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ampG Gene of *Pseudomonas aeruginosa* and Its Role in β-Lactamase Expression▽

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In enterobacteria, the *ampG* gene encodes a transmembrane protein (permease) that transports 1,6-GlcNAc-anhydro-MurNAc and the 1,6-GlcNAc-anhydro-MurNAc peptide from the periplasm to the cytoplasm, which serve as signal molecules for the induction of *ampC* β-lactamase. The role of AmpG as a transporter is also essential for cell wall recycling. *Pseudomonas aeruginosa* carries two AmpG homologues, AmpG (PA4393) and AmpGh1 (PA4218), with 45 and 41% amino acid sequence identity, respectively, to *Escherichia coli* AmpG, while the two homologues share only 19% amino acid identity. In *P. aeruginosa* strains PAO1 and PAK, inactivation of *ampG* drastically repressed the intrinsic β-lactam resistance while *ampGh1* deletion had little effect on the resistance. Further, deletion of *ampG* in an *ampD*-null mutant abolished the high-level β-lactam resistance that is associated with the loss of AmpD activity. The cloned *ampG* gene is able to complement both the *P. aeruginosa* and the *E. coli ampG* mutants, while that of *ampGh1* failed to do so, suggesting that PA4393 encodes the only functional AmpG protein in *P. aeruginosa*. We also demonstrate that the function of AmpG in laboratory strains of *P. aeruginosa* can effectively be inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), causing an increased sensitivity to β-lactams among laboratory as well as clinical isolates of *P. aeruginosa*. Our results suggest that inhibition of the AmpG activity is a potential strategy for enhancing the efficacy of β-lactams against *P. aeruginosa*, which carries inducible chromosomal *ampC*, especially in AmpC-hyperproducing clinical isolates.

Pseudomonas aeruginosa is an opportunistic pathogen that causes nosocomial pneumonia, urinary tract infections, and secondary bacteremia associated with burn wounds (41, 50). *P. aeruginosa* also plays a primary role in the morbidity and mortality of patients with cystic fibrosis (CF) by chronically colonizing the lungs of these patients (40). Nearly 80% of patients with CF become infected with *P. aeruginosa* by early adulthood, and a majority of them succumb to an infection caused by this microorganism (8, 12, 22).

β-Lactam antibiotics, mainly broad-spectrum cephalosporins, are among the major antibiotics being used to treat pseudomonas infections. Prolonged use of antipseudomonal β-lactams can result in multiple-β-lactam-resistant *P. aeruginosa* mutants that show high levels of AmpC β-lactamase production leading to therapeutic failures (9, 20, 31, 43, 44, 45).

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Chromosomally located inducible *ampC* is present in most *Enterobacteria* (except in *Escherichia coli* and *Shigella*, where this gene is noninducible due to the lack of *ampR*) and *P. aeruginosa* (2, 30). The process of AmpC regulation is intimately linked to peptidoglycan recycling (35). In *Citrobacter freundii* and *Enterobacter cloacae*, a number of genes are involved in AmpC induction (25, 32): *ampG*, which encodes a transmembrane protein and functions as a specific permease for the transport of 1,6-GlcNAc-anhydro-MurNAc and the 1,6-GlcNAc-anhydro-MurNAc peptide, the signal molecules involved in *ampC* expression (4, 6, 7, 18, 31); *ampR*, which encodes a DNA-binding protein belonging to the LysR superfamily (13), with two regulatory states (in the absence of a β-lactam inducer, AmpR binds to the UDP-MurNAc pentapeptide to promote the formation of an AmpR-DNA complex that represses *ampC* transcription, and [ii] in the presence of a β-lactam antibiotic, peptidoglycan fragments accumulate in the cytoplasm [4, 39, 51] and the 1,6-anhydro-MurNAc tripeptide (or pentapeptide) competitively displaces the UDP-MurNAc pentapeptide and converts AmpR into an activator, triggering the *ampC* expression or production of the β-lactamase [17]); *ampD*, which encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase and specifically hydrolyzes the 1,6-anhydro-MurNAc peptide, thus acting as a repressor for *ampC* expression (15, 27); and *ampE*, which encodes a cyto-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
Escherichia coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* (*argF-lacZYA*)*U169* ϕ80d*lacZ*	42
S17-1	*hsdR pro recA*; contains *RP4-2-Tc*::*Mu* integrated into the chromosome	47
SNO301-1	*ampD1 ampG*	45
Pseudomonas aeruginosa		
PAO1	Reference strain; genome completely sequenced	48
PAO1ΔDDh2Dh3	PAO1 deleted of the three *ampD* homologues	21
PAK	Reference strain	David Bradley
PAO1ΔG	PAO1 deleted of *ampG* (PA4393)	This study
PAO1ΔGh1	PAO1 deleted of *ampGh1* (PA4218)	This study
PAO1ΔGGh1	PAO1 deleted of both *ampG* (PA4393) and *ampGh1* (PA4218)	This study
PAKΔG	PAK deleted of *ampG* (PA4393)	This study
PAKΔGh1	PAK deleted of *ampGh1* (PA4218)	This study
PAKΔGGh1	PAK deleted of both *ampG* (PA4393) and *ampGh1* (PA4218)	This study
PAO1ΔDDh2Dh3G	PAO1ΔDDh2Dh3 deleted of *ampG* (PA4393)	This study
PAO1ΔDDh2Dh3Gh1	PAO1ΔDDh2Dh3 deleted of *ampGh1* (PA4218)	This study
Plasmids		
pGEMT	PCR cloning vector	Promega
pEC1C	*ampC ampR* from *E. cloacae*; Cm^r	16
pGKS273-5	*ampG* region from *E. coli* JRG582; Km^r	28
pUCP24	pUC18-derived broad-host-range vector; Gm^r	52
pUCP26	pUC18-derived broad-host-range vector; Tc^r	52
pEX18Tc	Counter selectable plasmid carrying *sacB* marker; *oriT*; Tc^r	14
pEXAG	pEX18Tc carrying *ampG* deletion construct; Tc^r	This study
pEXAGh1	pEX18Tc carrying *ampGh1* deletion construct; Tc^r	This study
pZY0901	*ampG* gene of PAO1 cloned into pUCP24; Gm^r	This study
pZY0908	*ampGh1* gene of PAO1 cloned into pUCP24; Gm^r	This study

[a] Antibiotic resistance markers: Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Tc, tetracycline.

plasmic membrane protein, thus acting as a sensory transducer molecule required for *ampC* induction (16), though the exact role of AmpE is not fully understood.

A recent study demonstrated that *ampC* expression in *P. aeruginosa* is coordinately repressed by three AmpD homologues (21). The three AmpD homologues are responsible for a stepwise *ampC* upregulation, ultimately leading to constitutive hyperexpression of the chromosomal cephalosporinase and high-level β-lactam resistance (21). Among clinical isolates of *P. aeruginosa*, loss of *ampD* function often accounts for the β-lactam-resistant phenotype (21, 29, 46). More recently, β-lactam-resistant *P. aeruginosa* strains where the β-lactamase overproduction can be attributed to partial or full derepression of PBP4 and/or by sequential deletion of the *ampD* homologues have been isolated (33, 46). Blockage of NagZ, a glycoside hydrolase, represses both the intrinsic β-lactam resistance and the high-level antipseudomonal β-lactam resistance that is associated with the loss of AmpD activity (1).

It has been shown that the inactivation of *ampG* by mutation or deletion confers noninducible and low-level β-lactamase expression to the bacterial cell (23, 24, 26, 28). In this report, we demonstrate that *P. aeruginosa* carries only one functional AmpG protein, which is essential for the expression of AmpC. We further demonstrate that a proton motive force inhibitor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), can effectively inhibit AmpG function in *P. aeruginosa*, rendering sensitivity to β-lactam through suppression of *ampC* expression. These results indicated that inhibition of AmpG activity could be an effective strategy for enhancing the efficacy of β-lactam antibiotics against Gram-negative pathogens carrying inducible chromosomal *ampC* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The laboratory strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strains PAO1 and PAK were used as wild types.

Cloning of *ampG* homologues from *P. aeruginosa*. Using PAO1 genomic DNA as a template, the two *ampG* homologues, PA4393 (*ampG*) and PA4218 (*ampGh1*), were PCR amplified with the primers listed in Table 2. PCR products were digested with BamHI-HindIII or EcoRI-HindIII and ligated into the same sites on pUCP24, resulting in pZY0901 and pZY0908, respectively.

Deletion of the *ampG* homologues in *P. aeruginosa*. Knockout mutants for the *ampG* homologues were constructed in accordance with the procedure described previously (11). With the use of purified PAO1 genomic DNA as a template, upstream and downstream 1-kb fragments of the PA4393 and PA4218 were amplified by PCR with the primers listed in Table 2. Upstream fragments were digested with EcoRI-BamHI while downstream fragments were digested with BamHI-HindIII, and corresponding upstream and downstream fragments were ligated into the EcoRI-HindIII sites of pEX18Tc (14) through a three-way ligation, creating plasmids pEXAG and pEXAGh1, respectively. These two plasmids were introduced into *E. coli* strain S17-1 and conjugated into PAO1, PAK, or PAO1ΔDDh2Dh3 to generate single crosses. Double crosses were then selected on Luria agar containing 5% sucrose. The resulting deletions of the *ampG* homologues were confirmed by PCR. A double *ampG* homologue mutant was further constructed from the single deletion mutants by the same procedure.

Antibiotic susceptibility tests. Bacterial MICs were determined for each antibiotic by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (5). For broth microdilution, serial 2-fold dilutions of the β-lactam antibiotics in Mueller-Hinton broth (MHB) were delivered into a 96-well plate. Each inoculum contained about 10^4 cells in 100 μl

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]	PCR product length (bp)	Use
AG-FB	GGGATCCCCTGCACAACGACAGGGTGGACATACG	1,250	Cloning of PA4393 (*ampG*)
AG-RHB	GAAGCTTTCAAGATCTGTGCTGCTCGGCGTTCTGGTGTC		
AGh1-FR	GGAATTCGTCACCGGAGACCACCATGCTTGAG	1,882	Cloning of PA4218 (*ampGh1*)
AGh1-RH	GAAGCTTAGGTGGAACGGCCACGCTAGCAACA		
AG-FUR	GGAATTCGAACCAGTGTTCGTCGAAGAAGCGATC	988	PA4393 upstream fragment
AG-RUB	GGGATCCGCGCACTCTAACCGCTCTACTTCGCTG		
AG-FDB	GGGATCCGGCGAGAATGAAAAAGGCCGGCATTCG	1,012	PA4393 downstream fragment
AG-RDH	GAAGCTTCCAGGCCCGGAAACGTCGCCCCACGG		
AGh1-FUR	GGAATTCTGGCGCTGTTCGACCTGCATGGCCTG	1,023	PA4218 upstream fragment
AGh1-RUB	GGGATCCGCTCAAGCATGGTGGTCTCCGGTGAC		
AGh1-FDB	GGGATCCGGCGCTGCCGATAGGCGCGCAGCGCC	1,166	PA4218 downstream fragment
AGh1-RDH	GAAGCTTCGCGGCGAACTGCAGATGATCCTGCTC		

[a] Underlined are restriction endonuclease sites. Primer sequences are based on the published PAO1 genome sequence (46).

taken from starter cultures grown to an optical density at 600 nm of 0.5. The measurements of MICs in the presence of the CCCP were carried out by preparing 96-well plates containing serial dilutions of β-lactam antibiotics in 48.8 μl of MHB, followed by the addition of 1.2 μl of CCCP (10 mM in dimethyl sulfoxide [DMSO]) to give a final concentration 120 μM. These broths were then inoculated with 50 μl of the desired bacterial culture. The MIC was defined as the lowest concentration of antibiotic that prevented the bacterial growth after 16 to 20 h of incubation at 37°C. All MICs were determined in triplicate.

β-Lactamase activity assays. The assays were performed as described previously (49). *E. coli* and *P. aeruginosa* cells were induced for 1 h with 4 μg/ml cefoxitin and for 2 h with various concentrations of cefoxitin, respectively. Crude cell extracts were prepared by sonication, and β-lactamase activity was quantified in a UV spectrophotometric assay with 100 μM nitrocefin (Calbiochem, San Diego, CA) as a substrate (49). Specific activity of β-lactamase was expressed as nanomoles of nitrocefin hydrolyzed at 30°C per min per milligram of protein. The protein content of crude extracts was determined by using bicinchoninic acid (BCA) protein assay reagent (Pierce) with bovine serum albumin as a standard. All the induction experiments were performed in triplicate, and the results represent averages for the three experiments.

RESULTS

Role of *ampG* homologues in *ampC* expression and β-lactam resistance. A search of the *P. aeruginosa* genome database (http://www.pseudomonas.com) for the homologues of *ampG* sequences of *E. coli* revealed the presence of two *ampG* homologues, PA4393 (*ampG*) and PA4218 (*ampGh1*). These two gene products shared 45% and 41% amino acid sequence identity, respectively, to AmpG of *E. coli*, while the two AmpG homologues share only 19% amino acid identity. To demonstrate the roles of *ampG* homologues in β-lactam resistance, the two *ampG* homologues of *P. aeruginosa*, *ampG* and *ampGh1*, were deleted individually or together in strains PAO1 and PAK and a highly β-lactam-resistant triple *ampD*-null mutant, PAO1ΔDDh2Dh3 (21), creating PAO1ΔG, PAO1ΔGh1, PAO1ΔGGh1, PAKΔG, PAKΔGh1, PAKΔGGh1, PAO1ΔDDh2Dh3G, and PAO1ΔDDh2Dh3Gh1, respectively (Table 1). Deletions of *ampG* in the PAO1 (PAO1ΔG), PAK (PAKΔG), and PAO1ΔDDh2Dh3 (PAO1ΔDDh2Dh3G) strains led to significant decreases in MICs for ampicillin (8-, 16-, and 64-fold, respectively). The MICs of ceftazidime were not different for PAO1ΔG, PAO1ΔGh1, PAKΔG, and PAKΔGh1, compared to the levels for the respective parental strains, PAO1 and PAK. Similar results were observed in PAO1ΔGGh1 and PAKΔGGh1, double mutants of the two *ampG* homologues (Table 3). Notably, inactivation of *ampG* in strain PAO1ΔDDh2Dh3 led to a 32-fold decrease in ceftazidime MICs. However, deletions of *ampGh1* in PAO1ΔGh1, PAKΔGh1, and PAO1ΔDDh2Dh3Gh1 resulted in no change in their MICs for ampicillin and ceftazidime.

The MICs of cefotaxime for PAO1ΔG, PAO1ΔGh1, PAKΔG, and PAKΔGh1 were slightly reduced or did not change compared to those for the wild-type strains (PAO1 and PAK). Also, loss of *ampGh1* in PAO1ΔDDh2Dh3 resulted in almost no change of MIC for cefotaxime. However, it is noticeable that the MIC of cefotaxime for PAO1ΔDDh2Dh3 with the *ampG* mutation was 128-fold lower than that without the *ampG* mutation and remained below CLSI susceptibility breakpoints (Table 3).

Since AmpG activity is required to produce the signal molecules for AmpC induction, inactivation of *ampG* in *P. aeruginosa* should block β-lactam-mediated induction of *ampC* expression. Indeed, strains PAO1ΔG, PAKΔG, and PAO1ΔDDh2Dh3G all lost inducible expression of β-lactamase activity compared to the levels for their respective parental strains. In fact, at 50 μg/ml cefoxitin, these mutant strains underwent cell lysis, yet no β-lactamase activity could be detected in the supernatants or cell-associated fraction. Cefoxitin at lower nonlysing concentrations of 1 μg/ml, 10 μg/ml, and 25 μg/ml were further tested for *ampC* induction. In the wild-type strain PAK, *ampC* expression gradually enhanced with the increase of cefoxitin concentration (Table 4). However, cefoxitin-induced β-lactamase levels were not significantly different from the basal levels in all of *ampG*-defective mutants, indicating that β-lactamase is no longer inducible in these mutants.

As shown previously (21), PAO1ΔDDh2Dh3 exhibits high-basal-level and constitutive β-lactamase production (stably derepressed). Notably, inactivation of *ampG* in the PAO1ΔDDh2Dh3 background yielded a dramatic decrease in the basal *ampC* expression level, with 1,242-fold-lower β-lactamase activity (Table 3). The decreased *ampC* expression resulting from the loss of the *ampG* gene was clearly reflected by the reductions of the MICs for ampicillin, ceftazidime, and cefotaxime.

PA4393 but not PA4218 encodes a functional AmpG protein in *P. aeruginosa*. The ability of PA4393 to complement the *ampG* mutant in *P. aeruginosa* was further tested. A plasmid expressing PA4393 (pZY0901) was transformed in PAO1ΔG, PAKΔG, and PAO1ΔDDh2Dh3G. MIC determination and β-lactamase induction assays (Table 3) revealed that PA4393

TABLE 3. MICs and specific activities for AmpC in the indicated strains

Strain	MIC (μg/ml)			β-Lactamase activity[a]	
	Ampicillin	Cefotaxime	Ceftazidime	Noninduced	Induced[b]
PAO1	256	16	1	4 ± 0.5	879 ± 54
PAK	512	8	1	6 ± 0.3	1,219 ± 65
PAO1ΔDDh1Dh2	1,024	512	32	3,380 ± 462	3,438 ± 367
PAO1ΔG	32	8	1	3 ± 0.3	0
PAO1ΔGh1	256	16	1	–	–
PAO1ΔGGh1	32	8	1	–	–
PAO1ΔG/pUCP24	32	8	1	–	–
PAO1ΔG/pZY0901	1,024	256	16	52 ± 4	1,303 ± 87
PAO1ΔG/pZY0908	32	8	1	–	–
PAKΔG	32	8	1	2 ± 0.2	0
PAKΔGh1	512	8	1	–	–
PAKΔGGh1	32	8	1	–	–
PAKΔG/pUCP24	32	4	1	–	–
PAKΔG/pZY0901	512	512	32	1,095 ± 65	2,573 ± 239
PAO1ΔDDh2Dh3G	16	4	1	3 ± 0.1	0
PAO1ΔDDh2Dh3Gh1	1,024	256	32	–	–
PAO1ΔDDh2Dh3G/pUCP24	16	4	1	–	–
PAO1ΔDDh2Dh3G/pZY0901	1,024	512	32	6,656 ± 326	8,130 ± 779
SNO301-1/pEC1C/pUCP26	16	<2	–	43 ± 2	38 ± 2
SNO301-1/pEC1C/pGKS273-5	1,024	64	–	1530 ± 80	3600 ± 90
SNO301-1/pEC1C/pZY0901	1,024	32	–	161 ± 8	3250 ± 110

[a] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. –, not tested.
[b] Induction was carried out with 50 μg of cefoxitin per ml for 2 h.

from PAO1 complemented the *ampG* mutant, resulting in high levels of ampicillin resistance as well as parental levels of β-lactamase activities. Also, the MICs of cefotaxime and ceftazidime for strains PAO1/pZY0901 and PAK/pZY0901 increased significantly compared to those for PAO1 and PAK, likely due to a high-copy-number *ampG* gene carried on pZY0901.

The mutation and complementation analysis described above clearly demonstrates that PA4393 carries a functional *ampG* gene. However, the fact that *ampGh1* deletion had no effect on the MICs suggested that this gene either is not expressed or does not code for a functional AmpG protein. To determine the ability of *ampGh1* to complement the *ampG* mutation, an *ampGh1* expression plasmid, pZY0908, was transformed into PAO1ΔG. The *ampGh1* gene in this plasmid is preceded by a *lac* promoter from the vector. As shown in Table 3, the MICs of ampicillin, cefotaxime, and ceftazidime for mutant cells containing plasmid pZY0908 were not different from those for cells carrying either the control plasmid pUCP24 or no plasmid, indicating that PA4218 (*ampGh1*) does not code for a functional AmpG protein.

PA4393 is able to complement an *E. coli ampG* mutant. The *P. aeruginosa ampG* gene was further tested for its ability to complement *E. coli ampG* mutant strain SNO301-1. Since *E. coli* strains do not contain *ampR*, which is necessary for β-lactamase expression (36), the *E. coli* strain SNO301-1 was first transformed with plasmid pEC1C carrying the *ampC* and *ampR* genes of *E. cloacae* (34). As a positive control, plasmid pGKS273-5 carrying the *ampG* gene of *E. coli* behind a *tac* promoter was transformed into SNO301-1/pEC1C. As the data in Table 3 show, the control strain of SNO301-1 containing both pEC1C and pUCP26 exhibited low levels of resistance to ampicillin and cefotaxime and a low basal level of β-lactamase activity which is noninducible. Introduction of the *ampG* genes of *E. coli* and *P. aeruginosa*, as expressed from plasmids pGKS273-5 and pZY0901, respectively, resulted in high levels of β-lactam resistance and hyperinducible β-lactamase expression (Table 3). However, the basal level β-lactamase activity was 9-fold higher in SNO301-1/pEC1C/pGK273-5 than in SNO301-1/pEC1C/pZY0901 (Table 3) while induced β-lactamase activities reached similar levels. The differences in the

TABLE 4. Specific activities of AmpC in *P. aeruginosa* strains with indicated concentrations of inducer

Strain	β-Lactamase activity[a]			
	No inducer	Inducer (1 μg/ml)	Inducer (10 μg/ml)	Inducer (25 μg/ml)
PAO1	4 ± 0.5	36 ± 10	165 ± 29	372 ± 66
PAK	6 ± 0.3	152 ± 12	747 ± 65	1,068 ± 83
PAO1ΔDDh1Dh2	3,380 ± 462	2,981 ± 382	3,007 ± 415	3,022 ± 394
PAO1ΔG	3 ± 0.3	3 ± 1.1	10 ± 1.3	0
PAKΔG	2 ± 0.2	2 ± 0.3	5 ± 0.8	0
PAO1ΔDDh1Dh2G	3 ± 0.1	4 ± 1	8 ± 0.6	0

[a] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. The inducer used was cefoxitin.

TABLE 5. Susceptibilities of *P. aeruginosa* strains and their mutants to ampicillin and cefotaxime in the presence or absence of CCCP[a]

Strain	MIC (μg/ml)			
	Ampicillin		Cefotaxime	
	−I	+I	−I	+I
PAO1	256	128	16	8
PAK	512	256	8	8
PAO1ΔDDh2Dh3	1,024	64	512	64
PAO1ΔG	32	32	8	4
PAO1ΔGh1	256	256	16	8
PAO1ΔGGh1	32	32	8	4
PAO1ΔG/pUCP24	32	32	8	4
PAO1ΔG/pZY0901	1,024	512	256	128
PAKΔG	32	32	8	4
PAKΔGh1	512	256	8	8
PAKΔGGh1	32	32	8	4
PAKΔG/pUCP24	32	32	4	4
PAKΔG/pZY0901	512	32	512	256
PAO1ΔDDh2Dh3G	16	16	4	4
PAO1ΔDDh2Dh3Gh1	1,024	64	256	64
PAO1ΔDDh2Dh3G/pUCP24	16	16	4	4
PAO1ΔDDh2Dh3G/pZY0901	1,024	32	512	64

[a] The bacterial strains were treated with (+I) or without (−I) 120 μM CCCP.

basal-level β-lactamase activity might be due to the poor recognition of the *P. aeruginosa ampG* promoter in *E. coli*.

Inhibition of AmpG by CCCP. Given our findings that the genetic inactivation of *ampG* reduces β-lactam resistance in PAO1, PAK, and PAO1ΔDDh2Dh3, we speculated whether inhibition of AmpG could suppress bacterial resistance to β-lactams in *P. aeruginosa*. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) has previously been shown to prevent the uptake of 1,6-GlcNAc-anhydro-MurNAc and 1,6-GlcNAc-anhydro-MurNAc peptides by AmpG in *E. coli* (4), but its effect on antibiotic resistance has never been investigated. CCCP itself exhibited an antimicrobial property on *P. aeruginosa*, with MICs of 250 μM for PAO1 and 400 μM for PAK (data not shown). We tested the efficacy of ampicillin with the combined use of CCCP at concentrations lower than its MIC. The presence of 120 μM CCCP enhanced the efficacy of ampicillin against PAO1, PAO1ΔGh1, PAK, PAKΔGh1, PAO1ΔDDh2Dh3, and PAO1ΔDDh2Dh3Gh1 2- to 8-fold (data not shown). Noticeably, a combination of 120 μM CCCP with cefotaxime resulted in an 8-fold reduction in the MIC for PAO1ΔDDh2Dh3 and a 4-fold reduction in the MIC for PAO1ΔDDh1Dh2Gh1 (Table 5). The MICs of ampicillin and cefotaxime for the PAO1ΔG, PAKΔG, and PAO1ΔDDh2Dh3G strains were not significantly different in the presence and absence of CCCP, which might be due to low MICs for these strains. The *ampG* mutants transcomplemented with pZY0901 produced sensitivity profiles similar to those for parental strains (Table 5). Thus, these results suggest that CCCP can block AmpG activity to the point that AmpC production is suppressed, resulting in increased antimicrobial activity.

To further illustrate whether CCCP can reduce AmpC expression by blocking AmpG activity, we also tested β-lactamase activities in the presence of 120 μM CCCP in PAO1ΔDDh1Dh2, PAO1ΔDDh1Dh2G, and PAO1ΔDDh1Dh2G/pZY0901. AmpC production in strains PAO1ΔDDh1Dh2 and PAO1ΔDDh1Dh2G/pZY0901 decreased 3-fold in the presence of CCCP (Table 6), indicating an inhibitory role for CCCP in the AmpG function of *P. aeruginosa*.

TABLE 6. Specific activities of AmpC in *P. aeruginosa* in the presence or absence of CCCP[a]

Strains	β-Lactamase activity[b]	
	−I	+I
PAO1ΔDDh2Dh3	3,380 ± 462	1,222 ± 145
PAO1ΔDDh2Dh3G	3 ± 0.3	3 ± 0.7
PAO1ΔDDh2Dh3G/pZY0901	6,656 ± 326	2,797 ± 442

[a] The bacterial strains were cultured with (+I) or without (−I) 120 μM CCCP.
[b] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

CCCP confers increased sensitivity to ampicillin in clinical isolates of *P. aeruginosa*. As a result of chronic colonization, *P. aeruginosa* isolates from CF patients often accumulate multiple mutations, causing various phenotypic changes, including mucoid colony morphology (10) and multidrug resistance (37). Eighteen randomly chosen CF isolates of *P. aeruginosa* were tested for their susceptibility to ampicillin in the presence or absence of 120 μM CCCP. In the absence of CCCP, 2 isolates were susceptible to ampicillin (MIC < 64), 11 were moderately resistant (MIC between 256 to 512), and 5 were highly resistant to ampicillin (MIC > 1,024) (Table 7). Interestingly, 10 out of the 18 isolates, representing over 55% of the randomly chosen isolates, displayed CCCP-dependent susceptibility to ampicillin, with decreases MICs ranging from 2-fold to over 128-fold (Table 7).

To test if the CCCP-mediated increase of sensitivity to ampicillin was due to the inhibitory effect of CCCP on efflux pumps, we tested the MICs of chloramphenicol and tetracycline. In the presence of CCCP, 4 isolates showed 4-fold decreases in chloramphenicol MICs (Table 7). Only 2 out of the 4 isolates produced similar effects on susceptibility to ampicil-

TABLE 7. Effect of CCCP on the susceptibility of CF isolates of *P. aeruginosa* to ampicillin, chloramphenicol, and tetracycline[a]

Strain	MIC (μg/ml)					
	Ampicillin		Chloramphenicol		Tetracycline	
	−I	+I	−I	+I	−I	+I
1	512	512	256	64	8	8
2	256	256	256	256	8	8
3	64	64	256	64	8	8
4	512	512	32	32	8	8
5	512	512	32	32	8	8
6	>1,024	>1,024	8	8	8	8
7	256	8	8	8	8	8
8	256	8	32	32	16	8
9	512	8	16	16	8	8
10	>1,024	8	32	8	8	8
11	512	8	32	32	8	8
12	>1,024	>1,024	8	8	8	8
13	>1,024	8	32	8	8	8
14	512	8	16	8	8	8
15	>1,024	8	8	8	8	8
16	16	8	8	8	8	8
17	512	8	8	8	8	8
18	256	256	32	32	16	8

[a] The bacterial strains were treated with (+I) or without (−I) 120 μM CCCP.

lin. However, the MICs of tetracycline for all of the strains were not significantly different in the presence and absence of CCCP (Table 7). These results further affirm that CCCP-mediated increase of sensitivity to ampicillin is through the inhibitory effect of CCCP on AmpG and not the efflux pumps.

DISCUSSION

In Gram-negative bacteria carrying inducible *ampC* genes on their chromosomes, the induction mechanism is directly linked to peptidoglycan recycling (35). *ampG* encodes a transmembrane protein that functions as a permease for 1,6-GlcNAc-anhydro-MurNAc peptides (7, 23). AmpG activity is required for the peptidoglycan monomers to enter the cytoplasm and be recycled and ultimately reincorporated into the peptidoglycan. Given that AmpG is responsible for the transport of the AmpC-inducing signal molecule (23, 28), blockage of AmpG activity may provide a novel strategy for enhancing the efficacy of β-lactams against bacteria carrying inducible *ampC*. AmpG inhibition would result in the suppression of both intrinsic *ampC* expression and the *ampC* hyperexpression caused by *ampD* mutations.

AmpG was originally identified as being required for induction of AmpC (β-lactamase) in *E. cloacae* (23). Recently, the topology of AmpG was investigated (3). Ten membrane-spanning segments were identified, and four other hydrophobic segments remained in the cytoplasm: two of these were too short to span the membrane, and the other two contained a mid-segment proline (3). Our experimental results indicated that PA4393 is the only functional *ampG* gene in the *P. aeruginosa* genome and that the gene product of PA4218 does not have AmpG function. Ablation of AmpG in *P. aeruginosa* either via genetic deletion or by the use of a proton motive force inhibitor (CCCP) significantly reduced resistance to β-lactams. The susceptibilities of PAO1ΔG and PAKΔG to ampicillin increased 8- to 16-fold. This is consistent with the observation of reduced AmpC production. The most profound effect on β-lactam resistance was observed when *ampG* was inactivated in PAO1ΔDDh2Dh3, a triple *ampD*-null mutant previously shown to exhibit the complete derepression of *ampC* and high-level resistance to antipseudomonal β-lactams (21). As shown previously (21), PAO1ΔDDh2Dh3 displayed high-level resistance to all antipseudomonal β-lactams tested except imipenem (a carbapenem resistant to hydrolysis by AmpC) compared to the level of resistance displayed by PAO1. This observation highlighted the requirement of AmpG activity for induction of AmpC expression in *P. aeruginosa* and indicated that the loss of AmpG activity can effectively reverse the significantly high-level-antipseudomonal-β-lactam-resistance phenotype of a mutant that is completely deficient in AmpD activity.

It is known that CCCP is an inhibitor of proton motive force and the resistance-nodulation-division efflux pump (38). A previous study also demonstrated that CCCP is an AmpG permease-specific inhibitor, affecting cell wall recycling (4), but its antimicrobial function has never been explored. The use of CCCP in combination with ampicillin and cefotaxime attenuated the resistance to these antibiotics close to the level of resistance by the *ampG* mutants (PAO1ΔG, PAKΔG, and PAO1ΔDDh2Dh3ΔG) (Table 5). Such a profound effect on PAO1ΔDDh2Dh3ΔG provides good support for targeting of AmpG with inhibitors. Although CCCP was found to increase the significant efficacy of ampicillin against PAO1ΔDDh2Dh3, it did not enhance the efficacy of ampicillin against PAO1 and PAK to the same extent. Comparison of these results to those achieved by genetic inactivation of *ampG* in these strains (Table 3) suggests that CCCP may not be able to completely inhibit endogenous AmpG, and thus, partial inhibition of AmpG cannot sufficiently prevent the transport of 1,6-GlcNAc-anhMurNAc and 1,6-GlcNAc-anhMurNAc peptides into the cytosol and the low basal level of AmpC. However, CCCP also has no obvious effect on cefotaxime when used against PAKΔG/pZY0901 and PAO1ΔG/pZY0901. It is possible that a weak *ampC*-inducing activity of the cefotaxime led to unremarkable CCCP inhibition (53).

To elucidate the role of the *P. aeruginosa ampG* product in regulation of AmpC expression, complementation studies of *P. aeruginosa ampG* mutants and the *E. coli ampG* mutant were performed with a cloned *P. aeruginosa ampG* gene. In these mutants, AmpC is constitutively produced at a low basal level and is noninducible. When *P. aeruginosa ampG* was expressed in PAO1 and PAK, the resulting strains produced moderate levels of the β-lactamase in the absence of an inducer and overproduced it in the presence of an inducer (Table 4). The expression of *P. aeruginosa ampG* also made these mutants highly resistant to ampicillin (Table 3). When *P. aeruginosa ampG* was expressed in *E. coli* SNO301-1/pEC1C, the *ampC* gene moderately produced β-lactamase in the absence of an inducer and overproduced it in the presence of the inducer (Table 3). Expression of *P. aeruginosa ampG* also made the *E. coli ampG* mutant highly resistant to β-lactam antibiotics (Table 3). These results indicated that the cloned *P. aeruginosa ampG* gene expresses a functional AmpG protein. These findings also suggest that *P. aeruginosa* AmpG functions as a permease and transports the 1,6-GlcNAc-anhMurNAc peptides, the signal molecule for induction of AmpC expression, from the periplasm to the cytoplasm (15, 18, 19, 23). The basal level of β-lactamase expression was 9-fold higher in cells expressing *E. coli ampG* than in cells expressing *P. aeruginosa ampG*. This difference is likely due to the weak *ampG* promoter of *P. aeruginosa* in *E. coli*, compared to the *ampG* gene that is under a strong *tac* promoter in plasmid pGKS273-5 (45).

An unexplained observation is that the basal level of β-lactamase activity in PAKΔG expressing *ampG* (PAKΔG/pZY0901) was 180-fold higher than that in PAO1 (PAO1ΔG/pZY0901) (Table 3), as if PAKΔG had an additional *ampD* defect. Although this possibility is not ruled out completely, it is unlikely, due to the following reasons: first, the PAKΔG mutant was independently generated twice, and tests of three mutants from each resulted in identical mutant phenotypes; second, the *ampG* and *ampD* gene loci are not physically linked, so an *ampD* mutation is unlikely to be introduced while the *ampG* mutant is generated; third, throughout the selection process, the bacterial cells were not subjected to selection by β-lactams (selected by tetracycline), so there was no selection pressure for *ampD* mutation.

Overall, AmpG is a valid target for the antimicrobial approach for a number of reasons. First, AmpG is essential for *ampC* (β-lactamase) expression and thus β-lactam resistance. Second, AmpG is a transmembrane protein and thus easier to

target the periplasmic portion, eliminating the need for a drug to penetrate inside the bacterial cytosol. Third, as part of the cell wall-recycling complex, AmpG is highly conserved among bacteria. Fourth, the effectiveness of AmpG inhibition as an antipseudomonal approach was further demonstrated by the CCCP-mediated increases in β-lactam efficacy obtained with laboratory as well as CF isolate strains of *P. aeruginosa* (Table 7). The mechanism of drug resistance in those CF isolates is not known at the present time, but a large proportion of these isolates (>55%) were responsive to CCCP-mediated "sensitization" to β-lactam, while having no effect on the MICs of chloramphenicol and tetracycline, demonstrating an efflux pump-independent mechanism. It is thus worthwhile to pursue better chemicals that show higher levels of specificity as well as inhibitory activity against AmpG while showing no cytotoxicity to humans.

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