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An *ampD* Gene in *Pseudomonas aeruginosa* Encodes a Negative Regulator of AmpC β-Lactamase Expression

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The *ampD* and *ampE* genes of *Pseudomonas aeruginosa* PAO1 were cloned and characterized. These genes are transcribed in the same orientation and form an operon. The deduced polypeptide of *P. aeruginosa ampD* exhibited more than 60% similarity to the AmpD proteins of enterobacteria and *Haemophilus influenzae*. The *ampD* product transcomplemented *Escherichia coli ampD* mutants to wild-type β-lactamase expression.

Most *Pseudomonas aeruginosa* strains, like nearly all members of the family *Enterobacteriaceae*, express an inducible chromosomally encoded AmpC β-lactamase (cephalosporinase) (27), which is placed in class C of Ambler's classification (1) and which is in Bush's group 1 (3). In enterobacteria, the regulation of AmpC β-lactamase expression is intimately linked to cell wall recycling and involves three genes; *ampR*, which encodes a transcriptional regulator of the LysR family; *ampG*, which encodes a transmembrane permease; and *ampD*, which encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase hydrolyzing 1,6-anhydromuropeptides (9, 12, 15, 25). In the absence of a β-lactam inducer, AmpR is repressed by the murein precursor UDP-MurNAc-pentapeptide (uridine-pyrophosphoryl-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanyl-D-alanine) (13). Since β-lactams interfere with murein synthesis, their actions lead to an increased periplasmic accumulation of degradation products such as 1,6-anhydromuropeptides, which are the signal molecules for β-lactamase induction (5, 11). *ampG* transports these products from periplasm to cytoplasm, where they are cleaved by AmpD, which acts as a negative regulator of AmpC β-lactamase expression (5, 11, 15, 25). In *ampD* mutants, the constitutive overproduction of AmpC β-lactamase is accompanied by an accumulation of aM-tripeptide (monosaccharide-tripeptide, 1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid) and aM-pentapeptide (monosaccharide-pentapeptide, 1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanyl-D-alanine) in the cytoplasm (5, 11). Jacobs et al. (13) suggested that the aM-tripeptide could be the AmpR-activating ligand, since this product can relieve in vitro the repressed state of AmpR, resulting in the activation of β-lactamase expression. However, potential interactions of the aM-pentapeptide with AmpR have not been investigated. Another gene, *ampE*, which encodes a transmembrane protein, forms an operon with *ampD*, but this gene is not involved in β-lactamase expression (10, 19, 24).

In *P. aeruginosa*, the inducible expression of AmpC β-lactamase is also under the control of the transcriptional regulator AmpR (21). To further elucidate the induction process, the *ampD* and *ampE* genes of this organism were cloned and characterized, and complementation analysis was performed with *Escherichia coli ampD* mutants with the cloned *P. aeruginosa ampD* and *ampE* genes. A part of this work was presented before (16).

The strains and plasmids used in this study are described in Table 1. *E. coli* DH5α was used as the host for construction and propagation of recombinant plasmids. Bacterial cells were grown in tryptic soy broth or tryptic soy agar (Difco Laboratories, Detroit, Mich.). When required, 50 μg of kanamycin/ml, 30 μg of chloramphenicol/ml, and various concentrations of cefotaxime, ampicillin, and cefoxitin were added (Sigma-Aldrich Canada, Oakville, Ontario, Canada).

Recombinant DNA techniques were performed essentially by standard procedures (26). To clone the *ampD* and *ampE* genes of *P. aeruginosa* PAO1, two degenerated oligonucleotide primers adapted to the *P. aeruginosa* codon usage and derived from two conserved regions of the *E. coli*, *Citrobacter freundii*, and *Enterobacter cloacae* AmpD amino acid sequences (10, 14, 19) were synthesized on a 394 DNA/RNA synthesizer (PE Applied Biosystems, Foster City, Calif.). The sequences of the primers were as follows: AmpD1, 5′-CGCTGCCSCCSGGCG ARTTCG-3′; and AmpD2, 5′-CGGGGCCSGGGTCGGTCT TGC-3′. A 400-bp DNA fragment was amplified by PCR with the *Taq* DNA polymerase (Promega, Madison, Wis.) and a

TABLE 1. Characteristics of the bacterial strains and plasmids used in this study

E. coli strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
DH5α	*deoR supE44* Δ(*lacZYA-argFV169*) (ϕ80 d*lac*ZΔM15) $F^-\lambda^-$ *hsdR17* ($r_k^-m_k^+$) *recA1 endA1 gyrA96 thi-1 relA1*	26
JRG582	Δ(*ampDE*)2	18
STC172	*ampD11E*$^+$	10
Plasmids		
pEC1C	Cm^r; *ampC ampR* from *E. cloacae*	23
pNH5	Km^r; *ampD* from *E. coli*	10
pBGS19$^+$	Km^r f1 Ori *lacPOZ*	28
pHUL4DE-PH	Km^r; *ampDE* region of *P. aeruginosa* PAO1 cloned into pBK-CMV	This study
pHUL4DE-1	Km^r; *ampDE* + ORF-1 from *P. aeruginosa* PAO1 cloned into pBGS19$^+$	This study
pHUL4DE-2	Km^r; *ampDE* from *P. aeruginosa* PAO1 cloned into pBGS19$^+$	This study
pHUL4D	Km^r; *ampD* from *P. aeruginosa* PAO1 cloned into pBGS19$^+$	This study

[a] Cm, chloramphenicol; Km, kanamycin.

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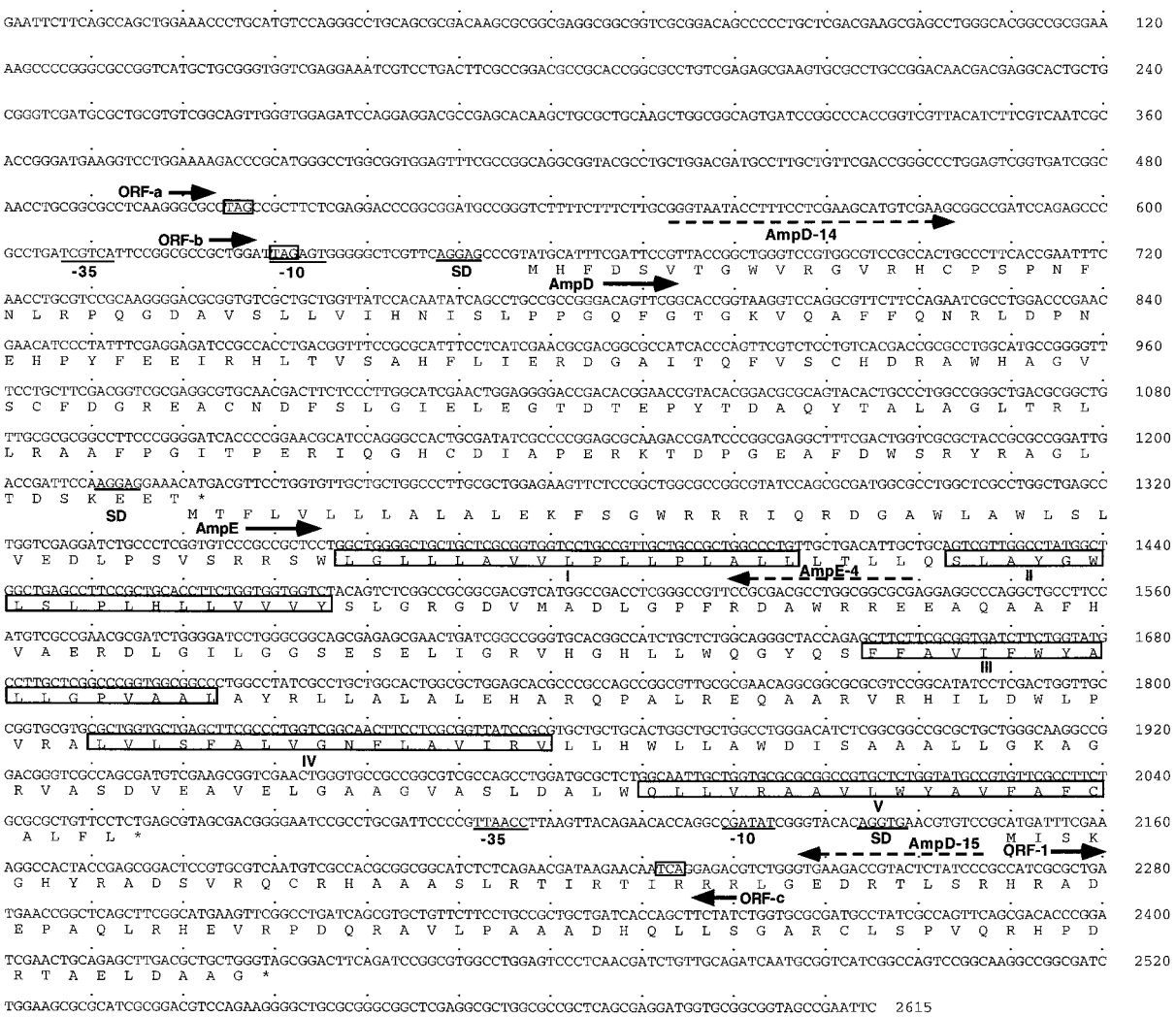

FIG. 1. Nucleotide sequence of the *P. aeruginosa ampD* and *ampE* genes with ORF-1 and predicted amino acids. The amino acids are presented according to the one-letter code. The putative SD sequences and the potential −10 and −35 regions of promoters are underlined. The stop codons are shown by asterisks. The five transmembrane domains of AmpE are boxed and named I, II, III, IV, and V. The stop codons for the potential ORF-a, ORF-b, and ORF-c are boxed. The oligonucleotide primers used for PCR amplification are shown by dashed arrows.

P. aeruginosa PAO1 lysate, the latter of which was prepared by the freezing-and-boiling method (30). This DNA fragment was used as a probe to screen a λ Zap Express genomic library of *P. aeruginosa* PAO1. Phage screening and in vivo plasmid excision were performed according to the instructions of the manufacturer (Stratagene, La Jolla, Calif.). A single phage containing a 6.7-kb genomic insert was selected, and plasmid pBK-CMV was excised out of the phage and named pHUL4DE-PH. The *ampD* gene was located on a 2.6-kb *Eco*RI fragment of pHULDE-PH, which was cloned into the *Eco*RI site of pBGS19$^+$ vector to generate plasmid pHUL4DE-1. Both strands of this DNA fragment were sequenced with the Deaza sequencing kit (Pharmacia Biotech, Baie d'Urfé, Québec, Canada) on a Pharmacia LKB Macrophor or the ABI Prism dye terminator cycle sequencing kit with AmpliTaq DNA polymerase, FS (PE Applied Biosystems), on a 373 DNA sequencer (PE Applied Biosystems). Sequence analysis, alignment, the homology study, G + C content calculation, and molecular mass prediction were done with the Genetics Computer Group software package version 9.0 (Madison, Wis.). The PSORT software was used to predict protein localization sites (22).

Sequence analysis of this DNA fragment revealed three complete open reading frames (ORFs) (Fig. 1). The first 567-bp ORF is located 657 nucleotides downstream of the *Eco*RI site and encodes a 188-amino-acid polypeptide (AmpD) with a predicted molecular mass of 21 kDa. A potential Shine-Dalgarno (SD) sequence (AGGAG) (8) and the consensus −10 (TAGAGT) and −35 (TCGTCA) regions of bacterial promoters (20) were identified 5, 23, and 45 nucleotides upstream of the *ampD* ATG start codon, respectively. Following *ampD*, a second ORF (AmpE) of 837 nucleotides, which consists of 278 amino acids with a predicted molecular mass of 31 kDa, was found. The *ampE* ATG start codon overlaps the *ampD* TGA termination codon and is preceded by a potential SD sequence (AGGAG) located 5 nucleotides upstream. This strongly suggests that these two genes form an operon, as described for *E. coli* (10, 19). The G + C contents of *ampD* and *ampE* were calculated to be 64 and 68%, respectively, which are typical for *P. aeruginosa* (32). Finally, 92 nucleotides downstream of the *ampE* TGA stop codon, a 282-bp ORF (ORF-1), which comprises 93 amino acids with a predicted molecular mass of 10 kDa (Fig. 1), was identified. This ORF is transcribed in the

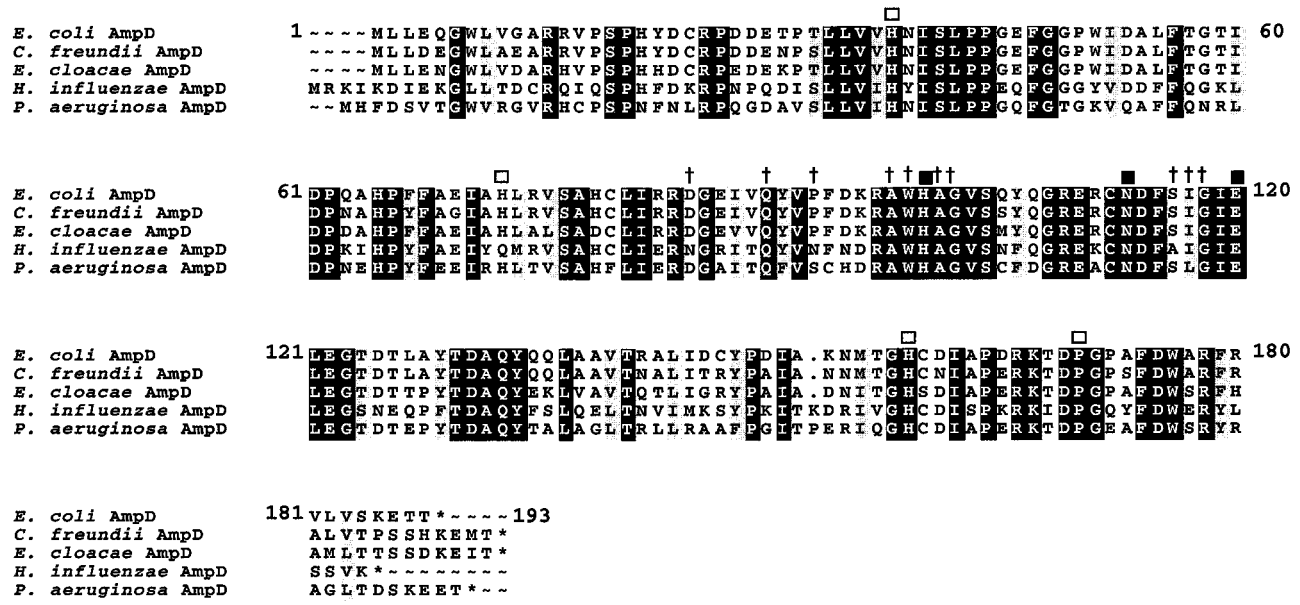

FIG. 2. Alignment of the amino acid sequence of *P. aeruginosa* PAO1 AmpD with those of the *E. coli*, *C. freundii*, *E. cloacae*, and putative *H. influenzae* AmpD proteins. The similar and identical amino acids are lightly and darkly shaded, respectively. The crosses and the open squares indicate the amino acids conserved in the core and outside region of the *Bacillus* cell wall hydrolases, respectively. The solid squares show the amino acids strictly conserved in various cell wall hydrolases (12).

same orientation as *ampDE* and is preceded by a potential SD sequence (AGGTG) as well as the −10 (CGATAT) and −35 (TTAACC) promoter-like sequences at 11, 25, and 52 nucleotides upstream of its ATG start codon, respectively. The product of ORF-1, like AmpD, possesses the features of a cytoplasmic protein (22). Three other potential ORFs (ORF-a, ORF-b, and ORF-c) from different reading frames, which are incomplete at the 5′ end, were also identified on each side of *ampDE* (Fig. 1). However, these ORFs, like ORF-1, showed no significant homology to any sequence in the GenBank database.

The predicted *P. aeruginosa* AmpD protein exhibited 65, 63, 62, and 62% similarity to the *E. coli*, *C. freundii*, *E. cloacae*, and putative *Haemophilus influenzae* AmpD proteins, respectively (7, 10, 14, 19). Amino acid sequence alignment of these AmpD proteins revealed many conserved motifs (Fig. 2). The conserved core region as well as the four strictly identical residues outside of this region, which relate the AmpD proteins of enterobacteria to the cell wall hydrolases of *Bacillus* spp. (12), were found in the *P. aeruginosa* AmpD protein. The deduced *P. aeruginosa* AmpE protein possesses the features of a cytoplasmic membrane protein (22) with five transmembrane-spanning domains (Fig. 1). It showed a low degree of similarity (33%) to its homolog in *E. coli* (10, 19) and seems to be less conserved than AmpD. Indeed, this difference with regard to AmpE is underlined by the fact that despite the identification of a putative *ampD* sequence in the genome sequence database of *H. influenzae*, no *ampE* sequence could be identified (7).

To determine the role of the *P. aeruginosa ampD* and *ampE* gene products, complementation analyses were performed with *E. coli* strains JRG582 [Δ(*ampDE*)2] and STC172 (*ampD11E*$^+$) (10, 18) with the cloned *P. aeruginosa ampD* and *ampE* genes. The MICs of β-lactam antibiotics for transformants were determined by the broth microdilution method as described previously (31). Induction assays and β-lactamase activity measurements were performed as described previously (31). Two plasmids were constructed to do a complementation study. Plasmid pHUL4DE-2, which contains the *ampDE* operon and the 105- and 209-nucleotide regions located upstream and downstream of this operon, respectively, was constructed as follows: a 1,714-bp DNA fragment was amplified by PCR with the *Pfu* DNA polymerase (Stratagene) and the oligonucleotide primers AmpD-14 (5′-GGGAATTCCTTTCCTCGAAGCATGTCG-3′) and AmpD-15 (5′-GGGATAGAGTACGGTCTTC-3′) (Fig. 1). This DNA fragment was cloned into the *Sma*I site of pBGS19$^+$ vector. Plasmid pHUL4D, which contains the complete *ampD* gene and the first 317 nucleotides encoding AmpE, was constructed by cloning a 984-bp DNA amplification product into the *Sma*I site of pBGS19$^+$ vector. This fragment was amplified as described above with the oligonucleotide primers AmpD-14 and AmpE-4 (5′-CGCCGCCAGGCGTCGCG-3′) (Fig. 1). The sequences of all cloned PCR DNA fragments were confirmed by complete resequencing.

Since *E. coli* strains do not contain *ampR* (24), the *E. coli* strains STC172 and JRG582 were first transformed with plasmid pEC1C carrying *ampC* and *ampR* of *E. cloacae* (23). The data in Table 2 show that STC172/pEC1C exhibits a high basal β-lactamase activity and is hyperinducible, while JRG582/pEC1C has a fully derepressed phenotype, as shown previously (10). Both of these constructs were highly resistant to ampicillin and cefotaxime. The *ampD* genes of both *E. coli* and *P. aeruginosa*, as expressed from pNH5 and pHUL4D, respectively, transcomplemented the *E. coli ampD* and *ampDE* mutants to low-level β-lactam resistance and wild-type β-lactamase expression (low basal level and inducibility) (Table 2). This shows that the cloned *P. aeruginosa ampD* gene expresses a functional AmpD protein in *E. coli* cells. These data, as well as the high homology observed among the AmpD proteins, strongly suggest that *P. aeruginosa* AmpD acts as an *N*-acetyl-anhydromuramyl-L-alanine amidase, which leads to a decreased amount of anhydromuropeptide, the signal molecule for β-lactamase expression (5, 9, 11, 12). The induced/noninduced ratio of β-lactamase activity was more than 7.5 times lower in cells producing the *E. coli* AmpD than that in cells containing *P. aeruginosa* AmpD, and this could be explained by the presence of a very strong promoter behind *E. coli ampD*.

TABLE 2. MICs of β-lactam antibiotics and specific activities of *E. cloacae* AmpC in *E. coli* STC172 and JRG582 containing the *E. coli ampD* and *P. aeruginosa ampD* and *ampE* genes

Strain	Relevant genotype	MIC (μg/ml)		β-Lactamase activity[a]		Fold increase in activity vs noninduced
		Ampicillin	Cefotaxime	Noninduced	Induced[b]	
STC172/pEC1C/pBGS19$^+$	*ampC*$^+$ *ampR*$^+$ *ampD11 ampE*$^+$	1,024	32	3,260 ± 190	23,490 ± 1,240	7.2
STC172/pEC1C/pNH5	*ampC*$^+$ *ampR*$^+$ *ampD*$^+$ *ampE*$^+$	8	0.5	59 ± 3	86 ± 5	1.5
STC172/pEC1C/pHUL4D	*ampC*$^+$ *ampR*$^+$ *ampD*$^+$ *ampE*$^+$	128	2	116 ± 5	2,420 ± 130	21
STC172/pEC1C/pHUL4DE-2	*ampC*$^+$ *ampR*$^+$ *ampD*$^+$ *ampE*$^+$	128	1	113 ± 5	2,930 ± 199	26
STC172/pEC1C/pHUL4DE-1	*ampC*$^+$ *ampR*$^+$ *ampD*$^+$ *ampE*$^+$ ORF-1	64	4	175 ± 7	1,260 ± 60	7.2
JRG582/pEC1C/pBGS19$^+$	*ampC*$^+$ *ampR*$^+$ Δ(*ampDE*)2	1,024	64	103,280 ± 6,230	109,400 ± 7,110	1.1
JRG582/pEC1C/pNH5	*ampC*$^+$ *ampR*$^+$ *ampD*$^+$	8	<0.5	216 ± 14	481 ± 29	2.2
JRG582/pEC1C/pHUL4D	*ampC*$^+$ *ampR*$^+$ *ampD*$^+$	128	8	226 ± 16	8,670 ± 510	38
JRG582/pEC1C/pHUL4DE-2	*ampC*$^+$ *ampR*$^+$ *ampD*$^+$ *ampE*$^+$	128	8	414 ± 39	7,050 ± 400	17
JRG582/pEC1C/pHUL4DE-1	*ampC*$^+$ *ampR*$^+$ *ampD*$^+$ *ampE*$^+$ ORF-1	256	2	241 ± 19	3,970 ± 300	17

[a] All of the induction experiments were performed in duplicate, and the results presented are averages of two determinations. β-Lactamase activity is expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.
[b] Induction was carried out with 4 μg of cefoxitin per ml.

The MICs, as well as the basal and induced levels, of β-lactamase were almost the same for cells containing AmpD as those for cells containing AmpD and AmpE. This strongly suggests that similar to the *E. coli* protein, *P. aeruginosa* AmpE has no effect on β-lactamase expression (24). This protein, like its homolog in *E. coli* (10, 19), has the features of an integral inner membrane protein, but its role in the bacterial cell is still unknown.

Expression of ORF-1 together with *ampDE* from plasmid pHULDE-1 reduced by more than 1.7-fold the induced level of β-lactamase in both *E. coli ampD* and *ampDE* mutants, compared to that in strains producing AmpD and AmpE. This suggests that the product of this ORF, which has the features of a cytoplasmic protein, may affect β-lactamase expression in the presence of a β-lactam inducer, perhaps by interacting directly with AmpD or by acting as a regulator of *ampD* expression, or perhaps by a new unknown mechanism. Further experiments are needed to explore the role of this ORF.

The high degree of homology among the various AmpD proteins shows that AmpD of *P. aeruginosa* is in its evolution very close to its homologs in enterobacteria, and they probably share a common mechanism of regulation of AmpC β-lactamase expression and murein metabolism. In our approach to characterizing the mechanism of regulation of AmpC β-lactamase production in *P. aeruginosa*, a putative *ampG* gene was also cloned and characterized (17). This further strengthens the close relationships between the *P. aeruginosa* and enterobacterial AmpC β-lactamase induction mechanism and cell wall recycling.

In enterobacteria, three out of four phenotypes of altered β-lactamase expression have been associated with mutations in *ampD* (2, 6, 10, 14, 19, 29). In clinical and laboratory isolates of *P. aeruginosa*, three phenotypes of altered β-lactamase expression have also been described (4, 27), and a study is in progress to characterize the *ampD* gene in some of these isolates.

Nucleotide sequence accession number. The nucleotide sequence data for the *ampD* and *ampE* genes appear in the GenBank database under accession no. AF082575.

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