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Enzymatic Logic of Anthrax Stealth Siderophore Biosynthesis: AsbA Catalyzes ATP-Dependent Condensation of Citric Acid and Spermidine

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Bacillus anthracis is a Gram-positive, spore-forming bacterium that has attracted exceptional interest because of its potential use as a biowarfare agent. Inhalation or ingestion of *B. anthracis* spores leads to systemic anthrax, which is characterized by an initial intracellular phase, where the spores germinate and proliferate, followed by massive bacteraemia and sepsis, which is rapidly lethal.[1] The high levels of mortality if not treated and the possibility of making genetically modified antibiotic-resistant strains, makes *B. anthracis* a major threat in a bioterrorism environment.[2] Therefore, the development and synthesis of new antibiotics active against *B. anthracis* is of much current interest.

As for many other pathogens, iron is an essential nutrient for *B. anthracis*. Iron is tightly bound and highly regulated in eukaryotes, and iron bioavailability for invading pathogens is therefore very low, making its assimilation a considerable challenge for the pathogen and an early defensive barrier for the host. The production, excretion, and reabsorption of small molecules with high affinity for iron, called siderophores, is a common strategy used by pathogenic bacteria to meet their iron requirements.[3] Many siderophores are biosynthesized by members of the well-known nonribosomal peptide synthetase (NRPS) multienzyme family, including enterobactin, vibriobactin, yersiniabactin, and mycobactin.[4] Biochemical studies of these siderophore-assembling NRPS systems has led recently to the synthesis of an effective inhibitor of mycobactin biosynthesis that inhibits *Mycobacterium tuberculosis* growth.[5] On the other hand, many other siderophores are biosynthesized by a nonribosomal peptide synthetase-independent siderophore (NIS) pathway, involving a novel family of putative synthetases about which much less is currently known.[6] Very recently, the first biochemical study of an enzyme belonging to this family DesD, which catalyzes the key oligomerization-macrocyclization reactions in desferrioxamine biosynthesis, has been reported.[7]

Two gene clusters (*asbABCDEF* and *bacACEBF*) responsible for siderophore biosynthesis have been identified in *B. anthracis*.[8] While *bacACEBF* encodes an NRPS-dependent pathway for bacillibactin biosynthesis present in all studied *Bacillus* species, *asbABCDEF* encodes a unique combination of NRPS and NIS synthetase enzymes responsible for the biosynthesis of petrobactin **1** (Figure 1),[8−11] which is only produced by pathogenic *Bacillus* species.[9] Recent genetic and biochemical experiments have investigated the role of the *asbABCDEF* genes and the AsbC, AsbD, and AsbE proteins in the assembly of **1**, leading to a proposed biosynthetic pathway.[10,11] Although bacillibactin production is not necessary for *B. anthracis* growth, production of **1** is required for growth in iron-depleted media and virulence in a mouse model.[8] Very recently, it has been shown that, in contrast to bacillibactin, **1** is not bound by siderocalin, thus allowing it to evade the mammalian immune system.[12] These data suggest that inhibitors of the biosynthesis and uptake of **1** may be useful antianthrax agents.

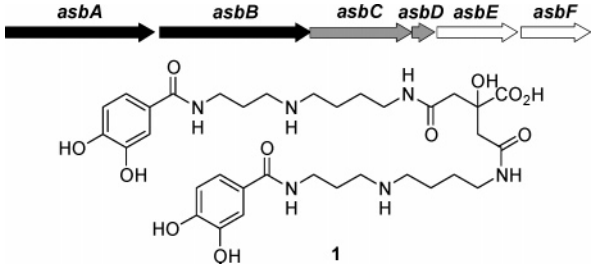
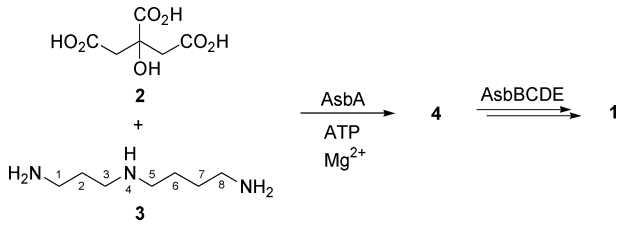


Figure 1. Structure of petrobactin **1** and organization of the *asbABCDEF* gene cluster in *B. anthracis* that directs its biosynthesis. Genes encoding NIS synthetases are in black, and genes encoding NRPS-like enzymes are in gray.

Scheme 1. Revised Pathway for Petrobactin **1** Biosynthesis Showing the Reaction Catalyzed by AsbA



Here we report the first biochemical study of AsbA, one of two NIS synthetases involved in the assembly of **1**. Our results prompt us to propose a revised pathway for the biosynthesis of **1**.

Sequence analysis of AsbA has shown that it is a "type A" NIS synthetase,[6] suggesting it catalyzes condensation of citric acid **2** with spermidine **3** (Scheme 1) or its N^1-3,4-dihydroxybenzoyl derivative. To test this hypothesis, we cloned *asbA* into pET-YSBLIC3C and overexpressed it in *E. coli* BL21star(DE3). The resulting soluble N-terminal His_6-tagged derivative of AsbA was purified from cell free extracts using Ni-NTA chromatography (see Supporting Information). Because it was unclear whether **3** or its N^1-3,4-dihydroxybenzoyl derivative was a substrate for AsbA, we initially investigated the trapping of a presumed citryl phosphate/nucleotidylate intermediate with hydroxylamine.[13] Incubation of AsbA with **2**, hydroxylamine, ATP, and Mg^{2+} generated a citryl hydroxamate that could be detected by time-dependent increase in absorption at 540 nm on addition of $FeCl_3$.[13] No activity was detected with enzyme inactivated by boiling, with nucleotide triphosphates (NTPs) other than ATP, or in the absence of Mg^{2+} (see Supporting Information).

Having established that AsbA catalyzes the ATP-dependent activation of a carboxyl group of **2**, as previously proposed,[6] we next examined whether the enzyme could catalyze ATP-dependent condensation of **2** with **3** or its 3,4-dihydroxybenzoyl derivative.

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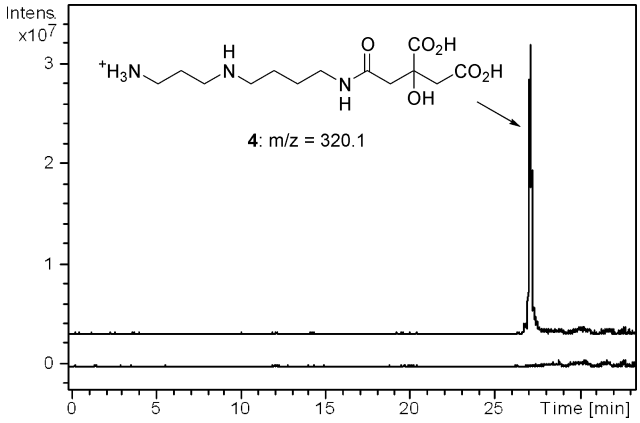

Figure 2. Extracted ion chromatograms at $m/z = 320.1$ from LC−MS analysis of incubations of **2**, **3**, ATP, and Mg^{2+} with AsbA (top trace) and heat-inactivated AsbA (bottom trace).

LC−MS analysis of a mixture of **2**, **3**, ATP, and Mg^{2+} incubated with AsbA at 37 °C for 90 min identified a new compound with $m/z = 320.1$ that was absent in control incubations with inactivated AsbA, or lacking Mg^{2+}. The identity of this compound isolated by semipreparative HPLC from a large-scale incubation was confirmed as N^8-citryl-spermidine **4** (Figure 2) by HRMS (calculated for $C_{13}H_{26}N_3O_6^+$, 320.1816; found, 320.1817), as well as MS/MS and ^{1}H NMR comparisons with a chemically synthesized authentic standard. In contrast, no conversion of various concentrations of chemically synthesized N^1-(3,4-dihydroxybenzoyl)-spermidine to N^1-(3,4-dihydroxy-benzoyl)-N^8-citryl-spermidine could be detected upon incubation with citric acid, ATP, Mg^{2+}, and AsbA at 37 °C for 90 min.

To determine what ATP is converted to in the AsbA-catalyzed condensation of **2** with **3**, we used continuous coupled assays for AMP and ADP formation.[14] These assays showed time-dependent formation of the former but not the latter. No time-dependent AMP formation was observed in incubations with N^1-(3,4-dihydroxybenzoyl)-spermidine in place of spermidine or α-ketoglutaric acid in place of **2**, providing further evidence that AsbA has substrate specificity for **2** and **3** (see Supporting Information). These data are consistent with the reaction of enzyme bound **2** and ATP to form an acyl−adenylate intermediate that undergoes nucleophilic attack at its activated carboxyl group by N^8 of **3** to form **4** (Scheme 1), but do not rule out other mechanistic possibilities.

Sherman and co-workers have recently shown that AsbC catalyzes ATP-dependent loading of 3,4-dihydroxybenzoic acid onto the holo-carrier protein AsbD.[11] They also showed that AsbE can catalyze transfer of the 3,4-dihydroxybenzoyl group from AsbD to **3**. However, N^1-(3,4-dihydroxybenzoyl)-spermidine, the intermediate that would be required for assembly of **1**, is only a minor product of this reaction. The major product appears to be the regioisomer N^8-(3,4-dihydroxybenzoyl)-spermidine. These data together with data on compounds accumulated in mutants lacking individual genes from the *asbABCDEF* gene cluster[10] led Sherman and co-workers to propose a pathway for the biosynthesis of **1** involving AsbA-catalyzed condensation of N^1-(3,4-dihydroxybenzoyl)-spermidine (formed by the action of AsbC, AsbD, and AsbE) with **2** to give N^1-(3,4-dihydroxybenzoyl)-N^8-citryl-spermidine, followed by AsbB-catalyzed condensation of this intermediate with a second molecule of N^1-(3,4-dihydroxybenzoyl)-spermidine. Our data show that this pathway cannot be correct; transfer of the 3,4-dihydroxybenzoyl group from AbsD to N^1 of **3** must occur after AsbA-catalyzed formation of **4** (Scheme 1). It is likely that N^1-(3,4-dihydroxybenzoyl)-spermidine, which is observed to accumulate in a Δ*asbA* mutant of *B. anthracis*,[10] is a shunt metabolite resulting from the biochemically demonstrated relaxed specificity of AsbE.[11] The order of the steps in assembly of **1** following formation of **4**, that is, acylation of N^1 of **4** with 3,4-dihydoxybenzoyl-AbsD and condensation of **4** with **3** or N^1-(3,4-dihydroxybenzoyl)-spermidine, remains to be established. Further experiments to investigate the substrate tolerance and specificity, respectively, of AsbE and AsbB will be required to do this. It is notable that the first step in the assembly of **1** defined here differs from the early steps of vibriobactin biosynthesis, where N^1 of norspermidine is acylated with a 2,3-dihydroxybenzoyl group prior to acylation of N^4 and N^7 with other acyl groups.[15]

In conclusion, we have carried out the first biochemical studies of AsbA, a key enzyme in petrobactin **1** biosynthesis that catalyzes the ATP-mediated condensation of citric acid **2** with spermidine **3**. This is the first enzyme from the type A subfamily of the NIS synthetases to be characterized and the results confirm the bioinformatics-derived prediction that type A enzymes catalyze NTP-dependent condensation of citric acid with a variety of amines and alcohols.[6] This study provides the fundamental knowledge required to design a high-throughput screen for AsbA inhibitors that may lead to the development of new antianthrax antibiotics targeting petrobactin assembly.

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Supporting Information Available: Complete ref 2, experimental procedures, SDS-PAGE analysis of His_6-AsbA expression and purification, the results of the enzyme activity assays, and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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