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

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# Metallomics

Integrated biometal science

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3 **The pathogen *Pseudomonas aeruginosa* optimizes the production of the siderophore**  
4 **pyochelin upon environmental challenges**  
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New Article Online  
DOI: 10.1039/C9MT00029A

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**Abstract**View Article Online  
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Siderophores are iron chelators produced by bacteria to access iron, an essential nutrient. The pathogen *Pseudomonas aeruginosa* produces two siderophores, pyoverdine and pyochelin, one with a high affinity for iron, and the second with a lower affinity, respectively. Furthermore, the production of both siderophores involve a positive auto-regulatory loop: the presence of the ferri-siderophore complex is essential to engage their large production. It was thus hard to consider a role for pyochelin in drastic competitive environments where the host or the environment 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 absence of pyoverdine, and thus higher difficulty to access iron, the bacteria is able to produce pyochelin independently of the presence of ferri-pyochelin. The regulation of the pyochelin pathway appeared more complex than expected with a more intricate tuning between repression and activation. Consequently, when the bacteria cannot produce pyoverdine it is 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.

## Significance to Metalloomics

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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 the 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 bacteria survival, especially during infections where the host deprive the bacteria from iron. Here we provide insights on the regulation of pyochelin production, that help to characterize its role and importance, and bring further information on the battle for iron during an infection.

## Introduction

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Iron is absolutely required for 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<sup>1,2</sup>. Siderophores chelate iron in the extracellular 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 for iron. A nutritional immune response is setup by the host to deprive bacteria from nutrients like iron<sup>3,4</sup>, mainly through the production of different iron chelators like transferrin or lipocalin. As counter measures, certain bacteria can produce different types of siderophore, fine tune their production in this adverse environment, or hijack host heme<sup>5</sup> and siderophores produced by other organisms<sup>5,6</sup>.

*P. aeruginosa* is an opportunist pathogen involved in different nosocomial infections, and/or in severe infections for patient 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 ( $10^{32} \text{ M}^{-1}$ )<sup>7</sup> than pyochelin ( $10^{28} \text{ M}^{-2}$ )<sup>8</sup>. 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<sup>9</sup>.

PVD and PCH production by *P. aeruginosa* is highly regulated by iron bioavailability. Both siderophores production are embedded into so-called positive autoregulation loop in which PVD-Fe and PCH-Fe are required for their own synthesis (Figure 1).

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

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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<sup>19</sup>. Once  
PCH is synthesized and excreted in the extra-cellular environment, PCH chelates iron (Figure  
1). The PCH-Fe complex is imported back into the periplasm via the specific outer membrane  
transporter, FptA<sup>20</sup>, and into the cytoplasm through the permease FptX<sup>21</sup>. In the cytoplasm, the  
PCH-Fe complex binds and activates the transcription regulator PchR<sup>19,22</sup>: the PCH-Fe is  
required for its own synthesis (Figure 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 are 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<sup>23</sup>. *P. aeruginosa*  
Fur is part of the tetramer sub family of Fur proteins and at high iron concentrations, Fur

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3 tetramer is activated by Fe<sup>2+</sup>, that split into dimer upon DNA interaction and represses the  
4 expression of both PVD and PCH pathways<sup>24,25</sup>. Fur directly binds the promoter of the  
5 transcription regulators of the two pathways (*pvdS*, *fpvI*, *fpvR* and *pchR*) and inhibits their  
6 production<sup>26-27</sup>. Fur may as well bind the promoter of other genes from the PCH pathway, but  
7 not of the PVD pathway<sup>19,28</sup>.  
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15 In the present work, we investigated whether the expression of the PCH pathway can evade the  
16 requirement of PCH-Fe, as is it the case for the PVD pathway. We showed that the expression  
17 of the PCH pathway can indeed evade the requirement of PCH-Fe only when PVD is not  
18 produced anymore, in a double mutant  $\Delta pvdF\Delta pchA$  or in a  $\Delta pvdS$  mutant, and simultaneous  
19 presence of iron competitors. We detailed also the regulation of the PCH pathway expression,  
20 and discussed the significance of these results.  
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## Materials and Methods

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### Strains used, growth conditions and chemicals

The *P. aeruginosa* and *Escherichia coli* strains and plasmids used in this study are listed in Table S1. Primers used are listed in Table S2. *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, 5g/l; K<sub>2</sub>HPO<sub>4</sub>, Merck, 1.4g/L; MgSO<sub>4</sub>, Merck, 0.25g/L) or DMEM (Gibco) / Fetal Bovine Serum (Gibco). When required, gentamycin (30 µg.ml<sup>-1</sup>) were added to *P. aeruginosa* cultures. The cells are then pelleted by centrifugation and re-suspended in fresh medium, and the resulting suspension was diluted to the OD<sub>600nm</sub> required. The range of OD<sub>600nm</sub> of the cultures, from which normalized values are obtained, are given (Table S3). Desferrioxamine was purchased from Sigma, and deferasirox synthesised as described by Steinhauser and collaborators<sup>29</sup> (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.

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

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3 primers (Table S2) allowing an overlapping with a second PCR fragment encompassing the  
4 open reading frame of mcherry. A second PCR was generated using the two first PCR fragment  
5 as template to obtain the transcriptional reporter fragment. This fragment was trimmed by  
6 digestion with *EcoRI* and *HindIII* or *BamHI* and inserted between the sites for these enzymes  
7 in pSEVA631 (<http://seva.cnb.csic.es>) and bacteria were transformed with this vector.  
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### 17 CAS assay

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19 4,4 mg of sulfosalicylic acid are added to 5 mL de CAS solution (0.6 mM HDTMA; 0.14 mM  
20 CAS; 10  $\mu$ M Fe; 1 M piperazine, pH 5.6). 100  $\mu$ l of this solution are dispensed into 96-well  
21 plates and 100  $\mu$ l of the molecule to be tested added. The molecules tested were dissolved in  
22 MeOH at a concentration of 200  $\mu$ M. After 3h of incubation at room temperature, the OD<sub>630nm</sub>  
23 are taken. The reddish solution (without competitor) turn blue when the competitor hijack iron.  
24 The mobilization capacity is estimated as the ratio between the absorbance of the well in which  
25 the molecule is added, and the control well (100  $\mu$ l MeOH added). Low values means high  
26 competition. These experiments were reproduced 3 times.  
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### 43 Real-time quantification of fluorescence intensity

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45 The cells were cultured overnight in CAA medium, pelleted by centrifugation, re-suspended in  
46 fresh CAA medium, and the resulting suspension was diluted to obtain an OD<sub>600nm</sub> of 0.01 units.  
47 200  $\mu$ l of the suspension per well were dispensed into a 96-well plate (Greiner, U-bottomed  
48 microplate). The plate was incubated at 30°C, with shaking, in a Tecan microplate reader  
49 (Infinite M200, Tecan) for measurements of OD<sub>600nm</sub> and mCherry (excitation/emission  
50 wavelengths: 570 nm/610 nm) fluorescence at 30 min intervals, for 24h. When required, iron  
51 (500 nM or 5  $\mu$ M), or gentamycin (30  $\mu$ g/ml) was added. When using reporter plasmids carrying  
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mcherry ORF, control experiments were conducted with a plasmid carrying mcherry ORF without any promoter. Results were obtained from three biological replicates. Values at 20h 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<sub>600nm</sub> of 0.1. The cells were then incubated, with vigorous shaking, at 30°C for 8 h. The OD<sub>600nm</sub> was taken to measure cell growth. The OD<sub>400nm</sub> was also taken from 1 ml of supernatant to measure the production of pyoverdine ( $\epsilon_{pvd} = 19000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ). Pyochelin was then extracted twice from 1ml of supernatant (acidified with 50  $\mu\text{l}$  of citric acid 1M) with 500  $\mu\text{l}$  of ethyl acetate. The absorbance at 320 nm was taken, and the pyochelin concentration calculated ( $\epsilon_{pch} = 4300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ). As a control, we further compared the absorbance of PVD/PCH purified from our different strains to 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<sup>30</sup>. Briefly, overnight cultures of strains grown in LB or CAA medium were pelleted, re-suspended and diluted in fresh medium to obtain an OD<sub>600nm</sub> of 0.1 units. RNAs were purified as described previously<sup>31</sup>. We then reverse transcribed 1  $\mu\text{g}$  of total RNA with a High-Capacity RNAto-cDNA Kit, in accordance with the manufacturer's instructions (Applied Biosystems). The amounts of specific complementary DNAs were assessed in a StepOne Plus instrument

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3 (Applied Biosystems) with Power Sybr Green PCR Master Mix (Applied Biosystems) and the  
4 appropriate primers (Table S2). The house keeping gene *uvrD* was used for normalization. View Article Online  
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8 Results, obtained from at least three biological replicates, are expressed as a ratio (fold change)  
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10 relative to the reference conditions. P-values were < 0.05  
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### 16 Iron uptake

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18  $^{55}\text{FeCl}_3$  was obtained from Perkin Elmer Life and Analytical Sciences (Billerica, MA, USA),  
19 in solution, at a concentration of 71.1 mM, with a specific activity of 10.18 Ci/g. Siderophore-  
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21  $^{55}\text{Fe}$  complexes were prepared at  $^{55}\text{Fe}$  concentrations of 20  $\mu\text{M}$ , with a siderophore:iron  
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23 (mol:mol) ratio of 20:1. Iron uptake assays were carried out three times, as previously  
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25 described<sup>32</sup>.  
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### PVD purification

1 mL of overnight LB culture of *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,  
incubated at 30°C under shaking condition (220 rpm). After 24 h of growth, 7.5 mL of this  
culture was transferred in an Erlenmeyer flask containing 1 L of sterile succinate medium. After  
24 h of incubation at 30°C under shaking condition (220 rpm), the culture was centrifuged  
(2664 g for 40 min). The supernatant was filtered twice with glass microfiber filters (Whatman,  
GF/C) and once on nitrocellulose filter (0.45  $\mu\text{m}$  porosity). The amount of pyoverdine present  
in the solution was estimated by measuring the absorbance at 400 nm. Siderophore containing  
supernatant was loaded, after acidification (pH 6.0), on XAD-4 column, washed with two

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3 volumes of purified water and eluted with one volume of 50% ethanol. The eluate was  
4 concentrated under vacuum on a rotary evaporator and lyophilized.  
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### 11 Electrophoretic mobility shift assay (EMSA)

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14 EMSA experiments were performed as previously described<sup>24</sup>. DNA radiolabelling was  
15 performed by incubating 20-30 nM DNA for 30 min at 37°C in the presence of 1 unit of T4  
16 polynucleotide kinase (NEB) and 0,5 µL of gamma ATP at 1 mCi /mmol. Labelled DNA was  
17 diluted 10 times in binding buffer (20 mM BisTrisPropane pH 8.5, 100 mM KCl, 3 mM MgCl<sub>2</sub>,  
18 10 µM MnCl<sub>2</sub>, 5% v/v glycerol and 0.01% Triton X-100), desalted on G25 mini spin column  
19 and stored at -20°C. EMSA were performed with 200-250 pM of freshly prepared radiolabelled  
20 DNA incubated 30 min at 25°C with different concentrations of protein in a binding buffer.  
21 After 30 min incubation at room temperature, 10 µL of each sample were loaded on 8 %  
22 polyacrylamide (29/1) gel in TA buffer (40 mM Tris acetate pH 8.2) with 10% of glycerol  
23 supplemented with 10µM of appropriate metal or in TAE buffer for EDTA treatment (40 mM  
24 Tris acetate pH 8.2 and 2mM EDTA). The gel was pre-run 30 min at 100 V in TA buffer  
25 supplemented with 100 µM of appropriate metal or in TAE buffer. Mobility shifts were revealed  
26 by exposing the gels (2h to 12h at RT) on a storage phosphor screen (GE healthcare) and  
27 quantified with a cyclone phosphoimager (Perkin Elmer). Results have been reproduced three  
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### 52 Proteomics analysis.

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55 Bacteria were grown in CAA medium as described above. After the first overnight culture in  
56 CAA medium, the bacteria were diluted in 10 mL CAA to an OD<sub>600nm</sub> of 0.1 and incubated for  
57 8 h at 30°C. For the digestion and cleanup steps, the same strategy was used as previously  
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3 described<sup>33</sup>. For the shotgun proteomics assays, 1 ug of peptides of each sample were subjected  
4 to LC-MS (liquid chromatography-mass spectrometry) analysis using the same approach as  
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8 previously described<sup>33</sup>.  
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## Results and Discussion

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### PCH-Fe is required for PCH synthesis

PchR is an AraC-type of transcription factors, which in most cases require a co-factor. A previous study showed through *in vitro* studies that PCH-Fe, and not PCH alone, could be the co-factor<sup>19</sup>. 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 gene from the PCH pathway in two mutants of this pathway. The first,  $\Delta pchA$ <sup>21</sup>, is unable to synthesize PCH, through the deletion of *pchA*, a gene encoding an enzyme involved in PCH biosynthesis, Figures 1 and S1. The second,  $\Delta fptA$ <sup>21</sup>, is unable to import PCH-Fe, through the deletion of *fptA*, a gene encoding a transporter responsible for the import of PCH-Fe. We measured by qRT-PCR the level of transcription in the strains PAO1 WT,  $\Delta pchA$ , and  $\Delta fptA$ , after 8h of growth in CAA, a strongly iron-depleted medium<sup>34</sup>. The expression of the genes from the PCH pathways was repressed in the strain  $\Delta pchA$  (Figure 2), highlighting the importance of PCH in the induction of its own synthesis. The repression of the genes from the PCH pathway in the  $\Delta fptA$  mutant (Figure 2) further showed that not only the siderophore, but the ferri-siderophore is required for the activation of the PCH pathway expression. These *in vivo* results comfort 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 $\Delta pvdF\Delta pchA$ mutant

To investigate the regulation pattern of the PCH pathway genes, we first measured by qRT-PCR the level of transcription of the genes of the PVD and PCH pathway in the strains PAO1

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3 WT,  $\Delta pvdF$ <sup>31</sup> (a strain unable to produce PVD, through the deletion of *pvdF*, a gene encoding  
4 an enzyme involved in PVD biosynthesis, Figures 1 and S1) and  $\Delta pchA$ <sup>21</sup>, (a strain unable to  
5 produce PCH, through the deletion of *pchA*, a gene encoding an enzyme involved in PCH  
6 biosynthesis, Figures 1 and S1) after 8h of growth in CAA, a strongly iron-depleted medium<sup>34</sup>.  
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8 For the PVD pathway, we have chosen to follow the transcription of the mRNAs encoding for  
9 *pvdA* and *pvdJ*, involved in PVD biosynthesis, *fpvA*, involved in the import of PVD-Fe across  
10 the outer membrane, as well as the sigma/anti-sigma factors *pvdS*, *fpvI*, and *fpvR* regulating the  
11 expression of the proteins of the PVD pathway. For the PCH pathway, the expression of the  
12 mRNAs encoding for *pchE*, an enzyme involved in PCH synthesis, *fptA* and *fptX*, the two  
13 transporters involved in the import of the PCH-Fe complex, and the transcription factor *pchR*  
14 were monitored. The house keeping gene *uvrD* was used for normalization.

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16 In a strain deprived of PVD ( $\Delta pvdF$ ), the transcription of the different genes from the PVD and  
17 PCH pathway was weakly affected (< 3 fold changes) when grown in CAA (Figure 2A & B).  
18 These results are in line with the literature, and an ‘evasion’ mechanism allowing a basal  
19 production of PVD in the absence of PVD-Fe<sup>11,12</sup>. Indeed, the induction of gene expression by  
20 PVD-Fe through the auto-regulatory loop (Figure 1) requires cells to secrete a basal level of  
21 PVD to switch on the autoregulation loop of the PVD pathway. In CAA, it even appears that  
22 the expression of the PVD pathways in the  $\Delta pvdF$  strain is almost similar to that in WT PAO1,  
23 suggesting that the production of PVD could be far beyond ‘basal’ production, even in the  
24 absence of PVD-Fe uptake. This production may thus not only serve to switch on the auto-  
25 regulatory loop, but could also save the production of PVD, and help the uptake of iron even in  
26 strong PVD-Fe import difficulties.

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28 In a strain deprived of PCH, the PVD pathway is expressed as in WT PAO1, while the genes  
29 from the PCH pathway were only poorly transcribed in the absence of PCH production (> 100  
30

fold difference) (Figure 2A & B), except that of *pchR*, which transcription is only regulated by Fur, and not stimulated by the PCH-Fe complex. Such results are in agreement with the knowledges on this auto-regulatory loop, for which PCH-Fe is important to induce the expression of the pathway<sup>21,22</sup>.

We then used a double mutant  $\Delta pvdF\Delta pchA$ , deficient in PVD and PCH production (Figure S1). 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, Figure 2A & B), in the double mutant  $\Delta pvdF\Delta pchA$  compared to the WT PAO1, was 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 only switch on when the bacteria faces strong difficulties in iron uptake (here absence of both PVD-Fe and PCH-Fe), and could help saving iron uptake in these conditions. It also suggest that the regulation of PCH production is more complex than expected.

### **The transcription of the genes of the PCH pathway in the double $\Delta pvdF\Delta pchA$ mutant depends on PchR**

To understand how the PCH pathways expression is restored in the  $\Delta pvdF\Delta pchA$  strain, we checked whether Fur could be involved in the phenotype, then if PchR or another transcription factor was involved in the phenotype. If in the  $\Delta pchA$  mutant, the iron content was only faintly decreased compared to wt, in the double mutant  $\Delta pchA\Delta pvdF$ , the iron content was almost two-fold lower<sup>34</sup>. Would this decrease in iron concentration affect Fur activity, and thus the

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3 activation of the PchR pathway? To verify this hypothesis, we compared the proteome of the  
4 double mutant  $\Delta pchA\Delta pvdF$  and the wt PAO1, to check whether some genes usually repressed  
5 by Fur could be upregulated in this context. Nevertheless, among the genes up-regulated in the  
6 double mutant  $\Delta pchA\Delta pvdF$ , we have not found genes already assigned as Fur-dependant  
7 (Table S4). To study the second hypothesis (involvement of PchR), we created a triple mutant  
8  $\Delta pvdF\Delta pchA\Delta pchR$  by deleting *pvdF*, *pchA* and *pchR*. In this mutant grown in CAA, the genes  
9 encoding for FptX, PchE, and PchR (as a control) belonging to the PCH pathway, were poorly  
10 transcribed (Figure S2). These results strongly suggest that the production of the proteins of the  
11 PCH pathway expression in the double mutant  $\Delta pvdF\Delta pchA$  depends on PchR even if no PCH  
12 is produced and consequently no PCH-Fe can interact with PchR.

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To confirm that PchR induced directly the expression of the genes from the PCH pathway in  
the  $\Delta pvdF\Delta pchA$  strain, we checked whether the sequence recognized by PchR (PchR box, see  
Figure 3A and 4C) of promoters from the PCH pathway genes was involved in their  
transcription in the double mutant  $\Delta pvdF\Delta pchA$ . Two genes were specifically followed up,  
*pchD*, which encodes an enzyme involved in the biosynthesis of PCH, and *fptA*, which is  
involved in the transport of PCH-Fe in the periplasm. Four transcriptional fusion plasmids were  
created, encoding the mCherry ORF fused to the promoter of *pchD* or *fptA*, that contained the  
potential determinants for recognition by PchR and Fur [p(*pchD*FurPchR) and p(*fptA*FurPchR)  
plasmids] or only by Fur [p(*pchD*Fur) and p(*fptA*Fur) plasmids] as schematised in Figure 3A.  
WT PAO1, the double mutant  $\Delta pvdF\Delta pchA$  (no PVD and PCH synthesis), but also  $\Delta pchA$  (no  
PCH synthesis) and  $\Delta pchR$  (no PCH synthesis and no PchR expression) were transformed with  
these plasmids, and mCherry production 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(*pchD*FurPchR) and p(*fptA*FurPchR)] was similar in WT PAO1 and in the double mutant  
 $\Delta pvdF\Delta pchA$  (or even greater in the latter), much weaker in the  $\Delta pchA$  mutant and only a faint

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3 expression was observed in the  $\Delta pchR$  mutant (Figure 3B). The production of mCherry from  
4 the plasmids in which no PchR box was present [p(*pchDFur*) and p(*fptAFur*)] was always below  
5 the control experiment and were thus not represented in Figure 3B.  
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11 It was previously shown that PCH-Fe was important to activate PchR and trigger the expression  
12 of the PCH pathway<sup>19,22</sup>. Here we showed that the PCH pathway remains still weakly expressed  
13 in the single mutant  $\Delta pchA$ , which is unable to produce PCH. We further showed that this  
14 production depended on PchR, on the recognition of the PchR box and its binding to the  
15 promoter regions. These results are in line with previous studies showing that PchR alone was  
16 still binding, even if very weakly, the promoter of some PCH genes<sup>19</sup>. Thus, there could be a  
17 ‘basal’ PCH production, even in the absence of PCH. This basal production might be crucial to  
18 switch on the autoregulation loop, for which PCH-Fe is required for a full activation of PchR,  
19 and a full activation of PCH synthesis.  
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51 In the double mutant, we further showed that the restoration of the expression of the PCH  
52 pathway depended as well on PchR, and on the recognition of the PchR box. Indeed, either the  
53 chromosomal deletion of the *pchR* gene, or the deletion of the PchR box from the transcriptional  
54 reporter plasmids diminished drastically, or even abolished the expression of the PCH effectors.  
55 Thus, the high level of PCH pathway expression in the double mutant  $\Delta pvdF\Delta pchA$  is due to  
56 an induction by PchR, but the precise mechanism remains unknown.  
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### Two Fur boxes lies in the promoters of the PCH effectors

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52 In order to get insight into this mechanism of regulation, we investigated the direct repression  
53 of both *pchD* and *fptA* genes by the global iron-responsive regulator Fur. Fur is a global  
54 transcriptional repressor, which is activated by Fe<sup>2+</sup>. It senses any increase in intracellular iron  
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3 concentrations and transduces this signal into regulation of gene expression, View Article Online  
DOI: 10.1039/C9MT00029A mainly as a  
4 repressor.  
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8 In addition to a PchR box, a Fur box has been identified in the promoter regions of *pchD* and  
9 *fptA* genes (<https://www.pseudomonas.com/>, schematised in Figure 3A). We thus checked  
10 whether the promoter sequence of these genes were indeed recognized by Fur using Electro  
11 mobility shift assay (EMSA). A PCR product, encompassing the *pchD* promoter (-135 to -1  
12 from the AUG) with its Fur and PchR boxes was designed, and its capacity to bind Fur studied.  
13 Since Fe<sup>2+</sup>-Fur complexes are not stable, Mn<sup>2+</sup>-Fur complexes are usually used<sup>24</sup>. This complex  
14 was able to bind to the promoter, and surprisingly, two bands were observed, suggesting that  
15 two sets of Fur molecules were binding to the PCR product (Figure 4A). An oligonucleotide  
16 covering only the Fur box of the promoter, and not the PchR box, was then used. In that case,  
17 one band was observed, suggesting that the PchR box also contained determinants for Fur  
18 recognition (Figure 4B). Consequently, the promoter region of *pchD* may contain 2 different  
19 sites containing Fur boxes. By aligning the sequences of the Fur and PchR boxes of *pchD* with  
20 the Fur box of *pchR*, some determinants of the Fur promoter consensus were indeed retrieved  
21 in both Fur and PchR boxes of the *pchD* promoter (Figure 4C). We further validated the  
22 presence of two Fur boxes in the promoter of another gene from the PCH pathway, *fptA*. Again,  
23 some nucleotide determinants of Fur boxes were found. EMSA assays confirmed the binding  
24 of Fur to this sequence. In that case, a second band might also be observed right on the top of  
25 the first band, but only at high concentration of Fur (500 μM) (Figure S3).  
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50 It thus appeared that the PchR box is recognized by PchR, and supports activation of gene  
51 expression at low iron concentration, when PchR is produced. This box may as well be  
52 recognized by Fur and, when iron concentration increases, supports repression of gene  
53 expression. To validate this hypothesis, we studied the expression of a transcriptional reporter  
54 plasmid that contained only an active PchR box, and no active Fur box. We thus used a plasmid,  
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3 p(*pchDFur<sub>mut</sub>*PchR) (Figure 3A), derived from p(*pchDFur*PchR). This plasmid contained a 3  
4 nucleotide mutation in the Fur box<sup>35</sup> to inhibit its sensitivity to iron (residues encircled in Figure  
5 4C). This plasmid was used in a wt,  $\Delta$ *pchA* and  $\Delta$ *pchR* background. These constructs allowed  
6 an evaluation of the contribution of PchR + PCH-Fe + Fur, PchR + Fur or Fur only, respectively,  
7 to the activation/repression of the PchR box by following the production of the reporter  
8 mCherry. We measured mCherry production after 20 h of growth in CAA, in the presence of  
9 increasing concentrations of iron. As shown in Figure 4D, the production of mCherry was very  
10 high in the wt background, confirming the importance of PCH-Fe in the activation of PchR,  
11 whereas without PCH-Fe, the expression was much lower, as shown above, and even weaker  
12 in a  $\Delta$ *pchR* background. When the concentration of iron increased, Fur got activated, and then  
13 either repressed the production of PchR in the wt and  $\Delta$ *pchA* background, and/or repressed  
14 directly the production of mCherry by binding the PchR box of the reporter construction. As a  
15 consequence, the PCH pathway (and thus mcherry) was less expressed. This inhibition is more  
16 pronounced in the wt, in which this pathway was fully activated thanks to PCH-Fe.  
17 Nevertheless, it is not possible to distinguish between a direct repression of the PchR box of the  
18 reporter and/or a repression of PchR production. In the  $\Delta$ *pchR* mutant, no PchR is produced.  
19 The inhibition of Mcherry production that is still observed is thus due to a repression by Fur.  
20 This inhibition confirms *in vivo* the repression activity of Fur on the PchR box. There are thus  
21 two sequences that trigger a sensitivity to iron concentration, probably through Fur, in the  
22 promoter region of different genes from the PCH pathway, one of them lying on top of the PchR  
23 binding site.  
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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 genes expression by PchR, and repression by Fur, depending on the intracellular iron concentration. The precise conditions in which these two factors are competing will still require further investigation.

**PCH production was repressed by desferrioxamine and deferasirox, in WT PAO1 but not in mutant  $\Delta pvdS$  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 environment in which this may occur? It is known that in cystic fibrosis patient, the bacteria can acquire mutation in the *pvdS* gene (PvdS being the sigma factor activating PVD production), and PVD production be abolished, or drastically diminished<sup>36</sup>. It is known that *P. aeruginosa* can use iron from heme, or heterologous siderophores<sup>6</sup>, but could it also use 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 been 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 *pvdS* deletion mutant ( $\Delta pvdS$ ), 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<sup>37-38</sup>. Desferrioxamine derives from a siderophore, and may be used by *P. aeruginosa* as iron source. Hence, ferri-desferrioxamine is probably recognized by the Fox system<sup>39</sup>. This chelator was indeed transported into *P. aeruginosa* (Figure S4A), and did not present toxic effect in the range of concentration tested (Figure S4B). Deferasirox on another side, is a man-made chemical iron chelator. It might not be transported, but we were not able to confirm that (Figure S4A). However, opposite to desferrioxamine, deferasirox becomes toxic at high concentration (>10 mM, Figure S4B). We then compared the iron chelation capacity of the different siderophore/chelator. We used the CAS assay

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3 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 blue according to the competition with the chelator. Thus, this  
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Assay provide an idea of the affinity and competition capacity of the different molecules tested. According to this test, desferasirox binds slightly less efficiently iron than PCH, and desferrioxamine has a better mobilization capacity (Figure S4C). These results reflects previous results, that desferrioxamine could mobilize iron from ferritin better than PCH and even PVD<sup>40</sup>. PCH may thus weakly compete with desferrioxamine and/or desferasirox, 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  $\mu$ M-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 (Figure 5A & C). This behaviour was expected, as the addition of iron chelators deprive the medium from 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  $\Delta pvdS$  mutant, PchE-mCherry expression was not decreasing as expected. This expression did almost not decrease when using deferasirox, (Figure 5A), while it decreased more slightly when using desferrioxamine (Figure 5C), that can be used as iron source

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 *fptA* (*p(fptA)FurPchR*), see above) fused to the ORF of *mcherry* was used, in WT PAO1 and  $\Delta pvdS$  backgrounds. To check whether the effect on PCH production was a general effect due to iron

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3 depletion, another transcriptional plasmid was used. It contains the promoter region of *pvdF*  
4 (involved in the biosynthesis of PVD) fused to the ORF of mcherry, p(*pvdF* promoter). The  
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6 addition of any of the chelators did not affect the expression of mCherry in these control  
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8 conditions (Figure 5B & D). Next, the results obtained with the plasmid p(*fptAFurPchR*)  
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10 confirmed those from the chromosomal fusion, the expression of mCherry decreased in the WT  
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12 PAO1 background but not in the  $\Delta pvdS$  background with deferasirox (Figure 5B) or more  
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14 slightly with desferrioxamine (Figure 5D). It was still possible that different PCH-Fe intake  
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16 capacity could lead to this phenotype. To discard this possibility, we measured the expression of  
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18 the reporter gene in deletion mutants of *fptA* (responsible for PCH-Fe intake),  $\Delta fptA$ , and  $\Delta pvdS\Delta fptA$ .  
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20 If PCH-Fe intake was involved in the phenotype observed, then the deletion of *fptA*, and thus PCH-Fe  
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22 intake, should lead to an almost identical expression of the PCH pathway whatever the environment.  
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24 This is not what we observe (Figure S4D). In the double mutant  $\Delta pvdS\Delta fptA$ , there is a decrease of the  
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26 reporter gene expression at higher competitor concentration. These results discard higher PCH-iron  
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28 uptake from being responsible of increasing PCH production.  
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32 Thus, while the presence of competitors for iron chelation inhibits the expression of the PCH  
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34 pathway in WT PAO1, *P. aeruginosa* may circumvent this inhibition in a  $\Delta pvdS$  mutant.  
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36 Altogether, when the bacteria faces strong difficulties in iron import (either in a  $\Delta pvdF\Delta pchA$   
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38 mutant, or in a  $\Delta pvdS$  mutant and simultaneous presence of iron chelators), they still manage to  
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40 express their siderophore pathways even if their production are embedded in auto-regulatory  
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### 45 **PCH production was inhibited in DMEM-serum medium in WT PAO1 but not in $\Delta pvdS$** 46 47 **context**

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49 We then tested the effect of mammalian serum, which contains iron chelators like transferrin,  
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51 and contribute to deprive the infection sites from iron. Transferrin is a poorer iron chelator than  
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53 PCH<sup>41</sup>, that was confirmed by the CAS assay (Figure S4C). *P. aeruginosa* strains were grown  
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3 in DMEM, a rich medium commonly used to grow mammalian cell, enriched with mammalian  
4 proteins (by bovine foetal serum supplementation), which comprises transferrin, and/or other  
5 iron chelating molecules. Since mCherry measurement (as performed above) could be biased  
6 in DMEM medium due to phenol red, we only measured the production of PCH in this medium  
7 and compared it to the production in CAA. The production of PCH in DMEM-serum decreased  
8 drastically (Figure 6), which may be due both to higher iron content, and, as described above,  
9 to the presence of transferrin that could outcompete PCH for iron binding. Nevertheless, the  
10 production of PCH increased strongly in the absence of PVD (Figure 6). Thus, as with the  
11 addition of desferrioxamine or deferasirox, the PCH pathway was specifically  
12 restored/overexpressed even in the presence of human chelators, when *P. aeruginosa* was  
13 unable to produce PVD.

### 14 **PVD and PCH did not compete for iron in CAA**

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16 The restoration of the PCH pathway expression in the  $\Delta pvdS$  mutant in the presence of  
17 competitors (deferasirox, desferrioxamine or serum), could also be due to the absence of PVD  
18 production. Since PVD has a higher affinity for iron than PCH<sup>7,8</sup>, it is more efficient than PCH  
19 in scavenging iron when this metal becomes rare and should inhibit the induction of the PCH  
20 pathway. Nevertheless, we did not observe any competition between PVD and PCH, leading to  
21 a decrease of PCH production when WT PAO1 cells were grown in CAA. Indeed, we did not  
22 observe differences in PCH production in PAO1 versus  $\Delta pvdS$  (Figure S1). Similarly, only a  
23 slight overexpression of the chromosomal fusion *pchE-mcherry* was observed in  $\Delta pvdS$  mutant  
24 cells versus PAO1 (Figure 7A). Thus, the pyoverdinin that is produced all over the experiment  
25 does not inhibit the expression of the PCH pathway.

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3 It is still possible that a competition may happen, but only if PVD is added at the beginning of  
4 the experiment. We checked this hypothesis by adding 20, 40, 60, and 120  $\mu\text{M}$  of purified PVD  
5 (that ranged from 1/5 to slightly more than one equivalent of PVD produced at the end of the  
6 culture) at the beginning of WT PAO1 and  $\Delta pvdS$  mutant cultures, both strains carrying a  
7 chromosomal fusion *pchE-mcherry*. The expression of PchE-Mcherry was measured after 5h,  
8 10h and 20h of growth (Figure 7B, C). In these conditions, as shown in the literature,<sup>42,43</sup> we  
9 observed a decrease of the PCH pathway expression, that reflected a competition for iron  
10 between PVD and PCH. This decrease of the PCH pathway expression was quite similar in the  
11 WT PAO1 and  $\Delta pvdS$  mutant. These results confirmed that the pyoverdinin produced by the WT  
12 PAO1 cells during the experiment did not induce an inhibition of the PCH pathway. Thus, PVD  
13 is able to inhibit PCH synthesis, but only if it is present from the beginning of the experiment.  
14 The low level of PCH production in the presence of deferasirox, desferrioxamine, or serum in  
15 the WT PAO1 strain is only due to the addition of the iron chelators at the beginning of the  
16 experiment and not due to a competition with PVD produced by the cells. Consequently, the  
17 restoration of PCH production in a  $\Delta pvdS$  strain is not due to a lack of competition with PVD.  
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## Conclusions

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Since iron is of utmost importance for life, bacteria 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 bacteria, the production of both siderophores are embedded into positive auto-regulation loops, built from different modules/proteins. Nevertheless, the comparison between these two systems has only been marginally studied, mainly because of a lack of knowledges 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 ‘classical’ AraC type of transcription factor, which requires an inducer. It was previously shown that the 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 manage 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), depicted Figure S5. Along with other studies, our results highlighted the complexity of these intricate regulation mechanisms between the PVD and PCH pathways, and Fur regulation that allow a fine tuning response to various iron/siderophore/chelator ratio conditions. 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

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3 be clarify is the fate of PCH-Fe once it enters into the cell through FptX. Indeed, free PCH-Fe New Article Online  
DOI: 10.1039/D0MT00029A  
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5 may trigger an oxidative stress (*i.e.* Adler *et al.* <sup>44</sup>). How does PchR get access to this PCH-Fe,  
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7 and may this unknown mechanism add a layer of regulation on PCH expression? May this  
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9 mechanism even modify the regulation balance between PchR / Fur? PVD production is already  
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11 known to be regulated at the level of the membrane through sigma/anti-sigma factors, would it  
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13 be the case for PCH? These subtle adaptations of siderophore production under varied stresses  
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15 certainly deserve further investigation to fully appreciate iron uptake.  
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20 It further appears that the positive auto-regulation of both siderophore production takes similar  
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22 routes than that of quorum sensing molecules. The comparison would be interesting to follow  
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24 up, by considering siderophore as sensing molecules. Indeed, the iron content of the media is  
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26 often a signal for lifestyle changes (at low low iron content, the bacteria switch to biofilm for  
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28 example). Further comparison between both systems –iron import and quorum sensing- should  
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30 also improve our understanding of the other functions that may be fulfilled by these iron import  
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32 systems.  
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37 These results may also help to reconsider the roles and importance of PCH. *P. aeruginosa* is  
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39 often responsible of chronic infection in lung infection from patient suffering from cystic  
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41 fibrosis. The sequence of different strains from chronically infected patients revealed that *P.*  
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43 *aeruginosa* accumulated mutations in the promoter of some genes, like *pvdS*, leading to the  
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45 absence of PVD production<sup>36</sup>. In such environment, the bacteria may force the production of  
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47 PCH, that pleads for a non-negligible role of PCH during infections. Such a conclusion is also  
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49 attested by recent studies, in which the deletion of the PCH system affected the virulence of the  
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51 bacteria in a mouse infection model<sup>45</sup>, or highlighting the role of PCH in oxidative stresses<sup>46</sup>.  
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54 Thus, the tug of war for iron between host and bacteria seems ever more complex, with the  
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56 bacteria optimizing all its iron import panoply to get access to iron.  
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## Authors contribution

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

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**Table S1.** Strains and plasmids used in this studyView Article Online  
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Strains and plasmids	Collection ID	Relevant characteristic	references
<b>Strains</b>			
<i>E. coli</i> Top10		F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR ) endA1 λ	Invitrogen
<i>E. coli</i> HB101		Helper strain carrying pME487	
PAO1 ATCC15692	Wild-type		47
Δ <i>pvdF</i>	PAS263	PAO1; <i>pvdF</i> chromosomally deleted	31
Δ <i>pchA</i>	PAS282	PAO1; <i>pchA</i> chromosomally deleted	21
Δ <i>pvdF</i> Δ <i>pchA</i>	PAS283	PAO1; <i>pvdF</i> and <i>pchA</i> chromosomally deleted	33
Δ <i>pvdF</i> Δ <i>pchA</i> Δ <i>pchR</i>	PAS285	PAO1; <i>pvdF</i> , <i>pchA</i> and <i>pchR</i> chromosomally deleted	48
Δ <i>pvdS</i>	PAS386	PAO1; <i>pvdS</i> chromosomally deleted	48
PAO1 <i>pchEmcherry</i>	PAS195	PAO1; <i>mcherry</i> fused to 3' of <i>pchE</i>	21
Δ <i>pvdSpchEmcherry</i>		PAO1; <i>pvdS</i> chromosomally deleted, <i>mcherry</i> fused to 3' of <i>pchE</i>	48
Δ <i>fptA</i>		PAO1; <i>fptA</i> chromosomally deleted	21
Δ <i>pvdS</i> Δ <i>fptA</i>		PAO1; <i>pvdS</i> and <i>fptA</i> chromosomally deleted	21
<b>plasmids</b>			
<b>Expression plasmids</b>			
pSEVA631		Expression plasmid carrying a Gm cassette	<a href="http://seva.cnb.csic.es">http://seva.cnb.csic.es</a>
pSEVA631- <i>p(pchDFurPchR)</i>	pAYC3	Fur box of <i>fptA</i> promoter fused to <i>mcherry</i> ORF	This study
pSEVA631- <i>p(fptAFurPchR)</i>	pAYC4	Fur + PchR box of <i>fptA</i> promoter fused to <i>mcherry</i> ORF	This study
pSEVA631- <i>p(pchDFur)</i>	pAYC5	Fur box of <i>pchD</i> promoter fused to <i>mcherry</i> ORF	This study

pSEVA631-p( <i>fptAFur</i> )	pAYC6	Fur + PchR box of <i>pchD</i> promoter fused to mcherry ORF	35	View Article Online DOI: 10.1039/D0MT00029A
pSEVA631- <i>pvdF</i> promoter	pPF4	<i>pvdF</i> promoter fused to mcherry ORF	This study	
pSEVA631-p( <i>pchD</i> Fur <sub>mut</sub> PchR)	pAF35	pAYC5 carrying a replacement CATT in GTAA (see Figure 4)	35	

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

Oligonucleotides	Sequences 5'-3'	Used for the following construct
<b>Cloning</b>		
<i>fptA</i> Fur Box Fwd	ATATATGAATTCGGTCGTCGTCGCAGAGC	pAYC3
<i>fptA</i> Fur Box Rev	CCATGTTA TCCTCCTCGC CCTTGCTCAC CAT CAGGTTT TCCTGTAGCC CGGG	pAYC3
<i>fptA</i> PchR Box & Fur Box Fwd	CAAAGAATTCGGCGAGGAAAGTTCCGCGAC G	pAYC4
<i>fptA</i> PchR Box & Fur Box Rev	CATGTTATCCTCCTCGCCCTTGCTCACCATCG TTCGAACAGCCTCGGGG	pAYC4
<i>pchD</i> Fur Box Fwd	CAAAGAATTCGAAACGCCGAAGAATTTCTCC CCTC	pAYC6
<i>pchD</i> Fur box Rev	GAAGGCATAA CTGGAACCCT CGATGGTGAG CAAGGGCGAG GAGGATAACA TGG	pAYC6
<i>pvdF</i> Fwd	ATATATGAATT CCTGGCGCAG GCTTTCG	pPF4
<i>pvdF</i> Rev	CCATGTTA TCCTCCTCGC CCTTGCTCAC CAT TTCATCATT CCAGGAGTGG	pPF4
mCHERRY Fwd	GTGAGCAAGGGCGAGGAGGATAACATG	pAYC and pPF series
mcherry hindIII rev	CTCCAAGCTTTTACTTGTACAGCTCGTCCATG CCGC	pAYC and pPF series
<b>qRT-PCR</b>		
FpvR F1	GGTCGAACTGGACCTCAATAC	

FpvR R1	CGTGGGTGACATGGAAGTAG
PvdS F1	CAGGCGCTCGAACAGAAATA
PvdS R1	CGTAGTTGATGTGCGAGGTT
FpvI F1	CAGATCGCCGAACATCTCAATA
FpvI R1	CACCCGACAGTGCTTCATC
uvrD F	CTACGGTAGCGAGACCTACAACAA
uvrD R	GCGGCTGACGGTATTGGA
PvdA R qRT	TGGTATTCGCGCAGCAAAC
PvdA F qRT	CGCCGAAGTTCACCGATCT
fpvA F qRT	AGCCGCCTACCAGGATAAGC
fpvA R qRT	TGCCGTAATAGACGCTGGTTT
fptA F qRT	GCGCCTGGGCTACAAGATC
fptA R qRT	CCGTAGCGGTTGTTCCAGTT
fptX F qRT	CCCTGGGTGGTCAAGTTCCT
fptX R qRT	CGGCGCGACCAGTGA
pvdJ F qRT	CGTGGCCGCGATATGG
pvdJ R qRT	CTCTTCAGGCTGACTTCGATACC
pchE F qRT	GGCAATGGCAAGGTCGAT
pchE R qRT	CACCGGGCGTTTGAGAAC
qRT-PCR-PchR-Fw	CGGCTTTCGCAAGGTGTT
qRT-PCR-PchR-Rv	AGGCGGTATTCCTGCAGGTA

Table S3

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range OD <sub>600</sub>	Growth conditions		
OD	no additives 0.25-0.3	0,5 μM iron 0.6-0.7	5 μM iron 1-1.1
OD	Desferrioxamine (0-10 mM) 0.2-0.3	Deferasirox (0-4 mM) 0.1-0.3	
OD	exjade (0-1 mM) in <i>ΔpvdSΔfptA</i> 0.01-0.3		

**Figure 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 in the environment, it chelates iron, and the 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 set the PCH auto-regulation loop. All PchR proteins are believed to respond to this regulation loop (red arrow). The positive auto-regulation loop is represented as 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 in 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 set the PVD auto-regulation 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 as a purple arrow.

**Figure 2. Expression of genes from the PVD and PCH pathway in WT PAO1 and in isogenic mutants.** (A) Analysis of changes in transcription of different genes from the PVD pathway (*pvdA*, *pvdJ*, *fpvA*, *pvdS*, *fpvR*, and *fpvI*), and (B) from the PCH pathway (*pchE*, *fptX*, *fptA*, and *pchR*). Reverse transcription qPCR was performed on *P. aeruginosa* WT PAO1 and

its isogenic mutants  $\Delta pchA$  (unable to produce PCH),  $\Delta fptA$  (unable to import PCH-Fe),  $\Delta pvdF$  (unable to produce PVD),  $\Delta pvdF\Delta pchA$  (unable to produce both siderophores), grown in CAA medium. Results are given as a ratio between the values obtained for the mutant over those obtained for the WT PAO1 strain, in logarithmic scale. Bars with the same letter are not significantly different ( $p > 0.05$ , one-way ANOVA, three biological replicates)

**Figure 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 *pchR* box is represented, as the *mcherry* ORF (in black, *pchD* derived promoters; in grey *fptA* derived promoters. (B) The production of mCherry from transcriptional reporter plasmids encompassing the *pchD* (black) or *fptA* (grey) promoters, in WT PAO1,  $\Delta pchA$ ,  $\Delta pchA\Delta pchF$ , or  $\Delta pchR$  backgrounds. mCherry production has been measured after 20 h of growth in CAA, and results are given as a ratio between fluorescence intensity and  $OD_{600nm}$ . PchE-mCherry expression is followed at 610 nm (excitation at 570 nm).

**Figure 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 *pchD* promoter in the presence of Fur or (B) of an oligonucleotide covering only the Fur box. (C) Alignment of the two regions from the *pchD* and *fptA* 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<sup>19</sup>. The start codon is highlighted in bold italics at the end of the sequences. The Fur box of the *pchR* promoter is given as comparison. For this sequence, the start of transcription is indicated by an arrow. The consensus Fur box is also given and the palindrome indicated.

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3 The encircled sequence CATT from the *pchD* promoter has been mutated in GTAA to inactivate  
4 the Fur box in the plasmid  $p(pchDFur_{mut}PchR)^{30}$ . (D) The production of mCherry from the  
5 transcriptional reporter plasmid  $p(pchDFur_{mut}PchR)$  has been followed, in a PAO1 wt,  $\Delta pchA$   
6 and  $\Delta pchR$  background. mCherry production has been measured after 20 h of growth in CAA,  
7 at increasing iron concentration added at the beginning of the growth. Results are given as a  
8 ratio between fluorescence intensity and  $OD_{600nm}$ . PchE-mCherry expression is followed at 610  
9 nm (excitation at 570 nm). Stars indicate that the values are significantly different ( $p < 0.05$ ,  
10 Kruskal-Wallis test or one-way ANOVA).  
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**Figure 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, C), or into transcriptional reporter plasmids (*p(fptAFurPchR)* and *p(pvdF promoter)*(B, D). mCherry fluorescence has been monitored after 20 h of growth, in the presence of different concentrations of deferasirox (A, B) or desferrioxamine (C, D), in WT PAO1 (black), or  $\Delta pvdS$  (grey) background. PchE-mCherry expression is followed at 610 nm (excitation at 570 nm). Results are normalized against the expression of mCherry/ $OD_{600nm}$  in the absence of deferasirox, or desferrioxamine, and given in percent.

**Figure 6.** Production of PCH after 8 h of growth in CAA and DMEM-serum by PAO1 and  $\Delta pvdS$  strains. Results were normalized against the production of PCH by PAO1 grown in CAA. PCH concentration was monitored at 320 nm.

**Figure 7. Effect of the presence of PVD on the PCH pathway expression.** (A) Monitoring of the expression of PchE-mCherry fluorescence in a wt PAO1 (black) or  $\Delta pvdS$  context (grey)

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3 during 24h of growth in CAA. **(B, C)** Monitoring of the expression of PchE-mCherry  
4 View Article Online  
DOI: 10.1039/C9MT00029A  
5 fluorescence after 5, 10 and 20 h of growth in CAA in the WT PAO1 **(B)** and  $\Delta pvdS$  **(C)**  
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7 backgrounds. Different amounts of PVD have been added at the beginning of the experiment  
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9 to assess its effect on PchE-mCherry expression. PchE-mCherry expression is followed at 610  
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11 nm (excitation at 570 nm).  
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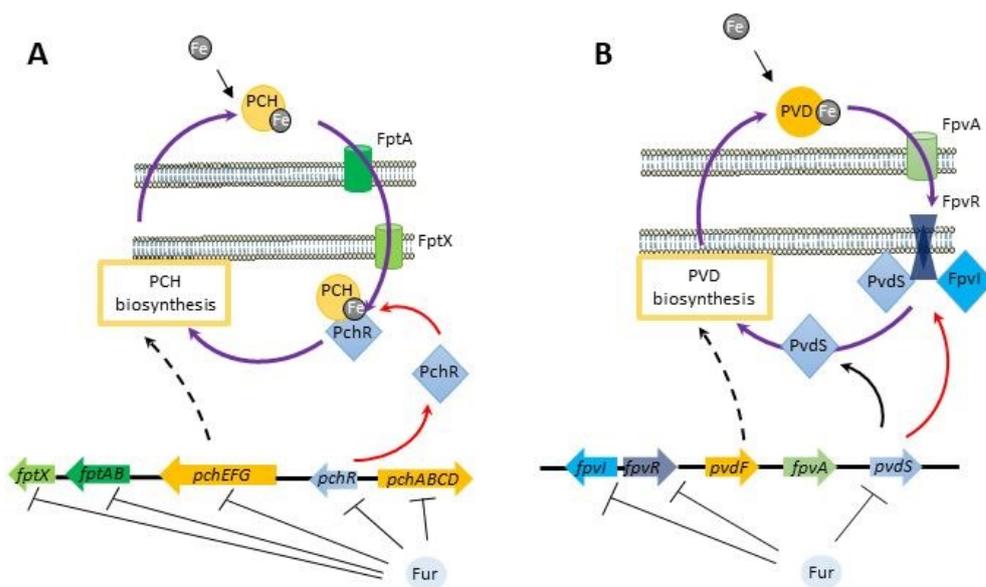


Figure 1

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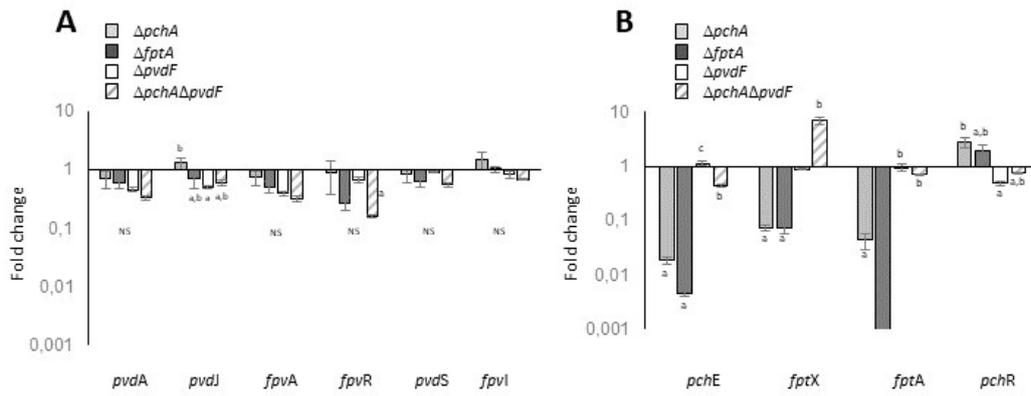


Figure 2

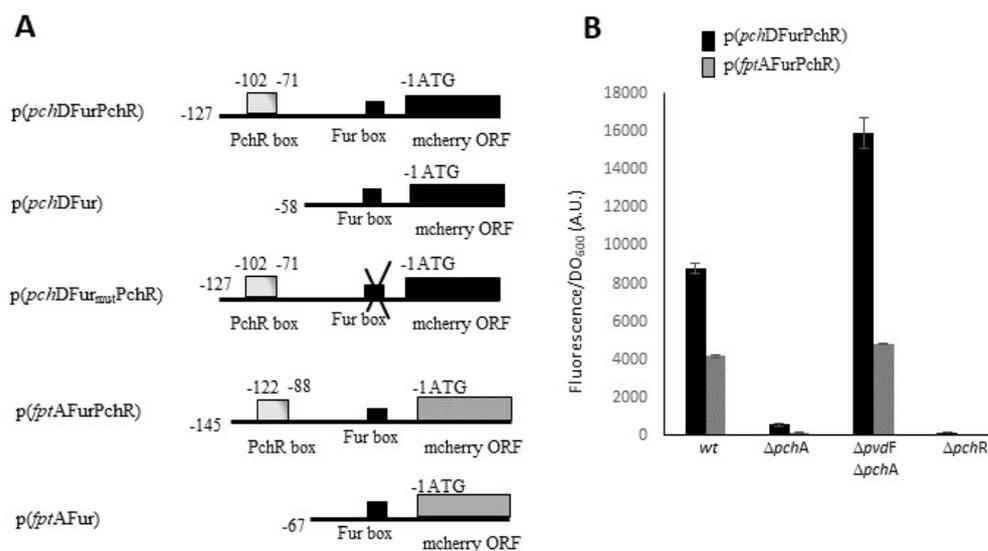


Figure 3

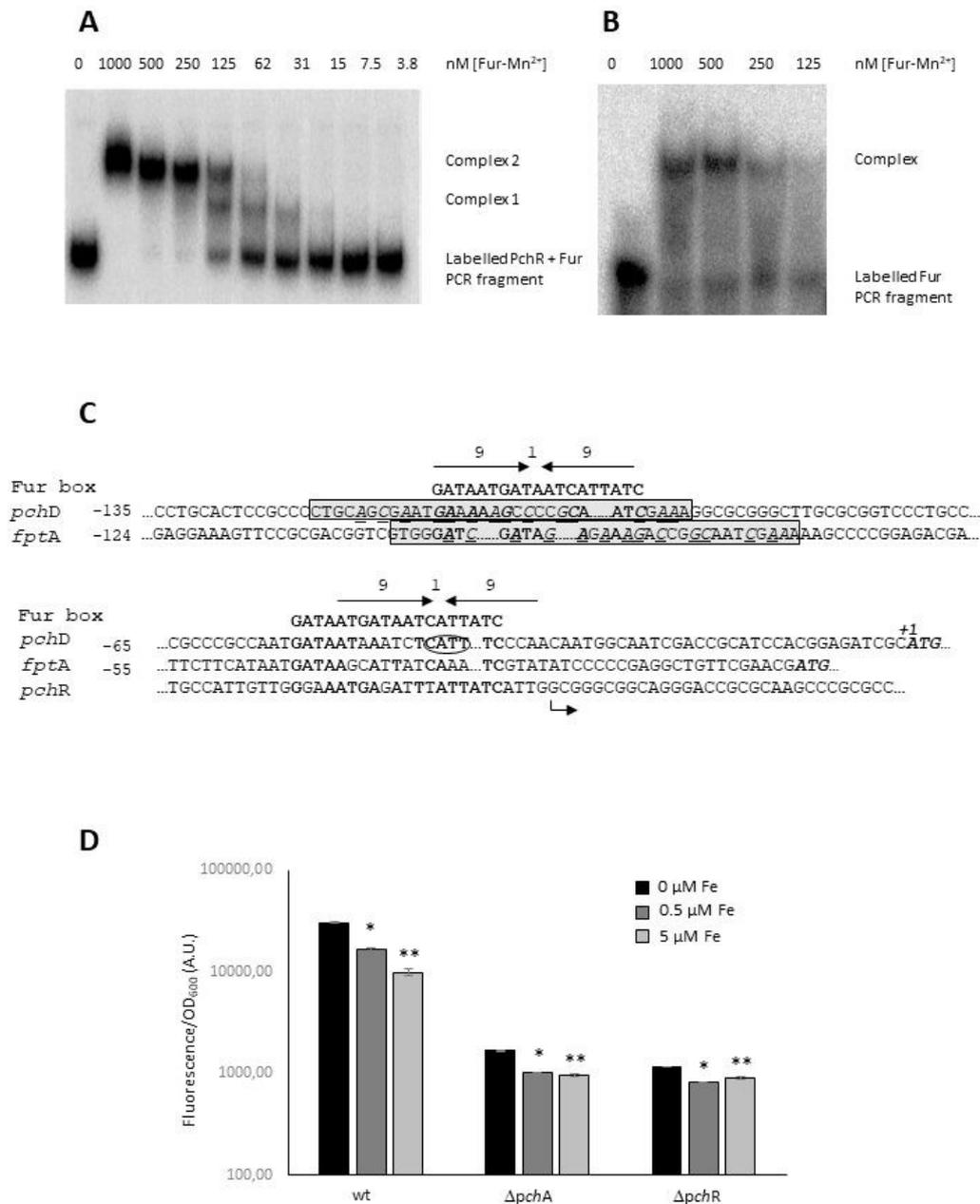


Figure 4

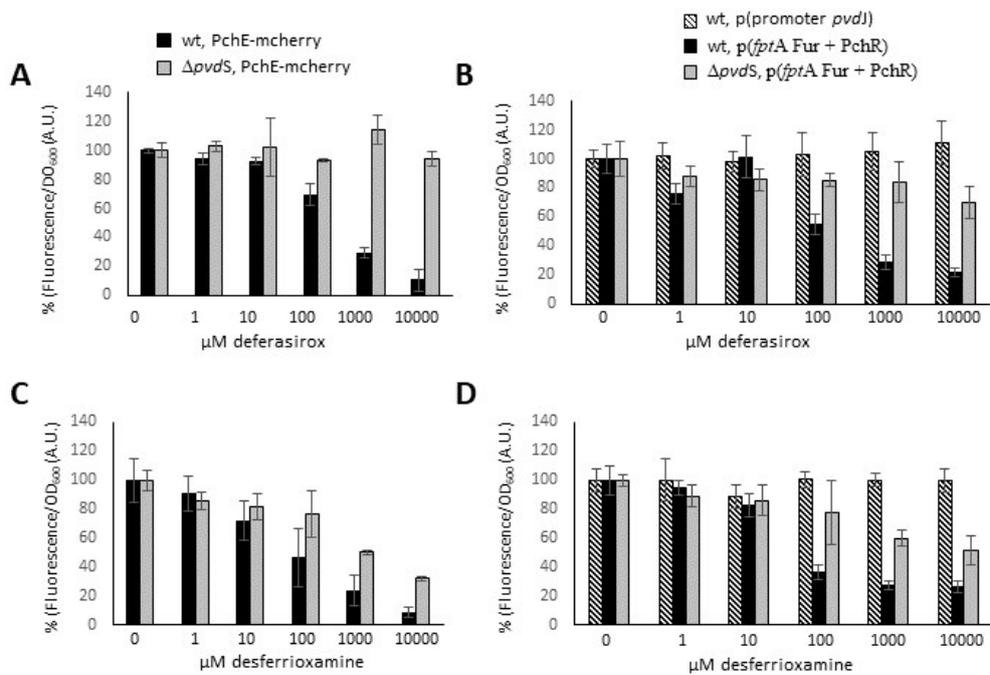


Figure 5

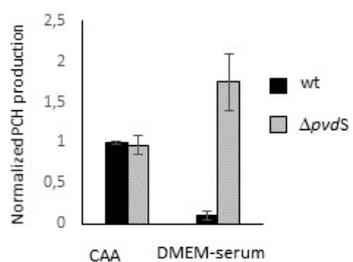


Figure 6

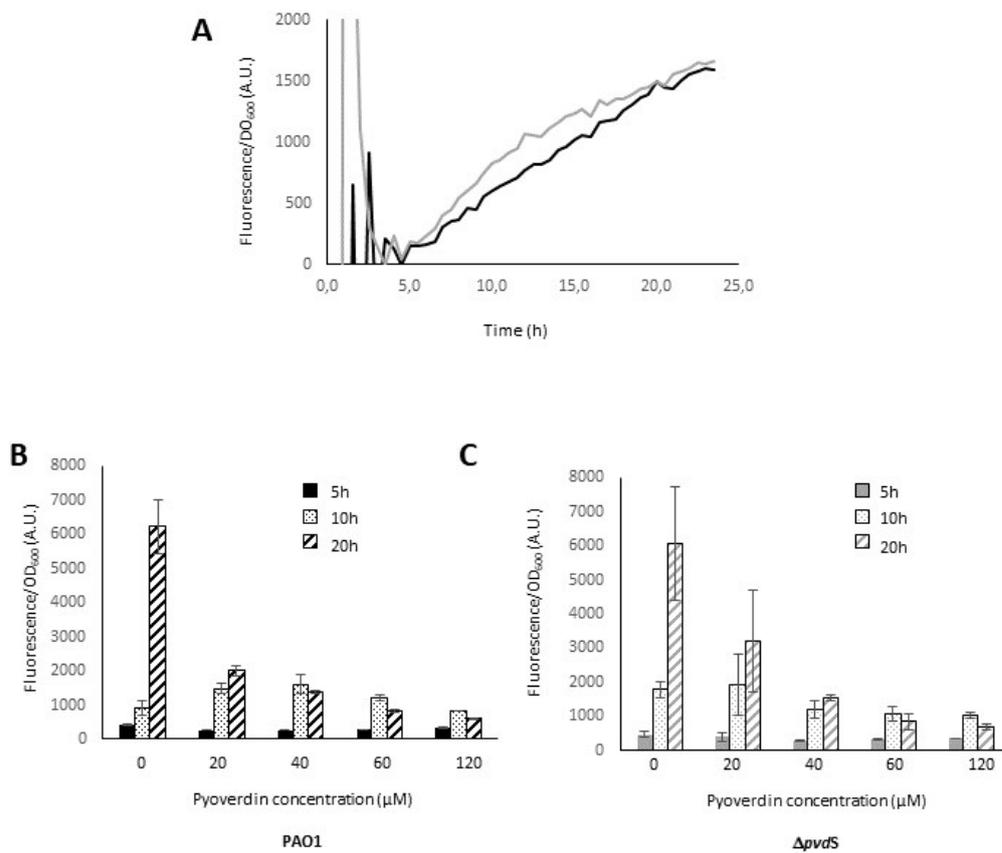


Figure 7