FUNCTIONAL CHARACTERIZATION OF MEXXY AND OPMG IN AMINOGLYCOSIDE EFFLUX IN *PSEUDOMONAS AERUGINOSA*

Rungtip Chuanchuen[1], Wechsiri Wannaprasat[1] and Herbert P Schweizer[2]

[1]Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand;
[2]Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA

Abstract. MexXY is an active efflux system that contributes to intrinsic resistance to aminoglycosides in *Pseudomonas aeruginosa.* MexXY can function in combination with OprM in aminoglycoside efflux but may also functionally associate with another as yet unidentified outer membrane channel. The possible role of OpmG as a third component of MexXY in aminoglycoside efflux was investigated by construction of unmarked Δ*opmG* mutants. Loss of OpmG did not have any impact on minimum inhibitory concentrations for aminoglycosides regardless of the presence of *oprM*, indicating that MexXY does not interact with OpmG in aminoglycoside efflux. In a clinical isolate PAJ010, Δ(*mexXY*) enhanced streptomycin susceptibility but neither Δ*oprM* nor *opmG* could, suggesting that MexXY functionally associates with an unidentified outer membrane protein for aminoglycoside efflux. Expression of an *opmG-lacZ* transcriptional fusion revealed that OpmG expression was neither constitutive nor inducible by gentamicin. Growth rates of wildtype *P. aeruginosa* and Δ*opmG* mutant derivatives were not different, indicating that expression of *opmG* is not essential for *P. aeruginosa* growth.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that can cause infections in both humans and animals. Treatment of *P. aeruginosa* infection is a therapeutic challenge as a result of the pathogen's intrinsic resistance to a wide range of antimicrobial agents. Aminoglycosides (AMGs) are a vital component of antipseudomonal chemotherapy with potent activity (Poole, 2005). However, *P. aeruginosa* clinical isolates that are resistant to AMGs have been isolated (Vogne *et al*, 2004; Wolter *et al*, 2004).

Broad-spectrum intrinsic resistance in *P. aeruginosa* results from the synergy of a low-outer membrane permeability, which reduces antibiotic influx, and active mutidrug efflux pumps that effectively extrude antibiotics from the cells (Schweizer, 2003). To date, at least six multidrug efflux systems belonging to the resistance-nodulation-cell division (RND) efflux system have been characterized in *P. aeruginosa*. These include MexAB-OprM (Poole *et al*, 1993), MexCD-OprJ (Poole *et al*, 1996), MexEF-OprN (Koehler *et al*, 1997), MexXY (Aires *et al*, 1999), MexJK (Chuanchuen *et al*, 2002), and MexGHI-OpmD (Aendekerk *et al*, 2002). Among these efflux systems, only MexXY efflux pump has been implicated in AMG-resistance (Masuda *et al*, 2000a). MexX is a periplasmic-membrane fusion protein that is associated with MexY, an inner membrane drug-proton antiporter. As the RND type efflux system functions as a tripartite system, the *mexXY* operon does not contain a gene for an outer membrane protein channel and can func-

Correspondence: Dr Rungtip Chuanchuen, Faculty of Veterinary Science, Chulalongkorn University, Pathumwan, Bangkok 10300, Thailand.
Tel: 66 (0) 2218-9578; Fax: 66 (0) 2218-9577
E-mail: rchuanchuen@yahoo.com

tionally cooperate with OprM of MexAB-OprM for AMG efflux (Masuda *et al,* 2000a,b). MexXY-OprM also effluxes nonaminoglycosides tetracycline, erythromycin and fluoroquinolone (Masuda *et al,* 2000b). However, many authors have suggested that another as yet unidentified outer membrane protein is likely the native one for MexXY (Westbrock-Wadman *et al*, 1999). *P. aeruginosa* has more than 160 outer membrane proteins (Hancock and Brinkman, 2002) and there are 18 members of the OprM family present in the genome (Hancock and Brinkman, 2002). Of these OprM homologs, OpmG, Opml and OpmB have been proposed to co-function with MexXY (Murata *et al*, 2002; Jo *et al*, 2003). Among these outer membrane proteins, OpmG has been shown to be a major outer membrane channel involved in AMG efflux and it was proposed as the most likely candidate for the OMP channel of MexXY (Jo *et al,* 2003).

The aims of this study were to investigate a possible cooperation of OpmG with MexXY in AMG efflux. Function and expression of OpmG was also examined. These results will help to expand knowledge on the molecular architecture of multidrug efflux systems that lack outer membrane proteins.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains used in this study are shown in Table 1. Bacteria were grown at 37ºC on Luria Bertani (LB) agar, LB broth (Difco, BD Diagnostic Systems, MD) or in Mueller-Hinton broth (MHB; Difco). Antibiotics used in selective medium for *Escherichia coli* were ampicillin, 100 μg/ml; gentamicin (EM SCIENCE, Gibbstown, NJ), 15 μg/ml and spectinomycin, 150 μg/ml. Those used for *P. aeruginosa* were carbenicillin (Gemini Bio-Products, Woodland, CA), 500 μg/ml; gentamicin, 50 μg/ml and spectinomycin, 300 μg/ml. All other antibiotics were purchased from Sigma-Aldrich (St Louis, MO).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by the standard two-fold microdilution technique according to Clinical and Laboratory Standards Institute guidelines (CLSI) formerly NCCLS (NCCLS, 1998). Experiments were repeated on two independent occasions.

General DNA methodology

All routine DNA procedures were performed as previously described (Hoang *et al*, 1998; Sambrook and Russell, 2001). Preparation of chromosomal and plasmid DNA was performed using ISOQUICK Nucleic acid extraction kit (ORCA Research, Bothell, WA) and QIAprep® Mini-spin kit (Qiagen, Valencia, CA) respectively. DNA fragments were purified using the QIAQuick Gel Extraction kit (Qiagen).

Generation of unmarked deletion mutants

Unmarked deletions of *opmG* were generated using the Flp/*FRT* recombinase technology as previously described (Hoang *et al,* 1998). Gene replacement vectors were pPS1281 for Δ*opmG* and pPS1366 for Δ*oprM* (Chuanchuen *et al*, 2005). The resulting mutants were confirmed to contain the proper deletions by PCR and nucleotide sequencing analyses. For complementation, the OpmG-expressing plasmid pPS1313 (Chuanchuen *et al* 2005) and its vector control pVLT35 (de Lorenzo *et al*, 1993) were transferred into Δ*opmG* mutants.

Construction of a plasmid encoding *opmG-lacZ* transcriptional fusion

DNA sequence encompassing the *opmG* promoter region was PCR amplified from PAO1 genomic DNA using primers containing base mismatches (indicated by lowercase letters in the primer sequences) that introduced new restriction sites after amplification. The primer opmGpromup (5′-CGCCgAaTTCAA GGTGCTGATCTTCGTCG-3′′) introduced an *Eco*RI site (underlined) upstream of the *opmG*

Table 1
Bacterial strains and plasmid used in the study.

Strain or plasmid	Relevant properties	Source or reference
Strain		
PAO1	Laboratory wildtype	Watson and Holloway, 1978
PAK	Clinical isolate of wild-type invasive strain	Chiron
PAO267	PA3579 with Δ(*mexAB-oprM*)	Chuanchuen *et al,* 2001
PAO280	PA267 with Δ(*mexXY*)	Chuanchuen *et al,* 2001
PAJ003	PAO1 with Δ*opmG*	This study
PAJ004	PAK with Δ*opmG*	This study
PAJ010	Veterinary clinical isolates	This study
PAJ086	PAO267 with Δ*opmG*	This study
PAJ089	PA3579 with Δ*opmG*	This study
PAJ092	PAJ010Δ*opmG*	This study
PAJ093	PAJ010Δ*oprM*	This study
PAJ094	PAJ010Δ*oprM*Δ*opmG*	This study
PAJ095	PAKΔ*oprM*	This study
PAJ096	PAKΔ*oprM*Δ*opmG*	This study
Plasmid		
PCR2:1	Ap^r ; a cloning vector for PCR products	Invitrogen
pVLT35	Sp^r; a low copy number cloning vector	de Lorenzo *et al,* 1993
pTZ120	Cb^r, *lacZ* fusion vector	Chuanchuen *et al,* 2005
pJS009	Ap^r; pCR2.1 with 1,636-bp DNA fragment containing *opmG* promoter from PAO1	This study
pJS010	Ap^r; pTZ120 with 1,410-bp DNA fragment containing *opmG* promoter from pJS009	This study
pPS1313	Sp^r; pVLT35 carrying *opmG* under P_{tac}	Chuanchuen *et al,* 2005
pPS1281	Ap^r, Gm^r: pEX18Ap containing Δ*opmG*::*FRT-*Gm^r fragment	Chuanchuen *et al,* 2005
pPS1366	Ap^r, Gm^r: pEX18Ap containing Δ*oprM*::*FRT-*Gm^r fragment	Chuanchuen *et al,* 2005

Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Gm^r, gentamicin resistance; Sp^r, spectinomycin resistance; *FRT*, *Flp*-recombinase target; P_{tac}, *E. coli lac* operon and *trp* operon hybrid promoter.

start codon and ribosome-binding site. The primer opmGpromdown (5′-GTCCAaGCTt GTGCCCGCCTGGAGG introduced a *Hin*dIII site (underlined) downstream of *opmG*. The PCR product was cloned into pCRII-2.1 (Invitrogen, CA) and then the *Eco*RI-*Hin*dIII fragment containing the *opmG* promoter was ligated between the same sites of pTZ120 (Chuanchuen *et al,* 2005) to form pJS010. The correct orientation of insertion was confirmed by nucleotide sequencing. The fusion plasmid was transferred into PAO280 (Chuanchuen *et al*, 2001). β-Galactosidase activity was measured in M9-glucose medium in the presence and absence of gentamicin (0.4 μg/ml) every hour for up to 12 hours as previously described (Miller, 1992).

Reverse transcription-PCR

Total RNA was isolated from *P. aeruginosa* isolates using Qiagen RNeasy Mini kit (Qiagen) and treated with RNase-free DNaseI (Invitrogen). Primers used were mexYRTup (5′-AGCTACAACATCCCTA-3′) and mexYRTdown (5′AGCACGTTGATCGAGAAG-3′). cDNA was

synthesized from 1 μg of total RNA. PCR assay was performed in a final volume of 25 μl containing 5 μl of DNA, 10 pmoles of each primer and 12.5 μl of 2.5X PCR buffer (Eppendorf® MasterMix; Hamburg, Germany). Reaction mixture was incubated for 5 minutes at 95ºC, followed by 30 cycles of 30 seconds at 95ºC, 30 seconds at 65ºC and 30 seconds at 72ºC and a final step of 10 minutes at 72ºC.

Growth curve analysis

Cells were grown overnight at 37ºC with shaking in LB medium containing spectinomycin where needed. Growth rate was measured in the presence and absence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were inoculated at an initial absorbance at 540 nm of 0.05. Optical density of the culture was recorded at 1-hour interval for 10 hours.

RESULTS

Influence of OpmG on aminoglycoside efflux

A possible role of OpmG as the third component required for formation of a MexXY functional tripartite efflux system was assessed by determination of MICs for AMGs in *P. aeruginosa* strains lacking *opmG* (Table 2). The *opmG* gene was deleted in two reference strains, PAO1 and PAK, and in a clinical isolate PAJ010. PAK is a laboratory-adapted clinical strain previously used in the study of the outer membrane protein efflux channel and PAJ010 was randomly selected from our strain collection. Strains PAJ003 (PAO1Δ*opmG*), PAJ004 (PAKΔ*opmG*) and PAJ092 (PAJ010Δ*opmG*) exhibited MIC values that were not different from their parents. Deletion of *oprM* in PAJ004 yielded Δ*oprM*Δ*opmG* mutants (*ie*, PAJ096) with reduced MICs (4-fold) for gentamicin and neomycin, and the same double deletions in PAJ092 (*ie*, PAJ094) resulted in a reduced MIC (8-fold) for only gentamicin. Disruption of *opmG* in PA0267 did not affect AMG susceptibility.

Effects of OpmG on growth rates

Function of OpmG was examined by growth curve analysis. Growth rates of PAO1, PAK and their Δ(*mexXY*) derivatives (*ie*, PAJ003 and PAJ004, respectively) were analyzed. Loss of *opmG* was also complemented with the *opmG*

Table 2
AMG susceptibility of *P. aeruginosa* mutants lacking selected genes.

Strain	Genotypes[a]	MIC (μg/ml)[b]				
		GEN	KAN	STR	SPC	NEO
PAO1	*mexAB-oprM mexXY opmG*	1.6	64	16	512	16
PAJ003	*mexAB-oprM mexXY* Δ*opmG*	1.6	64	16	512	16
PAO267	Δ(*mexAB-oprM*) *mexXY opmG*	0.2	32	32	512	4
PAJ086	Δ(*mexAB-oprM*) *mexXY* Δ*opmG*	0.2	32	16	512	4
PAK	*mexAB-oprM mexXY opmG*	1.6	64	16	512	16
PAJ004	*mexAB-oprM mexXY* Δ*opmG*	1.6	64	32	512	16
PAJ095	*mexAB-*Δ*oprM mexXY opmG*	0.4	32	16	512	4
PAJ096	*mexAB-*Δ*oprM mexXY* Δ*opmG*	0.4	16	16	512	4
PAJ010	*mexAB-oprM mexXY opmG*	1.6	64	32	512	8
PAJ092	*mexAB-oprM mexXY* Δ*opmG*	3.2	64	16	512	8
PAJ093	*mexAB-*Δ*oprM mexXY opmG*	0.4	64	32	128	16
PAJ094	*mexAB* Δ*oprM mexXY* Δ*opmG*	0.4	64	32	128	16

[a]Only genotypes relevant to the expression of the pumps under study are listed.

[b]Values in boldface indicate MICs for the parent strains that are at least 4-fold different from those for the corresponding D(*mexXY*) derivatives

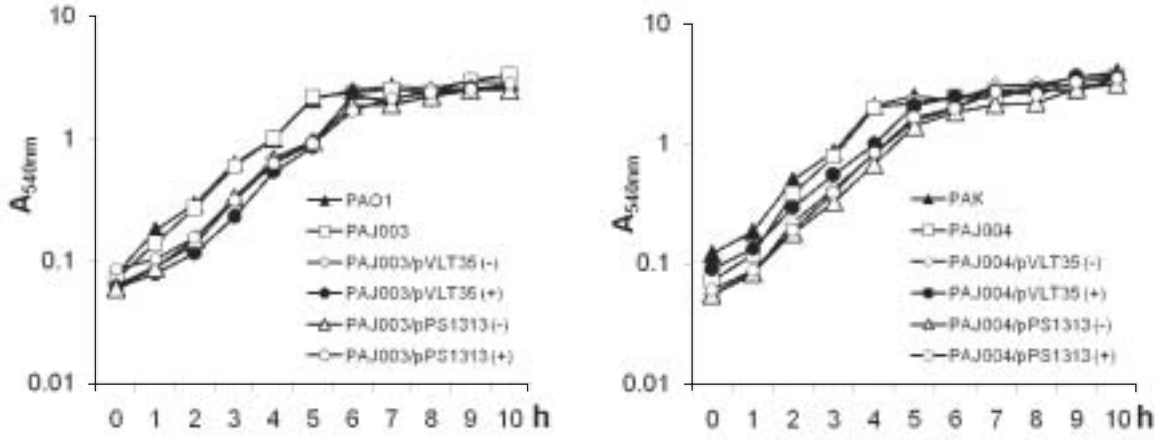

Fig 1–Growth of *P. aeruginosa* PAO1, PAK, PAJ010 and their Δ*opmG* mutant derivatives. Cells were inoculated at an initial absorbance at 540 nm of 0.05 in LB medium containing spectinomycin where needed. Optical density of the culture was measured in the presence (+) and absence (-) of 1 mM IPTG at 1-hour interval for 10 hours.

gene from pPS1313. Growth rates of all strains were the same either in the presence and absence of IPTG (Fig 1).

Expression of an *opmG-lacZ* transcription fusion

Expression of an *opmG-lacZ* transcriptional fusion was measured in a Δ(*mexXY*) mutant PAO280. β-Galactosidase activity of PAO280 containing the *opmG-lacZ* fusion plasmid and that containing pTZ120 control vector was not different, regardless of the presence of gentamicin at subinhibitory concentration (Fig 2). No PCR amplification was found when transcription of *opmG* in PAO1 was analyzed by reverse transcription analysis (data not shown).

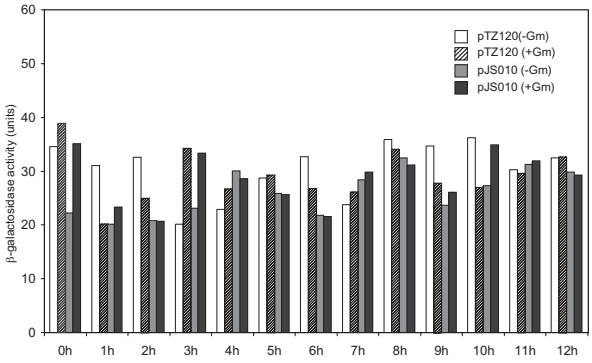

Fig 2–Expression of *opmG-lacZ*. Cells of PAO280 [Δ(*mexAB-oprM*) Δ(*mexXY*)] containing a plasmid-encoded *opmG-lacZ* (pJS010) and the vector control (pTZ120) were grown at an initial absorbance at 540 nm of 0.05 in M9-glucose medium supplemented with 100 μg/ml carbenicillin. β-Galactosidase activity was measured in triplicate samples in the presence and absence of 0.4 μg/ml gentamicin at 1-hour interval for 12 hours.

DISCUSSION

Even though several OMP candidates have been proposed to interact with MexXY, OMP(s) other than OprM interacting with these proteins in clinical isolates is not yet known. In this study, unmarked deletion mutants were generated to avoid possible undesired effects resulting from truncated protein. Loss of *opmG* did not affect the aminoglycoside MICs in the wildtype stains and their Δ*oprM* isogenic derivatives indicating that OpmG most likely does not functionally associate with MexXY in these strains. Our previous study showed that PAO267 [PAO1Δ*mexZ* Δ(*mexAB-oprM*)] was

susceptible to AMGs (Chuanchuen *et al,* 2001). In the present study, deletion of *opmG* in PA0267 did not further reduce AMG susceptibility supporting the non-functional association with MeXY. Interestingly, deletion of *mexXY* in PAJ010 enhanced (4-fold) the susceptibility to streptomycin but individual deletion of *oprM* (*ie*, PAJ093) and *opmG* (*ie*, PAJ092) did not have an impact on streptomycin MICs. Loss of both *oprM* and *opmG* (*ie*, PAJ094) also had a negligible influence. In this instance, it confirms that MexXY is able to interact with yet another OMP(s) to mediate, at least, streptomycin efflux.

Several outer membrane proteins function as channels for substrates needed for cell growth. These proteins allow substrates to cross outer membrane at a rate sufficient to support growth (Hancock *et al*, 1990). Since loss of OpmG does not affect growth rates of *P. aeruginosa*, it indicates that expression of *opmG* is not essential for cell growth.

OpmG was previously shown to be involved in AMG efflux (Jo *et al,* 2002, unpublished); however, it is shown in this study that this outer membrane protein does not functionally associate with MexXY. It is possible that OpmG may function as the efflux channel for other efflux systems. If this were true, then OpmG should be constitutively expressed. The results from determination of *opmG* expression indicate that OpmG is not constitutively produced and thus is unlikely to be available as an outer membrane channel protein for efflux systems, including MexXY. However, the *mexXY* operon is induced by exposure to many of its AMG substrates (Masuda *et al,* 2000a). Thus, it is possible that the presence of AMGs may be needed for the production of OpmG. Since β-galactosidase activity observed in the presence and absence of gentamicin was comparable (Fig 2), it indicates that expression of *opmG* is not induced by gentamicin. Taken together, the data confirmed our findings that OpmG is not functionally associated with MexXY in AMG efflux and OpmG and is not essential for growth.

In light of the results obtained, it confirms that MexXY can functionally utilize unidentified outer membrane proteins to efflux AMGs. Many characterized RND efflux systems contain outer membrane channel proteins that belong to the OprM family (Schweizer, 2003). Given the number of OprM homologs that have to be yet studied, it is possible that another candidate could be found within the OprM family. These findings warrant further studies (Schweizer, 2003). Since multidrug efflux pumps are the major resistance mechanisms in *P. aeruginosa*, inhibition of multidrug efflux system expression will help to reduce the level of intrinsic resistance leading to more susceptible strains. Currently, several efflux pump inhibitors have been investigated in order to improve and potentiate the activity of antibiotic substrates (Renau *et al*, 1999; Lomovskaya *et al*, 2001). Understanding of molecular architecture of multidrug efflux systems will benefit the future design of antibiotics and also of inhibitors of the systems.

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