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Molecular Characterization of the Gene Encoding a New AmpC β-Lactamase in a Clinical Strain of *Acinetobacter* Genomic Species 3

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A presumptive chromosomal cephalosporinase (pI, 9.0) from a clinical strain of *Acinetobacter* genomic species 3 (AG3) is reported. The nucleotide sequence of this β-lactamase shows for the first time the gene encoding an AmpC enzyme in AG3. In addition, the biochemical properties of the novel AG3 AmpC β-lactamase are reported

The bacterial genus *Acinetobacter* consists of strictly aerobic gram-negative coccobacilli, which are oxidase negative, nonmotile, nitrate negative, and nonfermentative.

Studies based on DNA-DNA hybridization have led to the description of 23 DNA homology groups, or genomospecies, within the genus *Acinetobacter* (5, 7, 11, 15, 21, 26). Seven species of this genus have been named and are included in the *Index of the Bacterial and Yeast Nomenclatural Changes* (19): (i) *A. calcoaceticus*, or genomospecies 1; (ii) *A. baumannii*, or genomospecies 2; (iii) *A. haemolyticus*, or genomospecies 4; (iv) *A. junii*, or genomospecies 5; (v) *A. johnsonii*, or genomospecies 7; (vi) *A. lwoffii*, or genomospecies 8; and (vii) *A. radioresistens*, or genomospecies 12. Groups 1 (*A. calcoaceticus*), 2 (*A. baumannii*), and the as-yet-unnamed and closely related genomic DNA groups 3 and 13TU (often referred to as the *A. calcoaceticus-A. baumannii* complex) are the most frequent species among clinical isolates, particularly *A. baumannii* and *Acinetobacter* genomospecies 3 (AG3) (5, 6, 13, 26).

Earlier studies showed statistically significant differences between the distributions in Hong Kong and Europe of genomic DNA groups of isolates obtained from blood cultures and various superficial carriage sites (10). Indeed, in some Hong Kong hospitals, AG3 strains accounted for up to 40% of the blood culture isolates (10). Antimicrobial susceptibilities also differed significantly among members of the *A. calcoaceticus-A. baumannii* complex, which indicates diversity in the molecular mechanisms involved in the antimicrobial resistance observed (14, 29). In keeping with this trend, *A. baumannii* was the most resistant genospecies.

The most common mechanism of the resistance of *A. baumannii* to β-lactam antibiotics is attributed to the presence of β-lactamases encoded either by the chromosome or by plasmids (2). Several class A, B, and D β-lactamases (1, 2, 3, 22, 23, 27), as well as chromosome-mediated cephalosporinases (pI, >8) (2, 3) (which confer different resistance phenotypes to *A. baumannii*) have been described. However, only one *ampC* gene (AmpC of RYC52763) encoding an AmpC β-lactamase (4) and two allelic variants (ABAC-1 and ABAC-2) (17) have been reported so far for *A. baumannii* strains.

Regarding β-lactam resistance in the AG3 group, two metalloenzymes, VIM-2 and IMP-4, have been identified in Hong Kong and Korea (9, 30).

The aim of this study was to elucidate the mechanisms associated with the resistance to β-lactam antibiotics shown by a clinical strain of *Acinetobacter* genomic DNA group 3

TABLE 1. MICs of β-lactams for clinical strain AJC68081, *E. coli* TG1, *E. coli* TG1 (pBE-1), and *E. coli* TG1 (pGER1)

Antibiotic	MIC (μg/ml)[a]			
	AJC68081 (produces AmpC of AG3)	*E. coli* TG1	*E. coli* TG1 (pBE-1)[b]	*E. coli* TG1 (pGER1)[c]
Amoxicillin	>256	3	256	128
Amoxicillin + clavulanate[d]	6	2	24	32
Piperacillin	>256	0.38	64	8
Cephalothin	>256	3	>256	>256
Cefuroxime	>256	1.5	>256	>256
Cefoxitin	>256	2	128	2
Cefotaxime	256	0.023	2	4
Cefotaxime + clavulanate[d]	0.19	0.023	1	1
Ceftazidime	32	0.064	0.75	16
Ceftazidime + clavulanate[d]	0.19	0.064	0.5	8
Cefepime	32	0.016	0.047	0.25
Aztreonam	12	0.032	0.25	1
Imipenem	1	0.125	0.38	0.125
Meropenem	0.75	0.008	0.023	0.012
Clavulanic acid	8–16	ND	ND	ND
Sulbactam	16–32	ND	ND	ND

[a] ND, not done.
[b] Transformant producing AmpC β-lactamase of AG3.
[c] Transformant producing AmpC β-lactamase of *A. baumannii.*
[d] Clavulanate was used at 4 μg/ml.

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(AJC68081) identified by amplified ribosomal DNA restriction analysis (28) and isolated from a wound exudate of a patient treated at the Juan Canalejo Hospitalary Complex.

The susceptibility testing of the AJC68081 strain was performed by a microdilution method following the recommendations of the National Committee for Clinical Laboratory Standards (20). Antibiotics were kindly provided by their manufacturers as powders of fixed potency. MICs were confirmed by the E-test. The antibiotic susceptibility profiles of all strains included in this study are shown in Table 1.

The β-lactamases were analyzed by isoelectric focusing as described by Matthew et al. (18). The sonicated extract of strain AJC68081 contained a single β-lactamase with a pI of ca. 9.0, which may correspond to that of a chromosomal cephalosporinase. Alkaline lysis of the bacteria (24) did not result in plasmid isolation.

The possibility of a certain degree of homology between the present AG3 cephalosporinase and the previously reported *ampC* gene of *A. baumannii* allowed us to design oligonucleotides that could specifically amplify our target AG3 cephalosporinase gene by PCR. Chromosomal DNA from strain AJC68081 was purified according to standard protocols (MasterPure DNA purification kit; Epicentre, Madison, Wis.). Five hundred nanograms of the AG3 chromosome was used as a template to be amplified by PCR with the two oligonucleotides ampC1 forward (5′-TAGTATTCGTCGTTAGAAAACAAT) and ampC2 reverse (5′-GCTTAGGATATGTTTGGTTCTT) (Sigma-Genosys Ltd., Cambridge, United Kingdom), which hybridize in the untranslated regions of the *A. baumannii* (RYC52763) *ampC* gene (4). The PCR was performed under the following conditions: denaturation, 10 min at 94°C; amplification, 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; and elongation, 16 min at 72°C. An aliquot (20 μl) of the sample was subjected to electrophoresis in a 1.0% agarose gel. The gel showed an amplified product, detected by ethidium bromide staining (50 mg/liter), of 1.3 kb, which was the expected size of the *ampC* gene in relation to that in *A. baumannii*. For further analysis, the 1.3-kb amplicon was ligated into pGEM-T easy vector (Promega Corporation, Madison, Wis.), and the recombinant plasmids were introduced into *Escherichia coli* TG1 by transformation with $CaCl_2$ (24). The selection of transformants on Luria-Bertani agar plates supplemented with ampicillin (50 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG, 25 μg/ml; Roche Diagnostics, Mannheim, Germany), and 5′-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 100 μg/ml; Roche Diagnostics) resulted in several white colonies, all carrying an identical recombinant plasmid. After alkaline lysis of the transformant (24), enzymatic digestion of the purified pGEM-T plasmid with EcoRI

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                                    TAG TAT TCG TCG TTA GAA AAC AAT TAT TGT GAC      33
ATT ATT TCA ATG AGT TAT CTA TTT TTG TCG TGT ACA GAG GAG CTA ATC      81
                                                   S.D.
ATG CGA TTT AAA AAA ATT TCT TGT CTA CTT TTA TCC CCG CTT TTT ATT     129
 m   r   f   k   k   i   s   c   l   l   l   s   p   l   f   i
TTT AGT ACC TCA ATT TAT GCG GGC AAT ACA CCA AAA GAC CAA GAA ATT     177
 f   s   t   s   i   y   a   G   N   T   P   K   D   Q   E   I
AAA AAA CTG GTA GAT CAA AAT TTT AAA CCA TTA TTA GAA AAA TAT GAT     225
 K   K   L   V   D   Q   N   F   K   P   L   L   E   K   Y   D
GTA CCA GGT ATG GCT GTG GGT GTT ATT CAA AAT AAT AAA AAG TAT GAA     273
 V   P   G   M   A   V   G   V   I   Q   N   N   K   K   Y   E
ATG TAT TAT GGT CTT CAA TCT GTT CAA GAT AAA AAA GCC GTA AAT AGC     321
 M   Y   Y   G   L   Q   S   V   Q   D   K   K   A   V   N   S
AGT ACC ATT TTT GAG CTA GGT TCT GTC AGT AAA TTA TTT ACC GCG ACA     369
 S   T   I   F   E   L   G   S   V   S   K   L   F   T   A   T
GCA GGT GGA TAT GCA AAA AAT AAA GGA AAA ATC TCT TTT GAC GAT ACG     417
 A   G   G   Y   A   K   N   K   G   K   I   S   F   D   D   T
CCT GGT AAA TAT TGG AAA GAG CTA AAA AAT ACA CCG ATT GAT CAA GTT     465
 P   G   K   Y   W   K   E   L   K   N   T   P   I   D   Q   V
AAC TTA CTT CAA CTC GCG ACG TAT ACA AGT GGC AAC CTC GCT TTA CAA     513
 N   L   L   Q   L   A   T   Y   T   S   G   N   L   A   L   Q
TTT CCA GAT GAA GTA CAA ACA GAT CAA CAA GTT TTA ACT TTT TTC AAA     561
 F   P   D   E   V   Q   T   D   Q   Q   V   L   T   F   F   K
GAC TGG CAA CCT AAA AAC CCA ATC GGT GAA TAC AGA CAA TAT TCA AAT     609
 D   W   Q   P   K   N   P   I   G   E   Y   R   Q   Y   S   N
CCA AGT ATT GGC CTA TTT GGA AAG GTT GTG GCT TTG TCT ATG AAT AAA     657
 P   S   I   G   L   F   G   K   V   V   A   L   S   M   N   K
CCT TTC GAC CAA GTC TTA GAA AAA ACA ATT TTT CCG GCC CTT GGC TTA     705
 P   F   D   Q   V   L   E   K   T   I   F   P   A   L   G   L
AAA CAT AGC TAT GTA AAT GTA CCT AAG ACC CAG ATG CAA AAC TAT GCT     753
 K   H   S   Y   V   N   V   P   K   T   Q   M   Q   N   Y   A
TTT GGC TAT AAC CAA GAA AAT CAG CCG ATT CGA GTT AAC CCC GGC CCA     801
 F   G   Y   N   Q   E   N   Q   P   I   R   V   N   P   G   P
CTT GAT GCC CCA GCA TAC GGC GTC AAA TCG ACA CTA CCC GAC ATG TTG     849
 L   D   A   P   A   Y   G   V   K   S   T   L   P   D   M   L
AGT TTT ATT CAT GCC AAC CTT ACC CCA CAG AAA TAT CCG ACA GAT ATT     897
 S   F   I   H   A   N   L   T   P   Q   K   I   P   T   D   I
CAA CGG GCA ATT AAT GAA ACA CAT CAA GGG TTC TAT CAA GTC GGC ACC     945
 Q   R   A   I   N   E   T   H   Q   G   F   Y   Q   V   G   T
ATG TAT CAG GCA CTT GGT TGG GAA GAG TTT TCT TAT CCG GCA ACG TTA     993
 M   Y   Q   A   L   G   W   E   E   F   S   Y   P   A   T   L
CAA ACT TTA TTA GAC AGT AAT TCA GAA CAG ATT GTG ATG AAA CCT AAT    1041
 Q   T   L   L   D   S   N   S   E   Q   I   V   M   K   P   N
AAA GTG ACT GCT ATT TCA AAG GAA CCT TCA GTT AAG ATG TAC CAT AAA    1089
 K   V   T   A   I   S   K   E   P   S   V   K   M   Y   H   K
ACT GGC TCA ACC AAC GGT TTC GGA ACA TAT GTG GTC TTT ATT CCT AAA    1137
 T   G   S   T   N   G   F   G   T   Y   V   V   F   I   P   K
GAA AAT ATT GGC TTA GTC ATG TTA ACC AAT AAA CGT ATT CCA AAT GAA    1185
 E   N   I   G   L   V   M   L   T   N   K   R   I   P   N   E
GAG CGC ATT AAG GCA GCT TAT GCT GTG CTG AAT GCA ATA AAG AAA TAA    1233
 E   R   I   K   A   A   Y   A   V   L   N   A   I   K   K   -
CCG TTT TAG CCA AAA ACA AAA AGA GAA ATG GAT AAT CCC ATT TCT CTT    1281
TTT TAT GCA CTT ATT CTT TAA AAG AAC CAA ACA TAT CCT A              1321
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FIG. 1. Nucleotide sequence of the 1.3-kb fragment. The amino acid sequence deduced for AmpC G3 β-lactamase is shown on the line below the nucleotide triplets. The ATG and TAA shown in boldface type represent the initiation and termination codons, respectively. A putative Shine-Dalgarno (S.D.) ribosomal recognition site is indicated. The positions of the primers used to sequence the gene and to detect the *ampC* gene in several AG3 strains are indicated by arrows. The positions of the primers used for amplification and further cloning of the gene are indicated by discontinuous arrows. The putative sequence of the signal peptide is indicated by boldface type and lowercase letters. The β-lactamase active site SVSK, the conserved triad KTG, and the typical class C motif YXN are shown in boldface type (12).

TABLE 2. Percent identity between amino acid sequences of AG3 AmpC and other class C β-lactamases

β-Lactamase[a]	% Identity with:							
	AG3 AmpC	*A. baumannii* AmpC	*Aeromonas hydrophila* AmpC	CMY-1	*Serratia marcescens* AmpC	*Aeromonas sobria* AmpC	FOX-5	*Pseudomonas aeruginosa* AmpC
AG3 AmpC	100	97	46	45	45	44	44	42
A. baumannii AmpC		100	46	45	43	44	43	40
A. hydrophila AmpC			100	82	48	79	75	57
CMY-1				100	48	77	72	56
S. marcescens AmpC					100	48	49	50
A. sobria AmpC						100	77	56
FOX-5							100	55
P. aeruginosa AmpC								100

[a] EMBL accession numbers: AG3 AmpC, CAE00827; *A. baumannii* AmpC, CAB77444; *A. hydrophila* AmpC, CAB76925; CMY-1, CAA63264; *S. marcescens* AmpC, CAB69829; *A. sobria* AmpC, CAA56561; FOX-5, AAG12974; *P. aeruginosa* AmpC, AAM08943.

released a 1.3-kb DNA insert, confirming the success of the cloning procedure. The nucleotide sequence of the insert was elucidated by sequencing it with the *Taq* DyeDeoxiTerminator cycle sequencing kit before analysis with an automatic DNA sequencer (377 Abi-Prims, Perkin-Elmer). The entire sequence of the fragment was 1,321 bp long and contained one open reading frame (Fig. 1) of 1,152 bp (383 amino acids long). GenBank database searches with this open reading frame revealed similarities to several class C chromosome-mediated β-lactamases (Table 2). At a protein level, the highest similarities detected (98.69 to 97.65%) were with the AmpC β-lactamases AmpC ABAC-1, AmpC ABAC-2, and AmpC RYC52763 from *A. baumannii*, respectively (4, 17). Comparison of the amino acids in the AmpC β-lactamases of AG3 with those in ABAC-1, ABAC-2, and AmpC RYC52763 from *A. baumannii* yielded five, eight, and nine amino acid differences, respectively (4, 17). The nucleotide sequence of the promoter region of the gene revealed two substitutions (A to G) at positions −21 and −53 with respect to that of *ampC* in *A. baumannii* (4). The importance of these changes in the regulation of the expression of the AmpC enzyme remains unknown.

To determine the MICs and the biochemical properties of the β-lactamase encoded by the 1.3-kb DNA insert, the insert was cloned in the pBGS18$^-$ plasmid (25) (which carries a kanamycin resistance gene), resulting in the pBE-1 plasmid. The β-lactam patterns of resistance to amoxicillin, cephalothin, cefuroxime, and cefoxitin and, to a lesser extent, amoxicillin-clavulanic acid and piperacillin of several transformants containing the pBE-1 plasmid were identical, whereas the MICs of ceftazidime, cefotaxime, cefepime, aztreonam, and carbapenems were slightly higher than those for the host *E. coli* TG1 strain (Table 1).

Chromosomal AmpC β-lactamases may be inducible in some gram-negative rods, and genes such as the repressor *ampR* and *ampD* may be involved in this pathway (16). The sequences of the flanking regions of the gene encoding the AmpC β-lactamase of *A. baumannii* did not show any homology with the *ampR* gene. Similarly, induction experiments with cefoxitin (at one-half the MIC) performed with the original AJC68081 strain did not show an increase in the synthesis of the AmpC β-lactamase, measured as specific enzymatic activity (in micromoles of nitrocefin hydrolyzed per minute per microgram of protein) when the inducer was added (159 ± 38 μmol/min/μg of protein without the inducer, 145 ± 15 μmol/min/μg of protein in the presence of inducer). These experiments indicated that the AG3 AmpC β-lactamase in this strain is noninducible.

To purify the AmpC enzyme, the bla_{ampC} gene was cloned in the pGEX-6P-1 vector, which allows a fusion protein between glutathione *S*-transferase and the AmpC enzyme. The β-lactamase was purified at homogeneity with the GST gene fusion system (Amersham Pharmacia Biotech, Europe GmbH) according to the manufacturer's directions. With sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the purified protein appeared as a band of ca. 40 kDa (≥95% purity) (data not shown). Kinetic experiments were performed as previously described (4). The specific activity of semipurified AmpC β-lactamase was 49,000 μmol of nitrocefin/min/μg of protein. The relative enzymatic efficiency (V_{max}/K_m) values indicated that cephalothin was hydrolyzed with higher hydrolytic efficiency than ampicillin, as expected for a class C β-lactamase (Table 3). This enzyme also showed moderate hydrolytic activity against cefuroxime and cefoxitin and very little hydrolytic activity against cefotaxime and imipenem; therefore, we could not obtain reliable K_m and V_{max} values for these antibiotics. Hydrolytic activity against ceftazidime was not detected. Fifty percent inhibitory concentrations were calculated as previously reported (4) (Table 3).

A PCR assay was performed with seven genotypically different AG3 strains (repetitive extragenic palindromic-PCR tested) to study the presence of the *ampC* gene. 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 oligonucleotide, 0.5 μg of the chromosomal DNA, and 2.5 U of Ecotaq polymerase (Group 3, Vigo, Spain). The primers for the *ampC*-coding region, P1 forward (5′-ACTTACTTCAACTCGCGACG) and P2 reverse (5′-TAAACACCACATATGTTCCG), were used in the amplification reaction. Amplification conditions were as follows: 10 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, followed by a final step 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). The *ampC* gene was disseminated among

TABLE 3. Kinetic parameters of the *Acinetobacter* genomic species 3 AmpC β-lactamase

Antibiotic	K_m (μM)	Relative V_{max} (%)[a]	V_{max}/K_m	Relative V_{max}/K_m (%)[a]	Hydrolysis rate (μmol/min/μl)[b]	Relative hydrolysis rate (%)[a]	IC_{50} (μM)[c]
Ampicillin	53	100	31	100	420	100	
Cephalothin	100	20,000	3330	10,600	28,400	6,770	
Cefoxitin	1	6	152	482	111	26	
Cefuroxime	52	46	15	47	63	15	
Cefotaxime	ND[d]	ND	ND	ND	19	4	
Imipenem	ND	ND	ND	ND	9	2	
Ceftazidime	ND	ND	ND	ND	<0.1	NH[e]	
Clavulanic acid							>250
Sulbactam							13

[a] Normalized with respect to value for ampicillin (taken as 100%).
[b] Hydrolysis rates were determined by using 100 μM concentrations of the indicated substrates.
[c] IC_{50}, 50% inhibitory concentration.
[d] ND, not done.
[e] NH, no hydrolysis detected.

all of the AG3 isolates (data not shown). This result strongly suggests that AmpC β-lactamase may play a role in the β-lactam resistance of AG3. Moreover, sequence identities among the *ampC* genes of the seven AG3 isolates studied were between 85 to 95% with respect to the *ampC* gene of *A. baumannii*.

The susceptibility of the clinical strain of AJC68081 to the combination of cefotaxime and ceftazidime with clavulanic acid is also remarkable (with a clavulanic acid MIC of 8 to 16 mg/liter [Table 1]). This unusual phenomenon has been detected in several strains of *A. baumannii* and AG3 and is independent of the presence of any extended-spectrum β-lactamases, which are well inhibited by clavulanic acid (8). It is thought that this finding may be related to penicillin-binding protein alterations which increase the susceptibility to clavulanic acid (unpublished data).

Another important consideration is the role of the β-lactamase under study in resistance to β-lactams in AG3. The MICs for clinical strain AJC68081 were higher than those for the *E. coli* transformant expressing the G3 AmpC enzyme (Table 1). An explanation for this result could be that other antibiotic resistance mechanisms, such as a loss or a reduction of porin expression, a constitutively basal expression of some efflux pump, or penicillin-binding protein modifications, operate at the same time in *A. baumannii* and/or AG3, as no other β-lactamases have been detected in clinical strain AJC68081.

The results obtained in the present study show that this AmpC β-lactamase (i) may play an important role in β-lactam resistance in AG3, (ii) was not inducible when cefoxitin was added (at one-half the MIC) and thus can be considered noninducible, and (iii) shows a typical cephalosporinase substrate profile, corresponding to that of a class C β-lactamase.

In summary, we report for the first time the cloning, sequencing, and analysis of the *ampC* gene and the biochemical characterization of the AmpC β-lactamase from a clinical strain of AG3.

Nucleotide sequence accession number. The GenBank accession number for the AmpC β-lactamase of AG3 is AJ575184.

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