Mailing address:
P. Tulkens
UCL 73.70 av. Mounier 73
1200 Brussels - Belgium
tulkens@facm.ucl.ac.be

P1731

Phenotypic and genotypic detection of Mex efflux pumps in *P. aeruginosa*

N. Mesaros, N. Caceres, F. Van Bambeke, M.P. Mingeot-Leclercq and P.M. Tulkens

Unité de Pharmacologie cellulaire et moléculaire, Université catholique de Louvain - Brussels - Belgium

Objectives : Infections by *P. aeruginosa* are difficult to manage because of its frequent multiresistance. Multidrug resistance is often associated with active efflux by several transporters, among which MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM possess a particularly large substrate specificity. Precocious detection of this mechanism of resistance by the clinical microbiologist appears worthwhile in this context.

Methods : we use 4 *P. aeruginosa* strains previously published as presenting resistance associated to 1 of these 4 Mex pumps. MIC were determined by the microdilution method for 3 antibiotics selected for their differential recognition by the 4 Mex pumps. Expression of inducible pumps (MexC, MexF) was looked for by RT-PCR and overexpression of constitutive pumps (MexB, MexXY), by QC-RT-PCR.

Results : MIC were measured by the microdilution method in the absence or in the presence of 50 mg/L Phe-Ala-ß-Naphtylamide (inhibitor of Mex transporters – PAßNA). MIC of carbenicillin was markedly increased in MexAB-OprM overproducer, that of erythromycin, in MexCD-OprJ producer and that of gentamicin, in the MexXY-OprM producer. Erythromycin MIC was increased to a lower extent in the MexEF-OprN producer. In all cases, MIC came back to basal values in the presence of PAßNA. Semiquantitative RT-PCR confirmed the expression of MexC and of MexE. The QC-RT-PCR (Quantitative-Competitive-RT-PCR) demonstrates a 6-fold overexpression of MexA and a 8-fold overexpression of MexX.

Conclusions: Resistance due to active efflux by Mex pumps can be easily detected by a simple phenotypic characterization and further confirmed by molecular biology approaches.

INTRODUCTION

Pseudomonas aeruginosa is primarily a nosocomial pathogen, responsible for opportunistic infections. These are of great concern, because *P. aeruginosa* presents intrinsic as well as acquired resistance to a wide variety of antimicrobial agents. In this context, polyspecific efflux pumps play a central role in the multiresistance of *P. aeruginosa* towards both antibiotics and antiseptics.

Seven "Mex-type" efflux pumps able to transport various antimicrobial agents have been characterized in *P. aeruginosa,* but four of them (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) have been shown to contribute to clinically significant resistance levels (Llanes *et al*., 2004). Inhibitors of Mex efflux pumps have been described, among which Phenyl-Alanine-ß-Naphtylamide restores antibiotic activity (at least *in vitro*) in strains resistant by overexpression of efflux pumps (Lomovskaya *et al*., 2001).

In this context, the development of rapid and reliable methods for the early detection of this efflux pumps in clinical isolates may positively assist for the selection of appropriate therapeutic agents in a given patient and also for the screening of resistance mechanisms in epidemiological surveys. These could include both phenotypic (such as changes in MIC) and genotypic characterization (modification in gene expression level).

ACKNOWLEDGMENTS:

NM is recipient of a fellowship from the program Prospective Research in Brussels

NC is postdoctoral fellow, and FVB chercheur qualifié of the Belgian Fonds National de la Recherche Scientifique

We thank P. Plésiat (Besançon, France) for the kind gift of the strains

OBJECTIVES

To develop rapid and reliable methods for phenotypic and genotypic characterization of efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) in *Pseudomonas aeruginosa.*

METHODS

Strains and growth conditions: we used *P. aeruginosa* wild type (PAO1), *P. aeruginosa* overexpressing MexAB-OprM (*nalB* mutant) (Kohler *et al*., 1997), *P. aeruginosa* overexpressing MexCD-OprJ (*EryR* mutant) (M. Michea-Hamzehpour *et al*., 1995), *P. aeruginosa* overexpressing MexEF-OprN (*PAO7H* mutant) (Kohler *et al*., 1997) and *P. aeruginosa* overproducing MexXY-OprM (Hocquet *et al*., 2003). The strains were grown over-night on Muller-Hinton broth (MHB) at 37°C under aerobic conditions and gentle agitation (100 rpm).

MICs: Minimal inhibitory concentrations (MIC's) were determined by the broth microdilution method according to NCCLS guidelines. Inocula of $5x10^5$ bacteria/ml in exponential growth were used. Measurements were done in the absence and in the presence of 50 mg/ml of the pump inhibitor Phe-Ala-ß-Naphtylamide (PAßNA).

RNA extraction: Total bacterial RNA (tRNA) was isolated for each strain from 1.5 ml of late-log-phase *P. aeruginosa* cultures and treated with Rnase-free DNase (1U of enzyme/µg RNA for 20 min at room temperature). All samples were checked for DNA contamination. Five µg of tRNA was used in RT-PCR reactions.

Quantification of *mexA* and *mexX* expression level: The genes of interest were amplified from their respective cDNA with primers -F and -R. Internal competitors were generated by PCR for each gene in the presence of primer 40mer (F+internal 20 pb) and -R primer (see below). During the PCR reactions, decreasing amounts of internal competitors (1000 ag to 5 ag) were co-amplified with the same amount of target cDNA (1 µl). PCR reactions were performed in triplicates. The two amplified products were separated in 1.7% agarose, stained with ethidium bromide, and quantified by densitometry. The concentration of internal competitor DNA at which the two amplimers show equal intensity was assigned as the concentration of target cDNA.

Detection of *mexC* and *mexE* expression: To check the expression of *mexC* and *mexE* genes we used a semi-quantitative RT-PCR technique. The RNA was prepared as described for *mexA* and *mexX* and the PCR products were separeted and stained as described for *mexA* and *mexX*. To check the integrity of cDNA's, we have amplified also the *mexA* gene.

REFERENCES

- Llanes *et al*., Clinical Strains of Pseudomonas aeruginosa Overproducing MexAB-OprM and MexXY-OprM Efflux Pumps Simultaneously. 2004; AAC,48:1797-1802
- Lomovskaya *et al*., Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: novel agents for combination therapy. 200; AAC, 45(1):105-116
- Kohler *et al*., Differential Selection of Multidrug Efflux System by Quinolones in *P. aeruginosa*. 1997; AAC, 41:2540-2543
- M. Michea-Hamzehpour *et al*., OprK and OprM Define Two Genetically Distinct Multidrug Efflux Systems in *Pseudomonas aeruginosa*.1995; AAC,39:2392-2396
- Kohler *et al*., Characterization of MexEF-OprN, a Positively Regulated Multidrug Efflux System of *Pseudomonas aeruginosa*. 1997; Mol.Microbiol,23:345-354
- Hocquet *et al*., MexXY-OprM Efflux Pump is Necessary for Adaptive Resistance of *Pseudomonas aeruginosa*. 2003; AAC,47:1371-1375

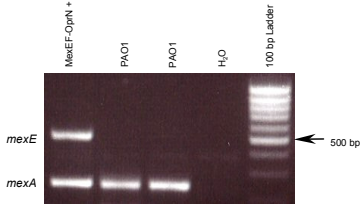
RESULTS: phenotypic characterization

MICs of *P. aeruginosa* strains used in this study.
The table shows the MIC measured in absence or in presence of 50 mg/L Phe-Ala-ß-Naphtylamide, an efflux pumps inhibitor (PAßNA).

drugs	PAO1	MexAB-OprM+	MexCD-OprJ+	MexEF-OprN+	MexXY-OprM+
Carbenicillin	32(3)	128(3)	16(3)	16(3)	32(3)
Carbenicillin+PAßNA	32(3)	32(3)	16(3)	16(3)	16(3)
Erythromycin	256(3)	256(3)	>1024(3)	128(3)	256(3)
Erythromycin+PAßNA	<16(3)	<16(3)	< 16(3)	<16(3)	<16(3)
Gentamicin	0.5(3)	0.5(3)	0.5(3)	0.5(3)	4(3)
Gentamicin+PAßNA	0.5(3)	0.5(3)	0.5(3)	0.5(3)	0.5(3)

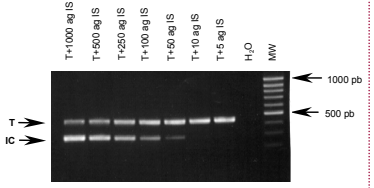
RESULTS: genotypic characterization

Detection of MexE/MexC expression by RT-PCR: MexEF and MexCD are not expressed in wild-type cells, so that amplification of the corresponding mRNA denotes resistance. We illustrate here RT-PCR amplification products of *mexE* (mexA, which is constitutively expressed, is used here as positive control for the RT-PCR reaction).

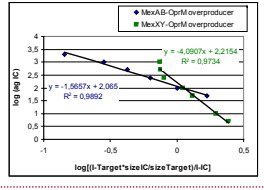


Quantification of mexA/MexX expression by QC-RT-PCR:

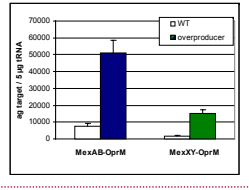
MexAB and MexXY overexpression can be quantified by QC-RT-PCR. We illustrate here the analysis for *mexX* in MexXY-OprM overproducer. Varying amounts of internal competitor (IC) (1000 ag to 5 ag) were co-amplified with 1 µl target cDNA (T) for 30 cycles. Ladder in the right side is 100 bp marker.



Quantification of *mexA* gene in MexAB-OprM overproducer and *mexX* gene in MexXY-OprM overproducer. The ratio between the intensity of two bands (T and IC) was plotted in log scale as a function of known amount of IS. The amount of IS for which the intensity of two bands was equal, was taken as target cDNA concentration.



Expression of mexAB-oprM and mexXY-oprM genes in wild type and overproducer strains. The expression of the *mexB* gene is more important than that of the *mexX* gene in the wild type strain. The expression of the *mexB* gene is increased about 6fold and that of that of the *mexX* gene, about 8-fold in overproducer strains as compared to a wild type strain.



CONCLUSIONS

- **we developed fast and robust methods for the identification of MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM efflux pumps in *P. aeruginosa* using phenotypic and genotypic approaches, that could be setup for routine clinical assessments.**
- **phenotypic characterization is easily obtained by measuring the influence of an efflux pump inhibitor on the MIC of adequately selected antibiotics.**
- **the basal gene expression level in a wild type strain is higher for *mexA* gene than *mexX* gene**
- **the expression of the gene encoding *mexA* in the MexAB-OprM efflux pump is around 6-fold higher in an overproducer strain than in a wild type strain**
- **the expression of the gene encoding *mexX* in the MexXY-OprM efflux pump is around 8-fold higher in an overproducer strain than in a wild type strain**