

1 **Pseudopaline, a staphylopine-like metallophore involved in zinc and nickel**
2 **uptake in *Pseudomonas aeruginosa***

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27

28 ABSTRACT

29 Metal uptake is vital for all living organisms. In metal scarce conditions, a common bacterial
30 strategy consists in the biosynthesis of metallophores, their export in the extracellular medium
31 and the recovery of a metal-metallophore complex through dedicated membrane transporters.
32 Staphylopine is a recently described metallophore distantly related to plant nicotianamine that
33 contributes to the broad-spectrum metal uptake capabilities of *Staphylococcus aureus*. Here,
34 we characterize a four genes operon (*PA4837–PA4834*) in *Pseudomonas aeruginosa* involved
35 in the biosynthesis and trafficking of a staphylopine-like metallophore named pseudopaline.
36 Pseudopaline differs from staphylopine with regard to the stereochemistry of its histidine
37 moiety associated to an alpha ketoglutarate moiety instead of pyruvate. *In vivo*, the
38 pseudopaline operon is regulated by zinc through the Zur repressor. The metal-uptake
39 property of the pseudopaline system appears different from that of staphylopine with a
40 predominant effect on nickel uptake, and on zinc uptake in metal scarce conditions mimicking
41 a chelating environment, thus reconciling the regulation of the *cnt* operon by zinc with its
42 function as a zinc importer under metal scarce conditions.

43

44 SIGNIFICANCE

45 Zinc is an essential micronutrients for bacteria, particularly important at the host-pathogen
46 interface where the host tends to sequester metals in a so called nutritional immunity
47 framework, and the pathogenic bacterium increases its metal uptake efforts in order to keep
48 up with its metal requirements. Here we reveal a novel metallophore, named pseudopaline,
49 which is synthesized and exported by *Pseudomonas aeruginosa* and serves for the uptake of
50 nickel in metal poor media, and for the uptake of zinc in metal scarce conditions that mimic
51 the chelating environment that presumably prevails within a host.

52

53 INTRODUCTION

54 Divalent metals (Mn, Fe, Co, Ni, Cu and Zn) are essential micronutrients for all life forms,
55 and acquisition of these metals is therefore vital, particularly for bacterial pathogens in the
56 context of host-pathogen interactions. Indeed, there is a competition between the host, which
57 tends to sequester metals in a so called nutritional immunity framework, and the pathogenic
58 bacterium, which increases its metal uptake efforts in order to keep up with its metal
59 requirements (1, 2). Most pathogenic bacteria produce metallophores for metal uptake, with
60 siderophores being the most well-characterized metallophore family (3). Siderophores are
61 synthesized within the cell through non ribosomal peptide synthases (NRPS) or through a
62 NRPS independent system (NIS) and then are exported in the extracellular medium where
63 they scavenge iron. Extracellular iron siderophore complexes can be recognized and actively
64 transported into the periplasm by TonB dependent transporters (TBDT) in Gram-negative
65 bacteria, and usually ABC transporters in both Gram-negative and Gram-positive bacteria are
66 used for the crossing of the cytoplasmic membrane. There are many variations on this
67 common theme and, for example, some bacteria do not produce a specific type of siderophore
68 although they are able to use it for iron import (4). The siderophore pathway could also
69 prevent toxic accumulation of metals, which was particularly studied in the case of
70 *Pseudomonas aeruginosa* (5, 6). *P. aeruginosa* synthesizes two types of siderophores with
71 high iron affinity, pyochelin and pyoverdine, the latter being a demonstrated virulence factor
72 (7).

73 Metallophores specific for the uptake of metals other than iron have also been described, such
74 as the chalcophore methanobactin involved in copper uptake in methane-oxidizing bacteria (8,
75 9). Manganesophore have not been described as such, although TseM, a protein effector
76 secreted through a Type VI secretion system, was shown to play an important role in TBDT-
77 dependent manganese uptake in *Burkholderia thailandensis* (10). There is also indirect
78 evidence for the existence of a nickelophore in *Escherichia coli*, although it has still to be
79 identified (11). Free histidine could also be used as a nickelophore *in vivo* for nickel uptake in
80 various bacteria (12, 13). Yersiniabactin, initially described as a siderophore, also exhibits
81 zincophore properties in *Yersinia pestis* (14, 15). Coelibactin, described in *Streptomyces*
82 *coelicolor*, may also represent a zincophore as it is synthesized by a NRPS under the control
83 of Zur, a zinc responsive repressor (16).

84 Staphylopine is a nicotianamine-like molecule that was recently described as a metallophore
85 with remarkable broad-spectrum specificity (17). In *Staphylococcus aureus*, staphylopine is
86 synthesized through the action of three soluble enzymes (SaCntKLM). SaCntK transforms L-

87 histidine in D-histidine, SaCntL transfers an aminobutyrate moiety from S-
88 adenosylmethionine (SAM) onto D-histidine, and SaCntM reductively condensates the
89 product of SaCntL (called xNA) with pyruvate. The staphylopine biosynthesis and trafficking
90 pathway is responsible for zinc, copper, nickel, cobalt and iron uptake, depending on the
91 growth conditions, and this system contributes to the virulence and fitness of *S. aureus* (17–
92 19). The *S. aureus cnt* operon is partly conserved in *P. aeruginosa*, where homologues of the
93 *cntL* and *cntM* genes are found, albeit with 20-30% sequence identity at the protein level.
94 Upstream of *cntL*, a gene codes a predicted outer membrane protein belonging to the TBDT
95 family, and downstream of *cntM*, a gene codes for a predicted inner membrane protein
96 belonging to the EamA or DMT family (drug/metabolite transporter; Figure S1).
97 Transcriptomic approaches revealed that this gene cluster was highly expressed in a burn
98 wound model (20). This last gene was also identified as part of a novel siderophore pathway
99 that appeared vital for the growth of *P. aeruginosa* in airway mucus secretion (AMS) (21).
100 Finally, through a transcriptomic study of a Zur deficient strain, these four genes were found
101 in the top five regulated units, although most of them were annotated as hypothetical (22).
102 Here, we show that the four above-mentioned genes (here named *cntO*, *cntL*, *cntM* and *cntI*;
103 see supplementary table S1 for correspondence with locus tag in PAO1, PA7 and PA14
104 strains of *P. aeruginosa*) are part of an operon that is regulated by zinc level through the Zur
105 repressor. Using biochemistry and metabolomics approaches, we prove that the two
106 biosynthetic enzymes (PaCntL and PaCntM) synthesize a novel metallophore, which we
107 named pseudopaline, and which differs from staphylopine by the presence of a D-histidine
108 moiety instead of L-histidine, and an α -ketoglutarate moiety instead of a pyruvate. A *cntL*
109 mutant strain is shown to be unable to synthesize pseudopaline and is impaired in its ability to
110 import nickel in a minimal media, supplemented or not with nickel. Under more stringent
111 conditions where a chelator such as EDTA is added to a minimal succinate (MS) medium, a
112 condition that presumably mimics the chelating environment prevailing within a host or in
113 AMS, we show evidence that the *cntL* mutant strain is unable to import zinc, therefore
114 reconciling the regulation of this operon by zinc with its function as a zinc importer
115 functioning in metal scarce conditions.

116

117 **Results and discussion**

118 **The *cnt* operon of *P. aeruginosa* is regulated by zinc level through the zinc-responsive** 119 **regulator Zur**

120 *In silico* analysis of the *cnt* gene cluster of *P. aeruginosa* PA14 strain indicated two
121 overlapping open reading frames between *cntL* and *cntM* and between *cntM* and *cntI*,
122 classically observed in operonic structures (Figure S1). Further screening of the upstream *cnt*
123 sequence for promoter regions using Bprom software (23), revealed a $\sigma 70$ promoter in the 200
124 base-pairs upstream from the annotated *cntO* ATG codon (Figure S1). Interestingly, a putative
125 Zur binding box “GTTATagtATAtC” can be identified overlapping the -10 box of the
126 predicted $\sigma 70$ promoter, (22, 24). This *in silico* analysis supports an operonic organization of
127 the four *cnt* genes and strongly suggests a transcriptional activation of this operon under zinc
128 depletion through the Zur repressor (25, 26). In order to test this hypothesis, we performed
129 RT-PCR experiments using as templates RNA and cDNA generated from a WT PA14 strain
130 grown in minimal succinate (MS) medium known to contains low levels of metals, including
131 zinc (5). The successful amplification of the four *cnt* gene transcripts (Figure S1) indeed
132 indicated their induction when cells were grown in a MS medium. The specific amplification
133 of the three *cnt* intergenic regions confirmed that the four *cnt* genes are co-transcribed in one
134 single transcript and therefore constitute an operon.

135 To validate at the protein level the transcriptional regulation of the *cnt* genes, we followed by
136 immunoblotting the PaCntL production under various growth conditions. In this respect, we
137 constructed a *cntL* mutant strain producing a chromosomally encoded V5-tagged PaCntL
138 ($\Delta cntL::cntL_{V5}$). In this strain, the recombinant *cntL_{V5}* gene was placed under the predicted *cnt*
139 promoter region and inserted at the *att* site of the *P. aeruginosa* genome. In agreement with
140 our transcriptional data, immunoblotting experiments indicated that, the recombinant
141 PaCntL_{V5} is only produced in MS medium and not in a rich medium such as the LB medium
142 (Figure 1A). Presumably, this is due to the low metal content of the MS medium as compared
143 to the LB medium. We then tested whether the *cntL* transcription was subject to metal
144 repression by checking PaCntL_{V5} production in MS medium supplemented with various
145 concentrations of the most representative metals. Dot-blot experiments showed a specific loss
146 of PaCntL_{V5} production in MS medium supplemented with as low as 0.1 μ M of ZnSO₄. An
147 addition of iron, nickel or cobalt at concentrations equivalent or above the one found in LB
148 rich medium (5) has no negative effect on PaCntL_{V5} production (Figure 1B). The hypothesis
149 of a Zur repressor regulating the *cnt* operon was then tested through the construction of a
150 PA14 $\Delta cntL::cntL_{V5}$ *zur*⁻ strain. PaCntL_{V5} was still produced in the *zur* mutant strain grown in

151 LB or MS media supplemented with 1 μ M of ZnSO₄, conditions in which Zur normally exerts
152 its repressor activity (Figure 1C). Taken together, these data therefore demonstrate that the *cnt*
153 operon of *P. aeruginosa* is negatively regulated by zinc, most probably through the binding of
154 a Zn-Zur repressor complex onto the predicted Zur binding motif identified in the σ 70
155 promoter, thus preventing the recruitment of RNA-polymerase.

156

157 ***In vivo* detection and characterization of a PaCntL-dependent metallophore in the** 158 **extracellular medium of *P. aeruginosa***

159 We constructed a PA14 mutant strain lacking PaCntL ($\Delta cntL$) and compared the composition
160 of the intra- and extra-cellular contents of wild type and $\Delta cntL$ strains grown under the
161 previously defined *cnt* inducible conditions. Extracellular samples were analysed by
162 hydrophilic interaction liquid chromatography (HILIC) with detection by inductively coupled
163 plasma mass spectrometry (ICP-MS) and electrospray ionization mass spectrometry (ESI-
164 MS). HILIC/ICP-MS data revealed the presence of a molecule complexed with nickel and
165 zinc in the supernatant of the WT strain, which was absent in the *cntL* mutant strain (Figure
166 2). ESI-MS investigation of the metabolites eluting at this same elution volume showed
167 unambiguously the presence of typical nickel and zinc isotopic patterns indicating the
168 presence of a free metallophore with a molecular mass of 386 Da (Figure 2). Using the
169 accurate mass and a molecular formula finder software we proposed the C₁₅N₄O₈H₂₀ empiric
170 formula for the ligand in complex with nickel or zinc (Figure 2, inset for the nickel chelate).
171 This ligand corresponds to a new metallophore produced by *P. aeruginosa* in a *cntL*-
172 dependent manner. Comparison of its elemental composition with that of staphylopine (328
173 Da) revealed the presence of two additional carbons and two oxygen atoms, suggesting the
174 use of an α -ketoglutarate (α KG) moiety instead of pyruvate as found in staphylopine. The
175 fragmentation of this metallophore in gas-phase confirmed this hypothesis (Figure S2). We
176 propose to name this new metallophore pseudopaline, to recall its origin from *P. aeruginosa*
177 and its belonging to the nopaline family of opine (27).

178

179 ***In vitro* reconstitution of the pseudopaline biosynthetic pathway catalysed by PaCntL** 180 **and PaCntM**

181 We have recently shown that the PaCntL/M orthologs in *S. aureus* (SaCntL/M) are
182 sequentially involved in the biosynthesis of the staphylopine metallophore, using a D-
183 histidine that is produced by the histidine racemase enzyme SaCntK (17). One of the main
184 difference between the *cnt* operons of *P. aeruginosa* and *S. aureus* is however the absence of

185 a *cntK* gene upstream of the *cntL-M* genes in *P. aeruginosa*. This observation led to the
186 possibility of using directly L-histidine instead of D-histidine. In order to investigate the
187 properties of CntL and CntM of *P. aeruginosa*, the corresponding genes were cloned,
188 heterologously expressed in *E. coli* and their products purified for further biochemical
189 analysis. Gel filtration experiments showed that PaCntL could form a complex with PaCntM
190 (Figure S3), although this interaction was not observed between SaCntL and SaCntM. With
191 regard to PaCntL, we used thin layer chromatography (TLC) separation to follow the carboxyl
192 moiety of a carboxyl-¹⁴C-labelled S-adenosine methionine (SAM) substrate, co-incubated
193 with either L- or D-histidine (Figure 3A). Only the incubation with L-histidine led to a novel
194 band corresponding to a reaction intermediate that we propose to name yNA. We
195 demonstrated subsequently that PaCntM preferentially bound to NADH and not to NADPH
196 (Figure 3B), contrary to SaCntM that showed a preference for NADPH. We then used TLC to
197 visualize the PaCntLM reaction products under various *in vitro* conditions using all the
198 putative substrates (Figure 3C). Unexpectedly, the co-incubation of both enzymes with their
199 most probable substrates (L-histidine, NADH and α KG) did not lead to the formation of an
200 additional radiolabelled product as for the case of staphylopine biosynthesis (17) (Figure 3C).
201 One possibility was therefore that the product of PaCntM was migrating at the same position
202 as the yNA in the conditions used during the TLC separation. We therefore decided to study
203 the same co-incubations by HILIC/ESI-MS, following the mass expected for the yNA
204 intermediate and the pseudopaline found in the extracellular fraction of *P. aeruginosa* grown
205 in MS medium. These experiments confirmed that the incubation of PaCntL with SAM and
206 L-histidine led to the formation of the yNA reaction intermediate (Figure 3D, top), and most
207 of all, revealed the production of pseudopaline when co-incubating PaCntL, PaCntM and their
208 proposed substrates (SAM, L-histidine, NADH and α KG; Figure 3D, bottom). Co-incubations
209 using alternative substrates of PaCntM (pyruvate or NADPH) only led to the production of
210 yNA. Interestingly, pseudopaline and yNA eluted at the same volume in these HILIC-ESI/MS
211 experiments, showing that their physical properties are very similar, as suggested by our
212 previous TLC experiments.

213 Pseudopaline is therefore biosynthesized in two steps: first, a nucleophilic attack of one α -
214 aminobutyric acid moiety from SAM onto L-histidine catalysed by PaCntL to produce the
215 reaction intermediate yNA, and second, a NADH reductive condensation of the yNA
216 intermediate with a molecule of α KG catalysed by PaCntM to produce pseudopaline (Figure
217 3E). Pseudopaline differs from staphylopine by the stereochemistry of the histidine moiety
218 (L- and D- respectively) and by the presence of an α KG moiety instead of pyruvate in

219 staphylopine. The biosynthesis of a specific metallophore by different bacteria recalls the
220 chemical evolution of a large diversity of siderophore in a chemical rivalry to get access to
221 one's own pool of metal (28). Indeed, once in the extracellular medium, secreted
222 metallophores are a common good, and a privileged access presumably gives a selective
223 advantage.

224

225 **Pseudopaline is involved in nickel and zinc uptake, depending on the chelating** 226 **properties of the media**

227 In order to address the involvement of pseudopaline in metal uptake *in vivo*, we compared the
228 intracellular concentration of various metals in PA14 WT, *ΔcntL* and *ΔcntL::cntL* strains.
229 Cells were grown in pseudopaline-synthesis conditions determined above (MS medium) and
230 the intracellular metal concentration was measured by ICP-MS. Under these growth
231 conditions we observed a significant 90% reduction of intracellular nickel concentration in the
232 *ΔcntL* mutant strain (Figure 4A), which was mostly recovered in the complemented strain.
233 The levels of all the other metals were not changed in the *ΔcntL* mutant strain compared to the
234 WT strain (data not shown). A similar 90% reduction in intracellular nickel concentration was
235 also observed when the culture was supplemented with 1μM NiCl₂ (Figure S4), thus
236 confirming that nickel uptake was predominantly performed by pseudopaline in these metal-
237 poor media. We were intrigued by the apparent contradiction between the clear *cnt* operon
238 regulation by zinc, and the absence of any effect on zinc uptake. A possible explanation is that
239 the effect of *cnt* could be masked by the effect of a zinc ion importer such as the ZnuABC
240 zinc transport system described in *P. aeruginosa* (22). In an attempt to discriminate between
241 both transport systems, we sequestered free metal ions by supplementing the growth medium
242 with increasing concentrations of EDTA, a chelating agent for divalent metals. Interestingly,
243 although we did not observe any effect using 10 μM EDTA, the supplementation with 100μM
244 EDTA ultimately revealed a pseudopaline-dependent zinc uptake, with a 60% decrease of
245 intracellular zinc content in the *ΔcntL* mutant strain in comparison with the WT strain (Figure
246 4B). The complemented strain accumulated zinc to a level comparable to the WT. In these
247 chelating conditions the pseudopaline-dependent nickel import is abolished (Figure 4A),
248 hence proving a direct link between pseudopaline and zinc uptake in metal scarce conditions
249 with competing zinc chelators. These conditions may prevail at the host-pathogen interface
250 where metal binding proteins such as calprotectin are produced by the host (29, 30), or in
251 AMS where metals are complexed in a nutritional immunity framework (1, 21).

252

253 **Model of pseudopaline synthesis and transport pathway in *P. aeruginosa***

254 We next investigated the putative roles of the two membrane proteins that are found in the *cnt*
255 operon of *P. aeruginosa* by determining the pseudopaline level in the extracellular and
256 intracellular fractions of WT and mutant strains (Figure 5A and 5B, respectively). With
257 regard to PaCntO, we found a small decrease in the extracellular content of pseudopaline in
258 the $\Delta cntO$ mutant strain in comparison with the WT strain. However, we also found that this
259 $\Delta cntO$ mutant strain was partly impaired in nickel accumulation (Figure S5). Altogether, and
260 because PaCntO belongs to the TBDT family of extracellular transporter, its most probable
261 role is in the import of pseudopaline-metal complexes, although it is not excluded that other
262 proteins of this family could participate in this process. Next, we noted a large decrease in the
263 extracellular pseudopaline level in the $\Delta cntI$ mutant strain in comparison with the WT strain,
264 with a concomitant increase in the intracellular space, consistent with a role of PaCntI in
265 pseudopaline export. It is also interesting to note that a $\Delta cntI$ mutant strain is virtually unable
266 to grow in AMS (21). The most probable scenario is that this mutant is deficient in metal
267 content, including zinc, but pseudopaline accumulation in the cytoplasmic space actually
268 worsens the situation by chelating an already poorly available metal. This assumption is
269 supported by our finding that a double $\Delta cntL\Delta cntI$ mutant suppresses the detrimental growth
270 defect of the single $\Delta cntI$ mutant strain, *ie* the absence of pseudopaline restores the normal
271 growth of a mutant devoid of the pseudopaline exporter (Figure S6). A model recapitulating
272 the pseudopaline pathway is shown in Figure 5.

273 It is interesting to note the differences and similarities between staphylopine and pseudopaline
274 and between their respective biosynthetic pathways (Figure S7). On one hand, pseudopaline
275 differs from staphylopine by the incorporation of a L-histidine instead of a D-histidine moiety
276 in staphylopine, thus explaining the absence of amino acid racemase in *P. aeruginosa*.
277 Another particularity of pseudopaline is the use of an α KG moiety instead of pyruvate as
278 substrate for the second reaction mediated by PaCntM. Together this leads to two species-
279 specific metallophores that might give a selective advantage in a competing environment. The
280 fact that staphylopine and pseudopaline belong to Gram-positive and Gram-negative bacteria
281 has important consequences on their respective transport mechanisms across the two types of
282 bacterial envelopes. Although the transporters of staphylopine are well identified, the outer
283 membrane exporter pseudopaline and inner membrane importer of the pseudopaline-metal
284 complex remains to be discovered (Figure 5). Recycling of the metallophore could also take
285 place in *P. aeruginosa*, as recently exemplified in the case for pyoverdine (31). An interesting

286 aspect of this work is the discovery of two different pathways for the export of these
287 nicotianamine-like bacterial metallophores. Whereas *S. aureus* uses a protein belonging to the
288 MFS family (SaCntE) for staphylopine export, *P. aeruginosa* uses a protein belonging to the
289 DMT family of transporters, with PaCntI possessing two predicted EamA domains for
290 pseudopaline export. In the view of their importance in the growth or virulence of these
291 important human pathogens (19, 21), they could emerge as attractive targets for novel
292 antibiotic development.

293

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305

306 **References**

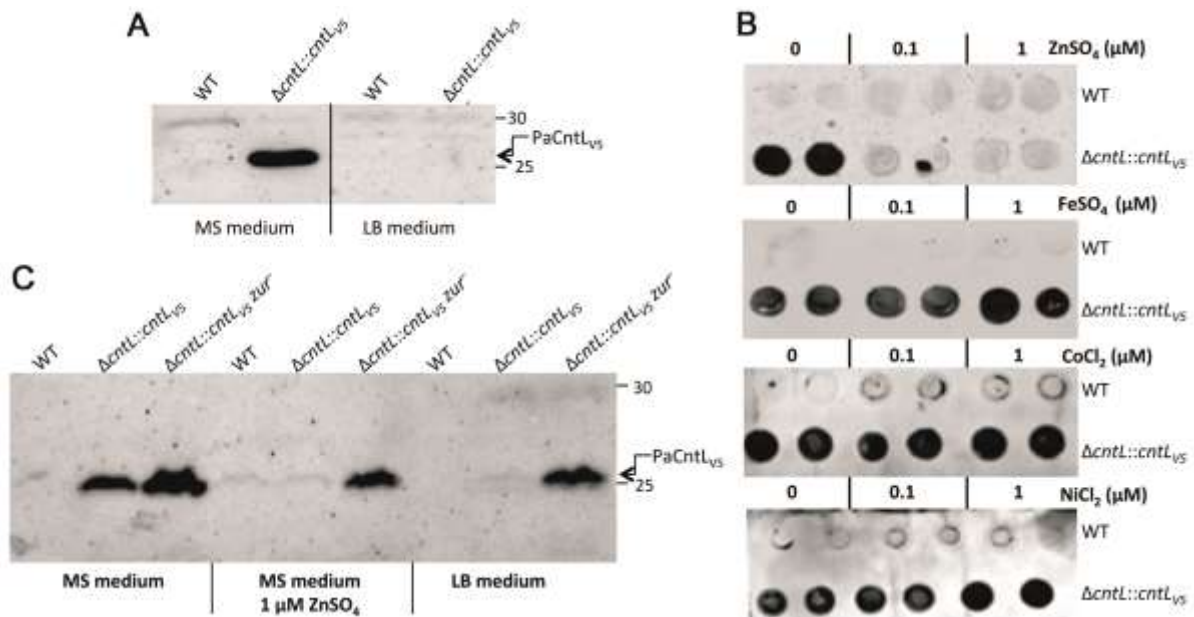
- 307 1. Hood MI, Skaar EP (2012) Nutritional immunity: transition metals at the pathogen–
308 host interface. *Nat Rev Microbiol* 10(8):525–537.
- 309 2. Weinberg ED (1975) Nutritional Immunity: Host’s Attempt to Withhold Iron From
310 Microbial Invaders. *JAMA* 231(1):39–41.
- 311 3. Budzikiewicz AG (2010) Siderophores from bacteria and from fungi. *Iron Uptake and*
312 *Homeostasis in Microorganisms* (Cornelis, P. and Andrews, S. C.), pp 1–16. Caister
313 Academic Press.
- 314 4. Cornelissen CN, Hollander A (2011) TonB-Dependent Transporters Expressed by
315 *Neisseria gonorrhoeae*. *Front Microbiol* 2:117.
- 316 5. Cunrath O, Geoffroy VA, Schalk IJ (2016) Metallome of *Pseudomonas aeruginosa*: a
317 role for siderophores. *Environ Microbiol* 18(10):3258–3267.
- 318 6. Schalk IJ, Hannauer M, Braud A (2011) New roles for bacterial siderophores in metal
319 transport and tolerance. *Environ Microbiol* 13(11):2844–2854.
- 320 7. Minandri F, et al. (2016) Role of Iron Uptake Systems in *Pseudomonas aeruginosa*
321 Virulence and Airway Infection. *Infect Immun* 84(8):2324–2335.
- 322 8. Kim HJ, et al. (2004) Methanobactin, a copper-acquisition compound from methane-
323 oxidizing bacteria. *Science* 305(5690):1612–1615.
- 324 9. Dassama LMK, Kenney GE, Ro SY, Zielazinski EL, Rosenzweig AC (2016)
325 Methanobactin transport machinery. *Proc Natl Acad Sci U S A* 113(46):13027–13032.
- 326 10. Si M, et al. (2017) Manganese scavenging and oxidative stress response mediated by
327 type VI secretion system in *Burkholderia thailandensis*. *Proc Natl Acad Sci U S A*
328 114(11):E2233–E2242.
- 329 11. Cherrier MV, Cavazza C, Bochot C, Lemaire D, Fontecilla-Camps JC (2008)
330 Structural characterization of a putative endogenous metal chelator in the periplasmic nickel
331 transporter NikA. *Biochemistry (Mosc)* 47(38):9937–9943.
- 332 12. Lebrette H, et al. (2015) Novel insights into nickel import in *Staphylococcus aureus*:
333 the positive role of free histidine and structural characterization of a new thiazolidine-type
334 nickel chelator. *Metallomics*. doi:10.1039/C4MT00295D.
- 335 13. Shaik MM, Cendron L, Salamina M, Ruzzene M, Zanotti G (2014) *Helicobacter*
336 *pylori* periplasmic receptor CeuE (HP1561) modulates its nickel affinity via organic
337 metallophores. *Mol Microbiol* 91(4):724–735.
- 338 14. Bobrov AG, et al. (2014) The *Yersinia pestis* Siderophore, Yersiniabactin, and the
339 ZnuABC system both contribute to Zinc acquisition and the development of lethal septicemic

- 340 plague in mice. *Mol Microbiol*. doi:10.1111/mmi.12693.
- 341 15. Bobrov AG, et al. (2017) Zinc transporters YbtX and ZnuABC are required for the
342 virulence of *Yersinia pestis* in bubonic and pneumonic plague in mice. *Met Integr Biometal*
343 *Sci* 9(6):757–772.
- 344 16. Kallifidas D, et al. (2010) The zinc-responsive regulator Zur controls expression of the
345 coelibactin gene cluster in *Streptomyces coelicolor*. *J Bacteriol* 192(2):608–611.
- 346 17. Ghsein G, et al. (2016) Biosynthesis of a broad-spectrum nicotianamine-like
347 metallophore in *Staphylococcus aureus*. *Science* 352(6289):1105–1109.
- 348 18. Remy L, et al. (2013) The *Staphylococcus aureus* Opp1 ABC transporter imports
349 nickel and cobalt in zinc-depleted conditions and contributes to virulence. *Mol Microbiol*
350 87(4):730–743.
- 351 19. Ding Y, Fu Y, Lee JC, Hooper DC (2012) *Staphylococcus aureus* NorD, a Putative
352 Efflux Pump Coregulated with the Opp1 Oligopeptide Permease, Contributes Selectively to
353 Fitness In Vivo. *J Bacteriol* 194(23):6586–6593.
- 354 20. Bielecki P, et al. (2011) In-vivo expression profiling of *Pseudomonas aeruginosa*
355 infections reveals niche-specific and strain-independent transcriptional programs. *PloS One*
356 6(9):e24235.
- 357 21. Gi M, et al. (2015) A novel siderophore system is essential for the growth of
358 *Pseudomonas aeruginosa* in airway mucus. *Sci Rep* 5:14644.
- 359 22. Pederick VG, et al. (2015) ZnuA and zinc homeostasis in *Pseudomonas aeruginosa*.
360 *Sci Rep* 5:13139.
- 361 23. Solovyev, V, Salamov A (2011) Automatic Annotation of Microbial Genomes and
362 Metagenomic Sequences. *Metagenomics and Its Applications in Agriculture, Biomedicine and*
363 *Environmental Studies* (Li, R.W.), pp 61–78. Nova Science Publishers.
- 364 24. Pawlik M-C, et al. (2012) The zinc-responsive regulon of *Neisseria meningitidis*
365 comprises 17 genes under control of a Zur element. *J Bacteriol* 194(23):6594–6603.
- 366 25. Patzer SI, Hantke K (2000) The zinc-responsive regulator Zur and its control of the
367 znu gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. *J Biol Chem*
368 275(32):24321–24332.
- 369 26. Ellison ML, et al. (2013) The transcriptional regulator Np20 is the zinc uptake
370 regulator in *Pseudomonas aeruginosa*. *PloS One* 8(9):e75389.
- 371 27. Thompson J, Donkersloot JA (1992) N-(carboxyalkyl)amino acids: occurrence,
372 synthesis, and functions. *Annu Rev Biochem* 61:517–557.
- 373 28. Barber MF, Elde NC (2015) Buried treasure: evolutionary perspectives on microbial

- 374 iron piracy. *Trends Genet TIG* 31(11):627–636.
- 375 29. Nakashige TG, et al. (2016) The Hexahistidine Motif of Host-Defense Protein Human
376 Calprotectin Contributes to Zinc Withholding and Its Functional Versatility. *J Am Chem Soc*
377 138(37):12243–12251.
- 378 30. Subramanian Vignesh K, Deepe GS (2016) Immunological orchestration of zinc
379 homeostasis: The battle between host mechanisms and pathogen defenses. *Arch Biochem*
380 *Biophys* 611:66–78.
- 381 31. Ganne G, et al. (2017) Iron Release from the Siderophore Pyoverdine in *Pseudomonas*
382 *aeruginosa* Involves Three New Actors: FpvC, FpvG, and FpvH. *ACS Chem Biol*
383 12(4):1056–1065.
- 384 32. Braud A, Hannauer M, Mislin GLA, Schalk IJ (2009) The *Pseudomonas aeruginosa*
385 pyochelin-iron uptake pathway and its metal specificity. *J Bacteriol* 191(11):3517–3525.
- 386 33. Jeong J-Y, et al. (2012) One-step sequence- and ligation-independent cloning as a
387 rapid and versatile cloning method for functional genomics studies. *Appl Environ Microbiol*
388 78(15):5440–5443.
- 389 34. Viarre V, et al. (2009) HxcQ liposecretin is self-piloted to the outer membrane by its
390 N-terminal lipid anchor. *J Biol Chem* 284(49):33815–33823.
- 391 35. Kaniga K, Delor I, Cornelis GR (1991) A wide-host-range suicide vector for
392 improving reverse genetics in gram-negative bacteria: inactivation of the blaA gene of
393 *Yersinia enterocolitica*. *Gene* 109(1):137–141.
- 394 36. Liberati NT, et al. (2006) An ordered, nonredundant library of *Pseudomonas*
395 *aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci U S A* 103(8):2833–
396 2838.
- 397 37. Hoang TT, Kutchma AJ, Becher A, Schweizer HP (2000) Integration-proficient
398 plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of
399 reporter and expression strains. *Plasmid* 43(1):59–72.
- 400 38. Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of
401 plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A*
402 76(4):1648–1652.
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407 **Figures and figure legends**

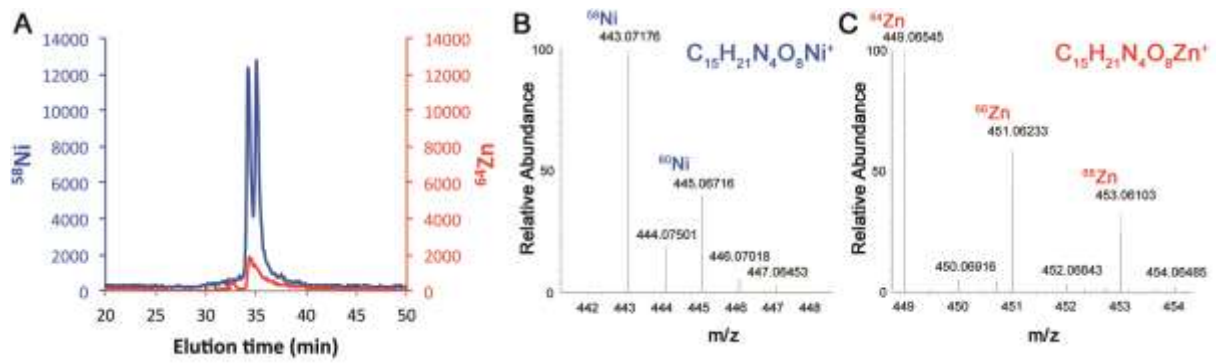


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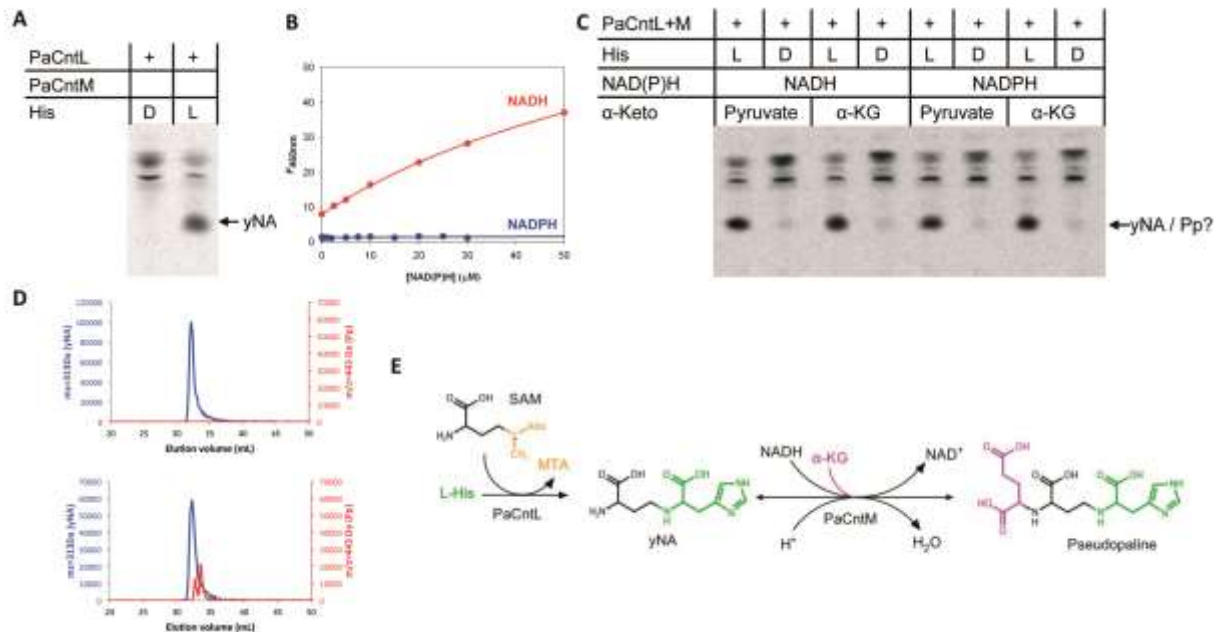
409 Figure 1: PaCntL production under various growth conditions. (A) Immunoblotting using
410 antibody directed against the V5 epitope for revealing PaCntL_{V5} production under poor (MS)
411 and rich (LB) media. (B) Dot-blot revealing the Pa-CntL_{V5} production in MS medium
412 supplemented by divalent metals. (C) Immunoblot detection of PaCntL_{V5} production in PA14
413 WT and Zur deficient strains (*zur*⁻) in various growth conditions.

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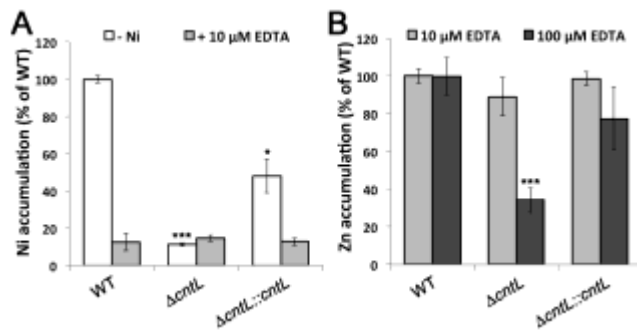


416
417 Figure 2: *In vivo* PaCntL-dependent detection of a nickel or zinc-bound metallophore in the
418 extracellular fraction of *P. aeruginosa*. (A) HILIC/ICP-MS chromatogram of metal-bound
419 metabolites. (B) HILIC-ESI/MS mass spectrum of a Ni-metallophore complex in the
420 extracellular fraction of the WT strain but absent in the $\Delta cntL$ mutant. (C) HILIC-ESI/MS
421 mass spectrum of a Zn-metallophore complex in the extracellular fraction of the WT strain but
422 absent in the $\Delta cntL$ mutant. The empirical molecular formula of the CntL-dependant Ni- or
423 Zn-metallophore complexes were deduced from the exact masse.
424



425
 426 Figure 3: *In vitro* reconstitution of the pseudopaline biosynthesis pathway. (A) TLC
 427 experiment using PaCntL and [¹⁴C]-SAM showing that PaCntL discriminates between D- and
 428 L-histidine substrate with the production of the reaction intermediate (noted yNA) only
 429 visible when using L-histidine. (B) Titration of NADPH (blue) and NADH (red) binding to
 430 PaCntM (5μM) followed by fluorescence resonance energy transfer. Fitting of the data
 431 obtained for NADH led to a K_d of 30μM. (C) TLC separation of reaction products incubating
 432 [¹⁴C]-SAM using purified enzymes (PaCntL and PaCntM), different source of α-ketoacid
 433 (pyruvate or α-KG), cofactor (NADH or NADPH) and histidine (L-His or D-His). (D)
 434 HILIC/ESI-MS chromatograms of putative reaction products using PaCntL incubated with L-
 435 histidine, revealing the production of the yNA intermediate (top), and a mix of PaCntL and
 436 PaCntM incubated with all their putative substrate (SAM, L-histidine, NADH and α-
 437 Ketaoglutarate), revealing the specific detection of pseudopaline in this case (red trace,
 438 bottom). (E) Summary of the PaCntL/M-dependent biosynthesis pathway for the assembly of
 439 pseudopaline from L-his, SAM, NADH and α-KG.

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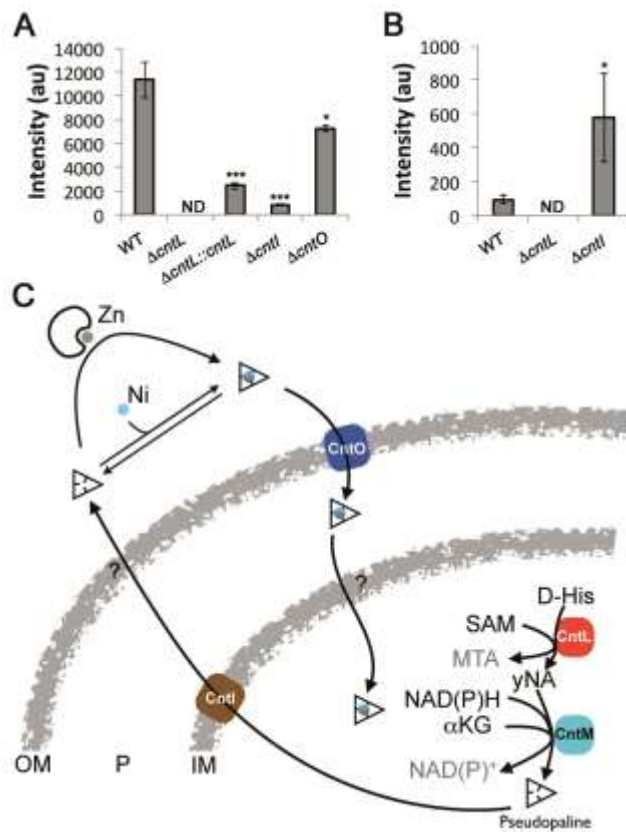


441

442 Figure 4: Pseudopaline is involved in nickel uptake in minimal media and in zinc uptake in
443 chelating media. Intracellular nickel (A) or zinc (B) levels measured by ICP-MS in WT, $\Delta cntL$
444 and $\Delta cntL::cntL$ strains grown in MS medium supplemented or not with 10 or 100 μ M EDTA.
445 Error bars, mean \pm s.d. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as compared to the WT.

446

447



448

449 Figure 5: Model of pseudopaline synthesis, secretion and metal uptake in *P. aeruginosa*. (A)

450 Extracellular detection of pseudopaline in the extracellular fraction of WT and mutant strains.

451 Error bars, mean ± s.d. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as compared to the WT. (B)

452 Intracellular detection of pseudopaline in the intracellular fraction of WT and mutant strains.

453 ND: Not Detectable. (C) Model of pseudopaline production, secretion and recovery of nickel

454 or zinc. Outer membrane (OM), inner membrane (IM), periplasm (P).

455

456

457 **Materials and methods**

458 *Bacterial strains, plasmids and growth conditions*

459 Bacterial strains, vectors and plasmids used in this study are listed in Table S2. *E. coli* strains
460 were grown aerobically with shaking at 37°C in Luria-Broth (LB) with antibiotics as required
461 (50 µg ml⁻¹ ampicillin (Ap), 25 µg ml⁻¹ kanamycin (Kan), 25 µg ml⁻¹ tetracycline (Tc), 15 µg
462 ml⁻¹ gentamicin (Gm), 30 µg ml⁻¹ streptomycin (Sm)). The *E. coli* strains CC118λpir and
463 SM10 were respectively used to propagate pKNG101 derivatives mutator plasmids and Mini-
464 CTX1 plasmids. Recombinant plasmids were introduced in *P. aeruginosa* by triparental
465 mating using pRK2013 and transconjugants selected on *Pseudomonas* isolation agar (PIA,
466 Difco Laboratories) supplemented with antibiotics as required (500 µg ml⁻¹ carbenicillin (Cb),
467 150 µg ml⁻¹ Gm, 2000 µg ml⁻¹ Sm, 200 µg ml⁻¹ Tc). All the *P. aeruginosa* strains used in this
468 study were derivatives of the parental PA14 strain. *P. aeruginosa* strains were grown
469 aerobically with horizontal shaking at 37°C with antibiotics as required (150 µg ml⁻¹ Cb, 50
470 µg ml⁻¹ Gm, 500 µg ml⁻¹ Sm, 50 µg ml⁻¹ Tc). Growths were performed in TSB rich medium
471 (Difco), minimal succinate (MS) medium (32) or chemically defined media (CDM) (18).
472 When specified, Nickel on a NiCl₂ 6H₂O form, or Ethylenediaminetetraacetic acid (EDTA)
473 were added to the media. Growth was monitored by OD600 measurement.

474

475 *Plasmid construction*

476 All plasmids constructed in this study were obtained by the one-step sequence- and ligation-
477 independent cloning (SLIC) method described in ref (33). All PCR primers employed for
478 plasmid construction are listed in Table S3. Genomic DNA was isolated and purified with
479 Pure Link genomic DNA minikit (Invitrogen). PCR reactions for cloning were performed by
480 using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Inc (NEB)) and the
481 products sequenced to verify the absence of any mutation (GATC-biotech).

482

483 *Construction of cntL, cntI, cntO and cntI/L deletion mutant strains of P. aeruginosa*

484 Two DNA fragments corresponding to upstream and downstream regions of *cntL*, *cntI* or *cntO*
485 genes were amplified from PA14 chromosomal DNA with PCR primers SL1/2 & SL3/4 for
486 *cntL*; SL12/13 & SL14/15 for *cntO* and SL19/20 & SL20/21 for *cntI* (Table S3). Upstream
487 and downstream regions were ligated by overlapping PCR and cloned into linearized
488 pKNG101 by the SLIC method. The resulting constructs were transformed into *E. coli*
489 CC118λpir and introduced into *P. aeruginosa* PA14 by conjugation. The strains in which the
490 chromosomal integration event occurred were selected on *Pseudomonas* isolation agar Gm

491 plates. Excision of the plasmid, resulting in the deletion of the chromosomal target gene was
492 performed after selection on Luria-Bertani (LB) plates containing 6% sucrose. Clones that
493 became sucrose resistant and Sm sensitive were confirmed to be deleted for the gene of
494 interest by PCR analysis. The $\Delta cntI \Delta cntL$ double mutant strain was constructed by knocking-
495 out *cntL* in the *cntI* mutant strain.

496

497 *Construction of P. aeruginosa strains with cntL_{V5} and cntL alleles inserted at aat site.*

498 DNA fragments corresponding to the *cnt* promoter region (see sequence Figure S1) and
499 *cntL_{V5}* or *cntL* alleles were generated by PCR from PA14 chromosomal DNA with PCR
500 primers SL7/8 & SL9/10 or SL9/50 (Table S3). Upstream and downstream regions were
501 ligated by overlapping PCR and cloned by the SLIC method in Mini-CTX1 vector. The
502 resulting plasmid was introduced into *P. aeruginosa* PA14WT and PA14 zur^- strains by
503 conjugation. The recombinant clones containing the mini-CTX1 inserted at the *attB* locus on
504 the *P. aeruginosa* genome were selected on tetracycline-containing PIA. Excision of the
505 unwanted plasmid DNA sequences (genes and associated promoter sequences that might
506 interfere with expression of genes cloned into the MCS) located between the *FRT* sites
507 (present on the mini-CTX1) was achieved by expressing Flp recombinase from a conjugative
508 plasmid, pFLP2 which was introduced into *P. aeruginosa* PA14 by conjugation. The *P.*
509 *aeruginosa* PA14 clones containing the pFLP2 were selected on carbenicillin-containing PIA.
510 Finally, selection for pFLP2 deficient strains was done after selection on LB plates containing
511 6% sucrose. Colonies that became sucrose resistant and CB^S have lost the pFLP2 plasmid.

512

513 *RNA isolation and RT-PCR reactions*

514 Total RNA was prepared from *P. aeruginosa* strain PA14 mid-log phase cultures grown in
515 MS medium using the SV Total RNA Isolation System (Promega). Contaminating DNA was
516 removed by digestion with Dnase I (RTS Dnase kit - Ozyme). DNA-free total RNA was then
517 used as a template for reverse transcriptase reactions using the Superscript III reverse
518 transcriptase and random hexamers as described by the manufacturer (Invitrogen). Intragenic
519 regions of *cntO*, *L*, *M* and *I* (see regions 1 to 4 Figure 1) were respectively amplified with
520 primer couples SL42/43, SL44/45, SL46/47, SL48/49. Intergenic regions upstream *cntO*,
521 between *cntO* and *cntL*, between *cntL* and *cntM*, between *cntM* and *cntI* and downstream *cntI*
522 (see regions 5 to 9 figure 1) were respectively amplified with primer couples SL38/39,
523 SL32/33, SL34b/35b, SL36/37, SL40/41.

524

525 *Protein detection by immunoblotting*

526 PA14 Δ *cntL::cntL*_{V5} was grown at 37°C in MS medium or Luria-Broth (LB) medium. When
527 optical density at 600 nm (OD₆₀₀) reached 0.4 to 0.6 a volume of culture equivalent to 2
528 OD₆₀₀ units was centrifuged for 2 min at 2.000 g. The pellet corresponding to whole cells was
529 resuspended in 1X SDS-PAGE loading buffer containing β -mercaptoethanol and heated for
530 10 min at 95 °C. Proteins samples equivalent 0.1 OD₆₀₀ units were separated by SDS-PAGE.
531 Electrophoresis was performed using 12% SDS-polyacrylamide gel at room temperature and
532 25 mA/gel. Immunoblotting was performed as previously described (34), with rabbit primary
533 and peroxidase-conjugated secondary antibodies respectively directed against V5 epitope
534 (Bethyl/interchim) (dilution 1:5,000) and rabbit IgG (Sigma) (dilution 1:25000). The
535 peroxidase reaction was developed by chemiluminescence (Pierce), scanned and analyzed
536 with ImageQuant LAS 4000 camera and TL analysis software (GE Healthcare Life sciences).

537

538 *Protein detection by dot blot*

539 A nitrocellulose membrane was incubated 5 min with transfer buffer and dried at room
540 temperature for 3 min. A 5 μ L drop of SDS-PAGE protein sample (equivalent to 0.5 OD₆₀₀
541 units) was loaded onto nitrocellulose membranes. After drying, the proteins were transferred
542 on the nitrocellulose for 5min at 80V and 0.1A using a Fast Blotter System (Pierce).
543 Immunoblotting was performed with SNAP i.d.[®] 2.0 Protein Detection System. The membrane
544 was blocked 5 min in TBS (1X) Tween 20 (0.1%) skim milk (0.5%), washed 4 times with
545 TBS-Tween, incubated 10 min in TBS-Tween-milk with primary rabbit antibody directed
546 against the V5 epitope (dilution 1:5,000), washed 4 times with TBS-Tween, incubated 10 min
547 in TBS-Tween-milk with anti-rabbit peroxidase-conjugated antibody and the peroxidase
548 reaction was developed by chemiluminescence, scanned with ImageQuant LAS 4000 camera
549 and analyzed by the ImageQuant TL analysis software.

550

551 *Viability assay on plate*

552 Pre-culture of PA14 strains were performed overnight in MS medium under horizontal
553 shaking at 37°C. The next day, a culture of fresh MS medium is inoculated by the pre-culture
554 at OD₆₀₀ of 0.1 and incubated for 6 hours under horizontal shaking at 37°C. Cultures were
555 then adjusted to OD₆₀₀ of 1 and subjected to 10% serial dilutions in fresh MS medium. 10 μ l
556 of culture samples were spotted on MS 5% agar plate and incubated overnight at 37°C.

557

558 *Sample preparation for analysis by HILIC/ICP-MS, HILIC/ESI-MS and determination of*
559 *metal concentrations by ICP-MS*

560 All the *P. aeruginosa* strains derivatives of the parental PA14 strain were grown aerobically
561 with horizontal shaking at 37°C in freshly made Minimal Succinate (MS) medium. All media
562 used in this study were filtered at 0.22µm with polycarbonate units before used, and stored at
563 4°C away from light in polycarbonate bottles. Pre-culture of 20mL were inoculated from fresh
564 MS-5% agar plates and grown until late exponential phase in polycarbonate erlenmeyers.
565 Culture of 25mL were inoculated at OD=0.1 and grown for 8h in freshly made MS or CDM
566 media supplemented or not with EDTA or nickel before inoculation. After 8h, OD₆₀₀ were
567 measured before cells were harvested by centrifugation (2,000g, 30min, 4°C). The
568 supernatant was collected, filtered and stored at -80°C. The pellet was washed twice with
569 1.3mL MS media + 1mM EDTA followed by a wash with 1.3mL MS media. OD₆₀₀ were
570 measured, and cells ruptured by successive sonication cycles. The lysates were then
571 centrifuged at 16,000g for 30min at 4°C and supernatants were collected and stored at -80°C.
572 These cell lysate and supernatant fractions were used for analysis of metal complexes using
573 HILIC/ICP-MS and HILIC/ESI-MS as described below. For metal quantitation, growth of
574 WT and mutant strains was done as described above. After 9h of growth, OD₆₀₀ was
575 measured before cells were harvested by centrifugation (2,000g, 30min, 4°C). The pellet was
576 washed 2 times with 1.3mL MS media + 1mM EDTA followed by a wash with 1.3mL MS
577 media. After the OD₆₀₀ was measured, cells were dried overnight at 95°C. The metal
578 quantification was determined by inductively coupled plasma mass spectrometry as described
579 elsewhere (17).

580

581 *Analysis of metal complexes using HILIC/ICP-MS and HILIC/ESI-MS*

582 Microbore HILIC separations were performed using an Agilent 1100 capillary HPLC system
583 (Agilent, Tokyo, Japan) coupled either to ICP-MS detection (7500cs instrument, Agilent) or
584 to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).
585 The column used for HILIC separation was a TSK gel amide 80 (250 mm × 1 mm i.d., 5 µm)
586 from Tosoh Biosciences (Stuttgart, Germany). Gradient elution, at a flow rate of 50 µl min⁻¹,
587 was carried out using eluent A, acetonitrile, and eluent B, 5 mM ammonium formate (pH 5.5).
588 Samples were diluted with acetonitrile and water to obtain a 1:2, sample to acetonitrile ratio,
589 and centrifuged. A 7 µl aliquot of the supernatant was injected into the HILIC column each
590 time. To get accurate masses during HILIC/ESI MS analysis, MS and MS/MS spectra were
591 recalibrated offline using precursor/fragment ions with known formula. Putative metal species

592 were fragmented during a subsequent chromatographic run with collision induced dissociation
593 (CID) mode at 35% energy. Signals were recorded at m/z corresponding to pseudopaline
594 complexes with Ni and Zn ($C_{15}H_{20}N_4O_8Ni^+$ and $C_{15}H_{20}N_4O_8Zn^+$ respectively). Detailed
595 procedures for chromatographic and MS analyses were described elsewhere (17).

596

597 *Protein cloning, expression and purification*

598 The *cntL* gene of *P. aeruginosa* P14 strain was amplified from genomic DNA and cloned in
599 the vector pET22b⁺ using the NdeI and XhoI restriction sites. This lead to a protein fused with
600 a C-Terminal His₆ tag. The *cntM* gene of *P. aeruginosa* P14 strain was amplified from
601 genomic DNA and cloned in the vector pET-TEV using the NdeI and XhoI restriction sites.
602 This lead to a protein fused with a N-Terminal His₆ tag that could eventually be cleaved by
603 TEV protease. Both vectors were transformed into *E. coli* BL21 strains for protein expression.
604 The strain carrying the pET22b⁺*cntL* plasmid were allowed to grow in LB medium containing
605 100 µg/ml ampicilline to an OD₆₀₀ of 0.7 before inducing expression with 1mM Isopropyl-β-
606 D-thiogalactopyranoside (IPTG) followed by 20 hours incubation at 16°C. The strain carrying
607 the pET-TEV*cntM* plasmid were allowed to grow in autoinducible medium containing 100
608 µg/ml ampicilline for overnight growth at 37°C. In both cases, the cells were pelleted,
609 resuspended in buffer A (50 mM phosphate, 450 mM NaCl, 20 mM imidazole, pH 8.0), and
610 disrupted with a French Press at 7 Mpa. The resulting soluble fraction was loaded on a Nickel
611 charged column (HisTrap column, GE Healthcare) and the protein was eluted by an imidazole
612 step gradient (50 mM wash and 200 mM elution). Gel filtration experiments were done using
613 a Hiload 26/60 superdex200 column (GE Healthcare) using buffer B (50mM HEPES, 50mM
614 NaCl, pH7.0)

615

616 *Fluorimetry and TLC experiments*

617 Fluorescence resonance energy transfer (FRET) experiments were performed using a Varian
618 Cary Eclipse spectrofluorimeter with an excitation wavelength of 280 nm (tryptophan
619 excitation, emission at 340 nm that is transferred to the NAD(P)H and recording of the
620 emission at 460nm. Ligand binding was determined from the partial enhancement of this
621 fluorescence emission. The amplitude of the FRET was fitted with a simple binding model
622 using SigmaPlot software. TLC experiments were done as described elsewhere (17).

623