Role of MexZ and PA5471 in transcriptional regulation of *mexXY* in *Pseudomonas aeruginosa*

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MexXY, a drug efflux pump in *Pseudomonas aeruginosa*, confers resistance to aminoglycoside antibiotics. We recently reported that MexZ binds to the promoter region of the *mexXY* operon. Electrophoretic mobility shift assay (EMSA) using recombinant MexZ and oligonucleotide probes prepared from the intergenic region between *mexZ* and *mexX* revealed that MexZ binds to a 20 bp palindromic sequence. Culture of *P. aeruginosa* in the presence of tetracycline induced higher levels of MexX and MexZ, as measured by immunoblotting and EMSA, than in the absence of antibiotics. When MexZ was expressed by a *mexZ* expression plasmid, the plasmid-borne MexZ repressed drug-induced MexX production, further confirming that MexZ acts as a repressor of the *mexXY* operon. PA5471 protein has been reported to be essential for drug-induced MexXY production. Similarly to that report, we observed that plasmid-borne PA5471 induced both MexX and MexZ production in PAO1 cells. Interestingly, interaction between MexZ and PA5471 was observed in a yeast two-hybrid assay. Furthermore, EMSA and *in vitro* transcription assays revealed that interaction between PA5471 and MexZ reduced MexZ DNA-binding ability, leading to *mexXY* transcription. These findings contribute to the understanding of the molecular mechanisms underlying the transcriptional regulation of *mexZ* and *mexXY* by drug-induced PA5471 expression.

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INTRODUCTION

Pseudomonas aeruginosa is a major opportunistic pathogen in patients with chronic respiratory disease or in immunocompromised hosts. This bacterium shows intrinsic and acquired resistance to structurally and functionally diverse antimicrobial agents. Among several drug-resistance mechanisms of bacteria (McDermott *et al.*, 2003; Poole, 2002b; Wright, 2003), drug efflux pumps are notable in that the transporters export a wide range of xenobiotics, including antimicrobial agents (Poole, 2005a). Among the efflux-pump family, the resistance-nodulation cell-division family transporters are unique to Gram-negative bacteria, and they contribute to the efflux of, and resistance to, antibiotics (Poole, 2002a). To date, seven different resistance-nodulation cell-division efflux pumps have been recognized (Mima *et al.*, 2005; Poole, 2005a),

and among them, MexAB-OprM and MexXY-OprM play major roles in the natural antimicrobial resistance of *P. aeruginosa* (Poole, 2001). Although these pumps share common substrates (Masuda *et al.*, 2000a; Okamoto *et al.*, 2002), production of MexXY contributes largely to aminoglycoside resistance in *P. aeruginosa* (Hocquet *et al.*, 2006; Masuda *et al.*, 2000a, b; Okamoto *et al.*, 2002; Poole, 2005b). Furthermore, their expression profiles are different: MexAB-OprM is constitutively produced in wild-type *P. aeruginosa* and overexpressed on mutation of the regulatory gene, whereas MexXY is produced in response to antimicrobial agents that target the ribosome (Jeannot *et al.*, 2005; Masuda *et al.*, 2000b).

We previously reported that MexZ binds to the *mexZ*–*mexX* intergenic DNA that contains the putative *mexXY* promoter (Matsuo *et al.*, 2004). Because of its structural homology to other efflux-pump repressors, such as AmrR in *Burkholderia pseudomallei* and MtrR in *Neisseria gonorrhoeae* (Hagman *et al.*, 1995; Klein *et al.*, 1991; Ma

Abbreviations: EMSA, electrophoretic mobility shift assay; HRP, horseradish peroxidase.

et al., 1996), MexZ is assumed to be a repressor of *mexXY* transcription, and the following results support this assumption (Llanes *et al.*, 2004; Morita *et al.*, 2006; Vogne *et al.*, 2004; Westbrook-Wadman *et al.*, 1999): (i) experimentally derived and clinical isolates of aminoglycoside-resistant *P. aeruginosa* had a mutation in *mexZ* or *mexZ*–*mexX* intergenic DNA (Llanes *et al.*, 2004; Vogne *et al.*, 2004); and (ii) expression of plasmid-borne MexZ reduced the expression of *mexY* mRNA (Hocquet *et al.*, 2006).

Morita *et al.* (2006) studied the role of the PA5471 gene in MexXY expression (Morita *et al.*, 2006) and found that antibiotic-mediated MexXY induction was compromised in strains with an impaired PA5471 gene (Morita *et al.*, 2006). Like MexXY expression, PA5471 expression is drug inducible, and its presence seems to be necessary for drug-induced MexXY expression. Therefore, PA5471 somehow contributes to the transcription of *mexXY*, probably by modulating the function of MexZ. However, it is not known how PA5471 interacts with MexZ to promote the transcription of *mexXY*.

To elucidate the molecular mechanisms of MexXY induction in *P. aeruginosa*, we studied the expression of MexX and MexZ, the binding of MexZ to the *mexX*

promoter, and the interaction between MexZ and PA5471 in a laboratory strain and a clinically isolated *P. aeruginosa* strain.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Bacterial cells were cultured in Luria broth [LB; 10 g tryptone (Becton Dickinson), 5 g yeast extract (Becton Dickinson) and 10 g NaCl per litre H₂O] and on LB agar [LB containing 1.5 % (w/v) agar] at 37 °C. The medium was supplemented with antibiotics as needed. Clinically isolated strain #21 of *P. aeruginosa* was employed to assess the mobility of MexZ–DNA complexes. The pEX1.8 plasmid and its derivatives were maintained in *Escherichia coli* and *P. aeruginosa* PAO1 cells in medium supplemented with 100 µg ampicillin (Sigma) ml^{−1} and 200 µg carbenicillin (CAR) (Wako) ml^{−1}, respectively.

Preparation and manipulation of DNA. Standard protocols described by Ausubel *et al.* (1995) were used for restriction endonuclease digestion, ligation, transformation, plasmid isolation and agarose gel electrophoresis (Ausubel *et al.*, 1995). Plasmid DNAs were prepared from *E. coli* by using the Nucleospin Plasmid kit (Macherey-Nagel) or the Plasmid Midi kit (Qiagen) according to the protocols provided by the manufacturers. The genomic DNA of *P. aeruginosa* was extracted by following the protocol of Barcak *et al.* (1991). The endonuclease-digested DNA fragments were purified

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics*	Reference or source
<i>E. coli</i>		
DH5α	F [−] ϕ80dlacZΔM15 Δ(<i>lacZYA</i> – <i>argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K [−] m _K ⁺) <i>supE44 λ</i> [−] <i>thi-1 gyrA96 relA1</i>	Toyobo
BL21(DE3)	F [−] <i>ompT hsdS_B</i> (r _B [−] m _B [−]) <i>gal dcm</i> (DE3)	Novagen
<i>P. aeruginosa</i>		
PAO1	Standard strain	NCTC 12934
#21	Clinically isolated strain with mutation in <i>mexZ</i> C ¹⁴ (ACC→ATC); mutated MexZ unable to bind DNA	This study
<i>S. cerevisiae</i>		
AH109	<i>MATa trp1-901 leu2-3 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3 GAL2_{UAS}-GAL2_{TATA}-ADE2 URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ</i>	Clontech
Plasmids		
pCR-Blunt II-TOPO	Cloning vector; Km ^r	Invitrogen
pET22b(+)	His ₆ tag expression vector; Ap ^r	Novagen
pET-MexZ	pET22b(+): <i>mexZ</i> cloned from NCTC 12934	This study
pET-PA5471	pET22b(+): PA5471 cloned from PAO1	This study
pEX1.8	Ap ^r /Cb ^r ; high-copy-number cloning and expression vector; contains both ColE1 <i>ori</i> and <i>P. aeruginosa ori</i> ; carries <i>lacI</i> ^q and a P _{lac} promoter for expression of gene insert	Pearson <i>et al.</i> (1997)
pEX-MexZ	pEX1.8:: <i>mexZ</i> cloned from PAO1	This study
pEX-MexZ/6His	pEX1.8:: <i>mexZ</i> with His ₆ -tag cloned from PAO1	This study
pEX-PA5471	pEX1.8:: PA5471 cloned from PAO1	This study
pACT2	Vector expressing protein fused to amino acids GAL4 AD (amino acids 768–881), and an HA epitope tag; Ap ^r	Clontech
pGBKT7	Vector expressing proteins fused to amino acids 1–147 of the GAL4 DNA-binding domain (DNA-BD); Km ^r	Clontech
pACT-MexZ	pACT2 AD:: <i>mexZ</i> cloned from PAO1	This study
pGBKT-PA5471	pGBKT7 BD:: PA5471 cloned from PAO1	This study

*Ap^r, ampicillin resistant; Km^r, kanamycin-resistant; Cb^r, carbenicillin resistant.

with a Sephaglas BandPrep kit (GE Healthcare). PCR products were purified with a StrataPrep PCR Purification kit (Stratagene). Competent *P. aeruginosa* cells were prepared as described by Smith & Iglewski (1989). Competent *E. coli* BL21(DE3) cells and Competent High DH5 α cells were purchased from Novagen and Toyobo, respectively.

Plasmid construction and transformation. All primers used for cloning of *mexZ* or PA5471 are shown in Table 2. To construct pCR-MexZ(EP) plasmids, *mexZ* and PA5471 were amplified by PCR with the primer set E-mexZ-s/P-mexZ-as; for plasmids pCR-MexZ(NS), pCR-PA5471(EP), pCR-PA5471(NX), and pCR-MexZ(ES) similar amplifications were performed with N-mexZ-s/S-mexZ-as, E-PA5471-s/P-PA5471-as, N-PA5471-s/X-PA5471-as, and E-mexZ-s/S-mexZ-as, respectively, by a standard PCR method (Ausubel *et al.*, 1995). The PCR products were cloned into plasmid pCR-BluntII-TOPO (Invitrogen) for nucleotide sequencing and further plasmid construction. To construct plasmid pET-MexZ, pCR-MexZ(NS) was digested with *NdeI* and *Sall*. The resulting DNA fragment was purified from agarose gel and cloned into the *NdeI/XhoI* site of pET22b(+). To construct pEX-MexZ, pCR-MexZ(EP) was digested with *EcoRI* and *PstI*. The resulting DNA fragment was gel-purified and cloned into the *EcoRI/PstI* site of pEX1.8. To construct pEX-MexZ/6His, His-tagged *mexZ* was amplified by PCR with the primer set E-mexZ-s/P-mexZ-his-as and pET-MexZ as the template. To construct pEX-PA5471 and pGBKT-PA5471, pCR-PA5471(EP) was digested with *EcoRI* and *PstI*. The resulting DNA fragment was gel-purified and cloned into the *EcoRI/PstI* site of pEX1.8 and pGBKT7. To construct pET-PA5471, pCR-PA5471(NX) was digested with *NdeI* and *XhoI*. The resulting DNA fragment was gel-purified and cloned into the *NdeI/XhoI* site of pET22b(+). To construct pACT2-MexZ, pCR-MexZ(ES) was digested with *EcoRI* and *Sall*. The resulting DNA fragment was gel-purified and inserted into the *EcoRI/XhoI* site of pACT2. *P. aeruginosa* was transformed with the plasmids by electroporation as described by Smith & Iglewski (1989) using a Gene Pulser II and a Pulse Controller II (Bio-Rad). *Saccharomyces cerevisiae* strain AH109 cells, used for yeast two-hybrid assay, were transformed by the lithium acetate method, as described in the manufacturer's protocol.

Recombinant proteins. *E. coli* BL21(DE3) cells harbouring recombinant MexZ (reMexZ) or recombinant PA5471 (rePA5471) were grown at 37 °C while being rotated at 160 r.p.m.; 1 mM IPTG was added when the OD₆₀₀ reached 0.6, and the cells were incubated for an additional 3 h. The bacterial cells were disintegrated by sonic oscillation in buffer A [50 mM sodium phosphate (pH 7.5), 300 mM NaCl and 20% (v/v) glycerol]. The cell lysate was centrifuged at 100 000 g at 4 °C for 60 min, and the clear supernatant was applied to a HisBind Resin column (Novagen). After being washed with buffer A three times, the column was eluted with buffer A containing 300 mM imidazole, and the eluate was dialysed against buffer B [20 mM Tris/HCl (pH 7.5), 0.2 mM EDTA, 1 mM DTT, 1 mM MgCl₂, 100 mM NaCl and 20% (v/v) glycerol].

Antibodies. To raise polyclonal antibody against reMexZ, the protein preparation was mixed with Freund's complete adjuvant and injected into a rabbit. Serum samples were collected 80 days after injection, and the anti-reMexZ antibody was affinity-purified through CNBr-Sepharose 4B columns coupled with reMexZ. Anti-MexX antibody was prepared as previously reported (Matsuo *et al.*, 2004). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody was supplied by GE Healthcare. Preimmune rabbit IgG was obtained from Upstate Biotechnology.

Detection of mutations in the *mexZ* gene and *mexZ-mexX* intergenic DNA. The clinically isolated clone #21 was subjected to sequencing analysis to detect possible mutations in the *mexZ* gene and *mexZ-mexX* intergenic DNA. The DNA fragment corresponding to the *mexZ-mexX* region was PCR amplified with the primer set mexZ-X-s/mexZ-as with genomic DNA isolated from clone #21 as a template. The DNA fragments were cloned into pCR-BluntII-TOPO (Invitrogen) and subjected to sequence analysis.

Preparation of *P. aeruginosa* cell extracts. *P. aeruginosa* cells were cultured in 2 ml LB for 16 h at 37 °C and then centrifuged at 20 000 g at 4 °C for 5 min; the pellet was then suspended in 100 μ l lysis buffer [10 mM Tris/HCl (pH 7.5), 10 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA and 0.5 mM DTT]. Cells were disintegrated by sonic oscillation with 15 repeated bursts of 1 min each, total 15 min, at

Table 2. Primer sequences used for DNA amplification

Primer	Sequence (5'→3')*	Purpose
E-mexZ-s	<u>GAATTC</u> ATGGCCAGGAAAAACCAAAG; <i>EcoRI</i>	PCR cloning of <i>mexZ</i>
N-mexZ-s	CATATGGTGGCCAGGAAAAACCAAAGAG; <i>NdeI</i>	PCR cloning of <i>mexZ</i>
P-mexZ-as	<u>CTGCAGT</u> CAGGCGTCCGCCAGCAACA; <i>PstI</i>	PCR cloning of <i>mexZ</i>
S-mexZ-as	<u>GTGCACT</u> AAGGCGTCCGCCAGCAACAGGTAGGGAG; <i>Sall</i>	PCR cloning of <i>mexZ</i>
P-mexZ-his-as	<u>CTGCAGG</u> CAGCCGGATCTCAGT; <i>PstI</i>	PCR cloning of <i>mexZ/6His</i>
E-PA5471-s	<u>GAATTC</u> ATGGGCAACTACATCAAGCCTTTGTCCGC; <i>EcoRI</i>	PCR cloning of PA5471
N-PA5471-s	CATATGATGGGCAACTACATCAAGCCTTTGTCCGC; <i>NdeI</i>	PCR cloning of PA5471
P-PA5471-as	<u>CTGCAGT</u> CATCGGCAGCACTCCCCAC; <i>PstI</i>	PCR cloning of PA5471
X-PA5471-as	<u>CTCGAGG</u> CATCGGCAGCACTCCCCACGGGTCTTGTA; <i>XhoI</i>	PCR cloning of PA5471
U1	CTTTGGTTTTCTGGCCACTGAACGT	EMSA probe
D1	GTCCATTGGATGTGCATGGGTG	EMSA probe
mexZ-X for Seq	GTCCATTGGATGTGCATGGGTG	Sequence analysis
mexZ-as for Seq	CTGCAGTCAGGCGTCCGCCAGCAACA	Sequence analysis
T7-185-MexX-s	CTAATACGACTCACTATAGG GAGAAACGCCAATTAATTAATC	<i>In vitro</i> transcription assay
MexX-us	TCACTGGCCCGCCGGCGAGGC	<i>In vitro</i> transcription assay

*Underlined sequences indicate restriction sites; the corresponding restriction endonuclease is indicated. The bold sequence indicates the T7 promoter region.

power setting 10 with a Sonifier 450D (Branson Ultrasonics) with a cup horn in which cold water (4 °C) was circulated. Intact cells were removed by centrifugation at 20 000 *g* at 4 °C for 5 min. The amount of protein was determined by the Bradford assay (Bio-Rad), and the final protein concentration of each sample was adjusted to 10 mg ml⁻¹ with lysis buffer for electrophoretic mobility shift assay (EMSA) and immunoblotting assays.

EMSA, competition assay and supershift assay. EMSA was carried out as described by Ausubel *et al.* (1995). Briefly, the probes were prepared by the annealing of four sets of complementary oligonucleotides (Fig. 1). Probe(-183/+17) was prepared by a standard PCR method and gel purification. Binding of recombinant protein and cell extract was performed at 20 °C for 30 min in 10 µl binding buffer [10 mM Tris/HCl (pH 8.0), 100 mM NaCl, 16 mM MgCl₂, 0.5 mM EDTA, 14% (v/v) glycerol and 1 mM DTT] containing 500 fmol of each probe end-labelled with [γ -³²P]ATP (GE Healthcare), 1 µg poly(dI-dC) (Amersham Biosciences) and 1 µg BSA. For the competition assay, a 50-fold excess of an appropriate unlabelled probe was added to the binding reaction mixtures. For the supershift assay, the recombinant protein and cell extract were preincubated with a rabbit IgG or an anti-MexZ antibody for 20 min before being mixed with the probe. Gel electrophoresis was carried out at 120 V for 2 h at 4 °C in 0.5 × TBE buffer [45 mM Tris/HCl (pH 8.0), 45 mM boric acid and 1 mM EDTA] on a 5% (w/v) polyacrylamide gel.

Immunoblotting assay. The extracts from *P. aeruginosa* cells were subjected to SDS-PAGE. The proteins were transferred onto Immobilon-P membranes (Millipore). After blocking non-specific binding sites with 5% (w/v) skim milk in TBS buffer [10 mM Tris/

HCl (pH 7.5) in 150 mM NaCl] for 2 h at room temperature, the membrane was probed for 1 h with primary antibody diluted 1:4000 in TBS containing 0.1% (w/v) skim milk. The membrane was next probed for 1 h with a 1:10 000 dilution of the secondary HRP-conjugated anti-rabbit IgG antibody. After the membrane had been washed three times with TBS containing 0.05% (v/v) Tween 20, the immunoreactive protein bands were visualized by using ECL Plus Western blotting Reagents (GE Healthcare) and an LAS-3000 mini luminescent image analyser (Fujifilm).

In vitro transcription assay. A 1396 bp DNA fragment containing a phage T7 promoter at its 5' end, the 185 bp 5'-flanking sequence of *mexX* (including the MexZ-binding site) and the *mexX* gene was amplified from *P. aeruginosa* PAO1 genomic DNA by PCR using KOD plus (Toyobo) and the primer set T7-185-MexX-s/MexX-us. The PCR product was gel-purified with a Sephadex BandPrep kit (GE Healthcare), and quantified with a GeneQuantII spectrophotometer (Pharmacia Biotech). *In vitro* transcription from this template was conducted with a CUGA7 *in vitro* transcription kit (Nippon Genetech) according to the manufacturers' protocol, except that the amount of the template was reduced to 5 pg (final concentration 15 nM), and half the suggested amount of CUGA7 enzyme solution was used. To assess the impact of the MexZ repressor on transcription from these templates, purified MexZ was added to the reaction mixtures. To assess the impact of PA5471 on MexZ repressor activity in this assay, purified PA5471 was also added to the reaction mixtures. A control reaction was also carried out with PA5471 alone. In all instances, reactions were allowed to proceed for 2 h at 37 °C. After DNase digestion for 30 min, the RNA products were purified by using Isogen (Nippon Gene) and ethanol precipitation. Purified RNA products were resuspended in 20 µl diethylpyrocarbonate-treated

Fig. 1. MexZ binds to a 20 bp palindromic sequence. The nucleotide sequence of the *mexZ-mexXY* intergenic region is shown. The translation initiation site of MexX is designated +1. Thin arrows indicate the PCR primers (sense primer U1 above, anti-sense primer D1 below) used for the preparation of probe(-183/+17). Thick arrows indicate the translation start codons for MexZ and MexX. The sequences of the oligonucleotide probes for EMSA are bold underlined. The 20 bp palindromic sequence is boxed.

water, mixed with the same volume of 8 M urea sample buffer, heated to 55 °C for 5 min, and then separated by electrophoresis in 4% (w/v) polyacrylamide gel in 1× TBE buffer. Size-separated RNA products were visualized by ethidium bromide staining.

Yeast two-hybrid assay. A yeast two-hybrid assay was performed with Matchmaker GAL4 Two-Hybrid System 2 (Clontech Laboratories). We constructed full-length MexZ and PA5471 expression vectors, pACT-MexZ and pGBKT-PA5471. *S. cerevisiae* AH109 cells were transformed with derivatives of both pACT2 and pGBKT7 by the lithium acetate method, as described in the manufacturer's protocol. Transformants were inoculated on selective minimal plates [SD medium containing 2% (w/v) agar and lacking leucine and tryptophan] and grown for 4 days at 30 °C to select clones transformed by both derivatives. Then, colonies were picked up and grown for 4 days at 30 °C on selective maximal plates [SD medium containing 2% (w/v) agar and lacking adenine, histidine, leucine and tryptophan] to select clones expressing the reporter genes *ADE* and *HIS3*.

RESULTS AND DISCUSSION

Refining of the MexZ-binding site

We reported previously that MexZ binds to the sequence between −104 and −66 bp upstream of the MexXY translation initiation site (Matsuo *et al.*, 2004). For further characterization of MexZ binding to the *mexX* promoter, EMSA was performed using reMexZ and four types of ³²P-labelled oligonucleotide probe. The nucleotide sequences of each probe are shown in Fig. 1. Although reMexZ bound to both probe(−115/−88) and probe(−105/−78) (Fig. 2a, lanes 1 and 2), a competition assay revealed that reMexZ bound specifically to probe(−105/−78) (lane 6), whereas the binding to probe(−115/−88) seemed to be non-specific (lane 5). Therefore, we used probe(−105/−78) to examine the DNA-binding ability of MexZ in the following EMSA. Probe(−105/−78) coded for a 20 bp palindromic sequence (Fig. 1). Using a higher dose of reMexZ elicited another, much slower-mobility, band (as seen in Fig. 2e, lanes 5, 7 and 8, arrowhead). Although excessive accumulation of radiolabelled probes in the adjacent lanes sometimes interfered with the bands and made them hard to recognize (as seen in Fig. 2b, lane 7), we used reMexZ in EMSA at a dose that showed only a single band.

Promotion of MexZ production by tetracycline, and MexZ binding to *mexXY* promoter region

MexXY is not produced in *P. aeruginosa* cells when the bacteria are grown in the absence of antibiotics. Tetracycline (TET) and other ribosome-targeting antibiotics promote MexXY production, even at subinhibitory concentrations (Jeannot *et al.*, 2005). The following lines of evidence on the regulation of MexXY production have been presented to date. The *mexZ* gene, located upstream of *mexXY*, encodes a repressor protein that suppresses transcription of *mexXY* (Dean *et al.*, 2003; Llanes *et al.*, 2004; Morita *et al.*, 2006; Vogne *et al.*, 2004; Westbrook-Wadman *et al.*, 1999). In fact, purified MexZ has been

shown by gel-shift assay to bind with a DNA fragment from the *mexZ*–*mexX* intergenic promoter region (Matsuo *et al.*, 2004). A computer-aided similarity search of MexZ has revealed that MexZ is a member of the TETR family of repressors (Aires *et al.*, 1999; Westbrook-Wadman *et al.*, 1999), which forms a homodimer for DNA binding (Aramaki *et al.*, 1995; Grkovic *et al.*, 2001; Hillen & Berens, 1994). We therefore hypothesized that MexZ represses the transcription of *mexXY*, except in the presence of an appropriate inducer molecule such as TET. To verify this hypothesis, we performed EMSA using the lysate from *P. aeruginosa* PAO1 cells grown in the absence of TET. Low-mobility bands, which probably corresponded to protein–DNA-complex bands, were faintly detected (Fig. 2b, lane 2). However, when EMSA was performed using the lysate from PAO1 cells grown in the presence of TET, the low-mobility bands were more clearly seen than in EMSA using lysate from the cells grown without antibiotics (Fig. 2b, lane 3). These TET-inducible protein–DNA complexes showed slightly higher mobility than the reMexZ–DNA complex (Fig. 2b, lane 1). Since reMexZ has a His₆ tag, an effect of the His₆ tag on the mobility of reMexZ could not be ruled out. To examine the effect of the His₆ tag on mobility, we conducted EMSA using lysates of the clinically isolated *P. aeruginosa* #21 transfected with pEX-MexZ or pEX-MexZ/6His. Because this strain had a mutation in *mexZ* (Table 1), endogenous MexZ was detectable by immunoblotting assay when cells were grown without antibiotics (Fig. 3, lane 5) but was unable to bind DNA (Fig. 2b, lane 4), even when cells were cultured with TET (data not shown). The locations of the shifted bands seen in the lanes of reMexZ and pEX-MexZ/6His, and of the TET-inducible protein band and pEX-MexZ, were identical (Fig. 2b, lanes 1 and 6, and lanes 3 and 5, respectively). Then, to compare the precise binding sequences of the TET-inducible protein and reMexZ, EMSA was performed using six different mutant oligonucleotide probes as cold competitors (Fig. 2c, upper panel). The mutated probes m2, m4 and m6 had only small effects on reMexZ binding to probe(−105/−78) DNA (Fig. 2c, lower panel), indicating that the nucleotide sequences present in these mutated bases were not important for reMexZ binding. A similar result was observed in a competition assay using the lysate from PAO1 cells grown with TET (data not shown). These results were consistent with the result that all the probes, except probe(−105/−78), failed to bind to reMexZ because they lacked the nucleotide sequences important for DNA binding (Figs 1 and 2a).

In the next experiment, the presence or absence of MexZ in the PAO1 cell lysates was assayed by immunoblotting with anti-MexZ antibody. MexZ was undetectable in PAO1 cells grown without TET (Fig. 2d, Fig. 3, lane 1). TET-induced production of MexZ paralleled the production of MexX and increased in a time-dependent manner (Fig. 2d). We then performed a supershift assay using this antibody. The protein–DNA-complex bands seen in EMSA using reMexZ

Fig. 2. Characterization of MexZ binding to the *mexX* promoter region. (a) EMSA indicates that recombinant MexZ (reMexZ) binds to probe(-115/-88) (lane 1) and probe(-105/-78) (lane 2), but not to probe(-95/-68) (lane 3) or probe(-85/-58) (lane 4). The competition assay indicated that reMexZ bound to probe(-105/-78) with the highest affinity (lane 6). (b) EMSA indicates reMexZ binding to probe(-105/-78) (lanes 1 and 7, white arrow). When *P. aeruginosa* PAO1 was cultured in Luria broth without antibiotic agents, a shifted band was faintly detected by EMSA using probe(-105/-78) (lane 2, solid arrow). This shifted band was detected more clearly upon culture with tetracycline (TET) lysate (lane 3, solid arrow). To examine the effect of the His₆ tag on mobility, EMSA was conducted using lysates of the clinically isolated *P. aeruginosa* #21 and its pEX-MexZ or pEX-MexZ/6His transformants. Endogenous MexZ of #21 could not bind to probe(-105/-78) (lane 4). The locations of the shifted bands seen in the lanes of pEX-MexZ (lane 5, solid arrow) and pEX-MEXz/6His (lane 6, white arrow) were identical to those of the TET-induced protein band (lane 3) and reMexZ (lane 1), respectively. (c) The sense oligonucleotide sequence of probe(-105/-78) is shown (upper panel). The arrows indicate bp substitutions in mutated oligonucleotide probes (m1, m2, m3, m4, m5 and m6). A competition assay was performed by using reMexZ and the mutated probes as cold competitors (lower panel). The same result was obtained with an extract from TET-stimulated PAO1 cells. (d) MexX and MexZ expression, examined by immunoblotting using antibodies against MexX and MexZ. At the indicated times after incubation in LB medium containing TET (2 $\mu\text{g ml}^{-1}$), extracts from PAO1 cells were subjected to immunoblotting. (e) By addition of an anti-MexZ antibody, both reMexZ-DNA complexes and TET-induced protein-DNA complexes (lanes 1-4 and 5-8, respectively, white arrow) were dose-dependently supershifted (lanes 2, 3, 4, 6, 7 and 8, solid arrow). As a negative control, preimmune rabbit IgG was employed in the assay (data not shown). All experiments were performed two or three times, with similar results.

Fig. 3. Effect of tetracycline and recombinant MexZ on MexX expression: immunoblotting analysis of MexX and MexZ expression in *P. aeruginosa* PAO1 cells, clinically isolated strain #21, and their transformants. As a control for the expression level of MexZ, recombinant MexZ (25 ng) was employed. MexX and MexZ were not expressed in PAO1 cells cultured in Luria broth without antibiotic agents (lane 2). TET (2 $\mu\text{g ml}^{-1}$) induced both MexX and MexZ expression (lane 3). Plasmid-borne MexZ repressed the TET-induced MexX expression (lane 5). Carbenicillin (CAR, 200 $\mu\text{g ml}^{-1}$) and transformation with a pEX1.8 mock plasmid (lane 4) had no detectable effect on MexX and MexZ expression in TET⁺ PAO1 cells. MexX and MexZ were expressed in clinically isolated *P. aeruginosa*, #21 (lane 6), when the bacteria were grown in the absence of antibiotics. Endogenous MexZ of #21 was not capable of DNA binding. Plasmid-borne MexZ and histidine-tagged MexZ cloned from PAO1 repressed the expression of MexX in #21 (lanes 7 and 8, respectively).

and the lysate from TET-stimulated PAO1 cells were dose-dependently supershifted by the addition of anti-MexZ antibody (Fig. 2e, lanes 2, 3, 4, 6, 7 and 8). The supershifted band in Fig. 2e, lane 6, is slightly fainter than that in lane 7. This is because some DNA-protein complexes were trapped in the upper parts of the well owing to the high protein concentration. A control experiment using pre-immune rabbit IgG showed no detectable supershift (data not shown). Another, much slower-mobility band is seen in lanes 3, 4, 5, 7 and 8. This slower-mobility band was often seen when the binding reaction was conducted in a more concentrated solution (see Fig. 4d, lane 2). We did not pursue the question of whether this slower-mobility band had a functional meaning.

Piecing together these results showed that the TET-induced protein was identical to MexZ. We concluded that TET stimulates MexZ production in *P. aeruginosa* PAO1, as reflected by the increased binding in EMSA and by the results of the immunoblotting analysis. Because MexZ is a member of the TetR family and it has been reported that one of the TetR family proteins, TtgR of *Pseudomonas putida*, has the ability to bind to several structurally unrelated molecules (Teran *et al.*, 2006), we checked the possibility of direct interaction between MexZ and antibiotics, especially TET, by EMSA. However, addition of TET to the binding reaction solutions could not alter the shifted band of the reMexZ-DNA complex (data not shown).

MexZ-mediated repression of *mexXY* transcription

It has been reported that TET stimulates MexXY production (Jeannot *et al.*, 2005) and induces the transcription of *mexZ* (Dean *et al.*, 2003; Morita *et al.*, 2006) in PAO1 cells. Our results also revealed that TET stimulated the production of MexZ, which binds with *mexXY* promoter DNA (Fig. 2b, Fig. 3, lane 3). Several reports have shown that some repressors of bacterial efflux pumps are auto-regulated (Sanchez *et al.*, 2002a, b; Teran *et al.*, 2006) and we thought this might be the case here. To clarify the role of MexZ again, we transformed PAO1 cells with the *mexZ* expression plasmid pEX-MexZ and examined the effects of plasmid-borne overproduced MexZ on MexX production; we observed that the plasmid-borne MexZ repressed MexX production in TET-stimulated PAO1 cells (Fig. 3, lane 5). A mock plasmid and CAR, which was used to select plasmids containing PAO1 cells, exerted no detectable effect on MexX and MexZ production (Fig. 3, lane 4). Thus, the role of MexZ as a repressor was firmly verified. Although TET stimulates both MexX and MexZ expression in PAO1 cells, TET-induced MexX expression may be regulated by a transcription mechanism that remains to be characterized.

Effect of PA5471 on MexX expression and interaction with MexZ

Recently, Morita *et al.* (2006) reported that ribosome-targeting antibiotics induce the production of cytoplasmic PA5471 protein, which seems to be indispensable for the production of MexXY proteins (Morita *et al.*, 2006). However, the precise function of PA5471 in the regulation of *mexXY* remains to be elucidated.

When PAO1 cells were transformed with the PA5471 expression plasmid pEX-PA5471, the plasmid-borne PA5471 promoted the production of both MexZ and MexX (Fig. 4a, lane 3), confirming the results of Morita *et al.* (2006). We performed an *in vitro* transcription assay using reMexZ, rePA5471 and a DNA fragment that contains a phage T7 promoter at its 5' end, the *mexZ*-*mexX* intergenic sequence (including the MexZ-binding site) and the *mexX* gene. *mexX* transcription from the DNA fragment was considerably suppressed by reMexZ (Fig. 4b, lane 2) and slightly suppressed by rePA5471 (lane 5). The slight suppression due to rePA5471 (lane 5) might have been caused by the presence of residual uneluted cell extract components, such as RNases, because the rePA5471 was affinity-purified by resin. Suppression by reMexZ was reduced by the addition of rePA5471 (lanes 3 and 4), indicating that the PA5471-induced transcription was MexZ-dependent.

To further examine the possibility of interaction between PA5471 and MexZ, we performed a yeast two-hybrid assay of AH109 yeast cells. We constructed full-length MexZ and PA5471 expression vectors, pACT-MexZ and pGBK-

Fig. 4. PA5471 binds to MexZ and relieves *mexX* repression. (a) PAO1 cells were transformed with the PA5471 expression plasmid pEX-PA5471. Plasmid-borne PA5471 stimulated both MexX and MexZ expression (lane 3), as did TET (lane 2). (b) To elucidate whether PA5471 functionally inactivates MexZ, an *in vitro* transcription assay was performed. reMexZ inhibited transcription of the DNA fragment containing the MexZ-binding site (lane 2). This repression was dose-dependently reduced by the addition of recombinant PA5471 (rePA5471) (lanes 3 and 4). (c) To characterize the possible association between MexZ and PA5471, a yeast two-hybrid assay was performed. As a negative control, pACT-mock and pGBKT-mock were used. Transformants containing both pACT-MexZ and pGBKT-PA5471 grew on maximal selective plates [SD medium containing 2% (w/v) agar and lacking adenine, histidine, leucine, and tryptophan], indicating interaction between MexZ and PA5471. (d) To examine the possibility that PA5471 binds to the *mexX* promoter region, EMSA was performed with probe(−183/+17). reMexZ bound to the *mexX* promoter region (bp −183 to +17) (lane 2, white arrow), but rePA5471 did not (lanes 3–8). (e) Recombinant PA5471 inhibited TET-induced MexZ binding to probe(−105/−78) (white arrow). Similar results were obtained using reMexZ.

PA5471, and then transformed the AH109 yeast cells with them. Yeast cells transformed with pACT and pGBK derivatives were selected using plates lacking leucine and tryptophan (Fig. 4c, upper panel). Because the interaction between MexZ and PA5471 could reconstitute GAL4 function, leading to the transcription of reporter genes *ADE* and *HIS3*, only cells transformed with pACT-MexZ and pGBK-PA5471 could grow on the plates lacking adenine, histidine, leucine and tryptophan (Fig. 4c, lower panel). Thus, the interaction between MexZ and PA5471 was confirmed.

We also performed EMSA using probe(−183/+17), which has a 203 bp 5′-flanking sequence of the *mexX* gene (Fig. 1), and recombinant proteins. Although reMexZ was capable of binding the probe (Fig. 4d, lane 2), rePA5471 failed to show binding (lanes 3–8). Because the shifted band of MexZ could not be observed in EMSAs performed with lysate from PA5471 overexpressing mutant (data not shown), in which the production of MexZ was increased,

we examined the interaction between probe(−105/−78), PA5471 and MexZ by EMSA using extracts from TET-stimulated PAO1 cells. Interestingly, rePA5471 inhibited the binding of MexZ to probe(−105/−78) in a dose-dependent manner (Fig. 4e). Similar results could be obtained using reMexZ. Because immunoblotting analysis showed that the amount of MexZ in the cell lysate was far lower than in the amount of reMexZ used in our experiments (Fig. 3, lanes 1 and 3), it seemed that much more PA5471 was required to reduce the binding of MexZ in the cell lysate than in the recombinant form. However, because these two experiments were conducted under different conditions (with respect to protein concentration and composition of reaction mix) and the influence of the His₆-tag in reMexZ on the interaction with PA5471 could not be excluded, we thought it was inappropriate to compare these EMSA results. All these results revealed that PA5471 interacts with MexZ, blocks its function, and facilitates transcription of the *mexXY* operon.

Transcriptional regulation of *mexXY* by the interaction of these proteins can be explained by comparison with the negative regulation of TetR-*tetA*, which is commonly used as a tool for selective target-gene regulation (Forster *et al.*, 1999; Freundlieb *et al.*, 1997; Gossen *et al.*, 1995). In short, expression of TetA is under the tight control of TetR (Hillen & Berens, 1994; Schnappinger & Hillen, 1996) because high-level production of TetA is lethal for bacterial cells (Eckert & Beck, 1989). Reduction of the affinity of TetR for *tetO*, which is accomplished by TetR and a tetracycline-magnesium complex, leads to the transcription of both *tetA* and *tetR* (Bertrand *et al.*, 1983). Similarly, without antibiotics, production of MexXY is tightly controlled by MexZ. Although production of MexZ was not detected by immunoblotting, binding of MexZ to the *mexZ*-*mexX* intergenic promoter region could be seen in our EMSA even when cells were grown without antibiotics. MexZ loses its DNA-binding ability by forming a complex with drug-induced PA5471. This leads to the transcription of *mexXY* and *mexZ*, which was proved by the TET-induced parallel production of MexZ with MexX in immunoblotting (Fig. 2d). The production level of MexZ then becomes high enough to weaken the effect of PA5471, and transcriptional regulation of *mexXY* and *mexZ* by MexZ occurs again. By this regulatory system, the production levels of MexXY and MexZ can be maintained at a certain level as long as PA5471 is present. In fact, production of MexX and MexZ did not change at or after 12 h culture with TET in our immunoblotting analysis (data not shown). Once the PA5471-inducer molecule, such as TET, disappears, downregulation of *mexX* and *mexZ* promptly occurs. Using immunoblotting, we could observe parallel reduction of TET-induced MexX and MexZ production in PAO1 cells after removal of the TET from the culture medium (data not shown). This regulation mechanism is quite similar to the regulation of MexAB-OprM transcription by MexR, which interacts with PA3719, leading to loss of its DNA-binding ability (Daigle *et al.*, 2007). However, the existence of shifted bands in our EMSAs when we used a lysate of PAO1 cells cultured with TET may seem contradictory if MexXY production is promoted by reducing the amount of MexZ bound to the promoter region. When the regulatory system described above is active, the production level of MexZ in each cell at each time point should fluctuate slightly higher or lower than equal to negate the effect of PA5471 to maintain the production level of MexX. The increase in binding of MexZ seen in the EMSA using PAO1 cultured with TET could be explained by slightly excess amounts of MexZ in some cells. In fact, when we conducted EMSA with lysates of PAO1 cells exposed to TET for 1–6 h at hourly intervals, the shifted bands of MexZ gradually became apparent with time (data not shown). There may be an alternative explanation for the existence of shifted bands in EMSAs using a lysate of cells cultured with TET. MexZ may be able to bind to antibiotics, such as TET, and the MexZ-antibiotic complex may be inactive as for other members of the TetR family. Because TET was not added

to the EMSA binding reaction, TET in the cell lysate was diluted and might not have been present at a high enough concentration to influence the binding ability of MexZ. However, as previously described in this report, addition of TET to the binding reaction solutions could not alter the shifted band of reMexZ-DNA complex in our EMSAs. A previous report showing that *P. aeruginosa* cells lacking PA5471 but possessing MexZ could not induce the expression of MexX by drug stimulation (Morita *et al.*, 2006) also implied low probability of this direct interaction.

Conclusion

This PA5471-mediated transcriptional regulation mechanism seems to correspond to the production of similar cytotoxic signals in response to the administration of several different antibiotics, rather than corresponding to a signal specific to each, although PA5471 may play another initial role in maintaining cell homeostasis, and the drug-efflux function of MexXY may be secondary to an unknown essential function. Elucidation of the transcriptional regulation of the PA5470-5471 operon might give us insight into the physiological function of the whole system.

Thus, we clarified the interaction between PA5471 and the MexZ-regulated expression of MexXY. However, the precise change in MexZ triggered by the interaction with PA5471 that leads to DNA-binding disability remains to be elucidated. The contribution of MexXY to aminoglycoside resistance in clinically isolated strains differs among strains, even when the *mexX* is highly expressed (Sobel *et al.*, 2003). We also surveyed the expression levels of MexX and MexZ in clinically isolated aminoglycoside-resistant strains. The finding that almost all the aminoglycoside-resistant strains expressed MexX, but that expression of MexZ in these strains was diverse (data not shown) is consistent with the results of a previous report (Sobel *et al.*, 2003). Functional assays and further examination of the expression profiles of PA5471 in these MexX-expressing AG-R strains are in progress in our laboratory.

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