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Stepwise Upregulation of the *Pseudomonas aeruginosa* Chromosomal Cephalosporinase Conferring High-Level β-Lactam Resistance Involves Three AmpD Homologues

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Development of resistance to the antipseudomonal penicillins and cephalosporins mediated by hyperproduction of the chromosomal cephalosporinase AmpC is a major threat to the successful treatment of *Pseudomonas aeruginosa* infections. Although *ampD* inactivation has been previously found to lead to a partially derepressed phenotype characterized by increased AmpC production but retaining further inducibility, the regulation of *ampC* in *P. aeruginosa* is far from well understood. We demonstrate that *ampC* expression is coordinately repressed by three AmpD homologues, including the previously described protein AmpD plus two additional proteins, designated AmpDh2 and AmpDh3. The three AmpD homologues are responsible for a stepwise *ampC* upregulation mechanism ultimately leading to constitutive hyperexpression of the chromosomal cephalosporinase and high-level antipseudomonal β-lactam resistance, as shown by analysis of the three single *ampD* mutants, the three double *ampD* mutants, and the triple *ampD* mutant. This is achieved by a three-step escalating mechanism rendering four relevant expression states: basal-level inducible expression (wild type), moderate-level hyperinducible expression with increased antipseudomonal β-lactam resistance (*ampD* mutant), high-level hyperinducible expression with high-level β-lactam resistance (*ampD ampDh3* double mutant), and very high-level (more than 1,000-fold compared to the wild type) derepressed expression (triple mutant). Although one-step inducible-derepressed expression models are frequent in natural resistance mechanisms, this is the first characterized example in which expression of a resistance gene can be sequentially amplified through multiple steps of derepression.

Pseudomonas aeruginosa is a ubiquitous versatile environmental microorganism that is the leading cause of opportunistic human infections (40). This pathogen is frequently involved in acute nosocomial infections, especially affecting patients in intensive care units (ICUs) with mechanical-ventilation-associated pneumonia or burn wound infections, both processes associated with a high mortality rate (41). *P. aeruginosa* is also the major cause of chronic respiratory infections in patients with cystic fibrosis and other underlying chronic respiratory diseases (12).

P. aeruginosa resistance development during antimicrobial therapy, mediated by the selection of mutations in certain chromosomal genes, is a frequent problem with major clinical consequences, especially when affecting critical patients in ICUs or in chronically colonized patients, where this problem is amplified because of the high prevalence of hypermutable strains (5, 7, 17, 29, 35). The most relevant mechanism for development of resistance to antipseudomonal penicillins (such as ticarcillin or piperacillin) and cephalosporins (such as ceftazidime or cefepime) is selection of mutations leading to hyperproduction of the chromosomal cephalosporinase AmpC (11, 26). AmpC is a group I class C β-lactamase present in most *Enterobacteriaceae* and in *P. aeruginosa* and other nonfermenting gram-negative bacilli (4, 27). With the exception of *Escherichia coli* and shigellae, β-lactamase is produced at low basal levels but its expression is inducible by certain β-lactams, especially cefoxitin and imipenem. The activity of the antipseudomonal penicillins and cephalosporins against *P. aeruginosa* is based on the fact that although these compounds are certainly hydrolyzed by AmpC, they are very weak inducers of this chromosomal β-lactamase (27, 28). Nevertheless, during treatment with β-lactams, resistant mutants showing high levels of AmpC production are frequently selected, leading to therapeutic failure (11, 17).

There are several genes involved in *ampC* induction, a process that is intimately linked to peptidoglycan recycling (32). This system was first characterized in *Enterobacteriaceae* (*Enterobacter cloacae* and *Citrobacter freundii*) and later found to be conserved also in *P. aeruginosa* (22, 30). 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 (14, 24); *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, 21); *ampD*, which encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase that hydrolyzes 1,6-anhydromurapeptides, acting as a repressor of *ampC* expression (13, 25); 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 (15).

Mutational inactivation of *ampD* is the main mechanism found to lead to the constitutive hyperproduction (derepression) of AmpC, and consequently to β-lactam resistance, in *Enterobacteriaceae* (25, 39). The accumulated 1,6-anhydro-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference or source
P. aeruginosa		
PAO1	Reference strain completely sequenced	40
PAΔD	PAO1 Δ*ampD*::*lox*	This work
PAΔDh2	PAO1 Δ*ampDh2*::*lox*	This work
PAΔDh3	PAO1 Δ*ampDh3*::*lox*	This work
PAΔDDh2	PAO1 Δ*ampD*::*lox* Δ*ampDh2*::*lox*	This work
PAΔDDh3	PAO1 Δ*ampD*::*lox* Δ*ampDh3*::*lox*	This work
PAΔDh2Dh3	PAO1 Δ*ampDh2*::*lox* Δ*ampDh3*::*lox*	This work
PAΔDDh2Dh3	PAO1 Δ*ampD*::*lox* Δ*ampDh2*::*lox* Δ*ampDh3*::*lox*	This work
E. coli		
XL1-Blue	F′::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15 recA1 endA1 gyrA96* (Nal^r) *thi hsdR17*(r_K^- m_K^-) *mcrB1*	3
S17-1	RecA *pro* (RP4-2Tet::Mu Kan::Tn*7*)	37
Plasmids		
pGEMT	PCR cloning vector	Promega
pUCP24	Gm^r; pUC18-based *Escherichia-Pseudomonas* shuttle vector	42
pUCPAD	Gm^r; pUCP24 containing PAO1 *ampD* gene	18
pUCPADE	Gm^r; pUCP24 containing complete PAO1 *ampDE* operon	18
pUCPADh2	Gm^r; pUCP24 containing PAO1 *ampDh2* gene	This work
pUCPADh3	Gm^r; pUCP24 containing PAO1 *ampDh3* gene	This work
pEX100Tlink	Ap^r *sacB*; pUC19-based gene replacement vector with MCS[a]	36
pUCGm*lox*	Ap^r Gm^r; pUC18-based vector containing *lox*-flanked *aacC1* gene	36
pCM157	Tc^r; *cre* expression vector	31
pEXADGm	pEX100Tlink containing 5′ and 3′ flanking sequence of *ampD*::Gm*lox*	This work
pEXADh2Gm	pEX100Tlink containing 5′ and 3′ flanking sequence of *ampDh2*::Gm*lox*	This work
PEXADh3Gm	pEX100Tlink containing 5′ and 3′ flanking sequence of *ampDh3*::Gm*lox*	This work

[a] MCS, multiple cloning site.

murapeptides produced by *ampD* inactivation presumably bind to AmpR, converting it into an activator of *ampC* expression (16).

Although the regulation of *ampC* in *P. aeruginosa* is not well understood, *ampD* inactivation has been previously found to lead to a partially derepressed phenotype characterized by increased AmpC production but retaining further inducibility (23). We demonstrate that *ampC* expression is coordinately repressed by three *ampD* homologues, the previously described *ampD* gene (22) plus two additional homologous genes, PA5485 and PA0807, from the completely sequenced PAO1 strain (40), here designated *ampDh2* and *ampDh3*, respectively. These three AmpD homologues are responsible for a stepwise *ampC* upregulation mechanism ultimately leading to the constitutive hyperexpression (more than 1,000-fold) of the chromosomal cephalosporinase and high-level (clinically relevant) antipseudomonal β-lactam resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and antibiotic susceptibility testing. The wild-type *Pseudomonas aeruginosa* strain used in this work was the completely sequenced reference strain PAO1 (40). The PAO1 mutant derivatives constructed, the plasmids used or constructed, and the *E. coli* strains used in this work are described in Table 1. The MICs of the antipseudomonal β-lactams ceftazidime, cefepime, ticarcillin, piperacillin, piperacillin-tazobactam, aztreonam, imipenem, and meropenem were determined in Müller-Hinton agar plates with Etest strips (AB Biodisk, Solna, Sweden) by following the manufacturer's recommendations.

Cloning of *ampD* homologues *ampDh2* and *ampDh3*. The *P. aeruginosa ampD* homologues *ampDh2* (PA5485) and *ampDh3* (PA0807) were detected by homology searches with the BLAST program at www.ncbi.nlm.nih.gov/BLAST. For cloning of *ampDh2* and *ampDh3*, the PAO1 wild-type genes were PCR amplified with the primers described in Table 2. PCR products were ligated to plasmid pGEM-T to obtain pGTADh2 or pGTADh3, respectively, which were transformed into *E. coli* XL1-Blue made competent by $CaCl_2$. Transformants were selected in 50 μg/ml ampicillin MacConkey agar plates. The *ampDh2* and *ampDh3* genes obtained from three independent experiments were fully sequenced to ascertain the absence of mutations in the cloned fragments produced during PCR amplification. The BigDye Terminator Kit (PE-Applied Biosystems) was used to perform the sequencing reactions, which were analyzed with an ABI Prism 3100 DNA sequencer (PE-Applied Biosystems). Plasmid DNAs from pGTADh2 and pGTADh3 digested with BamHI were ligated to plasmid pUCP24 digested with the same enzyme to obtain plasmids pUCPADh2 and pUCPADh3, 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.

Inactivation of *P. aeruginosa ampD* homologues. PAO1 *ampD* (PAΔD), *ampDh2* (PAΔDh2), and *ampDh3* (PAΔDh3) knockout mutants, as well as the three double mutants (*ampD ampDh2* [PAΔDDh2], *ampD ampDh3* [PAΔDDh3], and *ampD2 ampDh3* [PAΔDh2Dh3]) and the triple mutant (*ampD ampDh2 ampDh3* [PAΔDDh2Dh3]), were constructed by following the procedure previously described by Quénée et al. (36) for gene deletion and antibiotic marker recycling in *P. aeruginosa*. Upstream and downstream PCR products (Table 2) of *ampD*, *ampDh2*, or *ampDh3* were digested with either BamHI or EcoRI and HindIII and cloned by three-way ligation into pEX100Tlink with the HindIII site deleted and opened by EcoRI and BamHI to obtain plasmids pEXTAD, pEXTADh2, and pEXTADh3, which were transformed into *E. coli* XL1-Blue. Transformants were selected in 50 μg/ml ampicillin MacConkey agar plates. The *lox*-flanked gentamicin resistance cassette (*aacC1*) obtained by HindIII restriction of plasmid pUCGm*lox* was cloned into the single site for this enzyme formed by ligation of the two flanking fragments, producing plasmids pEXTADGm, pEXTADh2Gm, and pEXTADh3Gm, which were transformed into *E. coli* XL1- Blue. Transformants were selected in 50 μg/ml ampicillin–20 μg/ml gentamicin MacConkey agar plates. These plasmids were then transformed into *E. coli* helper strain S17-1. The PAΔD, PAΔDh2, and PAΔDh3 PAO1 mutants were generated by, respectively, introducing pEXTADGm, pEXTADh2Gm, and pEXTADh3Gm from *E. coli* S17-1 by conjugation and selection for double recombinants with 5% sucrose–30 μg/ml gentamicin–1 μg/ml cefotaxime Luria-Bertani (LB) agar plates. Double recombinants were checked first by screening for ticarcillin (250 μg/ml) susceptibility and afterwards by PCR amplification. For removal of the gentamicin resistance cassettes, plas-

TABLE 2. Primers used in this work

Primer	Sequence (5′–3′)[a]	PCR product size (bp)	Use
ADh2-FBHI	TCGGATCCGCCTGACCGCGCTGCCC	815	Cloning of *ampDh2*
ADh2-RBHI	TCGGATCCTCAGGAAGTCGGCACCGCC		
ADh3-FBHI	TCGGATCCCTTGGCCGGCCCCTGAAC	960	Cloning of *ampDh3*
ADh3-RBHI	TCGGATCCGCGACGACCTGAGCGACG		
AD-F0ERI	TCGAATTCGTCCTGACTTCGCCGGAC	523	AmpD inactivation
AD-R3HDIII	TCAAGCTTCACGGACCCAGCCGGTAAC		
AD-F2HDIII	TCAAGCTTGCCTGGACCCGAACGAAC	512	
AD-RBHI	TCGGATCCGAGGGCAGATCCTCGACCAG		
ADh2-FBHI	TCGGATCCGCCTGACCGCGCTGCCC	470	AmpDh2 inactivation
ADh2-R2HDIII	TCAAGCTTGATGCCGTGGCGCTTGGC		
ADh2-F3HDIII	TCAAGCTTGGAAGCGCCTCGCCGACG	438	
ADh2-R0ERI	TCGAATTCGGACGCAAGAAGGGCGGC		
ADh3-FBHI	TCGGATCCCTTGGCCGGCCCCTGAAC	443	AmpDh3 inactivation
ADh3-R3HDIII	TCAAGCTTGTCGGGGAAGGTGAACACG		
ADh3-F3HDIII	TCAAGCTTGAAGAACGTGGTCGGCCAC	443	
ADh3-RERI	TCGAATTCGCGACGACCTGAGCGACG		
ACrnaF	GGGCTGGCCTCGAAAGAGGAC	246	Quantification of *ampC* mRNA
ACrnaR	GCACCGAGTCGGGGAACTGCA		
ADrnaF	CGCGCATTTCCTCATCGAACGC	251	Quantification of *ampD* mRNA
ADrnaR	TCGCAGTGGCCCTGGATGCG		
AErnaF	CCGCTGCACCTTCTGGTGGTG	246	Quantification of *ampE* mRNA
AErnaR	GCCACCGGGCCGAGCAAGGCA		
ADh2rnaF	TCGCTGGGCATCCTCACCCAC	248	Quantification of *ampDh2* mRNA
ADh2rnaR	TCAGCGCCTGGATCTGTGCTTC		
ADh3rnaF	TGGTGCTGCACTACACCGCGC	246	Quantification of *ampDh3* mRNA
ADh3rnaR	CGAGGTTGACGATCTCGATGCC		

[a] Sites for restriction endonucleases are underlined. Primer sequences were obtained from the published PAO1 genome (40).

mid pCM157 was electroporated into the different mutants as previously described (38). Transformants were selected in 250 μg/ml tetracycline LB agar plates. One transformant for each mutant was grown overnight in 250 μg/ml tetracycline LB broth in order to allow expression of the *cre* recombinase. Plasmid pCM157 was then cured from the strains by three successive passages on LB broth. Selected colonies were then screened for tetracycline and gentamicin susceptibility. Finally, the knockout mutants were checked by PCR amplification and sequencing to ascertain that the corresponding genes were properly disrupted. The three double mutants and the triple mutant were then constructed from the single mutants sequentially by the same procedure.

Quantification of β-lactamase activity. β-Lactamase specific activity (nanomoles of nitrocefin hydrolyzed per minute per milligram of protein) was determined spectrophotometrically on crude sonic extracts from strain PAO1 and the seven above-described *ampD* mutants as previously described (18). To determine the β-lactamase specific activity after induction, before the preparation of the crude sonic extracts, the strains were grown in the presence of 50 μg/ml cefoxitin for 3 h. Alternatively, when specifically indicated, induction experiments were performed by incubation in the presence of ceftazidime (0.5 or 20 μg/ml). In all cases, the mean β-lactamase activity values obtained in three independent experiments were considered. The phenotypic determination of AmpC inducibility was performed by assessing the presence of antagonism between imipenem and ceftazidime disks in Müller-Hinton agar plates.

Quantification of the expression of *ampC*, *ampD*, *ampE*, *ampDh2*, and *ampDh3*. The levels of expression of *ampC*, *ampD*, *ampE*, *ampDh2*, and *ampDh3* were determined by real-time PCR in strain PAO1 and the seven *ampD* mutants with and without cefoxitin induction. Total RNA from logarithmic-phase-grown cultures (with and without 50 μg/ml cefoxitin) was obtained with the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and adjusted to a final concentration of 50 ng/μl. A 500-ng sample of purified RNA was then used for one-step reverse transcription and real-time PCR amplification with the QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Hilden, Germany) in a SmartCycler II (Cepheid, Sunnyvale, CA). The primers listed in Table 2 and previously described RpsL-1 and RpsL-2 (34) were used for amplification of *ampC*, *ampD*, *ampE*, *ampDh2*, *ampDh3*, and *rpsL* (used as a reference to calculate the relative amount of mRNA). In all cases, the mean values of relative mRNA expression obtained in three independent duplicate experiments were considered.

Complementation assays. For complementation experiments, plasmids pUCPADh2 and pUCPADh3; previously described plasmids pUCPAD and pUCPADE, harboring the PAO1 wild-type *ampD* gene and the complete *ampDE* operon, respectively (18); and plasmid pUCP24 (as a control) were electroporated into the different *ampD* mutants or PAO1. Transformants were selected in 50 μg/ml gentamicin LB agar plates. Ceftazidime MICs and β-lactamase activity were determined to evaluate the complementation of the AmpC hyperproduction phenotypes.

RESULTS

***ampDh2* and *ampDh3* are functional *ampD* homologues.** Figure 1 shows a ClustalW multiple-sequence alignment of the predicted amino acid sequences of AmpDh2 and AmpDh3 compared to the AmpD proteins from *P. aeruginosa* and several *Enterobacteriaceae*. AmpDh2 and AmpDh3 were 27 and 25% identical to *P. aeruginosa* AmpD and 26 and 26% identical to *E. cloacae* AmpD, respectively. The percentage of identity between AmpDh2 and AmpDh3 was 40%. PCR and sequencing of *ampDh2* and *ampDh3* from 10 different clonal types of *P. aeruginosa* clinical strains confirmed that both genes are highly conserved in this species. Furthermore, both AmpDh2 and AmpDh3 contain all of the residues previously found to be essential for *C. freundii* AmpD catalytic activity, including His34, His154, Lys162, and Asp164 (9). *ampDh2* is located in the chromosome of PAO1, between the alginate production regulatory gene *kinB* (PA5484) and PA5486, coding for a protein of unknown function, whereas *ampDh3* is located between PA0806 and PA0808, both encoding theoretical proteins of unknown function.

As previously noted (23), inactivation of *ampD* in strain PAO1 (PAΔD) led to a partially derepressed phenotype characterized by increased AmpC production but retaining further inducibility. This phenotype was associated with an

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AmpD-E.col.    ------MLLE--QGWLVGARRVPSPHYDCRPDDETPTLLVVHNISLPPGEFGGPWIDALF 52
AmpD-C.fre.    ------MLLD--EGWLAEARRVPSPHYDCRPDDENPSLLVVHNISLPPGEFGGPWIDALF 52
AmpD-E.clo     ------MLLE--NGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALF 52
AmpD-P.aer.    ------MHFDSVTGWVRGVRHCPSPNFNLRPQGDAVSLLVIHNISLPPGQFGTGKVQAFF 54
AmpDh2-P.aer.  MRSFVLLAFTLSLLAGCSSGPRLNTDYTSANQDSRVQFIVLHYTSTDLPHS----LGILT 56
AmpDh3-P.aer.  -------MLTIDY----------NSYRTTTPYGKRVRFLVLHYTALDFAAS----VKALT 39
                      :              ..       ..   ::*:*  :          :  :

AmpD-E.col.    TGTIDPQAHPFFAEIAHLRVSAHCLIRRDGEIVQYVPFDKRAWHAGVSQYQGRERCNDFS 112
AmpD-C.fre.    TGTIDPNAHPYFAGIAHLRVSAHCLIRRDGEIVQYVPFDKRAWHAGVSSYQGRERCNDFS 112
AmpD-E.clo.    TGTIDPDAHPFFAEIAHLALSADCLIRRDGEVVQYVPFDKRAWHAGVSMYQGRERCNDFS 112
AmpD-P.aer.    QNRLDPNEHPYFEEIRHLTVSAHFLIERDGAITQFVSCHDRAWHAGVSCFDGREACNDFS 114
AmpDh2-P.aer.  HGGVS--AHYLIGD-DEP-----------ATVYRLVDENRRAWHAGVSEWQGRTWLNATS 102
AmpDh3-P.aer.  TGAAS--AHYLIPAPHDPSYKAAGFK--GQRIFNLVAEEDRAWHAGVSGWARRDNLNDTS 95
                 .  .   *  :    .              : . *  . ******** :  *   *  *

AmpD-E.col.    IGIELEGTD----------TLAYTDAQYQQLAAVTRALIDCYPDIAKN-MTGHCDIAPDR 161
AmpD-C.fre.    IGIELEGTD----------TLAYTDAQYQQLAAVTNALITRYPAIANN-MTGHCNIAPER 161
AmpD-E.clo.    IGIELEGTD----------TTPYTDAQYEKLVAVTQTLIGRYPAIADN-ITGHSDIAPER 161
AmpD-P.aer.    LGIELEGTD----------TEPYTDAQYTALAGLTRLLRAAFPGITPERIQGHCDIAPER 164
AmpDh2-P.aer.  IGIEIVNQGYRDTPQGRVWYP-FSEAQIQALIPLLKDIAKRH-GITPDRIIGHSDIAPGR 160
AmpDh3-P.aer.  IGIEIVNLARDDD--GVFTFPDYERSQINALKQLAKNILQRYPDMTPKNVVGHSDIAVGR 153
               :***: .               :  :*   *  : . :   .  :: . : **.:**  *

AmpD-E.col.    KTDPGPAFDWARFRVLVS----KETT------------------------------- 183
AmpD-C.fre.    KTDPGPSFDWARFRALVTPSSHKEMT--------------------------------- 187
AmpD-E.clo.    KTDPGPAFDWSRFHAMLTTSSDKEIT--------------------------------- 187
AmpD-P.aer.    KTDPGEAFDWSRYRAGLTDS--KEET--------------------------------- 188
AmpDh2-P.aer.  KVDPGPLFPWKRLADAGLVPWPKPGELARRLAELN-GQLPDVRWFQQQLARHGYLVPQTG 219
AmpDh3-P.aer.  KSDPGPKLPWKELYEAGIGAWYDDATRDRYREGFERDGLPPRADLLEAFRLYGYALPATV 213
               * ***  : * .          .

AmpD-E.col.    ------------------------------------------
AmpD-C.fre.    ------------------------------------------
AmpD-E.clo.    ------------------------------------------
AmpD-P.aer.    ------------------------------------------
AmpDh2-P.aer.  ELEKDTRDVIGAFQMKYRPARFDGEPDLETAALLLAVPTS--- 259
AmpDh3-P.aer.  D-DAYFASLLRAFQMHFRPENYDGALDVETAAILYALNEKYPA 255
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FIG. 1. ClustalW multiple-sequence alignment of *P. aeruginosa* AmpDh2 and AmpDh3 and the previously described AmpD proteins from *P. aeruginosa*, *E. coli*, *E. cloacae*, and *C. freundii*. Conserved residues previously found to be essential for *C. freundii* AmpD catalytic activity (9) are in bold. Dashes indicate gaps. Asterisks, colons, and periods indicate identical, conserved, and semiconserved residues, respectively.

eightfold increase in the ceftazidime MICs. As shown in Table 3, the plasmids harboring *ampDh2* (pUCPADh2) and *ampDh3* (pUCPADh3) completely transcomplemented the AmpC hyperproduction phenotype of PAΔD as readily as those harboring the regular *ampD* gene (pUCPAD) and the complete *ampDE* operon (pUCPADE), demonstrating that they both have functional *N*-acetyl-anhydromuramyl-L-alanine amidase activity.

TABLE 3. Complementation of the AmpC hyperproduction phenotype of the PAO1 *ampD* mutant (PAΔD) with plasmids harboring wild-type *ampD* (pUCPAD), *ampDE* (pUCPADE), *ampDh2* (pUCPADh2), and *ampDh3* (pUCPADh3)

Strain	Ceftazidime MIC (μg/ml)	Avg β-lactamase sp act[a] ± SD	
		Basal	Induced
PAO1	1	1	52 ± 13
PAΔD	8	76 ± 18	250 ± 45
PAΔD(pUCP24)	8	60 ± 21	260 ± 52
PAΔD(pUCPAD)	1	1.7 ± 0.9	89 ± 32
PAΔD(pUCPADE)	1	1.4 ± 0.6	39 ± 14
PAΔD(pUCPADh2)	1	1.5 ± 0.5	73 ± 25
PAΔD(pUCPADh3)	1	2.7 ± 1.3	38 ± 16

[a] Relative values of β-lactamase activity considering 1 the basal activity of the reference strain PAO1 (19 nmol of nitrocefin hydrolyzed/min/mg of protein). For induction experiments, the strains were grown in the presence of 50 μg/ml cefoxitin for 3 h.

Role of *ampD* homologues in *ampC* regulation and β-lactam resistance. Table 4 shows the levels of *ampC* expression under basal and cefoxitin-induced conditions and the susceptibilities to the antipseudomonal β-lactams of strains PAO1 and the seven *ampD* single, double, and triple mutants. *ampD* inactivation in strain PAO1 (PAΔD) led to a 60-fold increase in *ampC* expression, reaching 150-fold under cefoxitin induction, and was associated with a significant increase, albeit not surpassing CLSI nonsusceptibility breakpoints, of the MICs of all of the antipseudomonal penicillins and cephalosporins. The resistance increase was highest for piperacillin, piperacillin-tazobactam, and ceftazidime and lowest for ticarcillin and cefepime. As for the highly β-lactamase hydrolysis-resistant carbapenems, the MICs of imipenem were not significantly affected but those of meropenem were considerably raised, albeit they remained far from the nonsusceptibility breakpoints. On the other hand, the inactivation of neither *ampDh2* nor *ampDh3* (PAΔDh2 and PAΔDh3) significantly modified *ampC* expression or the antipseudomonal β-lactam MICs. Furthermore, the *ampDh2-ampDh3* double inactiva-

TABLE 4. Levels of *ampC* expression under basal and cefoxitin-induced conditions and MICs of antipseudomonal β-lactams for strain PAO1 and seven *ampD* mutants

Strain	Avg level of *ampC* expression[a] ± SD		MIC (μg/ml)[b]							
	Basal	Induced	CAZ (≤8–>16)	FEP (≤8–>16)	TIC (≤64–>64)	PIP (≤64–>64)	PIP-TZ (≤64–>64)	ATM (≤8–>16)	IMP (≤4–>8)	MER (≤4–>8)
PAO1		43 ± 9	1	1	12	2	4	1	1.5	0.38
PAΔD	60 ± 19	152 ± 38	8	4	32	64	96	6	2	1
PAΔDh2	NC[c]	48 ± 15	0.75	1	8	1.5	3	1	1	0.38
PAΔDh3	NC	55 ± 5	1	1	12	3	4	1.5	1	0.38
PAΔDh2Dh3	2.3 ± 0.14	81 ± 26	0.75	1	6	2	3	0.5	1.5	0.38
PAΔDDh2	62 ± 9	234 ± 58	12	12	256	48	64	16	1	0.38
PAΔDDh3	191 ± 52	1,014 ± 297	48	32	>256	>256	>256	16	1.5	2
PAΔDDh2Dh3	1,020 ± 87	1,105 ± 88	48	32	>256	>256	>256	24	1.5	2

[a] Relative amount of *ampC* mRNA compared to PAO1 basal expression. Induction experiments were carried out with 50 μg/ml cefoxitin.
[b] CAZ, ceftazidime; FEP, cefepime; TIC, ticarcillin; PIP, piperacillin; PIP-TZ, piperacillin-tazobactam; ATM, aztreonam; IMP, imipenem; MER, meropenem. CLSI susceptibility and resistance breakpoints for each antibiotic are shown in parentheses (5a).
[c] NC, no change in *ampC* mRNA level compared to PAO1 basal expression.

tion (PAΔDh2Dh3) only slightly increased *ampC* basal and induced expression (twofold compared to PAO1) but did not produce any increase in β-lactam resistance, rather the opposite; MICs were 1 dilution lower than those of some of the antibiotics for PAO1 (Table 4).

This apparent lack of effect of the *ampD* homologues on the regulation of the chromosomal cephalosporinase and β-lactam resistance drastically changed when the *ampD ampDh2* (PAΔDDh2) and *ampD ampDh3* (PAΔDDh3) double mutants were analyzed. Regarding *ampC* expression, PAΔDDh2 caused only a modest increase in the inducibility of β-lactamase compared to PAΔD, whereas PAΔDDh3 caused a dramatic increase in both the basal and induced *ampC* levels (Table 4). The effect of PAΔDDh2 on β-lactam resistance was variable; it greatly increased the cefepime, aztreonam, and ticarcillin MICs, modestly increased those of ceftazidime, and modestly reduced those of piperacillin (with and without tazobactam) and meropenem compared to PAΔD. On the other hand, PAΔDDh3 dramatically increased the MICs (surpassing the clinical resistance breakpoints) of all of the antipseudomonal penicillins and cephalosporins and further increased the MICs of the carbapenem β-lactam meropenem. Finally, the triple mutant PAΔDDh2Dh3 caused a dramatic increase in the basal *ampC* expression level (1,000-fold compared to PAO1, 17-fold compared to PAΔD), which was not further cefoxitin inducible, showing that, finally, complete derepression was reached. Initially surprising, the antipseudomonal β-lactam MICs were not significantly raised for the completely derepressed triple mutant compared to the double mutant PAΔDDh3. The explanation for this apparently odd finding was found to be actually simple: subinhibitory concentrations of the, in theory, weak AmpC inducer β-lactams such as ceftazidime induced PAΔDDh3 AmpC production to values 1,000-fold higher than that of PAO1 (reaching the level of complete derepression) just as well as the potent inducer cefoxitin, as shown in Table 5.

Model for stepwise upregulation of *ampC* and high-level β-lactam resistance. From the above-described results, it can be deduced that upregulation to full derepression of the expression of *P. aeruginosa ampC* is achieved by a three-step escalating mechanism rendering four relevant expression states (shown in Fig. 2) and resistance phenotypes (pictures shown in Fig. 3): basal-level inducible expression (PAO1 wild type), moderate-level hyperinducible expression with increased antipseudomonal β-lactam resistance (PAΔD), high-level hyperinducible expression with high-level β-lactam resistance (PAΔDDh3), and very high-level derepressed expression not further increasing the already high-level β-lactam resistance (PAΔDDh2Dh3).

Interestingly, as shown in Table 6, any of the three *ampD* homo-

TABLE 5. Basal and cefoxitin- and ceftazidime-induced β-lactamase activities of double mutant PAΔDDh3 and triple mutant PAΔDDh2Dh3

Strain	Avg β-lactamase sp act[a] ± SD			
	Basal	Cefoxitin (50 μg/ml)	Ceftazidime (0.5 μg/ml)	Ceftazidime (20 μg/ml)
PAO1	1	52 ± 13	1.6 ± 0.2	NA[b]
PAΔDDh2Dh3	976 ± 165	977 ± 114	1,050 ± 125	995 ± 85
PAΔDDh3	210 ± 37	1,010 ± 35	780 ± 64	1,088 ± 122

[a] Relative values of β-lactamase activity considering 1 the basal activity of reference strain PAO1 (19 nmol of nitrocefin hydrolyzed/min/mg of protein).
[b] NA, not applicable (since the MIC for PAO1 is 1 μg/ml).

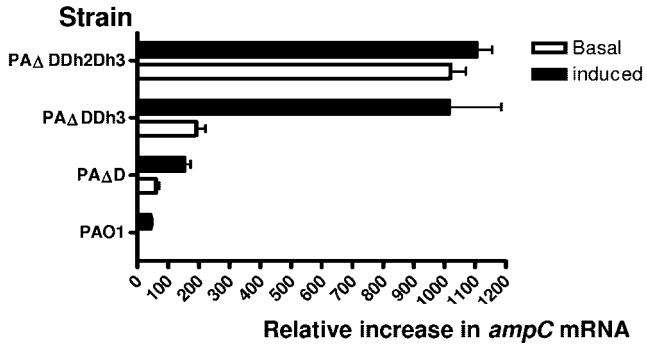

FIG. 2. Representation of the three-step escalating mechanism rendering four relevant expression states: basal-level inducible expression (PAO1), moderate-level hyperinducible expression (PAΔD), high-level hyperinducible expression (PAΔDDh3), and very high-level derepressed expression (PAΔDDh2Dh3).

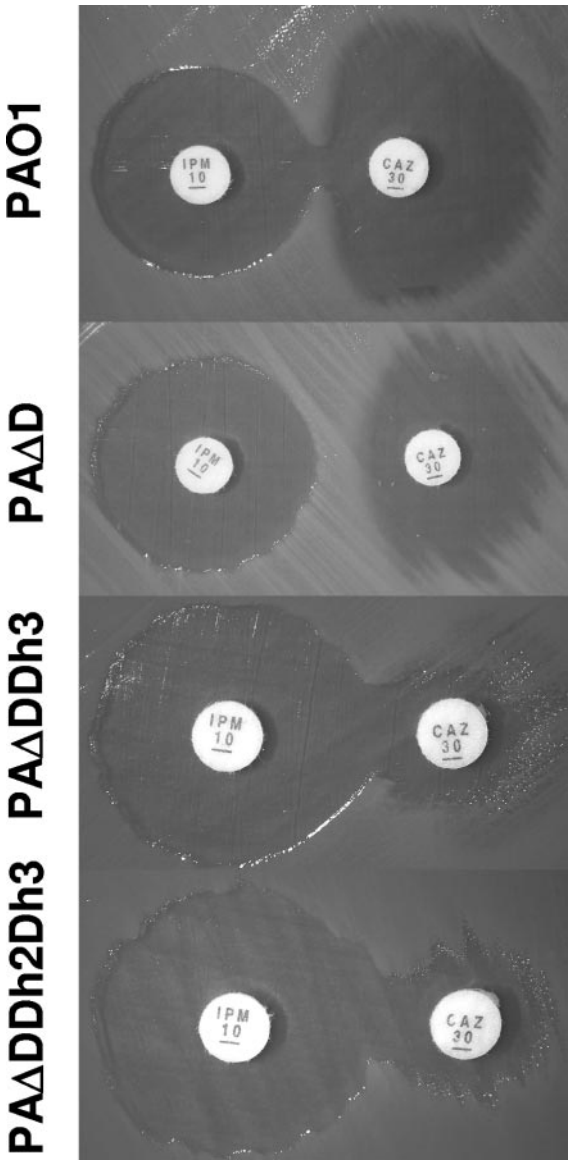

FIG. 3. Assessment of AmpC inducibility and antipseudomonal β-lactam resistance phenotypes with imipenem (IPM) and ceftazidime (CAZ) disks. PAO1, highly ceftazidime susceptible, AmpC-inducible phenotype; PAΔD, moderately ceftazidime resistant, AmpC-inducible phenotype; PAΔDDh3, highly ceftazidime resistant, apparently derepressed phenotype (produced by ceftazidime induction of AmpC to the maximum production level); PAΔDDh2Dh3, highly ceftazidime resistant, AmpC-derepressed phenotype.

logues, when produced from the high-copy-number pUCP24 derivatives (pUCPAD, pUCPADh2, and pUCPADh3), returned the very high-level derepressed expression and high-level β-lactam resistance of PAΔDDh2Dh3 to wild-type PAO1 levels, showing that the multiple-step upregulation model responds to quantitative rather than qualitative differences among the three *ampD* homologues involved.

Expression of *ampD* homologues is not regulated by AmpC inducers. The levels of expression of *ampD*, *ampE*, *ampDh2*, and *ampDh3* genes from strain PAO1 were quantified under basal and cefoxitin-induced conditions to find out if the expression of any of them was modified (up- or downregulated) by incubation in the presence of AmpC inducers, but no significant differences in the levels of mRNA were detected (data not shown). Similarly, the inactivation of any of the *ampD* homologues did not modify the expression of the other *ampD* genes (i.e., the expression of *ampDh2* or *ampDh3* was not modified in PAΔD compared to PAO1, and the same for the other combinations), showing that the expression of the different *ampD* homologues apparently is not interregulated. Finally, *ampD* inactivation did not cause an increase in its own transcription, which would be consistent with the constitutive expression of this gene. In contrast, the transcription of *ampDh2* or *ampDh3* was slightly increased when the respective gene was inactivated: a two- to fivefold increase in *ampDh2* or *ampDh3* mRNA was observed for all of the single, double, and triple *ampDh2* (PAΔDh2, PAΔDDh2, PAΔDh2Dh3, and PAΔDDh2Dh3)- or *ampDh3* (PAΔDh3, PAΔDDh3, PAΔDh2Dh3, and PAΔDDh2Dh3)-inactivated mutants, respectively, compared to wild-type PAO1. These results suggest that the expression of *ampDh2* and *ampDh3* is inducible.

DISCUSSION

Development of resistance to the antipseudomonal penicillins and cephalosporins mediated by hyperproduction of the chromosomal cephalosporinase AmpC is a major threat to the successful treatment of *P. aeruginosa* infections, especially those affecting critical patients in ICUs or in chronically colonized patients such as those suffering from cystic fibrosis. Although *ampD* inactivation has been previously found to lead to a partially derepressed phenotype (23) and a few natural *ampD* mutants have been characterized (1, 18), the regulation of *ampC* in *P. aeruginosa* is far from well understood. In this work, we show that the regulation of the *P. aeruginosa* cephalosporinase is likely the most sophisticated repression-derepression system described in the microbial world so far, finding that *ampC* expression is coordinately repressed by three *ampD* homologues. These three AmpD homologues are responsible for a stepwise *ampC* upregulation mechanism ultimately leading to constitutive hyperexpression (more than 1,000-fold) of the

TABLE 6. Complementation of the AmpC hyperproduction phenotype of the PAO1 *ampD* triple mutant (PAΔDDh2Dh3) with plasmids harboring wild-type *ampD* (pUCPAD), *ampDE* (pUCPADE), *ampDh2* (pUCPADh2), and *ampDh3* (pUCPADh3)

Strain	Ceftazidime MIC (μg/ml)	Avg β-lactamase sp act[a] ± SD	
		Basal	Induced
PAO1	1	1	52 ± 13
PAΔDDh2Dh3	48	976 ± 165	977 ± 114
PAΔDDh2Dh3(pUCP24)	48	932 ± 80	1,096 ± 94
PAΔDDh2Dh3(pUCPAD)	1	2.9 ± 0.6	37 ± 7
PAΔDDh2Dh3(pUCPADE)	0.75	1.6 ± 0.5	32 ± 8
PAΔDDh2Dh3(pUCPADh2)	1.5	2.4 ± 0.6	119 ± 23
PAΔDDh2Dh3(pUCPADh3)	1.5	2.1 ± 0.4	47 ± 11

[a] Relative values of β-lactamase activity considering 1 the basal activity of reference strain PAO1 (19 nmol of nitrocefin hydrolyzed/min/mg of protein). For induction experiments, the strains were grown in the presence of 50 μg/ml cefoxitin for 3 h.

chromosomal cephalosporinase and high-level (clinically relevant) antipseudomonal β-lactam resistance. Although one-step inducible-derepressed expression models are frequent in natural resistance mechanisms, this is the first characterized example in which expression can be sequentially amplified through multiple steps of derepression.

Development of resistance by AmpC hyperproduction is also a major resistance threat in *Enterobacteriaceae* such as *E. cloacae* and *C. freundii*, where the role of AmpD as a repressor of β-lactamase expression was actually first characterized (13, 25). In principle, it is generally accepted that in *Enterobacteriaceae*, AmpC is regulated by a one-step inducible-derepressed expression model in which constitutive hyperproduction is reached by AmpD inactivation. Nevertheless, homology searches of the databases with the complete sequences of *E. coli* and other members of the family *Enterobacteriaceae* revealed the presence of one, and just one, *ampD* homologue in addition to the regular *ampD* gene. In the light of this finding, it is tempting to speculate that *ampC* regulation in *Enterobacteriaceae* actually may respond to a two-step inducible-derepressed expression model. Previous findings showing a semiconstitutive AmpC hyperproduction phenotype in *C. freundii ampD* mutants may support this hypothesis (25).

Further studies are necessary to elucidate the in vivo dynamics of the AmpC derepression mediated by the three AmpD homologues during the treatment of *P. aeruginosa* infections with β-lactams and the interplay between the three described *ampC* repressors and other physiological functions, since the regulation of the chromosomal β-lactamases is intimately linked to cell wall recycling, which may modulate bacterial virulence (8, 22). The presence of up to three AmpD homologues in *P. aeruginosa* may certainly be beneficial for this microorganism because in addition to allowing it to acquire different levels of β-lactamase expression and β-lactam resistance, it may, in the *ampD* single mutant or in the *ampD ampDh2* or *ampD ampDh3* double mutant (partially derepressed phenotypes), permit hyperproduction of the cephalosporinase without disrupting the cell wall recycling process.

Interestingly, one of the *ampD* homologues (*ampDh2*) is located just contiguous to the alginate production regulatory genes *algB* and *kinB* (40). The reciprocal interaction between the *ampC* regulatory machinery and other cellular processes is indeed an issue of high potential relevance. For instance, Nuñez et al. (33) found that the *ampDE* operon of *Azotobacter vinelandii* is involved in alginate production and bacterial encystment. In this sense, Bagge et al. (2) found that the most potent AmpC inducer, imipenem, increased not only the expression of the chromosomal β-lactamase but also that of the genes coding for alginate biosynthesis in *P. aeruginosa* biofilms. It is noteworthy that alginate hyperproduction is a key factor in the development of *P. aeruginosa* chronic infections such as those of cystic fibrosis patients (12). Finally, it remains to be elucidated whether, in addition to their role as *ampC* repressors, the three AmpD homologues affect the expression of the gene encoding the recently described *P. aeruginosa* oxacillinase OXA-50 or PoxB (10, 19). This β-lactamase has been recently shown to be negatively regulated by AmpR, which is the opposite of the observed effect on *ampC* expression (20).

In summary, we describe the highly sophisticated mechanism of stepwise upregulation of the *P. aeruginosa* chromosomal cephalosporinase ultimately leading to constitutive hyperexpression of AmpC and high-level antipseudomonal β-lactam resistance. This system is the first characterized example in which the expression of a resistance mechanism can be sequentially amplified through multiple steps of derepression.

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