Biometals (2008) 21:581–589
DOI 10.1007/s10534-008-9144-9

Petrobactin is produced by both pathogenic and non-pathogenic isolates of the *Bacillus cereus* group of bacteria

Andrew T. Koppisch · Suraj Dhungana · Karen K. Hill · Hakim Boukhalfa · Henry S. Heine · Leslie A. Colip · Raymond B. Romero · Yulin Shou · Lawrence O. Ticknor · Babetta L. Marrone · Larry E. Hersman · Srinivas Iyer · Christy E. Ruggiero

Received: 20 February 2008 / Accepted: 23 April 2008 / Published online: 6 May 2008

Abstract Petrobactin is the primary siderophore synthesized by *Bacillus anthracis* str Sterne and is required for virulence of this organism in a mouse model. The siderophore's biosynthetic machinery was recently defined and gene homologues of this operon exist in several other *Bacillus* strains known to be mammalian pathogens, but are absent in several known to be harmless such as *B. subtilis* and *B. lichenformis*. Thus, a common hypothesis regarding siderophore production in *Bacillus* species is that petrobactin production is exclusive to pathogenic isolates. In order to test this hypothesis, siderophores produced by 106 strains of an in-house library of the *Bacillus cereus* sensu lato group were isolated and identified using a MALDI-TOF-MS assay. Strains were selected from a previously defined phylogenetic tree of this group in order to include both known pathogens and innocuous strains. Petrobactin is produced by pathogenic strains and innocuous isolates alike, and thus is not itself indicative of virulence.

Keywords Siderophores · Iron · *Bacillus cereus* · *Bacillus anthracis* · *Bacillus thuringiensis*

A. T. Koppisch · S. Dhungana · K. K. Hill · L. A. Colip · R. B. Romero · Y. Shou · B. L. Marrone · L. E. Hersman · S. Iyer
Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA

A. T. Koppisch (✉)
Department of Biology, Northern Arizona University, 500 S. Beaver St, Flagstaff, AZ 86004, USA
e-mail: koppisch@lanl.gov

H. Boukhalfa · C. E. Ruggiero
Chemistry Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA

H. S. Heine
Division of Bacteriology, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, MD 21702, USA

L. O. Ticknor
Decision Application Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA

Introduction

The ability of pathogenic bacteria to acquire iron has been linked to their virulence (Byers and Arceneaux 1998; Griffiths 1999; Koehler 2000). Certain Gram-negative bacteria have extremely specialized membrane proteins which directly extract iron from the iron transport protein of the mammalian host, transferrin (Dhungana et al. 2005). The majority of bacteria, however, produce iron specific chelating agents termed siderophores, that can sequester iron from various iron containing proteins (Dionis et al. 1991; Neilands 1995). The success of a pathogen within a host depends on its ability to acquire iron, which depends on the siderophore's thermodynamic and kinetic ability to obtain iron. Therefore,

siderophore biosynthesis has been increasingly recognized as a determinant of virulence in many pathogenic bacteria, including *B. anthracis* (De Voss et al. 2000; Cendrowski et al. 2004; Dale et al. 2004; Ferreras et al. 2005; Quadri 2007).

B. anthracis str Sterne produces two siderophores when cultured in iron-deficient media (Fig. 1) (Koppisch et al. 2005). The primary siderophore is the unusual 3,4-dihydroxybenzoate-containing siderophore petrobactin (Barbeau et al. 2002). In addition, a secondary siderophore, bacillibactin, is also produced. Bacillibactin, as its name suggests, has been observed in culture extracts of several species of *Bacillus*, including *B. subtilis* and *B. lichenformis* (May et al. 2001). When *B. anthracis* str Sterne is grown under physiologically-relevant conditions (37°C, 5% CO_2), production of bacillibactin is suppressed while production of petrobactin is not (Koppisch et al. 2005). Unlike bacillibactin, petrobactin is required for virulence of *B. anthracis* in a mouse model (Cendrowski et al. 2004) and is capable of evading the mammalian immune system (Abergel et al. 2006). Abergel et al. showed that siderocalin, which is a protein in mammalian immune response dedicated to sequestering siderophores, binds bacillibactin but is incapable of binding petrobactin. Moreover, petrobactin production has been observed in three isolates of the Sterne strain and one toxigenic strain of *B. cereus*, but absent in a strain of *B. thuringiensis* known to be harmless to humans (Wilson et al. 2006). Given all of this, it has been reasonably hypothesized that petrobactin is only produced by the pathogenic species (Wilson et al. 2006; Oves-Costales et al. 2007). A great deal of effort in many laboratories has been invested to determine factors, genetic or otherwise, which can distinguish the pathogenic isolates from innocuous strains. Thus, the hypothesis that petrobactin is only synthesized by pathogenic isolates is especially intriguing as this would essentially provide a convenient signature for the determination of harmful species. However, most laboratories have limited access to multiple strains within the *B. cereus* sensu lato group and as such, a comprehensive examination of this hypothesis including both known pathogens and environmental isolates has never been conducted. In this manuscript, we have examined petrobactin production in over 100 strains from an in-house library selected to represent all branches of the *B. cereus* sensu lato group phylogenetic tree. In doing so, we aimed to unequivocally determine if this metabolite is exclusive to known pathogens, and thus useful as a biomarker of virulence.

Materials and methods

General methods

All chemical reagents were purchased from Aldrich. All *Bacillus* strains except Ames and Vollum isolates were from an in-house strain library at Los Alamos Natl. Laboratory. All strains were cultured with protocols appropriate for their specific biosafety levels (BSL-1, 2 or 3). MALDI-TOF 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. Atomic absorption (AA) spectroscopy was performed on a Perkin–Elmer AAnalyst 600 graphite furnace atomic absorption spectrometer.

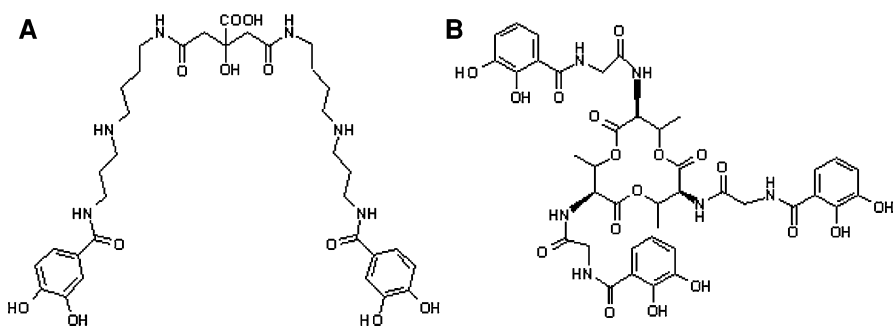


Fig. 1 Chemical structures of siderophores produced by *B. anthracis* str. Sterne. (**a**) Petrobactin, (**b**) bacillibactin

Preparation of iron-free media

We observe that reliable siderophore identification in *Bacillus* strains is greatly facilitated by the removal of trace amounts of Fe within the growth media. Media preparation follows that reported previously. Briefly, 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 7.0, 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 is known to bind Ca^{2+} and Mg^{+} ions (as reported by the manufacturer), and we have observed attenuated growth 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 verified that this media has <0.2 μM Fe.

Bacterial strains and growth conditions

Starter cultures of all *B. cereus* group isolates were made by inoculating a single colony (grown on nutrient agar) directly into 25 ml of iron-free media. The cultures were grown for 48 h at ambient conditions and at 37°C supplemented with 5% CO_2. After bacterial growth, the culture media was sterilized with 0.22 μM Steriflip filters. For BSL-2 and BSL-3 strains, 0.5 ml of the media was plated onto nutrient agar plates and incubated at 37°C for a further 48 h. If no colonies were detected after this time, the media was released for further manipulation in BSL-1 laboratories.

Siderophore isolation and analysis

The siderophore screen in this report is an adaptation of a procedure previously used for the large-scale purification of catecholate siderophores in our laboratory. Briefly, approximately 0.5 g of XAD-2 resin was added to sterile filtered CA media in a 50 ml falcon tube and the cap affixed tightly. The resin was mixed with media on a rotary platform overnight at 4°C, at which time it was removed and the medium decanted. The resin was then washed with 3 × 50 ml of ddH_2O. All remaining ddH_2O was removed from the resin and 1 ml of neat methanol was added, and the resin was agitated for 30 s on a vortex mixer. Approximately 2 μl of methanol eluent was applied directly to an individual well on a stainless steel MALDI plate, and overlayed (1:1 v/v) with freshly prepared α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA. Upon drying, the samples were analyzed using standard MALDI conditions. The presence of petrobactin was assayed in positive ionization mode using standard instrument settings.

AFLP data analysis

AFLP data of all strains in this study has been previously reported and data analysis of the microbial DNAs from the strains in this study was performed as previously described (Hill et al. 2004). Briefly, similarities between samples were measured using the Jaccard coefficient. Dendrograms were produced using the similarity matrix and the unweighted pair-group mean average method (F. J. Rohlf, NTSYS-PC numerical taxonomy and multivariate analysis system, version 1.8; Exeter Software, Setauket, N.Y.). Principal components for the AFLP fingerprint data were derived. The first and second and the first and third principal components were plotted with characters relating to the ten major clusters seen on the UPGMA dendrograms. All statistical data manipulations were done by using codes developed in S-Plus (Data Analysis Products Division, MathSoft, Seattle, Wash.).

Results

As stated previously, we selected strains in the *B. cereus* sensu lato group to include strains which

are both closely and more distantly related to *B. anthracis*. With the exception of *B. thuringiensis* 97–27, all strains of this species were isolated from soil and are considered innocuous (as evidenced by their biological safety level 1 designation by the U.S. Centers of Disease Control). The strains of *B. cereus* examined included those isolated from contaminated food products as well as various environmental isolates. Hill et al reported that a significant number of the toxigenic *B. cereus* isolates are more closely related to the *B. anthracis* isolates than those strains isolated from the environment, and this is also represented in the phylogenetic tree presented here (Fig. 2) (Hill et al. 2004). All *B. anthracis* isolates examined are pathogenic and occupy one subbranch of the tree.

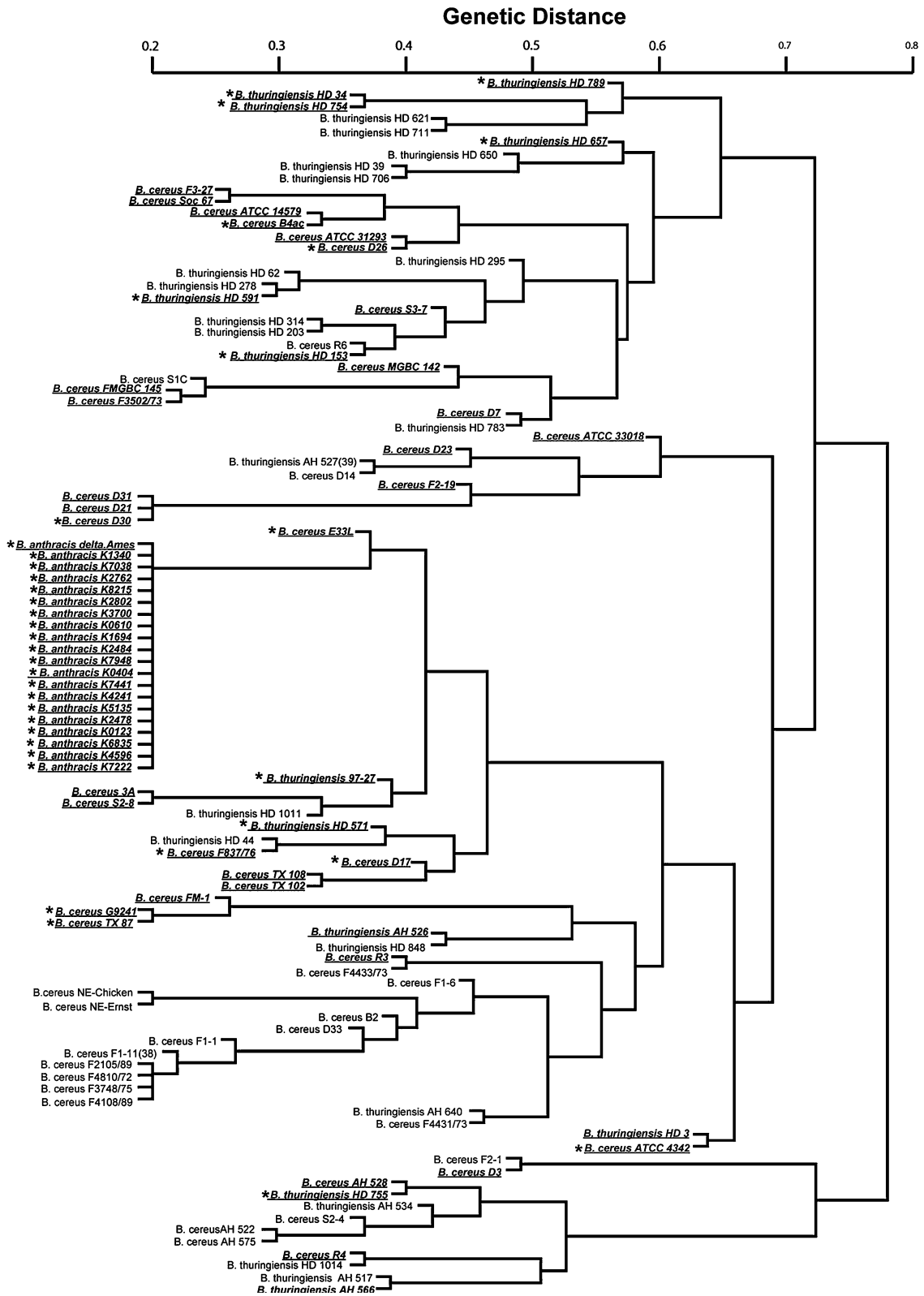
Our assay for petrobactin is amended from a protocol designed for the large-scale (multi-milligram) isolation of this metabolite and is designed to enable us to efficiently process a large number of bacterial samples (Koppisch et al. 2005). We observe our assay to reliably detect siderophores in culture at solution concentrations down to 20 nM. For comparison, under culture conditions similar to those reported here *B. anthracis* Sterne produces and accumulates petrobactin to concentrations of 3 μM or more (Koppisch et al. 2005). Thus, our assay is able to detect solution phase petrobactin concentrations that are approximately 150 fold less than that observed for the Sterne strain.

Including the *B. anthracis* subbranch, approximately 63% of the strains examined produced petrobactin (12/34 *B. thuringiensis* strains, 34/52 *B. cereus* strains, 20/20 *B. anthracis* strains), and like other *Bacillus* strains (May et al. 2001; Rey et al. 2004) all of the screened isolates also produced bacillibactin when grown under ambient conditions. Similar to that observed for the Sterne strain (Koppisch et al. 2005), bacillibactin production in all *B. anthracis* isolates examined was suppressed upon growth at 37°C with 5% CO_2 supplementation. The majority of the strains could be further grouped based on the ability to produce petrobactin, either primarily or as a singular component of a mixture of metabolites isolated in our screen (Figs. 2, 3). Furthermore, the ability to produce petrobactin is found in strains of every biological safety level (BSL 1, 2, and 3) designation examined (Fig. 4, Table 1). The chemical identities of the metabolites isolated along with petrobactin in some of the strains have not been identified, however they are likely not functional derivatives of petrobactin as no iron-binding ability is detected by them as measured with CAS assay reagent. Additionally, they are not consistent with conceivable shunt products of the petrobactin biosynthetic pathway (Lee et al. 2007; Oves-Costales et al. 2007) nor observed photochemical degradation products of this siderophore (Barbeau et al. 2002).

Fig. 2 Petrobactin production among *B. cereus* group isolates. Strains underlined/italicized are observed to produce petrobactin, and those further marked with an asterisk are observed to produce it as their primary siderophore

Discussion

The *B. cereus* group of bacteria encompasses the species *B. cereus*, *B. thuringiensis*, and *B. anthracis* (Sneath 1986). *B. thuringiensis* is known for its production of proteins that have insecticidal properties, and *B. cereus* isolates have been responsible for diarrheal and emetic outbreaks (Kramer and Gilbert 1989; Drobniewski 1993). *B. anthracis* is the causative agent of the potentially lethal disease anthrax. Given that the proliferation of *B. anthracis* is dictated in part by the ability of this organism to efficiently acquire iron from its host, the role of siderophores in virulence is evident (Cendrowski et al. 2004). The enzymes responsible for petrobactin biosynthesis are members of a family of proteins termed non-ribosomal peptide synthetase-independent siderophore (NIS) synthases, and other members of this family are known to produce siderophores important for infection in a number of pathogenic species (Challis 2005). Comparative genetic analysis also shows the petrobactin biosynthetic cluster is present in several known pathogenic *Bacillus* isolates but absent in *B. subtilis* and *B. lichenformis*. A phylogenetic analysis of these strains using fluorescent amplified fragment length polymorphism (AFLP) revealed the genetic diversity of this collection (Hill et al. 2004). One branch contained all of the *B. anthracis* strains, and some potential pathogenic strains of *B. cereus* and *B. thuringiensis* including *B. cereus* E33L, and *B. thuringiensis* 97–27, which is one of the few *B. thuringiensis* strains isolated from a human wound (Hernandez et al. 1999). All of the


Genetic Distance
0.2
0.3
0.4
0.5
0.6
0.7
0.8
* B. thuringiensis HD 789
*B. thuringiensis HD 34
* B. thuringiensis HD 754
B. thuringiensis HD 621
B. thuringiensis HD 711
* B. thuringiensis HD 657
B. thuringiensis HD 650
B. thuringiensis HD 39
B. thuringiensis HD 706
B. cereus F3-27
B. cereus Soc 67
B. cereus ATCC 14579
*B. cereus B4ac
B. cereus ATCC 31293
* B. cereus D26
B. thuringiensis HD 295
B. thuringiensis HD 62
B. thuringiensis HD 278
* B. thuringiensis HD 591
B. cereus S3-7
B. thuringiensis HD 314
B. thuringiensis HD 203
B. cereus R6
* B. thuringiensis HD 153
B. cereus MGBC 142
B. cereus S1C
B. cereus FMGBC 145
B. cereus F3502/73
B. cereus D7
B. thuringiensis HD 783
B. cereus ATCC 33018
B. cereus D23
B. thuringiensis AH 527(39)
B. cereus D14
B. cereus F2-19
B. cereus D31
B. cereus D21
*B. cereus D30
* B. cereus E33L
* B. anthracis delta.Ames
*B. anthracis K1340
*B. anthracis K7038
*B. anthracis K2762
*B. anthracis K8215
*B. anthracis K2802
*B. anthracis K3700
*B. anthracis K0610
*B. anthracis K1694
*B. anthracis K2484
*B. anthracis K7948
* B. anthracis K0404
* B. anthracis K7441
* B. anthracis K4241
* B. anthracis K5135
* B. anthracis K2478
* B. anthracis K0123
* B. anthracis K6835
* B. anthracis K4596
* B. anthracis K7222
* B. thuringiensis 97-27
B. cereus 3A
B. cereus S2-8
B. thuringiensis HD 1011
* B. thuringiensis HD 571
B. thuringiensis HD 44
* B. cereus F837/76
* B. cereus D17
B. cereus TX 108
B. cereus TX 102
B. cereus FM-1
* B. cereus G9241
*B. cereus TX 87
B. thuringiensis AH 526
B. thuringiensis HD 848
B. cereus R3
B. cereus F4433/73
B. cereus F1-6
B.cereus NE-Chicken
B. cereus NE-Ernst
B. cereus B2
B. cereus D33
B. cereus F1-1
B. cereus F1-11(38)
B. cereus F2105/89
B. cereus F4810/72
B. cereus F3748/75
B. cereus F4108/89
B. thuringiensis AH 640
B. cereus F4431/73
B. thuringiensis HD 3
*B. cereus ATCC 4342
B. cereus F2-1
B. cereus D3
B. cereus AH 528
*B. thuringiensis HD 755
B. thuringiensis AH 534
B. cereus S2-4
B. cereusAH 522
B. cereus AH 575
B. cereus R4
B. thuringiensis HD 1014
B. thuringiensis AH 517
B. thuringiensis AH 566

strains in this branch produced petrobactin as their primary siderophore. *B. anthracis* strains are genetically monomorphic (Keim et al. 1997, 2000), and the siderophores produced by this species is another illustration of this. However, we did not observe any correlation between strains capable of producing petrobactin and genetic relationship to *B. anthracis*, nor any relationship between petrobactin production and known pathogenicity/toxogenicity of strains. While all of the known mammalian pathogens did produce the siderophore, numerous innocuous soil-residing isolates of *B. cereus* and *B. thuringiensis* strains located in every phylogenetic branch are also fully capable of producing petrobactin. Furthermore, the amount of petrobactin produced was quantified for selected BSL-1 *B. thuringiensis* strains (HD 34, HD 591, HD 754, and HD 755). Accumulation of the siderophore in the culture media was observed to occur in comparable amounts (within experimental error) of those produced by *B. anthracis* Sterne grown under identical conditions. Although it is worth noting that all of the strains examined which are known to be capable of developing into potentially lethal infections in mammals (the *B. anthracis* isolates, *B. thuringiensis* 97–27, *B. cereus* G9241) do produce petrobactin as their main siderophore, our data shows that petrobactin production is widely distributed throughout the *B. cereus* group and is not exclusive to known pathogenic isolates.

While our results show petrobactin production is not indicative of virulence in and of itself, it is certainly a trait that is required for pathogenesis of these species in mammals (Casadevall 2006). A great deal of effort has been undertaken to characterize environmental *Bacillus* isolates (Keim et al. 2000; Hill et al. 2004; Hoffmaster et al. 2004; Daffonchio et al. 2006; Han et al. 2006; Sergeev et al. 2006) with a particular focus on those which have the potential to develop anthrax-like pathogenicity via acquisition of other virulence traits (either through inheritance or engineering). Identification of the petrobactin production phenotype in soil-residing strains provides a further means to aid these efforts.

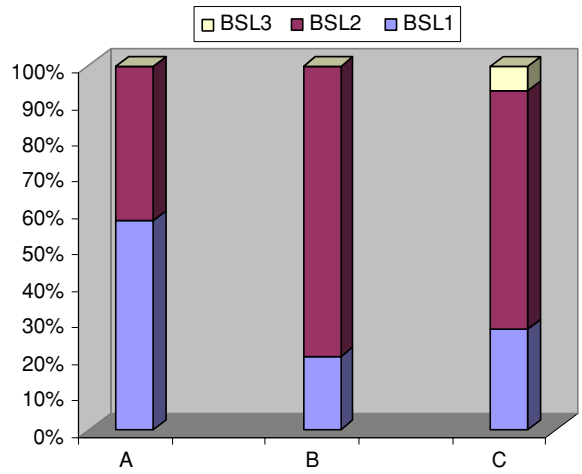


Fig. 4 Percentage of strains in each biological safety level designation and their siderophore production phenotypes. (**a**) No petrobactin, (**b**) petrobactin in a mixture of metabolites, (**c**) petrobactin alone

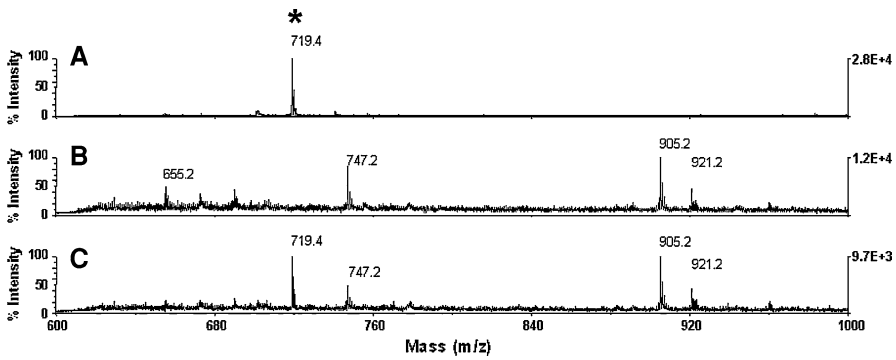


Fig. 3 Representative MALDI-MS (positive ionization mode) results for three strains which show each of the siderophore production phenotypes. (**a**) Petrobactin alone (K7741), (**b**) no petrobactin (D14), (**c**) petrobactin in a mixture of metabolites (F2-19). Petrobactin is marked with an asterisk (*). Bacillibactin is observed to be produced by all three of these strains when grown under ambient conditions (data not shown)

Table 1 List of strains that fall under each siderophore production category

Strain ID	Species	Strain ID	Species
Strains which do not produce petrobactin			
AH 522	*B. cereus*	**HD 39**	*B. thuringiensis*
AH 534	*B. thuringiensis*	**HD 44**	*B. thuringiensis*
AH 575	*B. thuringiensis*	**HD 62**	*B. thuringiensis*
AH 621	*B. thuringiensis*	**HD 87**	*B. thuringiensis*
AH 640	*B. thuringiensis*	**HD 203**	*B. thuringiensis*
AH 517	*B. thuringiensis*	**HD 278**	*B. thuringiensis*
AH 527	*B. thuringiensis*	**HD 295**	*B. thuringiensis*
B2	*B. cereus*	**HD 314**	*B. thuringiensis*
D14	*B. cereus*	**HD 621**	*B. thuringiensis*
D33	*B. cereus*	**HD 650**	*B. thuringiensis*
F1-1	*B. cereus*	**HD 706**	*B. thuringiensis*
F1-6	*B. cereus*	**HD 711**	*B. thuringiensis*
F1-11	*B. cereus*	**HD 783**	*B. thuringiensis*
F2-1	*B. cereus*	**HD 848**	*B. thuringiensis*
F2105/89	*B. cereus*	**HD 1011**	*B. thuringiensis*
F3748/75	*B. cereus*	**HD 1019**	*B. thuringiensis*
F4108/89	*B. cereus*	NE-Chicken	*B. cereus*
F4431/73	*B. cereus*	NE-Ernst	*B. cereus*
F4433/73	*B. cereus*	R6	*B. cereus*
F4810/72	*B. cereus*	S1C	*B. cereus*
Strains which produce petrobactin as one component of a mixture of metabolites			
ATCC 33018	*B. cereus*	MGBC 142	*B. cereus*
AH 526	*B. thuringiensis*	MGBC 145	*B. cereus*
AH 528	*B. cereus*	R3	*B. cereus*
AH 566	*B. thuringiensis*	R4	*B. cereus*
D3	*B. cereus*	S2-4	*B. cereus*
D7	*B. cereus*	S2-8	*B. cereus*
D12	*B. cereus*	S3-7	*B. cereus*
D23	*B. cereus*	Soc 67	*B. cereus*
D31	*B. cereus*	TX 102	*B. cereus*
F2-19	*B. cereus*	TX 108	*B. cereus*
F3-27	*B. cereus*	3A	*B. cereus*
F3502/73	*B. cereus*	4342	*B. cereus*
FM-1	*B. cereus*	14579	*B. cereus*
HD 3	*B. thuringiensis*	31293	*B. cereus*
HD 754	*B. thuringiensis*		
HD 755	*B. thuringiensis*		
Strains which produce petrobactin as their primary siderophore			
Ames	*B. anthracis*	F837/76	*B. cereus*
BA 155	*B. anthracis*	G9421	*B. cereus*
BA 156	*B. anthracis*	**HD 34**	*B. thuringiensis*
BA 160	*B. anthracis*	**HD 153**	*B. thuringiensis*
BA 161	*B. anthracis*	**HD 571**	*B. thuringiensis*

Table 1 continued

Strain ID	Species	Strain ID	Species
BA 162	*B. anthracis*	**HD 591**	*B. thuringiensis*
BA 169	*B. anthracis*	**HD 657**	*B. thuringiensis*
BA 170	*B. anthracis*	**HD 789**	*B. thuringiensis*
BA 172	*B. anthracis*	**HD 754**	*B. thuringiensis*
BA 176	*B. anthracis*	**HD 755**	*B. thuringiensis*
B4ac	*B. cereus*	E33L	*B. cereus*
D17	*B. cereus*	TX87	*B. cereus*
D26	*B. cereus*	**Vollum**	*B. anthracis*
D30	*B. cereus*	4342	*B. cereus*
		97–27	*B. thuringiensis*

Biosafety level 1 designated strains are in bold, BSL-2 in normal text, and BSL-3 strains in bold and underlined

Acknowledgements R.B.R. is supported by the NSF-REU Bioscience and Biotechnology PUSH program for northern New Mexico. We thank Drs. David Fox and Cindy Browder for helpful discussions. This work was supported by a Laboratory Directed Research and Development exploratory research grant. Los Alamos National Laboratory is operated by the University of California for the U.S. Department of Energy under contract W-7405-ENG-36.

References

Abergel RJ, Wilson MK, Arceneaux JE, Hoette TM, Strong RK, Byers BR, Raymond KN (2006) Anthrax pathogen evades the mammalian immune system through stealth siderophore production. Proc Natl Acad Sci U S A 103:18499–18503

Barbeau K, Zhang G, Live DH, Butler A (2002) Petrobactin, a photoreactive siderophore produced by the oil-degrading marine bacterium *Marinobacter hydrocarbonoclasticus*. J Am Chem Soc 124:378–379

Byers BR, Arceneaux JE (1998) Microbial iron transport: iron acquisition by pathogenic microorganisms, vol 35. Marcel Dekker Inc., New York, NY

Casadevall A (2006) Cards of virulence and the global virulome for humans. Microbe 1:359–364

Cendrowski S, MacArthur W, Hanna P (2004) *Bacillus anthracis* requires siderophore biosynthesis for growth in macrophages and mouse virulence. Mol Microbiol 51:407–417

Challis GL (2005) A widely distributed bacterial pathway for siderophore biosynthesis independent of nonribosomal peptide synthetases. Chembiochem 6:601–611

Daffonchio D, Raddadi N, Merabishvili M, Cherif A, Carmagnola L, Brusetti L, Rizzi A, Chanishvili N, Visca P, Sharp R, Borin S (2006) Strategy for identification of *Bacillus cereus* and *Bacillus thuringiensis* strains closely related to *Bacillus anthracis*. Appl Environ Microbiol 72:1295–12301

Dale SE, Doherty-Kirby A, Lajoie G, Heinrichs DE (2004) Role of siderophore biosynthesis in virulence of *Staphylococcus aureus*: identification and characterization of genes involved in production of a siderophore. Infect Immun 72:29–37

De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y, Barry CE III (2000) The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. Proc Natl Acad Sci U S A 97:1252–1257

Dhungana S, Anderson DS, Mietzner TA, Crumbliss AL (2005) Kinetics of iron release from ferric binding protein (FbpA): mechanistic implications in bacterial periplasm-to-cytosol Fe^{3+} transport. Biochemistry 44:9606–9618

Dionis JB, Jenny HB, Peter HH (1991) Therapeutically useful iron chelators. In: Winkelmann G (ed) Handbook of microbial iron chelates. CRC Press, Boca Raton, FL, pp 15–64

Drobniewski FA (1993) *Bacillus cereus* and related species. Clin Microbiol Rev 6:324–338

Ferreras JA, Rye JS, Di Lello F, Tan DS, Quadri LEN (2005) Small molecule inhibition of siderophore biosynthesis in *Mycobacterium tuberculosis* and *Yersinia pestis*. Nat Chem Biol 1:29–32

Griffiths E (1999) Iron in biological systems, 2nd edn. John Wiley, Chichester, UK

Han CS, Xie G, Challacombe JF, Altherr MR, Bhotika SS, Bruce D, Campbell CS, Campbell ML, Chen J, Chertkov O, Cleland C, Dimitrijevic M, Doggett NA, Fawcett JJ, Glavina T, Goodwin LA, Hill KK, Hitchcock P, Jackson PJ, Keim P, Kewalramani AR, Longmire J, Lucas S, Malfatti S, McMurry K, Meincke LJ, Misra M, Moseman BL, Mundt M, Munk AC, Okinaka RT, Parson-Quintana B, Reilly LP, Richardson P, Robinson DL, Rubin E, Saunders E, Tapia R, Tesmer JG, Thayer N, Thompson LS, Tice H, Ticknor LO, Wills PL, Brettin TS, Gilna P (2006) Pathogenomic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis*. J Bacteriol 188: 3382–3390

Hernandez E, Ramisse F, Cruel T, le Vagueresse R, Cavallo JD (1999) *Bacillus thuringiensis* serotype H34 isolated from human and insecticidal strains serotypes 3a3b and H14 can lead to death of immunocompetent mice after

pulmonary infection. FEMS Immunol Med Microbiol 24:43–47

Hill KK, Ticknor LO, Okinaka RT, Asay M, Blair H, Bliss KA, Laker M, Pardington PE, Richardson AP, Tonks M, Beecher DJ, Kemp JD, Kolsto AB, Wong AC, Keim P, Jackson PJ (2004) Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis* isolates. Appl Environ Microbiol 70:1068–1080

Hoffmaster AR, Ravel J, Rasko DA, Chapman GD, Chute MD, Marston CK, De BK, Sacchi CT, Fitzgerald C, Mayer LW, Maiden MC, Priest FG, Barker M, Jiang L, Cer RZ, Rilstone J, Peterson SN, Weyant RS, Galloway DR, Read TD, Popovic T, Fraser CM (2004) Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proc Natl Acad Sci U S A 101:8449–8454

Keim P, Kalif A, Schupp J, Hill K, Travis SE, Richmond K, Adair DM, Hugh-Jones M, Kuske CR, Jackson P (1997) Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J Bacteriol 179:818–824

Keim P, Price LB, Klevytska AM, Smith KL, Schupp JM, Okinaka R, Jackson PJ, Hugh-Jones ME (2000) Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J Bacteriol 182:2928–2936

Koehler TM (2000) Gram-positive pathogens. ASM Press, Washington, DC

Koppisch AT, Browder CC, Moe AL, Shelley JT, Kinkel BA, Hersman LE, Iyer S, Ruggiero CE (2005) Petrobactin is the primary siderophore synthesized by *Bacillus anthracis* str. Sterne under conditions of iron starvation. Biometals 18:577–585

Kramer JM, Gilbert RJ (1989) *Bacillus cereus* and other *Bacillus* species. Marcel Dekker, Inc., New York

Lee JY, Janes BK, Passalacqua KD, Pflegler BF, Bergman NH, Liu H, Hakansson K, Somu RV, Aldrich CC, Cendrowski S, Hanna PC, Sherman DH (2007) Biosynthetic analysis of the petrobactin biosynthetic pathway in *Bacillus anthracis*. J Bacteriol 189:1698–1710

May JJ, Wendrich TM, Marahiel MA (2001) The dhb operon of *Bacillus subtilis* encodes the biosynthetic template for the catecholic siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin. J Biol Chem 276:7209–7217

Neilands J (1995) Siderophores: structure and function of microbial iron transport compounds. J Biol Chem 270:26723

Oves-Costales D, Kadi N, Fogg MJ, Song L, Wilson KS, Challis GL (2007) Enzymatic logic of anthrax stealth siderophore biosynthesis: AsbA catalyzes ATP-dependent condensation of citric acid and spermidine. J Am Chem Soc 129:8416–8417

Quadri LE (2007) Strategic paradigm shifts in the antimicrobial drug discovery process of the 21st century. Infect Disord Drug Targets 7:230–237

Rey MW, Ramaiya P, Nelson BA, Brody-Karpin SD, Zaretsky EJ, Tang M, Lopez de Leon A, Xiang H, Gusti V, Clausen IG, Olsen PB, Rasmussen MD, Andersen JT, Jorgensen PL, Larsen TS, Sorokin A, Bolotin A, Lapidus A, Galleron N, Ehrlich SD, Berka RM (2004) Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus* species. Genome Biol 5:R77

Sergeev N, Distler M, Vargas M, Chizhikov V, Herold KE, Rasooly A (2006) Microarray analysis of *Bacillus cereus* group virulence factors. J Microbiol Methods 65:488–502

Sneath PHA (1986) Endospore-forming Gram-positive rods and cocci, vol 2. Williams and Wilkins Co., Baltimore, MD

Wilson MK, Abergel RJ, Raymond KN, Arceneaux JE, Byers BR (2006) Siderophores of *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis*. Biochem Biophys Res Commun 348:320–325