World J Microbiol Biotechnol (2014) 30:2681–2687
DOI 10.1007/s11274-014-1692-2

ORIGINAL PAPER

The effects of active efflux pumps on antibiotic resistance in *Pseudomonas aeruginosa*

Huseyin Agah Terzi · Canan Kulah · İhsan Hakkı Ciftci

Received: 20 April 2014 / Accepted: 18 June 2014 / Published online: 26 June 2014

Abstract In this study, we investigated the roles of active efflux pumps in antibiotic resistance. The transcription efflux pump genes were analyzed by real-time polymerase chain reaction (qPCR) to determine their role in drug resistance. Antibiotic sensitivity testing was carried out using the Vitek 2 automated system (bioMérieux, France). Isolates were divided into four groups according to their resistance status: multiple-drug resistant (MDR), isolated carbapenem resistant (ICR), isolated quinolone resistant (IQR), and carbapenem and quinolone resistant (CQR). Transcript levels of *mexB*, *mexD*, *mexF*, *and mexY* were analyzed by qPCR using a LightCycler instrument (Roche, Germany). The genetic similarity between isolates was determined using arbitrarily primed PCR (AP-PCR). Among the 50 isolates investigated, the frequency of genes classified as overexpressed were 88 % for *mexD*, 76 % for *mexB*, 46 % for *mexF*, and 40 % for *mexY*. Within the MDR group, *mexB* was overexpressed in 15 of 22 isolates, *mexD* in 20 of 22, *mexF* in 15 of 22, and *mexY* in 19 of 22. In the ICR group, isolates *mexB* and *mexD* were each overexpressed in five isolates. *mexD* overexpression was observed in all seven CQR isolates. Within the IQR group, *mexB* and *mexD* were overexpressed in all 12 isolates. *mexF* overexpression was detected in 7 of 12 isolates in this group. 18 distinct banding patterns were determined by AP-PCR. Increased transcription of *mexB* was directly correlated with meropenem resistance in the majority of isolates tested, while MexCD-OprJ and MexEF-OprN were related to quinolone resistance; the MexCD-OprJ efflux pump was also related to multidrug resistance. Increased transcription of *mexY* may contribute to the gentamicin resistance.

Keywords *Pseudomonas aeruginosa* · Resistance mechanism · Efflux pumps · qPCR · AP-PCR

H. A. Terzi (✉) · İ. H. Ciftci
Department of Medical Microbiology, Sakarya University Training and Research Hospital, Sakarya, Turkey
e-mail: agah.terzi@yahoo.com

C. Kulah
Department of Medical Microbiology, Bülent Ecevit University, School of Medicine, Zonguldak, Turkey

Introduction

The rise of antibiotic resistance is an increasingly important threat, particularly for infections caused by *Pseudomonas aeruginosa*. One of the primary mechanisms driving this resistance is the overexpression of efflux pump systems, which enable resistance to a wide range of drugs with different constitutional features. (Strateva and Yordanov 2009)

The RND family of efflux pumps, including MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, represent an important set of efflux systems in *P. aeruginosa*, with a broad range of drug specificities. MexAB-OprM was the first efflux pump found to target multiple classes of drugs, including fluoroquinolones, tetracyclines, chloramphenicol, β-lactams and β-lactamase inhibitors, macrolides, novobiocin, trimethoprim, and sulfonamides. (Kohler et al. 1996; Li et al. 1995, 1998). MexCD-OprJ exhibits a high degree of sequence similarity to MexAB-OprM, and has also been shown to extrude a variety of antimicrobial agents, including fluoroquinolones, β-lactams, chloramphenicol, tetracycline, novobiocin, trimethoprim, and macrolides (Poole et al. 1996). Other efflux pumps within this family exhibit more narrow spectra of activity; β-lactams are poor substrates for MexCD-OprJ

and MexEF-OprN, though MexEF-OprN is able to export fluoroquinolones, chloramphenicol, and trimethoprim. (Kohler et al. 1997). MexXY-OprM displays properties similar to those of other efflux systems, with a substrate profile that includes fluoroquinolones, specific ß-lactams, aminoglycosides, tetracycline, chloramphenicol, and erythromycin (Poole 2002; Schweizer 2003). The contributions of the remaining RND efflux pumps to resistance, and the mechanisms governing their expression, have yet to be fully elucidated (Lister et al. 2009).

In this study, we investigated the roles of active efflux pumps in antibiotic resistance. The transcription efflux pump genes *mexB*, *mexD*, *mexF*, *mexY*, were analyzed by real-time polymerase chain reaction to determine their role in drug resistance.

Materials and methods

Bacterial isolates and antibiotic sensitivity testing

Fifty *P. aeruginosa* clinical isolates were collected from clinical samples routinely sent to our laboratory between November 2010 and November 2011. Species identification was performed using conventional methods. Antibiotic sensitivity testing was carried out using the Vitek 2 automated system (bioMérieux, France). Isolates were divided into four groups according to their resistance status: multiple-drug resistant (MDR), isolated carbapenem resistant (ICR), isolated quinolone resistant (IQR), and carbapenem and quinolone resistant (CQR).

Minimum inhibitory concentration (MIC) testing

Minimum inhibitory concentrations of ceftazidime, gentamicin, piperacillin, ciprofloxacin, imipenem and meropenem were determined using the Vitek 2 system. The DensiCheck 2 system (bioMérieux, France) was used to calibrate the turbidity of samples to 0.5 McFarland standard. MIC values of ≥32 μg/ml for ceftazidime, ≥16 μg/ml for gentamicin, ≥128 μg/ml for piperacillin, ≥4 μg/ml for ciprofloxacin, ≥16 μg/ml for imipenem and ≥16 μg/ml for meropenem were defined as resistant, based upon Vitek 2 automated system.

Quantitative real-time PCR

Transcript levels of *mexB*, *mexD*, *mexF*, and *mexY* were analyzed by real-time polymerase chain reaction (qPCR) using a LightCycler instrument (Roche Diagnostics, Germany). Total RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics, Germany), and converted into cDNA using the Transcriptor High-fidelity cDNA Synthesis Kit (Roche Diagnostics, Germany) for qPCR. The quality and purity of the RNA obtained was evaluated spectrophotometrically (Maestrogen Nanodrop, USA). As a result of evaluation, required volume was calculated for 100 ng cDNA. Quantitative PCR was performed in capillary glass using a LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Germany), with primers specific for *mexB*, *mexD*, *mexF*, *mexY*, and *rpsL* (Table 1).

Control cDNA was obtained from *P. aeruginosa* strain PAO1. Amplification was carried out in triplicate using three cDNA samples for all isolates under the following conditions: initial denaturation for 10 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 20 s, annealing at 68 °C for 10 s, and elongation at 72 °C for 15 s. A final melting curve analysis was performed using a single read at 90 °C.

Arbitrarily primed PCR (AP-PCR)

To evaluate the similarities among strains, AP-PCR was performed using an M13 primer with the sequence 5′-GAGGGTGGCGGTTCT-3′. PCR was carried out under the following conditions: 2 cycles of 94 °C for 5 min, 40 °C for 5 min, and 72 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 40 °C for 1 min, and 72 °C for 2 min. Amplification products were identified by agarose gel electrophoresis; similarities among isolates were evaluated by comparing the band profiles.

Evaluation of gene expression

Transcription data were analyzed using the LightCycler Relative Quantification software. Relative expression values (R) were determined using the '$\Delta\Delta C_t$' method; the gene encoding the ribosomal protein RpsL was used as a reference for all target genes (Pfaffl et al. 2004). *P. aeruginosa* strain PAO1 was used as a standard for normalization of relative mRNA levels.

Strains were considered positive for *mexD*, *mexF*, or *mexY* overexpression when the corresponding mRNA level was ≥tenfold higher than that of PAO1. Borderline strains were defined as expression fivefold and tenfold that of the PAO1 control; all strains exhibiting ≤fivefold increased expression were defined as negative. Strains were considered positive for *mexB* overexpression when the corresponding mRNA level was ≥threefold higher than that of PAO1, negative if ≤twofold, and borderline if two- to three-fold higher (Xavier et al. 2010; Cabot et al. 2011).

Primer dimmers and other artifacts were evaluated by melting curve analysis. To confirm that specific amplification had occurred, melting curves of each amplicon were assessed and compared to Tm values obtained using PAO1 DNA as the template.

Table 1 PCR primers used in this study

Gene	Primer	Sequence (5′–3′)	Product size (bp)	References
mexB	mexB-F	CAAGGGCGTCGGTGACTTCCAG	273	Oh et al. (2003)
	mexB-R	ACCTGGGAACCGTCGGGATTGA		
mexD	mexD-F	GGAGTTCGGCCAGGTAGTGCTG	236	Oh et al. (2003)
	mexD-R	ACTGCATGTCCTCGGGGAAGAA		
mexF	mexF-F	CGCCTGGTCACCGAGGAAGAGT	254	El Amin et al. (2005)
	mexF-R	TAGTCCATGGCTTGCGGGAAGC		
mexY	mexY-F	TGGAAGTGCAGAACCGCCTG	270	Oh et al. (2003)
	mexY-R	AGGTCAGCTTGGCCGGGTC		
rpsL	rpsL-F	GCTGCAAAACTGCCCGCAACG	250	Oh et al. (2003)
	rpsL-R	ACCCGAGGTGTCCAGCGAACC		

Results

Antibiotic sensitivity testing

Clinical isolates were divided into four groups based on their drug sensitivity profiles: MDR, ICR, CQR, and IQR. MDR isolates exhibited resistance to ceftazidime, piperacillin, imipenem, and gentamicin. All ICR isolates were resistant to imipenem; an additional three isolates (33 %) also exhibited meropenem resistance. CQR isolates were resistant to imipenem and levofloxacin. IQR isolates were resistant to ciprofloxacin and levofloxacin, but were sensitive to all other drugs.

Minimum inhibitory concentration (MIC) testing

Minimum inhibitory concentration values were determined for all isolates. MDR isolates exhibited MICs of ≥64 μg/ml for ceftazidime, ≥128 μg/ml for piperacillin, ≥16 μg/ml for imipenem, and ≥16 μg/ml for gentamicin. ICR isolates exhibited MICs of ≥16 μg/ml for imipenem. CQR isolates exhibited MICs of ≥16 μg/ml for imipenem and ≥4 μg/ml for ciprofloxacin. The fourth group, IQR, exhibited ciprofloxacin MICs ≥4 μg/ml.

Gene expression

The relative mRNA expression levels of the *mexB*, *mexD*, *mexF*, *mexY* genes were determined by qPCR (Table 2). Among the 50 isolates investigated, the frequency of genes classified as overexpressed were 88 % for *mexD*, 76 % for *mexB*, 46 % for *mexF*, and 40 % for *mexY* (Table 3).

Within the MDR group, *mexB* was overexpressed in 15 of 22 isolates, *mexD* in 20 of 22, *mexF* in 15 of 22, and *mexY* in 19 of 22. Borderline overexpression of *mexB* was detected in three isolates. In the ICR group, isolates *mexB* and *mexD* were each overexpressed in five isolates. Borderline overexpression of *mexB* was detected in one isolate. No *mexF* overexpression was observed in six of nine isolates. No *mexY* overexpression was observed in any of the ICR isolates. *mexD* overexpression was observed in all seven CQR isolates. Six isolates exhibited *mexB* overexpression, with the seventh exhibiting borderline overexpression. One isolate overexpressed *mexF*; *mexY* overexpression was not seen in this group. Within the IQR group, *mexB* and *mexD* were overexpressed in all 12 isolates. *mexF* overexpression was detected in 7 of 12 isolates, with borderline overexpression in 4 isolates. *mexY* overexpression was detected in one isolate.

AP-PCR analysis

The genetic similarity between isolates was determined using AP-PCR and comparing band profiles; 18 distinct banding patterns were identified (Fig. 1).

Discussion

Pseudomonas aeruginosa is a clinically important pathogen capable of developing resistance during treatment. Of particular concern is the recent increase in the number of nosocomial infections caused by this pathogen, many of which are characterized by resistance to multiple drugs (Pechere and Kohler 1999; McGowan 2006). The goal of this study was to evaluate the molecular mechanisms underlying drug resistance in *P. aeruginosa*.

Among the RND efflux pumps, MexAB-OprM has the broadest substrate profile for β-lactam antibiotics, with an ability to export carbapenems and meropenem, though not imipenem due to the absence of a specific heterocyclic side chain in the chemical structure of imipenem that is recognized by MexAB-OprM (Poole and Srikumar 2001; Bonfiglio et al. 2002).

Previous studies found that *mexB* overexpression was highest among meropenem-nonsusceptible isolates (Cabot

Table 2 Gene transcription levels for all *P.aeruginosa* isolates

Isolate	Resistance phenotype	AP-PCR category	Relative gene expression (Fold PAO1)			
			mexB[i]	*mexD*[ii]	*mexF*[iii]	*mexY*[iv]
1	1	1	9	1.003	182	4.416
2	1	2	21	19.410	175	7.785
3	1	2	133	90.833	2.143	36.636
4	1	2	290	24.294	3.554	32.232
5	1	2	50	8.384	227	5.595
6	1	2	222	26.038	1.011	385
7	1	3	31	25.064	157	262
8	1	4	22	11.658	543	7.202
9	1	4	0.2	44	2.5	347
10	1	4	10	2.125	0	368
11	1	4	148	1.282	2.548	312
12	1	4	14	1.401	74	4.852
13	1	4	0.2	0.7	0.004	0,52
14	1	4	43	30.205	13712	2.873
15	1	4	14	72	34	4.882
16	1	4	20	2.650	372	396
17	1	4	69	10.894	93	3.379
18	1	4	2.4	100	11	241
19	1	4	2.6	270	0	21
20	1	5	0.6	22	0	1.6
21	1	6	0.3	4	0	204
22	1	6	2.4	76	0	0
23	2	7	17	1.7	0	0
24	2	8	0.6	3.5	0.3	0
25	2	9	3.4	217	0	0
26	2	10	14	21	0	0
27	2	11	4.4	4	0	0
28	2	12	1.3	11	0	0
29	2	12	2.2	8.6	0	0
30	2	12	1.9	41	0	0
31	2	12	3	80	0.1	0.2
32	3	13	13.7	466	0	0.4
33	3	13	6.5	151	4	0.1
34	3	13	4.4	47	2.5	0
35	3	14	11	261	4	0.5
36	3	14	4.1	40	3	0
37	3	14	7.1	652	17	1.9
38	3	14	2.2	129	6,9	0.5
39	4	15	3.5	47	8.4	3.4
40	4	15	10	201	81	4.1
41	4	15	4.2	47	6.7	0.5
42	4	15	11	324	30	3
43	4	15	5	150	56	11.4
44	4	15	3.2	48	5	0.6
45	4	15	6.1	85	20	3.2
46	4	15	5.4	97	11	3.5
47	4	15	4.1	57	10	6.5
48	4	16	3.7	79	14	3
49	4	17	4	74	8.9	2.1
50	4	18	3.1	44	2.8	1.6

Resistance phenotype: classification of *P.aeruginosa* isolates based upon antibiotic sensitivity

AP-PCR category: classification of *P.aeruginosa* isolates following AP-PCR analysis

i: Representing MexAB-OprM efflux pump

ii: Representing MexCD-OprJ efflux pump

iii: Representing MexEF-OprN efflux pump

iv: Representing MexXY-OprM efflux pump

Table 3 Overexpression of resistance genes in isolates with distinct resistance profiles

Phenotype	Isolate number (%)	mexB	mexD	mexF	mexY
MDR	22 (44)	15 (68.1)	20 (90.9)	15 (68.1)	19 (86.3)
ICR	9 (18)	5 (55.5)	5 (55.5)	0	0
CQR	7 (14)	6 (85.7)	7 (100)	1 (14.2)	0
IQR	12 (24)	12 (100)	12 (100)	7 (58.3)	1 (8.3)
Total	50 (100)	38 (76)	44 (88)	23 (46)	20 (40)

MDR multiple drug resistant, *ICR* ısolated carbapenem resistant, *CQR* carbapenem and quinolone resistant, *IQR* ısolated quinolone resistant

et al. 2011). This observation was consistent with a report of meropenem resistance in 62.5 % of isolates overexpressing *mexB* (Xavier et al. 2010). Here, we identified 35 isolates (70 %) that exhibited meropenem resistance, with *mexB* overexpressed in 25 of these (71 %); 4 additional isolates exhibited borderline overexpression. Among all efflux genes studied, the frequencies of overexpression were 88 % for *mexD* (44/50), 76 % for *mexB* (38/50), 46 % for *mexF* (23/50), and 40 % for *mexY* (20/50). Of the 35 meropenem-resistant isolates not exhibiting *mexB* overexpression, 6 overexpressed *mexD*.

In a study, increased transcription of *mexB* has been found to be closely associated with meropenem resistance. One high-level meropenem-resistant isolate showed no significant change in the *mexB* mRNA level; however, sequencing revealed the presence of a *nalB* mutation, which likely accounted for the resistance phenotype (El Amin et al. 2005). However, the authors failed to detect the overexpression of other efflux pump genes, such as *mexF* and *mexY*, which may have contributed to drug resistance, nor did they determine the effects of efflux inhibitors on drug susceptibility. Therefore, they concluded that while most isolates possess several resistance mechanisms, resistance related to efflux systems is only a small part of the full resistance profile of each isolate (El Amin et al. 2005).

Expression of *mexCD-oprJ* is controlled by *nfxB*, a negative regulator of *mexCD-oprJ* (Poole et al. 1996). Mutations within *nfxB* are believed to alter the repressor activity of NfxB, leading to hyperexpression of *mexCD-oprJ* in so-called *nfxB*-type mutants (Poole et al. 1996; Shiba et al. 1995). Giske et al. (2008) reported decreased transcription of *mexD* in 25 of the 27 carbapenem-resistant isolates, compared to only 2 overexpressors. Oh et al. (2003) determined that MexCD-OprJ was overexpressed in 5 of 20 fluoroquinolone-resistant clinical strains of *P. aeruginosa.* They concluded that the effect of MexCD-OprJ overexpression on antibiotic resistance in strains expressing high levels of *MexCD-OprJ* was most likely significant, since both strains exhibited high fluoroquinolone MICs, which could not be explained by only topoisomerase mutations.

In this study, *mexD* overexpression was detected in 44 of 50 clinical *P. aeruginosa* isolates (88 %), including all of the IQR isolates. *mexD* was also overexpressed in 100 % of the CQR isolates, one of which also overexpressed both *mexB* and *mexF*. Therefore, this pump likely plays an important role in quinolone resistance.

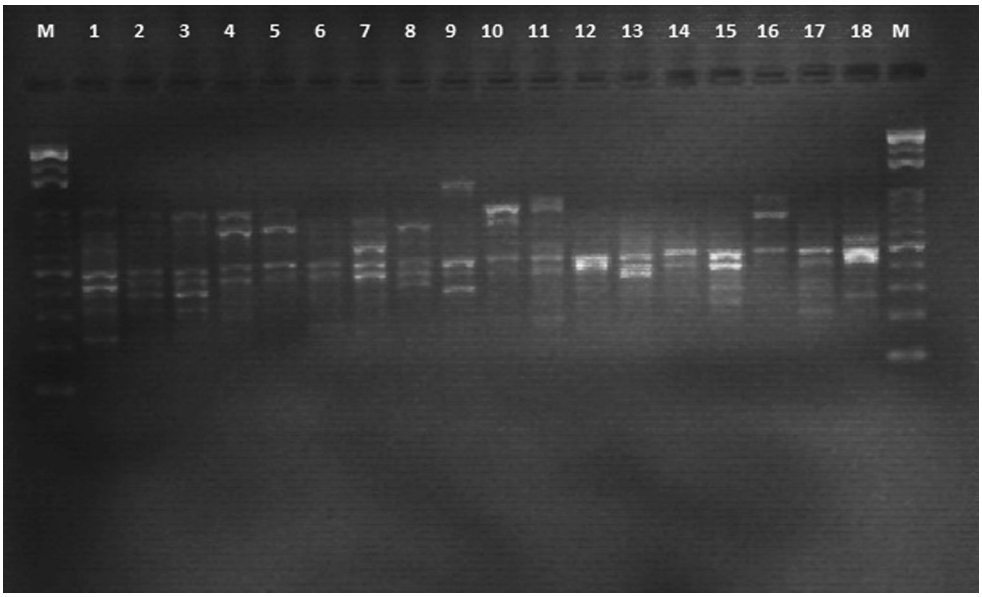

Fig. 1 Agarose gel electrophoresis of amplification products following AP-PCR. *1–18* amplification product patterns

The MexXY-OprM efflux system has been increasingly recognized as one of the primary determinants of aminoglycoside resistance in *P.aeruginosa*. (Nikaido and Pages 2012; Poole 2012) In a study with twenty-two multiresistant *P. aeruginosa* isolates, the most frequent efflux system was MexXY-OprM (82 %). (Henrichfreise et al. 2007) In our study, *mexY* overexpression was detected in 20 of 50 clinical *P. aeruginosa* isolates (40 %). Within the MDR group, *mexY* overexpressed in 19 of 22 (86 %). Some studies, including this work, show that the impact of MexXY-OprM on resistance in *P. aeruginosa* may have been underestimated so far (Hocquet et al. 2006; Islam et al. 2004).

mexF overexpression was observed in 7 of 12 IQR isolates, with borderline overexpression seen in an additional 4 isolates. Overexpression of the MexEF-oprN efflux pump is therefore also related to quinolone resistance.

Certain efflux pumps, such as *mexCD-oprJ*, have been shown to be induced in response to clinically important disinfectants, indicating an important role for MexCD-OprJ in intrinsic *P. aeruginosa* multidrug resistance in hospitals in which certain disinfectants are used (Morita et al. 2003). In our study, *mexD* overexpression was detected in 20 of 22 multidrug-resistant isolates, and in all 12 IQR isolates, indicative of widespread *mexD* overexpression in resistant strains.

Taken together, the data presented here indicate that a number of gene expression profiles associated with drug resistance. Increased transcription of *mexB* was directly correlated with meropenem resistance in the majority of isolates tested, while MexCD-OprJ and MexEF-OprN were related to quinolone resistance; the MexCD-OprJ efflux pump was also related to multidrug resistance. Increased transcription of *mexY* may contribute to the gentamicin resistance. Therefore, the mechanisms regulating the expression of these efflux genes deserve special attention.

Acknowledgments This study was granted approval by the local Ethics in Research Committee.

Conflict of interest None declared.

References

Bonfiglio G, Russo G, Nicoletti G (2002) Recent developments in carbapenems. Expert Opin Investig Drugs 11(4):529–544

Cabot G, Ocampo-Sosa AA, Tubau F, Macia MD, Rodriguez C, Moya B, Zamorano L, Suarez C, Pena C, Martinez-Martinez L, Oliver A, the Spanish Network for Research in Infectious Diseases (REIPI) (2011) Overexpression of AmpC and efflux pumps in *Pseudomonas aeruginosa* ısolates from bloodstream ınfections: prevalence and ımpact on resistance in a Spanish Multicenter Study. Antimicrob Agents Chemother 55(5):1906–1911

El Amin N, Giske CG, Jalal S, Keijser B, Kronvall G, Wretlind B (2005) Carbapenem resistance mechanisms in *Pseudomonas aeruginosa:* alterations of porin OprD and efflux proteins do not fully explain resistance patterns observe in clinical isolates. APMIS 113(3):187–196

Giske CG, Buaro L, Sundsfjord A, Wretlind B (2008) Alterations of porin, pumps, and penicillin-binding proteins in carbapenem resistant clinical ısolates of *Pseudomonas aeruginosa*. Microb Drug Resist 14(1):23–30

Henrichfreise B, Wiegand I, Pfister W, Wiedemann B (2007) Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. Antimicrob Agents Chemother 51(11):4062–4070

Hocquet D, Nordmann P, El Garch F, Cabanne L, Plesiat P (2006) Involvement of the MexXY–OprM efflux system in emergence of cefepime resistance in clinical strains of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 50(4):1347–1351

Islam S, Jalal S, Wretlind B (2004) Expression of the MexXY efflux pump in amikacin-resistant isolates of *Pseudomonas aeruginosa*. Clin Microbiol Infect 10(10):877–883

Kohler T, Kok M, Michea-Hamzehpour M, Plesiat P, Gotoh N, Nishino T, Curty LK, Pechere JC (1996) Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 40(10):2288–2290

Kohler T, Michea-Hamzehpour M, Henz U, Gotoh N, Curty LK, Pechere JC (1997) Characterization of MexE–MexF–OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol Microbiol 23(2):345–354

Li XZ, Nikaido H, Poole K (1995) Role of *mexA–mexB–oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 39(9):1948–1953

Li XZ, Zhang L, Srikumar R, Poole K (1998) Beta-lactamase inhibitors are substrates for the multidrug efflux pumps of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 42(2):399–403

Lister PD, Wolter DJ, Hanson ND (2009) Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev 22(4):582–610

McGowan JE Jr (2006) Resistance in nonfermenting gram negative bacteria: multidrug resistance to the maximum. Am J Infect Control 34(5 Suppl 1):29–37

Morita Y, Murata T, Mima T, Shiota S, Kuroda T, Mizushima T, Gotoh N, Nishino T, Tsuchiya T (2003) Induction of mexCD-oprJ operon for a multidrug efflux pump by disinfectants in wild-type *Pseudomonas aeruginosa* PAO1. J Antimicrob Chemother 51(4):991–994

Nikaido H, Pages JM (2012) Broad-specificity efflux pumps and their role in multi drug resistance of Gram-negative bacteria. FEMS Microbiol Rev 36(2):340–363

Oh H, Stenhoff J, Jalal S, Wretlind B (2003) Role of efflux pumps and mutations in genes for topoisomerases II and IV in fluoroquinolone-resistant *Pseudomonas aeruginosa* strains. Microb Drug Resist 9(4):323–328

Pechere JC, Kohler T (1999) Patterns and modes of beta-lactam resistance in *Pseudomonas aeruginosa*. Clin Microbiol Infect 5(Suppl 1):15–18

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol Lett 26(6):509–515

Poole K (2002) Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. Curr Pharm Biotechnol 3(2):77–98

Poole K (2012) Efflux-mediated antimicrobial resistance. In: Dougherty TJ, Pucci MJ (eds) Antibiotic discovery and development, vol 1. Springer, New York, pp 349–395

Poole K, Srikumar R (2001) Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. Curr Top Med Chem 1(1):59–71

Poole K, Gotoh N, Tsujimoto H, Zhao Q, Wada A, Yamasaki T, Neshat S, Yamagishi J, Li XZ, Nishino T (1996) Overexpression of the mexC–mexD–oprJ efflux operon in nfxB-type multidrug resistant strains of *Pseudomonas aeruginosa*. Mol Microbiol 21(4):713–724

Schweizer HP (2003) Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet Mol Res 2(1):48–62

Shiba T, Ishiguro K, Takemoto N, Koibuchi H, Sugimoto K (1995) Purification and characterization of the *Pseudomonas aeruginosa* NfxB protein, the negative regulator of the *nfxB* gene. J Bacteriol 177(20):5872–5877

Strateva T, Yordanov D (2009) *Pseudomonas aeruginosa*—a phenomenon of bacterial resistance. J Med Microbiol 58(Pt 9):1133–1148

Xavier DE, Picao RC, Girardello R, Fehlberg LC, Gales AC (2010) Efflux pumps expression and its association with porin down-regulation and β-lactamase production among *Pseudomonas aeruginosa* causing bloodstream infections in Brazil. BMC Microbiol 10:217