Impact of FiuA Outer Membrane Receptor Polymorphism on the Resistance of *Pseudomonas aeruginosa* toward Peptidoglycan Lipid II-Targeting PaeM Pyocins

Libera Latino,^a Delphine Patin,^a Dimitri Chérier,^a Thierry Touzé,^a Christine Pourcel,^a Hélène Barreteau,^a

Dominique Mengin-Lecreulx^a

^aInstitute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ Paris-Sud, Université Paris-Saclay, Gif-sur-Yvette, France

ABSTRACT Certain *Pseudomonas aeruginosa* strains produce a homolog of colicin M, namely, 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 60 clinical isolates. Seven of these strains, including PAO1, were susceptible to only one variant (2 to PaeM1 and 5 to PaeM2), and 11 were affected by both. 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 the 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 differences between the PaeM variants resulted from a polymorphism of both the pyocin and the outer membrane receptor FiuA. Moreover, we found that a third component, TonB1, a protein involved in iron transport in *P. aeruginosa*, working together with FiuA and the ExbB/ExbD complex, was directly implicated in this discrimination.

IMPORTANCE Bacterial antibiotic resistance constitutes a threat to human health, imposing the need for identification of new targets and development of new strategies to fight multiresistant pathogens. Bacteriocins and other weapons that bacteria have themselves developed to kill competitors are therefore of great interest and a valuable source of inspiration for us. Attention was paid here to two variants of a colicin M homolog (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 FiuA, PaeM pyocins, *Pseudomonas aeruginosa*, TonB, bacteriocins, cell wall, colicin M, lipid II, peptidoglycan, receptor structure-function

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

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

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emergence of resistant bacteria under conditions of 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 that are active against resistant bacterial pathogens thus requires the development of detailed basic research on the physiology of microorganisms and on metabolic pathways that are essential and specific to the bacterial world. The bacterial cell wall peptidoglycan polymer that protects cells against internal osmotic pressure constitutes one such 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) that 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 similar structural organizations in three distinct N-terminal, central, and C-terminal domains involved in their translocation, receptor binding, and cytotoxic activity, respectively, their modes of action differ, including pore formation in the inner membrane, DNA or RNA degradation, and 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 were shown to be present in only 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 to 289), which 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 to be 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 (>95%) decrease 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, the 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

FIG 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 ESPrpt 3.0. The triangle indicates 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 to 289) (10). On the basis of 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 above the sequences. Accession numbers are as follows: [ERZ09841.1](#) (PaeM1 from JJ692) and [SKA49653.1](#) (PaeM2 from DSM50071).

(12) compared to the other two PaeM variants produced by strains NCTC10332 and JJ692. PaeM4 was demonstrated to parasitize the HxC 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 the growth of PAO1 at all (6, 12). In the present study, we analyzed the polymorphism of the FiuA receptor in *P. aeruginosa* and demonstrated the essential role that it plays in determining the susceptibility or resistance of a panel of strains to the two PaeM_{JJ692} and PaeM_{NCTC10332} pyocin variants (here designated 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 of and the resistance of certain strains to these pyocins.

RESULTS

Purification and biochemical characterization of PaeM1 and PaeM2 variants. It was earlier shown that 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-residue 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 protein (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 (see Fig. S1A in the supplemental material). 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) in testing performed under 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 to 130), which are known to be involved in the translocation and reception steps for these bacteriocins, respectively, as established previously for ColM and other colicins (1, 15, 16). Only six

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

<i>P. aeruginosa</i> strain(s)	Susceptibility to PaeM1	Detection of PaeM1/ImM1 genes	Susceptibility to PaeM2 ^a	Detection of PaeM2/ImM2 genes
DET08, C1-14, C3-20, C4-17, C7-6, C7-18, C10-13, Pcyll-41, Pcyll-64, Pcyll-66, Pcyll-76	+	—/—	+	—/—
13i, Pcyll-55	+	—/—	—	—/—
C1-11, C5-6, C8-15, PAO1, Pcyll-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, Pcyll-10, Pcyll-18, Pcyll-54, Pcyll-63, Pcyll-81, Pcyll-82, Pcyll-86	—	—/—	—	—/—
JJ692, 14i, 20H, Pcyll-48	—	+/+	—	—/—
DSM50071	—	—/—	—	+/+

^aSusceptibility 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.

differences (in most cases, conservative) were found in the C-terminal region corresponding to the activity domain, consistent with the similar specific enzyme activities 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 tested here on a higher number of *P. aeruginosa* strains, including the reference PAO1 and PA14 strains, the susceptible DET08 strain, PaeM1- and PaeM2-producing strains JJ692 and DSM50071, and 60 other clinical isolates listed in Table 1. Only 12 of these strains, other than DET08, appeared to be susceptible to PaeM1 on 2YT medium agar plates in tests using the previously described spot killing assay (6, 10): six (C1-14, C3-20, C4-17, C7-6, C7-18, and C10-13) were isolated from cystic fibrosis patients, five (Pcyll-41, Pcyll-55, Pcyll-64, Pcyll-66, and Pcyll-76) were isolated from burn patients, and one (13i) was isolated from an intensive care unit patient (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- μ l spot for most of the susceptible strains that we tested (as shown in Fig. 2A for C4-17, which was chosen here as a representative strain). In most cases, the inhibition zones were rather turbid compared to the very clear zones observed with ColM on *E. coli* cell lawns.

FIG 2 Spot killing assay of PaeM variants and mutants on *P. aeruginosa* susceptible strain C4-17. Spots (5 μ l) of undiluted (0.6 mg/ml) or serially 10-fold-diluted wild-type or mutant PaeM1 (A) and PaeM2 (B) protein stocks (from left to right) were deposited on lawns of representative strain C4-17, which is susceptible to both PaeM variants. Growth inhibition zones were observed after 24 h of incubation at 37°C.

The absence of an 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 spectra of PaeM2 toward the same panel of *P. aeruginosa* strains. As shown in Table 1, many of the strains susceptible to PaeM1, namely, DET08, C1-14, C3-20, C4-17, C7-6, C7-18, C10-13, Pcyll-41, Pcyll-66, Pcyll-64, and Pcyll-76, were also inhibited by PaeM2. 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 Pcyll-55) appeared to be resistant to PaeM2, and several strains other than PAO1 that were resistant to PaeM1 but susceptible to PaeM2 (C1-11, C5-6, C8-15, and Pcyll-57) were also identified (Table 1).

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 that they produce by coexpression of 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 PCR of PaeM-encoding genes in the chromosome of all these strains was thus performed. Only three strains (14i, 20H, and Pcyll-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, 16 were positive by PCR for PaeM1 but none 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 were tested. We previously showed that mutations to alanine of aspartate residues D241 and D244 of PaeM1 led to production of 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 then 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 representative strain C4-17, but the inhibition zones were much more turbid than those seen with the wild-type PaeM1 and were detected with a 100-fold-larger amount of the mutant protein. No zone of inhibition was visible in tests of either the D241A mutant or the double mutant under 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). In tests of this mutant 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 presence of the siderophore-binding receptor FiuA (11, 12) strongly suggested that the differences that we observed in the

FIG 3 Clustering of *P. aeruginosa* strains using *in vivo* and *in silico* VNTR analysis data. For the multilocus variable-number tandem-repeat (VNTR) analysis (MLVA), *P. aeruginosa* strains 6077 (the original strain from which *exoU*-containing island A was transferred to strain JJ692) and DSM50071 were used as PaeM1/ImM1 gene- and PaeM2/ImM2 gene-containing reference strains, respectively. Green squares, *P. aeruginosa* strains belonging to the CC235 clonal population; red triangles, *P. aeruginosa* strains PCR positive for the PaeM1-ImM1 gene couple; blue triangles, *P. aeruginosa* strains PCR positive for the PaeM2-ImM2 gene couple; empty triangles, *in silico* search for the PaeM1-ImM1 (red) or PaeM2-ImM2 (blue) gene couple in some *P. aeruginosa* genomes.

antibacterial spectrum of the two PaeM variants could have resulted 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, and DET08), or as susceptible to PaeM1 only (13i and PcyII-55) or to PaeM2 only (PAO1 and PcyII-57) or to neither (PA14 and PcyII-10) were PCR amplified and sequenced. The *fiuA* gene sequences were shown to differ 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 to be five residues larger than that of the PaeM-producing strains (802 versus 797 residues), due to insertions of a SYYA motif and an asparagine residue at positions 417 to 420 and position 481 in the *FiuA*_{PAO1} sequence, respectively (Fig. 4). As shown in Fig. 4, 22 additional changes were observed between the *FiuA*_{PAO1} and *FiuA*_{JJ692} protein sequences, most of them being clustered in the central region (residues 390 to 510). However, only six residues differed between the *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 the PAO1 strain, 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 closely those of the DSM50071 and JJ692 strains (Fig. 4).

FIG 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 PA01 (susceptible to both PaeM variants). (Continued on next page)

FIG 5 Predicted FiuA_{PAO1} (A) and FiuA_{PA14} (B) receptor structures. Views of the FiuA_{PAO1} (A) and FiuA_{PA14} (B) siderophore receptor structures are drawn as ribbons. The plug and the transmembrane β -barrel domains are colored yellow and blue, respectively. The cytoplasmic turns and extracellular loops are colored green. In panel 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 panel B, the few (six) amino acid changes between FiuA_{PA14} and FiuA_{JJ692} are shown in orange. Structure prediction was performed using Phyre2 (17) on the basis of the crystal structures of FpvA (PDB entry 1XKH) and FptA (1XKW) receptors. The figure was generated with PyMOL (DeLano Scientific).

Considering the two PaeM-resistant strains PA14 and Pcyll-10, it was noteworthy that the FiuA_{PA14} receptor exhibited only six amino acid changes compared to FiuA_{JJ692} and eight compared to FiuA_{DSM50071} (Fig. 4), while the FiuA_{Pcyll-10} receptor was significantly different (91% identity).

To get an idea of the localization of these different amino acid changes in the three-dimensional (3D) structure of the receptor, a structural model of FiuA_{PAO1} was predicted using Phyre2 (17) and the previously elucidated crystal structures of siderophore receptors FpvA (18) and FptA (19), 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, i.e., 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 in comparisons of FiuA_{PAO1} to FiuA receptors of strains that are susceptible to the two PaeM variants were localized within two extracellular loops, namely, L4 and L5, suggesting that these regions might determine the specificity to PaeM1 and PaeM2 pyocins (Fig. 5). With the exceptions of the differences in the amino acid sequences at position 412 to 413 and position 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 susceptibility of the bacterium to PaeM variants. The impact of the variability of the FiuA sequence on the suscep-

FIG 4 Legend (Continued)

only to PaeM2); PA14 (resistant to both PaeM1 and PaeM2); 13i and Pcyll-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 above the sequences. Accession numbers are as follows: [SIP50767.1](#) (FiuA_{Pcyll-10}), [MK574015](#) (FiuA_{Pcyll-57}), [NP_249161.1](#) (FiuA_{PAO1}), [SCM60368.1](#) (FiuA_{PA14}), [ER216319.1](#) (FiuA_{JJ692}), [MK574017](#) (FiuA_{Pcyll-55}), [MK574013](#) (FiuA_{C4-17}), [MK574012](#) (FiuA_{C3-20}), [MK574014](#) (FiuA_{DET08}), [MK574016](#) (FiuA_{13i}), and [AKO84658.1](#) (FiuA_{DSM50071}).

FIG 6 Impact of FiuA receptor structure on the susceptibility of PAO1 strain to PaeM variants. The *fiuA* genes from different representative strains (PAO1, 13i, Pcyll-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 μ l of undiluted or serially 10-fold-diluted purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones were observed after 24 h of incubation at 37°C.

tibility 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 the resulting 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 to 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. The results also showed that the susceptibility of the strain 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 Pcyll-10 and PA14 led to distinct phenotypes; while expression of FiuA_{Pcyll-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_{Pcyll-10} receptor could not allow internalization of these bacteriocins into cells, at least in the PAO1 background, was consistent with the resistant phenotype of the Pcyll-10 strain. However, the demonstration that the FiuA_{PA14} receptor was perfectly functional in this context, allowing the 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 these pyocins and what the molecular determinants of the selective susceptibility of PAO1 strain to the PaeM2 variant are were 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 the JJ692 and DSM50071 strains were cloned into the pUCP24Nco vector and then expressed in the PAO1 background. As shown in Fig. 6G, the PAO1 strain expressing the *fiuA*_{JJ692} gene exhibited increased susceptibility to PaeM2 but, more importantly, was now inhibited by the PaeM1 variant too. Expression of the *fiuA* gene from the DSM50071 strain

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

provided similar results, with the susceptibility acquired by the strain to the two Paem variants even increased (Fig. 6H). These findings demonstrated that 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 and 6077), within 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 the colicinogenic strains that express both colicin and immunity genes close together on plasmids, we hypothesized earlier 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, referred to here as proteins ImM1 and ImM2, respectively, showed 84% sequence identity (Fig. 7A). On the basis of this homology and their similar (predicted) membrane topologies, these proteins were recently classified as integral membrane proteins belonging to the PmiA group by Ghequire and coworkers (20), and those authors showed that PmiA_{NCTC10332} (here named 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 tested here using the C4-17 strain, which is susceptible to both Paem variants, as the indicator strain. Transformation of this strain by the ImM1-expressing pUCP24 plasmid enabled it to resist both Paem1 and Paem2 (Fig. 7B). The ImM2 gene was similarly cloned, and its expression completely 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

FIG 8 Impact of FiuA polymorphism on the susceptibility of strain PA14 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 μ l of undiluted or serially 10-fold-diluted purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones were observed after 24 h of incubation at 37°C.

to the PaeM variants (Table 1). The *ImM1* gene was detected in only three strains, 14i, 20H, and Pcyll-48, and was in all cases coupled with the *PaeM1* gene (Table 1). As described for the *PaeM* genes, the search of the *ImM1* gene was also done in VNTR-selected strains. This gene was detected in 16 of 33 genomes screened and was 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* gene and/or the *PaeM2* gene in the collection of *PaeM*-resistant strains tested here.

Deciphering PA14 strain resistance to PaeM pyocins. As seen with about two-thirds of the *P. aeruginosa* strains tested in this work, the PA14 reference strain appeared to be resistant to both *PaeM* variants. Curiously, only a few amino acid changes were observed in comparisons of the FiuA protein sequence of this strain 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 the 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 multicopy plasmid remained totally resistant to these pyocins (Fig. 8). That the *PaeM* receptor activity of *FiuA*_{PA14} could be visualized in the PAO1 genetic background but not the PA14 genetic background resulted in our 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 results described above prompted us to analyze whether the PAO1 and PA14 strains might also exhibit some differences in the subsequent steps allowing import of these pyocins in the periplasm. As seen with 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 reimported 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). In contrast 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

FIG 9 Implication of TonB1 protein in the import of PaeM variants in strain PAO1. A PAO1 *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 μ l of undiluted or serially 10-fold-diluted purified protein stocks (0.6 mg/ml) on bacterial lawns. Growth inhibition zones were observed after 24 h of incubation at 37°C.

PaeM variants and whether it could eventually play a role in the resistance of the PA14 strain to these pyocins were 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, overexpression 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.

An alignment of *tonB1* gene sequences from various *P. aeruginosa* strains then revealed that the TonB1 protein sequence also displayed some variability in this bacterial species. In particular, the TonB1 protein from strain PA14 exhibited some specific differences compared to proteins from other strains, 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 coexpression 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 coexpression 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 but became susceptible to PaeM1 as well 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 the PAO1 and DSM50071 strains revealed 29 differences, several being clustered within the same ALTPASYAAASD motif localized in the central protein region (residues 412 to 424 in FiuA_{PAO1}). In FiuA_{DSM50071}, the corresponding sequence appeared as GITAGTAHA and was shorter by 4 residues (deletion of the SYAA motif). Whether the

FIG 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 the Clustal 2.1 program, and graphic visualization was obtained using ESPrnt 3.0. Amino acid numbers are indicated above 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 the experiments whose results are shown in panels B and C, the susceptibility of the strain to the PaeM pyocins was tested by spotting 5 μ l of undiluted or serially 10-fold-diluted purified protein stocks (0.6 mg/ml) on the bacterial lawns. Growth inhibition zones were observed after 24 h of incubation at 37°C. Accession numbers are as follows: [AAG08916.1](#) (TonB1_{PAO1}) and [SCM65789.1](#) (TonB1_{PA14}).

variability of this particular sequence could impact the receptor selectivity and host strain susceptibility to the two PaeM variants was then investigated. A modified version of the FiuA_{PAO1} receptor in which the ALTPASYAAASD motif was replaced by GITAG TAHA was generated by site-directed mutagenesis. As shown in Fig. 6D, PAO1 cells expressing this modified version of the receptor (designated “FiuA_{delPAO1}”) were then found to be 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 in testing of colicins, should specifically interact with the receptor and/or the translocation machinery (1). The demonstration (described above) that the PAO1 strain became susceptible to PaeM1 following either 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,

FIG 11 Susceptibility to chimeric Paem1/Paem2 proteins of PAO1 strain expressing different FiuA receptors. (A) Seven chimeric proteins (Paemchim1 to Paemchim7) were generated by partially or totally replacing the N-terminal reception/translocation region of Paem1 by the corresponding sequences of Paem2. An eighth chimeric protein, Paemchim8, was generated and resulted from 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. Spots (5 μ l) 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 of incubation at 37°C.

Paemchim2, and Paemchim3) proteins resulted from fusions of residues 1 to 48, 1 to 110, and 1 to 129 (i.e., the first residues) of Paem2 to residues 49 to 289, 111 to 289, and 130 to 289 of Paem1, respectively. These junctions were chosen based on alignments of the Paem sequences, which revealed some hot spots of divergence between the two variants (Fig. 1). As we have previously shown that the C-terminal and independently functional activity domain of Paem1 started around residues 127 to 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 PAO1 strains, each expressing one 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 the PAO1 strain. The first 48 residues of PaeM2 were apparently not sufficient to allow parasitizing of FiuA_{PAO1} receptor by PaeM1. The PaeM2 sequence consisting of residues 1 to 110 could fulfill this role, but its efficiency was lower than that of the full-length (residues 1 to 129) putative translocation/reception domain, indicating that the sequence of residues 117 to 128 of PaeM2 that is particularly divergent between the two variants (Fig. 1) is crucial for 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 the FiuA receptors from the JJ692 and DSM50071 strains were less selective than their FiuA_{PAO1} homolog. Four additional chimeric proteins (PaeMchim4 to PaeMchim7) which carried different other combinations of motifs found in the PaeM1 and PaeM2 N-terminal domains were designed and purified (Fig. S1B). None of them inhibited growth of PAO1 cells (or did so only very poorly in the case of PaeMchim6), but all were active on the strain expressing the FiuA_{DSM50071} receptor. Among them, only the PaeMchim7 protein exhibited potent activity with respect to the PAO1 strain expressing the FiuA_{JJ692} ortholog, as did the PaeMchim1 to PaeMchim3 chimeric proteins. The levels of impact of these N-terminal protein sequence modifications on the cytotoxic activity of the pyocins could thus differ 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 did; i.e., it 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 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., in the compartment where the peptidoglycan lipid II precursor is translocated and where it remains transiently accessible before being used for polymerization steps by the penicillin-binding proteins. The only physical barrier that they have to cross is the outer membrane of susceptible cells, and their antibacterial activities and spectra 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 reinternalized 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 immunity of the cell. 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 does 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 showed here that the FiuA receptors

from PaeM-producing strains were functionally active and able to internalize their own PaeM pyocins. Previous studies had already shown 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 demonstrated here that the antibacterial spectra of PaeM1 and PaeM2 bacteriocins were 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 (strains 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. The data 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 ALTPASYAAASD 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 in analysis of the resistance of the PA14 reference strain. 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 ColM receptor FhuA in *E. coli*, *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. 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 the PAO1 strain. These results suggested that FiuA_{PA14} and TonB1_{PAO1} proteins can interact and conjointly contribute to PaeM import into PA14 cells, in contrast 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 become 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 of translocation of the pyocins to the PA14 periplasm.

It was noteworthy, in considering the already known lipid II-targeting bacteriocins,

that not all the cognate immunity partners had been 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 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 previously named PmiA by Ghequire and coworkers (20). On the basis of 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 demonstrated here 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 analyses of the patterns of VNTR similarity to the PaeM-producing strains. Interestingly, most of the PaeM1-producing strains identified in this study belonged to clonal complex 235 (CC235) as deduced from VNTR genotyping (28) (Fig. 3). It is known that *P. aeruginosa* has mostly a nonclonal population structure, but multilocus sequence typing (MLST) revealed that a few sequence types (STs) are distributed worldwide and have been designated 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, and E6130952) and found that in all cases the PaeM1-ImM1 gene couple was located in 80-kb *exoU*-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 analysis was run using the sequence covering the latter gene couple, only two hits matched with 100% identity, namely, the *P. aeruginosa* strains designated DSM50071 and NCTC10332, which in fact represent the same strain, suggesting that this variant is very rare because it may have derived from 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 the ability of bacteriocins to adsorb on the bacterial surface and efficiently inhibit bacterial growth depends not only 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 roles 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. The presence of only a few amino acid changes in these proteins as well as in the PaeM pyocin was sufficient to modify the susceptibility of the strains and their selectivity with respect to the two PaeM variants. The data thus generated not

only contribute to our general knowledge on the *P. aeruginosa* physiology but will also certainly provide new insights into 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* DH5 α (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 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 medium was supplemented with agar (Sigma-Aldrich) at 1.6% (wt/vol) and at 0.7% for the top layer. When required, ampicillin, kanamycin, chloramphenicol, and gentamicin were added at final concentrations of 100, 50, 25, and 10 μ g/ml, respectively. For *P. aeruginosa* strains, the gentamicin concentration used ranged from 10 to 50 μ g/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 (Biomed) using Expand-Fidelity polymerase (Roche), and DNA fragments were purified using a Wizard PCR Preps DNA purification kit (Promega). The standard procedures for endonuclease digestions, ligation, and agarose gel electrophoresis were used as previously described by Sambrook et al. (36). pET2160 vector allowing high-level expression of proteins with a C-terminal 6 $\times$ His tag was previously described (6), as was multicopy *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 a QuikChange II XL site-directed mutagenesis kit (Stratagene) and the appropriate oligonucleotides listed in Table S1 in the supplemental material. All of the mutations introduced in plasmids were confirmed by DNA sequencing (Eurofins).

VNTR analysis. Strains were genotyped using the multilocus variable-number tandem-repeat analysis (MLVA) technique (40). It consists in the PCR amplification and analysis of 15 VNTRs whose sizes differ 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 using average linkages (UPGMA) algorithm. Strains differing at a maximum of 3 loci are clustered into clonal complexes (CC). The CC number corresponds to the multilocus 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 subjected to PCR amplification using the PaeM2-for and PaeM2-rev oligonucleotides shown in Table S1, which 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, which allows expression of the 289-residue 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 PaeM2-mut1 and PaeM2-mut2 oligonucleotides described in Table S1. The putative PaeM1 immunity gene (EXA14, here designated the ImM1 gene) that is located within *exoU*-containing island A of the *P. aeruginosa* JJ692 chromosome (6, 14) and its homolog (ImM2) detected in the genome of strain DSM50071 were subjected to PCR amplification using the ImM-for and ImM-rev primers, which 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 to further increase the number of restrictions sites present in the polylinker sequence, a NcoI site was introduced in this plasmid such 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 pUCP24-mut1 and pUCP24-mut2 primers (Table S1). Seven chimeric PaeM proteins were also generated in which sequences of different sizes of the PaeM1 N-terminal regions 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 PaeMchim-rev oligonucleotides indicated in Table S1. In the case of PaeMchim1, for instance, the following two gene fragments were first amplified: one fragment amplified by the use of PaeM2-for and PaeMchim1-rev primers and the pLL17 plasmid as the template and another fragment amplified by the use of PaeMchim1-for and T7term primers and the pMLD245 plasmid as the template. These two fragments were purified and hybridized together, and a third PCR was performed using this mixture as the template and oligonucleotides PaeM2-for and T7term as primers. The resulting fragment was then purified, treated by the use of NcoI and HindIII, and inserted into the pET2160 vector, yielding the pMLD601 plasmid. The same procedure was used for generating the plasmids expressing PaeMchim2

to PaeMchim7 (pMLD602 to pMLD605, pMLD618, and pMLD619, respectively). An eighth chimeric protein (PaeMchim8; pMLD627 plasmid) was also generated in which the N-terminal region of PaeM1 (residues 1 to 129) was fused to the C-terminal activity domain of PaeM2 (residues 130 to 289), i.e., in an inverse configuration compared to PaeMchim3 (Fig. 11A). The *fiuA* genes encoding PaeM outer membrane receptor variants were amplified from different *P. aeruginosa* strains, namely, PAO1, JJ692, DSM50071, PA14, Pcyll-10, 13i, and Pcyll-55, using the FiuA-for and FiuA-rev oligonucleotides listed 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, pMLD596, pMLD598, pMLD622, pMLD626, pMLD628, and pMLD629 plasmids, respectively. A similar plasmid, pMLD623, expressing a FiuA_{PAO1} mutant protein in which the ALTPASYAAASD sequence (residues 412 to 424) was replaced by the smaller GITAGTAHA motif found in FiuA receptors of some other strains such as DSM50071 (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 and pMLD653 expressing *fiuA*_{PA14}-*tonB1*_{PAO1} 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 the use of 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) and PaeM_{DSM50071} (PaeM2) and 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 *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 (OD₆₀₀) 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 8,000 × *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 MgCl₂ and 10 mM 2-mercaptoethanol (buffer A), and cells were subsequently disrupted by sonication (Bioruptor 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 His₆-tagged PaeM proteins were purified on nickel-nitrilotriacetate-agarose (Ni²⁺-NTA-agarose) 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 ultrafiltration (10K Amicon Ultra-15 filters; Millipore), and dialyzed overnight against buffer A supplemented with 10% glycerol. Protein concentrations were determined with a NanoDrop apparatus (Thermo Scientific), and sodium dodecyl sulfate-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 an overnight culture of the bacterial strain to be tested (ca. 10⁷ 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 allowed to dry for a few minutes under the hood. Then, 5 to 10 μl of serial dilutions in buffer A of the PaeM protein stocks was spotted on the top agar layer and allowed to dry under the hood. Plates were incubated for 24 h at 37°C, and the sensitivity of the strains and the efficiency of the pyocins were judged from the clear zones of inhibition observed at the spot position.

Lipid II hydrolase activity 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 ¹⁴C-radiolabeled lipid II (120 Bq), 20 mM MgCl₂, 10 mM 2-mercaptoethanol, 150 mM NaCl, 0.2% *n*-dodecyl-β-D-maltoside (DDM), and PaeM protein (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) using LK6D silica gel plates (Whatman) and 1-propanol-ammonium hydroxide-water (6:3:1 [vol/vol]) as the mobile phase. The latter two 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 Ni²⁺-NTA-agarose from Qiagen. The *meso*-diaminopimelic acid-containing ¹⁴C-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. A QuikChange II XL kit (Stratagene) was used for site-directed mutagenesis experiments. All other materials were of reagent grade and were obtained from commercial sources.

Data availability. 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*₁₃₁), and MK574017 (*FiuA*_{PCyII-55}). Accession numbers of other genes are indicated in the text and the figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00164-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We declare no conflicts of interest.

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