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The pathogen *Pseudomonas aeruginosa* optimizes the production of the siderophore pyochelin upon environmental challenges†

Olivier Cunrath,‡§[ab] Gwenaëlle Graulier,§[ab] Ana Carballido-Lopez,[ab] Julien Pérard,[c] Anne Forster,[ab] Valérie A. Geoffroy,[ab] Pamela Saint Auguste,[d] Dirk Bumann,[d] Gaetan L. A. Mislin,[ab] Isabelle Michaud-Soret,[c] Isabelle J. Schalk[ab] and Pierre Fechter*[ab]

Siderophores are iron chelators produced by bacteria to access iron, an essential nutrient. The pathogen *Pseudomonas aeruginosa* produces two siderophores, pyoverdine and pyochelin, the former with a high affinity for iron and the latter with a lower affinity. Furthermore, the production of both siderophores involves a positive auto-regulatory loop: the presence of the ferri-siderophore complex is essential for their large production. Since pyochelin has a lower affinity for iron it was hard to consider the role of pyochelin in drastic competitive environments where the host or the environmental microbiota produce strong iron chelators and may inhibit iron chelation by pyochelin. We showed here that the pyochelin pathway overcomes this difficulty through a more complex regulating mechanism for pyochelin production than previously described. Indeed, in the absence of pyoverdine, and thus higher difficulty to access iron, the bacteria are able to produce pyochelin independently of the presence of ferri-pyochelin. The regulation of the pyochelin pathway appeared to be more complex than expected with a more intricate tuning between repression and activation. Consequently, when the bacteria cannot produce pyoverdine they are able to produce pyochelin even in the presence of strong iron chelators. Such results support a more complex and varied role for this siderophore than previously described, and complexify the battle for iron during *P. aeruginosa* infection.

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Significance to metallomics

Iron is a key element for life; however it is poorly available at physiological pH. To import iron, bacteria evolved siderophores, small molecules secreted by bacteria to chelate iron and trigger its import. The pathogen *Pseudomonas aeruginosa* produces two siderophores, pyoverdine and pyochelin. Their production is at the heart of bacterial survival, especially during infections where the host deprives the bacteria of iron. Here we provide insights into the regulation of pyochelin production, which help to characterize its role and importance, and provide further information on the battle for iron during an infection.

Introduction

Iron is absolutely required for the growth of most living organisms, including bacteria, in which it is a cofactor for many enzymes involved in fundamental biological processes. However, iron availability in various aerobic environments is severely limited: iron(II) is rapidly oxidized to iron(III) in the presence of oxygen and precipitates. For bacteria, one of the more commonly used strategies to acquire iron involves the synthesis of siderophores and their release into the environment in order to chelate iron.[1,2] Siderophores chelate iron(III) in the extra-cellular environment, and the resulting ferri-siderophore complex is then actively transported back into the cell. During an infection, a battle for iron is engaged between the host and the bacteria resulting in an even lower bioavailability of iron. A nutritional immune response is set up by the host to deprive bacteria of nutrients like iron,[3,4] mainly through the production of different iron chelators like transferrin

[a] *Université de Strasbourg, UMR7242, ESBS, Bld Sébastien Brant, F-67413 Illkirch, Strasbourg, France. E-mail: P.Fechter@unistra.fr*
[b] *CNRS, UMR7242, ESBS, Bld Sébastien Brant, F-67413 Illkirch, Strasbourg, France*
[c] *Université de Grenoble Alpes, CNRS, CEA, IRIG, Laboratoire de Chimie et Biologie des Métaux, 38000, Grenoble, France*
[d] *Focal Area Infection Biology, Biozentrum, University of Basel, Basel, Switzerland*
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‡ Department of Zoology and Department of Biochemistry, University of Oxford, Oxford, UK.
§ Co-first author.

or lipocalin. As counter measures, certain bacteria can produce different types of siderophores, fine-tune their production in this adverse environment, or hijack host heme[5] and siderophores produced by other organisms.[5,6]

P. aeruginosa is an opportunistic pathogen involved in different nosocomial infections and/or in severe infections in patients suffering from cystic fibrosis or from major burns. This pathogen produces two major siderophores, pyoverdine (PVD) and pyochelin (PCH). PVD chelates ferric iron with a higher affinity (10^{32} M^{-1})[7] than pyochelin (10^{28} M^{-2}).[8] Since PCH has a lower affinity for ferric iron, it has often been considered as a secondary siderophore, produced mainly to spare PVD, and its role during infections remains unclear.[9]

PVD and PCH production by *P. aeruginosa* is highly regulated by iron bioavailability. The production of both siderophores involves so-called positive autoregulation loops in which PVD–Fe and PCH–Fe are required for their own synthesis (Fig. 1).

The expression of the PVD pathway is induced by two Sigma factors, PvdS and FpvI (reviewed by Llamas and collaborators[11]). While FpvI only induces the expression of the outer membrane transporter, FpvA, PvdS induces the expression of most other proteins of the pathway. Both Sigma factors are bound by an anti-Sigma factor, FpvR, which keeps the two Sigma factors inactive.[11,12] This regulating mechanism also needs that PVD chelates iron, and that the formed PVD–Fe complex is imported back into the periplasm *via* the specific outer membrane transporter, FpvA.[13] This uptake process induces a change of conformation of the N-terminal end of FpvA which interacts with the anti-Sigma factor FpvR.[14,15] As a consequence, PvdS and FpvI are released into the cytoplasm, and the expression of the PVD pathway is induced[10–16] (Fig. 1). Nevertheless, FpvR does not bind the whole pool of PvdS and FpvI, and a part of this pool is still found free in the cytoplasm.[12,17,18] This 'evasion' mechanism allows the synthesis of the first PVD molecules required to activate the autoregulatory loop.[11,12]

The PCH pathway is activated by a transcription factor, PchR, which belongs to the AraC-type transcription regulator family: to be active, it requires a cofactor, the PCH–Fe complex.[19] Once PCH is synthesized and excreted into the extra-cellular environment, PCH chelates iron (Fig. 1). The PCH–Fe complex is imported back into the periplasm *via* the specific outer membrane transporter, FptA,[20] and into the cytoplasm through the permease FptX.[21] In the cytoplasm, the PCH–Fe complex binds and activates the transcription regulator PchR to trigger transcription of the different genes involved

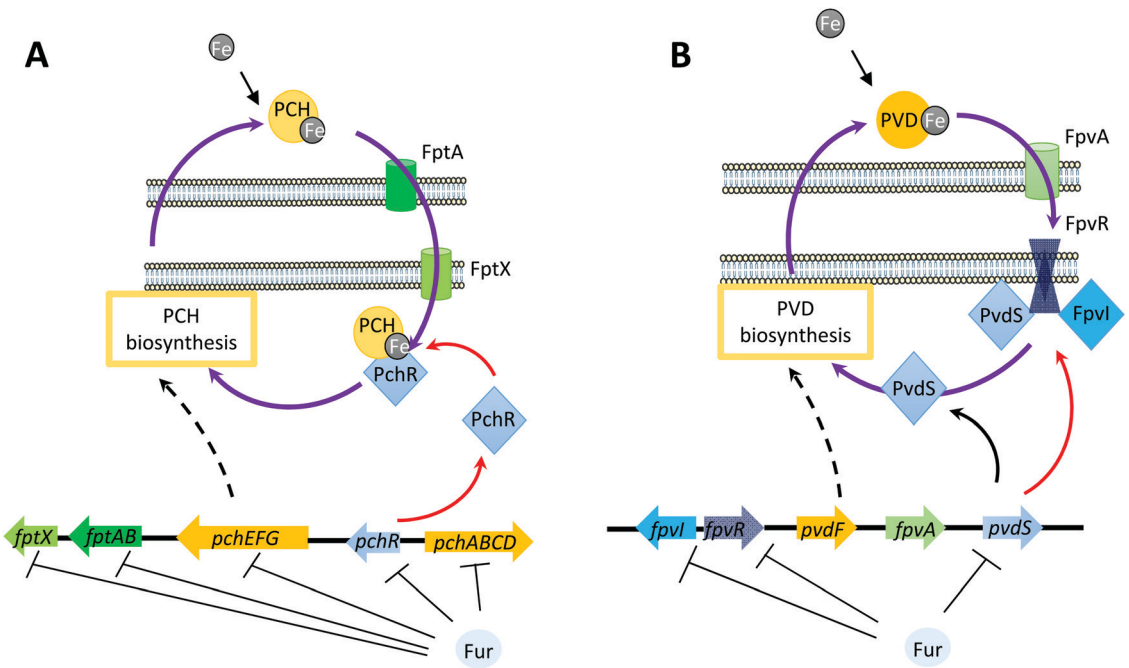

Fig. 1 Schemes of the auto-regulation loops involved in *P. aeruginosa* PCH and PVD pathways. (A) Ferri-pyochelin (PCH–Fe) import system. Once PCH is produced and excreted into the environment, it chelates iron, and PCH–Fe is imported back into the cytoplasm *via* the outer membrane transporter FptA and the permease FptX. In the cytoplasm, the PCH-Fe complex binds and activates the transcription regulator PchR, which activates the expression of the genes of the PCH pathway and consequently the production of PCH. This mechanism sets the PCH autoregulation loop. All PchR proteins are believed to respond to this regulation loop (red arrow). The positive auto-regulation loop is represented by a purple arrow. (B) Ferri-pyoverdine (PVD-Fe) import system. Two Sigma factors PvdS and FpvI induce the expression of most proteins from the system. Once excreted into the environment, PVD chelates iron. PVD-Fe uptake *via* the outer membrane transporter FpvA induces the release of PvdS and FpvI from FpvR, which was keeping the two transcription factors inactive. Consequently the expression of the PVD pathway is activated. This mechanism sets the PVD autoregulation loop. A red arrow indicates the PvdS pool of proteins that are subjected to this regulatory loop. Nevertheless, a part of the PvdS pool does not bind FpvR, and directly activates the expression of genes from the PVD pathway, so that PVD can be produced even in the absence of PVD-Fe. The positive auto-regulation loop is represented by a purple arrow.

in the PCH pathway:[19,22] the PCH–Fe complex is required for its own synthesis (Fig. 1). In contrast to the PVD pathway, no 'evasion' mechanism has been described so far for the PCH pathway. Thus it is not known how the first PCH molecules are produced, and whether PCH could be produced in conditions in which no PCH–Fe is present.

In replete conditions, *i.e.* enough iron is present in the medium, the expression of the two pathways is repressed by the iron-responsive global transcription regulator Fur.[23] *P. aeruginosa* Fur is part of the tetramer subfamily of Fur proteins and at high iron concentrations, the Fur tetramer is activated by Fe^{2+}, which splits into dimers upon DNA interaction and represses the expression of both PVD and PCH pathways.[24,25] Fur directly binds the promoter of the transcription regulators of the two pathways (*pvd*S, *fpv*I, *fpv*R and *pch*R) and inhibits their production.[26,27] Fur may as well bind the promoter of other genes of the PCH pathway, but not of the PVD pathway.[19,28]

In the present work, we investigated whether the expression of the PCH pathway can evade the requirement of PCH–Fe, as it is the case for the PVD pathway. We showed that the expression of the PCH pathway can indeed evade the requirement of PCH–Fe only when PVD is not produced anymore, in a double mutant Δ*pvd*FΔ*pch*A or in a Δ*pvd*S mutant, and in the simultaneous presence of iron competitors. We detailed also the regulation of the PCH pathway expression, and discussed the significance of these results.

Materials and methods

Strains used, growth conditions and chemicals

P. aeruginosa and *Escherichia coli* strains and plasmids used in this study are listed in Table S1 (ESI†). Primers used are listed in Table S2 (ESI†). *E. coli* strains were routinely grown in LB (Difco) at 37 °C. *P. aeruginosa* strains were first grown overnight at 30 °C in LB broth and were then washed, re-suspended and cultured overnight at 30 °C in CAA (casamino acids, Becton Dickinson, 5 g L^{-1}; K_2HPO_4, Merck, 1.4 g L^{-1}; $MgSO_4$, Merck, 0.25 g L^{-1}) or DMEM (Gibco)/Fetal Bovine Serum (Gibco). When required, gentamycin (30 μg mL^{-1}) was added to *P. aeruginosa* cultures. The cells are then pelleted by centrifugation and re-suspended in fresh CAA medium, and the resulting suspension was diluted to the $OD_{600\,nm}$ required. The range of $OD_{600\,nm}$ of the cultures, from which normalized values are obtained, are given in Table S3 (ESI†). Desferrioxamine was purchased as a mesylate salt from Sigma, and deferasirox was synthesised as described by Steinhauser and collaborators[29] (purity checked by mass spectrometry).

Reporter plasmid construction

All enzymes for DNA manipulation were purchased from Fermentas and were used in accordance with the manufacturer's instructions. *E. coli* strain TOP10 (Invitrogen) was used as the host strain for all plasmids. The DNA fragments from *P. aeruginosa* used for cloning were amplified from the genomic DNA with Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific). The primers used are listed in Table S2 (ESI†).

For the construction of transcriptional reporter plasmids, the promoters of the genes of interest were amplified from the chromosomal DNA of *P. aeruginosa* PAO1 by PCR with specific primers (Table S2, ESI†) allowing an overlapping with a second PCR fragment encompassing the open reading frame of m*cherry*. A second PCR was generated using the two first PCR fragments as a template to obtain the transcriptional reporter fragment. This fragment was trimmed by digestion with *Eco*RI and *Hind*III or *Bam*HI and inserted between the sites for these enzymes in pSEVA631 (http://seva.cnb.csic.es) and bacteria were transformed with this vector.

CAS assay

4.4 mg of sulfosalicylic acid was added to 5 mL of CAS solution (0.6 mM HDTMA; 0.14 mM CAS; 10 μM $FeCl_3$; 1 M piperazine, pH 5.6). 100 μL of this solution was dispensed into 96-well plates and 100 μL of the molecule to be tested was added. The molecules tested were dissolved in MeOH at a concentration of 200 μM. After 3 h of incubation at room temperature, the $OD_{630\,nm}$ was measured. The reddish solution (without competitor) turned blue when the competitor hijacked iron. The mobilization capacity was estimated as the ratio between the absorbance of the well in which the molecule is added and the absorbance of the control well (100 μL MeOH added). Low values mean high competition. These experiments were repeated 3 times.

Real-time quantification of fluorescence intensity

The cells were cultured overnight in CAA medium, pelleted by centrifugation, re-suspended in fresh CAA medium, and the resulting suspension was diluted to obtain an $OD_{600\,nm}$ of 0.01 units. 200 μL of suspension per well was dispensed into a 96-well plate (Greiner, U-bottomed microplate). The plate was incubated at 30 °C, with shaking, in a Tecan microplate reader (Infinite M200, Tecan) for measurements of $OD_{600\,nm}$ and mCherry (excitation/emission wavelengths: 570 nm/610 nm) fluorescence at 30 min intervals, for 24 h. When required, iron (500 nM or 5 μM) or gentamycin (30 μg mL^{-1}) was added. When using reporter plasmids carrying the mcherry ORF, control experiments were conducted with a plasmid carrying the mcherry ORF without any promoter. The results were obtained from three biological replicates. Values at 20 h of growth were taken, and used for histograms. *P*-Values were < 0.05.

Siderophore production

Overnight cultures of strains grown in DMEM or CAA medium were pelleted, re-suspended and diluted in fresh medium to obtain an $OD_{600\,nm}$ of 0.1. The cells were then incubated, with vigorous shaking, at 30 °C for 8 h. The $OD_{600\,nm}$ was taken to measure cell growth. The $OD_{400\,nm}$ was also taken from 1 mL of supernatant to measure the production of pyoverdine (ε_{pvd} = 19 000 M^{-1} cm^{-1}). Pyochelin was then extracted twice from 1 mL of supernatant (acidified with 50 μL of 1 M citric acid) with 500 μL of ethyl acetate. The absorbance at 320 nm was taken, and the pyochelin concentration was calculated

(ε_{pch} = 4300 M^{-1} cm^{-1}). As a control, we further compared the absorbance of PVD/PCH purified from our different strains with that of a PVD or PCH-deficient strain, to avoid any interference with other components from the media. These extractions were carried out three times.

Quantitative real-time PCR

Specific gene expression was measured by RT-qPCR, as previously described.[30] Briefly, overnight cultures of strains grown in LB or CAA medium were pelleted, re-suspended and diluted in fresh medium to obtain an $OD_{600\,nm}$ of 0.1 units. RNA was purified as described previously.[31] We then reverse transcribed 1 μg of total RNA using a high-capacity RNA to-cDNA Kit, in accordance with the manufacturer's instructions (Applied Biosystems). The amount of specific complementary DNA was assessed using a StepOne Plus instrument (Applied Biosystems) with Power Sybr Green PCR Master Mix (Applied Biosystems) and the appropriate primers (Table S2, ESI†). The housekeeping gene *uvr*D was used for normalization. The results, obtained from at least three biological replicates, are expressed as a ratio (fold change) relative to the reference conditions. *P*-Values were <0.05.

Iron uptake

$^{55}FeCl_3$ was obtained from PerkinElmer Life and Analytical Sciences (Billerica, MA, USA), in solution, at a concentration of 71.1 mM, with a specific activity of 10.18 Ci g^{-1}. Siderophore-^{55}Fe complexes were prepared at a ^{55}Fe concentration of 20 μM, with a siderophore : iron (mol/mol) ratio of 20 : 1. Iron uptake assays were carried out three times, as previously described.[32]

PVD purification

1 mL of overnight LB culture of wt *P. aeruginosa* (ATCC 15692) was centrifuged (5 min at 9871 g). The pellet was inoculated in succinate medium, and incubated at 30 °C for 24 h with shaking (220 rpm). The pellet, washed twice with 1 mL of sterile succinate medium, was suspended in 1 mL of sterile succinate medium and inoculated in 15 mL of sterile succinate medium and incubated at 30 °C under shaking conditions (220 rpm). After 24 h of growth, 7.5 mL of this culture was transferred into an Erlenmeyer flask containing 1 L of sterile succinate medium. After 24 h of incubation at 30 °C under shaking conditions (220 rpm), the culture was centrifuged (2664*g* for 40 min). The supernatant was filtered twice using glass microfiber filters (Whatman, GF/C) and once through a nitrocellulose filter (0.45 μm porosity). The amount of pyoverdine present in the solution was estimated by measuring the absorbance at 400 nm. The siderophore containing supernatant was loaded, after acidification (pH 6.0), on a XAD-4 column, washed with two volumes of purified water and eluted with one volume of 50% ethanol. The eluate was concentrated under vacuum on a rotary evaporator and lyophilized.

Electrophoretic mobility shift assay (EMSA)

EMSA experiments were performed as previously described.[24] DNA radiolabelling was performed by incubating 20–30 nM DNA for 30 min at 37 °C in the presence of 1 unit of T4 polynucleotide kinase (NEB) and 0.5 μL of gamma ATP at 1 mCi $mmol^{-1}$. Labelled DNA was diluted 10 times in binding buffer (20 mM bis-tris Propane pH 8.5, 100 mM KCl, 3 mM $MgCl_2$, 10 μM $MnCl_2$, 5% v/v glycerol and 0.01% Triton X-100), desalted on a G25 mini spin column and stored at −20 °C. EMSA was performed with 200–250 pM of freshly prepared radiolabelled DNA incubated for 30 min at 25 °C with different concentrations of protein in a binding buffer. After 30 min of incubation at room temperature, 10 μL of each sample was loaded on an 8% polyacrylamide (29/1) gel in TA buffer (40 mM Tris acetate pH 8.2) with 10% of glycerol supplemented with 10 μM of appropriate metal or in TAE buffer for EDTA treatment (40 mM Tris acetate pH 8.2 and 2 mM EDTA). The gel was pre-run for 30 min at 100 V in TA buffer supplemented with 100 μM of appropriate metal or in TAE buffer. Mobility shifts were revealed by exposing the gels (2 h to 12 h at RT) on a storage phosphor screen (GE Healthcare) and quantified using a cyclone phosphoimager (PerkinElmer). The results were reproduced three times.

Proteomics analysis

Bacteria were grown in CAA medium as described above. After the first overnight culture in CAA medium, the bacteria were diluted in 10 mL of CAA to an $OD_{600\,nm}$ of 0.1 and incubated for 8 h at 30 °C. For the digestion and cleanup steps, the same strategy was used as previously described.[33] For the shotgun proteomics assays, 1 μg of peptides of each sample was subjected to LC-MS (liquid chromatography-mass spectrometry) analysis using the same approach as previously described.[33]

Results and discussion

PCH–Fe is required for PCH synthesis

PchR is an AraC-type of transcription factor, which in most cases requires a co-factor. A previous study showed through *in vitro* studies that PCH–Fe, and not PCH alone, could be the co-factor.[19] As a consequence, as for PVD, the Ferri-siderophore would be required for its own synthesis. We confirmed these *in vitro* results by measuring the level of transcription of genes from the PCH pathway in two mutants of this pathway. The first, Δ*pch*A,[21] is unable to synthesize PCH, due to the deletion of *pchA*, a gene encoding an enzyme involved in PCH biosynthesis, Fig. 1 and Fig. S1 (ESI†). The second, Δ*fpt*A,[21] is unable to import PCH–Fe, due to the deletion of *fpt*A, a gene encoding the outer membrane transporter responsible for the import of PCH–Fe. We measured by qRT-PCR the transcription of the mRNAs encoding for PchE, an enzyme involved in PCH synthesis, FptA and FptX, the two transporters involved in the import of the PCH–Fe complex, and the transcription factor *pch*R in the strains wt PAO1, Δ*pch*A, and Δ*fpt*A, after 8 h of growth in CAA, a strongly iron-depleted medium.[34] The transcription of the genes from the PCH pathway was repressed in the strain Δ*pch*A (Fig. 2), highlighting the importance of PCH in the induction of its own synthesis. The repression of the

transcription of the genes from the PCH pathway in the Δ*fpt*A mutant (Fig. 2) further showed that not only the siderophore, but also the ferri-siderophore is required for the activation of the PCH pathway expression. These *in vivo* results confirm those obtained *in vitro* by Michel *et al.* (2005), and clearly establish an auto-regulation of the PCH production through PCH–Fe.

The genes of the PCH pathway are transcribed in a double Δ*pvd*FΔ*pch*A mutant

To investigate the regulation pattern of the PCH pathway genes, we first measured by qRT-PCR the level of transcription of genes of the PVD and PCH pathway in the strains PAO1 WT, Δ*pvd*F[31] (a strain unable to produce PVD, due to the deletion of *pvdF*, a gene encoding an enzyme involved in PVD biosynthesis, Fig. 1 and Fig. S1, ESI†) and Δ*pch*A,[21] (a strain unable to produce PCH, due to the deletion of *pchA*, a gene encoding an enzyme involved in PCH biosynthesis, Fig. 1 and Fig. S1, ESI†) after 8 h of growth in CAA, a strongly iron-depleted medium.[34]

For the PVD pathway, we have chosen to follow the transcription of the mRNAs encoding for *pvd*A and *pvd*J, involved in PVD biosynthesis, *fpv*A, involved in the import of PVD–Fe across the outer membrane, as well as the sigma/anti-sigma factors *pvd*S, *fpv*I, and *fpv*R regulating the expression of the proteins of the PVD pathway. For the PCH pathway, the expression of the mRNAs encoding for *pch*E, *fpt*X, *fpt*A, and *pch*R was monitored. The housekeeping gene *uvr*D was used for normalization.

In a strain deprived of PVD (Δ*pvd*F), the transcription of the different genes from the PVD and PCH pathway was weakly affected (<3 fold changes) when grown in CAA (Fig. 2A and B). These results are in line with the literature and an 'evasion' mechanism allowing a basal production of PVD in the absence of PVD–Fe.[11,12] Indeed, the induction of gene expression by PVD–Fe through the auto-regulatory loop (Fig. 1) requires cells to secrete a basal level of PVD to switch on the autoregulation loop of the PVD pathway. In CAA, it even appears that the expression of the PVD pathways in the Δ*pvd*F strain is almost similar to that in WT PAO1, suggesting that the production of PVD could be far beyond 'basal' production, even in the absence of PVD–Fe uptake. This production of PVD in the absence of PVD–Fe may thus help to switch on the autoregulatory loop, and also to keep some production of PVD, even when the bacteria faces serious difficulties in PVD–Fe import.

In a strain deprived of PCH, the PVD pathway is expressed as in wt PAO1, while the genes from the PCH pathway were only poorly transcribed in the absence of PCH production (>100 fold difference) (Fig. 2A and B), except that of *pch*R, whose transcription is only regulated by Fur, and not stimulated by the PCH–Fe complex. Such results are in agreement with previous studies showing that PCH–Fe induces the expression of the pathway.[21,22]

We then used a double mutant Δ*pvd*FΔ*pch*A, deficient in PVD and PCH production (Fig. S1, ESI†). We also measured the level of mRNA expression of both pathways and surprisingly, the genes from both PVD and PCH pathways were still strongly transcribed. Only weak differences of expression of these mRNAs (<3 fold changes compared to WT PAO1, Fig. 2A and B), in the double mutant Δ*pvd*FΔ*pch*A compared to the WT PAO1, were observed.

Our results showed that, as for the PVD autoregulation loop, there is an 'evasion' mechanism that would allow PCH synthesis even in the absence of PCH–Fe. This evasion is switched on only when the bacteria face strong difficulties in iron uptake (here the absence of both PVD–Fe and PCH–Fe), and could help supplying bacteria with iron in these conditions. It also suggests that the regulation of PCH production is more complex than expected.

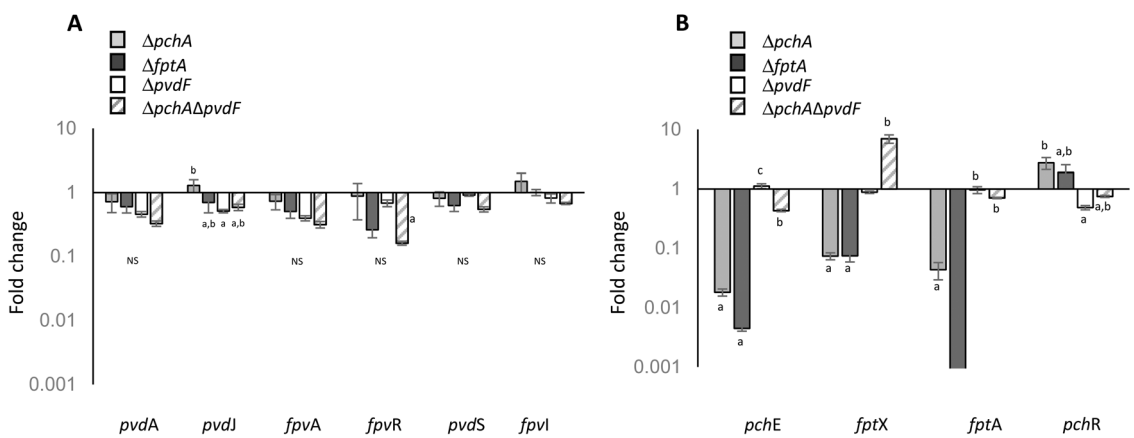

Fig. 2 Transcription of genes from the PVD and PCH pathway in WT PAO1 and in isogenic mutants. (A) Analysis of changes in the transcription of different genes from the PVD pathway (*pvd*A, *pvd*J, *fpv*A, *pvd*S, *fpv*R, and *fpv*I), and (B) from the PCH pathway (*pch*E, *fpt*X, *fpt*A, and *pch*R). Reverse transcription qPCR was performed on *P. aeruginosa* WT PAO1 and its isogenic mutants Δ*pch*A (unable to produce PCH), Δ*fpt*A (unable to import PCH-Fe), Δ*pvd*F (unable to produce PVD), and Δ*pvd*FΔ*pch*A (unable to produce both siderophores), grown in CAA medium. The results are given as a ratio between the values obtained for the mutant and those obtained for the WT PAO1 strain, on a logarithmic scale. Bars with the same letter are not significantly different ($p > 0.05$, one-way ANOVA, three biological replicates).

The transcription of the genes of the PCH pathway in the double Δ*pvd*FΔ*pch*A mutant depends on PchR

To understand how the PCH pathway expression is restored in the Δ*pvdF*Δ*pchA* strain, we checked whether Fur could be involved in the phenotype, and then whether PchR or another transcription factor was involved in the phenotype. In a previsous study, we have shown that in the Δ*pch*A mutant, the iron content was only faintly decreased compared to wt, whereas in the double mutant Δ*pch*AΔ*pvd*F, the iron content was almost two-fold lower.[34] Would this decrease in iron concentration affect Fur activity and thus the activation of the PchR pathway? To verify this hypothesis, we compared the proteome of the double mutant Δ*pch*AΔ*pvd*F and the wt PAO1, to check whether some genes usually repressed by Fur could be upregulated in this context. Nevertheless, among the genes up-regulated in the double mutant Δ*pch*AΔ*pvd*F, we did not find genes already assigned as Fur-dependent (Table S4, ESI†). To study the second hypothesis (involvement of PchR), we created a triple mutant Δ*pvd*FΔ*pch*AΔ*pch*R by deleting *pvdF*, *pchA* and *pchR*. In this mutant grown in CAA, the genes encoding for FptX, PchE, and PchR (as a control) belonging to the PCH pathway were poorly transcribed (Fig. S2, ESI†). These results strongly suggest that the production of the proteins of the PCH pathway in the double mutant Δ*pvd*FΔ*pch*A depends on PchR even if no PCH is produced and consequently no PCH–Fe can interact with PchR.

To confirm that PchR induced directly the expression of the genes from the PCH pathway in the Δ*pvdF*Δ*pchA* strain, we checked whether the sequence recognized by PchR (PchR box, see Fig. 3A and 4C) of promoters from the PCH pathway genes was involved in their transcription in the double mutant Δ*pvdF*Δ*pchA*. Two genes were specifically followed up, *pch*D, which encodes an enzyme involved in the biosynthesis of PCH, and *fpt*A, which is involved in the transport of PCH–Fe in the periplasm. Four transcriptional fusion plasmids were created, encoding the m*cherry* ORF fused to the promoter of *pch*D or *fpt*A, that contained the potential determinants for recognition by PchR and Fur [p(*pch*DFurPchR) and p(*fpt*AFurPchR) plasmids] or only by Fur [p(*pch*DFur) and p(*fpt*AFur) plasmids] as schematised in Fig. 3A. wt PAO1, the double mutant Δ*pvd*FΔ*pch*A (no PVD and PCH synthesis), but also Δ*pch*A (no PCH synthesis) and Δ*pch*R (no PCH synthesis and no PchR expression) were transformed with these plasmids, and mCherry production was measured after 20 h of growth in CAA. mCherry produced from the plasmid encoding its ORF fused to the promoter containing the PchR boxes [p(*pch*DFurPchR) and p(*fpt*AFurPchR)] was similar in WT PAO1 and in the double mutant Δ*pvd*FΔ*pch*A (or even greater in the latter), much weaker in the Δ*pch*A mutant and only faintly expressed in the Δ*pch*R mutant (Fig. 3B). The production of mCherry from the plasmids in which no PchR box was present [p(*pch*DFur) and p(*fpt*AFur)] was always below the control experiment and was thus not represented in Fig. 3B.

It was previously shown that PCH–Fe was important to activate PchR and trigger the expression of the PCH pathway.[19,22] Here we showed that the PCH pathway remains still weakly expressed in the single mutant Δ*pch*A, which is unable to produce PCH. We further showed that this production depended on PchR, on the recognition of the PchR box and its binding to the promoter regions. These results are in line with previous studies showing that PchR alone was still binding, even if very weakly, the

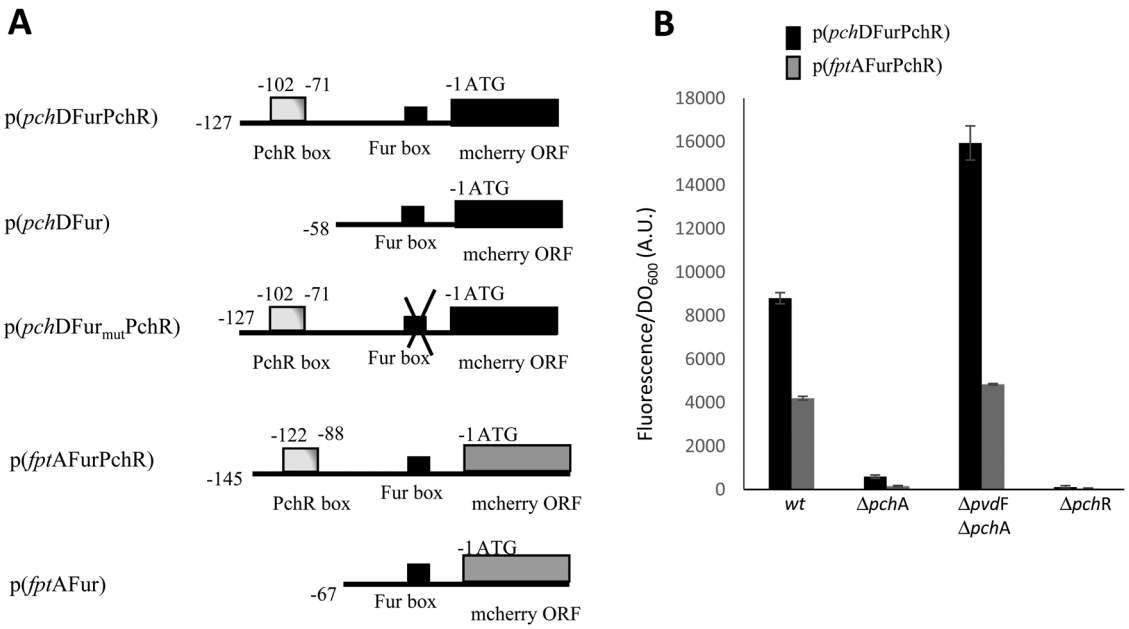

Fig. 3 Expression of transcriptional reporter genes from the PCH pathway in wt PAO1 and isogenic mutants. (A) Schematic representation of the transcriptional reporters used in this study. The *pch*R box is represented, as the *mcherry* ORF (in black, *pch*D derived promoters; in grey, *fpt*A derived promoters). (B) The production of mCherry from transcriptional reporter plasmids encompassing the *pch*D (black) or *fpt*A (grey) promoters, in wt PAO1, Δ*pch*A, Δ*pch*AΔ*pch*F, or Δ*pch*R backgrounds. mCherry production has been measured after 20 h of growth in CAA, and the results are given as a ratio between fluorescence intensity and $OD_{600\,nm}$. PchE-mCherry expression is followed at 610 nm (excitation at 570 nm).

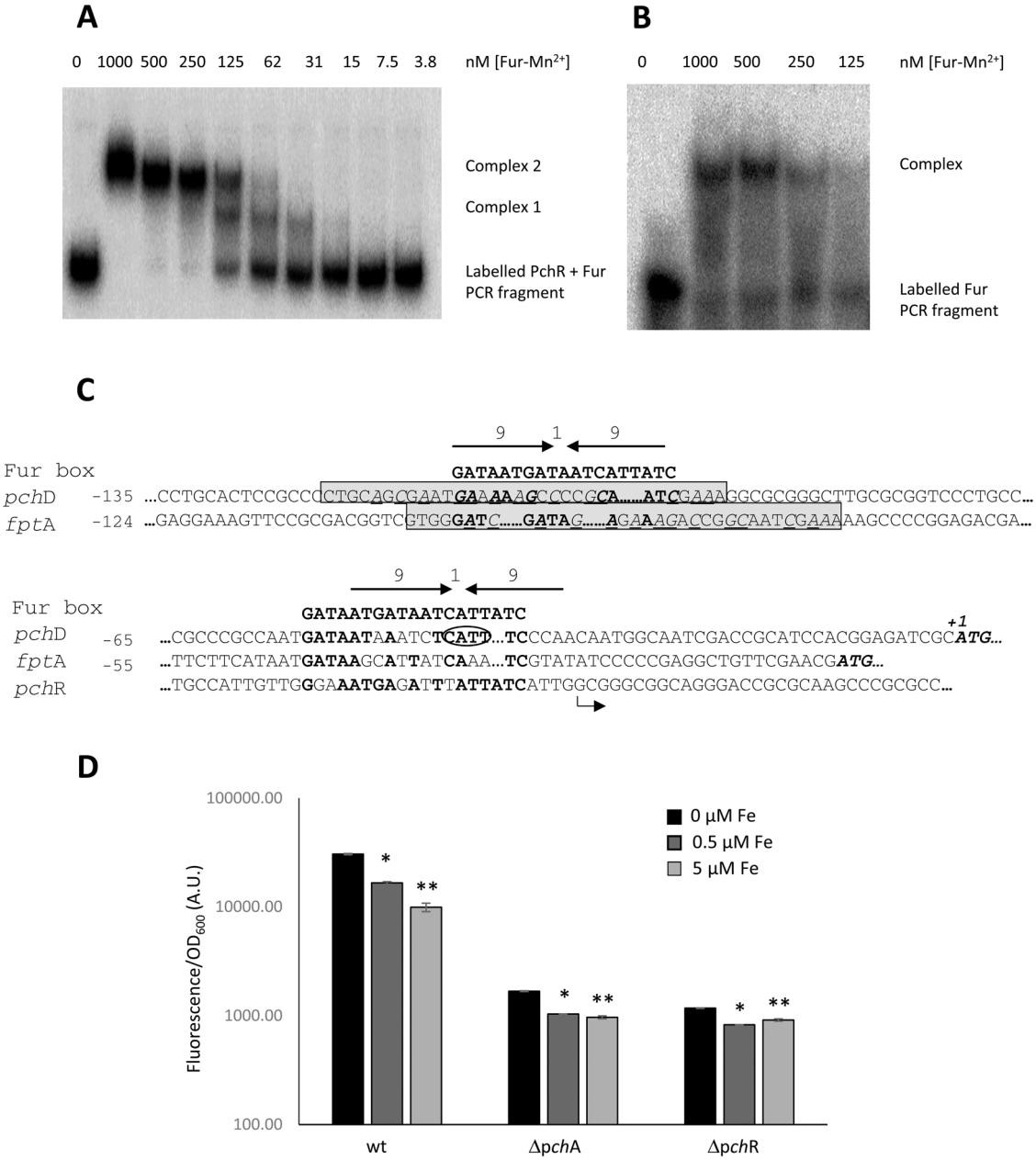

Fig. 4 Fur binding on gene promoters from the PCH pathway. (A) Electrophoretic mobility shifts of a PCR fragment encompassing the PchR and Fur boxes (−145 to −1) of the *pch*D promoter in the presence of Fur or (B) of an oligonucleotide covering only the Fur box. (C) Alignment of the two regions from the *pch*D and *fpt*A promoters containing putative Fur determinants, highlighted in bold. One is included into the sequence recognized by PchR (grey boxes), and one overlaps the TATA box. The nucleotides conserved in the PchR box are underlined.[19] The start codon is highlighted in bold italics at the end of the sequences. The Fur box of the *pch*R promoter is given for comparison. For this sequence, the start of transcription is indicated by an arrow. The consensus Fur box is also given and the palindrome is indicated. The encircled sequence CATT from the *pch*D promoter has been mutated in GTAA to inactivate the Fur box in the plasmid p(*pch*DFur$_{mut}$PchR).[30] (D) The production of mCherry from the transcriptional reporter plasmid p(*pch*DFur$_{mut}$PchR) has been followed, in a PAO1 wt, Δ*pch*A and Δ*pch*R background. mCherry production has been measured after 20 h of growth in CAA, at increasing iron concentration added at the beginning of the growth. The results are given as a ratio between fluorescence intensity and $OD_{600\,nm}$. PchE-mCherry expression is followed at 610 nm (excitation at 570 nm). Stars indicate that the values are significantly different ($p < 0.05$, Kruskal–Wallis test or one-way ANOVA).

promoter of some PCH genes.[19] Thus, there could be a 'basal' PCH production, even in the absence of PCH. This basal production might be crucial to switch on the autoregulation loop, for which PCH–Fe is required for a full activation of PchR, and a full activation of PCH synthesis.

In the double mutant, we further showed that the restoration of the expression of the PCH pathway depended as well on PchR and on the recognition of the PchR box. Indeed, either the chromosomal deletion of the *pch*R gene or the deletion of the PchR box from the transcriptional reporter plasmids diminished

drastically or even abolished the expression of the PCH effectors. Thus, the high level of PCH pathway expression in the double mutant Δ*pvdF*Δ*pchA* is due to an induction by PchR, but the precise mechanism remains unknown.

Two Fur boxes lie in the promoters of the PCH effectors

In order to get insight into this mechanism of regulation, we investigated the direct repression of both *pch*D and *fpt*A genes by the global iron-responsive regulator Fur. Fur is a global transcriptional repressor, which is activated by Fe^{2+}. It senses any increase in intracellular iron concentrations and transduces this signal into regulation of gene transcription, mainly as a repressor.

In addition to a PchR box, a Fur box has been identified in the promoter regions of *pch*D and *fpt*A genes (https://www.pseudomonas.com/, schematised in Fig. 3A). We thus checked whether the promoter sequence of these genes was indeed recognized by Fur using electrophoretic mobility shift assay (EMSA). A PCR product encompassing the *pch*D promoter (−135 to −1 from the AUG) with its Fur and PchR boxes was designed, and its capacity to bind Fur was studied. Since Fe^{2+}–Fur complexes are not stable, Mn^{2+}–Fur complexes are usually used.[24] This complex was able to bind to the promoter, and surprisingly, two bands were observed, suggesting that two sets of Fur molecules were binding to the PCR product (Fig. 4A). An oligonucleotide covering only the Fur box of the promoter, and not the PchR box, was then used. In that case, one band was observed, suggesting that the PchR box also contained determinants for Fur recognition (Fig. 4B). Consequently, the promoter region of *pchD* may contain 2 different sites containing Fur boxes. By aligning the sequences of the Fur and PchR boxes of *pch*D with the Fur box of *pch*R, some determinants of the consensus Fur promoter were indeed retrieved in both Fur and PchR boxes of the *pch*D promoter (Fig. 4C). We further validated the presence of two Fur boxes in the promoter of another gene from the PCH pathway, *fpt*A. Again, some nucleotide determinants of Fur boxes were found. EMSA assays confirmed the binding of Fur to this sequence. In that case, a second band might also be observed right on the top of the first band, but only at a high concentration of Fur (500 μM) (Fig. S3, ESI†).

It thus appeared that the PchR box is recognized by PchR, and supports activation of gene expression at low iron concentration, when PchR is produced. This box may as well be recognized by Fur and, when iron concentration increases, supports repression of gene expression. To validate this hypothesis, we studied the expression of a transcriptional reporter plasmid that contained only an active PchR box, and no active Fur box. We thus used a plasmid, p(*pch*DFur$_{mut}$PchR) (Fig. 3A), derived from p(*pch*DFurPchR). This plasmid contained a 3 nucleotide mutation in the Fur box[35] to inhibit its sensitivity to iron (residues encircled in Fig. 4C). This plasmid was used in a wt, Δ*pch*A and Δ*pch*R background. These constructs allowed an evaluation of the contribution of PchR in the presence of PCH–Fe and Fur, or of PchR and Fur or of Fur only, respectively, to the activation/repression of the PchR box by following the production of the reporter mCherry. We measured mCherry production after 20 h of growth in CAA, in the presence of increasing concentrations of iron. As shown in Fig. 4D, the production of mCherry was very high in the wt background, confirming the importance of PCH–Fe in the activation of PchR, whereas without PCH–Fe, the expression was much lower, as shown above, and even weaker in the Δ*pch*R background. When the concentration of iron increased, Fur got activated, and then either repressed the production of PchR in the wt and Δ*pch*A background, and/or repressed directly the production of mCherry by binding the PchR box of the reporter construction. As a consequence, the PCH pathway (and thus mcherry) was less expressed. This inhibition was more pronounced in the wt, in which this pathway was fully activated owing to PCH–Fe. Nevertheless, it is not possible to distinguish between the direct repression of the PchR box of the reporter and the repression of PchR production. In the Δ*pch*R mutant, no PchR is produced. The inhibition of Mcherry production that is still observed is thus due to a repression by Fur. This inhibition confirms *in vivo* the repression activity of Fur on the PchR box. There are thus two sequences that trigger sensitivity to iron concentration, probably through Fur, in the promoter region of different genes from the PCH pathway, one of them lying on top of the PchR binding site.

Altogether, these results showed that the regulation of the PCH pathway is far more complex than expected, and that there might be a balance between induction of gene expression by PchR and repression by Fur depending on the intracellular iron concentration. The precise conditions in which these two factors are competing still requires further investigation.

PCH production was repressed by desferrioxamine and deferasirox, in wt PAO1 but not in the mutant Δ*pvd*S context

What is the role of the restoration of the PCH pathway expression when both PVD and PCH are not expected to be produced? Are there natural environments in which this may occur? It is known that in cystic fibrosis patients, the bacteria can acquire mutation in the *pvd*S gene (PvdS being the sigma factor activating PVD production), and PVD production be abolished or drastically diminished.[36] It is known that *P. aeruginosa* can use iron from heme, or heterologous siderophores,[6] but what about the PCH pathway? According to the regulation of PCH production, PCH–Fe should be required to fully activate its production. Nevertheless, in a competitive environment (presence of other iron chelators), PCH may not be able to chelate iron anymore, and consequently not be produced either. We show here that it is not the case.

We compared the expression of the PCH system in wt PAO1 and in the *pvd*S deletion mutant (Δ*pvd*S), in the presence of competitors for iron chelation. We first tested the effect of two iron chelators, desferrioxamine and deferasirox. These iron chelators are FDA approved, used in iron overload illness like hemochromatosis or thalassemia, and already tested to potentiate the inhibition of *P. aeruginosa* infection by other drugs.[37,38] Desferrioxamine is a siderophore, and is used by *P. aeruginosa* as an iron source. Hence, Ferri-desferrioxamine is recognized by the Fox system.[39] This chelator was indeed transported into

P. aeruginosa (Fig. S4A, ESI†), and did not present toxic effects in the range of concentrations tested (Fig. S4B, ESI†). Deferasirox, on the other hand, is a man-made chemical iron chelator. It might not be transported, but we were not able to confirm that (Fig. S4A, ESI†). However, opposite to desferrioxamine, deferasirox becomes toxic at high concentration (>10 mM, Fig. S4B, ESI†). We then compared the iron chelation capacity of the different siderophores/chelators. We used the CAS assay for this purpose. This assay is based on the chelation of iron by CAS, which turns reddish. When a competitor is added, the colour turns to blue according to the competition between CAS and the chelator. Thus, this assay provides an idea of the affinity and competition capacity of the different molecules tested. According to this test, deferasirox binds slightly less efficiently iron than PCH, and desferrioxamine has a better mobilization capacity (Fig. S4C, ESI†). These results reflect previous ones showing that desferrioxamine could mobilize iron from ferritin better than PCH and even PVD.[40] PCH may thus weakly compete with desferrioxamine and/or deferasirox, especially when these chelators are added from the beginning of the experiment at high concentration. Different concentrations of the two FDA approved iron chelators were first added to the medium (1 μM–10 mM, 2 orders of magnitude higher than the maximal concentration of PVD or PCH produced in CAA), and we studied their effect on the PCH pathway expression. We measured the expression of a protein from the PCH pathway that was chromosomally tagged with mCherry (PchE-mCherry) after 20 h of growth, in the presence of these competitors. The expression of PchE-mCherry decreased with the addition of the two competitors in the wt PAO1 background (Fig. 5A and C). This behaviour was expected, as the addition of iron chelators deprives the medium of available iron, hinders the formation PCH–Fe and thus the activation of the PCH auto-regulation loop. Since the production of mCherry decreased similarly with both iron chelators, it showed that they both induced a similar inhibition of PCH synthesis, and can thus be considered as efficient competitors to PCH. Nevertheless, in the Δ*pvd*S mutant, PchE-mCherry expression did not decrease as expected. This expression almost did not decrease when using deferasirox (Fig. 5A), while it decreased more slightly when using desferrioxamine (Fig. 5C), which can be used as an iron source.

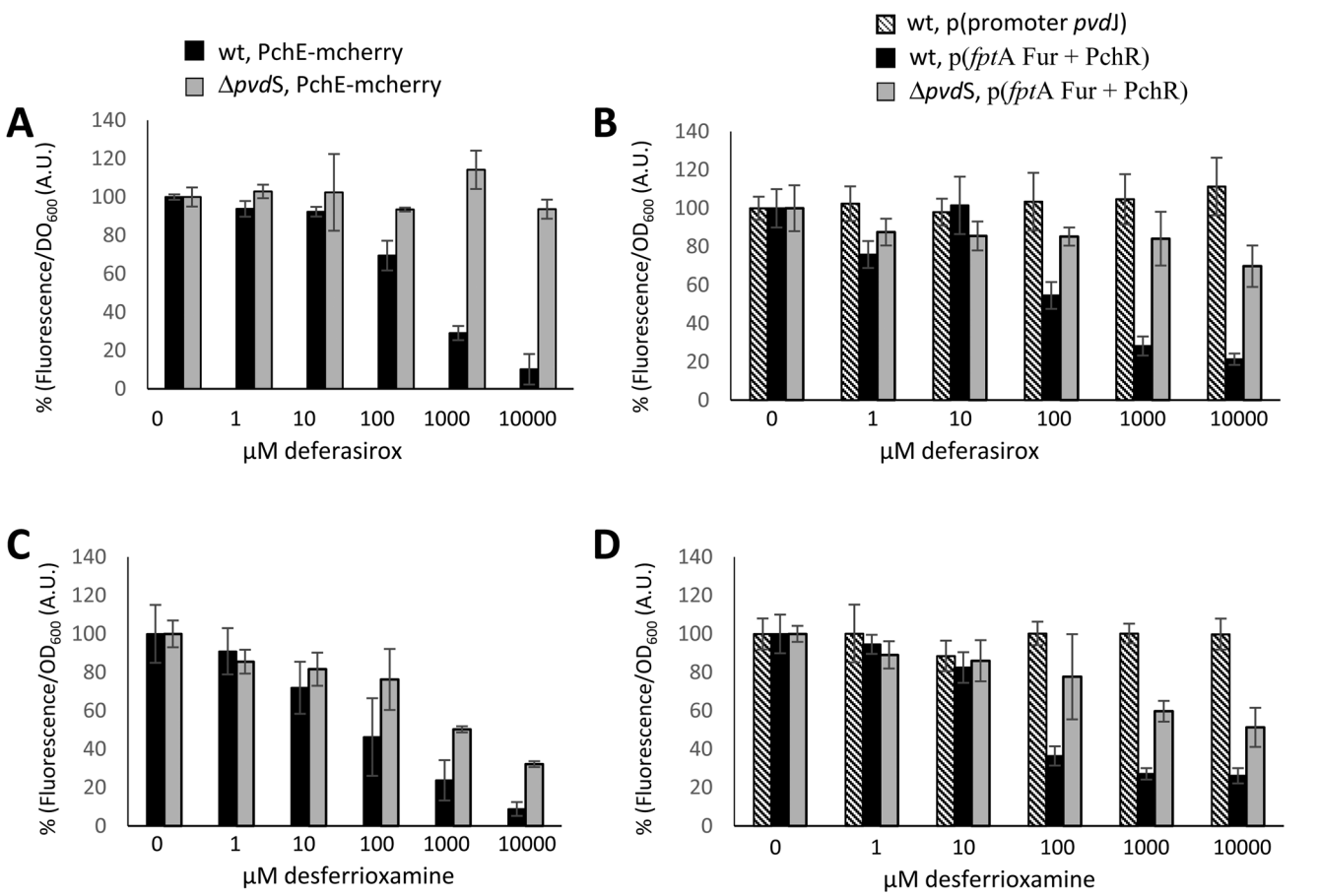

Fig. 5 Effect of iron chelators on PCH pathway expression. The mCherry ORF gene has been inserted into the chromosome of PAO1, at the 3′ end of *pchE* (A and C), or into transcriptional reporter plasmids (p(*fpt*AFurPchR) and p(*pvd*F promoter)) (B and D). mCherry fluorescence has been monitored after 20 h of growth, in the presence of different concentrations of deferasirox (A and B) or desferrioxamine (C and D), in the wt PAO1 (black) or Δ*pvd*S (grey) background. PchE-mCherry expression is followed at 610 nm (excitation at 570 nm). The results are normalized against the expression of mCherry/$OD_{600\,nm}$ in the absence of deferasirox, or desferrioxamine, and given in percent.

To validate the effect of these iron chelators on the expression of different genes from the PCH pathways, the transcriptional reporter plasmid containing the whole promoter of *fpt*A (p(*fpt*AFurPchR), see above) fused to the ORF of m*cherry* was used, in wt PAO1 and Δ*pvd*S backgrounds. To check whether the effect on PCH production was a general effect due to iron depletion, another transcriptional plasmid was used. It contained the promoter region of *pvd*F (involved in the biosynthesis of PVD) fused to the ORF of m*cherry*, p(*pvd*F promoter). The addition of any of the chelators did not affect the expression of mCherry in these control conditions (Fig. 5B and D). Next, the results obtained with the plasmid p(*fpt*AFurPchR) confirmed those from the chromosomal fusion: the expression of mCherry decreased in the wt PAO1 background but not in the Δ*pvd*S background with deferasirox (Fig. 5B) or more slightly with desferrioxamine (Fig. 5D). It was still possible that different PCH–Fe intake capacity could lead to this phenotype. To discard this possibility, we measured the expression of the reporter gene in deletion mutants of *fpt*A (responsible for PCH–Fe intake), Δ*fpt*A, and Δ*pvd*SΔ*fpt*A. If PCH–Fe intake was involved in the phenotype observed, then the deletion of *fpt*A, and thus PCH–Fe intake, should lead to an almost identical expression of the PCH pathway whatever the environment. This was not what we observed (Fig. S4D, ESI†). In the double mutant Δ*pvd*SΔ*fpt*A, there was a decrease of the reporter gene expression at higher competitor concentration. These results discard higher PCH–iron uptake from being responsible for increasing PCH production.

Thus, while the presence of competitors for iron chelation inhibits the expression of the PCH pathway in wt PAO1, *P. aeruginosa* may circumvent this inhibition in a Δ*pvd*S mutant. Altogether, when the bacteria face strong difficulties in iron import (either in a Δ*pvd*FΔ*pch*A mutant or in a Δ*pvd*S mutant and in the simultaneous presence of iron chelators), they still manage to express their siderophore pathways even if their production involves auto-regulatory loops.

PCH production was inhibited in DMEM–serum medium in WT PAO1 but not in the Δ*pvd*S context

We then tested the effect of mammalian serum, which contains iron chelators like transferrin and contributes to deprive the infection sites of iron. Transferrin is a poorer iron chelator than PCH,[41] which was confirmed by the CAS assay (Fig. S4C, ESI†). *P. aeruginosa* strains were grown in DMEM, a rich medium commonly used to grow mammalian cells, enriched with mammalian proteins (by bovine foetal serum supplementation), which comprise transferrin and/or other iron chelating molecules. Since mCherry measurement (as performed above) could be biased in DMEM medium due to phenol red, we only measured the production of PCH in this medium and compared it to the production in CAA. The production of PCH in DMEM–serum decreased drastically (Fig. 6), which may be due both to higher iron content, and, as described above, to the presence of transferrin that could outcompete PCH for iron binding. Nevertheless, the production of PCH increased strongly in the absence of PVD (Fig. 6). Thus, as with the addition of desferrioxamine or deferasirox, the PCH pathway was specifically restored/overexpressed even in the presence of human chelators, when *P. aeruginosa* was unable to produce PVD.

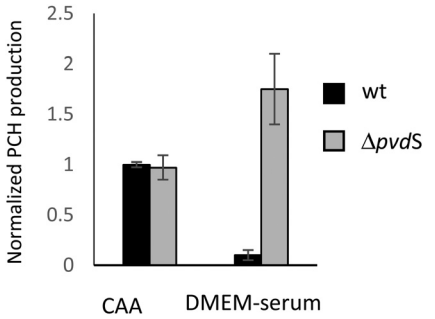

Fig. 6 Production of PCH after 8 h of growth in CAA and DMEM–serum by wt PAO1 and Δ*pvd*S strains. The results were normalized against the production of PCH by PAO1 grown in CAA. PCH concentration was monitored at 320 nm.

PVD and PCH did not compete for iron in CAA

The restoration of the PCH pathway expression in the Δ*pvd*S mutant in the presence of competitors (deferasirox, desferrioxamine or serum) could be also due to the absence of PVD production. Since PVD has a higher affinity for iron than PCH,[7,8] it is more efficient than PCH in scavenging iron when this metal becomes rare and should inhibit the induction of the PCH pathway. Nevertheless, we did not observe any competition between PVD and PCH, leading to a decrease of PCH production when wt PAO1 cells were grown in CAA. Indeed, we did not observe differences in PCH production in PAO1 *versus* Δ*pvd*S (Fig. S1, ESI†). Similarly, only a slight overexpression of the chromosomal fusion *pch*E-mcherry was observed in Δ*pvd*S mutant cells *versus* PAO1 (Fig. 7A). Thus, the pyoverdine that is produced all over the experiment does not inhibit the expression of the PCH pathway.

It is still possible that a competition may occur, but only if PVD is added at the beginning of the experiment. We checked this hypothesis by adding 20, 40, 60, and 120 μM concentrations of purified PVD (that ranged from 1/5 to slightly more than one equivalent of PVD produced at the end of the culture) at the beginning of WT PAO1 and Δ*pvd*S mutant cultures, both strains carrying a chromosomal fusion *pch*E-mcherry. The expression of PchE-Mcherry was measured after 5 h, 10 h and 20 h of growth (Fig. 7B and C). In these conditions, as shown in the literature,[42,43] we observed a decrease of the PCH pathway expression, which reflected a competition for iron between PVD and PCH. This decrease of the PCH pathway expression was quite similar in the wt PAO1 and Δ*pvd*S mutant. These results confirmed that the PVD produced by the wt PAO1 cells during the experiment did not induce an inhibition of the PCH pathway. Thus, PVD is able to inhibit PCH synthesis, but only if it is present from the beginning of the experiment.

The low level of PCH production in the presence of deferasirox, desferrioxamine, or serum in the wt PAO1 strain is only due to the addition of the iron chelators at the beginning of the experiment and not due to a competition with PVD produced by

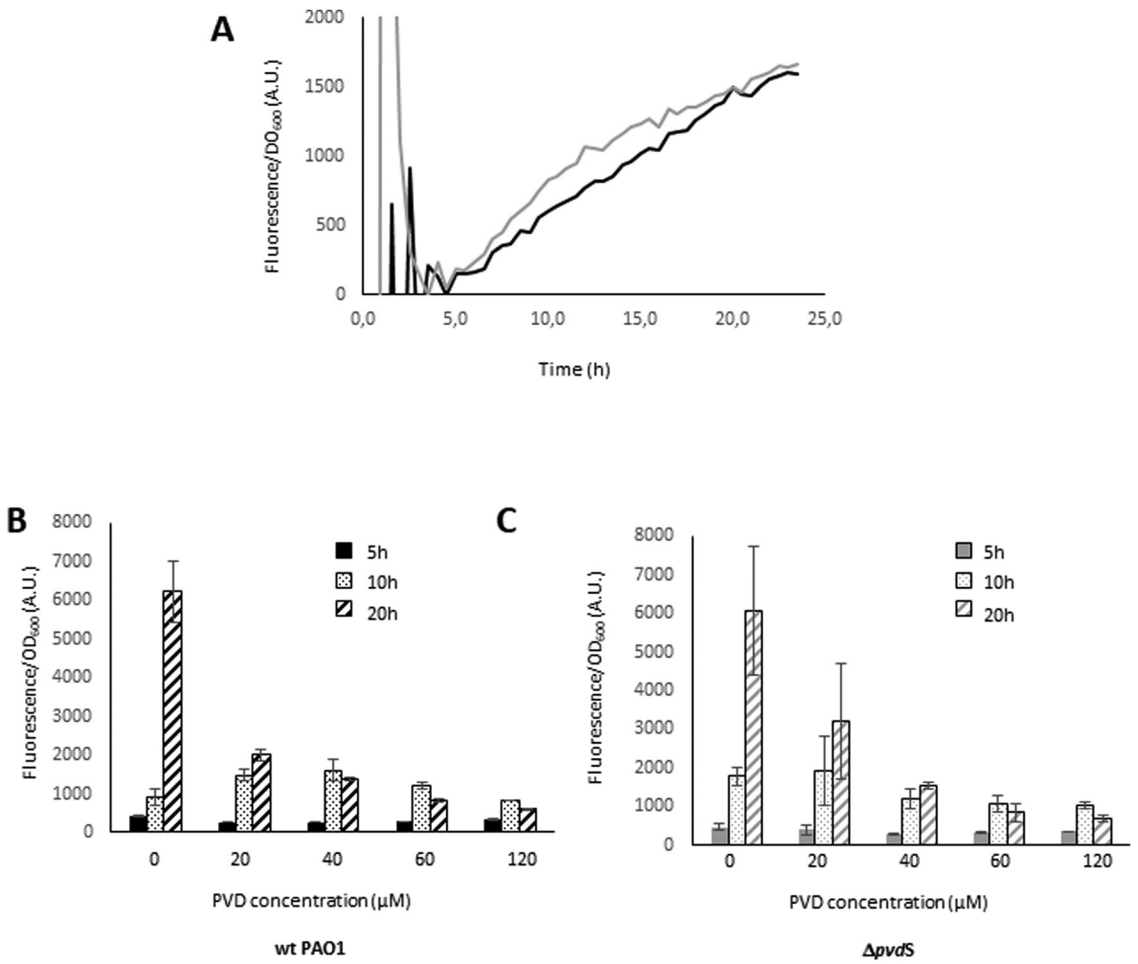

Fig. 7 Effect of the presence of PVD on the PCH pathway expression. (A) Monitoring of the expression of PchE-mCherry fluorescence in the wt PAO1 (black) or Δ*pvd*S context (grey) during 24 h of growth in CAA. (B and C) Monitoring of the expression of PchE-mCherry fluorescence after 5, 10 and 20 h of growth in CAA in the WT PAO1 (B) and Δ*pvd*S (C) backgrounds. Different amounts of PVD have been added at the beginning of the experiment to assess its effect on PchE-mCherry expression. PchE-mCherry expression is followed at 610 nm (excitation at 570 nm).

the cells. Consequently, the restoration of PCH production in the Δ*pvd*S strain is not due to a lack of competition with PVD.

Conclusions

Since iron is of utmost importance for life, bacteria have diversified their iron import strategies to adapt to ever changing environments. *P. aeruginosa* imports iron mainly through the production of two siderophores, PVD and PCH. In this bacterium, the production of both siderophores is associated with positive autoregulation loops, built from different modules/proteins. Nevertheless, the comparison between these two systems has only been marginally studied, mainly because of a lack of knowledge on the PCH system. We highlighted in this study a mechanism that could allow the bacteria to escape from the requirement of ferri-siderophore to switch on their respective pathway. Such an 'evasion' mechanism has already been described for the PVD pathway, and we showed that the PCH pathway can as well escape from PCH–Fe requirement.

The regulatory cascade leading to PCH production is based on PchR, a AraC type of transcription factor, which requires an inducer. It was previously shown that PCH–Fe, not PCH alone, was the inducible co-factor. Nevertheless, the regulation of this system proved to be more complex. Under strong difficulties to get access to iron (for example when both siderophores are not produced anymore), the cell manages to overcome the requirement of PCH for its own synthesis, probably through a mechanism based on the dual regulation of the PCH effector expression by an inducer (PchR) and a repressor (Fur), as depicted Fig. S5 (ESI†). Along with other studies, our results highlighted the complexity of these intricate regulation mechanisms between the PVD and PCH pathways, and Fur regulation. Nevertheless, further studies would be required to characterize more deeply the relative contribution of PchR *versus* Fur, and the role of PVD production/absence in this balance. ChIP experiments on PchR and Fur flag-tagged proteins for example could help to unravel some of these questions. Another point that should be clarified is the fate of PCH–Fe once it enters the cell through FptX. Indeed, free PCH–Fe may trigger an oxidative stress (Adler *et al.*[44]). How does PchR get access to this PCH–Fe, and may this unknown mechanism add a layer of regulation on PCH expression? May

this mechanism even modify the regulation balance between PchR and Fur? PVD production is already known to be regulated at the level of the membrane through Sigma/anti-Sigma factors, would it be the case for PCH? These subtle adaptations of siderophore production under varied stresses certainly deserve further investigation to fully appreciate iron uptake.

It further appears that the positive autoregulation of production of both siderophores takes similar routes to that of quorum sensing molecules. The comparison would be interesting to follow up, by considering siderophores as sensing molecules. Indeed, the iron content of the media is often a signal for lifestyle changes (at low iron content, the bacteria switch to biofilm for example). Further comparison between both systems – iron import and quorum sensing – should also improve our understanding of the other functions that may be fulfilled by these iron import systems.

These results may also help to reconsider the role and importance of PCH. *P. aeruginosa* is often responsible for chronic lung infection in patients suffering from cystic fibrosis. The sequence of different strains from chronically infected patients revealed that *P. aeruginosa* accumulated mutations in the promoter of some genes, like *pvd*S, leading to the absence of PVD production.[36] In such an environment, the bacteria may force the production of PCH, which pleads for a non-negligible role of PCH in infections. Such a conclusion is also attested by recent studies, in which the deletion of the PCH system affected the virulence of the bacteria in a mouse infection model,[45] or highlighted the role of PCH in oxidative stresses.[46] Thus, the tug of war for iron between host and bacteria seems to be ever more complex, with the bacteria optimizing all their iron import panoply to get access to iron.

Authors contribution

C. O. and G. G. performed most of the experiments, C. L. A. and F. A. designed and produced the plasmids used and transformed the different strains, G. V. purified pyoverdin, M. G. L. A. produced deferasirox, P. A. and M. S. I. designed and performed the EMSA, S. A. P. and B. D. designed and performed the proteomics analysis, S. I. J. and F. P. designed the experiments, and F. P. wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

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