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Role of *ampD* Homologs in Overproduction of AmpC in Clinical Isolates of *Pseudomonas aeruginosa*▽

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AmpD indirectly regulates the production of AmpC β-lactamase via the cell wall recycling pathway. Recent publications have demonstrated the presence of multiple *ampD* genes in *Pseudomonas aeruginosa* and *Escherichia coli*. In the prototype *P. aeruginosa* strain, PAO1, the three *ampD* genes (*ampD*, *ampDh2*, and *ampDh3*) contribute to a stepwise regulation of *ampC* β-lactamase and help explain the partial versus full derepression of *ampC*. In the present study, the roles of the three *ampD* homologs in nine clinical *P. aeruginosa* isolates with either partial or full derepression of *ampC* were evaluated. In eight of nine isolates, decreased RNA expression of the *ampD* genes was not associated with an increase in *ampC* expression. Sequence analyses revealed that every derepressed isolate carried mutations in *ampD*, and in two fully derepressed strains, only *ampD* was mutated. Furthermore, every *ampDh2* gene was of the wild type, and in some fully derepressed isolates, *ampDh3* was also of the wild type. Mutations in *ampD* and *ampDh3* were tested for their effect on function by using a plasmid model system, and the observed mutations resulted in nonfunctional AmpD proteins. Therefore, although the sequential deletion of the *ampD* homologs of *P. aeruginosa* can explain partial and full derepression in PAO1, the same model does not explain the overproduction of AmpC observed in these clinical isolates. Overall, the findings of the present study indicate that there is still an unknown factor(s) that contributes to *ampC* regulation in *P. aeruginosa*.

In *Pseudomonas aeruginosa*, the overproduction of AmpC β-lactamases, known phenotypically as derepression, can result in resistance to nearly all β-lactam antibiotics except the carbapenems. Historically, it was understood that the regulation of AmpC production required three proteins: AmpG, a plasma membrane-bound permease; AmpD, a cytosolic peptidoglycan-recycling amidase; and AmpR, the transcriptional regulator of AmpC (6). Derepression has been associated previously with defects within the *ampD* structural gene (6, 19) or decreased *ampD* expression (18). Derepression represents the inability of AmpR to keep *ampC* expression at constitutively low wild-type levels. Full derepression is the noninducible, high-level expression of *ampC*. Partial derepression occurs when an organism constitutively expresses *ampC* at higher levels than those in a wild-type strain but the gene can still be induced. There have been several publications indicating the absence of mutations in AmpG, AmpD, AmpR, and the *ampC-ampR* intergenic region in derepressed isolates of *P. aeruginosa* (1, 2, 10, 23).

A recent study presented data which demonstrated a mechanism for the levels of derepression observed in *P. aeruginosa* (7). In 2006, Juan and colleagues (7) identified two AmpD homologs in *P. aeruginosa*, AmpDh2 and AmpDh3. Using a series of *ampD* knockout clones, Juan et al. were able to show that together, the three AmpD proteins contribute to the stepwise upregulation of *ampC* in the wild-type strain PAO1. The loss of the *ampDh3* homolog gene in combination with the deletion of *ampD* (hereinafter referred to as *ampD1*) resulted in increased basal, although still inducible, expression of *ampC* compared to that in a strain with a single *ampD1* deletion. The loss of *ampC* inducibility (full derepression) was achieved only in a triple *ampD* mutant. The deletion of *ampD1* had the single greatest impact in terms of increased β-lactam MICs and high-level constitutive *ampC* expression. The loss of *ampDh3* together with *ampD1* increased β-lactam MICs and AmpC production more than the deletion of *ampDh2* together with *ampD1*. In summary, the effects of the different *ampD* genes on AmpC overproduction were as follows: *ampD1* > *ampDh3* > *ampDh2*.

The goal of this study was to examine the relative expression levels of the different *ampD* genes in PAO1 and determine whether sequence abnormalities and/or altered expression of the *ampD* genes could explain derepression in a panel of clinical isolates.

MATERIALS AND METHODS

Strains and susceptibility. *P. aeruginosa* strain PAO1 was used as the wild-type comparator strain. Nine clinical isolates of *P. aeruginosa* strains were examined, including four cystic fibrosis isolates (PA22, PA24, PA113, and PA367) and four non-cystic fibrosis clinical isolates (GB57, GB61, TX291, and TX292). A mutant, 164CD (5), derived from a clinical isolate by selection using ceftazidime, was also characterized. In the model system, the *ampD*-deficient *Escherichia coli* strain JRG582 (11) was used to test whether mutations in an *ampD* gene could complement the *ampD*-deficient phenotype (18). MICs of piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, and aztreonam were determined by agar dilution, Etest (AB Biodisk, Sweden), and/or broth microdilution according to CLSI recommendations (3).

RNA analysis using real-time RT-PCR. RNA was isolated from mid-logarithmic-phase cultures of *P. aeruginosa* by using TRIzol Max solution (Invitrogen, Carlsbad, CA) as described previously (18). Eight micrograms of total RNA was DNase treated as described previously (18). Reverse transcriptase PCR (RT-PCR) was performed using the QuantiTect SYBR green RT-PCR kit as instructed by the manufacturer (Qiagen, Valencia, CA) with 250 ng of template

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TABLE 1. Primers used in this study

Primer name[a]	Sequence (5′ to 3′)	Target	Purpose(s)[b]
PAampD1RTF1	GTGGTGGTCTACAGTCTCG	*ampD1*	R
PAampD1RTR1	CGAAGAAGCTCTGGTAGCC	*ampD1*	R
PAampDh2RT3F	CTACACCTCCACCGATCTG	*ampDh2*	R
PAampDh2RTR	GATCTCGATGCCGATCGAG	*ampDh2*	R
PAampDh3RTAF	CAGCTATCGCACCACTACC	*ampDh3*	R
PAampDh3RTB2R	CGACCAGGTTGAAGATGCG	*ampDh3*	R
SodBF1	CTCGAAGAGATCGTCAAGAG	*sodB*	R
SodBR1	CGGTAGTCGATGTAGTAGGC	*sodB*	R
PAampDF	GACGATGCCTTGCTGTTCG	*ampD1*	P, C, S
PAampDRseq	GATCCTCGACCAGGCTCAGC	*ampD1*	P, C, S
PAampDh2CF	GCACCACCTTCGACGGTCC	*ampDh2*	P, C, S
PAampDh2R	CGAGCCTTTCGTCCAGGTC	*ampDh2*	P, C, S
PAampDh3F	GACCGCTGCGAAAGGCTCTG	*ampDh3*	P, C, S
PAampDh3R	GTGCGACGGCATTCATGGC	*ampDh3*	P, C, S
PAampRCSeqF	GAACAGCAGTGTGGAAGCG	*ampR-ampC* intergenic region	P, S
PAampRSeqR2	CGAGAGCGAGATCGTTGC	*ampR-ampC* intergenic region	P, S
PAampRSeqIR	CAGCAGAGCACCGTCAGC	*ampR-ampC* intergenic region	S
PAampRSeqIF	GCGCTGGCGTTGTTCGAG	*ampR-ampC* intergenic region	S
ECamiDRTF	GGTCAGCTCGCATTATCTGG	*amiD*	R
ECamiDRTR	GAATCTGTGCCGGTTCAAACG	*amiD*	R
ECamiDF	GCAGTATGCTGATGGTTAGC	*amiD*	P, C, S
ECamiDR	GTCGTATTCGTTCCGTCC	*amiD*	P, C, S

[a] All primers with names beginning with PA and the SodBF1 and SodBR1 primers were designed using the PAO1 genome sequence (GenBank accession number NC_002516). Those with names beginning with EC were designed using the *E. coli* K-12 genome sequence (GenBank accession number NC_00913).
[b] R, RT-PCR; P, PCR; C, cloning; and S, sequencing.

RNA for each reaction. For the evaluation of the expression of *ampD* homolog genes, each strain was analyzed using five reactions: a no-RT control and the amplification of *ampD1*, *ampDh2*, *ampDh3*, and *sodB* (an endogenous control gene). Amplification was performed as described previously (18) using an annealing temperature of 53°C. The relative quantification of *ampD* and *ampC* expression in the nine clinical strains was calculated as described previously (13) using expression in PAO1 as the calibrator. For the determination of the ratio of expression levels within PAO1, a modification of the calculation described by Livak and Schmittgen (13) was employed such that the *sodB* threshold cycle (C_T) was used to normalize the average C_T value for each *ampD* gene and the *ampD1* expression level was used as the calibrator (set to 1) against which *ampDh2* and *ampDh3* expression levels were compared.

The induction of *ampC* expression was evaluated by real-time RT-PCR. Briefly, cells were grown to mid-logarithmic phase, at which time imipenem (Merck Research Laboratories, Rahway, NJ) was added at a concentration of one-fourth the MIC for each strain and cells were incubated at 37°C for 20 min (22). Relative *ampC* expression was quantified by real-time RT-PCR as described above using primers listed in Table 1. The coefficient of variation (the standard deviation divided by the mean) among results from different experiments was <10%.

In order to compare the levels of expression of the three *ampD* genes within PAO1, great care was taken to ensure similar amplification efficiencies for all three genes. Therefore, primers were designed to have similar (and often identical) nucleotide lengths, G+C contents, theoretical melting temperatures, and target amplicon lengths (Table 1). The amplification efficiency was experimentally verified with template DNA by real-time PCR. DNA was isolated from a 1.5-ml aliquot of 8-h PAO1 broth cultures by using the DNeasy blood and tissue kit (Qiagen, Valencia, CA). Each amplification reaction mixture included 250 ng of DNA. The QuantiTect SYBR green RT-PCR kit (Qiagen, Valencia, CA), minus RT, was used with an ABI Prism 7000 thermal cycler (Applied Biosystems, Foster City, CA) set to parameters described previously (18). The annealing temperature of 53°C provided the best amplification efficiency for all three genes. The coefficient of variation for the amplification efficiencies was <10%.

DNA isolation, amplification, purification, and sequencing. DNA was isolated as described previously (14). The DNA template was amplified by PCR using primers listed in Table 1. Amplified products were verified on a 1% agarose gel and prepared for sequencing using the Microcon YM-50 filter column (Millipore, Bedford, MA). Sequencing was performed by automated cycle sequencing using an ABI Prism 3100-Avant genetic analyzer. Sequence analyses were performed using the Vector NTI Advance version 10.3 software program (Invitrogen, Carlsbad, CA). *P. aeruginosa* sequences were compared to the PAO1 genome sequence (GenBank accession number NC_002516) and *E. coli* sequences were compared to the K-12 genome sequence (GenBank accession number NC_000913).

Cloning of *ampD* genes and determination of AmpD function. *ampD* mutations observed in the clinical isolates of *P. aeruginosa* were evaluated for their effects on AmpD function by using a previously described plasmid-based AmpD model system (18). Briefly, the AmpD model system uses a plasmid containing the inducible plasmid-carried *ampC* gene bla_{ACT-1}, which acts as the indicator for AmpD function. An AmpD-deficient strain of *E. coli* (JRG582) is transformed with this inducible genetic system, which results in a ceftazidime resistance phenotype for the strain. To test the significance of nucleotide variations of *ampD* genes and the corresponding amino acid variations identified during sequence analyses of clinical isolates (in this case, strains of *P. aeruginosa*), the *ampD* test genes were amplified by PCR, cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and subcloned into the XbaI and HindIII sites of pACYC184, the vector used in the model system (16). The *ampD*-deficient *E. coli* strain JRG582 (11) was transformed with the resulting constructs. The clones containing the *ampD* test genes were evaluated for the ability to complement the *ampD*-deficient *E. coli* strain by ceftazidime susceptibility testing using disk diffusion.

Comparison of the amino acid sequences of *P. aeruginosa* AmpDh2 and AmpDh3 and *E. coli* AmiD. An alignment of the amino acid sequences of *P. aeruginosa* AmpDh2 and AmpDh3 and *E. coli* AmiD was generated by the MAFFT (multiple alignment using fast Fourier transform) program (9) available at http://www.ebi.ac.uk/mafft/index.html. AmpDh2 and AmpDh3 amino acid sequences were examined for transmembrane helix domains by using the TMHMM version 2 prediction software available at http://www.cbs.dtu.dk/services/TMHMM/.

RESULTS

Expression studies. The relative expression of each *ampD* transcript in six independent PAO1 cultures was evaluated. The data were averaged and used for calculations as described in Materials and Methods, with *ampDh2* and *ampDh3* expression compared to that of *ampD1*. The ratio of the expression levels of the three genes, that of *ampD1* to that of *ampDh2* to that of *ampDh3*, was 1 to 0.24 to 10.7. The coefficient of

TABLE 2. Susceptibility phenotypes and associated *ampC* and *ampD* RNA expression for *P. aeruginosa* strains

Strain[a]	MIC (μg/ml) of (susceptibility phenotype for)[b]:					Level of *ampC* expression (CV)[c]		Level of *ampD1* expression (CV)[d]	Level of *ampDh2* expression (CV)[d]	Level of *ampDh3* expression (CV)[d]	*ampC* status[e]
	PIP	P/T	CAZ	ATM	FEP	Basal	Induced				
PAO1	4 (S)[f]	4 (S)	1 (S)	4 (S)	1 (S)	1 (0.02)	2,705 (0.04)	1.00 (<0.01)	1.00 (0.01)	1.00 (0.01)	WT
PA22	128 (R)	64 (S)	16 (I)	4 (S)	16 (I)	84 (0.05)	10,854 (0.01)	1.40 (<0.01)	1.10 (0.03)	1.10 (<0.01)	PD
PA367	64 (S)	64 (S)	32 (R)	4 (S)	16 (I)	65 (0.01)	11,800 (0.02)	0.59 (0.02)	0.76 (0.02)	0.51 (≤0.01)	PD
PA24	256 (R)	128 (R)	128 (R)	128 (R)	64 (R)	442 (0.04)	7,670 (0.02)	1.06 (0.03)	0.74 (0.03)	0.79 (<0.01)	PD
PA113	128 (R)	64 (S)	32 (R)	16 (I)	16 (I)	247 (0.01)	5,340 (0.01)	0.80 (0.02)	0.82 (0.02)	0.50 (0.02)	PD
GB57	>256 (R)[f]	>256 (R)[f]	>128 (R)[g]	>256 (R)[f]	>256 (R)[f]	5,853 (0.03)	12,677 (0.03)	0.57 (<0.01)	0.59 (0.02)	0.46 (<0.01)	FD
GB61	>256 (R)[f]	>256 (R)[f]	128 (R)[g]	48 (R)[f]	32 (R)[f]	1,616 (0.02)	6,597 (0.03)	0.92 (<0.01)	1.04 (0.02)	0.31 (0.05)	PD
TX291	256 (R)	>256 (R)[f]	32 (R)	64 (R)	32 (R)	1,919 (0.01)	6,994 (0.01)	0.93 (0.02)	5.20 (0.04)	0.75 (0.03)	PD
TX292	256 (R)	>256 (R)[f]	32 (R)	64 (R)	32 (R)	2,990 (0.01)	4,715 (0.05)	1.13 (<0.01)	4.00 (0.03)	1.08 (0.03)	FD
164CD	>1,024 (R)	>256 (R)[f]	128 (R)	>256 (R)[f]	>256 (R)[f]	6,468 (0.01)	5,526 (0.04)	1.17 (0.01)	1.89 (0.06)	0.83 (0.03)	FD

[a] Strains PA22, PA367, PA24, and PA113 are cystic fibrosis isolates; strains GB57, GB61, TX291, and TX292 are non-cystic fibrosis clinical isolates; 164CD is a mutant derived from a clinical isolate by selection using ceftazidime.

[b] Susceptibility data were determined and evaluated by agar dilution methodology (unless noted otherwise) as recommended by the CLSI (3). Phenotypic interpretation: S, susceptible; I, intermediate; R, resistant. PIP, piperacillin; P/T, piperacillin-tazobactam; CAZ, ceftazidime; ATM, aztreonam; FEP, cefepime.

[c] The levels of *ampC* expression in the presence and absence of imipenem were determined by real-time RT-PCR and compared to those in PAO1. The basal level of *ampC* expression in PAO1 was set at 1. CV, coefficient of variation.

[d] The level of *ampD* expression relative to that in PAO1 (which was set at 1.00) was determined by real-time RT-PCR. The coefficient of variation for the C_T values from at least two independent experiments is shown.

[e] *ampC* status based on *ampC* expression data; WT, wild type; PD, partially derepressed; FD, fully derepressed.

[f] The MIC was determined by Etest (AB Biodisk, Sweden).

[g] The MIC was determined by broth microdilution.

variation for the six cultures was <10% for each gene tested and ranged from 0.04 to 0.07.

Partial or full derepression of *ampC* RNA expression in the nine clinical isolates in the absence and presence of imipenem was evaluated. As indicated in Table 2, all of the strains exhibited elevations in basal *ampC* expression compared to that in PAO1, ranging from a 65-fold increase for PA367 to a 5,853-fold increase for GB57. All of the cystic fibrosis isolates, PA22, PA24, PA113, and PA367, were inducible and therefore had partially derepressed AmpC production. Of the non-cystic fibrosis isolates, strains GB57, TX292, and 164CD were fully derepressed, with a ≤2-fold change in *ampC* expression when exposed to imipenem. However, *ampC* RNA expression in strains GB61 and TX291 increased by ~4-fold in the presence of imipenem. Technically, these strains are considered to be partially derepressed.

The relative levels of expression of *ampD* RNA in the nine clinical strains were analyzed to determine whether decreased expression correlated with *ampC* overproduction. For most strains, no variation (≤2-fold) was observed in the expression of the *ampD* homologs. However, strains TX291 and TX292 expressed *ampDh2* at 5.2- and 4.0-fold-higher levels than that of the expression of PAO1 *ampDh2*, respectively (Table 2). In addition, a threefold decrease in *ampDh3* expression in GB61 was observed.

Sequence analysis and evaluation of AmpD function using the *E. coli* plasmid model system. *ampD1* and *ampDh3* from the wild-type *P. aeruginosa* strain PAO1 were able to complement the *ampD*-deficient *E. coli* strain, as indicated by a change in the ceftazidime zone diameter from 6 mm to 17 and 18 mm, respectively (Table 3). In contrast to the *ampD1* and *ampDh3* genes, PAO1 *ampDh2* failed to complement the *ampD*-deficient clone, resulting in no change in the ceftazidime zone diameter.

The three *ampD* homolog genes in nine clinical isolates were sequenced to identify mutations which may contribute to *ampC* overproduction (Table 3). Regardless of whether *ampC* was fully derepressed or partially derepressed (Tables 2 and 3), all of the clinical isolates encoded AmpD1 mutations. The most frequently observed mutation in AmpD1 was a glycine-to-alanine change corresponding to codon 148, which occurred in five of the nine isolates. Three of the isolates, one cystic fibrosis isolate and two non-cystic fibrosis isolates, contained an 11- to 12-nucleotide deletion at position 5064907 (relative to the PAO1 genome sequence [GenBank accession number NC_002516]) that resulted in either a frameshift mutation with the absence of a stop codon (strains TX291 and TX292) or a four-codon deletion (strain PA24). All nine strains encoded wild-type AmpDh2 and did not require the evaluation of *ampDh2* in the model. Six of the nine strains (67%) exhibited amino acid substitutions in AmpDh3. By using the PAO1 *ampD1* and *ampDh3* clones as controls, the *ampD1* and *ampDh3* mutations observed in the clinical isolates were evaluated for their effects on AmpD function.

The partially derepressed cystic fibrosis strains PA22 and PA367 carried identical mutations in *ampD1* and *ampDh3* (Table 3). Pulsed-field gel electrophoresis indicated that these strains were genetically related (unpublished data). The AmpD1 substitutions were a glycine-to-alanine change at position 148 and an aspartic acid-to-tyrosine change corresponding to codon 183. The AmpDh3 substitutions in strains PA22 and PA367 were an aspartic acid-to-glycine change at position 137 and an alanine-to-threonine change corresponding to codon 219. When tested in the model system, these mutations in *ampDh3* resulted in a ceftazidime zone diameter indicating resistance (10 mm). PA24 and PA113, both partially derepressed cystic fibrosis strains, also carried *ampD1* mutations as described above and *ampDh3* mutations (Table 3). The *ampDh3* genes in PA24 and PA113 carried a single mutation resulting in an Ala219Thr amino acid substitution.

Strain 164CD was a fully derepressed mutant derived from a clinical isolate by selection with ceftazidime (5) and carried

TABLE 3. Evaluation of *ampD* gene homologs from *P. aeruginosa* using the *E. coli* AmpD plasmid model system

Strain[a]	*ampC* status[b]	AmpD1 form or mutation(s)[c]	Diam (mm) of CAZ zone (susceptibility phenotype)[d]	AmpDh2 form or mutation(s)[c]	Diam (mm) of CAZ zone (susceptibility phenotype)[d]	AmpDh3 form or mutation(s)[c]	Diam (mm) of CAZ zone (susceptibility phenotype)[d]
PAO1[e]	WT	WT	17 (I)	WT	6 (R)	WT	18 (S)
PA22	PD	Gly148Ala, Asp183Tyr	8 (R)	WT	NT[f]	Asp137Gly, Ala219Thr	10 (R)
PA367	PD	Gly148Ala, Asp183Tyr	8 (R)	WT	NT	Asp137Gly, Ala219Thr	10 (R)
PA24	PD	12-nt deletion at nt 5064908; deletion of codons 144-147	6 (R)	WT	NT	Ala219Thr	9 (R)
PA113	PD	Gly148Ala, Asp183Tyr	8 (R)	WT	NT	Ala219Thr	9 (R)
GB57	FD	Val10Ile	10 (R)	WT	NT	WT	NT
GB61	PD	Gly148Ala	12 (R)	WT	NT	WT	NT
TX291	PD	11-nt deletion at nt 5064907; frameshift	11 (R)	WT	NT	Ala208Val	8 (R)
TX292	FD	11-nt deletion at nt 5064907; frameshift	11 (R)	WT	NT	WT	NT
164CD	FD	Leu32Pro, Gly46Ser, Gly148Ala	8 (R)	WT	NT	Ile67Thr	10 (R)

[a] *P. aeruginosa* strain from which *ampD* genes were amplified for analysis in the *E. coli* AmpD model system (18).
[b] *ampC* status based on *ampC* expression data presented in Table 2. Abbreviations are as defined in Table 2 footnote *e*.
[c] Nucleotides (nt) specifically mentioned in the table correspond to the PAO1 genome sequence (GenBank accession number NC_002516). WT, wild type.
[d] Ceftazidime (CAZ) disk diffusion data for cloned *P. aeruginosa ampD* genes within the *E. coli* AmpD model system (18). Failure to complement the *ampD*-deficient strain JRG582 was evaluated by the zone size for CAZ. CLSI *Enterobacteriaceae* breakpoints for CAZ were as follows: ≤14 mm, resistant (R); 15 to 17 mm, intermediate (I); and ≥18 mm, susceptible (S) (4).
[e] The *ampD* genes of PAO1 were tested in the model system as controls.
[f] NT, not tested because of the presence of the wild-type sequence.

unique mutations in both *ampD1* and *ampDh3* (Table 3). Together, the *ampD1* mutations resulted in ceftazidime resistance in the *ampD* model system, with a zone diameter of 8 mm. The mutation observed in AmpDh3 was an isoleucine-to-threonine change at position 67, which also resulted in ceftazidime resistance (the zone diameter was 10 mm) when tested in the model system.

Involvement of *E. coli amiD* in *ampDh2* complementation. Because PAO1 *ampDh2* failed to complement the AmpD-deficient phenotype of *E. coli* strain JRG582 in the model system, it was possible that *E. coli* possessed an *ampDh2* homolog. Recently, an AmpD homolog in *E. coli*, AmiD, has been identified (21). An alignment of the *P. aeruginosa* and *E. coli* AmpD homologs is presented in Fig. 1. AmiD of *E. coli* and AmpDh2 of *P. aeruginosa* share a lipobox motif sequence ($L^{14}AGC^{17}$) which is conserved in gram-negative bacteria (8, 21, 25). In addition, Lewenza et al. predicted that *ampDh3* (gene PA0807) encodes a transmembrane helix in the N-ter-

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EC AmiD     MRRFFWLVAAALLLAGCAGEKGIVEKEGYQLDTRRQAQAAYPRIKVLVIHYTADDFDSSL
PA AmpDh3   MLTI-------------------------DYNSYRTTTPYGKRVRFLVLHYTALDFAASV
PA AmpDh2   MRSFVLLAFTLSLLAGCS--------SGPRLNTDYTSANQDSRVQFIVLHYTSTDLPHSL
            *  :                          ::   :     *::.:*:***: *:  *:

EC AmiD     ATLTDKQVSSHYLVPAV-PPRYNGK----PRIWQLVPEQELAWHAGISAWRGATRLNDTS
PA AmpDh3   KALTTGAASAHYLIPAPHDPSYKAAGFKGQRIFNLVAEEDRAWHAGVSGWARRDNLNDTS
PA AmpDh2   GILTHGGVSAHYLIGDDEP----------ATVYRLVDENRRAWHAGVSEWQGRTWLNATS
              **   .*:***:                 ::.** *:  *****:* *     ** **

EC AmiD     IGIELENRGWQKSAGVKYFAPFEPAQIQALIPLAKDIIARY-HIKPENVVAHADIAPQRK
PA AmpDh3   IGIEIVNLA-RDDDGVFTFPDYERSQINALKQLAKNILQRYPDMTPKNVVGHSDIAVGRK
PA AmpDh2   IGIEIVNQGYRDTPQGRVWYPFSEAQIQALIPLLKDIAKRH-GITPDRIIGHSDIAPGRK
            ****: * . :.      :  :. :**:**  * *:*  *:  :.*..::.*:***  **

EC AmiD     DDPGPLFPWQQLAQQGIGAWPDAQRVNFYLAGRAPHTPVDTASLLELLARYGYDVKPDMT
PA AmpDh3   SDPGPKLPWKELYEAGIGAWYDDATRDRYREGFERDGLPPRADLLEAFRLYGYALPATVD
PA AmpDh2   VDPGPLFPWKRLADAGLVPWPKPGELARRLAELNGQ-LPDVRWFQQQLARHGYLVPQTGE
             **** :**:.* : *: .* .             .       : : :  :** :

EC AmiD     -PREQRRVIMAFQMHFRPTLYNGEADAETQAIAEALLEKYGQD
PA AmpDh3   -DAYFASLLRAFQMHFRPENYDGALDVETAAILYALNEKYPA-
PA AmpDh2   LEKDTRDVIGAFQMKYRPARFDGEPDLETAALLLAV----PTS
                  :: ****::**  ::*  * ** *:  *:
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FIG. 1. Alignment of *E. coli* (EC) AmiD and *P. aeruginosa* (PA) AmpDh2 and AmpDh3. Dashes indicate gaps, whereas asterisks, colons, and periods indicate identity, conserved substitution, and semiconserved substitution, respectively. The alignment was generated using the MAFFT program with the selection of a Clustal output. The lipobox sequence ($L^{14}AGC^{17}$) in *E. coli* AmiD and *P. aeruginosa* AmpDh2 is underlined. The transmembrane helix sequence in AmpDh3 is double underlined and was identified using the TMHMM version 2 prediction software (http://www.cbs.dtu.dk/services/TMHMM/).

minal sequence (Fig. 1), suggesting that AmpDh3 may insert into the cytoplasmic membrane (12). Overall, *E. coli* AmiD shows 50% similarity to *P. aeruginosa* AmpDh2 and 47% similarity to *P. aeruginosa* AmpDh3. Given these similarities between *ampDh2* of *P. aeruginosa* and *amiD* of *E. coli*, it is possible that within the AmpD model system, *P. aeruginosa ampDh2* genes cannot be evaluated due to the presence of wild-type *amiD*. Sequences and levels of expression of *amiD* in *E. coli* JRG582 were examined, and no differences from those in the wild-type *E. coli* strain K-12 259 (corresponding to GenBank accession number D90770) were observed.

Sequence analysis of *ampR* and the *ampR-ampC* intergenic region in GB57 and GB61. *P. aeruginosa* strains GB57 and GB61 had single amino acid substitutions in AmpD1, and both had wild-type AmpDh2 and AmpDh3 sequences. Therefore, the fully derepressed phenotype (Tables 2 and 3) of GB57 could not be explained through an AmpD mechanism. It was possible that mutations within *ampR* or the *ampR-ampC* intergenic region contributed to the high-level, noninducible RNA expression of *ampC*. Therefore, the *ampR* genes and the *ampR-ampC* intergenic regions in the fully derepressed strain GB57 and the partially derepressed strain GB61 were sequenced and compared. The *ampR-ampC* intergenic regions in GB61 and GB57 were both of the wild type, and the *ampR* sequence in GB61 was also of the wild type. However, mutations in the *ampR* structural gene of strain GB57 were observed and resulted in the following amino acid substitutions: Glu114Ala, Asp135Asn, Gly283Glu, and Met288Arg.

DISCUSSION

Derepressed phenotypes in *P. aeruginosa* in the absence of genetic mutations in the AmpC induction pathway have been observed previously (1, 2, 10). Juan et al. identified two *ampD* homologs in *P. aeruginosa* (*ampDh2* and *ampDh3*) and demonstrated that the deletion of all three *ampD* genes in the laboratory strain PAO1 was required for a fully derepressed phenotype. In addition, they found that with respect to *ampC* expression and β-lactam MICs, the relative importance of the AmpD proteins was as follows: AmpD1 > AmpDh3 > AmpDh2 (7). In this study, an analysis of the RNA expression patterns of the three *ampD* genes in PAO1 relative to one another showed that *ampDh3* was expressed at the highest level, followed by *ampD1* and then *ampDh2*. These data, together with the data generated by Juan et al., suggest that (i) the role of *ampDh2* in *ampC* regulation is minimal compared to those of *ampD1* and *ampDh3* and (ii) increased expression of *ampDh3* relative to that of *ampD1* and *ampDh2* in PAO1 may reflect the importance of the AmpDh3 enzyme in cell wall metabolism. Further experimentation is required to substantiate these observations.

In order to test the hypothesis of Juan et al. (7), the role of the three *ampD* genes in AmpC overproduction in nine clinical isolates with either partially or fully derepressed *ampC* expression was examined. All of the *ampD1* and *ampDh3* genes tested in the model encoded nonfunctional proteins, as indicated by a phenotype of ceftazidime resistance. But the sequence for *ampDh2* in all the isolates was of the wild type. This finding contradicts the requirement that all three *ampD* genes of *P. aeruginosa* PAO1 be deleted for the isolate to become fully derepressed (7). It was possible that *ampDh2* expression was altered in the clinical isolates, since a decrease in *ampD* gene expression has been associated previously with elevated levels of *ampC* expression in the presence of a wild-type AmpD sequence (18). However, instead of decreased *ampD* expression, the partially and fully derepressed strains TX291 and TX292 had elevated levels of *ampDh2* RNA expression. What role the elevated expression of *ampDh2* may play in the fully derepressed phenotypes of these strains is unclear.

Strain GB57 had the highest levels of *ampC* expression and was fully derepressed yet had only a single amino acid substitution in AmpD1. However, mutations in the structural gene *ampR* were observed and have been observed previously in other derepressed clinical isolates of *P. aeruginosa* (1, 15, 20). The Asp135Asn AmpR substitution observed in GB57 has been correlated previously with a large increase (16,000-fold) in β-lactamase activity (1). Therefore, it is possible that the combination of the mutations observed in both the *ampR* and *ampD1* genes was responsible for the 5,000-fold increase in basal-level *ampC* RNA expression and the fully derepressed phenotype.

Strains PA113 and GB61 highlight the need for further studies investigating alternative mechanisms in the *ampC* regulatory pathway. PA113 is a cystic fibrosis isolate which displayed a partially derepressed phenotype of *ampC* expression, with basal-level RNA expression 442-fold higher than that in PAO1. GB61 was also partially derepressed and expressed *ampC* RNA at a basal level 1,616 times higher than that in PAO1. The difference in the basal levels of expression between the two strains was fivefold. Although both strains had amino acid substitutions in AmpD1, the mutations in AmpDh3 differed. Strain PA113 had an amino acid substitution in AmpDh3, while GB61 had wild-type AmpDh3 but threefold-lower *ampDh3* RNA levels. Both strains carried wild-type *ampR* and *ampR-ampC* intergenic regions. Although the effect of *ampDh3* expression on *ampC* expression remains unknown, these data further indicate the complexity involved in the regulation of *ampC* gene expression.

The present study explored the impact of *ampD* homologs on *ampC* expression in clinical isolates and further substantiated the notion that the regulation of *ampC* β-lactamase in *P. aeruginosa* is exceedingly complex. These data support observations that additional mechanisms besides the known regulators of AmpC β-lactamase contribute to *ampC* regulation.

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