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# How the Presence of Hemin Affects the Expression of the Different Iron Uptake Pathways in *Pseudomonas aeruginosa* Cells

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**How the presence of hemin affects the expression of all iron-uptake pathways in *Pseudomonas aeruginosa* cells**

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3 **How the presence of hemin affects the expression of all iron-uptake pathways in**  
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5 ***Pseudomonas aeruginosa* cells**  
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41 **Keywords:** *Pseudomonas aeruginosa*, iron homeostasis, hemin (heam) uptake, iron uptake,  
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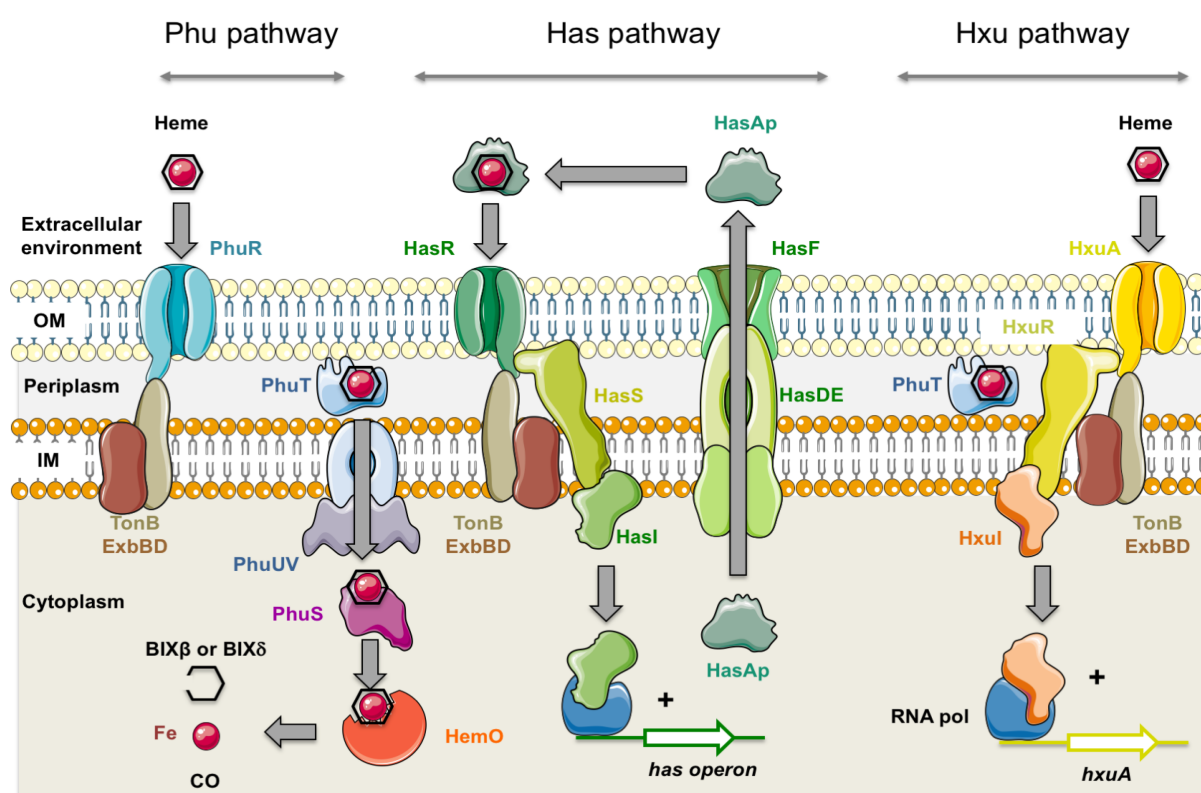
**ABSTRACT**

Iron is an essential nutriment for almost all organisms, but this metal is poorly bioavailable. During infection, bacteria access iron from the host by importing either iron or heme. *Pseudomonas aeruginosa*, a Gram-negative pathogen, secretes two siderophores, pyoverdine (PVD) and pyochelin (PCH), to access iron and is also able to use many siderophores produced by other microorganisms (called xenosiderophores). To access heme, *P. aeruginosa* uses three distinct uptake pathways, named Has, Phu, and Hxu. We previously showed that *P. aeruginosa* expresses the Has and Phu heme uptake systems, as well as the PVD- and PCH-dependent iron-uptake pathways, in iron-restricted growth conditions, using proteomic and RT-qPCR approaches. Here, using the same approaches, we show that physiological concentrations of hemin in the bacterial growth medium result in the repression of the expression of the proteins of the PVD- and PCH-dependent iron-uptake pathways, leading to less production of these two siderophores. This indicates that the pathogen adapts its phenotype to use hemin as an iron source rather than produce PVD and PCH to access iron. Moreover, the presence of both hemin and a xenosiderophore resulted in (i) the strong induction of the expression of the proteins of the added xenosiderophore-uptake pathway, (ii) repression of the PVD- and PCH-dependent iron-uptake pathways, and (iii) no effect on the expression levels of the Has, Phu, or Hxu systems, indicating that bacteria use both xenosiderophores and heme to access iron.

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3 Iron is essential for bacterial growth, virulence, and survival. This metal is used as an enzymatic  
4 cofactor and is required for many essential processes, such as deoxynucleotide biosynthesis,  
5 DNA replication, and respiration.<sup>1,2</sup> Iron can exist in two different oxidation states in biological  
6 systems, the ferric ( $\text{Fe}^{3+}$ ) or ferrous ( $\text{Fe}^{2+}$ ) forms, both playing an essential role in biological  
7 oxidation-reduction processes. In the human body, iron is tightly bound by high-affinity binding  
8 proteins, such as transferrin, lactoferrin, or ferritin, to prevent iron access to bacterial pathogens  
9 and, consequently, bacterial infections. This mechanism is called “nutritional immunity”.<sup>33</sup>  
10 However, most iron in the human body is included in heme, an aromatic tetrapyrrole  
11 macrocycle (protoporphyrin IX) that chelates one atom of iron. Heme is present in  
12 hemoproteins, such as hemoglobin and myoglobin.  
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26 The opportunistic human pathogen *Pseudomonas aeruginosa* is able to express three uptake  
27 pathways to access heme from the host as an iron source: the Has (Hemin assimilation system),  
28 Phu (*P*seudomonas heme utalization), and Hxu (Hemopexin utilization) pathways (Figure 1).  
29 This requires that *P. aeruginosa* expresses three different outer-membrane transporters to  
30 acquire heme from the environment: HasR, PhuR, and HxuA. These three transporters belong  
31 to the family of TonB-dependent transporters (TBDTs), as their uptake activity is regulated by  
32 the proton motive force of the inner membrane via the inner-membrane protein TonB.<sup>4</sup> HasR  
33 recognizes the HasAp-heme complex at the bacterial cell surface, as HasAp is a hemophore  
34 protein synthesized by *P. aeruginosa* and secreted into the extracellular environment by the  
35 HasDEF secretion system to scavenge heme from the environment.<sup>5</sup> HasAp of *P. aeruginosa*  
36 binds heme with a  $K_D$  of  $350 \pm 50$  nM affinity and afterwards interacts with HasR at the  
37 bacterial cell surface.<sup>6</sup> Once the HasR-HasAp-heme complex has been formed, heme is  
38 transferred from HasAp to the HasR transporter for uptake across the outer membrane.<sup>7-9</sup> Heme  
39 acquisition by the two other outer-membrane transporters, PhuR and HxuA, does not rely on a  
40 hemophore protein, as both transporters can directly bind heme.<sup>7,8</sup> Once in the periplasm, heme  
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transported by either HasR, PhuR, or HxuA, interacts with the periplasmic binding protein PhuT<sup>10</sup> for transfer to the ABC transporter PhuUV and uptake across the inner membrane. In the cytoplasm, heme binds to the cytosolic protein PhuS<sup>11</sup> and is transferred to the heme oxygenase HemO. The HemO protein cleaves heme to release biliverdin IX isoforms  $\beta$  (BIX $\beta$ ) or  $\delta$  (BIX $\delta$ ), carbon monoxide (CO), and iron.<sup>12,13</sup>



**Figure 1.** The three heme uptake pathways of *P. aeruginosa*. For more details, see the text in the introduction. OM, outer membrane; IM, inner membrane.

The transcription and expression of the genes of the Has system are regulated by an anti-sigma factor HasS and its corresponding ECF sigma factor HasI.<sup>14</sup> Once the heme has been transferred from HasAp to the TBDT HasR, the latter interacts through its periplasmic domains with the anti-sigma factor HasS, located in the inner membrane and spanning into the periplasm, to release the sigma transcriptional regulator HasI into the cytoplasm.<sup>14</sup> HasI then binds to RNA polymerase and upregulates transcription of the *hasR*, *hasA*, and *hasS* genes (*has* operon).<sup>13,14</sup>

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3 Moreover, BIX $\beta$  and BIX $\delta$  post-transcriptionally regulate HasAp translation and consequently  
4 the levels of this protein produced by *P. aeruginosa* cells.<sup>13,14</sup> The HxuA protein is also  
5 associated with an anti-sigma protein, HxuR, and an ECF sigma factor, HxuI. However,  
6 transcription of the genes of this system is activated in the presence of high concentrations of  
7 heme.<sup>15</sup> Has and Phu systems are both required for efficient utilization of heme in *P.*  
8 *aeruginosa*, with PhuR being the major heme transporter and HasR acting as a high affinity  
9 heme sensor of hemin present in the bacterial environment through its interaction with HasA  
10 and has an additional role in heme uptake.<sup>7,16</sup> HxuA will play a role only at higher heme  
11 concentrations.<sup>15</sup>

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24 In parallel with these heme-uptake pathways, *P. aeruginosa* is also able to access ferrous iron  
25 via the FeoABC inner-membrane transporter<sup>17</sup> and ferric iron by producing two siderophores,  
26 pyoverdine (PVD) and pyochelin (PCH).<sup>18,19</sup> Once they have scavenged iron in the bacterial  
27 environment, the ferric forms of these two siderophores are transported back across the outer  
28 membrane by their specific outer-membrane transporters, FpvA and FpvB for PVD-Fe<sup>3+</sup>,<sup>20,21</sup>  
29 and FptA for PCH-Fe<sup>3+</sup>.<sup>22</sup> These transporters also belong to the TBDT family and their uptake  
30 activity is regulated by the inner-membrane protein TonB. Iron is removed from PVD in the *P.*  
31 *aeruginosa* periplasm by a mechanism involving iron reduction and from PCH in the bacterial  
32 cytoplasm by an unknown mechanism.<sup>23,24</sup> *P. aeruginosa* can also use xenosiderophores  
33 (siderophores produced by other microorganisms) to acquire ferric iron in a siderophore-piracy  
34 strategy.<sup>25,26</sup> Indeed, the genome of *P. aeruginosa* contains various genes encoding TBDTs  
35 involved in ferri-xenosiderophore uptake. For example, *P. aeruginosa* is able to import ferric  
36 iron using the catechol xenosiderophore enterobactin (ENT) via the TBDTs PfeA and PirA,<sup>27-</sup>  
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30 and vibriobactin via FvbA.<sup>31</sup> It can also use the hydroxamate siderophores nocardamine  
(desferrioxamine E or NOCA) and desferrioxamine B (DFOB), transported by FoxA,<sup>32,33</sup> and  
ferrichrome, transported by FiuA.<sup>34,35</sup> *P. aeruginosa* also uses mycobactin and

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3 carboxymycobactin as xenosiderophores via the outer-membrane transporter FemA,<sup>36</sup>  
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5 aerobactin, rhizobactin, and schizokinen by ChtA,<sup>37</sup> and citrate by FecA.<sup>38</sup> Finally,  
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7 catecholamine neurotransmitters, such as dopamine, L-DOPA, epinephrine, and  
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9 norepinephrine, are able to chelate iron and efficiently import this nutrient into *P. aeruginosa*  
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11 cells *via* the outer-membrane transporters PiuA and PirA.<sup>26</sup>  
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15 Transcription of all the various genes encoding proteins involved in these ferric iron-uptake  
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17 pathways via siderophores is regulated either by sigma and anti-sigma factors, two-component  
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19 systems or transcriptional regulators of the AraC family.<sup>36,39,40</sup> The bacteria activate the  
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21 expression of the iron- or heme-uptake pathway(s) that are the most effective, depending on the  
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23 environment of the bacteria.<sup>26</sup> Previous proteomic and transcriptomic studies of our group have  
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25 shown how *P. aeruginosa* adapts the expression of its various iron-uptake pathways to  
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27 xenosiderophores.<sup>26,35,41</sup> Moreover, Hijazi *et al.* showed that the absence of PVD and PCH  
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29 biosynthesis increases the susceptibility of *P. aeruginosa* to Gallium-protoporphyrin (a toxic  
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31 analogue of heme), suggesting an increase in *hasR* and *phuR* transcription<sup>42</sup> and a regulatory  
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33 link between hemin acquisition systems via the HasR and PhuR proteins and PVD and PCH  
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35 biosynthesis.  
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41 An increasing amount of data show that siderophores can be used to import antibiotics into  
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43 bacteria in a Trojan-horse strategy: antibiotics are covalently attached to siderophores and  
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45 transported into the pathogens during the ferric-siderophores uptake process.<sup>43–45</sup> Such a  
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47 strategy increases the capacity of antibiotics to cross the bacterial envelope and accumulate in  
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49 the bacterial inner space. Cefiderocol, one such siderophore-antibiotic conjugate developed by  
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51 Shionogi, has been approved by the European Medicine Agency in April 2020 for the treatment  
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53 of infections due to aerobic Gram-negative organisms.<sup>46,47</sup> One major bottleneck limiting the  
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55 development of siderophore-antibiotic conjugates is that bacteria are equipped with several iron  
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57 uptake strategies, heme and ferri-siderophores uptake pathways. To make vectorization of  
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3 antibiotics by siderophores successful, it is important to understand how bacteria select and  
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5 synchronize the expression of their different iron import pathways in response to different iron  
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7 sources. For example, several studies highlighted that multiple *P. aeruginosa* iron uptake  
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9 mechanisms are active in chronic cystic fibrosis (CF) infection, with heme uptake pathways  
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11 playing a crucial role in adaptation of *P. aeruginosa* to this host environment.<sup>16,48–52</sup>  
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14 Vectorisation of antibiotics by siderophores needs the expression during infections of the  
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16 uptake pathways target by the siderophore-antibiotic conjugates.  
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20 Here, we investigated how a physiological concentration of heme affects the expression of  
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22 proteins of the various iron- and heme-uptake pathways present in the genome of *P. aeruginosa*  
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24 using differential proteomic and RT-qPCR approaches. We also tested the effect of the presence  
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26 of both heme and two catecholate iron chelators (TCV, an iron chelator used for antibiotic  
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28 vectorisation and ENT, a siderophore produced by *E. coli*) or heme and two hydroxamate  
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30 xenosiderophores (NOCA and DFOB both produced by actinomycetes) using the same  
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32 approaches. The presence of heme alone repressed the expression of the PVD- and PCH-  
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34 dependent iron-uptake pathways and slightly induced the expression of *hasR*, the TBDT of the  
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36 *has* system (the *has* and *phu* heme uptake pathways being already strongly expressed in iron-  
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38 restricted condition without heme<sup>26</sup>). The presence of heme with ENT, TCV, NOCA or DFOB,  
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40 induced the expression of the corresponding xenosiderophore-dependent iron uptake pathways,  
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42 the PVD- and PCH-dependent iron-uptake pathways were more strongly repressed than in the  
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44 presence of heme alone, and the Has and Phu heme uptake pathways have their expression  
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46 levels not affected by the presence of the tested xenosiderophores compared to the growth of  
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51 *P. aeruginosa* in CAA medium.  
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## RESULTS AND DISCUSSION

### Effect of 0.25 $\mu$ M hemin on the transcription and expression of *hasR*, *phuR*, and *hxuA*.

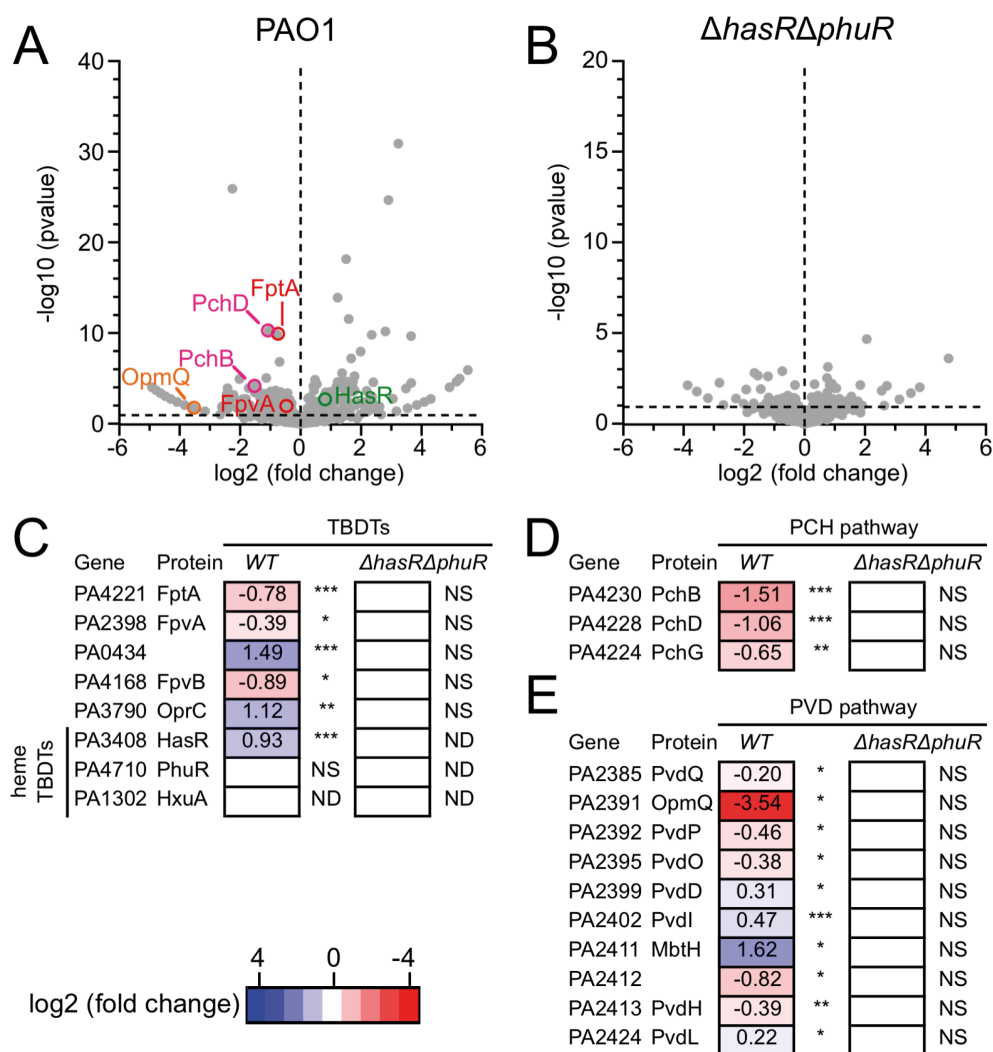
Previously, we have shown that the growth of *P. aeruginosa* in the absence of hemin in CAA medium (an iron-restricted medium with an iron concentration of approximately 20 nM<sup>53</sup>) induces the expression of proteins of the PVD- and PCH-dependent iron-uptake pathways, as well as HasR and PhuR, with log<sub>2</sub> fold changes in their expression of 5.96 and 5.62, respectively, relative to growth in LB medium (Table S1).<sup>26</sup> These previous data clearly show that HasR and PhuR expression is already strongly induced in iron-starved *P. aeruginosa* cells (grown in CAA), even if no hemin is present in the bacterial environment (Table S1). In addition, when grown in CAA medium, *P. aeruginosa* also expresses ChtA, the TBDT of the siderophores aerobactin, rhizobactin and schizokinen,<sup>37</sup> PirA, a ferri-catechol siderophore outer membrane TBDT, two TBDTs for which the siderophore transported is still unknown, encoded by PA0434 and PA3268, and OprC, a TBDT involved in Cu uptake<sup>54</sup> (Table S1). Moreover, we have also shown that the presence of xenosiderophores in *P. aeruginosa* growth media often strongly induces the expression of the proteins of their corresponding uptake pathways.<sup>26,32,35,36,41,45</sup>

Here, we first investigated the effect of 0.25  $\mu$ M hemin on the expression of the TBDT genes *hasR*, *phuR* and *hxuA*, involved in hemin uptake in *P. aeruginosa* cells, using differential proteomic approaches. The intracellular concentration of hemin in eukaryotic cells is estimated to be between 0.025 and 0.3  $\mu$ M,<sup>55</sup> and the K<sub>D</sub> of HasAp for hemin of 350  $\pm$  50 nM,<sup>6</sup> leading us to choose the concentration of 0.25  $\mu$ M hemin for our experiments. PAO1 was grown in CAA medium, with or without 0.25  $\mu$ M hemin, for 8 h and analyzed by differential proteomics (Figure 2). The 8-h cultures were also analyzed by RT-qPCR to follow the transcription of the *hasR* and *phuR*, genes (Figure 3). Outer-membrane transporters are very easily detected by proteomic techniques, often better than any other proteins involved in iron acquisition

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3 pathways.<sup>26,32,35,36,41,45</sup> Thus, TBDTs can be used as indicators to follow variations in expression  
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5 of iron-uptake pathways in *P. aeruginosa* cells.  
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8 The addition of 0.25  $\mu\text{M}$  hemin to the CAA growth medium resulted in a small additional  
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10 induction of the expression of HasR in PAO1 cells, with a  $\log_2$  fold change of 0.93 (Figure 2)  
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12 and PhuR and HxuA expression was not modified. In addition, we observed no significant  
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14 changes in the expression of the other genes encoding proteins of these three heme-uptake  
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16 pathways (presented in Figure 1). RT-qPCR data showed only slight upregulation of  
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18 transcription for *hasR* after 8 h ( $\log_2$  fold change of 0.99). In conclusion, the heme-uptake  
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20 pathways involving HasR and PhuR are already expressed at high levels in iron-restricted  
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22 conditions (CAA medium), even if no hemin is present in the bacterial environment.<sup>26</sup> The  
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24 addition of 0.25  $\mu\text{M}$  hemin to CAA growth media has only a minor additional impact on the  
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26 transcription and expression of *hasR* and no effect on *phuR* gene transcription. The additional  
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28 small induction of the transcription of *hasR* is due to the presence of hemin at 0.25  $\mu\text{M}$  and the  
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30 activation of transcription of the genes of this Has pathway by the anti-sigma and sigma factors  
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32 HasS and HasI after formation of the HasR-HasAp-hemin complex at the bacterial cell  
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34 surface.<sup>7,56</sup> There is no such transcriptional regulation for the Phu pathway and the expression  
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36 of the Hxu pathway is only activated at higher hemin concentrations<sup>15</sup> (Figure 1). Moreover,  
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38 growth assays in CAA medium of PAO1 and  $\Delta\textit{hasR}\Delta\textit{phuR}$  strains showed that the Has and Phu  
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40 heme-uptake pathways are mostly involved in hemin acquisition at 0.25  $\mu\text{M}$  hemin and not the  
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42 Hxu pathway (Figure S1). Indeed, PAO1 grown in the presence of 0.25  $\mu\text{M}$  hemin showed a  
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44 clear growth stimulation compared to the growth in the absence of hemin. Besides, in the  
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46 absence of hemin, PAO1 has a similar growth curve as a  $\Delta\textit{hasR}\Delta\textit{phuR}$  mutant in the presence  
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48 or absence of hemin, indicating that in our growth conditions only the Has and Phu systems are  
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50 involved in hemin acquisition and not the Hxu pathway. According to Otero-Asman *et al.*,  
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52 expression of the Hxu pathway is induced at higher hemin (20  $\mu\text{M}$ ) concentrations and probably  
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imports heme when present at higher concentrations in the bacterial environment.<sup>15</sup> In the host, during infection, bacteria growth under iron restriction conditions due to the inflammation-dependent iron sequestration response,<sup>3</sup> and in such iron-restricted conditions, the Has and Phu heme uptake pathways must be expressed and probably not the Hxu pathway. Indeed, gene expression analysis showed that heme is a critical source of iron during prolonged infection of the CF lung.<sup>16,48–52</sup>



**Figure 2: Differential proteomic analyses of *P. aeruginosa* PAO1 grown in the presence of TCX or TCX and heme. A.** PAO1 cells PAO1 and  $\Delta hasR\Delta phuR$  cells grown in CAA minimal medium and in the presence of 0.25  $\mu$ M heme. PAO1 (A) or  $\Delta hasR\Delta phuR$  (B) strains were grown in CAA medium, with or without heme (0.25  $\mu$ M), for 8 h and differential proteomic expression analyzed. The average values measured in CAA medium without heme were plotted against the average values in CAA supplemented with heme. C-E. Heat maps of the TBDTs showing a change in their expression (C), as well as

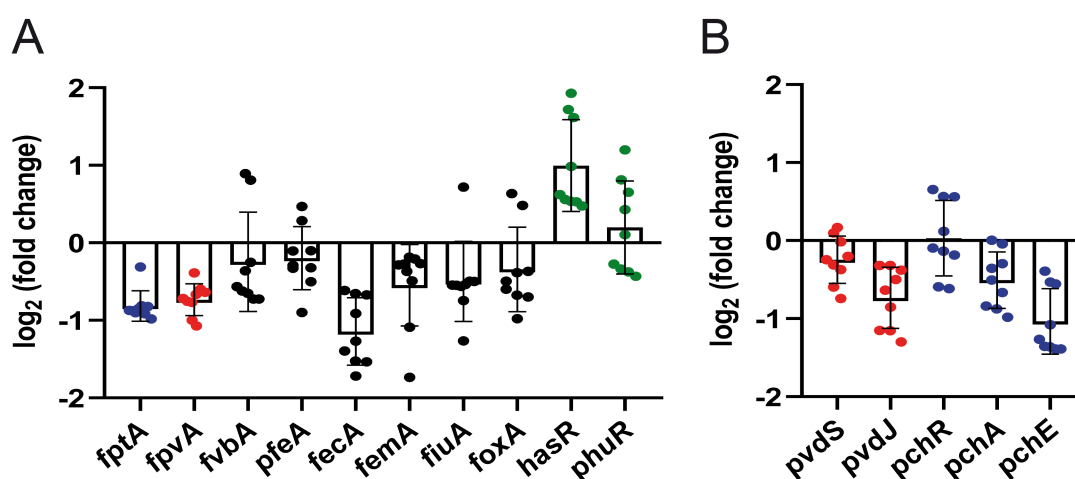
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3 proteins involved in PCH- (D) and PVD-dependent (E) iron-uptake pathways. In these heat  
4 maps, we chose to show HasR, PhuR and HxuA and the proteins for which a change in the level  
5 of expression was observed in one of the two conditions analyzed (PAO1 or  $\Delta hasR\Delta phuR$ ).  
6 The darker the shade of blue, the more expression of the protein is induced; the darker the shade  
7 of red, the more expression of the protein is repressed. NS: data not significant; ND: not  
8 detected (no peptide corresponding to the protein was detected by proteomic); \*p < 0.05, \*\*p  
9 < 0.01, and \*\*\*p < 0.001  
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#### 14 **The presence of 0.25 $\mu$ M hemin represses the transcription and expression of genes**

15 **involved in siderophore-dependent iron-uptake pathways.** The differential proteomic assay  
16 on PAO1 grown in CAA in the absence or presence of 0.25  $\mu$ M hemin described above also  
17 showed that the presence of a low concentration of hemin significantly downregulates the  
18 expression of genes encoding enzymes involved in PCH biosynthesis, such as PchB, PchD, and  
19 PchG (Figure 2). We observed an equivalent effect for the genes encoding proteins of the PVD  
20 pathway, with the outer membrane protein OpmQ of the efflux system PvdRT-OpmQ being the  
21 most highly affected, with a log<sub>2</sub> decrease in expression of 3.54-fold (PvdRT-OpmQ is involved  
22 in the secretion of newly synthesized PVD and PVD following the delivery of iron into *P.*  
23 *aeruginosa* cells<sup>57-59</sup>). For the TBDTs involved in the import of ferri-PVD and ferri-PCH, the  
24 proteomic data showed a small repression of the expression of the *fpvB*, *fpvA*, and *fptA* genes  
25 encoding the outer-membrane transporters of ferri-PVD and ferri-PCH, respectively. We also  
26 followed by RT-qPCR changes in the transcription of *fpvA*, *fptA*, and *pvdJ* (a gene encoding an  
27 enzyme involved in PVD biosynthesis), *pchA* and *pchE* (two genes encoding enzymes involved  
28 in PCH biosynthesis), and *pvdS* and *pchR* (two transcriptional regulators of the PVD and PCH  
29 pathways, respectively)<sup>19</sup> (Figure 3). The data showed downregulation of the transcription of  
30 *fpvA*, *fptA*, *pvdJ*, *pchA*, and *pchE* in the presence of 0.25  $\mu$ M hemin. The transcription of *pvdS*  
31 and *pchR* was less strongly affected by the presence of hemin.  
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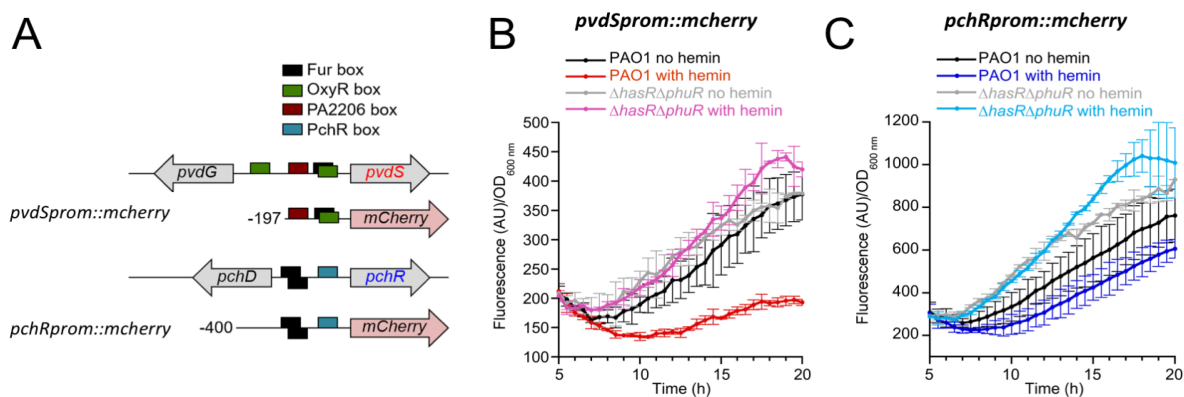
55 To validate the effect of hemin on the transcription and expression of the various genes and  
56 proteins involved in the PVD and PCH pathways, the promoter regions regulating transcription  
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of the *pvdS* and *pchR* genes were fused upstream of the *mCherry* gene to obtain the following reporter plasmids: *pvdSprom::mCherry* and *pchRprom::mCherry*. The *pvdS* promoter region contains a Fur box, a sequence interacting with the PA2206 LysR regulator and one with the regulator OxyR.<sup>60–62</sup> Fur is a transcriptional regulator responsible for controlling the intracellular concentration of iron in the bacteria and regulating negatively *pvdS* transcription.<sup>60,63</sup> PA2206 and OxyR, both contribute to an effective oxidative stress response in *P. aeruginosa* and are able to activate *pvdS* transcription under oxidative stress.<sup>61,62,64</sup> The *pchR* promoter region is composed of both a Fur box for transcriptional repression and a PchR box for transcriptional activation (PchR being the transcriptional AraC regulator of the PCH pathway).<sup>65</sup>



**Figure 3. Modulation of the transcription of genes encoding outer membrane transporters involved in iron or heme acquisition and genes of the PVD and PCH pathways in *P. aeruginosa* cells when grown in CAA medium with or without 0.25  $\mu$ M hemin.** *P. aeruginosa* PAO1 was grown as for the proteomic analyses in CAA medium, with or without 0.25  $\mu$ M hemin, for 8 h. Gene transcription was normalized to that of the housekeeping gene *uvrD* reference gene. The results show the ratio between the values obtained in the presence of hemin over those obtained in the absence of hemin. **A.** *fptA* encodes the TBDT of PCH, *fpvA* that of PVD, *fvbA* that of vibriobactin, *pfeA* that of enterobactin, *fecA* that of citrate, *femA* that of mycobactin and carboxymycobactin, *fiuA* that of ferrichrome, *foxA* that of DFOB and NOCA, and *hasR* and *phuR* that of hemin. **B.** *pvdJ* encodes an enzyme involved in PVD biosynthesis, *pchA* and *pchE* enzymes involved in PCH biosynthesis, *pvdS* a sigma factor regulating the transcription of the PVD pathway and *pchR* a transcriptional regulator of the PCH pathway. The data show the mean of three independent experiments. The data concerning genes of the PVD pathway are shown in red in both graphs, those of the PCH pathway in blue, those of the heme uptake pathways in green, and those encoding other outer membrane transporters in grey.

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6 *P. aeruginosa* PAO1 and  $\Delta hasR\Delta phuR$  cells were transformed with these plasmids. The growth  
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8 of PAO1 cells expressing mCherry, either under the *pvdS* promoter or the *pchR* promoter, in  
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10 CAA medium resulted in an increase in mCherry expression from both plasmids, but it was  
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12 two-fold greater from the *pchRprom::mCherry* plasmid than the plasmid carrying the *pvdS*  
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14 promoter (Figure 4B and 4C). Addition of 0.25  $\mu$ M hemin to the growth media almost  
15  
16 completely abolished the fluorescence for the strain carrying the *pvdS* promoter (regulating the  
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18 PVD pathways) on the plasmid, indicating strong repression of mCherry expression. We also  
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20 observed a decrease in fluorescence for the *pchR* promoter (regulating PCH pathway), but of a  
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22 lower amplitude, suggesting that mCherry expression was not completely abolished. These  
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24 results show that the presence of 0.25  $\mu$ M hemin in CAA medium represses the transcription  
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26 and expression of the various genes of the PVD- and PCH-dependent iron-uptake pathways in  
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28 PAO1 but with different efficiencies.  
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47 **Figure 4. Expression of mCherry under the control of *pvdS* or *pchR* promoters in *P.***  
48 ***aeruginosa* PAO1 cells grown in CAA medium with or without 0.25  $\mu$ M hemin. **A.****  
49 **Schematic representation of *pvdS* and *pchR* promoters and plasmid constructs used in the**  
50 **experiments of panels A and B. Fur boxes are shown in black, OxyR boxes are shown in green,**  
51 **PA2206 boxes are shown in brown and the PchR boxes on the *pchR* promoter are shown in**  
52 **blue. **B-C.** *P. aeruginosa* PAO1 or  $\Delta hasR\Delta phuR$  cells expressing mCherry under the control of**  
53 **the *pvdS* promoter (plasmid: *pvdS*prom::mCherry) (panel **B**) or *pchR* promoter (plasmid:**  
54 ***pchR*prom::mCherry) (panel **C**) were grown overnight in CAA medium. Then, the cells were**  
55 **resuspended in CAA medium with or without 0.25  $\mu$ M hemin and the mCherry fluorescence**  
56 **monitored during bacterial growth. Curves represent the averages of three independent**  
57 **experiments.**  
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3 In terms of the other siderophore-dependent iron-uptake pathways present in the *P. aeruginosa*  
4 genome, RT-qPCR analysis showed very slight downregulation of the transcription of all the  
5 genes encoding xenosiderophore outer-membrane transporters tested, *fvbA*, *pfeA*, *fecA*, *femA*,  
6 *fiuA* and *foxA*, but which may not be biologically significant (Figure 3). Finally, the proteomic  
7 data showed that the presence of 0.25  $\mu$ M hemin also slightly induced the expression of PA0434  
8 and OprC in PAO1 (OprC is a TBDT involved in Cu uptake) (Figure 2).  
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12 In conclusion, the presence of 0.25  $\mu$ M hemin in the environment slightly represses the  
13 expression of the PVD and PCH-dependent iron-uptake pathways in *P. aeruginosa*, as well as  
14 the expression of several xenosiderophore-dependent iron-uptake pathways. This indicates that  
15 the bacteria adapt their phenotype to access iron, mostly via heme uptake, when heme is the  
16 major iron source present in their environment. This decrease in the expression of the various  
17 siderophore-dependent iron-uptake pathways could be due to two different regulating  
18 mechanisms. One possibility is that fewer PVD-, PCH and xenosiderophore-Fe complexes are  
19 formed and able to activate the expression of the corresponding iron-uptake pathways via  
20 transcriptional regulators.<sup>36,39,40</sup> The second possibility is that the bacterial intracellular iron  
21 concentration increased, due to heme uptake, leading to repression of the transcription of the  
22 siderophore-dependent iron-uptake pathways via the Fur transcriptional regulator. When the  
23 intracellular iron concentration reaches a certain level, ferrous iron binds to Fur and the formed  
24 complex represses the transcription of iron-regulated genes, such as those encoding proteins  
25 involved in siderophore-dependent iron-uptake pathways.<sup>63</sup> However, with such a scenario, one  
26 should also see a decrease in the expression of the proteins involved in heme import. We  
27 observed no repression in the expression of the heme-uptake pathway, because transcription of  
28 the genes involved is induced by the presence of hemin via the HasI and HasR transcriptional  
29 regulators, this positive regulation (activation of transcription) counteracting probably the  
30 negative regulation (repression of transcription) by Fur.  
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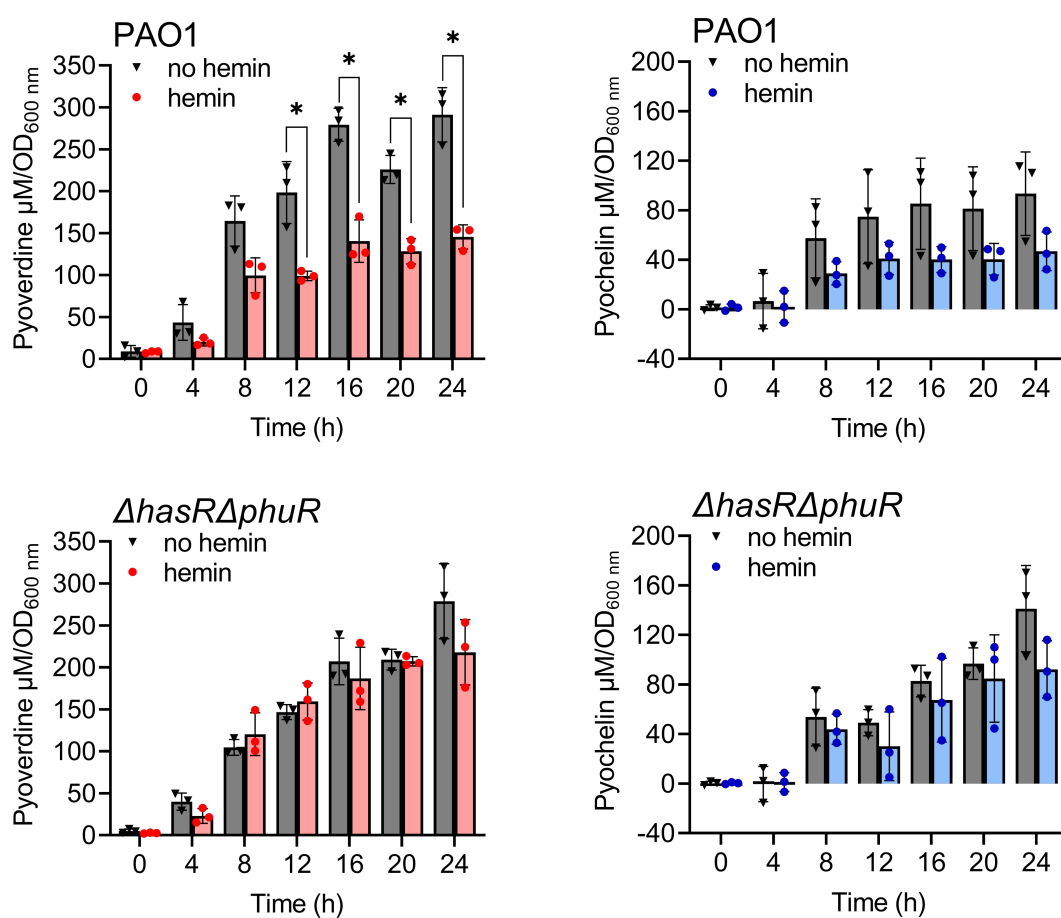
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6 **Repression of the expression of genes encoding proteins of the PCH- and PVD-dependent**  
7 **iron-uptake pathways by hemin is HasR and PhuR dependent.** We carried out differential  
8 proteomic analyses on the  $\Delta hasR\Delta phuR$  mutant, deleted for both the *hasR* and *phuR* genes and  
9 consequently unable to import hemin into *P. aeruginosa* cells by the outer-membrane  
10 transporters HasR and PhuR (Figure 2 and Figure S1). We no longer observed the repression  
11 of the transcription and expression of the genes of the PCH-, PVD-, or xenosiderophore-  
12 dependent iron-uptake pathways observed in PAO1 grown in the presence of hemin, in the  
13  $\Delta hasR\Delta phuR$  mutant. These data suggest that the repression of the expression of the genes  
14 encoding proteins of the PCH-, PVD- and xenosiderophore-dependent iron-uptake pathways  
15 by hemin requires the expression of *hasR* and *phuR* and the uptake of hemin by the bacteria  
16 and, consequently, involves Fur, the transcriptional regulator that senses the intracellular iron  
17 concentration.

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36 **The presence of 0.25  $\mu\text{M}$  hemin downregulates the production of PVD and PCH.** We  
37 further investigated the effect of hemin on the expression of genes encoding proteins involved  
38 in the PVD- and PCH-dependent iron-uptake pathways by monitoring the amount of PVD and  
39 PCH produced by bacteria grown in the absence or presence of hemin. PAO1 was grown in  
40 CAA medium with or without 0.25  $\mu\text{M}$  hemin and aliquots removed at various times, the  
41 bacteria pelleted, and PVD and PCH concentrations monitored in the growth media (Figure 5).  
42 We observed an increase in PVD and PCH production for PAO1 grown in the absence of hemin  
43 after 8 h of culture, which reached a plateau after 16 h of approximately  $290 \pm 35 \mu\text{M}/\text{OD}_{600 \text{ nm}}$   
44 for PVD and  $92 \pm 37 \mu\text{M}/\text{OD}_{600 \text{ nm}}$  for PCH. The presence of hemin decreased PVD production  
45 by 49% ( $147 \pm 33 \mu\text{M}/\text{OD}_{600 \text{ nm}}$ ) and that of PCH by 48% ( $47 \pm 13 \mu\text{M}/\text{OD}_{600 \text{ nm}}$ ) after 24 h of  
46 culture (Figure 5). Such repression of PVD production was not observed in the  $\Delta hasR\Delta phuR$

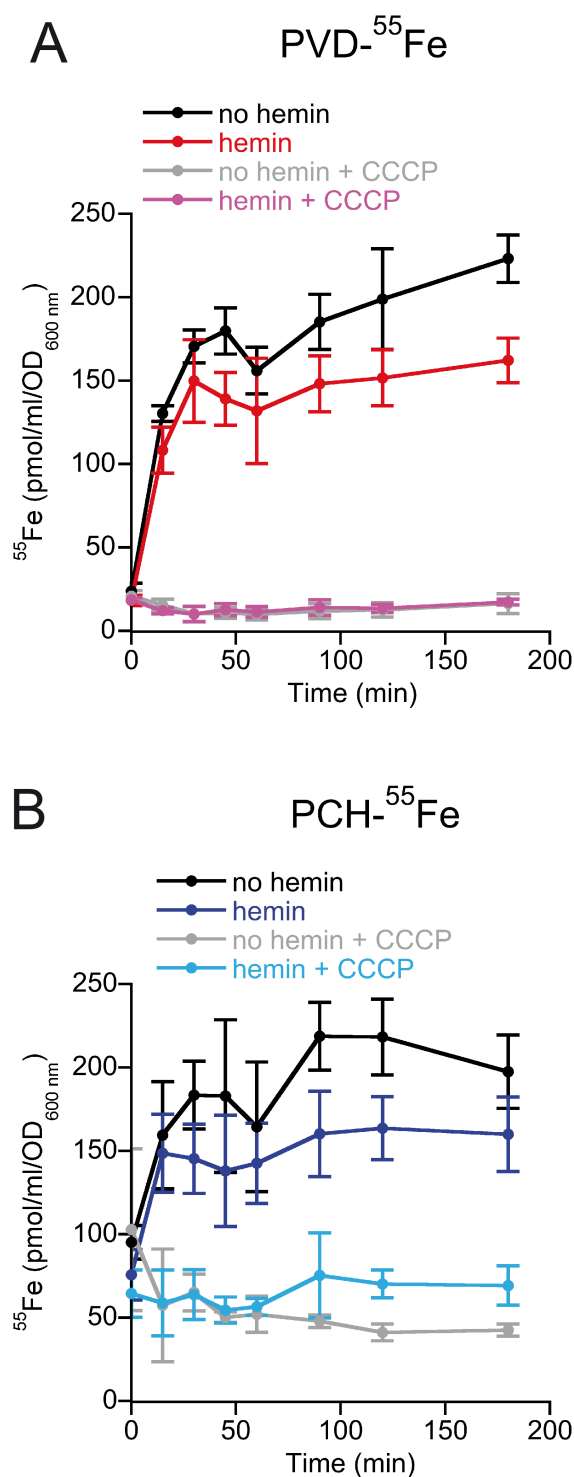
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3 mutant and it was much less for PCH production. These data for PVD and PCH production are  
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5 consistent with those for the transcription and expression of the PVD and PCH biosynthesis  
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7 genes observed in the absence or presence of 0.25  $\mu\text{M}$  hemin in PAO1 and  $\Delta\text{hasR}\Delta\text{phuR}$ .  
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9 Reduction in the expression of the enzymes involved in PVD and PCH biosynthesis in *P.*  
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11 *aeruginosa* PAO1 cells grown in the presence of hemin resulted in the production of less  
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13 siderophores. These data confirm that, in the presence of 0.25  $\mu\text{M}$  hemin, bacteria prefer to  
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15 access iron by importing hemin than to use their own siderophores, PVD and PCH, for ferric  
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17 iron import. This is consistent with the work of Smith and Wilks showing a significant reduction  
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19 of siderophore production by *P. aeruginosa* strains during the course of CF lung infection.<sup>50</sup>  
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27 **The presence of 0.25  $\mu\text{M}$  hemin downregulates  $^{55}\text{Fe}$  import by PVD and PCH.** As described  
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29 above, the presence of 0.25  $\mu\text{M}$  hemin slightly repressed the transcription and expression of the  
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31 genes encoding outer-membrane transporters FpvA, FpvB, and FptA. We evaluated its impact  
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33 on iron acquisition by the siderophores PVD and PCH by growing PAO1 in CAA minimal  
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35 medium in the absence or presence of 0.25  $\mu\text{M}$  hemin for 16 h. The cells were then used to  
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37 monitor  $^{55}\text{Fe}$  uptake via the siderophores PVD and PCH. PAO1 was incubated in the presence  
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39 of  $^{55}\text{Fe}$ -PVD or  $^{55}\text{Fe}$ -PCH complexes and aliquots removed at various times, the bacteria  
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41 pelleted, and the radioactivity in the bacteria counted. In the absence of hemin in the growth  
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43 culture, bacteria were able to acquire  $^{55}\text{Fe}$  via PVD and PCH, with uptake rates of  $223 \pm 14$   
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45 pmol/ml/OD<sub>600 nm</sub> for PVD and  $197.48 \pm 21.90$  pmol/ml/OD<sub>600 nm</sub> for PCH (Figure 6). When  
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47 PAO1 was grown in the presence of 0.25  $\mu\text{M}$  hemin, less  $^{55}\text{Fe}$  was taken up via PVD ( $162.18$   
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49  $\pm 13.36$  pmol/ml/OD<sub>600 nm</sub> after 3 h) and PCH ( $160.00 \pm 22.39$  pmol/ml/OD<sub>600 nm</sub> after 3 h)  
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51 (Figure 6A). Carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), an inhibitor of the inner-  
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53 membrane proton motive force, was used to verify that the  $^{55}\text{Fe}$  uptake observed was not a  
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55 consequence of iron diffusion across the bacterial membranes but due to the active uptake via  
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TBDTs. Indeed, CCCP inhibits all proton motive-dependent uptake in Gram-negative bacteria, such as the uptake of iron via siderophores.<sup>66</sup> In the presence of CCCP, no <sup>55</sup>Fe uptake by PVD or PCH was observed in the absence or presence of hemin in the bacterial culture (Figure 6), indicating that the uptake observed occurred via TBDTs and was not due to diffusion across porins.



**Figure 5. PVD and PCH production by *P. aeruginosa* PAO1 in the absence or presence of 0.25 μM hemin.** Bacteria were grown in CAA medium with or without 0.25 μM hemin. PVD (A) and PCH (B) production (μM/OD<sub>600 nm</sub>) was monitored during bacterial growth as described in materials and methods. The histogram values represent the averages of three independent experiments. The indicated p values as determined by a Student's *t* test (\* p < 0.05).



**Figure 6. Kinetics of <sup>55</sup>Fe uptake via PVD or PCH in *P. aeruginosa* PAO1 cells grown in the absence or presence of 0.25  $\mu$ M hemin.** The PAO1 strain was grown in CAA medium with or without hemin (0.25  $\mu$ M) for 16 h. Then, bacterial cells were washed in 50 mM Tris-HCl pH 8.0 and resuspended in the same buffer. Transport assays were initiated by adding 500 nM <sup>55</sup>Fe-PVD (A) or <sup>55</sup>Fe-PCH (B). Aliquots were removed at various times, centrifuged, and the radioactivity in the bacterial pellet measured. Results are expressed as atoms of <sup>55</sup>Fe transported per cell. Bacteria were also treated with 200  $\mu$ M of the protonophore CCCP to inhibit active iron uptake and visualize any iron diffusion across the bacterial membrane.

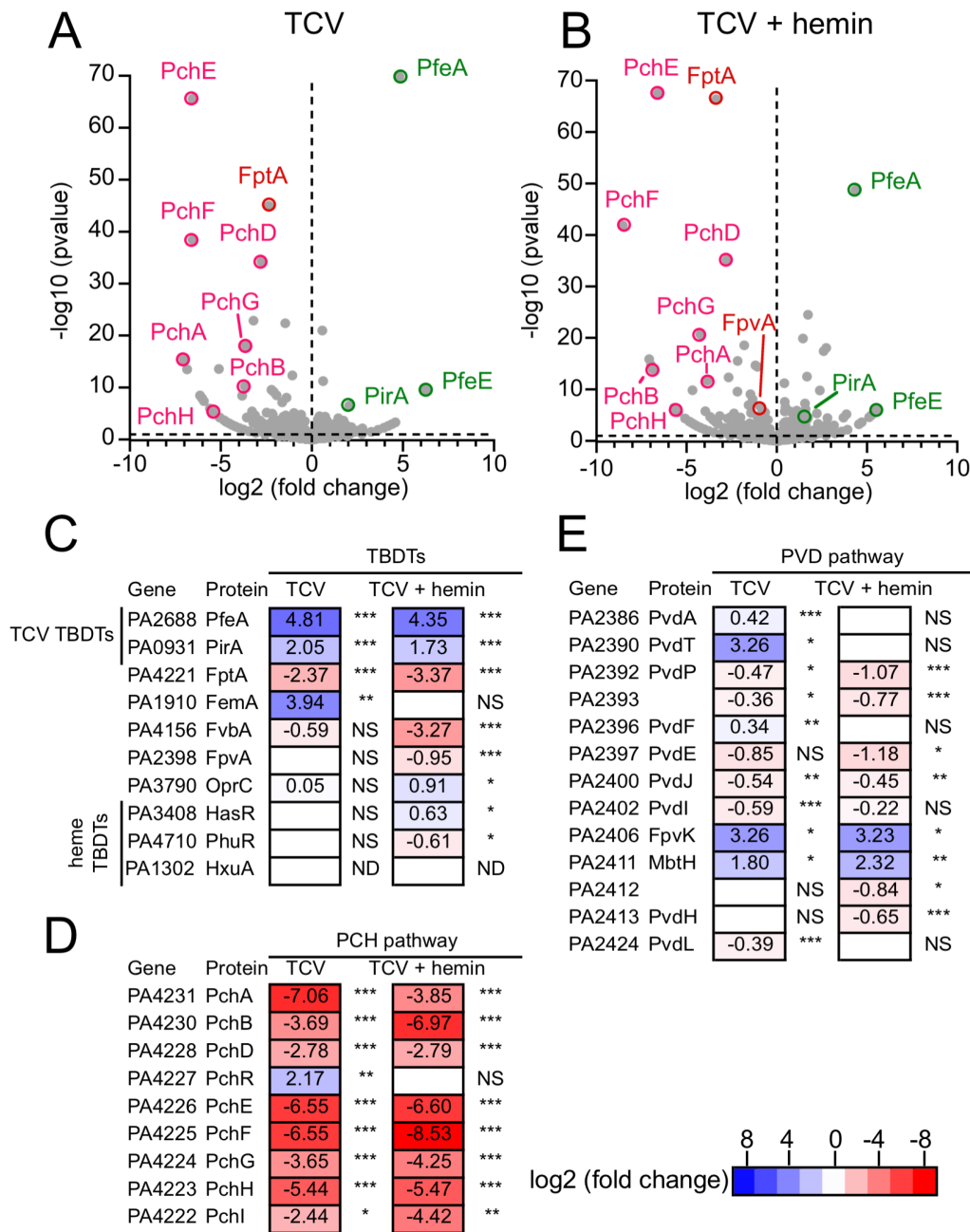
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3 In conclusion, the presence of hemin in the bacterial growth medium induces the partial  
4 repression of  $^{55}\text{Fe}$  uptake into *P. aeruginosa* cells by PVD and PCH due to the downregulation  
5 of *fpvA*, *fpvB*, and *fptA* transcription and expression of the corresponding transporters. Overall,  
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10 the data presented above show that the growth of *P. aeruginosa* cells in CAA medium in the  
11 presence of 0.25  $\mu\text{M}$  hemin results in expression of the Has and Phu pathways and strong  
12 repression of the PVD and PCH pathways (Table S1), but in a way that does not completely  
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15 abolish production of these two siderophores and iron uptake via these PVD- and PCH-  
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18 dependent pathways. It is likely that bacteria use both strategies to access iron in CAA medium  
19 supplemented with 0.25  $\mu\text{M}$  hemin, mainly hemin uptake and at a lesser extend ferric iron  
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22 uptake by PVD and PCH. If hemin becomes rare, the bacteria can easily and quickly switch to  
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25 iron access solely through their siderophores.  
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32 **Adaptation of the expression of the genes involved in the various iron-uptake pathways of**  
33 ***P. aeruginosa* PAO1 to the presence of hemin and xenosiderophores.** We explored the  
34 effects of a mixture of 0.25  $\mu\text{M}$  hemin and 10  $\mu\text{M}$  TCV, a synthetic catechol synthetic  
35 siderophore (Figure S3), and 0.25  $\mu\text{M}$  hemin and 10  $\mu\text{M}$  nocardamine (NOCA), a hydroxamate  
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38 siderophore (Figure S3), on *P. aeruginosa* PAO1 by differential proteomics. TCV is a synthetic  
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41 siderophore (Figure S3), on *P. aeruginosa* PAO1 by differential proteomics. TCV is a synthetic  
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44 analogue of the *E. coli*-produced siderophore enterobactin and is used in the literature for  
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47 antibiotic vectorization.<sup>45</sup> Previous studies have shown that the presence of TCV or TCV-  
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50 antibiotic compounds in the bacterial environment induces the transcription and consequently  
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53 the expression of the TBDT *pfeA*, which is the ferri-enterobactin outer-membrane transporter,  
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56 and *pfeE*, the esterase that hydrolyzes enterobactin to release bound iron.<sup>26</sup> Iron-loaded TCV or  
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59 TCV-antibiotic is imported, like ferri-ENT, by PfeA across the outer membrane.<sup>45,67</sup> NOCA  
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induces the transcription of *foxA* gene, FoxA being the TBDT internalizing the ferric form of  
NOCA.<sup>35</sup>

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3 As previously described, our differential proteomic data showed that the presence of 10  $\mu$ M  
4 TCV alone in the medium of PAO1 induces the expression of *pfeA* ( $\log_2$  fold change of 4.81),  
5 *pfeE* ( $\log_2$  fold change of 5.55), and *pirA*, another TBDT also involved in iron uptake via  
6 catechol xenosiderophores, ( $\log_2$  fold change of 2.05).<sup>26,41,45,67,68</sup> The presence of TCV also  
7 resulted in the repression of *fptA* expression ( $\log_2$  fold change of -2.37) and that of many genes  
8 encoding proteins of the PCH-dependent iron-uptake pathway (Figure 7A). In the presence of  
9 both 10  $\mu$ M TCV and 0.25  $\mu$ M hemin, the expression of *pfeA* and *pirA* was still induced with  
10 the same efficiency as with TCV alone. In addition, the expression of the various genes  
11 encoding proteins of the PCH pathway and *fvbA* (the TBDT of the catechol siderophore  
12 vibriobactin) were more highly repressed than in the presence of TCV alone. Taken together,  
13 these observations clearly show an additive effect of the 0.25  $\mu$ M hemin with the presence of  
14 10  $\mu$ M TCV (Figure 7B). In addition, we also observed slight repression of the expression of  
15 the outer-membrane transporter *fpvA* and no significant effect on the expression of the various  
16 other genes encoding proteins of the PVD pathway. By contrast, *hasR* expression increased  
17 slightly ( $\log_2$  fold change of 0.63; Figure 7C). No effect was observed on the expression of the  
18 genes encoding other proteins of the Has pathway or those of the Phu and Hxu pathways. RT-  
19 qPCR was used to confirm the proteomic data for *pfeA*, *hasR*, *fpvA* and *fptA* (Figure 8) and  
20 equivalent data were obtained. We also obtained similar results when *P. aeruginosa* was grown  
21 in the presence of 10  $\mu$ M enterobactin (ENT) or a mixture of 0.25  $\mu$ M hemin and 10  $\mu$ M ENT  
22 (Figure S4).  
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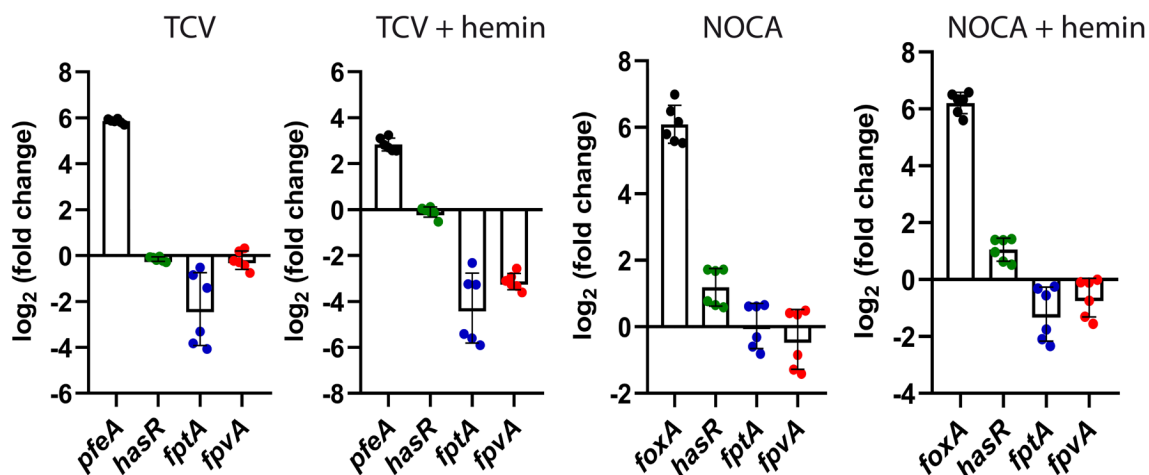
49 In the presence of 10  $\mu$ M NOCA alone, *foxA* expression was induced, as previously described  
50 ( $\log_2$  fold change of 8.43; Figure 9A).<sup>35</sup> In parallel, we observed downregulation of the  
51 expression of the PCH transporter *fptA* and other proteins of this pathway. Growth of the  
52 bacteria in the presence of 10  $\mu$ M NOCA and 0.25  $\mu$ M hemin (Figure 9) still resulted in an  
53 increase in the expression of *foxA* gene with, in addition, a small induction of *hasR* expression  
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3 (log<sub>2</sub> fold change of 0.75). On the contrary, we observed stronger repression of *fptA* expression  
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5 and that of various genes encoding proteins of the PCH pathway than with NOCA alone, as  
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7 well as slight repression of the expression of *fpvA* and *fpvB*, the two TBDTs of the PVD  
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9 pathway. These results were confirmed by RT-qPCR (Figure 8) and equivalent data were  
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11 obtained when *P. aeruginosa* was grown in the presence of 10 μM DFOB and 0.25 μM hemin  
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13 (Figure S5). The expression of all other genes encoding proteins of the PVD pathway (except  
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15 for *opmQ*) was less strongly affected by the presence of both hemin and NOCA.  
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**Figure 7. Differential proteomic analyses of *P. aeruginosa* PAO1 grown in the absence or presence of TCV or TCV and hemin.** **A.** PAO1 cells were grown in CAA medium with or without 10  $\mu$ M TCV for 8 h. The average values measured in CAA without TCV were plotted against the average values in CAA supplemented with NOCA. **B.** The same experiment was repeated with PAO1 cells grown in CAA with or without 10  $\mu$ M TCV and 0.25  $\mu$ M hemin. **C-E.** Heat maps of various TBDTs (**C**) or proteins involved in the PCH (**D**) or PVD pathways (**E**). In these heat maps, we chose to show HasR, PhuR and HxuA and the proteins for which a change in the level of expression was observed in one of the two conditions analyzed (TCV or TCV + hemin). The darker the shade of blue, the more expression of the protein is induced; the darker the shade of red, the more expression of the protein is repressed. NS: data not significant; ND: not detected (no peptide corresponding to the protein was detected by proteomic); \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$





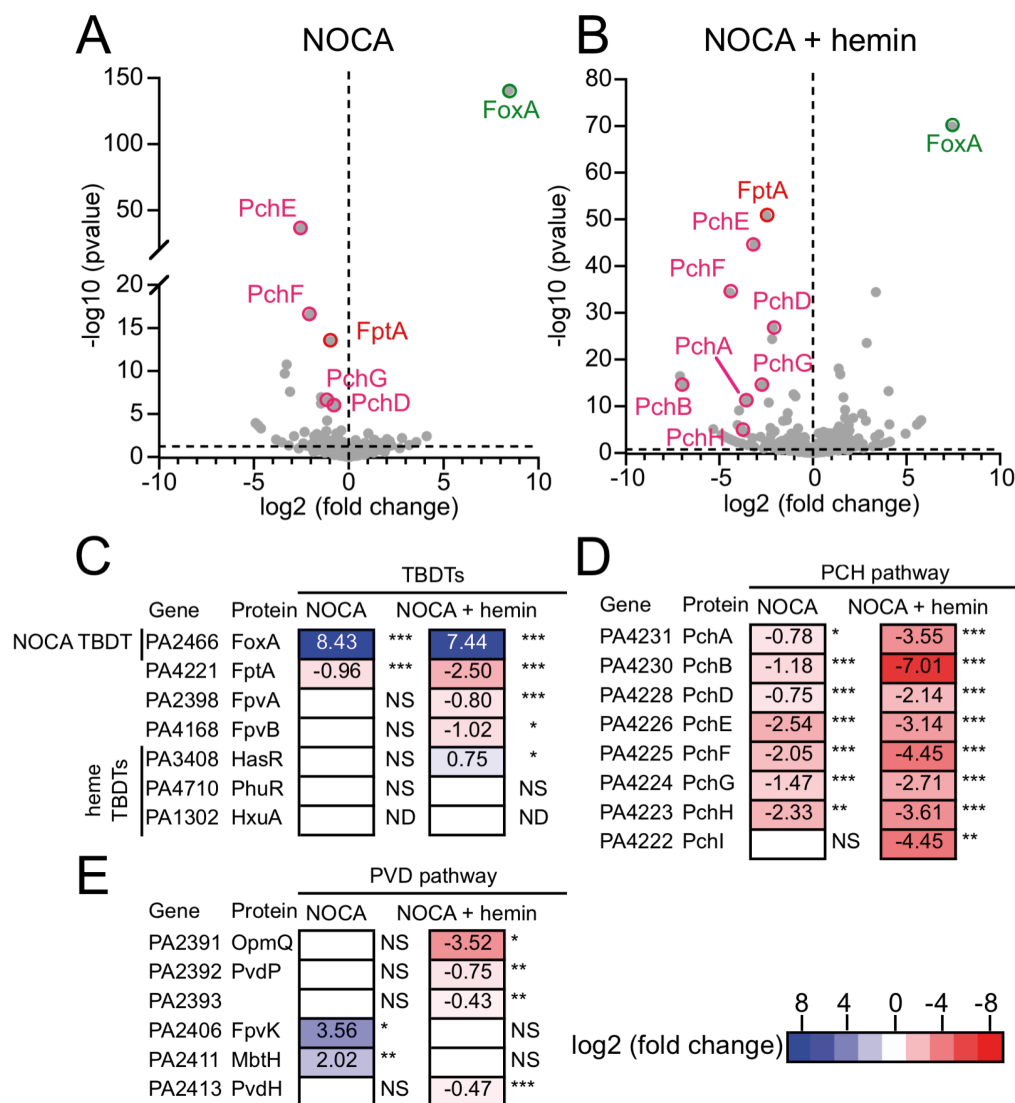
**Figure 8. Modulation of the transcription of *pfeA*, *foxA*, *hasR*, *fptA*, and *fpvA* in the absence or presence of TCV, TCV with hemin, NOCA, or NOCA with hemin.** *P. aeruginosa* PAO1 was grown in the presence of 10  $\mu$ M TCV, 10  $\mu$ M TCV with 0.25  $\mu$ M hemin, 10  $\mu$ M NOCA, or 10  $\mu$ M NOCA with 0.25  $\mu$ M hemin for 8 h. Gene transcription was normalized to that of the housekeeping *uvrD* reference gene. The results show the ratio between the values obtained in the presence of TCV, TCV with hemin, NOCA, or NOCA with hemin and those obtained in CAA. *fptA* encodes the TBDT of PCH, *fpvA* that of PVD, *pfeA* that of enterobactin, *foxA* that of NOCA and DFO-B, and *hasR* that of heme. The data show the mean of six independent experiments.

In summary, these results show that, in the presence of a mixture of hemin and a xenosiderophore, the xenosiderophore activates the expression of the gene encoding its corresponding TBDT. In addition, the expression of the genes of the PCH pathway is more strongly repressed than in the presence of only hemin or only a xenosiderophore, indicating that both iron sources (hemin or xenosiderophore having scavenged iron) have a cumulative effect in the phenotypic adaptation of *P. aeruginosa* concerning the expression of its various iron-uptake pathways. Such phenotypic adaptation of *P. aeruginosa* in the presence of hemin and a xenosiderophore indicates that the pathogen is able to access both hemin and ferric iron in such an environment. In this context, ferric iron uptake occurs via the xenosiderophore present rather than by PVD and PCH. This is a key point for the vectorization of antibiotics by siderophores.<sup>43–</sup>

<sup>45</sup> Such high phenotypic plasticity concerning the various iron-uptake pathways present in the

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3 *P. aeruginosa* genome is an asset in the development of siderophore-antibiotic conjugates and  
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5 the use of these uptake pathways to transport antibiotics into bacteria.  
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8 In the context of infection, *P. aeruginosa* grows under iron-restricted conditions and is also in  
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10 the presence of heme, which is mostly bound to hemoproteins, such as hemoglobin and  
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12 myoglobin. In such a context, based on the data presented here, the Has and Phu uptake  
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14 pathways are highly expressed, PVD- and PCH-dependent iron-uptake pathways are also  
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16 expressed but at a lower level compared to iron-restricted growth conditions in the absence of  
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18 heme. This is consistent with previous data showing that heme is a critical source of iron during  
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20 prolonged infection of the CF lung and that *P. aeruginosa* strains produce significantly less  
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22 siderophores during the course of CF lung infection.<sup>16,48–52</sup> In such an infection context,  
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24 according to the present data, if a catechol- or hydroxamate-type xenosiderophore is present or  
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26 a siderophore-antibiotic conjugate, its corresponding uptake pathway is certainly expressed,  
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28 despite the presence of heme. We previously showed that the presence of TCV-linezolid  
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30 induces the expression of the corresponding uptake pathway in *P. aeruginosa* in an infection  
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32 model (infection of epithelial cells).<sup>45</sup>  
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**Figure 9. Differential proteomic analyses of *P. aeruginosa* PAO1 grown in the absence or presence of NOCA or NOCA with hemin.** **A.** PAO1 cells were grown in CAA medium with or without 10  $\mu$ M NOCA for 8 h. The average values measured in CAA without NOCA were plotted against the average values in CAA supplemented with NOCA. **B.** The same experiment was repeated with PAO1 cells grown in CAA with or without 10  $\mu$ M NOCA and 0.25  $\mu$ M hemin. **C-E.** Heat maps of various TBDTs (**C**) or proteins involved in the PCH (**D**) or PVD pathways (**E**). In these heat maps, we chose to show HasR, PhuR and HxuA for the TBDTs and the transporters or proteins for which a change in the level of expression was observed in one of the two conditions analyzed (NOCA or NOCA + hemin). The darker the shade of blue, the more expression of the protein is induced; the darker the shade of red, the more expression of the protein is repressed. NS: data not significant; ND: not detected (no peptide corresponding to the protein was detected by proteomic); \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$

## CONCLUSION

As described in the introduction and summarized in Table S1, the genome of *P. aeruginosa* includes genes encoding at least 15 different iron or heme-uptake pathways.<sup>69</sup> They are not all expressed simultaneously and the bacteria do not express just one iron-uptake pathway at a time, but usually a combination of a few, those with the highest chance to scavenge iron or hemin from the growth environment.<sup>26,35,41,45</sup> Consequently, as we already discussed previously “the presence of a large panel of iron-acquisition pathways in the genome involves an even larger panel of possible phenotypes, with various combinations of expression of these iron-uptake pathways and with various expression levels, depending on the environmental stimuli”.<sup>26</sup> Data we previously obtained, using the same differential proteomic approach as used here, showed that the growth of *P. aeruginosa* in CAA versus LB resulted in the induction of the expression of the PVD- and PCH-dependent iron-uptake pathways, as well as the heme-uptake pathways Has and Phu, *chtA*, encoding the TBDT of the siderophores aerobactin, rhizobactin and schizobactin,<sup>37</sup> *pirA*, a gene encoding a ferri-catechol siderophore TBDT, two TBDTs for which the siderophore transported is still unknown, *PA0434* and *PA3268*, and *oprC*, a TBDT involved in Cu uptake<sup>54</sup> (Table S1). The presence of 0.25  $\mu$ M hemin in CAA growth medium slightly increased the expression of *hasR* and *phuR* and downregulated the expression of the genes encoding the proteins of the PVD- and PCH-dependent iron-uptake pathways, resulting in less production of PVD and PCH. However, efficient iron uptake via PVD and PCH was still possible in cells grown in CAA in the presence of hemin. Consequently, bacteria grown in CAA in the presence of hemin can both import hemin and access ferric iron via their siderophores PVD and PCH. In the simultaneous presence of hemin and a xenosiderophore, such as NOCA, DFOB, ENT, or the synthetic catechol chelator TCV, strong induction of the expression of the corresponding xenosiderophore-uptake pathways occurred, as well as repression of the PVD- and PCH-dependent iron-uptake pathways. Such expression of the

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3 xenosiderophore iron-uptake pathway is an asset for the vectorization of antibiotics by  
4 siderophores, as discussed above. Indeed, during an infection, even if heme is present in the  
5 environment of the pathogen, the bacteria will upregulate the expression of the uptake pathways  
6 of the siderophore-antibiotic conjugates if such compounds are present in their environment.  
7 Overall, these data provide precise insights on how *P. aeruginosa* adjusts the expression of its  
8 various iron-uptake pathways to match the presence of hemin and hemin with a  
9 xenosiderophore. These phenotypic patterns (described in Table S1) show amazing plasticity  
10 and can be finely modulated and adjusted by environmental stimuli, such as the presence of  
11 hemin or xenosiderophores.  
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## 27 **METHODS**

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30 **Chemicals.** Hemin chloride (hemin), desferrioxamine B (DFOB) and the protonophore  
31 carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma-Aldrich and  
32  $^{55}\text{FeCl}_3$  was purchased from Perkin Elmer. NOCA was purified as described.<sup>70</sup> Pyoverdine  
33 (PVD) was purified from *P. aeruginosa* PAO1 culture supernatants as previously described.<sup>71</sup>  
34 Pyochelin (PCH) was synthesized as previously described<sup>72</sup> as well as TCV.<sup>67</sup>  
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45 **Hemin preparation.** A stock solution of hemin (1mM) was prepared by dissolution in NaOH  
46 (0.1 M). Following filtration, the solution was diluted (1:1,000), and hemin concentration was  
47 determined at 385 nm using an extinction coefficient of 58,400 l.mol<sup>-1</sup>.cm<sup>-1</sup>.  
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55 **Bacterial strains and growth conditions.** Strain used are summarized in Table S2. Bacteria  
56 were first grown in LB medium overnight at 30°C. Cells were then washed and resuspended in  
57 iron-deficient CAA (casamino acid) medium containing 5 g l<sup>-1</sup> low-iron CAA (Difco), 1.46 g l<sup>-1</sup>  
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3 <sup>1</sup> K<sub>2</sub>HPO<sub>4</sub> · 3H<sub>2</sub>O and 0.25 g l<sup>-1</sup> MgSO<sub>4</sub> · 7H<sub>2</sub>O and were grown overnight at 30°C. To monitor  
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5 growth in the presence of siderophores, cells were resuspended again in fresh CAA medium at  
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7 an optical density of OD<sub>600 nm</sub> = 0.01 or 0.1 with or without 0.25 μM hemin in 96 well plates.  
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10 Plates were incubated at 30°C, with shaking every 15 min, in a microplate reader (Infinite  
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12 M200, Tecan). Growth was followed by measuring the optical density at 600 nm every 30 min,  
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14 for 20 h.  
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20 **Construction of *hasR* and *phuR* mutants.** Enzymes were obtained from ThermoFisher  
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22 Scientific. *Escherichia coli* TOP10 (Invitrogen) was used as the host strain for the plasmids.  
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24 Plasmid construction and primers (Table S4) were used as described previously.<sup>68</sup> Briefly,  
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26 1,400-bp inserts made up of the flanking sequences of either *hasR* or *phuR* were cloned into a  
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28 pEXG2 plasmid using HindIII and EcoRI restriction sites for *hasR* and *phuR*. Plasmids were  
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30 sequenced before being used to generate chromosomal mutations in a *P. aeruginosa* PAO1  
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32 strain as previously described.<sup>68</sup> Recombinant clones were isolated before being verified by  
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34 PCR and Sanger sequencing.  
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42 **Construction of *mCherry* reporter plasmids and *mCherry* expression monitoring.** Enzymes  
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44 were obtained from ThermoFisher Scientific. *Escherichia coli* SM10 (Invitrogen) was used as  
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46 the host strain for the plasmids. DNA fragments containing 197 bp of the *pvdS* promoter before  
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48 the ATG and 400 bp of the *pchR* promoter before the ATG were amplified by PCR. Sequence  
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50 encoding for mCherry was amplified by PCR using primers containing flanking region of *pvdS*  
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52 or *pchR* promoter (Table S4). A second PCR was used to generate a 908 bp fragment containing  
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54 the *pvdS* promoter or a 1111 bp fragment containing the *pchR* promoter in frame with mCherry  
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56 coding sequence. Each PCR product was purified, digested with BamHI and EcoRI for *pvdS*-  
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3 mCherry or with HindIII and EcoRI for *pchR*-mCherry, and then introduced into pSEVA631  
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5 vector. Recombinant clones were isolated before being verified by PCR and Sanger sequencing.  
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8 Bacterial growth and mCherry expression were followed by measuring the optical density at  
9  
10 600 nm and fluorescence at 610 nm (excitation at 570 nm), every 30 min, for 20 h. The mean  
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12 of three independent replicates were represented for each measurement.  
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18 **RNA extractions.** *P. aeruginosa* PAO1 cells were consecutively grown overnight in LB and  
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20 then in CAA medium. Afterwards, bacteria were pelleted and diluted again in CAA medium at  
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22 an OD<sub>600 nm</sub> of 0.1 and grown for 8 h with or without 0.25 μM hemin and/or 10 μM siderophore  
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24 (NOCA or TCV) at 30°C. Afterwards, 2.5 x 10<sup>8</sup> cells were mixed with two volumes of  
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26 RNeasy Protect Bacteria Reagent (Qiagen). Samples were lysed in Tris-EDTA pH 8.0 containing  
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28 15 mg/ml lysozyme (Sigma-Aldrich) for 15 minutes at 25°C. Then, lysates were homogenized  
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30 with QIAshredder kit (Qiagen) and total RNAs were extracted with RNeasy mini kit  
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32 (Qiagen). After treatment with DNase (RNase-Free DNase Set, Qiagen), RNAs were purified  
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34 with an RNeasy Mini Elute cleanup kit (Qiagen).  
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42 **RT-qPCR analysis.** 1 μg of RNA was reversed-transcribed with a High Capacity RNA to  
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44 cDNA Kit, in accordance with the manufacturer's instructions (Applied Biosystems). The  
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46 expression of genes was measured in a SpetOne Plus Instrument (Applied Biosystems), with  
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48 Power Sybr Green PCR Master Mix (Applied Biosystems) and the appropriate primers (listed  
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50 in Table S5). The *uvrD* expression was used as an internal control. For a given gene in each  
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52 strain, the transcript levels were normalized with respect to those for *uvrD* and were expressed  
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54 as a base two logarithms of the ratio (fold-change) relative to the reference conditions.  
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3 **Iron uptake.** Complexes of siderophore-<sup>55</sup>Fe were prepared like previously described<sup>73</sup> in 50  
4 mM Tris-HCl pH 8.0 buffer and with a siderophore:iron ratio of 20:1. *P. aeruginosa* PAO1  
5 cells were consecutively grown overnight in LB and then in CAA medium. Afterwards, bacteria  
6 were pelleted and diluted again in CAA medium at an OD<sub>600 nm</sub> of 0.1 and grown overnight  
7 with or without 0.25 μM hemin. After growth, bacteria were pellet, washed with 50 mM Tris-  
8 HCl pH 8.0, and diluted to an OD<sub>600 nm</sub> of 1.0. Half of the cells were incubated during 15 minutes  
9 with 200 μM CCCP at 30°C. Both samples of bacteria (treated or not with CCCP) were  
10 incubated with 500 nM siderophore-<sup>55</sup>Fe. At 0, 15, 30, 45, 60, 120 and 180 minutes, aliquots  
11 were removed, cells were harvested and radioactivity measured in the bacterial pellets.  
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27 **PVD and PCH measurement.** The production of PCH and PVD were measured as described  
28 previously.<sup>74</sup> PVD and PCH production were monitored using their characteristic absorbance  
29 at 400 nm (at neutral pH) and 320 nm respectively. Bacteria were centrifuged and PVD  
30 absorbance at 400 nm was directly measured in the supernatant. PCH was extracted from the  
31 supernatant with ethyl-acetate. 500 μl ethyl-acetate was added to 1 ml of bacterial supernatant  
32 acidified by solid citric acid until a pH 3.0, mixed and centrifuged to separate aqueous and  
33 organic phases. The concentration of PCH was determinate by measuring the absorbance at 320  
34 nm in the organic phase.  
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49 **Proteomic analysis.** *P. aeruginosa* PAO1 cells were consecutively grown overnight in LB and  
50 then in CAA medium. Afterwards, bacteria were pelleted and diluted again in CAA medium at  
51 an OD<sub>600 nm</sub> of 0.1 and grown for 8 h with or without 0.25 μM hemin and/or 10 μM siderophore  
52 (NOCA or TCV) at 30°C. The cells (5 x 10<sup>8</sup>) were harvested and used for proteomic analysis.  
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60 Each sample was prepared in biological triplicate for each cell-culture condition. Cell pellets



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3 were resuspended in 200  $\mu$ L lysis buffer (UTCT buffer containing 7 M urea, 2 M thiourea, 4%  
4 CHAPS, and 20 mM Tris-HCl pH 7.6) supplemented with nuclease and DNase. Protein  
5 concentrations were determined with the Bradford assay using bovine serum albumin as the  
6 standard. Proteins were further precipitated overnight with glacial 0.1 M ammonium acetate in  
7 100% methanol (5 volumes, - 20°C). After centrifugation at 12,000 x g and 4°C for 15 min, the  
8 resulting pellets were washed twice with 0.1M ammonium acetate in 80% methanol and further  
9 dried under vacuum (Speed-Vac concentrator). Pellets were resuspended in 100  $\mu$ L 50 mM  
10 ammonium bicarbonate and submitted to reduction (5 mM dithiothreitol, 95°C, 10 min) and  
11 alkylation (10 mM iodoacetamide, room temperature, 20 min). Proteins were finally digested  
12 overnight with 150 ng sequencing-grade trypsin (Promega). The proteomic datasets were  
13 obtained by injecting 750 ng of each peptide mixture into a Q-Exactive Plus mass spectrometer  
14 coupled to an EASY-nanoLC-1000 (Thermo-Fisher Scientific, USA) as described previously  
15 <sup>26</sup>. The raw data obtained were converted into .mgf files using Proteome Discoverer Daemon  
16 software (Thermo-Fisher Scientific, script “Export-to-mgf”, version 2.2). For both differential  
17 proteomic analyses, data were searched against the *P. aeruginosa* UniprotKB sub-database  
18 (UniprotKB release 2016\_12, taxon 208964, *P. aeruginosa* strain PAO1, 5564 forward protein  
19 sequences). Peptides and proteins were identified using the Mascot algorithm (version 2.5.1,  
20 Matrix Science, London, UK). The following parameters were used: (i) Trypsin/P was selected  
21 as the enzyme, (ii) two missed cleavages were allowed, (iii) methionine oxidation and  
22 acetylation of protein N-term were set as variable modifications and carbamidomethylation of  
23 cysteine as a fixed modification, (iv) mass tolerance for precursor ions was set at 10 ppm, and  
24 0.02 Da for fragment ions. Mascot data were further imported into Proline v1.4 software <sup>75</sup>.  
25 Proteins were validated against a Mascot pretty rank equal to 1 and a 1% FDR for both peptide  
26 spectrum matches (PSM score) and protein sets (Protein Set score). The total number of MS/MS  
27 fragmentation spectra was used to quantify each protein from at least three independent  
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3 biological replicates: this “BasicSC” value, calculated by proline, includes all PSMs of all  
4 peptides, including modified peptides (3 fixed and variable modifications) and the peptides  
5 shared by different protein sets. After column-wise normalization of the data matrix, the  
6 “BasicSC” spectral count values were submitted to a negative-binomial test using edgeR GLM  
7 regression by R (R v3.2.5). The statistical test was based on the published msmsTests R package  
8 available in Bioconductor to process label-free LC-MS/MS data by spectral counts <sup>76</sup>. For each  
9 identified protein, an adjusted P-value (adjp), corrected by the Benjamini–Hochberg procedure,  
10 was calculated, as well as the protein fold-change (FC). The MS data were deposited in the  
11 ProteomeXchange Consortium database via the PRIDE<sup>77</sup> partner repository with the dataset  
12 identifier: Project accession: PXD028818; Project DOI: 10.6019/PXD028818. Reviewer  
13 account details: Username: [reviewer\\_pxd028818@ebi.ac.uk](mailto:reviewer_pxd028818@ebi.ac.uk), Password: obn4O3Cj. Table S5  
14 shows the match between the figures, the proteomic files and samples used for the proteomic  
15 investigations.

## 39 SUPPORTING INFORMATION

40  
41 Table summarizing proteomic data obtained previously and in this study; table describing  
42 strains used in this study; table describing strains used in this study; table describing plasmids  
43 used in this study; 2 tables describing primers used in this study; a table showing the match  
44 between the figures, the proteomic files and samples used for the proteomic investigations;  
45 growth of PAO1 and  $\Delta hasR\Delta phuR$  cells in CAA in the absence an presence of hemin;  
46 modulation of the transcription of genes encoding TBDTs involved in iron or hemin acquisition in  
47  $\Delta hasR\Delta phuR$  cells when grown in CAA medium with or without 0.25  $\mu$ M hemin; chemical structures  
48 of siderophores used in the study; modulation of the transcription of genes of *P. aeruginosa* PAO1  
49 grown with ENT or with ENT and hemin; modulation of the transcription of genes of *P. aeruginosa*

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3 PAO1 grown with DFOB or DFPO with hemin; an Excel file with the other proteins having their  
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5 expression modulated by the growth conditions described in Figure 2, 7 and 9.  
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## 19 **AUTHOR CONTRIBUTIONS**

20  
21 V.N. and I.J.S. conceived the idea and designed the experiments. V.N. performed bacterial  
22  
23 cultures, <sup>55</sup>Fe uptake assays, siderophore measurements, RT-qPCR assays. L.K. and P.H.  
24  
25 performed proteomic assays and analyzes. M.M. and G.L.A.M. synthesized TCV. I.J.S., V.N.,  
26  
27 L.K. and P.H. wrote the manuscript.  
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## 34 **ABBREVIATIONS USED**

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37 CAA, casamino acid; CCCP, Carbonyl cyanide-*m*-chlorophenylhydrazone; DFOB,  
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39 desferrioxamine B; ENT, enterobactin ; NOCA, nocardamine ; PCH, pyochelin; PVD,  
40  
41 pyoverdine; RT-qPCR , quantitative reverse transcription polymerase chain reaction;  
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44 TBDT, TonB-dependent transporters; TCV, tris-catechol vector.  
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55  
56 instrumentation at the IBMC was funded by the University of Strasbourg, IdEx “Equipement  
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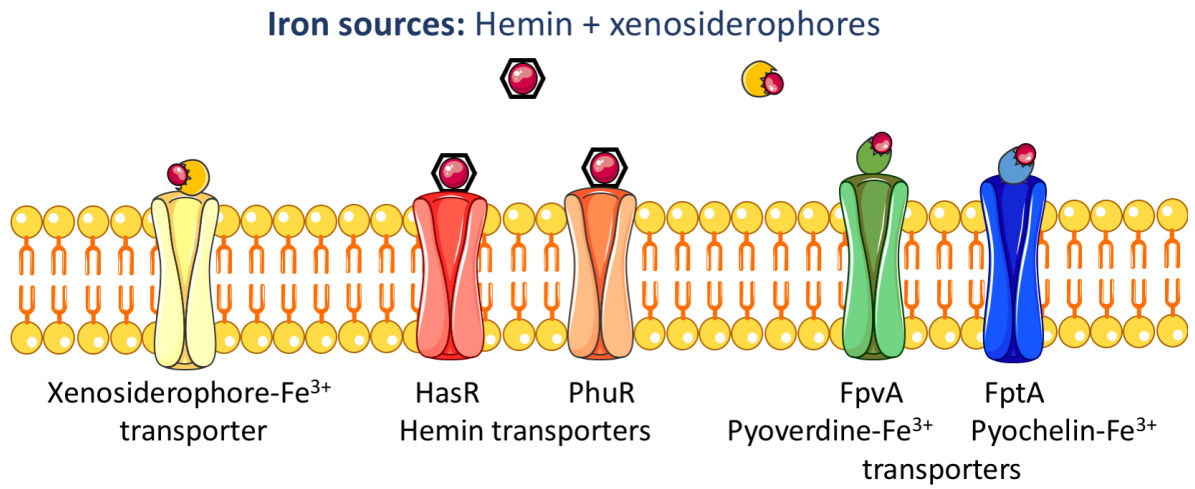
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**Transporters present in *P. aeruginosa* outer membrane**

Detected by differential proteomics and RT-qPCR