Benefit of Having Multiple *ampD* Genes for Acquiring β -Lactam Resistance without Losing Fitness and Virulence in *Pseudomonas aeruginosa*[▽]

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The inactivation of *ampD* in *Pseudomonas aeruginosa* leads to a partially derepressed phenotype, characterized by a moderately high level basal *ampC* expression that is still further inducible, due to the presence of two additional *ampD* genes in this species (*ampDh2* and *ampDh3*). The sequential inactivation of the three *ampD* genes was shown to lead to a stepwise upregulation of *ampC* expression, reaching full derepression in the triple mutant. To gain insight into the biological role of *P. aeruginosa* AmpD multiplicity, we determined the effects of the inactivation of the *ampD* genes on fitness and virulence. We show that, in contrast to what was previously documented for *Salmonella* spp., the inactivation of *ampD* in *P. aeruginosa* does not affect fitness or virulence in a mouse model of systemic infection. This lack of effect was demonstrated to be dependent on the presence of the additional *ampD* genes (*ampDh2* and *ampDh3*), since the double and the triple *ampD* mutants completely lost their biological competitiveness and virulence; full *ampC* derepression and disruption of the AmpD peptidoglycan recycling system itself are both found to cause a major biological cost. Furthermore, among the *ampD* genes, *ampDh3* is found to be the most relevant for virulence in *P. aeruginosa*. Therefore, as a consequence of the presence of additional *ampD* genes, partial *ampC* derepression mediated by *ampD* inactivation confers a biologically efficient resistance mechanism on *P. aeruginosa*.

Pseudomonas aeruginosa, as well as many other nonfermenting gram-negative bacilli and most members of the *Enterobacteriaceae*, produces a chromosomally encoded group I, class C cephalosporinase designated AmpC (2). Although AmpC is produced at very low basal levels in wild-type strains, its expression is highly inducible in the presence of certain β -lactams (β -lactamase inducers) such as cefoxitin or imipenem (23). In fact, the antipseudomonal penicillins (such as ticarcillin or piperacillin) and cephalosporins (such as ceftazidime or cefepime) are very weak AmpC inducers, despite the fact that they are hydrolyzed by this enzyme (23). For this reason, during treatment with these weak inducers, mutants showing constitutively high level AmpC production (AmpC-derepressed mutants) are frequently selected, leading to the failure of antimicrobial therapy (8, 14, 15, 22).

Several genes are involved in the regulation of *ampC* expression, a process that is intimately linked to peptidoglycan recycling (28). *ampG* encodes a transmembrane permease for 1,6-anhydromuropeptides, which are thought to be the signal molecules involved in AmpC induction, through interaction with the LysR-type transcriptional regulator AmpR (5, 12, 13, 18, 20). During regular bacterial growth, muropeptides are processed by the *N*-acetyl-anhydromuramyl-L-alanine amidase AmpD, which therefore plays two major roles: (i) it is required for the recycling of peptidoglycan catabolism products, and (ii) it prevents *ampC* induction (11, 21, 28). On the other hand,

during growth in the presence of strong β -lactamase inducers such as cefoxitin, muropeptides are generated and accumulate in the cytoplasm (due to the saturation of AmpD), leading to the AmpR-mediated induction of *ampC* expression (5, 12, 13, 20, 28). It is also well known that the mutational inactivation of AmpD leads to the accumulation of muropeptides and high-level *ampC* expression, even in the absence of β -lactamase inducers, producing the classical constitutively derepressed phenotype of AmpC production (21, 28). In fact, AmpD inactivation is the most frequently reported mechanism leading to AmpC hyperproduction and β -lactam resistance both in *Enterobacteriaceae* and in *P. aeruginosa* clinical isolates (1, 15, 19, 31).

The interplay between resistance mechanisms and bacterial fitness and virulence is a subject of growing interest. The latter are indeed key parameters for predicting the clinical relevance of the resistance mechanisms (effect on virulence), as well as their epidemiological transcendence, dependent on their capacity for persistence in bacterial populations once the selective pressure exerted by the antibiotics disappears (effect on fitness) (25, 26). Previous work with *Salmonella* spp., which lack the *ampC* gene but contain *ampD*, has revealed two interesting findings in this field: (i) the inactivation of *ampD* in *Salmonella* spp. is associated with a significant biological cost and decreased virulence, mediated by the accumulation of muropeptides (7), and (ii) high-level expression of *ampC* from a foreign plasmid also leads to significant decreases in virulence and in vivo fitness (27).

Interestingly, in contrast to the classical fully derepressed phenotypes observed in *Enterobacteriaceae*, the inactivation of AmpD in *P. aeruginosa* has been shown to lead to a partially derepressed phenotype, characterized by a moderately high

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TABLE 1. Strains and plasmids used or constructed

Strain or plasmid	Genotype/relevant characteristic(s) ^a	Reference or source
Strains		
<i>P. aeruginosa</i>		
PAO1	Reference strain, completely sequenced	Laboratory collection
PAΔDGm	PAO1 <i>ΔampD::Gmlox</i>	16
PAΔD	PAO1 <i>ΔampD::lox</i>	16
PAΔDh2Gm	PAO1 <i>ΔampDh2::Gmlox</i>	16
PAΔDh2	PAO1 <i>ΔampDh2::lox</i>	16
PAΔDh3Gm	PAO1 <i>ΔampDh3::Gmlox</i>	16
PAΔDh3	PAO1 <i>ΔampDh3::lox</i>	16
PAΔDDh2Gm	PAO1 <i>ΔampD::lox ΔampDh2::Gmlox</i>	16
PAΔDDh2	PAO1 <i>ΔampD::lox ΔampDh2::lox</i>	16
PAΔDDh3Gm	PAO1 <i>ΔampD::lox ΔampDh3::Gmlox</i>	16
PAΔDDh3	PAO1 <i>ΔampD::lox ΔampDh3::lox</i>	16
PAΔDDh2Dh3Gm	PAO1 <i>ΔampD::lox ΔampDh2::lox ΔampDh3::Gmlox</i>	16
PAΔDDh2Dh3	PAO1 <i>ΔampD::lox ΔampDh2::lox ΔampDh3::lox</i>	16
PAΔCGm	PAO1 <i>ΔampC::Gmlox</i>	This work
PAΔC	PAO1 <i>ΔampC::lox</i>	This work
PAΔDDh2Dh3CGm	PAO1 <i>ΔampD::lox ΔampDh2::lox ΔampDh3::lox ΔampC::Gmlox</i>	This work
PAΔDDh2Dh3C	PAO1 <i>ΔampD::lox ΔampDh2::lox ΔampDh3::lox ΔampC::lox</i>	This work
<i>E. coli</i>		
XL1-Blue	F'::Tn10 <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q <i>Δ(lacZ)</i> M15/ <i>recA1 endA1 gyrA96</i> (Nal ^r) <i>thi hsdR17</i> (r _K ⁻ m _K ⁻) <i>mcrB1</i>	Laboratory collection
S17-1	RecA <i>pro</i> (RP4-2Tet::Mu Kan::Tn7)	Laboratory collection
Plasmids		
pEX100Tlink	Ap ^r <i>sacB</i> ; pUC19-based gene replacement vector with an MCS	29
pUCGmlox	Ap ^r Gm ^r ; pUC18-based vector containing the <i>lox</i> -flanked <i>aacC1</i> gene	29
pCM157	Tc ^r ; <i>cre</i> expression vector	29
pEXACGm	pEX100Tlink containing 5' and 3' flanking sequences of <i>ampC::Gmlox</i>	This work

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

level basal *ampC* expression that is still further inducible in the presence of inducers (19). Recent studies have shown that the partial derepression of the *P. aeruginosa ampD* mutant is due to the presence of two additional *ampD* genes, designated *ampDh2* and *ampDh3*, in this species (16). Furthermore, the sequential inactivation of the three *ampD* genes has been shown to lead to a stepwise upregulation of *ampC* expression, reaching full derepression with very high level basal *ampC* expression (more than 1,000-fold compared to wild-type expression) in the triple mutant (16). Certainly, this is an interesting and complex system that needs to be studied further, since it is the first instance in which the expression of a chromosomally encoded resistance mechanism can be sequentially amplified through multiple steps of derepression. In this work, we explored the biological role of *P. aeruginosa* AmpD multiplicity in providing a mechanism for the sequential amplification of an antibiotic resistance gene and in avoiding the biological cost of the first-step resistant mutant (single *ampD* mutant).

MATERIALS AND METHODS

Bacterial strains, plasmids, and antibiotics. Laboratory strains and plasmids used or constructed in this work are listed in Table 1. We used the *P. aeruginosa* reference strain PAO1 and the previously constructed single *ampD* gene knockout mutants (PAΔD [*ampD*], PAΔDh2 [*ampDh2*], and PAΔDh3 [*ampDh3*]), double *ampD* mutants (PAΔDDh2 [*ampD ampDh2*] and PAΔDDh3 [*ampD*

ampDh3]), and triple *ampD* mutant (PAΔDDh2Dh3 [*ampD ampDh2 ampDh3*]) (16). These mutants had been constructed by following the procedure described by Quéneé et al. (29) for gene deletion and antibiotic resistance marker recycling in *P. aeruginosa* using the *cre-lox* system. By this procedure, two isogenic pairs of strains were obtained for each of the knockout mutants: one with and one without the (gentamicin) resistance marker (16). The antibiotics used (gentamicin, ampicillin, carbenillicin, and tetracycline) were purchased from Sigma-Aldrich.

Construction of *ampC* knockout mutants. The procedure for gene deletion and antibiotic resistance marker recycling in *P. aeruginosa* using the *cre-lox* system, described above, was used for the construction of the *ampC* knockout mutants of PAO1 (PAΔC) and of the triple *ampD* mutant (PAΔDDh2Dh3C). Briefly, upstream and downstream PCR products (Table 2) of *ampC* were digested with either BamHI or EcoRI and HindIII and then cloned by three-way ligation into pEX100Tlink deleted for the HindIII site and opened by EcoRI and BamHI to produce plasmid pEXAC, which was transformed into *Escherichia coli* strain XL1-Blue. Transformants were selected on LB agar (LBA) plates with 30 μg/ml ampicillin. The *lox*-flanked gentamicin resistance cassette (*aac1*) obtained by HindIII restriction of plasmid pUCGmlox was cloned into the single site for this enzyme formed by ligation between the two flanking fragments, producing plasmid pEXACGm, which was transformed into *E. coli* XL1-Blue. Transformants were selected on LBA plates with 30 μg/ml ampicillin and 5 μg/ml gentamicin. These plasmids were then transformed into the *E. coli* helper strain S17-1. Plasmid pEXACGm was transferred from *E. coli* S17-1 to PAO1 or PAΔDDh2Dh3 to produce the PAΔCGm or PAΔDDh2Dh3CGm mutant, respectively, after selection for double recombinants using LBA plates with 5% sucrose and 30 μg/ml gentamicin. Double recombinants were checked first by screening for susceptibility to carbenillicin (200 μg/ml) and afterwards by PCR amplification using primers AmpC-F-ERI and AmpC-R-BHI (Table 2). For the recycling of the gentamicin resistance cassettes (to yield the PAΔC and PAΔDDh2Dh3C mutants), plasmid pCM157 was electroporated into the differ-

TABLE 2. Primers used for the construction of the *ampC* knockout mutants

Primer	Sequence (5'–3') ^a	Location ^b	PCR product size (bp)
AmpC-F-ERI	TCGAATTCGCGCGCAGGGCGTTCAG	–186 to –169	415
AmpC-I-R-HDIII	TCAAGCTTCGTCCTCTTACGAGGCCAG ^c	210 to 229	
AmpC-I-F-HDIII	TCAAGCTTCAGGGCAGCCGCTTCGAC	366 to 384	432
AmpC-R-BHI	TGGATCCAGGTTGGCATCGACGAAG	779 to 798	

^a Restriction sites for endonucleases are underlined.
^b Relative to the *ampC* start codon.
^c A T→A substitution (boldfaced) was introduced to generate an early stop codon in the *ampC* sequence and therefore avoid the potential effect on fitness of the truncated AmpC enzyme.

ent mutants as previously described (30). Transformants were selected on LBA plates 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 three successive passages in LB broth. Selected colonies were then screened for susceptibility to tetracycline (250 µg/ml) and gentamicin (30 µg/ml) and were checked by PCR amplification and DNA sequencing.

In vitro competition experiments. In vitro competition experiments between each of the *ampD* mutants (harboring the gentamicin resistance marker) described above and PAO1 were performed. Additionally, competition experiments were performed between strains PAΔDDh2Dh3CGm and PAΔDDh2Dh3 (triple *ampD* mutants with and without *ampC* inactivated) and between strains PAΔCGm and PAO1 as controls. Exponentially growing cells of the corresponding mutant and wild-type strains in LB broth were mixed in a 1:1 ratio and diluted in 0.9% saline solution. Approximately 10³ cells from each of the mixtures were inoculated into eight 10-ml LB broth flasks and grown at 37°C and 180 rpm for 16 to 18 h, corresponding to approximately 20 generations. Serial 10-fold dilutions were plated in duplicate onto LBA alone and LBA with 15 µg/ml of gentamicin (LBA-Gm) in order to determine the total CFU and the CFU of the mutant, respectively, after overnight incubation at 37°C. Alternatively, when the difference in the number of colonies between the LBA and LBA-Gm plates was low (less than twofold), 100 randomly selected colonies from the LBA plates were replicated in LBA-Gm plates; mutant and wild-type colonies were recognized by the presence or absence of growth in these plates after overnight incubation. The competition index (CI) was defined as the mutant/wild-type ratio. CI values were calculated for each of the eight independent competitions, and the median values were recorded. Statistical analysis of the distribution of the CI values was performed using the Mann-Whitney U test. *P* values of <0.05 were considered to be statistically significant. During the standardization of the procedure, competition experiments between strains PAΔDGm (PAO1 *ampD* mutant harboring the Gm resistance cassette) and PAΔD (PAO1 *ampD* mutant not harboring the Gm resistance cassette) were performed; the CI values obtained (median CI, 0.95) ruled out any significant effect of the gentamicin resistance cassette on bacterial fitness. To assess the growth rates under non-competitive conditions, the doubling times of exponentially growing cells in LB broth at 37°C and 180 rpm were determined by plating serial 10-fold dilutions on LBA at 1-h intervals. Three independent experiments were performed for each of the mutants, and the results were compared with those for PAO1 using Student's *t* test.

Virulence and fitness in the mouse model of systemic infection. To assess the effect on virulence, approximately 5 × 10⁶ exponentially growing cells of PAO1, PAΔD, PAΔDh2, PAΔDh3, PAΔDDh2, PAΔDDh3, PAΔDDh2Dh3, or PAΔDDh2Dh3C were inoculated intraperitoneally into groups of 16 ICR(CD1) mice (Harlan Interfauna Ibérica, Barcelona, Spain) weighing 20 to 25 g, and mortality was monitored daily for 7 days. In vivo fitness was assessed by competition experiments in the mouse model of systemic infection. For this purpose, 1:1 mixtures of each of the mutant–wild-type pairs containing a total of approximately 5 × 10⁶ exponentially growing cells were inoculated intraperitoneally into eight 20- to 25-g ICR(CD1) mice. When indicated (see Results), competition experiments were also performed using a starting mutant/wild-type ratio of 10:1 or 10:10. Mice were sacrificed 24 h after inoculation, and their spleens were aseptically extracted and homogenized in 2 ml of 0.9% saline solution using the Ultra-Turrax T-25 disperser (IKA, Staufen, Germany). The number of CFU of each strain and the CI values were determined as described for in vitro competition experiments.

Sequencing of the *ampDh2* and *ampDh3* genes in AmpC-hyperproducing clinical *P. aeruginosa* isolates. A collection of 10 previously characterized pairs of isogenic clinical strains was further studied (15). Each pair of strains consisted of

an isolate showing wild-type AmpC production and a subsequent isogenic isolate showing AmpC hyperproduction, obtained after treatment with β-lactams (15). Each pair of clinical strains was recovered from a different patient, and each pair belonged to a different clone (15). Six of the 10 AmpC-hyperproducing isolates had previously been shown to contain *ampD*-inactivating mutations (15). In the present work, we explored whether these isolates may additionally contain inactivating mutations in *ampDh2* and/or *ampDh3*. For this purpose, these genes were amplified by PCR using primer pairs ADh2-FBHI–ADh2-RBHI and ADh3-FBHI–ADh3-RBHI, described previously (16), and were fully sequenced for the 10 pairs of isolates. Sequencing reactions were performed with the BigDye Terminator kit (Perkin-Elmer Applied Biosystems, Foster City, CA), and sequences were analyzed on an ABI Prism 3100 DNA sequencer (Perkin-Elmer Applied Biosystems). The resulting sequences were then compared (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with those previously reported for the wild-type PAO1 strain (16).

RESULTS

In vitro competition experiments. Figure 1 shows the CI values obtained for each of the in vitro competition experiments, and Table 3 summarizes the data on *ampC* expression and ceftazidime susceptibility, obtained as part of a previous work (16), as well as presenting the doubling time of each of the mutants studied. None of the single or double *ampD* mutants showed a significant modification of the growth rate; doubling times ranged from 28.3 to 37.6 min (30.0 min for PAO1) (Table 3). On the other hand, the triple *ampD* mutant (PAΔDDh2Dh3), in which AmpC was fully derepressed, showed a clearly reduced growth rate, with a doubling time of 78.2 min (*P* = 0.01 for comparison to PAO1) (Table 3). Con-

FIG. 1. Results of in vitro competition experiments. In vitro competitions were performed in LB broth flasks in which bacteria were grown at 37°C and 180 rpm for 16 to 18 h, corresponding to approximately 20 generations, as described in Materials and Methods. The CI values obtained for each of the eight independent experiments are plotted. The median CI values are shown in parentheses.

TABLE 3. *ampC* expression, ceftazidime susceptibilities, and doubling times for the mutants characterized

Strain	Avg level of <i>ampC</i> expression ^{a,b} ± SD		Ceftazidime MIC (μg/ml) ^b	Avg doubling time ^c ± SD
	Basal	Induced		
PAO1	1	43 ± 9	1	30.0 ± 8.3
PAΔD	60 ± 19	152 ± 38	8	28.3 ± 5.6
PAΔDh2	NC	48 ± 15	0.75	37.8 ± 4.8
PAΔDh3	NC	55 ± 5	1	34.4 ± 6.8
PAΔDDh2	62 ± 9	234 ± 58	12	33.2 ± 1.8
PAΔDDh3	191 ± 52	1,014 ± 297	48	35.2 ± 4.0
PAΔDDh2Dh3	1,020 ± 87	1,105 ± 88	48	78.2 ± 13.3*
PAΔDDh2Dh3C	NA	NA	1	37.6 ± 9.5
PAΔC	NA	NA	0.75	36.8 ± 3.4

^a Relative amount of *ampC* mRNA expression compared to basal expression in PAO1. Induction experiments were carried out with 50 μg/ml cefoxitin. NC, no change in *ampC* expression compared to PAO1 basal expression; NA, not applicable.

^b *ampC* expression and ceftazidime MIC data for PAO1, PAΔD, PAΔDh2, PAΔDh3, PAΔDDh2, PAΔDDh3, and PAΔDDh2Dh3 were obtained from reference 16.

^c For exponential growth in LB broth at 37°C and 180 rpm. *, $P = 0.01$ for comparison to PAO1.

sistent with the growth rates, the results for the PAΔDGm-PAO1 competition (median CI, 0.78; $P = 0.8$) showed that the inactivation of *ampD* in PAO1 was not associated with a significant fitness cost in vitro (Fig. 1). The *ampD ampDh2* double mutant was not associated with a significant cost, either (median CI for the PAΔDDh2Gm-PAO1 competition, 0.83), and the *ampD ampDh3* double mutant showed only a modest (though statistically significant [$P = 0.0003$]) reduction in fitness (median CI for the PAΔDDh3Gm-PAO1 competition, 0.61) (Fig. 1). On the other hand, the simultaneous inactivation of the three *ampD* genes resulted in a marked decrease in fitness, as shown by the median CI of 0.25 for the PAΔDDh2Dh3Gm-PAO1 competition ($P < 0.0001$). Nevertheless, the documented doubling time for the triple *ampD* mutant (double that for wild-type PAO1) should predict a much lower CI (approximately 0.001) in the competition experiments. The higher-than-expected CI might be explained by the fact that the differences in the growth rate between the triple *ampD* mutant and PAO1 were not that sharp in late-log phase (data not shown).

To explore whether *ampC* derepression was responsible for the reduced fitness of the triple *ampD* mutant, the corresponding *ampC* knockout mutants were constructed. As shown in Fig. 1, *ampC* derepression itself was found to be the main factor responsible for the in vitro cost documented, since inactivation of *ampC* restored the fitness of the triple *ampD* mutant to wild-type levels (median CI for the PAΔDDh2Dh3CGm-PAO1 competition, 0.80; $P = 0.9$). Similarly, the triple *ampD* mutant with *ampC* inactivated clearly outcompeted the triple *ampD* mutant (median CI for the PAΔDDh2Dh3CGm-PAΔDDh2Dh3 competition, 4.28; $P < 0.0001$) (Fig. 1). Furthermore, the inactivation of *ampC* in the triple *ampD* mutant restored its growth rate to wild-type levels (doubling time, 37.6 min) (Table 3). Control competition experiments between the PAO1 *ampC* mutant (PAΔC) and PAO1 ruled out any significant effect of *ampC* inactivation on fitness in the wild-type strain (median CI,

TABLE 4. Results of in vivo competition experiments in the mouse model of systemic infection

Competition	Median CI (range) ^a
PAΔDGm-PAO1	0.94 (0.67–1.12)
PAΔDh2Gm-PAO1	0.99 (0.66–1.68)
PAΔDh3Gm-PAO1	1.02 (0.26–4.2)
PAΔDDh2Gm-PAO1	0.23 (0.15–0.47)*
PAΔDDh3Gm-PAO1	<0.01 (<0.01–0.12)*
PAΔDDh2Dh3Gm-PAO1	<0.01 (<0.01)*
PAΔDDh2Dh3Gm-PAO1 (10:1)	<0.01 (<0.01)*
PAΔDDh2Dh3CGm-PAO1	<0.01 (<0.01–0.11)*
PAΔDDh2Dh3CGm-PAΔDDh2Dh3 (10:10)	12.5 (8.2–27.8)*

^a *, $P < 0.001$.

0.95) (Fig. 1). Similarly, the growth rate of PAΔC was not modified from that of PAO1 (Table 3). Overall, the results of the in vitro experiments show that *P. aeruginosa* can tolerate moderate overexpression (up to 2 log units) of AmpC without a significant effect on growth but that, in contrast, when the level of AmpC expression reaches a certain threshold (around 3 log units), fitness is significantly reduced.

Fitness and virulence in the mouse model of systemic infection. Results for the in vivo competition experiments in the mouse model of systemic infection are presented in Table 4. As shown, all the single *ampD* mutants retained full in vivo fitness relative to that of the wild-type strain, since none of them was outcompeted by PAO1 (median CI values, 0.94 to 1.02). On the other hand, the *ampD ampDh2* double mutant was associated with a significant biological cost (median CI, 0.23; $P < 0.001$), and the *ampD ampDh3* double mutant lost its biological competitiveness completely (median CI, <0.01). Accordingly, the *ampD ampDh2 ampDh3* triple mutant was completely outcompeted by PAO1, even when the competition experiments were performed with an initial 10:1 mutant/wild-type ratio (Table 4). Furthermore, in contrast to the results of in vitro competitions, *ampC* inactivation did not restore the in vivo fitness of the triple mutant at all (median CI for the PAΔDDh2Dh3CGm-PAO1 competition, <0.01), showing that AmpC derepression is not the main driver of the marked biological cost determined by the sequential inactivation of the *ampD* genes. Nevertheless, *ampC* derepression itself caused a significant reduction of in vivo fitness, since the triple mutant with *ampC* inactivated clearly outcompeted the triple mutant (median CI for the PAΔDDh2Dh3CGm-PAΔDDh2Dh3 competition, 12.5).

To assess the effect on virulence of the inactivation of the *ampD* genes, the mortality rates for groups of 16 mice inoculated with each of the strains studied were monitored daily for 7 days. As shown in Fig. 2, the inactivation of *ampD* or *ampDh2* did not modify the mouse survival curve at all. On the other hand, remarkably, the inactivation of *ampDh3* dramatically reduced mortality to 0% after 7 days, suggesting that this gene plays a major role in virulence. Accordingly, no mortality was observed for the *ampD ampDh3* double mutant (Fig. 2), the triple mutant, or the triple mutant with *ampC* inactivated (data not shown). Finally, the *ampD ampDh2* double mutant produced significantly ($P < 0.001$) lower mortality than either the *ampD* or the *ampDh2* single mutant (Fig. 2).

FIG. 2. Percentages of survival over time for groups of 16 mice inoculated intraperitoneally with 5×10^6 CFU of PAO1, PAD, PADh2, PADh3, PAADDh2, or PAADDh3. After 7 days, the percentage of survival for mice inoculated with strain PAADDh2Dh3 or PAADDh2Dh3C was 100% (data not included in the graph).

Sequencing of the *ampDh2* and *ampDh3* genes in AmpC-hyperproducing *P. aeruginosa* clinical isolates. The natural occurrence of *P. aeruginosa* double or triple *ampD* mutants was investigated in a collection of 10 previously characterized pairs of isogenic clinical strains (15). Each pair of strains consisted of an isolate showing wild-type AmpC production and a subsequent isogenic isolate showing AmpC hyperproduction, obtained after treatment with β -lactams (15). Each pair of clinical strains was recovered from a different patient, and each belonged to a different clone (15). Six of the 10 AmpC-hyperproducing isolates had previously been found to contain *ampD*-inactivating mutations, whereas the mechanisms leading to AmpC hyperproduction were unknown for the other 4 strains (15) (Table 5). The results of the sequencing of the *ampDh2* and *ampDh3* genes are shown in Table 5. Although some of the strains showed differences of one amino acid from the sequence of PAO1 (Table 5), these substitutions were always present in the wild-type isogenic isolates, suggesting that they are natural polymorphisms rather than inactivating mutations and therefore that double and triple *ampD* mutants are not frequent in the clinical setting.

DISCUSSION

The development of resistance by *Pseudomonas aeruginosa* during antimicrobial therapy, mediated by the selection of mutations in chromosomal genes, is a frequent problem with major clinical consequences (3, 6, 24). Particularly noteworthy among the mutation-mediated resistance mechanisms are those leading to the hyperproduction of the chromosomal cephalosporinase AmpC, which confers resistance to all the antipseudomonal penicillins and cephalosporins (23). Indeed, failure of treatment with β -lactams due to the selection of AmpC-hyperproducing mutants is a frequent outcome of *P. aeruginosa* infections (8, 15, 22). Furthermore, AmpC hyperproduction is one of the most prevalent resistance mechanisms documented among clinical *P. aeruginosa* strains, even among those isolated from patients with severe bloodstream infections (10), showing that the pathogenic capacity is not at all impaired. Along the same lines, AmpC hyperproduction is a frequent contributor to multidrug resistance in *P. aeruginosa* (9) and, combined with other mutational resistance mechanisms, is responsible for the origination of panresistant epidemic clones (4).

In contrast to the classical phenotype of full AmpC derepression generally reported for *Enterobacteriaceae*, *P. aeruginosa* clinical isolates most frequently show partially derepressed phenotypes (23). Indeed, the inactivation of AmpD in this species leads to a moderately high level basal *ampC* expression that is still further inducible in the presence of inducers, due to the production of two additional *N*-acetyl-anhydromuramyl-L-alanine amidases, AmpDh2 and AmpDh3 (16, 19). Homology searches using the available databases with the complete genome sequences of *E. coli* and other members of the *Enterobacteriaceae* revealed the presence of one, and only one, *ampD* homologue in addition to the regular *ampD* gene (16). This additional *N*-acetyl-anhydromuramyl-L-alanine amidase has recently been fully characterized in *E. coli*, where it has been designated AmiD (32). AmiD was shown to contain the signal sequence of outer membrane lipoproteins, and indeed its location in the outer membrane was demonstrated, in contrast to the cytoplasmic location of AmpD (32). *P. aeruginosa* AmpDh2 has the same signal sequence of outer membrane lipoproteins, suggesting that this amidase is the AmiD homologue. Therefore, AmpDh3 appears to be the extra amidase of *P. aeruginosa*. Indeed, AmpDh3 was previously shown to contribute much more than AmpDh2 to *ampC* repression in the *ampD* mutant background, thus playing a more relevant role in the characteristic partially derepressed phenotype of *P. aeruginosa* (16).

In this work we show that AmpD multiplicity in *P. aeruginosa* plays a major role in coupling the acquisition of antibiotic resistance with the maintenance of biological competitiveness and virulence. While full AmpC derepression is shown to impose a marked fitness cost both in vitro and in vivo, partial derepression, mediated by *ampD* inactivation in the presence of *ampDh2* and particularly *ampDh3*, does not modify biological competitiveness. Furthermore, independently of *ampC* expression, the inactivation of two or all three *ampD* genes im-

TABLE 5. Results of sequencing of the *ampD* genes from a collection of 10 ceftazidime-resistant, AmpC-hyperproducing clinical strains

Strain	<i>ampD</i> mutation ^a	Polymorphism ^b	
		AmpDh2	AmpDh3
BCL2A8	1-bp insertion (C) in nt 53	None	Ala219Thr
MCV1C6	None	None	Ala219Thr
FMC1H6	None	None	Ala208Val
JSG2A1	1-bp insertion (C) in nt 481	None	Ala219Thr
VFF2E2	None	None	Ala208Val
JCM2C2	1-bp deletion (C) in nt 449	Val40Ile	None
MSF2F5	None	None	None
MSC2A9	1-bp insertion (G) in nt 297	None	Ala219Thr
MQB1C5	C→T (stop codon) at nt 463	None	Ala219Thr
OFC2I4	Complete deletion of the <i>ampDE</i> region	None	None

^a Nucleotide (nt) numbers correspond to the PAO1 *ampD* sequence. *ampD* mutations were obtained from reference 16. None of the *ampD* mutations were present in the isogenic ceftazidime-susceptible isolates (16).

^b Amino acid numbers correspond to the PAO1 AmpDh2 or AmpDh3 sequence. All the polymorphisms detected were also present in the isogenic ceftazidime-susceptible isolates.

poses a marked biological cost in the mouse model, whereas in vivo fitness is not affected in any of the single *ampD* mutants. Nevertheless, remarkably, in contrast to the inactivation of *ampD* or *ampDh2*, *ampDh3* inactivation dramatically reduced the mortality of mice, suggesting that this gene plays a major role in virulence. The reason for the differential effect on virulence of *ampDh3* inactivation, compared to *ampD* or *ampDh2* inactivation, still remains to be elucidated. One of the possibilities that should be further explored is a potential role in cell wall degradation for the establishment of gaps in peptidoglycan, which are required for the assembly of type III and IV secretion systems (17).

Previous studies have shown that AmpD is required for the recycling of peptidoglycan catabolism products and that its inactivation leads to the accumulation of muropeptides (28). Furthermore, a recent work showed that the accumulation of muropeptides, determined by *ampD* inactivation, causes a marked reduction of in vivo fitness and virulence in *Salmonella enterica* (7). Indeed, this marked difference in the biological consequences of *ampD* inactivation between *Salmonella enterica* and *P. aeruginosa* is expected to be dependent on the presence of AmpDh3 in the latter species; the single *ampD* mutant of *Salmonella enterica* should be equivalent to the *P. aeruginosa ampD ampDh3* double mutant, which is completely impaired in fitness and virulence.

While full *ampC* derepression in *P. aeruginosa*, mediated by the sequential inactivation of the three *ampD* genes, confers high-level resistance to antipseudomonal β -lactams and partial derepression confers only moderate resistance, our results predict that the former phenotype should be very uncommon in the clinical setting, due to its high biological cost. Consistent with this hypothesis, none of the 10 natural AmpC-hyperproducing mutants characterized in this work seemed to contain inactivating mutations in *ampDh2* or *ampDh3*. Nevertheless, previous work shows that there are yet unidentified pathways, independent of the *ampD* genes, leading to high-level *ampC* expression, whose effects on fitness and virulence remain to be explored (15, 19).

In summary, we show that *P. aeruginosa* AmpD multiplicity plays a major role in coupling the acquisition of antibiotic resistance with the maintenance of biological competitiveness and virulence. Due to the presence of *ampDh2* and *ampDh3*, partial *ampC* derepression mediated by *ampD* inactivation confers a biologically efficient resistance mechanism on *P. aeruginosa*.

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