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High-throughput Tn-seq screens identify both known and novel *Pseudomonas putida* KT2440 genes involved in metal resistance

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1 full title:

2 **High-throughput Tn-seq screens identify both known and**
3 **novel *Pseudomonas putida* KT2440 genes involved in metal**
4 **resistance.**

5
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7 Parisot^{7*}, Christian Schori^{4,8*}, Christian H. Ahrens^{4,9}, Agnes Rodrigue¹ and
8 Erwan Gueguen^{1†}
9

10 Running title: *Pseudomonas putida* metal resistance genes Tn-seq screening

11
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13 alphabetical order.

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35 **Running head:** *Pseudomonas putida* genes important for metal resistance

36 **Keywords:** cadmium, zinc, copper, cobalt, stress, efflux, resistance, metal

37

38 **Abstract**

39 Chemical waste with toxic effects is released into the environment by industrial and urban
40 activities. *Pseudomonas putida*, a rhizosphere bacterium, harbors a wide variety of genes
41 capable of degrading hydrocarbons and xenobiotic compounds in its natural environment.
42 This bacterium harbors also a large set of metal resistance genes. Most studies that identify
43 genes involved in metal resistance in *P. putida* focus on over/underexpressed genes and may
44 miss other genes important for metal resistance whose expression does not change. In this
45 study, we used a Tn-seq approach to determine the essential genome of *P. putida* required for
46 growth in the presence of an excess of metals in a culture medium. Tn-seq enables the
47 detection of mutants with reduced or increased fitness in the presence of metal excess. We
48 validated our screen by identifying known metal resistance gene such as *czcA-1* (*PP_0043*),
49 *cadA-3* (*PP_5139*), *cadR* (*PP_5140*) and *pcoA2* (*PP_5380*). Their mutants were
50 underrepresented in the presence of zinc, cadmium (for *cadA-3* and *cadR*) or copper
51 respectively. In this study, we demonstrate by targeted mutagenesis and complementation
52 assay that *PP_5337* and *PP_0887* are putative transcriptional regulators involved in copper
53 and cadmium resistance, respectively, in *P. putida*. The study revealed the role of two genes,
54 *PP_1663* and *PP_5002*, in cadmium and cobalt resistance respectively. This is the first
55 evidence linking these genes to metal resistance and highlights the incomplete understanding
56 of metal resistance mechanisms in *P. putida*.

57

58 **Introduction**

59 *Pseudomonas putida* is an ubiquitous saprophytic bacterium that can utilize various sources
60 of carbon and energy. This soil microorganism has been widely used as an experimental
61 model to study the biodegradation of aromatic compounds or hydrocarbons (1, 2). It is able to
62 colonize various habitats and has been isolated from water, soil, and the plant rhizosphere,

63 sometimes polluted by various compounds (1, 3). The analysis of its complete genome
64 revealed that *P. putida* possesses a wide range of genes that are involved in metal
65 homeostasis or resistance. This suggests that the bacteria can survive in metal-polluted
66 environments (4).

67 Metals play a crucial role in several cellular processes of microorganisms. Certain metals,
68 such as nickel, cobalt, copper, and zinc, are essential nutrients. They function as stabilizers of
69 protein structures or bacterial cell walls, as catalysts for biochemical reactions, and help
70 maintain osmotic balance (5, 6). However, some metals can be highly toxic to cells. Metal
71 toxicity can occur in various ways, such as oxidative damage caused by the production of
72 reactive oxygen species, DNA damage, and protein damage due to the displacement of
73 essential metals from their native binding sites or binding to respiratory enzymes (5). While
74 some metals are essential for cellular function, they can also be toxic when present in excess.
75 Therefore, it is crucial to tightly regulate the concentration of metal ions in cells to maintain
76 optimal cellular activity. To maintain metal homeostasis, bacteria use various systems, such
77 as metal uptake/efflux, chelation, or sequestration (5, 6). Several systems have been identified
78 in *Pseudomonas putida* that confer resistance to heavy metals such as cadmium, zinc, and
79 cobalt. These systems include P-type ATPases (e.g., CadA for cadmium resistance or CzcA
80 for cadmium, zinc, and cobalt resistance), sequestration proteins (e.g., CopA for copper
81 resistance), uptake systems (e.g., ZnuB/C or NiKABCDE), regulators, and proteins involved
82 in redox mechanisms (4, 7, 8). Regarding metal stresses, a complete genome analysis has
83 shown that the *P. putida* genome contains 61 open reading frames that are probably involved
84 in metal tolerance or homeostasis, and seven more that are possibly involved in metal
85 resistance (4). Proteomic or transcriptomic technologies have been used to investigate the
86 response of *P. putida* to inhibitory concentrations of various metals (7–11). These studies
87 indicated that a significant number of genes in *P. putida* are responsible for maintaining
88 homeostasis, as well as tolerating and resisting various metals.

89 Although omics approaches (proteomics and transcriptomics) are considered powerful, they
90 have limitations in terms of detectability and typically only detect genes whose expression
91 levels significantly change between two conditions. These analyses may thus miss important
92 factors not affected by gene/protein level changes. Therefore, more comprehensive screens
93 are needed to identify new factors and ideally complete sets of genes involved in metal
94 resistance in *P. putida*. The screening of *P. putida* CD2 mutants obtained by Tn5 insertions
95 was also conducted in the presence of cadmium, which confirmed and completed the
96 overview of the *P. putida* stress responses (12, 13). However, the low saturation levels of the

97 Tn5 libraries suggests that some genes may have been missed during these analyses.
98 Additionally, the number of tested mutants was limited by the need to test each individual
99 mutant in every condition.

100 To gain a more comprehensive understanding of the genes necessary for metal resistance in
101 *P. putida*, we utilized a high-throughput sequencing of a saturated transposon library (Tn-seq)
102 (14, 15) in this study to screen tens of thousands of random insertion mutants of *P. putida* in
103 the presence of excess amounts of metal ions. Tn-seq is a powerful method that has
104 successfully been used to characterize essential genes in various conditions and many
105 different species. For example, it has been used to identify essential genes for human gut
106 colonization of *Bacteroides thetaiotaomicron* (16), mouse colonization by human pathogens
107 such as *Vibrio cholerae*, *Pseudomonas aeruginosa*, or *Streptococcus pneumoniae* (14, 17,
108 18), plant colonization by phytopathogens (19–21), tobramycin resistance genes of *P.*
109 *aeruginosa* (22), toxic compound resistance genes in *P. putida* (23) and identification of
110 genetic targets for improved tolerance of *P. putida* towards compounds relevant to lignin
111 conversion (24). This technique has also been also employed to identify gold, silver and
112 copper resistance genes in *Burkholderia cenocepacia* (25, 26). However, this technology has
113 not yet been employed to discover genes involved in metal resistance in *P. putida* KT2440.
114 By applying Tn-seq to screen a *P. putida* KT2440 mutant library in the presence of metals in
115 the culture medium, we identified numerous genes required for growth in a culture medium
116 rich in cobalt, copper, zinc (essential metals), or cadmium (a non-essential metal). Among
117 them were *czcA-1* (PP_0043), *cadA-3* (PP_5139), *cadR* (PP_5140) and *pcoA2* (PP_5380),
118 which are already known to be involved in zinc, cadmium, and copper resistance,
119 respectively, and thereby validating the approach employed. In addition, we discovered
120 several genes that were previously not associated with metal resistance and validated them
121 through in-frame deletion and complementation assays. Our findings demonstrate that
122 PP_1663 (Cd), *roxSR* (Cd), PP_5337 (Cu), and PP_5002 (Co) all are involved in metal
123 resistance in *P. putida*.

124

125 **Results and discussion**

126 **Characterization of *P. putida* KT2440 Himar1 transposon library**

127 Tn-seq screening has been performed numerous times with the opportunistic pathogen
128 *Pseudomonas aeruginosa*. An elegant strategy that has been developed involves the use of a
129 modified *Himar9* mariner transposon derivative carrying MmeI restriction sites in the

130 inverted repeats (IR) and a gentamicin resistance cassette between the IRs (18). The Mariner
131 transposon can specifically insert itself into the genome at TA sites. In *P. putida* KT2440,
132 129,002 TA sites can be targeted by this transposon. To generate a pool of approximately
133 1,000,000 colonies, we introduced by conjugation from *E. coli* the plasposon pSam_D-Gm
134 into *P. putida* KT2440 (18). Two technical replicates of the DNA libraries were created from
135 this pool and were subjected to high-throughput sequencing. The TPP software (27) was used
136 to determine the number of reads at each TA site. The sequencing of the two samples
137 detected 91,882 and 93,147 unique insertions into TA sites, with an average of 91 and 96
138 reads per TA, respectively (Table S3). The preparation of the Tn-seq library was highly
139 reproducible, with a Pearson correlation coefficient of 98%. The density of Tn insertions was
140 approximately 70% from our initial pool of mutants (Table S3), and the unique insertions
141 were distributed all around the chromosome (Fig. S1). These results indicate high quality and
142 coverage of our *P. putida* Tn-seq libraries.

143 The gene essentiality of the Tn-seq input libraries was next determined using the TRANSIT
144 software (27), which employs a Hidden Markov Model (HMM) method to predict essentiality
145 and non-essentiality for individual insertion sites (DeJesus & Ioerger 2013). The HMM
146 analysis identified 600 genes essential for growth on LB agar, representing 10.8% of the
147 genes of *P. putida* KT2440. 4458 genes were identified as non-essential genes (NE) (Table
148 S4).

149

150 **Screening of genes important for metal resistance.**

151 To identify new genes responsible for metal resistance in *P. putida* KT2440, we tested the
152 effects of copper chloride, zinc, cobalt, and cadmium on the growth of *P. putida*. We used LB
153 rich medium in our screens instead of minimal medium to prevent the loss of auxotrophic
154 mutants or biosynthesis pathways that could be important for metal resistance during growth.
155 To determine the optimal metal concentration, we grew *P. putida* KT2440 in LB rich medium
156 supplemented with varying concentrations of a metal ion solution. We compared the growth
157 of the WT strain with and without excess Cu, Zn, Cd, or Co under the same conditions used
158 for screening, i.e., in an Erlenmeyer flask with shaking at 30°C (Fig. S2). Under our
159 laboratory conditions, we found that a concentration of 10 µM cobalt chloride, 2.5 mM
160 copper chloride, 125 µM zinc chloride, and 12.5 µM cadmium chloride did not affect the
161 growth of the cultures during the exponential phase, compared to the growth of the WT strain
162 grown without an excess of these metals (Fig. S2). We hypothesized that under these

163 conditions, only homeostatic mechanisms will be selectively activated, rather than pleiotropic
164 responses to metal toxicity.

165 For the Tn-seq screening, biological replicates were performed to ensure the reproducibility
166 of the method. The cultures were inoculated with 10^7 bacteria from the mutant pool. After
167 twelve divisions in the presence of metals in the culture medium, at 28°C, the final pools of
168 mutants were collected. Sequencing of transposon insertion sites of the final pools, followed
169 by the TPP analysis, indicated highly reproducible results with a Pearson correlation
170 coefficient > 90% for each dataset (Table S3).

171 To test the statistical significance of the genes that contribute to *P. putida*'s loss or gain of
172 fitness in a metal-rich medium, we conducted a RESAMPLING (permutation test) analysis
173 using the TRANSIT software. We compared the results obtained from culture in LB to those
174 obtained from culture in LB with an excess of metal (Table S5). After applying Tn-seq to our
175 datasets and selecting only genes with an FDR adjusted p-value (q-value) ≤ 0.05 , we
176 identified 9 genes involved in cobalt resistance, 14 in copper resistance, 3 in zinc resistance,
177 and 8 in cadmium resistance. From these 28 genes, we applied an additional cutoff by
178 removing 3 genes with a mean read count in LB below 2 (less than 2 reads on average per
179 TA) and that are classified as essential or causing growth defects in LB. Finally, we retained
180 25 genes (Table 1). 23 genes were classified as non-essential in LB, while the remaining 2
181 were identified as causing growth defects and growth advantages. Some of these genes,
182 highlighted in bold, were previously known to be involved in metal resistance in *P. putida*,
183 thus confirming the validity of the Tn-seq approach. In the following sections, we discuss the
184 function of some of the genes we consider most important in relation to metal resistance.

185

186 **Analysis of the genes of *P. putida* required for metal resistance**

187

188 **Copper resistance.**

189 Genes required for copper resistance were identified using a subinhibitory concentration of
190 copper (see Figure S2). Copper is an essential metal required as a cofactor for electron
191 transport and redox enzyme systems in aerobic bacteria. In bacteria, copper exists in two
192 different states: the less toxic oxidized Cu(II) state, which can be transformed into the more
193 toxic reduced Cu(I) state under redox systems. Copper is toxic to cells because it can displace
194 other metals from essential complexes and bind to various biomolecules in an unspecific
195 manner. Fourteen candidate genes, listed in Table 1, were found to be possibly involved in
196 copper resistance. The function of these genes is discussed below.

197

198 *Inner membrane protein Cu⁺-ATPase*

199 The role of *P_{IB}-type ATPases* in copper resistance has been extensively studied in *P.*
200 *aeruginosa* PAO1. The transmembrane inner membrane protein *P_{IB}-type ATPase*, CopA, is
201 responsible for cytoplasmic Cu⁺ efflux. *P. aeruginosa* PAO1 has two homologous Cu⁺-
202 ATPases, CopA1_{PAO1} (PA3920) and CopA2_{PAO1} (PA1549). CopA1_{PAO1} was expressed in
203 response to high Cu⁺ (28–30), and its deletion induced copper sensitivity (31). However,
204 while CopA2_{PAO1} does not directly contribute to copper resistance, it does play a crucial role
205 in loading copper into cytochrome c oxidase subunits. Both enzymes export cytoplasmic Cu⁺
206 into the periplasm (31). In *P. putida* KT2440, only one copper Cu⁺-ATPase is present, which
207 is encoded by *PP_0586*. The protein is commonly referred to as CadA2 in *P. putida* KT2440.
208 However, we will use the name CopA1_{KT2440} in the further section in order to align with
209 naming and homologies, since *copA1*_{KT2440} is the orthologous gene of CopA1_{PAO1}. To ensure
210 that the two proteins were the same, we compared their 3D structures predicted by AlphaFold
211 (32) using the TM-Align algorithm (33). The structures were found to be superimposed (Fig.
212 S3). Previous research has demonstrated that CopA1_{KT2440} is highly produced in the presence
213 of copper in minimal salt media (7). Our Tn-seq screen in the presence of copper revealed
214 that *copA1*_{KT2440} mutants have a strong growth disadvantage (Table 1). In *P. aeruginosa*,
215 *copA1*_{PAO1} is positively regulated by CueR (PA4778) (28–30). The transcriptional regulation
216 of *copA1*_{KT2440} by CueR_{KT2440} has not been verified in *P. putida*. However, a putative cueR
217 binding site (ACCTTGCCTGCGTGGCAAGGT) is located in the promoter region of
218 *copA1*_{KT2440} as indicated in the RegPrecise database
219 (https://regprecise.lbl.gov/sites.jsp?regulog_id=5159 ; (34)), suggesting a direct regulation
220 like in *P. aeruginosa*.

221

222 *Outer membrane protein PcoB and putative periplasmic multi-copper oxidase PcoA*

223 In certain bacterial species, an outer membrane porin called PcoB appears to contribute to
224 periplasmic Cu⁺ efflux. *pcoB* is often co-localized with *pcoA* encoding a putative periplasmic
225 multi-copper oxidase (35). *pcoAB* were first identified as part of a copper resistance Cop
226 operon in the pPT23D plasmid of *Pseudomonas syringae* (36). For this reason, PcoA proteins
227 were sometimes mistakenly named CopA, despite being functionally distinct from the
228 previously described CopA proteins which are ATPases of the inner membrane (7, 36). In *P.*
229 *syringae*, PcoA and PcoB bind copper (36). In *P. aeruginosa*, the orthologous system named
230 *pcoA/B* (PA2065 and PA2064) is induced up to 70-fold in the presence of a high copper

231 sulfate concentration (28, 30). In *P. putida* KT2440, the orthologous genes of *PA2065* and
232 *PA2064* are *PP_5379* (*copB-2*) and *PP_5380* (*copA2*) respectively. The AlphaFold predicted
233 3D structure alignment of the orthologous proteins using the TM-Align algorithm confirms
234 that these proteins adopt the same conformation (Fig. S3). For clarity, we thus decided to
235 rename these *P. putida* KT2440 genes *pcoB-2* and *pcoA-2* respectively. Mutants of these
236 genes exhibit growth-defect phenotypes in the presence of copper (Table 1 and Fig 1). *P.*
237 *putida* KT2440 also has a second PcoAB system (*pcoA-1/pcoB-1*) encoded by
238 *PP_2204/PP_2205*, but mutants of these genes do not exhibit any growth-defects in the
239 presence of copper. As only the PcoA-2/B-2 system appears to be essential for copper
240 resistance, it is possible that the PcoA-1/B-1 system is not expressed under our laboratory
241 conditions or is less efficient than the PcoA-2/B-2 system.

242

243 After copper treatment, *pcoA-2* and *pcoB-2* are highly transcribed in *P. putida* KT2440, and
244 PcoB-2 accumulates in cells (7). In some strains of *E. coli* that harbor an episomal gene
245 cluster *pcoABCDRSE*, the Pco operon is mediated by the PcoRS two-component system. In
246 *P. aeruginosa* PAO1, PcoA/B expression is suppressed in the Δ *copR* strain (Teitzel et al.
247 2006; Miller et al. 2009; Quintana et al. 2017). In *P. putida* KT2440, we searched for the
248 consensus CopR binding site TGACANNNTGTNAT (30) and found it in the intergenic
249 region upstream of *copRI/CopSI* (*PP_2158/PP_2157*) and *pcoA-1/pcoB-1*
250 (*PP_2205/PP_2204*). We did not detect any putative CopR binding site within the promoter
251 region of *pcoA2/B2*, suggesting that these genes may be activated differently under copper
252 stress. *P. putida* has two CopR regulators, CopR1 (*PP_2158*) and CopR2 (*PP_5383*). The
253 proximity of *copR2* to *pcoA-2/B-2* suggest that CopR2 might regulate *pcoA2/B2*. However,
254 this hypothesis has not yet been investigated.

255

256 *The Cus system*

257 In *P. putida* KT2440, the genes *PP_5379* (*pcoB-2*) and *PP_5380* (*pcoA-2*) are located
258 adjacent to a cluster of five genes, *copR1/S1* and *cusCBAF*. *cusCBA* genes encode a putative
259 cation/proton antiporter that spans the outer and inner membranes and has been proposed to
260 be involved in copper and silver efflux (4). The two-component system CusR/S senses
261 periplasmic copper and regulates the Cus RND-type transport system in *E. coli* (37, 38).
262 However, our Tn-seq datasets did not reveal any potential role of the CusCBAF system and
263 CopRS two-component regulatory systems in copper resistance at the copper concentration

264 we used. This confirms an earlier observation showing that *cusC* expression is not activated
265 in the presence of copper (7). The *cusCBAF* operon, which was suggested to be involved in
266 copper resistance, may respond to a different concentration of copper.

267

268 **Cadmium resistance.**

269 Cadmium commonly forms cations with an oxidation state of II. Unlike zinc, cadmium has a
270 preference for binding to sulfur ligands, which can be problematic for proteins with disulfide
271 bonds. Zinc homeostasis and cadmium resistance mechanisms often overlap due to their
272 similarities. They share uptake and efflux transporters, as well as metal-responsive regulatory
273 proteins (4, 7, 39). Cadmium can be removed from the cytoplasm of bacterial cells through
274 various systems, including the P-type ATPase CadA-3 (PP_5139). CadA-3 is a homolog of
275 ZntA in *E. coli* (4, 40, 41). Our Tn-seq screening indicates that CadA-3 is involved in
276 cadmium resistance, with a strong negative \log_2FC of -4.99 (table 1 and fig. 1). It confirms
277 previous observations that CadA-3 confers resistance to Cd^{2+} , while CadA1 plays no role in
278 resistance to Cd, Zn, Cu, or Co in *P. putida* (39). The orthologous gene of *cadA-3* in *P.*
279 *putida* CD2 was previously shown to be a major determinant of cadmium resistance (12).

280 The *dsbA* gene, which is essential for cadmium resistance, was discovered with a negative
281 \log_2FC of -7.59 (Table 1 and Fig. 1). DsbA catalyzes the oxidation of disulfide bonds of
282 periplasmic proteins. As a result, the cysteine residues of DsbA become reduced, and the
283 protein must be oxidized by DsbB to be regenerated (42). Notably, a *dsbA* mutant was found
284 to be sensitive to cadmium and even zinc in both *E. coli* (43) and *Burkholderia cepacia* (44).
285 In contrast, a *dsbB* mutant did not appear to be essential for cadmium resistance under the test
286 conditions.

287 Our Tn-seq analysis showed that the gene encoding the RoxS sensor (PP_0887), which is part
288 of the RoxS-RoxR two-component system (PP_0887-PP_0888), is essential for cadmium
289 resistance. The two genes coding this two-component system are transcribed in a single unit
290 (45). The system belongs to the RegA/RegB family, where RegA functions as an integral
291 membrane sensor histidine kinase, and RegB is a sigma 54-dependent regulator. A whole-
292 genome transcriptional analysis was conducted to define the *P. putida* RoxS/RoxR regulon in
293 LB. The regulon includes genes involved in amino acid and sugar metabolism, the sulfur
294 starvation response, elements of the respiratory chain, and genes that participate in
295 maintaining the redox balance (45). Although a putative RoxR recognition element has been
296 identified in the promoters of genes regulated by this system (45), the specific genes that are
297 up or down regulated by RoxS/RoxR in response to cadmium are still unknown.

298

299 **Cobalt resistance.**

300 Cobalt is a transition metal with an oxidation state of II. It plays an essential role for
301 microorganisms as cofactors for diverse metalloenzymes. Cobalt toxicity is related to its
302 potential interference with iron and possibly manganese homeostasis. Bacteria typically use
303 efflux systems to survive in an environment with an excess of Co^{2+} . The cobalt resistance
304 system was poorly described in *Pseudomonas*, but it was studied in greater detail in other
305 organisms (46).

306 The genes of *P. putida* KT2440 involved in cobalt resistance were determined using a sub-
307 inhibitory concentration of cobalt. Our screen did not reveal any systems that cause cobalt
308 resistance in other bacteria and that exist in *P. putida*. The *czcCBA* RND system in
309 *Cupriavidus metallidurans* confers resistance to Cd^{2+} , Zn^{2+} , and Co^{2+} (47). Although the
310 CzcCBA system exists in KT2440, it was only reported to confer Zn^{2+} and Cd^{2+} resistance
311 (39). Additionally, it was discovered that CzcD, a member of the CDF family, confers cobalt
312 resistance in *Ralstonia sp.* Strain CH34, although to a lesser extent than the CzcCBA system
313 (48). A homolog of *czcD*, *PP_0026*, exist in *P. putida* KT2440, but it does not confer cobalt
314 resistance in our Tn-seq screening. Interestingly, our screen did not reveal a role of the MrdH
315 efflux pump (PP_2968), which is homologous to the RcnA efflux pump from *E. coli*.
316 Although cobalt induces *mrdH* activity, the efflux pump does not confer resistance to cobalt
317 (49).

318 Although our screening did not identify any genes encoding efflux pumps, we did identify
319 some genes that were not previously known to be involved in cobalt homeostasis. One of
320 these genes is *mgtA*, which encodes an ATP-dependent magnesium transporter that is
321 involved in the active transport of magnesium in cells. This gene has a positive $\log_2\text{FC}$ value,
322 indicating that the mutant confers a growth advantage in the presence of cobalt in the culture
323 medium. Although MgtA has not been experimentally characterized in *P. putida* or *E. coli*, its
324 ortholog in *Salmonella typhimurium* has been studied, where MgtA mediates magnesium
325 uptake (50, 51).

326

327 **Zinc resistance.**

328 Zinc has an affinity for ligands containing oxygen, nitrogen, or sulfur and is often used as an
329 enzyme cofactor in the cell. As mentioned for copper, zinc toxicity occurs with its ability to
330 replace another metal from enzymes or by forming complexes with other biomolecules. It
331 exists in cells mainly in the oxidized state Zn^{2+} . Zinc homeostasis is well documented and is

332 regulated by several processes: Zn²⁺ uptake regulation, sequestration by metallothioneins
333 (MT) and efflux system (52, 53). Our screen revealed three genes involved in zinc resistance:
334 *czcA-1* (PP_0043), *cadR* (PP_5140) and *pvdM* (PP_4213). The CzcCBA system has been
335 fully described in bacteria. It has been reported to confer Cd²⁺, Zn²⁺ and Co²⁺ resistance in *C.*
336 *metallidurans* and Cd²⁺ and Zn²⁺ resistance in *P. putida* KT2440 (39, 46). The identification
337 of the main component of the CzcCBA system, CzcA-1, confirms the validity of our
338 screening in the presence of zinc. The CzcCBA system may be predominant at the zinc
339 concentration used. At least five *czcA* genes have been described in *P. putida* KT2440 (4).
340 Our screen confirms a previous result showing that CzcCBA1 is the predominant CzcCBA
341 system in *P. putida* under laboratory conditions (39).

342

343

344 **Cross metal resistance**

345

346 *Pyoverdine*

347 Pyoverdine is the major siderophore in fluorescent *Pseudomonads*. The pyoverdine pathway
348 is complex, with 20 different proteins documented to be involved in its regulation, synthesis,
349 maturation, transport and uptake (54). Pyoverdine maturation starts with the transport of a
350 precursor (PVDIq) from the cytoplasm to the periplasm by the ABC transporter PvdE. PvdN
351 and PvdO are involved in the maturation of the pyoverdine precursor (55, 56). PvdM is
352 required for the oxidation of ferribactin by PvdP during periplasmic pyoverdine maturation
353 (57). The *pvdM,N,O,E* genes belong to an operon in *P. putida* KT2440 but not in *P.*
354 *aeruginosa* PAO1. The mature pyoverdine is able to chelate many metals, but with a lower
355 affinity than iron (Schalk & Guillon 2013), and could thus protect the cell from metal toxicity
356 (54, 58). In our screen, since all mutants grow in the same culture medium, a mutant
357 defective in the production of pyoverdine can be protected by the pyoverdine produced by the
358 other mutants. However, at least one gene of the *pvdMNOE* operon was found to be involved
359 in copper, cadmium or zinc resistance (Fig. 1 and Table 1). Although not statistically
360 significant for cadmium resistance, the *pvdO* gene has a log₂FC of -2.68, similar to the other
361 genes in the operon. Similarly, the *pvdN,O,E* genes have a log₂FC of -1.56, -1.26 and -2.01,
362 respectively, in the presence of zinc. The whole *pvdMNOE* operon seems to be important for
363 copper, zinc and cadmium resistance (Fig. 1, Table 1). This is consistent with the proposed
364 hypothesis that mature periplasmic storage of pyoverdine protects the bacterium from excess
365 metals other than iron by chelating these metals in the periplasm (54).

366

367 *gshA*

368 Among the genes involved in copper and zinc resistance, *gshA* (PP_0243) was found in our
369 Tn-seq screen. *gshA* encodes the glutamate cysteine ligase GshA, which forms the glutamyl-
370 cysteine from L-glutamate and is essential for copper resistance with a \log_2FC of -1.5 (Fig. 1
371 and Table 1). Glutamyl-cysteine is itself used by GshB to produce glutathione. Glutathione is
372 a key player in metal homeostasis in *E. coli* (59) and glutathione can buffer an excess of
373 intracellular copper in *Streptococcus pyogenes* (60). The thiol group and cysteine residues of
374 glutathione can directly bind to metal ions, protecting the cells from their deleterious
375 properties. *gshB* (PP_4993) had a negative \log_2FC but did not pass the statistical threshold for
376 copper resistance (\log_2FC of -1.04) (table S5). It is noteworthy that a mutant of the *proB* gene
377 (PP_0691), involved in proline biosynthesis, provides a growth advantage in the presence of
378 copper. *proB* encodes glutamate 5 kinase, which transforms L-glutamate into L-proline.
379 However, L-glutamate is also the substrate of the glutamate cysteine ligase, which is
380 produced by *gshA*. As the *gshA* gene appears to be essential for copper resistance, it is not
381 surprising that a *proB* mutant confers a growth advantage in presence of copper. Likewise, it
382 is not unexpected to detect *gshA* involved in a cross resistance since it has already been
383 described for copper (II), zinc (II) and cadmium (II) resistance in *E. coli* (Helbig et al. 2008).
384 However, it is the first time that *gshA* is described as being involved in cobalt resistance (59).

385

386 *CadR*

387 We also highlight the importance in metal resistance of the merR regulator CadR in metal
388 (PP_5140), which has a strong negative \log_2FC of -6.74 for cadmium resistance and of -4.31
389 for zinc resistance (table 1 and fig. 1). *cadR* is the neighbor gene of *cadA-3* (PP_5139),
390 which was also implicated in cobalt resistance but not zinc resistance in our screen (see upper
391 section). CadR regulates its own transcription and is known to respond to cadmium (61).
392 According to Canovas and colleagues, CadR was described as the putative regulator of *cadA-3*
393 (PP_5139), but this regulator does not regulate *cadA* in *P. putida* 06909 (61). Previous
394 mutational analysis indicated that *cadA-3* and *cadR* are partially responsible for zinc
395 resistance in *P. putida* 06909 (61). Although CadR preferentially binds to cadmium, it can
396 also weakly bind to zinc, resulting in less transcription activation compared to when it is
397 complexed with cadmium (62). In *P. aeruginosa*, CadR is constitutively bound to its
398 promoter and promptly activating *cadA* gene expression upon Zn binding. CadA is essential

399 for a timely induction of the CzcCBA efflux system (63). In our condition of an excess of
400 zinc ions, *cadA-3* was not required for zinc resistance.

401

402 **In-frame deletion mutants and complementation assays confirmed that *PP_1663* and**
403 ***roxSR* are required for Cd^{2+} , *PP_5337* for Cu^{2+} and *PP_5002* for Co^{2+} are required for**
404 **metal resistance.**

405

406 Our Tn-seq screen also identified several genes that were not previously known to be
407 associated with metal resistance. Among them, *PP_1663*, a gene encoding a putative
408 periplasmic protein of unknown function, was found to be involved in cadmium resistance
409 with the strongest $\log_2\text{FC}$ of -8.22. This gene is the ortholog of *PA0943* in *P. aeruginosa*
410 PAO1. A *PA0943* mutation rendered *P. aeruginosa* hypersensitive to the production of the
411 secretin XcpQ and altered the normal functioning of the Xcp protein export system (64). In *P.*
412 *putida* KT2440, The type II secretion system (Xcp) of *P. putida* is involved in the secretion
413 of phosphatase (65). We could also identify a new transcriptional regulator of the LysR
414 family, *PP_5337*, probably involved in copper resistance ($\log_2\text{FC}$ of -3.04). Analysis of its
415 regulon has not been performed yet. The Tn-seq screen in presence of cobalt also identified
416 the *PP_5002* gene with unknown function. The putative protein produced by *PP_5002*
417 contains a DUF971 domain which could be involved in Fe-S cluster assembly. The *PP_5002*
418 product could play a major role in iron homeostasis to counteract the deleterious effect of
419 cobalt on this equilibrium. Finally, we identified the RoxS sensor (*PP_0887*), which is part of
420 the RoxS-RoxR two-component system (*PP_0887-PP_0888*), as being essential for cadmium
421 resistance.

422 To confirm the role of these genes in metal resistance, we decided to go a step further by
423 performing in-frame deletions of these genes and selected other genes identified in our Tn-
424 seq screening. We also made mutants of *pcoA-2/B-2* for copper resistance, *czcA-1* for zinc
425 resistance, and *cadA-3* for cadmium resistance because they can be used as positive controls.
426 Since *roxS* (*PP_0887*) is in operon with *roxR* (*PP_0888*), a double mutant was constructed.
427 As several genes of the *pvdMNOE* operon were identified in our screening, we decided to
428 make the $\Delta pvdMNOE$ mutant. The genes that were deleted are underlined in Figure 1B.

429 First, to validate the Tn-seq results, we performed a competition experiment with the WT
430 strain and the mutants in a 1:1 ratio to calculate the fitness of the respective mutants
431 compared to the WT strain. We calculated a ratio in \log_{10} by dividing the number of colony-
432 forming units (cfu) of the mutant by the number of cfu of the WT strain after a co-culture of

433 the two strains. The experiment were performed in LB only or LB with a respective metal at
434 the identical concentration that was used in the Tn-seq screen. (Fig. 2). First, In LB only, all
435 tested mutants grew as well as the WT strain, except for the $\Delta dsbA$ and $\Delta gshA$ mutants,
436 which showed reduced growth fitness. In the presence of cadmium, we confirmed that the
437 $\Delta roxSR$, $\Delta pvdMNOE$, $\Delta cadR$, $\Delta dsbA$, ΔPP_{1663} , and $\Delta cadA-3$ mutants had a lower fitness
438 than the WT strain (Fig. 2A). The growth of the $\Delta dsbA$ and $\Delta cadA-3$ mutants in the presence
439 of cadmium was so low that the fitness could not be calculated. In the presence of copper, the
440 $\Delta pvdMNOE$, $\Delta copA1$, $\Delta gshA$, $\Delta pcoA2$, $\Delta pcoB2$, and ΔPP_{5337} mutants had a lower fitness
441 (Fig. 2B). When exposed to cobalt, only the ΔPP_{5002} mutant showed a significant fitness
442 defect. We were unable to confirm the sensitivity towards cobalt for the $\Delta pstC$, $\Delta gshA$,
443 $\Delta glnE$, and $\Delta prlC$ mutants (Fig. 2C). Finally, the mutants $\Delta cadR$, $\Delta pvdMNOE$, and $\Delta czcA-1$
444 exhibited lower fitness levels in the presence of zinc. However, the $cadA-3$ mutant showed no
445 sensitivity to zinc (Fig. 2D). This confirms our Tn-seq results. In conclusion, we also showed
446 that the $\Delta cadR$ mutant is sensitive to both cadmium and zinc and that the $\Delta pvdMNOE$ strain
447 is sensitive to both cadmium, zinc and copper. In general, all results confirm our Tn-seq
448 results, except for cobalt where only one gene (ΔPP_{5002}) could be validated.

449 Next, we focused our work on the four genes PP_{5337} , $roxSR$, PP_{1663} and PP_{5002}
450 because they had not been shown to be involved in metal resistance prior to our work. The
451 growth of these mutants was measured individually in LB liquid culture over time and
452 compared to the growth of the WT strain (Fig. 3A-G). No statistical difference in growth was
453 observed between the mutants and the WT strains. In contrast, in the presence of metal ions,
454 the mutants ΔPP_{5337} , $\Delta roxSR$, ΔPP_{1663} and ΔPP_{5002} showed a growth defect in LB
455 supplemented with Cu, Cd, Cd and Co, respectively (Fig. 3A, C, E, G).

456 To prove that the phenotypes of the mutants were in fact related to the deletion of the
457 respective target gene, we cloned the genes into the pJN105 plasmid under the control of the
458 arabinose-inducible promoter pBAD to perform a complementation assay. The WT and
459 mutants were grown in LB with arabinose to induce gene expression from the pJN105
460 plasmid. Cultures were performed in the presence of Cu, Cd, or Co (Fig. 3B, D, F, G).
461 Expression of the PP_{5337} , $roxSR$, PP_{1663} and PP_{5002} genes in the corresponding
462 mutant could at least partially suppress the growth defect caused by the metals. In particular,
463 these data demonstrated that PP_{1663} and $roxSR$ are novel genetic factors required for Cd^{2+} ,
464 PP_{5337} for Cu^{2+} and PP_{5002} for Co^{2+} metal resistance in *P. putida* KT2440. Taken
465 together, these results confirm that Tn-seq is (i) a reliable technique for identifying genes

466 involved in metal resistance in *P. putida* and ii) is able to confirm known genes and also
467 identify novel genes relevant for metal resistance.

468

469 **Concluding remarks**

470 Tn-seq has previously been used to comprehensively study the essential genomes of several
471 bacteria, sometimes in response to drugs (22, 66). However, there has been no Tn-seq
472 genome-wide study of factors necessary for Cu, Cd, Co and Zn resistance in *Pseudomonas*
473 *putida*. Motivated by previous studies, we ensured that we could rely on a completely
474 assembled, full genome sequence of our *P. putida* KT2440 strain in order to minimize the
475 risk to miss relevant genes (67). Overall, our approach was risky because *P. putida* has
476 multiple genetic determinants that affect its resistance to these metals. Functional redundancy
477 can be a challenge in this type of experimental approach. The absence of a genetic
478 determinant for metal-resistance may be compensated for by the expression of other metal-
479 resistant genes. We chose to work with sub-inhibitory concentrations of Cu, Cd, Co and Zn.
480 Our approach led to the identification of genes already known to be implicated in metal
481 resistance or homeostasis. The study has identified key genes involved in resistance, such as
482 *copA-1*, *pcoA-2*, and *pcoB-2* for copper, *cadA-3* and *cadR* for cadmium, and *czcA-1* for zinc.
483 This finding is also significant because it indicates that these genes do not have functional
484 redundancy. Miller and colleagues have demonstrated the response of *P. putida* to the
485 presence of cadmium and copper (7). Numerous transcriptional regulators, outer and inner
486 membrane proteins that form efflux channels and pumps, periplasmic proteins, and stress-
487 related proteins are involved. It is plausible that the genes identified in our screens are part of
488 the initial defense against these metals. If higher concentrations occur, other genes not
489 identified in our screens will come into play. To exemplify this vision, it is worth mentioning
490 the research conducted by Peng and his colleagues (11). They performed RNA-seq on *P.*
491 *putida* KT2440 grown under varying zinc concentrations. The authors found that the
492 transcriptome of *P. putida* was dependent on the concentration of zinc in the medium.
493 Specifically, at the lowest concentration tested (200 μ M in a semi-synthetic medium), only
494 PP_5139 and PP_0043 were overexpressed. These results support our observation that the
495 two genes are necessary for resistance to a zinc concentration of 125 μ M in LB.
496 Another example is the RND complex CzcCBA (PP_0043-PP_0045), known for its
497 resistance to zinc and cadmium (4). It appears that this system is dispensable for cadmium
498 resistance in our screen. It is possible that the *czcABC* system responds to a higher quantity of

499 metals and was therefore inactive in our conditions. This hypothesis is supported by the
500 identification of the *czCBA1* genes involved in resistance to 3mM cadmium resistance during
501 the screening of a Tn5 mutant library in *P. putida* CD2 (12). The difference in the
502 experiments in the two studies could explain this phenomenon. It would thus be interesting to
503 carry out a Tn-seq screen at higher metal concentrations. This would aid in identifying
504 additional genetic factors required for survival in environments with high levels of heavy
505 metals. Tn-seq has the added advantage of being able to identify genes that are not induced in
506 the presence of metal, making its results different from those obtained by transcriptomics or
507 proteomics.

508 Finally, our study allowed the identification of new important factors for metal resistance in
509 *P. putida*. Targeted in-frame mutagenesis and functional complementation prove that
510 *PP_1663* encoding a periplasmic protein and *roxSR* encoding a two-component system are
511 required for cadmium resistance. *PP_5337* is a new putative transcriptional regulator required
512 for copper resistance, and *PP_5002* an hypothetical protein required for cobalt resistance. To
513 better understand how these genes induces resistance to metals, further characterization is
514 necessary. For *RoxR* and *PP_5337*, a transcriptomic study should be conducted to identify
515 the genes that are regulated by these transcriptional regulators. In conclusion, our study
516 shows that there are still many studies to be carried out to fully understand the *P. putida*
517 resistome to metals.

518

519 **Methods**

520 **Bacterial strains and growth conditions.**

521 Bacterial strains, plasmids and oligonucleotides used in this study are described in Table S1
522 and Table S2. During the course of the project, we decided to re-sequence the genome of our
523 *P. putida* KT2440 strain present in our collection, referred to as PP1 (see supplementary
524 methods). The genome is registered under Genbank accession CP036494. The Average
525 Nucleotide Identity between this strain and *P. putida* KT2440 (Genbank AE015451.2) is
526 100% (<http://enve-omics.ce.gatech.edu/ani/>). The PP1 is thus referred as KT2440 strain in the
527 article.

528 *P. putida* and *E. coli* cells were grown at 28 and 37°C respectively in LB medium or 2YT
529 medium. When required, antibiotics were added at the following concentration: ampicillin,
530 100 µg/L, gentamicin, 30 µg/L for *P. putida* and 7 µg/L for *E. coli*, streptomycin, 100 µg/L.

531 Media were solidified with 1.5 g/L agar. During Tn-seq experiments, metals were used at a
532 subinhibitory concentration: CoCl₂ 10 μM, ZnCl₂ 125 μM, CuCl₂ 2.5 mM, CdCl₂ 12.5 μM.

533

534 **Construction of the transposon library**

535 *P. putida* strain KT2440 and *E. coli* MFDpir/pEGL55 were grown overnight in 2YT medium.
536 pEGL55 is a R6K suicide plasmid carrying the mariner transposon. 100 OD_{600nm} units of each
537 strain were mixed and centrifuged at 5000g for 10 min. The bacteria were resuspended in 1.2
538 mL of 2YT medium supplemented with diaminopimelic acid (300 μM) and plated on an
539 over-dried LB agar plate containing twice the normal concentration of agar. After 3 hours at
540 28°C, bacteria were collected and resuspended in 4 mL LB medium. A 20 μl aliquot was
541 diluted and plated on LB agar with gentamicin to estimate the efficiency of mutagenesis. The
542 other part was spread on 50 plates of LB agar with gentamicin and grown for 24 h at 28°C.
543 To confirm that the *P. putida* mutants had lost the plasmid, we performed colony PCR with
544 primers annealing to the *bla* gene of pEGL55. None of the 100 colonies tested produced a
545 PCR fragment, indicating loss of the plasmid in the bacteria tested. 800,000 mutants were
546 harvested in LB supplemented with 40% glycerol at -80°C. This library was directly
547 sequenced and represents the mutant pool in LB agar (see Table S4).

548 **DNA preparation for high-throughput sequencing**

549 To identify essential genes in LB or LB with metal, ~ 10⁷ mutants were inoculated in 25 mL
550 LB. The culture was then incubated at 28°C with shaking at 180 rpm. At OD₆₀₀ of 0.2, metals
551 were added independently at the following subinhibitory concentrations: cobalt 10 μM,
552 copper 2.5 mM, zinc 125 μM, and cadmium 12.5 μM. When OD₆₀₀ was 1.6, the culture
553 medium was diluted in the same medium with OD₆₀₀ of 0.03. This procedure was carried out
554 for 12 generations. The final pools of mutants were harvested by centrifugation of the culture
555 medium and stored at -80°C. DNA was extracted from aliquots of the bacterial suspension
556 using the Promega Wizard Genomic DNA Purification Kit. The next steps of the DNA
557 preparation methods were performed as described previously (20). Quality control of Tn-seq
558 DNA libraries (fragment size and concentration) and high-throughput sequencing on HiSeq
559 2500 (Illumina) were performed by MGX (CNRS sequencing service, Montpellier). 6 DNA
560 libraries were multiplexed on a flow cell. After demultiplexing, the total number of reads
561 ranged from 19 to 35 million (Table S3).

562 **Bioinformatics analysis.**

563 Raw reads from the fastQ files were first filtered using cutadapt v1.11 (Martin, 2011), and
564 only reads containing the mariner inverted left repeat

565 (ACAGGTTGGATGATAAGTCCCCGGTCTT) were trimmed and considered bona fide
566 transposon disrupted genes. The trimmed reads were then analyzed using a modified version
567 of the TPP script available in the TRANSIT software v2.0.2 (26447887). The mapping step
568 was modified to select only reads that mapped uniquely and without mismatch in the *P.*
569 *putida* KT2440 genome. The counting step was then modified to accurately count reads
570 mapping to each TA site in the reference genome according to the Tn-seq protocol used in
571 this study. Read counts per insertion were normalized using the LOESS method as described
572 in Zomer et al. (68). Next, we used the TRANSIT software (version 2.0) to compare the Tn-
573 seq datasets (27). Gene states obtained by TRANSIT after growth of the mutant bank of *P.*
574 *putida* KT24440 in LB agar and LB are presented in Table S4. Raw data of all datasets
575 analyzed by TRANSIT are presented in Table S5.

576 **Construction of the pKNG101 plasmids used for in-frame deletion in *P. putida* (Table**
577 **S1).** The 500 bp of DNA upstream and downstream of a target gene were amplified by PCR
578 (PrimeStar Max DNA Polymerase, Takara). The two 500 bp fragments were then fused by
579 overlapping PCR. The resulting 1 kbp DNA fragment was inserted between the BamHI/SpeI
580 restriction sites of pKNG101 by SLIC (69). Finally, the construct was transformed into
581 DH5 α π pir and verified by colony PCR and sequencing.

582 **Construction of the pJN105 plasmids used for complementation (Table S2).** The target
583 gene with native RBS was amplified by PCR (PrimeStar Max DNA Polymerase) from gDNA
584 of *P. putida* KT2440. The amplified fragment was inserted by SLIC between the SpeI and
585 SacI restriction sites of pJN105 and then transformed into DH5 α . The resulting plasmids
586 were validated by restriction mapping and sequencing.

587 **In-frame deletion mutant construction.**

588 To construct the in-frame deletion mutants of the genes underlined in Figure 2, the counter
589 selection method using the *sacB* gene was used (70). The suicide pKNG101 plasmid were
590 transferred from MFD π pir (71) to *P. putida* KT2440. The first recombination event was
591 selected on LB agar supplemented with streptomycin. Transconjugants were then plated on
592 LB agar without NaCl supplemented with 5% sucrose to allow the second recombination
593 event. In-frame deletions were then verified by PCR (Dreamtaq polymerase, Thermofisher).

594 **1 x 1 Competition assays.**

595 To compare the metal sensitivity of the mutants with the wild-type strain, 1 x 1 competition
596 experiments were performed as follows. First, to distinguish the mutants from the wild strain,
597 a GFP⁺ WT strain was constructed by inserting the constitutively expressed *gfp* gene into the

598 attTn7 site of the *P. putida* KT2440 chromosome using the pUC18-miniTn7-gfpmut3
599 plasmid
600 (72). The GFP⁺ strain grow as well as the WT (figure S4). Mutant and GFP⁺ WT strains were
601 grown separately in LB medium from an overnight culture in LB to OD₆₀₀ of 0.8. Bacteria
602 were then mixed in a 1:1 ratio at an initial OD₆₀₀ of 0.0125 in a 96-well plate containing 200
603 μL LB or LB with metal at a sub-inhibitory concentration. After 24 hours of growth at 28°C
604 in the Tecan M200 Pro with shaking, 5 μL of the cultures were used to inoculate a new 96-
605 well plate and placed under the same conditions. After a total of 48 hours of growth
606 (approximately 10 divisions), the bacteria were diluted and plated onto LB agar plates. After
607 48 hours at 28°C, GFP⁺ wild-type and mutant colonies were counted under blue light to
608 detect colony fluorescence. A ratio was then calculated by dividing the number of mutant
609 colonies by the number of wild-type colonies in each condition. The growth comparison
610 between a WT strain and a GFP⁺ strain in LB supplemented with different metals is shown in
611 Table S6.

612 **Individual growth in presence of metals.**

613 Single strain growth was performed in LB medium from an overnight culture in LB to an
614 OD₆₀₀ of 0.8. Bacteria were then inoculated at an initial OD₆₀₀ of 0.006 into a 96-well plate
615 containing 200 μL of LB or LB with metal at a sub-inhibitory concentration and placed at
616 28°C in the Tecan M200 Pro. OD₆₀₀ measurements were taken every 10 minutes after
617 shaking. Complementation assays were performed using the same protocol but with 0.2%
618 arabinose. Data are presented after 6.5 hours of growth.

619

620

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630

631 **Legend of figures**

632

633 **Table 1.** Metal resistance genes of *P. putida* KT2440 discovered by Tn-seq

634

635 **Figure 1. Genes involved in metal resistance according to the Tn-seq experiment.** (A)

636 Examples of negative selection revealed by Tn-seq. The graphs show the number of Tn-seq
637 reads at each location aligned to TA sites on the *P. putida* KT2440 genome. Results are
638 shown in LB only or LB with either Cu, Cd, Zn or Co. The regions with significantly fewer
639 reads are framed in red and the genes corresponding to these regions are indicated in red.

640 Data are averaged from biological replicates and normalized as described in Materials and
641 Methods. (B) Venn diagram of the genes with a positive or negative \log_2FC , indicating the
642 fitness difference between the test condition (LB with a excess of Cu, Cd, Co or Zn) and the
643 LB condition. Genes already known to be involved in metal resistance in *P. putida* are in
644 bold. The underlined genes were selected for in-frame deletion and further analysis.

645

646 **Figure 2. Competition between the wild type and the mutant strains of *P. putida* KT2440**

647 **in presence or not of a metal in excess.** Competitions were realized with an initial ratio of
648 1:1 in LB supplemented or not with a sub-inhibitory concentration of metals (cobalt 10 μM ,
649 zinc 125 μM , copper 2.5 mM, cadmium 12.5 μM). The respective final ratio was determined
650 as described in Methods and presented in Log_{10} . The experiment was realized four times. *
651 indicates a statistically significant difference relative to the absence of metal condition
652 ($p < 0.05$, Mann-Whitney U test).

653

654 **Figure 3. Individual growth cultures of the mutants and complementation assay.**

655 Individual growth of each mutant strain was performed in LB medium supplemented or not
656 with a sub-inhibitory concentration of metals (cobalt 10 μM , zinc 125 μM , copper 2.5 mM,
657 cadmium 12.5 μM) in a 96 well plate. OD at 600nm was measured over the time. Panels A, C,
658 E and F shows growth of the WT and the mutants in both conditions. Panels B, D, F and H
659 shows the functional complementation assay in presence of metal. The data represents the

660 mean of 4 replicates. The growth difference between the mutant and the WT (panels A, C, E
661 and F) or the complemented mutant and the WT in the presence of a metal (panels B, D, F
662 and H) is always statistically significantly different during the exponential phase ($p < 0.05$,
663 Mann-Whitney U test).

664

665 **Supporting information legends**

666

667 **Table S1. Bacterial strains and plasmids used in this study**

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

669 **Table S3. Tn-Seq analysis of *P. putida* KT2440.**

670 **Table S4. Data obtained by TRANSIT and HMM analysis after growth of the bank of**
671 **the *P. putida* KT2440 mutants in LB agar and after outgrowth in LB.**

672 **Table S5. Data obtained by TRANSIT and RESAMPLING analysis after growth in**
673 **presence of Co, Cd, Cu or Zn.**

674

675 **Figure S1. Mean read count of the Tn-seq experiment of *P. putida* KT2440 in LB agar**
676 **as a function of the genomic position after LOESS correction.**

677

678 **Figure S2. Determination of a metal's subinhibitory concentration for *P. putida***
679 **KT2440.**

680 10 mL of LB was inoculated at OD₆₀₀ of 0.03 from an overnight culture of *P. putida* KT2440
681 and placed in an Erlenmeyer flask at 28°C with shaking at 180 rpm. At OD₆₀₀ of 0.2, different
682 metal concentrations were added to the culture medium. OD₆₀₀ of the cultures were measured
683 over time.

684

685 **Figure S3. Predicted structures and cellular localizations of CopA, PcoA and pCoB**
686 **proteins of *P. putida* KT2440 and *P. aeruginosa* PAO1.**

687 Cellular localizations of the CopA (PP_0586 in *P. putida* ; PA3920 in *P. aeruginosa* PAO1),
688 PcoA (PP_5380 in *P. putida* ; PA2065 in *P. aeruginosa* PAO1) and PcoB proteins (PP_5379
689 in *P. putida* ; PA2064 in *P. aeruginosa* PAO1) are those indicated by the *Pseudomonas*
690 database (73). The 3D structures were predicted by AlphaFold (32) and compared using the
691 TM-Align algorithm (33). CopA is an inner-membrane copper-translocating P-type ATPase
692 composed of six transmembrane helices according to Uniprot (74). PcoA is a multi-copper

693 oxydase forming a globular protein in the periplasm. PcoB is a porin-like outer membran
694 protein with a α -barrel predicted structure. The figure was created www.BioRender.com

695

696 **Figure S4. Growth comparison between WT and GFP⁺ strain.**

697 The OD₆₀₀ over time of 200 μ L LB culture of *P. putida* KT2440 WT and its GFP⁺ derivative
698 was measured in a 96-well plate. The graphs are the mean of three replicates. No statistical
699 difference was detected.

700

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Table 1: Metal resistance genes of *P. putida* KT2440 discovered by Tn-seq

Metal ^a	Locus	Gene ^b	Function	State in LB ^c	No. of TAs	Mean read ^d		Log ₂ FC ^e	q-value ^f	orthologs in PAO1 ^g
						LB	LB + metal			
Co	PP_2645	mgtA	Magnesium transporter ATP-dependent	GD	24	6.1	78.9	3.70	0.00000	
	PP_5002	PP_5002	Hypothetical protein	NE	5	38.1	0.2	-7.31	0.00000	PA5055
	PP_0096	prlC	Oligopeptidase A	NE	30	126.0	23.4	-2.43	0.00000	
	PP_0340	glnE	Glutamate-ammonia-ligase	NE	37	28.1	6.5	-2.12	0.00000	
	PP_5328	pstC	Phosphate ABC transporter permease	NE	32	33.6	8.7	-1.95	0.00000	
	PP_0243	gshA	Glutamate-cysteine ligase	NE	28	16.5	5.6	-1.56	0.00000	
Cu	PP_0691	proB	Glutamate 5-kinase	NE	7	3.8	55.8	3.89	0.00000	
	PP_0586	cadA2 → copA1	Cadmium translocating P-type ATPase	NE	22	197.3	0.7	-8.19	0.00000	PA3920 = copA1
	PP_1735	htrB	Lipid A biosynthesis lauroyl acyltransferase	NE	10	23.6	0.1	-7.39	0.00000	
	PP_4216	pvdE	Pyoverdine ABC transporter ATP-binding protein/permease	NE	18	49.0	1.1	-5.45	0.00000	
	PP_4213	pvdM	Dipeptidase	NE	15	58.7	2.9	-4.35	0.00000	
	PP_4214	pvdN	Pyoverdine biosynthesis-like protein	NE	15	62.4	2.8	-4.49	0.00000	
	PP_4215	pvdO	Pyoverdine biosynthesis-like protein	NE	18	72.6	4.0	-4.18	0.00000	
	PP_2767	PP_2767	ABC transporter ATP-binding protein	NE	5	22.1	1.6	-3.81	0.00000	
	PP_5337	PP_5337	LysR family transcriptional regulator	NE	11	19.5	2.4	-3.04	0.00000	PA5428
	PP_5380	copA-2 → pcoA2	copper resistance protein A	NE	36	259.6	32.3	-3.01	0.00000	PA2065 = pcoA
PP_5379	copB-2 → pcoB2	copper resistance protein B	NE	17	113.1	33.5	-1.75	0.00000	PA2064 = pcoB	
PP_4194	gltA	Citrate synthase	NE	21	30.8	10.0	-1.62	0.00000		
PP_0243	gshA	Glutamate-cysteine ligase	NE	28	16.5	5.9	-1.50	0.00000		
PP_2328	cysH	Phosphoadenosine phosphosulfate reductase	GA	11	390.5	158.9	-1.30	0.00000		

	PP_5140	PP_5140 → cadR	MerR family transcriptional regulator	NE	9	181.6	9.1	-4.31	0.00000	PA3689
Zn	PP_4213	pvdM	Dipeptidase	NE	15	58.7	18.4	-1.67	0.00000	
	PP_0043	czcA-1	Cation efflux system protein	NE	51	211.3	90.6	-1.22	0.00000	PA2520 = czcA
	PP_1663	PP_1663	Hypothetical protein	NE	10	364.6	1.2	-8.22	0.00000	PA0943
	PP_0127	dsbA	Thiol:disulfide interchange protein	NE	8	32.2	0.2	-7.59	0.00000	
	PP_5140	PP_5140 → cadR	MerR family transcriptional regulator	NE	9	181.6	1.7	-6.74	0.00000	
	PP_5139	cadA-3	cadmium translocating P-type ATPase	NE	18	110.4	3.5	-4.99	0.00000	
	PP_4216	pvdE	Pyoverdine ABC transporter ATP-binding protein/permease	NE	18	49.0	4.1	-3.59	0.00000	
	PP_0887	PP_0887 → roxS	Sensor histidine kinase	NE	19	193.2	19.5	-3.31	0.00000	PA4494 = RoxS
Cd	PP_4213	pvdM	Dipeptidase	NE	15	58.7	7.4	-2.98	0.00000	
	PP_4214	pvdN	Pyoverdine biosynthesis-like protein	NE	15	62.4	8.6	-2.86	0.00000	

^a Metal tested for which a significant \log_2 FC has been calculated.

^b Name of the gene in the TIGR KT2440 genome of the *Pseudomonas* database (version 22.1, date: 2023-10-06). When named has evolved in other *Pseudomonas* species, new names of the genes have been indicated in order to align with homologies.

^c State of each gene in LB defined by the TRANSIT software using an Hidden Markov Model: NE, Non-Essential ; GD, Growth-Defect ; E, Essential ; GA, Growth-Advantage.

^d Mean reads per TA site for a gene in each growth condition.

^e Ratio of reads between the two conditions expressed in \log_2 .

^f P-values adjusted for multiple comparisons using the Benjamini-Hochberg procedure (See Transit manual).

^g Orthologous gene in *Pseudomonas aeruginosa* PAO1.

Figure 1

A

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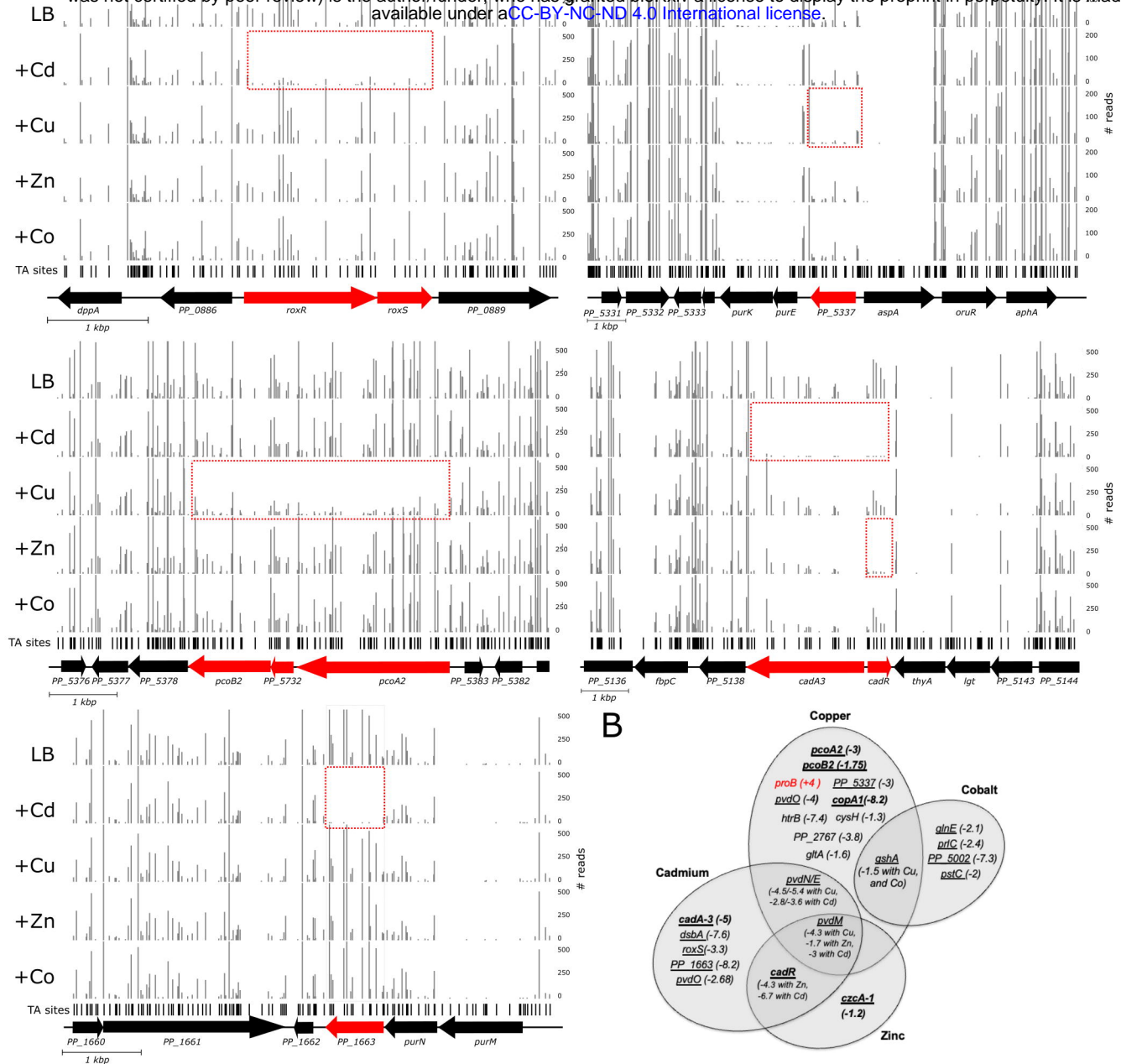


Figure 2

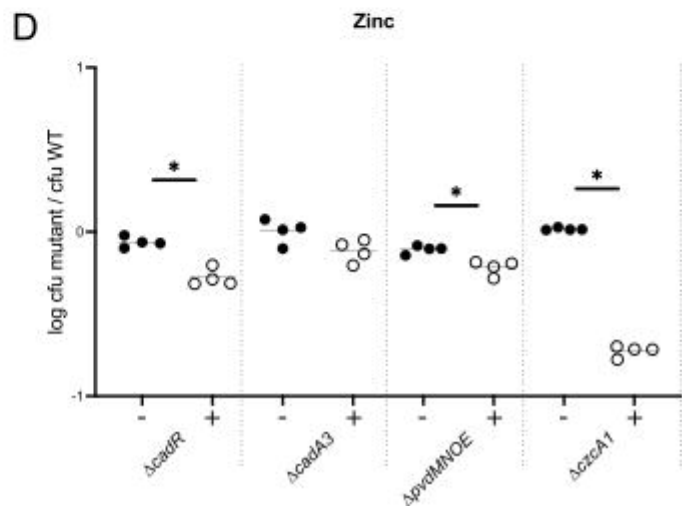
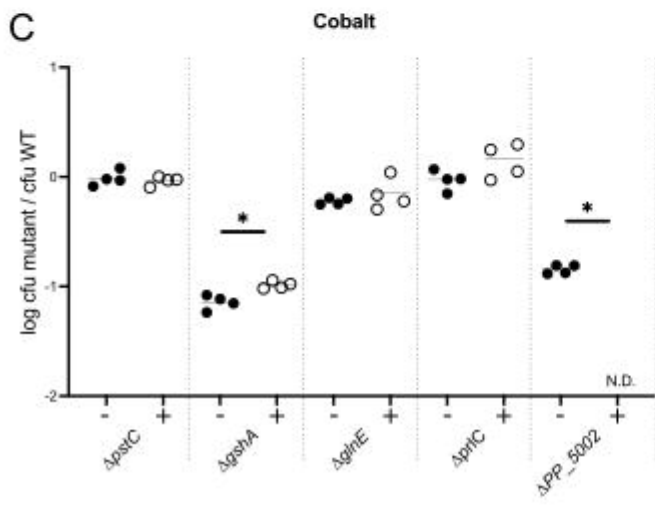
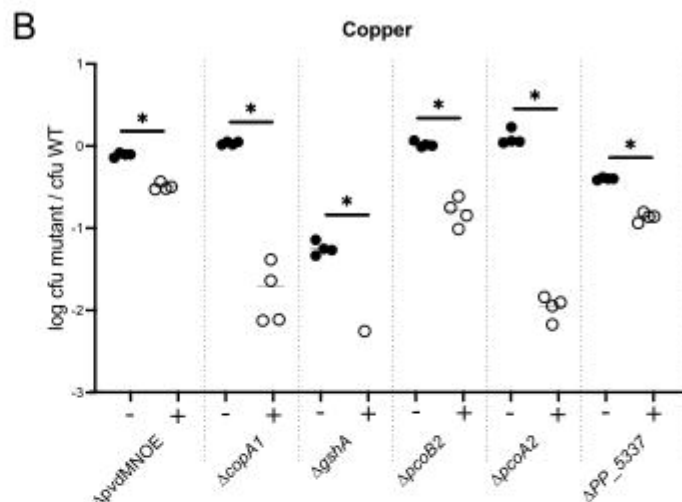
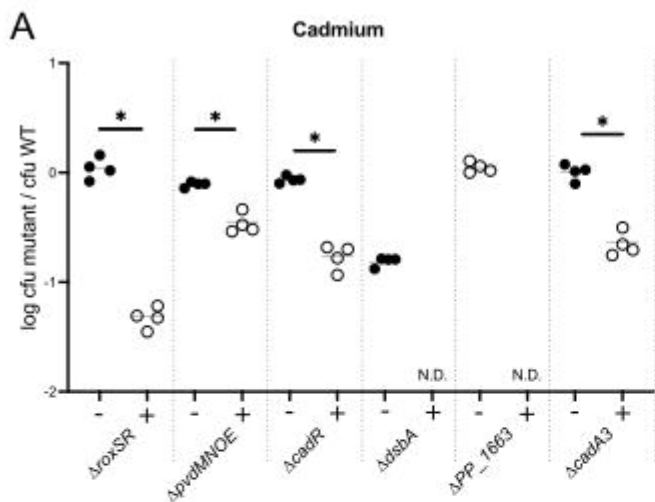
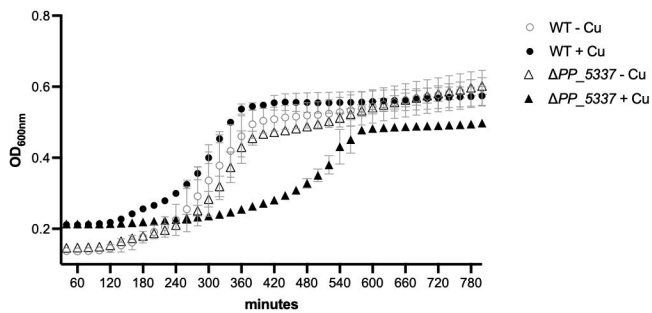
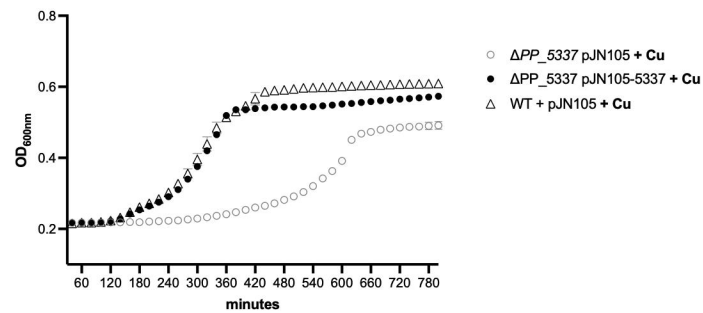


Figure 3

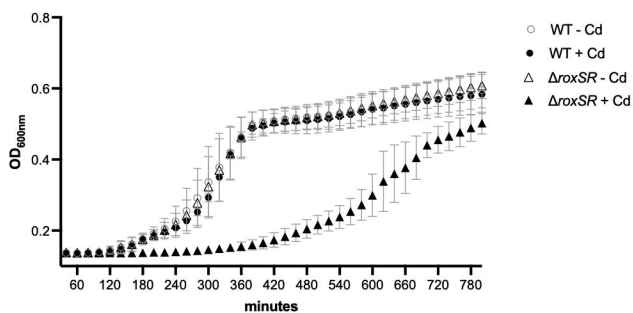
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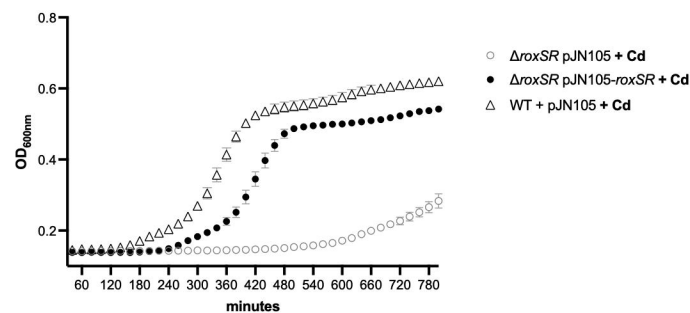
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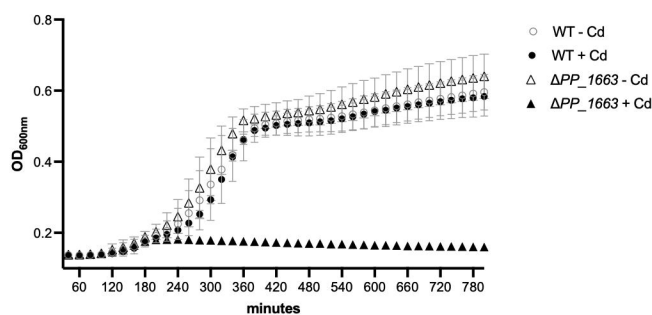
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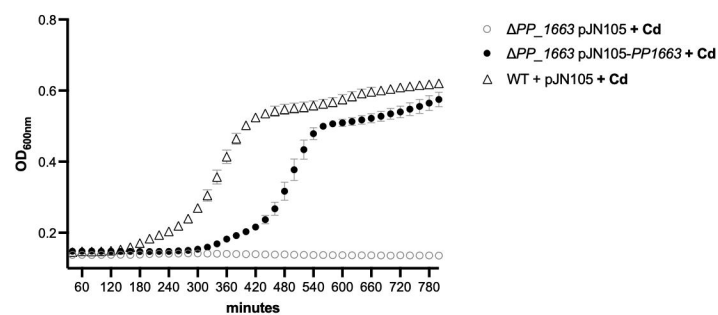
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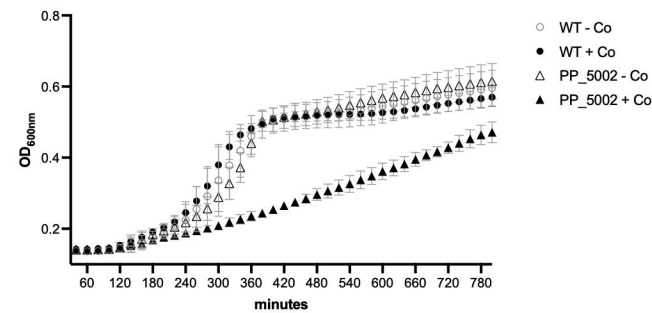
E



F



G



H

