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NOTE

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Knock-out of multidrug efflux pump MexXY-OprM results in increased susceptibility to antimicrobial peptides in *Pseudomonas aeruginosa*

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Abstract

Multidrug efflux systems of the resistance-nodulation-cell division family play a crucial role in resistance of *Pseudomonas aeruginosa* to a large variety of antibiotics. Here, we investigated the role of clinically relevant efflux pumps MexAB^{-}OprM, MexCD^{-}OprJ, and MexXY^{-}OprM in resistance against different cationic antimicrobial peptides (AMPs). Our results indicate that a knock-out in efflux pump MexXY-OprM increased susceptibility to some AMPs by two- to eightfold. Our data suggest a contribution of MexXY-OprM in resistance to certain AMPs in *P. aeruginosa*, which should be considered in the future development of new and highly active antimicrobial peptides to fight multidrug resistant infections.

KEYWORDS

antimicrobial peptides, efflux pump, MexXY-OprM, *Pseudomonas aeruginosa*

The Gram-negative bacterium *Pseudomonas aeruginosa* is one of the most important opportunistic human pathogens causing a wide range of severe chronic and nosocomial infections.[1] Due to a large arsenal of intrinsic resistance mechanisms, *P. aeruginosa* is inherently resistant to many commonly used antibiotics including aminoglycosides, fluoroquinolones, and ß-lactams. In addition to the very low permeability of its outer membrane and the expression of antibiotic cleaving enzymes, *P. aeruginosa* possesses several multidrug efflux systems of the RND family.[2] Among the various resistance-nodulation-cell division (RND) efflux pumps in *P. aeruginosa* are MexAB-OprM, MexCD-OprJ, and MexXY-OprM, which are all capable of exporting several different classes of antibiotics and are involved in multidrug resistance in laboratory strains and clinical isolates of *P. aeruginosa*.[3,4]

Antimicrobial peptides (AMPs) are an abundant and diverse group of molecules that are produced by many organisms as part of their first line defense. They are typically relatively short consisting of 10–60 amino acids, are positively charged with a net charge of +2 to +10 and have an amphiphilic character.[5] Since some of these peptides exhibit strong antimicrobial activities and their mechanism of action has been shown to address multiple

Abbreviations: Api, apidaecin; AMPs, antimicrobial peptides; CFU, colony forming unit; COL, colistin; *E. coli*, *Escherichia coli*; GRA, gramicidin A; HBD, human beta defensing; HPN, human neutrophil peptide; LPS, lipopolysaccharides; Mag, magainin 2; MH, Mueller Hinton; MIC, minimal growth inhibitory concentration; n.d, not determined; *P. aeruginosa*, *Pseudomonas aeruginosa*; PCR, polymerase chain reaction; PMB, polymyxin B; qRT-PCR, quantitative real-time polymerase chain reaction; RND, resistance-nodulation-cell division; WT, wild-type.

targets within the bacterial cell resulting in low resistance development, they are considered as a promising class of new antimicrobial agents, in particular, against multidrug resistant Gram-negative bacteria including *P. aeruginosa*.[5,6] Besides intracellular targets such as inhibition of protein synthesis or binding of nucleic acids, many antimicrobial peptides disrupt the bacterial cell membrane by transmembrane pore-formation which subsequently leads to cell death.[7]

Although the most relevant resistance mechanisms against polymyxins and AMPs in Gram-negative bacteria are membrane modification and proteolytic degradation, previous studies have also shown the involvement of transporters and efflux pumps in resistance to peptide antibiotics.[8,9] For example, it has been shown that the ABC transporter SapABCDF is a main transporter of AMPs in *Haemophilus influenza*, *Salmonella typhimurium*, and *Proteus mirabilis*.[8] In addition, the RND efflux pump MtrCDE has been shown previously to contribute to AMP resistance in the Gram-negative bacteria *Neisseria gonorrhoeae* and *Neisseria meningitides*.[10,11] More recently it has also been shown that the efflux pump MexXY-OprM contributes to the tolerance of *P. aeruginosa* to the peptide antibiotic colistin.[12] In this study, we investigated the role of clinically important RND efflux pumps MexAB-OprM, MexCD-OprJ, and MexXY-OprM in antimicrobial peptide resistance in *P. aeruginosa* by analyzing a set of efflux pump deficient and overexpressing strains.

To identify a role of RND efflux pumps in resistance to antimicrobial peptides in *P. aeruginosa*, we selected a set of efflux pump knock-out mutants and overexpressing strains (Table 1) and measured the minimal growth inhibitory concentrations (MICs) against a variety of natural and synthetic antimicrobial peptides (Table 2). The determination of MICs was performed as described previously,[18] the cells were incubated in Mueller Hinton (MH) broth at 37°C for 18 h. Among the synthetic peptides were IDR-1018,[19] 1037,[20] HHC-10,[21] and HHC-36[21] which are loosely based on linear bovine bactenecin variant Bac2A[21] and which have been reported previously to show a broad spectrum antibacterial activity against different pathogens including *P. aeruginosa*.[19,21,22] The MIC tests revealed a significant two- to eightfold increase in susceptibility against synthetic peptides Bac2a, 1037, HHC-10, and HHC-36 for the MexXY-deficient *P. aeruginosa* mutant K1525 (ΔMexXY-OprM) as well as for the triple mutant K2896 lacking all three efflux pumps MexAB-OprM, MexCD-OprJ, and MexXY-OprM in comparison with wildtype K767 (PAO1, Table 3). In contrast, no differences in MICs were observed between the tested strains of *P. aeruginosa* for indolicidin[23] and IDR-1018 (Table 3). In addition, MICs for gramicidin A, polymyxin B, and colistin did not reveal an increase in susceptibility in the triple mutant K2896 in comparison with WT K767 (Table 3). Furthermore, no differences in MICs were observed for IDR-1018, 1037, HHC-10, HHC-36, Bac2a, and indolicidin in the single knock-out mutants K1523 (ΔMexAB-OprM) and K1521 (ΔMexCD-OprJ) as well as for the MexAB-OprM overexpressing strain K1455, the MexCD-OprJ overexpressing strain K1536, and the MexXY overexpressing strain K2415 (Table 3). Overall, these MIC data suggested an involvement of the efflux pump MexXY-OprM in resistance to some antimicrobial peptides.

TABLE 1 Bacterial strains and plasmids used in this study.

Strain	Description	Characteristics[a] AB	CD	XY	Reference
P. aeruginosa strains					
K767	*P. aeruginosa* PAO1	+	+	+	[13]
K1455	K767 *nalB*	++	+	+	[14]
K1521	K767 Δ*mexCD-oprJ*	+	–	+	[15]
K1523	K767 Δ*mexB*	–	+	+	[16]
K1525	K767 Δ*mexXY*	+	+	–	[17]
K1536	K767 *nfxB*	+	++	+	[16]
K2415	K767 Δ*mexZ*	+	+	++	[17]
K2896	K767 Δ*mexB*Δ*mexCD* Δ*mexXY*	–	–	–	[15]
E. coli strains					
DH5α	Cloning strain	–	–	–	Invitrogen
DH5α-pJET1.2	Vector control strain	–	–	–	Invitrogen
DH5α-pJET1.2::*mexXY*	pJET1.2- harboring genes *mexXY* from *P. aeruginosa* PAO1	–	–	++	This study

[a]AB, *mexAB* possessing (+), deficient (–), or overexpression (++); CD, *mexCD* possessing (+), deficient (–), or overexpression (++); XY, *mexXY* possessing (+), deficient (–), or overexpression (++).

TABLE 2 Antimicrobial peptides used in this study.

Peptide	Amino acid sequence and structural features	No. of amino acids	Molecular weight (g/mol)	Net charge[a]
1037	KRFRIRVRV	9	1229.54	5.0
ApideacinIb	GNNRPVYIPQPRPPHPRL	18	2108.44	3.1
Bac2a	RLARIVVIRVAR-NH_2	12	1420.82	5.0
HHC-10	KRWWKWIRW	9	1444.75	4.0
HHC-36	KRWWKWWRR	9	1487.78	5.0
IDR-1018	VRLIVAVRIWRR	12	1536.94	4.0
Indolicidin	ILPWKWPWWPWRR-NH_2	13	1906.32	4.0
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	23	2466.94	3.1

[a]Net charge at pH 7.

TABLE 3 Antimicrobial peptide susceptibilities in *P. aeruginosa* PAO1 strains (MICs in μg/mL).

		MICs [μg/mL]										
Strain	Phenotype[a]	1037	HHC-36	HHC-10	Bac2a	Ind	IDR-1018	Api	Mag	PMB	GRA	COL
K767	WT	32	16	8	32	64	16	>128	>128	0.25	8	0.125
K1523	$AB^-CD^+XY^+$	32	16	8	32	64	16	>128	>128	0.25	n.d.	0.125
K1455	$AB^{++}CD^+XY^+$	32	16	8	32	64	16	>128	>128	0.125	n.d.	0.25
K1521	$AB^+CD^-XY^+$	32	16	8	32	64	16	>128	>128	0.125	n.d.	0.25
K1536	$AB^+CD^{++}XY^+$	32	16	8	32	64	16	>128	>128	0.125	n.d.	0.125
K1525	$AB^+CD^+XY^-$	**8**	**2**	**4**	**16**	64	16	>128	>128	0.25	8	0.125
K2415	$AB^+CD^+XY^{++}$	32	16	8	32	64	16	>128	>128	0.25	n.d.	0.25
K2896	$AB^-CD^-XY^-$	**8**	**2**	**4**	**16**	64	16	>128	>128	0.25	8	0.5

Abbreviations: Api, apidaecin 1b; COL, colistin; GRA, gramicidin A; Ind, indolicidin; Mag, magainin 2; n.d., not determined; PMB, polymyxin B.
[a]Possessing (+), deficient (−), or overexpression (++).

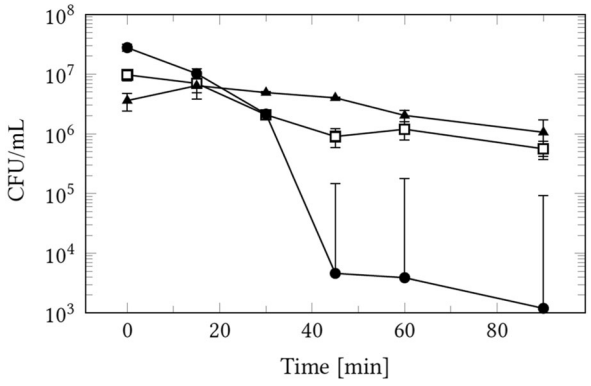

FIGURE 1 Time-dependent killing of *P. aeruginosa* K767, K1525, and K2415 by the AMP HHC-36. Mid-log phase bacterial cultures of *P. aeruginosa* mutants K1525 (closed circle), and K2415 (closed triangle) and their parental strain K767 (open square) were diluted in MH broth followed by the addition of AMP HHC-36 at 32 μg/mL. At the indicated time points, samples were serially diluted and plated on LB agar to determine CFUs. Experiments were performed in triplicate. The figure shows mean averages and SDs of one representative experiment ($n = 10$). CFU, colony forming unit; K767, PAO1 wild-type; K1525, Δ*mexXY*; K2415, *mexXY-oprM*-overexpressing.

To further examine this hypothesis, we monitored the time-dependent killing of *P. aeruginosa* WT, mutant K1525 (Δ*mexXY*), and *mexXY*-overexpressing strain K2415 in the presence of peptide HHC-36, which showed the strongest effect in our MIC analyses. The cell densities of bacterial cultures were adjusted to ~10^7 CFU/mL and HHC-36 was added at twofold MIC concentrations against WT (32 μg/mL) prior to the determination of CFUs after 0, 15, 30, 45, 60, and 90 min of incubation. The CFU counts for WT and K2415 still revealed cell numbers of 5.6×10^5 CFU/mL and 1.1×10^6 CFU/mL, respectively, after 90 min of incubation with HHC-36 (Figure 1). In contrast, at the end point of the experiment after 90 min, only 1.2×10^3 CFU/mL were detected in samples of efflux mutant K1525. Statistical significance of differences between CFU counts of K1525 and K767 as well as of K1525 and K2415 after 90 min was confirmed by a two-sided *t*-test for independent samples, resulting in p values < 0.001 in both cases.

In order to further verify these results, we amplified *mexXY* via PCR (primer sequences *mexXY*_forward: GAA CGTCCTCACAAGGGAAA, *mexXY*_reverse: GTGAACT

TABLE 4 Antimicrobial peptide susceptibilities in *E. coli* DH5-α strains (MICs in μg/mL).

Antibiotic/AMP	MIC [μg/mL]	
	E. coli* DH5α-pJET1.2::*mexXY	***E. coli* DH5α-pJET1.2-Control**
Gentamicin	0.125	0.06
HHC-36	0.5	0.125
IDR-1018	0.25	0.25
Apidaecin Ib	64	8
Magainin 2	4	4

GCTGTTGCCA GTC) and cloned these genes into expression vector pJET1.2 (Thermofisher Scientific) resulting in plasmid pJET1.2::*mexXY* (Table 1). Since *Escherichia coli* outer membrane protein TolC has been reported to adopt functions of *P. aeruginosa* OprM with respect to MexXY efflux pump functionality in *E. coli*[24] only the genes *mexX* and *mexY* were used for this cloning and heterologous expression in *E. coli*. Functionality of the heterologously expressed *mexXY* genes in *E. coli* was tested by MIC assays using gentamicin which is a known substrate of MexXY-OprM. The MIC value for gentamicin was consistantly two fold higher in *E. coli* pJET1.2::*mexXY* compared with the control strain *E. coli* pJET1.2-Control, which harbored a plasmid control. In subsequent experiments with cationic AMPs, heterologous *mexXY* expression decreased the susceptibility of *E. coli* to HHC-36, whereas the MIC of IDR-1018 was not affected (Table 4). These findings are in accordance with our results from the *P. aeruginosa* susceptibility tests, in which the MIC of HHC-36, but not of IDR-1018 was diminished in the MexXY-OprM knock-out mutant K1525. Moreover, the observation that the targeted expression of the *P. aeruginosa* MexXY efflux system increased bacterial resistance to HHC-36 even in *E. coli*, could suggest an active transport of HHC-36 by MexXY-OprM (or MexXY-TolC in the case of *E. coli*), however, further experiments are needed to confirm this activity.

Since we were not able to determine the MICs of natural peptides Apidaecin Ib[25] and Magainin 2[26] in *P. aeruginosa* due to the high intrinsic antimicrobial resistance to these agents (Table 3), we performed additional susceptibility tests with these peptides by using the more sensitive recombinant *E. coli* strains. For peptide ApidaecinIb, the MIC was eightfold higher in DH5α-pJET1.2::*mexXY* compared with the vector control strain (Table 4). In the case of Magainin 2, equal MICs of 4 μg/mL were determined for both *E. coli* strains.

Since MexXY-OprM expression has been shown to be induced by several of its substrates including the ribosome-targeting antibiotics gentamicin and erythromycin,[27] we analysed *mexX* gene expression in response to HHC-36 and IDR-1018 using qRT-PCR (primer sequences GAGTACAC CGAAGCGCAGAC and GGCTGGGAGAAGTTCACGTA) as described previously.[22] Cells were grown in MH broth to midlog phase followed by a 2 h incubation with 20 μg/mL HHC-36 and IDR-1018, respectively. The obtained c_t-values were normalized to the expression of the housekeeping gene *rpoD*. Samples were assayed three times in duplicate ($n \geq 6$). Untreated cultures served as controls. Quantification of relative gene expression levels by qRT-PCR revealed an upregulation of *mexX* gene expression by 3.9 ± 1.2 fold in the presence of HHC-36 in comparison with the untreated control. In contrast, *mexX* gene expression was not altered in response to IDR-1018 as indicated by the relative gene expression levels of 0.9 ± 0.2 compared with untreated cells.

In agreement with our results, previous work has indicated that MexAB-OprM is not involved in resistance to peptide antibiotics including polymyxin B and the human defense peptides LL-37, HBD-2, and HBD-3.[22,28,29] although it was shown that MexAB is involved in the development of phenotypic tolerance to colistin in *P. aeruginosa* biofilms.[30] Furthermore, it has been demonstrated that susceptibility of *P. aeruginosa* to LL-37 was independent of efflux pumps MexCD-OprJ, MexEF-OprN, MexXY-OprM, and MexGHI-OpmD.[22] However, our MIC and kill-curve data suggest that MexXY-OprM contributes to resistance against several short AMPs in *P. aeruginosa*. This is in alignment with a recent study by Puja and colleagues who demonstrated that MexXY-OprM contributes to resistance against the peptide antibiotic colistin in *P. aeruginosa*.[12] Interestingly, while this study found identical MICs against colistin for wildtype PAO1 and the corresponding MexXY-deficient mutant, kill-curve experiments clearly showed a much stronger effect of colistin on cells of the *mexXY* mutant strain compared with wild-type.[12] The authors suggested multiple reasons for how MexXY-OprM might be involved in colistin resistance. Among these were a connection of *mexXY* with genes associated with regulation and modification of LPS, and the export of LPS components or transport of other molecules to the bacterial surface which counteract the electrostatic binding of colistin. A link between LPS and MexXY has also been suggested previously.[31] In addition, Puja et al. suggested the possibility that colistin could be a direct substrate for MexXY-OprM.[12] Initially a previous study excluded polymyxin antibiotics as substrates for this efflux pump, however, this hypothesis was based mainly on MIC data and Puja et al. provided a more detailed study.[29]

To date, efflux-mediated resistance to antimicrobial peptides has been reported for several bacteria. In an early study, Shafer et al.[11] demonstrated that the *Neisseria gonorrhoeae* efflux system MtrCDE confers resistance to the cationic host defense peptides protegrin-1, LL-37, and tachyplesin-1, while Tzeng et al.[10] showed an involvement of MtrCDE in AMP resistance in *Neisseria meningitidis*. In *Yersinia enterocolitica* the temperature-sensitive inducible efflux pump and potassium antiporter system RosAB has been proposed to mediate adaptive resistance to polymyxin B by an active peptide export and, in addition, by an acidification of the cytoplasm.[32] In *Klebsiella pneumoniae* an enhanced susceptibility toward fluoroquinolone,

tetracycline, and aminoglycoside antibiotics as well as to human bronchoalveolar lavage fluid components HNP-1, HBD-1, and HBD-2 and to the bronchoalveolar lavage fluid itself was noticed upon knock-out of the inner membrane transporter protein AcrB of RND efflux system AcrAB-TolC.[33] In addition, this AcrAB-TolC knock-out was also correlated with a lowered resistance of *Klebsiella pneumoniae* against polymyxin B.[33]

In conclusion, our data indicate that the *P. aeruginosa* efflux pump system MexXY-OprM modulates susceptibility against synthetic and natural antimicrobial peptides including 1037, HHC-36, HHC-10, Bac2a, and Apidaecin Ib. Cationic AMPs represent one promising class of antibiotics effective against both Gram-positive and Gram-negative pathogens.[5] However, since our data demonstrate that MexXY-OprM is implicated in AMP resistance in *P. aeruginosa*, this characteristic should be considered in future developments of new and highly active antimicrobial peptides. In addition, more studies are necessary to obtain a better understanding of the molecular mechanism involved in efflux-mediated peptide resistance in *P. aeruginosa*.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

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