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Identification of a Gene Cluster That Directs Putrebactin Biosynthesis in *Shewanella* Species: PubC Catalyzes Cyclodimerization of *N*-Hydroxy-*N*-succinylputrescine

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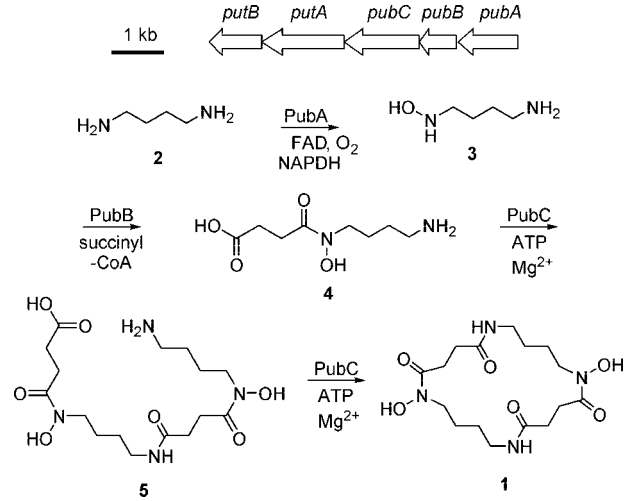
Siderophores are high-affinity ferric iron-chelating natural products that are biosynthesized and excreted by many microorganisms.[1] Iron is an essential nutrient for the growth and proliferation of virtually all microorganisms. However, direct ferric iron uptake is challenging for most microorganisms, because highly insoluble ferric oxide/hydroxide polymers are the predominant reservoir of iron in the environment and ferric iron is tightly bound to binding proteins in mammalian hosts, where its concentration is tightly regulated. Thus many saprophytic and pathogenic microorganisms depend on ferric-siderophore uptake systems for iron acquisition.[1]

Two main pathways for siderophore biosynthesis exist in microorganisms.[2,4] One pathway relies on nonribosomal peptide synthetase (NRPS) multienzymes that employ a thiotemplate mechanism for the assembly of structurally diverse iron chelators such as enterobactin, pyochelin and coelichelin, from amino and carboxylic acid building blocks.[2,3] The other pathway utilizes NRPS-independent siderophore (NIS) synthetases to catalyze ATP-dependent condensation reactions between dicarboxylic acids and diamines, diamine derivatives or amino alcohols, as well as between ω-aminocarboxylic acids.[4–6] While the biochemistry of NRPS-dependent siderophore biosynthetic pathways has been investigated for many years and is now generally well understood,[2] major advances in the biochemical understanding of NIS synthetases have only occurred very recently and much still remains to be learned about this fascinating and important enzyme family.[5,6]

We recently showed that desferrioxamine G_1 is assembled by ATP-dependent trimerization of *N*-hydroxy-*N*-succinylcadaverine (HSC) and that it is converted to desferrioxamine E by ATP-dependent macrocyclization.[6] These reactions are catalyzed by DesD, which is the first biochemically characterized member of a putative family of oligomerizing-macrocyclizing enzymes that forms a subset of the type C NIS sythetases.[4,6] BLAST searches revealed a gene (*pubC*), within a conserved cluster of genes (Scheme 1), in several sequenced *Shewanella* species including *Shewanella putrefaciens* that encodes a homologue of DesD (48−49% identity, 65−66% similarity).[6] Sequence comparisons of the *pubB* and *pubA* genes upstream of *pubC* showed that they encode proteins with 49% and 71% similarity, respectively, to DesC and DesB, which have been proposed to catalyze conversion of cadaverine to HSC in desferrioxamine biosynthesis.[7] The *putA* and *putB* genes, downstream of *pubC*, encode proteins with sequence similarity to TonB-dependent outer membrane ferric-siderophore receptors of Gram-negative bacteria and the cytoplasmic ferric-siderophore reductase FhuF of *E. coli*, respectively.[1,8] *S. putrefaciens* has been reported to produce putrebactin **1** (Figure 1), a macrocyclic-dimer of *N*-hydroxy-*N*-succinyl-putrescine (HSP).[9] Thus we hypothesized that *pubABC* encode the enzymes required to assemble putrebactin from putrescine. PubA could catalyze the O_2- and $FADH_2$-dependent hydroxylation of putrescine **2** to give *N*-hydroxyputrescine **3**; PubB could catalyze succinyl-CoA dependent succinylation of **3** to give HSP **4**; and PubC could catalyze ATP-dependent head-to-tail dimerization of HSP to give pre-putrebactin **5** and subsequent macrocyclization of **5** to give **1** (Scheme 1). We also hypothesized that *putAB* encode proteins involved in the uptake and utilization of ferric-putrebactin in *Shewanella* species.

Scheme 1. Organization of the Putrebactin **1** Biosynthetic Gene Cluster in *Shewanella* Species and Proposed Pathway for Putrebactin Biosynthesis



To examine the involvement of the *pubABC* genes in putrebactin biosynthesis and investigate the biochemical function of PubC, we

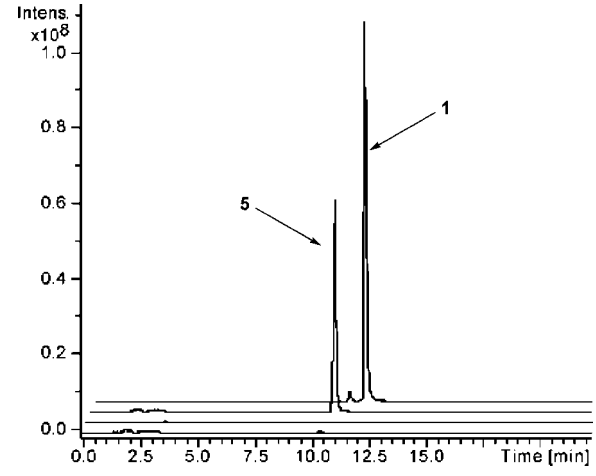


Figure 1. Extracted ion chromatograms (EICs) from LC−MS analysis of incubations of HSP **4**, ATP, Mg^{2+} with His_6-PubC from *Shewanella* sp. MR4. In order from top to bottom, they are: EIC at m/z = 373.1; EIC at m/z = 391.2; EIC at m/z = 373.1 from control reaction using heat-inactivated PubC; EIC at m/z = 391.2 from control reaction.

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cloned the *pubC* gene from *Shewanella* species MR-4 and MR-7 into pET151 and overexpressed it in *E. coli* BL21star(DE3). The resulting soluble His_6-PubC fusion proteins (which share 97% sequence identity) were purified from cell free extracts by Ni-NTA chromatography. Gel filtration analysis of the purified proteins indicated that they were dimers. We also synthesized HSP **4** in eight steps from 1-bromo-4-chloro-butane and *O*-benzyl-hydoxylamine using an analogous procedure to that recently reported for HSC.[6]

Each of the two His_6-PubC proteins was incubated with **4**, ATP, and Mg^{2+} for 5 min at 37 °C, and the reactions were stopped by precipitation of the enzyme with tricholoracetic acid. Positive ion LC−MS analyses of the reaction mixtures showed that two new compounds, absent in control reactions using enzymes inactivated by boiling, were formed, with $m/z = 391.2$ and 373.1, corresponding to the protonated ions of **5** and **1**, respectively (Figure 1). The compounds were purified from large-scale incubations of **4** with ATP, Mg^{2+}, and His_6-PubC from *Shewanella* sp. MR-4 by semipreparative reverse-phase HPLC. The molecular formulas of the ions generated from these compounds by ESI-TOF-MS were confirmed as $C_{16}H_{29}N_4O_6^+$ (calculated, 373.2082; found, 373.2084) and $C_{16}H_{31}N_4O_7^+$ (calculated, 391.2187; found, 391.2189), consistent with the assignment of $[\mathbf{1} + H]^+$ and $[\mathbf{5} + H]^+$, respectively, as the structures of these ions. The structure of **1** was unambiguously confirmed by 1- and 2-D NMR analyses. ESI-MS/MS analyses of **1** and the compound with $m/z = 391.2$ showed that they have closely related structures. Both produce a daughter ion with $m/z = 186.9$. For both compounds, this ion fragments to give ions with $m/z = 153.9$, 140.0, 123.1, 112.1. Thus, we assign structure **5** to the compound with $m/z = 391.2$.

To further investigate the mechanism of the PubC-catalyzed conversion of **4** to **1**, we used coupled assays for AMP/ADP and phosphate/pyrophosphate formation. These assays showed that ATP is converted to AMP and pyrophosphate during the reaction, strongly suggesting that PubC catalyzes reaction of **4** with ATP to form the corresponding acyl-adenylate and pyrophosphate. Nucleophilic attack of the amino group in a second molecule of **4** on this acyl adenylate would yield **5** and AMP. Analysis of the change in concentration of **5** and **1** with time in the PubC-catalyzed reactions of **4**, showed that **5** accumulates only transiently in the reaction, suggesting that it is a free intermediate in the formation of **1**. To test this hypothesis, we incubated **5** (partially purified from a large scale incubation of **4** with PubC, as described above) with ATP, Mg^{2+}, and His_6-PubC for 2 h and 40 min at 37 °C. LC−MS analysis of the reaction mixture, after addition of ferric iron to stop the reaction by precipitating the enzyme and to convert **5** and **1** to their ferric complexes, showed that **5** had been converted to **1**. In contrast, no conversion of **5** to **1** was observed in control reactions lacking ATP or with His_6-PubC inactivated by boiling prior to addition to the reaction mixture. These data provide strong support for the hypothesis that **5** is a free intermediate in the assembly of **1** from **4** and suggest that PubC catalyzes the reaction of ATP with the carboxyl group of **5** to form pyrophosphate and the corresponding acyl adenylate, which undergoes macrocyclization via intramolecular nucleophilic attack of the ω-amino group on carboxyl group of the adenylate, resulting in release of AMP and the formation of **1**.

We recently reported that DesD catalyzes the ATP- and Mg^{2+}-dependent assembly of the trimeric macrocycle desferrioxamine E from three molecules of HSC, a homologue of HSP **4** derived from cadaverine (1,5-diaminopentane) instead of putrescine **2**.[6] The linear trimer of HSC desferrioxamine G_1 was shown to be an intermediate in this reaction and mass spectrometric evidence for the transient accumulation of the linear dimer of HSC in the reaction was also obtained, suggesting that this may also be an intermediate in desferrioxamine E assembly.[6] The results reported here show that the dimeric macrocycle putrebactin **1** is assembled by ATP- and Mg^{2+}-dependent dimerization and subsequent macrocylization reactions of HSP **4** in *Shewanella* species. In analogy to the formation of desferrioxamine E from desferrioxamine G_1, putrebactin **1** is formed from the linear dimer **5** of HSP, via adenylation of the carboxyl group followed by intramolecular attack of the amino group on the carbonyl carbon of the resulting acyl adenylate. These reactions are catalyzed by PubC, an enzyme with nearly 50% sequence identity to DesD. The specific formation of a macrocyclic trimer and a macrocyclic dimer, respectively, from closely related substrates by two highly homologous enzymes is mechanistically intriguing. Detailed structural and kinetic studies of PubC and DesD will be required to reveal the molecular basis for control of substrate oligomerization by these enzymes.

The *pubC* gene resides within a conserved five-gene cluster in the genomes of *Shewanella* species that is postulated to direct the biosynthesis of **1** from putrescine, molecular oxygen, and succinyl-CoA, the uptake of the ferric complex of **1**, and the reductive removal of iron from ferric-putrebactin. PubA, PubB, and PubC are 46%, 40%, and 49% identical to the proteins encoded by *alcA*, *alcB,* and *alcC*, respectively. These genes are involved in the biosynthesis of alcaligin, a dihydroxylated derivative of putrebactin that is elaborated from putrescine in *Bordetella* species.[4] Also, PutA shows 40% sequence identity to FauA, the ferric-alcaligin outer membrane receptor of *Bordetella* species.[10] Thus, it seems likely that the biosynthesis of putrebactin and alcaligin, and the uptake of their ferric complexes may share many similiarities in *Shewanella* and *Bordetella* species.

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Supporting Information Available: Experimental procedures, SDS-PAGE analysis of His_6-PubC expression and purification, spectroscopic and enzyme assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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