Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR

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Abstract

In *Pseudomonas aeruginosa* many of the clinically relevant resistance mechanisms result from changes in gene expression as exemplified by the Mex drug efflux pumps, the AmpC β-lactamase and the carbapenem-specific porin OprD. We used quantitative real-time-PCR to analyze the expression of these genes in susceptible and antibiotic-resistant laboratory and clinical strains. In *nalB* mutants, which overexpress OprM, we observed a four- to eightfold increase in the expression of *mexA*, *mexB*, and *oprM* genes. *MexX* and *mexY* genes were induced eight to 12 times in the presence of 2 mg L^{-1} tetracycline. The *mexC/oprJ* and *mexE/oprN* gene expression levels were increased 30- to 250-fold and 100- to 760-fold in *nfxB* and *nfxC* mutants, respectively. We further found that in defined laboratory strains expression levels of *ampC* and *oprD* genes paralleled β-lactamase activity and OprD protein levels, respectively. Our data support the use of quantitative real-time-PCR chain reaction for the analysis of the antimicrobial resistance gene expression in *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa owes its intrinsic and acquired resistance to antimicrobial agents, biocides, organic solvents and heavy metals mostly to the expression of chromosomally encoded efflux pumps belonging to the so-called resistance-nodulation-cell division (RND) family of multidrug transporters (for a recent review, see Poole, 2004). For instance, the constitutively expressed MexAB–OprM (Poole *et al.*, 1993; Li *et al.*, 1995) and the substrate-inducible MexXY–OprM (Mine *et al.*, 1999; Ramos-Aires *et al.*, 1999) efflux systems contribute to the intrinsic resistance of *P. aeruginosa* to a large number of structurally unrelated antimicrobial agents. Mutational events occurring in cognate regulator genes result in the overexpression of these and other efflux pumps like MexCD–OprJ (Poole *et al.*, 1996a) and MexEF–OprN (Köhler *et al.*, 1997a). Moreover, the chromosomally encoded AmpC β-lactamase (Lodge *et al.*, 1990) and the APH(3′)-II aminoglycoside phosphotransferase (Okii *et al.*, 1983) confer resistance to β-lactams (penicillins, cephalosporins) and to the aminoglycosides kanamycin and neomycin, respectively. *Pseudomonas aeruginosa* may also limit the access of hydrophilic molecules by reducing expression of specific porin channels, like OprD, leading to carbapenem resistance (Trias *et al.*, 1989). It therefore appears that many important resistance mechanisms in *P. aeruginosa* result from changes in gene expression. Therefore, the comparison between resistance gene expression profiles between susceptible and antibiotic-resistant isolates should provide information on the underlying resistance mechanisms.

Western blot analysis has been used in the past to measure the expression level of efflux pump proteins and the OprD porin. However, this approach is time consuming and usually has a low sensitivity. The same arguments apply to the determination of AmpC expression levels by biochemical methods. We have therefore investigated the possibility of using quantitative real-time-PCR (qRT-PCR) to study the expression of antibiotic resistance genes in *P. aeruginosa*. This method has the advantage of being fast and sensitive and could be used in the clinical laboratory.

Materials and methods

Bacterial strains

All laboratory mutants were derived from the susceptible wild-type strain PAO1, here called PT5 (Table 1). The *nalB* mutant PT629 (Cosson *et al.*, 2002) overexpresses OprM as determined by western blot analysis (T. Köhler *et al.*,

Table 1. Bacterial strains

Strain	Phenotype	Genotype	Reference
PT5	PAO1 wild type		Lab collection
PT629	PT5*nalB*, overexpresses MexAB–OprM	4 bp deletion in *mexR*	Köhler *et al*. (1997b)
PT648	PT5*nfxB*, overexpresses MexCD–OprJ	2 bp deletion in *nfxB*	Cosson *et al*. (2002)
PT149	PT5*nfxC*, overexpresses MexEF–OprN	8 bp deletion in *mexT*	Köhler *et al*. (1997a)
PT364	PT5*oprD*::ΩTc, lacks OprD		Epp *et al*. (2001)
PT1102	PT5 imi-R, decreased OprD expression	ND	Perron *et al*. (2004)
PT1105	PT5 imi-R, decreased OprD expression	CzcS Val194Leu	Perron *et al*. (2004)
PT1108	PT5 imi-R, decreased OprD expression	CzcS Val194Leu	Perron *et al*. (2004)
PT1155	PT5 ctx-R, derepressed AmpC	ND	This study
PT1156	PT5 ctx-R, derepressed AmpC	ND	This study
302S	Susceptible blood isolate		Michéa-Hamzehpour *et al*. (1987)
302R9J	302S*nfxB*, overexpresses MexCD–OprJ	NfxB End200Cys	Unpublished
302R11J	302S*nfxB*, overexpresses MexCD–OprJ	1 bp deletion in *nfxB*	Unpublished
302R9N	302S*nfxC*, overexpresses MexEF–OprN	ND	Unpublished
302R11N	302S*nfxC*, overexpresses MexEF–OprN	ND	Unpublished
14	Susceptible clinical isolate		Ziha-Zarifi *et al*. (1998)
12	14*nalB*, overexpresses MexAB–OprM	MexR Asp8Glu	Ziha-Zarifi *et al*. (1998)
18	Susceptible clinical isolate		Ziha-Zarifi *et al*. (1998)
17	18*nalB*, overexpresses MexAB–OprM	1 bp deletion in *mexR*	Ziha-Zarifi *et al*. (1998)
96	Susceptible clinical isolate		Ziha-Zarifi *et al*. (1998)
109	96*nalB*, overexpresses MexAB–OprM	11 bp deletion in *mexR*	Ziha-Zarifi *et al*. (1998)

imi-R, imipenem-resistant; ctx-R, cefotaxime-resistant; ND, not determined; bp, base pair.

unpublished result). PT629 has a 4 base pair (bp) deletion at position 363 in the *mexR* gene, while strain PT648 (Köhler *et al*., 1997b) contains an AC deletion at codon 19 in *nfxB*, resulting in the overproduction of MexCD–OprJ (*nfxB* phenotype). The *nfxC* mutant PT149 (formerly PAO-7H) (Köhler *et al*., 1997a) overproduces MexEF–OprN because of the synthesis of a full-length *mexT* gene that is interrupted by an 8 bp DNA sequence in the susceptible wild-type strain PT5 (Köhler *et al*., 1999; Maseda *et al*., 2000). AmpC-derepressed strains PT1155 and PT1156 were selected by spreading *c*. 10^9 cells of PT5 on Luria–Bertani (LB) agar supplemented with 30 mg L^{-1} cefotaxime and incubating at 37 °C for 48 h. Clinical strains used in this study are shown in Table 1. The susceptible *Pseudomonas aeruginosa* clinical isolate 302S (designated as strain 1 in Michéa-Hamzehpour *et al*., 1987), was obtained from a blood culture of a patient hospitalized at the University Hospital Geneva. Resistant derivatives of this strain were selected in a murine peritonitis model using a single dose (25 mg kg^{-1}) of pefloxacin or ciprofloxacin (Michéa-Hamzehpour *et al*., 1987). Minimal inhibitory concentration (MIC) determinations and western blot analyses using antibodies against OprJ- and OprN-identified strains 302-R9J and 302-R11J as *nfxB* mutants, while strains 302-R9N and 302-R11N were *nfxC* mutants (data not shown). Three well-characterized pairs of isogenic susceptible and β-lactam-resistant clinical isolates (14/12, 18/17 and 96/109) (Table 1) were used for analysis of *mexA*, *mexB* and *oprM* expressions (Ziha-Zarifi *et al*., 1998). Strains were grown with agitation (250 r.p.m.) at 37 °C in LB medium.

Isolation of total RNA

Strains were inoculated from glycerol stocks into 2 mL LB medium and grown overnight (o/n) at 37 °C. Strains were subcultured in 5 mL LB medium and grown to mid-exponential phase (OD_{600} = 1.5–2.0). A 0.25 mL aliquot of this culture, corresponding to 5×10^8 cells, was added to 0.5 mL of RNAeasy bacteria protect solution (Qiagen, Hilden, Germany). Total RNA was isolated according to the instructions of the supplier. Residual DNA was eliminated by DNAse treatment using 20 U of RQ1 RNAse-free DNAse (Promega, Madison, WI). After removal of DNAse by phenol/chloroform extraction, RNA was precipitated and resuspended in 30 μL of RNAse-free H_2O. The final yield was *c*. 15 μg of RNA (260/280 ratio of > 1.8).

Synthesis of cDNA for RT-PCR

A reaction mix containing 1 μg of RNA was incubated in the presence of 250 ng random hexamers (Promega) and dNTPs (833 μM final concentration) in a total volume of 24 μL at 65 °C for 5 min. After chilling the mix on ice, 8 μL 5 × first-strand buffer, 4 μL of 25 mM $MgCl_2$ and 2 μL RNAsin RNAse inhibitor (Promega) were added. The reaction was preincubated for 10 min at 25 °C and then 2 min at 42 °C. The mix was divided into two 19 μL aliquots. To one aliquot, 1 μL (5 U) of ImPromII reverse transcriptase (Promega) was added; the other half served as a negative control to detect the amount of residual genomic DNA in the RNA preparations. Both samples were incubated at 42 °C for 50 min. Reverse transcriptase was inactivated by incubation at 70 °C

for 15 min. The cDNAs obtained were stored at $-20\,^{\circ}C$ until use.

RT-PCR analysis

The primers for the PCR amplification of cDNA were designed using the primer3 program available at the website (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and are shown in Table 2. A Rotor Gene RT-PCR machine (Corbett Research, Sydney, Australia; Model RG3000, software version 6) was used for the quantification of cDNA. Duplicate PCR reactions were performed using the SYBR Green Quantitect Kit (Qiagen). Three microliters of a 1 : 10 dilution of the cDNA was used in a total volume of 15 μL. After a 15 min activation of the modified Taq polymerase at 95 °C, 40 cycles of 20 s at 95 °C, 20 s at 60 °C and 30 s at 72 °C were performed. Data were acquired at 72 °C. A melt curve was run at the end of the 40 cycles to test for the presence of a unique PCR reaction product. To check for residual contaminating genomic DNA, control reactions without reverse transcriptase were analyzed in the same way using the rpsL-F/R primers. The amount of signal in the controls was usually close to the nontemplate control (NTC).

To correct for differences in the amount of starting material, the ribosomal *rpsL* gene was chosen as a housekeeping reference gene. Results are presented as ratios of gene expression between the target gene (target) and the reference gene (*rpsL*), which were obtained according to the following equation: $\text{Ratio} = (E_{\text{target gene}})^{\Delta C_t}_{\text{target(PT5}-\text{test strain)}} / (E_{rpsL})^{\Delta C_t}_{rpsL(\text{PT5}-\text{test strain})}$ (Pfaffl *et al.*, 2002), where E is the real-time PCR efficiency for a given gene and C_t the crossing point of the amplification curve with the threshold. An effect on gene expression was considered significant when the corresponding ratios were > 2.5 or < 0.4.

β-lactamase activity and *OprD* western blot analysis

β-lactamase activity was determined in cell-free extracts of LB-grown cultures using nitrocefin as a substrate. LB

Table 2. Primer sequences

Primer	5′-sequence-3′	Length (bp)	Position in gene	Product length (bp)
mexR1	CGCGAGCTGGAGGGAAGAAACC	22	217	150
mexR2	CGGGGCAAACAACTCGTCATGC	22	366	
mexA1	CGACCAGGCCGTGAGCAAGCAGC	23	375	316
mexA2	GGAGACCTTCGCCGCGTTGTCGC	23	668	
oprM1	GATCCCCGACTACCAGCGCCCCG	23	60	247
oprM2	ATGCGGTACTGCGCCCGGAAGGC	23	265	
nfxB1	CGCCTGATCAAGGAACACCTCACC	24	244	164
nfxB2	CGAAACACGCCTTTCTGCTGTCC	23	407	
mexC1	ATCCGGCACCGCTGAAGGCTGCG	23	284	344
mexC2	CGGATCGAGCTGCTGGATGCGCG	23	605	
mexC3	GTACCGGCGTCATGCAGGGTTC	22	1101	164
mexC4	TTACTGTTGCGGCGCAGGTGACT	23	1164	
oprJ1	GTTCCGGGCCTGAATGCCGCTGC	23	345	305
oprJ2	TCGCGGCTGACCAGGGTCTGACG	23	627	
mexZ1	GCATGGGCTTTCTCCGCCAGTGC	23	266	364
mexZ2	GCGTCCGCCAGCAACAGGTAGGG	23	629	
mexX1	TGAAGGCGGCCCTGGACATCAGC	23	302	326
mexX2	GATCTGCTCGACGCGGGTCAGCG	23	605	
mexT1	CAGCACCGCGGTGTTCCGCATCG	23	420	216
mexT2	ACGGTCTTGCGCTTGGCGTTGGC	23	635	
mexE4	CCAGGACCAGCACGAACTTCTTGC	24	944	114
mexE5	CGACAACGCCAAGGGCGAGTTCACC	25	831	
oprN1	CAACCGGGAGTGACCGAGGACCG	23	358	235
oprN2	TGCTCAGGGCAATCTTCTCGCGC	23	570	
ampC1	CGGCTCGGTGAGCAAGACCTTC	22	264	218
ampC2	AGTCGCGGATCTGTGCCTGGTC	22	481	
oprD1	ATCTACCGCACAAACGATGAAGG	23	772	156
oprD2	GCCGAAGCCGATATAATCAAACG	23	927	
oprD3	CTCGACGGCACCTCCGACAAGAC	23	287	232
oprD4	AGCCCTTCGAATTCGCTGCTCTG	23	518	
rpsL-F	GCAAGCGCATGGTCGACAAGA	21	35	201
rpsL-R	CGCTGTGCTCTTGCAGGTTGTGA	23	235	

bp, base pair.

Table 3. Relative expression levels of antibiotic resistance genes in laboratory strains as determined by qRT-PCR

	Strains				
Gene	PT5 *wt*	PT629 *nalB*	PT648 *nfxB*	PT149 *nfxC*	PT5+Tet
mexR	1.0	0.2	1.7	*0.2*	*0.3*
mexA	1.0	**6.1**	1.7	0.5	0.6
mexB	1.0	**5.0**	1.1	*0.2*	*0.4*
oprM	1.0	**4.2**	1.2	*0.4*	1.0
nfxB	1.0	0.9	**14.7**	0.7	1.6
mexC	1.0	1.3	**74.5**	**2.9**	**4.0**
oprJ	1.0	3.2	**33.4**	**2.9**	**3.8**
mexZ	1.0	0.2	2.3	*0.4*	**3.8**
mexX	1.0	1.9	1.6	*0.4*	**8.5**
mexY	1.0	0.9	1.8	*0.2*	**10.9**
mexT	1.0	1.2	2.1	1.9	1.7
mexE	1.0	2.4	**4.4**	**796.0**	**3.5**
oprN	1.0	1.5	**2.6**	**162.0**	**2.9**

Values represent fold change (mean of duplicate samples) in comparison with the transcription level in wild-type strain PT5.
In all cases the standard deviation for duplicate samples was less than 15% of the mean.
Values $\leq$ 0.4 are shown in italics, values $\geq$2.5 are shown in bold.
qRT-PCR, quantitative real-time-PCR chain reaction.

medium was inoculated 1 : 100 with o/n cultures. Where indicated, β-lactamase synthesis was induced by adding 500 mg L^{-1} benzyl penicillin during exponential growth. After 2 h of incubation, cells were harvested by centrifugation at 8 °C at 3200 ***g*** for 10 min. The pellet was washed twice and finally resuspended in cold 10 mM Na-phosphate buffer (pH 7.0). Cells were sonicated (5 × 20 pulses, power level 3 on a Branson sonicator) and centrifuged for 10 min at 15 500 ***g*** at 8 °C. The β-lactamase activity was determined by a kinetic assay in 10 mM Na-phosphate buffer (pH 7.0) using nitrocefin (0.1 mM final concentration) as the substrate. β-lactamase activity is expressed as the difference in absorption at OD_{482} per minute per milligram protein cell-free extract.

Western blots using a polyclonal OprD antibody were performed on total cell lysates as described previously (Perron *et al*., 2004).

Results and discussion

To assess the possibility of using qRT-PCR to detect antimicrobial resistance mechanisms in *Pseudomonas aeruginosa*, we measured gene expression of the four major Mex efflux pumps, the chromosomal AmpC β-lactamase and the porin OprD in both laboratory and well-characterized clinical strains (Table 1).

Expression analysis of *Mex* efflux pump genes

While genome analysis of *P. aeruginosa* has revealed the presence of 11 RND-type efflux pumps (www.pseudomonas.com), only the MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY pumps have been shown to be of clinical relevance. For each of these four efflux pump operons, we determined the expression of at least two pump genes and the corresponding regulator gene in isogenic susceptible (S) and antibiotic-resistant (R) *P. aeruginosa* strains.

We first analyzed gene expression of the constitutively expressed *mexAB–oprM* operon in laboratory strains PT5 and PT629 and in three pairs of isogenic S/R clinical isolates (14/12, 18/17, 96/109). The R strains were previously shown by western blot analysis to overexpress the OprM outer membrane protein (Ziha-Zarifi *et al*., 1998). Compared with the laboratory strain PT5, the isogenic *nalB* mutant PT629 showed a four- to sixfold higher expression of *mexA*, *mexB* and *oprM* (Table 3). These results are in agreement with a fourfold increase in *mexB* expression as determined by qRT-PCR in the *nalB* mutant OCR1 (Yoneda *et al*., 2005) and with the a 4.2-fold increase in the activity of a *mexA–phoA* reporter fusion in the *nalB* strain K1496 (Srikumar *et al*., 2000). Expression of the *mexR* regulator was dowregulated fivefold in the *nalB* mutant PT629, which is surprising considering the reported negative autoregulation of MexR (Poole *et al*., 1996b). In the three *nalB* clinical isolates, 12, 17 and 109, expression of *mexA*, *mexB* and *oprM* was four- to 10-fold higher than in PT5 (Table 4) and was comparable with the *nalB* laboratory strain PT629 (Table 3). A notable exception was the 35-fold higher *mexR* expression in the *nalB* strain 109, which is in agreement with the loss of negative autoregulation of *mexR* (Poole *et al*., 1996b).

In contrast to the constitutively expressed MexAB–OprM pump, the MexCD–OprJ efflux system (Poole *et al*., 1996a) can be induced by cationic ions like tetraphenylphosphonium chloride and ethidium bromide (Morita *et al*., 2001), but is expressed at clinically relevant levels only when

Table 4. Relative expression levels of antibiotic resistance genes in clinical isolates as determined by qRT-PCR

	Strains											
Gene	PT 5 *wt*	14	12 *nalB*	18	17 *nalB*	96	109 *nalB*	302S	302 R9J *nfxB*	302 R11J *nfxB*	302 R9N *nfxC*	302 R11N *nfxC*
mexR	1.0	0.7	0.5	0.7	1.4	*0.4*	**35.0**	0.6	1.3	*0.3*	*0.3*	0.8
mexA	1.0	1.3	**7.0**	1.5	**4.6**	0.9	**4.3**	1.6	1.3	1.4	1.3	1.5
mexB	1.0	2.4	**10.2**	1.9	**9.8**	1.5	**10.6**	0.8	0.9	0.8	*0.1*	0.7
oprM	1.0	1.7	**4.8**	1.2	**7.2**	1.1	**5.2**	*0.4*	*0.3*	*0.3*	*0.2*	*0.2*
nfxB	1.0	1.1	0.5	1.0	0.5	*0.4*	*0.4*	1.3	**41.2**	**34.8**	1.3	1.5
*mexC**	1.0	0.7	0.6	0.8	*0.2*	0.6	0.6	0.7	**272.3**	**339.4**	*0.4*	1.2
oprJ	1.0	0.7	0.7	0.9	*0.3*	*0.4*	*0.4*	0.5	**179.6**	**160.5**	*0.3*	0.6
mexT	1.0	**2.9**	1.5	**3.3**	1.6	1.0	1.6	**3.1**	**7.4**	2.4	2.0	**3.2**
mexE	1.0	**5.6**	**6.4**	**3.3**	1.4	1.7	1.6	1.2	5.5	1.3	**249.9**	**568.0**
oprN	1.0	2.4	**4.7**	1.9	0.7	0.9	0.8	1.0	2.9	1.1	**107.2**	**193.4**
mexZ	1.0	*0.1*	*0.1*	*0.05*	*0.02*	*0.05*	*0.06*	0.5	2.1	0.5	*0.4*	0.8
mexX	1.0	**8.7**	**11.0**	1.0	*0.4*	**6.9**	**7.5**	0.7	1.5	0.5	*0.2*	0.6
mexY	1.0	**8.2**	**6.5**	0.9	*0.1*	**5.2**	**5.2**	0.6	1.3	*0.4*	*0.4*	0.6
ampC	1.0	1.7	1.5	**2.5**	1.2	1.1	1.5	1.4	**7.5**	0.9	0.7	1.1
oprD	1.0	**2.7**	0.7	*< 0.01*	*< 0.01*	1.2	1.6	*0.07*	*0.07*	*0.02*	*0.04*	*0.01*

*mexC3/4 primers were used for strains derived from 302S and mexC1/2 primers for strains 14, 12, 18, 17, 96, 109.
Values represent fold change (mean of duplicate samples) in comparison with the transcription level in wild-type strain PT5.
In all cases the standard deviation for duplicate samples was less than 15% of the mean.
Values $\leq$ 0.4 are shown in italics, values $\geq$2.5 are shown in bold.
qRT-PCR, quantitative real-time PCR.

mutations arise in the cognate repressor gene *nfxB* (Hirai *et al*., 1987; Okazaki & Hirai, 1992; Morita *et al*., 2001).

As expected, the *nfxB* mutant PT648 derived from the laboratory strain PT5 showed a marked increase in both *mexC* (75-fold) and *oprJ* (33-fold) expressions (Table 3), which was accompanied by a 15-fold increase in the transcription of the repressor gene *nfxB* (Table 3). This can be explained by the loss of negative autoregulation of *nfxB* as observed by Shiba *et al*. (1995). We then tested two *nfxB* mutants derived from the clinical isolate 302S by exposure to pefloxacin in a mouse peritonitis model (Michéa-Hamzehpour *et al*., 1987). These strains showed MIC profiles reminiscent of *nfxB* mutants and overexpressed the OprJ outer membrane protein (data not shown). *MexC* and *oprJ* genes were overexpressed *c*. 300- and 170-fold, respectively Table 4). As in the laboratory mutant, *nfxB* expression in the clinical strains was derepressed (35- to 40-fold compared with PT5). Expression of the genes from the *mexAB–oprM*, *mexEF–oprN* and *mexXY* pumps were not significantly affected in these *nfxB* mutants (Table 4).

The *mexEF–oprN* operon (Köhler *et al*., 1997a) is positively regulated by the LysR-type activator protein MexT and is overexpressed in *nfxC* mutants (Köhler *et al*., 1999). In PT5, as well as in the PAO1 strain used for the genome sequencing project (Stover *et al*., 2000), the *mexT* structural gene is prematurely terminated by the presence of an 8 bp DNA sequence (Maseda *et al*., 2000) located near the 5′ end of *mexT* (Cosson *et al*., 2002). The *nfxC* mutant PT149 produces a full-length *mexT* resulting in the expression of *mexEF–oprN* (Maseda *et al*., 2000). As expected, expressions of *mexE* (796-fold) and *oprN* (162-fold) had increased drastically in PT149, while expression of *mexT* itself was not affected significantly (Table 3). In the two *nfxC* mutants, 302 R9N and 302 R11N, selected in the mouse after pefloxacin treatment (Michéa-Hamzehpour *et al*., 1987), gene inductions for *mexE* and *oprN* were at least 250- and 100-fold, respectively (Table 4), which are in agreement with the values observed in the laboratory strains. Again expression of *mexT* was not altered in the *nfxC* mutants, suggesting that *mexEF–oprN* expression occurs via activation of *mexT* at the genetic level (removal of 8 bp sequence), although activation at the protein level or through additional regulatory circuits cannot be excluded.

The *mexXY* operon does not contain a gene coding for an outer membrane protein but is able to recruit OprM of the MexAB–OprM efflux pump to form a functional tripartite efflux system (Mine *et al*., 1999; Ramos-Aires *et al*., 1999). MexXY–OprM is involved in the intrinsic and acquired resistance of *P. aeruginosa* to aminoglycoside antibiotics (Ramos-Aires *et al*., 1999). We induced the expression of this efflux pump gene by incubating the laboratory strain PT5 in the presence of 2 mg L^{-1} tetracycline as described previously (Masuda *et al*., 2000). Under these conditions, *mexX* and *mexY* expressions increased nine- and 11-fold, respectively (Table 3). The three- to fourfold inductions of *mexC–oprJ* and *mexE–oprN* genes by tetracycline are not expected to result in any effect on MICs given the more than 30-fold expression ratios for these pump genes in *nfxB* and

Table 5. Minimal inhibitory concentrations and β-lactamase expression for wild-type and cefotaxime-selected strains

Strains	CIP	CTX	CFA	AZT	β-lactamase activity	qRT-PCR fold increase for *ampC*
PT5	0.06	8	1	4	1.2 ± 0.2	1
PT5+BP*	ND	ND	ND	ND	16.2 ± 2.3	7.4
PT1155	0.06	128	4	4	13.3 ± 5.3	4.9
PT1156	0.06	256	16	8	290.1 ± 0.7	34.7

*BP, benzylpenicillin (500 mg L^{-1}) was added for induction of *ampC* in PT5.
CIP, ciprofloxacin; CTX, cefotaxime; CFA, ceftazidime; AZT, aztreonam; ND, not determined; qRT-PCR, quantitative real-time PCR.

nfxC mutants, respectively. These results are comparable with those obtained with aminoglycoside-resistant clinical isolates, which showed a six- to 15-fold increase in *mexY* (*amrB*) expression in comparison with PAO1, as estimated by semiquantitative RT-PCR (Westbrock-Wadman *et al.*, 1999), and with a fivefold increase in *mexY* expression in a *mexZ* mutant (Yoneda *et al.*, 2005). However, upregulation of *mexX* by 30- to 312-fold compared with PAO1 was recently reported in aminoglycoside-resistant clinical isolates (Llanes *et al.*, 2004). A decrease in the expression of both *mexX* and *mexY* genes in the *nfxC* mutants PT149 and 302 R9 could explain hypersusceptibility of this type of mutants to gentamicin and kanamycin (Fukuda *et al.*, 1990). Interestingly, both the β-lactam susceptible and resistant clinical isolates 14/12 and 96/109 showed a five- to 11-fold higher expression of *mexX* and *mexY* than the laboratory strain PT5 (Table 4). This would explain the previously reported elevated MICs (4–16 mg L^{-1}) of cefepime (Ziha-Zarifi *et al.*, 1998), a β-lactam transported not only by MexAB–OprM but also by the MexXY efflux pump (Ramos-Aires *et al.*, 1999). Low *mexZ* expression compared with PT5 was observed in the clinical isolates 14/12, 18/17 and 96/109 (Table 4). Reduced levels of the *mexZ* repressor gene could explain the increased expression of *mexXY* in strains 14/12 and 96/109, but surprisingly did not have any effect in strains 18/17. Hence, control of the *mexXY* operon in these strains might involve further regulatory elements, as previously suggested (Llanes *et al.*, 2004).

In summary, our data show qualitatively comparable results between gene and protein expression for *oprM*, *oprJ* and *oprN*. Together with the recently established quantitative correlation for *mexB* and *mexY* gene and protein expression (Yoneda *et al.*, 2005) our results support the use of qRT-PCR for the expression analysis of the four major efflux pumps in *P. aeruginosa*.

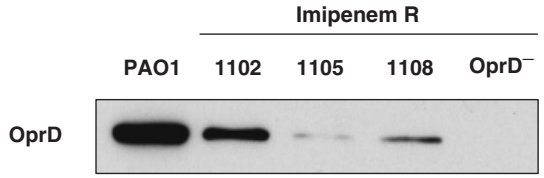
Analysis of *AmpC* expression

The chromosomal AmpC β-lactamase confers resistance to penicillins and most cephalosporins in *P. aeruginosa*. Its expression is inducible and can be partially or completely derepressed because of mutational events in regulatory loci (Langaee *et al.*, 2000). To extend our gene expression analysis, we evaluated whether expression of the *ampC* gene reflects β-lactamase activity. We selected on cefotaxime-containing plates two PT5 derivatives, called PT1155 and PT1156, which showed increased MICs to cefotaxime and ceftazidime. AmpC expression determined by qRT-PCR in these strains correlated with β-lactamase activity and MIC levels of cefotaxime. Expression of *ampC* could also be induced in the wild-type strain by the addition of benzylpenicillin (500 mg L^{-1}) (Table 5). As expected, *ampC* levels measured in the three clinical S/R pairs were comparable with those of PT5, confirming the absence of β-lactamase activity (Ziha-Zarifi *et al.*, 1998). Furthermore, we recently found a good correlation between *ampC* expression determined by qRT-PCR and the MIC profiles in more than 30 clinical isolates (A. Reinhardt *et al.*, unpublished data).

Analysis of *OprD* expression

Carbapenems, in particular imipenem and meropenem, enter *P. aeruginosa* cells via facilitated diffusion through the OprD porin (Trias *et al.*, 1989). Mutants resistant to these molecules are characterized by decreased amounts or complete loss of OprD (Trias *et al.*, 1989). We measured *oprD* gene expression levels by qRT-PCR and compared them with OprD protein levels as determined by western blot in whole-cell extracts from four carbapenem-resistant derivatives of the laboratory strain PT5 (Fig. 1). As expected, OprD protein was less abundant or undetectable in all imipenem-resistant strains. Furthermore, *oprD* gene expression levels paralleled the amount of OprD protein determined in total cell extracts (Fig. 1). In the clinical isolates 14/12 and 96/109 *oprD* levels were comparable with PT5, while those of the pair 18/17 decreased more than 100-fold. In agreement with these data, imipenem MICs were 1/0.5 mg L^{-1} for the first two pairs of strains while those of the latter pair were 4/4 mg L^{-1} (Ziha-Zarifi *et al.*, 1998).

Surprisingly, in the clinical isolate 302S and its derivatives we were initially unable to detect any *oprD* expression above the NTC background. We therefore repeated the qRT-PCR with another primer pair (oprD3/4), which yielded low but detectable amounts of *oprD* (Table 4). When the ratios were calculated with respect to strain 302S (ratio = 1), those of the two *nfxB* mutants were comparable (0.97 and 0.59) while



	PAO1	1102	1105	1108	OprD⁻
Fold change *oprD* expression	1.0	0.30	0.02	0.22	ND
Inhibition zone diameters (mm)	25	18	14	14	10

Fig. 1. Comparison between OprD expression in PT5 wild-type strain and in imipenem-resistant derivatives by western blot analysis and quantitative real-time PCR (qRT-PCR). Strains were grown to an $OD_{600\,nm} = 1$, and samples for total protein and RNA extraction were taken and treated as described in Materials and methods. Results for qRT-PCR are expressed relative to the amount of *oprD* in the wild-type strain PT5. Standard deviations for duplicate determinations were below 15% of the mean in two experiments. Imipenem susceptibility levels, expressed as inhibition zone diameters (mm), were taken from Perron *et al.* (2004). ND, not determined.

those of the *nfxC* mutants were four- to fivefold lower (0.28 and 0.19). These results are in agreement with the elevated imipenem MICs (4–8 mg L^{-1}) for the *nfxC* mutants compared with those of the susceptible strain 302S and its *nfxB* derivatives (0.5–1 mg L^{-1}).

OprD has been shown to be regulated both at the transcriptional (Ochs *et al.*, 1999) and the posttranscriptional level (Köhler *et al.*, 1999). A study comparing *oprD* gene and protein expression in 25 imipenem-resistant or susceptible clinical isolates showed decreased *oprD* gene expression in 17 out of 18 resistant isolates and 'normal' gene expression in six out of the seven susceptible isolates (Pirnay *et al.*, 2002), providing further support for a correlation between *oprD* gene expression and imipenem resistance.

General conclusions

In this study, we performed in 16 *Pseudomonas aeruginosa* isolates from both laboratory and clinical origin an expression analysis on 15 genes involved in antibiotic resistance. These included the four major *mex* efflux pump genes as well as *ampC* and *oprD*. Importantly, we observed that expression levels of these genes were comparable between the laboratory strain PT5 and the four susceptible clinical isolates (302S, 14, 18 and 96) with the notable exception of *oprD* in strain 302S. In both laboratory and clinical *nalB* mutants, we observed modest four- to 11-fold increases in the *expression of the mexAB–oprM and mexXY pumps*. In contrast, genes of the normally silent *mexCD–oprJ* and *mexEF–oprN* operons showed much higher expression ratios (30- to 796-fold) in *nfxB* and *nfxC* mutants, respectively. Since *mexAB–oprM* is already expressed constitutively in susceptible wild-type strains, a modest increase in gene transcription is apparently sufficient to further increase the resistance level. On the other hand, *mexCD–oprJ* and *mexEF–oprN* are not or weakly transcribed in susceptible strains and higher expression ratios of 70-fold for *mexC* and 250-fold for *mexE* seem to be required to observe an effect on the antibiotic susceptibility level.

Moreover, we found qualitative correlations between *ampC* expression determined by qRT-PCR and β-lactamase activity as well as for *oprD* expression and the amount of OprD protein estimated by western blot analysis in laboratory strains.

We are aware that identification of resistance mechanisms based on gene expression analysis might be complicated when several mechanisms affecting the same class of antibiotics are at work. Other caveats for this method are inherent to gene expression analysis in general, namely the fact that the presence of a transcript does not necessarily mean that it encodes a functional protein. Furthermore, posttranscriptional regulatory events influence protein expression and hence the phenotype. Certain genes may present hypervariable regions or mosaic structures like for instance *oprD*, which can acquire additional mutations and deletions in imipenem-resistant strains (Pirnay *et al.*, 2002; El Amin *et al.*, 2005). In this case it might be difficult to design a single universal primer pair for a given gene and negative results in the qRT-PCR should be confirmed with another primer pair, as shown in our study. However, this problem also arises during immunological protein detection as mosaic structures and polymorphisms might affect the protein structure preventing recognition by monoclonal antibodies.

In summary, our data strongly support the use of qRT-PCR as a rapid and sensitive method to determine the expression profile of antibiotic resistance determinants in *P. aeruginosa*. In the hospital setting, this method should allow a more targeted choice of antimicrobial treatments in particular once efflux pump inhibitors will be available.

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