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► To cite this version:

Libera Latino, Delphine Patin, Dimitri Chérier, Thierry Touzé, Christine Pourcel, et al.. Impact of FiuA outer-membrane receptor polymorphism on the resistance of *Pseudomonas aeruginosa* towards peptidoglycan lipid II-targeting PaeM pyocins. *Journal of Bacteriology*, 2019, 201 (13), 10.1128/JB.00164-19 . hal-02173140

HAL Id: hal-02173140

<https://hal.science/hal-02173140>

Submitted on 10 Nov 2020

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2 **Impact of FiuA outer-membrane receptor polymorphism on the resistance of**
3 ***Pseudomonas aeruginosa* towards peptidoglycan lipid II-targeting PaeM pyocins**

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11 Running title: PaeM and FiuA receptor polymorphism in *P. aeruginosa*

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15

16 **ABSTRACT**

17 Certain *Pseudomonas aeruginosa* strains produce a homolog of colicin M, named PaeM, that
18 specifically inhibits peptidoglycan biosynthesis of susceptible *P. aeruginosa* strains by
19 hydrolyzing the lipid II intermediate precursor. Two variants of this pyocin were identified
20 whose sequences mainly differed in the N-terminal protein moiety, *i.e.* the region involved in
21 the binding to the FiuA outer membrane receptor and translocation into the periplasm. The

22 antibacterial activity of these two variants, PaeM1 and PaeM2, was tested against various *P.*
23 *aeruginosa* strains comprising reference strains PAO1 and PA14, PaeM-producing strains
24 and sixty clinical isolates. Seven of these strains, including PAO1, were susceptible to only
25 one variant (two to PaeM1 and five to PaeM2) and eleven were affected by both of them. The
26 remaining strains, including PA14 and four PaeM1 producers, were resistant to both variants.
27 The differences in the antibacterial spectra of the two PaeM homologs prompted us to
28 investigate the molecular determinants allowing their internalization into *P. aeruginosa* cells,
29 taking the PAO1 strain that is susceptible to PaeM2 but resistant to PaeM1 as indicator strain.
30 Heterologous expression of *fiuA* gene orthologs from different strains into PAO1, site-
31 directed mutagenesis experiments and construction of PaeM chimeric proteins provided
32 evidence that the cell susceptibility and discrimination between PaeM variants resulted from
33 a polymorphism of both the pyocin and the outer membrane receptor FiuA. Moreover, we
34 found that a third component was directly implicated in this discrimination, TonB1, a protein
35 involved in iron transport in *P. aeruginosa*, working together with FiuA and the ExbB/ExbD
36 complex.

37 **IMPORTANCE**

38 Bacterial antibiotic resistance constitutes a threat to human health imposing identification of
39 new targets and development of new strategies to fight multi-resistant pathogens.
40 Bacteriocins and other weapons that the bacteria themselves developed to kill competitors are
41 therefore of great interest and a valuable source of inspiration for us. Attention was here paid
42 to two colicin M homologs (PaeM) produced by certain strains of *P. aeruginosa* that inhibit
43 the growth of their congeners by blocking cell-wall peptidoglycan synthesis. Molecular
44 determinants allowing recognition of these pyocins by the outer-membrane receptor FiuA
45 were identified and a receptor polymorphism affecting the susceptibility of *P. aeruginosa*

46 clinical strains was highlighted, providing new insights into the potential use of these pyocins
47 as an alternative to antibiotics.

48

49 **KEYWORDS:** *Pseudomonas aeruginosa*, bacteriocin, colicin M, lipid II, cell wall,
50 peptidoglycan, receptor structure-function, FiuA, TonB, PaeM pyocin.

51

52 **INTRODUCTION**

53 It is well established that bacterial resistance to antibiotics has become a serious problem
54 of public health that concerns almost all antibacterial agents and occurs in all fields of their
55 application. The two factors contributing to this phenomenon are the emergence of resistant
56 bacteria under the selective pressure of the antibiotics abundantly used in human and
57 veterinary medicine and the spread of resistant bacteria in the different ecosystems. The
58 urgent need for new antibacterial agents active against resistant bacterial pathogens thus
59 requires the development of detailed basic researches on the physiology of micro-organisms
60 and on metabolic pathways that are essential and specific to the bacterial world. The bacterial
61 cell-wall peptidoglycan polymer that protects cells against the internal osmotic pressure
62 constitutes such an interesting and validated target. Unfortunately, investment of
63 pharmaceutical companies in that field and the number of successful antibiotic discovery
64 programs and of novel drugs reaching clinical trials greatly diminished in recent years.

65 The strategies that are developed by the bacteria themselves to fight and eliminate
66 bacteria competing for the same ecological niches are very interesting in this respect and
67 could represent a great source of inspiration for us. The weapons (toxins) they produce allow
68 them to prevail within cell populations and fully express their virulence. Colicins are such
69 toxins produced by certain strains of *Escherichia coli* to kill competitors belonging to the
70 same or closely related species. Although they display a similar structural organization in

71 three distinct N-terminal, central and C-terminal domains involved in their translocation,
72 receptor binding, and cytotoxic activity, respectively, their mode of action is variable: pore
73 formation in the inner membrane, DNA or RNA degradation, or inhibition of cell wall
74 biosynthesis (1). Colicin M (ColM) is the smallest colicin identified to date (271 residues)
75 and the only one interfering with peptidoglycan synthesis (2, 3). We earlier demonstrated that
76 this colicin was an enzyme specifically catalyzing the degradation of the lipid II
77 peptidoglycan intermediate, thereby provoking the arrest of peptidoglycan synthesis and lysis
78 of targeted cells (2). This colicin parasitizes the FhuA outer-membrane receptor (4) and the
79 TonB/ExbB/ExbD import machinery to be internalized in the *E. coli* periplasm (1, 5).

80 Genes encoding putative homologs of the *E. coli* ColM were detected in the genomes of
81 some *Pseudomonas* species (*P. aeruginosa*, *P. syringae*, and *P. fluorescens*) and shown to be
82 present only in a limited number of virulent strains (6). These genes were cloned from the *P.*
83 *aeruginosa* JJ692, *P. syringae* pv. tomato DC3000, and *P. fluorescens* Q8r1-96 strains and
84 the corresponding proteins, named PaeM, PsyM and PflM, respectively, were purified and
85 their lipid II-degrading activity was characterized (6). Two other members of the same family
86 were also identified in *Pectobacterium carotovorum* (7, 8). The homology between these
87 different proteins was mainly observed in the second half of the protein sequence which
88 corresponds to the enzymatic activity domain. The lack of sequence similarity in the N-
89 terminal region was consistent with the fact that these bacteriocins parasitize receptors and
90 translocation machineries that are species-specific, explaining why they exhibit a narrow
91 range of antibacterial activity. These different ColM homologs, resulting from the fusion of a
92 conserved lipid II-targeting domain to a variable reception/translocation domain, thus
93 constitute a very interesting family of polymorphic toxins (9).

94 The PaeM bacteriocin produced by the *P. aeruginosa* JJ692 strain was characterized in
95 detail biochemically, functionally and structurally. It was crystallized and its structure

96 determined at 1.7 Å (10). Protein dissection experiments allowed us to more precisely
97 localize its activity domain (residues 134-289) that appeared to be independently functional
98 and 70-fold more active than the full-length protein in terms of enzymatic lipid II-degrading
99 activity (10). Site-directed mutagenesis of PaeM residues appearing as invariant or highly
100 conserved in this bacteriocin family showed that four of them, namely D241, D244, Y243
101 and R251, played an essential role in the catalytic process. Their mutation to alanine led to a
102 dramatic decrease (> 95%) of enzymatic activity and the loss of cytotoxicity toward the
103 DET08 strain, one of the few PaeM-susceptible *P. aeruginosa* strains identified at that time
104 (10).

105 More recently, novel PaeM-like pyocins, PaeM_{NCTC10332} and PaeM4, were identified in
106 some *P. aeruginosa* strains (11, 12). PaeM_{NCTC10332} shared 90% similarity with PaeM_{JJ692} and
107 only 20% identity with PaeM4 (13). The three bacteriocins were shown to target different
108 bacterial strains, with PaeM4 having a very broad spectrum of action (12) as compared to the
109 other two PaeM variants produced by strains NCTC10332 and JJ692. PaeM4 was
110 demonstrated to parasitize the HxuC heme receptor (PA1302) of *P. aeruginosa* strain PAO1,
111 while PaeM_{NCTC10332} targeted the FiuA ferrichrome receptor (11, 13). Surprisingly, PaeM
112 from JJ692 did not inhibit at all the growth of PAO1 (6, 12). In the present study, we
113 analyzed the polymorphism of the FiuA receptor in *P. aeruginosa* and demonstrated the
114 essential role it plays in determining the susceptibility or resistance of a panel of strains to the
115 two PaeM_{JJ692} and PaeM_{NCTC10332} pyocin variants (that will be designated here as PaeM1 and
116 PaeM2, respectively). We also localized regions of PaeM pyocins that participate in the
117 recognition of the receptor and the discrimination between the FiuA variants and we
118 demonstrated the essential role that the TonB1 protein plays in the import and the resistance
119 of certain strains to these pyocins.

120

121 RESULTS

122 **Purification and biochemical characterization of PaeM1 and PaeM2 variants.** It was
123 earlier shown that the 80-kb *exoU*-containing genomic island A present in the chromosome of
124 certain pathogenic strains of *P. aeruginosa* (such as JJ692 and 6077) contained a gene
125 (EXA13) encoding a 289-residues protein whose C-terminal domain exhibited significant
126 sequence homology with ColM (14). The corresponding protein (PaeM1) was purified and its
127 peptidoglycan lipid II-degrading activity was shown to be much higher (600-fold higher
128 k_{cat}/K_m ratio) than that of the *E. coli* ColM (2, 10). More recently, a gene was detected in the
129 genome of another *P. aeruginosa* strain, NCTC10332 (also named DSM50071) (11), that
130 encoded a PaeM2 variant displaying 90% sequence identity with PaeM1. In the present study,
131 this gene was cloned in the pET2160 expression vector and the PaeM2 protein was purified
132 to homogeneity in 6 × His-tagged form (calculated molecular weight: 33.2 kDa) with a
133 relatively good yield, *ca.* 3 mg/liter of culture (Fig. S1A). The lipid II-hydrolyzing activity of
134 the PaeM2 variant was confirmed *in vitro* and its specific activity, estimated at 12 ± 0.6
135 nmol/min/mg of protein, was quite similar to that of PaeM1 (13 ± 0.4 nmol/min/mg) when
136 tested in the same assay conditions.

137 As shown in Fig. 1, the amino acid sequences of PaeM1 and PaeM2 mainly differed (23
138 changes) in their N-terminal and central regions (residues 1-130) that are known to be
139 involved in the translocation and reception steps of these bacteriocins, respectively, as
140 established previously for ColM and other colicins (1, 15, 16). Only six differences, in most
141 cases conservative, were found in the C-terminal region corresponding to the activity domain,
142 consistent with the similar specific enzyme activity of the two PaeM variants. Whether the
143 amino acid variations observed in the N-terminal region of these proteins could affect their
144 recognition by the outer membrane receptors and translocation machineries, and thus their
145 antibacterial spectrum, was then questioned (see below).

146 **Antibacterial spectrum of PaeM1 and PaeM2 variants.** We earlier showed that the
147 PaeM1 protein did not exhibit antibacterial activity against *P. aeruginosa* reference strains
148 PAO1 and PA14 but inhibited the growth of two clinical isolates, namely NCK007 and
149 DET08, tested among 14 strains originating from two Paris hospitals (6, 10). PaeM1 was here
150 tested on a higher number of *P. aeruginosa* strains including the reference PAO1 and PA14
151 strains, the susceptible strain DET08, the PaeM1- and PaeM2-producing strains JJ692 and
152 DSM50071, and sixty other clinical isolates listed in Table 1. Only twelve of these strains,
153 other than DET08, appeared to be susceptible to PaeM1 on 2YT medium agar plates when
154 tested using the previously described spot killing assay (6, 10): six were isolated from cystic
155 fibrosis patients (C1-14, C3-20, C4-17, C7-6, C7-18, C10-13), five from burn patients (PcyII-
156 41, PcyII-55, PcyII-64, PcyII-66, PcyII-76) and one from an intensive care unit patient (13i)
157 (Table 1). The minimal concentration of PaeM1 required to observe a growth inhibition zone
158 on bacterial lawns was about 30 ng of PaeM1 per 5 μ l spot for most of the susceptible strains
159 we tested (as shown in Fig. 2A for C4-17 which was chosen here as representative strain). In
160 most cases, the inhibition zones were rather turbid as compared to the very clear zones
161 observed with ColM on *E. coli* cell lawns.

162 The absence of inhibitory effect of PaeM1 on strain PAO1 was rather surprising as the
163 growth of this strain was recently shown to be affected by PaeM2 (11). The aforementioned
164 differences of sequences existing in the N-terminal regions of the two PaeM variants
165 prompted us to analyze the activity spectrum of PaeM2 towards the same panel of *P.*
166 *aeruginosa* strains. As shown in Table 1, many of the strains susceptible to PaeM1 were also
167 inhibited by PaeM2, namely DET08, C1-14, C3-20, C4-17, C7-6, C7-18, C10-13, PcyII-41,
168 PcyII-66, PcyII-64 and PcyII-76. The minimal amount of PaeM2 needed to observe an
169 inhibition zone was 10 fold higher than that of PaeM1, i.e. about 300 ng per spot (as shown in
170 Fig. 2B for the representative strain C4-17). However, two PaeM1-susceptible strains (13i

171 and PcyII-55) appeared resistant to PaeM2, and several strains other than PAO1, that were
172 resistant to PaeM1 but susceptible to PaeM2, were also identified (C1-11, C5-6, C8-15, and
173 PcyII-57) (Table 1).

174 The PaeM1- and PaeM2-producing strains JJ692 and DSM50071 were not at all affected
175 by these two PaeM variants but this was somewhat expected as these strains should be
176 naturally protected from the bacteriocin they produce by co-expressing an immunity protein,
177 as is the case for colicinogenic *E. coli* strains (1). We hypothesized that some of the other
178 resistant strains identified in this work could also be producers of these bacteriocins. A search
179 by polymerase chain reaction (PCR) of PaeM-encoding genes in the chromosome of all these
180 strains was thus performed. Only three strains (14i, 20H and PcyII-48) encoded the PaeM1
181 gene and, as observed for PaeM-producing strains, were resistant to the two PaeM variants
182 (Table 1). In order to increase the chances of finding PaeM1- and PaeM2-producing strains,
183 an *in silico* variable number tandem repeat (VNTR) analysis was performed using available
184 genome sequencing data and the genotype profiles were compared to those of other strains
185 from our collection previously typed with the same markers. Strains 6077, the *exoU* island A-
186 containing strain from which the JJ692 strain derived (14), and DSM50071 were shown to
187 differ for most of the VNTRs analyzed (Fig. 3). Among the VNTR-selected strains, sixteen
188 were positive by PCR for PaeM1 but none of them possessed the PaeM2 gene (Fig. 3). The
189 latter PaeM1 amplicons were sequenced and all of them corresponded to the PaeM1 gene
190 sequence with an identity of 100%.

191 **Correlation between lipid II-degrading and antibacterial activities of PaeM variants.**

192 Point mutations of invariant residues known to play an essential role in the catalytic activity
193 of ColM-like enzymes were introduced in the two PaeM variants and their effects on the
194 enzymatic activity and cytotoxicity of these bacteriocins was tested. We previously showed
195 that mutations to alanine of aspartate residues D241 and D244 of PaeM1 led to proteins

196 exhibiting 0.6% and 4.9% of the enzymatic activity of the wild-type protein, respectively, and
197 that the D241A D244A double mutant was completely inactive both *in vitro* and *in vivo* on
198 strain DET08 (10). These single and double mutants of PaeM1 were now purified (Fig. S1A)
199 and tested on the larger panel of PaeM1-susceptible strains identified in the present study. As
200 shown in Fig. 2A, only the D244A mutant could still inhibit the growth of the representative
201 strain C4-17, but as compared to the wild-type PaeM1, inhibition zones were much more
202 turbid and detected with 100-fold higher amount of the mutant protein. No zone of inhibition
203 was visible when either the D241A mutant or the double mutant was tested in the same
204 conditions (Fig. 2A). In order to demonstrate that the D241 and D244 residues also played an
205 essential role in the lipid II-degrading activity of PaeM2, the PaeM2 D241A D244A mutant
206 protein was generated and purified (Fig. S1A). When this mutant was tested on the different
207 PaeM2-susceptible strains, no zones of growth inhibition were observed, as shown in Fig. 2B
208 for the C4-17 indicator strain. These data clearly confirmed that the cytotoxicity of the two
209 PaeM variants was correlated to their lipid II-degrading activity.

210 **Polymorphism of the PaeM receptor.** As mentioned above, several of the *P. aeruginosa*
211 strains tested in this work were susceptible to either PaeM1 or PaeM2, and some others were
212 inhibited by the two variants (Table 1). The recent demonstration that the import of PaeM2
213 was dependent on the siderophore-binding receptor FiuA (11, 12) strongly suggested that the
214 differences we observed in the antibacterial spectrum of the two PaeM variants could result
215 from modifications of the structure of this receptor. The *fiuA* genes of several strains
216 identified as susceptible to the two PaeM variants (C3-20, C4-17, DET08), or susceptible to
217 PaeM1 only (13i, PcyII-55), PaeM2 only (PAO1, PcyII-57), or none of them (PA14, PcyII-
218 10), were PCR amplified and sequenced. The *fiuA* gene sequence was shown to vary
219 significantly between these strains and to carry several amino acid changes that were mainly
220 localized in the central part of the protein sequence (Fig. 4). In particular, the FiuA receptor

221 from strain PAO1 appeared by five residues larger than that of the PaeM-producing strains
222 (802 *vs* 797 residues), due to insertions of a SYYA motif and an asparagine residue at
223 positions 417-420 and 481 in the FiuA_{PAO1} sequence, respectively (Fig. 4). As shown in Fig.
224 4, twenty-two additional changes were observed between the FiuA_{PAO1} and FiuA_{JJ692} protein
225 sequences, most of them being clustered in the 390-510 central region. However, only six
226 residues differed between FiuA_{JJ692} and FiuA_{DSM50071} receptors of PaeM-producing strains.
227 Interestingly, the PcyII-57 strain which was susceptible to PaeM2 but not PaeM1, as is
228 PAO1, encoded a FiuA protein that was almost 100% identical (only two changes) to that of
229 PAO1 (Fig. 4). In contrast, the FiuA sequence of strains 13i and PcyII-55, mainly susceptible
230 to PaeM1, and that of other clinical strains that were susceptible to the two PaeM variants,
231 such as DET08, C4-17 and C3-20, coded for quite similar FiuA receptors which resembled
232 more that of the DSM50071 and JJ692 strains (Fig. 4). When considering the two PaeM-
233 resistant strains PA14 and PcyII-10, it was noteworthy that the FiuA_{PA14} receptor exhibited
234 only six amino acid changes as compared to FiuA_{JJ692} and eight as compared to FiuA_{DSM50071}
235 (Fig. 4), while the FiuA_{PcyII-10} receptor was significantly different (91% identity).

236 To get an idea of the localization of these different amino acid changes in the 3D structure
237 of the receptor, a structural model of FiuA_{PAO1} was predicted using Phyre2 (17) and the
238 previously elucidated crystal structures of the FpvA (18) and FptA (19) siderophore receptors
239 which exhibit 35% and 25% sequence identity with FiuA, respectively. The resulting
240 structure appeared to be constituted of a transmembrane 22-stranded β -barrel domain
241 occluded by an N-terminal four-stranded β -sheet domain, the plug (Fig. 5). The β -strands of
242 the barrel were connected by short periplasmic turns and long extracellular loops.
243 Interestingly, most of the differences observed when comparing FiuA_{PAO1} to FiuA receptors
244 of strains that are susceptible to the two PaeM variants were localized within the two
245 extracellular loops L4 and L5, suggesting that these regions might determine the specificity

246 to PaeM1 and PaeM2 pyocins (Fig. 5). Except for differences in the amino acid sequences at
247 position 412-413 and 486 in FiuA_{DSM50071}, it was noteworthy that the few changes that
248 differentiated the FiuA_{PA14} receptor from its FiuA_{JJ692} and FiuA_{DSM50071} orthologs were
249 localized on the β -barrel domain and on the short periplasmic turns, i.e. in regions that are not
250 expected to directly interact with these pyocins (Fig. 5).

251 **Correlation between the FiuA structure and the bacterial susceptibility to PaeM**
252 **variants.** The impact of the variability of the FiuA sequence on the susceptibility of *P.*
253 *aeruginosa* strains to PaeM variants was then examined as follows. The different *fiuA* gene
254 orthologs mentioned above were cloned in the pUCP24Nco *E. coli/P. aeruginosa* shuttle
255 vector and these plasmids were introduced into the PAO1 strain, i.e. a reference strain
256 susceptible to PaeM2 only (Fig. 6A). The resulting transformants were then tested for their
257 susceptibility toward the two PaeM variants. First, we observed that the PAO1 strain became
258 much more susceptible to PaeM2 when transformed by the pUCP24Nco plasmid carrying its
259 own *fiuA*_{PAO1} gene (Fig. 6B), confirming that FiuA is indeed the receptor of this specific
260 bacteriocin. It also showed that the strain susceptibility can be significantly enhanced by
261 increasing the number of copies of this receptor per cell. The latter strain, however, remained
262 totally resistant to the PaeM1 variant. In contrast, the PAO1 strain became susceptible to the
263 PaeM1 variant following expression of the FiuA receptor from strain 13i (Fig. 6C), i.e. a
264 strain susceptible to PaeM1 only. Consistently, its susceptibility to PaeM2 was not
265 significantly increased in that case. Interestingly, expression of *fiuA* genes from the two
266 PaeM-resistant strains PcyII-10 and PA14 led to distinct phenotypes: while expression of
267 FiuA_{PcyII-10} did not increase the sensitivity of the PAO1 strain to PaeM variants (Fig. 6E),
268 expression of FiuA_{PA14} rendered this strain susceptible to PaeM1 and significantly increased
269 its susceptibility to PaeM2 (Fig. 6F). That the FiuA_{PcyII-10} receptor could not allow
270 internalization of these bacteriocins into cells, at least in PAO1 background, was consistent

271 with the resistant phenotype of the PcyII-10 strain. However, the demonstration that the
272 FiuA_{PA14} receptor was perfectly functional in this context, allowing PaeM1 variant to be
273 internalized and to exert its deleterious effects in the PAO1 strain, was rather surprising and
274 suggested that other factors may explain the natural resistance of the PA14 strain to this
275 bacteriocin. How the PA14 strain and the PaeM-producing strains resist to these pyocins and
276 what are the molecular determinants of the selective susceptibility of PAO1 strain to the
277 PaeM2 variant was thus investigated in more detail, as reported below.

278 **Elucidating the resistance of PaeM-producing cells.** In order to test if PaeM-producing
279 cells possessed a functional FiuA receptor, the *fiuA* genes of JJ692 and DSM50071 strains
280 were cloned into the pUCP24Nco vector and then expressed in the PAO1 background. As
281 shown in Fig. 6G, PAO1 strain expressing the *fiuA*_{JJ692} gene exhibited an increased
282 susceptibility to PaeM2 but, more importantly, was now inhibited by the PaeM1 variant too.
283 Expression of the *fiuA* gene from the DSM50071 strain provided similar results, with a
284 susceptibility acquired by the strain towards the two PaeM variants that was even increased
285 (Fig. 6H). These findings demonstrated that the strains JJ692 and DSM50071 encoded FiuA
286 receptors that were functionally active and capable of internalizing their own PaeM proteins.
287 The resistance mechanism thus occurred at a later step, after PaeM internalization. Generally,
288 bacteriocin-producing strains concomitantly express immunity proteins that protect them
289 from the deleterious activity of these toxins. The PaeM1-encoding gene was first identified in
290 the genome of some clinical strains of *P. aeruginosa* (JJ692, 6077), within the horizontally-
291 acquired and virulence determinant *exoU*-containing island A (6, 14). This gene named
292 EXA13 was followed by a short open reading frame of unknown function (EXA14)
293 theoretically coding for a 142-residue membrane protein displaying four predicted
294 transmembrane helices. By analogy with colicinogenic strains that express both colicin and
295 immunity genes close together on plasmids, we earlier hypothesized that EXA14 should code

296 for the immunity protein allowing self-protection of PaeM1-producing strains (6). In order to
297 definitively demonstrate the immunity function of this gene, EXA14-like genes were
298 amplified from several relevant *P. aeruginosa* strains and subsequently cloned in the pUCP24
299 vector. An alignment of the amino acid sequences of the putative immunity proteins from
300 strains JJ692 and DSM50071, that will be named here ImM1 and ImM2, respectively,
301 showed 84% sequence identity (Fig. 7A). Based on this homology and their similar
302 membrane topology (predicted), these proteins were recently classified as integral membrane
303 proteins belonging to the PmiA group by Ghequire and coworkers (20) and these authors
304 showed that PmiA_{NCTC10332} (named here ImM2) provided immunity against PaeM_{NCTC10332}
305 (PaeM2) to a susceptible *P. aeruginosa* strain (CPHL 12447). The capability of ImM1 and
306 ImM2 proteins to protect cells from the deleterious effects of the PaeM pyocins was here
307 tested using the C4-17 strain that is susceptible to both PaeM variants as indicator strain.
308 Transformation of this strain by the ImM1-expressing pUCP24 plasmid allowed it to resist
309 both PaeM1 and PaeM2 (Fig. 7B). The ImM2 gene was similarly cloned and its expression
310 totally protected C4-17 strain against PaeM1 and PaeM2, consistent with recent data from
311 Ghequire et al. (20). The occurrence of these immunity genes was then investigated by PCR
312 in the *P. aeruginosa* strain collection shown to be naturally resistant to the PaeM variants
313 (Table 1). The ImM1 gene was detected in only three strains, 14i, 20H and PcyII-48, and was
314 in all cases coupled with the PaeM1 gene (Table 1). As for PaeM genes, the search of ImM1
315 gene was also done in VNTR-selected strains. This gene was detected in sixteen out of thirty-
316 three genomes screened and shown in all cases to be associated with the cognate PaeM1 gene
317 (Fig. 3). No strain other than DSM50071 (alias NCTC10332) was shown to express the
318 ImM2 and/or PaeM2 gene in the collection of PaeM-resistant strains tested here.

319 **Deciphering PA14 strain resistance to PaeM pyocins.** As about two thirds of the *P.*
320 *aeruginosa* strains tested in this work, the reference strain PA14 appeared resistant to both

321 PaeM variants. Curiously, only few amino acid changes were observed when the FiuA
322 protein sequence of this strain was compared to that of PaeM-susceptible strains (Fig. 4). The
323 fact that the PAO1 strain became susceptible to PaeM1 following expression of the FiuA_{PA14}
324 receptor clearly indicated that the FiuA_{PA14} receptor was functional and that the latter few
325 amino acid changes did not abolish its capability to bind and internalize this pyocin. To
326 further identify the molecular determinants of PA14 resistance, this strain was transformed by
327 plasmids expressing either the *fiuA*_{DSM50071} gene or its own *fiuA*_{PA14} gene, i.e. two plasmids
328 conferring susceptibility to PaeM variants to the PAO1 strain. As shown in Fig. 8, PA14 cells
329 expressing the FiuA_{DSM50071} receptor became susceptible to the two PaeM variants,
330 suggesting that PA14 resistance resulted from a defective reception step, but surprisingly and
331 in contrast to what was observed in the PAO1 background (Fig. 6F), PA14 cells expressing
332 the FiuA_{PA14} receptor on the multi-copy plasmid remained totally resistant to these pyocins
333 (Fig. 8). That the PaeM-receptor activity of FiuA_{PA14} could be visualized in the PAO1 but not
334 the PA14 genetic background made us envisaging that some other steps needed by these
335 pyocins to get access to the lipid II target might not be operational in the PA14 strain.

336 **PaeM import and strain susceptibility depend on TonB1.** The above results prompted
337 us to analyze whether PAO1 and PA14 strains could also exhibit some differences in the
338 subsequent steps allowing import of these pyocins in the periplasm. As many other bacterial
339 pathogens, *P. aeruginosa* developed efficient systems to retrieve and transport iron from
340 natural sources. This is in particular achieved through the expression and release of two Fe³⁺-
341 chelating siderophores, pyochelin and pyoverdine, which are subsequently re-imported into
342 cells via the cognate FptA and FpvA outer membrane receptors, respectively (21). The latter
343 translocation event requires both the proton motive force and the TonB-ExbB-ExbD protein
344 machinery acting as energy coupler (22). Contrary to *E. coli* which possesses only one TonB
345 protein, *P. aeruginosa* expresses three *tonB* genes (*tonB1*, *tonB2* and *tonB3*) and *tonB1* was

346 shown to play the main role in the uptake of these siderophores (23-25). Whether the TonB1
347 protein was also responsible for the FiuA-dependent uptake of PaeM variants and could
348 eventually play a role in the PA14 strain resistance to these pyocins was thus tested. We
349 showed that a *tonB1*-null mutant of PAO1 was totally resistant to PaeM2 and that its
350 susceptibility to the pyocin was restored following transformation by a *tonB1*_{PAO1}-expressing
351 pUCP24 plasmid (Fig. 9). Furthermore, over-expression of different FiuA receptor orthologs
352 such as FiuA_{DSM50071} in the PAO1 Δ *tonB1* mutant did not change the resistant phenotype of
353 this strain (Fig. 9). These results clearly demonstrated that the TonB1 protein was absolutely
354 required for the import of PaeM pyocins into *P. aeruginosa* cells.

355 Then, an alignment of *tonB1* gene sequences from various *P. aeruginosa* strains revealed
356 that the TonB1 protein sequence also displayed some variability in this bacterial species. In
357 particular, the TonB1 protein from PA14 strain exhibited some specific differences as
358 compared to the others, differing from the TonB1_{PAO1} ortholog by eight amino acid changes
359 located in the C-terminal protein moiety (Fig. 10A). That these few changes could potentially
360 affect the capability of TonB1 to mediate PaeM import into PA14 cells was thus envisaged.
361 Transformation of the PaeM-resistant PA14 strain by a pUCP24 plasmid expressing the
362 *tonB1*_{PAO1} gene made this strain becoming slightly susceptible to PaeM1 (Fig. 10B). This
363 phenotypic change was not observed when the *tonB1*_{PA14} gene instead was present on the
364 plasmid (Fig. 10B), supporting the hypothesis that the few differences observed in the
365 TonB1_{PA14} protein structure played a role in the PA14 strain resistance. Interestingly,
366 however, the same *tonB1*_{PA14}-expressing pUCP24 plasmid restored the susceptibility of the
367 PAO1 *tonB1* mutant to the PaeM2 pyocin (Fig. 10C), demonstrating that the TonB1 protein
368 from the PA14 strain was functional in the PAO1 background. Moreover, we found that the
369 co-expression of the two FiuA_{PA14} and TonB1_{PAO1} genes on the same pUCP24 vector
370 rendered the PAO1 *tonB1* mutant susceptible to both the PaeM1 and PaeM2 variants, while

371 the co-expression of the FiuA_{PA14} and TonB1_{PA14} gene couple made it susceptible essentially
372 to PaeM2 (Fig. 10C). This result clearly showed that the FiuA_{PA14} protein can cooperate with
373 the TonB1_{PAO1} protein, but not with the TonB1_{PA14} protein, for internalization of PaeM
374 pyocins, thus explaining the PA14 strain resistance. The FiuA_{PAO1} receptor was
375 comparatively less restrictive, accepting both TonB1_{PAO1} and TonB1_{PA14} proteins for this
376 purpose.

377 **Deciphering PAO1 strain resistance to the PaeM1 variant.** The FiuA receptor of the
378 PAO1 strain could efficiently discriminate between the two PaeM variants as this strain was
379 susceptible only to PaeM2 and became susceptible to PaeM1 too following expression of
380 some other FiuA orthologs such as FiuA_{DSM50071} (Fig. 6H). As mentioned above and shown
381 in Fig. 4, the alignment of FiuA protein sequences from PAO1 and DSM50071 strains
382 revealed 29 differences, several being clustered within the same ALTPASYAAASD motif
383 localized in the central protein region (residues 412-424 in FiuA_{PAO1}). In FiuA_{DSM50071}, the
384 corresponding sequence appeared as GITAGTAHA and was by 4 residues shorter (deletion
385 of the SYYA motif). Whether the variability of this particular sequence could impact the
386 receptor selectivity and host strain susceptibility towards the two PaeM variants was then
387 investigated. A modified version of the FiuA_{PAO1} receptor in which the ALTPASYAAASD
388 motif was replaced by GITAGTAHA was generated by site-directed mutagenesis. As shown
389 in Fig. 6D, PAO1 cells expressing this modified version of the receptor (designated as
390 FiuA_{delPAO1}) were now susceptible to both PaeM variants, as this was the case when they
391 expressed the FiuA_{DSM50071} receptor. This finding clearly demonstrated the essential role
392 played by this particular motif in the capability of the FiuA_{PAO1} receptor to discriminate
393 between the two PaeM variants. The localization of this motif within one of the two
394 externally exposed loops in the predicted FiuA structure (loop 4 in Fig. 5) was perfectly
395 consistent with the phenomena observed.

396 **Chimeric PaeM proteins exhibit a modified antibacterial spectrum.** As reported
397 above, the amino acid sequences of the PaeM1 and PaeM2 variants differed mainly in the N-
398 terminal moiety of these proteins (Fig. 1), i.e. the region that based on the knowledge
399 previously acquired on colicins, should specifically interact with the receptor and/or the
400 translocation machinery (1). The above demonstration that the PAO1 strain became
401 susceptible to PaeM1 either following expression of FiuA receptors from some other strains
402 (JJ692 or DSM50071), or appropriate modification of its own FiuA_{PAO1} receptor, showed that
403 the FiuA receptor structure was the main factor allowing cells to discriminate between the
404 PaeM variants. To further identify the molecular determinants responsible for the strict
405 specificity of the FiuA_{PAO1} receptor for PaeM2 and the recognition between FiuA receptors
406 and PaeM variants in general, a series of chimeric PaeM1/PaeM2 proteins were constructed
407 in which N-terminal sequences of different lengths of the PaeM1 protein were replaced by
408 the corresponding sequences from PaeM2 (Fig. 11A). The first three PaeMchim1, 2 and 3
409 proteins consisted in fusions of the 1-48, 1-110 and 1-129 first residues of PaeM2 to the 49-
410 289, 111-289 and 130-289 sequences of PaeM1, respectively. These junctions were chosen
411 based on PaeM sequences alignment which revealed some hot spots of divergence between
412 the two variants (Fig. 1). As we previously showed that the C-terminal and independently-
413 functional activity domain of PaeM1 started around residues 127-134 (10), the PaeMchim3
414 construct thus theoretically contained the complete N-terminal reception/translocation
415 domain of PaeM2 fused to the C-terminal activity domain of PaeM1. These chimeric proteins
416 were expressed in *E. coli* cells, purified (Fig. S1B) and then tested on the PAO1 strain
417 expressing either of the three versions of FiuA receptor (FiuA_{PAO1}, FiuA_{JJ692} and
418 FiuA_{DSM50071}). As shown in Fig. 11B, the PAO1 strain expressing its own receptor, FiuA_{PAO1},
419 was not at all affected by PaeM1 or PaeMchim1, but turbid inhibition zones were observed
420 on the bacterial lawns when either PaeM2 or the hybrid proteins PaeMchim2 and PaeMchim3

421 were spotted. This demonstrated the functionality of these chimeric proteins and the ability of
422 the N-terminal domain of PaeM2 to allow internalization of the PaeM1 activity domain in
423 PAO1 strain. The first 48 residues of PaeM2 were apparently not sufficient to allow
424 parasitizing of FiuA_{PAO1} receptor by PaeM1. The 1-110 PaeM2 sequence could fulfill this
425 role but its efficiency was less than that of the full-length 1-129 putative
426 translocation/reception domain, indicating that the 117-128 PaeM2 sequence that is
427 particularly divergent between the two variants (Fig. 1) was crucial for an optimal binding of
428 PaeM1 to this receptor. This was consistent with the general concept that the central domain
429 of colicins and related bacteriocins is more specifically involved in this first step of reception
430 on the outer membrane of targeted cells (1). The same chimeric proteins were then tested on
431 PAO1 strains expressing the FiuA_{JJ692} or FiuA_{DSM50071} receptor. All of them inhibited growth
432 of these two strains as efficiently as the original PaeM1 and PaeM2 proteins (Fig. 11B),
433 confirming that FiuA receptors from the JJ692 and DSM50071 strains were less selective
434 than their FiuA_{PAO1} homolog. Four additional chimeric proteins PaeMchim4 to PaeMchim7
435 were designed and purified (Fig. S1B) which carried different other combinations of motifs
436 found in the PaeM1 and PaeM2 N-terminal domains. None of them inhibited growth of
437 PAO1 cells, or only very poorly for PaeMchim6, but all were active on the strain expressing
438 the FiuA_{DSM50071} receptor. Among them, only the PaeMchim7 protein exhibited a potent
439 activity on the PAO1 strain expressing the FiuA_{JJ692} ortholog, as did the PaeMchim1-3
440 chimeric proteins. The impact of these N-terminal protein sequence modifications on the
441 cytotoxic activity of the pyocins could thus vary significantly, depending on the strain and the
442 FiuA receptor considered. Finally, an eighth chimeric PaeM1/PaeM2 protein was constructed
443 in which the complete N-terminal translocation/reception domain of PaeM1 was fused this
444 time to the C-terminal activity domain of PaeM2. This PaeMchim8 protein behaved exactly

445 as the PaeM1 variant, i.e inhibited growth of PAO1 only when this strain expressed FiuA
446 receptors from PaeM1-susceptible strains (Fig. 11B).

447 All these data clearly highlighted the essential role played by the N-terminal domains of
448 PaeM proteins in the recognition of their specific FiuA receptors and in the determination of
449 the antibacterial spectrum of these bacteriocins.

450

451 **DISCUSSION**

452 Colicin M and its recently identified orthologs from *Pseudomonas* and *Pectobacterium*
453 species form a unique family of bacteriocins interfering with the integrity of the cell wall by
454 enzymatic degradation of peptidoglycan lipid intermediates (8, 9, 26). They exert their
455 deleterious activity in the periplasm of the targeted cells, *i.e.* the compartment where the
456 peptidoglycan lipid II precursor is translocated and remains transiently accessible before
457 being used for polymerization steps by the penicillin-binding proteins. The only physical
458 barrier they have to cross is the outer membrane of susceptible cells and their antibacterial
459 activity and spectrum will thus mainly depend on the presence or absence of the adequate
460 receptors and translocation machineries at the cell surface. ColM is known to be internalized
461 via the iron-siderophore transporter FhuA and the TonB-ExbB-ExbD import machinery (1, 4)
462 but how this colicin is released in the growth medium by the producing strains remains
463 unknown. Interestingly, expression of the ColM-encoding gene in *E. coli* cells was shown to
464 be toxic unless (i) the Cmi immunity protein was present or (ii) the FhuA or TonB system
465 proteins were absent (2). The latter finding demonstrated that the internally produced ColM
466 molecules should be first released in the external medium and then re-internalized through
467 FhuA to exhibit their cytotoxic effect. It also showed that the FhuA receptor of ColM-
468 producing strains remained “susceptible” to ColM and did not acquire any specific mutation
469 contributing to the cell immunity. As colicins are plasmid-encoded toxins, the colicinogenic

470 strains carrying these plasmids are self-protected by the encoded immunity proteins and
471 acquisition of these plasmids by other strains do not require selection of additional mutations
472 in their import systems. The situation is similar for PaeM-producing *P. aeruginosa* strains,
473 although the pyocin-encoding genes are located in the chromosome in that case. We here
474 showed that the FiuA receptors from PaeM-producing strains were functionally active and
475 able to internalize their own PaeM pyocins. Previous studies already showed that the PaeM2
476 variant from strain NCTC10332 (also named DSM50071), but not the PaeM_{JJ692} variant
477 (PaeM1), formed growth inhibition zones on wild-type PAO1 lawns (12). It was also reported
478 that the deletion of the FiuA receptor gene conferred resistance to PaeM2 in *P. aeruginosa*
479 PAO1 and to the chimeric bacteriocin PmnH in *P. fluorescens* LMG 1794 (11). Given the
480 high similarity between PaeM1 and PaeM2, it was rather surprising that the PaeM1 variant
481 produced by strain JJ692 did not target the same receptor as PaeM2 in *P. aeruginosa* PAO1.

482 In fact, we here demonstrated that the antibacterial spectrum of PaeM1 and PaeM2
483 bacteriocins was linked to the polymorphism of the FiuA receptor. Either of the two FiuA_{JJ692}
484 and FiuA_{DSM50071} receptors from PaeM1- and PaeM2-producing strains, respectively, allowed
485 internalization of both PaeM variants into PAO1 cells, consistent with the finding that
486 clinical strains susceptible to these two bacteriocins (DET08, C3-20 and C4-17) expressed a
487 receptor of this type. At least for the strains analyzed in the present work, expression of a
488 PAO1-type FiuA receptor was shown to be associated with PaeM2 susceptibility and PaeM1
489 resistance. It suggested that some of the few differences observed between the amino acid
490 sequences of these receptors were responsible for the lack of cytotoxicity of PaeM1 on PAO1
491 cells. The essential role played in this discrimination by the small region carrying a 4-residue
492 insertion (SYYA) that is localized on one of the extracellular loops of the FiuA_{PAO1} receptor
493 was then highlighted. Indeed, replacement of the ALTPASYAAASD motif from FiuA_{PAO1}
494 receptor by the shorter GITAGTAHA motif found at the same position in the FiuA_{DSM50071}

495 receptor sequence was sufficient to render PAO1 cells susceptible to both PaeM1 and PaeM2
496 variants. A different situation was encountered when analyzing the resistance of the reference
497 strain PA14. The FiuA receptor of this strain, which did not differ much from its FiuA_{JJ692}
498 and FiuA_{DSM50071} orthologs, failed to mediate PaeM internalization into PA14 cells while it
499 was perfectly functional in this respect when expressed in other genetic backgrounds such as
500 PAO1. This prompted us to identify which step downstream of the FiuA reception step
501 determined PA14 strain resistance to PaeM pyocins. As is the case for the ColM receptor
502 FhuA in *E. coli*, the *P. aeruginosa* receptor FiuA interacts with the TonB/ExbB/ExbD protein
503 complex to translocate siderophores into cells. In contrast to *E. coli*, *P. aeruginosa* possesses
504 three TonB proteins designated TonB1, TonB2 and TonB3. First, testing the susceptibility to
505 PaeM variants of PAO1 wild-type and *tonB1* mutant strains expressing different FiuA
506 receptors allowed us to demonstrate that the TonB1 protein was absolutely required for PaeM
507 import into *P. aeruginosa* cells. An alignment of TonB1 protein sequences from PAO1,
508 PA14 and some other strains tested in this study revealed that curiously only the TonB1_{PA14}
509 protein displayed some specific differences. We thus tested expression of the *tonB1*_{PAO1} gene
510 in the PA14 strain and found that this rendered the strain susceptible to PaeM pyocins, which
511 was consistent with the previous observation that the FiuA_{PA14} receptor was functional, i.e.
512 allowed internalization of both PaeM variants, when expressed in PAO1 strain. These results
513 suggested that FiuA_{PA14} and TonB1_{PAO1} proteins can interact together and conjointly
514 contribute to PaeM import into PA14 cells, contrary to the FiuA_{PA14}-TonB1_{PA14} couple. That
515 the expression of FiuA_{PAO1}, FiuA_{DSM50071} and some other receptors (except FiuA_{PA14}) also
516 made the PA14 strain becoming PaeM-susceptible suggested that the TonB1_{PA14} protein can
517 interact with these receptors. These different results thus highlighted the essential role played
518 by the TonB1 protein in the import of this class of pyocins and revealed that discrete
519 variations of FiuA or TonB1 sequences could impact their interaction and be responsible for

520 the loss of susceptibility of certain strains, as demonstrated here for PA14. Our data thus
521 strongly suggest that the changes observed in the periplasmic turns and β -barrel structure of
522 FiuA_{PA14}, compared to PaeM-susceptible receptors such as FiuA_{JJ692} or FiuA_{DSM50071}, are
523 responsible for this misrecognition of TonB1_{PA14} protein and thus for the impossibility for the
524 pyocins to be translocated to the PA14 periplasm.

525 It was noteworthy, when considering the already known lipid II-targeting bacteriocins,
526 that not all the cognate immunity partners were identified to date. The 3D crystal structure of
527 the Cmi protein that confers immunity to ColM-producing strains was previously determined
528 but its mode of action still remains to be elucidated (27). Immunity against *Pseudomonas*
529 ColM-like bacteriocins was predicted to be provided by integral membrane proteins
530 displaying four transmembrane helices that were named PmiA by Ghequire and coworkers
531 (20). Based on the position of its gene that is immediately distal to the PaeM1 gene, its
532 similarity (86% identity) with the already characterized (not annotated) immunity protein of
533 PaeM_{NCTC10332} (identical to PaeM_{DSM50071}), and its predicted C_{in}-N_{in} topology with four
534 transmembrane domains that is characteristic of immunity proteins belonging to the PmiA
535 group, we hypothesized and here demonstrated that ImM1 was the cognate immunity partner
536 of PaeM1 produced by *P. aeruginosa* strain JJ692. Considering the high level of sequence
537 similarity existing between the PaeM1 and PaeM2 proteins, as well as between the ImM1 and
538 ImM2 proteins, the complete or partial cross-resistance conferred to PaeM-susceptible strains
539 by these two immunity proteins was not so surprising.

540 The PaeM1-ImM1 gene couple was detected in about half of the strains selected by
541 VNTR-similarity pattern to the PaeM-producing strains. Interestingly, most of the PaeM1-
542 producing strains identified in this study belonged to the clonal complex CC235 as deduced
543 from VNTR genotyping (28) (Fig. 3). It is known that *P. aeruginosa* has mostly a non-clonal
544 population structure, but multi-locus sequence typing (MLST) revealed that a few sequence

545 types (STs) were worldwide distributed and designated as high-risk clones. One of these, the
546 ST235, was frequently associated with outbreaks and poor clinical outcomes (29-31). Strains
547 belonging to ST235 were resistant to fluoroquinolones, aminoglycosides and β -lactams and
548 were characterized by the presence of virulence factors such as a type III secretion system in
549 their chromosomes (14, 32). We looked at some of the publicly available genomes belonging
550 to ST235 (NCGM1900, NCGM1984, PB350, PB367, E6130952) and found that in all cases
551 the PaeM1-ImM1 gene couple was located in the 80-kb *exoU*-containing island A, as was the
552 case for *P. aeruginosa* strains JJ692 and 6077 (14). In contrast, none of the strains used in
553 this study carried the PaeM2-ImM2 gene couple. When a Blast was run using the sequence
554 covering the latter gene couple, only two hits matched with 100% identity, *P. aeruginosa*
555 strains DSM50071 and NCTC10332, which in fact are the same strain, suggesting that this
556 variant is very rare because it may have been derived by an horizontal gene transfer event
557 restricted to the sequence covering the PaeM-ImM gene couple.

558 It is well-known that *P. aeruginosa* relies on iron as a nutrient source and uses secreted
559 siderophores such as ferrichrome and ferrioxamine B to regulate its uptake (21). In *P.*
560 *aeruginosa*, the iron-siderophore complexes are transported across the outer membrane
561 primarily (80%) by the FiuA receptor and to a lesser extent (20%) by a secondary transporter
562 (27). The ability of PaeM variants to inhibit the bacterial growth may also be linked to the
563 expression levels of the corresponding target receptor. It is known that the FiuA receptor is
564 specifically induced under iron-restricted conditions in the presence of the ferrichrome
565 siderophore (28). This and the observation that PaeM2 produced clear inhibition zones on
566 PAO1 overexpressing its own FiuA receptor led us to think that bacteriocins ability to adsorb
567 on the bacterial surface and efficiently inhibit the bacterial growth not only depends on the
568 specific structure of the receptor but also on its expression level. This can be one of the
569 reasons why many of the clinical strains tested here were resistant to the two PaeM variants.

570 Alternatively, these strains may simply express a highly polymorphic FiuA receptor or a
571 TonB1_{PA14}-like protein. Further investigations are needed to decipher the corresponding
572 resistance mechanisms.

573 Our data highlighted the essential role played by the polymorphism of the FiuA receptor
574 and also by the TonB1 protein in the susceptibility of *P. aeruginosa* strains to the Paem
575 pyocins. Only a few amino acid changes in these proteins as well as in the Paem pyocin were
576 sufficient to modify the susceptibility of the strains and their selectivity towards the two
577 Paem variants. The data thus generated will not only contribute to our general knowledge on
578 the *P. aeruginosa* physiology but they will also certainly provide new insights on how these
579 different bacteriocins could potentially be exploited as an alternative to antibiotics for the
580 control of this pathogenic species which remains one of the leading causes of mortality from
581 nosocomial infections worldwide.

582

583 MATERIALS AND METHODS

584 **Bacterial strains and growth conditions.** *E. coli* DH5 α (Life Science Technologies,
585 Inc.) was used as the host strain in cloning experiments and the BL21(DE3)(pLysS) strain
586 (Novagen) was routinely used for the overproduction of Paem bacteriocins with pET
587 plasmids. Other strains used in this study are listed in Table 1. Clinical *P. aeruginosa* strains
588 were isolated from cystic fibrosis (CF) patients or from patients in intensive care units (ICU)
589 with different infection sites. Some of them were previously described by Llanes et al. (33)
590 and Larché et al. (28). The PAO1 *tonB1*-null mutant was obtained from Dr. Paolo Visca (34).
591 2YT rich medium (35) was used for growing cells and growth was monitored at 600 nm with
592 a Shimadzu UV-1601 spectrophotometer. For the preparation of plates, 2YT was
593 supplemented with agar (Sigma-Aldrich) at 1.6% (w/v), and 0.7% for the top layer. When
594 required, ampicillin, kanamycin, chloramphenicol and gentamicin were added at final

595 concentrations of 100, 50, 25 and 10 µg/ml, respectively. For *P. aeruginosa* strains, the
596 gentamicin concentration used varied from 10 to 50 µg/ml.

597 **General DNA techniques, plasmids and cell transformation.** PCR amplification of
598 genes from *E. coli* and *P. aeruginosa* chromosomes was performed in a Thermocycler 60
599 apparatus (Bio-med) using the Expand-Fidelity polymerase (Roche) and DNA fragments
600 were purified using the Wizard PCR Preps DNA purification kit (Promega). The standard
601 procedures for endonuclease digestions, ligation and agarose gel electrophoresis described by
602 Sambrook et al. were used (36). The pET2160 vector allowing high level expression of
603 proteins with a C-terminal 6 × His-tag was previously described (6), as well as the multi-copy
604 *E. coli-P. aeruginosa* shuttle vector pUCP24 (37). The pET2160-derivative plasmid
605 pMLD245 used for expression and purification of the Paem bacteriocin from strain JJ692
606 (Paem1) was previously described (6). Plasmids pTTB240, pTTB243 and pTTB38
607 expressing D241A, D244A, and D241A D244A mutants of Paem1, respectively, were
608 generated by mutagenesis of pMLD245 plasmid (10). *E. coli* cells were transformed with
609 plasmid DNA either by treatment with calcium chloride as described by Dagert and Ehrlich
610 (38) or by electroporation. Transformation of *P. aeruginosa* cells was done by
611 electroporation (39). Site-directed mutagenesis was performed by using the Quickchange II
612 XL site-directed mutagenesis kit (Stratagene) and appropriate oligonucleotides listed in Table
613 S1. All of the mutations introduced in plasmids were confirmed by DNA sequencing
614 (Eurofins).

615 **VNTR analysis.** Strains were genotyped using the multiple locus variable number of
616 tandem repeat (VNTR) analysis (MLVA) technique (40). It consists in the PCR amplification
617 and analysis of 15 VNTRs whose size varies in the different strains. The number of repeats at
618 each locus provides a code which is then used to perform a clustering analysis using the
619 Unweighted Pair Group Method with Arithmetic mean -UPGMA- algorithm. Strains

620 differing at a maximum of 3 loci are clustered into clonal complexes (CC). The CC number
621 corresponds to the multiple locus sequence typing (MLST) number.

622 **Construction of PaeM, ImM, FiuA and TonB1 expression plasmids.** The gene
623 encoding the PaeM bacteriocin homolog from *P. aeruginosa* strain DSM50071 (PaeM2) was
624 PCR-amplified using PaeM2-for and -rev oligonucleotides shown in Table S1 that introduced
625 NcoI and BglII restriction sites at the 5' and 3' gene extremities, respectively. The PCR
626 fragment was digested by these enzymes and then cloned between the same sites of the
627 pET2160 vector, resulting in plasmid pLL17 that allows expression of the 289-residues
628 PaeM2 protein with a C-terminal Arg-Ser-(His)₆ extension. A pLL42 derivative plasmid
629 expressing a D241A D244A mutated version of this PaeM2 protein was generated by site-
630 directed mutagenesis of pLL17 using the two oligonucleotides PaeM2-mut1 and -mut2
631 described in Table S1. The putative PaeM1 immunity gene (EXA14, here designated as
632 ImM1) that is located within the *exoU*-containing island A of *P. aeruginosa* JJ692
633 chromosome (6, 14) and its homolog (ImM2) detected in the genome of strain DSM50071
634 were PCR-amplified using the ImM-for and -rev primers that introduced BamHI and HindIII
635 restriction sites at the gene extremities, respectively (Table S1). These two genes were cloned
636 between the same sites of the pUCP24 *E. coli*-*P. aeruginosa* shuttle plasmid, yielding pLL18
637 and pLL19 plasmids, respectively. To optimize gene expression from the pUCP24 vector and
638 further increase the number of restrictions sites present in the polylinker sequence, a NcoI site
639 was introduced in this plasmid so that ATG initiation codons of the cloned genes were placed
640 exactly at the position of the initiation codon of the *lacZ* alpha peptide gene. This modified
641 vector pUCP24Nco was obtained by site-directed mutagenesis of pUCP24 using the two
642 pUCP24-mut1 and -mut2 primers (Table S1). Seven chimeric PaeM proteins were also
643 generated in which sequences of different sizes of PaeM1 N-terminal region were replaced by
644 corresponding sequences originating from the PaeM2 protein (as depicted in Fig. 11A). The

645 genes encoding these PaeMchim1 to PaeMchim7 proteins were generated by a two-step PCR
646 procedure using the PaeMchim-for and -rev oligonucleotides indicated in Table S1. In the
647 case of PaeMchim1 for instance, two gene fragments were first amplified: one fragment
648 using PaeM2-for and PaeMchim1-rev primers and the pLL17 plasmid as template and a
649 second fragment using the PaeMchim1-for and T7term primers and the pMLD245 plasmid as
650 template. These two fragments were purified, hybridized together, and a third PCR was
651 performed using this mixture as template and the oligonucleotides PaeM2-for and T7term as
652 primers. The resulting fragment was then purified, treated by NcoI and HindIII and inserted
653 in the pET2160 vector, yielding the pMLD601 plasmid. The same procedure was used for
654 generating the PaeMchim2 to PaeMchim7-expressing plasmids (pMLD602 to 605, 618 and
655 619, respectively). An eighth chimeric protein (PaeMchim8, pMLD627 plasmid) was also
656 generated in which the N-terminal region of PaeM1 (residues 1-129) was fused to the C-
657 terminal activity domain of PaeM2 (residues 130-289), i.e. an inverse configuration
658 compared to PaeMchim3 (see Fig. 11A). The *fiuA* genes encoding PaeM outer-membrane
659 receptor variants were amplified from different *P. aeruginosa* strains, namely PAO1, JJ692,
660 DSM50071, PA14, PcyII-10, 13i and PcyII-55 using the FiuA-for and FiuA-rev
661 oligonucleotides shown in Table S1. The resulting DNA fragments were cut by NcoI and
662 HindIII and inserted between the same sites of the pUCP24Nco vector, yielding the
663 pMLD599, 596, 598, 622, 626, 628 and 629 plasmids, respectively. A similar plasmid,
664 pMLD623, expressing a FiuA_{PAO1} mutant protein in which the ALTPASYAAASD
665 sequence (residues 412-424) was replaced by the smaller GITAGTAHA motif found in FiuA
666 receptors of some other strains such as DSM50071 (see Fig. 4), was created by PCR using
667 the FiuAdel-for and FiuAdel-rev oligonucleotides shown in Table S1. Plasmids expressing
668 the TonB1 protein that is required for the FiuA-dependent import of PaeM bacteriocins into
669 *P. aeruginosa* cells were also constructed. The *tonB1* genes from PAO1 and PA14 strains

670 were amplified using the TonB1-for and TonB1-rev oligonucleotides (Table S1) and the
671 resulting DNA fragments were cleaved by PciI and HindIII and inserted between the
672 compatible NcoI and HindIII sites of the pUCP24Nco vector, yielding the pLL58 and pLL59
673 plasmids, respectively. Plasmids pMLD652/653 expressing *fiuA*_{PA14-tonB1}_{PA01} and *fiuA*_{PA14-}
674 *tonB1*_{PA14} gene couples, respectively, were constructed as follows: first, the *fiuA* gene was
675 amplified from the PA14 chromosome using FiuA-for and FiuAtonB1-rev oligonucleotides
676 and the *tonB1* gene was amplified from PA01 and PA14 chromosomes using FiuAtonB1-for
677 and TonB1-rev oligonucleotides. These two fragments were hybridized together and a new
678 PCR was performed using FiuA-for and TonB1-rev as primers. The resulting fragments were
679 purified, treated by NcoI and HindIII and then inserted between the same sites in the
680 pUCP24Nco vector.

681 **Expression and purification of wild-type and mutant Paem proteins.** Recombinant
682 His-tagged proteins Paem_{JJ692} (Paem1), Paem_{DSM50071} (Paem2), the corresponding single or
683 double mutants (D241A, D244A and D241A D244A), as well as the eight chimeric Paem
684 proteins, were overproduced using the pET2160 derived plasmids described above and the *E.*
685 *coli* BL21(DE3)(pLysS) as the host strain. Exponentially-growing cultures (1 liter) were
686 incubated at 37°C until the optical density at 600 nm (OD₆₀₀) reached 0.8. Gene expression
687 was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) and
688 incubation was continued for 3 h at 37°C. Then, cells were harvested by centrifugation at
689 8000 × *g* for 10 min at 4°C and washed once with a 0.9% NaCl solution. Pellets were
690 resuspended in 10 ml of 20 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM
691 MgCl₂ and 10 mM 2-mercaptoethanol (buffer A) and cells were subsequently disrupted by
692 sonication (Bioblock Vibracell sonicator, model 72412). Insoluble proteins and bacterial
693 debris were removed by centrifugation at 200,000 × *g* (Beckman TL100 centrifuge) for 20
694 min at 4°C and the resulting supernatants were directly used for protein purification or were

695 stored at -20°C . The His₆-tagged PaeM proteins were purified on nickel-nitrilotriacetate
696 agarose (Ni²⁺-NTA) as recommended by the manufacturer (Qiagen). Briefly, crude soluble
697 protein extracts in buffer A were supplemented with 10 mM imidazole and 300 mM NaCl
698 and incubated for 1 h at 4°C with 2 ml of Ni-NTA resin preequilibrated in the same buffer.
699 The resin was then extensively washed with buffer A containing increasing concentrations of
700 imidazole (from 20 to 40 mM) and proteins were eluted with 100, 200 and 300 mM
701 imidazole. The relevant fractions were pooled, eventually concentrated by ultra-filtration
702 (10K Amicon Ultra-15 filters, Millipore), and dialyzed overnight against buffer A
703 supplemented with 10% glycerol. Protein concentrations were determined with a NanoDrop
704 (Thermo Scientific) and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-
705 PAGE) analysis of proteins was performed as described previously (41).

706 **Spot test killing assay.** The susceptibility of *E. coli* and *P. aeruginosa* strains to the
707 different PaeM protein variants purified in this work was tested using the classical spot
708 dilution assay (42). Briefly, an aliquot of overnight culture of the bacterial strain to be tested
709 (*ca.* 10^7 cells) was used to inoculate 3 ml of 2YT-top agar at 0.7%. The mixture was poured
710 on freshly prepared 2YT agar plates (30 ml) and let dry for a few min under the hood. Then,
711 5 to 10 μl of serial dilutions in buffer A of the PaeM protein stocks were spotted on the top
712 agar layer and let dry under the hood. Plates were incubated for 24 h at 37°C and the
713 sensitivity of strains and efficiency of pyocins was judged from the clear zones of inhibition
714 observed at the spot position.

715 **Lipid II hydrolase activity assay.** The enzymatic activity of PaeM variants was tested in
716 a reaction mixture containing, in a final volume of 10 μl , 100 mM Tris-HCl buffer, pH 7.5,
717 1.5 μM [¹⁴C]radiolabeled lipid II (120 Bq), 20 mM MgCl₂, 10 mM 2-mercaptoethanol, 150
718 mM NaCl, 0.2% *n*-dodecyl- β -D-maltoside (DDM), and PaeM protein (from 5 ng to 10 μg in
719 5 μl of buffer A). After 30 min of incubation at 37°C , the reaction was stopped by heating at

720 100°C for 1 min and the radiolabeled substrate (lipid II) and 1-PP-MurNAc(-pentapeptide)-
721 GlcNAc product were separated by thin-layer chromatography (TLC) on LK6D silica gel
722 plates (Whatman) using 1-propanol/ammonium hydroxide/water (6:3:1; v/v) as the mobile
723 phase. The two latter compounds migrated with R_f values of 0.7 and 0.3, respectively, and the
724 corresponding radioactive spots were quantified using a radioactivity scanner (model Multi-
725 Tracemaster LB285, EG&G Wallac/Berthold).

726 **Chemicals.** IPTG was obtained from Eurogentec, DDM detergent from Anatrace, and
727 Ni^{2+} -NTA agarose from Qiagen. The *meso*-diaminopimelic acid-containing [^{14}C]radiolabeled
728 lipid II was synthesized as described earlier (2). Antibiotics and reagents were from Sigma.
729 DNA ligase and restriction enzymes were obtained from New England Biolabs, and DNA
730 purification kits from Promega and Macherey-Nagel. Synthesis of oligonucleotides and DNA
731 sequencing were performed by Eurofins-MWG. The QuickChange II XL kit from Stratagene
732 was used for site-directed mutagenesis experiments. All other materials were reagent grade
733 and obtained from commercial sources.

734 **Accession numbers.** The *fiuA* gene sequences determined in the present study are
735 available under the following Genbank accession numbers: MK574012 (FiuA_{C3-20}),
736 MK574013 (FiuA_{C4-17}), MK574014 (FiuA_{DET08}), MK574015 (FiuA_{PcyII-57}), MK574016
737 (FiuA_{I3i}), and MK574014 (FiuA_{PcyII-55}). Accession numbers of other genes are indicated in
738 the text and the figure legends.

739

740 **ACKNOWLEDGMENTS**

741 We thank Alain Filloux, Paolo Visca, Jean-Luc Mainardi, and Michel Arthur for the gift of
742 *Pseudomonas aeruginosa* strains. This work was supported by the Fondation pour la
743 Recherche Médicale (DBF20160635737), the Centre National de la Recherche Scientifique
744 (UMR9198) and the Université Paris-Sud.

745 We declare no conflicts of interest.

746

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870 phatase specifically imported by *Escherichia coli*. J Biol Chem 283:25324-25331.

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874 **FIGURE LEGENDS**

875 **Figure 1. Sequence alignment of PaeM variants.** Amino acid sequences of PaeM1 from
876 strain JJ692 and PaeM2 from strain DSM50071 were aligned using the Clustal 2.1 program
877 and graphic visualization was obtained using ESPript 3.0. The black triangle localizes the be-
878 ginning of the activity domain. Dissection experiments indeed previously showed that the
879 isolated and independent lipid II-hydrolyzing domain of PaeM1 encompassed essentially the
880 C-terminal half of the protein (residues 134-289) (10). Based on the knowledge previously
881 acquired on the ColM bacteriocin (15, 43), the N-terminal (translocation) domain of PaeM
882 variants is thought to cover approximately the first 40 residues and the central (reception)
883 domain to extend between the latter two domains. Amino acid numbers are indicated on the
884 top of the sequences. Accession numbers: ERZ09841.1 (PaeM1 from JJ692), SKA49653.1
885 (PaeM2 from DSM50071).

886 **Figure 2. Spot killing assay of PaeM variants and mutants on *P. aeruginosa* susceptible**
887 **strain C4-17.** Five- μ l spots of undiluted (0.6 mg/ml) or serial 10-fold dilutions of wild-type

888 or mutant PaeM1 (A) and PaeM2 (B) protein stocks (from left to right) were deposited on
889 lawns of the representative strain C4-17 which is susceptible to both PaeM variants. Growth
890 inhibition zones were observed after 24 h of incubation at 37°C.

891 **Figure 3. Clustering of *P. aeruginosa* strains using *in vivo* and *in silico* VNTR analysis**
892 **data.** For the multiple locus variable number of tandem repeat (VNTR) analysis (MLVA), *P.*
893 *aeruginosa* strains 6077 (the original strain from which the *exoU*-containing island A was
894 transferred to strain JJ692) and DSM50071 were used as *PaeM1/ImM1*- and *PaeM2/ImM2*-
895 containing reference strains, respectively. Green squares, *P. aeruginosa* strains belonging to
896 the CC235 clonal population. Red triangles, *P. aeruginosa* strains PCR-positive for *PaeM1*-
897 *ImM1* gene couple. Blue triangles, *P. aeruginosa* strains PCR-positive for *PaeM2*-*ImM2* gene
898 couple. Empty triangles, *in silico* search for *PaeM1*-*ImM1* (red) or *PaeM2*-*ImM2* (blue) gene
899 couple in some *P. aeruginosa* genomes.

900 **Figure 4. Sequence alignment of FiuA receptors from selected *P. aeruginosa* strains.**
901 Amino acid sequences of FiuA outer-membrane receptor proteins from strains PcyII-10 and
902 PA14 (resistant to both PaeM variants), PcyII-57 and PAO1 (susceptible only to PaeM2),
903 PA14 (resistant to both PaeM1 and PaeM2), 13i and PcyII-55 (susceptible mainly to PaeM1),
904 C4-17, C3-20 and DET08 (susceptible to both PaeM variants), and JJ692 and DSM50071
905 (PaeM-producing strains) were aligned using the Clustal 2.1 program and graphic visualiza-
906 tion was obtained using ESPript 3.0. Amino acid numbers are indicated on the top of the se-
907 quences. Accession numbers: SIP50767.1 (FiuA_{PcyII-10}), MK574015 (FiuA_{PcyII-57}),
908 NP_249161.1 (FiuA_{PAO1}), SCM60368.1 (FiuA_{PA14}), ERZ16319.1 (FiuA_{JJ692}), MK574017
909 (FiuA_{PcyII-55}), MK574013 (FiuA_{C4-17}), MK574012 (FiuA_{C3-20}), MK574014 (FiuA_{DET08}),
910 MK574016 (FiuA_{13i}), AKO84658.1 (FiuA_{DSM50071}).

911 **Figure 5. Predicted FiuA_{PAO1} (A) and FiuA_{PA14} (B) receptor structures.** View of the
912 FiuA_{PAO1} (A) and FiuA_{PA14} (B) siderophore receptor structures drawn as ribbons. The plug

913 and the transmembrane β -barrel domains are colored in yellow and blue, respectively. The
914 cytoplasmic turns and extracellular loops are colored in green. In A, the differences observed
915 between the FiuA receptor of PAO1 and those of the strains susceptible to both PaeM vari-
916 ants are shown in red. In B, the few six amino acid changes between FiuA_{PA14} and FiuA_{JJ692}
917 are shown in orange. Structure prediction was made using Phyre2 (17), based on the crystal
918 structures of FpvA (PDB entry 1XKH) and FptA (1XKW) receptors. The figure was gener-
919 ated with PyMOL (DeLano Scientific).

920 **Figure 6. Impact of FiuA receptor structure on the susceptibility of PAO1 strain to**
921 **PaeM variants.** The *fiuA* genes from different representative strains (PAO1, 13i, PcyII-10,
922 PA14, JJ692 and DSM50071) were cloned in the pUCP24Nco vector and the PAO1 strain
923 was transformed by the plasmids thus generated. The susceptibility of the resulting strains to
924 the PaeM1 and PaeM2 pyocins was tested by spotting 5 μ l of undiluted or serial 10-fold dilu-
925 tions of the purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones
926 were observed after 24 h incubation at 37°C.

927 **Figure 7. Alignment of ImM immunity proteins (A) and their expression in C4-17**
928 **strain (B).** A, Amino acid sequences of ImM1 from strain JJ692 and ImM2 from strain
929 DSM50071 were aligned using the Clustal 2.1 program and graphic visualization was ob-
930 tained using ESPript 3.0. Amino acid numbers are indicated on the top of the sequences. B,
931 5- μ l spots of undiluted (0.6 mg/ml) or serial 10-fold dilutions of pure PaeM1 and PaeM2 pro-
932 tein stocks were deposited on lawns of strain C4-17 expressing either of the ImM1 or ImM2
933 genes on the pUCP24 vector. Growth inhibition zones were observed after 24 h incubation at
934 37°C.

935 **Figure 8. Impact of FiuA polymorphism on the susceptibility of PA14 strain to PaeM**
936 **variants.** *fiuA* genes from DSM50071 and PA14 strains were cloned in the pUCP24Nco vec-
937 tor. The PA14 strain was transformed by these plasmids and the susceptibility of the resulting

938 strains to PaeM1 and PaeM2 proteins was tested by spotting 5 μ l of undiluted or serial 10-
939 fold dilutions of the purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition
940 zones were observed after 24 h incubation at 37°C.

941 **Figure 9. Implication of TonB1 protein in the import of PaeM variants in PAO1 strain.**

942 A PAOI *tonB1*-null mutant was transformed with the pUCP24Nco plasmid expressing the
943 TonB1_{PAO1} protein or the FiuA_{DSM50071} receptor and the susceptibility of the resulting strains
944 to PaeM1 and PaeM2 proteins was tested by spotting 5 μ l of undiluted or serial 10-fold dilu-
945 tions of the purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones
946 were observed after 24 h incubation at 37°C.

947 **Figure 10. TonB1 protein sequences of PAO1 and PA14 strains (A) and their expression**
948 **in PA14 (B) and PAO1 Δ *tonB1* (C) backgrounds.** A, TonB1 protein sequences of strains

949 PAO1 and PA14 were aligned using Clustal 2.1 program and graphic visualization was ob-
950 tained using ESPript 3.0. Amino acid numbers are indicated on the top of the sequences. B,
951 Expression of the latter TonB1 proteins in the PA14 strain. C, Expression of TonB1_{PA14} and
952 of FiuA_{PA14}/TonB1_{PAO1} or FiuA_{PA14}/TonB1_{PA14} couples in the PAO1 Δ *tonB1* strain. In B and
953 C, the strain susceptibility to the PaeM pyocins was tested by spotting 5 μ l of undiluted or
954 serial 10-fold dilutions of purified protein stocks (0.6 mg/ml) on the bacterial lawns. Growth
955 inhibition zones were observed after 24 h incubation at 37°C. Accession numbers:
956 AAG08916.1 (TonB1_{PAO1}), SCM65789.1 (TonB1_{PA14}).

957 **Figure 11. Susceptibility to chimeric PaeM1/PaeM2 proteins of PAO1 strain expressing**

958 **different FiuA receptors.** A, Seven chimeric proteins PaeMchim1 to 7 were generated by
959 replacing partially or totally the N-terminal reception/translocation region of PaeM1 by the
960 corresponding sequences of PaeM2. An eighth chimeric protein PaeMchim8 was generated
961 which consisted in the fusion of the N-terminal region of PaeM1 to the C-terminal domain of

962 PaeM2. B, The susceptibility to these chimeric proteins of the PAO1 strain expressing FiuA
963 receptors from the indicated *P. aeruginosa* strains was tested by spot killing assay. 5- μ l spots
964 of undiluted (0.6 mg/ml) or 10-fold serially diluted protein stocks were deposited on bacterial
965 lawns. Spots of PaeM1 and PaeM2 natural proteins were also included as controls. Growth
966 inhibition zones were observed after 24 h incubation at 37°C.

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TABLE 1. Susceptibility to *PaeM* variants of *P. aeruginosa* strains used in this study.

<i>P. aeruginosa</i> strains	Susceptibility to <i>PaeM1</i> ^a	Detection of <i>PaeM1/ImM1</i>	Susceptibility to <i>PaeM2</i> ^a	Detection of <i>PaeM2/ImM2</i>
DET08, C1-14, C3-20, C4-17, C7-6, C7-18, C10-13, PcyII-41, PcyII-64, PcyII-66, PcyII-76	+	-/-	+	-/-
13i, PcyII-55	+	-/-	-	-/-
C1-11, C5-6, C8-15, PAO1, PcyII-57	-	-/-	+	-/-
6i, 10i, 23i, 24i, 25i, 26j, C1-7, C1-19, C2-10, C2-18, C3-2, C3-12, C3-13, C3-15, C3-18, C3-19, C4-8, C5-2, C5-11, C5-17, C5-18, C7-11, C7-12, C7-25, C8-11, C8-12, C8-17, C8-20, C9-12, C9-16, C10-1, C10-4, C10-5, C10-18, PA14, PcyII-10, PcyII-18, PcyII-54, PcyII-63, PcyII-81, PcyII-82, PcyII-86	-	-/-	-	-/-
JJ692, 14i, 20H, PcyII-48	-	+/+	-	-/-
DSM 50071	-	-/-	-	+/+

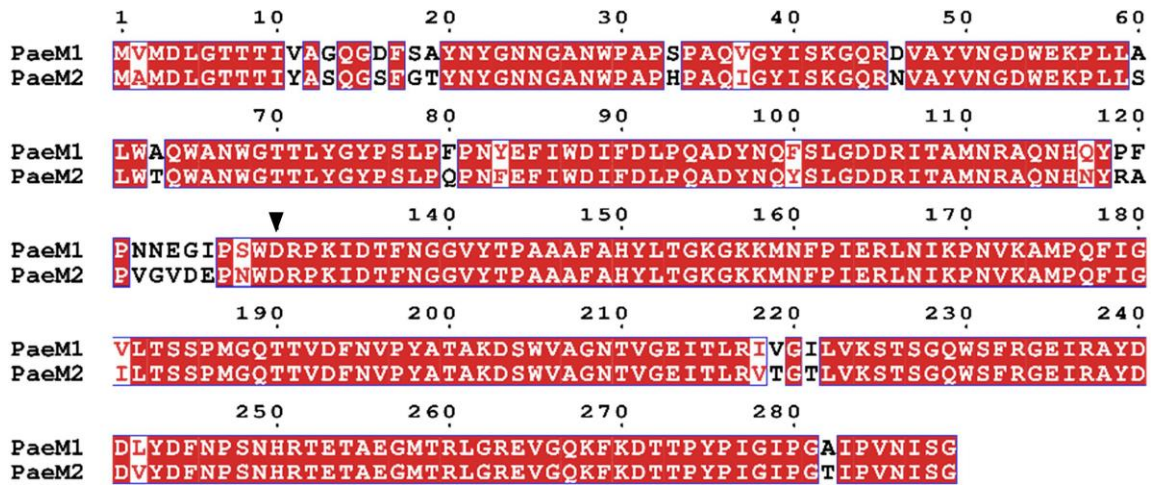
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972 ^a Susceptibility was tested using the spot killing assay described in Materials and Methods; -,
973 Resistant; +, susceptible; -/-, no *PaeM* and associated *ImM* genes detectable by PCR; +/+,
974 *PaeM* and *ImM* genes detected by PCR.

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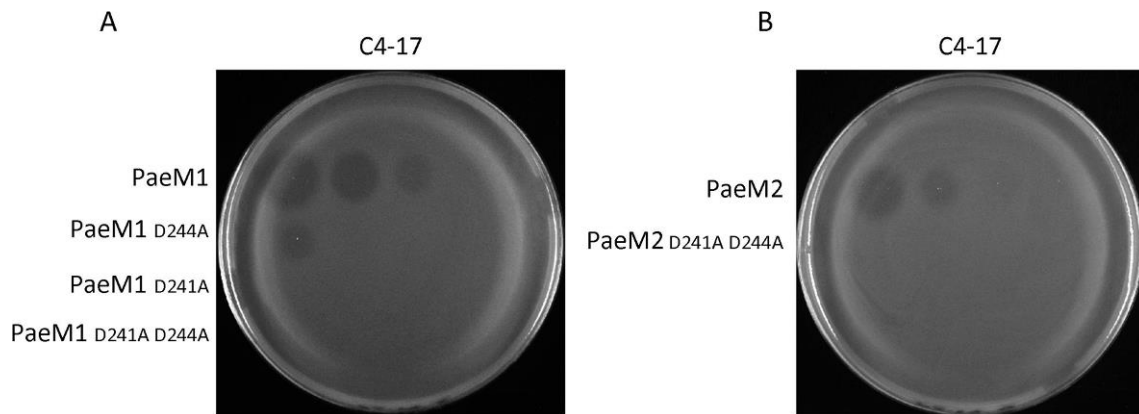
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Figure 1. Sequence alignment of PaeM variants. Amino acid sequences of PaeM1 from strain JJ692 and PaeM2 from strain DSM50071 were aligned using the Clustal 2.1 program and graphic visualization was obtained using ESPript 3.0. The black triangle localizes the beginning of the activity domain. Dissection experiments indeed previously showed that the isolated and independent lipid II-hydrolyzing domain of PaeM1 encompassed essentially the C-terminal half of the protein (residues 134-289) (10). Based on the knowledge previously acquired on the ColM bacteriocin (15, 43), the N-terminal (translocation) domain of PaeM variants is thought to cover approximately the first 40 residues and the central (reception) domain to extend between the latter two domains. Amino acid numbers are indicated on the top of the sequences. Accession numbers: ERZ09841.1 (PaeM1 from JJ692), SKA49653.1 (PaeM2 from DSM50071).

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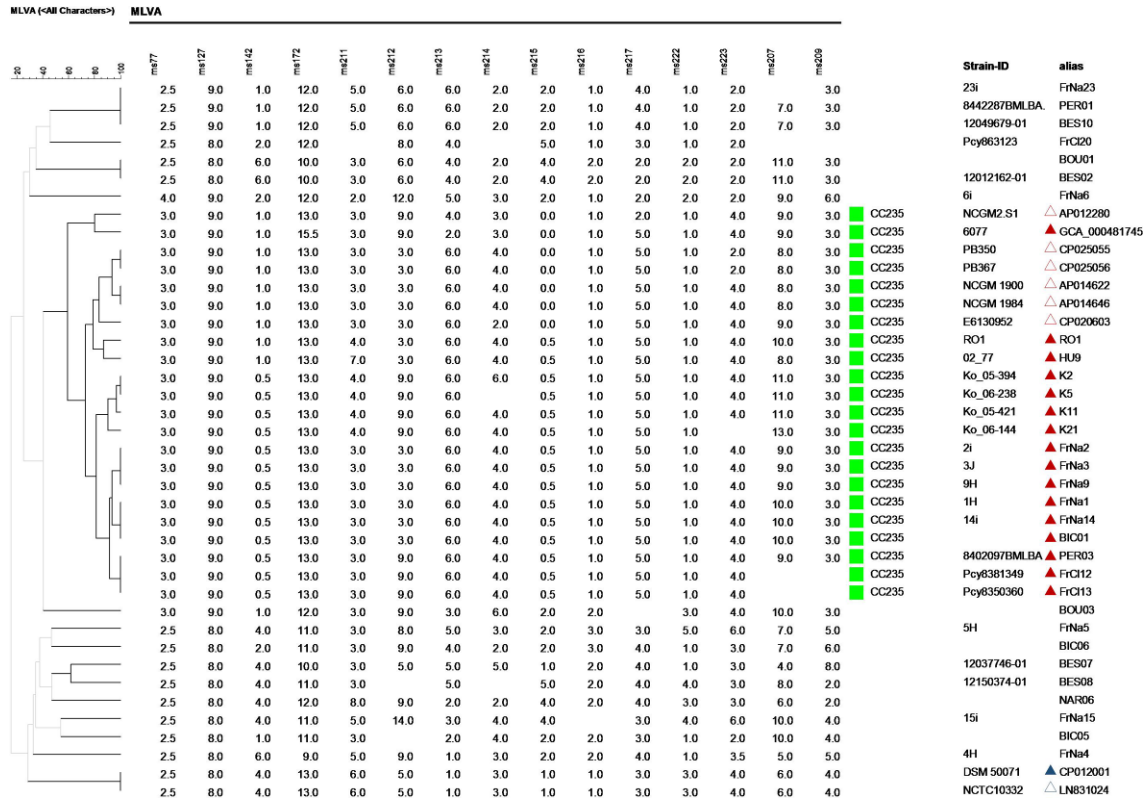


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997 **Figure 2. Spot killing assay of PaeM variants and mutants on *P. aeruginosa* susceptible**
998 **strain C4-17.** Five- μ l spots of undiluted (0.6 mg/ml) or serial 10-fold dilutions of wild-type
999 or mutant PaeM1 (A) and PaeM2 (B) protein stocks (from left to right) were deposited on
1000 lawns of the representative strain C4-17 which is susceptible to both PaeM variants. Growth
1001 inhibition zones were observed after 24 h of incubation at 37°C.

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Figure 3. Clustering of *P. aeruginosa* strains using *in vivo* and *in silico* VNTR analysis data. For the multiple locus variable number of tandem repeat (VNTR) analysis (MLVA), *P. aeruginosa* strains 6077 (the original strain from which the *exoU*-containing island A was transferred to strain JJ692) and DSM50071 were used as *Paem1/Imm1*- and *Paem2/Imm2*-containing reference strains, respectively. Green squares, *P. aeruginosa* strains belonging to the CC235 clonal population. Red triangles, *P. aeruginosa* strains PCR-positive for *Paem1-Imm1* gene couple. Blue triangles, *P. aeruginosa* strains PCR-positive for *Paem2-Imm2* gene couple. Empty triangles, *in silico* search for *Paem1-Imm1* (red) or *Paem2-Imm2* (blue) gene couple in some *P. aeruginosa* genomes.

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1 10 20 30 40 50 60 70 80 90 100

FiuAPcyII-10 MRPRTRFRPGLLALATLAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSABAVQGRFDAPGALREALKSGSGLVLENAG
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 FiuAP14 MRPRTRFRPGLLALATLAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSABAVQGRFDAPGALREALKSGSGLVLENAG
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 FiuAPcyII-57 GTYSLRKVPEDTSLQAMTVSGAEDDPLGPTSGVAKRSMGTGKTDPLETPRSISVATROQMTDRAVHNLDLAVRYMPGVVASSFGSDSRSDWLKVRG
 FiuAPA01 GTYSLRKVPEDTSLQAMTVSGAEDDPLGPTSGVAKRSMGTGKTDPLETPRSISVATROQMTDRAVHNLDLAVRYMPGVVASSFGSDSRSDWLKVRG
 FiuAP14 GTYSLRKVPEDTSLQAMTVSGAEDDPLGPTSGVAKRSMGTGKTDPLETPRSISVATROQMTDRAVHNLDLAVRYMPGVVASSFGSDSRSDWLKVRG
 FiuAJJ692 GTYSLRKVPEDTSLQAMTVSGAEDDPLGPTSGVAKRSMGTGKTDPLETPRSISVATROQMTDRAVHNLDLAVRYMPGVVASSFGSDSRSDWLKVRG
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 FiuA131 GTYSLRKVPEDTSLQAMTVSGAEDDPLGPTSGVAKRSMGTGKTDPLETPRSISVATROQMTDRAVHNLDLAVRYMPGVVASSFGSDSRSDWLKVRG
 FiuADSM50071 GTYSLRKVPEDTSLQAMTVSGAEDDPLGPTSGVAKRSMGTGKTDPLETPRSISVATROQMTDRAVHNLDLAVRYMPGVVASSFGSDSRSDWLKVRG

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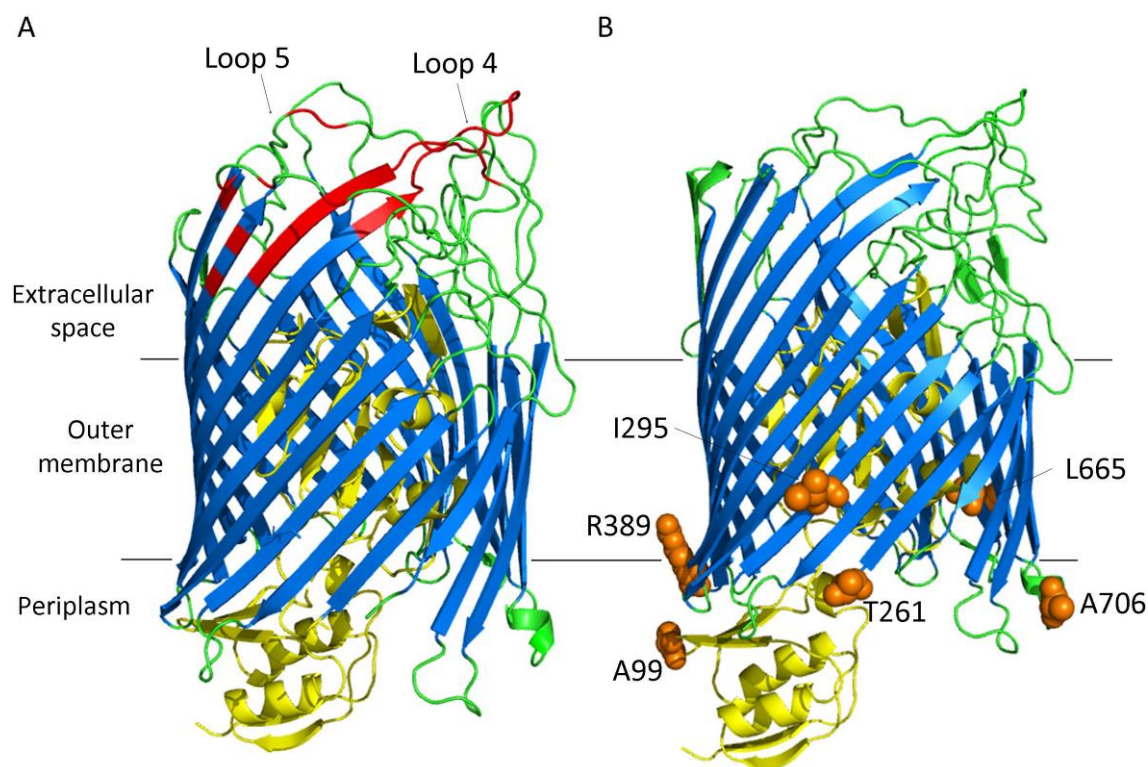
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1019 **Figure 4. Sequence alignment of FiuA receptors from selected *P. aeruginosa* strains.**

1020 Amino acid sequences of FiuA outer-membrane receptor proteins from strains PcyII-10 and
1021 PA14 (resistant to both PaeM variants), PcyII-57 and PAO1 (susceptible only to PaeM2),
1022 PA14 (resistant to both PaeM1 and PaeM2), 13i and PcyII-55 (susceptible mainly to PaeM1),
1023 C4-17, C3-20 and DET08 (susceptible to both PaeM variants), and JJ692 and DSM50071
1024 (PaeM-producing strains) were aligned using the Clustal 2.1 program and graphic visualiza-
1025 tion was obtained using ESPript 3.0. Amino acid numbers are indicated on the top of the se-
1026 quences. Accession numbers: SIP50767.1 (FiuA_{PcyII-10}), MK574015 (FiuA_{PcyII-57}),
1027 NP_249161.1 (FiuA_{PAO1}), SCM60368.1 (FiuA_{PA14}), ERZ16319.1 (FiuA_{JJ692}), MK574017
1028 (FiuA_{PcyII-55}), MK574013 (FiuA_{C4-17}), MK574012 (FiuA_{C3-20}), MK574014 (FiuA_{DET08}),
1029 MK574016 (FiuA_{13i}), AKO84658.1 (FiuA_{DSM50071}).

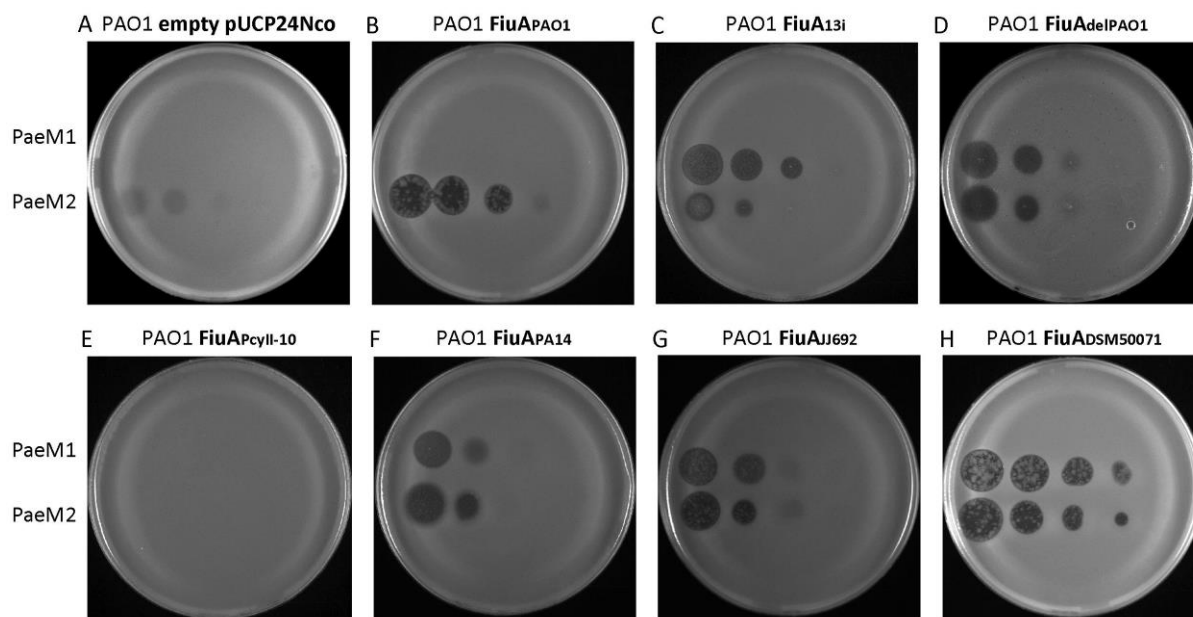
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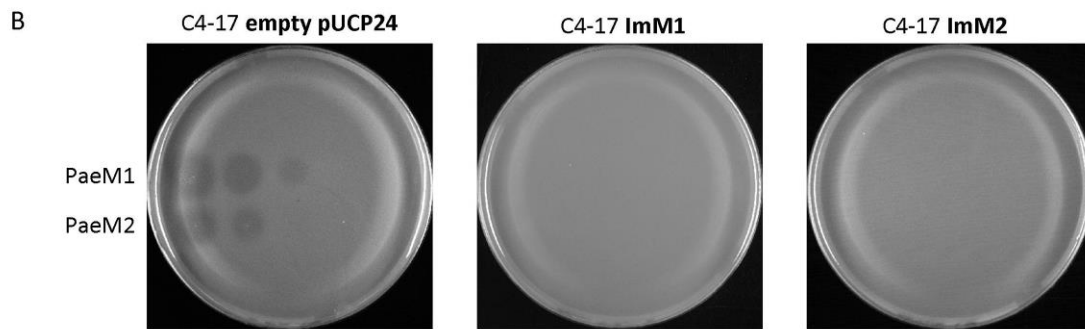
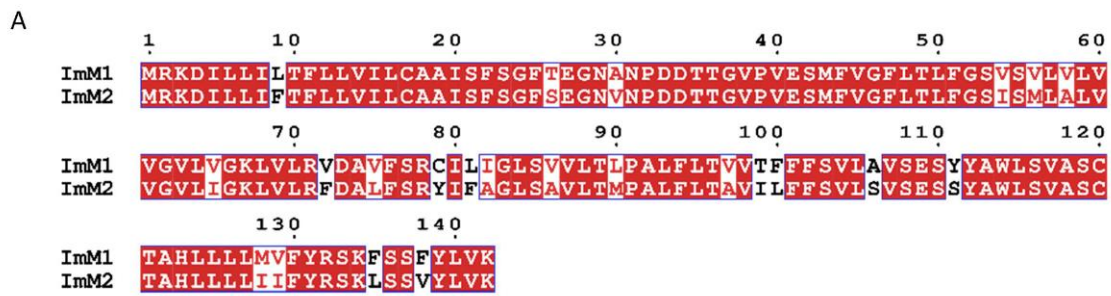
1033 **Figure 5. Predicted FiuA_{PAO1} (A) and FiuA_{PA14} (B) receptor structures.** View of the
1034 FiuA_{PAO1} (A) and FiuA_{PA14} (B) siderophore receptor structures drawn as ribbons. The plug
1035 and the transmembrane β-barrel domains are colored in yellow and blue, respectively. The
1036 cytoplasmic turns and extracellular loops are colored in green. In A, the differences observed
1037 between the FiuA receptor of PAO1 and those of the strains susceptible to both PaeM vari-
1038 ants are shown in red. In B, the six amino acid changes between FiuA_{PA14} and FiuA_{JJ692} are
1039 shown in orange. Structure prediction was made using Phyre2 (17), based on the crystal
1040 structures of FpvA (PDB entry 1XKH) and FptA (1XKW) receptors. The figure was generat-
1041 ed with PyMOL (DeLano Scientific).

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 1044 **Figure 6. Impact of FiuA receptor structure on the susceptibility of PAO1 strain to**
 1045 **Paem variants.** The *fiuA* genes from different representative strains (PAO1, 13i, PcyII-10,
 1046 PA14, JJ692 and DSM50071) were cloned in the pUCP24Nco vector and the PAO1 strain
 1047 was transformed by the plasmids thus generated. The susceptibility of the resulting strains to
 1048 the Paem1 and Paem2 pyocins was tested by spotting 5 μ l of undiluted or serial 10-fold dilu-
 1049 tions of the purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones
 1050 were observed after 24 h incubation at 37°C.

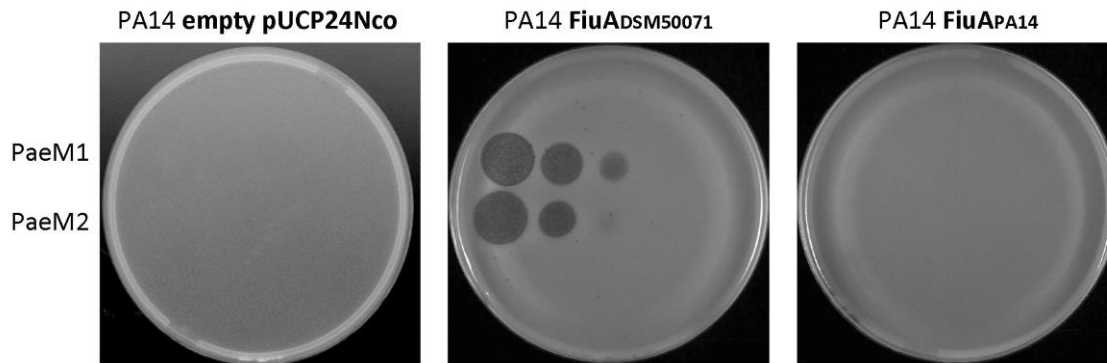
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Figure 7. Alignment of ImM immunity proteins (A) and their expression in C4-17 strain (B). A, Amino acid sequences of ImM1 from strain JJ692 and ImM2 from strain DSM50071 were aligned using the Clustal 2.1 program and graphic visualization was obtained using ESPript 3.0. Amino acid numbers are indicated on the top of the sequences. B, 5- μ l spots of undiluted (0.6 mg/ml) or serial 10-fold dilutions of pure Paem1 and Paem2 protein stocks were deposited on lawns of strain C4-17 expressing either of the ImM1 or ImM2 genes on the pUCP24 vector. Growth inhibition zones were observed after 24 h incubation at 37°C.

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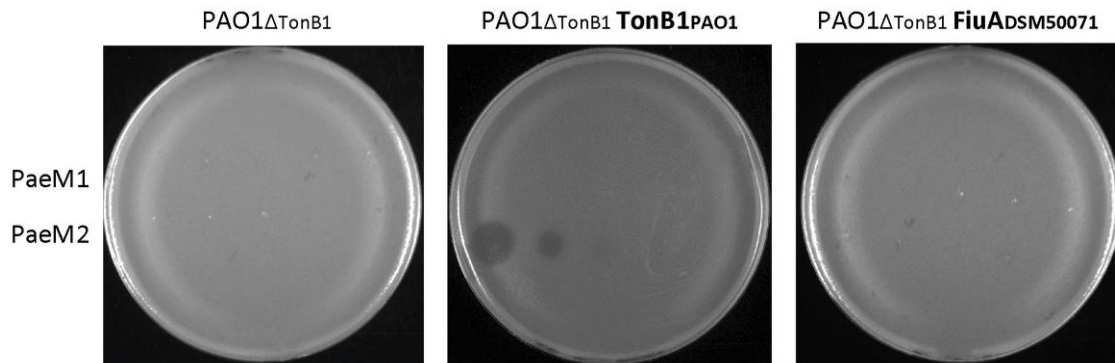


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1065 **Figure 8. Impact of FiuA polymorphism on the susceptibility of PA14 strain to Paem**
 1066 **variants.** *fiuA* genes from DSM50071 and PA14 strains were cloned in the pUCP24Nco vec-
 1067 tor. The PA14 strain was transformed by these plasmids and the susceptibility of the resulting
 1068 strains to Paem1 and Paem2 proteins was tested by spotting 5 μ l of undiluted or serial 10-
 1069 fold dilutions of the purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition
 1070 zones were observed after 24 h incubation at 37°C.

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Figure 9. Implication of TonB1 protein in the import of Paem variants in PAO1 strain.

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A PAOI *tonB1*-null mutant was transformed with the pUCP24Nco plasmid expressing the

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TonB1_{PAO1} protein or the FiuA_{DSM50071} receptor and the susceptibility of the resulting strains

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to Paem1 and Paem2 proteins was tested by spotting 5 μ l of undiluted or serial 10-fold dilu-

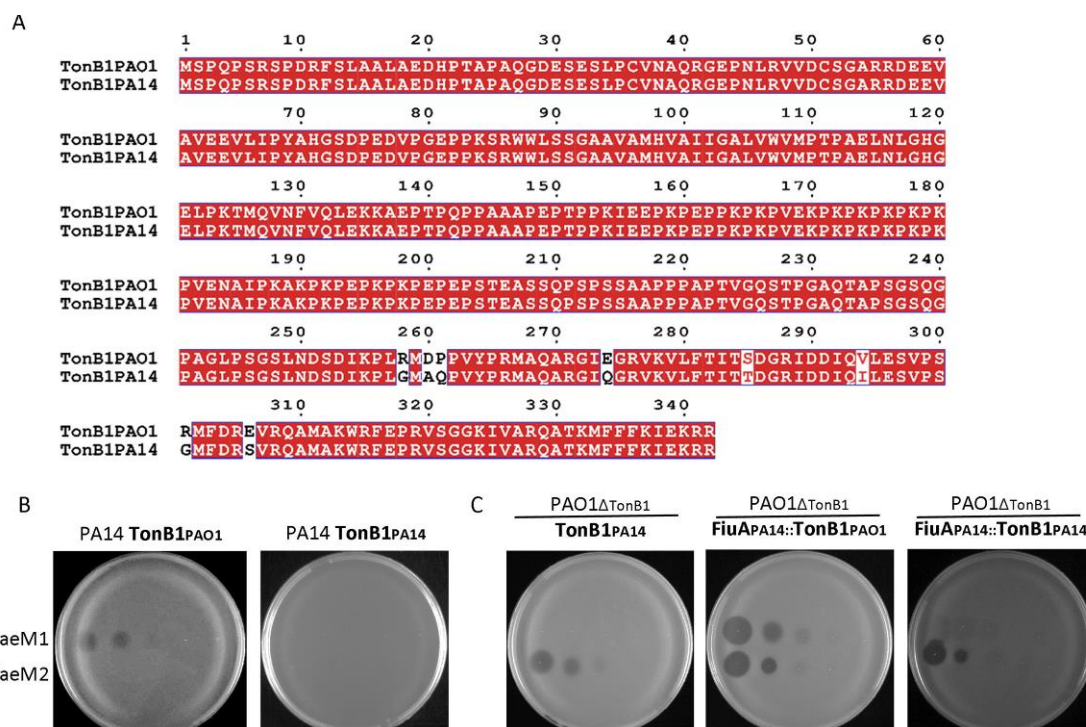
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tions of the purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones

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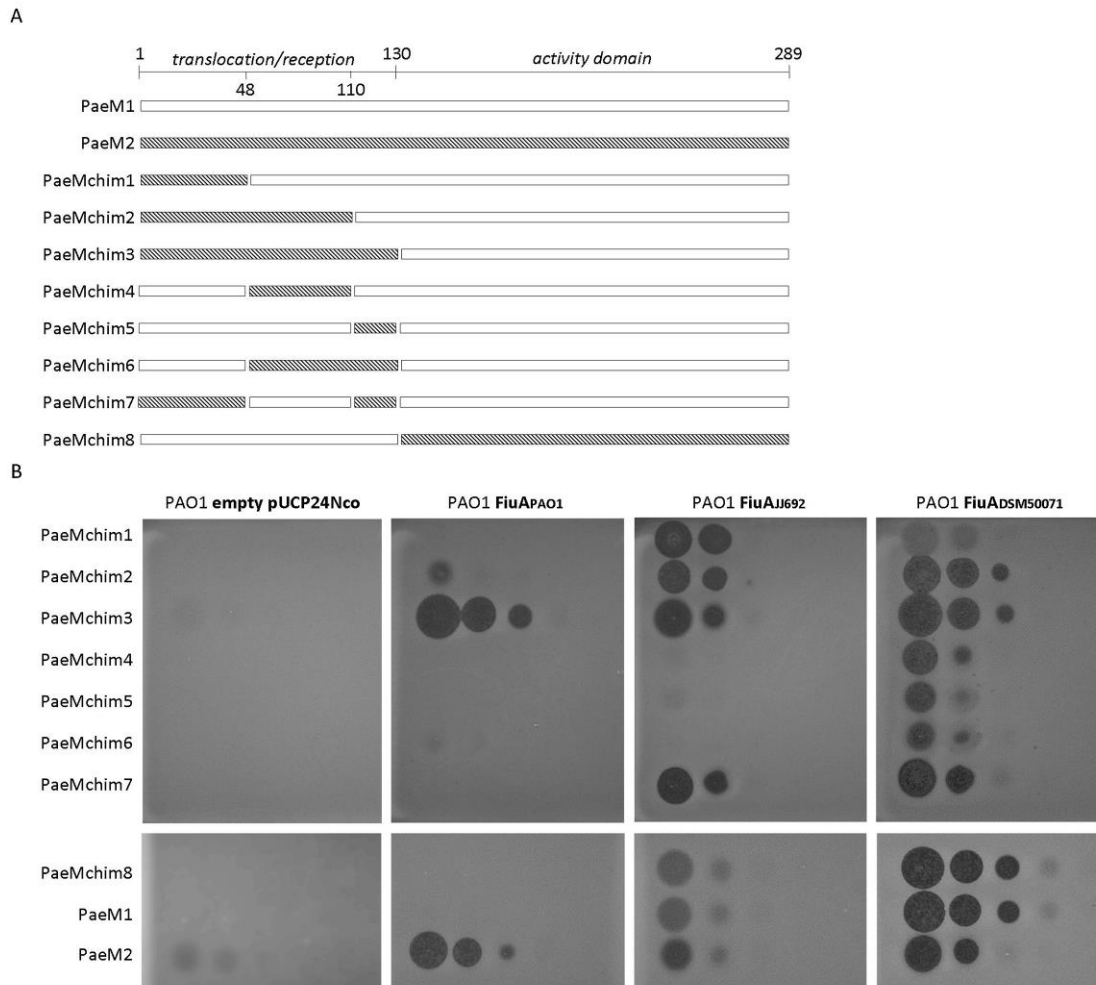
were observed after 24 h incubation at 37°C.

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1083 **Figure 10. TonB1 protein sequences of PAO1 and PA14 strains (A) and their expression**
1084 **in PA14 (B) and PAO1 Δ tonB1 (C) backgrounds.** A, TonB1 protein sequences of strains
1085 PAO1 and PA14 were aligned using Clustal 2.1 program and graphic visualization was ob-
1086 tained using ESPrpt 3.0. Amino acid numbers are indicated on the top of the sequences. B,
1087 Expression of the latter TonB1 proteins in the PA14 strain. C, Expression of TonB1_{PA14} and
1088 of FiuA_{PA14}/TonB1_{PAO1} or FiuA_{PA14}/TonB1_{PA14} couples in the PAO1 Δ tonB1 strain. In B and
1089 C, the strain susceptibility to the PaeM pyocins was tested by spotting 5 μ l of undiluted or
1090 serial 10-fold dilutions of purified protein stocks (0.6 mg/ml) on the bacterial lawns. Growth
1091 inhibition zones were observed after 24 h incubation at 37°C. Accession numbers:
1092 AAG08916.1 (TonB1_{PAO1}), SCM65789.1 (TonB1_{PA14}).

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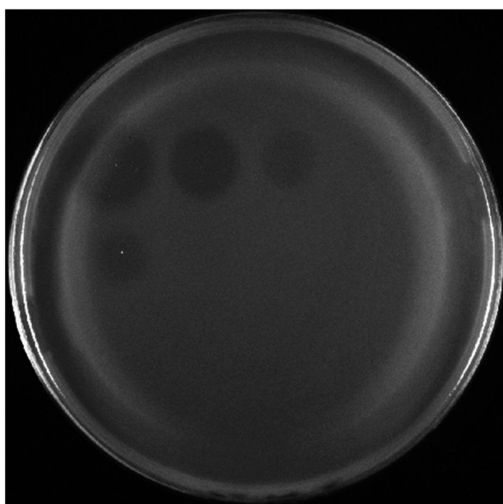
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Figure 11. Susceptibility to chimeric Paem1/Paem2 proteins of PAO1 strain expressing different FiuA receptors. A, Seven chimeric proteins Paemchim1 to 7 were generated by replacing partially or totally the N-terminal reception/translocation region of Paem1 by the corresponding sequences of Paem2. An eighth chimeric protein Paemchim8 was generated which consisted in the fusion of the N-terminal region of Paem1 to the C-terminal domain of Paem2. B, The susceptibility to these chimeric proteins of the PAO1 strain expressing FiuA receptors from the indicated *P. aeruginosa* strains was tested by spot killing assay. 5- μ l spots of undiluted (0.6 mg/ml) or 10-fold serially diluted protein stocks were deposited on bacterial lawns. Spots of Paem1 and Paem2 natural proteins were also included as controls. Growth inhibition zones were observed after 24 h incubation at 37°C.

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PaeM2	MAMD LGTTT IYASQGS	FTYNYGNNGANWPAP	HPAQVGYISKGQR	NVAYVNGDWEKPLLS			
	70	80	90	100	110	120	
PaeM1	LWAQWANWGTTLYGPSLP	FPNVEFIWDIFDLPQADYNQ	ESLGDDRITAMNRAQNHQ	YPF			
PaeM2	LWTQWANWGTTLYGPSLP	QPNVEFIWDIFDLPQADYNQ	YSLGDDRITAMNRAQNH	NYRA			
		140	150	160	170	180	
PaeM1	PNNEGI	PSWDRPKIDTFNGGVYTPAAAF	AHYLTGKGGKMNFP	PIERLN	IKPNVKAMPQ	FIG	
PaeM2	PVGVDE	PNWDRPKIDTFNGGVYTPAAAF	AHYLTGKGGKMNFP	PIERLN	IKPNVKAMPQ	FIG	
	190	200	210	220	230	240	
PaeM1	VLTS	SPMGQTTVDFNVPYATAKDSWVAGNTVGEITLR	IVGI	LVKSTSGQWSFRGEIRAYD			
PaeM2	ILTS	SPMGQTTVDFNVPYATAKDSWVAGNTVGEITLR	VTGT	LVKSTSGQWSFRGEIRAYD			
	250	260	270	280			
PaeM1	DL	YDFNPSNHRTETAEGMTRLGREVGQKFKD	TPYPIGIPGA	IPVNISG			
PaeM2	DV	YDFNPSNHRTETAEGMTRLGREVGQKFKD	TPYPIGIPGT	IPVNISG			

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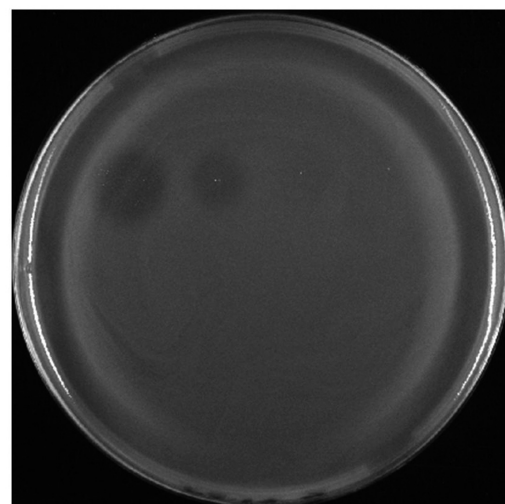
C4-17



PaeM1
PaeM1 D244A
PaeM1 D241A
PaeM1 D241A D244A

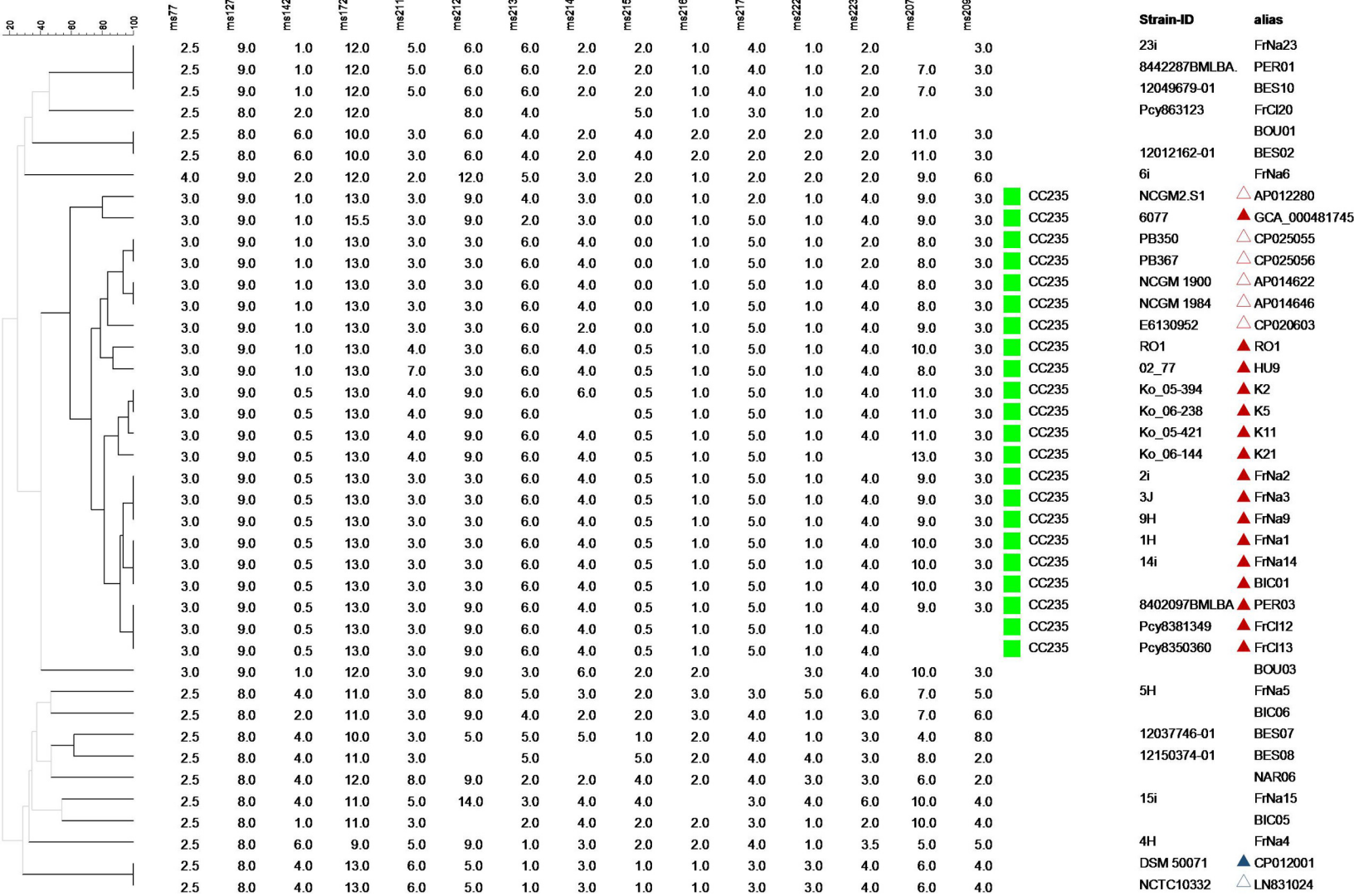
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C4-17



PaeM2
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MLVA (<All Characters>) MLVA



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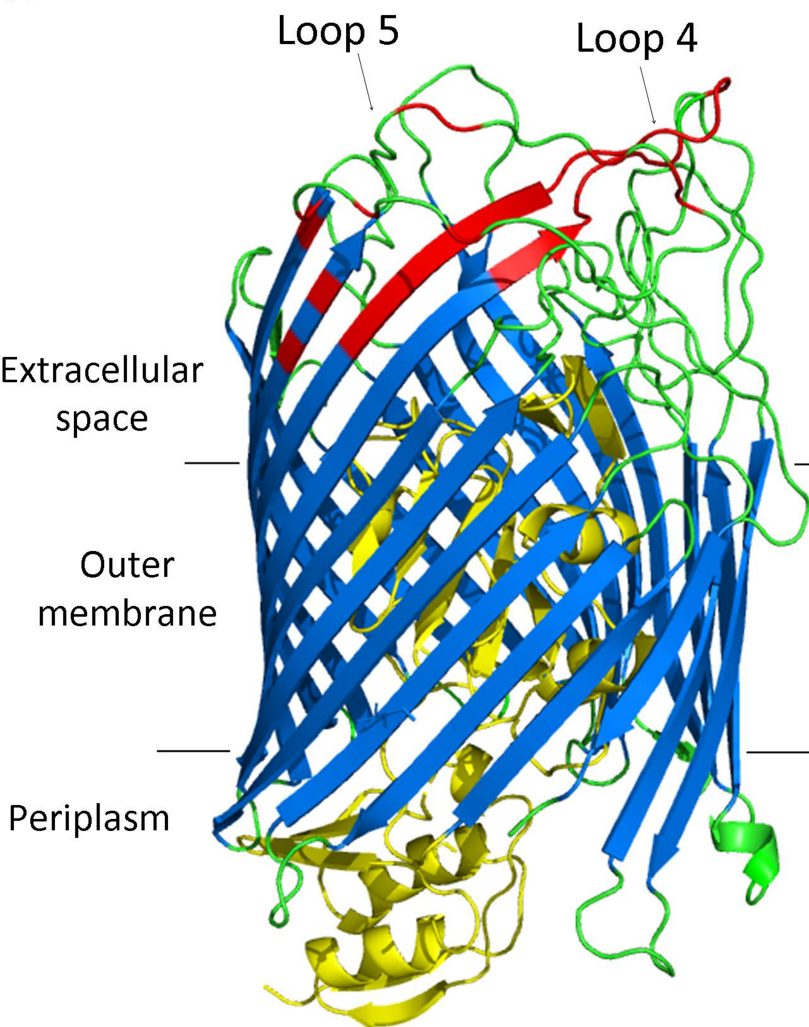
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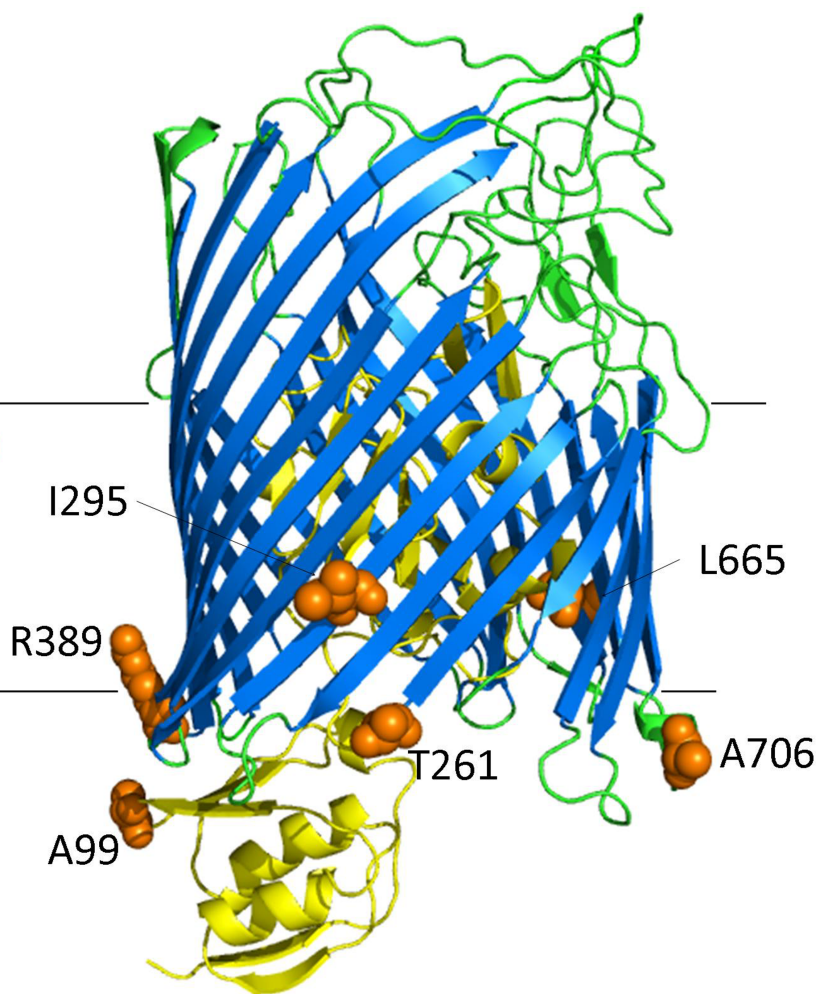
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FiuADET08 ASLWADYTWHEGLNGFSVGAGARYVGSYTDQANTYDGYAGSYTLYDAAVRYDLGQNLGSLKGVSAVANNNLNFNDKYLASCDCGYCYGQDRSVVGSV
FiuA13i ASLWADYTWHEGLNGFSVGAGARYVGSYTDQANTYDGYAGSYTLYDAAVRYDLGQNLGSLKGVSAVANNNLNFNDKYLASCDCGYCYGQDRSVVGSV
FiuADSM50071 ASLWADYTWHEGLNGFSVGAGARYVGSYTDQANTYDGYAGSYTLYDAAVRYDLGQNLGSLKGVSAVANNNLNFNDKYLASCDCGYCYGQDRSVVGSV

FiuAPcyII-10 SYKW
FiuAPcyII-57 SYKW
FiuAPA01 SYKW
FiuAPA14 SYKW
FiuAJJ692 SYKW
FiuAPcyII-55 SYKW
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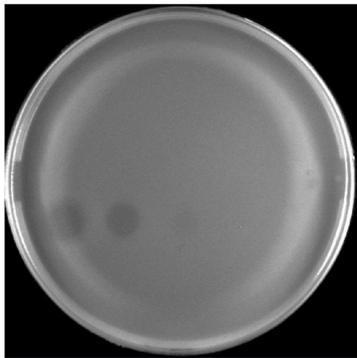
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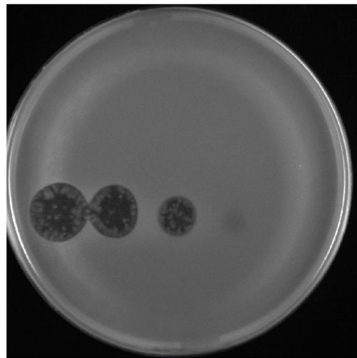
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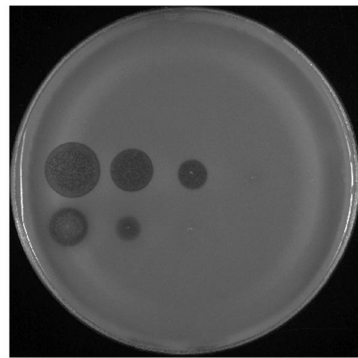
A PAO1 empty pUCP24Nco



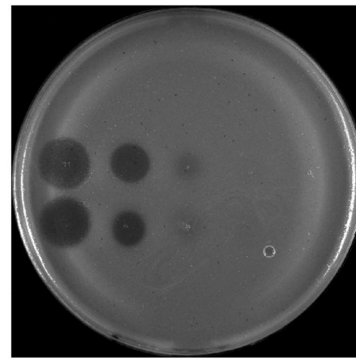
B PAO1 FiuA_{PAO1}



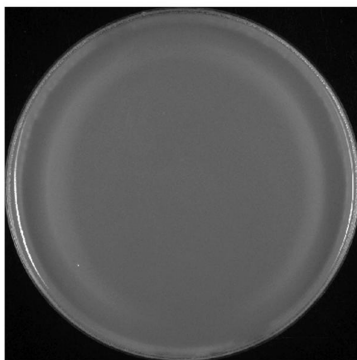
C PAO1 FiuA_{13i}



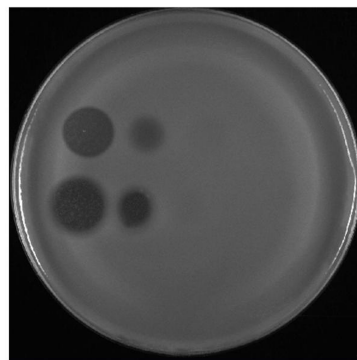
D PAO1 FiuA_{delPAO1}



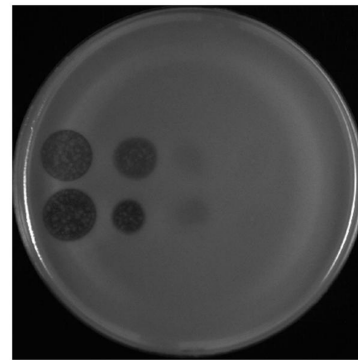
E PAO1 FiuA_{PcyII-10}



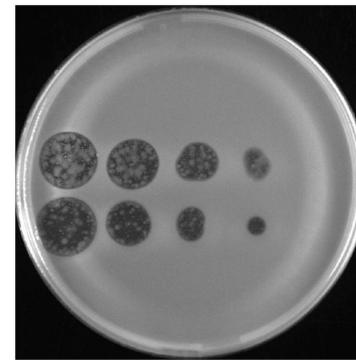
F PAO1 FiuA_{PA14}



G PAO1 FiuA_{J692}



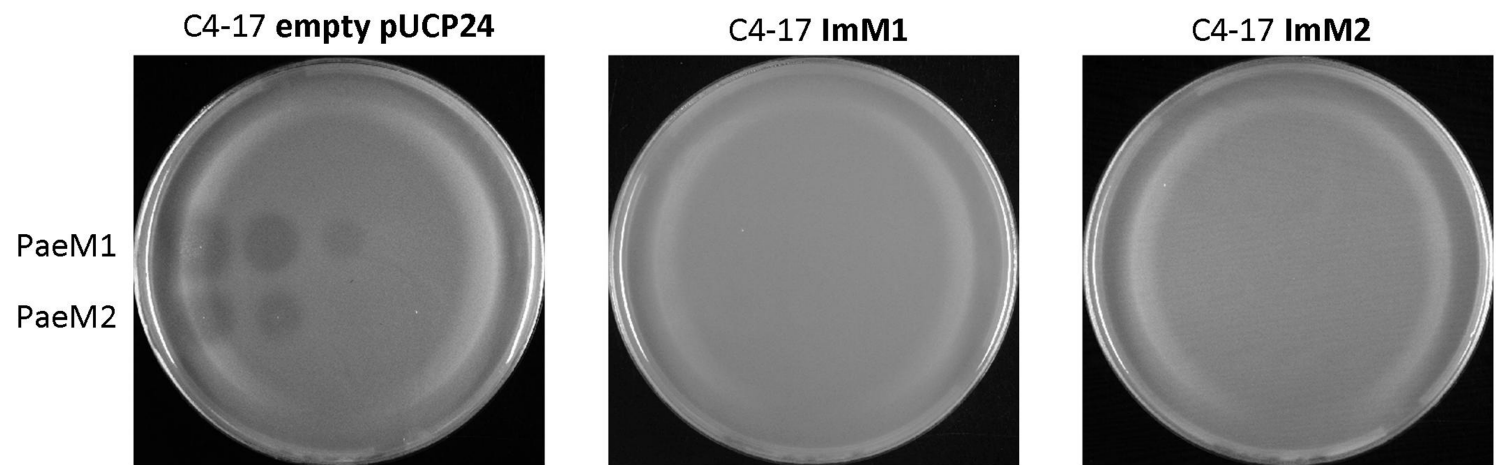
H PAO1 FiuA_{DSM50071}



A

	1	10	20	30	40	50	60
ImM1	MRKDILLI L T F L L V I L C A A I S F S G F T E G N A N P D D T T G V P V E S M F V G F L T L F G S V S V L V L V						
ImM2	MRKDILLI F T F L L V I L C A A I S F S G F S E G N V N P D D T T G V P V E S M F V G F L T L F G S I S M L A L V						
	70	80	90	100	110	120	
ImM1	V G V L V G K L V L R V D A V F S R C I L I G L S V V L T L P A L F L T V V T F F F S V I A V S E S Y Y A W L S V A S C						
ImM2	V G V L I G K L V L R F D A L F S R Y I F A G L S A V L T M P A L F L T A V I L F F S V I S V S E S S Y A W L S V A S C						
	130	140					
ImM1	T A H L L L L M V F Y R S K F S S F Y L V K						
ImM2	T A H L L L L I I F Y R S K L S S V Y L V K						

B



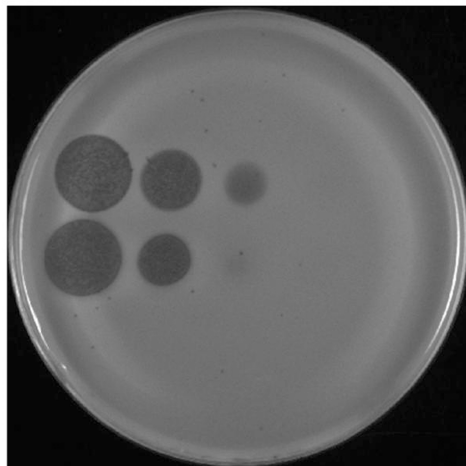
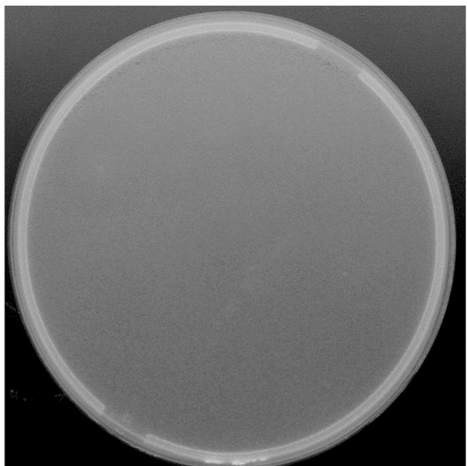
PA14 empty pUCP24Nco

PA14 FiuA_{DSM50071}

PA14 FiuA_{PA14}

PaeM1

PaeM2



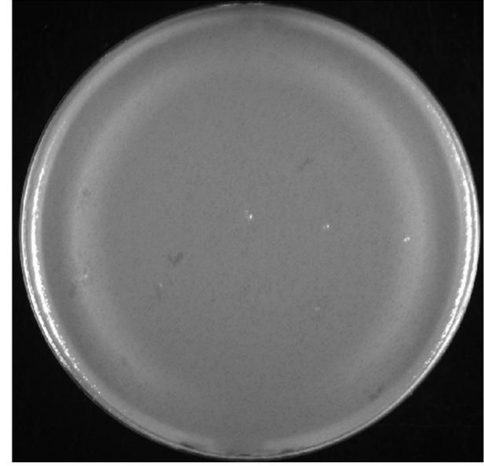
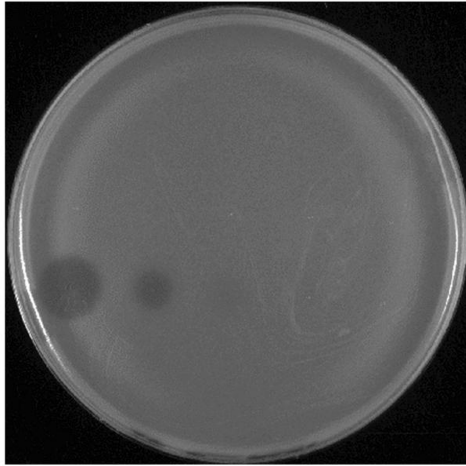
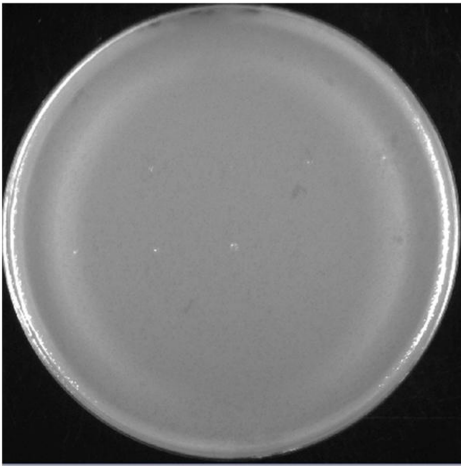
PAO1 Δ TonB1

PAO1 Δ TonB1 **TonB1**PAO1

PAO1 Δ TonB1 **FiuA**DSM50071

PaeM1

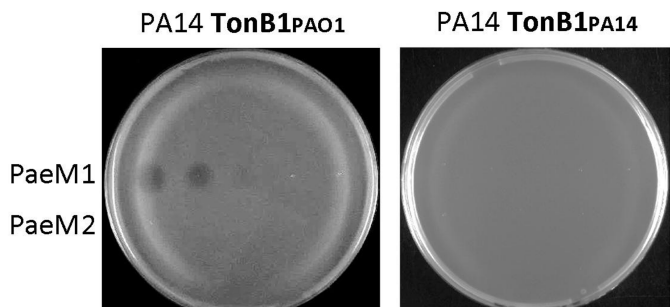
PaeM2



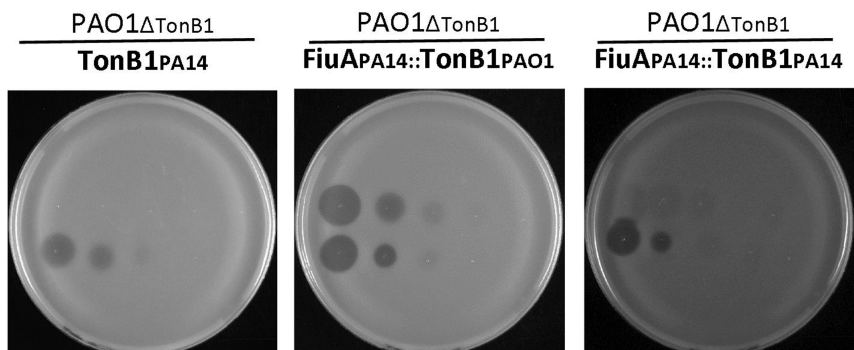
A

	1	10	20	30	40	50	60
TonB1PAO1	MSPQPSRSPDRFSLAALAEDHPTAPAQGDSESLPCVNAQRGEPNLRVVDCSGARRDEEV						
TonB1PA14	MSPQPSRSPDRFSLAALAEDHPTAPAQGDSESLPCVNAQRGEPNLRVVDCSGARRDEEV						
	70	80	90	100	110	120	
TonB1PAO1	AVEEVLIPYAHGSDPEDVPGPEPKSRWWLSSGAAVAMHVAIIGALVWVMPPTPAELNLGHG						
TonB1PA14	AVEEVLIPYAHGSDPEDVPGPEPKSRWWLSSGAAVAMHVAIIGALVWVMPPTPAELNLGHG						
	130	140	150	160	170	180	
TonB1PAO1	ELPKTMQVNFVQLEKKAETPQPPAAAEPTPPKIEEPKPEPKPKPVEKPKPKPKPKPK						
TonB1PA14	ELPKTMQVNFVQLEKKAETPQPPAAAEPTPPKIEEPKPEPKPKPVEKPKPKPKPKPK						
	190	200	210	220	230	240	
TonB1PAO1	PVENAIPKAKPKPEPKPKPEPEPSTEASSQSPSSAAPPAPTVGQSTPGAQTAPSGSQG						
TonB1PA14	PVENAIPKAKPKPEPKPKPEPEPSTEASSQSPSSAAPPAPTVGQSTPGAQTAPSGSQG						
	250	260	270	280	290	300	
TonB1PAO1	PAGLPSGSLNDS DIKPLRMDFPVYPRMAQARGIEGRVKVLF TITSDGRIDDIQVLESVPS						
TonB1PA14	PAGLPSGSLNDS DIKPLGMAQPVYPRMAQARGIQGRVKVLF TITTDGRIDDIQVLESVPS						
	310	320	330	340			
TonB1PAO1	RMFDR E VRQAMAKWRF EPRVSGGKIVARQATKMFFFKIEKRR						
TonB1PA14	GMFDRS VRQAMAKWRF EPRVSGGKIVARQATKMFFFKIEKRR						

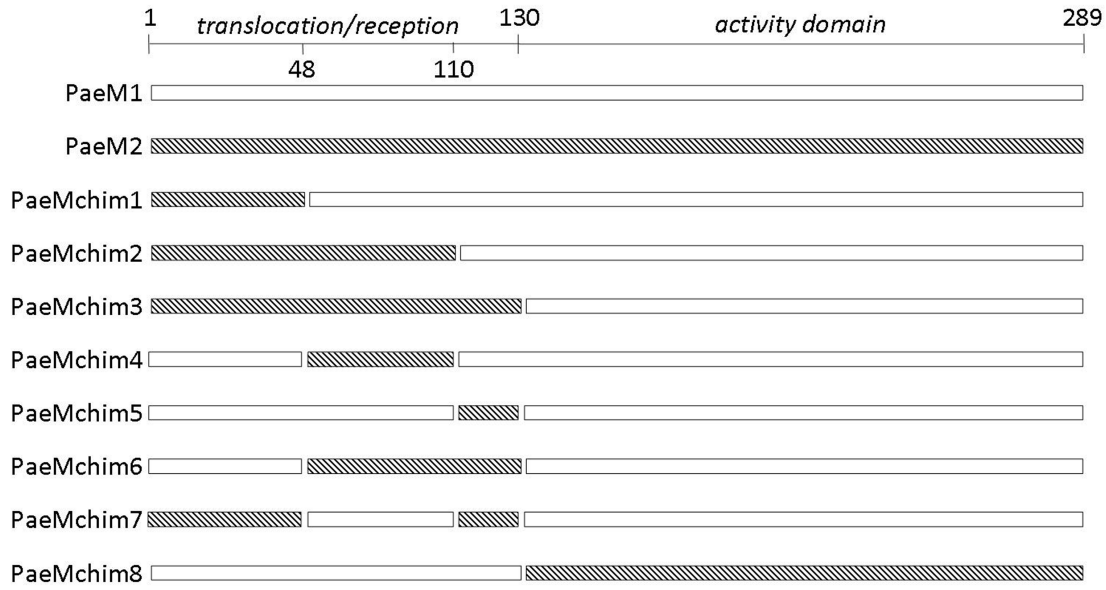
B



C



A



B

