

# Myxochelin Biosynthesis: Direct Evidence for Two- and Four-Electron Reduction of a Carrier Protein-Bound Thioester

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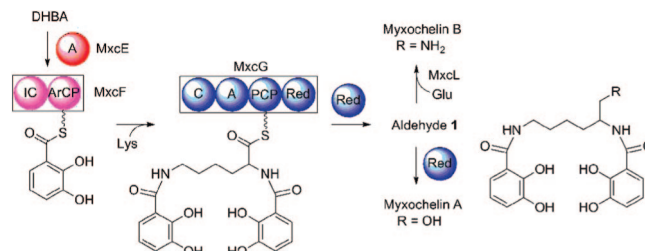
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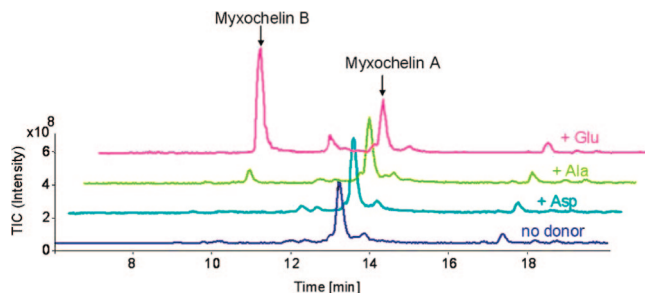
Iron serves as a cofactor for many enzymes and is thus a critical micronutrient in microbial metabolism.<sup>1</sup> Aerobic bacteria typically sequester iron from the environment through the production and secretion of low molecular weight, iron-chelating compounds called siderophores. Following recovery of the resulting  $\text{Fe}^{3+}$ -chelate complexes by specific transport systems,<sup>2</sup> the iron is released into the cell cytoplasm by hydrolysis.<sup>3</sup> Two siderophores are known to date from myxobacteria: the hydroxamate-type nannochelin<sup>4</sup> and the catechol-type myxochelins A and B.<sup>5,6</sup> The biosynthetic gene cluster for the myxochelins was recently cloned and sequenced from the myxobacterium *Stigmatella aurantiaca* Sg a15.<sup>6</sup> The cluster encodes four proteins (MxcCDEF) for synthesis of 2,3-dihydroxybenzoic acid (DHBA), a common structural feature of catechol-type siderophores, a nonribosomal peptide synthetase (NRPS) subunit MxcG, and a class III amino transferase, MxcL. One unusual feature of the NRPS MxcG is the presence of a C-terminal reductase (Red) domain, instead of a more typical thioesterase.

Together, these findings led to a biosynthetic proposal (Figure 1)<sup>6,7</sup> in which the assembled DHBA moiety tethered to the aryl carrier protein (ArCP) of MxcF is condensed successively with the two amino groups of lysine by the condensation (C) domain of MxcG. The resulting peptidyl carrier protein (PCP)-bound thioester is then reduced by the NAD(P)H-dependent Red domain to yield an aldehyde intermediate **1**, which can either undergo a further round of Red-catalyzed reduction to yield myxochelin A or be transaminated by MxcL to produce myxochelin B. Two-electron reductions of peptidyl carrier protein (PCP)-bound intermediates have been demonstrated in several pathways, including lysine biosynthesis in yeast<sup>8</sup> and formation of nostocyclopeptide in the cyanobacterium *Nostoc* sp. ATCC53789.<sup>9</sup> On the basis of sequence analysis, a similar enzyme is likely to function in the assembly of saframycin.<sup>10</sup> In contrast, evidence supports direct four-electron reduction to the corresponding alcohol by a terminal Red domain of NRPS subunit LtxA, during biosynthesis of lyngbyatoxin A.<sup>11</sup> To our knowledge, however, only the myxochelin pathway incorporates a single Red domain which appears to catalyze alternative two- or four-electron reduction of a common substrate. Strong evidence for Red-mediated four-electron reduction was provided by the reconstitution in vitro of myxochelin A biosynthesis by recombinant MxcE, F, and G, in the presence of ATP, NAD(P)H, Lys, and DHBA.<sup>7</sup> However, it has not been possible to date to re-establish the transamination reaction in vitro to yield myxochelin B nor to directly demonstrate the intermediacy of aldehyde **1** in the four-electron reductions of myxochelin and lyngbyatoxin.<sup>7,11</sup> We report here the successful reconstitution in vitro of the complete pathway to both myxochelins A and B, as well as isolation of the anticipated aldehyde intermediate **1**.

Recombinant MxcE, MxcF, and MxcG were produced as described earlier;<sup>7</sup> MxcF and MxcG were coexpressed with the broad specificity phosphopantetheinyl transferase MtaA to activate the ArCP and PCP domains, respectively, to their *holo* forms.<sup>12</sup>



**Figure 1.** Biosynthesis of myxochelins A and B, through a common aldehyde intermediate **1**.

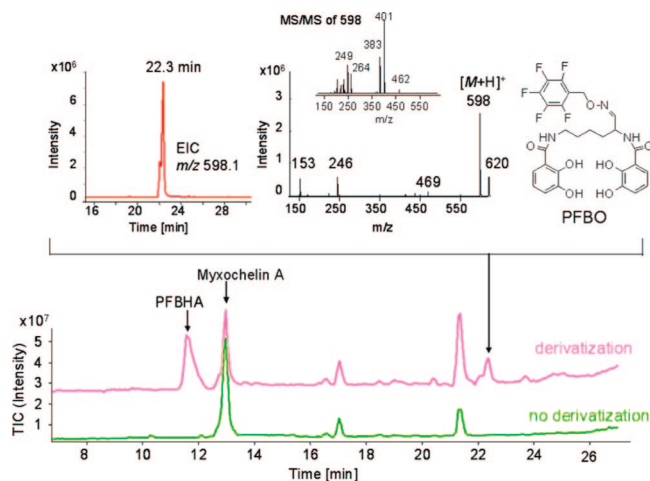


**Figure 2.** HPLC traces demonstrating production in vitro of myxochelins A and B, in the presence of various amino donors.

We obtained recombinant aminotransferase MxcL at high purity from *Escherichia coli*, both with an N-terminal His<sub>6</sub>-tag (40 mg/L) (Supporting Information) and as an intein-chitin binding domain fusion (5 mg/L).<sup>13</sup> Binding by purified MxcL of the cofactor pyridoxal 5'-phosphate (PLP) was confirmed by UV analysis ( $\lambda_{\text{max}}$  free PLP = 389 nm;  $\lambda_{\text{max}}$  enzyme-bound PLP = 410 nm) (Supporting Information). Formation of enzyme-bound pyridoxamine 5'-phosphate (PMP) as judged by UV was observed in the presence of glutamate ( $\lambda_{\text{max}}$  = 330 nm), consistent with the typical amino donor specificity of type III aminotransferases.<sup>14</sup>

We next attempted to reconstitute production of both myxochelins A and B in vitro. For this, we incubated MxcE, MxcF, MxcG, and MxcL (3 equiv relative to MxcG) together with Glu, ATP, NADPH, Lys, and DHBA. Incubations were performed for 1–6 h, extracted with ethyl acetate, concentrated, and analyzed by HPLC-ESI-MS. This analysis revealed the production of myxochelin A, as shown previously<sup>7</sup> (rt = 14 min;  $m/z$   $\text{MH}^+$  = 405) (Figure 2). However, we also detected a new peak with identical chromatographic behavior to an authentic sample of myxochelin B (rt = 11 min;  $m/z$   $\text{MH}^+$  = 404). We also showed that Ala serves as an alternative amino donor (ca. 10% of Glu), but that Asp is not accepted as a substrate (Figure 2).

Myxochelin B was produced at approximately twice the yield of myxochelin A, in contrast to the 10-fold excess of B which is typically seen in extracts of an alternative producer, *Myxococcus xanthus*. In principle, the relative amounts of myxochelins A and



**Figure 3.** HPLC traces demonstrating the formation of the PFBO derivative of the myxochelin aldehyde (pink). Inset shows the extracted ion chromatogram of the derivative, as well as MS and MS<sup>2</sup> analysis.

B should be controlled by how effectively the MxcG Red domain and MxcL compete for the common aldehyde intermediate **1**. This competition could occur on free aldehyde, so that the quantity of each product is controlled by the relative amounts of MxcL and MxcG in the cell. Alternatively, the substrate may be sequestered into a protein–protein complex formed between MxcG and MxcL; the precise configuration of the complex could determine the relative accessibility of the aldehyde to the two competing active sites. We aimed to distinguish between these possibilities by quantifying the relative production of myxochelins A and B in the presence of increasing amounts of MxcL (0.01–30×) relative to MxcG (1 μM) (Supporting Information). In the case of complex formation, we anticipated that the amount of myxochelin B would rise as functional complexes were formed but would plateau at approximately 10-fold relative to myxochelin A, once all of the MxcG was bound by MxcL. However, our analysis showed that myxochelin B was essentially the only product of the reaction at the highest concentrations of MxcL. Taken together, these data support a model in which the MxcL and the MxcG reductase compete directly for free aldehyde.

Detailed inspection of the HPLC-MS data also revealed a minor peak with a mass spectrum consistent with the expected aldehyde intermediate (rt = 12.7 min;  $m/z$  MH<sup>+</sup> = 403). The identity of the aldehyde was confirmed by high resolution mass spectrometry using a Thermo LTQ Orbitrap Hybrid FT mass spectrometer (calcd for

C<sub>20</sub>H<sub>22</sub>N<sub>2</sub>O<sub>7</sub> ([M + H]<sup>+</sup>) = 403.14998, found 403.15033, Δ = 0.352 mDa). Additional support for this assignment was obtained by derivatization of assay extracts with the aldehyde-selective reagent *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) (Supporting Information). The original aldehyde peak was absent from the derivatized samples, and instead a new peak was detected (rt = 22.3 min) with the expected mass for the pentafluorobenzylloxime (PFBO) derivative ( $m/z$  MH<sup>+</sup> = 598) (Figure 3). We further confirmed the identity of the derivatized aldehyde by HRMS (calcd for C<sub>27</sub>H<sub>24</sub>N<sub>3</sub>O<sub>7</sub>F<sub>5</sub> ([M + H]<sup>+</sup>) = 598.16072, found 598.15962, Δ = −1.098 mDa).

Taken together with earlier results,<sup>7</sup> the data reported here firmly establish the pathway proposed for the biosynthesis of myxochelins A and B in the myxobacterium *Stigmatella aurantiaca* and provide the first direct evidence for an aldehyde intermediate in the alternative two- or four-electron reduction of a PCP-bound thioester.

**Acknowledgment.** Funding was provided by the Alexander von Humboldt Foundation (K.J.W.), and the Deutsche Forschungsgemeinschaft (DFG). This communication is dedicated to the memory of Dr. Joe Spencer.

**Supporting Information Available:** Experimental details and analytical data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA8025278