

## Role of the Multidrug Efflux Systems of *Pseudomonas aeruginosa* in Organic Solvent Tolerance

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**Multidrug efflux pumps with a broad substrate specificity make a major contribution to intrinsic and acquired multiple antibiotic resistance in *Pseudomonas aeruginosa*. Using genetically defined efflux pump mutants, we investigated the involvement of the three known efflux systems, MexA-MexB-OprM, MexC-MexD-OprJ, and MexE-MexF-OprN, in organic solvent tolerance in this organism. Our results showed that all three systems are capable of providing some level of tolerance to organic solvents such as *n*-hexane and *p*-xylene. Expression of MexAB-OprM was correlated with the highest levels of tolerance, and indeed, this efflux system was a major contributor to the intrinsic solvent tolerance of *P. aeruginosa*. Intrinsic organic solvent tolerance was compromised by a protonophore, indicating that it is substantially energy dependent. These data suggest that the efflux of organic solvents is a factor in the tolerance of *P. aeruginosa* to these compounds and that the multidrug efflux systems of this organism can accommodate organic solvents, as well as antibiotics.**

Many organic solvents are toxic to microorganisms. Generally, toxicity of an organic solvent correlates inversely with the logarithm of its partition coefficient with *n*-octanol and water ( $\log P_{ow}$ ) (7, 14), at least for compounds with  $\log P_{ow}$  values between 1 and 5 (11). The toxicity of these compounds appears to be related to their ability to dissolve into biological membranes, disturbing the integrity of these structures and ultimately compromising their physiological function (for a review, see reference 11). Despite this, there have been numerous reports, particularly on members of the family *Pseudomonadaceae*, of strains demonstrating high-level tolerance of organic solvents (3, 14, 22), and in at least one case, this tolerance was attributed to active efflux of the organic solvent (15). Solvent tolerance has also been reported in *Escherichia coli* (2, 4), where it appears to be closely aligned with expression of the low-level multidrug resistance mediated by transcriptional activators encoded by the *marA* (6), *soxS* (24), and *robA* (23) genes. The *marA* gene forms part of an operon, *marRAB*, which is linked to the so-called multiple antibiotic resistance (Mar) phenotype (1). MarA-mediated multidrug resistance is known to involve the AcrAB-To1C multidrug efflux system (6, 8, 28), indicating that solvent tolerance in *E. coli*, too, likely involves solvent export (35). Consistent with this, increased expression of both AcrA and To1C has been demonstrated in organic solvent-tolerant mutants of *E. coli* (5).

*Pseudomonas aeruginosa* is an opportunistic human pathogen characterized by innate resistance to a variety of antimicrobial agents. This property is recognized to result mainly from the activity of broadly specific drug efflux systems (17, 18, 26, 31). Three such efflux systems have been described in *P. aeruginosa*, and they are encoded by the *mexA-mexB-oprM* (10, 19, 30, 31), *mexC-mexD-oprJ* (29), and *mexE-mexF-oprN* (16) operons. The MexA-MexB-OprM system has been demonstrated to contribute to the high intrinsic antibiotic resistance of this organism, and hyperexpression of the efflux genes is responsible for the elevated multidrug resistance of *nalB* mutants (19, 31, 32). MexC-MexD-OprJ and MexE-MexF-OprN

are apparently not expressed during growth under normal laboratory conditions but are expressed in *nfxB* (12, 29) and *nfxC* (9, 16) multidrug-resistant mutants, respectively. In light of the homology between AcrAB-To1C and the *P. aeruginosa* multidrug efflux systems (25), then, it was of interest to assess the involvement of the latter in organic solvent tolerance. We report here that the MexAB-OprM efflux system mediates intrinsic organic solvent tolerance in *P. aeruginosa* and that hyperexpression of this system in *nalB* mutants enhances such tolerance. Similarly, expression of the multidrug efflux systems MexCD-OprJ and MexEF-OprN also enhances solvent tolerance in this organism.

To study the role of multidrug efflux pumps in organic solvent tolerance, we used genetically defined efflux pump mutants (Table 1) and three organic solvents, *n*-hexane, *p*-xylene, and toluene (Table 2). Two approaches were employed to assess solvent tolerance. The first involved overlaying solvent (100%) onto 25-ml Luria-Bertani (LB) agar plates inoculated with bacteria as previously described (4). Briefly, stationary-phase LB broth cultures were diluted into the same medium to yield a suspension of approximately  $10^7$  cells/ml. A 5- $\mu$ l aliquot of the cell suspension was placed in duplicate on LB agar and allowed to dry before organic solvent was overlaid to a depth of approximately 2 mm. A variation of this method, termed efficiency of plating (EOP), was also employed (35). In this assay, 100  $\mu$ l of a cell suspension ( $10^7$  cells/ml) was spread over the surface of an LB agar plate which was subsequently overlaid with 1 ml of organic solvent. In both cases, the plates were sealed and growth was assessed following incubation at 30°C for 24 h. The second approach involved assessment of cell growth by measuring the increase in optical density at 660 nm ( $OD_{660}$ ) of a liquid culture supplemented with organic solvent. Briefly, stationary-phase cells were diluted into 30 ml of prewarmed (37°C) LB broth and incubated (with shaking) for 2 to 2.5 h at 37°C. At the early exponential phase of growth, organic solvent was added at a final concentration of 0.08 to 20% (vol/vol) and growth was monitored for a further 5 to 6 h. In some experiments, the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was included in the growth medium (20  $\mu$ M final concentration) to assess the influence of this energy inhibitor on the growth of *P. aeruginosa* in the presence and absence of organic solvents.

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TABLE 1. *P. aeruginosa* strains used in this study

Strain	Description	Reference
PAO1	Prototroph	
K1119	PAO1 $\Delta$ mexAB- <i>oprM</i>	20
OCR1	PAO1 <i>nalB</i>	21
ML5087	<i>ihv-220 thr-9001 leu-9001 met-9001 pur-67 aphA</i>	27
K1112	ML5087 <i>nalB</i>	33
K1110	ML5087 $\Delta$ <i>oprM</i>	20
K1121	ML5087 $\Delta$ mexAB- <i>oprM</i>	34
K1131	K1121 <i>nfxB</i>	20
K1115	ML5087 $\Delta$ mexAB- <i>oprM</i> $\Delta$ mexCD- <i>oprJ</i>	20
K1117	MexEF-OprN-hyperexpressing derivative of K1115	20
PAO6609	<i>met-9011 amiE200 rpsL pvd-9</i>	13
K1032	PAO6609 $\Delta$ mexAB- <i>oprM</i>	36

As shown in Table 2, all strains expressing wild-type levels of MexAB-OprM (PAO1, ML5087, and PAO6609), as well as the *nalB* mutants hyperexpressing MexAB-OprM (OCR1, and K1112), showed tolerance to *n*-hexane and *p*-xylene, but not to toluene, on agar plates. In EOP experiments, the latter strains elicited confluent growth while strains PAO1 and ML5087 yielded isolated colonies (Table 2), suggesting that *nalB* strains are better able to tolerate these solvents and, thus, that solvent tolerance in *P. aeruginosa* correlates with the level of expression of the MexAB-OprM efflux system. These isolated colonies did not appear to be solvent-tolerant mutants, as they elicited growth properties indistinguishable from those of the parental strains in liquid medium containing solvent (data not shown). The absence of a functional MexAB-OprM efflux system, due to deletion of either the entire *mexA-mexB-oprM* operon (in K1119 and K1032) or the *oprM* gene alone (in K1110), however, rendered these strains incapable of growth in the presence of any of the organic solvents tested (Table 2). Indeed, subsequent testing of the inoculation site after expo-

TABLE 2. Organic solvent tolerance of *P. aeruginosa* on agar plates

Strain	Relevant efflux phenotype <sup>a</sup>	Growth on <sup>a</sup> :		
		<i>n</i> -Hexane	<i>p</i> -Xylene	Toluene
PAO1	MexAB-OprM <sup>+</sup>	+ (S)	+ (S)	–
K1119	MexAB-OprM <sup>–</sup>	–	–	–
OCR1	MexAB-OprM <sup>++c</sup>	+ (C)	+ (C)	–
ML5087	MexAB-OprM <sup>+</sup>	+ (S)	+ (S)	–
K1110	MexAB <sup>+</sup> OprM <sup>–</sup>	–	–	–
K1112 <sup>d</sup>	MexAB-OprM <sup>++</sup>	+ (C)	+ (C)	–
K1131	MexAB-OprM <sup>–</sup>	–	–	–
	MexCD-OprJ <sup>+</sup>			
K1117	MexAB-OprM <sup>–</sup>	–	–	–
	MexEF-OprN <sup>+</sup>			
PAO6609	MexAB-OprM <sup>+</sup>	+	+	–
K1032	MexAB-OprM <sup>–</sup>	–	–	–

<sup>a</sup> Bacteria (5  $\mu$ l) were spotted onto LB agar, overlaid with organic solvent, and incubated overnight at 30°C as outlined in the text. +, growth; –, no growth. In parentheses are the results of EOP experiments in which 100- $\mu$ l cell cultures were plated on the surfaces of LB agar plates, overlaid with organic solvent, and likewise incubated overnight. C, confluent growth; S, single-colony growth. The log  $P_{ow}$  values of the solvents were as follows: *n*-hexane, 3.9; *p*-xylene, 3.1; toluene, 2.8.

<sup>b</sup> The status of those efflux systems (components) that are known to be expressed and, thus, potentially contribute to the organic solvent tolerance of the indicated strains is highlighted.

<sup>c</sup> High-level expression of MexAB-OprM.

<sup>d</sup> This strain yielded scattered colonies after 72 h of incubation in the presence of toluene.

sure to the solvents revealed that the bacteria applied to the plates were no longer viable, indicating that the solvents were bactericidal for these mutants. This was consistent with observations that exposure of these mutants to solvents in liquid medium precipitated a rapid decline in viable cell numbers, as assessed by using viable plate counts (data not shown).

Growth upon exposure to toluene occurred only for the *nalB* mutant *P. aeruginosa* K1112, a few toluene-tolerant colonies of which arose on plates after 72 h of incubation (Table 2). These were obviously mutants in that they subsequently displayed ready growth in the presence of toluene. Nonetheless, the fact that such mutants only arose from a strain already expressing elevated levels of MexAB-OprM suggests that this efflux system is able to accommodate toluene, thereby providing bacteria with a basal low-level tolerance from which mutants with high-level tolerance could be selected. Consistent with this, elevated production of MexAB-OprM was maintained in these mutants (assessed by Western immunoblotting of isolated cell envelopes with antiserum to OprM [20; data not shown]). That the solvent tolerance of so-called solvent-tolerant mutants can arise as a result of expression of multidrug efflux systems in *P. aeruginosa* was subsequently confirmed by the isolation of several mutants tolerant to 20% hexane (following serial passage of ML5087 in LB broth containing 3, 5, 7, 10, and, finally, 20% hexane) and the demonstration that >90% of these exhibited a multidrug resistance pattern indistinguishable from that of previously described *nalB* mutants (data not shown).

Cells were generally more sensitive to the effects of the organic solvents in liquid assays than in the plate assays. Consistent with its lower log  $P_{ow}$  value, *p*-xylene (log  $P_{ow}$  of 3.1) was more toxic than *n*-hexane (log  $P_{ow}$  of 3.9) and, thus, wild-type *P. aeruginosa* PAO1 was unable to grow in the presence of *p*-xylene at 1% (vol/vol) (Fig. 1C) or even 0.5% (vol/vol) (data not shown), although it grew well in hexane at 2% (vol/vol) (Fig. 1B). In contrast, *nalB* mutant strain OCR1 grew well and the  $\Delta$ mexAB-*oprM* mutant K1119 failed to grow at all in the presence of either solvent at these concentrations (Fig. 1B and C). Thus, solvent tolerance in liquid medium, as on solid medium, was enhanced by the presence of MexAB-OprM and was compromised by its absence. The failure to observe any differences in tolerance to hexane between PAO1 and its *nalB* derivative OCR1 (Fig. 1B), despite the qualitative differences seen on solid medium in the EOP experiments described above, probably reflected the levels of hexane used in the liquid-medium assays (2% [vol/vol]). Still, increasing the hexane level to 10% (vol/vol) in these assays also failed to discriminate between these strains, both of which grew quite well at this solvent concentration (data not shown). It seems likely, therefore, that the intrinsic levels of MexAB-OprM are more than sufficient to provide substantial tolerance to hexane, and only at much higher levels of hexane would differences between PAO1 and OCR1 be seen. Certainly, the differences on solid medium described above were observed when undiluted (i.e., 100%) hexane was used. Similarly, although PAO1 demonstrated tolerance to xylene on a solid medium, consistent with the expression of MexAB-OprM in this strain, neither PAO1 nor its *mexAB-oprM* deletion mutant K1119 grew in LB broth containing 0.5 to 1% (vol/vol) xylene. Indeed, we were unable to define a concentration of xylene which could discriminate between the wild-type strain and the MexAB-OprM<sup>–</sup> mutant in LB broth. These data highlight differences between the discriminating powers of the two assays and likely reflect unknown differences in the manner in which solvents interact with cells growing statically on solid surfaces versus shaken in liquid medium. Nonetheless, the obviously increased sensitivity of the *mexAB-oprM* deletion strains to organic sol-

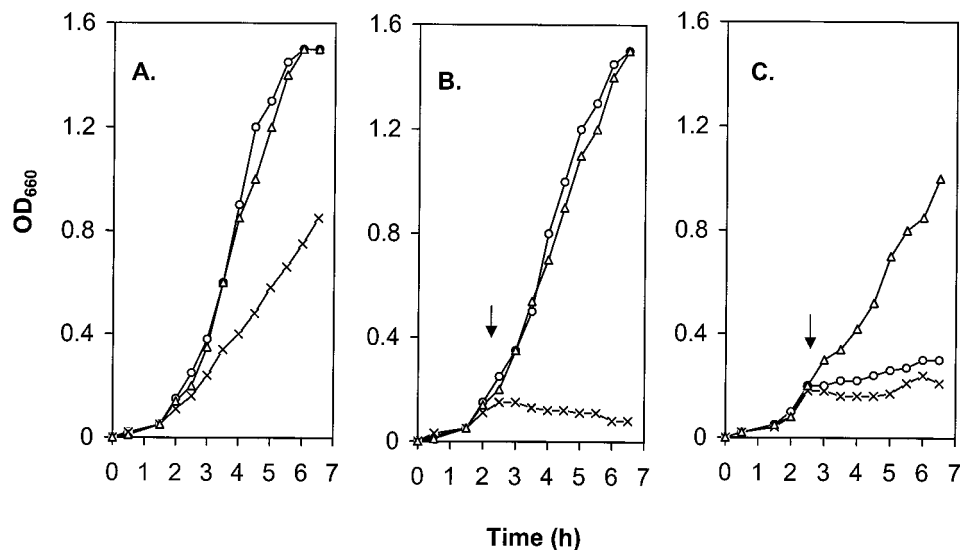


FIG. 1. Influence of the MexA-MexB-OprM efflux system on the growth of *P. aeruginosa* in the presence of organic solvents. *P. aeruginosa* PAO1 (○), K1119 ( $\Delta mexA-mexB-oprM$ ) (×), and OCR1 (*nalB*) ( $\Delta$ ) were grown in LB broth at 37°C to the early exponential phase, at which time (arrow) no solvent (A), *n*-hexane at 2% (vol/vol) (B), or *p*-xylene at 1% (vol/vol) (C) was added and growth determined by monitoring OD<sub>660</sub>.

vents is consistent with the notion that MexAB-OprM plays a significant role in intrinsic organic solvent tolerance in *P. aeruginosa*, just as it plays a major role in intrinsic multidrug resistance in this organism (17–20, 31). Interestingly, the growth rate of the *mexA-mexB-oprM* deletion mutant was reduced relative to that of the other two strains, even in the absence of solvent (Fig. 1A), although unlike solvent-containing cultures, solvent-free cultures of this mutant still elicited growth. It is likely, then, that some important cellular process (independent of multidrug and solvent efflux) is compromised as a result of the loss of MexAB-OprM in this organism.

The MexAB-OprM system functions as an energy-dependent exporter. Thus, any contribution of this efflux system to organic solvent tolerance (presumably via efflux) should be similarly energy dependent. To assess this, then, the influence of the protonophore CCCP, which was previously shown to compromise MexAB-OprM-mediated multidrug resistance and export (19), on the solvent tolerance of MexAB-OprM<sup>+</sup> strain PAO1 was assessed. Although PAO1 grew well in liquid medium in the presence of 0.2% (vol/vol) *p*-xylene (Fig. 2A), exposure of the cells to a concentration of CCCP (20 μM) which itself failed to adversely affect growth (Fig. 2A) almost completely abrogated the growth of this strain in the presence of 0.2% (vol/vol) *p*-xylene. Indeed, the effect of CCCP addition on the growth of PAO1 in the presence of xylene was comparable to the effect of deleting the *mexAB-oprM*-encoded efflux system (Fig. 2B), consistent with the idea that CCCP compromises MexAB-OprM activity and, thus, its contribution to solvent tolerance. Taken together, these results suggest that the MexAB-OprM efflux system exports organic solvents, as well as antibiotics.

The MexCD-OprJ and MexEF-OprN efflux systems are not expressed in wild-type cells, at least under standard laboratory conditions and in rich media (16, 29). To determine, therefore, if these systems could similarly contribute to organic solvent tolerance, strains hyperexpressing these efflux systems had to be examined. To overcome the contribution of MexAB-OprM to organic solvent tolerance, MexCD-OprJ and MexEF-OprN hyperexpression was selected in strains lacking MexAB-OprM. Strain K1121 lacks solvent tolerance as a result of the absence

of MexAB-OprM, and an *nfxB* derivative of this strain (K1131) failed to demonstrate tolerance to organic solvents (on solid media overlaid with solvent) at levels seen for MexAB-OprM<sup>+</sup> strain ML5087, despite the hyperexpression of MexCD-OprJ (Table 2). Still, K1131 grew substantially better than K1121 in LB broth supplemented with either 1% *n*-hexane (Fig. 3A) or 0.1% *p*-xylene (Fig. 3B), indicating that MexCD-OprJ hyperexpression did provide some measure of tolerance to these solvents. This was, however, markedly less than the 10% *n*-hexane or 1% *p*-xylene tolerance level seen in MexAB-OprM-hyperproducing strains. MexAB-OprM- and MexCD-OprJ-deficient strain K1115 was also sensitive to organic solvents in the solid-medium assay and remained so, despite the hyperexpres-

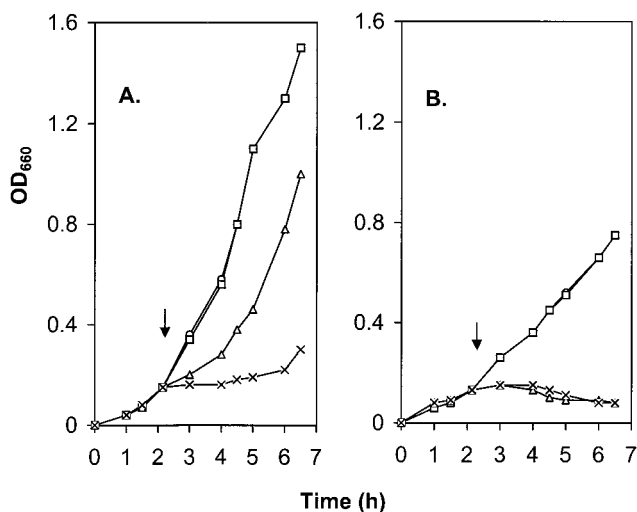


FIG. 2. Effect of CCCP on the organic solvent tolerance of *P. aeruginosa* PAO1 (A) and K1119 ( $\Delta mexA-mexB-oprM$ ) (B). Cells were grown in LB broth at 37°C to the early exponential phase, at which time (arrow) CCCP at 20 μM (□), *p*-xylene at 0.2% (vol/vol) ( $\Delta$ ), or CCCP at 20 μM and *p*-xylene at 0.2% (vol/vol) (×) were added and growth was determined by monitoring OD<sub>660</sub>. Control cultures (○, overlapped with the CCCP group [□]) received no supplementation.

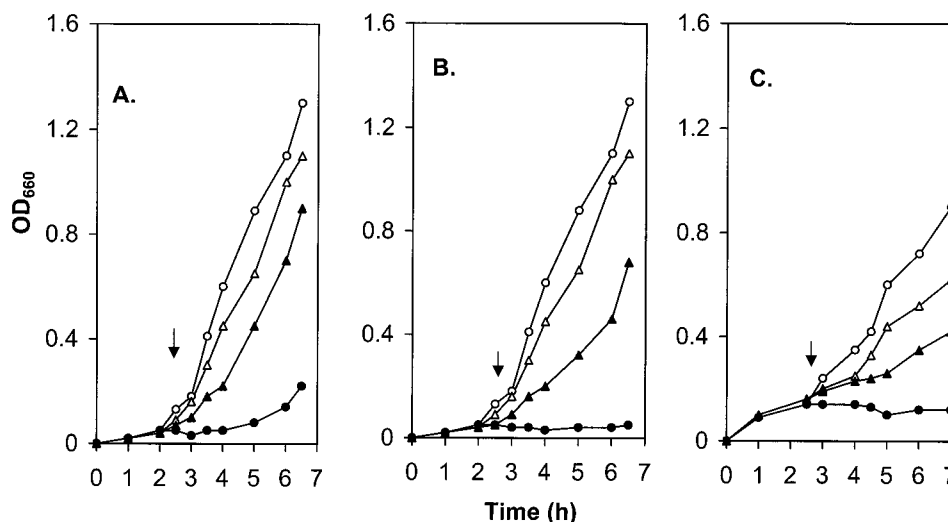


FIG. 3. Influence of the MexC-MexD-OprJ (A and B) and MexE-MexF-OprN (C) efflux systems on the growth of *P. aeruginosa* in the presence of organic solvents. (A and B) *P. aeruginosa* K1121 (MexCD-OprJ<sup>-</sup>) (○, ●) and K1131 (MexCD-OprJ<sup>+</sup>) (△, ▲) were grown in LB broth at 37°C to the early exponential phase, at which time (arrow) *n*-hexane (1% [vol/vol]; A, solid symbols) or *p*-xylene (0.1% [vol/vol]; B, solid symbols) was added and growth was determined by monitoring OD<sub>660</sub>. (C) *P. aeruginosa* K1115 (MexEF-OprN<sup>-</sup>) (○, ●) and K1117 (MexEF-OprN<sup>+</sup>) (△, ▲) were grown in LB broth at 37°C to the early exponential phase, at which time (arrow) *p*-xylene (0.08% [vol/vol]; solid symbols) was added and growth was determined by monitoring OD<sub>660</sub>. Growth of solvent-free cultures is represented by the open symbols in panels A, B, and C.

sion of MexEF-OprN (in K1117) (Table 2). Strain K1117 did, however, grow markedly better than K1115 in LB broth supplemented with a very modest 0.08% *p*-xylene (Fig. 3C), indicating that MexEF-OprN could contribute to a low-level tolerance to this solvent. No difference in tolerance to hexane at any concentration could be discerned between K1115 and K1117 (data not shown). Thus, all of the three known efflux systems in *P. aeruginosa* can contribute to organic solvent tolerance, although MexAB-OprM is by far the superior system for providing solvent tolerance. Given the known roles of these systems in drug export and the demonstration here that organic solvent tolerance could be compromised by an energy inhibitor, it is likely that these systems influence the solvent tolerance of *P. aeruginosa* by exporting the solvents out of the cell. Moreover, differences in tolerance levels afforded by each of the efflux systems likely reflect differences in the efficiency with which they accommodate the various organic solvents, reminiscent of differences in the abilities of these systems to accommodate the various antibiotics which are known to be substrates for these pumps (16, 19, 29, 30).

Mechanistically, it is unclear how the *P. aeruginosa* multi-drug efflux systems and similar efflux systems such as AcrAB-To1C, accommodate organic solvents. Antibiotics, being generally amphipathic molecules, are predicted to partition into the inner (most antibiotics) or outer ( $\beta$ -lactams) leaflet of the cytoplasmic membrane, from whence they are accessed by these efflux systems (25). Given that organic solvents are known to dissolve in lipid membranes and that their lethal effect likely involves compromising of the cytoplasmic membrane function, it is conceivable that these efflux systems also access organic solvents from within the bilayer as well. Still, it is unclear if the rate at which these solvents could be removed from the bilayer would be sufficient to ameliorate their toxic effects, and thus, the possibility that they are accessed prior to their dissolution in the cytoplasmic membrane cannot be ruled out. Nonetheless, these results appear to extend the known substrates for MexAB-OprM to organic solvents and once again serve to highlight the incredibly broad specificity exhib-

ited by this efflux system, which accommodates most classes of antibiotics, as well as dyes, detergents, and, now, organic solvents.

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