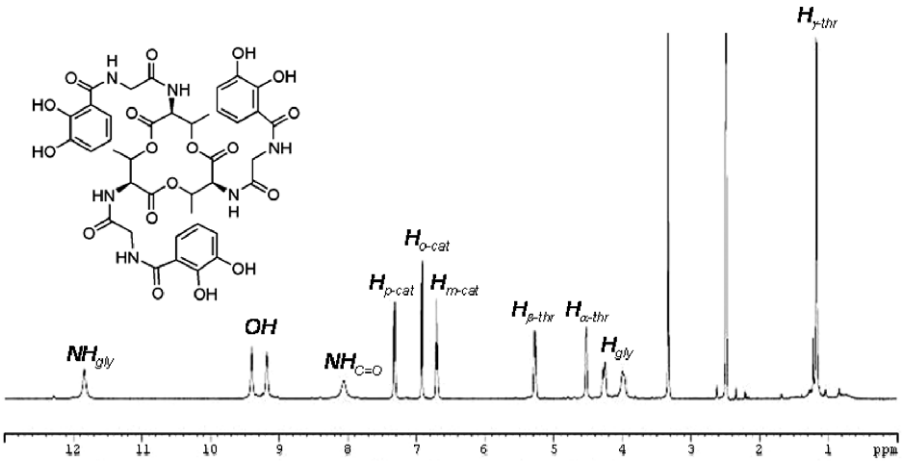

Fig. 2. ^{1}H NMR Spectra of bacillibactin purified from *B. cereus* in DMSO-d_6. Peaks are assigned directly on the spectra. Bacillibactin is composed of three types of fragments: the catechol iron binding moieties (cat), the threonine trilactone backbone (thr), and the glycine spacers (gly).