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Substrate-Dependent Utilization of OprM or OpmH by the *Pseudomonas aeruginosa* MexJK Efflux Pump

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MexJK requires OprM for erythromycin efflux but not for triclosan efflux. Deletion of 15 OprM family outer membrane proteins (OMPs) revealed that only the TolC homolog OpmH functions with MexJK for triclosan efflux. This is the first report of natural utilization of multiple OMPs by a given resistance nodulation cell division transporter/membrane fusion protein pair.

Efflux pumps of the resistance nodulation cell division family (RND) are associated with the intrinsic or acquired multidrug resistance phenotype of gram-negative bacteria (for recent reviews, see references 17 and 22). The RND pumps, as typified by AcrAB-TolC of *Escherichia coli* (8), form a tripartite complex spanning the entire cell envelope. High-resolution crystal structures of the outer membrane protein (OMP) component TolC (16), the RND membrane transporter AcrB (20, 26), and the *Pseudomonas aeruginosa* AcrA homolog MexA (1, 11) revealed a mechanism by which RND pumps can achieve this (7). Our previous studies showed that the MexJK efflux system required OprM for erythromycin efflux but not for triclosan efflux, and we reasoned that either MexJ and MexK were sufficient for triclosan efflux or MexJ and MexK interacted with an OMP other than OprM to form a functional triclosan efflux pump.

We previously determined that OprJ, OprM, and OprN did not function with MexJK to assemble an active triclosan efflux pump (4), although the respective OMP functioned with MexAB-OprM, MexCD-OprJ, and MexEF-OprN in triclosan efflux (2, 4). Because we suspected that an OMP of the 18-member OprM family might associate with MexJK, we decided to individually delete the genes encoding the remaining 15 members of this family in strain PAO327 constitutively expressing MexJK but not OprJ, OprM, and OprN. Although a comprehensive PAO1 transposon library was recently published which contains insertions in all OprM family genes (14), we decided to generate unmarked deletion mutants to avoid possible undesired effects resulting from truncated OMPs. The respective promoterless coding sequences were PCR amplified from PAO1 genomic DNA using gene-specific primers (unpublished data). Plasmid-borne deletions were generated and used to construct unmarked deletions in the PAO327 chromosome, utilizing published methods (12) (plasmids and strains used in this study are listed in Table 1; a comprehensive list of intermediate plasmids and strains is available from the authors). *opmJ* was deleted using a modified PCR-targeted method (5) in which the target gene is first cloned into a plasmid, followed by λRED-mediated recombination of a PCR-generated mutated copy of the gene (10). Because PCR amplification of the *oprN* gene proved futile for unknown reasons, an *oprN*::IS*lacZ*/hah-Tc transposon insertion from strain 8802 (14) was transferred to PAO327 using a previously described transformation procedure (9). Using these methods, we succeeded in deleting 14 of the 15 genes encoding the OprM family of OMPs and insertionally inactivating *oprN*. The resulting strains, except PAO375, contain well-defined unmarked deletion alleles and have between 21 and 395 codons deleted from the respective coding sequences. For complementation, the promoterless inserts were directionally cloned into pVLT35 (6), in which the DNA inserts are under the transcriptional control of the *E. coli tac* promoter and the *lac* repressor.

MICs for triclosan (KIC Chemicals, Armonk, NY) and erythromycin (Sigma, St. Louis, MO) were used as a measure of MexJK efflux pump activity in this panel of defined OMP mutants and their complemented derivatives. MICs were determined by the twofold broth microdilution technique (2). An agar incorporation method was used to determine triclosan MICs of >128 μg/ml (3). Deletion of only one gene, *opmH*, disrupted MexJK-mediated triclosan efflux, and overexpression of OpmH restored MexJK function to wild-type levels (Table 2). Deletion of *opmH* and overexpression of OpmH resulted in a marginal (twofold) decrease or increase, respectively, in the MIC of erythromycin, indicating that MexJK-OpmH may also be able to mediate some erythromycin efflux. Deletion of any of the other OprM family OMP genes and complementation with the corresponding genes expressed from the *tac* promoter did not have any significant effects on triclosan and erythromycin MICs, indicating that none of these OMPs can be utilized by MexJK to catalyze efflux of these antimicrobials. Reverse transcription-PCR analysis indicated that all genes were highly expressed in isopropyl-β-D-thiogalactopyranoside-induced cells, verifying that lack of complementation was not due to lack of expression (data not shown).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or properties[a]	Source or reference
PAO1	wild type	13
PAO325	Δ(*mexAB-oprM*)[b] *nfxB* Δ(*mexCD-oprJ*) Δ(*mexJKL*) Δ(*mexXY*) OpmH$^+$	4
PAO327	Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*) Δ(*mexXY*) *mexL* MexJ$^+$K$^+$	4
PAO346	PAO327 with Δ*opmD*302[c]	This study
PAO351	PAO327 with Δ*opmM*81	This study
PAO353	PAO327 with Δ*aprF*153	This study
PAO355	PAO327 with Δ*opmL*133	This study
PAO357	PAO327 with Δ*opmF*123	This study
PAO359	PAO327 with Δ*opmG*151	This study
PAO361	PAO327 with Δ*opmA*21	This study
PAO363	PAO327 with Δ*opmB*282	This study
PAO365	PAO327 with Δ*opmE*209	This study
PAO367	PAO327 with Δ*opmH*362	This study
PAO369	PAO327 with Δ*opmK*53	This study
PAO371	PAO327 with Δ*opmI*184	This study
PAO373	PAO327 with Δ*opmJ*177	This study
PAO375	PAO327 with *opmN*::IS*lacZ*/hah-Tc	This study
PAO377	PAO327 with Δ*opmQ*395	This study
PAO386	PAO325 with Δ*opmH*362	This study
PAO397	PAO386 with Δ(*mexEF-oprN*)	This study
8802	Tet^r; PAO1 with *opmN*::IS*lacZ*/hah-Tc	14
KG4510	Δ(*nfxB-mexCD-oprJ*) *mexR* Δ*oprM* Δ(*mexXY*)	21
pBSP II KS(−)/SK(−)	Ap^r; broad-host-range cloning vectors	23
pCR2.1	Ap^r; TA cloning vector for PCR fragments	Novagen
pVLT35	Sp^r/Sm^r; broad-host-range cloning vector	6
pPS1150	Ap^r; pBSP II SK(−) with 6,945-bp NotI *mexJKL* fragment	4
pPS1266	Ap^r; pCR2.1 with 1,673-bp *opmH* PCR fragment	This study
pPS1313	Sp^r/Sm^r; pVLT35 with 1,664-bp PstI-HindIII *opmH* fragment from pPS1266	This study
pPS1319	Ap^r; pCR2.1 with 1,563-bp EcoRV-HindIII *oprM* PCR fragment	This study
pPS1369	Ap^r; pCR2.1 SpeI(blunt)+XhoI with 4,369-bp SgfI(blunt)-XhoI *mexJK* fragment from pPS1150	This study
pPS1370	Ap^r; pBSP II KS(−) carrying a 4,394 bp BamHI-XbaI *mexJK* fragment from pPS1369	This study
pPS1371	Ap^r; pPS1370 BamHI(blunt)+HindIII with 1,530-bp EcoRV-HindIII *oprM* fragment from pPS1319	This study
pPS1372	Ap^r; pPS1370 BamHI(blunt)+HindIII with 1,659-bp PstI(blunt)-HindIII *opmH* fragment from pPS1266	This study
pPS1373	Ap^r; pPS1371 HindIII(blunt)+ClaI with 1,665-bp *Pst*I(blunt)-ClaI *opmH* fragment from pPS1389	This study
pPS1389	Ap^r; pBSP II KS(−) with 1,664-bp PstI-HindIII *opmH* fragment from pPS1266	This study
pPS1424	Ap^r; pBSP II KS(−)XhoI(blunt)+HindIII with 1,659-bp PstI(blunt)-HindIII *opmH* fragment from pPS1266	This study
pPS1425	Ap^r; pBSP II KS(−)XhoI(blunt)+HindIII with 1,530-bp *Eco*RV-HindIII *oprM* fragment from pPS1319	This study

[a] Abbreviations: Ap^r, ampicillin resistance; *FRT*, Flp recombinase target; Gm^r, gentamycin resistance; Sm^r/Sp^r, streptomycin/spectinomycin resistance; Tet^r, tetracycline resistance.
[b] All deletions are unmarked but contain an *FRT* scar insertion; e.g., Δ(*mexAB-oprM*)::*FRT*.
[c] Allele numbers indicate the number of codons deleted from the respective genes.

If OpmH is indeed the OMP used by MexJK for triclosan efflux, then OpmH must either be constitutively expressed or be coexpressed with MexJK. Utilizing previously described procedures (21), rabbit anti-OpmH polyclonal antiserum was developed by immunization with three oligopeptides, VGDT RIAFDERPATVKRN (amino acids 77 to 94), LNQSEQSRE GQRRQV (amino acids 341 to 355), and AEQLQSKPRQQY (amino acids 471 to 482), based on the deduced OpmH primary sequence (24). Membrane preparation and immunoblot analyses were performed as previously described (21). The anti-OpmH polyclonal antiserum did not cross-react with OprM, OprJ, OprN, OpmD, OpmE, and OpmB in membrane preparations of strains expressing these proteins constitutively but did react with native and histidine-tagged OpmH expressed in *P. aeruginosa* KG4510 (data not shown). Immunoblot analysis of total membrane proteins from PAO1 grown in LB medium (19) revealed growth phase-independent constitutive OpmH expression (Fig. 1). Similar observations were made when transcription of *opmH* was analyzed in LB-grown cells with an *opmH′-lacZ* transcriptional fusion and real-time PCR (data not shown). In both approaches, subinhibitory triclosan concentrations (8 μg/ml) did not significantly affect *opmH* expression, i.e., triclosan did not induce *opmH* transcription.

To further assess the roles of OprM and OpmH in MexJK-mediated erythromycin and triclosan efflux, we constructed hybrid operons containing *mexJK* alone, *mexJK-oprM*, *mexJK-opmH*, and *mexJK-oprM-opmH*, where constitutive transcription of all genes originated from the *mexJK* promoter. Plasmids containing these hybrid operons were transformed into strain PAO397, which carries deletions for five major efflux systems

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TABLE 2. Contribution of *oprM* and *opmH* to erythromycin and triclosan efflux

Strain/plasmid	OMP gene deletion/complementation	Efflux components expressed	MIC (μg/ml)[a]	
			Tri	Ery
PAO327[b]	Δ*oprJ* Δ*oprM*	MexJK, OpmH	128	16
PAO367	Δ*oprJ* Δ*oprM* Δ*opmH*362	MexJK	16	8
PAO367/pVLT35[c]	Δ*oprJ* Δ*oprM* Δ*opmH*362	MexJK	16	8
PAO367/pPS1313[c]	Δ*oprJ* Δ*oprM* Δ*opmH*362/*opmH*$^+$	MexJK, OpmH	128	16
PAO397/pBSP II-KS	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*	None	2	8
PAO397/pPS1370[d]	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*	MexJK	2	8
PAO397/pPS1371	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*oprM*$^+$	MexJK, OprM	2	32
PAO397/pPS1372	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*opmH*$^+$	MexJK, OpmH	>1,024	8
PAO397/pPS1373	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*oprM*$^+$ *opmH*$^+$	MexJK, OprM, OpmH	512	64
PAO397/pPS1424	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*opmH*$^+$	OpmH	8	8
PAO397/pPS1425	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*oprM*$^+$	OprM	2	8

[a] Erythromycin (Ery) MICs were determined using the broth microdilution method, and triclosan (Tri) MICs were determined using the agar incorporation method, except for PAO327 and the PAO367 series of strains, for which the microdilution method was used.

[b] The genotype of PAO327 is Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*) Δ(*mexXY*) *mexL* (MexJK constitutively expressed) and MexEF-OprN uninducible due to a *mexT* insertion (18).

[c] In pVLT35 and pPS1313, *opmH* transcription is driven from the *lacI* repressor-controlled P_{tac} promoter; media used for MIC determinations with cells harboring these two plasmids were therefore supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (Gold Biotechnology, St. Louis, MO).

[d] Hybrid *mexJK* (pPS1370), *mexJK-oprM* (pPS1371), *mexJK-opmH* (pPS1372), and *mexJK-oprM-opmH* (pPS1373) operons were assembled on pBSP II KS(−). pPS1371 contains *oprM*, 52 bp of its upstream region and 13 bp of its downstream region without transcriptional terminator. pPS1372 and pPS1373 contain *opmH*, 72 bp of its upstream region and 136 bp of its downstream region, including the transcriptional terminator. The recombinant plasmids were transformed into Δ(*mexAB-oprM*) Δ(*mexCD-oprJ*) Δ(*mexEF-oprN*) Δ(*mexLJK*) Δ(*mexXY*) Δ*opmH*362 strain PAO397, and efflux of the indicated antimicrobials was assessed by MIC determinations. On pPS1370–pPS1373, constitutive transcription of the cloned genes originates from the *mexJK* operon promoter (P_{JK}). pPS1424 and pPS1425 are based on pVLT35, and transcription of cloned genes is driven from the *lacI* repressor-controlled P_{lac} promoter; media used for MIC determinations with cells harboring these two plasmids were therefore supplemented with 1 mM IPTG.

(including *oprJ*, *oprM*, and *oprN*) and the Δ*opmH*362 mutation. MICs for erythromycin and tetracycline were determined as a measure of efflux of the respective antimicrobials (Table 2). Expression of all genes was verified by reverse transcription-PCR (data not shown). It is evident that MexJK requires OprM for erythromycin efflux and OpmH for triclosan efflux, since in the absence of OprM and OpmH neither erythromycin nor triclosan efflux is observed. As expected, simultaneous expression of OprM and OpmH led to efflux of both antimicrobials, although triclosan efflux was somewhat lower when compared to efflux by the MexJK-OpmH pump. This is perhaps due to competition of OpmH and OprM for formation of a functional MexJK-OpmH triclosan efflux pump. Erythromycin efflux was marginally (twofold) higher in the strain expressing both OprM and OpmH when compared to efflux observed in the MexJK-OprM-expressing strain. Expression of OprM alone had no effect, indicating that the observed triclosan and erythromycin efflux was not due to the mere presence of this OMP alone or its interaction with yet another efflux system. In contrast, overexpression of OpmH resulted in a significant (fourfold) increase in the MIC for triclosan, indicating that OprM may be able to interact with yet another efflux system to mediate triclosan efflux. Of note also is that the triclosan MICs for PAO367 (16 μg/ml) and PAO397 (2 μg/ml) are significantly different, but we do not yet understand the cause for this difference.

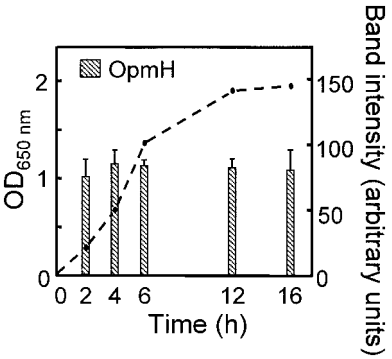

FIG. 1. Constitutive production of OpmH in *P. aeruginosa*. Cells of PAO1 were grown in LB medium, and aliquots were removed at the indicated time points after subculture. Total membranes (5 μg of protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to immunoblot analysis using anti-OpmH polyclonal antiserum. Relative expression levels of OpmH were determined by quantifying band intensities using the Scion Image program (Scion Corporation, Frederick, MD). Each bar represents the mean ± standard deviation of the intensity levels obtained in four experiments. The average optical density (650 nm) of the cultures used for sample preparation is overlaid.

The data presented here confirm that triclosan efflux in a strain expressing MexJK is mediated by a tripartite MexJK-OpmH efflux system. The constitutive expression and apparent absence of *opmH* cotranscribed efflux or secretion components confirm the previously published notion that the TolC homolog OpmH may play a more general role by interacting with more than one efflux system (15). However, the data presented here clearly indicate that these interactions may not be nonspecific, as previously suggested, but in some instances may rather depend on which substrate is being exported by the respective efflux system. How the OMP exerts its impact on substrate specificity of the pump remains unclear. One possibility is that the specific substrate export pathway, e.g., by opening of the exit pore, is formed only when all three proteins are properly assembled in the presence of the appropriate substrate. Another possibility is a substrate-induced assembly of the tripartite efflux pump, i.e., by recruitment of the appro-

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priate OMP, similar to what has previously been observed with the HlyBD-TolC protein export system (25).

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