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Short Communication

High-level resistance to meropenem in clinical isolates of *Pseudomonas aeruginosa* in the absence of carbapenemases: role of active efflux and porin alterations ☆Hussein Chalhoub ^a, Yolanda Sáenz ^b, Hector Rodriguez-Villalobos ^c, Olivier Denis ^d, Barbara C. Kahl ^e, Paul M. Tulkens ^a, Françoise Van Bambeke ^{a,*}^a Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium^b Centro de Investigación Biomédica de La Rioja (CIBIR), Logroño, Spain^c Laboratoire de microbiologie, Cliniques universitaires Saint-Luc, Brussels, Belgium^d Laboratoire de microbiologie, Hôpital Erasme, Université libre de Bruxelles, Brussels, Belgium^e University Hospital Münster, Münster, Germany

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ABSTRACT

High-level carbapenem resistance is worryingly increasing in clinical isolates and is often attributed to carbapenemase expression. This study aimed to determine the mechanisms leading to high-level meropenem resistance in six carbapenemase-negative *Pseudomonas aeruginosa* isolated from cystic fibrosis (CF) patients and seven carbapenemase-positive isolates from patients suffering from hospital-acquired pneumonia (HAP). MICs were determined in the absence or presence of L-arginine or glycine-glutamate as competitive substrates for OprD (OccD1) or OpdP (OccD3), respectively, or the efflux pump inhibitor Phe-Arg β-naphthylamide (PAβN). β-Lactamases were screened by phenotypic tests and/or PCR. The *oprD* gene and its promoter were sequenced; protein expression was evidenced by SDS-PAGE. *mexA*, *mexX*, *mexC* and *mexE* transcripts were evaluated by real-time and semiquantitative PCR. Meropenem/imipenem MICs were 64–128/16–32 mg/L and 128/128–256 mg/L in CF and HAP isolates, respectively; PAβN reduced meropenem MICs to 4–16 mg/L only and specifically in CF isolates; porin competitors had no effect on MICs. All isolates showed an increase in transcription levels of *mexA*, *mexX* and/or *mexC* and mutations in *oprD* leading to production of truncated proteins. AmpC-type cephalosporinases were overexpressed in CF isolates and VIM-2 was expressed in HAP isolates. Antibiotic exclusion from bacteria by concomitant efflux and reduced uptake is sufficient to confer high-level resistance to meropenem in isolates overexpressing AmpC-type cephalosporinases. As efflux is preponderant in these isolates, it confers a paradoxical phenotype where meropenem is less active than imipenem. Concomitant susceptibility testing of both carbapenems and rapid elucidation of the most probable resistance mechanisms is thus warranted.

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1. Introduction

Meropenem is widely used in the treatment of pulmonary exacerbations in cystic fibrosis (CF) patients infected by multidrug-resistant (MDR) *Pseudomonas aeruginosa*. Its consumption has promoted the emergence of high-level resistance, often ascribed to

expression of carbapenemases in hospital-acquired infections [1]. Upon screening of a collection of 333 *P. aeruginosa* isolates from CF patients, we observed meropenem minimum inhibitory concentrations (MICs) of ≥64 mg/L in six isolates that did not express carbapenemases. This study aimed to determine the mechanism(s) leading to this high-level meropenem resistance.

2. Materials and methods

2.1. Strains

A total of 13 carbapenem-resistant *P. aeruginosa* isolates were collected from two CF patients ($n = 6$ isolates) [2] and three patients ($n = 7$ isolates) suffering from hospital-acquired pneumonia (HAP) [3]. *Pseudomonas aeruginosa* PAO1 and derivatives thereof with overexpression

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or deletion of genes encoding efflux pumps or OprD were also studied (Table 1). Clonal relatedness was evaluated by pulsed-field gel electrophoresis (PFGE) following published recommendations [7].

2.2. Susceptibility testing

MICs were determined by broth microdilution following Clinical and Laboratory Standards Institute (CLSI) recommendations in cation-adjusted Mueller–Hinton broth (CA-MHB) (Becton Dickinson & Co., Franklin Lakes, NJ), in controlled conditions or in the presence of (i) 20 mg/L of the efflux pump inhibitor Phe–Arg β -naphthylamide (PA β N) combined with 1 mM MgSO₄ to strengthen the outer membrane, (ii) 10 mM L-arginine (L-Arg) (OprD [OccD1] substrate [8]) or (iii) 10 mM glycine–glutamate (Gly–Glu) (OprD [OccD3] substrate [8,9]). PA β N and porin substrates were used at the highest non-toxic concentration (no effect on bacterial growth). The CA-MHB was adjusted to pH 7.4 after addition of porin substrates. Susceptibility was categorised according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive criteria (http://www.euca.org/clinical_breakpoints/; accessed May 2016).

2.3. β -Lactamase screening and identification

Phenotypic screening for metallo- β -lactamases was performed by the double-disk synergy test (imipenem–0.5 M ethylene diamine

tetra-acetic acid (EDTA) pH 8–meropenem) [10]. The carbapenemase Nordmann–Poirel (Carba NP) and extended-spectrum β -lactamase (ESBL) NDP (Nordmann/Dortet/Poirel) tests, primarily developed for Enterobacteriaceae, were validated for *P. aeruginosa* using appropriate control strains and were used at first as a phenotypic screening test for carbapenemase(s) or for overproduction of AmpC-type cephalosporinases (not inhibited by clavulanic acid), respectively [4,5].

Detection of *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M} (groups 1, 2 and 9), *bla*_{VIM}, *bla*_{IMP}, *bla*_{KPC} and *bla*_{NDM} gene families was performed by real-time multiplex PCR using group-specific primers. Other genes encoding OXA (1, 2, 9, 10, 18, 20, 23, 24, 30, 48, 58 and 198), BEL (1–3), PER (1–5 and 7), GES (1–18) and VEB (1–7) enzymes were also detected by multiplex PCR (see [6,11] and the references cited therein).

2.4. Efflux pump expression

Strains were grown to late log phase [optical density at 620 nm (OD₆₂₀) = 1] in Luria–Bertani broth (Becton Dickinson & Co.) at 37 °C under agitation (130 rpm) and were harvested by centrifugation. Total RNA was isolated using an RNeasy Mini Kit (Stratag Molecular GmbH, Berlin, Germany) with residual DNA removed by RNase-Free DNase (Thermo Fisher Scientific, Waltham, MA). The absence of contaminating DNA was checked by PCR reactions for *rpsL* using RNA samples as a template. cDNA was obtained by reverse transcription of purified RNA and was used to evaluate the expression

Table 1

Minimum inhibitory concentrations (MICs) and gene expression levels in reference strains and in cystic fibrosis (CF) and hospital-acquired pneumonia (HAP) clinical isolates of *Pseudomonas aeruginosa*.

Reference strains and clinical isolates (with patient's ID code and date of collection)	β -Lactamases ^a	MIC (mg/L) ^b								Relative expression of genes encoding efflux pumps ^d		
		Meropenem				Imipenem				<i>mexA</i>	<i>mexX</i>	<i>mexC</i>
		Control	+10 mM L-Arg	+10 mM Gly-Glu	+20 mg/L PA β N ^c	Control	+10 mM L-Arg	+10 mM Gly-Glu	+20 mg/L PA β N ^c			
PAO1 wild-type	–	1	4	2	0.5	2	4	4	2	1.0	1.0	1.0
PAO1 Δ <i>oprD</i>	–	4	4	4	1–2	8	8	8	8	1.7	0.9	0.4
PAO1 Δ <i>mexAB::FRT</i>	–	0.25	ND	ND	0.064	2	ND	ND	ND	1.0	0.5	0.6
PAO1 Δ <i>mexAB-oprM, mexXY, mexEF-oprN, mexCD-oprJ, mexJK</i>	–	0.12	ND	ND	Toxic	1	ND	ND	0.5	0.07	2.1	0.0
PAO1 Δ <i>mexXY</i>	–	1	ND	ND	0.5	2	ND	ND	ND	1.9	1.0	2.0
PAO1 <i>MexAB–OprM</i> overproducer	–	4	ND	ND	1–2	2	ND	ND	ND	9.3	1.3	0.7
PAO1 <i>MexXY–OprM</i> overproducer	–	1	ND	ND	0.5	2	ND	ND	ND	1.5	7.7	2.0
PAO1 <i>MexEF–OprM</i> overproducer	–	1	ND	ND	0.5	2	ND	ND	ND	1.0	0.2	0.8
PAO1 <i>MexCD–OprM</i> overproducer	–	1	ND	ND	0.5	2	ND	ND	ND	1.2	0.4	7.5
128-CF (DAF69, 09/09/2010) ^e	AmpC-type	128	128	128	16	32	32	32	32	2.2	0.8	0.0
180-CF (DAF69, 04/10/2010) ^e	AmpC-type	64	64	64	16	16	16	16	16	3.7	1.7	2.3
238-CF (DAF69, 19/10/2010) ^e	AmpC-type	64	64	64	4	16	16	16	16	2.3	1.2	0.2
254-CF (DAF69, 26/10/2010) ^e	AmpC-type	64	64	64	8	16	16	16	16	14.3	3.1	2.7
306-CF (DAF69, 09/11/2010) ^e	AmpC-type	64	64	64	16	16	16	16	16	6.9	2.4	1.7
132-3-CF (132, 08/07/2012) ^f	AmpC-type	64	64	64	16	32	16	16	32	0.3	3.5	0.7
228-HAP (DS, 26/12/2005) ^e	VIM-2	128	128	128	128	128	128	128	128	2.3	1.0	1.0
218-HAP (DS, 26/01/2006) ^e	VIM-2	128	128	128	128	128	128	128	128	4.3	2.8	1.4
222-HAP (DS, 13/02/2006) ^e	VIM-2	128	128	128	128	128	128	128	128	6.5	4.3	13.6
240-HAP (OG, 08/04/2006) ^e	VIM-2	128	128	128	128	256	256	256	256	6.4	9.6	5.4
241-HAP (OG, 02/05/2006) ^e	VIM-2	128	128	128	128	256	256	256	256	2.8	1.6	0.9
258-HAP (ND, 10/08/2006) ^e	VIM-2	128	128	128	128	256	256	256	256	3.4	7.5	1.2
252-HAP (ND, 11/09/2006) ^e	VIM-2	128	128	128	128	256	256	256	256	3.7	4.8	1.3

L-Arg, L-arginine; Gly-Glu, glycine–glutamate; PA β N, Phe–Arg β -naphthylamide; ND, not determined.

^a AmpC-type overexpression (chromosomal and/or plasmid-mediated): phenotypic detection [4] using the extended-spectrum β -lactamase (ESBL) NDP (Nordmann/Dortet/Poirel) test compared with the wild-type strain PAO1 producing AmpC at very low basal levels; VIM-2: phenotypic detection [5] for carbapenemase(s) expression using the Carba NP test, with genotypic detection [6] for the *bla*_{VIM-2} gene (multiplex PCR).

^b European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (mg/L): meropenem, susceptible (S), ≤ 2 , resistant (R), >8 ; imipenem, S, ≤ 4 , R, >8 .

^c No toxicity detected at this concentration.

^d Mean values of transcripts levels obtained in three independent experiments performed in duplicate were considered. Increased gene expression compared with PAO1 (expression level set at 1.0) of ≥ 2 was considered significant and is shown in italic bold characters. Student's *t*-test and analysis of variance (ANOVA): $P < 0.05$ (*mexA*); $P < 0.01$ (*mexX* and *mexC*). PAO1 mutants overexpressing efflux pumps were used as controls.

^e From Erasme Hospital (Brussels, Belgium).

^f From University Hospital Münster (Münster, Germany).

of *mexE* by semiquantitative PCR and that of *mexA*, *mexX* and *mexC* by real-time PCR, with *rpsL* as a housekeeping gene and using the primers and conditions described in [Supplementary Appendix S1](#).

2.5. Porin expression and mutations

Outer membrane proteins were isolated and visualised by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) after Coomassie Brilliant Blue (Bio-Rad Laboratories, Hercules, CA) staining. Mutations in *oprD* and its promoter region were identified by PCR amplification and sequencing using the previously described primers and conditions, with the wild-type strain *P. aeruginosa* PAO1 used as a comparator [12].

3. Results

PFGE showed two different genotypes among isolates from CF patients ([Supplementary Fig. S1](#)). The same pulsotype was observed among all HAP isolates (three distinct patients over a 1-year period), corresponding to an epidemic clone in Belgian hospitals (see [13] and references cited therein). All isolates were multi-drug resistant (MDR; EUCAST criteria; [Supplementary Table S1](#)). They showed elevated MICs to meropenem (64–128 mg/L and 128 mg/L in CF and HAP isolates, respectively) and to imipenem, which were however less elevated in CF isolates (MICs of 16–32 mg/L) ([Table 1](#)).

Phenotypic and molecular screening tests for the production of carbapenem-hydrolysing enzymes in CF isolates showed neither carbapenemases nor ESBLs but overexpression of AmpC-type cephalosporinases (possibly associated with non-identified ESBLs, evidenced by the NDP test). In contrast, all HAP isolates were VIM-2 producers (known to increase MICs to 16 mg/L and >32 mg/L for meropenem and imipenem, respectively [12]). SDS-PAGE analysis showed no expression of the OprD porin ([Supplementary Fig. S2](#)). Sequencing showed no mutation in the promoter region of the gene, but several in the coding region ([Table 2](#)).

Ten isolates [the six CF isolates and four isolates (240, 241, 258 and 252) from two different HAP patients] showed nucleotide deletions and missense/nonsense mutations leading to a truncated (most probably inactive) porin of 117–295 amino acids (compared with 443 amino acids in the wild-type porin). Among all CF and HAP isolates, the different amino acid substitutions found in loops L1 (D43N, S57E and S59R), L2 (V127L), L3 (E185Q, P186G and

V189T), L4 (E202Q, I210A and E230K), L5 (S240T, N262T and T276A), L6 (A281G, K296Q, Q301E, R310E, G312R and A315G), L7 (L347M) and L8 (S403A and Q424E) were previously described both in carbapenem-resistant and -susceptible *P. aeruginosa* strains [15]. More specifically, the long loops L2 and L3 are important for the binding and/or entrance of imipenem [16]. However, a specific impact of loop-affecting mutations on imipenem activity could not be evidenced here, presumably due to the absence of expression of the truncated porin, the presence of other mutations, the concomitant expression of carbapenemases (HAP isolates only) and/or other carbapenem resistance mechanisms. Mutations tended to accumulate over time in isogenic isolates collected successively from the DAF69 CF patient (namely isolates 128 and 180 vs. isolates 238, 254 and 306).

The three last isolates (228, 218 and 222, originating from the same HAP patient) had a set of 20 amino acid substitutions and a deletion of two amino acid residues in loop 7 (L7-short) leading to the formation of a slightly shortened protein (441 amino acids). Truncation of L7 has been previously reported to confer hypersusceptibility to meropenem without affecting imipenem activity [14] but, again, this effect could not be evidenced here due to the concomitant expression of VIM-2 in these isolates. One new mutation was detected in loop L8 (R412P) of these three HAP clonal isolates, the impact of which on carbapenem susceptibility remains to be established.

[Table 1](#) shows the influence of porin expression on resistance to carbapenems (comparing the MICs of *P. aeruginosa* PAO1, PAO1 Δ *oprD* mutant and the clinical isolates). MICs were determined in control conditions and in the presence of L-Arg or Gly-Glu, considering that L-Arg is a competitor for OprD [8] and Gly-Glu is a competitor for OpdP (another member of the OccD porin family recently described as playing a minor role in carbapenem uptake by *P. aeruginosa* [9]). Deletion of OprD in PAO1 caused a 3 log₂ dilution increase in the MIC of both carbapenems. In *P. aeruginosa* PAO1, the MICs of carbapenems increased 2–4-fold and 2-fold in the presence of L-Arg and Gly-Glu, respectively. However, no effect was observed in PAO1 Δ *oprD* mutant and in clinical isolates. Thus, the alterations in porin expression/permeability observed could not suffice to explain the high-level resistance in CF isolates.

We therefore investigated the expression of efflux pumps in clinical isolates and their impact on carbapenem activity ([Table 1](#)). All but one isolate (CF isolate 132-3) showed a significant increase in

Table 2
Molecular characterisation of OprD protein in cystic fibrosis (CF) and hospital-acquired pneumonia (HAP) clinical isolates of *Pseudomonas aeruginosa*.

Strain origin	Identifier (PFGE pattern) ^a	Carbapenem susceptibility/resistance		OprD porin			
		Meropenem	Imipenem	No. of amino acids	Amino acid changes in protein sequence ^b	Loops affected	Insertion/deletion in <i>oprD</i> gene
Reference	PAO1 wild-type	S	S	443	—	—	—
Reference	PAO1 Δ <i>oprD</i>	S	R	0	—	—	—
CF	128 (P1a), 180 (P1b)	R	R	117	D43N, S57E, S59R, D118STOP ^c	L1	Deletion of 2 nt (312–313)
CF	238 (P1b), 254 (P1a), 306 (P1a)	R	R	295	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296STOP ^c	L1, L4, L5, L6	
CF	132-3 (P2)	R	R	228	D43N, S57E, S59R ^c	L1	Deletion of 1 nt (410)
HAP	228 (P3a), 218 (P3a), 222 (P3a)	R	R	441	V127L, E185Q, P186G, V189T, E202Q, I210A, E230K, S240T, N262T, T276A, A281G, K296Q, Q301E, R310E, G312R, A315G, L347M, S403A, R412P, Q424E	L2, L3, L4, L5, L6, L7, L8	Loop 7-short ^d
HAP	240 (P3b), 241 (P3b), 258 (P3b), 252 (P3b)	R	R	276	V127L, E185Q, P186G, V189T, E202Q, I210A, E230K, S240T, N262T, T276A, W277STOP ^c	L2, L3, L4, L5	

PFGE, pulsed-field gel electrophoresis; S, susceptible; R, resistant; nt, nucleotide(s).

^a See [Supplementary Fig. S1](#).

^b Substitutions were determined by comparison with the sequence of PAO1 (GenBank accession no. [AE004091](#)) that encodes a protein of 443 amino acids.

^c Premature stop codon.

^d Shortening of putative loop L7 previously reported [14].

the transcripts of *mexA*, and eight of them showed an increase for *mexX* compared with *P. aeruginosa* PAO1. Two CF and two HAP isolates also showed an increase in *mexC* expression levels, but none of the isolates expressed the *mexE* gene (Supplementary Fig. S3).

To assess the involvement of specific pumps in carbapenem efflux, MICs for derivatives of *P. aeruginosa* PAO1 with overexpression or deletion of genes encoding each of the putative candidates were determined. Overexpression of MexAB–OprM caused a 2 log₂ dilution increase in the meropenem MIC, whilst overexpression of each of the other pumps did not affect the MICs. Conversely, disruption of *mexAB* only or of the genes encoding five efflux pumps (*mexAB–oprM*, *mexXY*, *mexEF–oprN*, *mexCD–oprJ* and *mexJK*) reduced the meropenem MIC by 2 log₂ or 3 log₂ dilutions, respectively, designating MexAB–OprM as the main transporter for meropenem [12,17,18]. In contrast, the impact of efflux on imipenem activity was limited (only 1 log₂ dilution reduction in MIC for the mutant deleted for five efflux pumps). Table 1 also shows that the efflux pump inhibitor PAβN reduced the meropenem MICs by 2–4 log₂ dilutions in CF isolates but not in HAP isolates, emphasising a role for this mechanism of resistance in CF isolates. However, reversion of resistance was not complete upon addition of the efflux inhibitor, possibly due to incomplete inhibition of efflux at the concentration used and/or to the co-existence of other resistance mechanisms. PAβN did not modify the MIC of imipenem against clinical isolates.

4. Discussion

This study demonstrates that interplay between MexAB–OprM overexpression and OprD inactivation, combined with overexpression of AmpC-type cephalosporinases, is sufficient to confer high-level resistance to meropenem (MIC ≥ 64 mg/L) in *P. aeruginosa* from CF patients, in contrast to other studies showing an increase of meropenem MICs to ≤32 mg/L under similar conditions [17].

A key role of MexAB–OprM overexpression or chromosomal mutations (leading to both AmpC derepression and membrane impermeability) in high-level carbapenem resistance has already been described in CF isolates [18]. In line with previous work [19], the current data suggest that MexAB–OprM overexpression associated with OprD inactivation is probably sufficient to increase the meropenem MIC to ≥32 mg/L in CF isolates. Contrary to these authors, however, we found that imipenem was more active than meropenem against CF isolates, which is unusual [17] but has been described previously [15] for strains that do not express OprD. As meropenem MICs are brought back below those of imipenem in the presence of PAβN, we suggest that active efflux is the main reason for high-level resistance to meropenem in CF isolates. Building on earlier studies [19], the current work also identifies specific *oprD* mutations, including one that had not been previously described, and shows that these mutations can accumulate over the course of a chronic infection in isogenic isolates from the same patient, explaining an increase in resistance. This, moreover, underlines the change in expression of porins as a contributing factor for carbapenem resistance in Gram-negative bacteria [20].

These results confirm that high-level resistance to carbapenems in *P. aeruginosa* cannot be blindly ascribed to carbapenemase production, in particular among CF isolates in which active efflux appears to play an underestimated role. Concomitant susceptibility testing of both meropenem and imipenem is warranted and appropriate phenotypic tests may help in delineating the most probable mechanisms involved in carbapenem resistance.

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Ethical approval: Not required.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2016.09.012.

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Supplementary material**High-level resistance to meropenem in clinical isolates of *Pseudomonas aeruginosa* in the absence of carbapenemase: role of active efflux and porin alterations**

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Figure S1: Pulsed-field gel electrophoresis (PFGE) of *SpeI*-digested genomic DNA from *P. aeruginosa* isolates. P1-P3 correspond to different PFGE patterns; P1a and P1b as well as P3a and P3b are closely related.

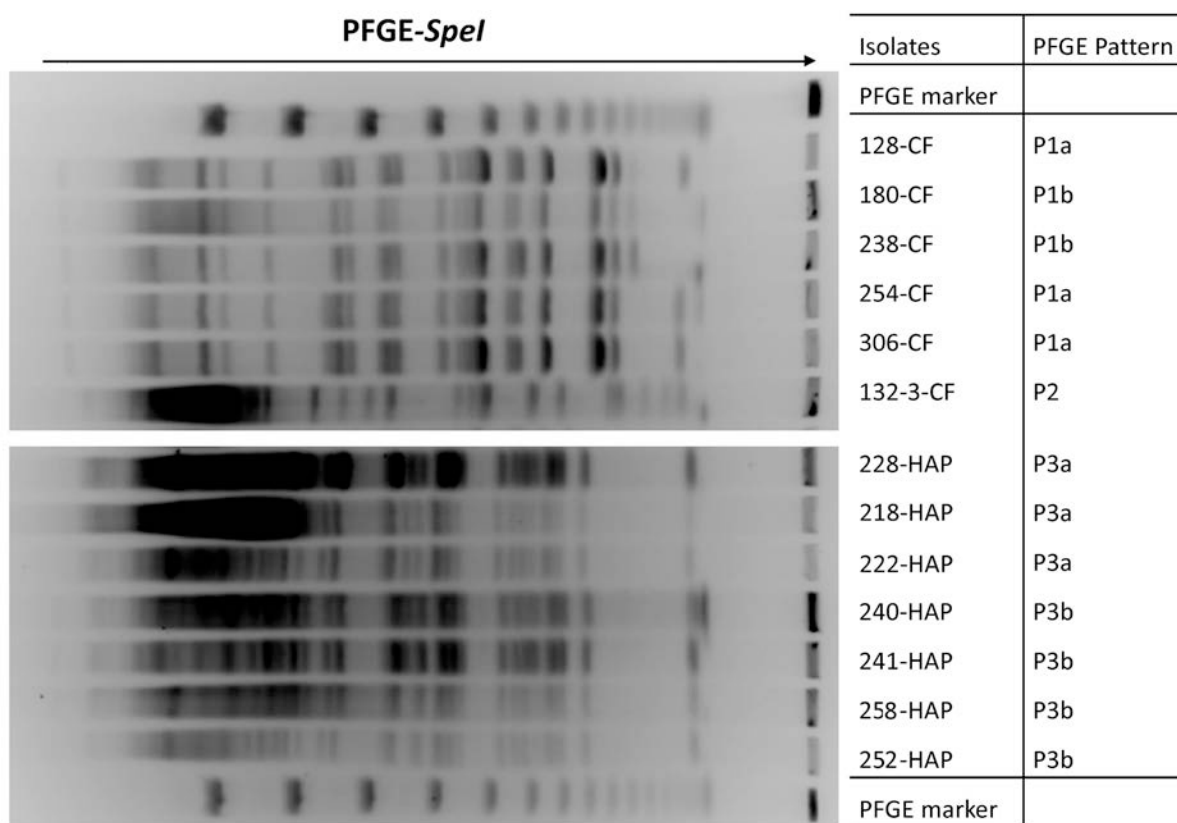


Figure S2: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie brilliant blue staining of the outer membrane proteins isolated from *P. aeruginosa* CF and HAP isolates. The WT strain PAO1 and its OprD mutant were included as positive and negative control strains. Gel1: CF-isolates; loaded as follows: lane1: protein Ladder; lane 2: PAO1; lane 3: PAO1- Δ OprD; lane 4: 128-CF; lane 5: 180-CF; lane 6: 238-CF; lane 7: 254-CF; lane 8: 306-CF; lane 9: 132-3-CF. Gel 2: HAP-isolates; loaded as follows: lane1: protein Ladder; lane 2: PAO1; lane 3: PAO1- Δ OprD; lane 4: 228-HAP; lane 5: 218-HAP; lane 6: 222-HAP; lane 7: 240-HAP; lane 8: 241-HAP; lane 9: 258-HAP; lane 10: 252-HAP.

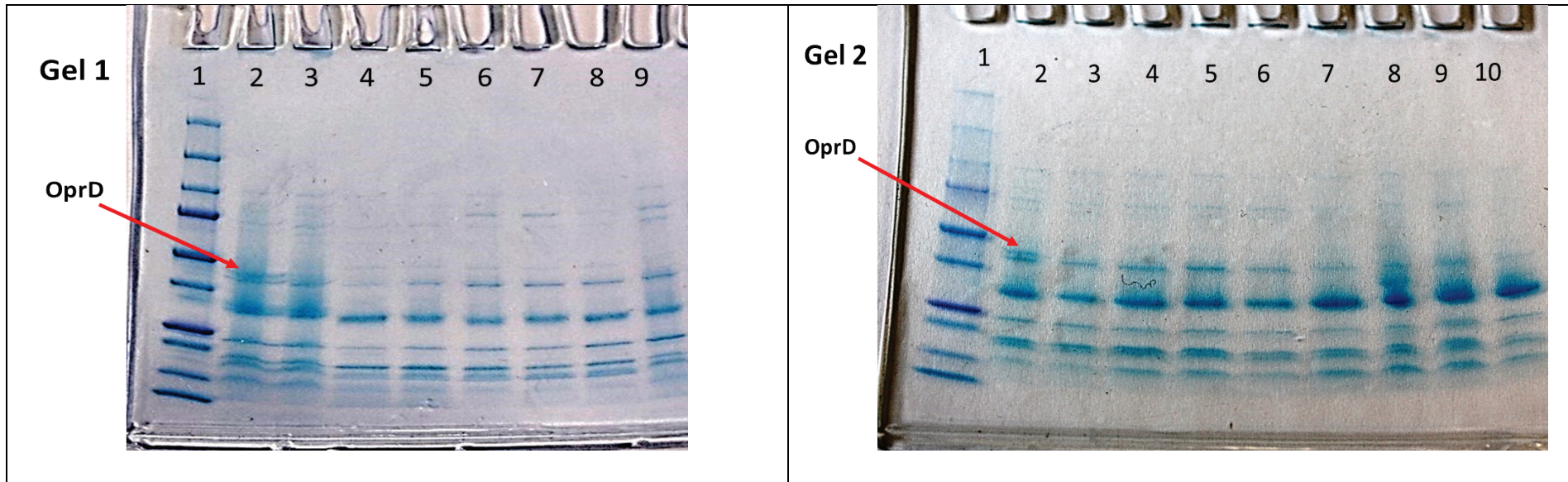
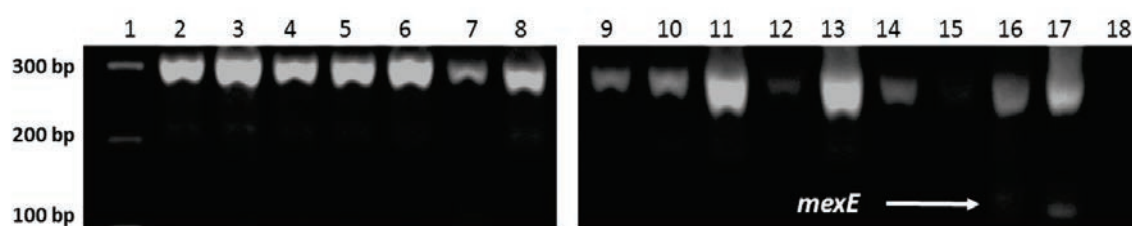


Figure S3: Detection of *mexE* expression by semi-quantitative RT-PCR. The figure shows the DNA-stained gels of amplified RT-PCR for CF and HAP isolates. The inducible target gene (*mexE*, 137 bp) is amplified together with the constitutive *mexA* gene (316 bp) used as internal positive control. Samples are loaded as follow: lane1: DNA ladder ; lane 2: PAO1 (negative control non-expressing *mexE* gene); lane 3: 128-CF; lane 4: 180-CF; lane 5: 238-CF; lane 6: 254-CF; lane 7: 306-CF; lane 8: 132-3-CF; lane 9: 228-HAP; lane 10: 218-HAP; lane 11: 222-HAP; lane 12: 240-HAP; lane 13: 241-HAP; lane 14: 258-HAP; lane 15: 252-HAP; lane 16: positive control with strain overexpressing *mexE* gene; lane 17: DNA template (positive control); lane 18: non template control.



Supplementary Table S1

Minimum inhibitory concentrations (MICs) of strains under study to other antibiotic classes

Reference strains and clinical isolates (with patient ID and date of collection)	MIC (mg/L) ^a							
	TIC	PIP	TZP	CAZ	CIP	AMK	TOB	CST
PAO1 Wild-type	32	4	4	2	0.5	4	1	4
PAO1 $\Delta oprD2$	32	4	4	2	0.25	4	1	2
PAO1 $\Delta mexAB::FRT$	2	0.5	0.5	1	0.12	1	1	4
PAO1 $\Delta mexAB-oprM, mexXY, mexEF-oprN, mexCD-oprJ, mexJK$	2	0.5	0.5	1	0.12	1	0.5	2
PAO1 $\Delta mexXY$	8	2	2	1	1	2	1	2
PAO1 MexAB-OprM overproducer	128	8	16	4	0.5	4	0.5	2
PAO1 MexXY-OprM overproducer	32	2	2	2	1	16	2	2
PAO1 MexEF-OprM overproducer	32	0.5	2	1	2	2	0.5	1
PAO1 MexCD-OprM overproducer	32	2	4	4	0.5	4	1	2
128-CF (DAF69, 09/09/2010) ^b	1024	1024	1024	1024	8	64	4	1
180-CF (DAF69, 04/10/2010) ^b	1024	1024	1024	1024	4	64	4	0.5
238-CF (DAF69, 19/10/2010) ^b	1024	1024	1024	1024	16	256	16	2
254-CF (DAF69, 26/10/2010) ^b	512	1024	1024	256	16	256	16	2
306-CF (DAF69, 09/11/2010) ^b	1024	1024	1024	512	16	64	4	2
132-3-CF (132, 08/07/2012) ^c	128	64	32	16	2	64	4	2
228-HAP (DS, 26/12/2005) ^b	1024	64	64	64	32	128	32	1
218-HAP (DS, 26/01/2006) ^b	1024	256	256	128	32	128	32	1
222-HAP (DS, 13/02/2006) ^b	1024	256	256	128	32	128	32	1
240-HAP (OG, 08/04/2006) ^b	1024	64	64	64	32	128	32	1
241-HAP (OG, 02/05/2006) ^b	1024	256	256	128	32	128	32	1
258-HAP (ND, 10/08/2006) ^b	1024	128	128	128	32	128	32	1
252-HAP (ND, 11/09/2006) ^b	1024	128	128	128	32	128	32	1

TIC, ticarcillin; PIP, piperacillin; TZP, piperacillin/tazobactam; CAZ, ceftazidime; CIP, ciprofloxacin; AMK, amikacin; TOB, tobramycin; CST, colistin; NA, not applicable (no I category).

^a European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints TIC, susceptible (S), ≤ 16 , resistant (R), > 16 ; PIP, S, ≤ 16 , R, > 16 ; TZP, S, ≤ 16 , R, > 16 ; CAZ, S, ≤ 8 , R, > 8 ; CIP, S, ≤ 0.5 , R, > 1 ; AMK, S, ≤ 8 , R, > 16 ; TOB, S, ≤ 4 , R, > 4 ; CST, S, ≤ 4 , R, > 4 .

^b From Erasme Hospital (Brussels, Belgium).

^c From University Hospital Münster (Münster, Germany).

Supplementary material

Primers and conditions used for semiquantitative and real-time PCR

a) Primers

1. Efflux systems

- *mexE*-Fwd: 5'-CGACAACGCCAAGGGCGAGTTCACC-3'
- *mexE*-Rev: 5'-CCAGGACCAGCACGAACTTCTTGC-3'
- *mexA*-Fwd: 5'-CGACCAGGCCGTGAGCAAGCAGC-3'
- *mexA*-Rev: 5'-GCGACAACGCGGCCGAAGGTCTCC-3'
- *mexX*-Fwd: 5'-TGAAGGCGGCCCTGGACATCAGC-3'
- *mexX*-Rev: 5'-CGCTGACCCGCGTCGAGCAGATC-3'
- *mexC*-Fwd: 5'-GTACCGGCGTCATGCAGGGTTC-3'
- *mexC*-Rev: 5'-TTACTGTTGCGGCGCAGGTGACT-3'

2. Housekeeping genes

- *rpsL*-Fwd: 5'-CGGCACTGCGTAAGGTATGC-3'
- *rpsL*-Rev: 5'-AGCAGGGTCGTTCGAAGTACG-3'

b) PCR conditions

Three min of cDNA denaturation at 95 °C, followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. A melt curve was run at the end of the real-time PCR cycles to check for the presence of a unique PCR reaction product.