Petrobactin biosynthesis: AsbB catalyzes condensation of spermidine with N^8-citryl-spermidine and its N^1-(3,4-dihydroxybenzoyl) derivative†

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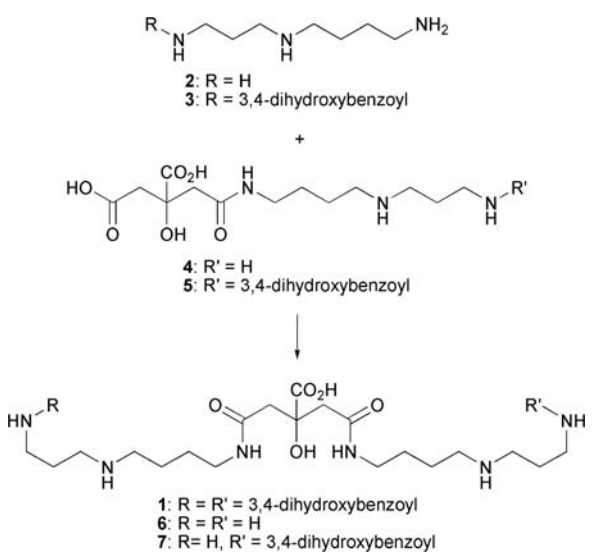
The AsbB enzyme, which is involved in the biosynthesis of the virulence-conferring siderophore petrobactin in *Bacillus anthracis*, is shown to catalyze efficient ATP-dependent condensation of spermidine, but not N^1-(3,4-dihydroxbenzoyl)-spermidine, with N^8-citryl-spermidine or N^1-(3,4-dihydroxbenzoyl)-N^8-citryl-spermidine, suggesting that N^1-(3,4-dihydroxbenzoyl)-spermidine is very unlikely to be a significant intermediate in petrobactin biosynthesis, contrary to previous suggestions.

Iron is an essential trace element for almost all living organisms. Despite being the fourth most abundant element on Earth's crust, its predominant presence as insoluble polymeric oxo-hydroxy complexes makes iron bioavailability very low in the environment. Therefore, in order to meet their iron requirements, many microorganisms have evolved the ability to produce and excrete small molecules with a high affinity for iron called siderophores.[1] Once a siderophore has scavenged iron from its environment, the ferric–siderophore complex is actively taken up by microbial cells.[1]

Many siderophores are biosynthesized by members of the extensively-studied non-ribosomal peptide synthetase (NRPS) multienzyme superfamily, *e.g.* mycobactin, enterobactin, vibriobactin and yersiniabactin.[2] On the other hand, a rapidly growing group of structurally-diverse siderophores is biosynthesized by an emerging family of new synthetases with no sequence or structural similarity to NRPSs *via* the so-called NRPS-independent siderophore (NIS) pathway.[3] NIS synthetases are classified as type A, type B or type C according to sequence similarity criteria.[3] A predictive model hypothesises that type A enzymes catalyse the condensation of one of the prochiral carboxyl groups in citric acid with amines or alcohols; type B enzymes catalyse the condensation of the γ-carboxyl group of α-ketoglutarate with amines; and type C enzymes catalyse either the condensation of a monoamide derivative of citric acid with an amine or alcohol, or the oligomerisation and macrocyclisation of ω-amino-carboxylic acids that contain a hydroxamate group.[3,4] Thus some type C enzymes are predicted to catalyse multiple iterations of the same type of condensation reaction, whereas others are predicted to catalyse only a single condensation. We recently reported biochemical investigations of AsbA, a type A NIS synthetase, and DesD, an "iterative" type C NIS synthetase, that catalyze key reactions in the biosynthesis of the *Bacillus anthracis* siderophore petrobactin **1** (Scheme 1) and the *Streptomyces* siderophores desferrioxamines B, G_1 and E, respectively.[5,6] AsbA and DesD were the first members of the NIS synthetase superfamily to be biochemically-characterized.

Because many pathogens depend on siderophore-mediated iron uptake from their hosts for growth and survival, the molecular machinery used by bacteria for the assembly, excretion and uptake of siderophores offers new targets for the development of novel antibiotics.[7] For example, effective inhibitors of mycobactin biosynthesis in *Mycobacterium tuberculosis* that inhibit growth under iron-limiting conditions have recently been reported.[8] Among the microorganisms that could be used for bioterrorism, *B. anthracis* is arguably one of the most dangerous.[9] The development of new antibiotics active against *B. anthracis* is therefore of much importance.

Two gene clusters (*asbABCDEF* and *bacACBEF*) have been reported to direct siderophore biosynthesis in *B. anthracis*.[10] *bacACBEF* encodes an NRPS-dependent pathway for the biosynthesis of bacillibactin, a siderophore present in all studied *Bacillus* species. On the other hand, *asbABCDEF*



Scheme 1 Possibile reactions catalysed by AsbB in petrobactin **1** biosynthesis.

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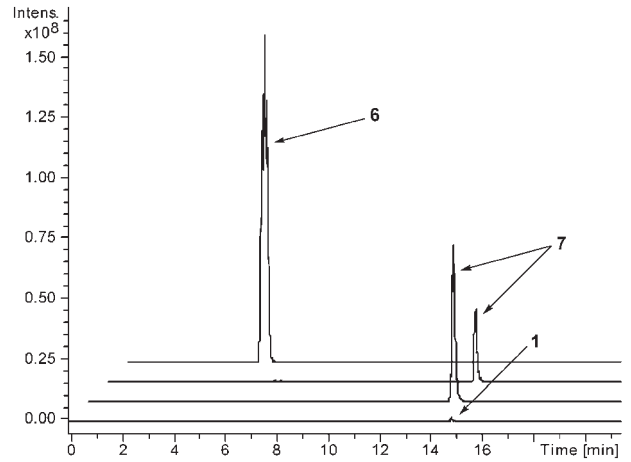


Fig. 1 Extracted ion chromatograms from LC-MS analyses, in positive ion mode, of incubations of AsbB, ATP, and Mg^{2+} with **4** and **2** (top trace, *m*/*z* 447.3), **4** and **3** (second trace, *m*/*z* 583.3), **5** and **2** (third trace, *m*/*z* 583.3), and **5** and **3** (bottom trace, *m*/*z* 719.3).

encodes a unique hybrid NRPS-NIS system for the assembly of **1**, which is present only in pathogenic *Bacillus* species.[11] While the production of bacillibactin is not necessary for *B. anthracis* growth, **1** has been shown to be required for growth in iron-depleted media and virulence in a mouse model,[10] probably due to its ability to evade the mammalian immune system.[12] These data suggest that inhibitors of the biosynthesis and uptake of **1** may be useful anti-anthrax agents.

Recent biochemical studies of the biosynthesis of **1** have shown that AsbC, a NRPS-like adenylating enzyme, catalyzes the ATP-dependent acylation of the phosphopantetheine thiol of the carrier protein AsbD with 3,4-dihydroxybenzoic acid.[13] They also showed that AsbE can catalyze the transfer of the 3,4-dihydroxybenzoyl group from AsbD to N^1 and N^8 of spermidine **2** to afford N^1-(3,4-dihydroxybenzoyl)-spermidine **3** and N^8-(3,4-dihydroxybenzoyl)-spermidine, respectively,[13] demonstrating that AsbE has relaxed substrate specificity. On the other hand, the NIS synthetase AsbA has been shown to catalyze the ATP-dependent condensation of citric acid with N^8 of spermidine **2** to afford N^8-citryl-spermidine **4**.[5] AsbA did not catalyze condensation of N^1-(3,4-dihydroxybenzoyl)-spermidine **3** with citrate, suggesting that the transfer of the 3,4-dihydroxybenzoyl group from AsbD to N^1 of spermidine occurs after AsbA-catalyzed formation of **4**. Here we report the first biochemical study of AsbB, the other NIS synthetase involved in the biosynthesis of **1**, and show that it preferentially catalyzes ATP-dependent condensation of N^8-citryl-spermidine **4** or N^1-(3,4-dihydroxybenzoyl)-N^8-citryl-spermidine **5** with spermidine **2**.

Sequence analysis of AsbB indicates that it is a type C NIS synthetase.[3] We therefore envisioned different possible roles for AsbB in petrobactin biosynthesis; it could catalyze condensation of either **4** or **5** with **2** or **3** (Scheme 1). To investigate these hypotheses, we cloned *asbB* into pET-YS-BLIC3C and overexpressed it in *E. coli* BL21star(DE3). The resulting soluble N-terminal His_6-tagged derivative of AsbB was purified from cell-free extracts using Ni-NTA and gel filtration chromatography. We synthesised **4** and **5** and

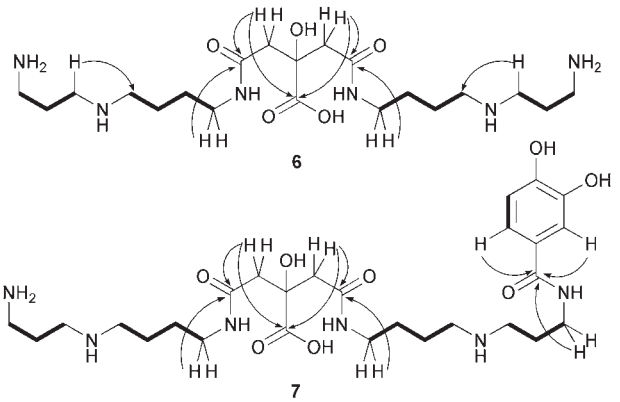


Fig. 2 Key correlations observed in the COSY (bold lines) and HMBC (arrows) spectra of **6** and **7**.

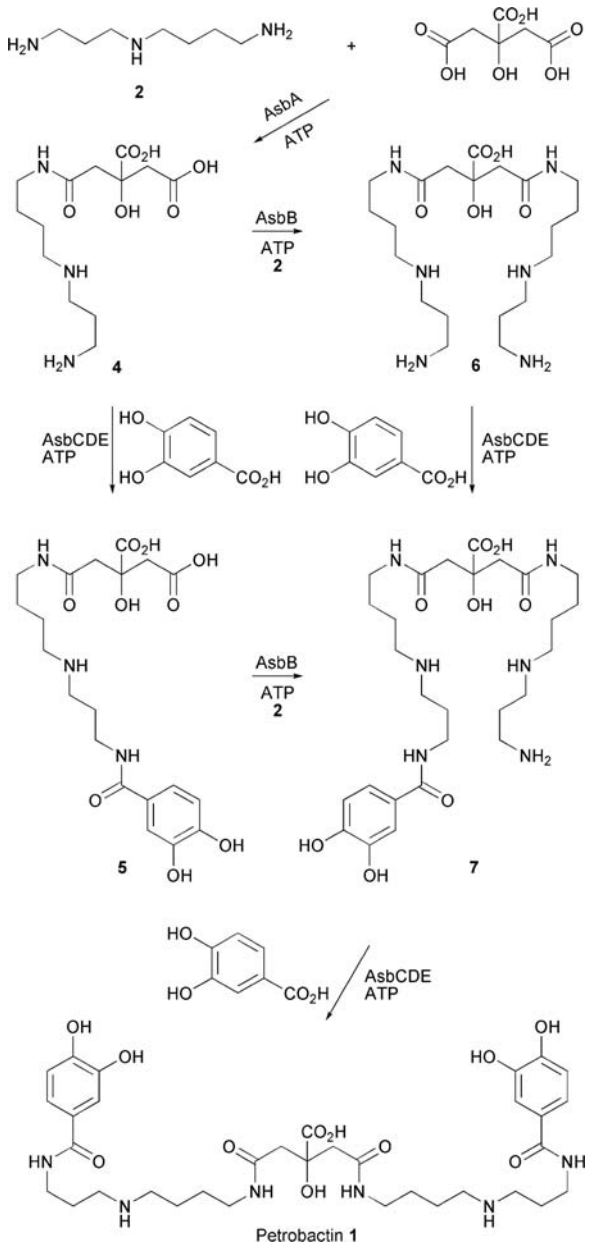
incubated equal quantities of each separately with equal quantities of **2** or synthetic **3**,[5] AsbB, a nucleotide triphosphate (NTP) and Mg^{2+} at 37 °C for 90 min. LC-ESI-MS analysis of the mixtures showed new compounds with *m*/*z* 447.3 for incubation of **4** with **2**, *m*/*z* 583.3 for incubations of **4** with **3** and **5** with **2**, and *m*/*z* 719.3 for incubation of **5** with **3** (Fig. 1). These new compounds were absent in incubations using heat-inactivated AsbB, lacking Mg^{2+}, or using NTPs other than ATP or GTP, suggesting that all four possible roles we envisioned for AsbB are, *a priori*, possible. Although LC-ESI-MS does not allow quantitation of the relative amounts of **1**, **6** and **7** produced in the reactions, qualitatively, **6** was formed in the largest amounts in these reactions, followed by **7** and then **1**. Only trace amounts of **1** could be detected in the reaction of **5** with **3**, suggesting that this is the least efficient of the reactions catalysed by AsbB. The ability of AsbB to use either ATP or GTP is not surprising, given the structural similarity of the purine bases in these NTPs. Large scale incubations of **2** with either **4** or **5**, AsbB, Mg^{2+} and ATP were carried out. **6** and **7** were isolated from these reactions by semi-preparative HPLC and their identities were confirmed by HRMS, MS-MS and 1-D and 2-D NMR experiments (Fig. 2).

To gain more insight into both the mechanism and the rate of the different AsbB-catalyzed reactions, we used continuous coupled assays for AMP and ADP formation.[14] These assays showed time-dependent formation of AMP but not ADP, consistent with the reaction of the enzyme-bound citrate derivatives **4** and **5** with ATP to form an acyl-adenylate intermediate that undergoes nucleophilic attack by **2** or **3** to form a new amide bond. To establish which of the four

Table 1 Relative rates of AMP formation catalysed by AsbB with different substrate combinations

Entry	Substrates	Relative rate[a]
1	**2** + **4**	57.10 ± 0.15
2	**2** + **5**	38.63 ± 5.76
3	**4**	1.12 ± 0.02
4	**3** + **4**	1.20 ± 0.15
5	**3** + **5**	1.00 ± 0.12
6	**5**	1.03 ± 0.13

[a] Rate values are the mean of three independent measurements and errors are quoted as ± one standard deviation.



Scheme 2 Revised pathway for petrobactin biosynthesis.

reactions catalysed by AsbB is the fastest, we measured the relative initial rates of AMP formation using saturating concentrations of ATP, **2** or **3**, and **4** or **5**. The rate of AMP formation from condensation of **2** with **4** or **5** was high relative to the background rate of AMP formation resulting from ATP hydrolysis (Table 1, entries 1–3 and 6). On the other hand, the rate of AMP formation from condensation of **3** with **4** or **5** was not significantly higher than the background rate of AMP formation (Table 1, entries 3–6). These data show that AsbB has a strong preference for **2** over **3** as a substrate, but a much less marked preference for **4** over **5**.

The inefficiency with which AsbB catalyzes condensation of **3** with **4** or **5**, together with our previously reported observation that AsbA catalyzes condensation of **2** but not **3** with citric acid to form **4**,[5] strongly suggests that **3** is unlikely to be a significant intermediate in petrobactin biosynthesis. Our data suggest a revised pathway for petrobactin biosynthesis in which **4**, **5**, **6** and **7** are all viable intermediates and AsbE functions as an acyltransferase that could transfer the 3,4-dihydroxybenzoyl group from the phosphopantetheine thiol of AsbD to the primary amino groups in **4**, **6** and **7**, rather than **2** as previously suggested[13] (Scheme 2).

AsbB is only the second type C NIS synthetase to be biochemically characterized. Our results confirm the bioinformatics-derived prediction that some type C enzymes can catalyze NTP-dependent condensation of a derivative of citric acid with amines.[3] DesD, another type C enzyme, has been shown experimentally to catalyse the ATP-dependent iterative condensation of ω-amino-carboxylic acids to form oligomeric products, some of which undergo subsequent ATP-dependent macrocylisation.[6] This raises an intriguing question for future research: how does AsbB avoid catalysing oligomerization (and subsequent macrocyclisation) of **4**? Clearly, the type C subfamily of NIS synthetases should be divided into two groups: The AsbB-like group, which catalyses a single condensation reaction and can therefore be thought of as ''modular'', and the DesD-like group, which catalyses multiple condensation reactions and can therefore be referred to as ''iterative''.

In conclusion, we have carried out the first biochemical study of AsbB, a key yet promiscuous enzyme in petrobactin biosynthesis. Our results provide the basic biochemical knowledge required to screen for inhibitors of AsbB, which may ultimately lead to the development of new antibiotics active against *B. anthracis* that abrogate petrobactin biosynthesis.

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