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Cloning, Nucleotide Sequencing, and Analysis of the Gene Encoding an AmpC β-Lactamase in *Acinetobacter baumannii*

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A clinical strain of *Acinetobacter baumannii* (strain Ab RYC 52763/97) that was isolated during an outbreak in our hospital and that was resistant to all β-lactam antibiotics tested produced three β-lactamases: a TEM-1-type (pI, 5.4) plasmid-mediated β-lactamase, a chromosomally mediated OXA-derived (pI, 9.0) β-lactamase, and a presumptive chromosomal cephalosporinase (pI, 9.4). The nucleotide sequence of the chromosomal cephalosporinase gene shows for the first time the gene encoding an AmpC β-lactamase in *A. baumannii*. In addition, we report here the biochemical properties of this *A. baumannii* AmpC β-lactamase.

Acinetobacter spp. are recognized as important opportunist pathogens mainly in immunocompromised patients (9, 10). Their contribution to nosocomial infection has increased over the past three decades (6, 7, 27), and several outbreaks of hospital infection have been reported worldwide (5; G. Bou, G. Cerveró, D. Malpica, M. Pérez-Vázquez, L. De Rafael, and J. Martínez-Beltrán, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. K-120, 1998). Though prevalent in nature (4) and generally regarded as commensals of human skin and of the human respiratory tract (1, 15), they have been implicated as the cause of serious infectious diseases such as pneumonia, urinary tract infection, endocarditis, wound infection, meningitis, and septicemia, involving mostly patients with impaired host defenses (6, 9, 30). Antimicrobial treatment of serious infections due to *Acinetobacter*, particularly those caused by *Acinetobacter baumannii*, is complicated by the widespread multidrug resistance pattern of the organism (8, 18). With respect to β-lactam antibiotics, the most common mechanism of resistance is due to the synthesis of β-lactamases encoded either by the chromosome or by plasmids (9). So far, several class A and class D plasmid-encoded β-lactamases conferring different phenotypes of resistance to *A. baumannii* have been described (9, 24, 31, 32; G. Bou, A. Oliver, and J. Martínez-Beltrán, submitted for publication). Likewise, different chromosomally mediated cephalosporinases (pI, >8) have also been reported (9, 25), and sometimes these cephalosporinases have been particularly difficult to visualize by isoelectric focusing (24). In any case, nucleotide sequencing of the *ampC* gene has never been described. The main objective of this work was to clone and sequence the gene encoding the chromosomally mediated AmpC β-lactamase from a multiresistant *A. baumannii* clinical strain (Ab RYC 52763/97) isolated during an outbreak at our hospital in 1997 (Bou et al., 38th ICAAC). A detailed description of its biochemical characteristics is also provided.

The susceptibility testing of the Ab RYC 52763/97 strain was performed by an agar dilution method, as recommended by the National Committee for Clinical Laboratory Standards (23), using Mueller-Hinton agar and an inoculum size of 10^4 CFU per spot. Antibiotics were kindly provided as powders of a fixed potency by their corresponding manufacturers. The antibiotic susceptibility profiles of all strains included in this study are shown in Table 1. The strain Ab RYC 52763/97 was highly resistant to all β-lactam antibiotics tested, with minimum inhibitory concentrations (MICs) of imipenem and meropenem being 128 and 256 μg/ml, respectively.

β-Lactamases were analyzed by isoelectric focusing as described by Matthew et al. (22). The sonicated extract of strain Ab RYC 52763/97 contained three β-lactamases, as follows: one yielded a band with a pI of 5.4 and was cloned after amplification with $bla_{\text{-TEM}}$ primers C1 (5′-GGGAATTCTCGGGGAAATGTGCGCGGAAC) and C2 (5′-GGGATCCGAGTAAACTTGGTCTGACAG) (TEM-1 type); a second focused at a pI of 9.0, and after cloning (clone pBMB-1), the gene nucleotide sequence showed homology with those of OXA-derived

TABLE 1. MICS of β-lactams

Antibiotic	MIC (μg/ml)[a]			
	A. baumannii RYC 52763/97	*E. coli* TG1	*E. coli* TG1 (pGER1)	*E. coli* TG1 (pGER2)
Ampicillin	>1,024	4	128	>1,024
Ampicillin plus clavulanic acid[b]	>1,024	4	64	128
Ampicillin plus sulbactam[b]	>1,024	2	16	>1,024
Ampicillin plus tazobactam[b]	>1,024	2	32	64
Ticarcillin	>1,024	4	32	>1,024
Cefazolin	>256	8	>256	>256
Cefuroxime	>256	4	>256	>256
Cefoxitin	>256	4	4	4
Cefotaxime	>256	≤0.125	4	8
Cefotaxime plus clavulanic acid	>256	≤0.125	2	4
Cefotaxime plus sulbactam	>256	≤0.125	0.25	2
Cefotaxime plus tazobactam	>256	≤0.125	0.5	1
Ceftazidime	>256	≤0.125	16	32
Ceftazidime plus clavulanic acid	>256	≤0.125	8	16
Ceftazidime plus sulbactam	>256	≤0.125	1	8
Ceftazidime plus tazobactam	>256	≤0.125	2	4
Cefepime	256	≤0.125	0.25	0.5
Aztreonam	>256	≤0.125	1	4
Imipenem	128	0.25	0.25	0.25
Meropenem	256	0.03	0.03	0.03

[a] Enzymes are as follows: for *A. baumannii* RYC 52763/97, AmpC plus a β-lactamase with a pI of 9.0 plus TEM-1; for *E. coli* TG1(pGER1), AmpC; and for *E. coli* TG1(pGER2), AmpC plus TEM-1. Clone *E. coli* TG1(pGER2) harbored the *ampC* gene in the pUC18 multicopy plasmid (containing the $bla_{\text{TEM-1}}$ resistance marker).

[b] Concentration of β-lactamase inhibitor was 4 μg/ml.

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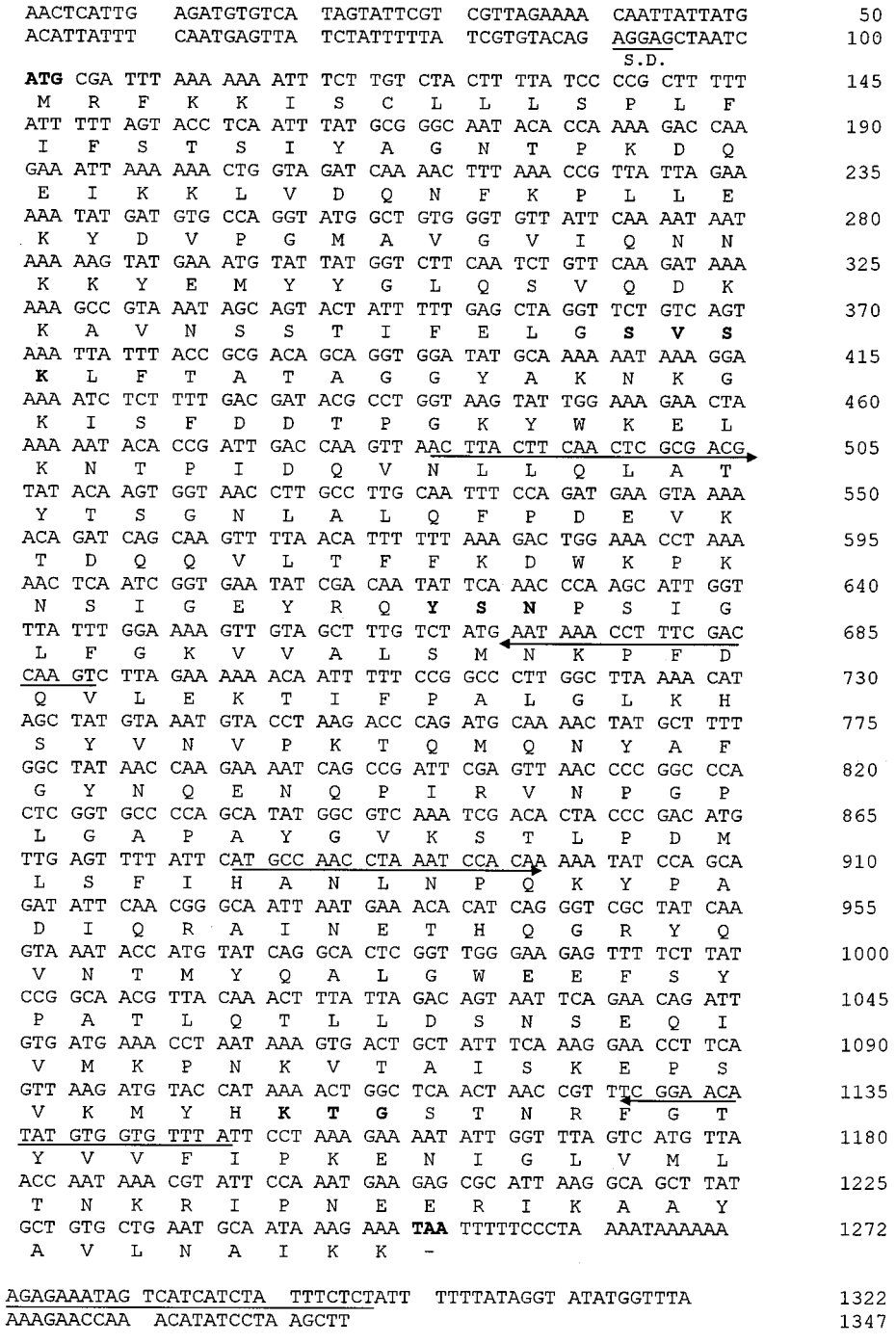

FIG. 1. Nucleotide sequence of the *ampC* gene. The deduced amino acid sequence of AmpC β-lactamase is shown below the nucleotide triplets. The boldface ATG and TAA represent the initiation and termination codons, respectively. A putative Shine-Dalgarno (S.D.) ribosomal recognition site (underlined) is indicated. The positions of the primers used to sequence the gene are indicated by underlining with arrowheads. The β-lactamase active site S-V-S-K, the conserved triad K-T-G, and the class C typical motif Y-X-N are presented in boldface. A putative terminator sequence is underlined.

β-lactamases (Bou et al., submitted); and the third focused at a pI of 9.4 and likely corresponded to a chromosomal cephalosporinase. With the *A. baumannii* clinical strain no transfer of resistance phenotype was observed in conjugation experiments. By alkaline lysis (28), a plasmid of 22 kb was isolated in this strain. By enzymatic restriction and ligation techniques (28) only a TEM-1 gene was found in this plasmid.

Chromosomal DNA of Ab RYC 52763/97 was prepared by the method of Grimont and Grimont (16), digested with *Hin*dIII (Boehringer-Mannheim, Mannheim, Germany), and ligated into the *Hin*dIII site of pBGS18$^-$ (29). Recombinant plasmids were introduced into *Escherichia coli* TG1 by transformation with $CaCl_2$ (28), and transformants were selected on Luria-Bertani agar plates supplemented with ampicillin (25 μg/ml) and kanamycin (50 μg/ml). Three *E. coli* transformants were obtained on selective medium supplemented with the antibi-

TABLE 2. Percent identity of amino acid sequences of *A. baumannii* AmpC and other class C β-lactamases

β-Lactamase (reference)	% Identity with:						
	A. baumannii AmpC	*A. sobria* AmpC	CMY-1	*S. marcescens* AmpC	FOX-2	FOX-3	*P. aeruginosa* AmpC
A. baumannii AmpC	100	42.3	41.8	41.7	41.3	41.0	40.5
A. sobria AmpC (26)		100	74.5	46.9	77.2	75.3	55.5
CMY-1 (2)			100	48.7	74.3	72.4	57.9
S. marcescens AmpC[a]				100	46.6	46.1	48.8
FOX-2 (3)					100	96.3	54.7
FOX-3 (20)						100	54.4
P. aeruginosa AmpC (19)							100

[a] EMBL accession no. AB008454.

otics mentioned above. They harbored a recombinant plasmid, designated pGER1, with an insert of about 2.2 kb.

The β-lactam susceptibility pattern of the three transformants was identical, displaying resistance to ampicillin, cefazolin, cefuroxime, and, to a lesser extent, ceftazidime, while the susceptibility to the remaining β-lactams tested was differently affected by the presence of the recombinant plasmid pGER1 (Table 1). The MICs of ticarcillin, cefotaxime, cefepime, and aztreonam for *E. coli* TG1(pGER1) were increased, whereas the MICs of cefoxitin, imipenem, and meropenem remained unchanged. Regarding the effect of the β-lactamase inhibitors sulbactam and tazobactam, the MICs of ampicillin, cefotaxime, and ceftazidime decreased by 4- to 16-fold, in contrast with the very slight effect obtained with clavulanic acid. By isoelectric focusing, the single band of β-lactamase activity in the *E. coli* transformant cofocused with the β-lactamase (pI, 9.4) of the wild-type strain (results not shown).

In order to perform the sequencing reactions the 2.2-kb insert from the plasmid pGER1 was cloned in the pUC18 multicopy plasmid (29), resulting in the plasmid pGER2. Sequencing was carried out with the *Taq* DyeDeoxiTerminator Cycle Sequencing Kit and with primers specific to the coding sequence, and the sequence was analyzed in an automatic DNA sequencer (377 Abi-Prims; Perkin-Elmer). The entire sequence of the fragment was determined twice for accuracy.

The sequenced fragment was 2,195 bp long and contained only one open reading frame (ORF) (Fig. 1). An ATG codon initiated an 1,152-bp ORF which ended with a TAA codon (383 amino acids long). The initiation codon was preceded by a Shine-Dalgarno ribosome-binding sequence, AGGAG. GenBank database searches with this ORF revealed similarities with several class C chromosomal and plasmid-mediated β-lactamases (Table 2). The highest identity (40.5 to 42.3%) was observed with the following β-lactamases: AmpC of *Aeromonas sobria*, CMY-1, AmpC of *Serratia marcescens*, FOX-2, FOX-3, and AmpC of *Pseudomonas aeruginosa*; 35 to 40% identity was seen for AmpC β-lactamases from *E. coli*, *Proteus stuartii*, *Yersinia enterocolitica*, *Morganella morganii*, *Enterobacter cloacae*, and *Citrobacter freundii*. The deduced peptide sequence contained conserved motifs found in serine β-lactamases (14), as follows: the SXSK motif of the active site of AmpC, the class C typical motif YXN, and the KTG domain. The nucleotide sequence of the flanking regions of the *ampC* gene (about 400 bp on each side) did not show inverted repeated sequences suggestive of the presence of a transposable element. This *ampC* gene was probably not inserted in an integron, since the 59-base element (specific to gene cassettes inserted in integron structures) was not observed on the flanking regions.

The synthesis of chromosomal AmpC β-lactamases in some gram-negative rods such as *E. cloacae*, *C. freundii*, and *P. aeruginosa* may be inducible and involves genes such as the repressor *ampR* and *ampD*. The first is located immediately upstream from the *ampC* gene and is divergently transcribed (19). Sequencing of the flanking regions of the gene encoding the AmpC β-lactamase produced by the Ab RYC 52763/97 strain did not show homology with the *ampR* regulatory gene. Induction experiments with cefoxitin (one-half of the MIC) performed with the *E. coli* transformant carrying the *ampC* gene and with the original Ab RYC 52763/97 strain did not show an increase in the synthesis of the AmpC β-lactamase, determined as specific enzymatic activity, when the inducer was added (data not shown). These experiments indicated that the *A. baumannii* AmpC β-lactamase in this strain is noninducible.

The substrate profile of the AmpC β-lactamase was determined with the enzyme partially purified by G-75 Sephadex (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Initial hydrolysis rates were monitored spectrophotometrically (UVIKON-930) at 25°C in 0.05 M phosphate buffer (pH 7.4), as described previously (12). The V_{max} values indicate that cephaloridine is hydrolyzed more quickly than ampicillin and that cefoxitin and imipenem are not hydrolyzed at detectable levels (Table 3). Inhibition studies showed that the remaining β-lactamase activities in the presence of 100 μM each inhibitor were as follows: clavulanic acid, 91%; sulbactam, 40%; and tazobactam, 45%. The 50% inhibitory concentrations (IC_{50}s) were obtained from semilogarithmic plots of enzymatic activity against inhibitor concentration. The IC_{50}s were 270 μM for clavulanic acid, 7 μM for sulbactam, and 8 μM for tazobactam. These results correlated with the decrease in the MICs of ampicillin, cefotaxime, and ceftazidime in combination with these inhibitors against *E. coli* TG1 harboring AmpC β-lactamase (pGER1).

TABLE 3. Kinetic and inhibition parameters of the *A. baumannii* AmpC β-lactamase[a]

Antibiotic	K_m (μM)	V_{max} (μmol/min/μl)	Relative V_{max}[b]	IC_{50} (μM)
Cephaloridine	960	5,000	100	
Ampicillin	38	380	7.6	
Ceftazidime	14	52	1.0	
Cefotaxime	7.0	25	0.5	
Cefoxitin		<1	<0.01	
Imipenem		<1	<0.01	
Clavulanic acid				270[c]
Sulbactam				7[c]
Tazobactam				8[c]

[a] Kinetic experiments were performed using a 7.45-mg/ml proteic extract with a specific activity of 0.064 μmol of nitrocefin hydrolyzed/min/μg of protein.

[b] Obtained by normalizing the value for each antibiotic to that for cephaloridine (set to 100).

[c] Nitrocefin (25 μg/ml) was used as the substrate following 10 min preincubation of enzyme and inhibitor.

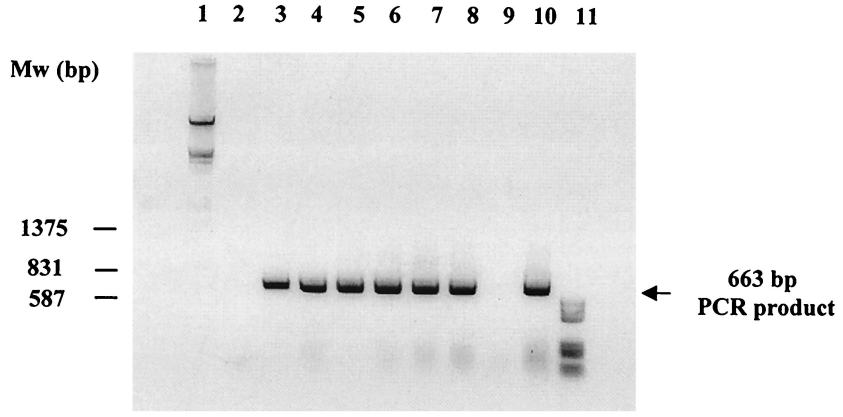

FIG. 2. Detection of the *ampC* gene in different *A. baumannii* isolates. The amplified products were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide (50 μg/ml). Lanes 1 and 11, DNA molecular weight markers III and V, respectively (Boehringer-Mannheim); lane 2, negative control (genomic DNA of *Listeria monocytogenes* amplified with primers P1 and P2); lanes 3 to 8, *A. baumannii* strains (Ab 1 to 6); lane 9, *A. junii* strain; lane 10, positive control (strain Ab RYC 52763/97).

In order to detect the *ampC* gene in different *A. baumannii* isolates, a PCR assay was performed. Six genotypically different *A. baumannii* strains (REP-PCR tested) with different antibiotic susceptibility patterns that had been collected in our hospital during the previous five years were used. The reactions were carried out with a 50-μl volume of a reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 2.0 mM magnesium chloride, 200 μM deoxynucleotide triphosphate, 50 pmol of each primer, 500 ng of the chromosomal DNA, and 2.5 U of *Taq* polymerase (Roche). The primers of the *ampC* coding region, P1 (5′-TAAACACC ACATATGTTCCG) and P2 (5′-ACTTACTTCAACTCGCG ACG), were used. Amplification reactions were submitted to the following program: initial denaturation (4 min at 94°C) followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C), and extension (2 min at 72°C), with a single extension of 10 min at 72°C. The amplified 663-bp product was resolved by electrophoresis in a 1.5% (wt/vol) agarose gel containing ethidium bromide (50 μg/ml). As shown in Fig. 2, the *ampC* gene was extensively spread between the six *A. baumannii* isolates but not in *Acinetobacter junii*. This result strongly suggests that the AmpC β-lactamase may play a role in the β-lactam resistance of *A. baumannii*.

The presence of chromosomal β-lactamases in the genus *Acinetobacter* was first suggested by Matthew and Harris (21). By examining β-lactamases from 30 strains of *Acinetobacter*, Joly-Guillou et al. (17) revealed a cephalosporinase with a pI of >8.0 in all these strains. Afterwards, Blechschmidt et al. (11) reported the purification and biochemical characterization of an extracellular β-lactamase with a pI of 9.3 produced by *Acinetobacter calcoaceticus*. Other attempts have been carried out in order to characterize the *A. baumannii* cephalosporinase biochemically (25), but nothing has been reported about the gene encoding the enzyme. The present study describes the genetic and biochemical characteristics of the *A. baumannii* AmpC β-lactamase. The following important features are noteworthy. (i) The amino acid sequence is closely related to the class C cephalosporinases, and the highest similarity was obtained with the *A. sobria* AmpC β-lactamase. (ii) The enzyme shows a typical cephalosporinase substrate profile (13). (iii) Enzymatic inhibition assays and antibiotic susceptibility studies showed a moderate inhibitory effect of sulbactam and tazobactam against *A. baumannii* AmpC β-lactamase. As for clavulanic acid, only a very slight inhibitory effect was observed. (iv) No AmpC β-lactamase induction was observed when cefoxitin was added, and no *ampR* sequences were found on the flanking regions of the *ampC* gene. Nevertheless, whether a regulatory AmpR-like protein gene exists in *A. baumannii*, although not adjacent to the *ampC* gene, remains to be elucidated.

Certainly, resistance to β-lactam antibiotics in *A. baumannii* is a multifactorial problem involving resistance mechanisms in addition to β-lactamases (9); thus, in our study, the β-lactam MICs conferred by the AmpC β-lactamase, even by means of a multicopy plasmid carried by *E. coli* TG1, did not reach the high MICs observed for the Ab RYC 52763/97 clinical strain (MICs conferred by the OXA-derived β-lactamase [Bou et al., submitted] are not relevant when compared with the *A. baumannii* clinical strain). Note also that the MICs of the carbapenems were not affected by the presence of the AmpC β-lactamase even in a multicopy plasmid. Nevertheless, the presence of the *ampC* gene in all six different *A. baumannii* strains tested suggests that the AmpC β-lactamase, in combination with other β-lactam resistance mechanisms, may play an important role in β-lactam resistance in *A. baumannii*.

In summary, we report here for the first time the cloning, sequencing, and analysis of the *ampC* gene and the biochemical characterization of the AmpC β-lactamase from an *A. baumannii* clinical strain.

Nucleotide sequence accession number. The nucleotide sequence of the *ampC* gene has been given the EMBL database accession no. AJ009979.

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