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► **To cite this version:**

Virginie Will, Véronique Gasser, Lauriane Kuhn, Sarah Fritsch, David Heinrichs, et al.. Siderophore specificities of the *Pseudomonas aeruginosa* TonB-dependent transporters ChtA and ActA. *FEBS Letters*, 2023, 10.1002/1873-3468.14740 . hal-04248262

**HAL Id: hal-04248262**

**<https://hal.science/hal-04248262>**

Submitted on 18 Oct 2023

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### Siderophore specificities of ChtA and ActA, two TonB-dependent transporters of *Pseudomonas aeruginosa*

Journal:	<i>FEBS Letters</i>
Manuscript ID	FEBSL-23-0596.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Will, Virginie; University of Strasbourg; CNRS Gasser, Véronique; University of Strasbourg; CNRS Kuhn, Lauriane; University of Strasbourg; CNRS Fritsch, Sarah; University of Strasbourg; CNRS Heinrichs, David; University of Western Ontario, Microbiology and Immunology Schalk, Isabelle; University of Strasbourg, UMR7242; CNRS, UMR7243
Keywords:	Siderophore, iron homeostasis, <i>Pseudomonas aeruginosa</i> , outer membrane transporters
Abstract:	Iron is an essential nutrient for the pathogen <i>Pseudomonas aeruginosa</i> . To access iron, the pathogen is able to express at least 15 different iron-uptake pathways, the vast majority involving small iron chelators called siderophores. <i>P. aeruginosa</i> produces two siderophores, but can also use many produced by other microorganisms. This implies that the bacterium expresses appropriate TonB-dependent transporters (TBDTs) at the outer membrane to import the ferric form of each of the siderophores used. Here, we show that the two $\alpha$ -carboxylate type siderophores rhizoferrin-Fe and staphyloferrin A-Fe are transported into <i>P. aeruginosa</i> cells by the TBDT ActA. Among the mixed $\alpha$ -carboxylate/hydroxamate type siderophores, we found aerobactin-Fe to be transported by ChtA and schizokinen-Fe and arthrobactin-Fe by ChtA and another unidentified TBDT.

1 **Siderophore specificities of ChtA and ActA, two TonB-dependent transporters of**  
2 ***Pseudomonas aeruginosa***

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23 **ABSTRACT**

24 Iron is an essential nutrient for the pathogen *Pseudomonas aeruginosa*. To access iron, the pathogen  
25 is able to express at least 15 different iron-uptake pathways, the vast majority involving small iron  
26 chelators called siderophores. *P. aeruginosa* produces two siderophores, but can also use many  
27 produced by other microorganisms. This implies that the bacterium expresses appropriate TonB-  
28 dependent transporters (TBDTs) at the outer membrane to import the ferric form of each of the  
29 siderophores used. Here, we show that the two  $\alpha$ -carboxylate type siderophores rhizoferrin-Fe and  
30 staphyloferrin A-Fe are transported into *P. aeruginosa* cells by the TBDT ActA. Among the mixed  $\alpha$ -  
31 carboxylate/hydroxamate type siderophores, we found aerobactin-Fe to be transported by ChtA and  
32 schizokinen-Fe and arthrobactin-Fe by ChtA and another unidentified TBDT.

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## 36 INTRODUCTION

37 Iron is an essential nutrient for the growth and virulence of *P. aeruginosa*, a ubiquitous bacterium and  
38 opportunistic pathogen often responsible for lung infections. *P. aeruginosa* is able to express and use  
39 at least 15 different iron acquisition pathways, each involving a specific set of proteins [1]. Among  
40 these import pathways, three involve different heme import strategies [2,3], one uses ferrous iron [4],  
41 and a large range use siderophores [1]. Siderophores are small molecules with a molecular mass  
42 between 200 and 2000 Da characterized by a very strong affinity for ferric iron [5]. They show an  
43 enormous diversity in their chemical structure, with certain siderophores exclusively possessing  
44 catecholate, hydroxamate, or  $\alpha$ -hydroxy-carboxylate residues for iron chelation, and many others  
45 exhibiting mixtures of different chelating functions [5].

46 Siderophores are synthesized by bacteria, fungi and plants and secreted into their environment to  
47 access iron. For Gram-negative bacteria, once siderophores have scavenged iron, their ferric  
48 complexes are transported back through the outer membrane by TonB-dependent transporters (TBDT)  
49 [6]. The uptake activity of TBDTs is dependent on a molecular motor, located in the inner membrane,  
50 composed of the three proteins: TonB, ExbB, and ExbD [7–10]. The structure of TBDTs is characterized  
51 by a  $\beta$  barrel, allowing insertion into the outer membrane, and a globular or plug domain that closes  
52 the lumen of the barrel [6]. A binding site is located on the extracellular side of the plug domain and is  
53 able to specifically recognize an iron-charged siderophore or a set of ferri-siderophores of very close  
54 chemical structure. The TonB-ExbB-ExbD protein complex is activated by the proton gradient through  
55 the inner membrane. As a consequence, the TonB protein interacts with the N-terminal periplasmic  
56 domain of the TBDTs to allow formation of a channel through the transporter and the import of ferri-  
57 siderophore complexes [7–10]. Depending on the pathway, the iron dissociates from the siderophore,  
58 either in the periplasm or cytoplasm, with transport of the ferri-siderophore complexes through the  
59 inner membrane via specific transporters in the second case [11].

60 *P. aeruginosa* produces two siderophores, pyoverdine and pyochelin [12], and can use many  
61 siderophores produced by other microorganisms (xenosiderophores) [1]. The ability of *P. aeruginosa*  
62 to use siderophores produced by other bacteria is due to the presence of numerous genes encoding  
63 TBDTs in its genome, each recognizing and importing a different iron-loaded xenosiderophore or a  
64 family of xenosiderophores of similar structure. The xenosiderophores that can be used by *P.*  
65 *aeruginosa* include mycobactins and carboxymycobactins (with FemA as the TBDT, [13]), enterobactin  
66 (PfeA and PirA for the TBDTs involved, [14,15]), vibriobactin (FvbA, [16]), nocardamine (FoxA, [17]),  
67 ferrichrome and ferrioxamine B (FiuA and FpvB, [18–20]), and even citrate (FecA, [21]). Recently, it  
68 has also been demonstrated that monocatechols, such as catecholamine neuro-mediators or plant  
69 derived catechols can be used as siderophores by *P. aeruginosa*, with PiuA and PirA as the TBDTs  
70 involved [22,23].

71 Here, we investigated the ability of *P. aeruginosa* to use two  $\alpha$ -carboxylate type siderophores,  
72 rhizoferrin (RHIZOF) and staphyloferrin A (STAPH A), and revisited the ability to use mixed  $\alpha$ -  
73 carboxylate/hydroxamate siderophores, aerobactin (AERO), arthrobactin (ARTHRO) and schizokinen  
74 (SCHIZO). RHIZOF is produced by Zygomycetes, such as *Mucor lusitanicus* [24], STAPH A by  
75 *Staphylococcus aureus* and many members of the coagulase-negative staphylococci [25]), AERO by *E.*  
76 *coli* and hypervirulent *Klebsiella pneumoniae* strains [26], ARTHRO by *Arthrobacter pascens* ATCC  
77 13346 [27], and SCHIZO by *Rhizobium* and *Bacillus megaterium* [28,29] (Figure 1). Previous studies had  
78 shown that AERO, SCHIZO and rhizobactin (a siderophore of the same family as RHIZOF) use the TBDT  
79 ChtA to import iron into *P. aeruginosa* cells [30]. As we had no access to rhizobactin, we tested here  
80 only RHIZOF (rhizoferrin). Here using  $^{55}\text{Fe}$  uptake and growth assays, we show that the TBDTs involved  
81 are: ActA ( $\alpha$ -carboxylate transporter A, gene PA3268) for RHIZOF and STAPH A, ChtA for AERO, and  
82 ChtA and another unidentified TBDT for SCHIZO and ARTHRO.

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84

## 85 MATERIALS AND METHODS

86 **Chemicals.** RHIZOF, AERO, ARTHRO and SCHIZO were purchased from EMC Microcollections  
87 (Tübingen, Germany), and STAPH A was synthesized as previously described [31]. The protonophore  
88 carbonyl cyanide m-chlorophenylhydrazone (CCCP) was purchased from Sigma-Aldrich and  $^{55}\text{FeCl}_3$  was  
89 purchased from Perkin Elmer.

90 **Bacterial strains, plasmids, and growth conditions.** The *P. aeruginosa* strains used in this study are  
91 listed in Table S1 in Supplemental Materials. The iron-deficient CAA (casamino acid) medium used is  
92 composed of  $5\text{ g l}^{-1}$  low-iron CAA (Difco),  $1.46\text{ g l}^{-1}$   $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , and  $0.25\text{ g l}^{-1}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .

93 **Plasmid and strain construction.** Enzymes were obtained from ThermoFisher Scientific. *Escherichia*  
94 *coli* TOP10 (Invitrogen) was used as the host strain for the plasmids. Plasmid construction and  
95 mutagenesis in the chromosomal genome of *P. aeruginosa* were performed as previously described  
96 [32]. Briefly, the blunt-end ligation strategy was used to insert 1400 bp of flanking sequences of the  
97 genes *chtA* or *actA* into the suicide vector pEXG2. The plasmids were then sequenced and used to  
98 introduce chromosomal mutations into the *P. aeruginosa*  $\Delta\text{pvdF}\Delta\text{pchA}$  strain. All generated mutants  
99 were verified by PCR and sequencing.

100 **Iron uptake.** Xenosiderophore- $^{55}\text{Fe}$  complexes were prepared at  $^{55}\text{Fe}$  concentrations of  $50\text{ }\mu\text{M}$ , with a  
101 xenosiderophore:iron (mol:mol) ratio of 20:1, as described previously. *P. aeruginosa* strains were  
102 successively grown overnight in LB broth, followed by overnight growth in CAA medium and, finally,  
103 overnight growth in CAA medium with either  $10\text{ }\mu\text{M}$  RHIZOF, STAPH A, AERO, ARTHRO or SCHIZO, all  
104 at  $30^\circ\text{C}$ . The bacteria were subsequently used to study  $^{55}\text{Fe}$  uptake kinetics, as described previously,  
105 in the presence or absence of  $200\text{ }\mu\text{M}$  CCCP (a proton motive force inhibitor) at a concentration of  
106 xenosiderophore- $^{55}\text{Fe}$  complexes of  $500\text{ nM}$ , with a xenosiderophore:iron (mol:mol) ratio of 20:1  
107 [33,34].

108

109 **Growth assays in iron-restricted conditions.** For *P. aeruginosa* growth assays in microplates, bacteria  
110 were grown as described above: a first overnight culture at 30°C in 5 mL LB broth, followed by washing  
111 of the bacteria and a second overnight culture in 10 mL CAA medium at 30°C. Bacteria were then  
112 washed, resuspended in CAA medium at an optical density of 0.01 at 600 nm, and distributed in the  
113 wells of a 96-well plate (Greiner, U-bottom microplate) in the presence or absence of 10 µM RHIZOF,  
114 STAPH A, AERO, ARTHRO or SCHIZO. The plate was incubated at 30°C, with shaking, in a TECAN  
115 microplate reader (Infinite M200, Tecan) and bacterial growth monitored at OD<sub>600 nm</sub>. The presented  
116 data are the mean of three replicates for each measurement.

117 **Proteomic analysis.** *P. aeruginosa*  $\Delta pvdF\Delta pchA$  cells were consecutively grown overnight in  
118 LB and then CAA medium. Bacteria were then pelleted and diluted again in CAA medium at an  
119 OD<sub>600 nm</sub> of 0.1 and grown for 8 h with or without 10 µM xenosiderophores at 30°C. The cells  
120 ( $5 \times 10^8$ ) were harvested and used for proteomic analysis. A biological triplicate was prepared  
121 for each sample for each cell-culture condition. Cell pellets were resuspended in 200 µL lysis  
122 buffer (UTCT buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, and 20 mM Tris-HCl pH 7.6)  
123 supplemented with nuclease and DNase. Protein concentrations were determined with the  
124 Bradford assay using bovine serum albumin as the standard. Proteins were further  
125 precipitated overnight with glacial 0.1 M ammonium acetate in 100% methanol (5 volumes, -  
126 20°C). After centrifugation at 12,000 x g and 4°C for 15 min, the resulting pellets were washed  
127 twice with 0.1 M ammonium acetate in 80% methanol and further dried under vacuum  
128 (Speed-Vac concentrator). Pellets were resuspended in 100 µL 50 mM ammonium  
129 bicarbonate and reduced (5 mM dithiothreitol, 95°C, 10 min) and alkylated (10 mM  
130 iodoacetamide, room temperature, 20 min). Proteins were finally digested overnight with 150  
131 ng sequencing-grade trypsin (Promega). The proteomic data sets were obtained by injecting 1000  
132 ng of each peptide mixture into a TripleTOF 5600 mass spectrometer (Sciex) coupled to an U3000-RSLC



133 liquid chromatography (Thermo-Fisher Scientific), as described previously. For both differential  
134 proteomic analyses, data were searched against the *P. aeruginosa* UniprotKB sub-database  
135 (UniprotKB release 2016\_12, taxon 208964, *P. aeruginosa* strain PAO1, 5564 forward protein  
136 sequences). Peptides and proteins were identified using the Mascot algorithm (version 2.5.1,  
137 Matrix Science, London, UK). The following parameters were used: (i) Trypsin/P was selected  
138 as the enzyme, (ii) two missed cleavages were allowed, (iii) methionine oxidation and  
139 acetylation of the protein N-terminus were set as variable modifications and  
140 carbamidomethylation of cysteine as a fixed modification, (iv) mass tolerance for precursor  
141 ions was set to 10 ppm and 0.02 Da for fragment ions. Mascot data were further imported  
142 into Proline v1.4 software [35]. Proteins were validated against a Mascot pretty rank equal to  
143 1 and a 1% FDR for both peptide spectrum matches (PSM score) and protein sets (Protein Set  
144 score). All MS/MS fragmentation spectra were used to quantify each protein from at least  
145 three independent biological replicates: this “BasicSC” value, calculated by Proline, included  
146 all PSMs of all peptides, including modified peptides (3 fixed and variable modifications) and  
147 the peptides shared by different protein sets. After column-wise normalization of the data  
148 matrix, the “BasicSC” spectral count values were subjected to a negative-binomial test using  
149 edgeR GLM regression of R (R v3.2.5) through the IPinquiry package [36]. The statistical test  
150 was based on the published msmsTests R package available in Bioconductor to process label-  
151 free LC-MS/MS data by spectral counts [37]. For each identified protein, an adjusted P-value  
152 (adjp), corrected by the Benjamini–Hochberg procedure, was calculated, as well as the protein  
153 fold-change (FC). The MS data were deposited in the ProteomeXchange Consortium database via the  
154 PRIDE [38] partner repository with the dataset identifier PXD043056 and 10.6019/PXD043056  
155 (Reviewer account details: Username: [reviewer\\_pxd043056@ebi.ac.uk](mailto:reviewer_pxd043056@ebi.ac.uk), Password: Ev8yglfz).

156 **Quantitative real-time PCR.** The expression of specific genes of interest was monitored by quantitative  
157 reverse transcription PCR (qRT-PCR) as described previously [32,39]. After a first overnight incubation  
158 in LB medium,  $\Delta pvdF\Delta pchA$  cells were grown in 10 mL CAA medium. Bacteria were then washed,  
159 resuspended in CAA medium at an optical density of 0.1 at 600 nm, and incubated in the presence of  
160 10 or 100  $\mu$ M RHIZOF, STAPH A, AERO, ARTHRO or SCHIZO, with shaking (220 rpm), at 30°C. After 8 h  
161 of incubation,  $2.5 \times 10^8$  cells were collected from the cultures and two volumes of RNAprotect Bacteria  
162 Reagent (Qiagen) was added. Total RNA was extracted from the pellet using the RNeasy Plus Mini Kit  
163 (Qiagen) following the manufacturer instructions. Total RNA (1  $\mu$ g) was reverse transcribed using the  
164 iScript cDNA Synthesis Kit (Biorad). Specific cDNA in our samples was quantified using a CFX Opus 96  
165 (Biorad), the appropriate primers (Table S3), and iTaq Universal SYBR Green Supermix (Biorad). The  
166 transcript level for each gene was normalized against those of *uvrD* and *rpsI* and are expressed as a  
167 log<sub>2</sub> ratio (fold-change) relative to the reference condition.

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171 **RESULTS AND DISCUSSION**

172 ***RHIZOF, STAPH A, AERO, ARTHRO and SCHIZO transport <sup>55</sup>Fe into P. aeruginosa cells.*** We investigated  
173 the ability of AERO, ARTHRO, SCHIZO, RHIZOF, and STAPH A to be used as xenosiderophores by *P.*  
174 *aeruginosa* and compared their efficiency of iron uptake by using <sup>55</sup>Fe. *P. aeruginosa*  $\Delta pvdF\Delta pchA$   
175 (Table S1 in Supplemental Materials), a strain unable to produce the siderophores PVD and PCH, was  
176 grown under iron-starvation conditions and in the presence of 10  $\mu$ M of the apo form (without iron)  
177 of one of the xenosiderophores to induce expression of the corresponding iron-uptake pathway.  
178 Indeed, previous studies have shown that *P. aeruginosa* is able to detect the presence of certain  
179 xenosiderophores in its environment and induce expression of the proteins belonging to the  
180 corresponding iron-uptake pathway in response [13,18,32,39–42]. The  $\Delta pvdF\Delta pchA$  mutant was used  
181 to avoid any iron uptake by the siderophores pyoverdine and pyochelin produced by *P. aeruginosa*.  
182  $\Delta pvdF\Delta pchA$  cells were then incubated in the presence of one of the five xenosiderophores in complex  
183 with <sup>55</sup>Fe and the radioactivity incorporated into the bacteria followed as a function of time. We  
184 observed <sup>55</sup>Fe accumulation for RHIZOF, STAPH A, AERO and SCHIZO, with very similar uptake rates of  
185 approximately 280-400 pmol.mL<sup>-1</sup>.OD<sub>600 nm</sub><sup>-1</sup> after a 3 h incubation (Figure 2A, B, C and E, kinetics in  
186 green). This is within the range that has been previously reported for pyoverdine and pyochelin  
187 [32,43,44]. The <sup>55</sup>Fe uptake rates for ARTHRO was clearly less efficient (Figure 2D, kinetic in green).  
188 <sup>55</sup>Fe accumulation was no longer observed for  $\Delta pvdF\Delta pchA$  cells pre-incubated with the protonophore  
189 CCCP for all five xenosiderophores (kinetics in yellow), demonstrating that the iron uptake observed  
190 for these compounds is dependent on the bacterial proton motive force. Therefore, the observed <sup>55</sup>Fe  
191 uptake was not due to passive diffusion across the outer membrane via porins or non-specific  
192 association with the cell surface, but rather to transport carried out by TBDTs. In conclusion, the five  
193 xenosiderophores RHIZOF, STAPH A, AERO, ARTHRO and SCHIZO, can transport iron into *P.*  
194 *aeruginosa* cells and act as xenosiderophores, but this uptake is clearly less efficient for ARTHRO  
195

196 ***None of these xenosiderophores significantly induces the transcription or expression of a TBDT.***

197 Previous studies have shown that the presence of certain xenosiderophores, such as enterobactin,  
198 nocardamine, or ferrichrome, in the environment of *P. aeruginosa* induces the expression of the  
199 corresponding TBDT by mechanisms involving sigma/anti-sigma factors, two-component systems, or  
200 AraC type transcriptional regulators [32,42]. Such induction of expression of the TBDT corresponding  
201 by its xenosiderophore often goes hand to hand with repression of the expression of the genes  
202 involved in the uptake of iron by pyochelin, whereas no effect is generally seen for the genes involved  
203 in iron uptake by pyoverdine [17,32,39,42,43].

204 Proteomic analyses of *P. aeruginosa*  $\Delta pvdF\Delta pchA$  grown in the presence or absence of 10  $\mu\text{M}$  of  
205 RHIZOF, STAPH A, AERO and SCHIZO, showed the induction of *chtA* and *actA* (PA3268) expression, with  
206 fold changes between 1.87 and 5.2, but with poor statistics suggesting that the expression induction  
207 observed may be an artefact (Figure S1, ARTHRO was not tested since the  $^{55}\text{Fe}$  uptake was low).  
208 Indeed, such induction of *chtA* and *actA* expression was not confirmed at the transcriptional level by  
209 qRT-PCR (Figure S2). For the qRT-PCR assay, we tested two concentrations of xenosiderophores, 10  
210 and 100  $\mu\text{M}$ , expecting that the transcription of *chtA* and/or *actA* could be induced by the higher  
211 concentration of xenosiderophores. The effect of ARTHRO on *chtA* and/or *actA* transcription was also  
212 tested in qRT-PCR. We observed no induction of the transcription of these two genes, even at 100  $\mu\text{M}$   
213 for all five xenosiderophores indicating these xenosiderophores are not able to induce the  
214 transcription and expression of *chtA* or *actA* when present in the environment of *P. aeruginosa*. ChtA  
215 has been previously reported to be involved in the uptake of iron via AERO, SCHIZO, and rhizobactin  
216 [30]. ActA is encoded by the gene PA3268, but the ligand transported by this transporter has not yet  
217 been identified. Surprisingly, *oprC* expression was repressed in the presence of 10  $\mu\text{M}$  RHIZOF, SCHIZO,  
218 or STAPH A, with fold changes between -5.6 and -2.5 relative to cells grown in the absence of any  
219 xenosiderophore (Figure S1). OprC is a TBDT involved in copper acquisition [45]. Such a reduction in  
220 *oprC* expression could not be confirmed at the transcriptional level by qRT-PCR (Figure S2), raising

221 doubts about its biological significance and the possibility that these xenosiderophores may import  
222 copper.

223 In conclusion, the proteomic and qRT-PCR data were not consistent concerning the induction of  
224 transcription and expression of *chtA* and *actA* by RHIZOF, STAPH A, AERO, ARTHRO or SCHIZO, making  
225 it difficult to draw any definitive conclusions. If expression of one of these TBDTs is induced by one of  
226 the xenosiderophores tested, it may be at a low level that is not easily detected by the approaches  
227 used here.

228

229 ***ActA imports iron via RHIZOF and STAPH A, the two  $\alpha$ -carboxylate type xenosiderophores.*** We  
230 further investigated the uptake pathway used by RHIZOF and STAPH A to import iron using a growth  
231 assay described previously [44] with the  $\Delta pvdF\Delta pchA$  strain grown under iron-restricted conditions  
232 (CAA medium containing approximately 20 nM iron, [46]). In this growth condition, the *P. aeruginosa*  
233 strain is unable to produce pyoverdine and pyochelin and probably accesses iron via citrate or imports  
234 ferrous iron after reduction. Addition of 10  $\mu$ M RHIZOF or STAPH A to this medium stimulated growth  
235 for both xenosiderophores tested, confirming that they can promote  $\Delta pvdF\Delta pchA$  growth by importing  
236 iron into the bacteria (Figure 3). In this assay, the presence of a xenosiderophore unable to import iron  
237 into *P. aeruginosa* cells completely inhibited growth, as previously shown for bacillibactin [44].

238 We used both the growth and  $^{55}\text{Fe}$  uptake assays to try to identify the TBDTs involved in the uptake of  
239 iron by these xenosiderophores. If a TBDT is involved, deletion of its gene should affect the growth of  
240 the bacteria, because no more iron uptake occurs. In the presence of RHIZOF and STAPH A, complete  
241 growth inhibition was observed when *actA* (PA3268) was deleted, clearly showing that ActA is the only  
242 TBDT that imports iron via these two xenosiderophores (Figure 3). Mutation of *chtA* had no effect on  
243 growth in the presence of RHIZOF, indicating that unlike rhizobactin [30], ChtA is not involved in iron  
244 acquisition by RHIZOF. Concerning the  $^{55}\text{Fe}$  assay, surprisingly, mutation of *actA* did not completely

245 abolish  $^{55}\text{Fe}$  uptake for either the RHIZOF- $^{55}\text{Fe}$  or STAPH A- $^{55}\text{Fe}$  complexes (69% and 39% inhibition,  
246 respectively, Figure 2), contrary to what would have been expected from the results of the growth  
247 experiments, and mutation of *chtA* had no effect on  $^{55}\text{Fe}$  import. The remaining  $^{55}\text{Fe}$  uptake observed  
248 for the  $\Delta pvdF\Delta pchA\Delta actA$  strain likely corresponds to the uptake of RHIZOF- $^{55}\text{Fe}$  and STAPH- $^{55}\text{Fe}$  via  
249 another pathway involving an unidentified TBDT. However, iron taken up via this uptake pathway  
250 appears to not be bioavailable for *P. aeruginosa*, as deletion of *actA* alone completely abolished  
251 growth in iron-restricted medium (Figure 3). One explanation could be that iron cannot be removed  
252 from RHIZOF and STAPH A in this second unidentified uptake pathway because it is unable to interact  
253 with the enzyme(s) involved in this process. Another possibility is that  $^{55}\text{Fe}$  in this second uptake  
254 pathway is blocked in the periplasm and unable to reach the cytoplasm because the two  
255 xenosiderophore- $^{55}\text{Fe}$  complexes are unable to be transported across the inner membrane.

256 Overall, these data show that ActA is the TBDT of the  $\alpha$ -carboxylate type siderophores RHIZOF and  
257 STAPH A. Another TBDT is apparently also able to import the two RHIZOF- $^{55}\text{Fe}$  and STAPH- $^{55}\text{Fe}$   
258 complexes across the outer membrane, but the iron is not bioavailable for *P. aeruginosa* cells. Contrary  
259 to what has been reported previously for rhizobactin [30], ChtA is not involved in iron acquisition by  
260 RHIZOF, which is surprising since both xenosiderophores have close chemical structures. It would be  
261 interesting to revisit the ability of ChtA to import iron via rhizobactin (unfortunately we were unable  
262 to get purified rhizobactin).

263

264

265 ***ChtA is the TBDT involved in the uptake of iron by AERO, ARTHRO, and SCHIZO, the three mixed  $\alpha$ -***  
266 ***carboxylate/hydroxamate type xenosiderophores.*** Using the same strategy as described above, we  
267 observed complete inhibition of bacterial growth in the presence of AERO when the *chtA* gene was  
268 deleted, indicating that ferri-AERO is transported into *P. aeruginosa* cells exclusively by this TBDT  
269 (Figure 4). In the presence of ARTHRO or SCHIZO, the growth of  $\Delta pvdF\Delta pchA\Delta chtA$  was only partially  
270 (54 and 45% respectively) affected by deletion of *chtA* compared to the growth of  $\Delta pvdF\Delta pchA$ .

271 Deletion of *actA* had no additional inhibitory effect on the growth of the  $\Delta pvdF\Delta pchA\Delta chtA\Delta actA$   
272 *mutant*. As complete inhibition of growth was not observed with deletion of either *chtA* or *actA*, at  
273 least one other unidentified TBDT is involved in the uptake of iron by ARTHRO and SCHIZO. Concerning  
274 the  $^{55}\text{Fe}$  assay, the deletion of *chtA* completely abolished the import of iron by AERO, perfectly  
275 consistent with the results observed in the growth experiment, confirming that ChtA is the only TBDT  
276 involved in iron import by this xenosiderophore. For ARTHRO and SCHIZO, the deletion of *actA* had no  
277 effect on  $^{55}\text{Fe}$  uptake, and the deletion *chtA* abolished 51 and 81% of  $^{55}\text{Fe}$  uptake respectively, a greater  
278 impact than that observed on bacterial growth. One explanation could be that the second unidentified  
279 TBDT involved is less efficient in importing iron but still allows the bacteria to survive in an iron-  
280 restricted environment. Such lower efficiency could come from lower expression of the TBDT involved  
281 or lower affinity of the Ferri-ARTHRO and ferri-SCHIZO complexes for the binding site of this TBDT.

282 Overall, these data show that ChtA transports the ferri forms of the mixed  $\alpha$ -carboxylate/hydroxamate  
283 siderophores AERO, ARTHRO and SCHIZO, confirming previous studies [30]. However, according to our  
284 data, another unidentified transporter is also involved in ferri-ARTHRO and ferri-SCHIZO transport.

285

286

## 287 **CONCLUSION**

288 Here, we show that *P. aeruginosa* can use RHIZOF, STAPH A, AERO, ARTHRO and SCHIZO as  
289 siderophores, expanding the number of xenosiderophores used by this pathogen. RHIZOF, STAPH A,  
290 AERO, and SCHIZO transport  $^{55}\text{Fe}$  with an efficiency close to that previously reported for pyoverdine,  
291 pyochelin, enterobactin, ferrichrome, and nocardamine [17,32].  $^{55}\text{Fe}$  uptake by ARTHRO was less  
292 efficient. The fact that this bacterium can use many xenosiderophores is an advantage for its survival  
293 in various microbial communities. For example, *S. aureus* and *P. aeruginosa* are frequently co-isolated  
294 from chronic infection, including the pulmonary infections of persons with cystic fibrosis (CF) and  
295 chronic wounds [47–51]. The ability of *P. aeruginosa* to use STAPH A to access iron may play a key role

296 in such co-infections. Recent studies have also shown that *S. aureus* inactivates *P. aeruginosa*  
297 siderophore pyochelin via methylation, indicating as well that competition for iron must be a tough  
298 battle between both pathogens in co-infections [52]. The two  $\alpha$ -carboxylate type xenosiderophores,  
299 RHIZOF-Fe and STAPH A-Fe, are transported into *P. aeruginosa* cells by ActA (PA3268), a TBDT for which  
300 the transported molecule had not been identified to date (Scheme 1). The gene PA3269, encoding an  
301 AraC type transcriptional regulator, is localized next to the *actA* gene, suggesting that *actA*  
302 transcription should involve a molecular mechanism similar to that of *fptA* (ferri-pyochelin TBDT).  
303 Indeed, PchR is a AraC transcriptional regulator capable to bind ferri-pyochelin transported into the  
304 bacteria by FptA, and inducing the transcription of *fptA* [41,53,54]. According the transcriptomic and  
305 proteomic data presented above, RHIZOF and STAPH A appear to be unable to efficiently induce *actA*  
306 transcription and expression.

307 For the mixed  $\alpha$ -carboxylate/hydroxamate type xenosiderophores, AERO-Fe is transported by ChtA (as  
308 previously described [30]) and ARTHRO-Fe and SCHIZO-Fe, by ChtA and another unidentified TBDT  
309 (Scheme 1). These two TBDTs, ActA and ChtA, have different binding selectivity: ActA is specific for  $\alpha$ -  
310 carboxylate type siderophores and ChtA for mixed  $\alpha$ -carboxylate/hydroxamate siderophores.

311

312

### 313 **ACKNOWLEDGMENTS**

314 Authors acknowledge the Centre National de la Recherche Scientifique (CNRS) for general  
315 financial support. The presented work was also supported by a grant from the CNRS (80/Prime  
316 2020), as well as with a grant from the Agence Nationale de la Recherche (ANR, grant number:  
317 ANR-22-CE44-0024-01). We also acknowledge the Interdisciplinary Thematic Institute (ITI)  
318 InnoVec (Innovative Vectorization of Biomolecules, IdEx, ANR-10-IDEX-0002). The mass  
319 spectrometry instrumentation at the IBMC was funded by the University of Strasbourg, IdEx  
320 "Equipement mi-lourd" 2015. VW had a fellowship from the Ministry of Research and



321 Education. Work in the Heinrichs laboratory was funded by Canadian Institutes of Health  
322 Research grant PJT-183848.

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324

## 325 **AUTHORS CONTRIBUTIONS**

326 VW carried out all the <sup>55</sup>Fe uptake assays, qRT-PCR, and bacterial growth assays, and prepared the  
327 figures of the manuscript; LK: carried out the proteomic experiments and analyses, writing – review &  
328 editing; SF: generated the mutants used in the manuscript; DEH: provided the staphyloferrin; GV:  
329 Conceptualization, supervised the biological experiments; IJS: Conceptualization, supervised the  
330 biological experiments, writing – review & editing, funding acquisition, project administration. All  
331 authors reviewed the manuscript.

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482 **LEGENDS**

483 **Figure 1. Chemical structure of rhizoferrin (RHIZOF), staphyloferrin A (STAPH A), aerobactin (AERO),**  
484 ***arthrobactin (ARTHRO) and schizokinen (SCHIZO).***

485  
486 **Figure 2. <sup>55</sup>Fe uptake by *P. aeruginosa* strains mediated by RHIZOF, STAPH A, AERO, ARTHRO and**  
487 **SCHIZO.** *P. aeruginosa*  $\Delta$ pvdF $\Delta$ pchA cells were grown in iron-restricted CAA medium in the presence  
488 of 10  $\mu$ M RHIZOF, STAPH A, AERO, ARTHRO, or SCHIZO, to induce expression of the corresponding  
489 uptake pathway. Afterwards, bacteria were incubated with 500 nM RHIZOF-<sup>55</sup>Fe, STAPH A-<sup>55</sup>Fe, AERO-  
490 <sup>55</sup>Fe, ARTHRO-<sup>55</sup>Fe, or SCHIZO-<sup>55</sup>Fe. The amount of <sup>55</sup>Fe taken up by the bacteria was measured as a  
491 function of time. As a control, the experiment was repeated in the presence of the protonophore CCCP  
492 (200  $\mu$ M). The experiment was repeated with the corresponding  $\Delta$ chtA and  $\Delta$ actA deletion mutants of  
493  $\Delta$ pvdF $\Delta$ pchA. Error bars were calculated from three independent biological replicates.

494  
495 **Figure 3. Growth of  $\Delta$ pvdF $\Delta$ pchA *P. aeruginosa* mutants in the presence of RHIZOF or STAPH A.**  
496  $\Delta$ pvdF $\Delta$ pchA and its corresponding  $\Delta$ chtA and  $\Delta$ actA deletion mutants were grown in CAA medium in  
497 the presence (growth kinetics in orange) or absence (kinetics in blue) of 10  $\mu$ M RHIZOF or STAPH A.  
498 Growth was followed by monitoring the optical density at 600 nm. Error bars were calculated from  
499 three independent biological replicates.

500  
501 **Figure 4. Growth of  $\Delta$ pvdF $\Delta$ pchA *P. aeruginosa* mutants in the presence of AERO, ARTHRO or SCHIZO.**  
502 The pyoverdine and pyochelin-deficient strain of *P. aeruginosa* ( $\Delta$ pvdF $\Delta$ pchA) and its corresponding  
503  $\Delta$ actA and  $\Delta$ chtA deletion mutants were used. Strains were grown in CAA medium in the presence  
504 (growth kinetics in orange) or absence (kinetics in blue) of 10  $\mu$ M AERO, ARTHRO or SCHIZO. Growth  
505 was followed by monitoring the optical density at 600 nm. Error bars were calculated from three  
506 independent biological replicates.

507



508 **Scheme 1. Iron acquisition by the xenosiderophores RHIZOF, STAPH A, AERO, ARTHRO and SCHIZO,**  
509 **in *P. aeruginosa*.** For more details see the text.

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For Review Only

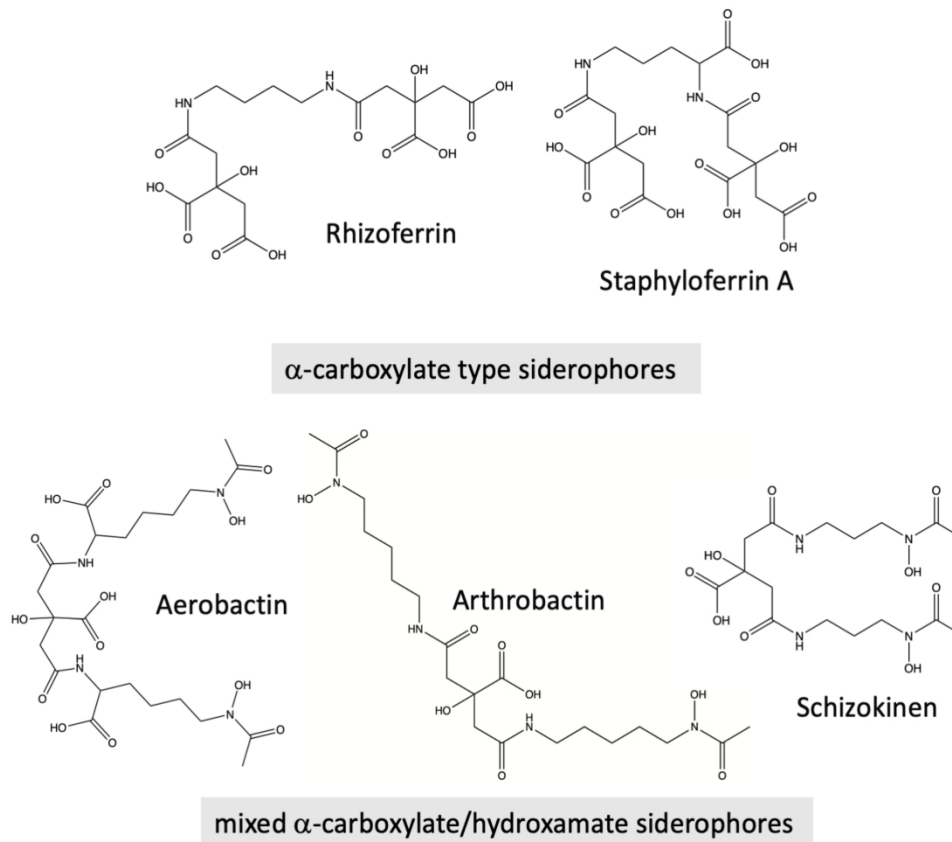


Figure 1. Chemical structure of rhizoferrin (RHIZOF), staphyloferrin A (STAPH A), aerobactin (AERO), arthrobactin (ARTHRO) and schizokinen (SCHIZO).

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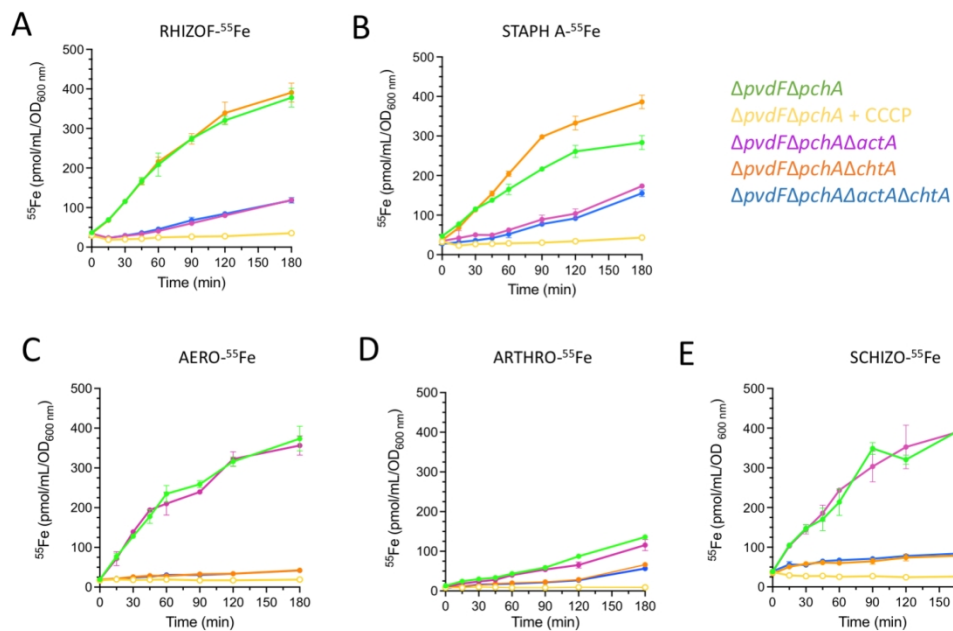
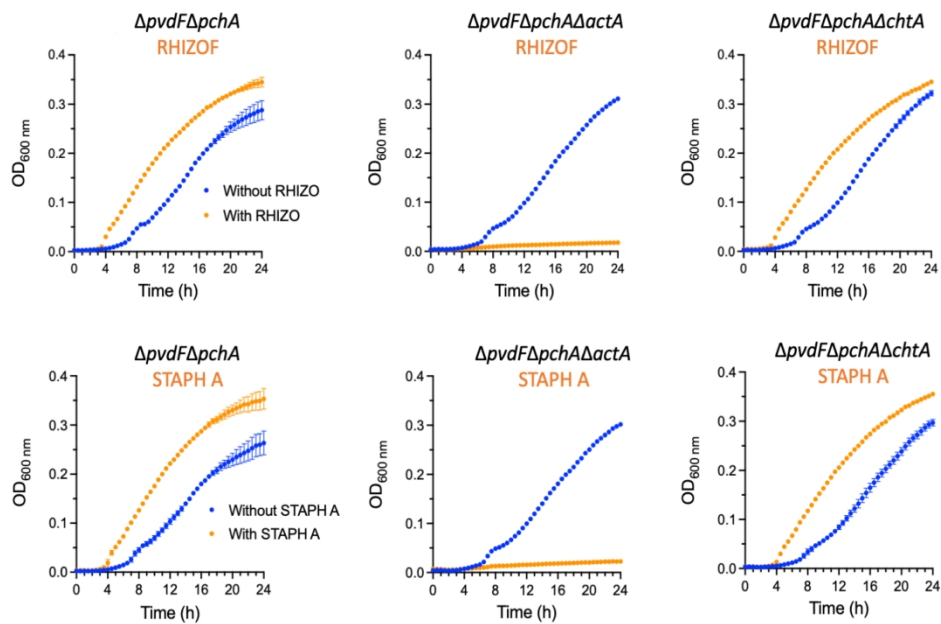


Figure 2.  $^{55}\text{Fe}$  uptake by *P. aeruginosa* strains mediated by RHIZOF, STAPH A, AERO, ARTHRO and SCHIZO. *P. aeruginosa*  $\Delta pvdF\Delta pchA$  cells were grown in iron-restricted CAA medium in the presence of 10  $\mu\text{M}$  RHIZOF, STAPH A, AERO, ARTHRO, or SCHIZO, to induce expression of the corresponding uptake pathway. Afterwards, bacteria were incubated with 500 nM RHIZOF- $^{55}\text{Fe}$ , STAPH A- $^{55}\text{Fe}$ , AERO- $^{55}\text{Fe}$ , ARTHRO- $^{55}\text{Fe}$ , or SCHIZO- $^{55}\text{Fe}$ . The amount of  $^{55}\text{Fe}$  taken up by the bacteria was measured as a function of time. As a control, the experiment was repeated in the presence of the protonophore CCCP (200  $\mu\text{M}$ ). The experiment was repeated with the corresponding  $\Delta chtA$  and  $\Delta actA$  deletion mutants of  $\Delta pvdF\Delta pchA$ . Error bars were calculated from three independent biological replicates.

159x104mm (600 x 600 DPI)



Caption : Figure 3. Growth of  $\Delta pvdF\Delta pchA$  *P. aeruginosa* mutants in the presence of RHIZOF or STAPH A.  $\Delta pvdF\Delta pchA$  and its corresponding  $\Delta chtA$  and  $\Delta actA$  deletion mutants were grown in CAA medium in the presence (growth kinetics in orange) or presence (kinetics in blue) of 10  $\mu$ M RHIZOF or STAPH A. Growth was followed by monitoring the optical density at 600 nm. Error bars were calculated from three independent biological replicates.

159x114mm (600 x 600 DPI)

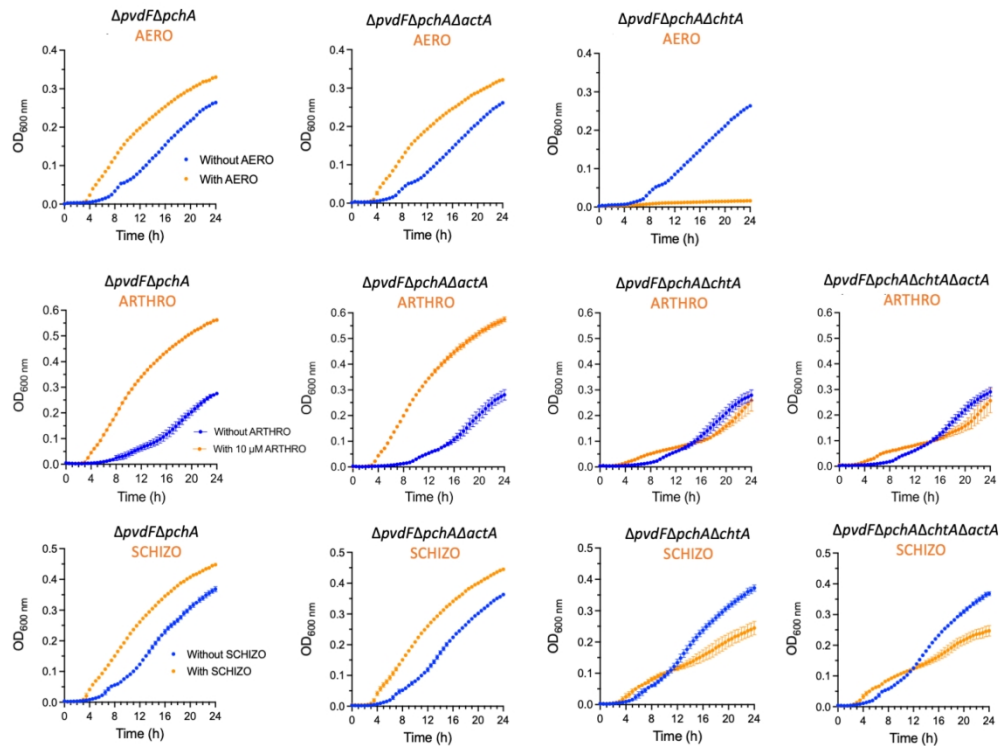
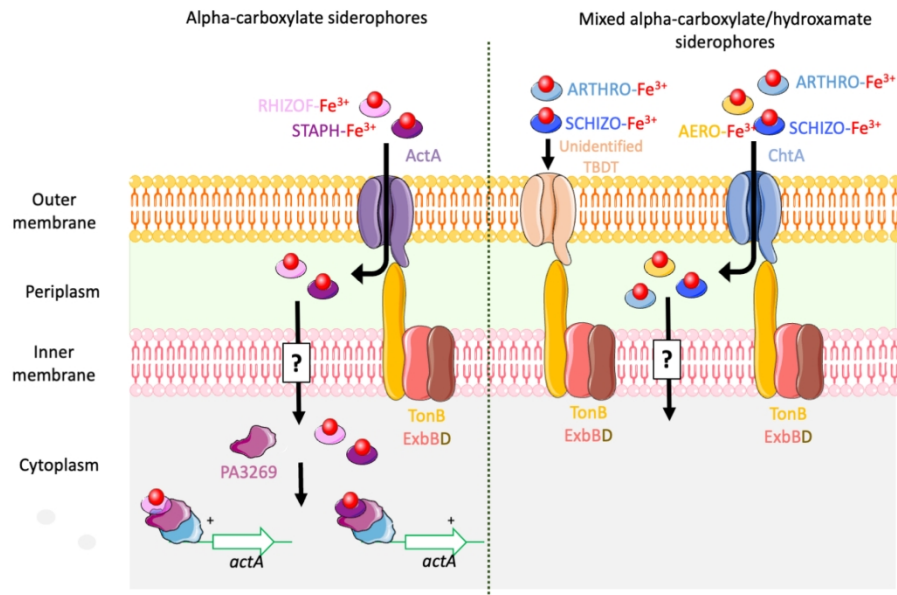


Figure 4. Growth of  $\Delta pvdF\Delta pchA$  *P. aeruginosa* mutants in the presence of AERO, ARTHRO or SCHIZO. The pyoverdine and pyochelin-deficient strain of *P. aeruginosa* ( $\Delta pvdF\Delta pchA$ ) and its corresponding  $\Delta actA$  and  $\Delta chtA$  deletion mutants were used. Strains were grown in CAA medium in the presence (growth kinetics in orange) or absence (kinetics in blue) of 10  $\mu$ M AERO, ARTHRO or SCHIZO. Growth was followed by monitoring the optical density at 600 nm. Error bars were calculated from three independent biological replicates.

169x129mm (600 x 600 DPI)



Scheme 1. Iron acquisition by the xenosiderophores RHIZOF, STAPH A, AERO, ARTHRO and SCHIZO, in *P. aeruginosa*. For more details see the text.

89x54mm (600 x 600 DPI)

## SUPPLEMENTAL MATERIAL

### Siderophore specificities of ChtA and ActA, two TonB-dependent transporters of *Pseudomonas aeruginosa*

Virginie Will<sup>1,2</sup>, Véronique Gasser<sup>1,2</sup>, Lauriane Kuhn<sup>3</sup>, Sarah Fritsch<sup>1,2</sup>, David E. Heinrichs<sup>4</sup> and Isabelle J. Schalk<sup>1,2\*</sup>.

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

Strains and plasmids	Collection ID	Relevant characteristics	Reference
<b><i>Pseudomonas aeruginosa</i></b>			
$\Delta pvdF\Delta pchA$	PAS283	PAO1; <i>pvdF</i> and <i>pchA</i> chromosomally deleted	[1]
$\Delta pvdF\Delta pchA\Delta chtA$	PAS653	PAO1; <i>pvdF</i> , <i>pchA</i> and <i>chtA</i> chromosomally deleted	This study
$\Delta pvdF\Delta pchA\Delta actA$	PAS635	PAO1; <i>pvdF</i> , <i>pchA</i> , and <i>actA</i> chromosomally deleted	This study
$\Delta pvdF\Delta pchA\Delta actA\Delta chtA$	PAS655	PAO1; <i>pvdF</i> , <i>pchA</i> , <i>actA</i> and <i>chtA</i> chromosomally deleted	This study
<b><i>Escherichia coli</i></b>			
TOP10		F- mcrA $\Delta$ (mrr-hsdRMS-mcrBC) $\phi$ 80lacZ $\Delta$ M15 $\Delta$ lacX74 nupG recA1 araD139 $\Delta$ (ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 $\lambda$ -	Invitrogen
<b>Plasmids</b>			
pEXG2	pEXG2	allelic exchange vector with pBR origin, gentamicin resistance, sacB	[2]
pEXG2 $\Delta chtA$	pSF07	pEXG2 carrying the sequence to delete <i>chtA</i>	This study
pEXG2 $\Delta actA$	pSF03	pEXG2 carrying the sequence to delete PA3268 ( <i>actA</i> )	This study

**Table S2. Primers used for the construction of the  $\Delta pvdF\Delta pchA\Delta chtA$ ,  $\Delta pvdF\Delta pchA\Delta actA$ , and  $\Delta pvdF\Delta pchA\Delta actA\Delta chtA$  strains. *actA* = gene PA3268.**

Oligonucleotides	Gene	Sequence
PA3268stop-550HindIII-F	<i>actA</i>	AAAAAAGCTTGGATCGAATACTTCACCGGGC
PA3268stop+117overlap-R	<i>actA</i>	GCACGGTCACCGTGGTCGCGTCGAACCTGAAGCTCGGA
PA3268start-97-F	<i>actA</i>	CGACCACGGTGACCGTGC
PA3268start-546XbaI-R	<i>actA</i>	AAAATCTAGACGTCCACTTGCACCGCCTG
PA4675Start-784 pb EcoRI	<i>chtA</i>	AAAAGAATTCAGTAACGCCCCAGACGAATCG
PA4675Start + 18 pb overlap	<i>chtA</i>	CAGGCTGTAGGTCCGCCCCCAGGGGAGCCGGGCG
PA4675Stop + 714 pb HindIII	<i>chtA</i>	AAAAAAGCTTTCAAACGAGGGGCATGCCATG
PA4675Stop -18 pb	<i>chtA</i>	GGGCGGACCTACAGCCTG

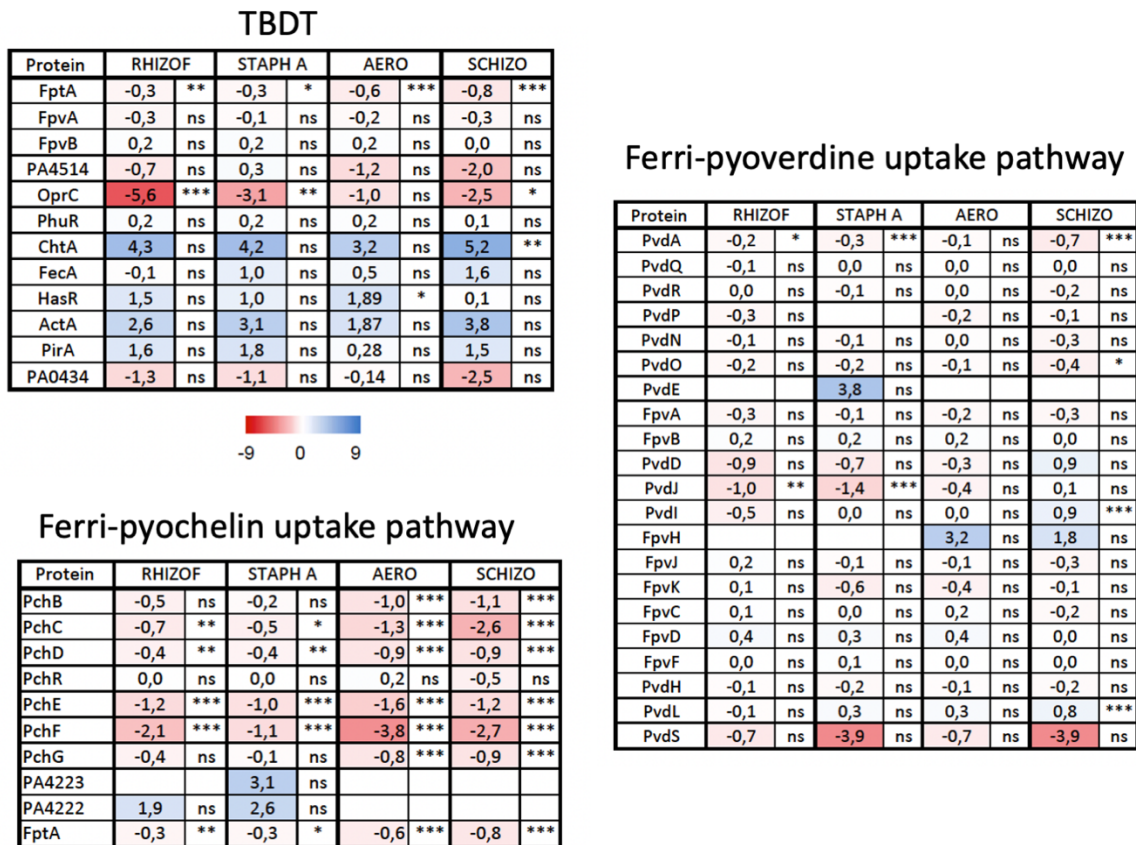


**Table S3. Primers used in this study for RT-qPCR.**

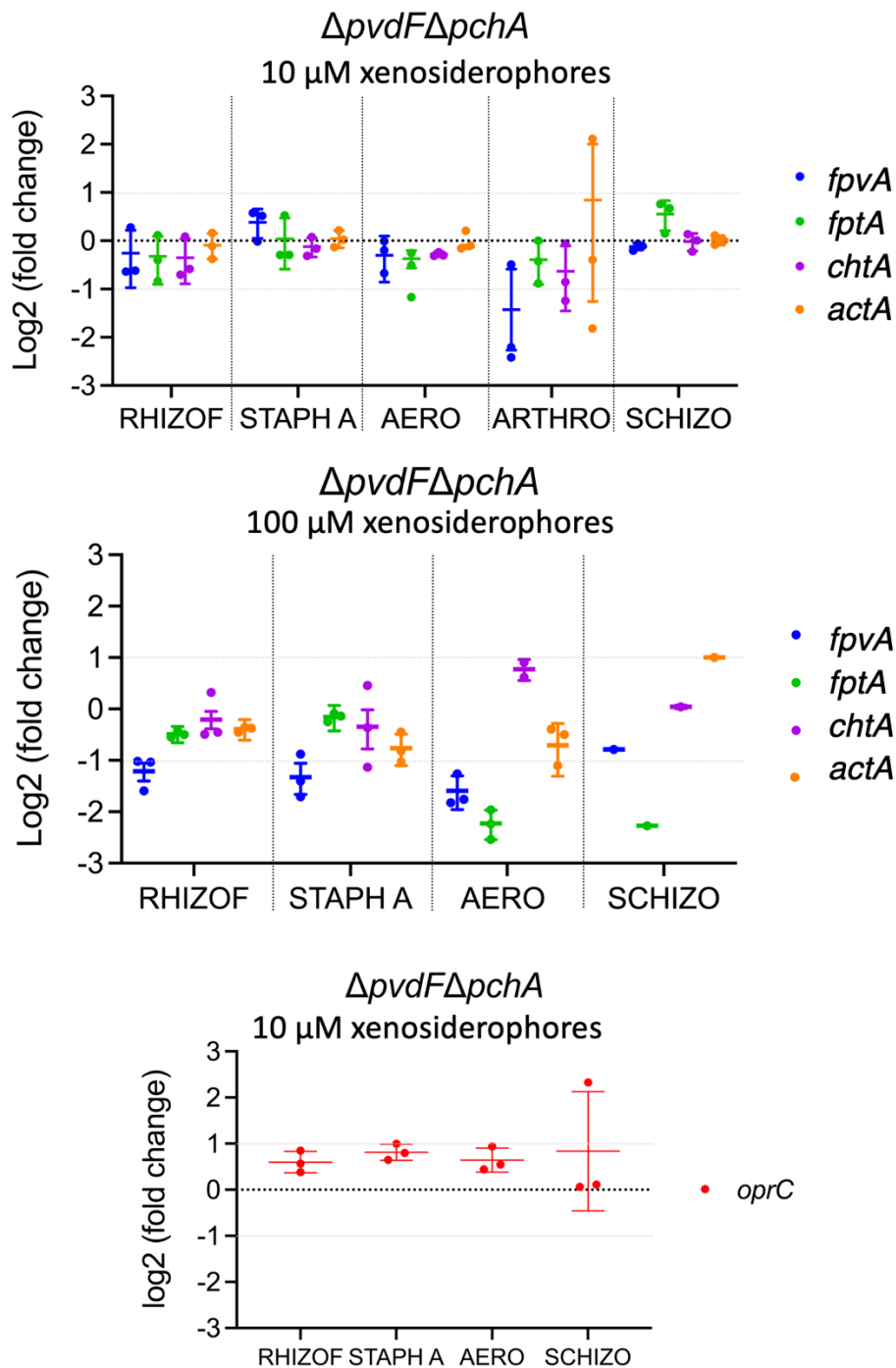
<b>Oligonucleotides</b>	<b>Sequence (5' to 3')</b>
<i>uvrD</i> F	CTACGGTAGCGAGACCTACAACAA
<i>uvrD</i> R	GCGGCTGACGGTATTGGA
<i>rpsL</i> F	TACACCACCACGCCGAAAA
<i>rpsL</i> R	CACCACCGATGTACGAGGAA
<i>fpvA</i> F	AGCCGCCTACCAGGATAAGC
<i>fpvA</i> R	TGCCGTAATAGACGCTGGTTT
<i>fptA</i> F	GCGCCTGGGCTACAAGATC
<i>fptA</i> R	CCGTAGCGGTTGTTCCAGTT
<i>oprC</i> F	GGACGGCTCGCAGTTCAA
<i>oprC</i> R	AGACCTGTGCCTCGACCTTCT
<i>chtA</i> F	AACGAGAAGATGGCCTTCAATC
<i>chtA</i> R	TCTCACCAGGGCCAGTTTC
<i>actA</i> F	CGTGCTGCTCAAGACCCATT
<i>actA</i> R	CGTAGGCGTCGTAGTAGTGGAA

**Table S3. TBDTs present in the *P. aeruginosa* PAO1 genome.**

Gene	Name	Ligand	Regulation	Reference
PA0151	-	-	Sigma/anti-sigma factors	[3]
PA0192	-	-		[3]
PA0434	-	-		[3]
PA0470	FiuA	Ferrichrome, ferrichrocin, Fusigen, Rhodotorulic acid	Sigma/anti-sigma factors	[4,5]
PA0781	-	-		[3]
PA0931	PirA	Enterobactin, mono catechol	Two component systems	[6,7]
PA1271	BtuB	Cobalamine		[3]
PA1302	HxuA	Haem	Sigma/anti-sigma factors	[8]
PA1322	PfuA	-		[3]
PA1365	-	-	Sigma/anti-sigma factors	[3]
PA1613	-	-		[3]
PA1910	FemA	Mycobactins, carboxymycobactins	Sigma/anti-sigma factors	[9]
PA1922	CirA	-		[3]
PA2057	SppR	-	Sigma/anti-sigma factors	[10]
PA2070	-	-		[3]
PA2089	-	-	Sigma/anti-sigma factors	[3]
PA2289	-	-		[3]
PA2335	OptO	-		[3]
PA2398	FpvA	Pyoverdine	Sigma/anti-sigma factors	[11]
PA2466	FoxA	Ferrioxamine B, Nocardamine	Sigma/anti-sigma factors	[4,12]
PA2590	-	-		[3]
PA2688	PfeA	Enterobactin	Two component systems	[13]
PA2911	-	-		[3]
PA3268	ActA	Rhizoferrin, Staphyloferrin A	AraC regulators	This study
PA3408	HasR	HasAp-haem	Sigma/anti-sigma factors	[14]
PA3790	OprC	Copper		[3]
PA3901	FecA	Citrate	Sigma/anti-sigma factors	[15]
PA4156	FvbA	Vibriobactin		[16]
PA4168	FpvB	Pyoverdine, ferrichrome, ferrioxamine B		[17,18]
PA4221	FptA	Pyochelin	AraC regulators	[19]
PA4514	PiuA	Mono catechol		[7]
PA4675	ChtA	Aerobactin, Arthrobactin Schizokinen		[20] This study
PA4710	PhuR	Haem		[14]
PA4837	CntO	Pseudopaline		[21]
PA4897	OptI	-	Sigma/anti-sigma factors	[3]



**Figure S1. A. Differential proteomic analyses of *P. aeruginosa*  $\Delta pvdF\Delta pchA$  grown in the presence of 10  $\mu$ M SCHIZO, STAPH A, AERO, or RHIZOF.**  $\Delta pvdF\Delta pchA$  strains were grown in CAA medium, with or without 10  $\mu$ M SCHIZO, STAPH A, AERO, or RHIZOF, for 8 h and differential proteomic expression analyzed. The average values measured in CAA medium without xenosiderophores were plotted against the average values in CAA supplemented with xenosiderophores. **A-C.** Heat maps of the TBDTs showing a change in their expression (A), as well as proteins involved in the pyochelin- (B) and pyoverdine-dependent (C) iron-uptake pathways. In these heat maps, we chose to show proteins for which a change in the level of expression was observed in one of the conditions analyzed: the darker the shade of red, the more the expression of the protein is repressed; the darker the shade of blue, the more the expression of the protein is induced. ns: data not significant; \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$



**Figure S2. Quantitative real-time PCR on *P. aeruginosa*  $\Delta pvdF\Delta pchA$  grown in the presence or absence of SCHIZO, STAPH A, AERO, ARTHRO, or RHIZOF.** The  $\Delta pvdF\Delta pchA$  strain was grown in the presence or absence of 10  $\mu\text{M}$  or 100  $\mu\text{M}$  SCHIZO, STAPH A, AERO, ARTHRO, or RHIZOF. Gene transcription was normalized using the *uvrD* and *rpsL* reference genes. The log<sub>2</sub> ratios between the values obtained in the presence of the xenosiderophores and the condition without xenosiderophores are shown. The data show the results of three independent experiments, as well as the mean of the experiment. *fpvA* encodes the TBDT of ferri-pyoverdine, *fptA* the TBDT of ferri-pyochelein, *fecA* the TBDT of ferri-citrate, and *oprC* a TBDT involved in copper uptake.

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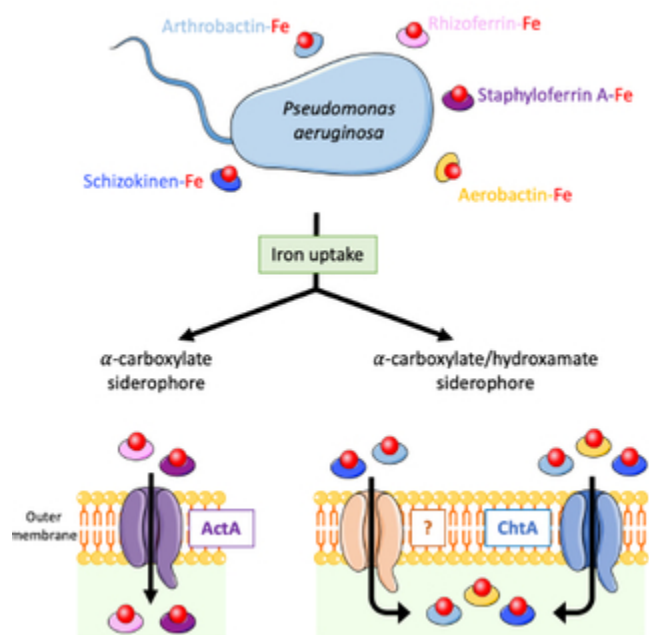
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For Review Only



13x13mm (600 x 600 DPI)



Iron is a key nutrient for the growth of almost all bacteria. The pathogen *Pseudomonas aeruginosa* is able to express at least 15 different iron acquisition pathways, each involving a specific outer membrane transporter. Most of these iron uptake pathways relay on small iron chelators (siderophores) produced by other microorganisms. We identified the outer membrane transporters involved in the uptake of iron via two  $\alpha$ -carboxylate siderophores and three mixed  $\alpha$ -carboxylate/hydroxamate siderophores.

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