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A Colicin M-Type Bacteriocin from *Pseudomonas aeruginosa* Targeting the HxuC Heme Receptor Requires a Novel Immunity Partner

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ABSTRACT Pyocins are bacteriocins secreted by *Pseudomonas aeruginosa*, and they assist in the colonization of different niches. A major subset of these antibacterial proteins adopt a modular organization characteristic of polymorphic toxins. They include a receptor-binding domain, a segment enabling membrane passage, and a toxin module at the carboxy terminus, which eventually kills the target cells. To protect themselves from their own products, bacteriocin-producing strains express an immunity gene concomitantly with the bacteriocin. We show here that a pyocin equipped with a phylogenetically distinct ColM toxin domain, PaeM4, mediates antagonism against a large set of *P. aeruginosa* isolates. Immunity to PaeM4 is provided by the inner membrane protein PmiC, which is equipped with a transmembrane topology not previously described for the ColM family. Given that strains lacking a *pmiC* gene are killed by PaeM4, the presence of such an immunity partner likely is a key criterion for escaping cellular death mediated by PaeM4. The presence of a TonB box in PaeM4 and enhanced bacteriocin activity under iron-poor conditions strongly suggested the targeting of a TonB-dependent receptor. Evaluation of PaeM4 activities against TonB-dependent receptor knockout mutants in *P. aeruginosa* PAO1 revealed that the heme receptor HxuC (PA1302) serves as a PaeM4 target at the cellular surface. Because other ColM-type pyocins may target the ferrichrome receptor FiuA, our results illustrate the versatility in target recognition conferred by the polymorphic nature of ColM-type bacteriocins.

IMPORTANCE The antimicrobial armamentarium of a bacterium is a major asset for colonizing competitive environments. Bacteriocins comprise a subset of these compounds. Pyocins are an example of such antibacterial proteins produced by *Pseudomonas aeruginosa*, killing other *P. aeruginosa* strains. A large group of these molecules show a modular protein architecture that includes a receptor-binding domain for initial target cell attachment and a killer domain. In this study, we have shown that a novel modular pyocin (PaeM4) that kills target bacteria via interference with peptidoglycan assembly takes advantage of the HxuC heme receptor. Cells can protect themselves from killing by the presence of a dedicated immunity partner, an integral inner membrane protein that adopts a transmembrane topology distinct from that of proteins currently known to provide immunity against such toxin activity. Understanding the receptors with which pyocins interact and how immunity to pyocins is achieved is a pivotal step toward the rational design of bacteriocin cocktails for the treatment of *P. aeruginosa* infections.

KEYWORDS protein antibiotic, pyocin, polymorphic toxin, toxin-immunity module, bacterial antagonism, TonB-dependent receptor, lipid II

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Bacteria secrete a variety of molecules to gain hold in competitive environments, and the active killing of neighboring cells is a key strategy facilitating the occupation of a certain niche (1). Antagonistic interactions between microorganisms occur in contact-dependent and contact-independent ways, and a tremendous variety of inhibitory mediators, including bacteriocins and antibiotics, have been described to date (2). Whereas the latter compounds usually exhibit a broad target spectrum, bacteriocin killing is confined to related bacteria, often belonging to the same genus or species as the producer strain (2–4). These ribosomally synthesized peptides and proteins have been well studied in Gram-negative bacteria in particular, with colicins (*Escherichia coli*) and pyocins (*Pseudomonas aeruginosa*) serving as model systems (5, 6). Given that bacteriocins allow treatment of bacterial infections in animals (7, 8) and that several bacteriocins, including colicins and certain pyocins, are amenable to large-scale production in plants (9–11), these compounds constitute promising leads for the development of new therapeutic agents (8, 12).

Pyocins differ greatly in size and mechanism of action, and to date several such antibacterial proteins have been identified in *P. aeruginosa* and other *Pseudomonas* species. Well-studied groups of *Pseudomonas* bacteriocins include R-type and F-type tailocins and lectin-like (L-type) bacteriocins; a third large group of *P. aeruginosa* bacteriocins consists of the S-type pyocins (6, 13). The latter proteins are equipped with a receptor-binding domain, a segment or domain enabling membrane passage, and a toxin domain at the carboxy terminus. To circumvent suicidal expression in producer strains, immunity genes are expressed concomitantly with the bacteriocin. The encoded immunity partners temporarily form a complex with the bacteriocin's toxin module (14) or are inserted in the inner membrane to enable bacteriocin secretion without causing harm to the producer cell (15, 16). Cognate immunity genes are usually located downstream of the bacteriocin genes, allowing efficient coexpression. In general, the encoded proteins selectively provide immunity to "matching" toxin partners only. To date, a large number of toxin functionalities have been described for these modular pyocins, carrying killer modules acting in the periplasm (pore formation or lipid II hydrolysis) or in the cytoplasm (DNase or RNase activities) (5, 6). S-type pyocins typically take advantage of TonB-dependent receptors to gain access to target cells. The latter outer membrane proteins (OMPs) facilitate the binding and transport of iron-bound siderophores to the periplasm (17, 18). This receptor hijacking by pyocins was characterized in detail for pyocin S2; part of the bacteriocin's receptor-binding domain displays structural similarity to the ferripyoverdine and induces the conformational changes needed for import of this siderophore by its receptor (the type I ferripyoverdine transporter FpvAI), tricking the transporter to initiate pyocin translocation (19). Other examples of such pyocin targets are the ferripyochelin transporter FptA (pyocin S5) and the type II ferripyoverdine transporter FpvAII (pyocin S3) (20, 21). Interestingly, different pyocin receptor-binding domains may be coupled to different toxin/immunity modules, validating these modular bacteriocins as a class of polymorphic toxins (6, 22, 23).

A subset of these modular bacteriocins in pseudomonads are those equipped with a ColM domain (24), a toxin module that was first identified in colicin M of *E. coli* (25, 26) but actually occurs in a wide variety of proteobacterial genera (27), including other gammaproteobacteria (*Pectobacterium*) (28), as well as betaproteobacteria (*Burkholderia*) (29). The ColM domain acts in the periplasm and provokes cellular killing through degradation of lipid II (30, 31). Based on phylogeny, 2 large subgroups of *Pseudomonas* ColM bacteriocins were previously discerned (27). For a number of *Pseudomonas* ColM bacteriocins (subtype α), piracy of the ferrichrome receptor FiuA was demonstrated (32), indicating an OMP that is also targeted by colicin M in *E. coli* (FhuA) (25), although the possibility that *Pseudomonas* bacteriocins from this subtype may also target other receptors cannot be excluded. Pectocins of *Pectobacterium* also belong to the ColMα subtype but target FusA, a TonB-dependent receptor mediating the uptake of iron-bound plant ferredoxin (33). Immunity to *Pseudomonas* ColMα bacteriocins is provided by integral membrane proteins named PmiA (for *Pseudomonas* ColM-type immunity, type A), consisting of 4 transmembrane helices (TMHs) (15). The actual mechanism by which these proteins provide immunity remains unclear. The cell surface target of

TABLE 1 Occurrence of the *paeM4* locus in *P. aeruginosa* genomes and susceptibility to PaeM4

paeM4 locus	Presence of *paeM4* locus in *P. aeruginosa* genomes (%)	No. (%) of strains with *paeM4* locus/total no. of strains in strain panel	No. of strains susceptible to PaeM4/no. of strains with *paeM4* locus in strain panel
paeM4-pmiC	3.04	2/75 (2.67)	0/2
pmiC (orphan)	27.88	33/75 (44.00)	2/33
Absent	69.08	40/75 (53.33)	40/40

Pseudomonas ColMβ bacteriocins remains unknown at this point, and such bacteriocin genes are lacking in *P. aeruginosa* genomes. Immunity to ColMβ-type bacteriocins was demonstrated in *Burkholderia* but not yet in *Pseudomonas* and is provided by an integral membrane protein with 3 TMHs (*Burkholderia*) or a periplasmic module anchored in the inner membrane (*Burkholderia* and *Pseudomonas*) (PmiB) (29). More recently, a phylogenetically distinct *P. aeruginosa* ColM bacteriocin, PaeM4, was functionally identified (10). Through inspection of the genomic context of *paeM4*, a downstream open reading frame (*pmiC*) in the opposite direction was retained as a candidate immunity partner (27).

In this study, we explore the occurrence of the *paeM4* locus in *P. aeruginosa* genomes, and we demonstrate the functionality of PmiC as a PaeM4 immunity partner. We show that PaeM4 does not use FiuA as a target receptor, in agreement with earlier suggestions (10), but takes advantage of another TonB-dependent OMP.

RESULTS AND DISCUSSION

Distribution of the pyocin M4 locus in *P. aeruginosa* genomes. Based on homology searches using the ColM domain of pyocin M4 (named PaeM4) from *P. aeruginosa* BL03 (10) and previously characterized ColM bacteriocins as search queries, pyocin genes with high sequence similarity (>99% pairwise amino acid [AA] identity of the encoded proteins) were retrieved from ~3% of the *P. aeruginosa* genomes (based on 2,665 assembled genomes) (Table 1). PaeM4 (342 AAs) is distinct from the less abundant PaeM (289 AAs, present in <1% of *P. aeruginosa* genomes) (Fig. 1), and thus far no *P. aeruginosa* isolate carrying both ColM-type pyocin genes could be identified. The ColM domains of PaeM (*P. aeruginosa* NCTC 10332) and PaeM4 (*P. aeruginosa* BL03) share 29% AA sequence identity, whereas their amino-terminal receptor-binding regions cannot be meaningfully aligned. The catalytic motif in the ColM domain of PaeM4 can be recognized as DxYD(x_5)QR, slightly deviating from the equivalent motif from colicin M and PaeM, DxYD(x_5)HR (27, 31, 34). However, it was found previously that limited variation in this sequence motif does not necessarily affect the bacteriocin function of ColM-type toxins (29). Furthermore, the presence of a proline-rich TonB box at the amino terminus of PaeM4 suggests that this pyocin likely targets a TonB-dependent receptor (27, 35), as was demonstrated previously for PaeM (32) and several other modular S-type pyocins (6).

The *paeM4* gene is part of a short region (~2.5 kb) with a GC content markedly lower than average (~46% versus ~67%); it consistently arises at the same locus but differs from the genomic position of *paeM*, which occurs in *exoU*-containing genomic island A (24). In contrast, this ColM-type pyocin is sandwiched between a gene encoding a β-ketoacyl synthase and the 3′,5′-bisphosphate nucleotidase gene *cysQ* (orthologues PA5174 and PA5175, respectively, in *Pseudomonas aeruginosa* PAO1) (Fig. 2). Downstream and in the opposite direction of *paeM4* is located a candidate immunity gene, previously termed *pmiC* (27). The encoded protein (232 AAs) is distinct from the PmiA ColM-type immunity partners; whereas the latter host 4 TMHs (15), PmiC adopts 6 predicted TMHs (Fig. 3A) (36). By genome analysis, a putative ColM immunity partner, PmiB, with a periplasmic domain was previously identified in a small set of pseudomonads; however, no *pmiB* immunity genes can be retrieved from *P. aeruginosa* genomes (27, 29) (Fig. 1). The *paeM4* locus may also be loaded with a transposase, such as in *P. aeruginosa* BL03, although this organization appears to be quite rare. In ~28% of *P. aeruginosa* genomes, such as in *P. aeruginosa* LESB58, only *pmiC* is present, accompa-

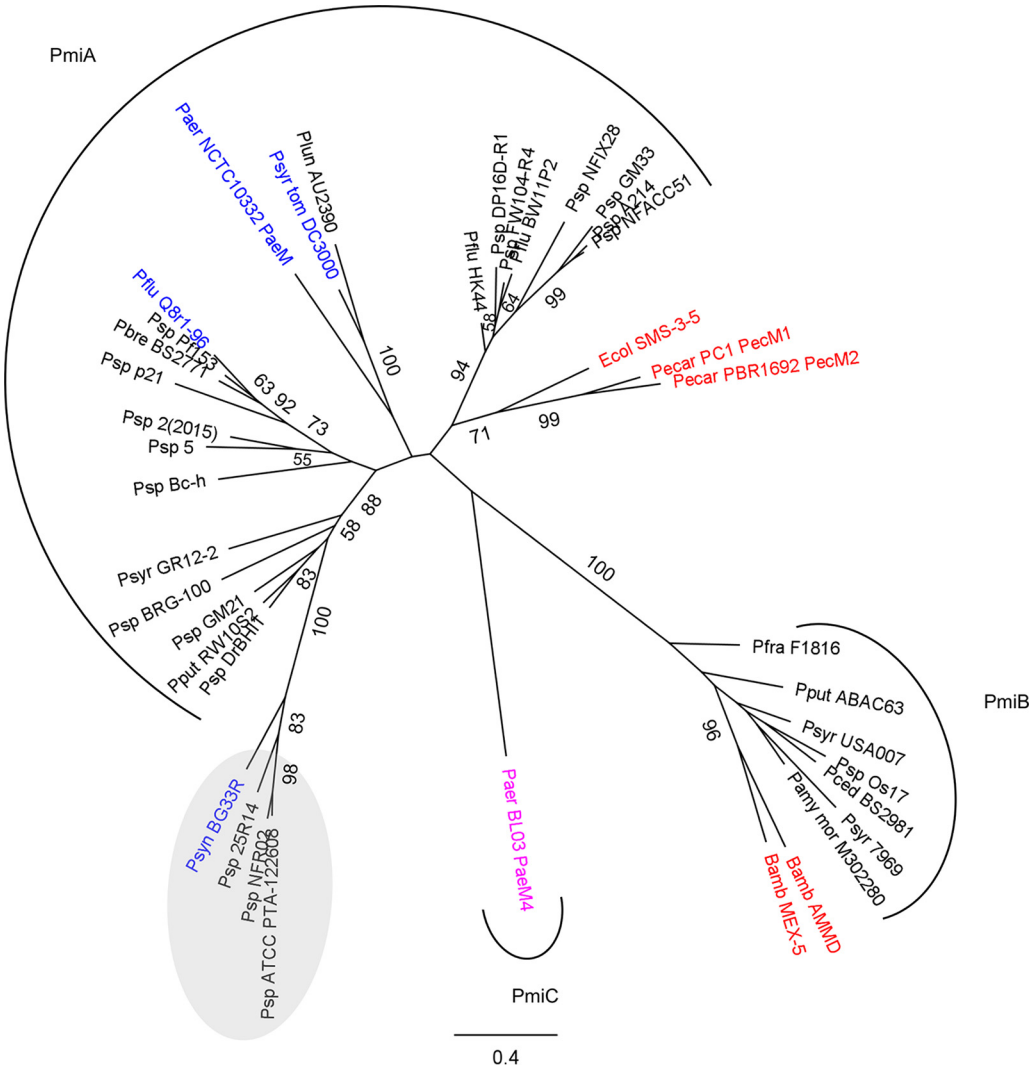

FIG 1 Phylogeny of ColM domains derived from characterized (blue) and putative (black) *Pseudomonas* bacteriocins and characterized bacteriocins in other genera (red). The distinct ColM-type bacteriocin from *P. aeruginosa* BL03 is shown in pink. In the maximum likelihood phylogenetic tree (PhyML, with the JTT substitution model) of ColM domains, highly homologous *Pseudomonas* sequences (>75% pairwise AA identity for full-length proteins) are represented by one sequence only. Bootstrap values (percentages of 1,000 replicates) higher than 50 are shown at the branches. The scale bar represents 0.4 substitutions per site. *Pseudomonas* representatives are grouped by arcs specifying the immunity partner present. Proteins retrieved from strains in which the ColM domain is part of a bacteriocin integrating 2 toxin domains are grouped in a gray oval. Genes encoding such hybrid bacteriocins are not accompanied by a (known) putative ColM-type immunity partner. Bamb, *Burkholderia ambifaria*; Ecol, *Escherichia coli*; Paer, *Pseudomonas aeruginosa*; Pamy mor, *Pseudomonas amygdali* pv. morsprunorum; Pbre, *Pseudomonas brenneri*; Pced, *Pseudomonas cedrina*; Pecar, *Pectobacterium carotovorum*; Pflu, *Pseudomonas fluorescens*; Pfra, *Pseudomonas fragi*; Plun, *Pseudomonas lundensis*; Pput, *Pseudomonas putida*; Psp, *Pseudomonas* sp.; Psyn, *Pseudomonas synxantha*; Psyr, *Pseudomonas syringae*; Psyr tom, *Pseudomonas syringae* pv. tomato.

nied by a short remnant of *paeM4* (Fig. 2). Immunity genes not accompanied by an adjacent full-length bacteriocin are generally referred to as orphans. Such orphan immunity genes were studied previously in the context of different polymorphic toxin systems mediating antagonism via cell-to-cell contact (37–39).

PaeM4 represents a broadly active bacteriocin. The *paeM4* gene (locus tag Q057_04089, from *P. aeruginosa* BL03) was cloned in expression vector pET28a(+). Previously, it was found that a coexpressed immunity gene protecting cells from colicin M toxin function is not needed when such bacteriocins are expressed in the cytoplasm of *E. coli* (24, 28, 29); therefore, the candidate immunity gene *pmiC* was not included in the construct. The bacteriocin gene was cloned to encode a carboxy-terminal His-

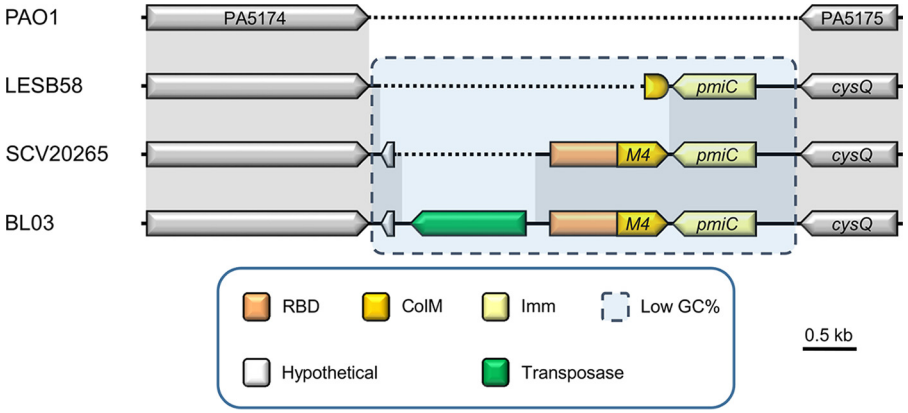

FIG 2 Representative genetic organizations of the *paeM4* locus in *Pseudomonas aeruginosa* strains. Synteny is represented by sequence conservation (gray shading). Genes are represented by arrows, and a *paeM4* fragment is shown as a shorter rounded shape. Functions of genes and gene parts (if known) are specified by color in the legend. The receptor-binding domain (RBD) of PaeM4 includes the TonB box that is part of a short region at the amino terminus of the encoded protein. The candidate immunity partner (Imm) PmiC is encoded downstream of *paeM4*, in the opposite direction. Dotted lines indicate the lack of an equivalent nucleotide sequence.

tagged protein, since an amino-terminal fusion product would interfere with the import-related function of the amino-terminal translocation segment of the pyocin (15, 34, 35). After transformation into *E. coli* BL21(DE3), pyocin gene expression was induced, cells were harvested, soluble proteins were isolated, and the recombinant

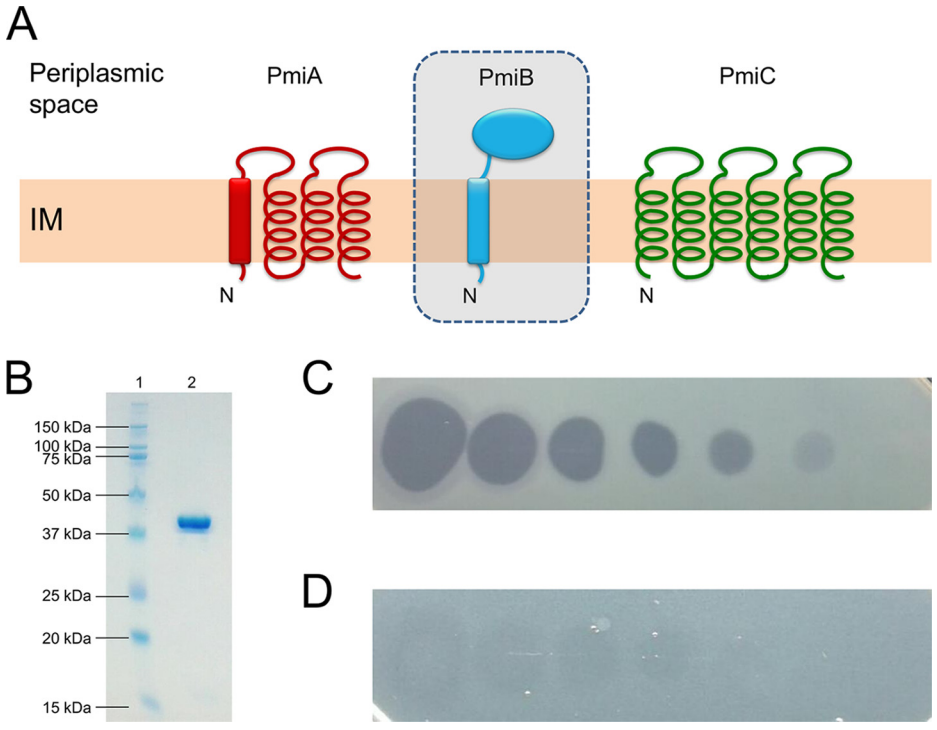

FIG 3 (A) Membrane topology model of ColM-type immunity proteins in pseudomonads. PmiA carries 4 predicted TMHs, the first of which may act as a Sec- or Tat-dependent signal sequence (rectangle). The putative ColM immunity partner PmiB (in shaded box) has a periplasmic domain and is anchored in the inner membrane (IM), but PmiB-encoding genes are absent from *P. aeruginosa* genomes. PmiC has 6 TMHs and occurs exclusively in *P. aeruginosa*. N indicates the amino terminus. *Pseudomonas* ColM-type immunity proteins were drawn as in reference 27. (B) SDS-PAGE gel of purified recombinant PaeM4. Lane 1, Kaleidoscope ladder, with sizes indicated in kilodaltons; lane 2, purified His-tagged PaeM4 (~41 kDa; predicted size, 39.99 kDa). (C and D) Spot assay of 10-fold serial dilutions of PaeM4 (initial concentration, 1 mg/ml) against *P. aeruginosa* Br993 on CAA medium, in the absence (C) or presence (D) of 50 μM $FeCl_3$.

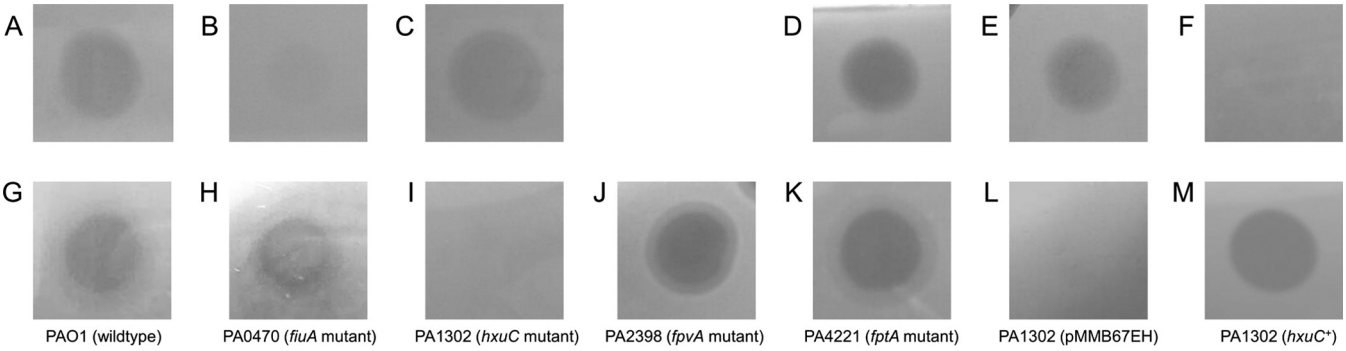

FIG 4 (A to C) Spot assays of PaeM4 (10 μl; 0.08 mg/ml) against *P. aeruginosa* PA7 equipped with pJB3Tc20 (empty plasmid) (control) (A), expressing $pmiC_{BL03}$ (B), or expressing $pmiA_{NCTC10332}$ (C). (D to F) Spot assays of PaeM (10 μl; 0.4 mg/ml) against *P. aeruginosa* PA7 equipped with pJB3Tc20 (empty plasmid) (D), expressing $pmiC_{BL03}$ (E), or expressing $pmiA_{NCTC10332}$ (F). (G to M) Spot assays of PaeM4 (10 μl; 1 mg/ml) against *P. aeruginosa* PAO1 and selected TonB-dependent transporter mutants, i.e., wild-type PAO1 (G), PA0470 (*fiuA*) (H), PA1302 (*hxuC*) (I), PA2398 (*fpvA*) (J), PA4221 (*fptA*) (K), PA1302 equipped with pMMB67EH (empty vector) (control) (L), and PA1302 expressing *hxuC* (pCMPG6293) (M). Three biological repeats and 3 technical repeats were performed for every test.

protein was purified (Fig. 3B). His-tagged pyocin displayed (weak) antagonistic activity against *P. aeruginosa* PAO1, as demonstrated earlier (10). A total of 56% of the strains (42/75 strains) in our *P. aeruginosa* test panel were killed by PaeM4 (Table 1; also see Table S1 in the supplemental material), a PaeM4 killing frequency very similar to that noted by Paškevičius and colleagues (53%) (10). Bactericidal activity was evident under iron-poor conditions (Casamino Acids [CAA] medium), whereas PaeM4 function was almost completely abrogated in the presence of iron (50 μM $FeCl_3$) (Fig. 3C and D). This finding suggests that PaeM4 takes advantage of an outer membrane receptor that is controlled by the ferric uptake regulator (Fur) (40). Similar to several other modular pyocins (20, 21, 32, 41, 42), this correlates with the targeting of a TonB-dependent receptor involved in iron acquisition (see above).

PmiC confers immunity to PaeM4 killing. The putative PaeM4 immunity partner, *pmiC* (locus tag Q057_04090, from *P. aeruginosa* BL03), was cloned in shuttle vector pJB3Tc20 and introduced into the PaeM4-susceptible strain *P. aeruginosa* PA7 (43) via electroporation. Via spot-on-lawn assays, transformants were tested for altered PaeM4 sensitivity. When equipped with *pmiC*, transformants were no longer susceptible to PaeM4, confirming the immunity function of PmiC (Fig. 4A and B). In contrast, when *pmiA*, providing immunity to PaeM from *P. aeruginosa* NCTC 10332 (15), was introduced into PA7, transformants remained as sensitive to PaeM4 as before (Fig. 4C). Conversely, *pmiC* could not immunize cells against the PaeM function in *P. aeruginosa* PA7 (15) (Fig. 4D to F). These observations are in line with the generally high specificity and selectivity of immunity partners in silencing toxin functions (14, 44–46).

In a next step, all isolates of the strain panel tested for PaeM4 susceptibility were evaluated for the presence of the *paeM4* locus via PCR, using a primer couple situated on the β-ketoacyl synthase gene (forward) and *cysQ* (reverse) (Table 1 and Fig. 2). Amplicon sizes of ~620 bp (based on the genome sequence of PAO1), ~2,020 bp (LESB58), and ~3,100 bp (SCV20265) corresponded to the absence and the presence of a partial and complete *paeM4* locus, respectively (gel electrophoresis data not shown). Partial *paeM4* loci (33/75 strains [44.00%]) were somewhat more abundant than expected from *P. aeruginosa* genome sequence data in GenBank (27.9%) (Table 1). Interestingly, all strains from the panel lacking *pmiC* were killed by PaeM4 (Table S1). Conversely, PaeM4-insensitive isolates were equipped with a partial or complete *paeM4* region, suggesting that the *pmiC*-encoded immunity is also functional *in vivo*. Two strains of our *P. aeruginosa* panel (CF_PA41 [47] and CPHL2000 [48]) were sensitive to PaeM4, despite carrying a *pmiC* orphan immunity gene (based on PCR screening). In the corresponding amplicons, no single-nucleotide polymorphisms (such as frameshifts) that might account for a loss of immunity function could be detected (data not shown). Overall, *pmiC* genes coupled to *paeM4* display a high degree of sequence conservation (>97% pairwise AA identity of the encoded proteins), whereas conservation with and

among *pmiC* orphans is lower (~76% AA identity with $PmiC_{BL03}$ and >93% AA identity among PmiC orphans). Previously, it was noted that homologous orphan pyocin immunity genes with significant sequence similarity may offer immunity to noncognate toxins (15, 49), suggesting the functionality of orphan *pmiC* genes. It should be emphasized that significant DNA sequence variation can be noted in the regions preceding the orphan *pmiC* genes, which may in turn exert transcriptional effects. The expression, functionality, and role of orphan (*pmiC*) immunity genes in strains escaping pyocin killing will be discussed in a separate publication.

PaeM4 targets an uptake system for heme. The presence of a TonB box at the amino-terminal end of PaeM4 and the observation that PaeM4 bacteriocin activity is significantly enhanced under iron-poor conditions strongly suggest the exploitation of a TonB-dependent receptor for target cell attachment and subsequent transfer to the periplasm. Strain PAO1, which is susceptible to PaeM4 and PaeM, contains a total of 36 TonB-dependent transporters (50), and defined mutants (51) of each of them were tested for altered pyocin susceptibility. As demonstrated previously (10), PAO1 mutant PA0470 (defective in the PaeM receptor, *fiuA*) was still sensitive to PaeM4 (Fig. 4G and H). This can be expected, given the quite different receptor-binding domains of PaeM and PaeM4. Other mutants also displayed PaeM4 susceptibility patterns similar to that of the wild-type strain, except for PA1302 (Fig. 4I to K; also see Table S1 in the supplemental material). The latter mutant is altered in the TonB-dependent receptor gene *hxuC*, which is involved in the uptake of heme, in line with the hypothesis regarding the targeting of an iron-dependent receptor by PaeM4. This OMP is part of the 3-gene cluster *hxuCBA*, which was previously studied in more detail in *Haemophilus influenzae* for the import of heme-hemopexin (52) and was shown to act as a virulence factor (53). The role of PA1302 was validated via complementation; when *hxuC* was cloned in shuttle vector pMMB67EH and transformed to PA1302, the PaeM4 susceptibility phenotype (spot assay) was restored (Fig. 4L and M).

The *hxuC* gene can be readily retrieved from all sequenced *P. aeruginosa* strains and appears to be part of the *P. aeruginosa* core genome (54). Furthermore, HxuC displays a high degree of conservation (>95% pairwise AA identity among orthologues in *P. aeruginosa*). Therefore, the presence of a *pmiC* immunity gene likely is the primary determinant accounting for PaeM4 resistance, a hypothesis that is supported by the results of the PaeM4 locus screening in this study. This observation may also be of future interest for designing bacteriocin cocktails (12). Preference should be given to the design of (chimeric) pyocins uniting (i) toxin modules for which immunity genes rarely occur and (ii) receptor-binding domains targeting TonB-dependent OMPs, which are part of the *P. aeruginosa* core genome and are highly conserved. In this perspective, the results obtained here suggest that the receptor-binding domain of PaeM4 constitutes an interesting candidate to include in the design of chimeric pyocins, in contrast to its toxin module (given the frequent occurrence of *pmiC*). Recently, it was shown that polymorphism in BamA, an insertase accounting for the assembly of proteins in the outer membrane, determines which *Pseudomonas* strains are killed by lectin-like bacteriocins (55). Also, polymorphism of the FiuA receptor was postulated to account for differences in susceptibility to the highly similar PaeM bacteriocins (from *P. aeruginosa* strains JJ692 and NCTC10332) (10, 12).

MATERIALS AND METHODS

Genome searches and phylogenetic analysis. Genes encoding putative ColM bacteriocins in *P. aeruginosa* (2,665 assembled *P. aeruginosa* genomes; data collection on 10 December 2017) were identified by BLAST homology searches using the National Center for Biotechnology (NCBI) nonredundant database. ColM domains of previously characterized ColM-type bacteriocins were used as a search query (Pfam PF14859) (27). Similarly, *P. aeruginosa* genomes were analyzed for the presence of genes encoding PmiC immunity proteins (no Pfam domain). Regions upstream and downstream of *paeM4* genes were analyzed further. progressiveMauve was used to align *paeM4* loci (56), multiple sequence alignments were generated with MUSCLE (proteins) (57) and MAFFT (DNA sequences) (58), and phylogenetic analysis was executed with PhyML (1,000 bootstrap replicates) using the JTT substitution model (59). Transmembrane regions and topology were predicted using TOPCONS (http://topcons.cbr.su.se) (60) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM) (36).

TABLE 2 Primers used in this study

Primer name	Sequence[a]	Purpose
PGPRB-10249	TGGCAGCAGCCAACTCAGCTT	Insert validation in pET28a
PGPRB-10250	TATAGGCGCCAGCAACCGCA	Insert validation in pET28a
PGPRB-10255	GCTCACTCATTAGGCACCC	Insert validation in pJB3Tc20
PGPRB-10256	GGTAACGCCAGGGTTTTC	Insert validation in pJB3Tc20
PGPRB-10409	AGCGGATAACAATTTCACACAGG	Insert validation in pMMB67EH
PGPRB-10410	TCTGTATCAGGCTGAAAATCTTCTCTC	Insert validation in pMMB67EH
PGPRB-10434	TGGCTA**CCATGG**GTCCAATGGAATTCCCTCCAACTAC	Cloning of $paeM4_{BL03}$ in pET28a
PGPRB-10436	TGGCTA**CTCGAG**CAACTTATCTCCAGTGCCTGAT	Cloning of $paeM4_{BL03}$ in pET28a
PGPRB-10440	TGGCTA**CTGCAG**CTTGCAGGACGTAGAATGCATGAG	Cloning of $pmiC_{BL03}$ in pJB3Tc20
PGPRB-10441	TGGCTA**GAATTC**CTATCTCTTGATAGGTGCTTTCTCATCAC	Cloning of $pmiC_{BL03}$ in pJB3Tc20
PGPRB-10448	GCCGCTGGTATACAAGAAGGA	Presence of *paeM4* locus in *P. aeruginosa* genomes
PGPRB-10449	GAGGAGTTCACCGTCAACGT	Presence of *paeM4* locus in *P. aeruginosa* genomes
PGPRB-10473	TGGCTA**GAATTC**AGTCACTTGGGGAGAGTTCCAGCATGAC	Cloning of *hxuC* in pMMB67EH
PGPRB-10474	TGGCTA**AAGCTT**CCAATACTTCATGCTCGGCGACAACCG	Cloning of *hxuC* in pMMB67EH

[a]Restriction sites incorporated in primers are shown in bold, as follows: AAGCTT, HindIII; CCATGG, NcoI; CTCGAG, XhoI; CTGCAG, PstI; GAATTC, EcoRI.

Bacterial strains and media. *P. aeruginosa* isolates were grown in LB medium (MP Biomedicals) or CAA medium (BD Bacto), and *E. coli* was grown in LB medium. *P. aeruginosa* PAO1 and transposon mutants altered in genes encoding (putative) TonB-dependent receptors were obtained from the Manoil laboratory (University of Washington) (51); other strains originated from an in-house collection and J. P. Pirnay (Queen Astrid Military Hospital, Belgium) (48). The presence of the transposon insert in the correct gene in each of the PAO1 mutants was tested and confirmed via PCR (data not shown). All strains used in this study are summarized in Table S1. Strains were routinely grown at 37°C, with shaking at 200 rpm. Plasmids were propagated in *E. coli* DH5α (BIOKÉ), and *E. coli* BL21(DE3) (VWR International) was used for the production of recombinant bacteriocins. Bacterial growth media were solidified with agar (1.5%; Invitrogen). Filter-sterilized (0.22-μm filters; Sarstedt) additives, i.e., kanamycin (50 μg/ml; Sigma-Aldrich), gentamicin (15 μg/ml; TCI Europe NV), tetracycline (15 to 150 μg/ml; Sigma-Aldrich), isopropyl β-D-thiogalactopyranoside (IPTG) (0.1 to 1 mM; Formedium), and $FeCl_3$ (50 μM; Sigma-Aldrich), were added when needed. Bacterial strains were stored on plates at 4°C or in glycerol (50% [vol/vol]; VWR International) at −80°C.

DNA methods and plasmid construction. Genomic DNA was extracted from overnight cell cultures using the Puregene Yeast/Bact. kit B (Qiagen), and synthetic genes and primers were obtained from IDT DNA. The gene encoding bacteriocin PaeM4 from *P. aeruginosa* BL03 (locus tag Q057_04089) and the candidate immunity gene *pmiC* from *P. aeruginosa* BL03 (locus tag Q057_04050) were PCR amplified with Q5 polymerase (New England BioLabs) using a T100 thermal cycler (Bio-Rad). Primers are listed in Table 2. Subsequently, PCR amplicons were purified with the GenElute PCR cleanup kit (Sigma-Aldrich) and digested with NcoI and XhoI (for *paeM4*) or PstI and EcoRI (for $pmiC_{BL03}$) (restriction enzymes were from New England BioLabs). After purification with the PCR cleanup kit, *paeM4* and *pmiC* were ligated in pET28a(+) and the shuttle vector pJB3Tc20 (61), respectively, using T4 DNA ligase (Invitrogen). Ligation reactions were performed overnight at 16°C. The following day, products were transformed into *E. coli* DH5α via heat shock. After selection on LB plates containing the appropriate antibiotic, transformants were validated for the presence of the insert via colony PCR using *Taq* polymerase (New England BioLabs) with the primer pairs PGPRB-10249/PGPRB-10250 (pET28a) and PGPRB-10255/PGPRB-10256 (pJB3Tc20). Plasmids with the insert were isolated with the Nucleospin Plasmid EasyPure kit (Macherey-Nagel) and sequence verified by Sanger sequencing (GATC Biotech, Constance, Germany). A similar procedure was followed for the cloning of PA1302 from *P. aeruginosa* PAO1 in pMMB67EH (62), using restriction enzymes EcoRI and HindIII. The pyocin-encoding plasmids pCMPG6283 (containing *paeM4*) and pCMPG6250 (containing *paeM* from *P. aeruginosa* NCTC10332), which was constructed previously (15), were transformed into *E. coli* BL21(DE3), with selection on kanamycin. Plasmids containing the immunity genes $pmiC_{BL03}$ (pCMPG6287) and $pmiA_{NCTC10332}$ (pCMPG6252), which was constructed previously (15), were electroporated to sucrose-competent (63) *P. aeruginosa* cells using a Gene-Pulser Xcell (Bio-Rad), with selection on tetracycline. pCMPG6293 ($hxuC_{PAO1}$ in pMMB67EH) was electroporated into *P. aeruginosa* PAO1 mutant PA1302, with selection on gentamicin. All plasmids used in this study are summarized in Table S2. Plasmids without inserts were used as controls in subsequent assays. All enzymes (DNA polymerases, restriction enzymes, and T4 DNA ligase) were used according to the supplier's specifications. The preparation of competent *E. coli* and heat shock transformation of *E. coli* were performed following standard procedures (64).

The presence of a partial or complete *paeM4* locus in strains from our *P. aeruginosa* strain panel was evaluated by PCR screening (in case no genomic information was available). Primers PGPRB-10463 and PGPRB-10464 were selected based on DNA sequence conservation in the *paeM4*-flanking genes PA5174 and PA5175 (*P. aeruginosa* PAO1). Q5 polymerase was used for PCR amplification. Fragments to be sequenced were purified with the PCR cleanup kit.

Expression and purification of His-tagged bacteriocin. Plasmids containing pyocin-encoding genes (PaeM [pCMPG6250] and PaeM4 [pCMPG6283]) were transformed into *E. coli* BL21(DE3) via heat shock. Recombinant His-tagged proteins were generated as follows. First, 5-ml volumes of overnight *E. coli* cultures were transferred to 500-ml Erlenmeyer flasks and incubated at 37°C until the optical density at 600 nm (OD_{600}) reached ~0.7. Next, cell cultures were cooled, supplemented

with IPTG (final concentration of 1 mM), and incubated overnight at 20°C, with shaking. The following day, cells were collected via centrifugation at 5,000 × *g* (20 min at 4°C) using a Beckman Coulter Avanti J-E ultracentrifuge, supernatants were discarded, and pellets were frozen overnight at −20°C. The next day, cell pellets were thawed, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole [pH 7.8]; VWR International and Sigma-Aldrich) (5 ml of buffer per 1 g of pellet), and sonicated using a Branson digital sonifier (amplitude of 20%; 10 cycles of 30 s on and 30 s off). Cell lysates were treated with Pierce universal nuclease (0.01 U/μl; Invitrogen) for 1 h at 37°C and then were centrifuged at 10,000 × *g* for 30 min at 4°C, to remove residual debris. Supernatants containing soluble proteins were filtered (0.22-μm filters) and validated for the presence of recombinant proteins by SDS-PAGE (Invitrogen), with subsequent Coomassie blue staining (InstantBlue; Expedeon). Samples containing His-tagged proteins were applied to 5-ml HisTrap HP columns (GE Healthcare) and purified by affinity chromatography using an Äkta Purifier (GE Healthcare). Bacteriocins were eluted with a linear gradient of imidazole (10 to 500 mM), using lysis buffer as the running buffer. Fractions that eluted at high imidazole concentrations were validated for the presence of recombinant pyocin by SDS-PAGE, using the Precision Plus Protein Kaleidoscope ladder (Bio-Rad) as a size standard, and were dialyzed with Tris buffer (50 mM, 200 mM NaCl [pH 7.5]; Sigma-Aldrich) overnight. Protein samples were subsequently concentrated using Vivaspin filters (Sartorius) and were purified via gel filtration on a HiLoad Superdex 200 preparative grade 16/600 column (GE Healthcare), using Tris buffer as the running buffer. Concentrations of purified bacteriocins were calculated by absorbance measurements at 280 nm, using a ND-1000 spectrophotometer (Thermo Scientific). Calculated molar extinction coefficients for the His-tagged pyocins PaeM and PaeM4 were 73,340 M^{-1} cm^{-1} and 77,810 M^{-1} cm^{-1}, respectively.

Bacteriocin spot assay. The bacteriocin activity of PaeM4 and PaeM was evaluated using the soft agar overlay assay (65). Purified and filter-sterilized recombinant proteins (10-μl volumes; 1 mg/ml) were spotted onto lawns of test bacteria. After air drying of the bacteriocin spots, petri dishes were incubated overnight. The following day, plates were evaluated for the presence of halos, i.e., zones lacking bacterial growth, which were indicative of antagonistic activity. Dialysis buffer was used as a negative control. Spot assays were performed with bacteriocin purified from 3 independent batches. All tests were routinely performed in triplicate (independent repeats) on CAA plates, which were supplemented with IPTG, tetracycline, or $FeCl_3$ if needed. For evaluation of the contributions of immunity genes and $FeCl_3$ to bacteriocin susceptibility, serial dilution series were used for comparison.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00716-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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