

## Expression in *Escherichia coli* of a New Multidrug Efflux Pump, MexXY, from *Pseudomonas aeruginosa*

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Received 20 July 1998/Returned for modification 22 September 1998/Accepted 2 December 1998

**Two new genes (*mexXY*) similar to *mexAB*, *mexCD*, and *mexEF* and mediating multidrug resistance were cloned from the chromosome of *Pseudomonas aeruginosa*. Elevated ethidium extrusion was observed with *Escherichia coli* cells harboring the plasmid carrying *mexXY*. This MexXY system confers higher resistance to fluoroquinolones than the MexAB and MexCD systems, and *E. coli* TolC or *P. aeruginosa* OprM is necessary for the function of the MexXY system.**

*Pseudomonas aeruginosa* shows significant degrees of intrinsic resistance to a wide variety of antimicrobial agents, including most  $\beta$ -lactams, fluoroquinolones, tetracycline, chloramphenicol, and erythromycin. This is a major problem in hospitals because *P. aeruginosa* is an important opportunistic pathogen and a leading cause of hospital-acquired infections. Three RND (resistance nodulation cell division) family drug efflux systems are known to exist in *P. aeruginosa*: MexAB-OprM (18), MexCD-OprJ (16), and MexEF-OprN (6). Recent studies have made it clear that these Mex systems, especially MexAB-OprM in wild-type cells, are mainly responsible for the intrinsic resistance of this organism to many antimicrobial agents (8, 9). Similar results demonstrating an involvement of the RND family multidrug efflux system AcrAB in mediating intrinsic resistance to many antimicrobial agents in *Escherichia coli* have been reported (11). Thus, it seems that RND family multidrug efflux systems are common in gram-negative bacteria and are responsible for intrinsic resistance to many antimicrobial agents. Here we report on a new multidrug efflux system, MexXY, of *P. aeruginosa*.

**Cloning of multidrug resistance genes.** *P. aeruginosa* PAO1 was used as a source of chromosomal DNA. *E. coli* KAM3 { $\Delta$ acrAB supE hsdD5 thi ( $\Delta$ lac-proAB)/F' [*traD36 proAB*<sup>+</sup> *lacI*<sup>q</sup> *lac*  $\Delta$ ZM15]}, a derivative of TG1, was used as a host for gene cloning (13, 19). *E. coli* KAM3 lacks *acrAB* genes and is therefore hypersensitive to many antimicrobial agents (14). Chromosomal DNA was prepared from cells of *P. aeruginosa* (1). The DNA was partially digested with *Sau3A1*. The DNA fragments ranging between 4 and 10 kbp were ligated into pBR322, which had been digested with *Bam*HI. Competent cells of *E. coli* KAM3 were transformed with the ligated hybrid plasmids (5) and spread onto agar plates containing L medium (7) and 1.5% agar plus one of the following antimicrobial agents: 12  $\mu$ g of ethidium bromide per ml, 10  $\mu$ g of erythromycin per ml, or 1  $\mu$ g of chloramphenicol per ml. The plates were incubated at 37°C for 1 day. The clones formed were picked. Plasmid DNA was prepared from each of the transformants by using a mini-prep kit (Qiagen Inc.) as suggested by the manufacturer. Competent cells of *E. coli* KAM3 were retransformed and spread on the same plates again. The plates were incubated at 37°C

for 1 day. Many colonies appeared on the plates. Plasmid DNA from each of the retransformants was prepared. The ethidium bromide plates gave us the largest number of candidates (43 candidates), followed by the erythromycin plates (8 candidates), and the chloramphenicol plates (3 candidates). We prepared and digested plasmids from all of these transformants. Three patterns were seen, one of which was similar to the restriction pattern of the *mexAB* region and a second one that was similar to that of the *mexCD* region. We confirmed that the former type was *mexAB* and the latter type was *mexCD* by partial sequencing. However, the third plasmid seemed to contain a novel drug resistance gene(s). We designated the new genes *mexXY* as described below. The new genes were identified from all three kinds of selection plates.

We measured the MICs of many antimicrobial agents with KAM3 cells harboring plasmids carrying each type of *mex* gene. Cells of KAM3/pTEM4 (carrying the *mexXY* genes) showed resistance to acriflavine, ethidium bromide, erythromycin, and fluoroquinolones (Table 1) and some degree of resistance to tetracycline, chloramphenicol, and kanamycin (data not shown). Cells of KAM3/pTUM3 (carrying the *mexAB oprM* genes [17]) showed lower resistance to most of the above-mentioned antimicrobial agents than did KAM3/pTEM4 cells (data not shown). Cells of KAM3/pTEM31 (carrying the *mexCD oprJ* genes [16]) showed higher resistance to acriflavine and ethidium bromide than KAM3/pTEM4 cells did but lower resistance to fluoroquinolones (data not shown). Thus, this indicates that the *mexXY* genes are multidrug resistance genes. One of the characteristics of the MexXY system is that this system conferred higher resistance to fluoroquinolones than other Mex systems did.

We measured ethidium efflux with KAM3/pBR322 cells and KAM3/pTEM4 cells to clarify whether the *mexXY* are genes for a multidrug efflux system. We observed a lower intracellular ethidium level before addition of an H<sup>+</sup> conductor, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), than after its addition with KAM3/pBR322 cells (Fig. 1). This indicates that an ethidium efflux pump driven by an electrochemical potential of H<sup>+</sup> is still present in KAM3 cells. We observed a much lower intracellular ethidium level before the addition of CCCP with KAM3/pTEM4 cells than with KAM3/pBR322 cells. The intracellular ethidium level increased after the addition of CCCP and reached the same level as that in the case of KAM3/pBR322 cells. Thus, it is clear that the *mexXY* genes confer to the cells H<sup>+</sup>-driven ethidium efflux ability.

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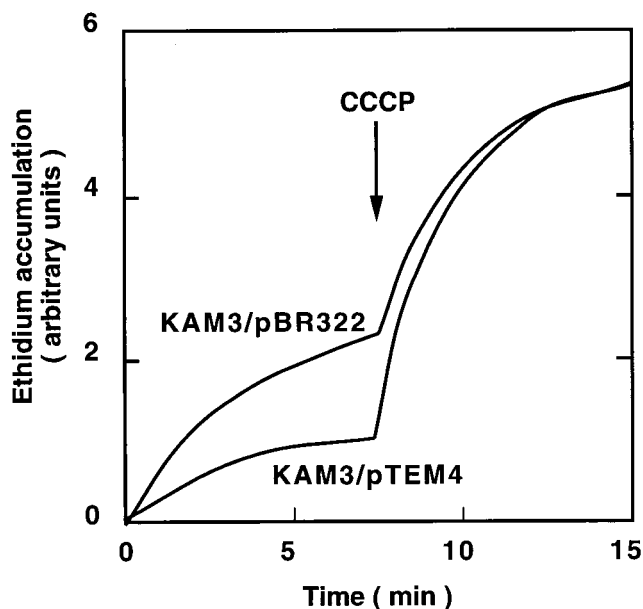


FIG. 1. Accumulation of ethidium in host cells and in transformed cells. *E. coli* KAM3/pBR322 and KAM3/pTEM4 cells were grown in L medium supplemented with 40 mM potassium lactate. Ethidium bromide was added to cell suspensions of KAM3/pBR322 and KAM3/pTEM4 at a final concentration of 10  $\mu$ M. Accumulation of ethidium was monitored continuously by measuring the fluorescence of ethidium in cells, at the excitation and emission wavelengths of 500 and 580 nm, respectively. After 7.5 min (arrow), CCCP was added to the suspensions at a final concentration of 100  $\mu$ M.

**Sequences of genes and products.** We determined the nucleotide sequence (20) of the DNA insert in pTEM4. We found two open reading frames (ORFs) oriented in the same direction preceded by Shine-Dalgarno sequences in the nucleotide sequence determined (Fig. 2). We designated the first ORF *mexX* and the second ORF *mexY*. Amino acid sequences were deduced from the *mexX* and *mexY* nucleotide sequences. The deduced MexX and MexY sequences consist of 389 and 1,046 residues, respectively. The calculated molecular masses are 41,444 and 113,116 Da, respectively. We found a promoter-like sequence in the upstream region from the *mexX* gene. It seems that *mexX* and *mexY* are in one operon. No other ORF was found in the region downstream (about 1 kbp in length) from the *mexY* gene.

**Characteristics of the primary structure.** A homology search of sequence databases (GenBank and SwissProt) revealed that MexX has 30 to 40% sequence identity and 40 to

60% similarity with MexA (17), MexC (16), MexE (6), AcrA (10), AcrE (12), and putative YhiU (15) and that MexY has 40 to 50% identity and 60 to 70% similarity with MexB (17), MexD (16), MexF (6), AcrB (10), AcrD (12), AcrF (12), and putative YhiV (15). Thus, it is clear that MexXY is a member of the RND family.

Hydropathy values were calculated along amino acid sequences of MexX and MexY by the method of Eisenberg et al. (3). Judging from the hydropathy patterns, MexX is a hydrophilic protein with one hydrophobic domain in its N-terminal region (data not shown). The N-terminal hydrophobic region seems to be a signal sequence (24). Immediately following the hydrophobic core region of the signal sequence is the sequence L-L-G-C, which is very similar to the sequence L-L-A-G-C, which has been reported to be a consensus sequence of the signal cleavage site of lipoproteins of gram-negative bacteria (14). Therefore, it is likely that the MexX is a lipoprotein anchored in the membrane by its lipid portion. It has been reported that AcrE (EnvC) is a lipoprotein of the cytoplasmic membrane of *E. coli* (21). On the other hand, MexY seems to be an intrinsic membrane protein with many hydrophobic domains.

**Requirement for TolC or OprM.** OprM, an outer membrane protein, is necessary for the function of the MexAB system. The *oprM* gene is located just downstream from the *mexAB* genes (18). OprJ is necessary for the function of MexCD. The *oprJ* gene is adjacent to the *mexCD* genes (16). There are only 400 bp between the termination codon of *mexY* and a *Bam*HI site, which is in the downstream end of the DNA region necessary for conferring drug resistance. No ORF corresponding to an outer membrane protein or to any other protein was found in this 400-bp region or in the downstream region (1 kbp in length) from *mexXY*. The MexXY system is functional in *E. coli* KAM3. This suggests that an outer membrane protein of *E. coli* such as TolC, which is required for the functioning of the AcrAB system (4), may be utilized by the MexXY system as the outer membrane component. We tested this possibility. *E. coli* N43 ( $F^-$  *lac ara mal xyl mtl gal rpsL acrA1 tolC::Tn10* and its parent N43 (4, 25) were used for this purpose. *E. coli* N43 lacks the AcrAB system like KAM3. Cells of N43 were hypersensitive to many antimicrobial agents, but, as anticipated, N43/pTEM4 became resistant to many antimicrobial agents (Table 1). Cells of N43 *tolC::Tn10* were also hypersensitive to many antimicrobial agents. However, N43 *tolC::Tn10*/pTEM4 cells were still hypersensitive to many antimicrobial agents (Table 1). This means that the MexXY system in N43 *tolC::Tn10* was not functional whereas that in N43 was functional. Thus, we conclude that TolC is necessary for the MexXY system to function in *E. coli*. It has been reported that MexCD functions in *E. coli* cells in conjunction with TolC (22). We also tested whether the TolC could be replaced with the OprM of *P. aeruginosa* in *E. coli*. Plasmid pTEM4 carrying the *mexXY* genes and plasmid pPMM2 carrying the *oprM* gene were used for this purpose. pPMM2 (vector, pACYC184) is a derivative of pTUM3 (pBR322) and carries *oprM* but not *mexAB*. Either pTEM4 or pPMM2 alone, or both, were introduced into N43 *tolC::Tn10* cells. The sensitivity to many antimicrobial agents was then tested. Introduction of both *mexXY* and *oprM* into N43 *tolC::Tn10* cells resulted in an increase in the MICs of many antimicrobial agents (Table 1). Introduction of either *mexXY* or *oprM* alone, however, resulted in no increase in the MICs. Thus, it seems that OprM forms a functional multidrug efflux pump together with MexXY in *E. coli* cells. It has been reported that OprM and OprJ are interchangeable (23). Thus, it seems that OprJ and OprN could be alternative outer membrane proteins for the MexXY system.

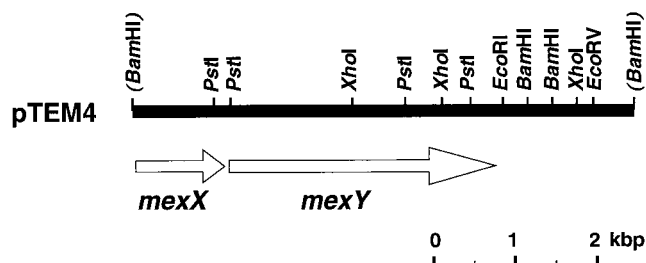


FIG. 2. Restriction map of DNA insert carried on pTEM4. The DNA region derived from the *P. aeruginosa* chromosomal DNA and carried on pTEM4 is shown as a horizontal solid bar. Restriction sites on pTEM4 are indicated. Locations of *mexX* and *mexY* are indicated by open arrows.

TABLE 1. Susceptibilities of study strains to different compounds and effect of *tolC* and *oprM* on the function of MexXY

Compound	MIC ( $\mu\text{g/ml}$ ) for <i>E. coli</i> strain tested						
	KAM3	KAM3/pTEM4 ( <i>mexXY</i> ) <sup>a</sup>	N43	N43/pTEM4 ( <i>mexXY</i> )	N43 <i>tolC</i> ::Tn10	N43 <i>tolC</i> ::Tn10/pTEM4 ( <i>mexXY</i> )	N43 <i>tolC</i> ::Tn10/ pTEM4/pPMM2 ( <i>mexXY oprM</i> )
Acriflavine	2	16	4	32	4	4	32
Ethidium bromide	1	32	4	64	4	2	32
Erythromycin	1	32	8	128	2	2	128
Norfloxacin	0.016	0.13	0.03	0.125	0.03	0.016	0.125
Ofloxacin	0.016	0.13	0.016	0.06	0.016	0.008	0.125
Tetraphenyl phosphonium	NT <sup>b</sup>	NT	16	512	16	16	NT

<sup>a</sup> Genes carried on the plasmids are shown in parentheses.

<sup>b</sup> NT, not tested.

However, since OprJ and OprN are not expressed in wild-type *P. aeruginosa* (6, 16), it is very likely that OprM is the most probable candidate to form a complex with MexXY and function as a multidrug efflux pump in wild-type *P. aeruginosa*. Very recently it has been reported that OprM can be expressed and function in a drug efflux capacity independent of MexAB in *P. aeruginosa* (26). This OprM-dependent and MexAB-independent system is responsible for resistance to quinolones, erythromycin, and tetracycline (26). This substrate specificity is the same as that of the MexXY system.

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases under accession no. AB015853.

We thank Joe A. Fralick for providing us with *E. coli* N43 and N43 *tolC*::Tn10 and Manuel F. Varela for critically reading the manuscript.

This work was supported in part by a grant from the Ministry of Education, Science, Sports, and Culture of Japan.

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