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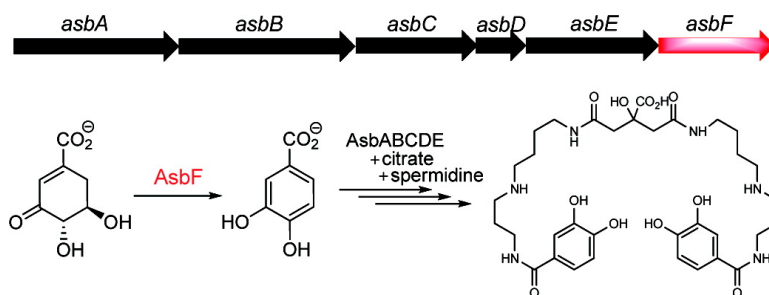
Rapid Report

The Missing Link in Petrobactin Biosynthesis: *asbF* Encodes a (#)-3-Dehydroshikimate Dehydratase

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The Missing Link in Petrobactin Biosynthesis: *asbF* Encodes a (–)-3-Dehydroshikimate Dehydratase[†]

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ABSTRACT: The siderophore petrobactin harbors unique 3,4-dihydroxybenzoyl iron-liganding groups. These moieties are known to be synthesized from shikimate pathway precursors, but no reports of the biosynthetic enzymes responsible for this conversion have been published. The gene encoding AsbF from *Bacillus thuringiensis* 97-27 was overexpressed in an *Escherichia coli* host. AsbF rapidly and efficiently transforms (–)-3-dehydroshikimate (DHS) into 3,4-dihydroxybenzoate ($k_{\text{cat}}^{\text{DHS}} = 217 \pm 10 \text{ min}^{-1}$; $K_{\text{m}}^{\text{DHS}} = 125 \pm 14 \mu\text{M}$) at 37 °C and has an absolute requirement for divalent metal. Finally, the pH versus $k_{\text{cat}}^{\text{DHS}}$ profile revealed two ionizable groups ($\text{pK}_{\text{a}1} = 7.9 \pm 0.1$, and $\text{pK}_{\text{a}2} = 9.3 \pm 0.1$).

The siderophore petrobactin is produced by the causative agent of anthrax (*Bacillus anthracis*) as well as numerous other pathogenic species in the *Bacillus cereus* sensu lato group (e.g., *Bacillus thuringiensis* 97-27 and *B. cereus* G9241) (1, 2). Although not a direct determinant of a *Bacillus* species pathogenicity (3), it has been demonstrated that petrobactin biosynthesis is essential for survival in its host (4). Petrobactin and its sulfonated derivative (5) are the only known siderophores to use the unusual 3,4-dihydroxybenzoyl (3,4-DHB) iron-chelating moieties. Siderocalin, a protein that was found to sequester most siderophores as part of the innate human immune response, is unable to bind iron-complexed petrobactin, and it has been postulated that the 3,4-DHB groups of petrobactin may play a significant role in the occlusion of the siderophore from the binding pocket. As such, petrobactin has been termed a “stealth” siderophore, as it not only evades the mammalian immune system itself but also facilitates the growth and proliferation of the producing bacteria in a mammalian host (6, 7). Thus, targeted inhibition of the petrobactin-specific biosynthetic enzymes is an attractive target for the development of a novel class of anti-anthrax therapeutics as there are no orthologous enzymes in humans.

Two gene clusters (*asbABCDEF* and *bacACBEF*) have been implicated in siderophore biosynthesis in *B. anthracis*

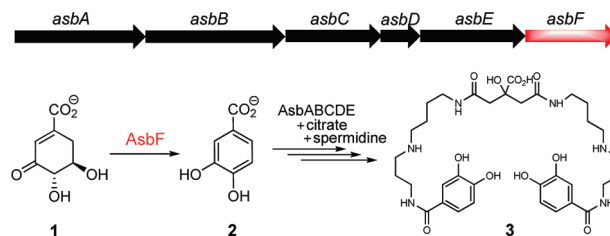


FIGURE 1: Conversion of (–)-3-DHS (1) to 3,4-DHB (2) by AsbF and subsequent incorporation into petrobactin (3) by the remaining Asb gene products. The gene encoding AsbF is highlighted in red and is part of the *asbABCDEF* gene cluster.

(4). The *bac* operon encodes the well-understood nonribosomal peptide synthetase (NRPS)-dependent enzymes responsible for the biosynthesis of the siderophore bacillibactin. Bacillibactin contains 2,3-catecholate groups and is produced by many *Bacillus* species. The *asb* operon, however, encodes both NRPS and NRPS-independent siderophore synthetase enzymes, which ultimately form the unique siderophore from three components: citrate, spermidine, and 3,4-DHB (8–11). The assembly of the individual petrobactin constituents has been assigned to five of the six gene products in the *asb* operon (*asbABCDEF*) and a function proposed for each. However, the role of the *asbF* gene product in petrobactin biosynthesis has remained an enigma until very recently (12).

As part of the ongoing efforts to unveil the biosynthetic route to petrobactin, we recently identified the major metabolic pathway responsible for the biosynthesis of the 3,4-DHB groups (12). Feeding studies with [^{13}C]glucose in *B. anthracis* strain Sterne provided a convenient handle for assessing isotopic enrichment at specific locations in the catecholate moiety, and the early steps of the shikimate pathway were identified as the primary source for 3,4-DHB production. We are the first to report the function and enzyme characterization of the final gene product from the *asbABCDEF* operon, AsbF, and identify the protein as a (–)-3-dehydroshikimate dehydratase (DHSase) for direct conversion of DHS to 3,4-DHB (Figure 1).

Genes encoding DHSases have been identified and, in some instances, isolated and partially characterized from *Neurospora* sp. (13, 14), *Aspergillus* sp. (15), *Acinetobacter* sp. (16), *Klebsiella* sp. (17), *Vigna mungo* (18), and *Pseudomonas* sp. (19). In all reported cases, the function of the enzyme was to enable microbes to catabolize hydroaromatic com-

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pounds (shikimate and quinate). In stark contrast, this is the first report of a DHSase implicated in an alternative pathway, specifically, petrobactin biosynthesis.

Initial BLAST searches queried against AsbF in all *Bacillus* strains revealed little homology (<30%) between known DHSases and genes encoding proteins of unknown function in sequenced members of the *B. cereus* sensu lato group. However, AsbF is predicted to share structural homology with members of apurinic/aprimidinic endonuclease family 2 (APendo2). Moreover, almost all of the characterized DHSases are also predicted to share homology with this same superfamily (Figure S1 of the Supporting Information). Since previous studies revealed deletion of *asbF* in *B. anthracis* was deleterious to the organism and a function was proposed for the remaining gene products in the *asb* operon (8–11), a logical question followed: what is the most straightforward pathway to 3,4-DHB? Our recent labeling studies implicated the early steps in the shikimate pathway as being responsible for the biosynthesis of the catecholate moiety in petrobactin (12); hence, DHS is directly converted to 3,4-DHB. Although all previously reported DHSases have been located as part of a quinate degradation pathway, we surmised that AsbF would logically be a DHSase responsible for 3,4-DHB biosynthesis.

Previous studies with DHSases derived from fungal sources have noted the difficulty of overexpressing and purifying active DHSase in a recombinant *Escherichia coli* host. Only Frost et al. (20) has overcome this barrier by cloning the gene encoding DHSase from *Klebsiella pneumoniae* (*aroZ*), which has a close evolutionary relationship with *E. coli*. Since our construct is of bacterial origin, we anticipated that expression of *asbF* would be more facile than in previous reports. The *asbF* sequence from *B. thuringiensis* 97-27 (Bt 97-27) was chosen for overexpression since it is the only strain isolated from a human wound among the otherwise nonpathogenic *B. thuringiensis* strains (21), and the encoded protein bears excellent sequence homology to the related pathogenic strains¹ and other petrobactin-producing isolates in the *B. cereus* sensu lato group (3). The Bt 97-27 AsbF was overproduced in *E. coli* and purified as an N-terminally His₆-tagged recombinant DHSase (rDHSase). The ~35 kDa recombinant protein was obtained in a yield of 80 mg/L of culture (Figure S2 of the Supporting Information).

Incubation of the purified Bt 97-27 rDHSase with DHS and divalent metal revealed a distinct increase in absorbance at 290 nm when compared to that of a control without enzyme (14). Large-scale incubation of the enzyme with DHS and subsequent product isolation and characterization by UV spectroscopy clearly revealed a compound with a spectral trace identical to that of a 3,4-DHB standard (Figure 2). The purified enzyme product was also confirmed by both ¹H and ¹³C NMR spectroscopy and high-resolution mass spectrometry to be 3,4-DHB (Supporting Information).

Although the first characterization of a DHSase occurred more than 50 years ago, nearly all reports are of fungal or plant origin. The lone exception was a DHSase cloned from the *K. pneumoniae* genome (20). Not surprisingly, detailed

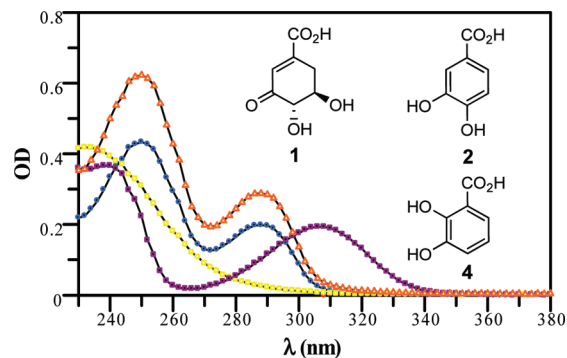


FIGURE 2: UV traces of (—) 3-DHS (1) (yellow), 3,4-DHB (2) standard (orange), 2,3-DHB (4) (purple), and the rDHSase-catalyzed product (blue).

characterization of the Bt 97-27 rDHSase revealed several interesting properties not previously observed in other systems. The Michaelis–Menten parameters were determined for rDHSase in the presence of the substrate, DHS, and Mg²⁺ as a cofactor using a modified procedure reported by Strømman (14) at two different temperatures (37 and 30 °C) and are reported in the Supporting Information. On the basis of these results, interesting comparisons between the aforementioned and related DHSases can be surmised. First, under optimal reaction conditions, the K_m^{DHS} is ~5-fold lower ($125 \pm 14 \mu\text{M}$ at 37 °C) than that of any other reported DHSase [~ 530 – $600 \mu\text{M}$ (Table 1 of the Supporting Information)]. Although no direct comparisons for the rate of turnover ($k_{\text{cat}}^{\text{DHS}} = 217 \pm 10 \text{ min}^{-1}$ at 37 °C) could be made between the Bt 97-27 enzyme and related DHSases, we found that the specific activity for this class of enzymes spanned more than 4 orders of magnitude (0.1–261 units/mg). The specific activity for the Bt 97-27 rDHSase fell on the lower end of the spectrum, which was expected since Bt 97-27 is more closely related to *K. pneumoniae* than the other organisms from which the DHSase was biochemically characterized and is reflected by the phylogenetic analysis of proposed and characterized DHSases alike.

Furthermore, when we examined the pH profile versus k_{cat} (Figure S4 of the Supporting Information), a large disparity in pH optimum for catalysis was observed when it was compared to those of both the *Neurospora crassa* and *V. mungo* DHSase pH profiles. In contrast to all DHSases, where activity was measured at pH 7.4–7.8, we found the pH optimum for the Bt 97-27 enzyme to be at pH 8.4–8.8 with less than 15% residual activity at pH 7.4. The bell-shaped pH versus $k_{\text{cat}}^{\text{DHS}}$ curve fit nicely to the equation for an enzyme with two ionizable groups ($\text{p}K_{\text{a}1} = 7.9 \pm 0.1$, and $\text{p}K_{\text{a}2} = 9.3 \pm 0.1$). Analysis of the recently deposited crystal structure of *B. anthracis* AsbF (Protein Data Bank entry 3dx5) with bound Mn²⁺ and product, 3,4-DHB, revealed Y₂₁₇ was in the proximity ($\sim 4 \text{ \AA}$) of C-6 of 3,4-DHB and is a putative residue directly involved in hydrogen abstraction of the substrate, DHS. This residue nicely correlates with our pH studies on the closely related Bt 97-27 enzyme, although detailed mechanistic studies are required to resolve the active site residues directly involved in catalysis. In addition, the rDHSase has a strict requirement for divalent metal as demonstrated by the complete loss of activity toward DHS when rDHSase was dialyzed in the presence of 1 mM EDTA (data not shown). Greater than 95% activity was restored when the rDHSase was incubated

¹ The AsbF from *B. thuringiensis* 97-27 ssp. konkukian shares near 100% sequence identity with the homologous enzymes from numerous *B. anthracis* isolates, including the Sterne strain.

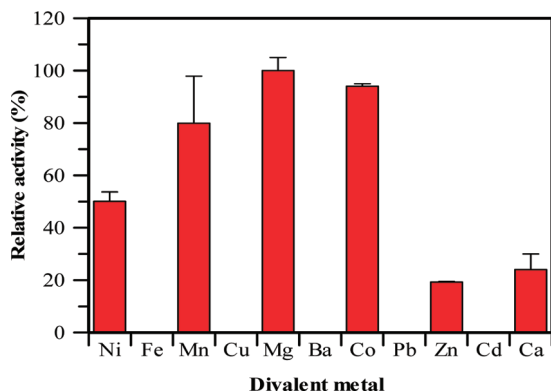


FIGURE 3: Metal dependency for direct conversion of DHS to 3,4-DHB by rDHSase. Relative rDHSase activities in the presence of divalent metal were normalized against rDHSase in the presence of 5 mM MgCl_2 . Experimental conditions are outlined in the Supporting Information.

with Mg^{2+} . Of the divalent metals tested, only Mg^{2+} , Mn^{2+} , and Co^{2+} provided maximal rates under the conditions that were employed. We also observed activity in the presence of Ni^{2+} and, to a much lesser extent, Zn^{2+} and Ca^{2+} (Figure 3). The Bt 97-27 rDHSase thus appears to be promiscuous with respect to its divalent metal requirement.

Finally, rDHSase was found to be heat intolerant and was rapidly deactivated ($t_{1/2} \sim 10$ min) when it was incubated at 46 °C. We observed a modest increase in stability in the presence of divalent magnesium salts (data not shown) as previously reported for DHSases.

Generally, bacterial pathogenesis involves three overarching steps: invasion, growth, and disease manifestation. Intervention at any single point requires, at minimum, a basal level of understanding of the enabling mechanism(s) for the pathogen to succeed in persisting and/or proliferating in the host. Here, we report and characterize a single enzyme that functions to aid growth of the pathogen as well as its ability to evade the host's immune response. Thus, this DHSase and homologues in related petrobactin-producing pathogenic bacteria may be considered new targets for the development of novel therapeutic agents.

In addition, amino acid sequence analysis predicts a classic TIM barrel-like fold shared by many enzymes and most likely reflects a common dependency for DHSases on divalent metal for substrate binding and catalysis, although a secondary role of divalent metal-dependent structural stabilization cannot be ruled out. AsbF appears to catalyze the same reaction as previously characterized DHSases, but the role of the *asbF*-encoded DHSase is thus far evolutionarily unique. Hence, AsbF represents the first report of a DHS dehydratase utilized in a noncatabolic pathway, specifically petrobactin biosynthesis, and should be placed into a newly defined class of DHSases based on function. Class I DHSases are directly involved in aromatic catabolism, while

class II DHSases are specifically utilized in petrobactin biosynthesis. Detailed mechanistic investigations are in progress.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Experimental details for rDHSase production, characterization, and product identification. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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