Effect of carbapenems on the transcriptional expression of the *oprD*, *oprM* and *oprN* genes in *Pseudomonas aeruginosa*

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The effects of imipenem and meropenem on the transcriptional expression of resistance-related genes *oprD*, *oprM* and *oprN* in *Pseudomonas aeruginosa* were studied by quantitative real-time PCR. Four strains were examined: the type strain PT5 (PAO1), its derivatives M7 and PT149, and a clinical isolate, PaKT3. The derivative M7 is a *nalB* mutant, overexpressing the MexAB–OprM pump, and the derivative PT149 is a *nfx*C-type mutant, overexpressing the MexEF–OprN pump while it is down-regulated for the OprD protein. After 18 h incubation in broth, the cultures were divided into three portions. Two were supplemented with antibiotics and the other was left antibiotic-free as the control. After a further 45 min incubation, total RNA was isolated from the strains by guanidine denaturation and acid-phenol/chloroform extraction. DNA-free total RNAs were immediately reverse-transcribed by MMuLV reverse transcriptase. Concentrations of mRNAs obtained by quantitative PCR were expressed relative to uninduced portions of the strains. The results showed that *oprD* was relatively stable against carbapenem antibiotics. *oprM* was induced significantly by imipenem in only one strain and *oprN* was induced by imipenem in most of the strains. The responses at the mRNA level found here were unexpected and suggested a chaotic, unpredictable regulatory mechanism.

INTRODUCTION

Pseudomonas aeruginosa poses a significant health threat because of its ability to develop resistance to structurally unrelated antibiotics, leading to multiple drug resistance (MDR). In *P. aeruginosa*, MDR is often linked to the altered regulation of specific efflux pumps and porins (Poole, 2000, 2001). So far, four efflux pumps, all of the Resistance-Nodulation-Division (RND) type, namely MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY–OprM, and an outer-membrane porin (OprD) have been shown to be strongly associated with antibiotic resistance (Poole, 2001).

Pumps belonging to the Mex family exhibit a broad range and substrate-specific affinity for antibiotics (Aires *et al.*, 2002). Tetracycline, chloramphenicol, quinolones, most penicillins, most cephems (except ceftazidime) and meropenem are among the substrates of the so-called pumps (Masuda *et al.*, 2000). Porin D, on the other hand, behaves as a portal for the entry of imipenem and is linked with resistance to this antibiotic (Ochs *et al.*, 1999; Yoneyama & Nakae, 1993).

These pumps and porins play a vital role in the adaptation of *P. aeruginosa* to environmental conditions as well. For example, porin D facilitates the uptake of basic amino acids (Ochs *et al.*, 1999), and efflux pumps from the Mex family regulate quorum sensing and a number of other virulence determinants (Evans *et al.*, 1998; Kohler *et al.*, 2001). In other words, these pumps and the outer-membrane porin OprD are engaged in extremely important functions, affecting both antimicrobial resistance and potential to cause disease.

The regulation of these operons and how they respond to antibiotic challenge at the mRNA level have not been fully explained. To this end, we studied the effect of two carbapenem antibiotics, imipenem and meropenem, on the transcriptional expression of the *oprD*, *oprM* and *oprN* genes of four selected strains of *P. aeruginosa*.

METHODS

Strains. Four strains were used in the study: the type strain *P. aeruginosa* PT5 (PAO1), two mutants of this, PT149 and M7 (Kohler *et al.*, 1997), and a clinical isolate, PaKT3, that is fully susceptible to β-lactam antibiotics. According to protein expression studies, PT149 is a *nfxC*-type mutant strain which overproduces MexEF–OprN and is down-regulated for OprD, while M7 is a *nalB* mutant overproducing the pump MexAB–OprM.

The MICs of imipenem and meropenem were determined by the E-test method (Oxoid) and are as follows: PT5, 1/0·38 (imipenem/meropenem, μg ml^{-1}); PT149, 2/0·5; M7, 1/0·5; and PaKT3, 1/0·19.

DNA and RNA studies. All plastic materials were made RNase-free by treatment with diethyl pyrocarbonate (DEPC). To protect it from degradation, purified RNA was transcribed to cDNA immediately or was stored at 4 °C for a maximum of 1 h before reverse transcription. Cultures were grown in Mueller–Hinton broth (Oxoid) at 37 °C.

Induction by carbapenems was as follows. Bacteria were incubated in 30 ml broth for 18 h and cultures were divided into three equal portions. Imipenem and meropenem were added to two separate tubes and the remaining tube was left antibiotic-free as the control. Both the antibiotic-supplemented and control tubes were incubated for an additional 45 min before RNA isolation.

Induction experiments were carried out at low and high antibiotic concentrations. The low concentrations for imipenem and meropenem were 1 and 0·5 μg ml^{-1}, respectively; the high concentrations were 16 and 8 μg ml^{-1}, respectively. Experiments were repeated three times and the results were evaluated together.

In the second stage of the study, induction with imipenem and meropenem was tested using exponentially growing bacteria. First, growth plots in similar incubation conditions were obtained and showed that in these conditions the strains grew actively up to 13 h of the incubation period. As a result, inductions at this stage were tested at 10 h. Incubation and induction conditions were exactly the same as for the first stage experiment. However, imipenem and meropenem were incorporated at sub-MIC levels (imipenem, 0·5 μg ml^{-1}; meropenem, 0·25 μg ml^{-1}) and the induction periods were 45 min and 3 h.

Total RNA was isolated from bacteria using a commercial kit (EZ-RNA, Biological Industries) according to the manufacturer's instructions. The method is based on guanidine denaturation and acid-phenol/chloroform extraction of total RNA. An additional chloroform extraction step, however, was included to improve purity. The integrity of the RNA was confirmed by agarose gel (2·5 %) electrophoresis and, finally, total RNA was dissolved in 40 μl DEPC-treated double-distilled water. Reverse transcriptions were performed using random hexamer primers, 100 IU MMuLV (MBI Fermentas) and 8 μl RNA in 20 μl final volume at 42 °C for 2 h.

Real-time PCR. The sequences of the studied genes were obtained from the gene bank of the National Library of Medicine, and primers were designed with the aid of OLIGO software (version 5.0) (Molecular Biology Insights). The sequences of the primers are shown in Table 1.

PCR was performed using a LightCycler instrument (Roche Diagnostics), in capillary glass tubes with the LightCycler FastStart DNA Master SYBR Green I kit (Roche). All work was carried out on desktop coolers (pre-cooled to 4 °C). Master mixtures were prepared in accordance with the manufacturer's recommendations but the final concentrations of Mg and primers were 2·5 mM and 50 pmol (each), respectively.

Control cDNA was obtained from *P. aeruginosa* PT5 and the primers of the control reactions were *proC* gene specific. Six controls obtained by twofold dilutions were always included. The concentration of the highest control was approximately 1 μg ml^{-1}.

The concentrations of the studied cDNA preparations were adjusted on the LightCycler instrument. Briefly, for every sample, 1 μl cDNA and 9 μl SYBR Green I (same concentration as indicated for the PCR assay by the manufacturer) were mixed in capillary tubes. After an incubation step of 95 °C for 5 min, the fluorescence emissions were read at 55 °C using the real-time fluorometry (RTF) facility of the LightCycler instrument to enable comparison of the amounts of cDNA. Subsequently the cDNA concentrations were adjusted to a level between the highest and the first dilution of the control cDNA. This adjustment was critical to obtain good amplification.

An arbitrary value of 1·600 E+3 was given to the highest concentration of control cDNA and twofold dilutions down to 0·050 E+3 were included. After 5 min of an initial 'activation and denaturation' step at 95 °C, PCR was accomplished in 13 s at 95 °C, 10 s at 58 °C, 15 s at 72 °C and with a single read after 5 s at 85 °C for 45 cycles.

Evaluation of real-time PCR. Melting curve analysis was employed to check for the existence of primer dimers and other artefacts. Consequently, only dimer- and artefact-free amplifications were included in the analysis. The Second Derivate Maximum algorithm of the LightCycler software was used to calculate the concentrations. The software drew a standard curve by plotting the crossing cycle number versus the logarithm of given concentrations for each control sample.

For every sample, six genes were studied, namely *proC*, *rpoD*, *oprB*, *oprD*, *oprM* and *oprN*, and *proC* and *rpoD* genes served as the internal controls of the test strains (Savli *et al.*, 2003). Raw concentration values were corrected according to the geometric means of the *proC* and *rpoD* genes.

Outer-membrane proteins. Cells growing in Mueller–Hinton broth for 18 h were harvested by centrifugation at 1500 ***g*** for 30 min at 8 °C, suspended in 30 mM Tris/HCl (pH 8·0), and then broken in a sonicator for 2 min. Unbroken cells were removed by centrifugation at 4 °C and membranes were pelleted by centrifugation at 100 000 ***g*** for 1 h at 4 °C and suspended in the same buffer. The inner membrane was solubilized by adding sodium *N*-lauroylsarcosinate to the suspension at a final

Table 1. Primers used in the real-time PCR quantification studies

Gene	Forward	Reverse
proC	5′ CAG GCC GGG CAG TTG CTG TC 3′	5′ GGT CAG GCG CGA GGC TGT CT 3′
rpoD	5′ GGG CGA AGA AGG AAA TGG TC 3′	5′ CAG GTG GCG TAG GTG GAG AA 3′
oprB	5′ CGA GGG CGA GGA CTT CAA CAG C 3′	5′ CTC CAG GTT CGA CGG GTT CTG C 3′
ampC	5′ AGA TTC CCC TGC CTG TGC 3′	5′ GGC GGT GAA GGT CTT GCT 3′
oprD	5′ TCC GCA GGT AGC ACT CAG TTC 3′	5′ AAG CCG GAT TCA TAG GTG GTG 3′
oprM	5′ CCA TGA GCC GCC AAC TGT C 3′	5′ CCT GGA ACG CCG TCT GGA T 3′
oprN	5′ GCG CGA GAA GAT TGC CCT GAG 3′	5′ GCG GCG AAA GGT CCA CTG TCA 3′

concentration of 1 % and incubating for 30 min at room temperature. The outer membrane was pelleted by centrifugation at 40 000 ***g*** for 40 min at 4 °C and then suspended in the buffer. The proteins were analysed by SDS-PAGE with 10·7 % (w/v) acrylamide and 0·3 % (w/v) *N*,*N*′-methylene-bis-acrylamide in the running gel. Samples for SDS-PAGE were treated with 2 % SDS–5 % 2-mercaptoethanol at 100 °C for 5 min and then subjected to electrophoresis at a constant 60 V for 2 h.

Study design and statistical analysis. Twelve independently obtained cDNA samples of PT5 were diluted twofold up to six dilutions and used as controls in different experiments. First, the raw concentration data of the control dilutions were evaluated statistically to assess the magnitude of sampling errors and all other application deviations. Second, housekeeping genes *proC* and *rpoD* were statistically analysed for stability to validate their use as internal controls. Third, the normalized values of induced target genes were compared against uninduced controls for the relative expression levels of mRNA.

Statistical analyses were performed with the aid of the statistical package SPSS (version 9.0). Gene stability was calculated by Spearman correlation coefficients on the raw concentration data (Savli *et al.*, 2003).

Relative comparisons of the resistance genes were made after correcting the crossing point (CP) values by the geometric mean of *proC* and *rpoD* genes with the aid of a freely distributed Excel applet REST (Pfaffl *et al.*, 2002). This applet calculates the relative expression of a gene, which is defined as the expression ratio of a target gene against its control by the Pair-Wise Fixed Reallocation Randomization test. The details are described elsewhere (Pfaffl *et al.*, 2002).

RESULTS

Descriptive statistical data from the observed versus expected values of the serial dilutions are shown in Table 2. In this analysis, the standard deviations of the observed values were within ±0·5 of the means. Therefore, higher variations in the relative comparisons were accepted as significant. The second analysis was performed on correlations between the raw concentrations of the studied genes. This comparison revealed that *proC* and *rpoD* had the highest correlation coefficients (0·911). This high correlation validated the use of these genes as internal controls in these experiments. Subsequently, in relative comparisons, raw data were normalized using geometric means of these genes.

Relative comparisons of the mRNA from uninduced strains were obtained against the type strain PT5 (Fig. 1). This revealed the up-regulation of *oprM* in M7 and up-regulation of *oprN* in PT149, as expected (Table 3). However, the mRNA

Table 2. Observed versus expected quantities of the *proC* gene from the serially diluted cDNA of PT5

Values are the copy number.

Expected*	Observed				
	Mean	SE	SD	Minimum	Maximum
1600	1482·58	54·48	188·71	1191·00	1831·00
800	740·68	38·37	132·92	540·60	908·20
400	446·83	18·19	63·00	346·30	548·90
200	269·63	15·03	52·07	190·80	372·40
100	98·32	10·41	36·06	49·66	163·90
50	45·39	3·46	11·99	31·26	70·42

*Expected concentrations were assigned arbitrarily.

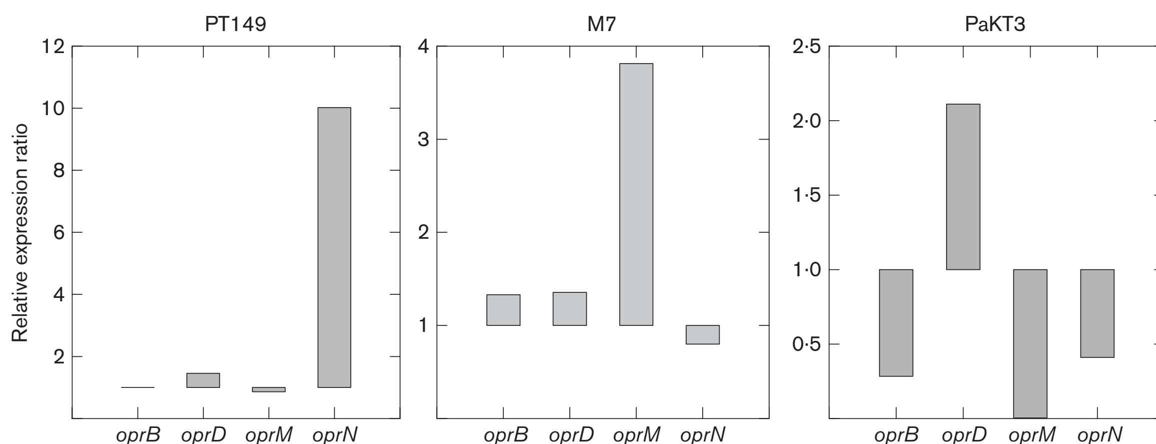

Fig. 1. Anchor bar chart (anchor set point, 1) of *oprB*, *oprD*, *oprM* and *oprN* genes relative to the corresponding genes of the type strain PT5 (PAO1).

Table 3. Expression ratios and the significance of studied genes relative to the type strain PT5 in uninduced conditions

Expression ratios and *p* values were calculated by the Pair-Wise Fixed Reallocation Randomization test.

	Relative expression ratio (*p*)			
	oprB	***oprD***	***oprM***	***oprN***
M7	1·330 (0·672)	1·355 (0·457)	3·814 (0·035)	0·800 (0·694)
PT149	0·997 (0·970)	1·460 (0·249)	0·864 (0·327)	10·026 (0·033)
PaKT3	0·283 (0·001)	2·111 (0·057)	0·003 (0·001)	0·410 (0·001)

level of *oprD* was not down-regulated in PT149. On the other hand, the outer-membrane fractions in the SDS-polyacrylamide gel of PT149 and M7 were consistent with the previous findings (Kohler *et al.*, 1997), such as the OprD protein was down-regulated in PT149 while the OprM protein was up-regulated in M7 (*nalB* mutant). Interestingly, both bands were absent in PaKT3 (Fig. 2).

The relative expressions of mRNAs from induced versus uninduced bacteria are shown in Table 4. This showed three important features: first, imipenem especially at the high concentration, induced *oprN* in most instances; second, imipenem, and to a lesser extent meropenem, induced *oprM* in PaKT3 but not in others; and last, *oprD* was not significantly affected by antibiotic pressure.

These unexpected results prompted us to repeat the experiments under sub-MIC levels of antibiotics over two time periods, i.e. 45 min and 3 h (Table 5). The *ampC* gene was strongly induced by imipenem as expected, and *oprD* again was not down-regulated under imipenem pressure. However, imipenem and meropenem in most instances increased *oprM* and more strikingly increased *oprN* transcriptions.

DISCUSSION

We tested the responses of the *oprB*, *oprD*, *oprM* and *oprN* genes of *P. aeruginosa* to carbapenem antibiotics at the mRNA level. Genes *oprD*, *oprM* and *oprN* are antimicrobial resistance related and *oprB* is a carbohydrate gateway (Wylie & Worobec, 1995). The latter was included to serve as a control for cellular status. We showed that *oprB* was not significantly affected by the inducing conditions even with the high levels of carbapenems. In other words, it is highly probable that the inducing conditions in this study did not cause sharp reactions in the cellular metabolism of *P. aeruginosa* within the initial 45 min.

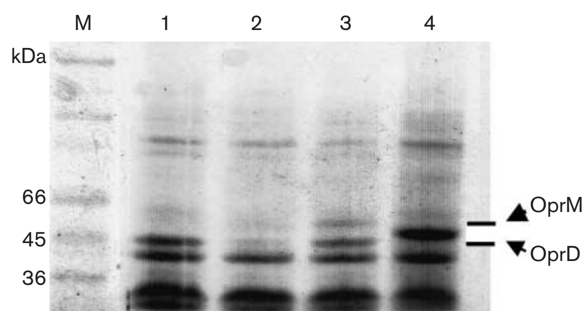

Fig. 2. SDS-PAGE analysis of the outer-membrane proteins of the studied strains; the outer membranes were prepared and solubilized in 2 % SDS–5 % 2-mercaptoethanol at 100 °C for 5 min. Lanes: M, molecular mass marker; 1, PT5 (type strain); 2, PT149 (*nfxC*-type mutant); 3, M7 (*nalB* mutant); 4, PaKT3.

It has been shown in outer-membrane diffusion studies that OprD is the principal portal of entry for imipenem (Huang & Hancock, 1996; Perez *et al.*, 1996). Confirming these experiments, imipenem-resistant isolates, in some studies, have been found to be deficient in the OprD protein (Trias *et al.*, 1989; Yoneyama & Nakae, 1993). Therefore, a relation between the deficiency of OprD and imipenem resistance is evident. To our knowledge, this relationship has not been previously studied at the mRNA level. We tested this relationship in four selected strains, one of which, PT149, is a mutant with down-regulation of the OprD protein. Interestingly, *oprD* mRNA was not down-regulated in this strain. The disagreement between protein and mRNA levels of *oprD* in PT149 has been shown by indirect methods previously and was explained by the existence of a post-transcriptional regulation pathway (Kohler *et al.*, 1997). However, one might expect a negative response (down-regulation) of *oprD* mRNA among the other three strains during imipenem pressure, which did not appear to occur. These experiments failed to show a significant relationship between *oprD* mRNA levels and imipenem or meropenem induction in the tested strains. This study, therefore, provided further evidence for the existence of a post-transcriptional regulatory pathway for OprD.

Another significant finding was the absence of the band corresponding to OprD in PaKT3 despite the slightly increased level of its mRNA. This strain was a fully susceptible clinical isolate and so the absence of the OprD band needs to be explored in further studies.

The highly inducible nature of OprM in PaKT3 is worthy of note. In uninduced conditions, OprM mRNA was down-regulated by a factor of 0·003. In other words, *oprM* was almost undetectable in PaKT3, which is consistent with the absence of the corresponding protein band in the SDS-PAGE

Table 4. mRNA levels of induced strains relative to uninduced control strains

Values are the copy number.

Antibiotic concn*	Strain	Imipenem/meropenem			
		oprB	*oprD*	*oprM*	*oprN*
High	PT5	0·94/0·71	1·47/1·18	0·98/0·96	0·59/0·43
	M7	0·46/0·34	0·76/0·66	1·04/0·59	0·44/0·48
	PT149	1·10/0·80	1·64/1·06	1·49/1·30	2·55/1·56
	PaKT3	1·14/1·67	1·70/2·54	13·59/1·96	3·85/2·28
Low	PT5	1·70/2·65	2·05/1·66	0·89/0·98	4·80/6·29
	M7	0·65/0·93	2·10/2·23	0·84/0·87	1·04/1·32
	PT149	0·87/2·33	0·92/1·41	0·82/1·10	1·44/1·27
	PaKT3	1·69/1·46	1·79/1·28	9·13/3·04	2·71/1·31

*High, imipenem 16 μg ml^{-1} and meropenem 8 μg ml^{-1}; low, imipenem 1 μg ml^{-1} and meropenem 0·5 μg ml^{-1}.

Table 5. Relative mRNA levels of induced strains at exponentially growing phase

Values are the copy number.

Incubation time*	Strain	Imipenem/meropenem†			
		ampC	*oprD*	*oprM*	*oprN*
45 min	PT5	43·34/1·39	2·78/2·25	3·62/2·16	0·24/0·20
	M7	37·37/1·08	1·02/1·04	1·60/4·21	0·15/0·24
	PT149	52·45/1·52	2·22/0·52	4·70/1·43	2·33/0·98
	PaKT3	3·30/2·60	3·74/3·05	1·29/3·70	1·06/1·61
3 h	PT5	18·64/2·23	2·06/9·13	0·66/1·29	0·27/0·22
	M7	23·72/1·61	1·44/1·97	0·71/0·57	393·60/1360·50
	PT149	1·06/0·76	0·31/1·97	0·13/1·78	0·68/60·98
	PaKT3	1·53/0·08	1·54/1·46	1·98/1·14	3·08/10·99

*Incubation times under antibiotic pressure.

†Antibiotic concentrations are at sub-MIC levels: imipenem 0·5 μg ml^{-1} and meropenem 0·125 μg ml^{-1}.

analysis. When induced by imipenem it was up-regulated significantly. Imipenem failed to induce OprM in other test strains. The fact that *oprM*, in contrast to the other strains, was highly inducible in PaKT3 could be explained by the existence of a defective repressor gene, *mexR*. Sequence analysis, however, failed to show any insertion or deletion in the *mexR* region (data not shown) of PaKT3.

The mRNA of *oprN* was induced by imipenem in all strains, except M7. This was also unexpected, because it has been demonstrated that imipenem is not a substrate of the MexEF–OprN pump (Maseda *et al.*, 2000).

Induction experiments under sub-MIC levels of antibiotics at the exponentially growing phase further revealed the unpredictable and unexpected reaction of these porin and pump genes in *P. aeruginosa*.

This study provided interesting data on the mRNA responses of *P. aeruginosa* to carbapenem antibiotics. Imipenem induced *oprM* in one strain but not in others, *oprD* stayed relatively stable, and the responses of *oprN* were variable. In other words, transcriptional responses in *P. aeruginosa* were unpredictable and chaotic. Further studies are therefore needed to understand the pathways of mRNA regulation of resistance-related genes in *P. aeruginosa* during antibiotic pressure.

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