Gene cloning and characteristics of the RND-type multidrug efflux pump MuxABC-OpmB possessing two RND components in *Pseudomonas aeruginosa*

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muxA-muxB-muxC-opmB (formerly PA2528-PA2527-PA2526-*opmB*), encoding a putative resistance nodulation cell division (RND)-type multidrug efflux pump system, was cloned from *Pseudomonas aeruginosa* PAO1. Introduction of *muxABC-opmB* into *P. aeruginosa* YM64, a drug-hypersusceptible strain, led to elevated MICs of aztreonam, macrolides, novobiocin and tetracycline. Since *muxB* and *muxC*, both of which encode RND components, were essential for function, MuxABC-OpmB is thought to be a drug efflux pump with four components. One novobiocin-resistant mutant, PMX725, isolated from *P. aeruginosa* PMX7 showed elevated resistance not only to novobiocin but also to aztreonam, macrolides and tetracycline. Increased mRNA expression of *muxABC-opmB* was observed in the mutant PMX725 compared with the parental strain. Sequencing analysis revealed that a single-nucleotide insertion had occurred in the deduced promoter region for *muxABC-opmB* in PMX725. In this study, we have characterized the last RND-type multidrug efflux pump predicted from the genome sequence in *P. aeruginosa*.

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INTRODUCTION

Pseudomonas aeruginosa naturally exhibits a high level of resistance to a wide variety of agents (e.g. antibiotics, detergents and dyes) toxic to micro-organisms. In addition, this organism easily acquires higher resistance to anti-pseudomonal agents by multiple mechanisms (Dubois *et al.*, 2008; Hocquet *et al.*, 2008; Ikonomidis *et al.*, 2008; Jalal *et al.*, 2000; Lomovskaya *et al.*, 1999; Pitout *et al.*, 2008; Sobel *et al.*, 2003). In the last few decades, emergence and spread of multidrug-resistant *P. aeruginosa* strains has been a great concern in clinical sites all over the world.

So far, several types of drug-resistant systems have been reported in *P. aeruginosa*. Among them, multidrug efflux pumps are now recognized to play an important role in the

intrinsic and acquired resistance to structurally unrelated antimicrobial agents in *P. aeruginosa*.

Bacterial drug efflux pumps are generally classified into five families: the resistance nodulation cell division (RND) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family, and the ATP-binding cassette (ABC) family. The genome project for *P. aeruginosa* was completed in 2000, and the genome information predicted that there would be genes encoding 11 RND-type multidrug efflux pumps, 20 MFS-type multidrug efflux pumps, two SMR-type multidrug efflux pumps, one MATE-type multidrug efflux pump, and one ABC-type multidrug efflux pump (Stover *et al.*, 2000). Among them, RND-type efflux pumps have been characterized in most detail.

In *P. aeruginosa* the RND-type efflux pump is thought to play an important role in the intrinsic resistance to antimicrobial agents from the result of gene disruptions (Morita *et al.*, 2001b). In several clinically isolated mutants of *P. aeruginosa*, higher expression of RND-type efflux pumps was observed (Hirai *et al.*, 1987; Rella & Haas, 1982; Wolter *et al.*, 2004). Of the 11 RND efflux pumps identified in this organism, 10 pumps, MexAB-OprM (Poole *et al.*,

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Abbreviation: RND, resistance nodulation cell division.

1993), MexCD-OprJ (Poole *et al.*, 1996), MexEF-OprN (Kohler *et al.*, 1997), MexGHI-OpmD (Aendekerk *et al.*, 2002; Sekiya *et al.*, 2003), MexJK (Chuanchuen *et al.*, 2002), MexMN (Mima *et al.*, 2005), MexPQ-OpmE (Mima *et al.*, 2005), MexVW (Li *et al.*, 2003), MexXY (Mine *et al.*, 1999; Westbrook-Wadman *et al.*, 1999) and TriABC-OpmH (Mima *et al.*, 2007), have been experimentally confirmed, and their properties have been reported. The RND-type efflux pump usually consists of three components to fulfil the function properly and includes an inner-membrane component (RND component), a periplasmic component (MFP component), and an outer-membrane component (OMP component) (Touze *et al.*, 2004), and the three-dimensional structures of MexA, MexB and OprM have been reported (Akama *et al.*, 2004a, b; Higgins *et al.*, 2004; Sennhauser *et al.*, 2009). However, regarding the most recently analysed RND efflux pump in *P. aeruginosa*, TriABC-OpmH, it has been reported that this system needs four components: an RND component, two MFP components and an OMP component (Mima *et al.*, 2007).

PA2528-PA2527-PA2526-*opmB* encodes components for a possible RND-type efflux pump. This would be the last hitherto uncharacterized RND-type multidrug efflux pump in *P. aeruginosa*. The PA2528-PA2527-PA2526-OpmB

system seems to possess two RND components. This type of RND-type efflux pump has been reported in *Escherichia coli* (MdtABCD) (Baranova & Nikaido, 2002; Nagakubo *et al.*, 2002), but PA2528-PA2527-PA2526-OpmB in *P. aeruginosa* has not yet been characterized.

Here we report on the characterization of PA2528-PA2527-PA2526-OpmB (renamed as MuxA-MuxB-MuxC-OpmB) and on the isolation and properties of mutants that hyper-express MuxABC-OpmB.

METHODS

Bacteria and growth. Bacterial strains and plasmids used in this study are listed in Table 1. Cells were cultured at 37 °C under aerobic conditions. Bacterial strains were grown in L medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, pH 7.0) (Lennox, 1955). Appropriate concentrations of antibiotics were added when required. PAI plates (2 % Bacto peptone, 1 % K₂SO₄, 0.03 % cetyltrimethylammonium bromide, 14.7 mM MgCl₂, 1 % glycerol, 1.5 % agar) were used to select *P. aeruginosa* cells. *E. coli* cells carrying pUCP20T (Schweizer, 1991; West *et al.*, 1994), pEX100T (Hoang *et al.*, 1998), or their derivatives were cultured in L medium with 100 µg ampicillin ml⁻¹. *P. aeruginosa* cells carrying pUCP20T (Schweizer, 1991; West *et al.*, 1994) or its derivatives were cultured in L medium with 100 µg carbenicillin ml⁻¹.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
<i>P. aeruginosa</i>		
PAO1	Wild-type	Mine <i>et al.</i> (1999)
YM44	PAO1 Δ mexAB Δ mexCD- <i>oprJ</i> Δ mexEF- <i>oprN</i> Δ mexXY	Li <i>et al.</i> (2003)
YM64	PAO1 Δ mexAB- <i>oprM</i> Δ mexCD- <i>oprJ</i> Δ mexEF- <i>oprN</i> Δ mexXY	Morita <i>et al.</i> (2001b)
PMX6	YM64 Δ mexHI- <i>opmD</i> Δ pmpM	He <i>et al.</i> (2004)
PMX7	PMX6 Δ mexVW	This study
PMX725	PMX7 derivative, novobiocin-resistant mutant	This study
KN7	PMX7 Δ muxABC- <i>opmB</i> ::Gm ^r , GFP	This study
KN725	PMX725 Δ muxABC- <i>opmB</i> ::Gm ^r , GFP	This study
<i>E. coli</i>		
KAM32	Δ acrB Δ ydjE Δ (lac-pro) <i>supE</i> <i>thi</i> <i>hsd</i> Δ 5/F' <i>traD</i> 36 <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> Δ M15	Chen <i>et al.</i> (2002)
SM10 <i>lacI</i> ^q	<i>thi-1</i> <i>thr</i> <i>leu</i> <i>tonA</i> <i>lacy</i> <i>supE</i> <i>recA</i> ::RP4-2-Tc::Mu (Km ^r) <i>lacI</i> ^q	Schweizer (1994)
Plasmids		
pEX100T	Broad-host-range gene replacement vector; <i>sacB</i> Ap ^r Cb ^r	Hoang <i>et al.</i> (1998)
pPS858	Ap ^r Gm ^r ; source of Gm ^r -GFP fragment flanked by FRT sites	Hoang <i>et al.</i> (1998)
pUCP20T/21T	Broad-host-range cloning vector; Mob ⁺ Ap ^r Cb ^r	Schweizer (1991), West <i>et al.</i> (1994)
pMUX2	pUCP20T derivative carrying <i>muxABC-opmB</i> with original promoter	This study
pMUX3	pUCP20T derivative carrying <i>muxABC-opmB</i> without original promoter	This study
pMUX4	pMUX2 Δ muxB	This study
pMUX5	pMUX2 Δ muxC	This study
pMUX6	pMUX2 Δ opmB	This study
pMUX8	pUCP21T derivative carrying <i>muxABC-opmB</i> with original promoter	This study
pMUX82	pUCP21T derivative carrying <i>muxABC-opmB</i> having T insertional mutation in the promoter region from PMX725	This study
pTAJ2	pUCP20T derivative carrying <i>mexVW</i>	Li <i>et al.</i> (2003)
pTAJ4	pEX100T derivative for disruption of <i>mexVW</i>	This study
pEMU2	pEX100T derivative for disruption of <i>muxABC-opmB</i>	This study

Construction of plasmids. Chromosomal DNA for a PCR template was prepared from cells of *P. aeruginosa* PAO1 (Chen & Kuo, 1993). The gene cloning for PA2528-PA2527-PA2526-*opmB*, designated *muxABC-opmB*, was performed in two steps (the primers used in this study are listed in Table 2). A PCR product containing the first half of *muxABC-opmB* was generated with Mux forward 1 and Mux reverse 1 primer pairs. The amplified fragment contains a putative promoter in the upstream region of *muxA*. Mux forward 2 and Mux reverse 2 primer pairs were used to clone the latter half of the operon. The amplified DNA fragment of the first half was digested with *EcoRI* and *KpnI* and ligated with pUCP20T, which was treated with *EcoRI* and *KpnI*. The resulting plasmid was designated pMUX92 (Fig. 1). The latter-half fragment of the *muxABC-opmB* operon was also cloned in pUCP20T [pMUX93 (Fig. 1)]. The pMUX92 plasmid was digested with *KpnI* and *HindIII* ligated to the *KpnI*–*HindIII* fragment (6.9 kbp) from pMUX93. The resulting plasmid carrying the whole region of *muxABC-opmB* was named pMUX2. Cells of *E. coli* KAM32 (Chen *et al.*, 2002) were used for the plasmid construction. The plasmid constructs were transferred into *P. aeruginosa* cells for analysis.

For the construction of pMUX3, carrying *muxABC-opmB* but not the putative original promoter region, we used Mux forward 3 and Mux reverse 1 primer pairs for PCR. The product was digested with *EcoRI* and *KpnI*, and ligated with pUCP20T (pMUX94; Fig. 1). Plasmid pMUX3 was constructed by a similar procedure, using pMUX94 instead of pMUX92.

To investigate whether two RND components and an OMP component are necessary for the function of the efflux pump, we constructed pMUX4 (Δ *muxB*), pMUX5 (Δ *muxC*) and pMUX6 (Δ *opmB*). Plasmid pMUX2 was digested with *SmaI* and *KpnI* and blunted with T4 DNA polymerase (TaKaRa). Then the fragment was self-ligated, and the resulting plasmid was named pMUX4. Plasmid pMUX93 was digested with *NotI* and *XhoI* and blunted with T4 DNA polymerase. The fragment was self-ligated. Then the self-ligated plasmid was digested with *KpnI* and *HindIII* and inserted into the *KpnI*–*HindIII* site of pMUX92. The resulting plasmid was named pMUX5. The 0.32 kbp *MefI* fragment was deleted from pMUX2. The resulting plasmid was named pMUX6. The mRNA expression of the ORFs in the downstream region of the deleted gene was confirmed by RT-PCR in *P. aeruginosa* transformed with pMUX4 or pMUX5.

We also constructed pMUX8 and pMUX82 in order to investigate the effect of mutation at the promoter region of the *muxABC-opmB*

operon. The strategy to construct pMUX8 and pMUX82 was common except for the PCR used to amplify the first half of *muxABC-opmB*. Primers Mux forward 1 and Mux reverse 1 were used to amplify the first half of *muxABC-opmB* in PMX7 or PMX725. Each PCR product was digested with *EcoRI* and *KpnI* and ligated into pUCP21T (Schweizer, 1991; West *et al.*, 1994). Then the plasmid was digested with *Eco52I* to remove the *Eco52I* fragment (0.69 kbp). The plasmid deleted of the *Eco52I* fragment was digested with *KpnI* and *XhoI*, and the *KpnI*–*XhoI* fragment (1.2 kbp) from pMUX2 was ligated to the corresponding site. The plasmid digested with *HindIII* and *KpnI* was ligated with the *HindIII*–*KpnI* fragment (6.7 kbp) from pMUX2. We designated them as pMUX8 and pMUX82, possessing *muxABC-opmB* and the promoter region from PMX7 and PMX725, respectively.

Drug-susceptibility testing. The MICs of various antimicrobial agents were determined in Mueller–Hinton broth (Difco) by the twofold dilution method according to CLSI recommendations (CLSI, 2006). Cells in the test medium (10^5 cells ml^{−1}) were incubated at 37 °C for 24 h, and the growth was subsequently measured. Rokitamycin was a kind gift from Asahi Kasei Co. (Tokyo, Japan). The testing was repeated at least three times.

Isolation of novobiocin-resistant mutants. Approximately 10^9 cells of *P. aeruginosa* PMX7 were spread onto L plates containing 256 µg novobiocin ml^{−1}, which is eight times the MIC for *P. aeruginosa* PMX7. Five large colonies and approximately 100 small colonies appeared on the plate after 24 h incubation at 37 °C. We chose the five large colonies and one of the small colonies and analysed them.

RT-PCR. RNA preparations and reverse transcriptional PCR were performed according to the manufacturer's protocols. Briefly, total bacterial RNA was isolated from cells grown to an OD₆₅₀ of 0.7 using the RNeasy Mini kit (Qiagen). Residual DNA was removed by the treatment with RNase-Free DNase (Promega). One nanogram of DNase-treated RNA was used as a template for one reaction using the Qiagen OneStep RT-PCR kit (Qiagen). Primer pairs to detect mRNA are listed in Table 2. Expression of the *rpsL* gene was used as an internal control. The products were separated by 3% agarose X gel (Nippongene) electrophoresis and visualized with ethidium bromide.

Gene disruption in *P. aeruginosa*. Plasmid pTAJ4, used to disrupt the *mexVW* genes, was constructed after several steps. First, pTAJ2 (Li *et al.*, 2003) treated with *SaII* was ligated with *SaII* fragments

Table 2. Primers used in this study

Primer	Sequence (5' to 3')	Application
Mux forward 1	GGGGAATTCGGCGGAATGCCTAACTATCG	Gene cloning
Mux reverse 1	GAAGGGCGTGACAGACTCA	Gene cloning
Mux forward 2	ACGCCACCCTGATTCCCA	Gene cloning
Mux reverse 2	CCCAAGCTTCCGACACGTATTCACCTCCACA	Gene cloning
Mux forward 3	CCCGAATTCAGCGCCTCCGTCTAAGCC	Gene cloning
<i>muxA</i> -RT-F	TGGTCAAGGTGCTGTTCCA	RT-PCR
<i>muxA</i> -RT-R	GCTATCGAGTCCTCGGCATAC	RT-PCR
<i>muxB</i> -RT-F	TCCTGATTTCCGGCTTCGTC	RT-PCR
<i>muxB</i> -RT-R	GGCATGGCCAGGTAGAGTAG	RT-PCR
<i>muxC</i> -RT-F	TCGAGAAGGACGACAAGCAC	RT-PCR
<i>muxC</i> -RT-R	GGGTGACGATTAGCAGCACA	RT-PCR
<i>opmB</i> -RT-F	GCACGACTACTCGACCAA	RT-PCR
<i>opmB</i> -RT-R	GCGGATCTGTTCTGTCATCA	RT-PCR
<i>rpsL</i> -RT-F	GCAACTATCAACCAGCTGGTG	RT-PCR
<i>rpsL</i> -RT-R	GCTGTGCTCTTGCAGTTGTG	RT-PCR

Fig. 1. Map of plasmids and location of ORFs. ORFs are indicated by thick black arrows. Regions of chromosomal DNA present in each recombinant plasmid are indicated by bold black lines. The white bar in pMUX82 indicates the mutant-type promoter region derived from the mutant *P. aeruginosa* PMX725. The positions of the primers are indicated by thin arrows above the ORFs. *Plac* with a small arrow indicates the *lac* promoter on the vector and its direction. F1, Mux forward 1; F2, Mux forward 2; F3, Mux forward 3; R1, Mux reverse 1; R2, Mux reverse 2.

(1.9 kbp) containing the gentamicin-resistance marker (*aacC1*) and sandwiched between two FRT (Flp recombinase target) sites from pPS858 (Hoang *et al.*, 1998). The resulting plasmid pTAJ3 was digested with *Afl*II and blunted; thereafter, the fragment was digested with *Sph*I. The fragment produced (4.6 kbp) was ligated with pEX100T (Hoang *et al.*, 1998) that had been digested with *Sma*I and *Sph*I. The resultant plasmid was named pTAJ4, containing *mexVW* disrupted with the insert of *aacC1* (9.9 kbp).

Plasmid pMUX2 was digested with *Sall* and the fragment (5.0 kbp) was self-ligated. Then the self-ligated plasmid was digested with *Eco*RI and *Hind*III. The *Eco*RI–*Hind*III fragment (0.8 kbp) was collected, and both ends were blunted with T4 DNA polymerase. The fragment was ligated with the *Sma*I site in pEX100T (pEMU1). The *Sall* fragment (1.9 kbp) from pPS858 was ligated into pEMU1 that had been treated with *Sall*. The resulting plasmid for *muxABC-opmB* disruption was named pEMU2.

We used the method of Hoang *et al.* (1998) to disrupt *mexVW* or *muxABC-opmB*. The plasmid for gene disruption was transferred from *E. coli* SM10 *lac*^F (Schweizer, 1994) into *P. aeruginosa* by conjugation. The *mexVW* locus was disrupted using pTAJ4 in *P. aeruginosa* PMX6 (He *et al.*, 2004), and the resulting strain was named *P. aeruginosa* PMX7. *P. aeruginosa* KN7 and KN725, which are *muxABC-opmB* gene disruptants, were constructed from *P. aeruginosa* PMX7 and PMX725, respectively. The genotypes of the resultant mutants were confirmed by PCR.

RESULTS

Drug susceptibility of *P. aeruginosa* possessing *muxABC-opmB* on a plasmid

Eleven RND-type efflux systems were predicted to be present on the *P. aeruginosa* PAO1 genome ([http://](http://www.pseudomonas.com/index.jsp)

www.pseudomonas.com/index.jsp). So far, 10 of these efflux systems have been characterized. Here we cloned the last hitherto uncharacterized genes for the putative RND-type efflux pump PA2528-PA2527-PA2526-*opmB* by PCR (Fig. 1). These genes appear to compose an operon that includes the *opmB* gene. *OpmB* has been previously reported to be an OMP component that cooperates with the MexAB, MexCD and MexXY multidrug efflux pumps (Murata *et al.*, 2002). We renamed the PA2528-PA2527-PA2526 genes as *muxA*, *muxB*, *muxC*, respectively. The *muxA* gene seems to encode an MFP component. Both *muxB* and *muxC* seem to encode RND components. BLAST search showed that homologues for MuxABC were present in other bacteria. The most similar homologue among already characterized multidrug efflux pumps was MdtABC in *E. coli* (Baranova & Nikaido, 2002; Nagakubo *et al.*, 2002). MuxA showed high similarity with MdtA, which is an MFP component (40 % identity and 78 % similarity). MuxB and MuxC showed significantly high similarity with the RND components MdtB and MdtC (65 % identity and 91 % similarity between MuxB and MdtB, 61 % identity and 88 % similarity between MuxC and MdtC).

To investigate the contribution of MuxABC-*OpmB* to resistance to several antimicrobial agents, pMUX2 carrying *muxABC-opmB* was introduced into cells of *P. aeruginosa* YM64 (Morita *et al.*, 2001b). Since four major RND-type efflux pump genes are deleted in YM64, it is easy to assess differences in drug susceptibilities. *P. aeruginosa* YM64/pMUX2 showed increased MICs for aztreonam, novobiocin, tetracycline, erythromycin, kitasamycin and rokitamycin (Table 3). Increases in MICs were not observed with oleandomycin and clarithromycin, which are categorized as 14-membered macrolides (data not shown).

A promoter-like sequence is present in the cloned fragment in pMUX2, and this putative promoter and the *lac* promoter on the vector are present in tandem in this plasmid. Since a potent repressor in *P. aeruginosa* cells might repress the gene expression even if the gene is cloned in a multicopy plasmid, we constructed pMUX3, carrying *muxABC-opmB* without the putative promoter. In this plasmid *muxABC-opmB* is present just downstream of the *lac* promoter. *P. aeruginosa* YM64 transformed with pMUX3 showed the same MIC values of several antibiotics as YM64/pMUX2 (data not shown).

Necessity for two RND components, MuxB and MuxC

Since MuxABC shared sequence similarity with MdtABC, an RND-type efflux pump of *E. coli*, the properties of these two systems might be similar. In *E. coli*, it has been reported that both of the two RND components, MdtB and MdtC, were essential for its full activity (Baranova & Nikaido, 2002; Nagakubo *et al.*, 2002). To investigate the necessity for MuxB and MuxC, we constructed pMUX4 (Δ *muxB* from pMUX2) and pMUX5 (Δ *muxC* from pMUX2) (Fig. 1). The MICs of several drugs such as

Table 3. OpmB is indispensable for the function of the MuxABC-OpmB efflux pump

Antimicrobial agent		MIC ($\mu\text{g ml}^{-1}$)				
		YM64/pUCP20T	YM64/pMUX2	YM64/pMUX6	YM44/pUCP20T	YM44/pMUX6
	<i>muxABC</i>	—	+	+	—	+
	<i>opmB</i>	—	+	—	—	—
	<i>oprM</i>	—	—	—	+	+
Aztreonam		0.25	1	0.13	0.25	0.25
Imipenem		1	1	1	2	1
Norfloxacin		0.06	0.06	0.06	0.13	0.13
Novobiocin		32	512	8	32	16
Gentamicin		0.25	0.25	0.25	0.25	0.25
Chlortetracycline		1	2	0.5	2	1
Doxycycline		0.5	1	0.5	2	1
Tetracycline		0.5	1	0.25	1	1
Erythromycin		16	32	8	64	32
Kitasamycin		64	256	32	256	128
Rokitamycin		64	512	32	128	128
Chloramphenicol		2	2	2	4	2

aztreonam and novobiocin in *P. aeruginosa* YM64 transformed with pMUX4 or pMUX5 were the same as those in the control cells, YM64/pUCP20T (data not shown). We should take into account a possible polar effect on the genes located downstream of the disrupted gene. Therefore we measured mRNA expression of the genes that are downstream of the deleted ORF. No change was observed in the expression of the genes adjacent to the deleted gene (data not shown). Therefore, we conclude that both of the two RND components are essential for the function of the MuxABC-OpmB pump.

We also constructed a plasmid with *muxA* deleted from pMUX3 to test the necessity of MuxA for the pump function. *P. aeruginosa* YM64 transformed with this plasmid did not show increased MICs of aztreonam and novobiocin (data not shown). Thus, we conclude that MuxA is also essential for the pump.

Necessity for OMP component OpmB

The RND-type efflux pump requires an OMP component for function (Aires *et al.*, 1999; Fralick, 1996). The *opmB* gene encoding an OMP component is present in the downstream region of *muxABC*. We showed that introduction of *muxABC-opmB* into cells of *P. aeruginosa* resulted in elevated MICs of several antibiotics (Table 3). This suggested that OpmB could function with MuxABC. We investigated, therefore, whether OpmB is really essential for the function with MuxABC. We constructed a plasmid pMUX6 carrying an intact *muxABC* region and a disrupted *opmB* gene (Fig. 1). *P. aeruginosa* YM64 transformed with pMUX6 did not show increased MICs of antimicrobial agents tested compared with the control YM64/pUCP20T (Table 3). Thus, we conclude that OpmB is essential for the pump function of MuxABC. It should be pointed out that we observed somewhat decreased MICs of

aztreonam, novobiocin, tetracycline, erythromycin, kitasamycin and rokitamycin with YM64/pMUX6 compared to the control (Table 3). This suggests that OpmB also functions with other multidrug efflux pump(s) that extrude these antibiotics. The possible reason is discussed later.

YM64 host cells are defective for *oprM*, which codes for OprM, a major OMP component for RND-type multidrug efflux pumps in *P. aeruginosa*. Since OpmB showed high similarity with OprM, we tested the possibility that MuxABC can use OprM instead of OpmB, by using YM44, an OprM active strain, as host. Contrary to our expectation, however, we observed no increase in the MICs of antimicrobial agents tested with YM44/pMUX6 (possessing *muxABC* and *oprM*) compared with YM44/pUCP20T (control possessing *oprM*) (Table 3).

These results support the view that OpmB cannot be replaced with OprM for the function of the MuxABC system.

Isolation of novobiocin-resistant mutants

It seemed possible to isolate mutants that show elevated expression of the *muxABC-opmB* operon or elevated function of the MuxABC-OpmB pump. For this purpose, we used *P. aeruginosa* PMX7 ($\Delta\text{mexAB-oprM}$, $\Delta\text{mexCD-oprJ}$, $\Delta\text{mexEF-oprN}$, ΔmexXY , $\Delta\text{mexHI-opmD}$, ΔmexVW , ΔpmpM). Since novobiocin is a good substrate for the MuxABC-OpmB pump (Table 3), we tried to isolate novobiocin-resistant mutants from PMX7. The MIC of novobiocin in all six mutants was elevated, and was eight times higher than that in the parental PMX7. Four of them showed elevated MIC of only novobiocin (data not shown). However, in two remaining mutants, the MICs of aztreonam, erythromycin, kitasamycin and rokitamycin

were also elevated (Table 4). We named one of these multidrug-resistant mutants as PMX725. PMX725 grew better in the presence of tetracycline than PMX7 (data not shown) although the MIC of tetracycline in these two types of cells was the same (Table 4).

Since novobiocin, aztreonam, erythromycin and tetracycline are substrates for the MuxABC-OpmB multidrug efflux pump, PMX725 is presumed to be a mutant with respect to this pump. In order to test whether *muxABC-opmB* is responsible for elevated resistance to these antibiotics in PMX725, we disrupted *muxABC-opmB* in the mutant. As expected, the construct KN725, a disruptant of *muxABC-opmB* derived from PMX725, showed reduced MICs of the antibiotics compared with the parental strain PMX725 (Table 4). Moreover, the result of the RT-PCR measurement revealed that the expression of *muxA* increased in PMX725 (Fig. 2). These results indicate that the increased resistance to novobiocin, aztreonam and erythromycin in the mutant PMX725 was caused by the increased expression of *muxABC-opmB*.

Interestingly, KN7, a deficient mutant of *muxABC-opmB* genes, was more susceptible to novobiocin, kidasamycin and rokitamycin (which are substrates for the MuxABC-OpmB pump) than the parental PMX7 (Δ *mexAB-oprM*, Δ *mexCD-oprJ*, Δ *mexEF-oprN*, Δ *mexXY*, Δ *mexHI-opmD*, Δ *mexVW*, Δ *pmpM*) (Table 4). In addition, an RT-PCR experiment revealed that *muxA* was expressed in the parental PMX7 (Fig. 2). These results also indicate that *muxABC-opmB* is expressed in the parent, and is responsible for resistances to these antibiotics in the parental PMX7.

Identification of the mutation in PMX725

In order to identify the mutation in *P. aeruginosa* PMX725 in which expression of the *muxA* gene was elevated (Fig. 2), we determined the DNA sequence of the putative promoter region of *muxA* in PMX725. A single nucleotide insertion

Fig. 2. mRNA expression of *muxA* in *P. aeruginosa*. Expression of *muxA* (upper) and *rpsL* (lower; internal control) was measured by the RT-PCR method. Lanes: 1, marker (pUC19 digested with *Msp* I); 2, PMX7; 3, PMX725. The PCR cycles were 30 for *muxA* and 24 for *rpsL*.

was found adjacent to the putative -10 region for the *muxABC-opmB* operon in the PMX725 strain (Fig. 3).

To confirm whether the insertion of T adjacent to the possible -10 region of the putative promoter for *muxA* in PMX725 is responsible for increased expression of the *muxABC-opmB* operon, we cloned *muxABC-opmB* from PMX725 with the mutated promoter region (pMUX82). We compared the expression levels of the operon by RT-PCR. Expression of *muxA*, *muxB*, *muxC* and *opmB* in KN7/pMUX82 (mutant type) was clearly higher than that in KN7/pMUX8 (wild-type) (data not shown). Based on these results, we conclude that the insertion of T in the putative promoter region of *muxABC-opmB* enhanced expression of the genes in PMX725, and that the putative promoter would be the real promoter for the genes.

DISCUSSION

In this study we cloned the PA2528-PA2527-PA2526-*opmB* genes encoding an RND-type multidrug efflux pump and renamed PA2528, PA2527, PA2526 as *muxA*, *muxB* and *muxC*, respectively. This pump system needs four components, two RND components (MuxB and MuxC), one MFP component (MuxA) and one OMP component (OpmB). MuxABC-OpmB is the only RND-type pump that requires two RND components in *P. aeruginosa*. We surveyed genome databases to find genes encoding two RND-component-like proteins in an operon, and found homologues of MuxB and MuxC in *Ralstonia* [e.g. *R. solana*-

Table 4. Contribution of MuxABC-OpmB to the resistance to antimicrobial agents

Antimicrobial agent	MIC ($\mu\text{g ml}^{-1}$)			
	PMX7	PMX725	KN7	KN725
Aztreonam	0.25	0.5	0.25	0.25
Norfloxacin	0.03	0.03	0.03	0.03
Novobiocin	32	256	4	4
Gentamicin	0.25	0.25	ND*	ND*
Tetracycline	0.5	0.5	0.5	0.5
Erythromycin	8	16	8	8
Kitasamycin	32	128	8	8
Rokitamycin	32	256	16	16
Chloramphenicol	8	8	4	4

*ND, Not determined.

Fig. 3. Nucleotide sequence of wild-type and mutant-type promoter region of *muxABC-opmB*. The upper row indicates the nucleotide sequence in wild-type PAO1, and the lower row indicates that in PMX725. The deduced promoter region is shown as -35 and -10 , and the single nucleotide insertion (T) near the putative -10 region is indicated by a box.

cearum (Salanoubat *et al.*, 2002), *R. pickettii* (NC_010678)], *Chromobacterium* [e.g. *C. violaceum* (Brazilian National Genome Project Consortium, 2003)], *Pseudomonas* [e.g. *P. putida* (YP_001267510), *P. entomophila* (Vodovar *et al.*, 2006), *P. fluorescens* (Paulsen *et al.*, 2005), *P. syringae* (Almeida *et al.*, 2009)], *Burkholderia* [e.g. *B. dolosa* (YP_002098778), *B. thailandensis* (YP_443379), *B. pseudomallei* (YP_002106659), *B. cenocepacia* (YP_001764468)] among others. The function of these homologues, however, has not been investigated. Thus, it is unclear whether they are drug efflux pumps.

MuxABC shows high similarity with MdtABC from members of the *Enterobacteriaceae* (Baranova & Nikaido, 2002; Kube *et al.*, 2008; Nagakubo *et al.*, 2002; Nishino & Yamaguchi, 2001; Nishino *et al.*, 2007; Parkhill *et al.*, 2001a, b). It has been reported that MdtABC elevated the resistance levels against cholate, SDS and novobiocin in *E. coli* (Baranova & Nikaido, 2002; Nagakubo *et al.*, 2002). We tried to measure the MICs of cholate and SDS in *P. aeruginosa* YM64/pUCP20T (control) and YM64/pMUX2 (carrying *muxABC-opmB*), but were unable to because the cells grew at the solubility ranges of cholate and SDS.

We detected the mRNA of *muxABC-opmB* in cells of wild-type *P. aeruginosa* PAO1 (data not shown). Therefore it seemed possible that the MuxABC-OpmB pump contributes to the intrinsic resistance of *P. aeruginosa* against novobiocin, and to the macrolides to some extent. However, we were unable to detect a change in the MIC of novobiocin when we disrupted the *muxABC-opmB* genes in *P. aeruginosa* PAO1 (data not shown). The reason why an MIC change was not detected in the *mux*-deleted cells could be that potent novobiocin efflux pumps such as MexAB-OprM and MexCD-OprJ are active enough in PAO1 (Masuda *et al.*, 2000; Schweizer, 2003) to mask the defect of MuxABC-OpmB.

Our results indicated that the elevated MIC of novobiocin in mutant PMX725 was caused by a mutation in the promoter of the *muxABC-opmB* operon. A similar result was recently reported for *triABC* in *P. aeruginosa* (Mima *et al.*, 2007). These authors reported that a single nucleotide substitution in the predicted promoter -35 sequence elevated the expression of *triABC*. It has also been reported in various bacteria that a mutation in a promoter region enhanced gene expression related to drug resistance and that the cells showed increased drug resistance (Huang *et al.*, 2004; Kaatz *et al.*, 1999; Siu *et al.*, 2003). Therefore, it seems to be quite a common phenomenon that mutations in the promoter region elevate drug resistance levels in bacteria.

Lack of an OMP component of the RND-type pump confers a severe effect on the resistances to antibiotics in bacteria, and it is sometimes more serious for cells than the lack of an RND component for a drug efflux pump (Morita *et al.*, 2001a; Sulavik *et al.*, 2001). Therefore, we investigated whether the decrease in the MIC of novobiocin in KN7, the whole *muxABC-opmB* of which is disrupted,

was mainly caused by the deletion of *opmB* alone. The MIC of novobiocin was measured in KN7 transformed with a plasmid carrying *opmB* but not *muxABC*. We observed no change in the MIC value by the introduction of *opmB* (data not shown). Thus, it is clear that not only OpmB, but also MuxABC, are necessary for the observed novobiocin resistance. It should be pointed out, however, that we observed an interesting phenomenon. In YM64/pMUX6 and YM44/pMUX6, the MICs of several antimicrobial agents were lower than the controls (Table 3). pMUX6 carries *muxABC* but not *opmB* (Table 1). This means that overexpression of MuxABC without OpmB conferred elevated susceptibilities to several antimicrobial agents in cells of YM64 and YM44. One possible reason is that overproduced MuxABC may recruit an OMP component from another functional efflux pump (or pumps). It seems unlikely that MuxABC and the recruited OMP would exhibit drug-resistance activity. Meanwhile, the efflux system that has lost its OMP component would no longer function as a pump. It is likely that possible candidates include certain efflux pumps from the MF family. EmrAB, an MF family multidrug efflux pump from *E. coli*, has been reported to function with the OMP component TolC (Lewis, 2000), and genes for its orthologue are present in the genome of *P. aeruginosa* (PA5159-PA5160). We are now investigating the function of all the deduced MF-type multidrug efflux pumps in *P. aeruginosa*. Other candidates are also possible.

In this report, we have characterized the last RND-type multidrug efflux pump predicted from the genome sequence in *P. aeruginosa*. All the putative RND-type multidrug efflux pumps predicted from the genome sequence have been shown to be responsible for resistance to antimicrobial agents or metal ions. Thus, the primary characterization of all RND-type efflux pumps in *P. aeruginosa* PAO1 has been completed. We now have some understanding of the substrate specificities for all of the *P. aeruginosa* RND pumps and of their contributions to intrinsic resistance to antimicrobial agents in *P. aeruginosa*. It should be noted, however, that such properties of each of the RND pumps became clear only under laboratory conditions. The expression patterns of each efflux pump in biofilm or in the human body might be different from those observed under laboratory conditions. It would thus be important to know the expression profiles and properties of each pump in biofilm or in the human body, where treatment of infectious disease by *P. aeruginosa* becomes necessary.

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