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Journal:	<i>FEBS Letters</i>
Manuscript ID	Draft
Wiley - Manuscript type:	Research Letter
Date Submitted by the Author:	n/a
Complete List of Authors:	ROCHE, Béatrice; Universität Basel Department Biozentrum, Infection Biology Focal Area Mislin, Gaëtan; CNRS-Universite de Strasbourg, UMR7242 schalk, isabelle; CNRS-Universite de Strasbourg, UMR7242
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Identification of the fatty acid coenzyme-A ligase FadD1 as an interacting partner of FptX in the *Pseudomonas aeruginosa* pyochelin pathway

Béatrice Roche^{1,2,3*}, Gaëtan L.A. Mislin^{1,2} and Isabelle J. Schalk^{1,2}

¹. CNRS, UMR7242, ESBS, Bld Sébastien Brant, F-67412 Illkirch, France.

². Université de Strasbourg, UMR7242, ESBS, Bld Sébastien Brant, F-67412 Illkirch, France.

³. Present address: Biozentrum, University of Basel, CH-4056 Basel, Switzerland

* = correspondence should be addressed to Béatrice Roche (beatrice.roche@unibas.ch)

Abstract

Pseudomonas aeruginosa is one of the most important nosocomial bacteria emerging as a highly multidrug-resistant pathogen. *P. aeruginosa* produces two siderophores including pyochelin to fulfill its need for iron during infections. We know that both outer and inner membrane proteins FptA and FptX are involved in the ferri-pyochelin uptake, but this process requires increasing molecular and biochemical knowledge. Here, using bacterial two-hybrid and copurification assays we identified the fatty acid coenzyme-A ligase FadD1 as a novel interacting partner of the inner membrane transporter FptX, and found that FadD1 may play a role in pyochelin production. We managed to purify the FadD1-FptX inner membrane complex and obtained low-resolution 3D models, opening the way for future high-resolution structures.

Keywords: *Pseudomonas aeruginosa*; pyochelin siderophore; iron uptake; coenzyme-A ligase; membrane complex.

Introduction

Pseudomonas aeruginosa is a major human pathogen naturally resistant to many antibiotics due to the low permeability of its envelope. The situation is particularly critical as *P. aeruginosa* belongs to the World Health Organization list of antibiotic-resistant priority pathogen [1]. The success of bacteria to colonize the host relies mostly on their ability to obtain adequate supplies of the nutrients that are essential for growth and virulence. To circumvent the low bioavailability of iron in the host during infections, *P. aeruginosa* produces the two siderophores pyoverdine (PVD) and pyochelin (PCH) [2-4]. Siderophores are small iron chelating compounds produced by bacteria under iron limitation conditions [5]. Previous studies have shown an upregulation of the PCH pathway in clinical isolates from cystic fibrosis patients [6-7] and reported a potential role of PCH in the inflammatory response [8].

PCH is synthesized by non-ribosomal peptide synthetases in the bacterial cytoplasm and then secreted into the extracellular medium by an unknown mechanism [9]. Once in the bacterial environment, PCH chelates ferric iron leading to the formation of ferri-PCH complexes. Only two proteins involved in the ferri-PCH uptake have been described so far: the specific TonB-dependent outer membrane transporter FptA [10-12], and the inner membrane protein FptX [13-15]. Much attention has been paid to structural studies of FptA, and X-ray structure of FptA bound to ferri-PCH provided valuable information for the design of PCH-antibiotic conjugates [16-17]. In the context of antibiotic resistance, conjugation of antibiotics with siderophore is a promising drug delivery strategy exploiting iron uptake systems as gates to carry out antibiotics into the bacteria [18-19]. Such Trojan horse strategy can provide increased penetration of drugs previously unable to cross the cell wall of bacteria [4]. But we currently lack biochemical/structural data on the inner membrane transporter FptX predicted to have 12 transmembrane segments.

Here, we explored potential new actors involved in the ferri-PCH uptake pathway by using a genome-wide screening approach. We identified the fatty acid coenzyme-A ligase FadD1 as a new and previously undescribed interacting partner of FptX, forming an inner membrane complex. Deletion of *fadD1* led to a decrease in PCH production, suggesting a role of FadD1 in biosynthesis and/or secretion of this siderophore.

We also managed to express and purify FptX and could establish low-resolution 3D models of FptX, FadD1 and of the FadD1-FptX complex by negative-stain electron microscopy (EM).

Materials and methods

Chemicals and growth media.

X-Gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside) and IPTG (Isopropyl- β -D thiogalactoside) were purchased from Euromedex. ONPG (2-Nitrophenyl- β -D galactopyranoside), sodium oleate, and N-lauroylsarcosine sodium salt were obtained from Sigma-Aldrich. Detergent n-Dodecyl- β -D-maltopyranoside (DDM) was purchased from Anatrace. LB (Lennox) and LB agar medium were both purchased from Difco and were used as nutriment-rich medium in all experiments. Bacteria were routinely grown at 30°C in LB broth (Difco) or in iron-restricted medium (CAA) as previously described [15]. Media were supplemented as necessary with ampicillin (Ap; 100 $\mu\text{g.mL}^{-1}$), kanamycin (Kan; 50 $\mu\text{g.mL}^{-1}$), gentamicin (Gm; 30 $\mu\text{g.mL}^{-1}$), chloramphenicol (Cm; 50 $\mu\text{g.mL}^{-1}$), IPTG (0.5 mM). The strains used in this study are listed in Table S1.

Plasmids construction.

All DNA amplifications for cloning were performed with Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific) using genomic DNA of PAO1 strain as template. Restriction enzymes and T4 DNA ligase were purchased from ThermoFisher Scientific. All enzymes were used in accordance with the manufacturer's instructions. Oligonucleotides are listed in Table S2. *Escherichia coli* strain Top10 was used as the host strain for all plasmids. Absence of mutations was checked by DNA sequencing (GATC).

Expression plasmids. Plasmid p33-fptX(His₆) was constructed by PCR amplification of the coding region of *fptX* by using the primer pairs 970/969. The PCR product was then digested with EcoRI and HindIII enzymes and ligated into the EcoRI/HindIII linearized pBAD33 vector. Plasmid p24-fadD1(StrepII) was constructed by PCR amplification of the coding region of *fadD1* by using the primer pairs 1157/1158. The

PCR product was then digested with NcoI and HindIII enzymes and ligated into the NcoI/HindIII linearized pBAD24 vector.

Two-hybrid plasmids. DNA fragments encoding proteins of interest were fused to the two fragments T25 and T18 of *Bordetella pertussis* adenylate cyclase carried on the pKT25, pKNT25, pUT18C and pUT18 vectors. The DNA regions encoding *fptX* and *fadD1* were amplified by PCR by using primer pairs 951/952 and 1037/1038, respectively. PCR products were then digested with XbaI and KpnI enzymes and ligated into the XbaI/KpnI linearized pKT25, pKNT25, pUT18C and pUT18 vectors.

Bacterial two-hybrid screen.

Four independent two-hybrid libraries (pUT18CPAO1Lib, pUT18PAO1Lib, pUT18+1PAO1Lib and pUT18+2PAO1Lib) of PAO1 genome were tested independently. The pKNT25-fptX vector was used as the bait and the two-hybrid screen was performed as described in Houot *et al.*, 2012 [20].

Bacterial two-hybrid assay.

The adenylate cyclase-based bacterial two-hybrid technique was used as previously published [21].

Western blot analysis.

Proteins were applied to SDS-PAGE acrylamide gels and transferred onto nitrocellulose membranes. FptX(His₆) and FadD1(StrepII) proteins were immunodetected with the anti-His (1:3,000 ; GeneTex) and the StrepMAB (1:1,000 ; IBA) antibodies, respectively. Immunoblots were developed by using horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies (1:10,000 ; GE Healthcare), followed by chemiluminescence detection.

Expression and purification of FptX.

Top10 *E. coli* cells were transformed with the p33-fptX(His₆) vector. Cells were grown at 37°C with shaking until they reached an OD₆₀₀ ≈ 0.5, and the expression of *fptX* gene was induced with 0.01 % arabinose for

4 h at 37°C. Cell pellets were resuspended in 50 mM Tris-HCl pH 8, 100 mM NaCl, supplemented with EDTA-free protease inhibitor (Roche). After sonication, the cell suspension was clarified by centrifugation at 7,700 g for 15 min at 4°C. Membrane fraction was then collected by ultracentrifugation at 118,000 g for 40 min at 4°C. After mechanically homogenized, membranes were solubilized in 50 mM Tris-HCl pH 8, 200 mM NaCl, 1 % DDM at 4°C overnight. The suspension was clarified by ultracentrifugation at 118,000 g for 40 min at 4°C. The supernatant was loaded onto a 1-mL HisTrap HP column (GE Healthcare) equilibrated with buffer A (50 mM Tris-HCl pH 8, 500 mM NaCl, 0.1 % n-Dodecyl- β -D-maltopyranoside (DDM), 20 mM imidazole). FptX was eluted in buffer A containing 500 mM imidazole. Central fraction of the peak was loaded onto a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 50 mM Hepes pH 7.5, 50 mM NaCl, 0.025 % DDM. FptX was eluted as a single peak and the central fraction was immediately used for EM sample preparation.

Expression and purification of FadD1.

Top10 *E. coli* cells were transformed with the p24-fadD1(StrepII) vector. Cell growth, gene induction and membrane preparation were done as described for FptX. The supernatant was loaded onto a 5-mL StrepTrap HP column (GE Healthcare) equilibrated with buffer A (50 mM Tris-HCl pH 8, 200 mM NaCl, 0.1 % DDM). FadD1 was eluted in buffer A containing 2.5 mM D-Desthiobiotin. Peak fractions were pooled and loaded onto a Superdex 200 10/300 GL (GE Healthcare) column equilibrated with 50 mM Hepes pH 7.5, 50 mM NaCl, 0.025 % DDM. FadD1 was eluted as a single peak and the central fraction was immediately used for EM sample preparation.

Expression and purification of the FadD1-FptX complex.

Top10 *E. coli* cells were transformed with the p33-fptX(His₆) and p24-fadD1(StrepII) vectors. Cell growth, gene induction and membrane preparation were done as described for FptX. The supernatant was loaded onto a 1-mL StrepTrap HP column (GE Healthcare) equilibrated with buffer A (50 mM Tris-HCl pH 8, 50 mM NaCl, 0.1 % DDM). The FadD1-FptX complex was eluted in buffer A supplemented with 2.5 mM

D-Desthiobiotin (Sigma Aldrich) into a 1-mL HisTrap HP column (GE Healthcare). The column was washed in buffer A and the FadD1-FptX complex was eluted in buffer A supplemented with 250 mM imidazole. Central fraction of the peak was then loaded onto a Superdex 200 column 10/300 GL (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 8, 50 mM NaCl, 0.025 % DDM. The FadD1-FptX complex was eluted as a single peak, and the central fraction was immediately used for EM sample preparation.

EM processing of FptX, FadD1 and the FadD1-FptX complex.

Determination of FadD1-FptX complex, FptX and FadD1 proteins structure was made by negative-stain EM. 5 μL of FadD1-FptX complex (28 $\text{ng}\cdot\mu\text{L}^{-1}$), FptX (23 $\text{ng}\cdot\mu\text{L}^{-1}$) and FadD1 (6.25 $\text{ng}\cdot\mu\text{L}^{-1}$) sample was fixed with 0.6 μL glutaraldehyde 1%. The sample was then spotted to 300 meshes Cu grids covered with a carbon film (Euromedex CF300-CU-050). After 60s of absorption, each sample was stained with 2% uranyl acetate. For the FadD1-FptX complex, the sample was washed with one drop of 50 mM Tris pH 8, 50 mM NaCl before staining with 2% uranyl acetate. Images were recorded automatically using the SerialEM software on a FEI Tecnai F20 microscope operating at a voltage of 200 kV and a defocus range of 500 nm, using a Gatan US1000 detector at a nominal magnification of 50,000X, yielding a pixel size of 2.12. A dose rate of 20 electrons per square angstrom per second, and an exposure time of 10s were used. For the FadD1-FptX complex, a total of 58,034 particles were selected from 3,000 independent images and extracted within boxes of 200 pixels using Relion2. For FptX, a total of 38,746 particles were selected from 1,500 independent images and extracted within boxes of 192 pixels using Relion2. For FadD1, a total of 102,978 particles were selected from 1,500 independent images and extracted within boxes of 120 pixels using Relion2. The defocus value was estimated, and the contrast transfer function was corrected using CTF estimation in Relion2. We used two rounds of reference-free 2D class averaging to clean up the selected data set. High-populated classes displaying high-resolution features were conserved during the procedure with a final data set of 30,355 particles for the FadD1-FptX complex, 33,001 particles for FptX and 61,553 particles for FadD1. An initial 3D model was generated in EMAN2. Relion2 auto-refine procedure was used

to obtain a final reconstruction at 33-Å, 26-Å and 17-Å resolution for FadD1-FptX, FptX and FadD1, respectively, after masking and without symmetry constraints. Resolutions are based on the “gold standard” Fourier shell correlation (FSC) (Fig. S3). UCSF Chimera was used to display three-dimensional reconstructions.

Iron uptake assays and cell fractionation.

Cells were cultured overnight in LB medium at 30°C, and afterwards at 30°C overnight in CAA medium. Then, they were diluted in fresh CAA medium and grown at 30°C overnight. Bacteria were washed with 50 mM Tris-HCl at pH 8 and diluted to an OD_{600nm} of 1. The PCH-⁵⁵Fe complex was prepared as previously described [22]. Cells were incubated 30 min with 200 nM PCH-⁵⁵Fe at 30°C. After incubation, cells were centrifuged for 8 min at 6, 700 g to obtain the supernatant as the extracellular medium, and the cell pellet which was resuspend in Tris-sucrose EDTA (Tris-HCl 0.2 M pH 8, EDTA 1 mM, sucrose 20 %). Spheroplasts were obtained by adding 200 µg.mL⁻¹ lysozyme (Euromedex) to the suspension and incubated at 4°C for 1 h. The suspension was centrifuged for 8 min at 6,700 g at 4°C to obtain the spheroplast pellet and the periplasmic fraction (supernatant). The supernatant was ultracentrifuged (120, 000 g, 40 min, 4°C) to clarify the periplasmic fraction. Spheroplast pellet was washed with Tris-HCl 0.2 M pH 8, sucrose 20 %, and resuspend in cold water by vortexing. The suspension was then incubated with benzonase for 1 h at 37°C. The cytoplasmic fraction was isolated by ultracentrifugation (120,000 g, 40 min, 4°C). Pellet was washed twice with cold water and resuspended in Tris-HCl 0.2 M pH 8, sucrose 20 % to obtain the bacterial membranes.

Deletion mutant construction.

For the deletion of both *fadD1* and *fadD2* genes, PCR was used to generate a 500 bp DNA fragment upstream of *fadD2* and a 500 bp DNA fragment downstream of *fadD1*, by using the primer pairs 1280/1281 and 1282/1151, respectively. Each PCR product was linked by overlapping PCR and products were digested with HindIII and EcoRI and cloned into the suicide vector pEXG2, yielding pEXG2Δ*fadD1*Δ*fadD2*.

Deletion in the chromosomal genome of *P. aeruginosa* was generated by transferring the suicide vector from *E. coli* SM10 strains into recipient strains allowing the plasmid to integrate into the chromosome, with selection for gentamicin resistance. A second crossing-over event excising the vector was selected using NaCl-free LB plates supplemented with 5 % sucrose. Deletions were confirmed by sequencing using external primers.

PCH production.

Cells were cultured overnight in LB medium at 37°C, and afterwards at 30°C overnight in iron-deficient CAA medium. They were then diluted to 0.1 OD_{600nm} units in fresh CAA medium and incubated at 30°C for 16 h. The extracellular medium was separated from the cells by centrifugation at 7,700 g for 15 min. The pH of the supernatant was acidified by adding 1 M citric acid and PCH was extracted with dichloromethane. Pyochelin concentration was determined by measuring the absorbance at 320 nm ($\epsilon = 4,300 \text{ L.mol}^{-1}.\text{cm}^{-1}$). Results are expressed as a function of PCH produced (μM) to bacterial growth (OD_{600nm}).

Significance tests

Student's *t* test was used for two-group comparisons using unpaired two-tailed *t* test. Analyses were performed using GraphPad Prism 8.

Results

Identification of FptX interacting partners. FptX is proposed as the inner membrane protein involved in ferri-PCH uptake in *P. aeruginosa* [13-15]. In order to find other proteins involved in this pathway, we looked for FptX-interacting partners by using a bacterial two-hybrid genome wide screening. The pKNT25-fptX bait plasmid was combined with the *P. aeruginosa* PAO1 fragment prey libraries constructed in the pUT18 vector by Houot *et al.*, 2012 [20]. Blue colonies reflecting a protein interaction with FptX were identified, and the DNA sequence inserts contained in the pUT18 derivative vectors were further sequenced.

The prey fragments corresponded either to the 253 first amino acids of the fatty acid coenzyme-A ligase FadD1, or to the 120 first amino acids of PA1316, a putative major facilitator superfamily transporter. Only FadD1 was identified multiple times throughout this screen and the interaction between FadD1 and FptX was validated by cloning the entire proteins in two-hybrid vectors (Fig. 1A). The interaction was also confirmed by co-expressing FadD1 with a StrepII tag and FptX with a His₆ tag at the carboxy terminus in *E. coli*. Total membranes were isolated and solubilized using the detergent DDM. The complex was specifically isolated by a two-step affinity chromatography and the presence of FadD1(StrepII) (63 kDa) and FptX(His₆) (44 kDa) was confirmed by immunoblot analysis (Fig. 1B and C). The overall FptX(His₆) distribution on SDS-PAGE reveals several bands ranging from 44 to 150 kDa, suggesting multimerization of the protein. Altogether, these results revealed a new complex between the inner membrane transporter FptX and the fatty acid coenzyme-A ligase FadD1.

FadD1 is involved in PCH production rather than ferri-PCH uptake. This interaction between FptX and FadD1 must involve a biological role for FadD1 in the PCH pathway. Based on the role of FptX in ferri-PCH uptake [15], we first investigated the possible involvement of FadD1 in iron uptake *via* PCH. *P. aeruginosa* has in its genome two genes encoding closely related enzymes for the fatty acid metabolism, FadD1 and FadD2, which exhibit 60 % identity [23]. We deleted the two *fadD* genes to avoid any functional redundancy. PCH-⁵⁵Fe uptake assays were performed using a *P. aeruginosa* strain unable to produce PVD and PCH ($\Delta pvdF\Delta pchA$) in order to control the PCH concentration and to have no iron uptake by PVD, the other siderophore produced by *P. aeruginosa*. Cells were grown under iron-restricted conditions, harvested and incubated in the presence of 200 nM PCH-⁵⁵Fe. After 30 min incubation, cells were pelleted and the cytoplasmic, periplasmic and membrane fractions isolated by cell fractionation and the radioactivity monitored in each cell compartment (Fig. 2).

In the $\Delta pvdF\Delta pchA$ control strain, most of the radioactivity (53 %) was found in the cytoplasm, 8 % in the periplasm, 18 % associated to the membranes and 21 % still in the extracellular medium (Fig. 2), while for the $\Delta fptX$ mutant most of ⁵⁵Fe was not imported into the bacteria and found in the extracellular medium

(Fig. 2). In the absence of FptX, the ferri-PCH complex cannot enter the cells, which is related to previous data showing that the expression of *fptA*, encoding for the outer membrane transporter, is drastically decreased in an $\Delta fptX$ mutant [24]. Regarding the $\Delta fadD1D2$ mutant, a very similar ^{55}Fe repartition was observed compare to the control strain, indicating that FadD1 plays no role in iron acquisition by PCH. We next investigated the effect of *fadD1D2* mutations on PCH production. WT and the corresponding mutant strains were grown under iron-starvation conditions and PCH was extracted from growth medium and its concentration determined using its characteristic absorbance at 320 nm. A 1.4-, 2.4-, and 4.4-fold decrease in PCH concentration in the growth medium was observed for the $\Delta fadD1D2$, $\Delta fptX$ and $\Delta fptX\Delta fadD1D2$ mutants respectively, compared to the wild type PAO1 strain (Fig. 3), suggesting a role of FadD1 in PCH production and/or secretion.

Biochemical and architectural analysis of FptX, FadD1 and the FadD1-FptX complex.

In contrast to the available X-ray structure of the outer membrane transporter FptA [11], structural data are still lacking for the inner membrane transporter FptX. The determination of the 3D structure of this polytopic inner membrane protein remains very challenging because of the low expression levels of FptX even in overproducing bacterial systems. Consequently, we then investigated the architecture of FptX, FadD1 and the FadD1-FptX complex by negative-stain EM. First, the proteins were produced and purified from *E. coli* membranes and their functional integrity was established by assessing the binding activity to ferri-PCH for FptX and the FadD1-FptX complex, and the fatty acid coenzyme-A ligase activity for FadD1 (Fig. S1). EM data set were collected, and reference-free two-dimensional class revealed characteristic views of the FadD1-FptX complex, FptX and FadD1 (Fig. S2). The EM data set was used to reconstruct a 33-Å, 26-Å and 17-Å resolution three-dimensional (3D) model of the FadD1-FptX complex, FptX and FadD1 proteins, respectively (Fig. 4). FadD1-FptX complex is ~230 Å in height and ~150 Å in diameter. FptX is ~225 Å in height and ~120 Å in diameter, and FadD1 is ~115 Å in height and ~90 Å in diameter. The FptX EM model was then docked into the EM volume of the FadD1-FptX complex, showing that FptX occupies most of the EM envelope of the complex (Fig. 4). However, at this low resolution and according to the small volume of

FadD1, the electron density may not have sufficiently distinctive features for an unambiguous placement of FadD1 in the EM volume complex.

Discussion

The data presented here give new insights in the *P. aeruginosa* PCH siderophore pathway. Previous studies had shown that FptA and FptX are the outer and inner membrane transporters involved in iron uptake by the siderophore PCH [10-15]. Both proteins also play a role in PCH production: in $\Delta fptA$ and $\Delta fptX$ mutants no more PCH-Fe is transported into bacteria, affecting the PCH-mediated signaling cascade, and hence PCH production [14,24].

Here, the bacterial two-hybrid screen represented a powerful tool as it identified for the first time a fatty acid coenzyme-A ligase enzyme, FadD1, as an interacting partner of the inner membrane transporter FptX. FadD1 is described as a fatty acid coenzyme-A ligase involved in fatty acid metabolism in *P. aeruginosa* [23]. To assess the physiological significance of the FadD1-FptX complex, we explored the impact of FadD1 on both ferri-PCH uptake and PCH production. We showed that FadD1 has no impact on iron uptake by PCH but is required for optimum PCH production. FadD1 may play a role in biosynthesis and/or secretion of PCH, but the data do not allow to discriminate between these two biological processes. Our findings suggest a potential acylation of PCH. FadD1 might load a fatty acid chain to PCH, allowing PCH to be anchored to the inner membrane during its synthesis as described previously for PVD, ferrichrome or mycobactin [25-27]. For mycobactin, the fatty acid ligase FadD33 (also known as MbtM) was reported to transfer a lipophilic aliphatic chain to the ϵ -amino group of the lysine mycobactin core [27]. In the case of PVD, the biosynthesis begins with an acylation introducing a myristic or myristoleic group carried out by the first module of the PvdL non-ribosomal peptide synthetase [26]. The presence of a myristic or myristoleic acid group retains the PVD precursor at the membrane during its biosynthesis. For both PVD and mycobactin, the acylation of siderophore promotes plasma membrane localization. Further experiments are still necessary to identify the role of an acylation of PCH by FadD1. Moreover, currently nothing is known concerning the proteins and the molecular mechanisms involved in PCH secretion as well as in the

molecular mechanism allowing iron release from PCH.

FptX was reported to be a homolog of the transporter of rhizobactin RhtX, belonging to the major facilitator superfamily (MFS) transporters [13]. Currently, structure determination of such inner membrane proteins remains a difficult task and presents a challenge for expression and purification. Here, we established for the first time a low-resolution 3D model of FptX, its interacting partner FadD1 and of the FadD1-FptX complex by negative-stain EM. Due to the lack of information concerning the various domains of FptX and the low resolution of the EM reconstruction, it was impossible to clearly define a topology of the complex. Altogether, these results highlight a new actor in the PCH pathway and suggest that this pathway would probably be more complex than what has been described so far. In addition, our efforts to purify and initiate low resolution EM-models pave the way for future high-resolution studies for structure-based drug design to counter antibiotic resistance, one of the biggest public health challenges of our time.

Acknowledgement

Authors acknowledge the Centre National de la Recherche Scientifique (CNRS) for general financial support. BR would like to thank Roche Pharmaceutical Research and Early Development Basel for their financial support via the Roche Postdoctoral Fellowship (RPF) Program. We acknowledge the support and the use of resources of the French Infrastructure for Integrated Structural Biology (FRISBI) ANR-10-INBS-05, "Project ID 52", and of Instruct-ERIC, "PID 2886". Specifically, we would like to thank Christine Ruhlmann and Corinne Crucifix (IGBMC, Strasbourg) for help with EM sample preparation and EM data collection, and Veronique Mallouh (IGBMC, Strasbourg) for help with data processing. We acknowledge Eric Durand (LISM, Marseille) for fruitful EM processing discussion. We acknowledge Christophe Bordi (LISM, Marseille), Laetitia Houot (LISM, Marseille) and Aurélia Battesti (LCB, Marseille) for kindly providing the PAO1 two-hybrid libraries and the two-hybrid vectors, and Laurent Loiseau (LCB, Marseille) for providing pBAD vectors.

Author contributions

BR and IS designed the experiments. BR performed the experiments. GM provided the PCH siderophore. BR wrote the manuscript. IS helped to improve the article. All authors discussed and commented on the manuscript.

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Figure legends

Fig. 1. Interaction between FptX and FadD1. (A) Bacterial two-hybrid assay. The *fadD1* and *fptX* genes were cloned into the two-hybrid pUT18C or pKNT25 vectors and corresponding vectors were co-transformed in DHM1 cells that were spotted on Xgal-IPTG LB agar plates. A blue color reflects interaction between chimeric proteins while white color attests the absence of interaction. RetS was reported to form dimers and is used as a positive control [28]. The experiments were done in triplicate and a representative result is shown. (B) Two-step affinity chromatography. Solubilized bacterial membranes overexpressing FadD1(StrepII) and FptX(His₆) proteins were submitted to a two-step affinity chromatography: loading into a StrepTrap HP column (1), elution with desthiobiotin into a HisTrap HP column (2), and final elution with imidazole (3). (C) Immunoblot analysis of the fractions corresponding to the different chromatography steps using anti-His (α -His) and anti-StrepII (α -StrepII) antibodies.

Fig. 2. ⁵⁵Fe distribution in *P. aeruginosa* cell compartments after incubation with PCH-⁵⁵Fe. Strains were grown in CAA medium and then incubated in the presence of 200 nM PCH-⁵⁵Fe during 30 min. Afterwards, the cells were then pelleted; the extracellular medium, periplasm, cytoplasm and membrane fractions isolated as described in the Materials and methods, and the amount of ⁵⁵Fe present monitored. The results are expressed as the percentage of pmol of ⁵⁵Fe incubated with the cells. The errors bars represent the standard deviation of the mean of three independent experiments.

Fig. 3. Amount of PCH secreted by PAO1 and its corresponding mutants. Cells were grown in CAA medium and PCH was extracted from the growth medium as described in Materials and Methods and its

concentration was determined by measuring the absorbance at 320 nm. The errors bars represent the standard deviation of the mean of six independent experiments. Student's t-tests were performed between data (****P < 0.0001).

Fig. 4. Architecture of the purified proteins. 33-Å 3D reconstruction of the FadD1-FptX complex (A), 26-Å 3D reconstruction of FptX (B) and 17-Å 3D reconstruction of FadD1(C) without symmetry applied. The maps are displayed in different views. (D) Fitting of FptX (orange) in the EM volume of the FadD1-FptX complex (grey) without symmetry applied.

B

Extracellular medium
 Periplasm
 Cytoplasm
 Membranes

Supplementary data

Identification of the fatty acid coenzyme-A ligase FadD1 as an interacting partner of FptX in the *Pseudomonas aeruginosa* pyochelin pathway

Béatrice Roche^{1,2,3*}, Gaëtan L.A. Mislin^{1,2} and Isabelle J. Schalk^{1,2}

¹. CNRS, UMR7242, ESBS, Bld Sébastien Brant, F-67412 Illkirch, France.

². Université de Strasbourg, UMR7242, ESBS, Bld Sébastien Brant, F-67412 Illkirch, France.

³. Present address: Biozentrum, University of Basel, CH-4056 Basel, Switzerland

* = correspondence should be addressed to Béatrice Roche (beatrice.roche@unibas.ch)

Materials and methods

Binding of PCH-⁵⁵Fe.

10 nM of purified FptX or the complex was incubated with various amount of PCH-⁵⁵Fe in 500 µL of 50 mM Hepes pH 7.5, 50 mM NaCl, 0.025 % DDM at room temperature for 2 h. The solution of PCH complexed with ⁵⁵Fe was prepared with a siderophore/iron (mol/mol) ratio of 20:1. Incubation was stopped by filtering the assay mixture over GF/B filters (Whatman, presoaked in 0.1 % polyethyleneimine to reduce the non-specific retention). The filters were then rapidly washed three times with 1 mL of 50 mM Tris pH 8 and counted for radioactivity in a scintillation mixture. The experiment was repeated in the absence of the purified protein. For each PCH-⁵⁵Fe concentration, the non-specific retention was then subtracted from the total radioactivity monitored in the presence of the protein.

Measurement of fatty acyl-CoA synthetase activity of FadD1.

The activity of purified FadD1 was assessed using Ellman's reagent to monitor the consumption of the free thiol group of Co-A due to FadD1-catalyzed acyl-CoA ligation [1]. This colorimetric assay quantifies the

instantaneous free thiol concentration via absorbance readings at 412 nm. A color change is produced upon the binding of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) to the free thiol groups of CoA-SH. Progressive loss of signal at 412 nm is indicative of CoA-SH consumption. Reactions were prepared with 40 µg of purified FadD1 in a reaction buffer containing final concentrations of 150 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 2 mM EDTA, 0.1 % Triton X-100, 5 mM ATP, 0.5 mM co-enzyme A and sodium oleate (0 and 200 µM). To perform the reaction, each mixture was assembled containing all components above (excluding CoASH). The reaction mixture was prewarmed at 37°C for 2 min. The reaction was initiated with the addition of CoASH (final concentration 0.5 mM) that was pre-incubated at 37°C for 2 min, quickly mixed, and incubated at 37°C during the course of the reaction. 70 µL aliquots of the reaction were taken at different intervals and mixed with 600 µL of 0.4 mM DTNB (dissolved in 0.1 M potassium phosphate at pH 8). $A_{412\text{nm}}$ values were acquired after a 5-minute color development period at room temperature. Control reaction mixture lacking protein was also prepared. The previously published extinction coefficient of 13,600 M⁻¹.cm⁻¹ for DTNB was used to compute specific activity [1].

Figure legends

Fig. S1. Purification and biochemical characterization of FptX, FadD1 and the FadD1-FptX complex. (A, D and G) Size-exclusion chromatography analysis of the purified FptX (A), FadD1 (D) and the FadD1-FptX complex (G) (continuous line) on a Superdex 200 10/300 GL. The dead volume is indicated as the V₀. The arrow indicates the position of the fraction corresponding to FptX (A), FadD1 (D) and the FadD1-FptX complex (G). (B and H) Immunoblot analysis of the purified FptX (B) and the FadD1-FptX complex (H) using anti-His (α-His) and anti-StrepII (α-Strep) antibodies. (C and I) Binding of PCH-⁵⁵Fe to purified FptX (C) and FadD1-FptX complex (I). Purified proteins were incubated with 10, 100 or 200 nM of PCH-⁵⁵Fe complex. The non-specific retention was subtracted from the total of radioactivity monitored in the presence of the proteins. (E) SDS-PAGE of the purified FadD1 protein analyzed by Instant blue™ staining. (F) FadD1 enzymatic assay was conducted using DTNB to monitor CoA-SH consumption at 412 nm with increasing

amount of sodium oleate as substrate. A loss of signal at 412 nm is indicative of FadD1 activity. The errors bars represent the standard deviation of the mean of three independent experiments.

Fig. S2. Representative electron micrograph showing FadD1-FptX (A), FptX (D) and FadD1 (G) particles present in several orientations. Scale bar, (A and G) 50 nm, (D) 20nm. Representative views of FadD1-FptX (B), FptX (E) and FadD1 (H). Typical class averages of FadD1-FptX (C), FptX (F) and FadD1 (I).

Fig. S3. FSC curves of FptX (A), the FadD1-FptX complex (B) and FadD1 (C) reconstruction without symmetry applied. The FSC curves were calculated in Relion using the masked reconstruction of FptX, the FadD1-FptX complex and FadD1, respectively.

79 **Table S1.** Strains and plasmids used in this study.

Strains		Characteristics	Reference
<i>Escherichia coli</i>	ID collection		
TOP10	2	F ⁻ , <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>), φ80 <i>lacZ</i> , Δ <i>M15</i> , Δ <i>lacX74</i> , <i>recA1</i> , <i>araD139</i> , Δ(<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> , <i>endA1</i> , <i>mup</i>	Invitrogen
<i>E. coli</i> DHM1	281	F ⁻ , <i>cya</i> ⁻ , <i>recA1</i> , <i>endA</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> , <i>spoT1</i> , <i>rfbD1</i> , <i>cya-854</i>	[2]
<i>Pseudomonas aeruginosa</i>			
PAO1	43	Wild-type strain	[3]
Δ <i>fptX</i>	PAS218	PAO1; <i>fptX</i> chromosomally deleted	[4]
Δ <i>fadD1D2</i>	PAS431	PAO1; <i>fadD1</i> and <i>fadD2</i> chromosomally deleted	This study
Δ <i>fptX</i> Δ <i>fadD1D2</i>	PAS410	PAO1; <i>fadD1</i> , <i>fadD2</i> and <i>fptX</i> chromosomally deleted	This study
Δ <i>pvdF</i> Δ <i>pchA</i>	PAS213	PAO1; <i>pvdF</i> and <i>pchA</i> chromosomally deleted	[4]
Δ <i>pvdF</i> Δ <i>pchA</i> Δ <i>fptX</i>	PAS273	PAO1; <i>pvdF</i> , <i>pchA</i> and <i>fptX</i> chromosomally deleted	[4]
Δ <i>pvdF</i> Δ <i>pchA</i> Δ <i>fadD1D2</i>	PAS428	PAO1; <i>pvdF</i> , <i>pchA</i> , <i>fadD1</i> and <i>fadD2</i> chromosomally deleted	This study
Δ <i>pvdF</i> Δ <i>pchA</i> Δ <i>fptX</i> Δ <i>fadD1D2</i>	PAS413	PAO1; <i>pvdF</i> , <i>pchA</i> , <i>fptX</i> , <i>fadD1</i> and <i>fadD2</i> chromosomally deleted	This study
Plasmids	ID collection	Characteristics	Reference
pKNT25	264	P15 origin, Plac, C-terminal T25 cyclase fragment, Kan ^R	[2]
pUT18C	265	ColE1 origin, Plac, N-terminal T18 cyclase fragment, Ap ^R	[2]
pBAD33	270	pBAD33 plasmid, Cm ^R	[5]
pBAD24	271	pBAD24 plasmid, Ap ^R	[5]
pUT18CPAO1lib	294	pUT18C containing PAO1 genomic library as a C-terminal T18 fusion	[6]
pUT18PAO1lib	295	pUT18C containing PAO1 genomic library as an N-terminal T18 fusion	[6]
pUT18+1PAO1lib	296	pUT18+1 containing PAO1 genomic library as an N-terminal T18 fusion	[6]
pUT18+2PAO1lib	297	pUT18+2 containing PAO1 genomic library as an N-terminal T18 fusion	[6]
pKNT25-fptX	268	pKNT25 plasmid containing <i>fptX-cyaAT25</i> fusion	This study
pUT18C-fadD1	293	pUT18C plasmid containing <i>cyaAT18-fadD1</i> fusion	This study
p33-fptX(His ₆)	272	pBAD33 plasmid expressing fptX Cter-fused to 6His tag	This study
p24-fadD1(StreptII)	311	pBAD24 plasmid expressing fadD1 Cter-fused to StreptII tag	This study
pEXG2 delfadD1D2	330	suicide vector for deleting <i>fadD1</i> and <i>fadD2</i>	This study

80

81 **Table S2.** Oligonucleotides used in this study.

Name	ID collection	Sequence (5' → 3')
pKT25-fptXXbaI	951	CCGGTCTAGAGATGCTTGAGCTGTACCGCCACCGC
pKT25-fptXXpnl	952	CCGGGGTACCTCAGGCCTCTCCGCCCGCCCTT
pKT25-fadD1UP	1037	CCGGTCTAGAGATGATCGAAAACCTCTGGAAGGAC
pKT25-fadD1DO	1038	CCGGGGTACCCGCTTCTGGCCCGCTTCTTCAGCTC
fptX-UPeCoRI	970	CCGGAATTCAAGGAGATATACATATGCTTGAGCTGTACCGCCACCGC
fptX-DOHindIII	969	CCGGAAGCTTTCAGGCCTCTCCGCCCGCCCTT
fadD1-UPp24	1157	CCGGCCATGGCCATCGAAAACCTCTGGAAGGACAAG
fadD1-DOSStrepCter	1158	CCGGAAGCTTTCATTTTTCGAAGTGC GGTTGGCTCCAGCTAGCCTTCTGGCCCGCTTCTCAGCT
DelfadD1-500UPHindIII	1150	CCGGAAGCTTGCTTCAATGTCTACCCCAACGAGATCGAAG
DelfadD1-500DO EcoRI	1151	CCGGGAATTCGGGAAGGAGTCTGGTCGGTCACTAGGCTT
Del fadD1-ATG rev	1152	TGGGTACTTGTCTTCCAGAAGTTTTTCGATCAT
Del fadD1-STOP for	1153	TCTGGAAGGACAAGTACCCACTGCGCGACGAAGAGCTGAAGAAA
Del fadD2-500UPHindIII	1280	CCGGAAGCTTTTGCCCCAGCCGCCCTGGTCGGCGAAGTGG
Del ATG-fadD2 rev	1281	TTCAAGTTGCATTTATCTTGTCTCTTACCTG
Del STOP-fadD1fadD2 for	1282	GTAAGAGGACAAGAATAAATGCAACCTGAAAAGCGGGCCAGAAGTAAGTCCGC

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D **E** **F** **G** **H** **I**