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AmpG Inactivation Restores Susceptibility of Pan-β-Lactam-Resistant *Pseudomonas aeruginosa* Clinical Strains▽

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Constitutive AmpC hyperproduction is the most frequent mechanism of resistance to the weak AmpC inducers antipseudomonal penicillins and cephalosporins. Previously, we demonstrated that inhibition of the β-*N*-acetylglucosaminidase NagZ prevents and reverts this mechanism of resistance, which is caused by *ampD* and/or *dacB* (PBP4) mutations in *Pseudomonas aeruginosa*. In this work, we compared NagZ with a second candidate target, the AmpG permease for GlcNAc-1,6-anhydromuropeptides, for their ability to block AmpC expression pathways. Inactivation of *nagZ* or *ampG* fully restored the susceptibility and basal *ampC* expression of *ampD* or *dacB* laboratory mutants and impaired the emergence of one-step ceftazidime-resistant mutants in population analysis experiments. Nevertheless, only *ampG* inactivation fully blocked *ampC* induction, reducing the MICs of the potent AmpC inducer imipenem from 2 to 0.38 μg/ml. Moreover, through population analysis and characterization of laboratory mutants, we showed that *ampG* inactivation minimized the impact on resistance of the carbapenem porin OprD, reducing the MIC of imipenem for a PAO1 OprD mutant from >32 to 0.5 μg/ml. AmpG and NagZ targets were additionally evaluated in three clinical isolates that are pan-β-lactam resistant due to AmpC hyperproduction, OprD inactivation, and overexpression of several efflux pumps. A marked increase in susceptibility to ceftazidime and piperacillin-tazobactam was observed in both cases, while only *ampG* inactivation fully restored wild-type imipenem susceptibility. Susceptibility to meropenem, cefepime, and aztreonam was also enhanced, although to a lower extent due to the high impact of efflux pumps on the activity of these antibiotics. Thus, our results suggest that development of small-molecule inhibitors of AmpG could provide an excellent strategy to overcome the relevant mechanisms of resistance (OprD inactivation plus AmpC induction) to imipenem, the only currently available β-lactam not significantly affected by *P. aeruginosa* major efflux pumps.

The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains severely compromises the selection of appropriate treatments and is therefore associated with significant morbidity and mortality (21, 34). Indeed, one of the most striking features of *P. aeruginosa* is its extraordinary capacity for developing resistance to almost any available antibiotic by the selection of mutations in chromosomal genes (24, 28). Among the mutation-mediated β-lactam resistance mechanisms, particularly noteworthy are those leading to the constitutive overexpression of the inducible chromosomal cephalosporinase AmpC, which confers resistance to penicillins, cephalosporins, and monobactams (7, 14). Additionally, mutations that lead to the repression or inactivation of the porin OprD, acting synergistically with inducible or constitutively overexpressed AmpC, confer resistance to carbapenems (8, 26, 37).

AmpC is a chromosomally encoded group I, class C cephalosporinase produced by *P. aeruginosa*, as well as many other nonfermenting Gram-negative bacilli and most *Enterobacteriaceae* (3). Although AmpC is produced at very low basal levels in wild-type strains, its expression is inducible in the presence of certain β-lactams (β-lactamase inducers), such as cefoxitin and imipenem (27). In fact, the activity of the antipseudomonal penicillins (such as ticarcillin and piperacillin), cephalosporins (such as ceftazidime and cefepime), and monobactams (such as aztreonam) relies on the fact that they are very weak AmpC inducers, since they too are hydrolytically inactivated by this enzyme (27). For this reason, during treatment with these weak inducers, mutants showing constitutive high-level AmpC production (AmpC derepressed mutants) are frequently selected, leading to the failure of antimicrobial therapy (7, 13, 14, 24, 25).

There are several genes involved in the regulation of *ampC* expression, a process that was first investigated in the *Enterobacteriaceae* and found to be intimately linked to peptidoglycan recycling (33, 36). *ampG* encodes an inner membrane permease for GlcNAc-1,6-anhydromuropeptides, which are peptidoglycan catabolites that, upon entry into the cytosol, are processed by the β-*N*-acetylglucosaminidase, known as NagZ, to generate 1,6-anhydromuropeptides (4, 18, 40). The 1,6-anhydromuropeptide products of NagZ are thought to induce AmpC production by interacting with the LysR-type transcriptional regulator AmpR (2, 6, 11, 12, 22). During regular bacterial growth, 1,6-anhydromuropeptides are processed by the *N*-acetyl-anhydromuramyl-L-alanine amidase AmpD, avoiding *ampC* induction (10, 20, 36). On the other hand, during growth in the presence of strong β-lactamase inducers, large amounts of muropeptides are generated and accumulate in the cytoplasm,

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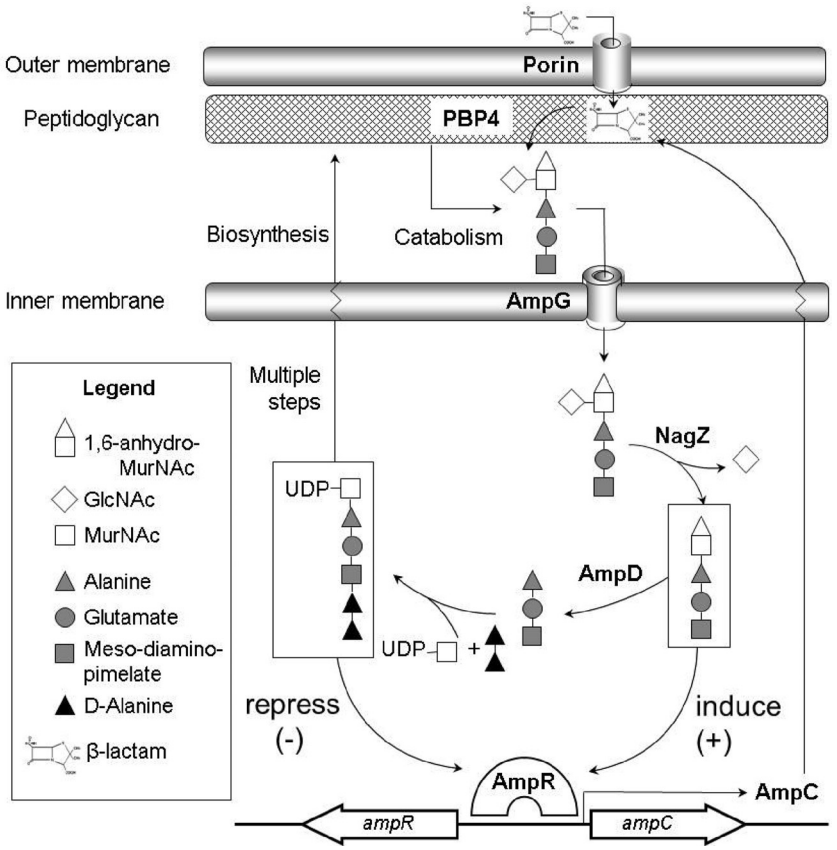

FIG. 1. Schematic representation of the interplay between peptidoglycan recycling, *ampC* regulation, and β-lactam resistance.

which leads to the AmpR-mediated induction of *ampC* expression (6, 11, 12, 22). It is also well-known that the mutational inactivation of AmpD leads to the accumulation of 1,6-anhydromuropeptides and high-level *ampC* expression, even in the absence of β-lactamase inducers, producing the classical constitutively derepressed phenotype of AmpC production (23).

Pseudomonas aeruginosa AmpG (17, 43), AmpR (16), NagZ (1), and AmpD (19) homologues have been identified. Further studies showed that *P. aeruginosa* has 3 *ampD* genes (*ampD*, *ampDh2*, and *ampDh3*) and that their sequential inactivation leads to a stepwise upregulation of *ampC* expression, reaching full derepression with very high level basal *ampC* expression in the triple mutant (15). Recent work showed, however, that one-step high-level resistance in *P. aeruginosa* frequently results, in clinical strains, from the inactivation of *dacB*, encoding the nonessential penicillin-binding protein 4 (PBP4) (31). The inactivation of PBP4 was shown to give rise to a complex β-lactam resistance response, triggering overproduction of the chromosomal β-lactamase AmpC and the specific activation of the CreBC (BlrAB) two-component regulator (31). A schematic representation of the interplay between peptidoglycan recycling, *ampC* regulation, and β-lactam resistance is shown in Fig. 1.

Development of strategies for combating these resistance mechanisms is crucial for preserving the activity of needed β-lactam antibiotics (29). Given that NagZ removes GlcNAc to produce the 1,6-anhydromuropeptides (4, 40), inhibitors of this enzyme have been shown to mitigate AmpC-driven resistance (39). In previous studies, we have demonstrated that inactivation or direct inhibition of NagZ in *P. aeruginosa* prevents and reverts resistance to the weak AmpC inducers antipseudomonal penicillins and cephalosporins driven by constitutive overexpression of AmpC caused by either AmpD or PBP4 mutations (1, 42). NagZ inactivation also attenuated the high-level resistance of the AmpD-PBP4 double mutant, although wild-type susceptibility was not fully restored (42). Additionally, NagZ inactivation did not block *ampC* inducibility in the presence of the strong inducer cefoxitin (42). The molecular basis for this incomplete inhibition of the AmpC regulatory pathway remains unclear, although the possibility that it could arise from the interaction of the accumulated NagZ substrates (GlcNAc-1,6-anhydromuropeptides) with AmpR cannot be ruled out. In any event, we speculate that blocking peptidoglycan recycling earlier in the pathway could be more efficient. Thus, in this work we comparatively evaluated NagZ with a second candidate target, AmpG, in a collection of *P. aeruginosa* laboratory mutants and well-characterized pan-β-lactam-resistant clinical strains.

MATERIALS AND METHODS

Strains, plasmids, and susceptibility testing. The bacterial strains and plasmids used or constructed in this study are listed in Table 1. MICs of ceftazidime, cefepime, aztreonam, piperacillin-tazobactam, imipenem, meropenem, and ciprofloxacin were determined using Etest strips (AB Biodisk, Solna, Sweden) on Mueller-Hinton (MH) agar, according to the manufacturer's recommendations.

TABLE 1. Strains and plasmids used or constructed

Strain or plasmid	Genotype/relevant characteristics[a]	Reference or source
P. aeruginosa		
PAO1	Reference strain completely sequenced	Laboratory collection
PAΔD	PAO1 Δ*ampD*::*lox*	15
PAΔdB	PAO1 Δ*dacB*::*lox*	31
PAdacB	1A1 spontaneous *dacB* mutant (W273X) of PAO1	31
PAdacBΔD	PadacB Δ*ampD*::*lox*	31
PAΔR	PAO1 Δ*ampR*::*lox*	31
PAΔC	PAO1 Δ*ampC*::*lox*	30
PAΔnZ	PAO1 Δ*nagZ*::*lox*	42
PAΔDnZ	PAO1 Δ*ampD*::*lox* Δ*nagZ*::*lox*	42
PAΔdBnZ	PAO1 Δ*dacB*::*lox* Δ*nagZ*::*lox*	42
PAdacBΔDnZ	PadacB Δ*ampD*::*lox* Δ*nagZ*::*lox*	42
PAΔG	PAO1 Δ*ampG*::*lox*	This work
PAΔDG	PAO1 Δ*ampD*::*lox* Δ*ampG*::*lox*	This work
PAΔdBG	PAO1 Δ*dacB*::*lox* Δ*ampG*::*lox*	This work
PAdacBΔDG	PadacB Δ*ampD*::*lox* Δ*ampG*::*lox*	This work
PAOD1	Spontaneous *oprD* null mutant (W65X) of PAO1	32
PAOD1ΔnZ	PAOD1 Δ*nagZ*::Gm *lox*	This work
PAOD1ΔG	PAOD1 Δ*ampG*::Gm *lox*	This work
JSG2A1	Pan-β-lactam resistant *P. aeruginosa* clinical strain	14
JSG2A1ΔnZ	JSG2A1 Δ*nagZ*::Gm *lox*	This work
JSG2A1ΔG	JSG2A1 Δ*ampG*::Gm *lox*	This work
MQB1C5	Pan-β-lactam-resistant *P. aeruginosa* clinical strain	14
MQB1C5ΔnZ	MQB1C5 Δ*nagZ*::Gm *lox*	This work
MQB1C5ΔG	MQB1C5 Δ*ampG*::Gm *lox*	This work
OFC2I4	Pan-β-lactam-resistant *P. aeruginosa* clinical strain	14
OFC2I4ΔnZ	OFC2I4 Δ*nagZ*::Gm *lox*	This work
OFC2I4ΔG	OFC2I4 Δ*ampG*::Gm *lox*	This work
E. coli		
XL1-Blue	F′::Tn*10 proA*$^+$ *proB*$^+$ *lacI*^{q} Δ(*lacZ*)M15/*recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* (r_k^- m_k^-) *mcrB1*	Laboratory collection
S17.1	*recA pro* (RP4-2Tet::Mu Kan::Tn*7*)	Laboratory collection
Plasmids		
pUCP26	Tc^r, pUC-18-based *Escherichia-Pseudomonas* shuttle vector	41
pUCP26ampG	pUC26 containing PAO1 *ampG* gene	This work
pEX100Tlink	Ap^r *sacB*, pUC19-based gene replacement vector with an MCS	38
pEX18Tc	Tc^r *sacB*, pUC18-based gene replacement vector with an MCS	9
pUCGmlox	Ap^r Gm^r, pUC18-based vector containing the *lox*-flanked *aacC1* gene	38
pCM157	Tc^r, *cre* expression vector	38
pEXnZ	pEX100Tlink containing 5′and 3′ flanking sequence of *nagZ*	42
pEXnZGm	pEX100Tlink containing 5′and 3′ flanking sequence of *nagZ*::Gm *lox*	42
pEXTcΔampG::Gm	pEX18Tc containing 5′and 3′ flanking sequence of *ampG*::Gm *lox*	This work

[a] MCS, multiple cloning site; Ap^r, ampicillin resistant; Gm^r, gentamicin resistant; Tc^r, tetracycline resistant.

Additionally, MICs of cefoxitin were determined by microdilution in 100 μl of cation-adjusted MH broth following CLSI guidelines (5). The phenotypic determination of AmpC inducibility was performed by assessing MH agar plates for the presence of antagonism between imipenem and ceftazidime disks (separated 5 to 30 mm) as previously described (15).

Construction of *nagZ*- and *ampG*-knockout mutants. The *nagZ*- and *ampG*-knockout mutants generated in this study from several PAO1 derivative mutants and pan-β-lactam-resistant clinical strains are shown in Table 1. Knockout mutants were constructed following well-established procedures (15, 31) based on the *cre-lox* system for gene deletion and antibiotic resistance marker recycling in *P. aeruginosa* (38). The previously constructed plasmid pEXnZGm (Δ*nagZ*::Gm) (42) was used as the donor for the generation of *nagZ*-knockout mutants. For the construction of *ampG*-knockout mutants, upstream and downstream PCR products (Table 2) of *ampG* (using PAO1 DNA as the template) were digested with

TABLE 2. Primers used for cloning and construction of *ampG* knockout mutants

Primer	Sequence (5′–3′)[a]	PCR product size (bp)	Use
AGF1-ERI	GATATAGAATTCCGGTCGCGGCGGCACCATCTG	823	*ampG* inactivation
AGR1-XbI	TATATCTCTAGAGCTGGCGGGAGACTTGTAGGC		
AGF2-XbI	GATATATCTAGATACGTCACCGCGGTGATGGGC	621	
AGR2-HD3	TATATCAAGCTTGTGCTGATCCTGCTGTTCCGC		
AGF-ERI	GATATAGAATTCAAGAAGGAGATATACATATGACTCAGCAATCCTGG	1,785	*ampG* cloning
AGR-HD3	TATATCAAGCTTTCAGTGCTGCTCGGCGTTCTGGTGTCCC		

[a] Restriction sites for endonucleases are underlined.

either EcoRI or HindIII and XbaI and cloned by a three-way ligation into pEX18Tc (9). The resulting plasmid (pEXTcΔampG) was transformed into *Escherichia coli* NM522, and transformants were selected on LB agar plates supplemented with 5 μg/ml tetracycline. The *lox*-flanked gentamicin resistance cassette (*aac1*) obtained by XbaI restriction of plasmid pUCGmlox was cloned into the single site for this enzyme formed by the ligation of the two flanking fragments. The resulting plasmid (pEXTcΔampG::Gm) was again transformed into *E. coli* NM522, and transformants were selected on LB agar plates supplemented with 5 μg/ml tetracycline and 5 μg/ml gentamicin. The plasmid was then transformed into the *E. coli* S17.1 helper strain. The different *nagZ*- or *ampG*-knockout mutants were then generated by conjugational transfer of pEXnZGm or pEXTcΔampG::Gm from *E. coli* S17.1 to the corresponding *P. aeruginosa* strains, followed by selection of double recombinants on LB agar containing 10% sucrose and 30 μg/ml gentamicin. Double recombinants were checked first by screening for carbenicillin (for pEXnZGm) or tetracycline (for pEXTcΔampG::Gm) susceptibility and then by PCR amplification and sequencing. For the recycling of the gentamicin resistance cassettes, plasmid pCM157 was electroporated into the different mutants. Transformants were selected on LB agar plates supplemented with 250 μg/ml tetracycline. One transformant for each mutant was grown overnight in LB broth with 250 μg/ml tetracycline in order to allow the expression of the *cre* recombinase. Plasmid pCM157 was then cured from the strains by successive passages on LB broth. Selected colonies were then screened for tetracycline (250 μg/ml) and gentamicin (30 μg/ml) susceptibility and checked by PCR amplification and DNA sequencing.

Cloning of wild-type *ampG* gene and complementation experiments. The wild-type PAO1 *ampG* gene was PCR amplified using the primers listed in Table 2. The PCR product was digested with EcoRI and HindIII and ligated into plasmid pUCP26. The ligation reaction was used to transform chemically competent *E. coli* NM522 cells, and transformants were selected on LB agar supplemented with 5 μg/ml tetracycline. The recombinant plasmid was isolated from a single transformant, and its presence was verified by restriction analysis and DNA sequencing. The resulting *ampG* expression plasmid (pUCPampG) and pUCP26 (control vector), were electroporated into the *ampG*-knockout mutant of PAO1 (PAΔG). Cefoxitin MICs were determined in triplicate by the broth microdilution method in 100 μl of cation-adjusted Mueller-Hinton broth. Tetracycline (50 μg/ml) was added to the broth in order to maintain the complementation plasmid.

Quantification of basal and induced *ampC* expression. The relative levels of *ampC* mRNA were determined by real-time reverse transcription-PCR (RT-PCR) following previously described protocols (15). Briefly, total RNA from logarithmic-phase-grown cultures (with and without 50 μg/ml of cefoxitin) was obtained with an RNeasy minikit (Qiagen, Hilden, Germany). Fifty nanograms of purified RNA was then used for one-step reverse transcription and real-time PCR, using a QuantiTect SYBR green reverse transcription-PCR kit (Qiagen, Hilden, Germany) in a SmartCycler II apparatus (Cepheid, Sunnyvale, CA). Previously described conditions and primers were used (15). The *rpsL* housekeeping gene was used to normalize the expression levels, and results were always referred to PAO1 basal expression. All RT-PCRs were performed in duplicate, and the mean values of mRNA expression resulting from three independent experiments were considered in all cases.

Population analysis of ceftazidime and imipenem susceptibility and resistance emergence. Serial dilutions of 10-ml overnight cultures (MH broth) of PAO1, PAΔnZ, or PAΔG were seeded on MH agar plates containing 0, 0.12, 0.25, 0.5, 1, 2, 4, 8, or 16 μg/ml of ceftazidime or imipenem. Colonies growing after 24 h of incubation were counted, to plot the numbers of CFU at each antibiotic concentration. All experiments were performed in triplicate, and the results are shown as mean values ± standard deviations.

Characterization of pan-β-lactam-resistant clinical isolates. Three isolates resistant to all β-lactams tested (including penicillins, cephalosporins, monobactams, and carbapenems), each recovered from a different intensive care unit patient as part of a previous study (14), were used. Each isolate belonged to a different clone, and all were known to overexpress *ampC* due to *ampD* and/or *dacB* (PBP4) mutations (14, 31). The involvement of efflux pump overexpression in the resistance phenotype was also explored in this work. For this purpose, the expression of the genes encoding the four major *P. aeruginosa* efflux pumps, MexAB-OprM (*mexB*), MexCD-OprJ (*mexD*), MexEF-OprN (*mexF*), and MexXY-OprM (*mexY*), was determined by real-time RT-PCR following previously described protocols (35). Briefly, total RNA from logarithmic-phase-grown cultures was obtained with an RNeasy minikit (Qiagen, Hilden, Germany). Fifty nanograms of purified RNA was then used for one-step reverse transcription and real-time PCR, using a QuantiTect SYBR green reverse transcription-PCR kit (Qiagen, Hilden, Germany) in a SmartCycler II apparatus (Cepheid, Sunnyvale). Previously described conditions and primers were used (35). The *rpsL* housekeeping gene was used to normalize the expression levels, and results were always referred to PAO1 basal expression. All RT-PCRs were performed in duplicate, and the mean values of mRNA expression resulting from three independent experiments were considered in all cases. Overexpression was considered when the corresponding mRNA level was at least 3-fold (*mexB*) or 10-fold (*mexD*, *mexF*, *mexY*) higher than that for PAO1. The involvement of *oprD* inactivation in carbapenem resistance was explored through PCR amplification, followed by sequencing, using previously described primers and conditions (8).

RESULTS AND DISCUSSION

Comparison of AmpG and NagZ as targets to suppress AmpC-driven β-lactam resistance. In previous work (1, 42), we

TABLE 3. MICs and basal and induced *ampC* expression in the studied strains

Strain[a]	MIC (μg/ml)[b]							Relative mRNA level[c]	
	CAZ	FEP	IMP	MER	PTZ	ATM	FOX	Basal	Induced[d]
PAO1	1	1	2	0.5	3	3	2,048	1	78 ± 34
PAΔnZ	1	1	1	0.38	3	3	1,024	−2.1 ± 1.5	469 ± 230
PAΔG	1	1	0.38	0.38	3	2	64	−1.1 ± 0.4	1.9 ± 1.0
PAΔD	8	4	2	1.5	32	8	4,096	47 ± 9.5	134 ± 11
PAΔDnZ	2	2	1	0.75	6	3	1,024	1.0 ± 0.4	483 ± 26
PAΔDG	1	1.5	0.38	0.5	3	1.5	64	−1.5 ± 0.6	1.6 ± 0.8
PAΔdB	24	12	2	0.75	64	16	2,048	51 ± 16	232 ± 67
PAΔdBnZ	1.5	1.5	1	0.5	4	3	1,024	3.0 ± 1.9	661 ± 374
PAΔdBG	1	0.75	0.38	0.38	2	1.5	64	−1.1 ± 1.3	1.2 ± 0.9
PAdacBΔD	96	32	2	2	>256	48	4,096	1,770 ± 414	1,950 ± 480
PAdacBΔDnZ	4	2	1.5	0.75	24	6	1,024	40 ± 17	906 ± 80
PAdacBΔDG	0.75	1	0.38	0.25	1.5	1.5	64	1.7 ± 0.6	1.8 ± 1.5
PAΔR	1.5	1.5	0.5	0.25	4	4	64	3.8 ± 0.4	3.3 ± 0.8
PAΔC	1	1	0.5	0.25	3	3	64	NA	NA

[a] PAO1 mutants: PAΔnZ, *nagZ*; PAΔG, *ampG*; PAΔD, *ampD*; PAΔDnZ, *ampD-nagZ*; PAΔDG, *ampD-ampG*; PAΔdB, *dacB*; PAΔdBnZ, *dacB-nagZ*; PAΔdBG, *dacB-ampG*; PAdacBΔD, *dacB-ampD*; PAdacBΔDnZ, *dacB-ampD-nagZ*; PAdacBΔDG, *dacB-ampD-ampG*; PAΔR, *ampR*; PAΔC, *ampC*. Complementation of the PAO1 *ampG* mutant (PAΔG) with plasmid pUCPampG fully restored wild-type cefoxitin MICs.

[b] CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MER, meropenem; PTZ, piperacillin-tazobactam; ATM, aztreonam, FOX, cefoxitin.

[c] Relative amount of *ampC* mRNA compared to PAO1 basal levels ± standard deviation. *ampC* expression data for *nagZ* mutants obtained in previous work (42) were included for comparative purposes. NA, not applicable.

[d] Induction experiments were carried out with 50 μg/ml of cefoxitin.

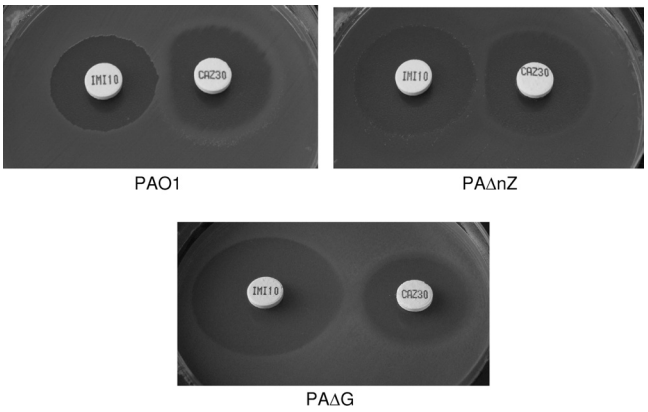

FIG. 2. Double-disk (imipenem-ceftazidime) AmpC induction test with strains PAO1, PAΔnZ, and PAΔG.

showed that the inactivation of *nagZ* fully restored susceptibility and basal *ampC* expression of *ampD* or *dacB* single mutants. Major reductions of MICs and *ampC* expression were also observed for the *ampD-dacB* double mutant, although wild-type levels were not fully reached. Furthermore, we showed that *nagZ* inactivation had little effect on *ampC* inducibility. In order to identify effective targets for suppressing AmpC-driven resistance in *P. aeruginosa*, here we compared the effects of *ampG* and *nagZ* inactivation, and the results are shown in Table 3. As shown, *ampG* inactivation fully restored susceptibility and basal *ampC* expression in *ampD* and *dacB* single and double mutants. More importantly, in contrast to *nagZ* inactivation, it fully blocked AmpC inducibility, as evidenced by *ampC* expression data (Table 3), results of the imipenem-ceftazidime double-disk AmpC induction test (Fig. 2), or the marked hypersusceptibility of the *ampG* mutants to the AmpC inducers imipenem and cefoxitin.

Blocking *ampC* induction through AmpG inactivation prevents and reverts imipenem resistance driven by lack of OprD expression. The results presented above suggested that both NagZ and AmpG are necessary for resistance to the weak inducers, including antipseudomonal penicillins and cephalosporins, acquired through AmpD or PBP4 mutations that lead to constitutive AmpC overexpression. Indeed, through a population analysis of ceftazidime susceptibility (Fig. 3A), we documented that the behaviors of *nagZ* and *ampG* mutants are essentially identical: deletion of either gene impaired the emergence of one-step ceftazidime-resistant mutants at concentrations of ≥8 μg/ml (susceptibility breakpoint), in sharp contrast to results for wild-type PAO1, in which mutants were still readily selected at concentrations of at least 16 μg/ml. On the other hand, major differences were observed between *nagZ* and *ampG* mutants in the population analysis of susceptibility to the potent AmpC inducer imipenem (Fig. 3B). While PAO1 imipenem-resistant mutants were detected at concentrations of up to 8 μg/ml (resistance breakpoint), *nagZ* inactivation significantly increased imipenem susceptibility and reduced the highest concentration yielding mutants by 4-fold (to 2 μg/ml). Nevertheless, remarkably, this effect was far more intense for the *ampG* mutant, which yielded no imipenem-resistant mutants at concentrations above 0.5 μg/ml (16-fold lower than that for PAO1).

These results suggested that *ampG* inactivation might minimize the impact of the most relevant imipenem resistance mechanism in *P. aeruginosa*, the inactivation of the carbapenem porin OprD. To test this possibility, we constructed and analyzed the *nagZ* and *ampG* mutants of a previously generated *oprD* mutant of PAO1 (32). Indeed, as shown in Table 4, imipenem MICs of the PAOD1 *oprD* mutant (>32 μg/ml) were reduced to 6 μg/ml in the *nagZ* mutant and to 0.5 μg/ml (below wild-type PAO1 MICs) in the *ampG* mutant. These results clearly indicate that inducible *ampC* expression (which is blocked through AmpG inactivation) is necessary for OprD inactivation-driven imipenem resistance, in agreement with previous evidence (26).

Comparison of AmpG and NagZ as targets for restoring susceptibility in pan-β-lactam-resistant *P. aeruginosa* clinical strains. *P. aeruginosa* pan-β-lactam resistance in the clinical

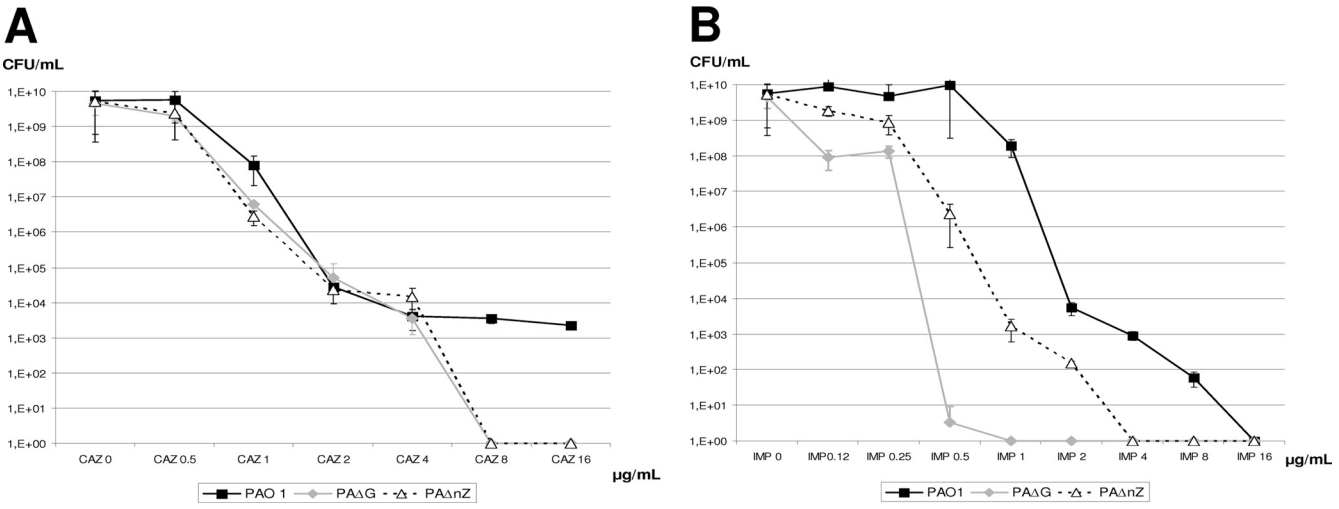

FIG. 3. Population analysis of PAO1, PAΔnZ, and PAΔG ceftazidime (CAZ) (A) and imipenem (IMP) (B) susceptibility. Overnight cultures of PAO1, PAΔnZ, or PAΔG were plated on MH agar containing 0, 0.12, 0.25, 0.5, 1, 2, 4, 8, and 16 μg/ml of ceftazidime or imipenem, and the CFU counts were enumerated after 24 h of incubation. The results are shown as mean values of 3 experiments ± standard deviations.

TABLE 4. MICs of *nagZ* and *ampG* mutants of pan-β-lactam-resistant *P. aeruginosa* clinical strains

Strain[a]	Resistance mechanism				MIC (μg/ml)[d]						
	AmpD[b]	PBP4[b]	OprD	Efflux[c]	CAZ	FEP	IMP	MER	PTZ	ATM	CIP[e]
PAO1					1.5	1.5	2	0.38	2	4	0.094
PAOD1			W65X		1.5	1.5	>32	2	1.5	4	0.094
PAOD1ΔnZ					1.5	1.5	6	2	2	2	0.094
PAOD1ΔG					1.5	1.5	0.5	2	2	2	0.094
JSG2A1	Ins. 1 bp (C) in nt 481	T428P	Δ*oprD*	*mexB* (6.5-fold)	192	96	>32	>32	>256	128	3
JSG2A1ΔnZ					8	16	>32	>32	24	32	3
JSG2A1ΔG					3	8	1.5	24	8	32	3
MQB1C5	Q155X		W339X	*mexB* (14-fold) *mexY* (21-fold)	24	24	>32	>32	96	64	1
MQB1C5ΔnZ					3	16	>32	>32	12	32	1
MQB1C5ΔG					3	8	2	>32	12	32	0.75
OFC2I4	Δ*ampDE*	M200I, del. D201	Δ*oprD*	*mexY* (12-fold) *mexF* (10-fold)	48	24	>32	>32	>256	32	0.38
OFC2I4ΔnZ					3	12	>32	6	16	4	0.38
OFC2I4ΔG					1.5	8	1.5	3	3	4	0.38

[a] *nagZ* (ΔnZ) and *ampG* (ΔG) mutants of the OprD mutant of PAO1 (PAOD1) and pan-β-lactam-resistant clinical strains JSG2A1, MBQ1C5, and OFC3I4.
[b] Mutations in *ampD* and *dacB* (PBP4) were documented in previous studies (14, 31). Ins., insertion; nt, nucleotide; del., deletion.
[c] Relative expression of efflux pump-encoding genes compared to that in wild-type PAO1. Breakpoints used for defining overexpression were ≥3-fold for *mexB* and ≥10-fold for *mexY*, *mexD*, and *mexF*.
[d] CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MER, meropenem; PTZ, piperacillin-tazobactam; ATM, aztreonam, CIP, ciprofloxacin.
[e] Ciprofloxacin MICs were included as a control to evaluate the specificity of the effect of *nagZ* and *ampG* inactivation on β-lactam susceptibility.

setting is known to frequently result from the interplay of AmpC hyperproduction, OprD inactivation, and the overexpression of efflux pumps (24, 28). Thus, we evaluated the effect of *nagZ* and *ampG* inactivation in three different clones of well-characterized pan-β-lactam-resistant *P. aeruginosa* clinical isolates. These strains were already known to overexpress *ampC* due to *ampD* and/or *dacB* (PBP4) mutations (14, 31). In this work, we documented that all three isolates additionally presented *oprD*-inactivating mutations and overexpressed one or several efflux pumps (Table 4). Both *nagZ* and *ampG* inactivation notably increased β-lactam susceptibility in the clinical strains, although important differences were again observed (Table 4). A marked effect on ceftazidime and piperacillin-tazobactam susceptibility was observed in both cases, although knocking out *ampG* had a greater effect in the two *ampD-dacB* double mutants. Again, the sharpest differences were observed for imipenem, with *ampG* inactivation fully restoring wild-type susceptibility for the three isolates. Susceptibility to meropenem, cefepime, and aztreonam was also enhanced, although to a lower extent, consistent with the higher impact of efflux pump overexpression on the activity of these antibiotics. It should thus be noted that imipenem is likely the optimal candidate for combination with future potential AmpG inhibitors, given that this strategy impairs the interplay of the most relevant mechanism of resistance (OprD inactivation and AmpC induction) to this antibiotic, which is the only currently available β-lactam not significantly impacted by any of the *P. aeruginosa* major efflux pumps (24).

Concluding remarks. In summary, we show that NagZ and AmpG are excellent targets for reverting and preventing the emergence of resistance to the weak AmpC inducers antipseudomonal penicillins and cephalosporins, driven by AmpC overexpression due to an AmpD and/or DacB mutation. Indeed, we have already shown the potential utility of small-molecule NagZ inhibitors for this purpose (1, 42), and research directed to the identification of AmpG inhibitors is ongoing. The latter strategy is highly encouraging, since we show that *ampG* inactivation fully blocks *ampC* induction, additionally minimizing the impact of OprD inactivation on imipenem resistance both in laboratory mutants and in pan-β-lactam-resistant clinical strains.

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