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## Opportunistic use of catecholamine neurotransmitters as siderophores to access iron by *Pseudomonas aeruginosa*

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**Running title:** *P. aeruginosa* cells use catecholamines to access iron

22

23 **ABSTRACT**

24 Iron is an essential nutrient for bacterial growth and the cause of a fierce battle between the  
25 pathogen and host during infection. Bacteria have developed several strategies to access  
26 iron from the host, the most common being the production of siderophores, small iron-  
27 chelating molecules secreted into the bacterial environment. The opportunist pathogen  
28 *Pseudomonas aeruginosa* produces two siderophores, pyoverdine and pyochelin, and is also  
29 able to use a wide panoply of xenosiderophores, siderophores produced by other  
30 microorganisms. Here, we demonstrate that catecholamine neurotransmitters (dopamine, L-  
31 DOPA, epinephrine, and norepinephrine) are able to chelate iron and efficiently bring iron  
32 into *P. aeruginosa* cells via TonB-dependent transporters (TBDTs). Bacterial growth assays  
33 under strong iron-restricted conditions and with numerous mutants showed that the TBDTs  
34 involved are PiuA and PirA. PiuA exhibited more pronounced specificity for dopamine uptake  
35 than for norepinephrine, epinephrine, and L-DOPA, whereas PirA specificity appeared to be  
36 higher for L-DOPA and norepinephrine. Proteomic and qRT-PCR approaches showed *pirA*  
37 transcription and expression to be induced in the presence of all four catecholamines.  
38 Finally, the oxidative properties of catecholamines enable them to reduce iron, and we  
39 observed ferrous iron uptake via the FeoABC system in the presence of L-DOPA.

41

42 **INTRODUCTION**

43 Iron availability is very low for bacteria during infection due to the sequestration of iron by  
44 host proteins, such as transferrin, lactoferrin and ferritin. Thus, most bacteria access iron by  
45 relying on the production and secretion of siderophores, low molecular weight organic  
46 compounds with an extremely high affinity for ferric iron (Hider and Kong, 2011). These  
47 compounds scavenge iron from the bacterial environment before being transported back  
48 into bacteria via outer-membrane transporters, of which the uptake activity is regulated by  
49 an interaction with an inner-membrane protein called TonB (Schalk and Guillon, 2013).  
50 These outer-membrane transporters are commonly called TonB-dependent transporters  
51 (TBDT).

52 Siderophores produced by bacteria can have widely varying chemical structures, but all  
53 chelate ferric iron via either catecholate, hydroxamate, carboxylate functions, salicylate or  
54 heterocycle (Hider and Kong, 2011). The most highly studied siderophores are probably the  
55 tris-catecholates, which include the archetypal siderophore enterobactin (Pollack and  
56 Neilands, 1970; O'Brien and Gibson, 1970), as well as protochelin, corynebactin,  
57 salmochelin, and bacillibactin (Cornish and Page, 2000; May *et al.*, 2001; Bluhm *et al.*, 2002;  
58 Müller *et al.*, 2009). These siderophores behave as hexadentate ligands that can chelate  
59 ferric iron through the hydroxyl moieties of their three catechol functions, forming  
60 octahedral complexes (Raymond *et al.*, 2003).

61 Catecholate-containing molecules also have major functions in metazoans, with, for  
62 example, catecholamines, such as dopamine, norepinephrine, and epinephrine, acting as  
63 neurotransmitters in vertebrates (Eisenhofer *et al.*, 2004). However, they also exhibit a wide  
64 array of functions across all kingdoms, to the extent that they have been hypothesized to be

65 part of an ancient evolutionary chemical language used by all life forms, much like the  
66 genetic code (Roshchina, 2016). Although the question of the evolutionary origin of  
67 catecholamines remains unresolved, it is clear that this class of molecules is ancient and  
68 widespread. Indeed, in mammals, they are not confined solely to the central nervous system  
69 but can be found in the gastrointestinal tract, peripheral sympathetic nerve endings, and  
70 bloodstream (Eisenhofer *et al.*, 2004).

71 The existence of a link between catecholamines and infection was proposed after the  
72 observation of a correlation between high levels of circulating catecholamines and serious  
73 infections (Groves *et al.*, 1973; Jones *et al.*, 1988). Cases of sepsis have been noted from the  
74 beginning of the therapeutic use of purified adrenaline in the 1940s (Cooper, 1946; Evans *et*  
75 *al.*, 1948). The first hypotheses concerning this link concerned the immunomodulatory role  
76 of adrenaline and norepinephrine, until Lyte and Ernst studied the impact of  
77 neurotransmitters on bacterial growth and virulence in the 1990s (Lyte and Ernst 1992; Lyte  
78 *et al.* 1997). The ability of catecholamines to stimulate growth and enhance virulence  
79 appears to be widespread among both Gram-negative and Gram-positive bacteria  
80 (Verbrugghe *et al.*, 2012). One hypothesis to explain this effect is the detection of  
81 catecholamines by two-component systems, thus allowing the pathogen to recognize an  
82 abnormal inflammatory state in their host (Reading *et al.*, 2009; Hughes *et al.*, 2009).  
83 Another is that catecholamines may be used as siderophores or agents to facilitate the  
84 acquisition of iron by pathogens, as these molecules are capable to form ferric complexes via  
85 their catechol group (Siraki *et al.*, 2000; Dichtl *et al.*, 2019). Consistent with this second  
86 hypothesis, it has been shown that *Bacillus subtilis* (Miethke and Skerra, 2010), *Bordetella*  
87 *bronchiseptica* (Anderson and Armstrong, 2008), and *E. coli* (Burton *et al.*, 2002) are able to  
88 use norepinephrine for iron import. *Bacillus subtilis* assimilates norepinephrine-Fe through

89 the ferrous iron uptake pathway FeuABC (Miethke and Skerra, 2010). Moreover,  
90 norepinephrine-Fe has been shown to facilitate iron acquisition from transferrin in  
91 *Bordetella bronchiseptica* in a TonB-dependent fashion and activate the transcription of the  
92 enterobactin TBDT *bfeA* (Anderson and Armstrong, 2008). In addition, the  
93 enterohemorrhagic strain of *Escherichia coli* O157:H7 is able to sense the presence of  
94 epinephrine and norepinephrine in its environment through the QseC/B two-component  
95 system involved in quorum sensing (Hughes *et al.*, 2009) and displays increased iron  
96 acquisition in the presence of both norepinephrine and transferrin (Freestone *et al.*, 2003).  
97 At last, more recently, it was shown that the solute binding lipoprotein PiuA from the Fe  
98 acquisition ABC transporter PiuBCDA, binds tetradentate norepinephrine-Fe complexes,  
99 (Zhang *et al.*, 2020).

100 Despite recent interest in metabolic crosstalk between microorganisms and their hosts  
101 (Belkaid and Hand, 2014; Yoo *et al.*, 2020), very little information is available concerning the  
102 clinically relevant pathogen *P. aeruginosa*. The few studies undertaken have demonstrated  
103 that the PA14 strain shows increased virulence when cultivated with norepinephrine, a  
104 result that could be replicated by adding FeCl<sub>3</sub> to the culture medium, suggests a link  
105 between norepinephrine and an increase in bacterial intracellular iron concentrations  
106 (Hegde *et al.*, 2009). It has been proposed that catecholamines have the ability to facilitate  
107 iron scavenging from transferrin by reducing iron(III) to soluble iron(II), for which transferrin  
108 has a very low affinity (Sandrini *et al.*, 2010). However, *P. aeruginosa* has been shown to be  
109 capable of scavenging iron from transferrin without the assistance of such molecules, as the  
110 ability to produce the high-affinity siderophore pyoverdine and protease elastase is  
111 sufficient (Doring *et al.*, 1988; Meyer *et al.*, 1996). Consequently, it is still unknown whether

112 catecholamine involvement in iron acquisition is biologically relevant or whether the  
113 observations described above were artifacts caused by the limitation of available models.  
114 No attempts have yet been made to determine whether ferric forms of catecholamine  
115 compounds can enter *P. aeruginosa* cells and directly provide this opportunistic pathogen  
116 with iron. This pathogen is able to import ferrous iron ( $\text{Fe}^{2+}$ ) via the FeoABC uptake system,  
117 access heme using three heme acquisition pathways, and access ferric iron ( $\text{Fe}^{3+}$ ) using  
118 siderophores (Cornelis and Dingemans, 2013). *P. aeruginosa* produces two siderophores,  
119 pyoverdine (PVD) and pyochelin (PCH) (Schalk *et al.*, 2020), but it is also able to use  
120 numerous siderophores produced by other bacteria present in its environment in a  
121 “siderophore piracy” strategy (Cornelis and Dingemans, 2013; Perraud *et al.*, 2020). *P.*  
122 *aeruginosa* has in its genome 34 genes encoding TBDTs, with twelve of them being involved  
123 in iron acquisition (Winsor *et al.*, 2016). For a third of these transporters, the siderophore or  
124 molecule transported has not been identified.

125 Here, we show that dopamine, L-DOPA, epinephrine, and norepinephrine (Figure 1) are all  
126 able to chelate iron.  $^{55}\text{Fe}$  uptake assays, bacterial growth tests under iron-restricted  
127 conditions, and the use of numerous mutants showed that these molecules transport iron in  
128 *P. aeruginosa* cells via the TBDTs PirA and PiuA. Proteomic and qRT-PCR approaches also  
129 showed that *pirA* transcription and expression is stimulated by the presence of  
130 catecholamines in the bacterial environment, also indicating that PirA is involved in iron  
131 acquisition via catecholamine compounds. Finally, ferrous uptake was also observed in the  
132 presence of L-DOPA, involving the FeoABC system.

134 **RESULTS**

135 **Catecholamines chelate iron via their catechol function.** Catecholamines are known to  
136 form complexes with iron (Melin *et al.*, 2015), but their affinity for iron is unknown. We used  
137 the CAS-Fe (Chrome azurol S) test, developed by Neilands and Schwyn (Schwyn and  
138 Neilands, 1987), to assess the ability of several catecholamines to chelate iron (Figure 1). In  
139 this test, the ternary complex chrome azurol S-Fe<sup>3+</sup>-hexadecyltrimethylammonium bromide,  
140 with absorbance at 630 nm, serves as an indicator (Schwyn and Neilands, 1987), as its color  
141 changes from blue to orange when a strong chelator removes the iron from the dye.  
142 Dopamine, L-DOPA, epinephrine, and norepinephrine were able to scavenge iron from CAS  
143 with equivalent efficiency, whereas normetanephrine, an O-methylated structural analogue  
144 of norepinephrine, was not (Figure 2A). These data confirm that catecholamines are able to  
145 chelate iron(III) through their catechol moiety. Comparison of the ability of catecholamines  
146 to scavenge iron from CAS with that of pyoverdine or pyochelin (the two siderophores  
147 produced by *P. aeruginosa* to access iron (Schalk *et al.*, 2020)) clearly showed these  
148 monocatechol-containing compounds to have a much lower affinity for iron than the two  
149 siderophores produced by *P. aeruginosa* ( $k_a$  of  $10^{32}$  M<sup>-1</sup> and  $10^{18}$  M<sup>-2</sup> for pyoverdine and  
150 pyochelin respectively (Albrecht-Gary *et al.*, 1994; Brandel *et al.*, 2012)). Comparison of the  
151 ability to scavenge iron from CAS between the catecholamines themselves showed  
152 norepinephrine to have the highest affinity, followed by epinephrine, dopamine, and L-  
153 DOPA.

154 We also tested the ability of these catecholamines to compete for iron with pyoverdine. Apo  
155 pyoverdine is fluorescent at 447 nm (excitation at 400 nm) and this fluorescence is  
156 quenched when the siderophore chelates iron (Schalk *et al.*, 2002; Folschweiller *et al.*, 2002).  
157 We used this spectral property to compare the ability of the catecholamines to remove iron

158 from 10  $\mu\text{M}$  pyoverdine-Fe (Perraud *et al.*, 2020). The same assay was also carried out using  
159 a previously described tris-catechol compound (TCV), which has an ability to compete for  
160 iron with pyoverdine similar to that of the tris-catechol enterobactin produced by *E. coli*  
161 (Gasser *et al.*, 2016). As previously described, TCV efficiently removed iron from pyoverdine:  
162 all 10  $\mu\text{M}$  of pyoverdine was in the apo form in the presence of 17  $\mu\text{M}$  of TCV (Figure 2B).  
163 Norepinephrine, dopamine, and epinephrine were clearly less efficient than TCV in this  
164 competition assay: 20 mM of these mono-catechols removed iron from only 20 to 25% of  
165 the 10  $\mu\text{M}$  of ferri-pyoverdine present in the assay. Normetanephrin was unable to scavenge  
166 iron from pyoverdine, which was expected as this molecule is O-methylated. L-DOPA was  
167 unable to remove iron from pyoverdine-Fe but was able to do so from CAS-Fe (Figure 2A),  
168 indicating that L-DOPA has a lower affinity for iron than norepinephrine, dopamine, and  
169 epinephrine.

170 Overall, these data show that norepinephrine, dopamine, epinephrine, and L-DOPA are able  
171 to chelate iron but with affinities clearly lower than those of pyoverdine and siderophores  
172 such as tris-catechols.

173  
174 **Catecholamines are able to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  at physiological pH.** Catecholamines are  
175 easily oxidized in the presence of Lewis acids such as  $\text{Fe}^{3+}$ . Thus, we investigated whether  
176 catecholamines can reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  using ferrozine, a specific  $\text{Fe}^{2+}$  chelator that forms a  
177 stable magenta-colored complex solely with  $\text{Fe}^{2+}$ , with a specific absorption peak at 562 nm  
178 (Stookey, 1970). Incubation of the catecholamines with  $\text{Fe}^{3+}$  and ferrozine resulted in a slow  
179 increase in absorbance at 562 nm, corresponding to ferrozine- $\text{Fe}^{2+}$  formation, for all  
180 molecules except normetanephrine (Figure 2C). The ability of ferrozine to trap  $\text{Fe}^{2+}$  indicates  
181 that a fraction of  $\text{Fe}^{3+}$  was reduced to  $\text{Fe}^{2+}$  in the presence of catecholamines other than

182 normetanephrine. However, this fraction only represented approximately 10% of the Fe<sup>3+</sup>  
183 reduced by dithiothreitol (DTT), and the kinetics of reduction were also much slower than  
184 that observed with DTT. The inability of normetanephrine to reduce iron was certainly due  
185 to O-methylation of the catechol function.

186

187 ***Catecholamines are able to promote <sup>55</sup>Fe-acquisition by P. aeruginosa.*** We followed the  
188 kinetics of <sup>55</sup>Fe accumulation by *P. aeruginosa* cells incubated in the presence of  
189 catecholamine-<sup>55</sup>Fe complexes to determine whether dopamine, L-DOPA, epinephrine, and  
190 norepinephrine are able to promote iron assimilation by the bacteria (Figure 3). The uptake  
191 assays were carried out using a *P. aeruginosa* strain that is unable to produce pyoverdine or  
192 pyochelin ( $\Delta pvdF\Delta pchA$ , Table 1SM in Supplemental Materials) to ensure that the observed  
193 transport of iron was due to the catecholamines and not the siderophores produced by the  
194 bacteria. We included a control condition in which the strain  $\Delta pvdF\Delta pchA$  was preincubated  
195 with the proton-motive force inhibitor carbonyl cyanide-m-chlorophenylhydrazone (CCCP)  
196 for each of the catecholamines tested. This made it possible to distinguish TonB-dependent  
197 transport from nonspecific interactions with the bacterial cell surface or diffusion of  
198 complexed-<sup>55</sup>Fe into the bacteria through porins. Indeed, as CCCP inhibits the inner  
199 membrane proton gradient, it blocks any transport involving TBDTs (Clément *et al.*, 2004).  
200 Moreover, *P. aeruginosa* cells are able to sense the presence of siderophores produced by  
201 other bacteria (xenosiderophores) in their environment and, in response, express the  
202 proteins (among others the TBDTs) required to access iron via these xenosiderophores  
203 (Llamas *et al.*, 2006; Llamas *et al.*, 2008; Perraud *et al.*, 2020). Such a mechanism may also  
204 exist for catecholamines. Thus, the bacteria used in this <sup>55</sup>Fe uptake assay were grown in

205 CAA (iron-restricted medium) in the presence of 10  $\mu$ M of one of the four catecholamines to  
206 induce the expression of the potential proteins needed to access iron via these molecules.  
207 We observed  $^{55}\text{Fe}$  accumulation over time for all four catecholamines (Figure 3), with an  
208 efficiency of approximately 40% of that of pyoverdine (pyoverdin- $^{55}\text{Fe}$  uptake was carried  
209 out in parallel, Fig 1SM in Supplemental Materials) and of the same order as that we  
210 previously observed for ferrichrome ((Normant *et al.*, 2020), an xenosiderophore used by *P.*  
211 *aeruginosa*).

212 Except for dopamine,  $^{55}\text{Fe}$  uptake was largely inhibited in the presence of CCCP, indicating  
213 that the observed transport is dependent on the proton gradient of the bacterial inner  
214 membrane and is thus TonB-dependent, as for any iron uptake by siderophores.  
215 Furthermore, the inhibition of  $^{55}\text{Fe}$  accumulation by CCCP in the presence of epinephrine, L-  
216 DOPA, or norepinephrine also rules out reduction of ferric to ferrous iron during the 3 h of  
217 the uptake assay, as it is more soluble and can either diffuse into bacteria or be imported by  
218 the FeoABC system.

219 For dopamine, the accumulation of  $^{55}\text{Fe}$  was independent of the inner-membrane proton  
220 gradient (Figure 3), indicating either diffusion of the complex into the bacteria, a nonspecific  
221 interaction of the ferric complex with the bacterial cell surface, or the reduction of iron  
222 followed by diffusion or uptake by the inner membrane FeoAB transporter. A study of the  
223 effects of dopamine in the context of the pathophysiology of Parkinson's disease showed the  
224 ability of this compound to form adducts with peroxidized fatty acids via its primary amine  
225 (Liu *et al.*, 2008), which could explain our  $^{55}\text{Fe}$  assimilation data. However, we cannot  
226 exclude that such a nonspecific interaction or the diffusion of dopamine- $^{55}\text{Fe}$  through porins  
227 did not mask weak TonB-dependent transport. Indeed, the amount of  $^{55}\text{Fe}$  associated with  
228 bacteria was slightly higher for dopamine than the other molecules tested.

229 We next attempted to identify the TBDT(s) involved in iron acquisition by these  
230 catecholamines. We used a *P. aeruginosa* strain that is unable to produce pyoverdine or  
231 pyochelin and mutated for several TBDTs involved in iron uptake via catechol-type  
232 siderophores ( $\Delta pvdF\Delta pchA\Delta pfeA\Delta pirA\Delta piuA\Delta fvbA\Delta PA0434$ , Table 1SM in Supplementary  
233 materials). This mutant is deleted for: *pfeA* and *pirA*, two TBDTs involved in ferri-  
234 enterobactin uptake (Ghysels *et al.*, 2005); *piuA*, a TBDT involved in the uptake of  
235 BAL30072-Fe, a siderophore-beta-lactam compound developed by Basilea (Moynié *et al.*,  
236 2017)); *fvbA*, a TBDT involved in the uptake of ferri-vibriobactin, a tris-catechol type  
237 siderophore (Elias *et al.*, 2011)), and PA0434, a TBDT for which the siderophore has not been  
238 identified but for which the expression is induced by the presence of ferri-vibriobactin  
239 (Perraud *et al.*, 2020). Deletion of none of these five genes had an effect on the iron uptake  
240 kinetics using epinephrine, norepinephrine, dopamine, or L-DOPA as chelators, suggesting  
241 either the involvement of none of these TBDTs in this biological process or the involvement  
242 of several TBDT(s). The involvement of several TBDTs in the import of iron by a siderophore  
243 has also been shown for pyoverdine (FpvA and FpvB, (Ghysels *et al.*, 2004)), enterobactin  
244 (PfeA and PirA, (Ghysels *et al.*, 2005), ferrichrome (FiuA and another unidentified TBDT,  
245 (Normant *et al.*, 2020)), and desferioxamine (FoxA and another unidentified TBDT (Normant  
246 *et al.*, 2020)). One TBDT can take over uptake if the other is not expressed.

247 In conclusion, these data clearly show that L-DOPA, epinephrine, norepinephrine, and  
248 dopamine are able to promote iron transport into *P. aeruginosa* cells that are unable to  
249 produce pyoverdine or pyochelin and that such transport is clearly TonB-dependent for L-  
250 DOPA, epinephrine, and norepinephrine and apparently not for dopamine. However, we  
251 were unable to identify the transporters involved using this approach.

252

253 ***The presence of catecholamines in P. aeruginosa growth media induces the expression of***  
254 ***the TBDT PirA.*** As already mentioned above, *P. aeruginosa*, like many other bacterial  
255 species, is able to use siderophores produced by other bacteria (xenosiderophores) to access  
256 iron, in a strategy called siderophore piracy (Traxler *et al.*, 2012; Galet *et al.*, 2015). This goes  
257 hand in hand with the presence of at least one gene in the bacterial genome encoding a  
258 TBDT for each xenosiderophore that can be used, as well as, genes encoding inner-  
259 membrane transporters and enzymes involved in xenosiderophore-iron dissociation  
260 mechanisms. The transcription of these genes are often upregulated by the presence of the  
261 xenosiderophores themselves in the bacterial environment (Dean *et al.*, 1996; Llamas *et al.*,  
262 2006; Llamas *et al.*, 2008; Llamas *et al.*, 2014), and can be easily followed using a differential  
263 proteomic approach that we previously developed (Perraud *et al.*, 2020). Thus, we studied  
264 the adaptation of the proteome of *P. aeruginosa* to the presence of 10  $\mu$ M of the  
265 catecholamines or normetanephrine. For these experiments, we used a strain that is unable  
266 to produce pyoverdine or pyochelin ( $\Delta pvdF\Delta pchA$ ), as the catecholamines have a low affinity  
267 for iron and compete poorly with pyoverdine for iron chelation (Figures 2A and 2B).  
268 Cells were grown in iron-restricted casaminoacid medium (CAA medium, iron concentration  
269 of 20 nM according to previous work of our team (Cunrath *et al.*, 2016)), with or without one  
270 of the five compounds tested, and analyzed by proteomics and RT-qPCR. The proteomic data  
271 (Figure 4) show very strong induction of the expression of the TBDT PirA in the presence of  
272 epinephrine, as well as weaker induction of the expression of this transporter in the  
273 presence of norepinephrine and L-DOPA. PirA has been described in the literature as a  
274 secondary TBDT for iron uptake by enterobactin in *P. aeruginosa* (Ghysels *et al.*, 2005).  
275 Neither normetanephrine nor dopamine were able to induce the expression of this  
276 transporter, consistent with the observation that normetanephrine is unable to chelate

277 ferric iron and dopamine to carry out a TonB-dependent transport of iron. We also observed  
278 a slight decrease in the expression of the ferric citrate-transporter *FecA* in the presence of  
279 dopamine, epinephrine, and L-DOPA. No strong repression of the expression of the various  
280 proteins involved in pyoverdine and pyochelin (the two siderophores produced by *P.*  
281 *aeruginosa*) uptake pathways was observed.

282 RT-qPCR (qualitative reverse transcription polymerase chain reaction) (Figure 5) confirmed  
283 that epinephrine, norepinephrine, and L-DOPA are able to induce the transcription of *pirA*,  
284 whereas normetanephrine is not. Dopamine also appeared to slightly induce *pirA*  
285 transcription, but apparently with a level of protein synthesis that was not detected by the  
286 proteomic approach. We also observed increased transcription of *feoB*, a gene involved in  
287 the assimilation of iron(II), for dopamine, L-DOPA, epinephrine, and normetanephrine, but  
288 there was no detectable induction of the expression of the FeoAB system by differential  
289 proteomics. Normetanephrine is unable to bind or reduce ferric iron. Thus, the observed  
290 increase in *feoB* transcription may not be associated with the induction of these genes by  
291 the catecholamines to import the ferrous iron produced by these chelators. Surprisingly, the  
292 transcription of *fecA* was not significantly reduced, in contrast to the results obtained by  
293 differential proteomics. We also observed a decrease in the transcription of *fptA*, the gene  
294 encoding the TBDT for pyochelin, in the presence of epinephrine and norepinephrine,  
295 indicating that these two compounds apparently compete more efficiently for iron with  
296 pyochelin than L-DOPA or dopamine. Finally, the transcription of *fpvA*, the TBDT of the  
297 pyoverdine pathway, was not repressed because none of the catecholamines tested were  
298 able to compete for iron with this siderophore.

299 Overall, these data show that the presence of epinephrine, norepinephrine, L-DOPA, or, to a  
300 lower extent, dopamine in the bacterial growth media induces the transcription and

301 expression of the TBDT PirA, suggesting that this transporter is involved in iron assimilation  
302 by these compounds in *P. aeruginosa* cells. The induction was the strongest with  
303 epinephrine. In addition, as *pirA* transcription is regulated by a two-component system  
304 (PirS/PirR), the ferri-catecholamine complex is necessarily able to interact with the sensor  
305 PirS, located in the cytoplasmic membrane, demonstrating an ability of the molecules to  
306 cross the outer membrane of *P. aeruginosa* and activate the sensor at the inner membrane

307

308 **Deletion of *piuA* and, to a lower extent, *pirA* strongly inhibits bacterial growth in the**

309 **presence of catecholamines.** Epinephrine, norepinephrine, and L-DOPA appear to induce

310 PirA expression. Thus, we sought to determine whether the deletion of *pirA*, and eventually

311 other genes encoding TBDTs, would have an inhibitory effect on *P. aeruginosa* grown in iron-

312 restricted conditions in the presence of catecholamines. As already described, we performed

313 the growth test in iron-restricted growth medium (CAA medium), in which mutants with

314 gene deletions are routinely tested: if a gene deletion leads to growth inhibition, the

315 mutated gene is involved in iron assimilation. In this test, we used the mutants  $\Delta pvdF\Delta pchA$

316 (unable to produce the siderophores pyoverdine and pyochelin) and a corresponding

317 collection of mutants deleted for genes encoding TBDTs (Table 1SM in Supplementary

318 Materials). The various strains were grown in CAA medium in the absence or presence of 10

319  $\mu\text{M}$  of one of the catecholamine compounds or normetanephrine. Only the data of mutants

320 showing growth inhibition are presented in Figure 6. Surprisingly, deletion of *pirA* (strain

321  $\Delta pvdF\Delta pchA\Delta pirA$ , Table 1SM) had no impact on the growth of *P. aeruginosa* in the presence

322 of catecholamines, despite the induction of transcription and expression of this gene

323 observed by proteomics and qRT-PCR, indicating that at least one other transporter can

324 perform ferri-catecholamine uptake when PirA is not expressed.

325 Surprisingly, this experiment revealed 60% inhibition of bacterial growth for the *piuA* mutant  
326 ( $\Delta pvdF\Delta pchA\Delta piuA$ ) in the presence of dopamine, a delay in growth in the presence of L-  
327 DOPA, epinephrine and norepinephrine, and no effect when normetanephrine was present.  
328 These data indicate that PiuA is involved in iron acquisition via catecholamine compounds  
329 and, despite the data obtained when following  $^{55}\text{Fe}$  uptake, dopamine also appears to  
330 transport iron via a TBDT. The fact that normetanephrine, the O-methylated catecholamine  
331 analogue, had no impact on  $\Delta pvdF\Delta pchA\Delta piuA$  growth strongly suggests that the observed  
332 growth inhibition or delay was directly dependent on the iron-chelating or iron-reducing  
333 properties of the four other catecholamines. PiuA is a TBDT for which the natural ligand(s)  
334 have not been yet identified. Based on *P. aeruginosa* genome *piuA* transcription, it is not  
335 regulated by a sigma factor or two-component system and therefore cannot be induced by  
336 the presence of the catecholamine molecules, unlike *pirA* (Winsor *et al.*, 2016). Altogether,  
337 our results indicate, that PiuA is involved in the uptake of iron by epinephrine,  
338 norepinephrine, dopamine, and L-DOPA. PiuA in *P. aeruginosa* is a TBDT and not a solute  
339 binding lipoprotein associated to the ABC transporter PiuBCDA as in *Streptococcus*  
340 *pneumoniae* (Zhang *et al.*, 2020), both proteins have the same name but do not belong to  
341 the same protein family.

342 Catecholamines are easily oxidized and their quinone derivatives are potentially disruptive  
343 to redox systems. Thus, we also verified that the growth impairment we observed with the  
344 *piuA* mutation was not due to hypothetical hypersensitivity to oxidative stress of the strains,  
345 but rather to the sequestration of iron by the catecholamines, rendering the nutrient  
346 unusable by the bacteria because their transporter, PiuA, was not expressed. We therefore  
347 grew the  $\Delta pvdF\Delta pchA$  and  $\Delta pvdF\Delta pchA\Delta piuA$  strains, either in the presence of one of the  
348 catecholamines or in the presence of one of the catecholamines and enterobactin, a very

349 strong iron chelator. Enterobactin is a tris-catechol siderophore that displays one of the  
350 highest known affinities for iron(III) amongst siderophores ( $K_d = 10^{-52}$  M, (Harris *et al.*, 1979)  
351 and would easily be able to out-compete catecholamines for iron. In the presence of  
352 enterobactin, the catecholamines would theoretically be unable to compete for iron  
353 chelation and consequently no further iron acquisition should occur via PiuA but only via  
354 PfeA and PirA, the two TBDTs of ferri-enterobactin (Dean and Poole, 1993; Ghysels *et al.*,  
355 2005). We observed no significant differences in growth between the two conditions for  
356  $\Delta pvdF\Delta pchA$  cells, either in the presence of only catecholamines or catecholamines and  
357 enterobactin. As expected, we observed almost no growth of the  $\Delta pvdF\Delta pchA\Delta piuA$  mutant  
358 in the presence of the catecholamines alone, but the growth was restored by enterobactin,  
359 because the bacteria could access iron via this siderophore and its transporters, PfeA and  
360 PirA. This confirms that the inhibitory effect observed in the presence of catecholamines for  
361 the *piuA*-deleted strain was due to the inability of the bacteria to access the iron pool  
362 chelated by catecholamines and not oxidative stress.

363 We observed further growth inhibition for dopamine and norepinephrine when both *piuA*  
364 and *pirA* were deleted, reaching the 60% inhibition observed with L-DOPA on  
365  $\Delta pvdF\Delta pchA\Delta piuA$ , suggesting that PirA is involved in iron acquisition via at least these two  
366 molecules. As growth inhibition was not complete for the double *piuA* and *pirA* mutant,  
367 especially for epinephrine, a third TBDT is likely be involved in iron assimilation via  
368 catecholamine compounds. Deletion of *pfeA*, the enterobactin TBDT, had no additional  
369 inhibitory effect (growth with  $\Delta pvdF\Delta pchA\Delta piuA\Delta pirA\Delta pfeA$ ). We also compared the growth  
370 between  $\Delta pvdF\Delta pchA\Delta piuA$  and  $\Delta pvdF\Delta pchA\Delta piuA\Delta feoABC$  strains, *feoABC* consisting of the  
371 three genes involved in ferrous iron uptake, to assess ferrous iron uptake in the presence of

372 the catecholamines. There was an additional effect for L-DOPA only, suggesting that this  
373 compound may reduce iron, which is then transported by the FeoABC system.

374 In conclusion, PiuA is involved in iron assimilation mostly via dopamine but also, at a lower  
375 level via norepinephrine, L-DOPA and epinephrine. PirA appears to be more highly involved  
376 in iron uptake via L-DOPA and norepinephrine. Finally, iron is apparently also weakly  
377 transported by FeoAB in the presence of L-DOPA.

378

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380 **DISCUSSION**

381 Catecholamines have already been reported to be capable of facilitating iron acquisition  
382 from transferrin (Sandrini *et al.*, 2010), but nothing is known about the possible use of  
383 catecholamines as siderophores for iron uptake by *P. aeruginosa*. Our data show that *P.*  
384 *aeruginosa* is able to use catecholamines to acquire this metal under iron-restricted  
385 conditions, even in the absence of proteins such as transferrin, through the use of TBDTs, as  
386 observed for all siderophores in Gram-negative bacteria. The growth assay we used under  
387 strong iron-restricted conditions with a  $\Delta pvdF\Delta pchA$  strain and its corresponding TBDT  
388 mutants show that both the TBDTs PirA and PiuA are involved in this uptake process (Figure  
389 7). PiuA appears to be more specific to dopamine but is also able to transport iron-loaded  
390 epinephrine, norepinephrine, and L-DOPA, whereas PirA appears to be more specific to L-  
391 DOPA and norepinephrine. Finally, ferrous iron is also apparently weakly transported by the  
392 ferrous iron transporter FeoAB in the presence of L-DOPA. Deletion of *piuA*, *pirA*, and *feoB*  
393 did not completely abolish iron uptake by catecholamines, suggesting that (i) one other  
394 TBDT is also involved, (ii) that catecholamines-Fe complexes diffusion via porins, especially in  
395 the case of dopamine, or (iii) that  $Fe^{3+}$  is rapidly exchanged with other ligands in the growth  
396 media like citrate.

397  
398 The identification of the *piuA* gene in the importation of ferri-catecholamine complexes into  
399 *P. aeruginosa* cells provides information concerning the ligand selectivity of this TBDT. To  
400 date, the only molecules described in the literature to be recognized by PiuA are the  
401 synthetic sideromycins BAL30072 and MC-1 from the pharmaceutical industry (Moynié *et al.*,  
402 2017). These sideromycins are based on vectorization by monohydroxypyridones,

403 considered to be analogues of monocatechols. PiuA may play a key role in iron acquisition  
404 via catecholamines by the pathogen during infections.

405  
406 The induction of transcription and expression of *pirA* in the presence of catecholamines  
407 indicates that this TBDT is able to import iron-loaded catecholamines into *P. aeruginosa* cells  
408 and that these compounds are able to interact with the two component system PirS/PirR  
409 (PirS being the inner-membrane sensor and PirA the transcriptional regulator (Llamas *et al.*,  
410 2008)) (Figure 7). As PirS is located in the inner membrane, catecholamines have to reach  
411 the bacterial periplasm to interact with this sensor and activate the transcription of *pirA*. Our  
412 proteomic results can also explain the induction of *pirA* transcription observed in *P.*  
413 *aeruginosa* cells in an acute murine pneumonia infection model: not only was the  
414 transcription of *pirA* induced, but also proteins of the pyoverdine and heme uptake  
415 pathways (Damron *et al.*, 2016). These results suggest that *P. aeruginosa* is able to use  
416 catecholamines as siderophores during an infection via the PirA transporter.

417  
418 A surprising result in our data was the non-involvement of the tris-catechol enterobactin and  
419 the vibriobactin TBDTs, PfeA, PirA, and FvbA, in the importation of the iron complexes of  
420 epinephrine, norepinephrine, dopamine, and L-DOPA. According to the literature, three  
421 TBDTs have been identified to be involved in the uptake of ferric-loaded catechol  
422 siderophores: PfeA and PirA for enterobactin-Fe and protochelin-Fe and FvbA for  
423 vibriobactin-Fe (Poole *et al.*, 1990; Ghysels *et al.*, 2005; Elias *et al.*, 2011; Gasser *et al.*, 2016),  
424 these compounds all being tris-catechols. The mode of iron chelation by epinephrine,  
425 norepinephrine, dopamine, and L-DOPA, as well as enterobactin and vibriobactin, involves  
426 catechol functions. However, catecholamines are based on a 3,4-dihydroxyphenyl backbone,

427 whereas enterobactin and vibriobactin are based on a 2,3-dihydroxyphenyl backbone,  
428 implying steric hindrance that could explain the lack of interaction with PfeA. In addition,  
429 enterobactin (and most catechol-type siderophores) presents a carboxyl in alpha of the  
430 catechol cycle, thus leaving the possibility of a change in iron coordination between a  
431 catecholate mode and a salicylate mode (Abergel *et al.*, 2006), which may be important for  
432 the interaction with the PfeA transporter *in vivo*. In addition, importantly, the stoichiometry  
433 of iron chelation by catecholamines theoretically involves three catecholamines for a ferric  
434 ion. However, in solution, the existence of a mixture between complexes with  
435 stoichiometries of 3:1 (catecholamine:Fe<sup>3+</sup>), 2:1, or even 1:1 with a molecule of water,  
436 citrate, or any other small molecule to complete the coordination cannot be excluded, as  
437 previously described for ferric complexes of pyochelin and citrate (Cobessi *et al.*, 2005;  
438 Mislin *et al.*, 2006; Silva *et al.*, 2009). Concerning enterobactin, only one form of ferric-  
439 enterobactin is formed, which is recognized by PfeA and, to a lesser extent, PirA. For the  
440 catecholamines, the existence of a mixture of ferric complexes with different chelation  
441 stoichiometries must have a certain impact on their recognition by the involved TBDTs and  
442 could explain why two different TBDTs, PirA and PiuA, with different binding selectivity, are  
443 involved in iron acquisition by these molecules. Moreover, the <sup>55</sup>Fe uptake assay used in this  
444 study was unable to show the involvement of PirA or PiuA in iron assimilation by  
445 catecholamines, whereas the growth assay with the  $\Delta pvdF\Delta pchA$  strain and its  
446 corresponding TBDT mutants was able to do so. Clearly, the sensitivity of the two assays is  
447 not the same for various reasons. The <sup>55</sup>Fe uptake assay lasted only 3 h, with cells in the  
448 stationary phase of growth, whereas the growth assay lasted 24 hours, with the cells being  
449 first in the exponential phase and then stationary phase of growth. Moreover, the  
450 concentration of iron and catecholamines was not the same in the two assays. The <sup>55</sup>Fe

451 uptake assay required a  $^{55}\text{Fe}$  concentration of 500 nM to detect iron accumulation in  
452 bacteria and, consequently, a catecholamine concentration of 200  $\mu\text{M}$  to avoid  $^{55}\text{Fe}$   
453 precipitation, which generates a high background of radioactive noise. In the growth assay,  
454 the iron concentration was approximately 20 nM (Cunrath *et al.*, 2016) and that of the  
455 catecholamines 10  $\mu\text{M}$ . These assays certainly involve complexes with different iron:catechol  
456 stoichiometries, which can also explain the different results obtained for these two assays. It  
457 would also be informative to investigate the effects of catecholamine degradation products  
458 (oxidation of catecholamines). Indeed, aminochrome and adrenochrome, oxidation products  
459 of epinephrine and dopamine, as well as dihydroxyindole, may also play a role in iron  
460 uptake, as the catechol group, involved in iron chelation, is still present in these molecules  
461 and probably more of these molecules are formed during the 48-h culture assay than in the  
462 3 h of  $^{55}\text{Fe}$  uptake monitoring.

463  
464 Despite these encouraging results, the molecular mechanisms involved in iron acquisition by  
465 catecholamines are far from being elucidated. There are likely a large number of proteins  
466 involved that have not been identified, such as inner-membrane transporters, and proteins  
467 involved in iron release from catecholamines. Nothing is currently known about the  
468 mechanism of iron release from the siderophores transported by the PirA and PiuA TBDTs.  
469 No genes encoding for an iron reductase or an inner membrane transporter are found next  
470 to *piA* and *pirA* on *P. aeruginosa* chromosome (Winsor *et al.*, 2016).

471 Moreover, the present study was carried out under *in vitro* conditions using planktonic  
472 cultures of *P. aeruginosa* to identify TBDTs involved in this uptake. Now that the TBDTs have  
473 been identified, it would be informative to determine what happens in an animal model that  
474 simulates a chronic *P. aeruginosa* infection. Moreover, the iron-reducing properties of

475 catecholamines need to be explored further, particularly for bacteria living in biofilms, a life  
476 form in which ferrous iron has a more preponderant role than in planktonic cultures. Finally,  
477 although catecholamines are unlikely to significantly compete with *P. aeruginosa*  
478 siderophores, this does not exclude an important biological role for these molecules under  
479 certain conditions, especially during chronic *P. aeruginosa* infections, for which it is known  
480 that spontaneous mutations of the PvdS regulator can cause the absence of pyoverdine  
481 secretion without, however, affecting the fitness of the bacteria. Such *pvdS* mutants may use  
482 catecholamines as siderophores.

483  
484 Although the study of the interactions between host catecholamines and pathogens is only  
485 in its early stages, our data represent a major breakthrough by showing that dopamine, L-  
486 DOPA, epinephrine, and norepinephrine are able to chelate iron and transport it efficiently  
487 into *P. aeruginosa* cells via active proton-motive force-dependent uptake. The TBDTs  
488 involved are PiuA and PirA, with the two transporters showing different uptake selectivity:  
489 PiuA is more specific to dopamine and PirA appears to be more specific to L-DOPA and  
490 norepinephrine. Ferrous iron uptake was also observed via the FeoABC system in the  
491 presence of L-DOPA (Figure 7). How ferrous iron is transported across the outer membrane  
492 stays unknown and probably occurs via porins. A possible candidate could be the homolog of  
493 OprG of *Acinetobacter baumani* (Catel-Ferreira *et al.*, 2016; Chevalier *et al.*, 2017).

494

496 **Material and Methods**

497 **Chemicals.** Dopamine HCl, L-norepinephrine HCl, L-epinephrine bitartrate, L-DOPA, DL-  
498 normetanephrine HCl, enterobactin and protonophore CCCP (carbonyl cyanide *m*-  
499 chlorophenylhydrazone) were purchase from Sigma-Aldrich. PVD was purified as previously  
500 described (Demange *et al.*, 1990). Catecholamines and normetanephrine were solubilized at  
501 100 mM in ultrapure water before use, with the exception of L-DOPA which was solubilized  
502 at 10 mM owing to its poor solubility in aqueous solution.  $^{55}\text{FeCl}_3$  was obtained from Perkin  
503 Elmer Life and Analytical Sciences (Billerica, MA, USA).

504  
505 **Growth condition.** The strains used are listed in Table SM1 in Supplementary Materials. *P.*  
506 *aeruginosa* was routinely precultured in LB medium for 24 h at 30°C with 220 rpm agitation  
507 before being transferred in iron-restricted CAA medium (casamino acid medium,  
508 composition: 5 g l<sup>-1</sup> low-iron CAA (Difco), 1.46 g l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub> 3H<sub>2</sub>O, 0.25 g l<sup>-1</sup> MgSO<sub>4</sub> 7H<sub>2</sub>O) and  
509 incubated overnight at 30°C. Iron concentration in CAA medium is about 20 nM (Cunrath *et*  
510 *al.*, 2016).

511  
512 **Plasmid and Strain Construction.** Enzymes were obtained from ThermoFisher Scientific.  
513 *Escherichia coli* strains SM10 or TOP10 (Invitrogen) were used as the host strains for the  
514 plasmids. Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific) was used in order  
515 to amplify fragments from a preparation of *P. aeruginosa* PAO1 DNA. Primers are listed in  
516 Table 3SM. A 1400 bp insert containing the 700 bp flanking sequences of the gene and the  
517 HindIII, EcoRI or XhoI restriction sites was amplified for the construction of the pEXG2  
518  $\Delta\text{PA0434}$ , pEXG2  $\Delta\text{piuA}$  and pEXG2  $\Delta\text{fvbA}$  plasmids. Both insert and empty pEXG2 plasmid  
519 were digested with appropriate restriction enzymes and inserts were subsequently cloned

520 into pEXG2 using cohesive end ligation with T4 DNA ligase. Mutations in the chromosomal  
521 genome of *P. aeruginosa* were generated as previously described, by sequentially  
522 introducing pEXG2 vectors using mating into *P. aeruginosa* PAO1  $\Delta pvdF\Delta pchA$  and selecting  
523 for deletion mutants before verification by PCR (polymerase chain reaction) and sequencing  
524 (Perraud *et al.*, 2018).

525

526 **Iron chelation assays.** The CAS (Chrome Azurol S, purchased from Sigma) solution was  
527 prepared according to Schwyn & Neilands as previously described (Perraud *et al.*, 2020)  
528 with the omission of sulfocalicylic acid. 100  $\mu$ L of the CAS-shuttle reagent at 7.5  $\mu$ M was  
529 incubated at room temperature in the presence of increasing concentrations of  
530 catecholamines or normetanephrine for 1 h. For each concentration of compound tested,  
531 the absorbance at 630 nm was monitored in a TECAN Infinite M200 plate reader.

532

533 **Iron scavenging from pyoverdine-Fe.** Pyoverdine was prepared in solution at 10 mM water  
534 and the catecholamines as described above. The competition assay with pyoverdine-Fe was  
535 carried out as described previously (Perraud *et al.*, 2020). PVD-Fe is prepared in solution at  
536 10  $\mu$ M in 100  $\mu$ L of 100 mM HEPES buffer pH 7.4, and incubated at 25 °C in the presence of  
537 increasing concentrations of catecholamines for 48 h. The fluorescence at 447 nm  
538 corresponding to formed apo pyoverdine was monitored (excitation at 400 nm) in a TECAN  
539 Infinite M200 plate reader.

540

541 **Iron reduction assay.** Iron(III) reduction in presence of catecholamines was monitored using  
542 ferrozine (Sigma), a specific Fe<sup>2+</sup> chelator. Ferrozine forms a stable magenta-colored solution  
543 with Fe<sup>2+</sup> and is therefore used as a reagent and indicator for Fe<sup>2+</sup>. The complex has an

544 absorption peak at 562 nm. A mix of 0.5 mM of ferrozine and 50  $\mu$ M of catecholamine in 100  
545 mM HEPES buffer (pH 7.4) was added to a 96-well flat plate (Greiner). Right before the start  
546 of the experience, 50  $\mu$ M of  $\text{FeCl}_3$  in HCl 0.1 N were added to the wells and the increase of  
547 absorbance at 562 nm, corresponding to the formation of ferrozine-Fe(II) complex, was  
548 monitored using a TECAN M200 microplate reader. The positive control was performed  
549 using 100 mM of the reducing agent dithiothreitol instead of catecholamine.

550  
551 **<sup>55</sup>Fe uptake assays.** The iron uptake assays were carried out as previously described (Gasser  
552 *et al.*, 2016) except that the catecholamine-<sup>55</sup>Fe complexes were prepared with a ratio of  
553 400:1 (chelator: <sup>55</sup>Fe) and for enterobactin-<sup>55</sup>Fe of 20:1 incubated for 15 minutes,  
554 catecholamines having a much lower affinity for iron compared to enterobactin and they  
555 chelate ferric iron with 3:1 stoichiometry. Concerning bacterial strains used, overnight  
556 cultures of *bacteria* cultivated in CAA medium were resuspended in 50 mM TrisHCl pH 8.0  
557 buffer at an OD<sub>600 nm</sub> of 1 and incubated with <sup>55</sup>Fe-catecholamine complexes at a final  
558 concentration of 500 nM for 3 h at 30°C. Aliquots were removed at different time, the  
559 bacteria pellet and radioactivity measured by scintillation counting.

560  
561 **Label-free proteomics analysis.** *P. aeruginosa* PAO1 cells grown in CAA medium were  
562 diluted to an OD<sub>600nm</sub> of 0.1 units and incubated in the absence or presence of 10  $\mu$ M  
563 dopamine, L-DOPA, epinephrine, norepinephrine or normetanephrine at 30°C for 8 h. 5.10<sup>8</sup>  
564 cells from each culture were used for proteomic analysis. Each sample was prepared in  
565 biological triplicate for each cell culture condition. Cell pellets were resuspended in 200  $\mu$ L of  
566 lysis buffer (UTCT buffer containing 7M urea, 2M thiourea, 4% CHAPS (3-[(3-  
567 cholamidopropyl)dimethylammonio]-1-propanesulfonate) and 20 mM Tris-HCl pH 7.6)

568 supplemented with nuclease and DNase. Protein concentrations were determined by Bradford  
569 assay using bovine serum albumin as standard. Proteins were further precipitated overnight  
570 with glacial 0.1 M ammonium acetate in 100 % methanol (5 volumes, -20°C). After  
571 centrifugation at 12.000 g and 4°C during 15 min, the resulting pellets were washed twice  
572 with 0.1 M ammonium acetate in 80% methanol and further dried under vacuum (Speed-Vac  
573 concentrator). Pellets were resuspended in 100 µL of 50 mM ammonium bicarbonate and  
574 submitted to reduction (5 mM Dithiothreitol, 95°C, 10 min) and alkylation (10 mM  
575 Iodoacetamide, room temperature, 20 min). Proteins were finally digested overnight with 150  
576 ng of sequencing-grade trypsin (Promega). The proteomic datasets were obtained by the  
577 injection of 5000 ng of each peptidic mixture on a Q-Exactive Plus mass spectrometer  
578 coupled to an EASY-nanoLC-1000 (Thermo-Fisher Scientific, USA) as described previously  
579 (Perraud *et al.*, 2020).

580 The raw data obtained were converted into .mgf files with Proteome Discoverer Daemon  
581 software (Thermo-Fisher Scientific, script “Export-to-mgf”, version 2.2). For both differential  
582 proteomic analyses, data were searched against the *P. aeruginosa* UniprotKB sub-database  
583 with a decoy strategy (UniprotKB release 2016\_12, taxon 208964, *P. aeruginosa* strain  
584 PAO1, 5564 forward protein sequences). Peptides and proteins were identified with Mascot  
585 algorithm (version 2.5.1, Matrix Science, London, UK). The following parameters were used:  
586 (i) Trypsin/P was selected as enzyme, (ii) two missed cleavages were allowed, (iii)  
587 methionine oxidation and acetylation of protein N-term were set as variable modifications and  
588 carbamidomethylation of cysteine as fixed modification, (iv) mass tolerance for precursor  
589 ions was set at 10 ppm, and at 0.02 Da for fragment ions. Mascot data were further imported  
590 into Proline v1.4 software (<http://proline.profiroteomics.fr/>) (Bouyssié *et al.*, 2020). Proteins  
591 were validated on Mascot pretty rank equal to 1, and 1% FDR (False Discovery Rate) on both  
592 peptide spectrum matches (PSM score) and protein sets (Protein Set score). The total number

593 of MS/MS fragmentation spectra was used to quantify each protein from at least three  
594 independent biological replicates: this “BasicSC” value calculated by Proline includes all  
595 PSMs of all peptides, including the modified peptides (3 fixed and variable modifications)  
596 and the peptides shared by different protein sets. After a normalization of the data matrix with  
597 the total number of spectra in one sample, the “BasicSC” spectral count values were  
598 submitted to a negative-binomial test using an edgeR GLM regression through R (R v3.2.5).  
599 The statistical test was based on the published msmsTests R package available in  
600 Bioconductor to process label-free LC-MS/MS data by spectral counts (Gregori *et al.*, 2019).  
601 For each identified protein, an adjusted P-value (adjp) corrected by Benjamini–Hochberg was  
602 calculated, as well as a protein fold-change (FC).

603

604 **Quantitative real-time PCR (qRT-PCR) on bacteria grown liquid culture.** qRT-PCR was used  
605 to follow specific gene transcription as previously described (Gross and Loper, 2009; Gasser  
606 *et al.*, 2016). Bacteria were grown as for the proteomic analyses for 8 h, at 30°C, in the  
607 presence or absence of 10 µM dopamine, L-DOPA, epinephrine, norepinephrine or  
608 normetanephrine and with vigorous shaking. An aliquot of  $2.5 \times 10^8$  cells from this culture  
609 was added to two volumes of RNAProtect Bacteria Reagent (Qiagen) and exactly the same  
610 protocol was used as previously described (Perraud *et al.*, 2020). Primer efficiency were  
611 determined using serially diluted genomic DNA and the double  $\Delta C_T$  method was used to  
612 analyze qPCR data.

613

614 **Growth screening in the absence and presence of catecholamines of different *P.***  
615 ***aeruginosa* T<sub>BDT</sub> mutants.** Overnight cultures of *P. aeruginosa*, grown in CAA medium, were  
616 seeded at an OD<sub>600 nm</sub> of 0.02 in 200 µL fresh CAA medium in the absence or presence of

617 norepinephrine, epinephrine, dopamine, L-DOPA or normetanephrine, in 96 well U-shaped  
618 plates (Greiner). Fresh, sterile-filtered aqueous solutions of the tested compounds were  
619 added at a final concentration of 10  $\mu$ M. OD<sub>600 nm</sub> was monitored in an Infinite M200 (TECAN,  
620 Austria) plate reader for 22 h, with regular agitation and incubation temperature set to 30°C.  
621 Growth rescue assays in presence of enterobactin were performed following the same  
622 procedure, with enterobactin being dissolved at 10 mM in dimethylsulfoxide and added at a  
623 final concentration of 10  $\mu$ M.

624

625

626

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633

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831 **Figure legends**

832

833 **Figure 1. Structure of dopamine, L-DOPA, epinephrine, norepinephrine, and**  
 834 **normetanephrine.** Iron chelating groups are shown in red.

835

836 **Figure 2. A. Ability of the various catecholamines to scavenge iron from CAS-Fe complexes.**

837 CAS-Fe (7.5  $\mu\text{M}$ ) was incubated with increasing concentrations of pyoverdine, pyochelin,  
 838 dopamine, L-DOPA, epinephrine, norepinephrine, and normetanephrine, as described in  
 839 Materials and Methods. CAS-Fe dissociation was followed by monitoring the absorbance at  
 840 630 nm. The data were normalized using  $(A_{\text{MEASURED}} - A_{\text{apoCAS}}) / (A_{\text{apoCAS}} - A_{\text{CAS-Fe}})$ ,  $A_{\text{MEASURED}}$  for  
 841 the absorbance measured for each experimental condition,  $A_{\text{apoCAS}}$  for the absorbance of  
 842 CAS without iron, and  $A_{\text{CAS-Fe}}$  for the absorbance of CAS loaded with iron.

843 **B. Ability of the various catecholamines to scavenge iron from pyoverdine-Fe complexes.**

844 PVD-Fe (10  $\mu\text{M}$ ) in 100 mM HEPES pH 7.4 was incubated with increasing concentrations of  
 845 TCV, dopamine, L-DOPA, epinephrine, norepinephrine, and normetanephrine, as described  
 846 in Materials and Methods, for 48 h (until equilibrium was reached), as described previously  
 847 (Perraud *et al.*, 2020). Metal-free PVD is fluorescent, with absorbance typically at 447 nm,  
 848 and PVD-Fe is not (Albrecht-Gary *et al.*, 1994; Folschweiller *et al.*, 2002). Apo PVD formation  
 849 was thus followed by monitoring fluorescent at 447 nm (excitation at 400 nm). The data  
 850 were normalized using the formula  $(F_{\text{MEASURED}} - F_{\text{PVD-Fe}}) / (F_{\text{PVD}} - F_{\text{PVD-Fe}})$ ,  $F_{\text{MEASURED}}$  being the  
 851 fluorescence measured for each experimental condition,  $F_{\text{PVD-Fe}}$  the fluorescence of 10  $\mu\text{M}$   
 852 PVD-Fe, and  $F_{\text{PVD}}$  the fluorescence of 10  $\mu\text{M}$  PVD.

853 **C. Kinetics of iron reduction.**  $\text{FeCl}_3$  (50  $\mu\text{M}$ ) was incubated in 100 mM HEPES buffer at pH

854 7.4, in the presence of 0.5 mM ferrozine (gray line), 0.5 mM ferrozine and 100 mM DTT

855 (black line), or 0.5 mM ferrozine and 50  $\mu$ M of each of the catecholamines (dopamine: blue  
856 line, L-DOPA: red, epinephrine: orange, norepinephrine: green, and normetanephrine: pink).  
857 The formation of ferrozine-Fe<sup>2+</sup> was followed at 562 nm every 60 s. The graph on the right is  
858 a zoom of the graph on the left.

859

860 **Figure 3. A. <sup>55</sup>Fe uptake by  $\Delta pvdF\Delta pchA$  and  $\Delta pvdF\Delta pchA\Delta pfeA\Delta pirA\Delta piuA\Delta fvbA\Delta PA0434$**

861 **cells in the presence of the catecholamines.** The strains were grown in CAA medium

862 supplemented with one of the catecholamines (dopamine, L-DOPA, epinephrine, or

863 norepinephrine) to activate the expression of the corresponding inducible uptake pathway.

864 Then, bacteria were harvested and incubated in 50 mM Tris-HCl pH 8.0 in the presence of

865 500 nM <sup>55</sup>Fe in complex with catecholamines. Since catecholamines have a low affinity for

866 iron relative to siderophores, they were mixed with <sup>55</sup>Fe in a 400:1 (catecholamines:<sup>55</sup>Fe)

867 ratio, as described in Materials and Methods (at a lower ratio, such as 20:1, a small amount

868 of iron precipitation was observed, affecting the <sup>55</sup>Fe-uptake data). Aliquots were removed

869 at various times, the bacteria pelleted, and the radioactivity retained in the cells measured.

870 In each panel, uptake by  $\Delta pvdF\Delta pchA$  cells is shown in blue and that by

871  $\Delta pvdF\Delta pchA\Delta pfeA\Delta pirA\Delta piuA\Delta fvbA\Delta PA0434$  cells in yellow. For each siderophore, the

872 uptake assays with  $\Delta pvdF\Delta pchA$  cells were also performed in the presence of the

873 protonophore CCCP (green curve) to differentiate proton-motive force-dependent transport

874 from diffusion or nonspecific interactions. For each curve, the data represent the mean of

875 three independent experiments.

876

877 **Figure 4. Analysis of changes in the expression of proteins involved in iron-uptake**

878 **pathways in *P. aeruginosa* cells grown under iron-limited conditions (CAA medium) in the**

879 **absence or presence of catecholamines. A.** Proteomic analyses were performed on the  
880 pyoverdine- and pyochelin-deficient *P. aeruginosa* strains ( $\Delta pvdF\Delta pchA$ ) grown overnight in  
881 CAA supplemented, or not, with 10  $\mu$ M dopamine, L-DOPA, epinephrine, norepinephrine, or  
882 normetanephrine. Average values measured in CAA in the absence of any supplementation  
883 with siderophores are plotted against average values measured in CAA supplemented with  
884 either 10  $\mu$ M catecholamines or normetanephrine. Median values represent the median of  
885 the relative intensity of each protein, normalized against all proteins detected by shotgun  
886 analysis ( $n = 3$ ). The proteins of the pyochelin pathway are represented by yellow dots, those  
887 of the pyoverdine pathway by green dots, and the TBDTs by blue dots. Sup Data 1 shows the  
888 detailed results of protein identification and quantitation.

889 **B.** Heat maps of various TBDTs and proteins involved in the pyochelin and pyoverdine iron  
890 uptake pathways: the darker the shade of blue, the higher the expression of the protein; the  
891 darker the shade of red, the more the expression of the protein is repressed. Only the  
892 proteins for which a change in the level of expression was observed are shown. FecA, FptA,  
893 FpvA, PA0434, PA3268, PfeA, PhuR, and PirA are TBDTs; FpvA, FpvC, PvdI, PvdL, and PvdQ  
894 are proteins of the pyoverdine-dependent iron-uptake pathway; FptA, PchB, PchC, PchD,  
895 PchE, and PchF are proteins of the pyochelin-dependent iron-uptake pathway.

896

897 **Figure 5. Analysis of changes in the transcription of genes encoding TBDTs involved in iron-**  
898 **uptake pathways in *P. aeruginosa* cells grown under iron-limited conditions (CAA medium)**  
899 **in the absence or presence of catecholamines.** RT-qPCR was performed on pyoverdine- and  
900 pyochelin-deficient *P. aeruginosa* strains ( $\Delta pvdF\Delta pchA$ ) grown in CAA medium for 8 h, as  
901 described in Materials and Methods, with or without 10  $\mu$ M dopamine, L-DOPA,  
902 epinephrine, norepinephrine, and normetanephrine. The data were normalized relative to

903 the reference gene *uvrD* and are representative of three independent experiments  
904 performed in triplicate ( $n = 3$ ). Results are presented as the ratio between the values  
905 obtained in the presence of the catecholamines or normetanephrine over those obtained in  
906 their absence. *fecA* encodes the TBDT of ferri-citrate, *feoB* the ferrous inner membrane  
907 transporter, *fptA* and *fpvA* the TBDTs of pyochelin and pyoverdine, respectively, *pfeA* and  
908 *pirA* the TBDT of ENT, and *piuA* the TBDT of an unknown xenosiderophore.

909

910 **Figure 6. Growth of different *P. aeruginosa* TBDT mutants in the absence or presence of 10**  
911  **$\mu\text{M}$  catecholamines.** The strains used are a pyoverdine and pyochelin mutant ( $\Delta pvdF\Delta pchA$ )  
912 and its corresponding TBDT deletion mutants. Strains were grown in CAA medium in the  
913 absence (Ctrl for control, kinetics in black) or presence of 10  $\mu\text{M}$  catecholamines or  
914 normetanephrine. Growth was followed by monitoring optical density (OD) at 600 nm.

915

916 **Figure 7: Transporters involved in iron acquisition by catecholamines in *P. aeruginosa*.**  
917 Catecholamines transport iron in *P. aeruginosa* cells via the TBDTs PirA and PiuA.  
918 Catecholamine-Fe complexes are able to induce *pirA* transcription probably by interacting in  
919 the bacterial periplasm with the sensor of the two-component system PfeS/PirR (PirA being  
920 the transcriptional regulator). In the presence of L-DOPA, ferrous uptake is observed  
921 involving the FeoABC system.

922

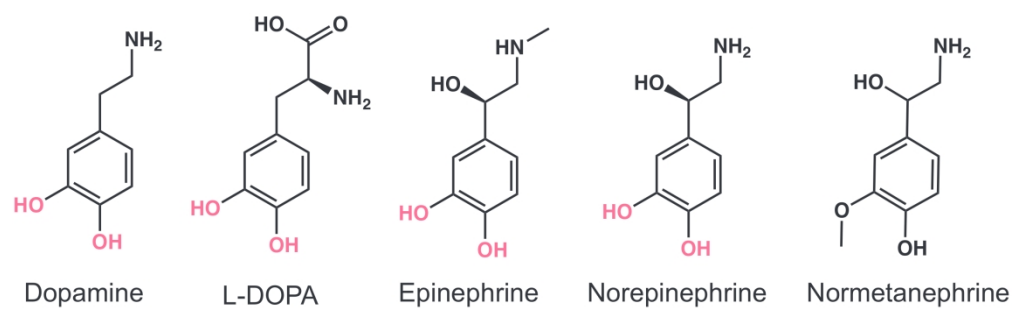


Figure 1. Structure of dopamine, L-DOPA, epinephrine, norepinephrine, and normetanephrine. Iron chelating groups are shown in red.

241x71mm (300 x 300 DPI)

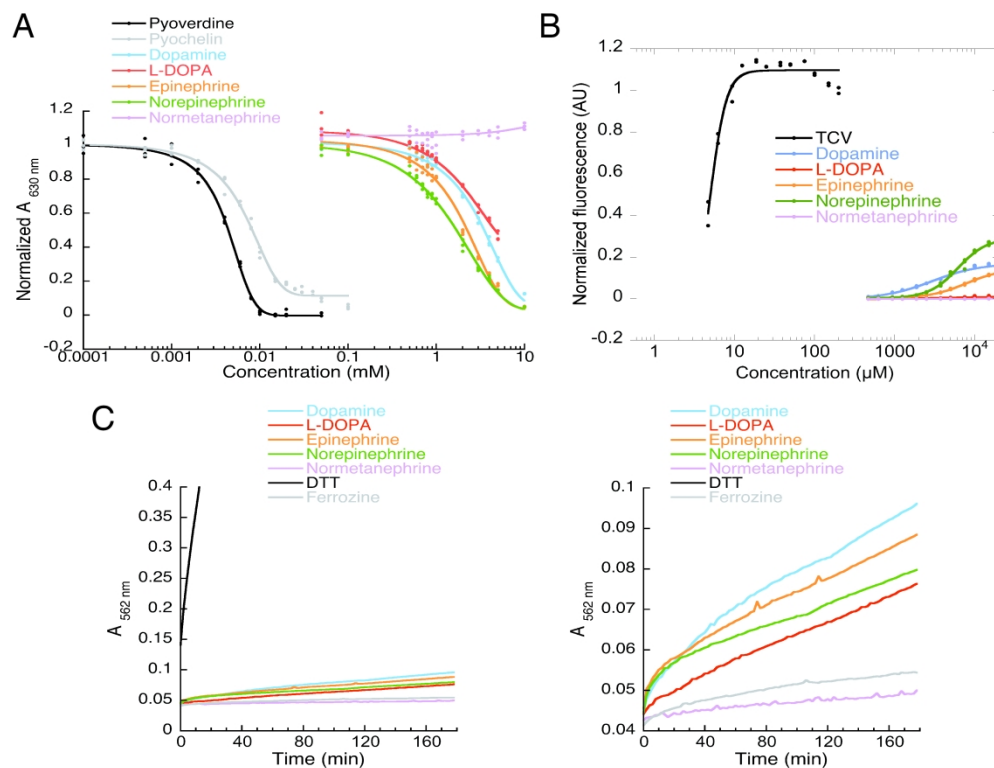


Figure 2. A. Ability of the various catecholamines to scavenge iron from CAS-Fe complexes. CAS-Fe ( $7.5 \mu\text{M}$ ) was incubated with increasing concentrations of pyoverdine, pyochelin, dopamine, L-DOPA, epinephrine, norepinephrine, and normetanephrine, as described in Materials and Methods. CAS-Fe dissociation was followed by monitoring the absorbance at 630 nm. The data were normalized using  $(A_{\text{MEASURED}} - A_{\text{apoCAS}})/(A_{\text{apoCAS}} - A_{\text{CAS-Fe}})$ ,  $A_{\text{MEASURED}}$  for the absorbance measured for each experimental condition,  $A_{\text{apoCAS}}$  for the absorbance of CAS without iron, and  $A_{\text{CAS-Fe}}$  for the absorbance of CAS loaded with iron. B. Ability of the various catecholamines to scavenge iron from PVD-Fe complexes. PVD-Fe ( $10 \mu\text{M}$ ) in 100 mM HEPES pH 7.4 was incubated with increasing concentrations of TCV, dopamine, L-DOPA, epinephrine, norepinephrine, and normetanephrine, as described in Materials and Methods, for 48 h (until equilibrium was reached), as described previously (Perraud *et al.*, 2020b). Metal-free PVD is fluorescent, with absorbance typically at 447 nm, and PVD-Fe is not (Albrecht-Gary *et al.*, 1994; Folschweiller *et al.*, 2002). Apo PVD formation was thus followed by monitoring fluorescent at 447 nm (excitation at 400 nm). The data were normalized using the formula  $(F_{\text{MEASURED}} - F_{\text{PVD-Fe}})/(F_{\text{PVD}} - F_{\text{PVD-Fe}})$ ,  $F_{\text{MEASURED}}$  being the fluorescence measured for each experimental condition,  $F_{\text{PVD-Fe}}$  the fluorescence of  $10 \mu\text{M}$  PVD-Fe, and  $F_{\text{PVD}}$  the fluorescence of  $10 \mu\text{M}$  PVD.

C. Kinetics of iron reduction.  $\text{FeCl}_3$  ( $50 \mu\text{M}$ ) was incubated in 100 mM HEPES buffer at pH 7.4, in the presence of 0.5 mM ferrozine (gray line), 0.5 mM ferrozine and 100 mM DTT (black line), or 0.5 mM ferrozine and 50  $\mu\text{M}$  of each of the catecholamines (dopamine: blue line, L-DOPA: red, epinephrine: orange, norepinephrine: green, and normetanephrine: pink). The formation of ferrozine- $\text{Fe}^{2+}$  was followed at 562 nm every 60 s. The graph on the right is a zoom of the graph on the left.

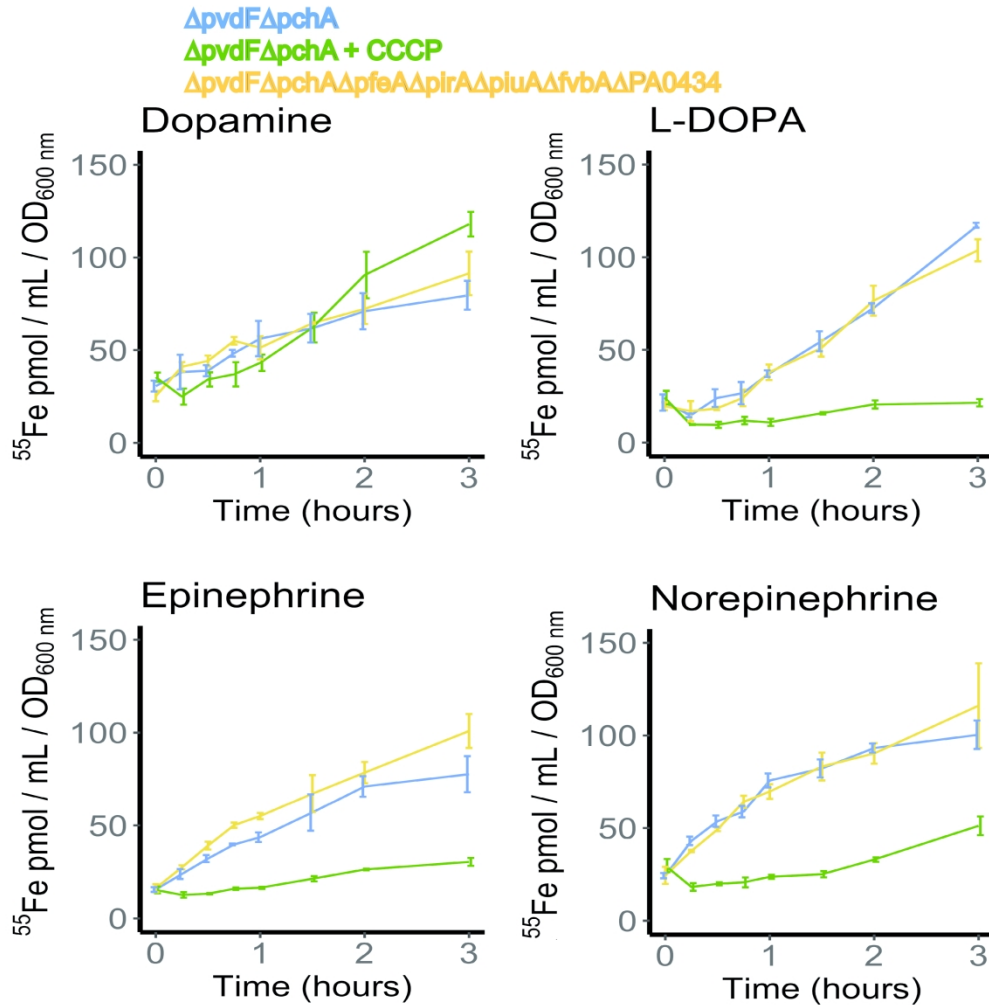


Figure 3. A. <sup>55</sup>Fe uptake by  $\Delta pvdF\Delta pchA$  and  $\Delta pvdF\Delta pchA\Delta pfeA\Delta pirA\Delta piuA\Delta fvbA\Delta PA0434$  cells in the presence of the catecholamines. The strains were grown in CAA medium supplemented with one of the catecholamines (dopamine, L-DOPA, epinephrine, or norepinephrine) to activate the expression of the corresponding inducible uptake pathway. Then, bacteria were harvested and incubated in 50 mM Tris-HCl pH 8.0 in the presence of 500 nM <sup>55</sup>Fe in complex with catecholamines. Since catecholamines have a low affinity for iron relative to siderophores, they were mixed with <sup>55</sup>Fe in a 400:1 (catecholamines:<sup>55</sup>Fe) ratio, as described in Materials and Methods (at a lower ratio, such as 20:1, a small amount of iron precipitation was observed, affecting the <sup>55</sup>Fe-uptake data). Aliquots were removed at various times, the bacteria pelleted, and the radioactivity retained in the cells measured. In each panel, uptake by  $\Delta pvdF\Delta pchA$  cells is shown in blue and that by  $\Delta pvdF\Delta pchA\Delta pfeA\Delta pirA\Delta piuA\Delta fvbA\Delta PA0434$  cells in yellow. For each siderophore, the uptake assays with  $\Delta pvdF\Delta pchA$  cells were also performed in the presence of the protonophore CCCP (green curve) to differentiate proton-motive force-dependent transport from diffusion or nonspecific interactions. For each curve, the data represent the mean of three independent experiments.

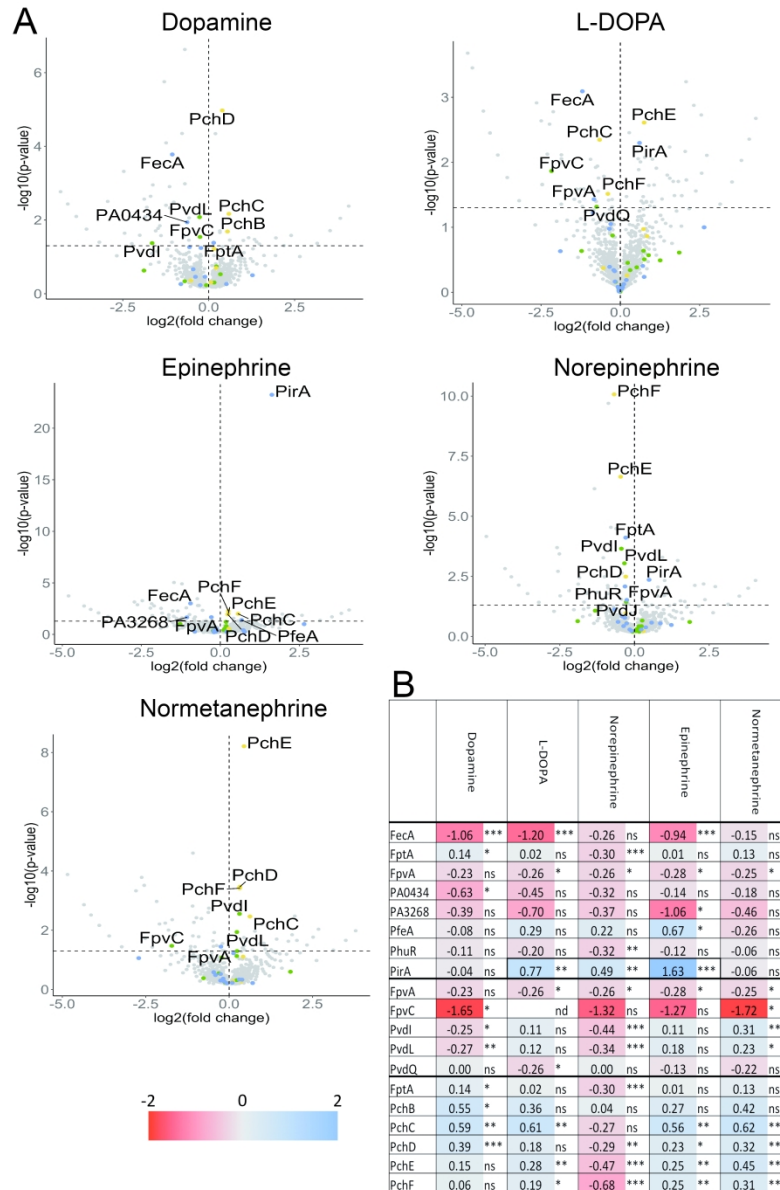


Figure 4. Analysis of changes in the expression of proteins involved in iron-uptake pathways in *P. aeruginosa* cells grown under iron-limited conditions (CAA medium) in the absence or presence of catecholamines. A.

Proteomic analyses were performed on the pyoverdine- and pyochelin-deficient *P. aeruginosa* strains ( $\Delta pvdF\Delta pchA$ ) grown overnight in CAA supplemented, or not, with 10  $\mu\text{M}$  dopamine, L-DOPA, epinephrine, norepinephrine, or normetanephrine. Average values measured in CAA in the absence of any supplementation with siderophores are plotted against average values measured in CAA supplemented with either 10  $\mu\text{M}$  catecholamines or normetanephrine. Median values represent the median of the relative intensity of each protein, normalized against all proteins detected by shotgun analysis ( $n = 3$ ). The proteins of the pyochelin pathway are represented by yellow dots, those of the pyoverdine pathway by green dots, and the TBDTs by blue dots. B. Heat maps of various TBDTs and proteins involved in the pyochelin and pyoverdine iron uptake pathways: the darker the shade of blue, the higher the expression of the protein; the darker the shade of red, the more the expression of the protein is repressed. Only the proteins for which a change in the level of expression was observed are shown. FecA, FptA, FpvA, PA0434, PA3268, PfeA, PhuR, and PirA are TBDTs; FpvA, FpvC, PvdI, PvdL, and PvdQ are proteins of the pyoverdine-dependent

iron-uptake pathway; FptA, PchB, PchC, PchD, PchE, and PchF are proteins of the pyochelin-dependent iron-uptake pathway.

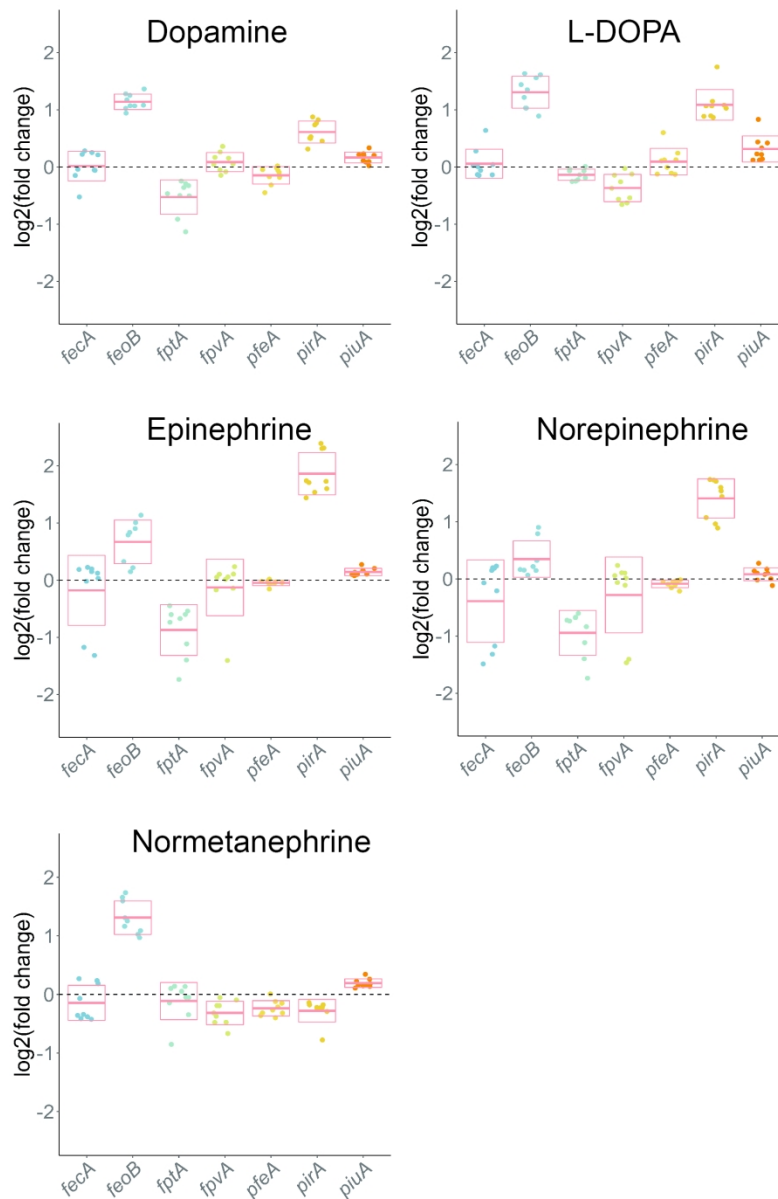


Figure 5. Analysis of changes in the transcription of genes encoding TBDTs involved in iron-uptake pathways in *P. aeruginosa* cells grown under iron-limited conditions (CAA medium) in the absence or presence of catecholamines. RT-qPCR was performed on pyoverdine- and pyochelin-deficient *P. aeruginosa* strains ( $\Delta pvdF\Delta pchA$ ) grown in CAA medium for 8 h, as described in Materials and Methods, with or without 10  $\mu$ M dopamine, L-DOPA, epinephrine, norepinephrine, and normetanephrine. The data were normalized relative to the reference gene *uvrD* and are representative of three independent experiments performed in triplicate ( $n = 3$ ). Results are presented as the ratio between the values obtained in the presence of the catecholamines or normetanephrine over those obtained in their absence. *fecA* encodes the TBDT of ferricitrate, *feoB* the ferrous inner membrane transporter, *fptA* and *fpvA* the TBDTs of pyochelin and pyoverdine, respectively, *pfeA* and *pirA* the TBDT of ENT, and *piuA* the TBDT of an unknown exsiderophore.

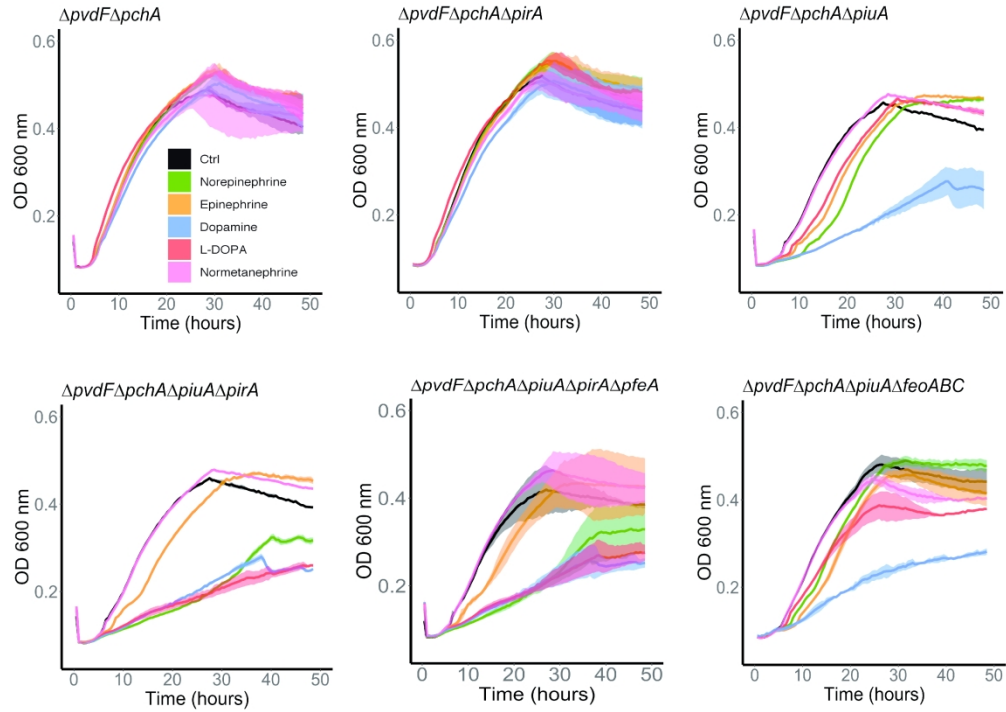


Figure 6. Growth of different *P. aeruginosa* TBDT mutants in the absence or presence of 10  $\mu$ M catecholamines. The strains used are a pyoverdine and pyochelin mutant ( $\Delta pvdF\Delta pchA$ ) and its corresponding TBDT deletion mutants. Strains were grown in CAA medium in the absence (Ctrl for control, kinetics in black) or presence of 10  $\mu$ M catecholamines or normetanephrine. Growth was followed by monitoring optical density (OD) at 600 nm.

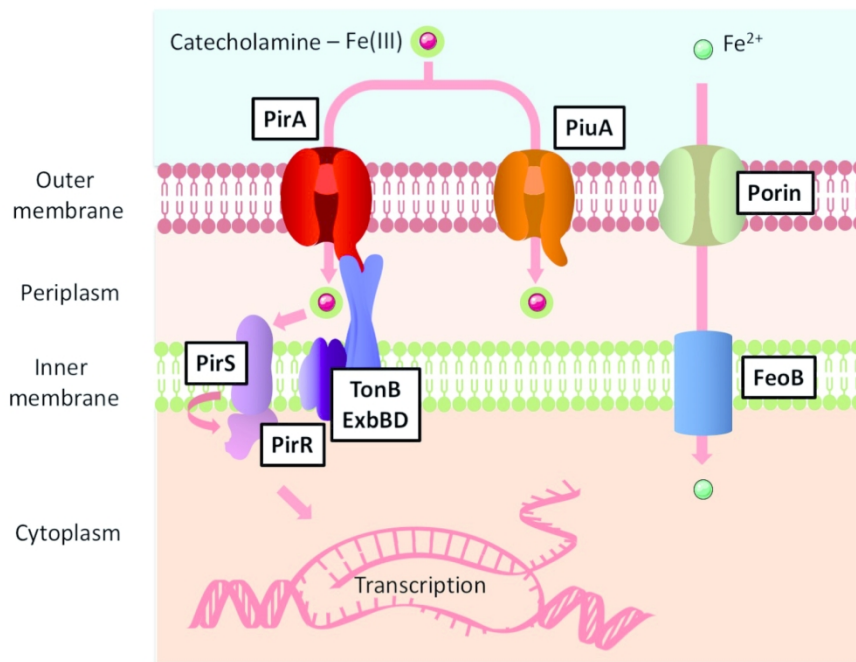


Figure 7: Transporters involved in iron acquisition by catecholamines in *P. aeruginosa*. Catecholamines transport iron in *P. aeruginosa* cells via the TBDTs PirA and PiuA. Catecholamine-Fe complexes are able to induce *pirA* transcription probably by interacting in the bacterial periplasm with the sensor of the two-component system PfeS/PirR (PirA being the transcriptional regulator). In the presence of L-DOPA, ferrous uptake is observed involving the FeoABC system.

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