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1 **Involvement of the *Pseudomonas aeruginosa* MexAB-OprM efflux**
2 **pump in the secretion of the metallophore pseudopaline**

3

4 Running title: MexAB-dependent pseudopaline secretion

5

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24 **ABSTRACT**

25 The ability for all organisms to acquire metals from their environment is essential for life. To
26 overcome the metal restriction imposed by the host's nutritional immunity, bacterial
27 pathogens exploits the use of small high metal affinity molecules called metallophores.
28 Metallophores are first synthesized in the cytoplasm, then secreted into the medium where
29 they sequester the metal. The metal-metallophore complex is then imported into the bacterium
30 following binding to dedicated cell surface receptors. Recently, a new family of
31 metallophores has been discovered in pathogenic bacteria called staphylopine in
32 *Staphylococcus aureus* and pseudopaline in *Pseudomonas aeruginosa*. Here, we are
33 expanding the molecular understanding of pseudopaline secretion/recovery cycle across the
34 double-membraned envelope of the Gram-negative bacterium *Pseudomonas aeruginosa*. We
35 first revealed that pseudopaline is secreted in a two-step process including export across the
36 inner membrane by the CntI exporter followed by a specific transport across the outer
37 membrane by the MexAB-OprM efflux pump. Such involvement of MexAB-OprM in
38 pseudopaline secretion, reveal a new natural function that extends its spectrum of functions
39 and therefore reasserts its interest as antibacterial target. We then addressed the fate of the
40 recovered metal-loaded pseudopaline by combining *in vitro* reconstitution experiments using
41 radio-labeled pseudopaline subjected to bacterial lysates, and *in vivo* phenotyping in absence
42 of pseudopaline transporters. Our data support the existence of a pseudopaline
43 degradation/modification mechanism, possibly involved in metal release following
44 pseudopaline recovery. All together our data allowed us to provide an improved molecular
45 model of secretion, recovery and fate of this important metallophore by *P. aeruginosa*.

46

47 **Keywords:** Metallophore; pseudopaline; *Pseudomonas aeruginosa*; efflux pump; MexAB-
48 OprM

49 **IMPORTANCE**

50 Pseudopaline is a broad spectrum metallophore produced and used by *Pseudomonas*
51 *aeruginosa* to supply the bacterium in metal in metal scarce environments. Here we are
52 investigating the pseudopaline transport/recovery cycle across the bacterial envelope. We are
53 first demonstrating that pseudopaline secretion in the medium is achieved by a specific efflux
54 pump, usually dedicated to the release of toxic compounds such as antibiotics, thus revealing
55 a new natural function for this efflux pump reasserting its interest as antibacterial target.
56 Additional experiments also revealing the existence of an intracellular pseudopaline
57 degradation mechanism providing new clues to another obscure step of the pseudopaline
58 cycle which is the intracellular metal liberation from the imported metal-pseudopaline
59 complex. All together our data allowed us to disclose important aspects of the secretion,
60 recovery and fate of this essential molecule used by *P. aeruginosa* to survive during infections
61 thus constituting new potential targets for antibacterial development.

62 INTRODUCTION

63 The availability of a diversity of metals is essential to support a variety of essential biological
64 functions and bacteria have evolved several mechanisms for their acquisition from
65 environmental sources. For this purpose, bacteria deploy multiple systems that govern both
66 influx and efflux of transition metals allowing cells to maintain a meticulously regulated pool
67 of metals in any circumstances. This is particularly relevant for pathogenic bacteria within a
68 host, where the concentration of free metals is kept extremely low by the host's nutritional
69 immunity framework, which has very likely evolved to limit the growth of microorganisms in
70 infected tissues (1, 2). Under such metal scarce conditions, the most common strategy used by
71 pathogenic bacteria to import metals involves the synthesis and release of metallophores
72 followed by the capture and import of the metal-metallophore complex (3).

73 *Pseudomonas aeruginosa* is a versatile opportunistic human pathogen, well equipped for
74 thriving in particularly metal limited environments (4, 5). Indeed, this pathogen is able to
75 survive under strong metal scarcity due to its ability to produce various metallophores. To
76 date, our knowledge of the metallophore-mediated metal uptake capabilities of *P. aeruginosa*
77 has been limited to two iron-specific siderophores, pyochelin and pyoverdine (6) and a
78 recently discovered broad-spectrum metallophore named pseudopaline (7). Pseudopaline is
79 distantly related to plant nicotianamine and belongs to the same opine-type broad-spectrum
80 metallophore family as staphylopine and yersinopine synthesized by *Staphylococcus aureus*
81 and *Yersinia pestis*, respectively (8-10). The *cntOLMI* genes of *P. aeruginosa* encode for the
82 proteins involved in pseudopaline biosynthesis and transport (7). They all cluster together in a
83 single operon negatively regulated by zinc through the Zur repressor (7, 11). The first gene of
84 the operon, *cntO* encodes for the OM component CntO, belonging to the TonB-Dependent
85 Transporter (TBDT) family dedicated to pseudopaline recovery from the environment (7, 12,
86 13). The second and third genes of the operon, respectively *cntL* and *cntM*, encode the two

87 cytoplasmic soluble enzymes CntL and CntM responsible for pseudopaline biosynthesis in a
88 two steps process. The first step, in which CntL produces the reaction intermediate yNA using
89 S-adenosine methionine (SAM) and L-histidine as substrates, is followed by a NADH
90 reductive condensation of the yNA intermediate with a molecule of α -ketoglutarate (α KG),
91 catalyzed by CntM to produce pseudopaline (7). The fourth gene of the *cnt* operon, *cntI*,
92 encodes the IM protein CntI belonging to the EamA or drug/metabolite transporter (DMT)
93 family involved in pseudopaline export from the cytoplasm to the periplasm after biosynthesis
94 (7).

95 CntI and CntO were shown to be essential for *P. aeruginosa* growth in Airway Mucus
96 Secretion (AMS) (4) and *P. aeruginosa*'s capacity to colonize mice (12) respectively.
97 Moreover, several transcriptomic analyses showed that *cnt* genes are up-regulated during lung
98 infection (14, 15). During chronic infection, polymorphisms within the promoter sequence of
99 the *cnt* operon were reported, leading to an altered Zur binding site and a less stringent
100 repression (16). Altogether, these pieces of information suggest that the involvement of the
101 Cnt machinery in zinc uptake in a scarce and chelated environment is crucial for *P.*
102 *aeruginosa* survival during lung infections of cystic fibrosis patients.

103 Nonetheless, the complete pseudopaline export and recovery pathway remains to be fully
104 elucidated. In particular it remains unknown how pseudopaline crosses the OM to be released
105 in the extracellular milieu. Several other metallophores rely on efflux pumps for their
106 secretion through the OM including the pyoverdine with PvdRT-OpmQ in *P. aeruginosa* (17,
107 18) or enterobactin with the modular AcrAB-, AcrAD-, and MdtBC-TolC RND efflux pump
108 in *Escherichia coli* (19).

109 In this study we are completing the understanding of the pseudopaline secretion pathway by
110 demonstrating that, following its export across the IM by CntI, its translocation across the OM
111 is accomplished by the tripartite RND efflux pumps MexAB-OprM, thus revealing the

112 existence of a two-step pseudopaline secretion system in *P. aeruginosa*. We have also
113 observed that the impaired secretion of pseudopaline in absence of its dedicated efflux pump
114 does not lead to periplasmic accumulation of pseudopaline in contrast to the cytoplasmic
115 accumulation observed in absence of IM exporter. We are providing experimental evidence
116 supporting the existence of a pseudopaline modification mechanism in the periplasm. This
117 unidentified pseudopaline modification mechanism may also be involved in physiological
118 pseudopaline alteration, leading to the release of the cognate metal following its recovery
119 from the extracellular medium by CntO.

120

121 **RESULTS**

122 **Pseudopaline is required for normal growth, biofilm formation and full extracellular** 123 **protease activity in minimal chelated media**

124 Pseudopaline is essential for *P. aeruginosa* growth in Airway Mucus Secretion (AMS) and
125 provides an advantage for mice colonization (4, 12). Several transcriptomic approaches
126 showed that the pseudopaline biosynthesis and transport machinery, encoded by the *cnt*
127 operon, is upregulated by *P. aeruginosa* in the lungs of Cystic Fibrosis (CF) patients (14, 15).
128 Similarly, growth in a minimal chelated medium (minimal succinate medium supplemented
129 with 100 μ M EDTA, MCM) induces a significant expression of the *cnt* operon compared to
130 the LB nutrient rich medium (figure S1). We previously showed that pseudopaline was
131 required for zinc uptake in MCM (7). Altogether, these pieces of information indicate that
132 during infection and growth in MCM, *P. aeruginosa* is starved for Zn, further validating
133 MCM as a medium mimicking the host Zn shortage condition. In order to evaluate the
134 implication of pseudopaline in physiological functions relevant for *P. aeruginosa*'s
135 pathogenesis, we compared different fitness parameters between a wild type and a $\Delta cntL$
136 strain, deficient for pseudopaline biosynthesis, during growth in MCM. We have observed

137 that both generation time and biofilm formation capacity are significantly affected in the
138 $\Delta cntL$ strain in comparison with the wild type (figure 1). We therefore suggest that the
139 pseudopaline-dependent fitness phenotypes observed in MCM are also occurring during
140 infection and therefore contribute to the pseudopaline requirement during infection. In
141 addition to fitness deficiencies, we also observed a significant reduction in the global
142 extracellular protease activity of the pseudopaline deficient strain (figure 1C). Taking into
143 account that around 6% of bacterial proteomes includes Zn metalloenzymes (20, 21), involved
144 in various cellular processes including translation, general metabolism or extracellular
145 protease activities, we consider that the observed phenotypes are due to an impairment of Zn
146 containing extracellular proteases. As a whole, these data indicate that several
147 metalloenzymes important for infections rely on pseudopaline-mediated Zn uptake and justify
148 the necessity of this metal uptake for *P. aeruginosa*'s fitness during lung infections occurring
149 in cystic fibrosis patients. Therefore, any new additional information on the molecular
150 mechanisms underlying this metal uptake pathway would be useful to understand it better and
151 envisage its inactivation.

152

153 **Pseudopaline export across the bacterial OM is mediated by the MexAB-OprM efflux** 154 **pump**

155 We demonstrated elsewhere that pseudopaline was secreted in the medium during bacterial
156 growth in MCM (7). While pseudopaline is exported across the IM by CntI, the machinery
157 involved in its export across the OM remains unknown. In a preliminary search for
158 pseudopaline OM export candidates, we selected four relevant RND efflux pumps of *P.*
159 *aeruginosa* which are known to accommodate a wide array of structurally unrelated
160 antibacterial molecules, namely MexAB-OprM, MexCD-OprJ, MexEF-OprN and

161 MexXY/OprM (22). To test the possible involvement of the four selected efflux pumps in the
162 pseudopaline secretion, we measured and compared the extracellular pseudopaline levels in a
163 set of PA14 cultures including a quadruple mutant strain lacking the four MexAB, CD, EF
164 and XY RND components, called PA14 Δ RNDs and additional control strains deficient in
165 pseudopaline synthesis (PA14 Δ cntL) or IM export (PA14 Δ cntI) (figure 2). The strains were
166 grown in MCM and pseudopaline content was measured by hydrophilic interaction liquid
167 chromatography (HILIC) – electrospray ionization mass spectrometry (ESI MS) in the culture
168 supernatant. As already reported by Lhospice *et al.*, (7), the PA14 Δ cntL strain lacking the
169 CntL enzyme shows no detectable pseudopaline in the supernatant while the PA14 Δ cntI strain
170 lacking the pseudopaline IM exporter CntI displays a substantial 90% decrease of the
171 extracellular pseudopaline content (figure 2). Remarkably, the quadruple PA14 Δ RNDs mutant
172 strain displays a similar major reduction of the extracellular pseudopaline content, thus,
173 indicating a similar extent of impairment in pseudopaline secretion in absence of the four
174 RND efflux pumps. Therefore, we conclude that at least one of the four RND components
175 selected is involved in pseudopaline translocation through the OM. We then focused our
176 investigations on MexAB-OprM since it is the major and the most ubiquitous, constitutively
177 expressed, RND efflux pump of *P. aeruginosa*. We first tested the involvement of the IM
178 module of the pump, the MexAB RND component, by measuring extracellular pseudopaline
179 content in the corresponding mexAB mutant strain (PA14 Δ mexAB). This strain showed a
180 similar reduced pseudopaline content to the PA14 Δ cntI and PA14 Δ RNDs strains, indicating
181 that the MexAB RND component of the tripartite efflux pump MexAB-OprM is required for
182 pseudopaline secretion. To confirm the involvement of this RND efflux pump in pseudopaline
183 secretion, we tested the contribution of its OM TolC-like component, OprM to the
184 extracellular pseudopaline recovery. Data presented figure 2 indicate that, in absence of
185 OprM, pseudopaline secretion is impaired to the same extend than in the MexAB deficient

186 strain. The similar impairment in pseudopaline secretion independently observed in strains
187 lacking MexAB or OprM, known to form the tripartite MexAB-OprM RND efflux pump,
188 revealed the exclusive involvement of this pump in pseudopaline secretion across the OM.

189

190 **The absence of pseudopaline OM exporter does not trigger intracellular pseudopaline**
191 **accumulation.**

192 Pseudopaline is synthesized in the cytoplasm and then exported across the IM into the
193 periplasm through CntI. Data presented in figure 2 show unambiguously that pseudopaline is
194 taken in charge in the periplasm by the MexAB-OprM tripartite RND efflux pump to
195 complete its secretion to the extracellular milieu, therefore constituting the second step of the
196 secretion process. As already noticed earlier and further confirmed by this study, the
197 intracellular pseudopaline measurement in absence of its IM exporter CntI revealed an
198 important cytoplasmic accumulation, which is associated with a toxic phenotype (Figure 3).
199 We showed that this phenotype was a consequence of pseudopaline accumulation since it is
200 suppressed in the double *cntL/cntI* mutant which does not produce pseudopaline (figure 3B).
201 In contrast, when pseudopaline OM export is impaired in strains lacking the MexAB or the
202 OprM component of the MexAB-OprM RND efflux pump involved in this process, we do not
203 detect any cellular accumulation (figure 3A) and did not observe any adverse effect on
204 bacterial growth. (figure 3B). Consequently, the combined secretion defect of a MexAB-
205 OprM deficient strain without periplasmic accumulation suggests that the periplasm is a
206 compartment which cannot accommodate pseudopaline accumulation. Two hypotheses
207 therefore arise: (i) either there is a regulative effect with a negative feedback on pseudopaline
208 synthesis or (ii) it exists a specific periplasmic machinery preventing pseudopaline
209 accumulation in this compartment.

210

211 **Absence of pseudopaline OM exporter does not affect *cnt* gene's expressions**

212 The simultaneous decrease of the pseudopaline content in the supernatant of a PA14 Δ mexAB
213 strain without a concomitant rise of pseudopaline's periplasmic content (figures 2 & 3) raises
214 the question of pseudopaline fate when it is not released in the milieu from the periplasm. In
215 order to explore the hypothesis of a regulation mechanism of the *cnt* operon when RND efflux
216 pumps are impaired, we examined the transcription level of the *cnt* operon in the different
217 genetic backgrounds (figure 4). We used qRT-PCR to measure the transcriptional level of
218 *cntO* and *cntM* genes in the PA14, PA14 Δ *cntI*, PA14 Δ mexAB and PA14 Δ RNDs strains in
219 MCM, a growth medium previously shown to induce pseudopaline production and secretion
220 (figure S1). We found that the expressions of *cntO* and *cntM* genes of the *cnt* operon are not
221 affected in the *cntI* mutant or in any RND efflux pump deletion combinations in comparison
222 with the wild type. This allowed us to rule out the possibility of a negative feedback on the *cnt*
223 genes expression when pseudopaline secretion is impaired. We therefore conclude that the
224 pseudopaline production level is not affected by the deletion of the genes involved in its
225 export across the bacterial envelope and the lack of extracellular pseudopaline observed in
226 absence of MexAB-OprM is truly due to a secretion defect.

227

228 **Evidence of an intracellular pseudopaline modification mechanism**

229 Since we could not detect periplasmic accumulation of pseudopaline when its export across
230 the OM was impaired, we hypothesized that the non-secreted pseudopaline is either degraded
231 or modified in the periplasm by a dedicated mechanism. To check this hypothesis, we took
232 advantage of the reconstituted *in vitro* pseudopaline biosynthesis pathway previously
233 developed (7) to follow the fate of radiolabeled pseudopaline under various conditions (figure

234 5). We used thin layer chromatography (TLC) to separate and visualize the radiolabeled
235 pathway intermediate yNA and pseudopaline when the radiolabeled substrate ^{14}C S-adenosyl-
236 methionine (SAM) is incubated with defined substrate and enzymes (figure 5A top). We then
237 followed by TLC the integrity of the *in vitro* synthesized radiolabeled pseudopaline upon
238 incubation with various concentrations of PA14 cell extracts generated under *cnt* induction
239 conditions. Data presented figure 5A reveal that the amount of radiolabeled pseudopaline
240 gradually decreased upon increasing amount of PA14 lysates, with a doubling of the band
241 corresponding to pseudopaline (figures 5A lines 4 to 6 and 5B lines 4 & 6), which reflects the
242 appearance of a modified form of pseudopaline. This suggests the existence of a pseudopaline
243 modification mechanism in the *P. aeruginosa* cellular soluble extracts. As a specificity
244 control, no degradation was observed when *E. coli* cell lysates are added to the reaction in the
245 same proportions (figures 5A lines 7 to 9 and 5B lines 8 & 9). Since pseudopaline
246 accumulates in the cytoplasm in absence of IM exporter but does not accumulate in the
247 periplasm in absence of OM exporter, our observation implies that the highlighted
248 modification mechanism is restricted to the periplasmic compartment. Interestingly, the fact
249 that pseudopaline modification is observed with both wild type PA14 and the PA14 Δ *mexAB*
250 lysates (figure 5B lines 4, 5 and 6, 7) suggests that this modification mechanism is
251 constitutive rather specifically induced upon pseudopaline accumulation in absence of its OM
252 exporter. We therefore propose that this pseudopaline modification process also occurs under
253 physiological conditions and is used for metal recovery from the pseudopaline-metal complex
254 imported *via* the CntO outer membrane importer (see model, figure 6).

255

256 **A model of pseudopaline secretion and recovery by *P. aeruginosa***

257 We described recently the metallophore pseudopaline, secreted by *P. aeruginosa* and involved
258 in zinc uptake under metal limiting conditions (7). Owing to the data reported in this study on
259 pseudopaline secretion and fate after recovery, we are now able to provide a detailed model of
260 secretion and recovery of this important metallophore by *P. aeruginosa* (figure 6). In this
261 updated model, following the synthesis of pseudopaline in the cytoplasm by the CntL and
262 CntM enzymes, the metallophore is then translocated into the periplasm through the CntI
263 exporter. As demonstrated in this study, the periplasmic pseudopaline uses the MexAB-OprM
264 RND efflux pump for the final release in the extracellular milieu. MexAB-OprM therefore
265 constitutes, together with CntI, a two-step secretion system that exports pseudopaline from the
266 site of its synthesis across the IM, periplasm and OM. Our data reveal the involvement of the
267 MexAB-OprM efflux pump in the secretion of a natural component made by the bacteria that
268 may constitute one of its primary substrates. In the extracellular medium, pseudopaline binds
269 Zn and Ni. It is then recognized by the OM TonB-dependent receptor CntO which allows its
270 import into the periplasm. Additional results generated in this study strongly suggest the
271 existence of a pseudopaline modification system involved in periplasmic pseudopaline
272 homeostasis and possibly in metal liberation. The free metal will then be available to bacterial
273 metalloproteins following its import into the cytoplasm by general importers such as ZnuA in
274 the case of zinc (11)

275

276 **DISCUSSION**

277 Pseudopaline is a metallophore synthesized and used by the opportunistic bacterial pathogen
278 *P. aeruginosa* to recover metals from metal scarce environments. This metallophore plays an
279 important role in bacterial survival during infection likely resulting from the lack of bio-
280 available metals in the host. The pseudopaline metal uptake pathway utilizes a complex

281 secretion and recovery cycle of the metallophore across the Gram-negative bacterial envelope.
282 We showed previously that pseudopaline export across the inner membrane and import across
283 the outer membrane are mediated by the CntI exporter and the CntO TBDT, respectively, both
284 encoded on the pseudopaline *cnt* operon together with the *cntL* and *cntM* biosynthetic genes.
285 In this study, we are extending our previous characterization of the pseudopaline cycle by
286 revealing the involvement of an RND efflux pump in this process. Our data demonstrate that
287 the MexAB-OprM efflux pump is responsible for the final secretion step of the metallophore
288 in the extracellular medium therefore filling an important gap in the pseudopaline cycle.

289 In the molecular mechanisms underlying pseudopaline secretion, MexAB-OprM forms
290 together with CntI an unprecedented two-step secretion system that drives pseudopaline from
291 the cytoplasm to the extracellular medium. The involvement of an efflux pump in the cellular
292 release of a metallophore has already been reported but in combination with different IM
293 exporters. The *E.coli* enterobactin is exported across the cytoplasmic membrane through the
294 IM major-facilitator transporter EntS (23) and then exported across the OM from the
295 periplasm through the OM channel TolC, an ortholog of the *P. aeruginosa* OprM (19).
296 Another example was reported in the closely related organism *Salmonella enterica* Sv
297 Typhimurium where the salmochelin is exported to the medium through the IroC-TolC
298 complex (24). This is also the case in *P. aeruginosa* where the siderophore pyoverdine is
299 secreted through the PvdRT-OpmQ efflux pump (17, 18). Here we unravel an unprecedented
300 combination involving an IM exporter belonging to the EamA or drug/metabolite transporter
301 (DMT) family and the MexAB-OprM RND efflux pump and, therefore, extend the spectrum
302 of efflux pump-associated two-step secretion systems involved in metallophores across Gram-
303 negative envelopes. On the basis of structural and functional studies, it was previously
304 described that the RND protein AcrB, a close homolog of the MexB protein, is a homotrimer
305 acting as a peristaltic pump, able to load its substrate in the periplasmic compartment before

306 secretion across the OM through the TolC channel (25). However, whether efflux pump-
307 dependent metallophores including pseudopaline are first exported to the periplasm, remains
308 to be determined.

309 So far, MexAB-OprM efflux functions have mostly been dedicated to the release of
310 exogenous toxic compounds such as antibiotics as well as a series of amphiphilic molecules,
311 disinfectants, dyes, solvents or detergents (26). Additional data indicate that MexAB-OprM
312 efflux pump is also releasing non-permeable autoinducers involved in bacterial quorum
313 sensing (27, 28). The present study highlights an additional function for the MexAB-OprM
314 efflux pump in endogenous metallophore secretion. Such involvement of MexAB-OprM in
315 the secretion of important small natural compounds has already been indirectly suggested
316 following the observation that a *mexAB* mutant is unable to kill mice that were deficient in
317 leucocytes, whereas the parent strain caused a fatal infection (29). The finding that MexAB-
318 OprM is essential for pseudopaline secretion, itself necessary to support *P. aeruginosa* growth
319 in airway mucus secretions (4) provide a possible additional explanation for the MexAB-
320 OprM requirement in virulence.

321 While at least 12 different functional RND efflux pumps have been described in *P.*
322 *aeruginosa*, our data reveal a specific and exclusive recognition of pseudopaline by MexAB-
323 OprM. In a recent study, Tsutsumi *et al.* revealed the structure of the complete MexAB-OprM
324 complex in the presence or absence of drugs at near-atomic resolution and proposed precise
325 mechanisms for substrate recognition and efflux supporting such specificity (25). In addition,
326 Ramaswany *et al.* recently performed an exhaustive atomic-level comparison of the main
327 putative effector recognition sites in the two RND modules MexB and MexY (30). They
328 pointed out specific signatures that may constitute selectivity filters such as mosaic-like
329 lipophilic and electrostatic surfaces of the binding pockets of MexB and MexY proteins
330 providing multifunctional binding sites for diverse substrates. Further structure function

331 investigations are necessary to understand MexB specificity toward pseudopaline. The
332 question, whether the natural endogenous compound pseudopaline is recognized and exported
333 in a same manner as the exogenous compounds requires further investigations.

334 In contrast to pyoverdine (17, 18) and enterobactin (31), we observe neither subsequent
335 accumulation, nor toxicity when pseudopaline export across the OM is impaired. We are
336 providing experimental data suggesting that the absence of pseudopaline accumulation in
337 these conditions is due to pseudopaline modification by a dedicated system specific to *P.*
338 *aeruginosa*. The presence and activity of such a pseudopaline modification mechanism in
339 other than abnormal pseudopaline periplasmic accumulation, suggests a function for this
340 machinery under physiological conditions in the wild type bacteria. The absence of encoded
341 specific machinery in the *cnt* operon to import the pseudopaline-metal complex through the
342 IM into the cytoplasm (as found for example in the *cnt* operon in *S. aureus* and *Y. pestis* (8))
343 is in line with the hypothesis of metal release from pseudopaline in the periplasm. We
344 therefore propose that the pseudopaline-mediated metal import pathway ends in the
345 periplasm, where the free metal will be distributed to various metalloproteases such as
346 exoproteases that fold in the periplasm before secretion in the medium by the type II secretion
347 system (32) or cytoplasmic metalloenzymes after metal transport through pseudopaline-
348 independent importers such as Znu ABC transporter described for zinc import (11). Further
349 experiments, out of the scope of this study, will be necessary to identify the determinants
350 underlying the pseudopaline's modification mechanism.

351 In the case of pyoverdine, it has been shown that it can bind and import in the periplasm a
352 large array of metals with different affinities, (33). In the same study, the authors
353 demonstrated that the PvdRT-OpmQ machinery is involved in the secretion of unwanted
354 siderophore-metal complexes, leading to specific integration of iron in the cellular pool.
355 Therefore, we hypothesize that, similarly, MexAB-OprM could play a role in the selectivity

356 of metals imported by pseudopaline. Investigation on this potential role would give an insight
357 on the nature of metal physiologically targeted by this machinery. Indeed, if the import of zinc
358 by pseudopaline is required for full extracellular metalloprotease activity (Figure 1), the
359 physiological relevance underlying Ni and Co import by pseudopaline remains to be
360 demonstrated.

361 Finally, other siderophores such as pyoverdine have been shown to be recycled (34), and this
362 could also be the case for pseudopaline. The absence of a recycling mechanism would lead to
363 a constant production of pseudopaline during infection, and would represent an important
364 investment of cellular resources. More research is needed in order to determine whether the
365 modification of pseudopaline we observed is reversible and allows recycling or, alternatively,
366 whether pseudopaline is fully degraded after uptake.

367

368 MATERIAL AND METHODS

369 Bacterial strains, plasmids and liquid growth conditions.

370 Bacterial strains, vectors and plasmids used in this study are listed in Table 1. *E. coli* strains
371 were grown aerobically with orbital shaking at 37 °C in Luria-Broth (LB) with antibiotics as
372 required (25 µg ml⁻¹ kanamycin (Kan), 25 µg ml⁻¹ tetracycline (Tc), 15 µg ml⁻¹ gentamicin
373 (Gm), 30 µg ml⁻¹ streptomycin (Sm)). The *E. coli* strains CC118λpir and SM10 were used to
374 propagate pKNG101 derivatives mutator plasmids. Recombinant plasmids were introduced
375 into *P. aeruginosa* by triparental mating using pRK2013 and transconjugants selected on
376 *Pseudomonas* isolation agar (PIA, Difco Laboratories) supplemented with antibiotics as
377 required (500 µg ml⁻¹ carbenicillin (Cb), 150 µg ml⁻¹ Gm, 2000 µg ml⁻¹ Sm, 200 µg ml⁻¹ Tc).
378 All *P. aeruginosa* strains used in this study were derivatives of the parental PA14 strain. *P.*
379 *aeruginosa* strains were grown aerobically with horizontal shaking at 37 °C with antibiotics

380 as required (150 $\mu\text{g ml}^{-1}$ (Cb), 50 $\mu\text{g ml}^{-1}$ (Gm), 500 $\mu\text{g ml}^{-1}$ (Sm), 50 $\mu\text{g ml}^{-1}$ (Tc)). Growths
381 were performed rich LB medium and in minimal chelated medium (minimal succinate media
382 (per liter: 30g K_2HPO_4 , 15g KH_2PO_4 , 5g $(\text{NH}_4)_2\text{SO}_4$, 1g MgSO_4 , 20g Succinic acid and 15.5g
383 NaOH, pH 7.0, MS) supplemented with 100 μM EDTA, MCM). MCM pre-culture of 20 mL
384 were inoculated from fresh MCM, 1.5% agar plates, grown until late exponential phase in
385 polycarbonate Erlenmeyer flasks in order to avoid any metal contamination. Growth was
386 monitored by OD_{600} measurement.

387 **Plasmid constructions.**

388 All plasmids constructed in this study except pKNG ΔoprM were generated by the one-step
389 sequence and ligation-independent cloning (SLIC) method described in (35). To construct the
390 suicide plasmid pKNG ΔoprM , the ΔoprM fragment was cloned into plasmid pCR-Blunt
391 according to manufacturer's instructions (Invitrogen) and then subcloned as BamHI/XbaI
392 fragment into the suicide vector pKNG-101. All PCR primers employed for plasmid
393 construction are listed in Table 2. Genomic DNA was isolated and purified with Pure Link
394 genomic DNA minikit (Invitrogen). PCR reactions for cloning were performed by using Q5®
395 High-Fidelity DNA Polymerase (New England Biolabs, Inc (NEB)). All the products
396 sequenced to verify the absence of any mutations (GATC-biotech).

397 **Construction of *P. aeruginosa* PA14 knock-out mutants**

398 PA14 ΔoprM was constructed from PA14 using overlapping PCRs with specific
399 iPA14*mexOprM* primers described in Table 2. Homologous recombination was carried out
400 between the 5' (512-bp) and 3' (539-pb) regions flanking *oprM* according to the protocol of
401 (36) modified by (37). The allelic exchange was verified by PCR and nucleotides sequencing
402 experiments confirmed the deletion of 1415-bp in the *oprM* locus. To construct the
403 pKNG101 $\Delta\text{cntOLMI}$, two DNA fragments corresponding to upstream and downstream
404 regions of *cntO* and *cntI* were amplified from PA14 chromosomal DNA with PCR primers

405 SL12/NG07 and NG08/SL22, respectively Upstream (639-bp) and downstream (517-bp)
406 regions were ligated by overlapping PCR and cloned into linearized pKNG101 by the SLIC
407 method. The resulting constructs were transformed into *E. coli* CC118 λ pir and introduced into
408 *P. aeruginosa* PA14 by conjugation. The strains in which the chromosomal integration event
409 occurred were selected on *Pseudomonas* isolation agar Gm plates. Excision of the plasmid,
410 resulting in the deletion of the chromosomal target gene was performed after selection on
411 Luria-Bertani (LB) plates containing 6% sucrose. Clones that became sucrose resistant and
412 Sm sensitive were confirmed to be deleted for the genes of interest by PCR analysis.

413 **Generation time, biofilm formation and extracellular protease activity measurements**

414 Generation time in minutes defines the time required by the bacterium to perform one cycle of
415 division. Generation time was calculated in exponential phase from the growth curves of six
416 independent biological replicates. Each over-night cultures were inoculated at OD₆₀₀ of 0,05
417 in fresh MCM in clear, flat bottom, 96-well plates (CorningTM Costar 3596). Growth was
418 recorded in a Tecan Spark Microplate Incubator/Reader (Thermo Fisher Scientific) for 24
419 hours at 37°C under continuous shaking and with 600nm absorbance readings every 30
420 minutes, corrected with the absorbance of non-inoculated media.

421 Biofilm formation was measured in clear, flat bottom, 24-well plates (CorningTM Costar
422 3526). Each over-night cultures were inoculated at OD₆₀₀ of 0,2 in 1 mL fresh MCM and
423 incubated at 30°C for 24 hours. Following incubation, planktonic bacteria and media were
424 rinsed away with non-sterile deionized water. The wells were filled with 0,1% crystal violet
425 solution and sat for 15min at RT and then washed with deionized water three times. Any
426 crystal violet staining on the bottom of the well were cleaned, and the plate was let to air-dry
427 overnight at room temperature. Crystal violet rings were then solubilized in 30% glacial acetic
428 acid. Adherent biofilm was quantified by measuring optical density of all samples at 550nm
429 and normalized to WT mean absorbance. Photos used for biofilm illustration where made

430 from biofilm experiments following the same method, but realized in borosilicate glass tubes
431 for microbiological cultures.

432 Global extracellular protease activity: Pre-culture of PA14 strains are inoculated from a fresh
433 1,5% agar MCM plate, and incubated overday in 20 mL MCM under horizontal shaking at
434 37°C. A 25 mL culture of fresh MCM is then inoculated by the pre-culture at OD₆₀₀ of 0,05
435 and incubated for 10 hours under horizontal shaking at 37 °C in polycarbonate baffled
436 Erlenmeyer flask (Corning 431407). Culture were centrifuged at 13000 xg for 15 min at 4°C
437 and supernatants was concentrated with Amicon Ultra-15 10000Da NMWL (Merk C7715)
438 and normalized to cell density. Total extracellular proteolytic activity was determined using
439 casein conjugated to an azo-dye as a substrate, following a modified procedure described by
440 Kessler *et al.*, (38). Briefly, 0,25 mL of concentrated and normalized supernatant was added
441 to 0,75 mL reaction buffer (0,05M Tris-HCl, 0,5 mM CaCl₂, pH 7,5, 3 mg.mL⁻¹ Azocasein
442 (Sigma A2765)) preheated at 37°C and vortexed. The reaction mixture was incubated at 37°C
443 for 15 min. The indigested substrate was precipitated by adding trichloroacetic acid to a final
444 3,3% and vortexed. The samples were then left at RT for 30 minutes, and centrifuged at
445 10000 xg for 20min. The absorbance was measured at 400 nm.

446 **Viability assay on plate.**

447 Pre-culture of PA14 strains were performed overnight in MCM under horizontal shaking at
448 37°C. The next day, a culture of fresh MCM is inoculated by the pre-culture at OD₆₀₀ of 0.05
449 and incubated for 9 hours under horizontal shaking at 37 °C. Cultures were then adjusted to
450 OD₆₀₀ of 1 and subjected to 10% serial dilutions in fresh MCM. 10 µl of culture samples were
451 spotted on MS 1.5% agar or agarose plate an incubated 30h at 37 °C.

452 **RNA Preparation and Reverse Transcription.**

453 RNAs were prepared from 25 mL PA14 culture in MCM in the exponential growth phase
454 after 10h shaking at 37°C. The cells were harvested and frozen at -80°C. Total RNAs were

455 isolated from the pellet using RNeasy mini Kit (Qiagen) according to the manufacturer's
456 instructions; an extra TURBO DNase (Invitrogen) digestion step was done to eliminate the
457 contaminating DNA. The RNA quality was assessed by the TapeStation 4200 system
458 (Agilent). RNA was quantified spectrophotometrically at 260 nm (NanoDrop 1000; Thermo
459 Fisher Scientific). For cDNA synthesis, 1 µg total RNA and 0.5 µg random primers
460 (Promega) were used with the GoScript™ Reverse transcriptase (Promega) according to the
461 manufacturer's instruction.

462 **Quantitative real-time PCR (qPCR)**

463 qPCR analyses were performed on a CFX96 Real-Time System (Bio-Rad, France). The
464 reaction volume was 15 µL and the final concentration of each primer was 0.5 µM. The
465 cycling parameters of the qRT-PCR were 98°C for 2 min, followed by 45 cycles of 98°C for 5
466 s, 60°C for 10 s. A final melting curve from 65°C to 95°C was added to check the specificity
467 of the amplification. To determine the amplification kinetics of each product, the fluorescence
468 derived from the incorporation of EvaGreen into the double-stranded PCR products was
469 measured at the end of each cycle using the SsoFast EvaGreen Supermix 2X Kit (Bio-Rad).
470 The results were analyzed using Bio-Rad CFX Manager software, version 3.1 (Bio-Rad). The
471 16S RNA and *uvrD* genes were used as a reference for normalizations. For each point a
472 technical duplicate was performed. The amplification efficiencies for each primer pairs were
473 comprised between 80 and 100%. All of the primer pairs used for qPCR are shown in the
474 table 2.

475 **Pseudopaline detection by HILIC - ESI-MS.**

476 All the *P. aeruginosa* strains were grown aerobically with horizontal shaking at 37 °C in
477 freshly made MCM. Cultures were then adjusted at OD = 0.05 in 25 mL freshly made MCM
478 and grown for 10 h. After 10 h, OD₆₀₀ was measured before cells were harvested by
479 centrifugation (4,000xg, 30 min, 4 °C). The supernatant was collected, filtered and stored at

480 -80°C . The pellet was washed three times with cold MCM and resuspended in 1.3 ml cold
481 MCM. OD_{600} were measured, and cells ruptured by successive sonication cycles (three times
482 30 sec at 60% of maximal power, keeping the solution cold (Brandson Sonifier 450
483 microtip)). The lysates were then centrifuged at $16,000\times g$ for 30 min at 4°C and supernatants
484 were collected, filtered at $0.22\ \mu\text{m}$, and stored at -80°C . These cell lysate and supernatant
485 fractions were analyzed to detect pseudopaline using HILIC/ESI-MS as previously described
486 (7). Pseudopaline levels were determined using the relative intensity of the m/z corresponding
487 to the pseudopaline-Ni complex in extracellular or intracellular fractions saturated with
488 nickel, in order to force nickel complex formation.

489 ***in vitro* pseudopaline modification monitoring by thin layer chromatography.**

490 Preparation of *P. aeruginosa* and *E. coli* cell lysates. Pre-culture of PA14 strains are
491 inoculated from a fresh MS + 1,5% agar plate, and incubated overday in 20 mL MS medium
492 under horizontal shaking at 37°C . The culture is then adjusted at $\text{OD}_{600} = 0,05$ with 100 mL
493 of fresh MCM and incubated for 10 hours under horizontal shaking at 37°C . *E. coli* MG1655
494 strain were grown in M63 media (per liter of water, 13.6 g KH_2PO_4 , 2 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g
495 MgSO_4 , glucose 0.2%, casamino acids 0.5%, pH 7), with the same culture time and
496 conditions. An aliquot corresponding to 100 OD_{600} units of each bacterial culture was
497 centrifuged for 10 min at $2,000\times g$. Cell pellets were resuspended in 10 mL 50 mM Tris-HCl
498 pH 7.6 and ruptured by successive sonication cycles (three times 30 sec at 60% of maximal
499 power, keeping the solution cold (Brandson Sonifier 450 microtip). The final lysate was
500 centrifuged at $2,000\times g$ for 15 min to remove unbroken cells and the supernatant was saved as
501 total cell lysate fraction.

502 Radiolabeled yNA and pseudopaline are first produced with the reaction of the purified
503 enzymes CntL (for yNA) or a mixture of CntL and CntM (for pseudopaline) with their
504 substrates and then incubated with cell lysates. For the production reaction, we used CntL

505 from *Yersinia pestis* (YpCntL) and CntM from *Paenibacillus mucillaginosus* (PmCntM) that
506 have been shown to be more stable and effective than their equivalent from *Pseudomonas*
507 *aeruginosa* (8). Cloning, expression and purification of PmCntM have already been described
508 (8). For YpCntL, the gene encoding the YpCntL protein was cloned in a pET-TEV plasmid
509 (synthetic DNA from GenScript). After transformation, *E. coli* BL21 strains are aerobically
510 cultivated with horizontal shaking in LB media supplemented with kanamycin at 50 mg.ml⁻¹.
511 These strains were grown at 37°C and, when the OD of the culture was ~0.6, IPTG was
512 added at a final concentration of 0.1 mM for induction. After overnight growth at 16°C, cells
513 were recovered by centrifugation at 5,000xg for 20 min at 4°C, resuspended in buffer (20 mM
514 Na₂HPO₄, 300 mM NaCl, 15 mM Imidazole, pH 7) and disrupted using a Constant cell
515 disruption system operating at 2 Kbar. Cell debris were removed by centrifugation at 8,000xg
516 for 20 min and the supernatants were centrifuged at 100,000xg for 45 min at 4°C to remove
517 cell wall debris and membrane proteins. The resulting soluble fraction was loaded on a nickel
518 affinity column (HisTrap 1 ml column, G.E. Healthcare), and the protein was eluted stepwise
519 with imidazole (50 mM for wash and 500 mM for elution). Elution fraction was adjusted to 1
520 mg/mL and cleaved by TEV protease in a ratio 1:80 w/w. After an overnight incubation, the
521 mixture was centrifuged at 10,000xg for 10 min and then loaded again on a nickel affinity
522 column. YpCntL was recovered in the flow through and then transferred into an imidazole-free
523 buffer (20 mM Na₂HPO₄, 300 mM NaCl, glycerol 10%, pH 7).
524 For the TLC assays, the purified enzymes are incubated at a final concentration of 2.5 µM
525 with carboxyl-[¹⁴C]-labeled SAM (2.5 µM), L-histidine (10 µM), NADPH (30 µM) and α-KG
526 (1 mM). The total volume was 100 µl in ammonium acetate buffer (100 mM, pH=7). The
527 mixtures are incubated for 10 or 15 min at 28°C. Cell lysates at different concentrations are
528 then added to the radiolabeled pseudopaline (50% of each, v/v), and these mixtures are
529 incubated for another 15 or 45 min at 28 °C. A same reaction is done with the addition of only

530 ammonium acetate buffer in the radiolabeled pseudopaline as a control. The reactions are
531 finally stopped by adding ethanol to a final concentration of 50% (v/v). An aliquot of 10 µl of
532 the reaction mixtures is spotted on HPTLC (High-Performance TLC) Silica Gel 60 Glass
533 Plates (Merck KGaA) after centrifugation at 16.000 g for 2 min. The products are then
534 separated by thin-layer chromatography by developing plate with a phenol:n-
535 butanol:formate:water (12 : 3 : 2 : 3 v/v) solvent system. HPTLC plates were dried and
536 exposed to a [¹⁴C]-sensitive imaging plate for 5 or 6 days. Imaging plates are then scanned on
537 a Typhoon FLA 7000 phosphorimager (GE Healthcare). The original TLC plates of *in vitro*
538 pseudopaline degradation by PA14 cell lysates experiments presented in figure 4 are
539 presented figure S2.

540 **Statistics.**

541 Statistics were determined using the Student's t-test function of excel using a bilateral model
542 and assuming equal variance when distribution of samples had normal distribution (figure 1).
543 For nonparametric data (figures 2 and 3) statistical significance was calculated using the
544 Wilcoxon rank sum test.

545

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553

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677

678 **Table1:** Bacterial strains, vectors and plasmids used in this study

Strains, vectors & plasmids	Descriptions	References
<i>E. coli</i>		
CC118 λ pir	$\Delta(ara-leu) araD \Delta lacX74 galE galK phoA20 thi-1 rpsE rpoB argE$ (Am) <i>recA1 RfR</i> (λ pir)	(36)
SM10	<i>thi-1, thr, leu, lacY, supE, recA::RP4-2-Tc::Mu</i> ; Km ^R	Laboratory collection
K-12	MG1655 (<i>F lambda ilvG rfb 50 rph 1</i>)	Laboratory collection
<i>P. aeruginosa</i>		
PA14	Wild type	(39)
PA14 $\Delta cntL$	<i>cntL</i> (PA14_63940) deletion mutant	(7)
PA14 $\Delta cntL::cntL$	PA14 $\Delta cntL$ harboring a wild type copy of <i>cntL</i> gene at <i>att</i>	(7)
PA14 $\Delta cntI$	<i>cntI</i> (PA14_63910) deletion mutant	(7)
PA14 $\Delta cntLI$	<i>cntL</i> & <i>cntI</i> deletion strain	(7)
PA14 $\Delta cntOLMI$	<i>cntO</i> (PA14_63960), <i>cntL</i> , <i>cntM</i> (PA14_63920) & <i>cntI</i> deletion strain	This work
PA14 ΔAB	<i>mexA</i> and <i>mexB</i> (PA14_05530 & PA14_05540) deletion mutant	(40)
PA14 $\Delta RNDs$	<i>mexAB</i> , <i>mexCD-oprJ</i> , <i>mexEF-oprN</i> and <i>mexXY</i> deletion mutant	(40)
PA14 $\Delta oprM$	<i>oprM</i> (PA14_05550) deletion mutant	This work
Vectors & plasmids		
pKNG101	Suicide vector SmR, <i>oriR6K</i> , <i>oriTRK2</i> , <i>mobRK2</i> , <i>sacBR+</i>	(36)
pKNG101 $\Delta cntL$	Suicide plasmid for <i>cntL</i> deletion	(7)
pKNG101 $\Delta cntI$	Suicide plasmid for <i>cntI</i> deletion	(7)
pKNG101 $\Delta cntOLMI$	Suicide plasmid for <i>cnt</i> operon deletion	This work
pKNG101 $\Delta oprM$	Suicide plasmid for <i>oprM</i> deletion	This work
pRK2013	Plasmid for triparental mating. KmR, ColE1, Tra+ Mob+	(41)
pET-TEV-Y $pcntL$	plasmid producing CntL	Synthetic DNA from GenScript
pET-TEV-P $mcntM$	plasmid producing CntM	(8)

679

680

681

682 **Table 2:** Oligonucleotides used in this study

Name	Sequence (5'-3')
NG1	CGGGCTGCAGGAATTCGGCTGGGCTGGTCGT
NG07	GTCGCGCCGGAGGCTCACATGGGAAATCGCACCAGAA
NG08	TTCTGGTGCGATTCCCATGTGAGCCTCCGGCGCGACCGG
SL01	CAGGTCGACGGATCCCCGGGGAAAAAGAAGAACGTGCTCACC
SL02	GGCCTTCTCCATGGCATGGCTTCCTGGCG
SL03	GCCATGCCATGGAGAAGGCCGGTCGATGA
SL04	TATGCATCCGCGGGCCCCGGGAGGTAGACCCTGCGCTTGAC
SL05	CCTTCAAGCCCAACGGCGGT
SL06	ACCACAGCCGCCGCATGGCG
SL12	CAGGTCGACGGATCCCCGGGGAAATGCAGCGGATCGAG
SL19	CAGGTCGACGGATCCCCGGGTCTACCCGGAGGGACCTATC
SL20	GGAGGCTCACTTCAGCAGGTCGAGCACCA
SL21	CTGCTGAAGTGAGCCTCCGGCGCGACCGG
SL22	TATGCATCCGCGGGCCCCGGGGCTGCTCTACAGCATCTCGAC
SL23	GACAGTTACCTGGACGTGGTC
SL24	CAAGCCTTTATCCGTTCCAA
<i>iPA14mexOprM-1</i>	TACGAAAGCTGGTCGATTCC
<i>iPA14mexOprM-2</i>	GCGATACCAGGAACGACAGCGGCTAC
<i>iPA14mexOprM-3</i>	TTCTGTCTGATCGCCTTCCGCGCCATG
<i>iPA14mexOprM-4</i>	ACTTCGACAATTTCCGGCAAC
<i>qRTcntOF</i>	ACGAACTGATCCTGCGTAGC
<i>qRTcntOR</i>	CGCCATTTCTCGTTGAACTC
<i>qRTcntMF</i>	TACGTGCACAGTCCGTTCTT
<i>qRTcntMR</i>	GCAACAGTTCGCTCAGCTC
<i>qRT16SF</i>	CAGCTCGTGTGTCGTGAGATGT

qRT16SR	GATCCGGACTACGATCGGTT
qRT $uvrDF$	CACGCCTCGCCCTACAGC
qRT $uvrDR$	GGATCTGGAAGTTCTGCTCAGC

683

684

685 **FIGURE LEGENDS**

686 **Figure 1: Impact of pseudopaline on generation time, biofilm formation capacity and**
687 **extracellular protease activity in MCM.** PA14 wild-type (WT), mutant (*ΔcntL*) and
688 complemented (*ΔcntL::cntL*) strains were grown in MCM. Generation time (A), biofilm
689 formation capacity (B) and extracellular protease activity (C) were measured as described in
690 the M&M section. A representative illustration of crystal violet rings obtained with the
691 different strains during the biofilm formation assay is presented panel B. Errors bar, mean ±
692 standard deviation (sd) of at least two independent biological replicates. *P<0.05, ***P<0.001
693 as compared to the wild type (WT).

694

695 **Figure 2: Extracellular pseudopaline content in absence of pseudopaline inner and outer**
696 **membrane exporters.** *P. aeruginosa* PA14 wild-type and mutant strains were grown for 10h
697 at 37 °C in MCM. The relative levels of extracellular pseudopaline in the supernatant were
698 measured by HILIC - ESI-MS. Errors bar, mean ± sd of at least three independent biological
699 replicates. *P<0.05, **P<0.01, ***P<0.001, as compared to the wild type (WT); ND: Not
700 Detectable, NS: Not Significant, Pp: pseudopaline.

701

702 **Figure 3: Intracellular pseudopaline content and phenotypes of *P. aeruginosa* PA14**
703 **strains lacking the pseudopaline inner and outer membrane exporters.** (A) Detection of
704 pseudopaline in the intracellular fractions of wild type and mutant strains. Strains were grown
705 for 10h at 37 °C in MCM. The relative levels of intracellular pseudopaline in the cell fraction
706 were measured by HILIC - ESI-MS. Errors bar, mean ± sd of at least three independent
707 biological replicates. *P<0.05 as compared to the wild type (WT); ND: Not Detectable. (B)
708 Viability of PA14 WT and mutant strains assessed by serial dilutions spotted on 1.5% agar
709 MCM plates.

710

711 **Figure 4: Expression of *cntO* and *cntM* genes in absence of pseudopaline inner and outer**
712 **membrane exporters.** Transcriptional activities were measured by RT-qPCR in PA14 wild
713 type and various mutant strains. The relative *16S RNA* and *uvrD* normalized transcriptional
714 expressions of *cntO* and *cntM* in various *cnt* and RNDs mutant strains compared to the wild

715 type strain is presented. The two red lines are indicating the two time up and down regulation
716 boundaries for a significant transcriptional effect. Experiments have been done in three
717 biological replicates each including three technical replicates.

718

719 **Figure 5: *in vitro* pseudopaline degradation by PA14 cell lysates.** (A top) Summary of the
720 CntL/M-dependent biosynthesis pathway for the assembly of yNA and pseudopaline from L-
721 his, SAM, NADH and α -KG. (A, B) Thin layer chromatography (TLC) separation of [14 C]-
722 SAM (S-adenozyl methionine) and reaction products (yNA and pseudopaline) after
723 incubation with defined enzymes, substrates and cofactor, followed or not by an incubation
724 with increased concentration of *E. coli*, PA14wt and PA14 Δ *mexAB* cell lysates. Incubation
725 times for pseudopaline production are 15 and 10 minutes for experiments performed in panels
726 A and B respectively. Incubation times with cell lysates are 15 and 45 minutes for
727 experiments performed in panels A and B respectively.

728

729 **Figure 6: Model of pseudopaline synthesis, secretion, and metal uptake in *P. aeruginosa*.**
730 See text for details. Outer membrane (OM), periplasm (P), inner membrane (IM), L-histidine
731 (L-His), S-adenozyl methionine (SAM), α -keto-glutarate (α -KG) and cofactor (NADH). The
732 dashed triangle around pseudopaline symbolizes pseudopaline periplasmic
733 degradation/modification.

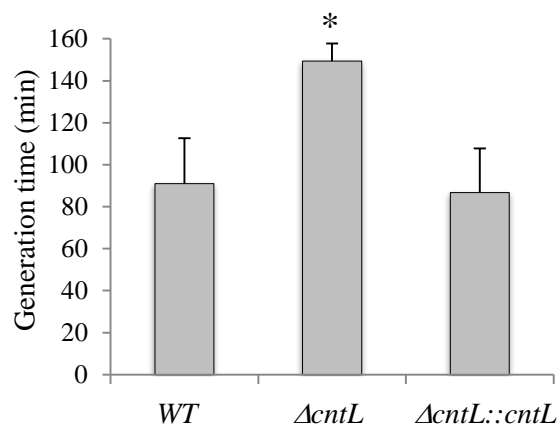
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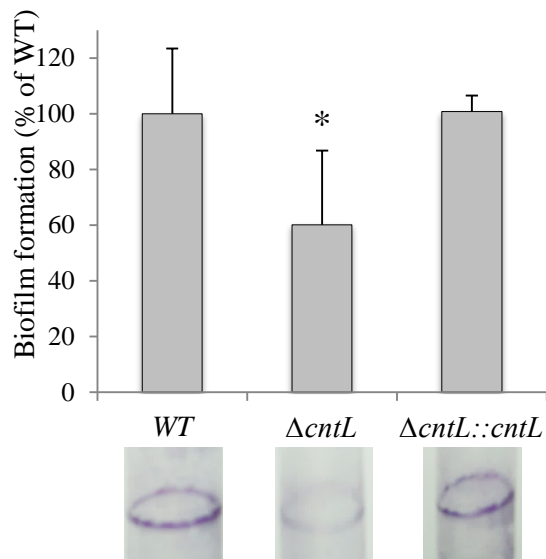
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A

Generation time in MCM

**B**

Biofilm formation in MCM

**C**

Global extracellular protease activity in MCM

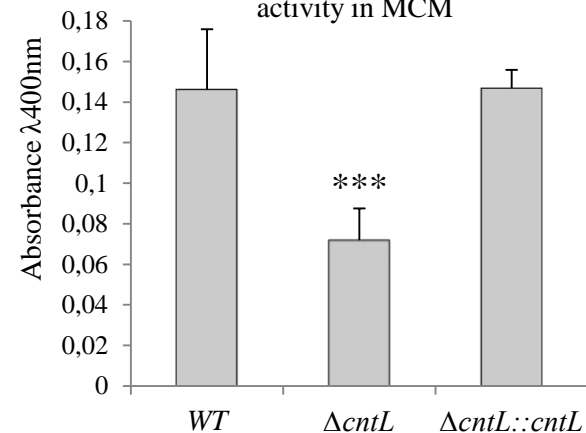


Figure 1: Impact of pseudopaline on generation time, biofilm formation capacity and extracellular protease activity in MCM. PA14 wild-type (WT), mutant ($\Delta cntL$) and complemented ($\Delta cntL::cntL$) strains were grown in MCM. Generation time (A), biofilm formation capacity (B) and extracellular protease activity (C) were measured as described in the M&M section. A representative illustration of crystal violet rings obtained with the different strains during the biofilm formation assay is presented panel B. Errors bar, mean \pm standard deviation (sd) of at least two independent biological replicates. * $P < 0.05$, *** $P < 0.001$ as compared to the wild type (WT).

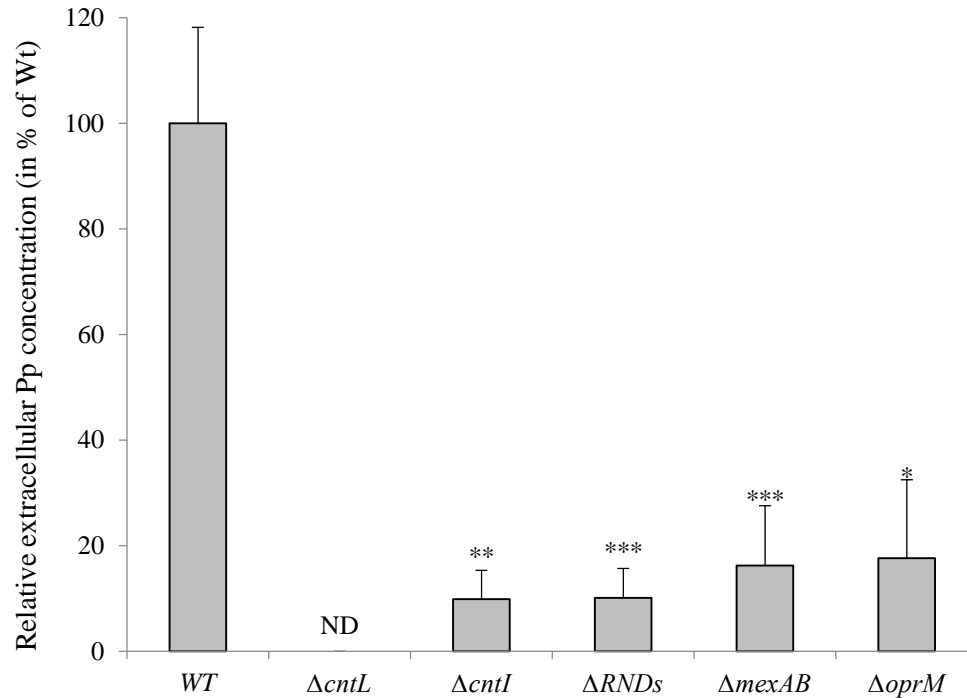


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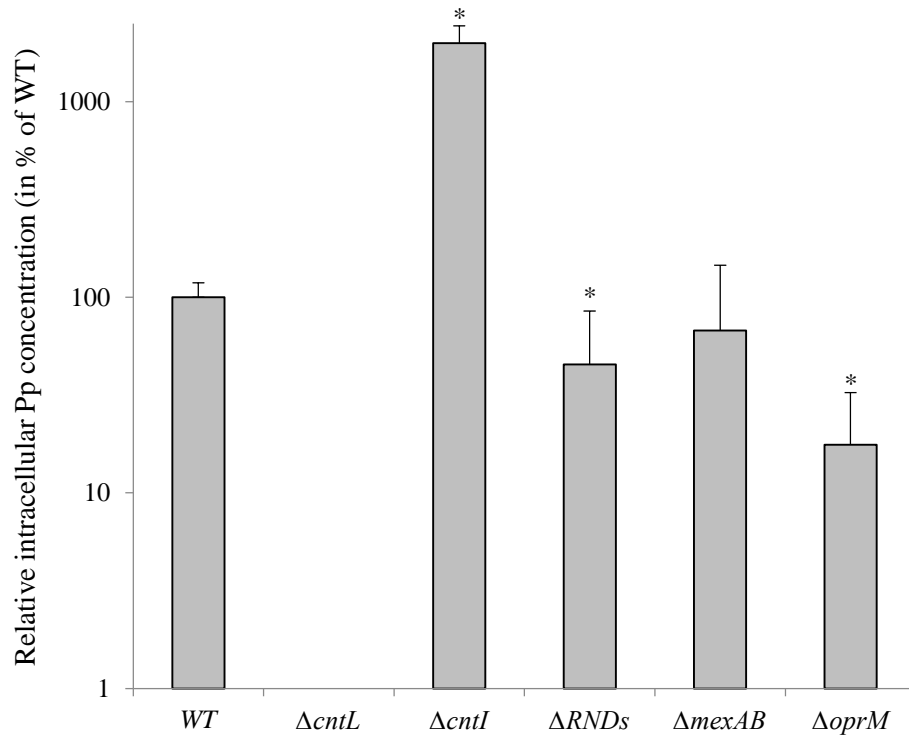
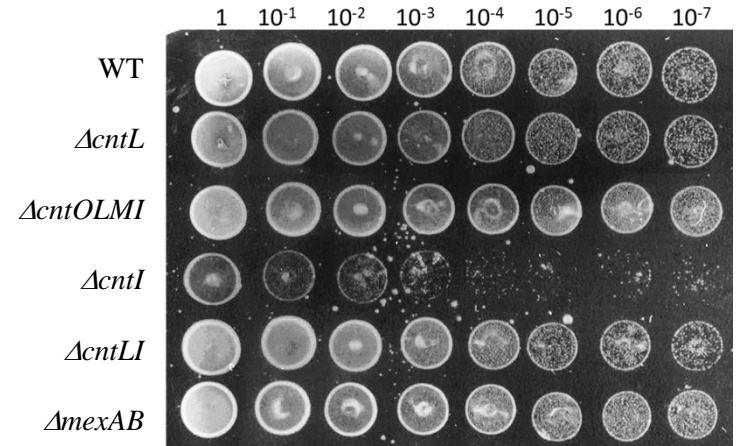
A**B**

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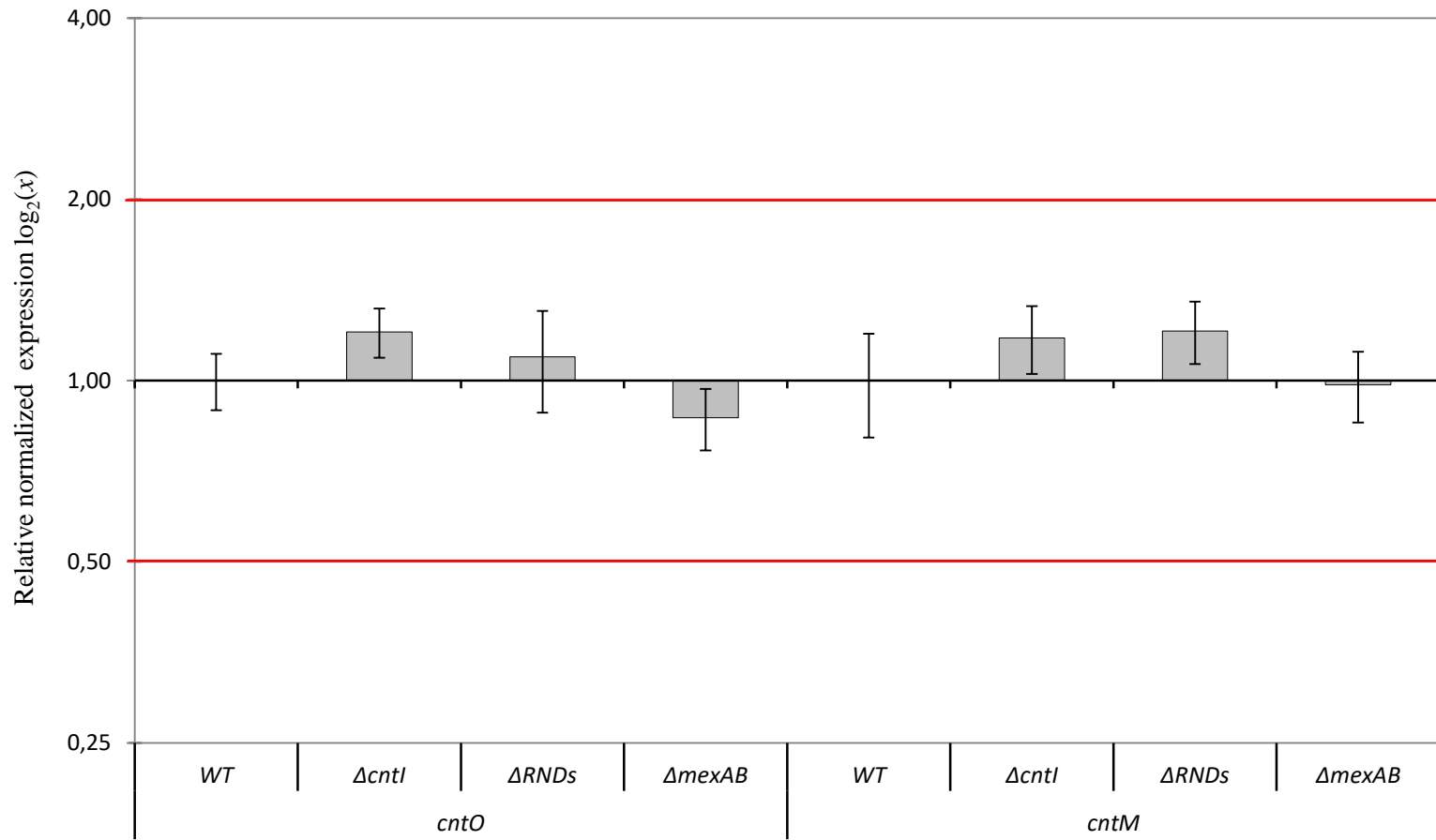


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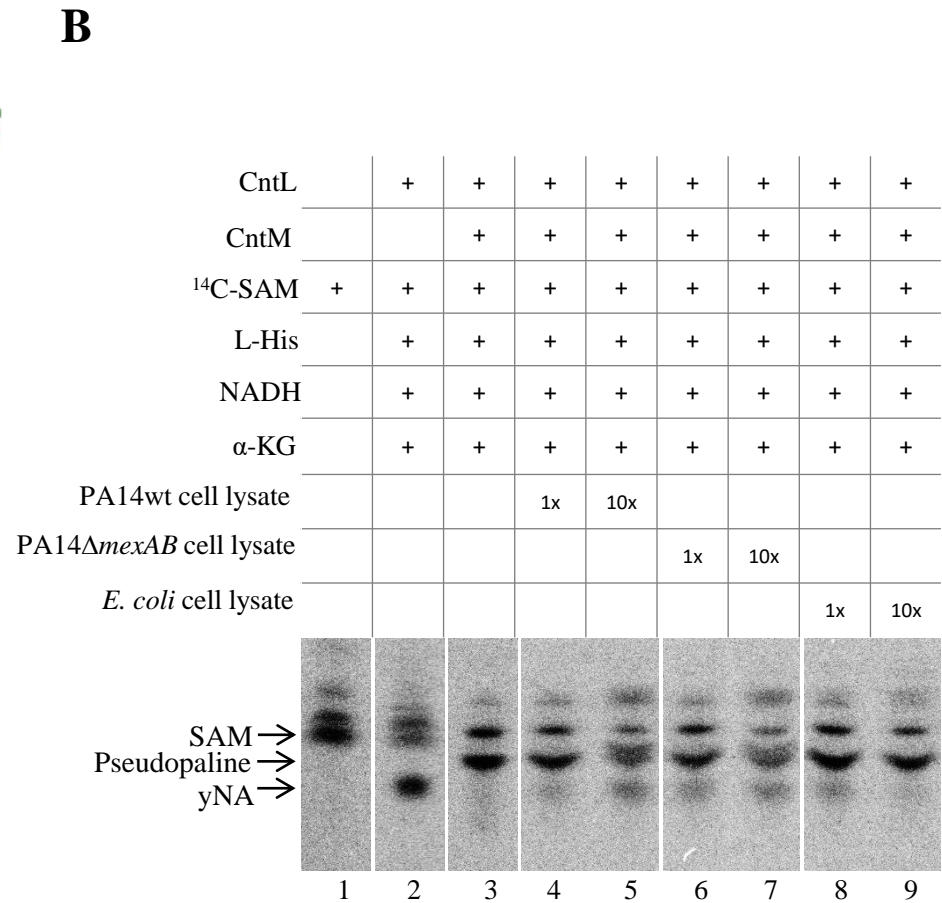
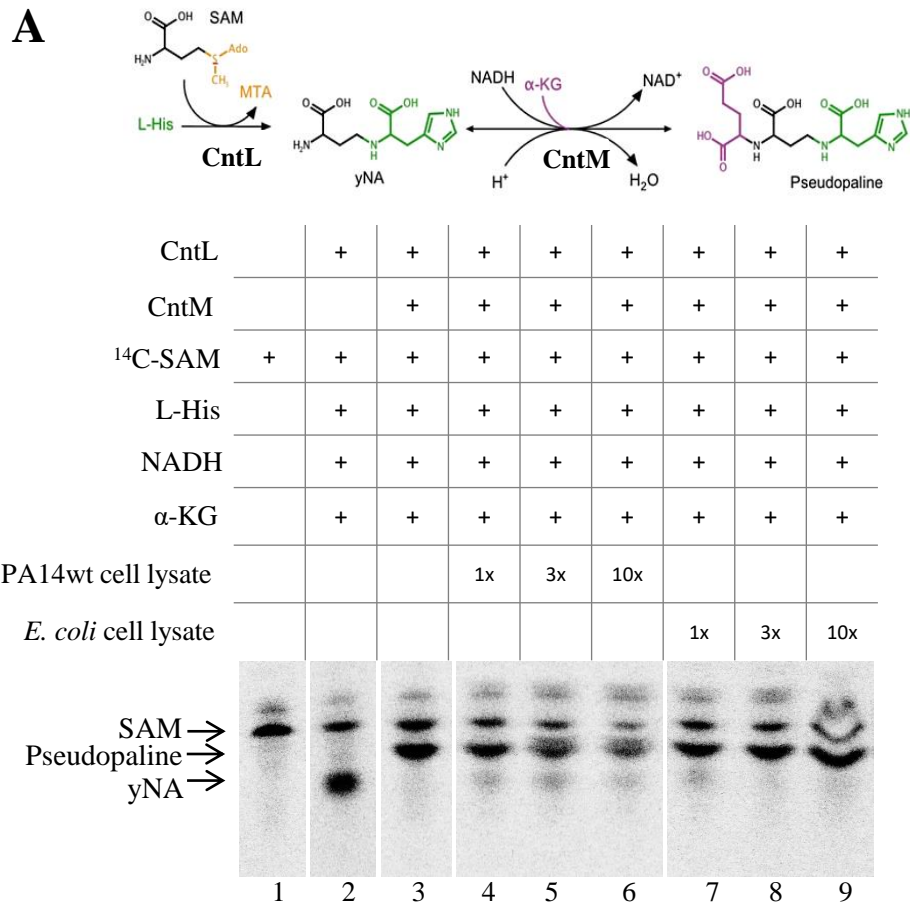


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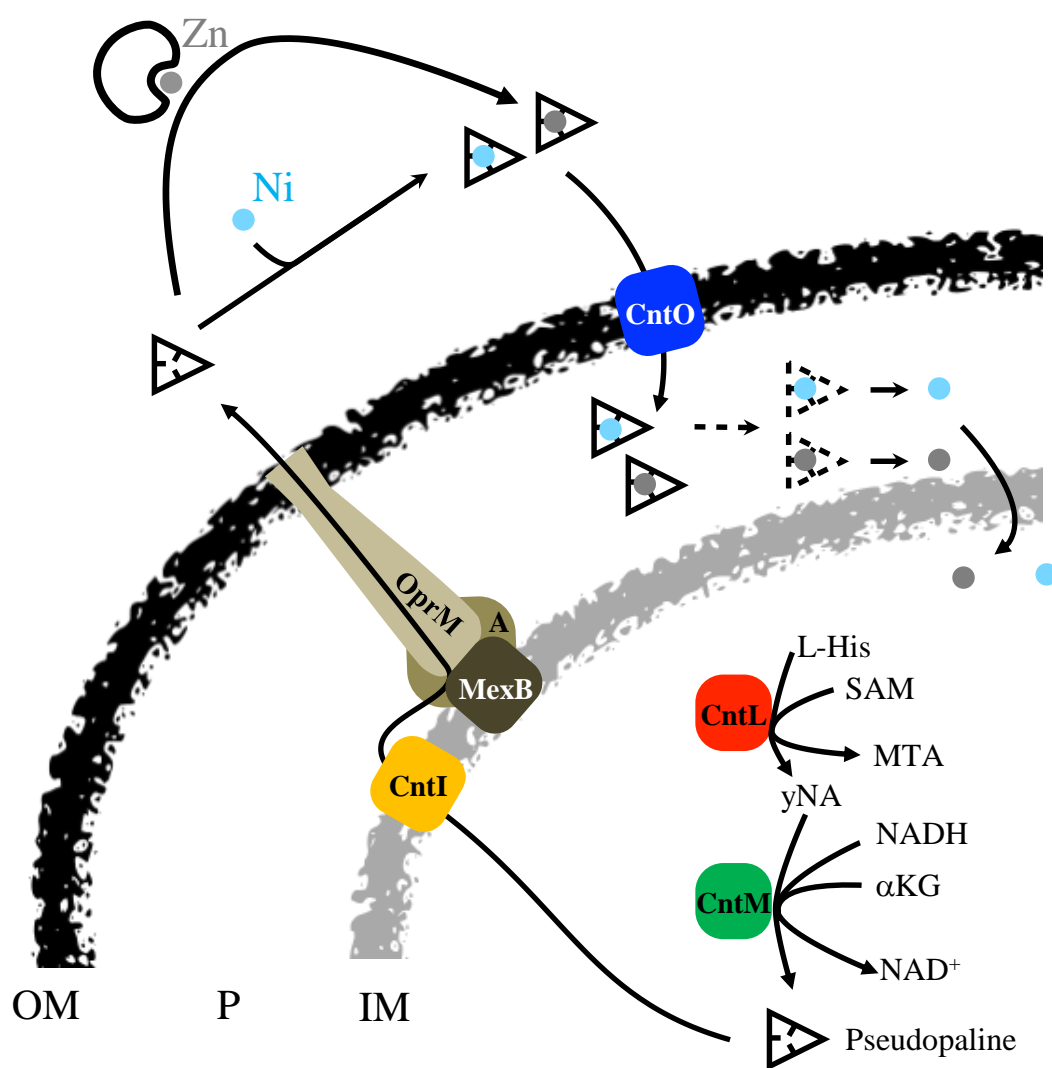


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