SUPPORTING INFORMATION

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

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Cloning of *mxcL*

The gene *mxcL* was amplified from plasmid pCBP-MxcL[1] by PCR using the following primers (introduced restriction sites are underlined): forward 5′-TTTAAAACATATGGATACGCACTCGAAGAGACACC-3′ (*Nde*I) and reverse 5′-TTTAAAAAGCTTTCAGAGAGACTGCAACACCTCTC-3′ (*Hind*III). PCR reactions were carried out in presence of 10% DMSO using *Phusion* polymerase (Finnzyme), as follows: 2 min at 97 °C (10 s at 97 °C in subsequent cycles), 15 s at 60 °C, 20 s at 72°C (30 cycles), then extension at 72 °C for 10 min. The PCR product was inserted between appropriate restriction sites of pET-28a(+) (Novagen) to yield expression construct pET28-*mxcL*. The insert was verified by sequencing.

Overexpression and purification of His_6-tagged MxcL

E. coli Rosseta2 (DE3)pLyS harbouring pET28-*mxcL* was cultured in 0.5 L LB medium supplemented with kanamycin (30 µg/mL) and chloramphenicol (34 µg/mL), and was grown at 37 °C with agitation until OD_{600} reached 0.5–1.0. Protein overexpression was induced by addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16 °C overnight. The cell pellet was collected and resuspended in lysis/binding buffer (50 mM Tris-HCl (pH 7.8), 200 mM NaCl, 10 mM imidazole, 20 µM PLP, 10% glycerol) and disrupted by passing through a French press (3×). The cleared lysate was loaded to a column packed with 1 mL Ni sepharose resin (GE Healthcare) and pre-equilibrated with 20 mL

lysis/binding buffer. The column was washed sequentially with 10 mL binding buffer and 10 mL washing buffer (50 mM Tris-HCl (pH 7.8), 200 mM NaCl, 60 mM imidazole, 20 µM PLP, 10% glycerol), before the protein was eluted with 4 mL elution buffer (50 mM Tris-HCl (pH 7.8), 200 mM NaCl, 200 mM imidazole, 20 µM PLP, 10% glycerol). Purified N-terminally His_6-tagged MxcL was exchanged into storage buffer (50 mM Hepes (pH 7.6), 100 mM NaCl, 20 µM PLP, 10% glycerol) using Amicon Ultra centrifugal filter units (Millipore), and flash-frozen in liquid N_2 for storage at –80 °C. Typically yields were 40 mg protein/L culture.

Spectrophotometric measurement of PLP-bound MxcL

Purified N-terminally His_6-tagged MxcL (10 mg/mL, 0.2 mM) was incubated with 0.2 mM PLP at 4 °C for 1 h and then at room temperature for 30 min. The protein was exchanged into assay buffer (50 mM Hepes (pH 7.6)) using a PD-10 column (GE Healthcare) and diluted to an appropriate concentration (0.8 mg/mL) before measurement. UV spectra were recorded between 260–620 nm on a SPECORD 50 UV/Vis spectrometer (Analytikjena). The amino donor glutamate was added to a concentration of 0.2 mM (Figure S-1).

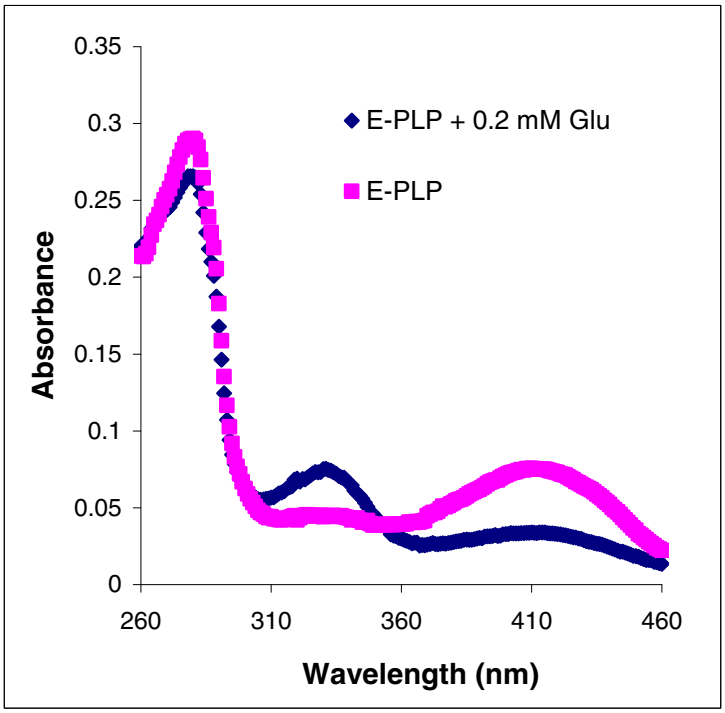

Figure S-1. UV spectra of PLP/PMP-bound MxcL.

Reconstitution *in vitro* of myxochelin biosynthesis

Enzymatic activity was assayed in a 50 µL reaction containing 50 mM Hepes (pH 7.6), 10 mM $MgCl_2$, 2 mM ATP, 2 mM NADPH, 2 mM 2,3-dihydroxylbenzoic acid (2,3-DHBA), 2 mM lysine, 2 mM glutamate, 0.2 µM MxcE, 0.4 µM MxcF, 1 µM MxcG, and 3 µM MxcL. Reactions were incubated at 37 °C for 1–6 hours. The proteins were precipitated by adding equal volume of acetone and frozen at –80°C for 30 min. The aqueous solution was analyzed on an ion trap mass spectrometer (HCT plus, Bruker Daltonics) fitted with an ESI source and coupled with a HP1100 HPLC system (Agilent). Samples were separated on a nucleodur C18 3µ RP column (125 × 2 mm) equipped with a pre-column (8 × 3 mm; Macherey & Nagel), using the following gradient at a flow rate of 0.4 mL/min: 0–2 min 5%

B, 2–32 min 5–95% B, 32–36 min 95% B, 36–39 min 95–5% B (buffer A: water containing 0.1% formic acid; buffer B: acetonitrile containing 0.1% formic acid). Mass spectra were acquired in positive mode (200–2000 Da; Myxochelin A ($[M+H]^+$ = 405.2; r.t. 13 min) and B ($[M+H]^+$ = 404.3; r.t. 11 min)). Under these conditions, myxochelin B was obtained at 2–5 fold excess relative to myxochelin A. To determine the amino donor for MxcL, glutamate was replaced by alanine or aspartate. To investigate the possible complex formation between MxcL and MxcG, the concentration of MxcL was varied (0.01, 0.05, 0.1, 0.2, 0.5, 0.8, 1.0, 2.0, 4.0, 6.0, 50 µM) while that of MxcE, MxcF, MxcG was fixed at 0.2, 0.4 and 1 µM, respectively. Reactions were incubated for 4 h and then 5 µL of each mixture was injected directly onto the column for LC-MS analysis. The relative production of myxochelin B and A (Figure S-2) was quantified by integrating peak areas in the respective extracted ion chromatogram.

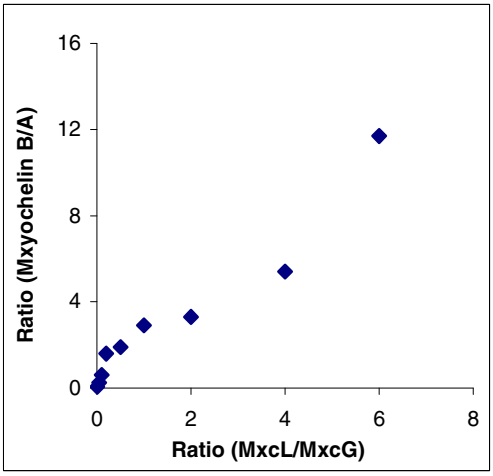

Figure S-2. Production of myxochelin B relative to A, as the molar ratio of MxcL:MxcG was increased. The data point from one incubation (MxcL/MxcG = 50, B/A = 67) was omitted from the graph for display purposes.

Detection of the aldehyde intermediate

Low amounts of the aldehyde intermediate could be detected by LC-ESI-MS from the enzyme reaction (1% compared to myxochelin B production) ($[M+H]^+$ = 403.2, r.t. 12.7 min). The aldehyde intermediate eluted at a very similar retention time to myxochelin A (r.t. 13.1 min), however the MS^2 data unambiguously confirmed its identity (Figure S-3). The reaction was scaled up to 500 µL and extracted with 500 µL ethyl acetate (3×). The organic phase was evaporated to dryness and redissolved in 100 µL methanol. The enzyme products (100 µL) were reacted with 5 µL of pentafluorobenzylhydroxylamine hydrochloride (PFBHA; 10 mg/mL) in MeOH at room temperature for 3 h, and analyzed by LC-ESI-MS.

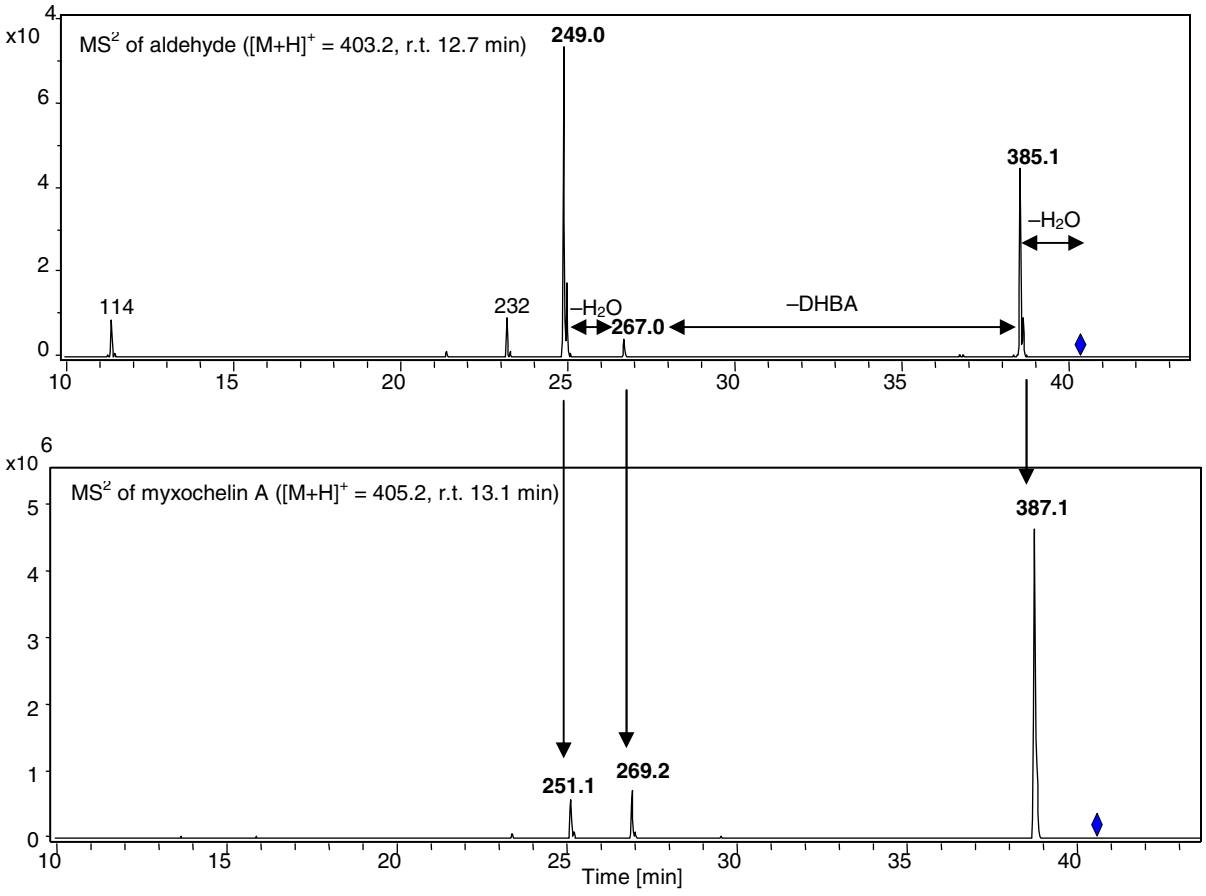

Figure S-3. MS^2 data of aldehyde intermediate compared to myxochelin A. The blue diamonds indicate the positions of the parent ions, and the arrows show corresponding fragments in the two spectra.

Reference

1. Gaitatzis, N.; Kunze, B.; Müller, R. *ChemBioChem* **2005**, *6*, 365–374.