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Inactivation of MuxABC-OpmB Transporter System in *Pseudomonas aeruginosa* Leads to Increased Ampicillin and Carbenicillin Resistance and Decreased Virulence

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Resistance-Nodulation-Cell Division (RND) pumps play important roles in bacterial resistance to antibiotics. *Pseudomonas aeruginosa* is an important human pathogen which exhibits high level resistance to antibiotics. There are total of 12 RND pumps present in the *P. aeruginosa* PAO1 genome. The recently characterized MuxABC-OpmB system has been shown to play a role in resistance to novobiocin, aztreonam, macrolides, and tetracycline in a multiple knockout mutation. In this study, we examined the expression levels of all the 12 RND pump gene clusters and tested the involvement of MuxABC-OpmB in pathogenicity. The results indicated that in addition to the four known constitutively expressed RND pumps, *mexAB-oprM*, *mexGHI-opmD*, *mexVW*, and *mexXY*, relatively high levels of expression were observed with *mexJK* and *muxABC-opmB* in the conditions tested. Inactivation of *muxA* in the *muxABC-opmB* operon resulted in elevated resistance to ampicillin and carbenicillin. The mutant also showed attenuated virulence in both *Brassica rapa pekinensis* and *Drosophila melanogaster* infection models. The decreased virulence at least in part was due to decreased twitching motility in the mutant. These results indicate that the RND pump MuxABC-OpmB is associated with ampicillin and carbenicillin susceptibility and also involved in pathogenesis in *P. aeruginosa*.

***Keywords*:** MuxABC-OpmB, β-lactam resistance, virulence, twitching motility, *P. aeruginosa*

Pseudomonas aeruginosa is an important human opportunistic pathogen. It exhibits high levels of intrinsic resistance to antibiotics, and consequently its infection is difficult to eradicate (Poole *et al.*, 1993, 2001; Li *et al.*, 1994). In recent years, multidrug resistant *P. aeruginosa* strains have become a great concern in hospitals all over the world.

It is known that the intrinsic resistance of *P. aeruginosa* mostly results from the low permeability of the outer membrane and the existence of multidrug efflux pumps (Hancock, 1997; Zgurskaya and Nikaido, 2000). Five families of efflux pumps have been identified: the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance nodulation cell division (RND) family, multidrug and toxic compounds extrusion (MATE) family, and ATP binding cassette (ABC) family (Schweizer, 2003). Among them, the RND family multidrug pumps play a major role in *P. aeruginosa* intrinsic antibiotic resistance. There are twelve RND-type efflux pumps in *P. aeruginosa* PAO1 genome and all of them have been characterized to some details in recently years. They include MexAB-OprM (Li *et al.*, 1995, 2003; Zhao *et al.*, 1998), MexCD-OprJ (Poole *et al.*, 1996, Linares *et al.*, 2005), MexEF-OprN (Kohler *et al.*, 1997; Linares *et al.*, 2005), MexGHI-OpmD (Aendekerk *et al.*, 2002, 2005; Sekiya *et al.*, 2003), MexJK-OprM (Chuanchuen *et al.*, 2002), MexMN (Li *et al.*, 2003), MexPQ-OpmE (Mima *et al.*, 2005), MexVW-OprM (Li *et al.*, 2003), MexXY-OprM (Mine *et al.*, 1999; Masuda *et al.*, 2000), CzcCBA (Rensing *et al.*, 1997; Hassan *et al.*, 1999; Perron *et al.*, 2004; Caille *et al.*, 2007), TriABC-OpmH (Mima *et al.*, 2007), and MuxABC-OpmB (Mima *et al.*, 2009). The MuxABC-OpmB was the last reported RND system in *P. aeruginosa*. It has been shown to increase resistance to aztreonam, macrolides, novobiocin, and tetracycline when introduced in a multiple knockout mutant (Mima *et al.*, 2009).

In this study, we measured the expression level of 12 RND pumps in *P. aeruginosa* PAO1 and investigated the involvement of MuxABC-OpmB in antibiotic resistance and in virulence using a *muxA* knockout mutant. The data indicating that six of the 12 RND pumps were constitutively expressed is presented, and the association of MuxABC-OpmB RND pump with ampicillin and carbenicillin resistance and with pathogenesis has been discussed.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study were listed in Table 1. Luria-Bertani (LB), Brian Heart Infusion (BHI), and *Pseudomonas* Isolate Agar (PIA) were used routinely. Defatted milk solid medium which is LB media supplemented with 2% defatted milk was used for measuring proteolytic activity and blood agar plates were used for measuring hemolytic activity. M8 medium (Sambrook

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Table 1. Bacterial strains and plasmids used in this study

Stain and Plasmid	Characteristics	Reference
Escherichia coli		
DH10B	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG /pMON14272 / pMON7124*	Invitrogen
SM10-*λpir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc:Mu, Kan^R, *λpir*	Simon *et al.* (1983)
Pseudomonas aeruginosa		
PAO1	Wild type	Holloway *et al.* (1994)
PAO(*muxA⁻*)	Mutant with a *lacZ*-Gen cassette inserted in PA2528; Gen^R	This study
PAO(*pilA⁻*)	Mutant with a *lacZ*-Gen cassette inserted in PA4525; Gen^R	This study
C2528	PAO*(muxA⁻)*complemented strain; PAO*(muxA⁻)attB*::PA2528	This study
PAM	PAO transposon mutant that shows 128 fold resistance for Car	Lab collection
Plasmids		
pEX18Tc	Broad-host-range gene replacement vector; *sacB*, Tet^R	Schweizer (1992)
pRK2013	Broad-host-range helper vector; Tra1 Kan^R	Figurski and Helinski (1979)
pZ1918-LacZ	Vector, source of the cassette containing promoterless *lacZ* and Gen^R (*lacZ*-Gen^R)	Schweizer (1993)
pM2528	knockout plasmid for constructing PAO(*muxA⁻*); Gen^R Tet^R	This study
pM4525	knockout plasmid for constructing PAO(*pilA⁻*); Gen^R Tet^R	This study
Mini-CTX1	Integration plasmid; Tet^R	Hoang *et al.* (2000)
pC2528	Complementation construct, mini-CTX1 with 1624bp fragment covering PA2528 and 313 bp upstream and 30 bp downstream regions; Tet^R	This study
pMS402	Reporter vector, *luxCDABE*; Kan^R Tmp^R	Duan *et al.* (2003)
pKD-*mexA*	pMS402 carrying the promoter region of *mexAB-oprM*; Kan^R Tmp^R	This study
pKD-*mexC*	pMS402 carrying the promoter region of *mexCD-oprJ*; Kan^R Tmp^R	This study
pKD-*mexE*	pMS402 carrying the promoter region of *mexEF-oprN*; Kan^R Tmp^R	This study
pKD-*mexG*	pMS402 carrying the promoter region of *mexGHI-opmD*; Kan^R Tmp^R	This study
pKD-*mexJ*	pMS402 carrying the promoter region of *mexJK*; Kan^R Tmp^R	This study
pKD-*mexM*	pMS402 carrying the promoter region of *mexMN*; Kan^R Tmp^R	This study
pKD-*mexP*	pMS402 carrying the promoter region of *mexPQ-opmE*; Kan^R Tmp^R	This study
pKD-*mexV*	pMS402 carrying the promoter region of *mexVW*; $Kan^R Tmp^R$	This study
pKD-*mexX*	pMS402 carrying the promoter region of *mexXY*; $Kan^R Tmp^R$	This study
pKD-*czcC*	pMS402 carrying the promoter region of *czcCBA*; Kan^R Tmp^R	This study
pKD-*triA*	pMS402 carrying the promoter region of *triABC*; Kan^R Tmp^R	This study
pKD-*muxA*	pMS402 carrying the promoter region of *muxABC-opmB*; $Kan^R Tmp^R$	This study

et al., 1989) supplemented with 0.2% Glucose, 2 mM $MgSO_4$, and 0.05% casamino acids was also used. Solid medium with 0.5% agar was used for swarming assays, and 1.0% agar for twitching motility assays. Swimming plates were LB agar with 0.3% agar. Cultures were grown at 37℃. When appropriate, kanamycin (Kan, 50 μg/ml), gentamicin (Gen, 15 μg/ml) and tetracycline (Tet, 15 μg/ml) were added for *Escherichia coli*; Trimethoprim (Tmp, 300 μg/ml), Gen (150 μg/ml), and Tet (300 μg/ml) were added for *P. aeruginosa*.

DNA manipulations

Enzymes used for DNA manipulations were obtained from Fermentas (Canada) and New England Biolabs (USA). PCRs were carried out using DingGuo Taq Plus DNA polymerase (DingGuo ChangSheng Corporation, China). BioDev-tech plasmid mini-prep kit, PCR product purification kit, and gel purification kits (Bioteke Corporation, China) were used according to manufacturer's instruction. Plasmids were constructed using standard techniques and were introduced into *E. coli* or *P. aeruginosa* by electroporation (Sambrook *et al.*, 1989; Choi *et al.*, 2006).

Construction of gene expression reporters

The promoter regions of all the 12 RND pump gene clusters were PCR amplified and cloned into pMS402 containing a promoterless *luxCDABE* reporter (Duan *et al.*, 2003) to monitor gene expression. The primers used are listed in Table 2. A *Bam*HI and an *Xho*I sites were incorporated in the primers to facilitate subsequent cloning. Genomic DNA of PAO1 was isolated following established procedures and used as the template for amplifying the promoter regions of the RND pumps. The PCR procedures used were as follows: 94℃ for 3 min; 30 cycles of 40 sec at 94℃, 40 sec at 56℃, and 1 min at 72℃; and the final step was 10 min extension at 72℃. A annealing temperature of 51°C was used for amplification of *mexCD-oprJ* and *czcCBA* promoter regions.

The PCR products were digested with *Bam*HI and *Xho*I, and cloned into pMS402 digested by the same enzymes. The recombinant plasmids were electroporated into DH10B strain using Eppendorf Electroporator 2510 (Germany), and transformants were selected on LB agar containing Kan at 50 μg/ml. After restriction analysis, the recombinant plasmids were transferred into PAO1 by electroporation.

Monitoring gene expression

The promoter activity of the RND pumps was measured as light production in the wells of a 96-well clear-bottom Costar plate (Corning Inc, USA) using a Perkin-Elmer Victor3 multilabel plate reader

Table 2. PCR primers used in constructing RND expression reporter plasmids

Primers	Sequence (5'→3')
mexA-f	AAACTCGAGGCCGAGTAAACCTAATG
mexA-r	CTGGATCCGGAACCAGTACACGCATGG
mexC-f	AAACTCGAGCAGGGTGGCCTTGCTTAC
mexC-r	CTGGATCCTGCACCGTCAGGACCTC
mexE-f	AATCTCGAGCATGTTCATCGGCGATCC
mexE-r	CAGGATCCAGGCGCTCAGGACCAGTA
mexG-f	AATCTCGAGTTGCCCGCAAGCTACAC
mexG-r	CAGGATCCAGAGCCAGTTGCTTTC
mexJ-f	AAACTCGAGGGCGATATTCAGCAGGAC
mexJ-r	CAGGATCCGGTACATGTGACACCTTC
mexM-f	AATCTCGAGCACGGCTCAGTATCGACTCC
mexM-r	CAGGATCCGATGCCGCTGACCTGTTG
mexP-f	AAACTCGAGCCGGACTTCCCTTCCTA
mexP-r	TGGATCCTGATGCGGATGAAGTGTCTG
mexV-f	AAACTCGAGGTTGCCGCTTCTACGACTG
mexV-r	CCGGATCCGATCTGCTGACGGATGGAGT
mexX-f	AATCTCGAGGCAGACCTCGATCTTGT
mexX-r	CTGGATCCAGCCCAGCAGGAATAGGG
czcC-f	AAACTCGAGATCCAACTATTGCCAGTCTC
czcC-r	CAGGATCCAGAGTCCCATCAGTAGCC
triA-f	AAACTCGAGCGTGCCTGGTGGAGATTC
triA-r	CAGGATCCAGCGACCTTTGGAATG
muxA-f	AACCTCGAGTTTCAACGGGTCGATCATCT
muxA-r	CCGGATCCATCACCAGGCCGATCAC

Underlined are *Xho*I and *Bam*HI restriction sites incorporated in the primers to facilitate cloning.

(USA). 2 μl overnight cultures of the reporter strains were diluted into 100 μl fresh medium and cultivated for additional 2 h before being used as inoculants. 5 μl of inoculants were introduced into the wells containing a total of 95 μl medium. The initial OD_{595} value in the wells was about 0.1. Filter-sterilized mineral oil (60 μl) was added to the wells to prevent evaporation during the assay. Promoter activity (light production) was measured every 30 min for 24 h. Bacterial growth was monitored at the same time by measuring OD_{595}. The strain carrying pMS402 was used as a control.

Construction of mutant strain PAO (*muxA*$^-$) and PAO (*pilA*$^-$)

To construct the *muxA* knockout mutant, a *sacB*-based gene replacement method was used (Hoang *et al.*, 1998). A knockout plasmid pM2528 was first constructed as follows. A 1,665 bp PCR fragment containing *muxA* was generated with primers *muxA*-mf (5'-CCTCGAGTTTCAACGGGTCGATCATCT-3'), and *muxA*-mr (5'-CGTAAGCTTCGGAACTGCTGGAACGACATC-3'). Underlined are *Ava*I and *Sph*I restriction sites incorporated in the primers to facilitate cloning. The PCR fragments were digested by these enzymes and ligated into pEX18-Tc plasmid (Schweizer, 1992) digested with the same restriction enzymes. Then the *lacZ*-Gen^R cassette from pZ1918-LacZ (Schweizer, 1993) was inserted into the *Pst*I site of the PCR fragment. The orientation of the *lacZ*-Gen^R cassette was determined by *Eco*RV digest.

For *pilA* mutant, a 1,100 bp PCR fragment containing *pilA* was generated with primers *pilA*-mf (5'-TGCCAAGCTGGAAGCTTCCG-3') and *pilA*-mr (5'-GGAATTCCAGCGACAGCTTGTTG-3') containing a *Hind*III and an *Eco*RI site respectively. The PCR product was cloned into pEX18-Tc using these restriction sites. The *lacZ*-Gen^R cassette was then inserted into the *Kpn*I site on the PCR fragment. The constructed plasmid was named pM4525.

Introduction of above knockout vectors into PAO1 was carried out by triparental mating (Ditta *et al.*, 1980). Briefly, 25 ml of overnight cultures of *E. coli* containing pM2528 or pM4525, *E. coli* containing pRK2013, and *P. aeruginosa* PAO1 were pelleted respectively and resuspended in PBS. The bacteria were mixed in a ratio of 1:1:1 in a micro centrifuge and then spotted onto a LB agar plate. After growing overnight at 37°C, the bacteria were scraped and resuspended in 1 ml of PBS. Dilutions were spread on PIA containing Gen at 150 μg/ml. Clones undergone second crossover was selected on LB containing 10% sucrose. Correct knockout unable to grow on PIA containing Tet but able to grow on LB containing 10% sucrose was verified by PCR analysis.

Drug susceptibility tests

MICs of antimicrobial agents were measured by two-fold dilutions of the agents in the wells of 96-well plates. Overnight bacterial cultures were diluted at 1:150 in LB and added to the wells containing different concentrations of antibiotics and incubated at 37°C for 24 h.

Disc-diffusion tests were also used to measure drug susceptibility. 10 μl of antibiotics solutions was spotted on 6 mm diameter filter-discs placed on LB agar plates spread with 4×10^6 CFU test bacterial cells. The plates were incubated at 37°C for 18 h. The antibiotic susceptibility was determined by measuring the diameters of the zones of clearance around the antimicrobial agents (Poole *et al.*, 1993).

β-Lactamase test

Test of β-lactamase was carried out as previously described with minor modifications (Perret, 1954). A solution containing 10,000 U of ampicillin or penicillin G per ml of phosphate buffer was prepared and dispensed in 0.5-ml small tubes. The test bacteria grown on LB agar for 18 to 24 h were suspended in the ampicillin or penicillin G solution and adjusted to make a density of about 10^9 cells/ml. After 0.5 h at 37°C and 1 h at room temperature, 50 μl starch indicator were added to the suspension, followed by mixing with 20 μl iodine reagent. A blue color immediately developed due to the reaction of the iodine with the starch. Persistence of the blue color for longer than 10 min constituted a negative test and indicated that the ampicillin molecules had not undergone β-lactam ring cleavage (Perret, 1954; Caille *et al.*, 2007). Rapid decolorization occurred if the penicillin was hydrolyzed suggesting the presence of β-lactamase activity.

Complementation of PAO (*muxA*$^-$) mutant

In an attempt to restore the function of MuxABC-OpmB in PAO (*muxA*$^-$), the *muxA* gene together with its promoter was integrated at the *attB* site on the chromosome using the mini-CTX1 system (Hoang *et al.*, 2000). The entire gene was PCR amplified together with a 313 bp upstream promoter region and 30 bp downstream region using primers C2528f (5'-GTGGAATTCGGCGCGACTGCGGAGC-3') and C2528r (5'-TCGGATCCGGCCGCAGGATGAACGGG-3'), containing an *Eco*RI and a *Bam*HI site (underlined) respectively. The product was ligated into mini-CTX1 to generated plasmid pC2528 and transferred into *E. coli* SM10-*λpir*. pC2528 was then transferred into PAO(*muxA*$^-$) by biparental mating. Integrants were selected on PIA containing Tet at 200 μg/ml and Gen at 50 μg/ml. The integrase on pC2528 promoted the integration of the vector into the *attB* site on the *P. aeruginosa* genome. The plasmid portion of pC2528 was resolved from the chromosome by introducing pFLP2 which carries an Flp recombinase gene (Hoang *et al.*, 2000). The resultant strain was designated C2528.

In vivo virulence assays

A Chinese cabbage (*Brassica pekinensis*) infection model based on the lettuce infection model by Rahme *et al.* (1997) and a fruit fly (*Drosophila melanogaster* Canton S) injection model (Chugani *et al.*, 2001) were used to compare the virulence of the *muxA* mutant and the wild type. PAO1 and the isogenic mutant PAO (*muxA*$^-$) were grown overnight at 37°C in LB. Cells were collected and washed in 10 mM $MgSO_4$. After diluted to 10^7 CFU/ml, 10 µl of the diluted cells was inoculated with a micropipette into the stems of Chinese cabbage. The plant stems were washed with 0.1% H_2O_2 before the inoculation. Inoculated stems were placed in a 15 cm diameter Petri dishes containing a filter impregnated with 10 mM $MgSO_4$ and kept in a growth chamber at 28°C with the symptoms monitored daily for 5 days (Rahme *et al.*, 1997). Same midrib of the cabbage was inoculated with the PAO1 for comparison.

The Drosophila assay was carried out as described previously (Chugani *et al.*, 2001). Test strains were grown overnight in LB medium and OD_{600} values were adjusted to 0.2 before use. 3-5 days old female fruit flies were injected with PAO1 and PAO (*muxA*$^-$) respectively at the dorsolateral thorax using 28-gauge needles. PBS was used as negative control and PAO (*pilA*$^-$) was used as non-twitching control. Each group had 100 flies. The injected flies were kept at 25°C in standard glass fly culture vials (10 flies per vial) containing 5 ml of 5% sucrose agar. Live and dead flies were counted hourly for 30 hours, and the virulence of bacterial strains was measured as the rate of killing.

Pseudomonas quinolone signal (PQS) molecule extraction and quantitation

The extraction of PQS was performed as described by Aendekerk *et al*. (2005) Test strains were cultured at 37°C for 18 h (OD_{600} approximately at 2.0). For the detection of extracellular PQS, 10 ml supernatants were extracted with equal acidified ethyl acetate. The organic phase was dried and resuspended in 50 µl methanol. For intracellular PQS, cells from 40 ml culture was resuspended in 20 ml LB and sonicated. The supernatant of the cell lysate was extracted using equal volume of acidified ethyl acetate and resuspended in 100 µl methanol after evaporation.

10 µl of the extracellular or intracellular PQS extracts were spotted on a silica gel plate (HaiLang, China) saturated in 5% KH_2PO_4 for 30 min and activated at 100°C for 1 h. The running solution used was dichloromethane and methanol at 95:5. PQS was visualized under long-wave UV light 365 nm in Syngene Bio-imaging systems (England).

Virulence factor and motility phenotyping assays

A series of virulence factors were tested according to previously described methods including pyocyanin production (Kurachi, 1958), protease assay (Brown and Foster, 1970; Wretlind *et al.*, 1977; Sokol *et al.*, 1979), hemolysin assay (Bettelheim, 1995), swimming (Bai *et al.*, 2007; Wilhelm *et al.*, 2007; Overhage *et al.*, 2008), swarming (Kohler *et al.*, 2000; Overhage *et al.*, 2008) and twitching motility (Alm and Mattick, 1995; Semmler *et al.*, 1999) in both PAO(*muxA*$^-$) and PAO1. Twitching motility was tested following the method described previously (Alm and Mattick, 1995; Semmler *et al.*, 1999). 2 µl of bacterial cells that were grown overnight at 37°C in LB and adjusted to OD_{600} of 0.5 were inoculated into underneath of M8 agar (1%). After incubated overnight at 37°C, the plates were left at room temperature for 2 more days. The zones of twitching were then visualized by staining with Coomassie brilliant blue R250 after the agar

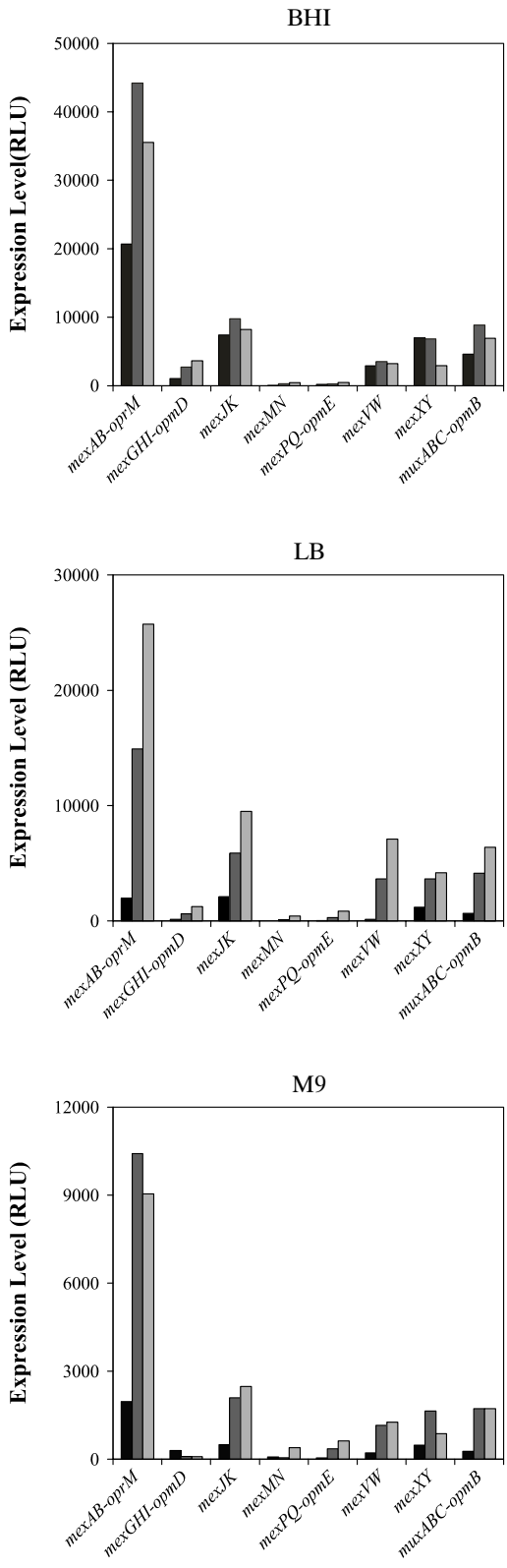

Fig. 1. The relative expression levels of the RND pumps at different growth phases in different media. RLU, relative light units (CPS/OD_{595}). Values for lag phase are shown in black bars, exponential phase in dark-grey and stationary phase in light-grey. No detectable light production was observed from the rest RND promoter reporters.

was removed.

Results

Comparison of expression levels of the RND pump genes There are total of 12 RND pump gene clusters in *P. aeruginosa* PAO1 genome. In order to understand overall expression in the RND family, systematical measurement of the expression levels of all the 12 pumps in PAO1 was carried out. The individual promoter regions of the 12 RND pump gene clusters were amplified by PCR and fused upstream of the promoterless *lux* reporter on plasmid pMS402 (Duan *et al.*, 2003), and the expression level of the 12 RND pump gene clusters in *P. aeruginosa* PAO1 were measured. The results indicated that in addition to the four known constitutively expressed RND pumps, *mexAB-oprM*, *mexGHI-opmD*, *mexVW*, and *mexXY*, relatively high levels of expression were observed for *mexJK* and *muxABC-opmB*, suggesting they are also constitutively expressed. No detectable activity was observed with the rest RND pump promoter reporters under the laboratory conditions tested (Fig. 1).

Increased drug resistance of PAO (*muxA*$^-$)

MuxABC-OpmB is the latest RND pump characterized in *P. aeruginosa*. To further investigate the contribution of MuxABC-OpmB to antimicrobial agent resistance in *P. aeruginosa*, a *muxA* knockout mutant was constructed and its susceptibilities to 13 different antibiotics were compared with those of the wild type strain PAO1. Somewhat surprisingly, the MIC of ampicillin increased 4 fold from 625 μg/ml in PAO1 to 2,500 μg/ml in the mutant, and the MIC of carbenicillin increased 10 fold from 100 μg/ml in PAO1 to 1,000 μg/ml in the mutant. Drug susceptibility was also tested using disc diffusion method and similar result was obtained (Fig. 2). Difference of susceptibility was not observed between PAO (*muxA*$^-$) and PAO1 with other antimicrobial agents, including azithromycin, chloramphenicol, cefotaxime, ciprofloxacin, erythromycin, kanamycin, oxytetracycline HCl, rifampicin, streptomycin, tetracycline, and vancomycin.

To examine if the increased resistance of the *muxA* mutant to ampicillin and carbenicillin was related to changed β-lactamase activity, the mutant was compared with the wild type in β-lactamase activity. The results (Table 3) indicate that the *muxA* mutant had higher β-lactam hydrolysis activity than PAO1, suggesting the PAO(*muxA*$^-$) had elevated β-lactamase activity in the culture.

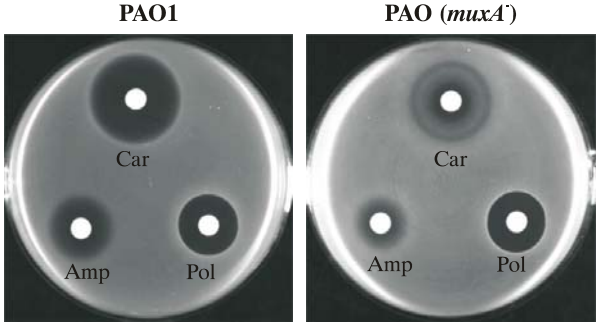

Fig. 2. Altered antibiotic susceptibility in PAO(*muxA*$^-$). Amp, ampicillin; Car, carbenicillin; Pol, polymyxin B. 500 μg Amp, 500 μg Car, and 50 μg Pol were added to the filter discs respectively. Pol was used as a negative control; no change in susceptibility to Pol was observed between PAO(*muxA*$^-$) and PAO1.

Table 3. Iodometric detection of penicillin hydrolysis by the β-lactamas

Strains	Penicillin substrate	Incubation time (sec)				
		5	10	20	60	120
PAO (*muxA*$^-$)	Ampicillin	0	+	++		
	Penicillin G	++	++	++		
PAO1	Ampicillin	0	0	0	+	++
	Penicillin G	0	0	0	+	++
PAM	Ampicillin	++				
	Penicillin G	++				
Control	Ampicillin	0	0	0	0	0
	Penicillin G	0	0	0	0	0

"++", extensive substrate hydrolysis indicated by decolorization; "+", partial hydrolysis; "0", no color change observed. Samples with no bacterial culture were used as negative controls which showed no color change within 20 min.

An attempt to complement the *muxA* mutant by inserting PCR amplified *muxA* at the *attB* site in the chromosome using the mini-CTX1 systems (Hoang *et al.*, 2000) was performed, but the result indicated the single *muxA* was unable to restore the wild type phenotype. This is probably due to the polar effect of the *lacZ*-Gen^R inserted in *muxA* on the downstream genes. Complementation using the whole fragment of *muxABC-opmB* in the *muxA* mutant was unsuccessful due to the whole gene cluster is about 11 kb, making error free PCR amplification and cloning impractical.

Lost twitching motility of PAO (*muxA*$^-$)

RND pumps in *P. aeruginosa* are not only important for antibiotic resistance but also important for pathogenesis (Hirakata *et al.*, 2002). RND pumps have been shown involved in the efflux of signal molecules (Pearson *et al.*, 1999; Kohler *et al.*, 2001; Aendekerk *et al.*, 2005) and virulence factors (Hirakata *et al.*, 2002). For RND pump MexGHI-OpmD, the *mexI* and *opmD* mutants showed increased resistance to several antibiotics and decreased virulence because this pump is connected with PQS production (Aendekerk *et al.*, 2005). To investigate whether the mutation of *muxA* affected the bacterial growth or virulence factor efflux, a series of virulence factor assays were measured.

As indicated by CFU counting, there was no obvious difference in the growth rate of the mutant compared to PAO1 (data not shown). The production of virulence factors including proteases, hemolysin, and pyocyanin showed no changes in the mutant. PQS production in the *muxA* mutant which lost MuxABC-OpmB function was also compared to that in PAO1. Both the extracellular and intracellular PQS levels were comparable for both strains, but no difference was observed (data not shown).

Being an important component of bacterial pathogenesis, motility in the mutant was also tested. While no difference in swimming or swarming motilities between the mutant and PAO1 was observed, PAO (*muxA*$^-$) was defective in twitching motility. The loss in twitching motility was similar to that observed for a *pilA* mutant (Fig. 3).

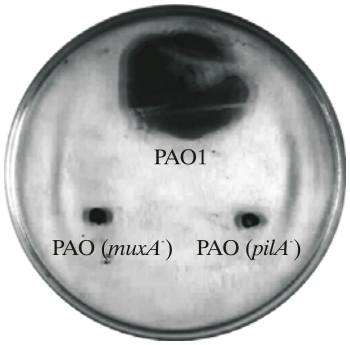

Fig. 3. Twitching motility. Decreased twitching motility in PAO (*muxA*⁻) and PAO (*pilA*⁻). PAO1 (upper) formed a hazy zone of cells spread underneath the agar while PAO (*muxA*⁻) (bottom left) and PAO (*pilA*⁻) (bottom right) lacked a zone.

Attenuated virulence of *muxA* mutation PAO (*muxA*⁻)

RND pumps in *P. aeruginosa* are not only important for antibiotic resistance but can also be important for pathogenesis (Hirakata *et al.*, 2002). The involvement of MuxABC-OpmB in bacterial virulence was investigated using both plant and animal models. The virulence of PAO (*muxA*⁻) was compared with that of the wild type strain PAO1 using *Brassica pekinensis* and *Drosophila melanogaster* Canton S hosts. As shown in Fig. 4, the PAO (*muxA*⁻) mutant showed attenuated virulence compared with PAO1 in both infection models. PAO (*muxA*⁻) caused obviously less severe necrosis and maceration than PAO1. *Drosophila melanogaster* Canton S infected with PAO (*muxA*⁻) also showed higher survival rate.

To verify that the loss of twitching motility in PAO (*muxA*⁻) directly contributed the decreased virulence a non-twitching *pilA* mutant PAO (*pilA*⁻) was constructed and used as a control in the virulence assays. As shown in Fig. 4, similar results were obtained for these two mutants. This observation is in agreement with the previous results showing twitching motility is involved in *P. aeruginosa* virulence (Hahn, 1997; Comolli *et al.*, 1999).

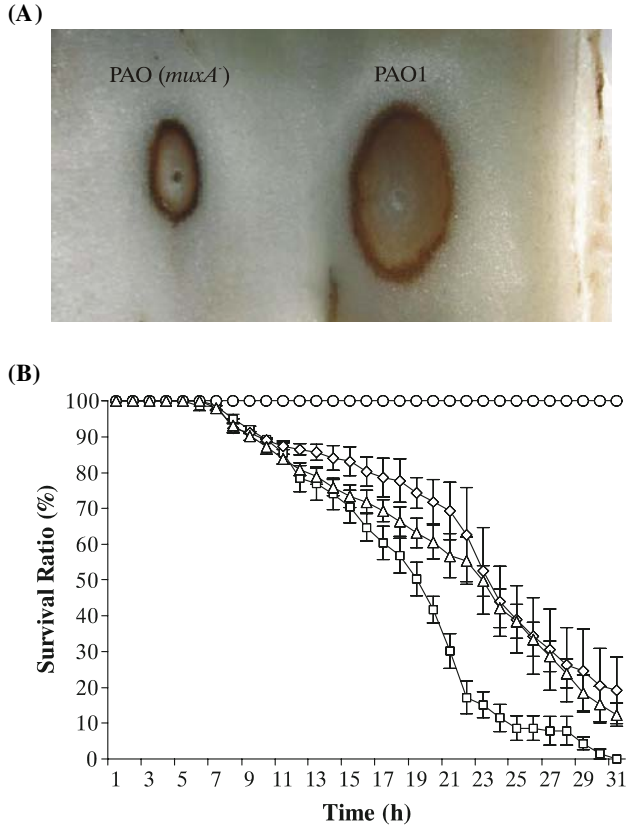

Fig. 4. Virulence of PAO (*muxA*⁻) and PAO1 in *Brassica pekinensis* and *Drosophila melanogaster* Canton S hosts. (A) Necrosis and maceration caused by PAO (*muxA*⁻) and PAO1 in *Brassica pekinensis*. (B) Survival of *Drosophila melanogaster* Canton S infected with PBS (circles), PAO (*muxA*⁻) (diamonds), PAO (*pilA*⁻) (triangles) and PAO1 (squares).

Discussion

There are total of 12 RND pump gene clusters in *P. aeruginosa* PAO1 genome. Not all of the RND pumps in *P. aeruginosa* play equal roles in the bacterium's intrinsic resistance to antibiotics. It has been reported that the expression of *mexAB-oprM* and *mexXY* is constitutive under lab conditions (Poole *et al.*, 1993, 2000; Middlemiss and Poole, 2004), and the expression of *mexGHI-opmD* and *mexVW* can be detected by RT-PCR (Aendekerk *et al.*, 2002; Li *et al.*, 2003). Consistent with these previous reports, our results indicate that the expression of these four RND pumps in PAO1 was detectable in the conditions tested. *mexAB-oprM* had highest level of expression, in agreement with the fact that *mexAB-oprM* is one of the most important RND pumps (Middlemiss and Poole, 2004). In contrast to the report that the *mexJK* pump is repressed by *mexL* in un-inducing conditions (Chuanchuen *et al.*, 2002), our expression data indicate that *mexJK* was also expressed in PAO1 together with the newly characterized *muxABC-opmB* in three different media used in our experiments. Differences in expression levels in different growth phases were observed (Fig. 1). It is possible that discrepancy between our data and the previous report was due to the differences in the sensitivity of the expression reporter systems used. However, a *mexL* mutant has been reported to be responsible for the high level of expression of *mexJK* and resistance to tetracycline, erythromycin and triclosan (Chuanchuen *et al.*, 2002). Difference in PAO strains used in different labs has been reported (Klockgether *et al.*, 2010); therefore, it is also possible that the difference observed was due to strain difference in these studies.

MuxABC-OpmB is a newly characterized RND efflux pump, and increased resistance to aztreonam, macrolides, novobiocin and tetracycline has been reported when this pump is over-expressed in a strain where four major RND pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY- OprM are abolished simultaneously (Mima *et al.*, 2009). Our data shows that this cluster was constantly expressed in the wild type PAO1. MIC test results indicate that a single *muxA* knockout mutant did not result in increased susceptibility to all the antibiotics tested including chloramphenicol, erythromycin and tetracycline. It seems that the involvement of MuxABC-

OpmB in antibiotic resistance only becomes evident when other RND pumps are absent. Surprisingly, the MICs for ampicillin and carbenicillin in the *muxA* mutant were actually elevated. This is somewhat similar to the previous observation that the elimination of *mexGHI-opmD*, i.e. *mexI* or *opmD* deletion, caused increased resistance to kanamycin, spectinomycin, carbenicillin, rifampicin, tetracycline and chloramphenicol (Aendekerk *et al.*, 2005). The increased resistance is due to the absence of PQS in these mutants (Aendekerk *et al.*, 2005). However, PQS production in the *muxA* mutant wasn't different from that in PAO1. Neither did bacterial growth change in the *muxABC-opmB* mutant, an observation different from the *mexGHI-opmD* mutants.

The attenuated virulence in *muxA* mutant indicates that the MuxABC-OpmB plays an important role in *P. aeruginosa* virulence. This adds another player to the RND pumps, i.e. MexAB-OprM, MexEF-OprN, MexGHI-OpmD, and MexXY-OprM, known to be involved in *P. aeruginosa* pathogenicity (Hirakata *et al.*, 2002; Aendekerk *et al.*, 2005). However, unlike the mechanism in the case of MexGHI-OpmD the involvement of MuxABC-OpmB pump in pathogenesis seems to be unrelated to PQS production. Among the virulence factors tested in the *muxA* mutant only twitching motility was changed when compared to PAO1. It has been shown that twitching motility, a pilus-mediated bacterial surface movement, was required for *P. aeruginosa* virulence (Hahn, 1997; Comolli *et al.*, 1999; Mattick, 2002; Alarcon *et al.*, 2009). It is plausible that the lost motility in the *muxA* mutant at least partially accounted for the attenuated virulence observed. However, the underling mechanism connecting MuxABC-OpmB and twitching motility needs to be explored. The involvement of MuxABC-OpmB in both antibiotic resistance and pathogenicity in *P. aeruginosa* warrants further investigation on this last characterized RND pump.

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