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Roles of MexXY- and MexAB-multidrug efflux pumps in intrinsic multidrug resistance of *Pseudomonas aeruginosa* PAO1

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To envisage the roles of MexXY- and MexAB-multidrug efflux pumps in the intrinsic multidrug resistance of wild-type strain *Pseudomonas aeruginosa* PAO1, we constructed mutants lacking either individual or both efflux pumps. A mutant lacking MexXY showed increased susceptibility to aminoglycosides, erythromycin, and tetracycline, but not to β -lactams, chloramphenicol, or quinolones. A mutant lacking MexAB showed increased susceptibility to β -lactams, chloramphenicol, and nalidixic acid, but not to aminoglycosides, erythromycin, tetracycline, or fluoroquinolones. A mutant lacking both MexXY and MexAB showed an increased susceptibility to all antimicrobial agents tested compared with the wild type. Very similar results were obtained with a mutant lacking MexAB-OprM and a mutant lacking both MexXY and MexAB-OprM. Thus it is clear that OprM is essential not only for the function of MexAB, but also for the function of MexXY. Furthermore, we found that each pump compensated to some extent for the lack of another pump with respect to the common substrates (tetracycline, quinolones, and cefpirome). The introduction of a plasmid carrying the *mexXY* genes into *P. aeruginosa* PAO1 cells increased the resistance to fluoroquinolones. This suggests that the *mexXY* genes could be involved in acquired resistance to fluoroquinolones in *P. aeruginosa* PAO1.

Key Words—— intrinsic drug resistance; MexAB; MexXY; multidrug efflux pump; *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is a well-known opportunistic pathogen and a leading cause of hospital-acquired infections. The wild-type strain of these bacteria shows significant degrees of intrinsic resistance to a wide variety of antibacterial agents, including most β -lactams, fluoroquinolones, tetracycline, chloramphenicol, and erythromycin. This property has been attributed to the impermeability of the outer membrane and

to the activity of several drug efflux systems (Li et al., 1995).

To date, four multidrug efflux systems encoded by structurally related genes have been reported: *mexAB-oprM* (Poole et al., 1993a, b), *mexCD-oprJ* (Poole et al., 1996), *mexEF-oprN* (Kohler et al., 1997), and *mexXY* (Mine et al., 1999). Among them, both MexAB-OprM and MexXY systems seem to contribute to intrinsic multidrug resistance in wild-type *P. aeruginosa*, but the MexCD-OprJ and MexEF-OprN systems do not (Aires et al., 1999; Kohler et al., 1997; Li et al., 1995; Poole et al., 1996; Yoneyama et al., 1997). It has been reported that the latter two multidrug efflux systems are functional in mutants and contribute to increased

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resistance to many antibacterial agents (Kohler et al., 1997; Poole et al., 1996).

Interestingly OprM can also contribute to antibiotic resistance independent of MexAB (Zao et al., 1998). We reported previously that the MexXY is functional only when OprM of *P. aeruginosa* or TolC of *E. coli* is present (Aires et al., 1999; Mine et al., 1999). These results strongly suggested that MexAB, MexXY both interact with OprM and form functional efflux pumps (i.e., MexAB-OprM and MexXY-OprM) in wild-type *P. aeruginosa*.

At present, the individual roles of MexXY and MexAB in the intrinsic multidrug resistance of wild-type *P. aeruginosa* are not fully understood. The presence of the two pumps and because many antibacterial agents are common substrates for the two pumps made our investigations difficult. Although several reports dealing with the role of MexAB are available, one must be careful in interpreting the results, because MexXY, besides MexAB, is present in wild-type cells. To elucidate the individual roles of MexXY and MexAB in multidrug resistance in the wild-type strain of *P. aeruginosa*, we constructed mutants lacking one or both of these multidrug efflux pumps. Here we report on the contribution of MexXY and MexAB to intrinsic multidrug resistance in *P. aeruginosa*.

Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 cells (Holloway, 1969) were used as prototypes in this study. *E. coli* KAM3 (Morita et al., 1998) or XL1-Blue (Bullock et al., 1987) were used as hosts when plasmids were constructed. *E. coli* SM10 *lacI^q* was used as a mobilizer strain (Schweizer, 1994; Simon et al., 1983).

Growth of cells. Bacterial cells were grown in L broth (Lennox, 1955) at 37°C unless otherwise indicated. *P. aeruginosa* cells were selectively grown in VBMM medium (Schweizer, 1991; Vogel and Bonner, 1956). *E. coli* cells cannot grow in this medium. Antibiotics were added to the selection media at the following concentrations (per milliliter): for *E. coli*, ampicillin sodium salt (60 µg), chloramphenicol (20 µg), gentamicin sulfate (10 µg), erythromycin (10 µg), ethidium bromide (10 µg); for *P. aeruginosa* PAO1, carbenicillin sodium salt (500 µg), gentamicin sulfate (200 µg); for *P. aeruginosa* YM2, carbenicillin sodium salt (500 µg),

gentamicin sulfate (15 µg); for *P. aeruginosa* YM3, carbenicillin sodium salt (20 µg), gentamicin sulfate (200 µg); for *P. aeruginosa* YM4, YM5, and YM6, carbenicillin sodium salt (20 µg), gentamicin sulfate (15 µg).

DNA manipulations. Plasmid DNA was purified with Qiagen-tip 100 (Qiagen, Hilden, Germany). DNA was digested with pertinent restriction enzymes (Nippon Gene (Tokyo, Japan), Roche Molecular Biochemicals (Tokyo, Japan), or New England Biolabs, SG4 OTY (UK)). DNA fragments were blunt-ended with T4 DNA polymerase (New England Biolabs) in the presence of 100 µM dNTPs. Plasmid DNA was introduced into the competent cells of *E. coli* strains, using the RbCl2 method (Hanahan, 1983).

Construction of plasmid pTEM424 for deletion of mexXY. First, a 4.3 kbp *EcoRV-EcoRV* fragment containing *mexXY* genes from pTEM4 (Mine et al., 1999) was subcloned between the *SmaI* site of pEX100T, a gene replacement vector containing *sacB*, *oriT*, and *bla* (Schweizer and Hoang, 1995), to construct pTEM423. Next, a 1.9 kbp *PstI-PstI* fragment containing a gentamicin resistant marker flanked by *FRT* sites from pPS858 (Hoang et al., 1998) was subcloned between the same sites of pTEM423 to construct pTEM424. Both *mexX* and *mexY* were deleted in the pTEM424.

Construction of plasmid pTUM325 for deletion of mexAB. First, a 4.4 kbp *SacI-SphI* fragment containing *mexA* and a partial *mexB* region from pTUM3, a derivative of pBR322 carrying *mexAB* (Mine et al., 1999) was subcloned between the *SacI* and *SphI* sites of pSTV28 (Takara Shuzo, Shiga, Japan) to construct pTUM323. Next, a 1.9 kbp *PstI-PstI* fragment containing a gentamicin-resistant marker flanked by *FRT* sites from pPS858 was subcloned between the same sites of pTUM323 to construct pTUM324. Then a 4.1 kbp *SalI-SphI* fragment containing a disrupted *mexAB* region from pTUM324 was subcloned between the *SmaI* and *SphI* sites of pEX100T to construct pTUM325. Both *mexA* and *mexB* were deleted in the pTUM325.

Construction of pTUM125 for deletion of mexAB-oprM. First, a 5.5 kbp *SacI-XhoI* fragment containing *mexAB* and a partial *oprM* from pTUM12, a derivative of pBR322 carrying *mexAB-oprM* (Mine, T., et al., unpublished), was subcloned between the *SacI* and *HindIII* sites of pSTV28 to construct pTUM123. Next, a 1.9 kbp *SalI-SalI* fragment containing a gentamicin-resistant marker flanked by *FRT* sites from pPS858 was subcloned between the same sites of pTUM123 to

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant properties	References
<i>P. aeruginosa</i>		
PAO1	wild type	Holloway, 1969
YM2	PAO1 derivative with <i>mexXY::FRT</i>	this study
YM3	PAO1 derivative with <i>mexAB::FRT</i>	this study
YM4	PAO1 derivative with <i>mexXY::FRT, mexAB::FRT</i>	this study
YM5	PAO1 derivative with <i>mexAB-oprM::FRT</i>	this study
YM6	PAO1 derivative with <i>mexXY::FRT, mexAB-oprM::FRT</i>	this study
Plasmid		
pTEM4	pBR322 derivative carrying <i>mexXY</i> genes	Mine et al., 1999
pTEM423	pEX100T derivative carrying <i>mexXY</i> genes	this study
pTEM424	pEX100T derivative carrying <i>mexXY</i> genes replaced with Gm ^r -GFP fragment flanked by <i>FRT</i> sites	this study
pTUM3	pBR322 derivative carrying <i>mexAB</i> genes	Mine et al., 1999
pTUM323	pSTV28 derivative carrying <i>mexA</i> and partial <i>mexB</i> gene	this study
pTUM324	pSTV28 derivative carrying <i>mexA</i> and partial <i>mexB</i> gene replaced with Gm ^r -GFP fragment flanked by <i>FRT</i> sites	this study
pTUM325	pEX100T derivative carrying <i>mexA</i> and partial <i>mexB</i> gene replaced with Gm ^r -GFP fragment flanked by <i>FRT</i> sites	this study
pTUM12	pBR322 derivative carrying <i>mexAB-oprM</i> genes	Mine, T., et al., unpublished
pTUM123	pSTV28 derivative carrying <i>mexAB</i> and partial <i>oprM</i> genes	this study
pTUM124	pSTV28 derivative carrying <i>mexAB</i> and partial <i>oprM</i> genes replaced with Gm ^r -GFP fragment flanked by <i>FRT</i> sites	this study
pTUM125	pEX100T derivative carrying <i>mexAB</i> and partial <i>oprM</i> genes replaced with Gm ^r -GFP fragment flanked by <i>FRT</i> sites	this study
pSTV28	vector (<i>ori</i> of pACYC184, cat of Tn9, multicloning site of pUC118)	TaKaRa Co.
pPS858	source of Gm ^r -GFP fragment flanked by <i>FRT</i> sites	Hoang et al., 1998
pEX100T	gene replacement vector	Schweizer and Hoang, 1995
pFLP2	source of yeast FLP recombinase	Hoang et al., 1998
pUCP20T	mobilizable broad-host-range cloning vector	West et al., 1994
pTEM433	pUCP20T derivative carrying <i>mexXY</i> gene followed by <i>tet</i> promoter	this study

FRT: Flp recombinase target (Hoang et al., 1998).

construct pTUM124. Then, a 4.2 kbp *Sspl*-*Sspl* fragment containing a disrupted *mexAB-oprM* region from pTUM124 was subcloned into the *Sma*I site of pEX100T to construct pTUM125. In the pTUM125, *mexA*, *mexB* and *oprM* were deleted.

Construction of pTEM433 carrying *mexXY*. First, a 5.8 kbp *Eco*RI-*Eco*RI fragment containing *mexXY* genes from pTEM4 (Mine et al., 1999) was subcloned between the *Eco*RI site of pUCP20T, one of the *Escherichia-Pseudomonas* shuttle vectors (West et al., 1994). The resultant plasmid pTEM433 carries *mexXY*, which is preceded by the *lac* promoter.

Gene replacement. Gene replacement was performed according to the method of Hoang. (Hoang et al., 1998).

Genomic Southern analysis. Chromosomal DNA was isolated by the CTAB (cetyltrimethylammonium bromide) method (Ausubel et al., 1991) and digested with *Eco*RI. Southern blotting (Southern, 1975) was performed by using the ECLTM direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech, Tokyo, Japan). A 4.8 kbp *Pac*I-*Bam*HI fragment containing the *mexXY* genes from pTEM422 and a 5.6 kbp *Sac*I-*Xho*I fragment containing the *mexAB-oprM* genes from pTUM122 were used as the probe. Hybond-N (Amersham Pharmacia Biotech) was used as the nylon membrane for the DNA transfer.

Drug susceptibility test. The minimum inhibitory concentrations (MICs) of various drugs were determined in Mueller-Hinton broth (Difco, Sparks, USA) by

Table 2. Changes in MICs in the mutants with deficient Mex systems.

Drug	MIC (μg/ml)						
	Strain	PAO1	YM2	YM3	YM5	YM4	YM6
	Deficiency		ΔMexXY	ΔMexAB	ΔMexAB ΔOprM	ΔMexXY ΔMexAB	ΔMexXY ΔMexAB ΔOprM
Nalidixic acid		64	64	16	4	4	4
Norfloxacin		1	0.5	0.5	0.25	0.25	0.25
Ofloxacin		1	1	0.5	0.12	0.25	0.12
Ciprofloxacin HCl		0.25	0.25	0.25	0.03	0.06	0.03
Spafloxacin		1	0.5	0.5	0.12	0.25	0.12
Levofloxacin		0.5	0.5	0.25	0.06	0.06	0.06
Carbenicillin sodium		32	32	1	1	1	1
Aztreonam		4	8	0.5	0.5	0.5	0.5
Cefoperazone HCl		4	8	0.5	0.5	0.5	0.5
Cefpirome		4	4	2	1	1	1
Chloramphenicol		32	64	8	2	4	2
Erythromycin		256	64	256	32	64	32
Kanamycin sulfate		256	64	256	64	64	64
Streptomycin sulfate		64	4	64	4	4	4
Gentamicin sulfate		8	0.5	8	1	0.5	0.5
Tetracycline HCl		32	8	16	1	1	0.5

the twofold dilution method (Japan Society of Chemotherapy, 1990). Cells in the test medium (10^5 cells/ml) were incubated at 37°C for 24 h, and thereafter the growth was judged.

Results and Discussion

Changes in MIC of antibacterial agents in mutants lacking MexXY or MexAB, or both

We measured the MICs of many antibacterial agents in mutants lacking either MexXY or MexAB, or both (Table 2). A mutant lacking MexXY showed a decrease in the MICs of streptomycin, gentamicin, erythromycin, and tetracycline, but not of the quinolones, β -lactams, or chloramphenicol. Mutants lacking MexAB showed a decrease in the MICs of nalidixic acid, β -lactams, chloramphenicol, and tetracycline, but not of other quinolones, aminoglycosides, or erythromycin. On the other hand, mutants lacking both MexXY and MexAB showed a large decrease in the MICs of a wide variety of antibacterial drugs: all quinolones tested, all β -lactams tested, erythromycin, aminoglycosides, and tetracycline. Mutants lacking MexAB-OprM or both MexXY and MexAB-OprM showed a very similar pattern and

extent of decrease in the MICs of antibacterial agents and were comparable to the MICs of the mutant lacking both MexXY and MexAB. These results strongly support the idea that OprM is a common component for MexXY and MexAB (Mine et al., 1999). In the absence of OprM, neither MexXY nor MexAB can function as a multidrug efflux pump or extrude their substrate drugs. Our results strongly support the view that the MexXY pump, besides the MexAB pump (Li et al., 1995; Yoneyama et al., 1997), contributes to the intrinsic resistance of *P. aeruginosa* to at least streptomycin, gentamicin, erythromycin, and tetracycline.

The MIC values for many antibacterial drugs were very similar between the mutant lacking both MexXY and MexAB and the mutant lacking both MexXY and MexAB-OprM (Table 2). Thus it seems that no other multidrug efflux pump except the two (MexXY and MexAB) utilizes the outer membrane protein OprM.

Our previous results obtained with MexXY expressed in *E. coli* cells indicated that MexXY made cells resistant to ethidium bromide and some other antibacterial dyes (Mine et al., 1999). We tested whether the MIC of ethidium bromide was changed in the mutant lacking the MexXY. However, we observed no sig-

Table 3. Changes in MICs due to the introduction of *mexXY* genes.

Drug	MIC ($\mu\text{g/ml}$)					
	Strain	PAO1	PAO1	YM4	YM4	YM6
		/pUCP20T	/pTEM433	/pUCP20T	/pTEM433	/pUCP20T
	Deficiency			ΔMexXY ΔMexAB	ΔMexXY ΔMexAB	ΔMexXY ΔMexAB ΔOprM
	Introduced	+MexXY		+MexXY		+MexXY
Nalidixic acid	64	64	8	32	4	2
Norfloxacin	1	4	0.5	2	0.25	0.25
Ofloxacin	1	8	0.12	4	0.06	0.12
Ciprofloxacin HCl	0.25	2	0.03	1	0.03	0.03
Spafloxacin	1	8	0.12	4	0.06	0.12
Levofloxacin	0.5	4	0.06	2	0.06	0.03
Aztreonam	4	4	0.5	0.5	0.5	0.5
Cefoperazone HCl	8	8	4	8	4	4
Cefpirome	4	32	1	16	1	1
Chloramphenicol	32	16	4	4	2	2
Erythromycin	256	512	64	512	32	32
Streptomycin sulfate	64	32	4	64	4	4
Gentamicin sulfate	8	4	0.5	8	0.5	0.5
Tetracycline HCl	32	64	1	32	0.5	1

nificant change in the MIC of the mutant of *P. aeruginosa* compared with its parent, PAO1 (data not shown). The MIC of ethidium bromide in PAO1 was higher than 256 $\mu\text{g/ml}$. Even in the mutant lacking both MexXY and MexAB, no significant change in the MIC for ethidium was detected. This suggests that *P. aeruginosa* PAO1 has another potent efflux system for ethidium.

Effect of introduction of *mexXY* genes into deficient mutants

We first introduced the *mexXY* genes into wild-type PAO1 cells (Table 3). We observed a 4 to 8 times increase in the MICs of fluoroquinolones and one β -lactam, cefpirome. These results suggest that a mutation in the *mexXY* genes could result in elevated resistance to fluoroquinolones and cefpirome in *P. aeruginosa*. In other words, fluoroquinolone-resistant mutants with a mutation in the *mexXY* genes or its regulatory gene could be clinically isolated. On the other hand, no clear change in the MIC was detected with erythromycin, streptomycin, gentamicin, or tetracycline, which are good substrates for MexXY-OprM. Perhaps the efflux

activity of MexXY-OprM for these antibiotics is nearly maximal in *P. aeruginosa*, and a significant increase in the efflux does not take place even if more MexXY is present in the cells. It is not likely that the amount of OprM is limited because we observed an increase in the MIC of other antibacterial agents, as described above.

Second, we introduced the *mexXY* genes into mutant cells lacking both MexXY and MexAB (Table 3). We observed 4- to 30-fold increases in the MICs of fluoroquinolones, a 16-fold increase with cefpirome, an 8-fold increase with erythromycin, a 16-fold increase with streptomycin and gentamicin, and a 32-fold increase with tetracycline. Thus it seems that overproduction of the MexXY compensates for the deficiency of MexAB of *P. aeruginosa*. It is clear that the MexXY is a multidrug efflux pump with very broad specificity. It is also clear that the substrate specificity is different between MexXY and MexAB, though some substrates are common.

Third, we introduced the *mexXY* genes into mutant cells lacking both MexXY and MexAB-OprM (Table 3). It was expected that this introduction would not result

in a change in the MIC of any drug because OprM is necessary for the function of MexXY (Mine et al., 1999). Indeed, we observed no significant change in the MIC of any drug tested between YM6/pUCP20T (lacking MexXY and MexAB-OprM) and YM6/pTEM433 (lacking the chromosomally encoded MexXY and MexAB-OprM, but possessing MexXY derived from a plasmid). These results strongly support that OprM is essential for the function of MexXY.

After the completion of this work, Masuda et al. (2000) reported similar results.

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