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Molecular Mechanisms of β-Lactam Resistance Mediated by AmpC Hyperproduction in *Pseudomonas aeruginosa* Clinical Strains

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The molecular mechanisms of β-lactam resistance mediated by AmpC hyperproduction in natural strains of *Pseudomonas aeruginosa* were investigated in a collection of 10 isogenic, ceftazidime-susceptible and -resistant pairs of isolates, each sequentially recovered from a different intensive care unit patient treated with β-lactams. All 10 ceftazidime-resistant mutants hyperproduced AmpC (β-lactamase activities were 12- to 657-fold higher than those of the parent strains), but none of them harbored mutations in *ampR* or the *ampC-ampR* intergenic region. On the other hand, six of them harbored inactivating mutations in *ampD*: four contained frameshift mutations, one had a C→T mutation, creating a premature stop codon, and finally, one had a large deletion, including the complete *ampDE* region. Complementation studies revealed that only three of the six *ampD* mutants could be fully transcomplemented with either *ampD*- or *ampDE*-harboring plasmids, whereas one of them could be transcomplemented only with *ampDE* and two of them (including the mutant with the deletion of the *ampDE* region and one with an *ampD* frameshift mutation leading to an *ampDE*-fused open reading frame) could not be fully transcomplemented with any of the plasmids. Finally, one of the four mutants with no mutations in *ampD* could be transcomplemented, but only with *ampDE*. Although the inactivation of AmpD is found to be the most frequent mechanism of AmpC hyperproduction in clinical strains, our findings suggest that for certain types of mutations, AmpE plays an indirect role in resistance and that there are other unknown genes involved in AmpC hyperproduction, with at least one of them apparently located close to the *ampDE* operon.

Pseudomonas aeruginosa is a ubiquitous versatile environmental microorganism that is the leading cause of opportunistic human infections (34). This pathogen is frequently involved in acute nosocomial infections, especially affecting patients in intensive care units (ICU) with mechanical-ventilation-associated pneumonia or burn wound infections, both of which are associated with a high mortality rate (26, 35). *P. aeruginosa* is also the major cause of chronic respiratory infections in patients with cystic fibrosis (CF) and other underlying chronic respiratory diseases (9, 25, 27).

P. aeruginosa resistance development during antimicrobial therapy, mediated by the selection of mutations in certain chromosomal genes, is a frequent problem with major consequences, especially when it affects critical patients in the ICU or chronically colonized patients, in whom this problem is amplified due to the high prevalence of hypermutable strains (5, 7, 10, 14, 23, 31). The most relevant mechanism for the development of resistance to the antipseudomonal penicillins (such as ticarcillin or piperacillin) and cephalosporins (such as ceftazidime) is the selection of mutations leading to the hyperproduction of the chromosomal cephalosporinase AmpC (8, 21, 23). AmpC is a group I, class C β-lactamase present in most *Enterobacteriaceae* and in *P. aeruginosa* and other nonfermenting gram-negative bacilli (2, 22). With the exception of those in *Escherichia coli* and *Shigella* spp., β-lactamase is produced at low basal levels, but its expression is inducible by certain β-lactams, specially cefoxitin and imipenem. During treatment with β-lactams, resistant mutants showing constitutive high levels of AmpC production are frequently selected, leading to therapeutic failure (8, 37).

There are several genes involved in *ampC* induction, a process that is intimately linked to peptidoglycan recycling (28). This system was first characterized for *Enterobacteriaceae* (*Enterobacter cloacae* and *Citrobacter freundii*) and was later found to be conserved also in *P. aeruginosa* (17, 18, 24). Of the genes involved, *ampR*, which is contiguous to *ampC* but divergently transcribed, encodes a transcriptional regulator of the LysR family that is required for β-lactamase induction (12, 20). *ampG* encodes a transmembrane protein that functions as a permease for 1,6-anhydromurapeptides, which are thought to be the signal molecules involved in *ampC* induction (6, 15). *ampD* encodes a cytosolic *N*-acetyl-anhydromuramil-L-alanine amidase that hydrolyzes 1,6-anhydromurapeptides, acting as a repressor of *ampC* expression (11, 19), and *ampE*, which forms the bicistronic *ampDE* operon together with *ampD*, encodes a cytoplasmic membrane protein thought to act as a sensory transducer molecule required for induction (13).

Mutational inactivation of *ampD* and specific point mutations on *ampR* are the main mechanisms found to lead to the hyperproduction of AmpC, and consequently to β-lactam resistance, in *Enterobacteriaceae* (16, 19, 33). As for *P. aeruginosa*, although *ampD* inactivation in a few strains and a specific point mutation in *ampR* (Asp135Asn) in one strain have also been associated with β-lactamase overproduction, the investigation of a limited number of mutant strains has revealed interesting differences, such as

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the absence of mutations either in *ampD* or in *ampR* in some AmpC-hyperproducing strains (1, 3, 18).

The objectives of the present work were (i) to characterize the mutations responsible for AmpC hyperproduction in a large collection of *P. aeruginosa* clinical strains isolated from ICU patients and (ii) to find out whether *ampE* (as part of the *ampDE* operon) has an indirect role in resistance or whether, on the contrary, only *ampD* is involved in the AmpC hyperproduction phenotype. For this purpose, complementation studies using plasmids harboring either *ampD* alone or the complete *ampDE* operon were conducted with all the ceftazidime-resistant strains.

MATERIALS AND METHODS

Bacterial strains and susceptibility testing. A total of 10 pairs of ceftazidime-susceptible and -resistant (≥8-fold increase in MICs) *P. aeruginosa* clinical isolates were studied. Each pair of isolates was sequentially recovered from clinical samples (bronchial aspirates and wound infections in 9 cases and 1 case, respectively) from each of the 10 patients admitted to the ICU between September 2002 and November 2003 as part of a study of the epidemiology of *P. aeruginosa* antibiotic resistance in this setting (14). For two of the patients, two sequential mutants, each with increased ceftazidime resistance, were recovered. The 10 patients from which the ceftazidime-resistant mutants were isolated had been previously treated or were under treatment with either ceftazidime, cefepime, or piperacillin-tazobactam. The paired isolates from each of the patients were documented to be isogenic by pulsed-field gel electrophoresis, and each of the 10 pairs studied was found to belong to a different clone in the previous work (14). Bacterial identification and initial susceptibility testing were performed with the Wider system (Francisco Soria Melguizo, Madrid, Spain) (4). Additionally, ceftazidime MICs were determined by the Etest method (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations.

β-Lactamase assays and determination of expression of efflux pumps. Specific β-lactamase activity (nanomoles of nitrocefin hydrolyzed per minute per milligram of protein) was determined spectrophotometrically on crude sonic extracts as previously described (30). For induction experiments, before the preparation of the crude sonic extracts, the strains were grown in the presence of 50 μg/ml cefoxitin for 3 hours. In all cases, the mean values of β-lactamase activity obtained in two independent experiments were considered. Based on the analysis of the results from the duplicate experiments, differences in β-lactamase activities above twofold were considered significant. To evaluate the possible presence of other β-lactamases in addition to AmpC in the studied strains, two different approaches were followed. (i) The inhibition of β-lactamase activity after the incubation of crude extracts for 15 min in 50 μM cloxacillin, a class C β-lactamase inhibitor (22), was tested. Representative β-lactamase extracts from classes A (TEM-1), B (VIM-2), C (PAO1 induced with cefoxitin), and D (OXA-40) were used as controls and for procedure standardization. β-Lactamase activities were measured after cloxacillin treatment as described above, and a >95% reduction of nitrocefin hydrolysis was considered a positive result for class C β-lactamases and negative for classes A, B, and D. (ii) Isoelectric focusing (IEF) of crude sonic extracts using Phast gels (pH gradient, 3 to 9) in a Phast system apparatus (Pharmacia AB, Uppsala, Sweden) was performed for all the ceftazidime-susceptible (with and without cefoxitin induction) and -resistant pairs of isolates.

The levels of expression of *mexB* and *mexF* were determined by real-time PCR for the 10 pairs of ceftazidime-susceptible and -resistant isolates and PAO1 (as a control) by following a modified protocol previously described by Oh et al. (29). Briefly, total RNA from logarithmic-phase-grown cultures was obtained with the RNeasy mini kit (QIAGEN, Hilden, Germany) and was adjusted to a final concentration of 50 ng/μl. Purified RNA (50 ng) was then used for one-step reverse transcription- and real-time PCR amplification using the QuantiTect SYBR green reverse transcription-PCR kit (QIAGEN, Hilden, Germany) in the SmartCycler II (Cepheid, Sunnyvale, CA). Previously described conditions and primers MxB-U and MxB-L, MxF-U and MexF-L, and RpsL-1 and RpsL-2 were used for the amplification of *mexB*, *mexF*, and *rpsL* (used as references to calculate the relative amounts of mRNA of efflux pump proteins), respectively (29). In all cases, the mean values of mRNA expression obtained in three experiments were considered.

PCR amplification and sequencing of *ampD*, *ampE*, *ampR*, and the *ampR-ampC* intergenic region. PCR amplification of *ampD*, *ampE*, and *ampR* (including the *ampR-ampC* intergenic region) was performed on whole DNA extracts (DNeasy tissue kit; QIAGEN, Hilden, Germany) from both the ceftazidime-susceptible and the ceftazidime-resistant isolates from each of the 10 pairs of *P. aeruginosa* strains. Primers ADF (5′-GTACGCCTGCTGGACGATG-3′) and ADR (5′-GAGGGCAGATCCTCGACCAG-3′) were used to amplify a 0.9-kb DNA fragment containing the complete *ampD* gene and its promoter region (17). AEF (5′-GCCTGGACCCGAACGAAC-3′) and AER (5′-TCAGAGGAA CAGCGCGCAG-3′) were used to amplify a 1.2-kb fragment containing the complete *ampE* gene (17), and ARF (5′-GTCGACCCAGTGCCTTCAGG-3′) and ARR (5′-CTCGAGAGCGAGATCGTTGC-3′) were used to amplify a 1.4-kb fragment containing *ampR* and the *ampR-ampC* intergenic region (24). Two independent PCR products for each isolate and gene were sequenced on both strands. The BigDye Terminator kit (PE-Applied Biosystems) was used for performing the sequencing reactions that were analyzed with the ABI Prism 3100 DNA sequencer (PE-Applied Biosystems).

Cloning of *ampD* and *ampDE* and complementation studies. For cloning *ampD*, the PAO1 wild-type gene was PCR amplified using primers ADF_{BHI} and ADR_{BHI} (the above-described primers ADF and ADR to which a tail containing a BamHI restriction site was added). For cloning the whole *ampDE* operon, the same forward primer (ADF_{BHI}) and the reverse primer AER_{BHI} (the above-described AER to which a tail containing a BamHI restriction site was added) were used. PCR products were ligated to plasmid pGEM-T to obtain pGTAD or pGTADE, which were transformed into the *E. coli* XL1-Blue strain made competent by $CaCl_2$. Transformants were selected in 50 μg/ml ampicillin-MacConkey agar plates. *ampD* or *ampDE* fragments obtained from three independent experiments were fully sequenced to ascertain the absence of mutations in the cloned fragments produced during PCR amplification. Plasmid DNA from pGTAD or pGTADE digested with either BamHI or EcoRI, respectively, was ligated to plasmid pUCP24 (36), which was digested with the same enzymes to obtain plasmids pUCPAD and pUCPADE, which were transformed into *E. coli* XL1-Blue. Transformants were selected in 20 μg/ml gentamicin-MacConkey agar plates. In both cases, recombinant plasmids with DNA inserts with an orientation opposite to that of the LacZ promoter were selected. Plasmids pUCPAD, pUCPADE, and pUCP24 were then electroporated into the different ceftazidime-resistant strains or PAO1 (as a control) as previously described (32). Transformants were selected in 50 μg/ml gentamicin–Luria-Bertani (LB) agar plates. Ceftazidime MICs and β-lactamase activity were determined to evaluate the complementation of the AmpC hyperproduction phenotype.

Nucleotide sequence accession number. The GenBank accession number for the *ampDE*-fused open reading frame of AmpC-hyperproducing strain JGS2A1 is DQ114494.

RESULTS

Ceftazidime MICs and the basal and induced β-lactamase activities of the 10 paired ceftazidime-susceptible and -resistant isogenic clinical strains are shown in Table 1. The increase in ceftazidime MICs of the resistant mutants ranged from 8- to 42-fold. For two of the patients, two-step ceftazidime-resistant mutants were sequentially recovered, and therefore, three isolates for each of the patients are shown in Table 1.

All the susceptible isolates had an inducible AmpC production phenotype, with induced/basal β-lactamase activity ratios ranging from 11 to 88. In all cases, ceftazidime resistance was associated with a significant increase in basal β-lactamase activities that ranged 12- to 657-fold higher than those of the susceptible strains, demonstrating the presence of an AmpC hyperproduction phenotype (Table 1). For both patients with two-step ceftazidime-resistant mutants, only the second resistant isolates (MBQ1C5 and OFC2I4) were associated with AmpC hyperproduction. Five of the 10 studied resistant mutants retained a certain degree of inducibility (induced/basal β-lactamase activity ratios were >2), whereas the other five were apparently fully derepressed mutants.

The presence of additional β-lactamases besides AmpC in the studied strains was ruled out by the cloxacillin inhibition

TABLE 1. Ceftazidime MICs, specific β-lactamase activity, results for complementation with pUCPAD and pUCPADE, and *ampD* mutations of the 10 pairs of ceftazidime-susceptible and ceftazidime-resistant isogenic clinical strains

Strain and plasmid	Treatment before resistance[a]	Ceftazidime MIC (μg/ml)	β-Lactamase sp act[b,c]		*ampD* mutation[d]
			Basal	Induced	
PAO1		1	1	52	
pUCP24		1	1	31	
pUCPAD		1	2	99	
pUCPADE		1	1	19	
BCL1J6	Pip-Tz + Tob	1	0.9	10	Frameshift mutation (1-bp insertion [C] in nt 53)
BCL2A8		8	37	34	
pUCP24		8	45	41	
pUCPAD		8	9	43	
pUCPADE		2	0.5	30	
MCV1C4	Fep + Tob	1	0.9	12	No mutations
MCV1C6		24	24	95	
pUCP24		24	19	103	
pUCPAD		24	9	133	
pUCPADE		24	7	45	
FMC1H1	Caz + Tob	1	3	53	No mutations
FMC1H6	Pip-Tz + Tob	16	412	842	
pUCP24		24	489	877	
pUCPAD		24	267	752	
pUCPADE		24	263	583	
JSG1H9	Imp + Tob	6	0.5	13	Frameshift mutation (1-bp insertion [C] in nt 481; leads to *ampDE* fusion)
JSG2A1	Pip-Tz + Tob	>256	258	184	
pUCP24		>256	272	246	
pUCPAD		>256	278	495	
pUCPADE		>256	9	95	
VFF2D5	Caz + Tob	1.5	2	34	No mutations
VFF2E2		32	26	103	
pUCP24		32	23	67	
pUCPAD		24	8	52	
pUCPADE		16	5	35	
JCM2B9	Pip-Tz + Tob	1.5	0.3	19	Frameshift mutation (1-bp deletion [C] in nt 449)
JCM2C2		24	197	224	
pUCP24		24	309	353	
pUCPAD		1	1	41	
pUCPADE		1	0.5	10	
MSF2F4	Imp + Tob	1.5	0.6	53	No mutations
MSF2F5	Caz + Tob	16	7	29	
pUCP24		16	8	30	
pUCPAD		12	6	19	
pUCPADE		1.5	2	20	
MSC2A5	Fep	1	2	52	Frameshift mutation (1-bp insertion [G] in nt 297)
MSC2A9	Caz	32	272	458	
pUCP24		32	165	331	
pUCPAD		1	1	73	
pUCPADE		1	1	12	
MQB1A10	Cip + Tob	1	2	9	Premature stop codon (C→T in nt 463 of *ampD*)
MQB1B2	Imp + AmK	4	3	141	
MQB1C5	Caz + Cip	32	162	350	
pUCP24		32	173	440	
PUCPAD		4	1	113	
pUCPADE		4	0.5	49	
OFC2G5	Fep + Tob	1	0.6	16	Complete deletion of *ampDE* region
OFC2H1	Cip + Imp	4	0.5	9	
OFC2I4	Caz + Tob	96	152	146	
pUCP24		96	206	204	
pUCPAD		96	158	145	
pUCPADE		96	6	24	

[a] Antibiotic treatments received before the isolation of the ceftazidime-resistant strain. Antibiotic abbreviations: Pip-Tz, piperacillin-tazobactam; Caz, ceftazidime; Fep, cefepime; Imp, imipenem; Tob, tobramycin; Amk, amikacin; Cip, ciprofloxacin.

[b] Relative values of β-lactamase activities, with 1 being considered the obtained basal activity for the reference strain PAO1 (19 nanomoles of nitrocefin hydrolyzed per minute per milligram of protein).

[c] Mean values from two independent experiments. Based on the analysis of the results from the duplicate experiments, only differences in β-lactamase activities above twofold are considered significant.

[d] Shown nucleotide numbers are those corresponding to the PAO1 *ampD* sequence.

test and IEF as described in Materials and Methods. Briefly, the β-lactamase activity of crude sonic extracts was inhibited by cloxacillin in all cases, and in IEF, no β-lactamase bands were detected in the basal extracts from ceftazidime-susceptible isolates and a single pI 8 to 9 β-lactamase band was detected in both cefoxitin-induced extracts from the susceptible isolates and basal extracts from the resistant mutants.

The contribution of the hyperproduction of efflux pumps to the ceftazidime resistance phenotype was explored as well in the 10 pairs of isolates. As expected, no significant differences were documented in the expression of *mexB* or *mexF* between the ceftazidime-susceptible isolates and the ceftazidime-resistant mutants hyperproducing AmpC. Nevertheless, for one the pairs (JSG1H9-JSG2A1), both isolates hyperexpressed MexAB-OprM (*mexB* mRNA levels were sixfold higher than those of PAO1), a finding that could explain the higher ceftazidime MICs (6 and >256 μg/ml for JSG1H9 and JSG2A1, respectively) than those for the other pairs of isolates (Table 1). Regarding the two cases in which two-step ceftazidime-resistant mutants were documented, the first-step mutation not related to AmpC hyperproduction was found to be associated with the hyperexpression of MexEF-OprN, showing strains MBQ1B2 and OFC2H1 to have 5- and 11-fold-higher levels of *mexF* mRNA, respectively, than those of their respective susceptible parent strains.

Characterization of the mutations leading to AmpC hyperproduction. The presence of mutations responsible for the AmpC hyperproduction phenotype was investigated by the sequencing of *ampD*, *ampE*, *ampR*, and the *ampC-ampR* intergenic region of both ceftazidime-susceptible and -resistant isolates. None of the resistant isolates contained any mutations in *ampR* or the *ampC-ampR* intergenic region compared with their isogenic susceptible isolates. On the other hand, 6 of the 10 AmpC-hyperproducing mutants contained inactivating mutations in *ampD* (Table 1). Interestingly, the five AmpC-hyperproducing mutants that retained certain inducibility included the four strains with no mutations in *ampD* but only one of the six strains with mutations in this gene. Four of the strains harbored *ampD* frameshift mutations produced by a 1-bp insertion or deletion (Table 1). Strain MQB1C5 contained a C→T mutation on nucleotide (nt) 463 of *ampD*, creating a premature stop codon. Finally, strain OFC2I4 had a large deletion, including the complete *ampDE* region, not present in its susceptible pair (OFC2G5) or in the isolate with an intermediate resistance level isolated from the same patient (OFC2H1). This deletion was associated with the loss of an approximately 25-kb DNA fragment from one of the SpeI restriction fragments in the pulsed-field gel electrophoresis analysis (not shown). PCR amplification using *ampD* and *ampE* primers as well as two sets of internal primers (one for each gene) consistently failed for strain OFC2I4. Finally, PCR mapping, using primers based on the published PAO1 sequence (34), of an 11-kb fragment surrounding the *ampDE* region (from nt 5057716 to 5068746 of the PAO1 sequence, including hypothetical proteins PA4517 to PA4524) confirmed as well that the complete region was conserved (identical structure to that of PAO1) in the susceptible strain and completely absent in OFC2I4.

Complementation of *ampD*-deficient mutants with plasmids harboring wild-type *ampD* (pUCPAD) or wild-type *ampDE* (pUCPADE). Complementation of the AmpC hyperproduction phenotype was evaluated by comparing the ceftazidime MICs and β-lactamase activities of transformant colonies harboring pUCPAD or pUCPADE with those of transformants harboring pUCP24. For strains JCM2C2, MSC2A9, and MQB1C5, ceftazidime MICs and basal β-lactamase activities were restored back to wild-type levels when the strains harbored either pUCPAD or pUCPADE (Table 1). On the other hand, the AmpC hyperproduction phenotype of strain BCL2A8 was transcomplemented only by pUCPADE and not by pUCPAD. The only apparent difference in the *ampD*-inactivating mutation from this strain compared to those of the above strains with a positive pUCPAD complementation is that the inactivating mutation is located much earlier in the coding sequence (Table 1).

Finally, two of the strains (JSG2A1 and OFC2I4) harboring inactivating mutations in *ampD* could not be fully transcomplemented either with pUCPAD or with pUCPADE (Table 1). Nevertheless, for both strains, a partial complementation of the increased β-lactamase activities was documented only with pUCPADE, but the partial complementation of β-lactamase activities was not accompanied by a reduction of ceftazidime MICs. The presence of additional mutations in secondary loci may be responsible for the obtained results, although the particularity of the documented *ampD* mutations for both strains could also explain the results. As for strain OFC2I4, harboring a large deletion including the complete *ampDE* region, the failure of plasmid pUCPADE to fully transcomplement the AmpC hyperproduction phenotype could suggest that a secondary locus involved in *ampC* expression is located close to *ampDE*, and therefore, a single genetic event (deletion) could be responsible for the phenotype. On the other hand, the *ampD* mutation of strain JSG2A1 was also particularly different from the other frameshift mutations documented. The 1-bp insertion in *ampD* from this strain was located in nt 481 close to the 3′ end of the 564-nt coding sequence of *ampD*. Since (i) *ampE* is read in the +3 frame compared to *ampD*, (ii) a stop codon is not created by the frameshift mutation, and (iii) the stop and start codons of both genes are overlapped, a single open reading frame containing *ampD* (with the last 28 codons modified) and the complete *ampE* gene is originated. Therefore, the coding sequence predicts the creation of a hybrid protein containing both AmpD (with the last 28 amino acids modified) and the regular AmpE. Given the different cellular locations of AmpD (cytosol) and AmpE (cytoplasmic membrane), the role of this potential hybrid protein is difficult to predict, although one of the possibilities, supported by the negative complementation results, could be that this hybrid protein is not functional for *ampC* repression and exhibits a dominant negative effect when wild-type AmpD/AmpE is present. To explore the possibility of this *ampD* mutation being dominant, the *ampDE* region of JSG2A1 was cloned as described for PAO1, and the resulting plasmid ($pUCPADE_{JSG}$) was transformed into the reference strain PAO1. Since neither the β-lactamase activity nor the ceftazidime MIC was modified in strain PAO1 harboring $pUCPADE_{JSG}$, the results were apparently inconsistent with the AmpC hyperproduction pheno-

type that was expected to occur if the mutation was truly dominant.

Complementation of AmpC-hyperproducing mutants with no mutations in the *ampDE* region with plasmid pUCPAD or pUCPADE. None of the strains for which *ampD*-inactivating mutations were not documented could be transcomplemented with pUCPAD, although a slight decrease in β-lactamase activity was documented for some of the mutants harboring the plasmid (Table 1). The slight decrease in the β-lactamase activities of the mutant strains was more apparent when the strains harbored pUCPADE, and remarkably, for one of them (MSF2F5), a positive complementation result was obtained with this plasmid, with ceftazidime MICs being restored to the wild-type levels.

DISCUSSION

Although specific point mutations in *ampR* may lead to the constitutive hyperexpression of AmpC, our results for the strains from ICU patients, together with those previously obtained in the CF setting (1, 3), strengthen the observation that this is a very infrequent mechanism of β-lactam resistance in natural strains of *P. aeruginosa*. On the other hand, the in vitro inactivation of *ampD* has been found to confer a partially derepressed phenotype in strain PAO1, leading to a moderate (approximately 35-fold-higher) basal level but hyperinducible AmpC expression (18). The involvement of *ampD* inactivation in AmpC hyperproduction has been previously studied in a limited number of clinical strains of *P. aeruginosa* (1, 18). Bagge et al. found the interruption of *ampD* by an insertion sequence in two CF AmpC-hyperproducing clinical isolates, but none of the three in vitro or in vivo ceftazidime-resistant variants from two other clinical strains contained any inactivating mutation in *ampD* (1). Similarly, Langaee et al. found no *ampD*-inactivating mutations in any of the four strains hyperproducing AmpC (18). Our results with a large collection of isogenic ceftazidime-susceptible and -resistant pairs of isolates from ICU patients treated with β-lactams show that *ampD* inactivation is the most frequent mechanism for AmpC hyperproduction in clinical strains but also reveal a complex multifactorial picture of the regulation of β-lactamase expression in *P. aeruginosa*.

Whereas the role of AmpD as a repressor of β-lactamase expression is clear, we still have very limited information on the role of the other component, AmpE, encoded by the bicistronic *ampDE* operon. *ampE* encodes a cytoplasmic membrane protein thought to act as a sensory transducer molecule required for induction (13). In addition to having this function, AmpE can modulate *ampC* repression in hyperproducing strains in the absence of inducers, as our results show for the first time. In this sense, for several mutant strains, including some with and some without *ampD*-inactivating mutations, the production of both AmpD and AmpE from a plasmid repressed β-lactamase expression more readily than the production of AmpD alone, reaching, in some cases, the extreme results of full complementation of the hyperproduction phenotype with pUCPADE in the absence of pUCPAD complementation. It seems clear that the role of AmpE in *ampC* repression is not decisive by itself, since the inactivation of *ampE* alone did not determine the derepression of *ampC* in the *E. coli* model of expression of the *E. cloacae* chromosomal β-lactamase used by Honore et al. (13). This finding is also supported by the fact that none of the 10 *P. aeruginosa* AmpC-hyperproducing mutants studied harbored inactivating mutations just in *ampE*. Nevertheless, our complementation results for the mutants with and without AmpD-inactivating mutations could be the consequence of an indirect role of AmpE in *ampC* repression/derepression; the polarity of *ampD*-inactivating mutations over *ampE* expression, the role of AmpDE fusion proteins produced by the 1-bp insertion of *ampD*-inactivating mutations (such as that of strain JSG2A1), and the potential effect of AmpE as a transducer molecule in the activity of other yet-unknown gene products involved in β-lactamase expression are among the important future research directions opened by this work. Regarding the last point, the completely sequenced chromosome of strain PAO1 (34) contains two additional *ampD* homologues (PA0807 and PA5485) for which implications in β-lactamase expression are currently being explored by our group. Furthermore, the lack of full complementation of the AmpC hyperproduction phenotype of strain OFC2I4 (harboring a large deletion, including the complete *ampDE* operon and surrounding regions) with pUCPAD or pUCPADE may suggest that other genes involved in the regulation of the chromosomal cephalosporinase are located close to the *ampDE* operon.

In summary, our findings show that AmpD inactivation is the most frequent mechanism leading to AmpC hyperproduction in *P. aeruginosa* clinical strains but also suggest that for certain types of mutations, AmpE plays an indirect role in β-lactam resistance and that there are several unknown genes involved in AmpC hyperproduction, with at least one of them apparently located close to the *ampDE* operon.

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