RESEARCH LETTER

Efflux system overexpression and decreased OprD contribute to the carbapenem heterogeneity in *Pseudomonas aeruginosa*

Alexandros Ikonomidis[1], Athanassios Tsakris[2], Maria Kantzanou[1], Nicholas Spanakis[2], Antonios N. Maniatis[1] & Spyros Pournaras[1]

[1]Department of Microbiology, Medical School, University of Thessaly, Larissa, Greece; and [2]Department of Microbiology, Medical School, University of Athens, Athens, Greece

Correspondence: Spyros Pournaras, Department of Microbiology, Medical School, University of Thessaly, Larissa 41110, Larissa, Greece. Tel.: +30 2410 682537; fax: +30 2410 681570; e-mail: pournaras@med.uth.gr

Received 12 September 2007; accepted 19 October 2007.
First published online 7 December 2007.

DOI:10.1111/j.1574-6968.2007.00997.x

Editor: Stephen Smith

Keywords
Pseudomonas aeruginosa; carbapenem; heteroresistance; mutants; OprD; efflux pump overexpression.

Abstract

Pseudomonas aeruginosa strains exhibiting a heterogeneous mode of growth against carbapenems have been described recently. This study investigated the underlying molecular mechanisms in four genetically unrelated *P. aeruginosa* clinical isolates that were previously characterized by population analyses as heterogeneously resistant against carbapenems. Mutant subpopulations of all four isolates had at least fourfold higher minimum inhibitory concentrations than those of native cells for imipenem and meropenem. The heterogeneous subpopulations, when compared with the respective native ones, had significantly increased transcription levels of the *mexB* and *mexY* genes ($P < 0.05$), whereas transcription levels of the *mexE* gene remained unchanged. They also exhibited significantly decreased expression of the *oprD* gene ($P < 0.05$) and decreased intensity of the protein band of the porin OprD. Upregulation of efflux systems, in part, and the decrease of OprD contribute to the heterogeneous growth against carbapenems in our *P. aeruginosa* clinical isolates.

Introduction

Pseudomonas aeruginosa is a clinically significant Gram-negative rod-shaped bacterium that may be selected and propagated within the hospital environment. Antimicrobial resistance in this species is a problem of growing concern and limits our therapeutic alternatives. Among β-lactams, carbapenems, mainly meropenem and imipenem, are potent agents for the treatment of infections due to multidrug-resistant *P. aeruginosa* isolates. However, diminished permeability, overexpression of the intrinsic efflux systems and production of carbapenem hydrolysing β-lactamases have been described as mechanisms that confer resistance to carbapenems in *P. aeruginosa* (Livermore & Woodford, 2006). It has also been proposed that drug dosing insufficient to eliminate *P. aeruginosa* mutants may contribute to the selection of carbapenem-resistant mutants among apparently susceptible populations (Tam *et al.*, 2005). In concordance with this observation, we have recently shown that carbapenem-heteroresistant subpopulations may exist among clinical *P. aeruginosa* isolates, and the phenotypic characteristics of this heterogeneous mode of growth were determined (Pournaras *et al.*, 2007). In the present study we investigated the molecular mechanisms that contribute to the expression of meropenem heterogeneity in these *P. aeruginosa* isolates.

Materials and methods

Strains and antibiotic susceptibilities

Four genetically unrelated, apparently imipenem- and meropenem-susceptible *P. aeruginosa* clinical isolates that were characterized previously as heterogeneously resistant to carbapenems (Pournaras *et al.*, 2007) were randomly selected and included in this study (isolates 1, 5, 6 and 10). These native populations as well as their heterogeneous subpopulations grown in the highest meropenem concentration in population analyses were tested in all experiments. Imipenem, meropenem, ceftazidime, gentamicin, amikacin, netilmicin and ciprofloxacin minimum inhibitory concentrations (MICs) of the native and the respective meropenem heterogeneous subpopulations were determined by agar dilution according to CLSI (2007). Increments of 2 mg L^{-1}

for concentrations ranging from 2 to 32 mg L^{-1} and of 8 mg L^{-1} from 32 to 64 mg L^{-1} were used to determine more precisely MICs of native and heterogeneous subpopulations.

DNA manipulations and PCR

PCR testing of the isolates for metallo-β-lactamase (MBL) genes bla_{IMP}, bla_{VIM} and bla_{SPM} was performed using consensus primers for each enzyme group (Pournaras *et al.*, 2005). The presence and expression of genes *mexB* and *mexY* that respectively represent the efflux systems MexAB-OprM and MexXY-OprM affecting meropenem activity and genes *mexE* and *oprD* (Dumas *et al.*, 2006) were investigated by qualitative and quantitative real-time reverse transcription-PCR (QRT-PCR). These measurements were made in triplicate using an MX3005P instrument (Stratagene, La Jolla, CA) with Brilliant SYBR Green (Qiagen, Hilden, Germany), following a previously described protocol (Maniati *et al.*, 2007). Gene expression in meropenem subpopulations was expressed as the fold increase relative to the respective native populations, which were used as controls. The single-copy housekeeping gene *rpsL* was used for normalization (Sobel *et al.*, 2003). Negative control QRT-PCR reactions with PCR amplification of RNA extract without being reverse transcribed were included in the quantitative assays to check for contaminating DNA.

Outer membrane protein detection and sequencing

Outer membrane protein (OMP) analysis was performed according to a previously described protocol (Fernandez-Cuenca *et al.*, 2003). Protein concentration was calculated by UV spectrophotometry at 280 nm. Electrophoresis of 100 μg of protein extract was performed using a Novex 10–20% Tricine Gel 1.0 mm Kit (Invitrogen) according to the manufacturer's instructions. Silver staining was achieved using a SilverQuest Staining Kit (Invitrogen).

After electrophoresis OMPs were transferred from the gel to PVDF nylon membranes (Immobilon^{TM}, Millipore) using a semidry transfer system (Yrdimes, Wealtec). The protein band corresponding to the molecular weight of porin OprD (*c.* 46 kDa) was cut from the membrane and protein sequencing by Edman degradation was performed using a 473 Applied Biosystems protein sequencer. Chemicals and methods were those recommended by the manufacturer.

Statistical analysis

The statistical significance of mean fold change in expression of the genes tested by QRT-PCR was determined via a paired *t*-test using GraphPad Prism Version 4.00 software (GraphPad Software, Inc.). The level of significance for all analyses was <0.05.

Results

MIC data for the native and mutant subpopulations for meropenem, imipenem, ceftazidime and antimicrobials that are putative substrates for the studied efflux systems (gentamicin, amikacin, netilmicin and ciprofloxacin) (Poole, 2004) are shown in Table 1. The native populations had meropenem and imipenem MICs ranging from 0.5 to 1 mg L^{-1} and from 1 to 4 mg L^{-1}, respectively. Meropenem and imipenem MICs of the mutant subpopulations ranged from 4 to 10 mg L^{-1} and from 14 to 22 mg L^{-1}, respectively, and remained stable after seven daily subcultures in antibiotic-free medium. Notably, mutant subpopulations of all four strains had at least fourfold higher MICs than those of native cells for both carbapenems tested. In all cases, native and mutant subpopulations had invariable low ceftazidime

Table 1. Susceptibility status and expression of genes *mexB*, *mexY*, *mexE* and *oprD* in meropenem mutants relative to the respective native strains

	Agar dilution MICs (mg L^{-1})							Fold of change in gene expression in the mutant subpopulations			
Isolate	MEM	IPM	CAZ	GEN	AMK	NET	CIP	*mexB*	*mexY*	*mexE*	OprD
1	0.5	1	2	6	8	8	0.25				
Mm of isolate 1	4	22	2	6	12	12	0.5	1.69 ± 0.08 ($P = 0.002$)	1.89 ± 0.05 ($P < 0.001$)	1.02 ± 0.03 (NS)	-1.38 ± 0.02 ($P < 0.001$)
5	1	2	2	8	32	24	0.5				
Mm of isolate 5	6	20	2	16	48	48	1	1.52 ± 0.11 ($P = 0.007$)	1.99 ± 0.13 ($P = 0.003$)	1.10 ± 0.09 (NS)	-1.44 ± 0.05 ($P = 0.002$)
6	1	4	4	4	4	2	0.25				
Mm of isolate 6	8	16	4	6	12	12	0.5	2.08 ± 0.07 ($P < 0.001$)	2.73 ± 0.16 ($P = 0.001$)	1.09 ± 0.07 (NS)	-1.29 ± 0.02 ($P < 0.001$)
10	0.5	1	2	4	12	6	0.12				
Mm of isolate 10	10	14	4	12	48	12	0.5	1.39 ± 0.02 ($P < 0.001$)	2.05 ± 0.06 ($P < 0.001$)	1.13 ± 0.11 (NS)	-1.30 ± 0.03 ($P = 0.002$)

Mm, meropenem mutant; IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; GEN, gentamicin; AMK, amikacin; NET, netilmicin; CIP, ciprofloxacin; NS, not significant.

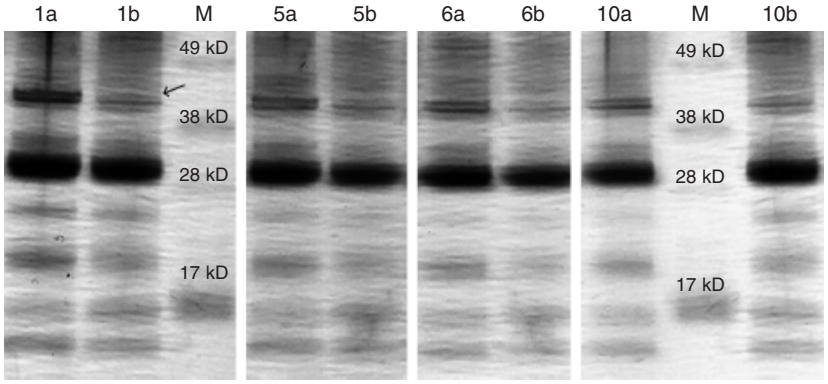

Fig. 1. Silver-stained OMP profile of the isolates included in this study. The letters a and b correspond to the native and meropenem heterogeneous populations, respectively, of each isolate. Arrows indicate porin OprD.

MICs ranging from 2 to 4 mg L^{-1}, indicating that *ampC* was probably not overexpressed.

All strains were negative for MBL production as shown by PCR but positive for the efflux pump coding genes tested as well as for the *oprD* gene. QRT-PCR revealed that the heterogeneous subpopulations in comparison with their native populations had increased levels of transcription of the *mexB* and *mexY* genes, ranging from *c.* 1.39- to 2.08-fold for the *mexB* gene and from 1.89- to 2.73-fold for the *mexY* gene. These differences in expression relative to the native populations were statistically significant in all four isolates and for both efflux genes ($P<0.05$) (Table 1). OMP analysis revealed lower intensity of an *c.* 46-kDa protein band in meropenem mutants compared with the native populations. Sequence analysis determined this band to be consistent with the porin OprD (Fig. 1). A decreased expression of the *oprD* gene (ranging *c.* from -1.29- to -1.44-fold) was also confirmed by QRT-PCR ($P<0.05$) but insignificant changes in expression of the *mexE* gene were detected ($P>0.05$) (Table 1).

Discussion

Heterogeneous growth against carbapenems has been previously demonstrated in clinical isolates of *P. aeruginosa* that were apparently susceptible to carbapenems (Pournaras *et al.*, 2007). Those findings may indicate that inappropriate use of carbapenems may lead to resistance development and possible therapeutic failure. In the present study, we investigated in meropenem heterogeneous subpopulations the transcription levels of genes representative for the efflux systems MexAB-OprM and MexXY-OprM that directly affect meropenem activity in *P. aeruginosa* (Poole, 2004). It has been shown that concomitant overproduction of two multicomponent Mex pumps in *P. aeruginosa* strain PAO1 produced additive resistance effects (Lee *et al.*, 2000). Mutant subpopulations in this study showed significantly increased levels of mRNA for both genes when compared with the respective native strains, suggesting their contribution to the reduced levels of meropenem susceptibility. Furthermore, the lower susceptibility of meropenem mutant subpopulations to antimicrobials such as amikacin, which might be exported by the respective efflux systems, also supports the upregulation of those genes.

SDS-PAGE along with protein sequencing analysis revealed a decreased quantity of OprD in meropenem mutant subpopulations, which was confirmed by its significantly reduced expression in QRT-PCR. Expression of *mexE* was also quantified, as overexpression of the *mexEF–oprN* operon has been associated with decreased *oprD* expression (Kohler *et al.*, 1999a). It was found that the decreased expression of *oprD* was not associated with overexpression of this operon, supporting previous observations that OprD is influenced not only by a single repressor mechanism but also other undefined mechanisms (Ochs *et al.*, 1999; Quale *et al.*, 2006).

A previous study (Kohler *et al.*, 1999b) has shown a moderate influx rate also of meropenem via OprD, indicating a possible contribution of this porin deficiency not only to imipenem but also to meropenem resistance; similar conclusions have been drawn from clinical isolates (Quale *et al.*, 2006). Thus, the cross-heteroresistance of mutant subpopulations, compared with the native ones, to both carbapenems can be attributed only to OprD deficiency. Nevertheless, a combined contribution of upregulated efflux pumps and decreased OprD may better explain heterogeneous growth of carbapenem. Lastly, it could be postulated that the observed simultaneous contribution of unrelated mechanisms, such as two efflux systems and OprD, to the carbapenem heterogeneity may be due to a selection, under strong antibiotic pressure, of strains with altered metabolic profile or hypermutator features (Macia *et al.*, 2005).

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