Novel Activators of the MexEF-OprN Multidrug Efflux System in *Pseudomonas aeruginosa* 8380

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Abstract

The spread of antibiotics and antibiotic-resistant bacteria in aquatic environments has become a major problem all over the world, and preventing the proliferation of resistant bacteria is one of the foremost concerns for global health. To achieve effective prevention, it is important to analyze antibiotic resistance genes and their expression mechanisms. *Pseudomonas aeruginosa* is a ubiquitous environmental gram-negative bacterium that adapts rapidly to antibiotic-contaminated environments, and harbors many unknown resistance-related genes in its genome. To identify new antibiotic resistance-related genes, randomly-sheared 8380 strain chromosomal DNA fragments were introduced into 8380 cells by using pUCP24. DNA fragments containing the *scfB* gene conferred resistance to various antibiotics and the resistant profile was similar to that of NfxC-type mutants expressing the MexEF-OprN efflux pump in an MexT-dependent manner. Wild-type 8380 cells did not express MexEF-OprN due to inhibition of MexT by active MexS. Since 8380 cells with introduction of the *scfB* gene expressed the MexEF-OprN efflux pump, but 8380 Δ *mexT* cells with introduction of this gene did not, it was demonstrated that *scfB* induced the MexEF-OprN efflux pump in an MexT-dependent manner. Moreover, overexpression of *scfF*, a homologue of *scfB*, induced MexEF-OprN expression. These results suggest that the genome of *P. aeruginosa* contains many unknown resistance-related genes.

Key words: *Pseudomonas aeruginosa*, antibiotics, multi-drug efflux pump, horizontal gene transfer

INTRODUCTION

A huge amount of antibiotics are used to treat patients all over the world. However, use of antibiotics is not confined to medical practice, and a much larger volume of these agents is actually employed to treat animals and as growth-promoting agents for animals and fish¹⁾. In Japan, the volume of antibiotics utilized for animals and fish is three-fold greater than for human therapy²⁾. Antibiotics enter aquatic environments such as rivers and sewage treatment plants due to incomplete metabolism by humans and animals³⁾. Therefore, multidrug-resistant

microorganisms develop in aquatic environments in areas where antibiotics are used, and the emergence and spread of such multidrug resistant bacteria has become universally recognized as a global public health menace³⁾⁻⁵⁾. While nobody understands how resistance genes are generated, horizontal gene transfer is well known to be one of the key factors involved in spreading resistance-related genes and resistant organisms in the environment and human body, because antibiotic resistance genes are often encoded by self-transferable plasmids and transposable elements³⁾. There is always a risk of unknown resistance-related genes in

self-transferable plasmids or transposable elements being transferred between related or unrelated bacteria and spreading easily around the world, as in the case of the vancomycin resistance gene operon⁶⁾. Microorganisms are thought to harbor many unknown resistance-related genes in their genomes because a number of resistant microorganisms emerge soon after each new antibiotic is released into the market, but there is insufficient information about most of the resistance-related genes in bacterial genomes and the environment.

Pseudomonas aeruginosa is a ubiquitous environmental Gram-negative bacterium that causes various opportunistic infections in immunocompromised hosts^{7),8)}. This bacterium shows resistance to a variety of structurally and functionally dissimilar antimicrobial agents, which can make chemotherapy difficult^{9)–11)}, and it is thought that many unknown resistance-related genes exist in the *P. aeruginosa* genome. It has been reported that the wide spectrum of antibiotic resistance displayed by this microorganism is mainly attributable to the production of resistance-nodulation-cell division (RND)-type efflux pumps, which extrude a variety of xenobiotics from the interior of cells to the external milieu^{10),12)}. Among these antibiotic efflux systems, MexAB-OprM and MexXY-OprM are expressed in wild-type *P.*

aeruginosa and contribute to intrinsic resistance, while ten other efflux pumps are quiescent in wild-type bacteria and their production is conditional^{13)–15)}. Currently, we lack information about the genes and signals that induce the expression of silent unknown resistance-related genes. In this study, to understand the expression of bacterial resistance and identify new resistance-related genes, we screened *P. aeruginosa* 8380 for unknown resistance-related genes¹⁶⁾.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions The bacterial strains and plasmids used in this study are listed in Table 1. Both *Escherichia coli* and *P. aeruginosa* were grown at 37°C in LB broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl per liter, adjusted to pH 7.0). pUCP24 is a broad host-range plasmid bearing the Gm^r marker.

DNA manipulation We manipulated recombinant DNA by using standard procedures described previously^{17),18)}. Chromosomal DNA was isolated from *P. aeruginosa* using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol.

Construction of *P. aeruginosa* strains with chromosomal deletion To construct the *P. aeruginosa* 8380 strain with chromosomal

Table 1 Bacterial strains and plasmids

Strains/plasmids	Relevant properties	Source (references)
Strains		
<i>P. aeruginosa</i>		
8380	Clinical strain	16, 22
8380C	3-bp deletion of the <i>mexS</i> gene, NfxC-type mutant of 8380	27
8380Δ <i>mexT</i>	Δ <i>mexT</i> gene of 8380	This study
<i>E. coli</i>		
HST08	<i>E. coli</i> strain for transformation	TaKaRa
S17-1	Mobilizer strain	
Plasmids		
pGMS1	Vector for gene disruption; Gm ^r <i>sacB</i> ⁺	20
pUCP24	<i>E. coli</i> - <i>P. aeruginosa</i> shuttle vector; Gm ^r	21
pSCFB	pUCP24 derivative carrying <i>scfB</i>	This study
pSCFC	pUCP24 derivative carrying <i>scfC</i>	This study
pSCFF	pUCP24 derivative carrying <i>scfF</i>	This study

mexT gene deletion, the chromosomal DNA flanking *mexT* was amplified by PCR and the products were inserted into the pGMS1 plasmid. Then a 1023-bp DNA fragment of the *mexT* upstream region and a 967-bp fragment of the *mexT* downstream region were amplified using primer pairs (inf-kmexT1/inv-mexT4 and inf-kmexT2/inv-mexT3, respectively) in the presence of 8380 strain chromosomal DNA as a template (Table 2). Linearized pGMS1 plasmid DNA was amplified by PCR using a primer pair (inf-vector1 and inf-vector2) (Table 2). Next, the 1023-bp and 967-bp PCR products were inserted into the respective cloning sites on the linearized pGMS1 plasmid DNA by the In-Fusion cloning technique, yielding pGMS1-Δ*mexT*. This plasmid was transferred to the *E. coli* mobilizer strain S17-1 and then to *P. aeruginosa* 8380 by conjugation, as reported earlier^{19,20}. Deletion of the *mexT* gene was confirmed by nucleotide sequencing of the PCR product (data not shown).

Preparation of an 8380 genomic DNA clone library and isolation of chloramphenicol-resistant colonies Chromosomal DNA from *P. aeruginosa* 8380 was partially digested with *Sau*3AI and separated by agarose gel electrophoresis. Partially shared fragments (3 to 4 kbp in size) were recovered and ligated with pUCP24²¹, followed by digested with *Bam*HI and treatment with alkaline

phosphatase. The ligation mixture was introduced into *E. coli* HST08 and transformants were selected by incubation on LB plates containing 10 μg/mL of gentamicin. Plasmid DNA was extracted from the transformants, and introduced into *P. aeruginosa* 8380. Subsequently, transformants were selected by incubation on LB plates containing 40 μg/mL of gentamicin, and colonies from these plates were streaked onto another LB plates containing 500 μg/mL of chloramphenicol. Plasmid DNA was extracted from chloramphenicol-resistant colonies, and the fragment containing chromosomal DNA was amplified by PCR using a primer pair (RV-M and M13-47) (Table 2). Then the PCR products were subjected to nucleotide sequencing.

Construction of recombinant plasmids To construct recombinant plasmids carrying PA8380_33440 (*scfB*), PA8380_33450 (*scfC*), and PA8380_02790 (*scfF*), the *scfB*, *scfC*, and *scfF* genes were amplified by PCR using the following primer pairs: inf-*scfB*F/inf-*scfB*R, inf-*scfC*F/inf-*scfC*R, and inf-*scfF*F/inf-*scfF*R, respectively (Table 2). These primers contained a 15 bp tag for In-Fusion HD Cloning. The PCR products were inserted into pUCP24 treated with *Bam*HI using In-Fusion® HD Cloning Kit. The resulting plasmids were designated as pSCFB, pSCFC, and pSCFF.

Other techniques The method of western

Table 2 Primers used in this study

Primers	Sequences
inf-kmexT1	5'-GCTATGACCATGATTCATCGCCGAGCAGGGTCATCTG-3'
inv-mexT4	5'-GCTGAGGATCCGTCACGCTGCGTTCGTGCATC-3'
inf-kmexT2	5'-TTTCCCAGTCACGACTCGACGTACTTGAGGAACACG-3'
inv-mexT3	5'-TGACGGATCCTCAGCATGTTTCATCGCGCATCC-3'
inf-vector1	5'-AATCATGGTCATAGCTGTTTCCTG-3'
inf-vector2	5'-GTCGTGACTGGGAAAACCCTGGCG-3'
RV-M	5'-GAGCGGATAACAATTTACACAGG-3'
M13-47	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'
inf- <i>scfB</i> F	5'-CGGTACCCGGGGATCTTGCGCAGCGGCGAATCCA-3'
inf- <i>scfB</i> R	5'-CGACTCTAGAGGATCGCTGAGAGGCCAGAACAAAGG-3'
inf- <i>scfC</i> F	5'-CGGTACCCGGGGATCGCCGATGGGAAACACATGCAGG-3'
inf- <i>scfC</i> R	5'-CGACTCTAGAGGATCCTGCCTCCGATTACCAGTTGC-3'
inf- <i>scfF</i> F	5'-CGGTACCCGGGGATCACTGAGGCGTCGCTCGTTTC-3'
inf- <i>scfF</i> R	5'-CGACTCTAGAGGATCCCTGCACCTGTCATGGAGC-3'

blot analysis has been described previously¹⁰⁾. MICs of antibiotics were determined in triplicate by the agar dilution method with Mueller-Hinton agar II (Difco) according to the method recommended by the Japanese Society of Chemotherapy. The antibiotics tested were ofloxacin (Sigma-Aldrich), ciprofloxacin (LKT Laboratories), chloramphenicol (Wako), imipenem (LKT Laboratories), carbenicillin (Wako), aztreonam (MP Biomedicals, LCC), kanamycin (Wako), and gentamicin (Nacalai Tesque).

RESULTS AND DISCUSSIONS

Cloning of *P. aeruginosa* fragments inducing the MexEF-OprN efflux pump Partially digested fragments (3 to 4 kbp) of total *P. aeruginosa* 8380 DNA were ligated with *Bam*HI-digested pUCP24. After transformation of the ligation mixture, ca. 24,000 transformants were obtained by incubation on LB plates containing 10 µg/mL of gentamicin. All of the transformants were gathered into test tubes, and plasmid DNAs were eluted for transformation of *P. aeruginosa* 8380. As a result, more than 16,000 transformants were obtained by incubation on LB plates containing 40 µg/mL of gentamicin. All of the colonies were subjected to replica-plating on LB plates with

500 µg/mL of chloramphenicol, after which only three colonies grew (CR01, CR02, and CR03). Following purification of plasmids from these three transformants, 8380 cells were re-transformed with those plasmids. All of the transformants thus obtained also showed resistance to chloramphenicol, suggesting that DNA fragments in the plasmids conferred resistance. Eighteen randomly selected transformants exhibited similar resistance to ciprofloxacin, ofloxacin, chloramphenicol, and imipenem (Table 3). Interestingly, these transformants did not show resistance to kanamycin, carbenicillin, or aztreonam (Table 3). This drug resistance profile was quite similar to that of NfxC-type mutants expressing the RND-type MexEF-OprN efflux pump, suggesting the possibility that the cloned DNA fragments induced that pump^{13), 22)–24)}.

To verify whether MexE protein was expressed in CR1, CR2, and CR3 cells, we performed an immunoblotting analysis using anti-MexE antibody. MexE was detected in samples from all of CR01, CR02, and CR03 cells at comparable levels to that found in an NfxC mutant, 8380C (Fig. 1). This finding strongly indicated that the DNA fragments induced expression of the MexEF-OprN efflux pump to confer antibiotic resistance.

Table 3 Antibiotic susceptibilities of strains harboring the *scfB* gene or *scfC* gene

Strains	MICs (µg/mL)							
	CHL	CIP	OFX	IPM	CAR	ATM	KAN	GEN
8380	128	0.125	1	1	32	4	64	2
8380(pUCP24)	128	0.125	1	1	32	4	64	>128
CR01	>1024	4.000	32	4	8	1	16	128
CR02	>1024	4.000	32	4	8	1	16	128
CR03	1024	2.000	16	2	16	2	32	>128
8380(p#01) No.1–6	>1024	4.000	32	4	8	1	16	128
8380(p#02) No.1–6	>1024	4.000	32	4	8	1	16	128
8380(p#03) No.1–6	1024	2.000	16	2	16	2	32	>128
8380(pSCFB)	>1024	4.000	32	4	8	1	16	>128
8380(pSCFC)	128	0.125	1	1	32	4	64	>128
8380Δ <i>mexT</i>	128	0.125	1	1	32	4	64	2
8380Δ <i>mexT</i> (pUCP24)	64	0.125	1	1	32	4	64	>128
8380Δ <i>mexT</i> (pSCFB)	64	0.125	1	1	32	4	64	>128
8380C	>1024	4.000	32	4	8	1	16	1

Abbreviations: CHL, chloramphenicol; CIP, ciprofloxacin; OFX, ofloxacin; IPM, imipenem; CAR, carbenicillin; ATM, aztreonam; KAN, kanamycin; GEN, gentamicin.

Analysis of the DNA fragments inserted into pUCP24

We determined the sequences of the DNA fragments inserted into pUCP24 (Fig. 2). Plasmids p#01 and p#02 (from CR01 and CR02, respectively) contained the same DNA fragment encoding three open reading frames, PA8380_33430 (*scfA*, *scfB*, and *scfC*). Plasmid p#03 from CR03 contained a fragment encoding four open reading frames from *scfB* to PA8380_33470 (*scfE*). Three plasmids shared two genes, *scfB* and *scfC*, suggesting that the products of these genes induced the *mexEF-oprN* operon. The deduced amino acid sequence of the polypeptide encoded by *scfB* showed 89% homology and 56% identity with *scfF* in *P. aeruginosa* 8380 and belonged to the antibiotic biosynthesis monooxygenase superfamily, which is involved in the biosynthesis of several antibiotics²⁵. On the other hand, the polypeptide encoded by *scfC* was a hypothetical protein annotated in a few *P. aeruginosa* strains.

To confirm that the *scfB* and *scfC* genes were responsible for induction of the MexEF-OprN efflux pump, plasmids pSCFB and pSCFC (containing the amplified *scfB* and *scfC* DNA fragments, respectively) were introduced into 8380 cells and the antibiotic susceptibility profile was assessed. It was found that the susceptibility profiles of 8380(pSCFC) cells and 8380(pUCP24) cells were comparable (Table 3). On the other hand, 8380(pSCFB) cells showed resistance to ciprofloxacin, ofloxacin, chloramphenicol, and imipenem, and these cells also expressed MexE protein (Fig. 3, Table 3). These findings demonstrated that the *scfB* gene induced the expression of MexEF-OprN and conferred resistance to multiple antibiotics.

In 8380 cells, we previously reported that MexS repressed the activity of MexT, which is an activator of the *mexEF-oprN* operon^{26, 27}. Hence, a defect of MexS leads to activation of MexT and subsequent expression of MexEF-OprN in 8380 cells. To understand the role of MexT in induction of the MexEF-OprN efflux pump by overexpression of *scfB*, we measured the susceptibility of *mexT*-defective 8380 cells transfected with pSCFB. We found that 8380Δ*mexT*(pSCFB) cells exhibited comparable antibiotic susceptibility to 8380Δ*mexT* and 8380Δ*mexT*(pUCP24) cells, and MexE

Fig. 1 Expression of MexE protein in the three transformants.

Total cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and bands were visualized by immunoblotting using anti-MexE antibody. The amount of protein applied was 10 µg per lane. Lanes: 1, 8380; 2, 8380(pUCP24); 3, CR01; 4, CR02; 5, CR03; 6, 8380C.

Fig. 2 Schematic gene map of the fragment inserted into the indicated plasmid.

Fig. 3 Expression of MexE protein and MexS protein in 8380 and 8380Δ*mexT* harboring pSCFB.

Total cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and bands were visualized by immunoblotting using anti-MexE and -MexS antibody. The amount of protein applied was 10 and 40 µg per lane for the MexE and MexS panels, respectively. Lanes: 1, 8380; 2, 8380(pUCP24); 3, 8380(pSCFB); 4, 8380Δ*mexT*; 5, 8380Δ*mexT*(pUCP24); 6, 8380Δ*mexT*(pSCFB); 7, 8380C.

protein expression was not detected in 8380Δ*mexT*(pSCFB) cells (Fig. 3, Table 3). Therefore induction of the MexEF-OprN efflux pump by *scfB* requires MexT, suggesting that this gene induce MexEF-OprN *via* activation of MexT. Next, we focused on involvement of *mexS* in expression of the MexEF-OprN efflux pump by detecting MexS in 8380 cells after introduction of pSCFB. Surprisingly, expression of the negative regulator MexS was not reduced in 8380(pSCFB) cells, but MexS was over-

expressed in these cells (like 8380C cells) compared with its expression in 8380 and 8380(pUCP24) cells (Fig. 3). Nevertheless, 8380(pSCFB) cells showed overexpression of the MexEF-OprN efflux pump. This result suggested that the *scfB* gene product blocked the inhibition of MexT by MexS. Why was expression of MexS protein increased in 8380(pSCFB) and 8380C cells? Köhler *et al.* previously reported that MexS expression was depressed its own expression in mediating the *mexT* gene product²⁸⁾. Therefore, 8380 cells with a defective *mexS* gene, such as the NfxC mutant, overexpress the *mexS* gene product *via* activation of MexT (Fig. 3), and overexpression of MexS in 8380(pSCFB) cells seems to result in activation of MexT.

MexEF-OprN overexpression by *scfF*, a homologue of *scfB* The *scfB* gene activated MexT, resulting in overexpression of the MexEF-OprN efflux pump. This finding encouraged us to analyze *scfF*, which is a homologue of *scfB*. To verify that *scfF* induces the MexEF-OprN efflux pump, we investigated antibiotic resistance and expression of MexE protein in 8380 cells transformed with the pSCFF plasmid bearing the *scfF* gene. Transformed 8380(pSCFF) cells exhibited stronger resistance to chloramphenicol, ofloxacin, ciprofloxacin, and imipenem compared with 8380(pUCP24) cells and had a similar resistance profile to that of 8380(pSCFB) cells, but showed less resistance to the tested antibiotics than 8380(pSCFB) and 8380C cells (Table 4). Moreover, 8380(pSCFF) cells expressed MexE protein, but its expression was lower than in 8380(pSCFB) cells (Fig. 4). These results strongly indicated that the *scfF* gene product also induced the MexEF-OprN efflux pump and conferred resistance to various antibiotics.

Many studies have shown a significant risk spreading antibiotic resistant microorganisms in medical practice^{29),30)}. In 2014 and 2015, World Health Organization (WHO) announced that antibiotics resistance would threaten the achievements of modern medicine and the world would be heading toward a post-antibiotic era unless preventive measure was taken on a global scale^{31),32)}. WHO also requested numerous international sections to control its emergence and spread, and to

Table 4 Effects of the *scfB* and *scfF* genes on antibiotic susceptibility

Strains	MICs (μg/mL)			
	CHL	CIP	OFX	IPM
8380	128	0.125	1	1
8380(pUCP24)	128	0.125	1	1
8380(pSCFB)	>1024	4.000	32	4
8380(pSCFF)	1024	1.000	4	2
8380C	>1024	4.000	32	4

Abbreviations: CHL, chloramphenicol; CIP, ciprofloxacin; OFX, ofloxacin; IPM, imipenem.

Fig. 4 Expression of MexE protein in 8380 and 8380Δ*mexT* harboring pSCFF.

Total cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and bands were visualized by immunoblotting using anti-MexE antibody. The amount of protein applied was 10 μg per lane. Lanes: 1, 8380(pUCP24); 2, 8380(pSCFB); 3, 8380(pSCFF).

develop novel antibiotics immediately. In response to their requests, six Japanese societies in the field of microbiology and medicine along with infectious disease have also announced their recommendation for the development of new antibiotics for nationals, Japanese government, Japanese pharmaceutical companies, and Japanese universities³³⁾. However, the development of them does not come off well because bacteria are ever evolving with relative ease and the developed antibiotics lose effectiveness despite the cost of researching and manufacturing effective ones is very high.

To suppress the spread of antibiotic resistant microorganisms, it is certainly necessary to cooperate with not only the clinical workers, but also the workers following the stockbreeding because antibiotics has been used more for animals and fish than human. In addition, the antibiotics are often detected in aquatic environments such as rivers and sewage even in Japan where antibiotics have been used with strict regulation in both fields. Therefore, the existence of antibiotics in the environment

promotes the emergence and spread of resistant microorganisms. In this paper, we identified new regulator genes inducing RND-type efflux pumps which contribute antibiotic resistance in *Pseudomonas aeruginosa*. We also found that the cells, which introduced the plasmids harboring these regulator genes, acquired a resistance to antibiotics. In the environment and human body, non-resistant microorganisms become resistant to antibiotics by transferring the plasmid, harboring resistance genes, derived from resistant microorganisms³⁰. Therefore, there is a possibility that the genes we found may always become prevalent resistance factors. Resistance genes and resistant microorganisms should be monitored regularly even in aquatic environments for suppressing the spread of the resistant cells. A further finding of novel factors inducing resistance to antibiotics should be conducted.

CONCLUSIONS

This study demonstrated that overexpression of *scfB* and its homologue *scfF* induced expression of the MexEF-OprN RND-type xenobiotic efflux pump in an MexT-dependent and MexS-independent manner. The proteins encoded by *scfB* and *scfF* each have an antimicrobial synthetic monooxygenase domain and may regulate gene expression through oxidation-reduction reactions. Both *scfB* and *scfF* could induce RND-type xenobiotic efflux pumps in other bacteria and confer resistance to antibiotics if transferred *via* self-transferable plasmids or transposable elements because most gram-negative bacteria have several RND-type xenobiotic efflux pumps. It is possible that the genome of each microorganism contains many antibiotic resistance-related genes that have not yet been discovered.

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