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Petrobactin is the primary siderophore synthesized by *Bacillus anthracis* str. *Sterne* under conditions of iron starvation

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Abstract

The siderophores of *Bacillus anthracis* are critical for the pathogen's proliferation and may be necessary for its virulence. *Bacillus anthracis* str. *Sterne* cells were cultured in iron free media and the siderophores produced were isolated and purified using a combination of XAD-2 resin, reverse-phase FPLC, and size exclusion chromatography. A combination of ^{1}H and ^{13}C NMR spectroscopy, UV spectroscopy and ESI-MS/MS fragmentation were used to identify the primary siderophore as petrobactin, a catecholate species containing unusual 3,4-dihydroxybenzoate moieties, previously only identified in extracts of *Marinobacter hydrocarbonoclasticus*. A secondary siderophore was observed and structural analysis of this species is consistent with that reported for bacillibactin, a siderophore observed in many species of bacilli. This is the first structural characterization of a siderophore from *B. anthracis*, as well as the first characterization of a 3,4-DHB containing catecholate in a pathogen.

Introduction

Iron is an essential requirement for the growth and proliferation of microbes, and the efficiency of its acquisition in bacteria is generally believed to be linked to their pathogenicity (Griffiths 1999). The biosynthesis of siderophores is one common strategy many microbes employ to obtain iron from their environment (Neilands 1995; Byers & Arceneaux 1998). These high-affinity iron chelates are biosynthesized by the cell and released to solublize and sequester Fe(III) and are then recognized by the host as an iron-siderophore conjugate by specific receptors on the cell surface (Faraldo-Gomez & Sansom 2003). Typically, siderophores are categorized by their iron-liganding groups, including catecholates, α-hydroxy carboxylates, hydroxamates, and mixed-types containing one or more of the aforementioned groups (Figure 1) (Matzanke 1991). A common trend, which is mirrored in terpene (Hemmerlin *et al.* 2003; Wu *et al.* 2004) and polyketide (Thomas 2001) secondary metabolites, is that microbes from the same genus frequently biosynthesize similar, if not identical siderophores. Aerobactin and enterobactin, for example, have been observed to be produced in members of several genera of enteric bacteria (Neilands & Nakamura 1991; Brackelsberg *et al.* 1997). While these siderophores are not necessarily exclusively produced by a particular genus, the production of similar siderophores by similar bacteria is likely a result of the high genome conservation within species, specifically in terms of siderophore biosynthetic machinery.

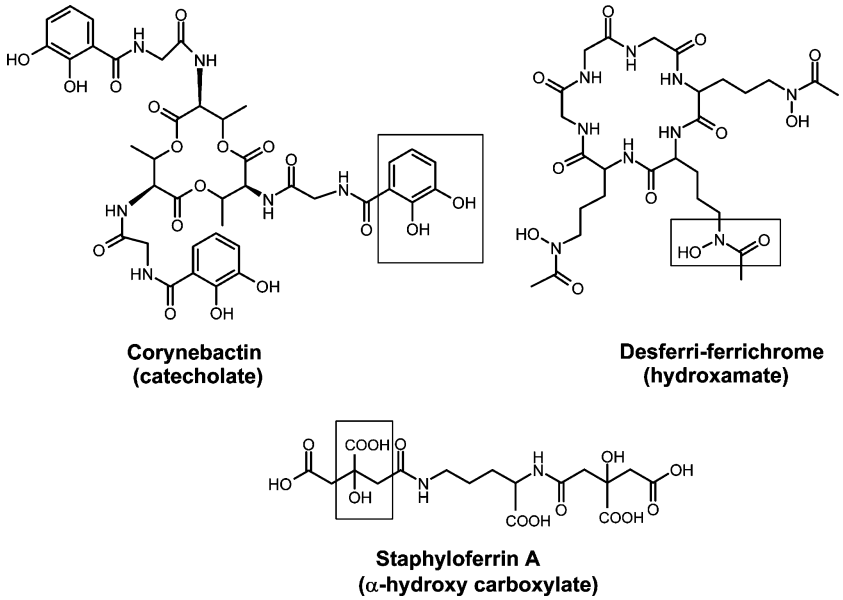


Figure 1. Representative siderophores with catechol, hydroxamate, and α-carboxy hydroxylate moieties.

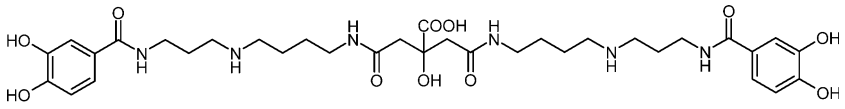
B. subtilis and *B. anthracis*, for instance, contain 63% identity within the ~4 kDa operon identified to be responsible for bacillibactin biosynthesis in the former (May *et al.* 2001).

B. anthracis, the etiological agent of anthrax, and has been the focus of a great deal of attention as a result of the terror attacks in the US in 2001 (Jernigan *et al.* 2001). Although relatively little has been reported at this time about the iron uptake mechanisms in this pathogen, genome sequencing projects have uncovered two possible biosynthetic operons in *B. anthracis* (Read *et al.* 2003). One contains homology (~45% similarity) to genes involved in aerobactin biosynthesis (termed "asb" operon; hydroxamate based) in *E. coli*, and the other has a significant degree of homology (63% identity, 75–86% similarity) to genes involved in bacillibactin biosynthesis (termed "bac" operon; catecholate based) in *B. subtilis* and other species of bacilli. One of these operons (asb) was shown to be essential for growth in macrophages and virulence in mice (Cendrowski *et al.* 2004). Siderophore biosynthesis has also shown promise as a novel antibiotic target against tuberculosis and plague (Ferreras *et al.* 2005). Thus, anti-anthrax strategies which target siderophore biosynthesis or uptake may be effective in preventing infection. The development of new anti-anthrax measures to counter exposure of this bioweapon to a larger populace is of utmost importance (Webb & Blaser 2002). In this report, we have isolated and characterized the primary siderophore synthesized by *B. anthracis* under conditions of iron starvation, and have identified it as petrobactin, which is an unusual 3,4-dihydroxybenzoate containing mixed-type siderophore previously only observed to be produced by an unrelated marine bacterium (Figure 2) (Barbeau *et al.* 2002). Although 3,4-dihydroxybenzoic acid has long been observed in *B. anthracis* culture media (Chao *et al.* 1967), and recently reported to be involved in the organisms siderophores (Garner *et al.* 2004), a complete multidentate 3,4-DHB containing siderophore has never been reported in this, or in any other pathogenic bacteria. A secondary siderophore was also observed, and structural analysis of this species is consistent with that reported for bacillibactin, a tris catecholate siderophore found in many species of bacilli (May *et al.* 2001).

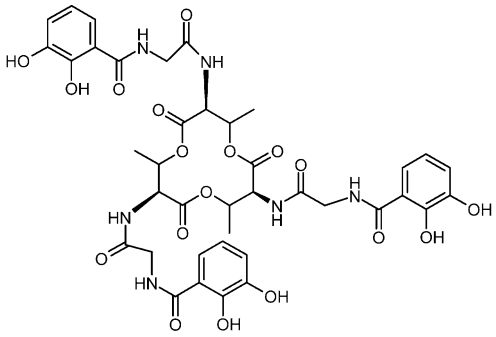
Materials and methods

General methods

All chemical reagents were purchased from Aldrich. Isolates of *B.anthracis* str. Sterne were kindly provided both by J. Pannucci (Los Alamos



Petrobactin



Bacillibactin

Figure 2. Chemical structures of petrobactin and bacillibactin.

Natl. Laboratory) and P. Jackson (Lawrence Livermore Natl. Laboratory). *B. subtilis* 6051 was kindly provided by K. Hill (Los Alamos Natl. Laboratory). *B. lichenformis* 11945 was kindly provided by L. Hersman (Los Alamos Natl. Laboratory). The Sterne strain of *B.anthracis* lacks one (p×02)g of two toxin-encoding plasmids harbored by fully virulent strains, and as a result of this does not represent a significant danger to healthy individuals. It is genetically identical to pathogenic strains of this bacterium with the exception of the aforementioned plasmid, however it requires no special treatment outside of standard BSL-2 protocols. Electrospray MS/MS was performed on an Applied Biosystems Q-Star quadrupole TOF mass spectrometer in positive ion mode. MALDI MS was performed on an Applied Biosystems Voyager MS using α-cyano-hydroxycinnamic acid as matrix (0.1–1 to 1:1 sample to matrix ratios), in both positive and negative ion modes. Surface assisted laser desorption ionization (SALDI-MS) was also performed on an Applied Biosystems Voyager MS in both positive and negative ion modes. ^{1}H and ^{13}C NMR were taken in D_2O on a JEOL GSX NMR spectrometer at 270 MHz and 72 MHz, respectively. Atomic absorption (AA) spectroscopy was performed on a Perkin–Elmer AAnalyst 600 graphite furnace atomic absorption spectrometer. Thin-layer chromatography was performed on cellulose (Whatman), and stained with a solution of 1% $FeCl_3$ in 0.5 M HNO_3.

Preparation of iron-free media

Siderophore production in *B. anthracis* is greatly facilitated by the removal of even trace amounts of Fe. All glassware and culture vessels were washed with 9 M HNO_3 for 1 h and rinsed with copious amounts of deionized water prior to use. Cultures were grown in a minimal media containing KH_2PO_4 (5 mM), K_2HPO_4 (5 mM), HEPES (100 mM), Adenine (15.5 μM), Uracil (12.5 μM), L-Tryptophan (40 μM), L-Cysteine (70 μM), Glycine (200 μM), Thiamine–HCl (30 μM), and Casamino acids (3.6 g/l). The pH of this solution was adjusted to approximately 7.2, and the media was then treated with CHELEX resin (10 g/l) for 1 h. The resin was then removed by filtration, and $CaCl_2{\cdot}2H_2O$ and $MgSO_4{\cdot}7H_2O$ were added to concentrations of 100 μM and 40 μM, respectively. CHELEX resin has a moderate affinity for Ca^{2+} and Mg^{+} ions, and we have observed attenuated growth of *B. anthracis* in this media if these salts are added prior to CHELEX treatment. All cultures were autoclaved for 20 min before adding 0.125 ml/l of a general trace metals stock solution containing $CuSO_4$ (70 mg/l), $MnSO_4{\cdot}H_2O$ (35 mg/l), $ZnCl_2$ (23 mg/l), $CaCl_2$ (1 g/l), $CoCl_2$ (18 mg/l), H_3BO_3 (7 mg/l), and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (60 mg/l). Additionally, the $MnSO_4{\cdot}H_2O$ concentration was augmented to 5 μM in the final solution, and a 20% sterile CHELEX-treated glucose solution (10 ml/l) was added. AA spectroscopy showed that this media has <0.2 μM Fe.

Bacterial strains and growth conditions

Starter cultures of *B. anthracis* Sterne were made by inoculating a single colony into 10 ml of Brain/Heart Infusion (BHI) media (Gibco) (12–13 μM Fe by AA spectroscopy). Upon observing confluent growth, 0.5 ml of this culture was used to inoculate 25 ml of iron free media. Two to three subsequent inoculations into iron-free subcultures were performed prior to large scale growth to minimize iron contaminations from the BHI media. Twenty-five milliliter of a confluent iron free subculture were used to inoculate 2 l, the cultures were covered in foil and shaken at RT for 12–24 h. After growth was completed, bacteria were removed via centrifugation and the media filter sterilized by passing through a 0.22 μm filter. Small-scale (25 ml) growths were performed in this same manner, and grown either at 23 °C or 37 °C in ambient air or 37 °C in air supplemented with 5% CO_2. Small-scale cultures of *B. subtilis* 6051 and *B. lichenformis* 11945 were also grown using these conditions.

Purification of petrobactin

Filtered media was acidified to pH = 5 and stirred with XAD-2 resin (15 g/l) for 12 h. The resin was then loaded into a plastic FPLC column and washed with ddH_2O, until the eluent from the column showed a minimal absorbance at 260 nM. Crude petrobactin was then eluted from the column with 25% methanol. The presence of petrobactin, and other iron-binding species was also tracked by analyzing fractions with the Chrome-azurol S assay (Schwyn & Neilands 1987). The methanol was removed with a rotary evaporator and the sample was then reduced to dryness via lyophilization to yield 3–5 mg of a yellow–brown powder. The crude material was then redissolved in a minimal amount of ddH_2O and purified over C18 silica using FPLC. A gradient of 0–100% AcCN/0.1% TFA in ddH_2O/0.1%TFA at 2 ml/min over 2 h was used to elute petrobactin as a single peak. After concentration, minor contaminants were removed by passing the material over a Biogel P2 fine column. Dried petrobactin was reconstituted in ddH_2O and eluted from the column (1 ml/h) in this same solvent. The eluent was monitored at 260 nM and concentration of the eluted material typically affords 1.5–2 mg of a white powder, identified as petrobactin. R_f 0.9 (12:5:3/1-propanol:water:acetic acid, cellulose). ^{1}H NMR (270 MHz, D_2O) δ 7.24 (m, 4H), 6.88 (d, 2H, J = 10 Hz), 3.44 (t, 4H, J = 6Hz), 3.18 (t, 4H, J = 6 Hz), 3.04 (m, 8H), 2.60 (dd, 4H, J = 12 Hz, 32 Hz), 1.96 (m, 4H), 1.66 (m, 4H), 1.54 (m, 4H). ^{13}C NMR (72 MHz, D_2O) δ 171.8, 170.7, 148.3, 144.2, 125.8, 120.7, 116.0, 115.2, 74.7, 47.4, 45.2, 44.9, 38.6, 36.7, 26.0, 25.7, 23.1. UV (H_2O, pH = 3) 230 (30,100 M^{-1} cm^{-1}), 257 (10,200 M^{-1} cm^{-1}), 291 (5400 M^{-1} cm^{-1}) nm. ESI-MS m/z calcd for $C_{34}H_{51}N_6O_{11}$ $(M+H)^+$ 719.3610, found 719.3522.

Formation of Fe(III)-petrobactin

Iron bound petrobactin was formed in two ways. Fe(III) chloride (80 μM) was added to a solution of petrobactin (80 μM) in water at pH 3, and the pH was raised by the addition of minimal amounts of 1 M NaOH. Spectra were taken at various pH and this continued until no visible difference was noted upon base addition (pH $\sim$ 8). Alternatively, petrobactin (80 μM) in 50 mM Tris, pH = 8, was titrated with a stock solution of Fe(III) chloride to 80 μM. UV (H_2O, pH = 8) 237 (29,300 M^{-1} cm^{-1}), 281, 312 (17,200 M^{-1} cm^{-1}), 480 (2300 M^{-1} cm^{-1}) nm.

Hydrolysis of petrobactin

Solid petrobactin (1 mg) was dissolved in 9 N HCl (1 ml) and placed in a boiling water bath for 2 h. Following acid treatment, the sample was diluted into 5 ml of water, quenched with sodium bicarbonate, and the pH was then lowered to 5–6. The sample was then lyophilized to dryness, resuspended in 10 ml of methanol, and the spectrum was recorded from 200 to 700 nm.

Isolation of bacillibactin

The isolation of bacillibactin from culture extracts followed the same basic procedure as that described in the purification of petrobactin, however, bacillibactin was observed to elute from XAD resin only in 100% methanol. We observed a significant loss of material and purity upon concentration in vacuo, as removal of the solvent yielded <1 mg of a yellow oil which contained bacillibactin, and further purification has proven

unsuccessful. MALDI-MS m/z calcd for $C_{39}H_{41}N_6O_{18}$ $(M-H)^-$ 881.7725, found 881.6449.

Hydrolysis of bacillibactin and SALDI-MS analysis

Partial acid hydrolysis of bacillibactin prior to concentration was accomplished by heating the methanol XAD eluate (1 ml) with an equal volume of 6 N HCl in a boiling water bath for 30 min. The fragments were analyzed using nanoporous silica thin films for SALDI-MS, constructed in-house at LANL (A. Dattelbaum and S. Iyer, unpublished results). These films can be used in commercially available MALDI-TOF instruments using standard operating procedures, and unlike ESI-MS, allow for simultaneous examination of molecular fragments in both positive and negative ion modes. The use of SALDI-MS also avoids sample interference from the ionization of MALDI-MS matrices. The resulting ions are consistent with what has been previously reported for negative mode ESI-MS/MS of bacillibactin (May *et al.* 2001), as well as an identical SALDI-MS treatment of authentic bacillibactin produced by *B. subtilis*. $(M+H)^+$: 194.04 (DHB-Gly). $(M-H)^-$: 292.82 (DHB-Gly-Thr), 248.88 ((DHB-Gly-Thr)-CO_2).

Results

Our primary objective is to determine the chemical structures of the siderophores produced by *B. anthracis*. Although the organism has gene operons which putatively encode both hydroxamate (asb operon) and catecholate (bac operon) siderophores, previous reports have demonstrated that culture broth of *B. anthracis* gave a negative result in the Czaky test, which assays for the presence of hydroxamate siderophores (Czaky 1948; Gilliam *et al.* 1981). This suggests that hydroxamate siderophores are either not produced by the organism, or produced at a level which is insufficient to yield a positive result using this assay. We employed a general strategy to purify the catechol siderophores from culture media using Amberlite XAD-2 resin. The amberlite XAD series of resins adsorb small aromatic and aliphatic molecules from aqueous solutions have found extensive use in the purification of catecholate siderophores. Upon batch binding and elution with methanol, we were surprised to find two dominant species by MS. The minor component of the two (typically 20–25% of the total amount of isolated catecholates by weight) showed an $(M-H)^-$ m/z of 881 Da, which is consistent with the predicted $(M-H)^-$ m/z for bacillibactin, while the major component (75–80% of the total isolated) showed a $(M+H)^+$ m/z of 719 Da. We observe the minor component to be unstable in the absence of solvent, and thus have not obtained adequate material for rigorous chemical structure analysis. However, both the observed m/z for this material as well as fragments obtained from a partial acid digestion are consistent with those reported for authentic bacillibactin (May *et al.* 2001). Following purification of the primary siderophore, Electrospray-MS/MS was used to fragment the material. The fragmentation pattern was identical to that reported for petrobactin, and included fragments for dihydroxybenzoate, spermidinyl, and citrate moieties (Figure 3). Both the ^1H and ^{13}C spectra for our isolated species agree well with that reported both for petrobactin isolated from *M. hydrocarbonoclasticus* culture media, and for chemically synthesized standards (Bergeron *et al.* 2003; Gardner *et al.* 2004; Hickford *et al.* 2004). Minor differences in the ^1H NMR spectra between our isolated material (in D_2O) and that reported for petrobactin (in DMSO) are consistent with changes expected from analysis in a protic rather than in an aprotic solvent. All exchangeable protons (phenolic, amide and acidic) in the compound, for example, are not visible in our spectra.

Synthetic standards of petrobactin and its 2,3-DHB containing homolog have been reported and characterized (Bergeron *et al.* 2003; Gardner *et al.* 2004), and these compounds are distinguishable both from their resonances and splitting pattern in the aromatic region of the ^1H NMR spectra. The splitting pattern we observe for the resonance at 6.88 ppm (d, J = 10 Hz) is consistent with its counterpart in 3,4-petrobactin (6.76 ppm, d, J = 10 Hz), but not for any observed in standards of 2,3-petrobactin (aromatic protons for this compound resonate at 6.69, 6.92, and 7.26 ppm and all appear as doublets of doublets, $J = \sim 1$ and 8 Hz, even in the presence of a deuterated protic solvent). Although this observation alone suggests that our isolated species contains 3,4-catecholates, differences in field strength of our spectrometer (270 MHz), relative to that used in the

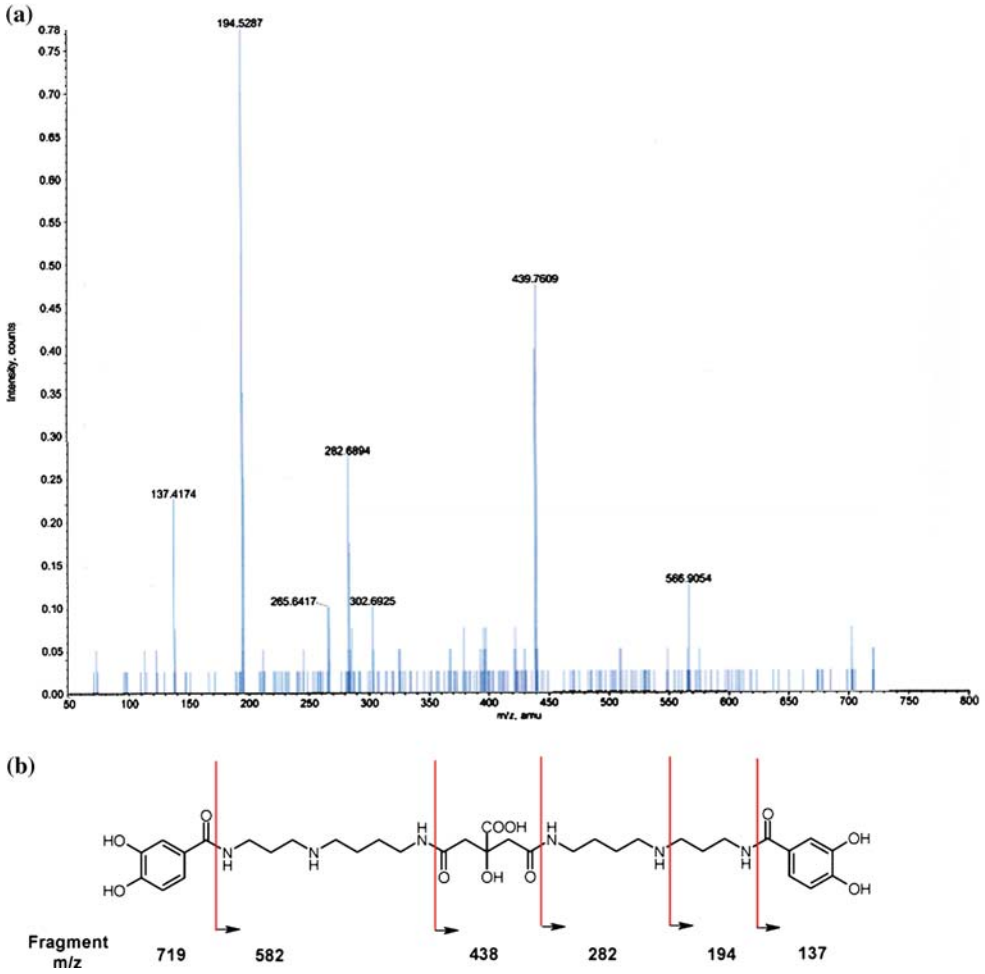


Figure 3. (a) ESI-MS/MS fragmentation pattern of isolated petrobactin. (b) Structure of petrobactin and m/z values for selected fragments. The mono-catechol containing species is not seen as a fragment (m/z = 582), but may be dehydrated or exist as a succinimide (m/z = 565) (see ref. 11).

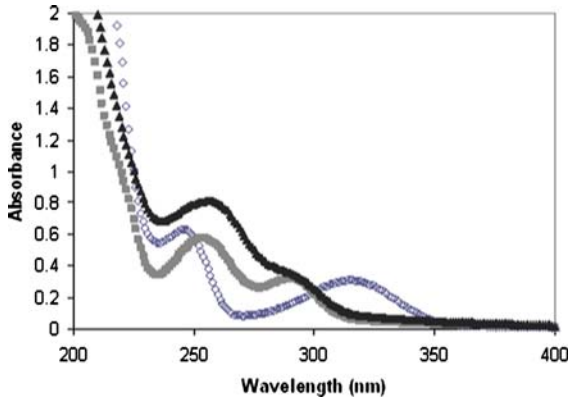


Figure 4. UV-Visible absorption spectrum of petrobactin hydrolysate (▲) as compared to standards of 2,3-DHB (◇) and 3,4-DHB (■).

aforementioned studies (500 MHz), prevent us from assigning all of the splitting patterns in the aromatic region. Thus, the UV-Vis spectroscopic profile of hydrolyzed petrobactin was obtained and compared to that for authentic 3,4-dihydroxybenzoic acid (Figure 4). The 2,3- and 3,4-DHB groups have drastically different UV-Visible absorbances, and this technique was used to establish the catecholate hydroxylation pattern in at least one siderophore from *B. anthracis* (Garner *et al*. 2004). Two main absorbances were observed in the UV region at 258 and 292 nm, which is consistent with that reported for 3,4-DHB (~260 and 295 nm), but not for 2,3-DHB (248 and 318 nm).

The majority of all catecholate siderophores which have been structurally characterized have been 2,3-dihydroxybenzoate containing species. In

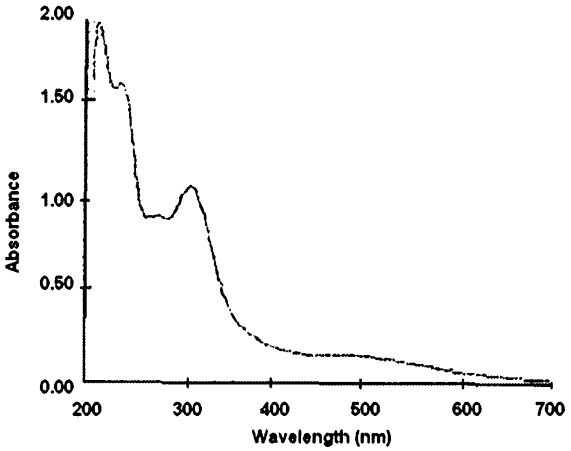


Figure 5. UV-Visible absorption spectrum of Fe(III)-petrobactin.

fact, only petrobactin, and a related siderophore, petrobactin sulfate, have been reported to contain the 3,4-dihydroxyl substitution pattern. Interestingly, 3,4-dihydroxybenzoic acid was reported to be observed in *B. anthracis* culture media in the early 1960s (Chao *et al*. 1967), and more recently implicated that a catecholate siderophore produced by *B. anthracis* also contained an 3,4-DHB moiety (Garner *et al*. 2004). Although the exact chemical structure of this species was not determined, its biosynthesis was shown to be under temperature control; the species was detectable when cultures were grown at 23 °C, but not when the organism was cultured at 37 °C in 5% CO_2. We observe petrobactin to be produced regardless of the culture temperature (Figure 6), however, bacillibactin is undetectable by MALDI-MS analysis in crude siderophore isolates of cultures grown at 37 °C/5% CO_2 (Figure 7). Interestingly, some bacillibactin is observed when cultures are grown at 37 °C in ambient air, which suggests its production may be influenced both by temperature and bicarbonate.

The UV-Vis absorption spectrum of Fe(III)-petrobactin that we observe is also consistent with previous reports (Figure 5). Another interesting feature of petrobactin is the siderophore becomes photolabile upon binding of Fe(III) (Barbeau *et al*. 2002). A light-catalyzed decarboxylation of the iron–siderophore conjugate was observed to yield a species with a $(M-H^+)$ m/z of 673 Da, with a concomitant change in the UV-Vis spectrum. We typically analyze purified samples of siderophores with MALDI-MS, and if the culture vessels are not covered during growth, one of the main contaminants observable after XAD elution has a $(M-H^+)$ m/z of 655 Da, which could be explained by a decarboxylation in addition to a dehydration in the MALDI matrix. Presumably, this species could arise from a photolysis caused by the remaining iron (<0.2 μM) present in the media. Additionally, we find that decarboxylation of petrobactin may also be afforded by excess base (as monitored by MALDI-MS). The UV-spectrum of the base-treated material titrated with Fe(III) is also consistent with that reported for the Fe(III)-photoproduct (data not shown).

Discussion

Siderophore biosynthesis, in addition to contributing actively to the growth of this *B. anthracis*, has

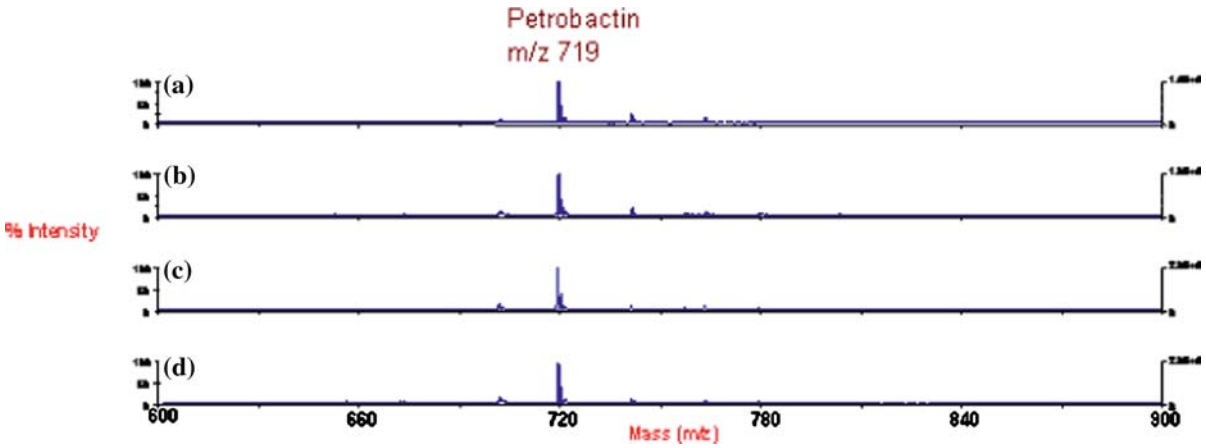


Figure 6. Positive ion MALDI-MS of crude XAD2 eluents of cultures grown under the following conditions: (a) 23 °C in ambient air. (b) 30 °C in ambient air. (c) 37 °C in ambient air. (d) 37 °C/5% CO_2 in air.

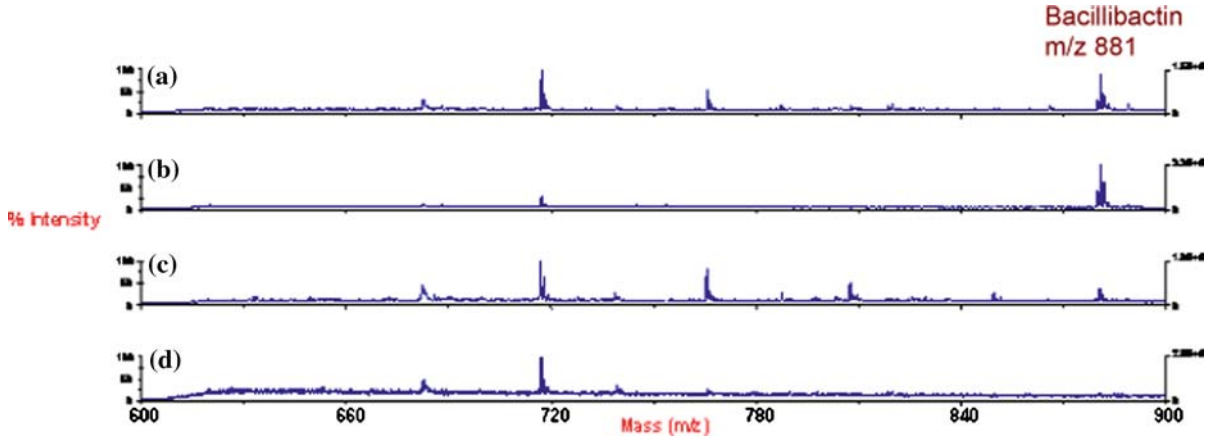


Figure 7. Negative ion MALDI-MS of crude XAD2 eluents of cultures grown under the following conditions: (a) 23 °C in ambient air. (b) 30 °C in ambient air. (c) 37 °C in ambient air. (d) 37 °C/5% CO_2 in air.

also been shown to be critical for virulence and survival in mammalian macrophages (Cendrowski *et al*. 2004). Using genetic deletions in *B. anthracis* str. *Ames*, the authors showed that bac$^-$ strains produced attenuated levels of catechol siderophore relative to the wild-type strain (by 41%), although its growth in iron-depleted media and virulence in mice were not affected. While a disruption of the bac operon would be expected to abolish bacillibactin synthesis, it would not be expected to attenuate petrobactin synthesis. Since we observe petrobactin to accumulate in the media to a larger extent than the *B. anthracis* bacillibactin, it is reasonable to assume the bacteria can use petrobactin for growth and virulence in the absence of bacillibactin.

As noted previously, the presence of free 3,4-DHB in culture extracts of *B. anthracis* has been observed for some time, and recently implicated to be incorporated into catechol siderophores of this organism. The observation that *B. anthracis* produces petrobactin is consistent with these observations, as petrobactin, along with a sulfated derivative, are the only reported examples of catecholate siderophores which contain 3,4-DHB groups instead of the ubiquitous 2,3-DHB reported for all other catecholates. A petrobactin is produced by *B. anthracis* str. Sterne isolates from two separate sources provided to us as single colonies on solid media. Thus, the possibility that petrobactin may be produced by a contaminating organism is unlikely. We do not observe petrobactin by MALDI-MS analysis in siderophore isolates of *Bacillus lichenformis* or *Bacillus subtilis* when these strains are grown under similar conditions as that of *B. anthracis*. Although it is not uncommon for oceanic bacteria to produce similar siderophores to terrestrial counterparts, the reasons why *B. anthracis* (highly dispersed soil bacterium) would only share this metabolic capability with *M. hydrocarbonoclasticus* (oil-degrading oceanic bacterium) and not with similar species of bacilli remains a mystery.

Another point of interest regards the bacillibactin from *B. anthracis*. Other species of bacilli are known to produce this trithreonine catecholate with 2,3-DHB groups. However, given that *B. anthracis* already incorporates 3,4-DHB into petrobactin, it is possible that the *B. anthracis* analogue of bacillibactin may contain these moieties as well. This hypothesis is supported by the observation that a temperature-controlled siderophore in *B. anthracis* has previously been shown to contain 3,4-DHB groups (Garner *et al*. 2004), and that we observe production of the putative bacillibactin to clearly be the catecholate species that is under temperature control. Further, structural identification efforts of this siderophore are currently under way.

The inhibition of siderophore biosynthesis has been recently reported to be a promising antibiotic strategy against tuberculosis and plague (Ferreras *et al*. 2005). Since petrobactin is the primary catecholate species produced by anthrax, its production is not repressed by conditions similar to that found in a human host, and is not known to be produced by any other species of bacilli, petrobactin synthesis may be an attractive target for novel anti-anthrax therapeutics.

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