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Biosynthesis of the pyoverdine siderophore of *Pseudomonas aeruginosa* involves precursors with a myristic or a myristoleic acid chain

Mélissa Hannauer [a], Mathias Schäfer [b], Françoise Hoegy [a], Patrick Gizzi [a], Patrick Wehrung [c], Gaëtan L.A. Mislin [a], Herbert Budzikiewicz [b,*], Isabelle J. Schalk [a,*]

[a] UMR 7242, Université de Strasbourg-CNRS, ESBS, Blvd. Sébastien Brant, F-67413 Illkirch, Strasbourg, France
[b] Institut für Organische Chemie der Universität zu Köln, Greinstr. 4, D-50939 Köln, Germany
[c] Service Commun d'Analyse, Faculté de Pharmacie, 74 route du Rhin, F-67401 Illkirch, France

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ABSTRACT

Pyoverdine I (PVDI) is the major siderophore produced by *Pseudomonas aeruginosa* to import iron. Biosynthesis of this chelator involves non-ribosomal peptide synthetases and other enzymes. PvdQ is a periplasmic enzyme from the NTN hydrolase family and is involved in the final steps of PVDI biosynthesis. A *pvdQ* mutant produces two non-fluorescent PVDI precursors with a higher molecular mass than PVDI. In the present study, we describe the use of mass spectrometry to determine the structure of these PVDI precursors and show that they both contain a unformed chromophore like ferribactin, and either a myristic or myristoleic chain that must be removed before PVDI is secreted into the extracellular medium.

1. Introduction

To assimilate iron, fluorescent *Pseudomonads* produce yellow-green fluorescent siderophores, called pyoverdines [1]. These iron chelators are all composed of three distinct structural parts: (i) a quinoline chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline, which confers color and fluorescence to the molecule; (ii) a strain-specific peptide comprised of 6–12 amino acids bound to the carboxylic group of the chromophore; and (iii) a dicarboxylic acid, amide or α-ketoglutaric acid attached to the NH_2 group of the chromophore [2]. The peptide moiety differs among strains by the number, composition and configuration of amino acids. Pyoverdine synthesis has been best described in the *Pseudomonas aeruginosa* strain PAO1, which produces pyoverdine I (PVDI, Fig. 1A) [3].

PVDI synthesis begins with the assembly of the peptide backbone in the cytoplasm, by four non-ribosomal peptide synthetases (NRPSs)—PvdL, PvdI, PvdJ, and PvdD—, leading to a non-fluorescent precursor called ferribactin [3,4]. The substrates for the NRPS enzymes are produced by a variety of other enzymes [3]. Several of these enzymes are well characterized, such as the ornithine hydroxylase PvdA [5], the aminotransferase PvdH [6], and the hydroxyornithine transformylase PvdF [7]. It has been recently proposed that PVDI synthesis starts with PvdL, which couples coenzyme A with a myristate fatty acid in an ATP-dependent reaction, and delivers the complex to L-Glu carried by a second module of PvdL [8]. Following this, D-Tyr and L-Dab are incorporated by the two other modules of PvdL, forming a tertrahydropyrimidine ring, which is a precursor of the dihydroxyquinoline chromophore [9]. After incorporation of all residues into the peptide backbone by NRPS, this cytoplasmic non-fluorescent precursor —called ferribactin— is transported across the inner membrane into the periplasm by a process involving PvdE, which is an "export" ABC transporter essential for PVDI production [10]. The PVDI chromophore appears to be synthesized from the peptide precursor by a multistep oxidative process [11], although the enzymes involved are yet to be identified. Mutation of the periplasmic enzymes PvdN [12], PvdO, and PvdP abolish production of PVDI [10], indicating that these proteins are involved in either PVDI formation or in a step preceding it. Mutation of periplasmic enzyme PvdQ results in the secretion of a PVDI precursor with a higher molecular mass than PVDI [10]. This precursor has been purified and was proposed to be a substrate of PvdQ [8], providing a direct link between PvdQ and the mechanism of myristate group removal by PvdQ before

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Abbreviations: PVDI, pyoverdine I; PVDIq, PVDI precursor produced by PAO1*pvdQ*; NRPS, non-ribosomal peptide synthetases; CA, collision activation; HPLC–MS, high performance liquid chromatography–mass spectrometry

* Corresponding authors. Address: UMR 7242, ESBS, Blvd. Sébastien Brant, BP 10412, F-67413 Illkirch, Strasbourg, France. Fax: +33 3 68 85 48 29 (I.J. Schalk).

E-mail addresses: aco88@uni-koeln.de (H. Budzikiewicz), isabelle.schalk@unistra.fr (I.J. Schalk).

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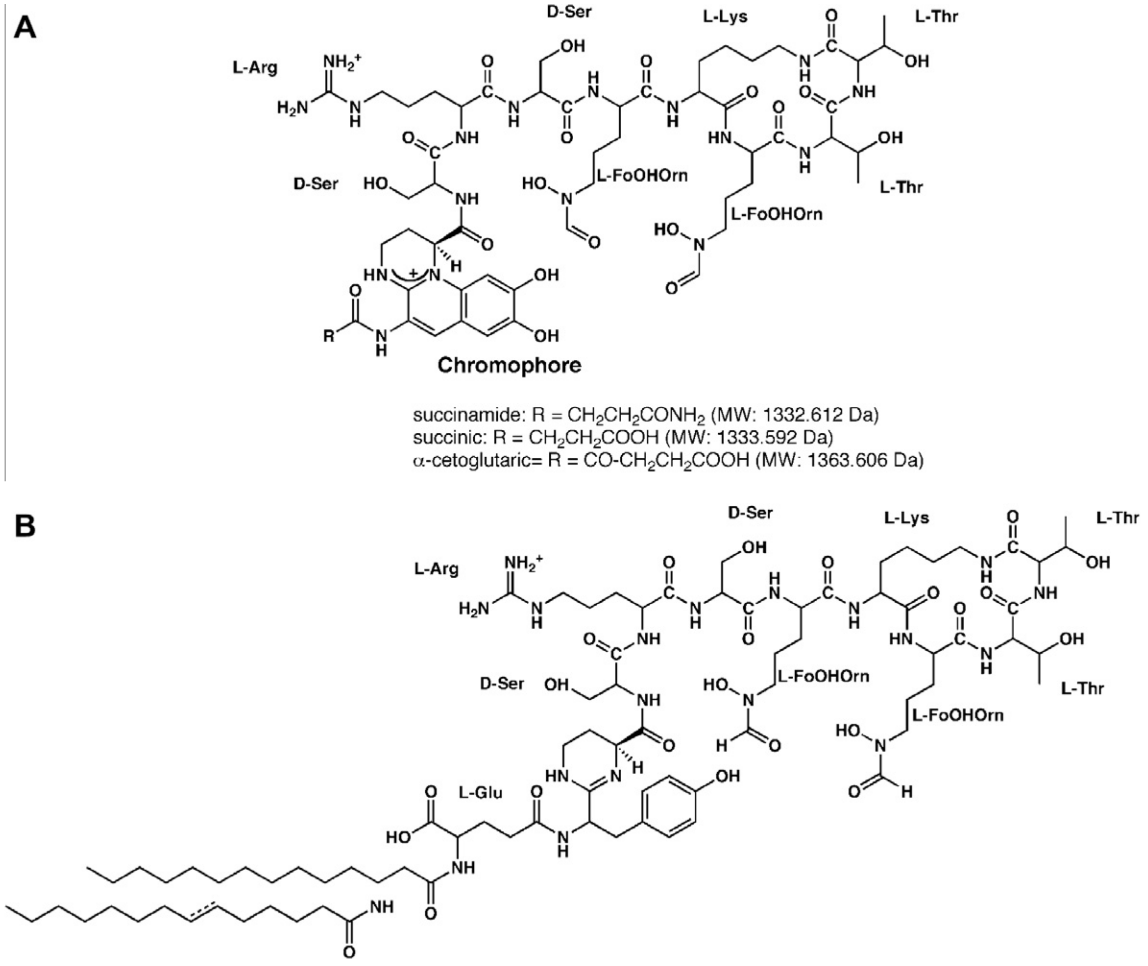

Fig. 1. Structure of PVDI isoforms produced by PAO1 (A) and PVDI precursors produced by PAO1*pvdQ* (B). The exact position of the double bond in tetradecenoic acid could not be precisely determined and might be in a different position.

secretion into the extracellular milieu. PvdQ belongs to the NTN hydrolase family [13] and was identified as a periplasmic quorum-quenching protein that cleaves acyl homoserine lactones [14]. Transport of newly synthesized PVDI from the periplasm into the extracellular medium involves PvdRT-OpmQ, an ATP-dependent efflux pump [15]. Analyses of *Pseudomonas* genomes suggest that there are analogous biosynthetic pathways for other pyoverdines in other strains and species [3,16].

In the present study, we used mass spectrometry to determine the structure of two PVDI precursors produced by a *pvdQ* mutant and showed that one has a myristate moiety —as previously suggested [8]— and the other has a myristoleate group. Both non-fluorescent precursors have an unformed chromophore with the fatty acid chains linked to the L-Glu residue—not to an Asp residue, as proposed by Gulick et al. [8].

2. Experimental procedures

2.1. Bacterial strains and growth

P. aeruginosa strains PAO1 [17] and PAO1*pvdQ* (*pvdQ* mutant described previously [18]) were grown at 30 °C in succinate medium (composition: K_2HPO_4; 3.0 g/l KH_2PO_4; 1.0 g/l $[NH_4]_2SO_4$; 0.2 g/l $MgSO_4{\cdot}7H_2O$; 4.0 g/l sodium succinate) with the pH adjusted to 7.0 by addition of NaOH.

2.2. Purification and characterization of PVDI and PVDI precursors released in the extracellular medium

PVDI and its precursors were purified on an octadecylsilane column (Lichroprep RP 18, 40–63 µm, Merck) as previously described [10]. For LC–MS measurements, samples were dissolved in water to a concentration of 10^{-5} M, injected onto an Agilent 1200 rapid resolution LC system fitted with a Thermo HypersilGold C18 column (1 × 3 × 1.9 cm), and analyzed in positive ESI mode performed on an Agilent 6520 Accurate mass QToF spectrometer. For structure determination, PVDI precursors were purified by HPLC (Gilson system with a photodiode array detector) on a Zorbax SB-C18 column (9.4 × 250 mm, Agilent) with a 0–100% acetonitrile gradient over 30 min at a flow rate of 3 ml/min.

2.3. Determination of the structure of PVDI precursors by mass spectrometry

Low-resolution data were obtained with a MAT 900 ST instrument with an EB-quadrupole ion trap (QIT) geometry and equipped with an ESI ion source (Finnigan MAT, Bremen, Germany). ESI experiments were conducted with a spray voltage of 3.6 kV, a capillary temperature of 230 °C, and a flow rate of 3 µl/min. The samples were dissolved in H_2O/CH_3OH 1:1. Fragmentation was induced by collision activation (CA) at ∼2×10^{-3} Pa He. High-reso-