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

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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

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

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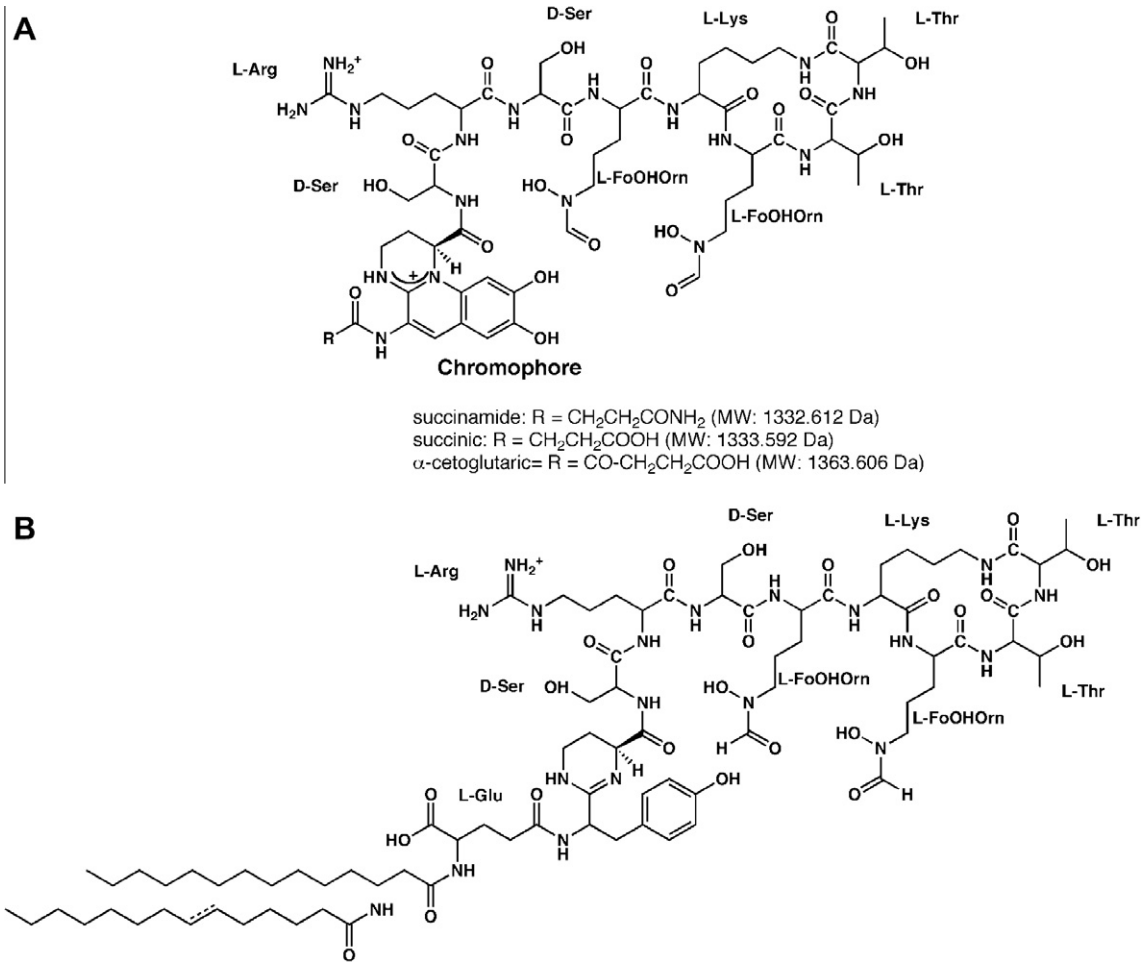

Fig. 1. Structure of PVDI isoforms produced by PAO1 (A) and PVDI precursors produced by PAO1*pvdQ* (B). The exact position of the double bond in tetradecenoic acid could not be precisely determined and might be in a different position.

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 ($1 \times 3 \times 1.9$ 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 $\sim 2 \times 10^{-3}$ Pa He. High-reso-

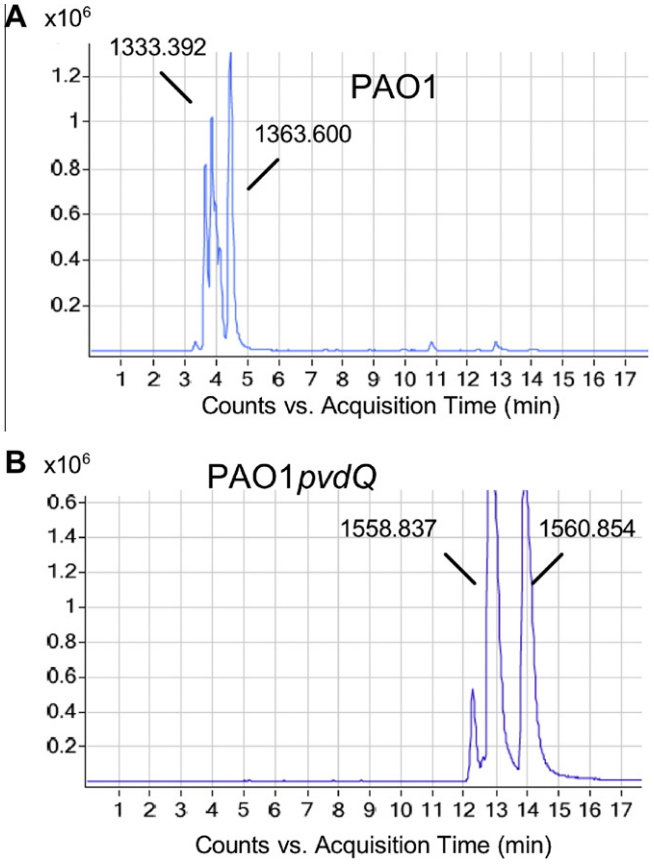

Fig. 2. HPLC–MS analysis and relative quantification of secreted PVDI by PAO1 (A) and PVDI precursors by PAO1*pvdQ* (B). Secreted PVDI and PVDI precursors were prepared as described in materials and methods, and analyzed by HPLC–MS. The MS base peak chromatograms are shown. The compounds of molecular mass of 1333.392 and 1363.600 produced by PAO1 are the succinamide and the α-ketoglutaric isoforms of PVDI, respectively.

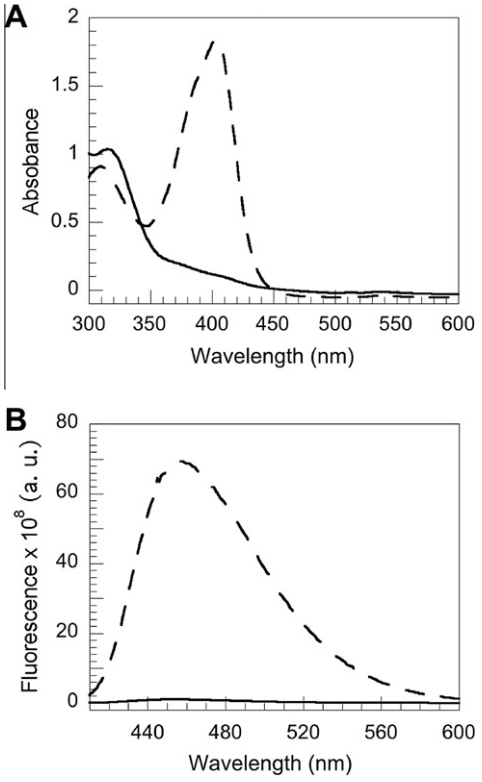

Fig. 3. Absorbance (A) and fluorescent (B) spectra of the PVDI precursor of *m/z* 1,560.85 Da (solid line) and PVDI (dashed line). UV spectra were obtained with 200 μM siderophore in 50 mM Tris–HCl pH 8.0. Fluorescent spectra were recorded in the same buffer with 2 μM siderophore (excitation wavelength 400 nm). Equivalent spectra have been obtained with the precursor A.

lution data were obtained with a LTQ FTMS-Orbitrap XL instrument (Fisher Scientific, Bremen, Germany).

3. Results

3.1. Spectral properties of PVDI precursors

We have previously described one precursor produced by PAO1*pvdQ* as a fluorescent compound of *m/z* 1560.85 [10]. In the present study, HPLC–MS analyses of the siderophores produced by PAO1*pvdQ* showed the production of two compounds, one of *m/z* 1560.85—which has been previously reported—and a second one of *m/z* 1558.83 (Fig. 2). Neither of the compounds—called A for *m/z* 1558.83, and B for 1560.85—were fluorescent and neither showed the absorbance at 400 characteristic of PVDI (Fig. 3). The fluorescence previously described as associated with precursor B [10] was due to a contamination, which was eliminated in the present study by HPLC purification. These two PVDI precursors must be two precursors with a unformed chromophore and do not appear to have the structure proposed recently by Gulick et al. [8].

3.2. Structure of PVDI precursors

Elucidation of the structure of compounds A and B derived from mass-spectrometry data was based on the known amino-acid sequence of PVDI isolated from *P. aeruginosa* ATCC 15692 [19,20]. To designate the peptide fragments, the nomenclature proposed by Roepstorff & Fohlmann [21] was used. The typical decomposition mode of acid-amide structures is the loss of a ketene moiety, viz. $[R–CH_2–CO–NH–R']^+ \rightarrow [R–CH{=}CO]^+ + [NH_2–R']$. The fragmentation behavior of pyoverdines has been reviewed [22,23] and the present mass spectral fragmentation data are assembled in Table 1.

The two compounds A and B, differ by one degree of unsaturation (2 H). The masses of their $[M+H]^+$ ions are 1559, 8419 Da (calc'd 1559, 8433 Da for $C_{70}H_{115}N_{18}O_{22}$) and 1561, 8590 Da (calc'd 1561, 8589 Da for $C_{70}H_{117}N_{18}O_{22}$) (Table 1, first line of data). In the following discussion ions of compound A will be reported, marked by * when shifted by 2 Da for compound B. CA of $[M+H]^+$ resulted in the loss of $C_{14}H_{26}O_2$ (probably ketene type elimination plus H_2O amounting to tetradecenoic acid; 1333, 6502 Da, calc'd 1333, 6499 Da for $C_{56}H_{89}N_{18}O_{20}$) followed by H_2O (1315, 6399 Da; calc'd 1315, 6393 Da for $C_{56}H_{87}N_{18}O_{19}$) and NH_3 (1298, 6133 Da, calc'd 1298, 6128 Da for $C_{56}H_{84}N_{17}O_{19}$) (Table 1, lines 2–4). The most important fragment for the present purpose is the ion *m/z* 1222, 6188, calc'd 1222, 6178 Da for $C_{51}H_{84}N_{17}O_{18}$ resulting from the ketene-type loss of $C_{19}H_{31}NO_4$, i.e., the Glu side chain substituted with of $C_{13}H_{25}CO$. The doubly protonated analog of *m/z* 1222 (*m/z* 611,5) is found with high abundance in the ion trap CA spectrum. Compound B shows an analogous behavior (loss of $C_{19}H_{33}NO_4$) (Table 1, fifth line).

All the ions reported [24] for a ferribactin chromophore with a Glu side chain and Ser as the first amino acid (*m/z* 86, 136, 170, 198, 303, 371, 416) are prominent in the quadrupol CA spectra of $[M+2H]^{2+}$ (*m/z* 780,5 and 781) for both compounds (Table 1, sixth line).

The ion *m/z* 642* (642, 3858; calc'd 642, 3866 for $C_{34}H_{52}N_5O_7$) indicates that the Glu-Chromophore-Ser part of the molecule contains the tetradecenoic (tetradecanoic) acid substituent (Table 1,

Table 1
Mass spectrometry data.

Compound A			Peptide sequence ions	Compound B		
Remarks	Mass	Ion		Ion	Mass	Remarks
1559,8419 (calc'd 1559,8433 for $C_{70}H_{115}N_{18}O_{22}$)	1559	$[M+H]^+$		$[M+H]^+$	1561	1561,8590 (calc'd 1561,8589 for $C_{70}H_{117}N_{18}O_{22}$)
1333,6502, calc'd 1333,6499 for $C_{56}H_{89}N_{18}O_{20}$	1333[1]	- $C_{14}H_{26}O_2$		- $C_{14}H_{28}O_2$	1333[1]	these ions coincide in mass with those from cpd. A
1315,6399; calc'd 1315,6393 for $C_{56}H_{87}N_{18}O_{19}$	1315	- H_2O		- H_2O	1315	
1298,6133, calc'd 1298,6128 for $C_{56}H_{84}N_{17}O_{19}$	1298	- NH_3		- NH_3	1298	
1222,6188, calc'd 1222, 6178 for $C_{51}H_{84}N_{17}O_{18}$	1222[1]	- $C_{19}H_{31}NO_4$		- $C_{19}H_{33}NO_4$	1222[1]	
[24]		86, 136, 170, 198, 305, 371, 416	Glu-Chr-Ser	86, 136, 170, 198, 305, 371, 416		[24]
	416[2]	a_1 - $C_{14}H_{26}O_2$	Ser	a_1 $C_{14}H_{28}O_2$	416[2]	
642,3858, calc'd 642,3866 for $C_{34}H_{52}N_5O_7$	642	a_1	Ser	a_1	644	
	670	b_1	Ser		67	
826,4828, calc'd 826,4826 for $C_{34}H_{64}N_9O_9$	826	b_2	Arg		828	
784,4611, calc'd 784,4608 for $C_{33}H_{62}N_9O_7$	784	- CH_2N_2 from Arg		CH_2N_2 from Arg	786	
	913	b_3	Ser		915	
1071,5830 calc'd 1071,5837 for $C_{50}H_{79}N_{12}O_{14}$	1071	b_4	FoOHOrn		1073	
	1054	- NH_3		- NH_3	1056	
1026,5618, calc'd 1026,5623 for $C_{49}H_{76}N_{13}O_{13}$	1026	- CO from FoOHOrn		- CO from FoOHOrn	1028	
	489	y_4"	(Lys...Thr)	$y_{4"}$	489	
		y_5"	FoOHOrn	y_5"		
		y_6"	Ser	y_6"		
	890	y_7"	Arg	y_7"	890	
959,4914; calc'd 959,4909 for $C_{38}H_{67}N_{14}O_{15}$	959	y_8" - $C_{14}H_{26}O_2$	Ser	y_8" - $C_{14}H_{28}O_2$	959	

Abbreviations: calc'd: calculated; FoOHOrn: N5-formyl-N5-hydroxy Orn; Chr: ferribactin chromophore; a, b, c: N-terminal, y″ C-terminal fragment ions according to the Roepstorff [20] nomenclature.

[1] The doubly protonated analogs of 1333 and 1222 (667 and 611,5) are found with high abundance in the ion-trap CA spectrum.

[2] The ions reported for a ferribactin chromophore with a Glu side chain and *m*/*z* 642 are prominent in the octopol CA spectrum of $[M+2H]^{2+}$ (780,5), the following ions in the CA ion-trap spectrum.

lines 7–8). In the ion trap CA spectrum the ion b_1 is found at 670*, b_2 at 826*, b_3 at 913*, b_4 at 1071, 5830* (calc'd 1071, 5837 for $C_{50}H_{71}N_{12}O_{14}$) with high abundance (loss of the lactame ring; accompanied by c_4'' at 1088*), evidently all containing the tetradecenoic (tetradecanoic) acid residue (Table 1, lines 9–13). The C-terminal ring fragment (y_4'') occurs at 489 followed by y_7'' (890) and y_8'' ([Ser-H_2O]$^+$) (959, 4914; calc'd 959, 4909 for $C_{38}H_{67}N_{14}O_{15}$), all of them without the fatty acid residue (Table 1, lines 16–20). The fragment ions mentioned are in agreement with the conclusion that compound A is the ferribactin with a Glu side chain corresponding to the PVDI ATCC 15692 substituted at the Glu side chain with tetradecenoic (tetradecanoic) acid.

4. Discussion

The biosynthesis of PVDI is a complex biological process, involving at least eleven different enzymes. The process starts in the cytoplasm and ends in the periplasm before secretion of PVDI into the extracellular medium by the efflux pump, PvdRT-OpmQ. Mutation of *pvdQ* —which codes for a periplasmic enzyme involved in PVDI synthesis— stops secretion of PVDI, and instead two precursors, A and B, of higher molecular weight, are secreted. The A and B precursors differ by only one degree of unsaturation. Both compounds do not have the characteristic absorbance of PVDI at 400 nm and are not fluorescent (Fig. 3), indicating that they have an unformed chromophore. Structure determination by mass spectrometry confirmed that compounds A and B contain the same unformed chromophore as ferribactin (Fig. 3), with the presence of a tetradecenoic acid (in compound A) or tetradecanoic acid (in compound B) on the L-Glu residue (Fig. 1B). However, the exact position of the double-bond in the tetradecenoic acid could not be precisely determined and may be in a different position, as proposed in Fig. 1B. The two structures proposed from the mass-spectra analyses are different from those of Gulick et al. [8] for compound B, who proposed an already formed dihydropyoverdine chromophore and an Asp residue instead of Glu.

As recently suggested by Gulick et al. [8], myristic or myristoleic acid is probably added to the L-Glu residue at the beginning of PVDI biosynthesis by PvdL. PvdL is atypical among PVDI NRPS because the initial module —PvdL-M1, predicted to function as a starter module— contains an unusual domain which is very similar to acyl coenzyme A ligases [9]. This suggests that PVDI synthesis begins with an acylation and that the precursor ferribactin is therefore most likely acylated. Consistent with this, the binding site of this PvdL-M1 module can bind to mysristate [8]. The periplasmic PvdQ acylase is also able to recognize tetradecanoic acid, suggesting that this enzyme is involved in the removal of this fatty-acid chain before secretion [8]. This is consistent with the observation in the present study, that a *pvdQ* mutant produces precursors with myristic or myristoleic acid still bound to the L-Glu residue. The physiological function of PvdQ is therefore not in the deacylation of the quorum sensing molecule 3-oxo-C12 as previously suggested [14] but in the deacylation of the PVDI precursor isolated in the present work.

The fact that a *pvdQ* mutant produces a PVDI precursors with a myristic or myristoleic group and an unformed chromophore suggests that periplasmic PvdQ enzyme removes the fatty-acid chain before cyclization of the chromophore. Therefore PVDI maturation in the periplasm includes an excision of the acylated fatty chain and a cyclization of the chromophore, which yields a fluorescent siderophore. Mutation of the periplasmic enzymes PvdN and PvdP abolished the secretion of fluorescent PVDI [10], indicating that these proteins are involved in either PVDI chromophore formation or in a step preceding it. The current model for PVDI periplasmic maturation thus proposes that PvdQ removes the acylated fatty chain of the non-fluorescent precursor after its export into the periplasmic space. PvdN and PvdP, of still unknown functions, would then be involved in the chromophore cyclization, yielding a fluorescent siderophore. The efflux pump PvdRT-OpmQ has been implicated in the delivery of newly synthesized PVDI from the periplasm into the extracellular medium [15].

The presence of a myristic or myristoleic acid group retains the PVDI precursor at the membrane during its biosynthesis. PVDI is a siderophore with a high affinity for iron [25] and previous studies showed that the precursor, PVDIq —called here compound B— was able to chelate ^{55}Fe and transport it into *P. aeruginosa* cells [10]. By retaining the PVDI precursors at the membrane through a myristoyl group, the siderophore does not diffuse throughout the cytoplasm or periplasm with the risk of chelating all metals present. This myristic chain is removed before cyclisation of the chromophore.

Compounds A and B are first examples of siderophore precursors with a fatty-acid chain that clearly retains the chelator bound to the bacterial membrane during synthesis. The only other siderophores with fatty-acid chains that have been reported in the literature are marine siderophores, which are secreted into the bacterial marine environment with the fatty chain [26]. Analyses of *Pseudomonas* genomes indicate that there are analogous biosynthetic pathways for other pyoverdines production in other strains and species [3,16]. Therefore, the data presented here for PVDI are probably valid for the synthesis of other pyoverdines produced by fluorescent pseudomonads.

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