HAL
open science

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

Libera Latino, Delphine Patin, Dimitri Chérier, Thierry Touzé, Christine Pourcel, Hélène Barreteau, Dominique Mengin-Lecreulx

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-02173140v1

Submitted on 10 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Libera Latino,[a,#] Delphine Patin,[a] Dimitri Chérier,[a] Thierry Touzé,[a] Christine Pourcel,[a] Hélène Barreteau,[a] and Dominique Mengin-Lecreulx[a,#]

[a]Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ Paris-Sud, Université Paris-Saclay, 91198 Gif-sur-Yvette, France.

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

Address correspondence to Libera Latino, libera.latino@i2bc.paris-saclay.fr or Dominique Mengin-Lecreulx, dominique.mengin-lecreulx@i2bc.paris-saclay.fr.

ABSTRACT

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

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

IMPORTANCE

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

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

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

INTRODUCTION

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

PaeM import and strain susceptibility depend on TonB1. The above results prompted us to analyze whether PAO1 and PA14 strains could also exhibit some differences in the subsequent steps allowing import of these pyocins in the periplasm. As many other bacterial pathogens, *P. aeruginosa* developed efficient systems to retrieve and transport iron from natural sources. This is in particular achieved through the expression and release of two Fe^{3+}-chelating siderophores, pyochelin and pyoverdine, which are subsequently re-imported into cells via the cognate FptA and FpvA outer membrane receptors, respectively (21). The latter translocation event requires both the proton motive force and the TonB-ExbB-ExbD protein machinery acting as energy coupler (22). Contrary to *E. coli* which possesses only one TonB protein, *P. aeruginosa* expresses three *tonB* genes (*tonB1*, *tonB2* and *tonB3*) and *tonB1* was

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

receptor sequence was sufficient to render PAO1 cells susceptible to both PaeM1 and PaeM2 variants. A different situation was encountered when analyzing the resistance of the reference strain PA14. The FiuA receptor of this strain, which did not differ much from its $FiuA_{JJ692}$ and $FiuA_{DSM50071}$ orthologs, failed to mediate PaeM internalization into PA14 cells while it was perfectly functional in this respect when expressed in other genetic backgrounds such as PAO1. This prompted us to identify which step downstream of the FiuA reception step determined PA14 strain resistance to PaeM pyocins. As is the case for the ColM receptor FhuA in *E. coli*, the *P. aeruginosa* receptor FiuA interacts with the TonB/ExbB/ExbD protein complex to translocate siderophores into cells. In contrast to *E. coli*, *P. aeruginosa* possesses three TonB proteins designated TonB1, TonB2 and TonB3. First, testing the susceptibility to PaeM variants of PAO1 wild-type and *tonB1* mutant strains expressing different FiuA receptors allowed us to demonstrate that the TonB1 protein was absolutely required for PaeM import into *P. aeruginosa* cells. An alignment of TonB1 protein sequences from PAO1, PA14 and some other strains tested in this study revealed that curiously only the $TonB1_{PA14}$ protein displayed some specific differences. We thus tested expression of the $tonB1_{PAO1}$ gene in the PA14 strain and found that this rendered the strain susceptible to PaeM pyocins, which was consistent with the previous observation that the $FiuA_{PA14}$ receptor was functional, i.e. allowed internalization of both PaeM variants, when expressed in PAO1 strain. These results suggested that $FiuA_{PA14}$ and $TonB1_{PAO1}$ proteins can interact together and conjointly contribute to PaeM import into PA14 cells, contrary to the $FiuA_{PA14}$-$TonB1_{PA14}$ couple. That the expression of $FiuA_{PAO1}$, $FiuA_{DSM50071}$ and some other receptors (except $FiuA_{PA14}$) also made the PA14 strain becoming PaeM-susceptible suggested that the $TonB1_{PA14}$ protein can interact with these receptors. These different results thus highlighted the essential role played by the TonB1 protein in the import of this class of pyocins and revealed that discrete variations of FiuA or TonB1 sequences could impact their interaction and be responsible for

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

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

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

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

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

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

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

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5α (Life Science Technologies, Inc.) was used as the host strain in cloning experiments and the BL21(DE3)(pLysS) strain (Novagen) was routinely used for the overproduction of PaeM bacteriocins with pET plasmids. Other strains used in this study are listed in Table 1. Clinical *P. aeruginosa* strains were isolated from cystic fibrosis (CF) patients or from patients in intensive care units (ICU) with different infection sites. Some of them were previously described by Llanes et al. (33) and Larché et al. (28). The PAO1 *tonB1*-null mutant was obtained from Dr. Paolo Visca (34). 2YT rich medium (35) was used for growing cells and growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer. For the preparation of plates, 2YT was supplemented with agar (Sigma-Aldrich) at 1.6% (w/v), and 0.7% for the top layer. When required, ampicillin, kanamycin, chloramphenicol and gentamicin were added at final

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

General DNA techniques, plasmids and cell transformation. PCR amplification of genes from *E. coli* and *P. aeruginosa* chromosomes was performed in a Thermocycler 60 apparatus (Bio-med) using the Expand-Fidelity polymerase (Roche) and DNA fragments were purified using the Wizard PCR Preps DNA purification kit (Promega). The standard procedures for endonuclease digestions, ligation and agarose gel electrophoresis described by Sambrook et al. were used (36). The pET2160 vector allowing high level expression of proteins with a C-terminal 6 × His-tag was previously described (6), as well as the multi-copy *E. coli-P. aeruginosa* shuttle vector pUCP24 (37). The pET2160-derivative plasmid pMLD245 used for expression and purification of the PaeM bacteriocin from strain JJ692 (PaeM1) was previously described (6). Plasmids pTTB240, pTTB243 and pTTB38 expressing D241A, D244A, and D241A D244A mutants of PaeM1, respectively, were generated by mutagenesis of pMLD245 plasmid (10). *E. coli* cells were transformed with plasmid DNA either by treatment with calcium chloride as described by Dagert and Ehrlich (38) or by electroporation. Transformation of *P. aeruginosa* cells was done by electroporation (39). Site-directed mutagenesis was performed by using the Quickchange II XL site-directed mutagenesis kit (Stratagene) and appropriate oligonucleotides listed in Table S1. All of the mutations introduced in plasmids were confirmed by DNA sequencing (Eurofins).

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

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

Construction of PaeM, ImM, FiuA and TonB1 expression plasmids. The gene encoding the PaeM bacteriocin homolog from *P. aeruginosa* strain DSM50071 (PaeM2) was PCR-amplified using PaeM2-for and -rev oligonucleotides shown in Table S1 that introduced NcoI and BglII restriction sites at the 5' and 3' gene extremities, respectively. The PCR fragment was digested by these enzymes and then cloned between the same sites of the pET2160 vector, resulting in plasmid pLL17 that allows expression of the 289-residues PaeM2 protein with a C-terminal Arg-Ser-$(His)_6$ extension. A pLL42 derivative plasmid expressing a D241A D244A mutated version of this PaeM2 protein was generated by site-directed mutagenesis of pLL17 using the two oligonucleotides PaeM2-mut1 and -mut2 described in Table S1. The putative PaeM1 immunity gene (EXA14, here designated as ImM1) that is located within the *exoU*-containing island A of *P. aeruginosa* JJ692 chromosome (6, 14) and its homolog (ImM2) detected in the genome of strain DSM50071 were PCR-amplified using the ImM-for and -rev primers that introduced BamHI and HindIII restriction sites at the gene extremities, respectively (Table S1). These two genes were cloned between the same sites of the pUCP24 *E. coli-P. aeruginosa* shuttle plasmid, yielding pLL18 and pLL19 plasmids, respectively. To optimize gene expression from the pUCP24 vector and further increase the number of restrictions sites present in the polylinker sequence, a NcoI site was introduced in this plasmid so that ATG initiation codons of the cloned genes were placed exactly at the position of the initiation codon of the *lacZ* alpha peptide gene. This modified vector pUCP24Nco was obtained by site-directed mutagenesis of pUCP24 using the two pUCP24-mut1 and -mut2 primers (Table S1). Seven chimeric PaeM proteins were also generated in which sequences of different sizes of PaeM1 N-terminal region were replaced by corresponding sequences originating from the PaeM2 protein (as depicted in Fig. 11A). The

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

were amplified using the TonB1-for and TonB1-rev oligonucleotides (Table S1) and the resulting DNA fragments were cleaved by PciI and HindIII and inserted between the compatible NcoI and HindIII sites of the pUCP24Nco vector, yielding the pLL58 and pLL59 plasmids, respectively. Plasmids pMLD652/653 expressing *fiuA*$_{PA14}$-*tonB1*$_{PA01}$ and *fiuA*$_{PA14}$-*tonB1*$_{PA14}$ gene couples, respectively, were constructed as follows: first, the *fiuA* gene was amplified from the PA14 chromosome using FiuA-for and FiuAtonB1-rev oligonucleotides and the *tonB1* gene was amplified from PAO1 and PA14 chromosomes using FiuAtonB1-for and TonB1-rev oligonucleotides. These two fragments were hybridized together and a new PCR was performed using FiuA-for and TonB1-rev as primers. The resulting fragments were purified, treated by NcoI and HindIII and then inserted between the same sites in the pUCP24Nco vector.

Expression and purification of wild-type and mutant PaeM proteins. Recombinant His-tagged proteins PaeM$_{JJ692}$ (PaeM1), PaeM$_{DSM50071}$ (PaeM2), the corresponding single or double mutants (D241A, D244A and D241A D244A), as well as the eight chimeric PaeM proteins, were overproduced using the pET2160 derived plasmids described above and the *E. coli* BL21(DE3)(pLysS) as the host strain. Exponentially-growing cultures (1 liter) were incubated at 37°C until the optical density at 600 nm (OD_{600}) reached 0.8. Gene expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) and incubation was continued for 3 h at 37°C. Then, cells were harvested by centrifugation at 8000 × *g* for 10 min at 4°C and washed once with a 0.9% NaCl solution. Pellets were resuspended in 10 ml of 20 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM $MgCl_2$ and 10 mM 2-mercaptoethanol (buffer A) and cells were subsequently disrupted by sonication (Bioblock Vibracell sonicator, model 72412). Insoluble proteins and bacterial debris were removed by centrifugation at 200,000 × *g* (Beckman TL100 centrifuge) for 20 min at 4°C and the resulting supernatants were directly used for protein purification or were

stored at -20°C. The His_6-tagged PaeM proteins were purified on nickel-nitrilotriacetate agarose (Ni^{2+}-NTA) as recommended by the manufacturer (Qiagen). Briefly, crude soluble protein extracts in buffer A were supplemented with 10 mM imidazole and 300 mM NaCl and incubated for 1 h at 4°C with 2 ml of Ni-NTA resin preequilibrated in the same buffer. The resin was then extensively washed with buffer A containing increasing concentrations of imidazole (from 20 to 40 mM) and proteins were eluted with 100, 200 and 300 mM imidazole. The relevant fractions were pooled, eventually concentrated by ultra-filtration (10K Amicon Ultra-15 filters, Millipore), and dialyzed overnight against buffer A supplemented with 10% glycerol. Protein concentrations were determined with a NanoDrop (Thermo Scientific) and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins was performed as described previously (41).

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

Lipid II hydrolase activiy assay. The enzymatic activity of PaeM variants was tested in a reaction mixture containing, in a final volume of 10 µl, 100 mM Tris-HCl buffer, pH 7.5, 1.5 µM [^{14}C]radiolabeled lipid II (120 Bq), 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 150 mM NaCl, 0.2% *n*-dodecyl-β-D-maltoside (DDM), and PaeM protein (from 5 ng to 10 µg in 5 µl of buffer A). After 30 min of incubation at 37°C, the reaction was stopped by heating at

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

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

Accession numbers. The *fiuA* gene sequences determined in the present study are available under the following Genbank accession numbers: MK574012 ($FiuA_{C3-20}$), MK574013 ($FiuA_{C4-17}$), MK574014 ($FiuA_{DET08}$), MK574015 ($FiuA_{PcyII-57}$), MK574016 ($FiuA_{13i}$), and MK574014 ($FiuA_{PcyII-55}$). Accession numbers of other genes are indicated in the text and the figure legends.

ACKNOWLEDGMENTS

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

We declare no conflicts of interest.

REFERENCES

1. Cascales E, Buchanan SK, Duché D, Kleanthous C, Lloubès R, Postle K, Riley M, Slatin S, Cavard D. 2007. Colicin biology. Microbiol Mol Biol Rev 71:158-229.
2. El Ghachi M, Bouhss A, Barreteau H, Touzé T, Auger G, Blanot D, Mengin-Lecreulx D. 2006. Colicin M exerts its bacteriolytic effect via enzymatic degradation of undecaprenyl phosphate-linked peptidoglycan precursors. J Biol Chem 281:22761-22772.
3. Schaller K, Höltje JV, Braun V. 1982. Colicin M is an inhibitor of murein biosynthesis. J Bacteriol 152:994-1000.
4. Braun V, Schaller K, Wolff H. 1973. A common receptor protein for phage T5 and colicin M in the outer membrane of *Escherichia coli* B. Biochim Biophys Acta 323:87-97.
5. Braun V, Patzer SI, Hantke K. 2002. Ton-dependent colicins and microcins: modular design and evolution. Biochimie 84:365-380.
6. Barreteau H, Bouhss A, Fourgeaud M, Mainardi JL, Touzé T, Gerard F, Blanot D, Arthur M, Mengin-Lecreulx D. 2009. Human- and plant-pathogenic *Pseudomonas* species produce bacteriocins exhibiting colicin M-like hydrolase activity towards peptidoglycan precursors. J Bacteriol 191:3657-3664.
7. Grinter R, Josts I, Zeth K, Roszak AW, McCaughey LC, Cogdell RJ, Milner JJ, Kelly SM, Byron O, Walker D. 2014. Structure of the atypical bacteriocin pectocin M2 implies a novel mechanism of protein uptake. Mol Microbiol 93:234-246.
8. Chérier D, Giacomucci S, Patin D, Bouhss A, Touzé T, Blanot D, Mengin-Lecreulx D, Barreteau H. 2016. Pectocin M1 (PcaM1) inhibits *Escherichia coli* cell growth and peptidoglycan biosynthesis through periplasmic expression. Antibiotics (Basel) 5:E36.

9. Ghequire MGK, Buchanan SK, De Mot R. 2018. The ColM family, polymorphic toxins breaching the bacterial cell wall. MBio 9:e02267-17.
10. Barreteau H, Tiouajni M, Graille M, Josseaume N, Bouhss A, Patin D, Blanot D, Fourgeaud M, Mainardi JL, Arthur M, van Tilbeurgh H, Mengin-Lecreulx D, Touzé T. 2012. Functional and structural characterization of PaeM, a colicin M-like bacteriocin produced by *Pseudomonas aeruginosa*. J Biol Chem 287:37395-37405.
11. Ghequire MG, Kemland L, Anoz-Carbonell E, Buchanan SK, De Mot R. 2017. A natural chimeric *Pseudomonas* bacteriocin with novel pore-forming activity parasitizes the ferrichrome transporter. MBio 8:e01961-16.
12. Paskevicius S, Starkevic U, Misiunas A, Vitkauskiene A, Gleba Y, Razanskiene A. 2017. Plant-expressed pyocins for control of *Pseudomonas aeruginosa*. PLoS One 12:e0185782.
13. Ghequire MGK, Ozturk B. 2018. A colicin M-type bacteriocin from *Pseudomonas aeruginosa* targeting the HxuC heme receptor requires a novel immunity partner. Appl Environ Microbiol 84:e00716-18.
14. Kulasekara BR, Kulasekara HD, Wolfgang MC, Stevens L, Frank DW, Lory S. 2006. Acquisition and evolution of the *exoU* locus in *Pseudomonas aeruginosa*. J Bacteriol 188:4037-4050.
15. Barreteau H, Bouhss A, Gérard F, Duché D, Boussaid B, Blanot D, Lloubès R, Mengin-Lecreulx D, Touzé T. 2010. Deciphering the catalytic domain of colicin M, a peptidoglycan lipid II degrading enzyme. J Biol Chem 285:12378-12389.
16. Helbig S, Braun V. 2011. Mapping functional domains of colicin M. J Bacteriol 193:815-821.

17. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nature Protocols 10:845-858.
18. Cobessi D, Celia H, Folschweiller N, Schalk IJ, Abdallah MA, Pattus F. 2005. The crystal structure of the pyoverdine outer membrane receptor FpvA from *Pseudomonas aeruginosa* at 3.6 Å resolution. J Mol Biol 347:121-134.
19. Cobessi D, Celia H, Pattus F. 2005. Crystal structure at high resolution of ferric-pyochelin and its membrane receptor FptA from *Pseudomonas aeruginosa*. J Mol Biol 352:893-904.
20. Ghequire MG, Kemland L, De Mot R. 2017. Novel immunity proteins associated with colicin M-like bacteriocins exhibit promiscuous protection in *Pseudomonas*. Front Microbiol 8:93.
21. Schalk IJ, Cunrath O. 2016. An overview of the biological metal uptake pathways in *Pseudomonas aeruginosa*. Environ Microbiol 18:3227-3246.
22. Noinaj N, Guillier M, Barnard TJ, Buchanan SK. 2010. TonB-dependent transporters: regulation, structure, and function. Annu Rev Microbiol 64:43-60.
23. Huang B, Ru K, Yuan Z, Whitchurch CB, Mattick JS. 2004. *tonB3* is required for normal twitching motility and extracellular assembly of type IV pili. J Bacteriol 186:4387-4389.
24. Zhao Q, Poole K. 2000. A second *tonB* gene in *Pseudomonas aeruginosa* is linked to the *exbB* and *exbD* genes. FEMS Microbiol Lett 184:127-132.
25. Takase H, Nitanai H, Hoshino K, Otani T. 2000. Requirement of the *Pseudomonas aeruginosa tonB* gene for high-affinity iron acquisition and infection. Infect Immun 68:4498-4504.
26. Touzé T, Barreteau H, El Ghachi M, Bouhss A, Barnéoud-Arnoulet A, Patin D, Sacco E, Blanot D, Arthur M, Duché D, Lloubès R, Mengin-Lecreulx D. 2012. Colicin M, a pepti-

doglycan lipid-II-degrading enzyme: potential use for antibacterial means? Biochem Soc Trans 40:1522-1527.

27. Gérard F, Brooks MA, Barreteau H, Touzé T, Graille M, Bouhss A, Blanot D, van Tilbeurgh H, Mengin-Lecreulx D. 2011. X-ray structure and site-directed mutagenesis analysis of the *Escherichia coli* colicin M immunity protein. J Bacteriol 193:205-214.

28. Larché J, Pouillot F, Essoh C, Libisch B, Straut M, Lee JC, Soler C, Lamarca R, Gleize E, Gabard J, Vergnaud G, Pourcel C. 2012. Rapid identification of international multidrug-resistant *Pseudomonas aeruginosa* clones by multiple-locus variable number of tandem repeats analysis and investigation of their susceptibility to lytic bacteriophages. Antimicrob Agents Chemother 56:6175-6180.

29. Hong JS, Yoon EJ, Lee H, Jeong SH, Lee K. 2016. Clonal dissemination of *Pseudomonas aeruginosa* sequence type 235 isolates carrying blaIMP-6 and emergence of blaGES-24 and blaIMP-10 on novel genomic islands PAGI-15 and -16 in South Korea. Antimicrob Agents Chemother 60:7216-7223.

30. Miyoshi-Akiyama T, Tada T, Ohmagari N, Viet Hung N, Tharavichitkul P, Pokhrel BM, Gniadkowski M, Shimojima M, Kirikae T. 2017. Emergence and spread of epidemic multidrug-resistant *Pseudomonas aeruginosa*. Genome Biol Evol 9:3238-3245.

31. Treepong P, Kos VN, Guyeux C, Blanc DS, Bertrand X, Valot B, Hocquet D. 2018. Global emergence of the widespread *Pseudomonas aeruginosa* ST235 clone. Clin Microbiol Infect 24:258-266.

32. Sato H, Frank DW, Hillard CJ, Feix JB, Pankhaniya RR, Moriyama K, Finck-Barbançon V, Buchaklian A, Lei M, Long RM, Wiener-Kronish J, Sawa T. 2003. The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III cytotoxin, ExoU. EMBO J 22:2959-2969.

33. Llanes C, Pourcel C, Richardot C, Plésiat P, Fichant G, Cavallo JD, Mérens A, Group GS. 2013. Diversity of β-lactam resistance mechanisms in cystic fibrosis isolates of *Pseudomonas aeruginosa*: a French multicentre study. J Antimicrob Chemother 68:1763-1771.

34. Minandri F, Imperi F, Frangipani E, Bonchi C, Visaggio D, Facchini M, Pasquali P, Bragonzi A, Visca P. 2016. Role of Iron Uptake Systems in *Pseudomonas aeruginosa* Virulence and Airway Infection. Infect Immun 84:2324-2335.

35. Miller JH. Experiments in Molecular Genetics. In: Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; 1972:431-435.

36. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; 1989.

37. West SE, Schweizer HP, Dall C, Sample AK, Runyen-Janecky LJ. 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene 148:81-86.

38. Dagert M, Ehrlich SD. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene 6:23-28.

39. Choi KH, Kumar A, Schweizer HP. 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods 64:391-397.

40. Vu-Thien H, Corbineau G, Hormigos K, Fauroux B, Corvol H, Clément A, Vergnaud G, Pourcel C. 2007. Multiple-locus variable-number tandem-repeat analysis for longitudinal survey of sources of *Pseudomonas aeruginosa* infection in cystic fibrosis patients. J Clin Microbiol 45:3175-3183.

41. Laemmli UK, Favre M. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J Mol Biol 80:575-599.

42. Pugsley AP, Schnaitman CA. 1978. Outer membrane proteins of *Escherichia coli*. VII. Evidence that bacteriophage-directed protein 2 functions as a pore. J Bacteriol 133:1181-1189.

43. Zeth K, Romer C, Patzer SI, Braun V. 2008. Crystal structure of colicin M, a novel phosphatase specifically imported by Escherichia coli. J Biol Chem 283:25324-25331.

FIGURE LEGENDS

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).

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

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

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.

Figure 4. Sequence alignment of FiuA receptors from selected *P. aeruginosa* strains. Amino acid sequences of FiuA outer-membrane receptor proteins from strains PcyII-10 and PA14 (resistant to both PaeM variants), PcyII-57 and PAO1 (susceptible only to PaeM2), PA14 (resistant to both PaeM1 and PaeM2), 13i and PcyII-55 (susceptible mainly to PaeM1), C4-17, C3-20 and DET08 (susceptible to both PaeM variants), and JJ692 and DSM50071 (PaeM-producing strains) 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. Accession numbers: SIP50767.1 ($FiuA_{PcyII-10}$), MK574015 ($FiuA_{PcyII-57}$), NP_249161.1 ($FiuA_{PAO1}$), SCM60368.1 ($FiuA_{PA14}$), ERZ16319.1 ($FiuA_{JJ692}$), MK574017 ($FiuA_{PcyII-55}$), MK574013 ($FiuA_{C4-17}$), MK574012 ($FiuA_{C3-20}$), MK574014 ($FiuA_{DET08}$), MK574016 ($FiuA_{13i}$), AKO84658.1 ($FiuA_{DSM50071}$).

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

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

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

Figure 7. Alignement 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-µl 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.

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

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

Figure 9. Implication of TonB1 protein in the import of PaeM variants in PAO1 strain. A PAOI *tonB1*-null mutant was transformed with the pUCP24Nco plasmid expressing the $TonB1_{PAO1}$ protein or the $FiuA_{DSM50071}$ receptor and the susceptibility of the resulting strains to PaeM1 and PaeM2 proteins was tested by spotting 5 µl of undiluted or serial 10-fold dilutions of the purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones were observed after 24 h incubation at 37°C.

Figure 10. TonB1 protein sequences of PAO1 and PA14 strains (A) and their expression in PA14 (B) and PAO1 Δ*tonB1* (C) backgrounds. A, TonB1 protein sequences of strains PAO1 and PA14 were aligned using 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, Expression of the latter TonB1 proteins in the PA14 strain. C, Expression of $TonB1_{PA14}$ and of $FiuA_{PA14}$/$TonB1_{PAO1}$ or $FiuA_{PA14}$/$TonB1_{PA14}$ couples in the PAO1 Δ*tonB1* strain. In B and C, the strain susceptibility to the PaeM pyocins was tested by spotting 5 µl of undiluted or serial 10-fold dilutions of purified protein stocks (0.6 mg/ml) on the bacterial lawns. Growth inhibition zones were observed after 24 h incubation at 37°C. Accession numbers: AAG08916.1 ($TonB1_{PAO1}$), SCM65789.1 ($TonB1_{PA14}$).

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-µl 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.

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

***P. aeruginosa* strains**	**Susceptibility to PaeM1[a]**	**Detection of *PaeM1*/*ImM1***	**Susceptibility to PaeM2[a]**	**Detection of *PaeM2*/*ImM2***
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	-	-/-	-	+/+

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

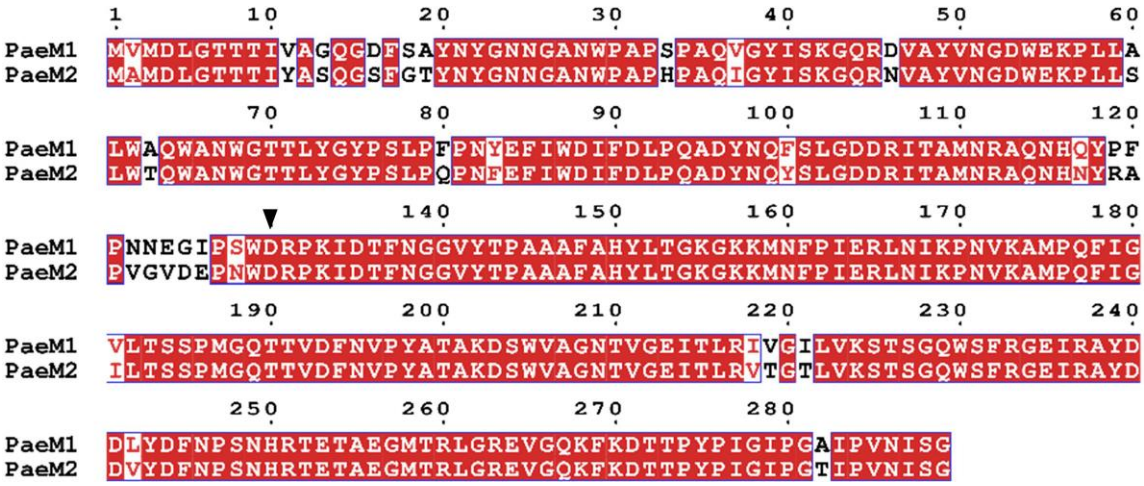


Figure 1. Sequence alignment of PaeM variants. Amino acid sequences of PaeM1 from strain JJ692 and PaeM2 from strain DSM50071 were aligned using the Clustal 2.1 program and graphic visualization was obtained using ESPript 3.0. The black triangle localizes the beginning of the activity domain. Dissection 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).

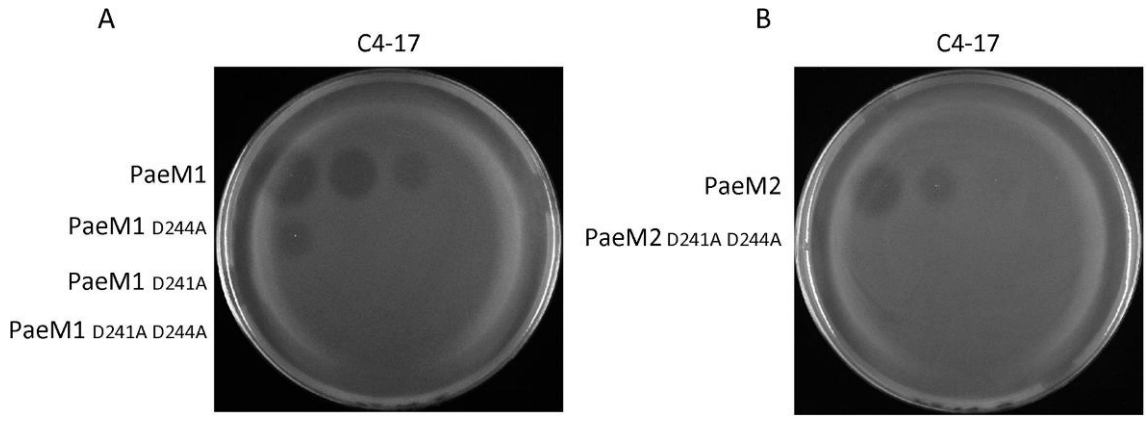


Figure 2. Spot killing assay of PaeM variants and mutants on *P. aeruginosa* susceptible strain C4-17. Five-µl spots of undiluted (0.6 mg/ml) or serial 10-fold dilutions of wild-type or mutant PaeM1 (A) and PaeM2 (B) protein stocks (from left to right) were deposited on lawns of the representative strain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

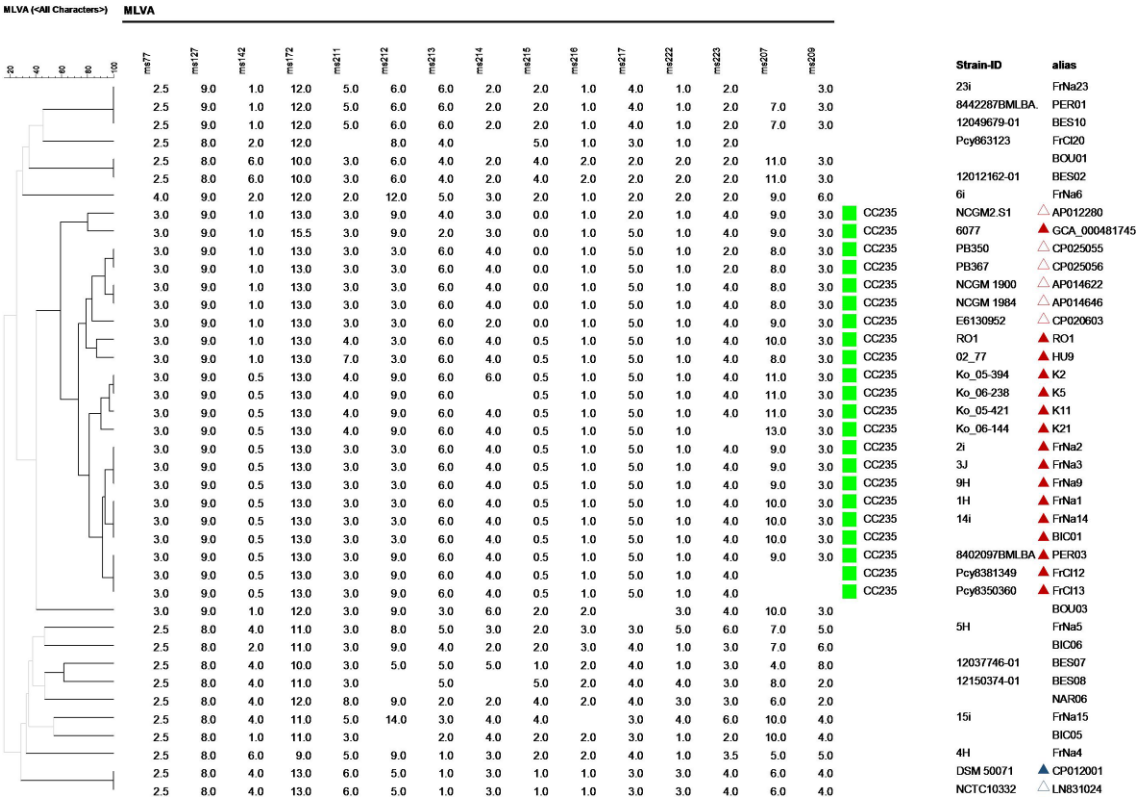


Figure 3. Clustering of *P. aeruginosa* strains using *in vivo* and *in silico* VNTR analysis data. For the multiple locus variable number of tandem repeat (VNTR) analysis (MLVA), *P. aeruginosa* strains 6077 (the original strain from which the *exoU*-containing island A was transferred to strain JJ692) 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.

1017

```
                1        10        20        30        40        50        60        70        80        90       100
FiuAPcyII-10    MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAPcyII-57    MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAPAO1        MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAPA14        MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAJJ692       MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENVG
FiuAPcyII-55    MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENVG
FiuAC4-17       MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAC3-20       MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuADET08       MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuA13i         MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuADSM50071    MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG

                110       120       130       140       150       160       170       180       190       200
FiuAPcyII-10    GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDSRSDWLKVRG
FiuAPcyII-57    GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuAPAO1        GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDSRSDWLKVRG
FiuAPA14        GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuAJJ692       GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuAPcyII-55    GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuAC4-17       GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuAC3-20       GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuADET08       GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDSRSDWLKVRG
FiuA13i         GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDSRSDWLKVRG
FiuADSM50071    GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHSLDDAVRYMPGVVASSFGSDTRSDWLKVRG

                210       220       230       240       250       260       270       280       290       300
FiuAPcyII-10    FKPTQFLDGLPMATGSYNNPKLETWNLERLALLRGPASSVYGQTPPGGMLDMVSLRPQGIASHEIKAEIGNYNHNQISFDSTGPIDDEGRFLYRLSGVVR
FiuAPcyII-57    FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAPAO1        FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAPA14        FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVTSHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRISGVVR
FiuAJJ692       FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAPcyII-55    FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAC4-17       FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEEKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAC3-20       FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuADET08       FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuA13i         FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuADSM50071    FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR

                310       320       330       340       350       360       370       380       390       400
FiuAPcyII-10    DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLSQFNRDDTAATSQFLPLRGTKYDAPFGKISHHKNLGDPDWDFYDRTYYWLGYSFERRINDVWQFRQN
FiuAPcyII-57    ESNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuAPAO1        DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuAPA14        DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuAJJ692       DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFEQRINDVWQFRQN
FiuAPcyII-55    DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFEQRINDVWQFRQN
FiuAC4-17       DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuAC3-20       DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuADET08       DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuA13i         DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuADSM50071    DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN

                410  Loop 4   430       440       450       460       470  Loop 5   490       500
FiuAPcyII-10    LRYLRNDLSFQTLTPTSSIDAASADGTLQRMTSVDEDISQFVVDNNFQADFETGALRHTLLLGIDHNRTRTDYTSIYNFTGVPPTNVNNPIYGQPIPKP
FiuAPcyII-57    LRYLRNDLSFQALTPASYYAAASDDGTLQRTSTSVDEDVSQFVMDNNFQADFDTGALRHTLLLGIDHNRTTNEYTSIFGFN.VPPTNINNPIYGQPIPKP
FiuAPAO1        LRYLRNDLSFQALTPASYYAAASDDGTLQRTSTSVDEDVSQFVVDNNFQADFDTGALRHTLLLGIDHNRTTNEYTSIFGFN.VPPTNINNPIYGQPIPKP
FiuAPA14        LRYLRNDLSFQAVTAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGIDHNRTDNQYTSIFGS..APPSNVNNPIYGQPIVKP
FiuAJJ692       LRYLRNDLSFQAVTAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGIDHNRTDNQYTSIFGS..APPSNVNNPIYGQPIVKP
FiuAPcyII-55    LRYLRNDLSFQAVTAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGIDHNRTDNQYTSIFGS..APPSNVNNPIYGQPIVKP
FiuAC4-17       LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGIDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP
FiuAC3-20       LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGIDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP
FiuADET08       LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGIDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP
FiuA13i         LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGIDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP
FiuADSM50071    LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGIDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP

                510       520       530       540       550       560       570       580       590       600
FiuAPcyII-10    SRSSAFYDYRQKTYQTGLYVQDQIALDNWRLTLGGREDWVHAGATFYNQNDATNTSRDKKFSGNAALSYIFDNGLAPYLSYAESFQPSTGADDIGSNKMF
FiuAPcyII-57    PRSDAYYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAPAO1        PRSDAYYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAPA14        DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAJJ692       DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAPcyII-55    DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAC4-17       DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAC3-20       DRSTAFYNYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuADET08       DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuA13i         DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuADSM50071    DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL

                610       620       630       640       650       660       670       680       690       700
FiuAPcyII-10    KPTEGKQWELGVKYQPVGSDSLFTAAVYDLRQENVRVTDNVNGTPTTSQAGEVKVTGLELEATSNVTDNLKVIGSYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAPcyII-57    KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAPAO1        KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAPA14        KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKLIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAJJ692       KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAPcyII-55    KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAC4-17       KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAC3-20       KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuADET08       KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuA13i         KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuADSM50071    KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ

                710       720       730       740       750       760       770       780       790       800
FiuAPcyII-10    ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAPcyII-57    ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAPAO1        ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAPA14        ASLWADYTWHEGALNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAJJ692       ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAPcyII-55    ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAC4-17       ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAC3-20       ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuADET08       ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuA13i         ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuADSM50071    ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV

FiuAPcyII-10    SYKW
FiuAPcyII-57    SYKW
FiuAPAO1        SYKW
FiuAPA14        SYKW
FiuAJJ692       SYKW
FiuAPcyII-55    SYKW
FiuAC4-17       SYKW
FiuAC3-20       SYKW
FiuADET08       SYKW
FiuA13i         SYKW
FiuADSM50071    SYKW
```

Figure 4. Sequence alignment of FiuA receptors from selected *P. aeruginosa* strains. Amino acid sequences of FiuA outer-membrane receptor proteins from strains PcyII-10 and PA14 (resistant to both PaeM variants), PcyII-57 and PAO1 (susceptible only to PaeM2), PA14 (resistant to both PaeM1 and PaeM2), 13i and PcyII-55 (susceptible mainly to PaeM1), C4-17, C3-20 and DET08 (susceptible to both PaeM variants), and JJ692 and DSM50071 (PaeM-producing strains) 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. Accession numbers: SIP50767.1 ($FiuA_{PcyII-10}$), MK574015 ($FiuA_{PcyII-57}$), NP_249161.1 ($FiuA_{PAO1}$), SCM60368.1 ($FiuA_{PA14}$), ERZ16319.1 ($FiuA_{JJ692}$), MK574017 ($FiuA_{PcyII-55}$), MK574013 ($FiuA_{C4-17}$), MK574012 ($FiuA_{C3-20}$), MK574014 ($FiuA_{DET08}$), MK574016 ($FiuA_{13i}$), AKO84658.1 ($FiuA_{DSM50071}$).

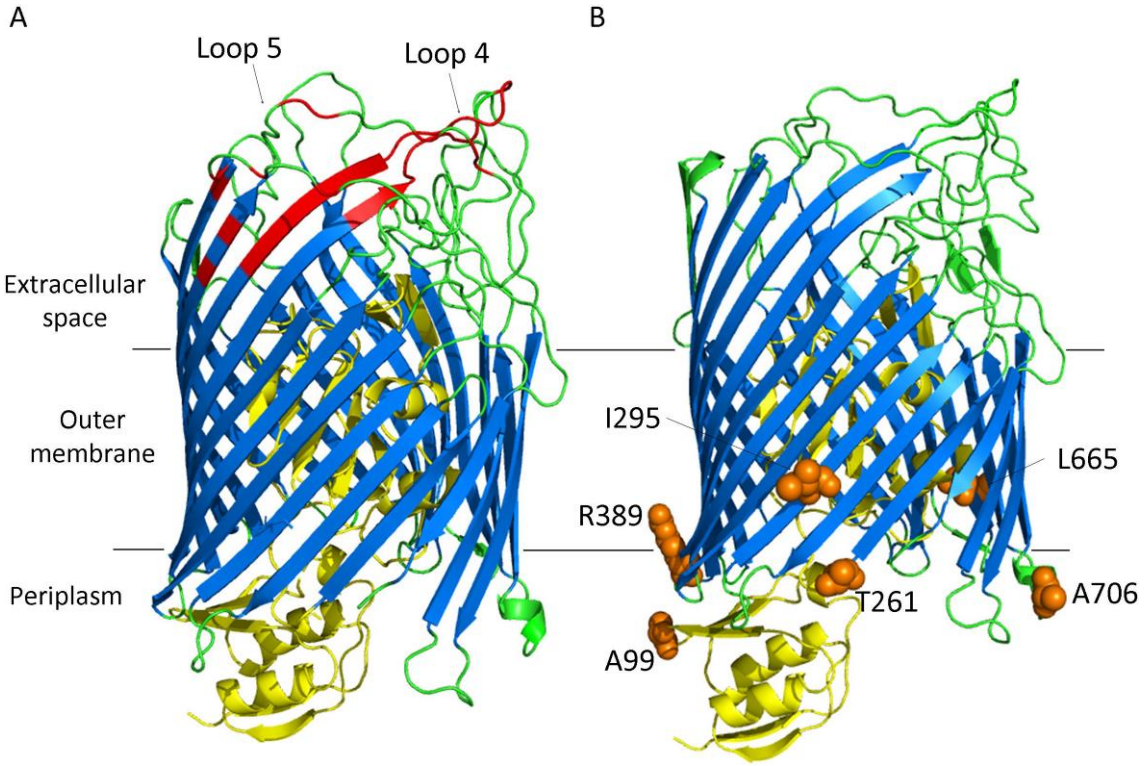


Figure 5. **Predicted $FiuA_{PAO1}$ (A) and $FiuA_{PA14}$ (B) receptor structures.** View of the $FiuA_{PAO1}$ (A) and $FiuA_{PA14}$ (B) siderophore receptor structures drawn as ribbons. The plug and the transmembrane β-barrel domains are colored in yellow and blue, respectively. The cytoplasmic turns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

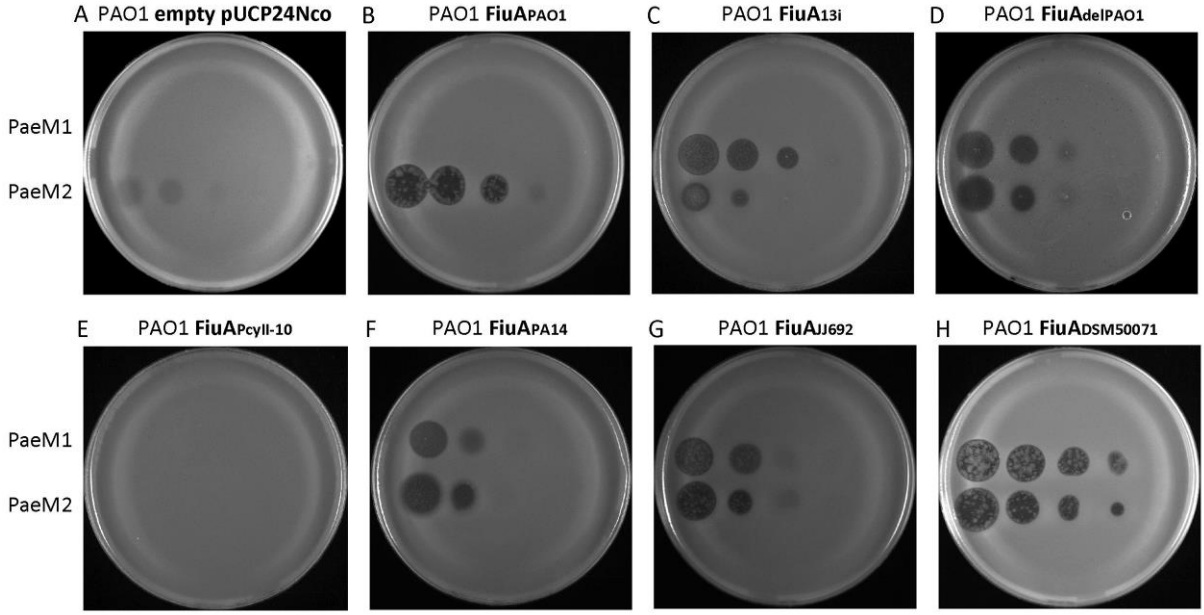


Figure 6. Impact of FiuA receptor structure on the susceptibility of PAO1 strain to PaeM variants. The *fiuA* genes from different representative strains (PAO1, 13i, PcyII-10, PA14, JJ692 and DSM50071) were cloned in the pUCP24Nco vector and the PAO1 strain was transformed by the plasmids thus generated.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

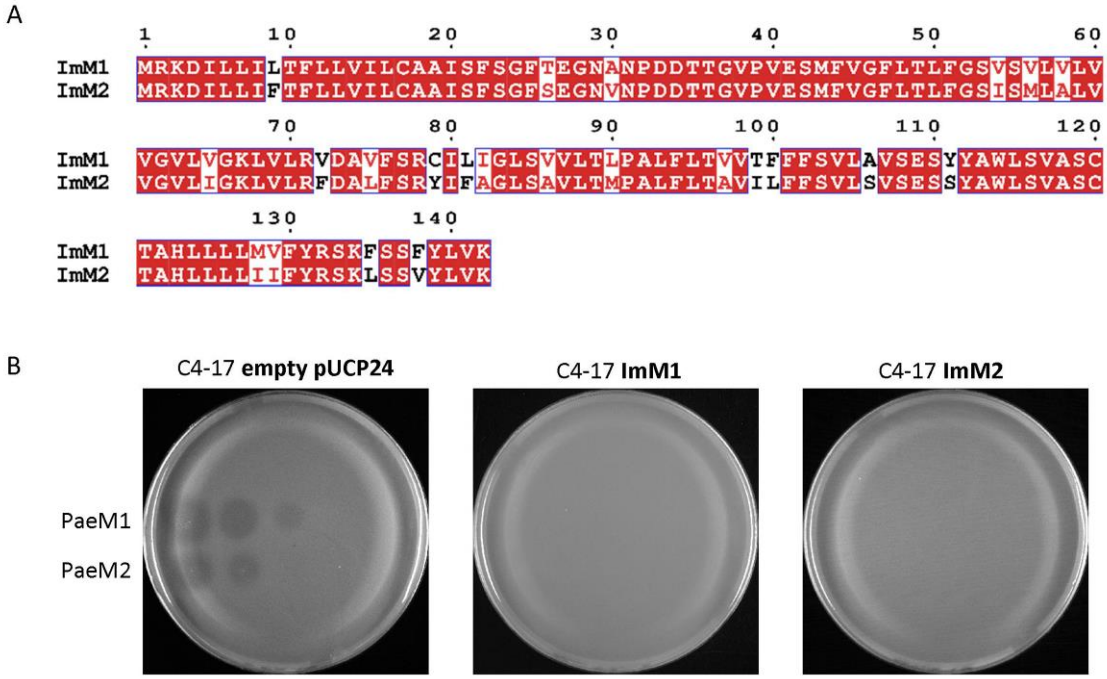


Figure 7. Alignement of ImM immunity proteins (A) and their expression in C4-17 strain (B). A, Amino acid sequences of ImM1 from strain JJ692 and ImM2 from strain DSM50071 were aligned using the Clustal 2.1 program and graphic visualization was obtained using ESPript 3.0. Amino acid numbers are indicated on the top of the sequences. B, 5-µl 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.

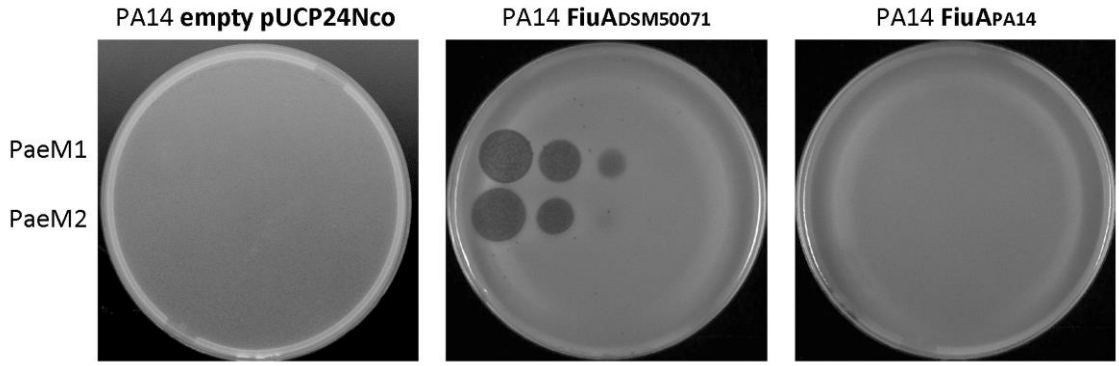


Figure 8. Impact of FiuA polymorphism on the susceptibility of PA14 strain to PaeM variants. *fiuA* genes from DSM50071 and PA14 strains were cloned in the pUCP24Nco vector. The PA14 strain was transformed by these plasmids and the susceptibility of the resulting strains to PaeM1 and PaeM2 proteins was tested by spotting 5 µl of undiluted or serial 10-fold dilutions of the purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones were observed after 24 h incubation at 37°C.

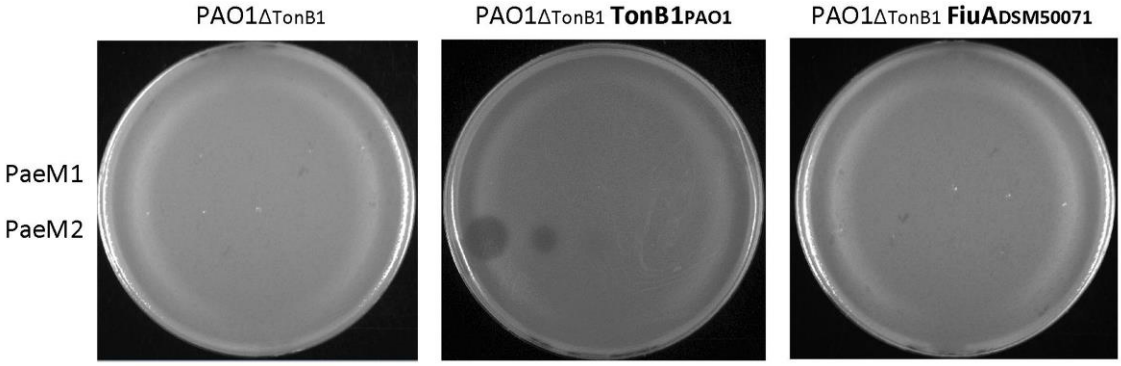


Figure 9. Implication of TonB1 protein in the import of PaeM variants in PAO1 strain. A PAOI *tonB1*-null mutant was transformed with the pUCP24Nco plasmid expressing the $TonB1_{PAO1}$ protein or the $FiuA_{DSM50071}$ receptor and the susceptibility of the resulting strains to PaeM1 and PaeM2 proteins was tested by spotting 5 µl of undiluted or serial 10-fold dilutions of the purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones were observed after 24 h incubation at 37°C.

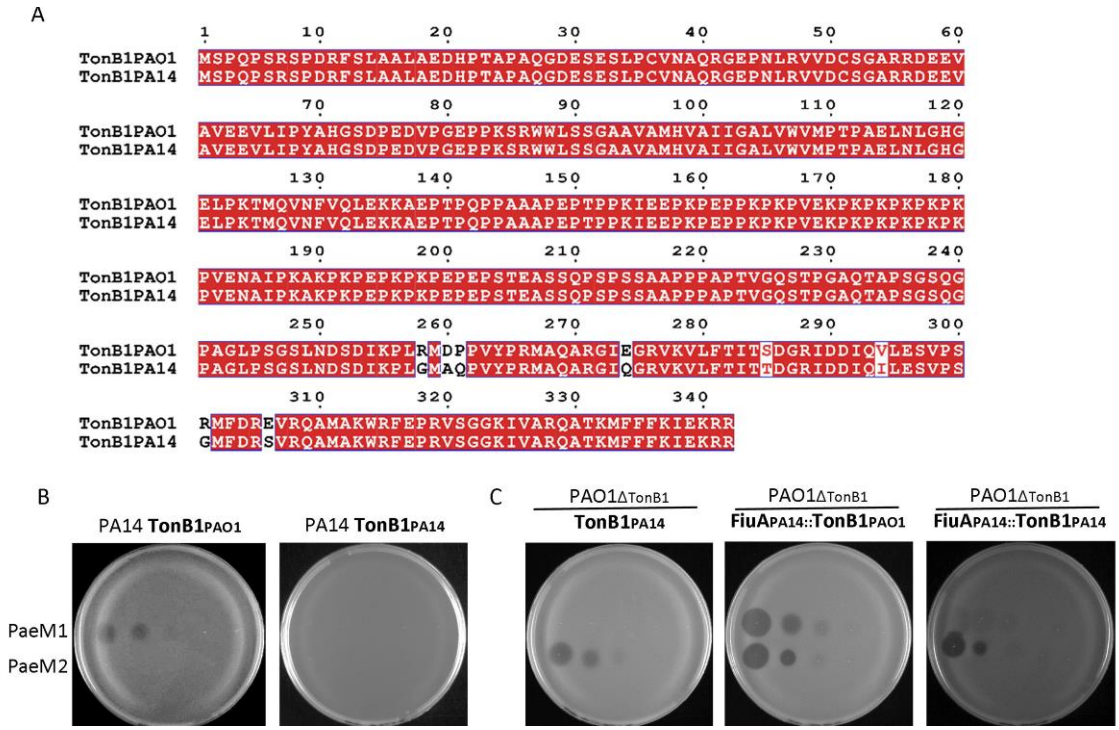


Figure 10. TonB1 protein sequences of PAO1 and PA14 strains (A) and their expression in PA14 (B) and PAO1 Δ*tonB1* (C) backgrounds. A, TonB1 protein sequences of strains PAO1 and PA14 were aligned using Clustal 2.1 program and graphic visualization was obtained using ESPript 3.0. Amino acid numbers are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

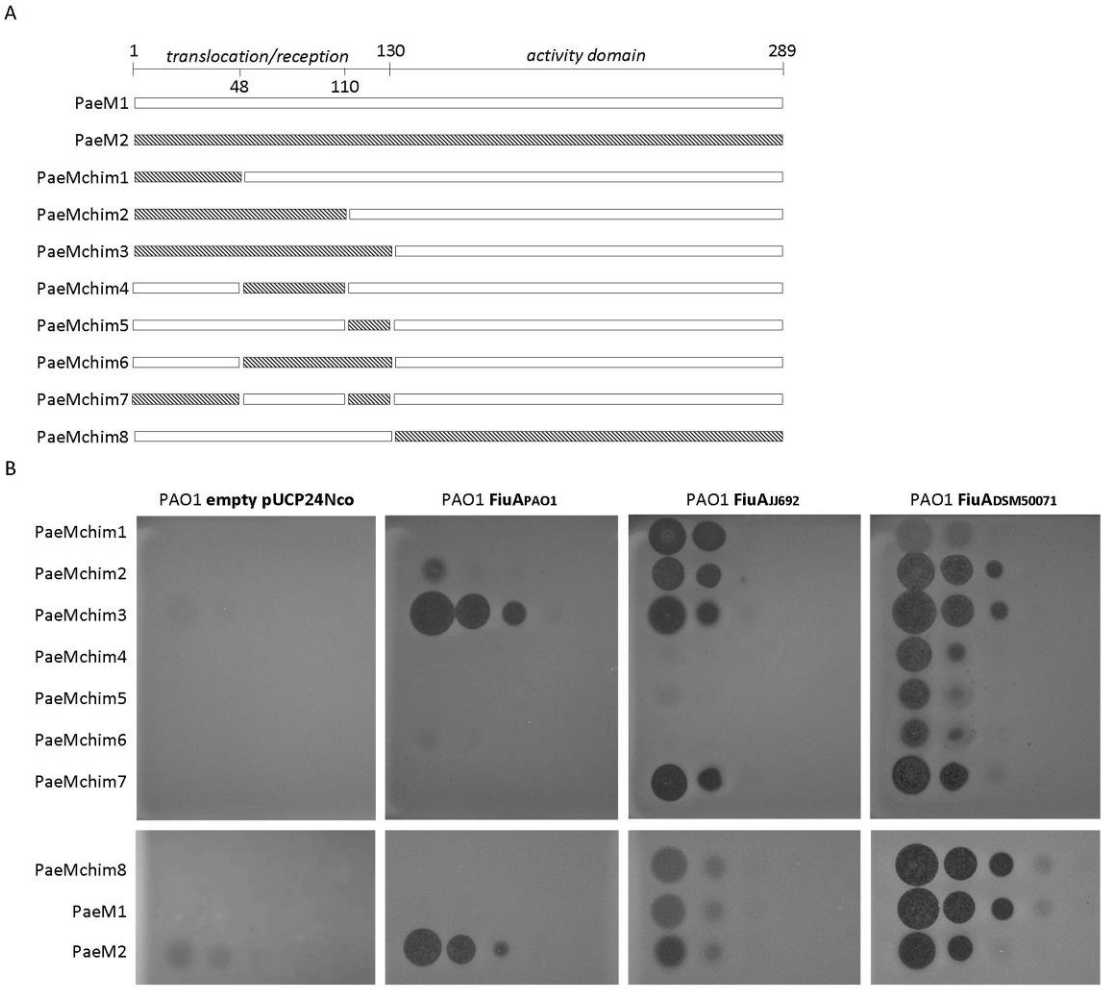
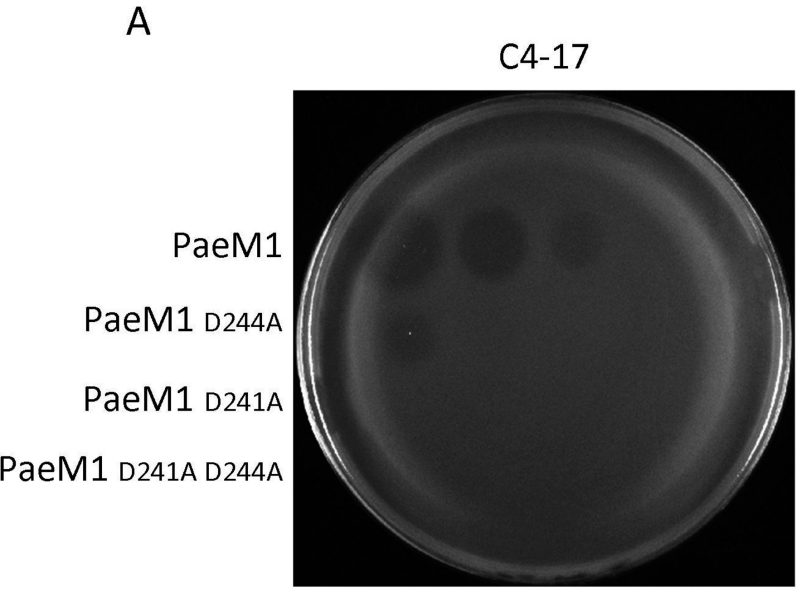
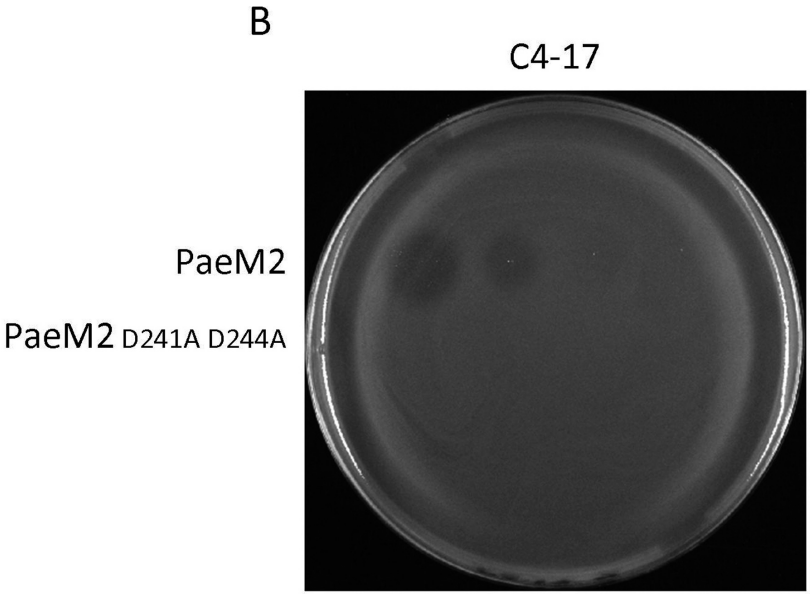


Figure 11. Susceptibility to chimeric PaeM1/PaeM2 proteins of PAO1 strain expressing different FiuA receptors. A, Seven chimeric proteins PaeMchim1 to 7 were generated by replacing partially or totally the N-terminal reception/translocation region of PaeM1 by the corresponding sequences of PaeM2. An 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-µl 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.

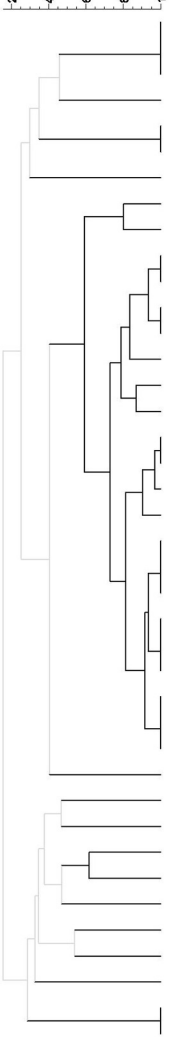
```
         1         10        20        30        40        50        60
PaeM1    MVMDLGTTTIVAGQGDFSAYNYGNNGANWPAPSPAQVGYISKGQRDVAYVNGDWEKPLLA
PaeM2    MAMDLGTTTIYASQGSFGTYNYGNNGANWPAPHPAQIGYISKGQRNVAYVNGDWEKPLLS

                 70        80        90       100       110       120
PaeM1    LWAQWANWGTTLYGYPSLPFPNYEFIWDIFDLPQADYNQFSLGDDRITAMNRAQNHQYPF
PaeM2    LWTQWANWGTTLYGYPSLPQPNFEFIWDIFDLPQADYNQYSLGDDRITAMNRAQNHNYRA

                           140       150       160       170       180
PaeM1    PNNEGIPSWDRPKIDTFNGGVYTPAAAFAHYLTGKGKKMNFPIERLNIKPNVKAMPQFIG
PaeM2    PVGVDEPNWDRPKIDTFNGGVYTPAAAFAHYLTGKGKKMNFPIERLNIKPNVKAMPQFIG

                 190       200       210       220       230       240
PaeM1    VLTSSPMGQTTVDFNVPYATAKDSWVAGNTVGEITLRIVGILVKSTSGQWSFRGEIRAYD
PaeM2    ILTSSPMGQTTVDFNVPYATAKDSWVAGNTVGEITLRVTGTLVKSTSGQWSFRGEIRAYD

                 250       260       270       280
PaeM1    DLYDFNPSNHRTETAEGMTRLGREVGQKFKDTTPYPIGIPGAIPVNISG
PaeM2    DVYDFNPSNHRTETAEGMTRLGREVGQKFKDTTPYPIGIPGTIPVNISG
```


A
C4-17
PaeM1
PaeM1 D244A
PaeM1 D241A
PaeM1 D241A D244A


B
C4-17
PaeM2
PaeM2 D241A D244A

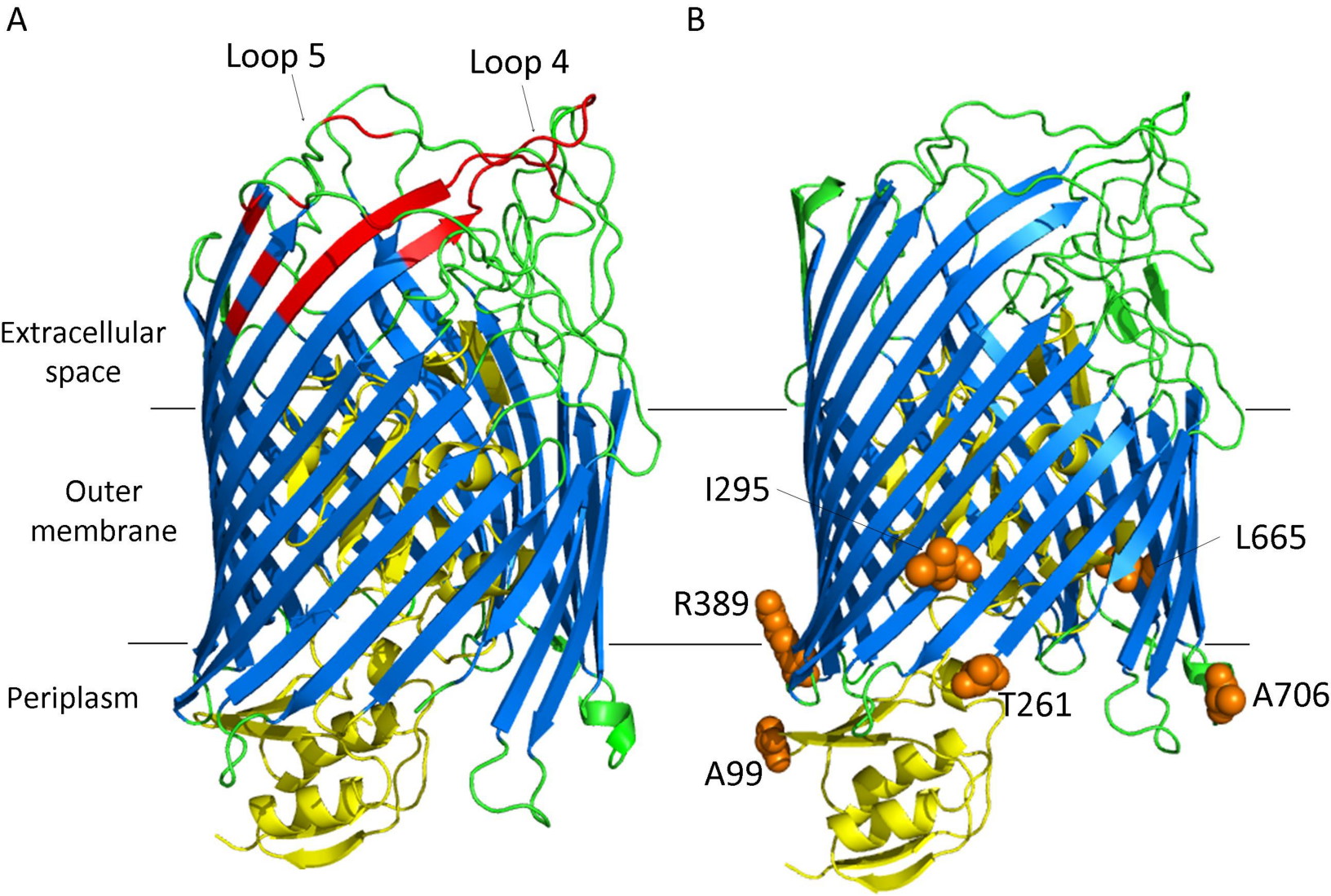
MLVA (<All Characters>)

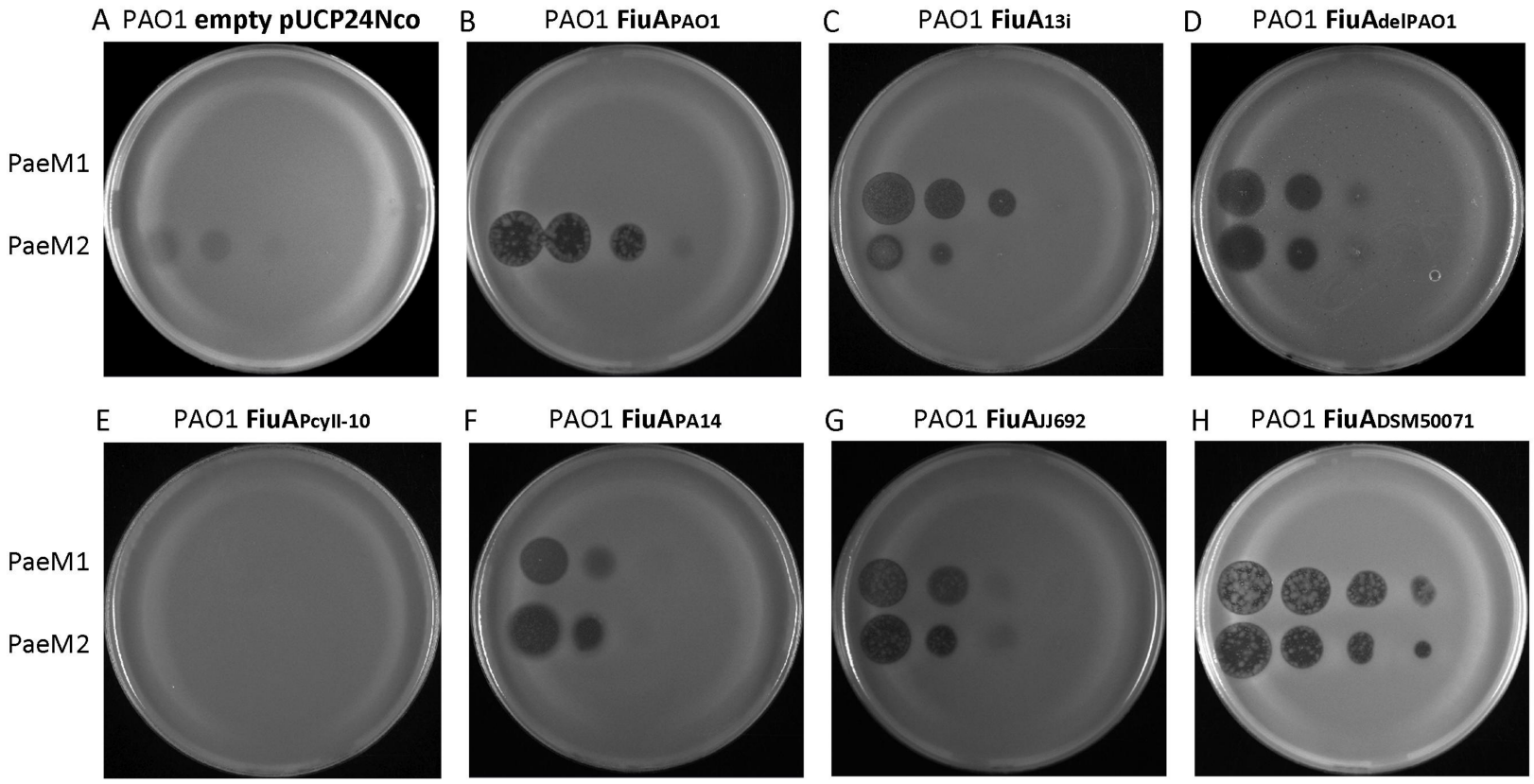
MLVA



Strain-ID	alias	ms77	ms127	ms142	ms172	ms211	ms212	ms213	ms214	ms215	ms216	ms217	ms222	ms223	ms207	ms209	
23i	FrNa23	2.5	9.0	1.0	12.0	5.0	6.0	6.0	2.0	2.0	1.0	4.0	1.0	2.0		3.0	
8442287BMLBA.	PER01	2.5	9.0	1.0	12.0	5.0	6.0	6.0	2.0	2.0	1.0	4.0	1.0	2.0	7.0	3.0	
12049679-01	BES10	2.5	9.0	1.0	12.0	5.0	6.0	6.0	2.0	2.0	1.0	4.0	1.0	2.0	7.0	3.0	
Pcy863123	FrCI20	2.5	8.0	2.0	12.0		8.0	4.0		5.0	1.0	3.0	1.0	2.0			
	BOU01	2.5	8.0	6.0	10.0	3.0	6.0	4.0	2.0	4.0	2.0	2.0	2.0	2.0	11.0	3.0	
12012162-01	BES02	2.5	8.0	6.0	10.0	3.0	6.0	4.0	2.0	4.0	2.0	2.0	2.0	2.0	11.0	3.0	
6i	FrNa6	4.0	9.0	2.0	12.0	2.0	12.0	5.0	3.0	2.0	1.0	2.0	2.0	2.0	9.0	6.0	
NCGM2.S1	△ AP012280	3.0	9.0	1.0	13.0	3.0	9.0	4.0	3.0	0.0	1.0	2.0	1.0	4.0	9.0	3.0	CC235
6077	▲ GCA_000481745	3.0	9.0	1.0	15.5	3.0	9.0	2.0	3.0	0.0	1.0	5.0	1.0	4.0	9.0	3.0	CC235
PB350	△ CP025055	3.0	9.0	1.0	13.0	3.0	3.0	6.0	4.0	0.0	1.0	5.0	1.0	2.0	8.0	3.0	CC235
PB367	△ CP025056	3.0	9.0	1.0	13.0	3.0	3.0	6.0	4.0	0.0	1.0	5.0	1.0	2.0	8.0	3.0	CC235
NCGM 1900	△ AP014622	3.0	9.0	1.0	13.0	3.0	3.0	6.0	4.0	0.0	1.0	5.0	1.0	4.0	8.0	3.0	CC235
NCGM 1984	△ AP014646	3.0	9.0	1.0	13.0	3.0	3.0	6.0	4.0	0.0	1.0	5.0	1.0	4.0	8.0	3.0	CC235
E6130952	△ CP020603	3.0	9.0	1.0	13.0	3.0	3.0	6.0	2.0	0.0	1.0	5.0	1.0	4.0	9.0	3.0	CC235
RO1	▲ RO1	3.0	9.0	1.0	13.0	4.0	3.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	10.0	3.0	CC235
02_77	▲ HU9	3.0	9.0	1.0	13.0	7.0	3.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	8.0	3.0	CC235
Ko_05-394	▲ K2	3.0	9.0	0.5	13.0	4.0	9.0	6.0	6.0	0.5	1.0	5.0	1.0	4.0	11.0	3.0	CC235
Ko_06-238	▲ K5	3.0	9.0	0.5	13.0	4.0	9.0	6.0		0.5	1.0	5.0	1.0	4.0	11.0	3.0	CC235
Ko_05-421	▲ K11	3.0	9.0	0.5	13.0	4.0	9.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	11.0	3.0	CC235
Ko_06-144	▲ K21	3.0	9.0	0.5	13.0	4.0	9.0	6.0	4.0	0.5	1.0	5.0	1.0		13.0	3.0	CC235
2i	▲ FrNa2	3.0	9.0	0.5	13.0	3.0	3.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	9.0	3.0	CC235
3J	▲ FrNa3	3.0	9.0	0.5	13.0	3.0	3.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	9.0	3.0	CC235
9H	▲ FrNa9	3.0	9.0	0.5	13.0	3.0	3.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	9.0	3.0	CC235
1H	▲ FrNa1	3.0	9.0	0.5	13.0	3.0	3.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	10.0	3.0	CC235
14i	▲ FrNa14	3.0	9.0	0.5	13.0	3.0	3.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	10.0	3.0	CC235
	▲ BIC01	3.0	9.0	0.5	13.0	3.0	3.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	10.0	3.0	CC235
8402097BMLBA	▲ PER03	3.0	9.0	0.5	13.0	3.0	9.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0	9.0	3.0	CC235
Pcy8381349	▲ FrCI12	3.0	9.0	0.5	13.0	3.0	9.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0			CC235
Pcy8350360	▲ FrCI13	3.0	9.0	0.5	13.0	3.0	9.0	6.0	4.0	0.5	1.0	5.0	1.0	4.0			CC235
	BOU03	3.0	9.0	1.0	12.0	3.0	9.0	3.0	6.0	2.0	2.0		3.0	4.0	10.0	3.0	
5H	FrNa5	2.5	8.0	4.0	11.0	3.0	8.0	5.0	3.0	2.0	3.0	3.0	5.0	6.0	7.0	5.0	
	BIC06	2.5	8.0	2.0	11.0	3.0	9.0	4.0	2.0	2.0	3.0	4.0	1.0	3.0	7.0	6.0	
12037746-01	BES07	2.5	8.0	4.0	10.0	3.0	5.0	5.0	5.0	1.0	2.0	4.0	1.0	3.0	4.0	8.0	
12150374-01	BES08	2.5	8.0	4.0	11.0	3.0		5.0		5.0	2.0	4.0	4.0	3.0	8.0	2.0	
	NAR06	2.5	8.0	4.0	12.0	8.0	9.0	2.0	2.0	4.0	2.0	4.0	3.0	3.0	6.0	2.0	
15i	FrNa15	2.5	8.0	4.0	11.0	5.0	14.0	3.0	4.0	4.0		3.0	4.0	6.0	10.0	4.0	
	BIC05	2.5	8.0	1.0	11.0	3.0		2.0	4.0	2.0	2.0	3.0	1.0	2.0	10.0	4.0	
4H	FrNa4	2.5	8.0	6.0	9.0	5.0	9.0	1.0	3.0	2.0	2.0	4.0	1.0	3.5	5.0	5.0	
DSM 50071	▲ CP012001	2.5	8.0	4.0	13.0	6.0	5.0	1.0	3.0	1.0	1.0	3.0	3.0	4.0	6.0	4.0	
NCTC10332	△ LN831024	2.5	8.0	4.0	13.0	6.0	5.0	1.0	3.0	1.0	1.0	3.0	3.0	4.0	6.0	4.0	

```
                1         10        20        30        40        50        60        70        80        90       100
FiuAPcyII-10    MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAPcyII-57    MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAPAO1        MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAPA14        MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAJJ692       MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENVG
FiuAPcyII-55    MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENVG
FiuAC4-17       MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuAC3-20       MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuADET08       MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuA13i         MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG
FiuADSM50071    MPRPTFRPGLLALAIALAVPAPFLHAAPAEQASVRDYRIAAGPLAGTLNRIAAQAGLVLTLDPALAEGRSAHAVQGRFDAPGALREALKGSGLELVENAG

                       110       120       130       140       150       160       170       180       190       200
FiuAPcyII-10    GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDSRSDWLKVRG
FiuAPcyII-57    GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDSRSDWLKVRG
FiuAPAO1        GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDSRSDWLKVRG
FiuAPA14        GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuAJJ692       GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuAPcyII-55    GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuAC4-17       GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuAC3-20       GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDTRSDWLKVRG
FiuADET08       GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDSRSDWLKVRG
FiuA13i         GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHNLDDAVRYMPGVVASSFGSDSRSDWLKVRG
FiuADSM50071    GTYSLRKVPEDTLSLQAMTVSGAEEDPLGPTSGYVAKRSMTGTKTDTPILETPRSISVATRQQMTDRAVHSLDDAVRYMPGVVASSFGSDTRSDWLKVRG

                       210       220       230       240       250       260       270       280       290       300
FiuAPcyII-10    FKPTQFLDGLPMATGSYNNPKLETWNLERLALLRGPASSVYGQTPPGGMLDMVSLRPQGIASHELKAEIGNYNHNQISFDSTGPIDDEGRFLYRLSGVVR
FiuAPcyII-57    FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAPAO1        FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAPA14        FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVTSHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRISGVVR
FiuAJJ692       FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAPcyII-55    FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAC4-17       FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEEKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuAC3-20       FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuADET08       FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuA13i         FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR
FiuADSM50071    FKPTQFLDGLPMAVGVYNNPKLETWNLERVALLRGPASSVYGQTPPGGMLDMVSLRPQNVASHEVKAEVGNYNHKQVSFDSTGPLDDEGRFLYRVSGVVR

                       310       320       330       340       350       360       370       380       390       400
FiuAPcyII-10    DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLSQFNRDDTAATSQFLPLRGTKYDAPFGKISHHKNLGDPDWDFYDRTYYWLGYSFERRINDVWQFRQN
FiuAPcyII-57    ESNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuAPAO1        DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuAPA14        DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuAJJ692       DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFEQRINDVWQFRQN
FiuAPcyII-55    DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFEQRINDVWQFRQN
FiuAC4-17       DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuAC3-20       DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuADET08       DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuA13i         DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN
FiuADSM50071    DSNTQVDHIDDKRYNIAPSLTWNIDDDTRLTFLGQFNRDDTGITSQFLPLRGTKYDAPFGKVSHHKNLGDPDWEYYDRTYYWLGYAFERRINDVWQFRQN

                       410       Loop 4    430       440       450       460       470       Loop 5    490       500
FiuAPcyII-10    LRYLRNDLSFQTLTPTSSIDAASADGTLQRMTTSVDEDISQFVVDNNFQADFETGALRHTLLLGIDHNRTRTDYTSIYNFTGVPPTNVNNPIYGQPIPKP
FiuAPcyII-57    LRYLRNDLSFQALTPASYYAAASDDGTLQRTSTSVDEDVSQFVMDNNFQADFDTGALRHTLLLGLDHNRTTNEYTSIFGFN.VPPTNINNPIYGQPIPKP
FiuAPAO1        LRYLRNDLSFQALTPASYYAAASDDGTLQRTSTSVDEDVSQFVVDNNFQADFDTGALRHTLLLGLDHNRTTNEYTSIFGFN.VPPTNINNPIYGQPIPKP
FiuAPA14        LRYLRNDLSFQAVTAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGLDHNRTDNQYTSIFGS..APPSNVNNPIYGQPIVKP
FiuAJJ692       LRYLRNDLSFQAVTAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGLDHNRTDNQYTSIFGS..APPSNVNNPIYGQPIVKP
FiuAPcyII-55    LRYLRNDLSFQAVTAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGLDHNRTDNQYTSIFGS..APPSNVNNPIYGQPIVKP
FiuAC4-17       LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGLDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP
FiuAC3-20       LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGLDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP
FiuADET08       LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGLDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP
FiuA13i         LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGLDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP
FiuADSM50071    LRYLRNDLSFQGITAG....TAHADGTLERSTTSVDEDVSQFVVDNNFQADFETGALRHTLLLGLDHNRTDNQYTSIFGS..APPSNVINPIYGQPIVKP

                       510       520       530       540       550       560       570       580       590       600
FiuAPcyII-10    SRSSAFYDYRQKTYQTGLYVQDQIALDNWRLTLGGREDWVHAGATFYNQNDATNTSRDKKFSGNAALSYIFDNGLAPYLSYAESFQPSTGADDIGSNKMF
FiuAPcyII-57    PRSDAYYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAPAO1        PRSDAYYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAPA14        DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAJJ692       DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAPcyII-55    DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAC4-17       DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuAC3-20       DRSTAFYNYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuADET08       DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuA13i         DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL
FiuADSM50071    DRSTAFYDYKQKTYQTGLYVQDQIALDNWRLTLGGREDWVHTGATFYNKGDATNTSRDKKFSGNAALSYIFDNGVAPYISYAESFQPTTGAD.MNSTSSL

                       610       620       630       640       650       660       670       680       690       700
FiuAPcyII-10    KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVRVTDNVNGTPTTSQAGEVKVTGIELEATSNVTDNLKVIGSYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAPcyII-57    KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAPAO1        KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAPA14        KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKLIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAJJ692       KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAPcyII-55    KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAC4-17       KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuAC3-20       KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuADET08       KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuA13i         KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ
FiuADSM50071    KPTEGKQWEIGVKYQPVGSDSLFTAAVYDLRQENVSVSQNIGGTPVTSQTGEVKVSGIELEATSNVTDNLKMIGAYSYADTEVKKGQYAGNRLQQAPRNQ

                       710       720       730       740       750       760       770       780       790       800
FiuAPcyII-10    ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAPcyII-57    ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAPAO1        ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAPA14        ASLWADYTWHEGALNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAJJ692       ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAPcyII-55    ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAC4-17       ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuAC3-20       ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuADET08       ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuA13i         ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV
FiuADSM50071    ASLWADYTWHEGTLNGFSVGAGARYVGSTYGDQANTYDGYAGSYTLYDAAVRYDLGQLNGSLKGVSVAVNANNLFNKDYLASCDGYYCYYGDQRSVVGSV

FiuAPcyII-10    SYKW
FiuAPcyII-57    SYKW
FiuAPAO1        SYKW
FiuAPA14        SYKW
FiuAJJ692       SYKW
FiuAPcyII-55    SYKW
FiuAC4-17       SYKW
FiuAC3-20       SYKW
FiuADET08       SYKW
FiuA13i         SYKW
FiuADSM50071    SYKW
```

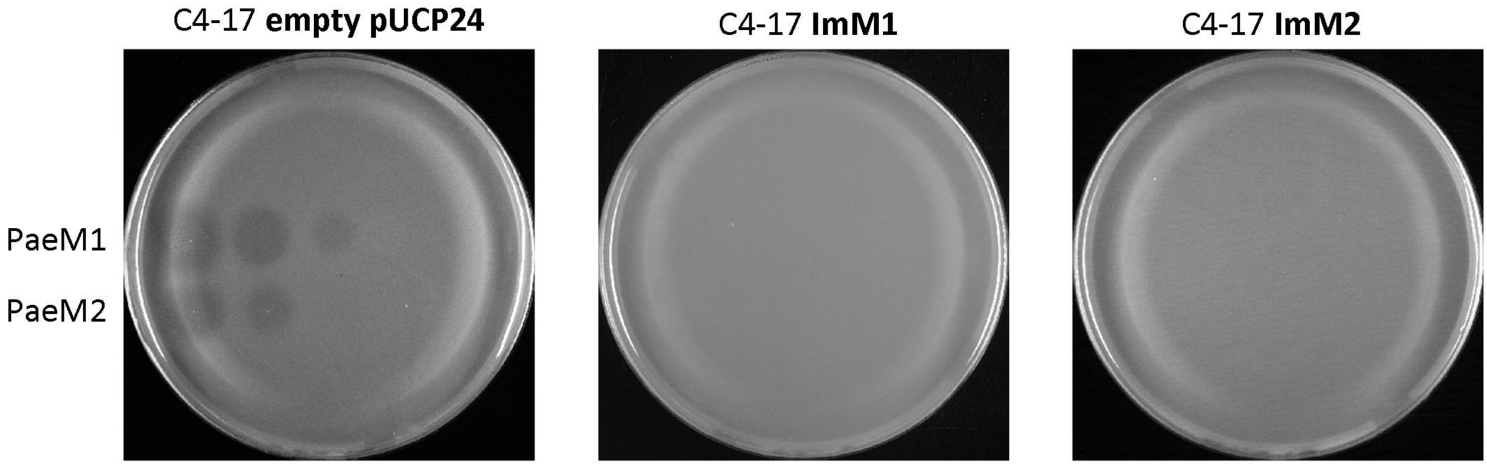

A
Loop 5
Loop 4
Extracellular space
Outer membrane
Periplasm
B
I295
L665
R389
T261
A706
A99

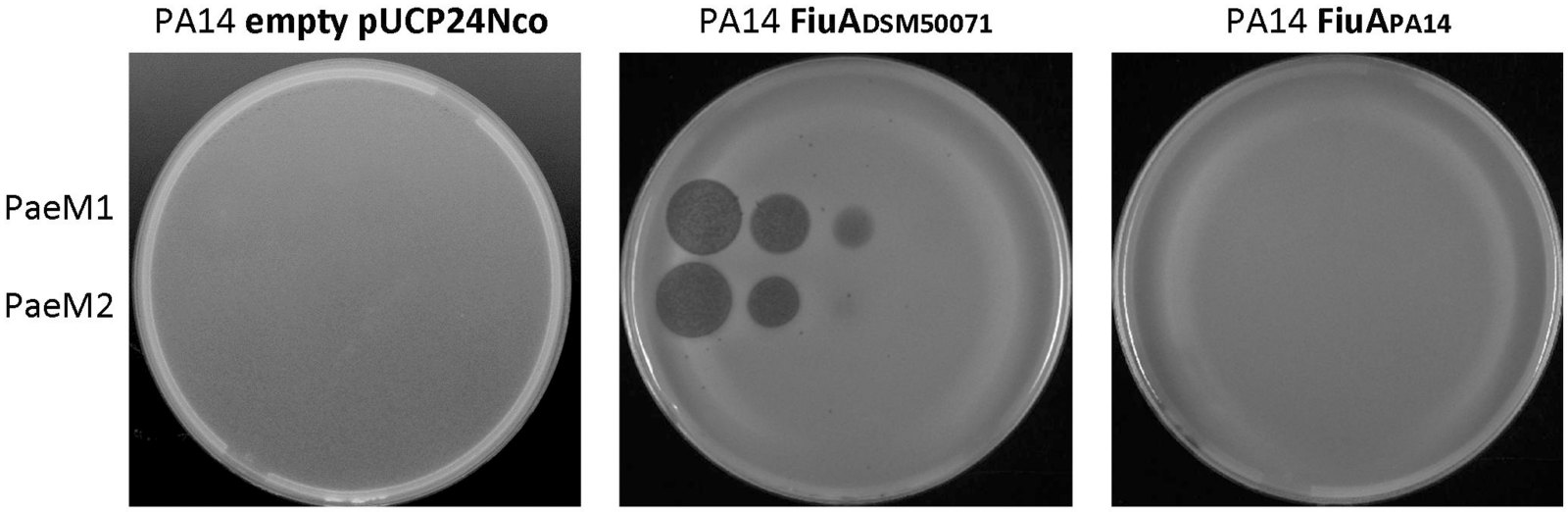

A PAO1 empty pUCP24Nco
B PAO1 FiuAPAO1
C PAO1 FiuA13i
D PAO1 FiuAdelPAO1
PaeM1
PaeM2
E PAO1 FiuAPcyII-10
F PAO1 FiuAPA14
G PAO1 FiuAJJ692
H PAO1 FiuADSM50071
PaeM1
PaeM2

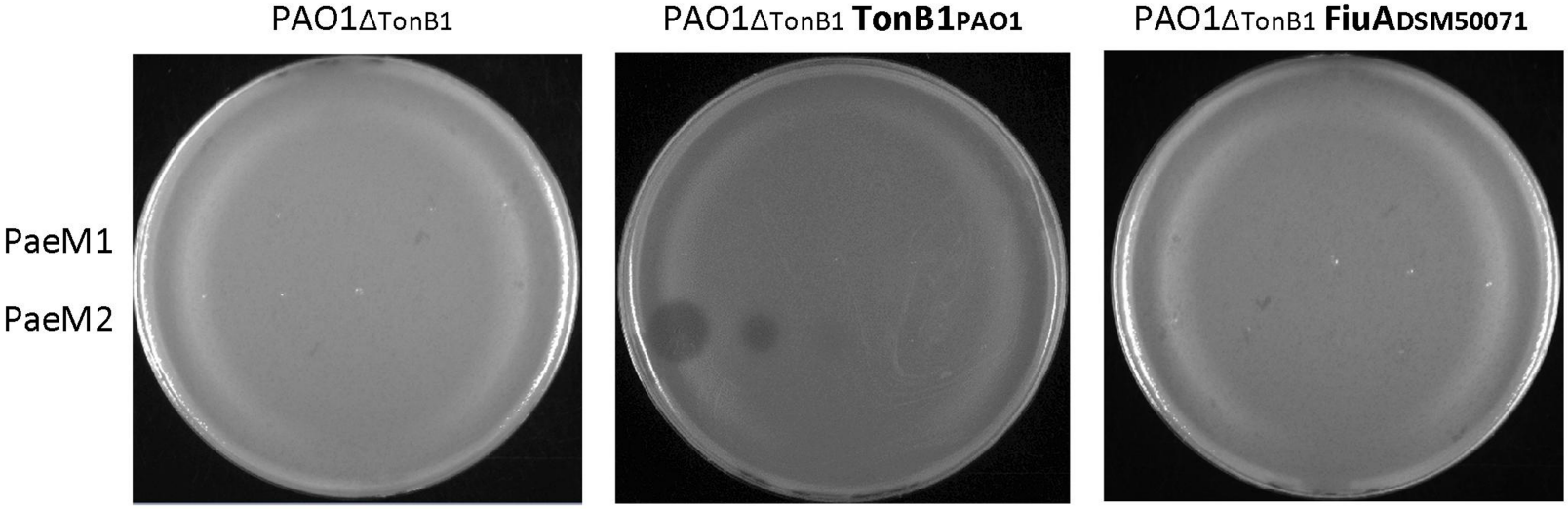
A

```
         1         10        20        30        40        50        60
ImM1     MRKDILLILTFLLVILCAAISFSGFTEGNANPDDTTGVPVESMFVGFLTLFGSVSVLVLV
ImM2     MRKDILLIFTFLLVILCAAISFSGFSEGNVNPDDTTGVPVESMFVGFLTLFGSISMLALV

                 70        80        90       100       110       120
ImM1     VGVLVGKLVLRVDAVFSRCILIGLSVVLTLPALFLTVVTFFFSVLAVSESYYAWLSVASC
ImM2     VGVLIGKLVLRFDALFSRYIFAGLSAVLTMPALFLTAVILFFSVLSVSESSYAWLSVASC

                130       140
ImM1     TAHLLLLMVFYRSKFSSFYLVK
ImM2     TAHLLLLIIFYRSKLSSVYLVK
```

B




PA14 empty pUCP24Nco
PA14 FiuADSM50071
PA14 FiuAPA14
PaeM1
PaeM2


PAO1ΔTonB1
PAO1ΔTonB1 TonB1PAO1
PAO1ΔTonB1 FiuADSM50071
PaeM1
PaeM2

A

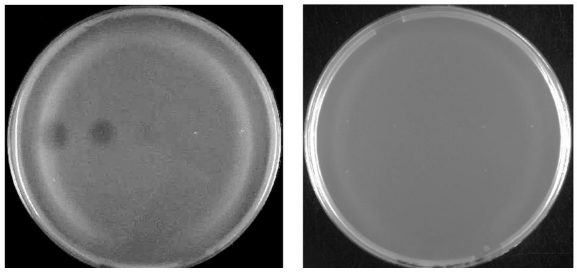
```
                1         10        20        30        40        50        60
TonB1PAO1       MSPQPSRSPDRFSLAALAEDHPTAPAQGDESESLPCVNAQRGEPNLRVVDCSGARRDEEV
TonB1PA14       MSPQPSRSPDRFSLAALAEDHPTAPAQGDESESLPCVNAQRGEPNLRVVDCSGARRDEEV

                          70        80        90       100       110       120
TonB1PAO1       AVEEVLIPYAHGSDPEDVPGEPPKSRWWLSSGAAVAMHVAIIGALVWVMPTPAELNLGHG
TonB1PA14       AVEEVLIPYAHGSDPEDVPGEPPKSRWWLSSGAAVAMHVAIIGALVWVMPTPAELNLGHG

                         130       140       150       160       170       180
TonB1PAO1       ELPKTMQVNFVQLEKKAEPTPQPPAAAPEPTPPKIEEPKPEPPKPKPVEKPKPKPKPKPK
TonB1PA14       ELPKTMQVNFVQLEKKAEPTPQPPAAAPEPTPPKIEEPKPEPPKPKPVEKPKPKPKPKPK

                         190       200       210       220       230       240
TonB1PAO1       PVENAIPKAKPKPEPKPKPEPEPSTEASSQPSPSSAAPPPAPTVGQSTPGAQTAPSGSQG
TonB1PA14       PVENAIPKAKPKPEPKPKPEPEPSTEASSQPSPSSAAPPPAPTVGQSTPGAQTAPSGSQG

                         250       260       270       280       290       300
TonB1PAO1       PAGLPSGSLNDSDIKPLRMDPPVYPRMAQARGIEGRVKVLFTITSDGRIDDIQVLESVPS
TonB1PA14       PAGLPSGSLNDSDIKPLGMAQPVYPRMAQARGIQGRVKVLFTITTDGRIDDIQILESVPS

                         310       320       330       340
TonB1PAO1       RMFDREVRQAMAKWRFEPRVSGGKIVARQATKMFFFKIEKRR
TonB1PA14       GMFDRSVRQAMAKWRFEPRVSGGKIVARQATKMFFFKIEKRR
```

B

PA14 **TonB1**$_{PAO1}$ | PA14 **TonB1**$_{PA14}$

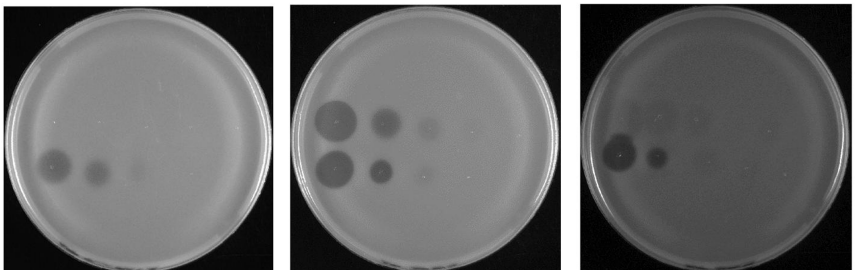
PaeM1

PaeM2

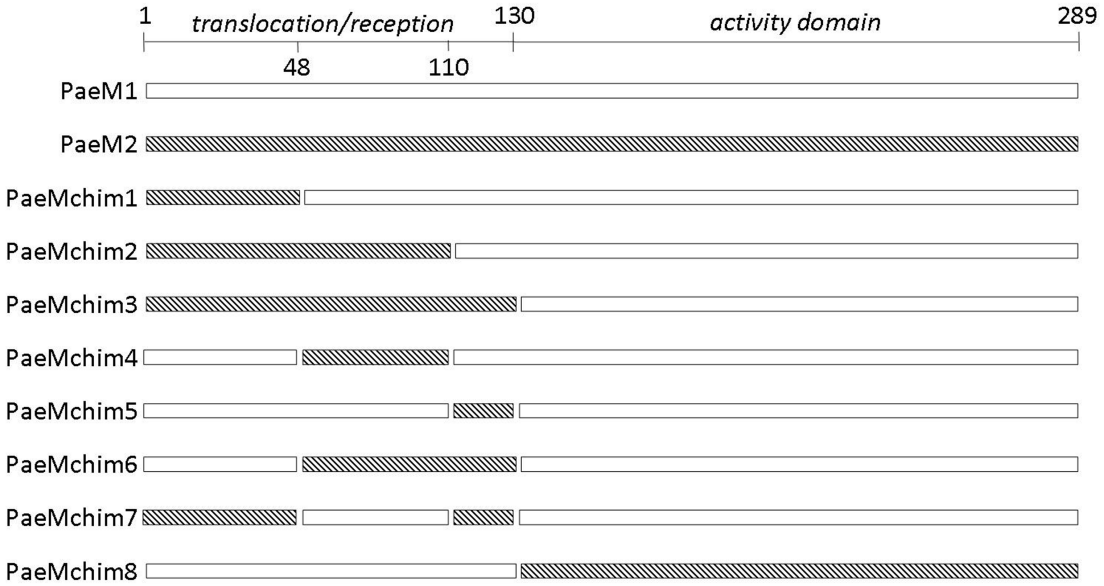


C

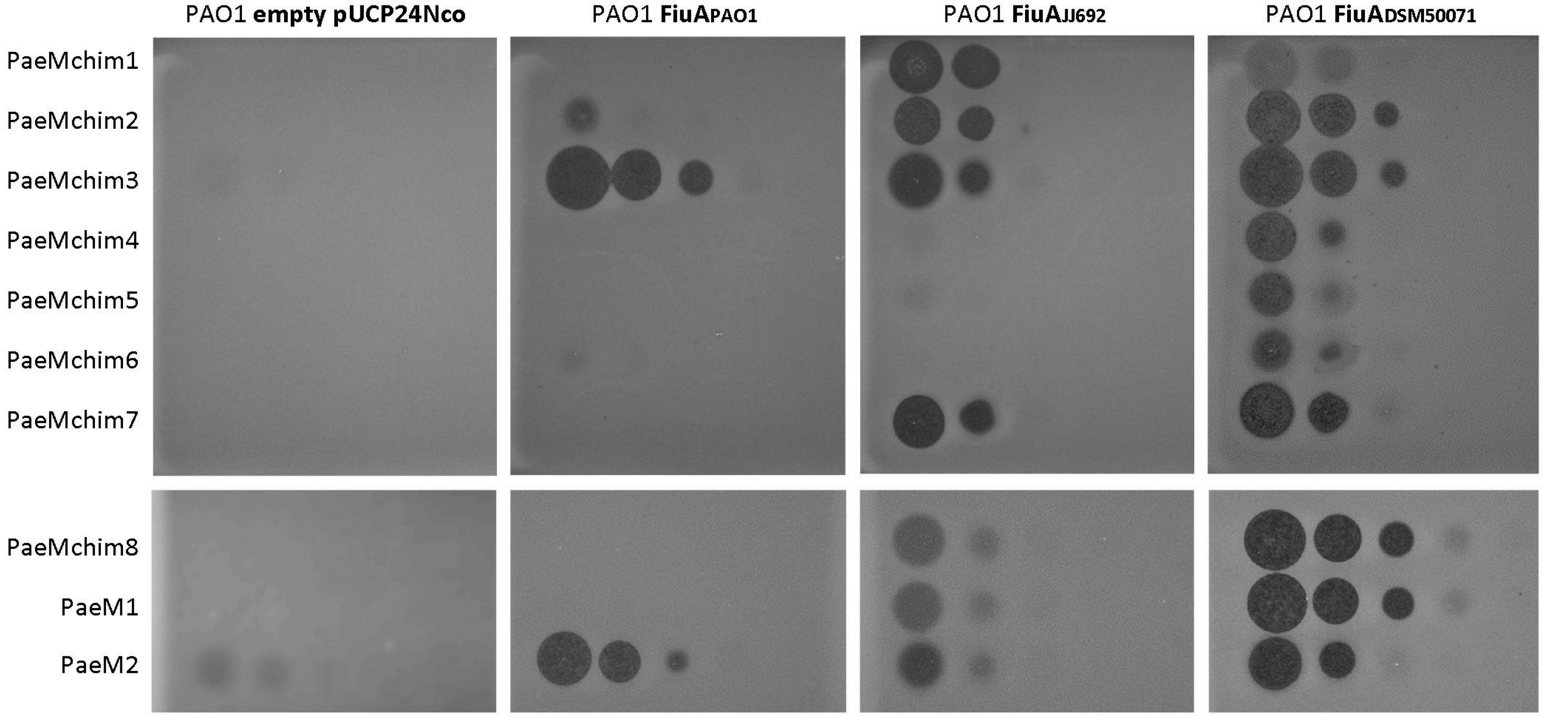
PAO1$_{\Delta TonB1}$ **TonB1**$_{PA14}$ | PAO1$_{\Delta TonB1}$ **FiuA**$_{PA14}$**::TonB1**$_{PAO1}$ | PAO1$_{\Delta TonB1}$ **FiuA**$_{PA14}$**::TonB1**$_{PA14}$



A


1
translocation/reception
130
activity domain
289
48
110
PaeM1
PaeM2
PaeMchim1
PaeMchim2
PaeMchim3
PaeMchim4
PaeMchim5
PaeMchim6
PaeMchim7
PaeMchim8

B


PAO1 empty pUCP24Nco
PAO1 FiuAPAO1
PAO1 FiuAJJ692
PAO1 FiuADSM50071
PaeMchim1
PaeMchim2
PaeMchim3
PaeMchim4
PaeMchim5
PaeMchim6
PaeMchim7
PaeMchim8
PaeM1
PaeM2