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Identification of Novel Genes Responsible for Overexpression of *ampC* in *Pseudomonas aeruginosa* PAO1

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The development of resistance to antipseudomonal penicillins and cephalosporins mediated by the chromosomal *ampC* gene in *Pseudomonas aeruginosa* is of clinical importance. We isolated piperacillin-resistant mutants derived from *P. aeruginosa* PAO1 and analyzed two mutants that had an insertion in *mpl* and *nuoN*. One mutant, YT1677, was resistant to piperacillin and ceftazidime and had an insertion in *mpl*, which encodes UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase. The other mutant, YT7988, showed increased MICs of piperacillin, ceftazidime, cefepime, and cefoperazone, and the insertion was mapped to *nuoN*, which encodes NADH dehydrogenase I chain N. Complementation experiments demonstrated that these mutations resulted in higher levels of resistance to β-lactams. The expression of genes reported to be involved in β-lactam resistance was examined by real-time PCR in YT1677 and YT7988 mutants. Overexpression was observed for only *ampC*, and other genes were expressed normally. Deletion of the *ampR* gene in YT1677 and YT7988 resulted in decreased expression of *ampC*, indicating that the mutations in YT1677 and YT7988 affected the expression of *ampC* through the function of AmpR.

Pseudomonas aeruginosa is a versatile Gram-negative bacterium that is isolated from soil, water, and most man-made environments, as well as from plant and animal tissue. It is a major opportunistic human pathogen (1). *P. aeruginosa* infection is of considerable importance in immunocompromised patients, who are less able to fight infections.

P. aeruginosa is naturally resistant to antibiotics and has many mechanisms to reduce its susceptibility to antibiotics, such as the overexpression of efflux pumps, decreased production of the outer membrane protein (D2 porin), overexpression of the chromosomally encoded AmpC cephalosporinase, modification of drugs, and mutation(s) at the target site of the drug. The bacteria also develop antibiotic resistance through the acquisition of resistance genes carried on mobile genetic elements (2, 3). It is well known that selection by penicillins, such as piperacillin (PIP), and cephalosporins, such as cefepime (FEP) or ceftazidime (CAZ), leads to hyperproduction of the chromosomally encoded AmpC cephalosporinase (4).

The AmpC cephalosporinase is a class C β-lactamase and is present in most *Enterobacteriaceae*, *P. aeruginosa*, and other Gram-negative organisms. The production of AmpC is induced by β-lactams, and this is the major mechanism of resistance to β-lactams without acquisition of an externally acquired resistance gene (5, 6). It is known that the overproduction of AmpC in *P. aeruginosa* results in resistance to nearly all β-lactam antibiotics, with the exception of the carbapenems (5, 7, 8).

In *P. aeruginosa*, the mechanism that controls AmpC cephalosporinase production is implicated in the control of various genes. The induction of the *ampC* gene is intimately connected to peptidoglycan recycling (9), but the regulation of *ampC* expression in *P. aeruginosa* is not well understood. AmpC production is repressed under normal cell wall recycling conditions, where cell wall components are recycled by the function of *ampD* and other enzymes. When peptidoglycan synthesis is inhibited by β-lactam antibiotics, the levels of cell wall components increase in the cytoplasm. These components then activate AmpR, which belongs to an LysR superfamily, which in turn switches on *ampC* expression (10). As AmpR binds to the intergenic region between *ampR* and *ampC*, mutations in this region and *ampR* could increase AmpC production (10–12). AmpG is a transmembrane protein that functions as a specific permease for 1,6-anhydromurapeptides, which are thought to be the signal molecules involved in *ampC* induction (13, 14). The *ampD* and *ampE* genes of *P. aeruginosa* PAO1 are transcribed in the same orientation and form an operon structure (15). AmpD is a cytosolic *N*-acetylmuramyl-L-alanine amidase that hydrolyzes 1,6-anhydromurapeptides, acting as a repressor for *ampC* expression (9). Inactivation of *ampD* in *P. aeruginosa* leads to the derepression of *ampC* (16). *P. aeruginosa* has three *ampD* genes, *ampD* and the homologues *ampDh2* and *ampDh3*, which are responsible for a stepwise *ampC* upregulation mechanism, and *ampC* expression is coordinately repressed by the three *ampD* genes (17). The deletion of the *ampD* genes is required for fully derepressed *ampC* expression. However, AmpD plays a much greater role in the repression of *ampC* expression than the two homologues (17). *P. aeruginosa* has another chromosomally encoded β-lactamase called PoxB. *poxB* is shown to be negatively regulated by AmpR, while *ampC* is positively regulated (18). Recent studies reported that the *dacB* gene was involved in *ampC* expression. *dacB* encodes penicillin binding protein 4 (PBP4), known to be a nonessential PBP (19). The inactivation of *dacB* results in a high level of β-lactam resistance and the overproduction of AmpC. This implies that PBP4 behaves as a trap for β-lactams (19). In addition, the *creD* gene was upregulated in *dacB* mutant strains (19). *creD* encodes an inner membrane protein and

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TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s) or description	Source or reference
P. aeruginosa strains		
PAO1	*P. aeruginosa*	Laboratory stock
PAO1 Δ*ampR*	PAO1 Δ*ampR*	This study
YT1677	PAO1 *mpl*::Tn*Mod*-OGm	This study
YT1678	PAO1 *mpl*::Tn*Mod*-OGm *attB*::*lacI*^{q}-P_{T7}-*mpl*	This study
1677 Δ*ampR*	PAO1 *mpl*::Tn*Mod*-OGmΔ*ampR*	This study
YT7988	PAO1 *nuoN*::Tn*Mod*-OGm	This study
YT7989	PAO1 *nuoN*::Tn*Mod*-OGm *attB*::*lacI*^{q}-P_{T7}-*nuoN*	This study
7988 Δ*ampR*	YT7988 Δ*ampR*	This study
E. coli strains		
DH5α λ*pir*	F$^-$ ϕ80 Δ*lacZ*(M15) *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* λ*pir*	Laboratory stock
CSH55	F$^-$ Δ(*lac-pro*) *supE nalA thi*	Laboratory stock
Plasmids		
pDS132	Suicide plasmid, *pir* dependent, CHL^r; *oriT oriV*$_{R6K}$ *mob*$_{RP4}$ *bla sacB*	33
pFLP2	Cb^r; source of Flp recombinase	34
pTn*Mod*-OGm	Minitransposon vector; pMB1 *oriR oriT* Tn*5 tnp*, GEN^r	25
pRK2013	*tra* region of RK2 cloned in ColE1; Tra$^+$, Mob$^+$, KAN^r	35
pYM101	Expression vector; *oriT*$^+$Tc^r, mini-CTX::*lacI*^{q}-P_{T7}-MCS[a]	26
pYT101	pYM101/*mpl*; *mpl* gene cloned in pYM101	This study
pYT102	pYM101/*nuoN*; *nouN* gene cloned in pYM101	This study
pYT106	pDS132/Δ*ampR*; *ampR* gene containing a deletion cloned in pDS132	This study

[a] MCS, multiple cloning site.

is regulated by the two-component regulator CreBC, and the CreBC system is suggested to be involved in β-lactam resistance in *P. aeruginosa* (19). The CreBC system has been well studied in *Escherichia coli*, and it has been shown to be a global regulator of gene expression involved in metabolic control (20). *nagZ* encodes β-*N*-acetyl-D-glucosaminidase, and inactivation of *nagZ* in *P. aeruginosa* attenuates β-lactam resistance (21). *nagZ* inactivation in the *dacB* or *ampD* mutant prevents overexpression of *ampC*. *nagZ* inactivation did not impair *ampC* inducibility (22). All the components of the described resistance mechanism, *dacB*, *ampC*, *ampD*, *ampR*, and *creBCD*, of *P. aeruginosa* have been found to be involved in the regulation of AmpC production and, thus, β-lactam resistance. Several systems appear to be involved in the expression of the *ampC* gene, and the complete picture of the regulatory system is still not clear.

We conducted this study to determine the regulation of overexpression of *ampC* in *P. aeruginosa* PAO1. We isolated PIP-resistant mutants of *P. aeruginosa* PAO1 by transposon mutagenesis and identified novel genes responsible for the expression of *ampC*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *P. aeruginosa* PAO1 was used as the parent strain. *Escherichia coli* strain DH5α λ*pir* was used as the host strain for plasmid transformation. *E. coli* strain CSH55 was used in conjugation experiments. Transposon insertion mutants YT1677 and YT7988 were derived from *P. aeruginosa* PAO1 by transposon mutagenesis with pTn*Mod*-OGm by selection using gentamicin (GEN). Both *P. aeruginosa* and *E. coli* were grown either in Luria-Bertani (LB) medium (Becton, Dickinson, Sparks, MD, USA) or antibiotic medium 3 (AB3) (Becton, Dickinson) at 37°C. Agar plates were prepared by the addition of 1.5% agar to the medium.

Determination of MICs and antibiotics. The MICs of the antibiotics were determined by the agar dilution method according to CLSI recommendations (23). Antibiotics used in the susceptibility test were cefepime (FEP), cefoperazone (CFP), ceftazidime (CAZ), piperacillin (PIP), imipenem (IPM), meropenem (MEM), aztreonam (ATM), and ciprofloxacin (CIP).

DNA manipulations and genetic techniques. Total DNA was prepared from 1 ml of overnight culture using Isoplant (Wako Pure Chemicals, Osaka, Japan), and plasmid DNA was prepared using a QIAprep Spin Miniprep Kit (Qiagen, Santa Clara, CA, USA). PCR amplifications were carried out with a Veriti 96-well Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA). EmeraldAmp PCR Master Mix (TaKaRa Bio, Shiga, Japan) was used to examine insert fragments by colony-directed PCR of the *E. coli* recombinant cells. PrimeSTAR GXL DNA Polymerase (TaKaRa Bio) and TaKaRa *Taq* Hot Start (TaKaRa Bio) were used for the amplification of fragments used for cloning and for DNA sequencing, respectively. PCR products for cloning and sequencing were extracted from the agarose gel by Wizard SV Gel and PCR cleanup systems (Promega Corp., Madison, WI, USA). Sequencing was performed with a BigDye Terminator cycle sequencing kit (Applied Biosystems) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems). DNA sequences were analyzed with Genetyx, version 10, software (Genetyx, Tokyo, Japan) and subjected to a homology search by the Genetyx homology search program, and a homology search using BLAST was performed through the NCBI website (http://www.ncbi.nlm.nih.gov/Tools/index.html). Transformation of competent *E. coli* cells was carried out using the calcium chloride method (24).

Transposon mutagenesis with pTn*Mod*-OGm and determination of the insertion site. A donor strain carrying pTn*Mod*-OGm and pRK2013 was constructed by conjugation on an agar plate with strain DH5α λ*pir* carrying pTn*Mod*-OGm and CSH55 carrying pRK2013. Conjugation on the agar plate was performed as follows: overnight cultures of donor and recipient strains were mixed at a ratio of 1 to 10, and 5 μl of the mixture was spotted onto an LB agar plate. After the liquid was absorbed onto the agar, the mating plate was incubated at 37°C for 8 h. The cells were scraped off the plate and resuspended in 1 ml of LB broth, and appropriate dilutions were plated on selective plates containing kanamycin (KAN) (40 μg/ml) and GEN (10 μg/ml). The resulting conjugants were used as the donor strain in transposon mutagenesis. Overnight cultures of the donor strain, DH5α λ*pir* carrying pTn*Mod*-OGm and pRK2013, and the recip-

TABLE 2 Primers used in this work

Function and target	Primer[a]	Sequence (5′→3′)	Position (5′→3′)[b]	Product length (bp)	Reference or source
Real-time PCR					
ampC	ampC1	CGGCTCGGTGAGCAAGACCTTC	264–285	218	36
	ampC2	AGTCGCGGATCTGTGCCTGGTC	460–481		
ampR	ampR/F	CGCGCCATCCCTTCATC	353–369	55	This study
	ampR/R	ATGTCGACGCGGTTGTTGT	389–407		
ampD	ampD/F	TCGCTGCTGGTTATCCACAA	91–110	77	This study
	ampD/R	ACCTTACCGGTGCCGAACT	149–167		
ampDh2	ampDh2/F	ACCGGCGAGCTGGAGAA	652–668	84	This study
	ampDh2/R	GGACGGTATTTCATCTGGAAAGC	713–735		
ampDh3	ampDh3/F	CTGACCATCGACTACAACAGCTATC	4–28	76	This study
	ampDh3/R	GGAAGCGCACGCGTTT	64–79		
ampG	ampG/F	ATCGACATGGGCTTCTCCAA	1204–1223	127	This study
	ampG/R	ACAGGATGGAGAGGATGCTGAA	1309–1330		
dacB	dacB/F	CCGCGACATCAACAAATACAGT	924–945	79	This study
	dacB/R	CGCCGATGGAGAGGAACA	985–1002		
creD	creDrna/F	CGGCGTGCTGCAGGATATCGC	114–134	251	19
	creDrna/R	TGTCGACGTGGTACAGGCGCG	344–364		
nagZ	nagZrna/F	CTTCGCTCGCAACATCGA	96–113	56	This study
	nagZrna/R	GAATGGCCGCACACAGTTC	133–151		
mexA	mexA/F	GTTCCCCAACCCGAACAAC	792–810	68	37
	mexA/R	TGACGCCTTCCTGCAACTG	859–841		
rpoD	rpoD/F	CCTGCCGGAGGATATTTCC	96–114	70	37
	rpoD/R	GATCCCCATGTCGTTGATCAT	165–145		
Sequencing					
Tn*Mod*-OGm	pTnGm/Lout	CTTTCCTGGTACCGTCGACATGC			This study
	pTnGm/Rout	TACAGTTTACGAACCGAACAGGC			
Cloning					
nuoN	PA2649 Eco/F	GGAATTCAGCCAGTGCTGGACATCT			This study
	PA2649 Bam/R	CGGGATCCGACGTCGATTTCGTGGAAGGT			
mpl	PA4020-SacI/F				
	PA4020-BamI/R	TTGCGAGCTCCATTCACAGCGCCTTCGAC			This study
		CGGGATCCAGGGTAATGCGTTCCGGA			
Δ*ampR*	ampR-SphI/F2	ACATGCATGCCTAGGCTTGCGCAGGATTTGCGGCA			This study
	ampR-2/R	CCGTCAGACGCGGTTGTTGTGGGTGGAC			
	ampR-SphI/2	ACATGCATGCTTCCAATCACAACCCCAACGCCTC			
	ampR-2/F	CGCGTCTGACGGTGCTCTGCTGCCCGG			

[a] F, forward; R, reverse.
[b] The positions given are from the first base of the coding sequences of the genes.

ient strain, PAO1, were mixed at a ratio of 10 to 1, and conjugation on an agar plate was performed as described above, except that the mating mixture was scraped off the plate using a toothpick and streaked on a selective plate containing GEN at a concentration of 40 μg/ml and tetracycline (TET) at a concentration of 10 μg/ml. Only two transconjugants were picked from each mating to avoid picking the same mutants, and these were purified by single-colony isolation on the same selective plate. The transconjugants were examined for their susceptibility to PIP by the agar dilution method (23), and the strain showing increased resistance to PIP was used for further analysis. To determine the insertion site of Tn*Mod*-OGm, the region flanking the insertion was cloned. Total DNA from the mutant strain with the desired phenotype was prepared using Isoplant, and the DNA was digested by EcoRI (Roche Diagnostics) as the transposon does not contain this restriction site. After digestion, the enzyme was inactivated by ethanol precipitation, and the DNA was self-ligated. A portion of the ligation mixture was used for the transformation of *E. coli* DH5α λ*pir*, selecting for gentamicin resistance (10 μg/ml). As Tn*Mod*-OGm carried the replication origin of plasmid pMB1, a circular DNA molecule with Tn*Mod*-OGm can be maintained as a plasmid in the *E. coli* cell (25). The resulting transformants carried a plasmid consisting of Tn*Mod*-OGm and the flanking region. The flanking region was sequenced with the primers pTnGm/Lout and pTnGm/Rout, which anneal to the distal region of Tn*Mod*-OGm (Table 2). Using the sequence obtained from the flanking DNA, the position of the insertion was determined by a BLAST search and a search of the online *Pseudomonas* Genome Database (http://www.pseudomonas.com/).

Complementation test. Wild-type genes were cloned into the expression vector pYM101, which can be integrated into the chromosome, and expression of the inserted gene was induced by the addition of IPTG. The *mpl* gene from *P. aeruginosa* PAO1 was amplified by PCR with primers PA4020-SacI/F and PA4020-BamI/R, which incorporate a SacI and BamHI site, respectively. The *nuoN* gene from the wild-type strain *P. aeruginosa* PAO1 was amplified by PCR with primers PA2649-Eco/F and PA2649-Bam/R, which incorporate an EcoRI and BamHI site, respectively (Table 2). The amplified DNA fragments were subcloned into expression vector pYM101 to create plasmid pYT101 carrying *mpl* and plasmid pYT102 carrying *nuoN*, respectively (Table 1). After confirmatory sequencing of the cloned fragments, each plasmid was introduced into DH5α λ*pir* by transformation. The transformants were selected on LB agar plates containing tetracycline (TET) at a concentration of 10 μg/ml. The plasmid pRK2013 was introduced into the transformant by conjugation with CSH55 carrying pRK2013 by incubation on an agar plate for 8 h. The conjugants were selected for KAN and TET resistance. The resulting strain was used as the donor strain for conjugation on the agar plate with

the insertional mutant strain YT1677 or YT7988 to construct a strain for the complementation test. Conjugants were selected on an agar plate containing TET and GEN at concentrations of 70 μg/ml and 10 μg/ml, respectively. Conjugants were expected to have either pYT101 or pYT102 integrated into the chromosomal *att* site. The pFLP2 plasmid was then introduced into the conjugants carrying pYT101 or pYT102 in the *att* site by conjugation with DH5α λ*pir* carrying pFLP2 and pRK2013 in order to eliminate the unwanted plasmid backbone sequences of pYM101 (26). The transconjugants were selected on LB agar plates containing carbenicillin (CAR) and GEN at concentrations of 200 μg/ml and 40 μg/ml, respectively. Curing of pFLP2 was carried out as described previously (27).

RNA analysis using real-time RT-PCR. Total RNA was isolated from *P. aeruginosa* strains using a FastRNA Pro Blue Kit (MP Biomedicals, Santa Ana, CA, USA). A 0.5-ml aliquot of overnight culture was added to 50 ml of LB broth, and cells were grown for 3 h at 37°C with or without 50 μg/ml cefoxitin (FOX). In an induction experiment with piperacillin (PIP), its concentration was 0.5× MIC for each strain. Cells were harvested by centrifugation, and the pellet was resuspended in 700 μl of 50 mM glucose–25 mM Tris-HCl (pH 8.0). Preparation of total RNA was performed according to the manufacturer's protocol. The RNA sample was treated with 50 units of DNase I (Roche) for 2 h at 37°C to remove contaminating DNA. DNase I was eliminated by phenol-chloroform extraction and ethanol precipitation. The pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated H_2O. cDNAs were made using PrimeScript RT Master Mix (TaKaRa Bio). Real-time PCR was performed with SYBR Premix *Ex Taq* (TaKaRa Bio) and a 7500 Fast Real-Time PCR System (Applied Biosystems). The primers used for real-time reverse transcription-PCR (RT-PCR) are listed in Table 2. The *rpoD* transcript was measured as an endogenous control to normalize the level of the transcripts of interest.

Inactivation of *ampR* in transposon mutants. To introduce a deletion into the *ampR* gene in *P. aeruginosa* strains PAO1, YT1677, and YT7988, a fragment containing a 91-bp deletion in *ampR* (Δ*ampR* fragment) was first cloned in plasmid pDS132. The deletion ran from bp 403 to bp 493 (from the first base of the *ampR* coding region), resulting in the appearance of the stop codon as the 135th codon and production of a truncated AmpR with a length of 134 amino acids. The wild-type AmpR is 296 amino acids in length. The Δ*ampR* fragment was obtained by overlapping PCR (24) as follows: the upstream DNA fragment of Δ*ampR* was obtained by PCR with the primers ampR-SphI/F2 and ampR-2/R, and the downstream DNA fragment was obtained by PCR with the primers ampR-2/F and ampR-SphI/2 (Table 2). The full-length Δ*ampR* fragment, which has an SphI recognition site at both ends, was obtained by PCR with both fragments obtained above and the primers ampR-SphI/F and ampR-SphI/R. The Δ*ampR* fragment was cloned into pDS132. The resulting plasmid was designated pYT106. In order to introduce pYT106 into strains PAO1, YT1677, and YT7988, the helper plasmid pRK2013 was transferred into DH5α λ*pir* carrying pYT106 by conjugation on an agar plate, and transconjugants were used as a donor strain in the conjugation on an agar plate with PAO1, YT1677, and YT7988. Transconjugants were selected on LB agar plates containing CHL (70 μg/ml) and TET (10 μg/ml).

Transconjugants were expected to have the plasmid integrated into the chromosome by a single homologous recombination through *ampR* sequences. To replace the wild-type *ampR* with the mutated allele (Δ*ampR*), a second crossover was required, which resulted in the elimination of plasmid DNA. Sucrose-resistant cells were selected from an overnight culture of the transconjugants. These could grow on a 5% sucrose plate and were expected to lose pDS132 DNA because pDS132 has a *sacB* gene conferring sucrose sensitivity to the host cell. A number of sucrose-resistant colonies were examined for the elimination of pDS132 DNA and the DNA sequence of the Δ*ampR* gene.

TABLE 3 MICs for mutant strains

Strain[b]	MIC (μg/ml)[a]							
	PIP	CAZ	FEP	CFP	IPM	MEM	ATM	CIP
PAO1	3.13	3.13	3.13	12.5	1.56	0.4	3.13	0.2
PAO1 Δ*ampR*	3.13	3.13	3.13	12.5	0.4	0.4	3.13	0.2
YT1677	50	6.25	3.13	400	1.56	0.8	3.13	0.2
YT1678[c]	6.25	1.56	3.13	12.5	1.56	0.8	3.13	0.2
1677 Δ*ampR*	3.13	0.8	1.56	6.25	0.4	0.4	3.13	0.2
YT7988	100	25	25	400	1.56	0.8	6.25	0.2
YT7989[c]	12.5	3.13	3.13	25	1.56	0.8	6.25	0.2
7988 Δ*ampR*	6.25	3.13	3.13	12.5	0.4	0.4	6.25	0.2

[a] PIP, piperacillin; CAZ, ceftazidime; FEP, cefepime; CFP, cefoperazone; IPM, imipenem; MEM, meropenem; ATM, aztreonam; CIP, ciprofloxacin.
[b] YT1678 and YT7989 are derivatives of YT1677 and YT7988 carrying intact *mpl* gene and *nuoN* gene under the control of inducible promoter with IPTG, respectively. PAO1 Δ*ampR*, 1677 Δ*ampR*, and 7988 Δ*ampR* represent strains PAO1, YT1677, and YT7988 with a deletion of *ampR*, respectively.
[c] The induction was carried out with 1.0 mM IPTG.

RESULTS

Transposon mutagenesis of *P. aeruginosa* PAO1 resulting in increased resistance to PIP. To identify the genes contributing to the expression of β-lactam resistance in *P. aeruginosa* PAO1, we isolated 10,000 insertional mutants carrying random insertions with the Tn*Mod*-OGm transposon from 5,000 independent experiments. Some mutant strains exhibited a higher resistance to PIP, and two strains named YT1677 and YT7988 were analyzed further. The results of a susceptibility test for these two strains are shown in Table 3. YT1677 showed increased resistance to PIP and CFP although there was little change in susceptibilities to CAZ, FEP, IPM, MEM, ATM, and CIP. YT7988 showed increased resistance to PIP, CAZ, FEP, and CFP while there was little change in the MICs other antibiotics to this strain. The DNA sequences of the flanking regions of Tn*Mod*-OGm in the mutant strains were determined to map the insertions. Using a BLAST search and the online *Pseudomonas* Genome Database, two Tn*Mod*-OGm insertions were mapped in two open reading frames (ORFs), PA4020 in YT1677 and PA2649 in YT7988. PA4020 is 1,356 bp in length and encodes the 451-amino-acid UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase (*mpl*), which shows a 60% identity and an 72% similarity with that of *E. coli* (accession number ELC33566.1). Tn*Mod*-OGm was mapped between bp 680 and 681 from the start codon of PA4020 in YT1677. PA2649 is 1,461 bp in length and encodes the 486-amino-acid NADH dehydrogenase I chain N (*nuoN*), showing a 64% identity and an 81% similarity with that of *E. coli* (accession number WP_001600231). Tn*Mod*-OGm was mapped between bp 230 and 231 from the start codon of PA2649 in YT7988.

Complementation test. We examined whether inactivation of PA4020 or PA2649 resulted in increased resistance to the β-lactam antibiotics tested. As described in Materials and Methods, PA4020 and PA2649 were cloned in the expression vector pYM101 to make pYT104 and pYT105, respectively. After conjugation, they were integrated into the ϕCTX attachment site on the chromosome of YT1677 and YT7988, respectively. After integration and the elimination of the plasmid backbone, the resulting strains were designated YT1678 (*mpl*) and YT7989 (*nuoN*). The expression of PA4020 (*mpl*) and PA2649 (*nuoN*) cloned in YT1678 and YT7989 was induced by the addition of 1 mM isopropyl-β-D-

TABLE 4 Expression of *ampC* and *ampR* genes in the mutants studied as determined by qRT-PCR

Induction treatment and strain[b]	*ampC* expression[a]		*ampR* expression[a]	
	Basal	Induced	Basal	Induced
With FOX[c]				
PAO1	1	48.1 ± 25.5	1	0.8 ± 0.4
PAO1 Δ*ampR*	1.1 ± 0.6	2.8 ± 1.6	ND	ND
YT1677	12.4 ± 8.2	9522.6 ± 4192.8	0.9 ± 0.3	1.7 ± 0.6
1677 Δ*ampR*	2.4 ± 2.5	3.2 ± 3.3	ND	ND
YT7988	1.7 ± 1.0	564.3 ± 112.1	0.9 ± 0.1	1.2 ± 0.6
7988 Δ*ampR*	2.4 ± 1.2	1.9 ± 1.0	ND	ND
With PIP[d]				
PAO1	1	5.0 ± 3.8	1	1.1 ± 0.4
YT1677	3.5 ± 2.5	16.9 ± 9.5	1.1 ± 0.6	1.8 ± 0.4
YT7988	2.9 ± 2.6	21.5 ± 5.5	1.3 ± 0.5	1.4 ± 0.6

[a] Relative amount of *ampC* or *ampR* mRNA compared to the PAO1 basal level ± standard deviation. All experiments were performed in triplicate. ND, not determined.
[b] PAO1 Δ*ampR*, 1677 Δ*ampR*, and 7988 Δ*ampR* represent strains PAO1, YT1677, and YT7988 with a deletion of *ampR*, respectively.
[c] Induction was carried out with 50 μg/ml cefoxitin (FOX).
[d] Induction was carried out with 0.5× MIC of piperacillin (PIP) for each strain.

thiogalactopyranoside (IPTG). The susceptibilities of these strains to antibiotics were examined after induction, and the results are shown in Table 3. After induction, the PIP MIC and CFP MIC of YT1678 decreased from 50 μg/ml to 6.25 μg/ml and from 400 μg/ml to 12.5 μg/ml, respectively, levels which were almost the same as those of the parent strain PAO1. Induction of YT7989 showed reduced MICs as follows: PIP, from 200 μg/ml to 12.5 μg/ml; CAZ, from 25 μg/ml to 3.13 μg/ml; FEP, from 25 μg/ml to 3.13 μg/ml; CFP, from 400 μg/ml to 25 μg/ml. Although MICs of PIP and CFP were still high, they were greatly reduced. These results indicate that the mutations in YT1677 and YT7988 were complemented by the wild-type genes cloned on the expression vector, which had integrated into the chromosome, indicating that mutations in YT1677 and YT7988 resulted in increased resistance to the β-lactam antibiotics tested.

Measurement of expression by real-time PCR. In order to analyze the mechanism of overexpression of β-lactam resistance, the expression of genes related to β-lactam resistance was examined by real-time quantitative RT-PCR (qRT-PCR). The overexpression of efflux pumps and the chromosomal AmpC cephalosporinase encoded by *ampC* are recognized as major mechanisms of resistance in *P. aeruginosa*. Therefore, the expression levels of *mexA*, a component of a major efflux pump in the MexAB-OprM complex, and the genes involved in the expression of *ampC*, namely, *ampD*, *ampDh*, *ampDh2*, *ampG*, *dacB*, *creD*, and *nagZ*, were examined. Compared with the levels of expression of these genes in PAO1, no gene except *ampC* in YT1677 and YT7988 showed any significant difference either with or without induction by FOX. YT1677 showed an elevated level (12.4-fold) of *ampC* expression without induction (basal), and *ampC* expression in YT1677 increased almost 200-fold compared with that of PAO1 after induction. In contrast, YT7988 showed small difference in *ampC* expression without induction (1.7-fold) although *ampC* expression in YT7988 increased 11.7-fold with induction compared with PAO1 (Table 4). The expression of *ampR*, a positive regulator of *ampC*, did not change in either mutant with or without induction (Table 4). Although an elevated expression of *ampC* was observed in YT1677 and YT7988 with or without induction with FOX, the level of expression in YT1677 was much higher than that in YT7988, which was more resistant to PIP than YT1677 (Table 3). Therefore, an induction experiment was carried out with PIP at a concentration of 0.5× MIC for each strain. As shown in Table 4, the levels of expression of *ampC* after induction with PIP in YT1677 (16.9-fold) and YT7988 (21.5-fold) were almost the same and lower than those with FOX. However, the levels were higher than the level in PAO1 (5.0-fold) in the case of induction with FOX. In addition, expression of *ampR* was also examined. As with FOX, the level of expression of *ampR* did not change significantly in either YT1677 or YT7988 with or without induction with PIP. These results indicated that the resistance to the β-lactam antibiotics tested resulted from the overexpression of *ampC* caused by the mutations and not from the overexpression of its positive regulator, *ampR*.

Overexpression of *ampC* is dependent on the function of *ampR*. AmpR is a cytosolic protein and is believed to play a major role as a positive regulator in the regulation of *ampC* expression. We therefore examined the requirement for AmpR in the overexpression of *ampC* in YT1677 and YT7988. Previously, the DNA sequence of the *ampR-ampC* coding region, including the regulatory sequences, was determined in YT1677 and YT7988. No mutation was found, excluding the possibility that a mutation(s) in the regulatory region and/or *ampR*-coding region resulted in the overexpression of *ampC* (data not shown). We also determined the DNA sequence of the *ampD* gene and its homologues as mutation here results in the accumulation of immature cell wall components because of a defect in the cell wall recycling system and in constitutive expression of *ampC*. No mutation was found in these genes, as in the case of the *ampR-ampC* region (data not shown). We attempted to inactivate *ampR* by introducing a deletion into the *ampR* locus in YT1677 and YT7988, as described in Materials and Methods. The resulting strains were designated 1677 Δ*ampR* and 7988 Δ*ampR*, respectively. The MICs of antibiotics for Δ*ampR* strains were examined, and the results are shown in Table 3. Similar to the results obtained with YT1678 and YT7989 in the complementation test, these Δ*ampR* strains restored the sensitivity to the antibiotics to which YT1677 and YT7988 were resistant. In addition, both 1677 Δ*ampR* and 7988 Δ*ampR* strains were more sensitive to IPM (0.4 μg/ml) than their parent strains and PAO1 (1.56 μg/ml). This result indicated that *ampR* was required for the resistance to the β-lactam antibiotics tested. The expression of *ampC* in the 1677 Δ*ampR* and 7988 Δ*ampR* strains was measured, and the results are shown in Table 4. The expression levels of *ampC* in 1677 Δ*ampR* and 7988 Δ*ampR* decreased although *ampC* in 1677 Δ*ampR* and 7988 Δ*ampR* still showed a level of expression 2- to 3-fold higher than that in PAO1 but the same as that observed in PAO1 Δ*ampR* (induced). These results indicate that the overexpression of *ampC* in YT1677 and YT7988 was dependent on the function of *ampR*.

DISCUSSION

YT1677 had a Tn*Mod*-OGm insertion in *mpl* encoding UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase, which is involved in the recycling of cell wall components. In *Escherichia coli*, *mpl* is a nonessential gene because *murC* encodes an enzyme that adds murein tripeptide to UDP-MurNAc (where MurNAc is N-acetylmuramic acid) and bypasses the defect in the cell wall component recycling system caused by the muta-

tion in *mpl* (28). Although *mpl* is not essential, deletion of *mpl* resulted in a 50% decrease in UDP-MurNAc-pentapeptide compared with the wild-type strain and an increase of L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate (29). This event may then lead to the accumulation of immature cell wall components, such as 1,6-anhydro-*N*-acetylmuramic oligopeptides. It is therefore reasonable to believe that immature cell wall components are accumulated in YT1677 in the absence of a β-lactam challenge as this strain has an insertion in *mpl* leading to the constitutive expression of *ampC*. In *Acinetobacter baylyi*, inactivation of *mpl* or *ampD* results in hypersensitivity to β-lactam antibiotics such as CAZ and PIP (30), and it is well known that inactivation of *ampD* results in the overexpression of *ampC* in *P. aeruginosa* (31). These results imply that there are variations in the cell wall component recycling system or *ampC* regulation system in Gram-negative bacteria.

YT7988 had an insertion of Tn*Mod*-OGm in the *nuoN* gene encoding the NADH dehydrogenase I chain N, and this mutation caused overexpression of *ampC* through the function of *ampR* but not through *ampR* overexpression, resulting in a decreased susceptibility to the β-lactam antibiotics tested. This is the first report describing the involvement of a *nuo* gene in resistance to β-lactam antibiotics although the involvement of *nuoG* in resistance to aminoglycosides has been reported (32). As the *nuo* genes produce NADH dehydrogenase I, which is involved in the biogenesis of energy, a defect in these genes might affect the function of the efflux pumps, which require energy. However, the susceptibility of YT7988 to quinolone (CIP), a substrate of the efflux pumps, did not change, indicating that *nuoN* mutation did not affect the biogenesis of energy (Table 3). Experiments with Δ*ampR* mutants were carried out to examine the possibility that the mutations in YT1677 and YT7988 resulted in overexpression of *ampC* through the pathway independent of AmpR. The results indicated that overexpression of *ampC* in YT1677 and YT7988 was dependent on AmpR function as an *ampD* mutation, for example, resulted in *ampC* overexpression through AmpR function (9). It was clear that the defect in *mpl* resulted in the overexpression of *ampC* through AmpR function because *mpl* is involved in the recycling of cell wall components. However, it was unclear how the defect in *nuoN* led to the overexpression of *ampC* through AmpR function. It is unlikely that immature cell wall components are accumulated without a β-lactam challenge in YT7988 because the expression of *ampC* in YT7988 was about 2-fold greater than in PAO1 in the absence of a β-lactam challenge. Although *ampC* overexpression was observed in the absence of β-lactam antibiotic in YT1677 (4- to 12-fold), it was observed in YT7988 only when β-lactam antibiotic was added to the medium. It is therefore suggested that inactivation of *nuoN* did not result in the accumulation of immature cell wall components in the absence of β-lactam antibiotic, but it might enhance the sensitivity of AmpR to the immature cell wall components generated in the presence of β-lactam antibiotic or might somehow enhance the activity of AmpR. It is also likely that the state of the cell wall might change and that, as a result of the *nuoN* mutation, more immature cell wall components accumulated in YT7988 than in PAO1 when β-lactam antibiotic was added. Although YT7988 exhibited a higher PIP MIC than YT1677, *ampC* expression in YT7988 was much lower than that in YT1677 in the presence of FOX (Tables 3 and 4). In the induction experiment with PIP, however, *ampC* expression in YT7988 was slightly higher than that in YT1677, and the levels of the expression in YT1677, YT7988, and PAO1 were lower than those in the induction experiments with FOX (Table 4). These results suggested that the inducibility effects of the two antibiotics were different and that the physiological states that resulted from the mutations were different. These results suggested that *ampC* did not play a major role in PIP resistance in YT7988 and that other genes were responsible. It is well known, however, that no gene except *ampC* is expected to confer high-level resistance to PIP without the acquisition of an external resistance gene and that *ampR* is a positive regulator of *ampC*. In this study, the *ampR* deletion mutant of YT7988 became as sensitive as PAO1 (Table 3), and its *ampC* transcription level decreased to that of PAO1 (Table 4). These results excluded the hypothesis and indicated that *nuoN* mutation in YT7988 resulted in high-level resistance to PIP by overexpression of *ampC*, as mentioned above, although it remains unclear how the *nuoN* defect resulted in *ampC* overexpression.

In conclusion, it is clear that there is a factor acting at AmpR or upstream of the AmpR function and that overexpression of *ampC* induced by the mutation in *nuoN* suggests the presence of a new regulatory mechanism for *ampC* expression or the cell wall recycling system.

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