## 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  $\beta$ -lactamase shows for the first time the gene encoding an AmpC enzyme in AG3. In addition, the biochemical properties of the novel AG3 AmpC  $\beta$ -lactamase are reported

The bacterial genus *Acinetobacter* consists of strictly aerobic gram-negative coccobacilli, which are oxidase negative, non-motile, 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  $\beta$ -lactam antibiotics is attributed to the presence of  $\beta$ -lactamases encoded either by the chromosome or by plasmids (2). Several class A, B, and D  $\beta$ -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  $\beta$ -lactamase (4) and two allelic variants (ABAC-1 and ABAC-2) (17) have been reported so far for *A. baumannii* strains.

Regarding  $\beta$ -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  $\beta$ -lactam antibiotics shown by a clinical strain of *Acinetobacter* genomic DNA group 3

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

	MIC (µg/ml) <sup>a</sup>							
Antibiotic	AJC68081 (produces AmpC of AG3)	E. coli TG1	<i>E. coli</i> TG1 (pBE-1) <sup>b</sup>	E. coli TG1 (pGER1) <sup>c</sup>				
Amoxicillin	>256	3	256	128				
Amoxicillin + clavulanate <sup>d</sup>	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 <sup>d</sup>	0.19	0.023	1	1				
Ceftazidime	32	0.064	0.75	16				
Ceftazidime + clavulanate <sup>d</sup>	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				

<sup>a</sup> ND, not done.

 $^{\it b}$  Transformant producing AmpC  $\beta\text{-lactamase}$  of AG3.

<sup>c</sup> Transformant producing AmpC β-lactamase of A. baumannii.

<sup>d</sup> Clavulanate was used at 4  $\mu$ g/ml.

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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
ATG	CGA	TTT	AAA	ААА	ATT	TCT	TGT	CTA	CTT	TTA	TCC	S CCG	.D. CTT	TTT	ATT	129
m	r	£	k	k	i	s	с	1	1	1	s	P	1	f	i	
TTT	AGT	ACC	TCA	ATT	TAT	GCG	GGC	AAT	ACA	CCA	ААА	GAC	CAA	GAA	ATT	177
£	ទ	t	s	i	У	a	G	N	т	P	к	D	Q	Е	I	
AAA	ААА	CTG	GTA	GAT	CAA	AAT	TTT	AAA	CCA	TTA	TTA	GAA	ААА	TAT	GAT	225
К	K	L	v	D	Q	N	F	к	Р	L	L	Е	к	Y	D	
GTA	CCA	GGT	ATG	GCT	GTG	GGT	GTT	ATT	CAA	AAT	AAT	ААА	AAG	TAT	GAA	273
v	Ρ	G	м	А	v	G	v	I	Q	N	N	к	к	Y	Е	
ATG	TAT	TAT	GGT	CTT	CAA	TCT	GTT	CAA	GAT	ААА	AAA	GCC	GTA	AAT	AGC	321
М	Y	Y	G	L	Q	s	v	Q	D	к	к	А	v	N	s	
AGT	ACC	ATT	TTT	GAG	CTA	GGT	TCT	GTC	AGT	AAA	TTA	TTT	ACC	GCG	ACA	369
S	т	I	F	Е	г	G	s	v	s	ĸ	г	F	т	А	т	
GCA	GGT	GGA	TAT	GCA	AAA	AAT	AAA	GGA	AAA	ATC	TCT	TTT	GAC	GAT	ACG	417
А	G	G	Y	A	к	N	к	G	к	I	s	F	D	D	т	
CCT	GGT	AAA	TAT	TGG	ААА	GAG	CTA	AAA	AAT	ACA	CCG	ATT	GAT	CAA	GTT	465
Р	G	к	Y	W	к	Е	L	к	Ν	т	Ρ	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	т	Y	т	s	G	N	L	А	$\mathbf{L}$	Q	
TTT	CCA	GAT	GAA	GTA	CAA	ACA	GAT	CAA	CAA	GTT	TTA	ACT	TTT	TTC	AAA	561
F	Р	D	Е	v	Q	т	D	Q	Q	v	L	т	F	F	к	
GAC	TGG	CAA	CCT	ААА	AAC	CCA	ATC	GGT	gaa	TAC	AGA	CAA	TAT	TCA	AAT	609
D	W	Q	P	K	Ν	₽	I	G	Е	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	$\mathbf{L}$	F	G	К	v	v	А	ь	s	М	N	К	
CCT	TTC	GAC	CAA	GTC	TTA	GAA	ААА	ACA	ATT	TTT	CCG	GCC	CTT	GGC	TTA	705
Ρ	F	D	Q	v	L	Е	K	т	I	F	Ρ	A	$\mathbf{L}$	G	L	
P AAA	F CAT	D AGC	Q TAT	V GTA	L AAT	e gta	K CCT	T AAG	I ACC	F CAG	P ATG	A CAA	L AAC	G TAT	L GCT	753
p aaa K	F CAT H	D AGC S	Q TAT Y	V GTA V	L AAT N	e gta v	K CCT P	T AAG K	I ACC T	F CAG Q	P ATG M	a caa Q	L AAC N	G TAT Y	L GCT A	753
P AAA K TTT	F CAT H GGC	D AGC S TAT	Q TAT Y AAC	V GTA V CAA	L AAT N GAA	e gta v aat	K CCT P CAG	T AAG K CCG	I ACC T ATT	F CAG Q CGA	P ATG M GTT	A CAA Q AAC	L AAC N CCC	G TAT Y GGC	L GCT A CCA	753 801
P AAA K TTT F	F CAT H GGC G	D AGC S TAT Y	Q TAT Y AAC N	V GTA V CAA Q	L AAT N GAA E	e gta v aat n	K CCT P CAG Q	T AAG K CCG P	I ACC T ATT I	F CAG Q CGA R	P ATG M GTT V	A CAA Q AAC N	L AAC N CCC P	G TAT Y GGC G	L GCT A CCA P	753 801
P AAA K TTT F CTT	F CAT H GGC G GAT	D AGC S TAT Y GCC	Q TAT Y AAC N CCA	V GTA V CAA Q GCA	L AAT N GAA E TAC	e gta v aat n ggc	K CCT P CAG Q GTC	T AAG K CCG P AAA	I ACC T ATT I TCG	F CAG Q CGA R ACA	P ATG M GTT V CTA	A CAA Q AAC N CCC	L AAC N CCC P GAC	g TAT Y GGC G ATG	L GCT A CCA P TTG	753 801 849
P AAA K TTT F CTT L	F CAT H GGC G GAT D	D AGC S TAT Y GCC A	Q TAT Y AAC N CCA P	V GTA V CAA Q GCA A	L AAT N GAA E TAC Y	e gta v aat N ggc g	K CCT P CAG Q GTC V	T AAG K CCG P AAA K	I ACC T ATT I TCG S	F CAG Q CGA R ACA T	P ATG M GTT V CTA L	A Q AAC N CCC	L AAC N CCC P GAC D	G TAT Y GGC G ATG M	L GCT A CCA P TTG L	753 801 849
P AAA K TTT F CTT L AGT	F CAT H GGC G GAT D TTT	D AGC S TAT Y GCC A ATT	Q TAT Y AAC N CCA P CAT	V GTA V CAA Q GCA A GCC	L AAT N GAA E TAC Y AAC	e gta v aat N ggc g ctt	K CCT P CAG Q GTC V ACC	T AAG K CCG P AAA K CCA	I ACC T ATT I TCG S CAG	F CAG Q CGA R ACA T AAA	P ATG M GTT V CTA L TAT	A Q AAC N CCC P CCG	L AAC N CCC P GAC D ACA	G TAT Y GGC G ATG M GAT	L GCT A CCA P TTG L ATT	753 801 849 897
P AAA K TTT F CTT L AGT S	F CAT H GGC GAT D TTT F	D AGC S TAT Y GCC A ATT I	Q TAT Y AAC N CCA P CAT H	V GTA V CAA Q GCA A GCC A	L AAT N GAA E TAC Y AAC N	E GTA V AAT N GGC G CTT L	K CCT P CAG Q GTC V ACC T	T AAG CCG P AAA K CCA P	I ACC T ATT I TCG S CAG Q	F Q Q CGA R ACA T AAA K	P ATG M GTT V CTA L TAT	A Q AAC N CCC P CCG P	L AAC N CCC P GAC D ACA	G TAT Y GGC G ATG M GAT D	L GCT A CCA P TTG L ATT I	753 801 849 897
P AAAA K TTTT F CTTT L AGT S CAAA	F CAT H GGC GAT D TTT F CGGG	D AGC S TAT Y GCC A ATT I GCA	Q TAT Y AAC N CCA P CAT H ATT	V GTA V CAA Q GCA A GCC A AAT	L AAT N GAA E TAC Y AAC N GAA	E GTA V AAT N GGC G CTT L ACA	K CCT P CAG Q GTC V ACC T CAT	T AAG K CCG P AAA K CCA P CAA	I ACC T ATT I CG CAG Q GGG	F CAG Q CGA R ACA T AAA K TTC	P ATG M GTT CTA L TAT I TAT	A Q AAC N CCC P CCG P CAA	L AAC N CCC P GAC D ACA T GTC	G TAT Y GGC G ATG GAT D GGC	L GCT A CCA P TTG L ATT I ACC	753 801 845 897 945
P AAA K TTTT F CTTT L AGT S CAA Q	F CAT H GGC GAT D TTT F CGG R	D AGC S TAT Y GCC A ATT I GCA A	Q TAT Y AAC N CCA P CAT H ATT I	V GTA V CAA Q GCA A GCC A AAT N	L AAT N GAA E TAC Y AAC N GAA E	E GTA V AAT N GGC G CTT L ACA T	K CCT P CAG GTC V ACC T CAT H	T AAG K CCG P AAA K CCA P CAA Q	I ACC T ATT I CAG S CAG Q GGG G G	F CAG Q CGA R ACA T AAA K TTC F	P ATG M GTT V CTA L TAT I TAT Y	A Q AAC N CCC P CCG P CAA Q	L AAC N CCC P GAC D ACA T GTC V	G TAT Y GGC G ATG M GAT D GGC G	L GCT A CCA P TTG L TTG I ACC T	753 801 849 897 945
P AAAA K TTTT F CTTT L AGT S CAA Q ATG	F CAT H GGC GAT D TTT F CGG R TAT	D AGC S TAT GCC A ATT I GCA A CAG	Q TAT Y AAC CCA P CAT H ATT I GCA	V GTA V CAA Q GCA A GCC A AAT N CTT	L AAT N GAA E TAC Y AAC N GAA E GGT	E GTA V AAT GGC G CTT L ACA T GG	K CCT P CAG Q GTC V ACC T CAT H GAA	T AAG CCG P AAA K CCA P CAA Q GAG	I ACC T ATT I CAG CAG GGG G TTT	F CAG Q CGA R ACA T AAA K TTC F TCT	P ATG M GTT V CTA L TAT I TAT Y TAT	A CAA Q AAC N CCCC P CCCG Q CCCG	L AAC P GAC D ACA T GTC V GCA	G TAT GGC G ATG GAT D GGC G ACG	L GCT A CCA P TTG L ATT I ACC T	753 801 849 945 993
P AAAA K TTTT F CTTT L AGT S CAAA Q ATG M	F CAT H GGC G GAT D TTT F CGG R R TAT Y	D AGC S TAT Y GCC A ATT I GCA A CAG Q	Q TAT Y AAC N CCA P CAT H ATT I GCA A	V GTA V CAA Q GCA A GCC A A AAT N CTT L	L AAT N GAA E TAC Y AAC N GAA E GGT G	E GTA V AAT N GGC G CTT L ACA T GG	K CCT P CAG GTC V ACC T CAT H GAA E	T AAG CCCG P AAAA K CCAA Q GAG E	I ACC T ATT I CAG GGG G GGG TTT F	F CAG Q CGA R ACA T T CA F TCT S	P ATG M GTT V CTA L TAT I TAT Y X	A Q AAC N CCC P CCG Q CCG P	L AAC P GAC D ACA T GTC V GCA A	G TAT Y GGC G ATG GAT GGC G ACG T	L GCT A CCA P TTG L ATT ACC T TTA L	753 801 849 945 993
P AAAA K TTTT F CTTT AGT S CAAA Q ATG M CAAA	F CAT H GGC G GAT D TTT F CGG R TAT Y ACT	D AGC S TAT Y GCC A ATT GCA GCA Q TTA	Q TAT Y AAC N CCA P CAT H ATT I GCA A TTA	V GTA V CAA Q GCA A GCC A AAT N CTT L GAC	L AAT N GAA E TAC Y AAC N GAA E GGT G AGT	E GTA V AAT N GGC G CTT L ACA T GG W AAT	K CCT P CAG GTC V ACC T CAT H GAA E TCA	T AAG K CCG P AAA K CCA P CAA Q GAG E GAA	I ACC T ATT I CAG G G G G G G G G TTT F CAG	F CAG Q CGA R ACA T AAA K TTC F TCT S ATT	P ATG M GTT V CTA L TAT I TAT Y TAT Y GTG	A Q AAC P CCCG P CCG Q CCCG P CCG	L AAC N CCCC P GAC D ACA T GTC V GCA A AAA	G TAT Y GGC G ATG GAT GAT GGC G ACG T CCT	L GCT A CCA P TTG L ATT ACC T TTA L AAT	753 801 849 897 945 992
P AAA K TTT F CTT AGT S CAA ATG ATG CAA Q	F CAT H GGC GAT D TTT F CGG R TAT Y ACT T	D AGC S TAT Y GCC A ATT I GCA A CAG Q TTA L	Q TAT Y AAC N CCA P CAT H ATT I GCA A TTA	V GTA V CAA Q GCA A GCC A AAT N CTT L GAC D	L AAT N GAA TAC Y AAC N GAA E GGT G AGT S	E GTA V AAT GGC G CTT L ACA T GG W AAT	K CCT P CAG GTC V ACC T CAT H GAA E TCA S	T AAG K CCCG P AAAA K CCCA P CAAA Q GAG E GAAA E	I ACC T ATT I CAG GGG G GGG G TTT F CAG Q	F Q CGA R ACA T AAA K TTC F TCT S ATT I	P ATG M GTT V CTA L TAT I TAT Y GTG V	A Q AAC N CCC P CCG P CAA Q CCG P ATG M	L AAC N CCCC P GAC D ACA T GTC V GCA A AAA K	G TAT Y GGC G ATG GAT D GGC G ACG T T CCT	L GCT A CCA P TTG L ATT I ACC T TTA L AAT N	753 801 845 897 945 993
P AAA K TTT F CTT AGT S CAA Q ATG AAA	F CAT H GGC G TT TT CGG R TAT Y ACT T GTG	D AGC S TAT Y GCC A ATT I GCA A CAG Q TTA L ACT	Q TAT Y AAC N CCA P CAT H ATT I GCA A <u>TTA</u> L GCT	V GTA V CAA Q GCA A GCC A AAT N CTT L GAC D ATT	L AAT N GAA E TAC Y AAC N GAA E GGT G AGT S TCA	E GTA V AAT GGC G CTT L ACA T GG W AAT N AAG	K CCT P CAG GTC V ACC T CAT H GAA E TCA S GAA	T AAG K CCG P AAA K CCA P CAA Q GAG E GAA E CCT	I ACC T ATT I CCG S CAG GGG G GGG G TTT F CAG Q CAG Q TCA	F CAG Q CGA R ACA T AAA K TTC F TCT S ATT I GTT	P ATG GTT V CTA L TAT I TAT Y GTG V AAG	A CAA Q AAC P CCC P CAA Q CCC Q CCA Q CCC Q ATG M ATG	L AAC P GAC D ACA T GTC V GCA A AAA K TAC	G TAT Y GGC G ATG GAT D GGC G ACG T CCT P CAT	L GCT A CCA P TTG L ATT TTA L AAT L AAT	753 801 849 945 993 1041
P AAAA K TTT F CTT AGT S CAA Q ATG M CAAA Q AAA K	F CAT H GGC G TTT F CGG R TAT Y ACT T GTG V	D AGC S TAT Y GCC A ATT I GCA A CAG Q TTA L ACT T	Q TAT Y AAC CCA P CAT H ATT I GCA A TTA L GCT A	V GTA Q GCA A GCC A AAT CTT L GAC D ATT I	L AAT N GAA E TAC Y AAC Y GAA GGA GGT G AGT S TCA	E GTA V AAT N GGC G CTT L ACA T GG W AAT N AAG K	K CCT P CAG Q GTC V ACC T CAT H GAA E CAT E CAT E	T AAG K CCG P AAA K CCA CAA Q GAG GAG E GAA E CCT P	I ACC T ATT I CAG GGG G GGG G TTT F CAG CAG CAG C AG S	F CAG Q CGA R ACA T AAA T TCC F TCCT S ATT I GTT V	P ATG GTT V CTA L TAT TAT TAT Y GTG V AAG K	A CAA Q AAC N CCCC P CCCG P CAA Q CCCG Q CCCG M ATG M	L AAC P GAC D ACA T GTC V GCA A AAA K TAC Y	G TAT Y GGC G ATG GAT GGT GGC G ACG ACG T CCT P CAT H	L GCT A CCA P TTG A TTG A A C T TTA L A AAT N AAA K	753 801 849 945 993 1041
P AAA K TTT F CTT AGT S CAA Q ATG Q AAA K ACT	F CAT H GGC G TTT F CGG R TTT Y ACT T T GTG V GGC	D AGC S TAT Y GCC A ATT I GCA A CAG Q TTA L ACT T CA	Q TAT Y AAC N CCA P CAT H ATT I GCA A TTA L GCT A	V GTA V CAA Q GCA A GCC A AAT L GAC D ATT I AAC	L AAT N GAA E TAC N AAC N GAA E GGT S TCA S GGT	E GTA V AAT N GGC G CTT L ACA T TGG W AAA N AAG K TTC	K CCT P CAG GTC V ACC T CAT H GAA S GAA E GGA	T AAAG K CCCG P AAAA K CCCA Q GAAG E GAAA E CCCT P ACA	I ACC T ATT I CAG GGG GGG GGG GTTT F CAG Q CAG CAG TCA	F CAG Q CGA R ACA T AAA K TTC F TCT S ATT I GTT V GTG	P ATG GTT V CTA TAT I TAT TAT Y GTG V AAG GTC	A CAA Q AAC P CCCG P CAA Q CCCG P ATG M ATG M	L AAC P GAC D ACA T GTC V GCA A AAA K TAC Y	G TAT Y GGC G ATG GAT D GGC G ACG ACG P CAT H CCT	L GCT P TTG L ATT I ACC T TTA AAC N AAA K AAA	753 801 849 945 993 1041 1085
P AAAA K TTTT F CTTT AGT AGT Q AAGT Q AAAA K ACT T	F CAT GGC GAT D TTT F CGG R TAT Y ACT T GTG V GGC <b>G</b>	D AGC S TAT Y GCC A ATT I GCA A CAG Q TTA L ACT T CA S	Q TAT Y AAC N CCA P CAT H ATT I GCA A TTA CC A C T	V GTA V CAA Q GCA A GCC A AAT CTT GAC D ATT I AAC	L AAT N GAA E TAC Y AAC N GAA E GGT G TCA S GGT G	E GTA V AAT GGC G CTT L L ACA T GG W AACA N AAG K TTC F	K CCT P CAG GTC V ACC T CAT H GAA E GAA E GAA G GA	T AAAG K CCCG P AAAA K CCAA P CAAA Q GAAG E CCT P ACA T	I ACC T ATT I TCG S CAG GGG G GGG G TTT F CAG CAG CAG TTT T TCA S TAT Y	F CAG Q CGA T ACA T AAA K TTC F TCT S ATT I GTT V GTG V	P ATG GTT V CTA TAT I TAT Y GTG V AAG GTC V	A CAA Q AAC P CCG P CCG P CAA Q CCG P ATG ATG M TTT F	L AAC P GAC D ACA T GTC V GCA A AAA K TAC Y ATT I	G TAT Y GGCC G ATG GAT D GGC G CCT P CAT H CCT P	L GCT A CCA P TTG ATT A ATT A ACC T TTA L AAA N AAA K K	753 801 849 945 991 1041 1085
P AAAA K TTT F CTT AGT CAA CAA CAA Q AAAA K ACT T GAA	F CAT GGC GAT D TTT F CGG R TAT T T GTG GTG GC G G AAT	D AGC S TAT GCC A A TT GCA A CAG Q TTA CAG Q TTA TCA S ATT	Q TAT Y AAC N CCA P CAT H ATT GCA A TTA GCC A A CC T GGC	V GTA Q GCA A GCC A A A CTT CTT L GAC D ATT I AAC N TTA	L AAT N GAA E TAC Y AAC N GAA GAA GGT G GTC	E GTA V AAT GGC G CTT L ACA T TGG W AAT N AAG K TTC F ATG	K CCT P CAG GTC V ACC T CAT H GAA E TCA S GAA E GGA G TTA	T AAG K CCG P AAA K CCA P CAA Q GAG E GAA E CCT P ACA T ACC	I ACC T ATT I TCG S CAG G G G G G G G G G G G G G C AG C A	F CAG Q CGA R ACA T T C T T C T C T C T C T C T C T C	P ATG GTT V CTA L TAT TAT Y GTG V AAG GTC K CGT	A CAA Q AAC P CCC P CCA Q CCG P CAA Q CCG M ATG M ATG M TTT F ATT	L AAC N CCCC P GAC D ACA T GTC V GCA A AAA K TAC Y ATT I CCA	G TAT Y GGCC G ATG GGC G G G C C C T P C C T H C C T H C C T P C A T	L GCT A CCA P TTG L ATT AATT TTA L AAA AAA K GAA	753 801 845 993 993 1041 1089 1137
P AAAA K TTT CTT CTT CTT AGT CAA CAA CAA Q AAAA K ACT T GAA E	F CAT GGC GAT D TTT F CGG R TAT T ACT T GTG V GGC G AAT N	D AGC S TAT Y GCC A ATT I GCA A CAG Q TTA L ACT T CA S ATT I I	Q TAT Y AAC CCA P CAT H ATT I GCA A CAT L GCA A C CAT T CAT CAT C CAT C CAT C CAT C CAT C CAT C CAT C CA C C CA C C CA C C CA C C CA C CA C CA C C CA C C C CA C C CA C C CA C C CA C C CA C C CA C C CA C C CA C C CA C C CA C C CA C C CA C C CA C C CA C C CA C	V GTA Q GCA A GCC A A AAT CTT GAC U AAT I AAC N TTA L	L AAT N GAA E TAC Y AAC N GAA E GGT GGT G GTC V	E GTA V AAT GGC G CTT L ACA T TGG W AAT N AAG K TTC F ATG	K CCT P CAG GTC V ACC T CAT H GAA E GAA E GGA G TTA L	T AAG K CCG P AAA K CCA P CAA Q GAG GAG GAA E CCT P ACA T ACC	I ACC T ATT I CCG S CAG G G G G G G G G G G G G G G G G G G	F CAG Q CGA R ACA T T AAA K TTC F TCT S ATT I GTT V GTG Q V AAA K	P ATG GTT V CTA TAT TAT TAT Y GTG GTG K GTC V CGT	A Q AAC N CCC P CCG P CCA Q CCG P CCA ATG M ATG M TTT F ATT I	L AAC P GAC D ACA T GTC V GCA A AAA K TAC Y ATT I CCA	G TAT Y GGC G ATG GAT D GGC G ACG CAT H CAT H CCT P CAT N	L GCT A CCA P TTG A TTG A A C T T A A A A A A A A A A A A A A A	753 801 845 897 945 993 1041 1089 1137
P AAAA TTT T CTT CTT AGT S AGT AAA A AAA A AAA A CAA A CAA CAA CAA CA	F CAT H GGC G TTT F CGG R TAT T Y GTG GTG V GGC G AAT N CGC	D AGC S TAT Y GCC A ATT I GCA A CAG Q TTA L ACT T CA S ATT I I ATT	Q TAT Y AAC CCA P CAT H ATT I GCA A CA A CA C T CA CA CA CA CA CA CA CA CA CA CA CA CA	V GTA Q GCA A GCC A AAT N CTT L GAC N TTA L GCA	L AAT N GAA E TAC Y AAC N GAA E GGT G GGT G GGT G GCT V QCT	E GTA V AAT N GGC G CTT L ACA T G G W AAG K TTC F ATG M TAT	K CCT P CAG GTC V ACC T CAT H GAA E S GAA E GGA G TTA L CTTA	T AAG K CCG P AAA K CCA P CAA Q GAG E GAG E CCT P ACA T ACC T	I ACC T ATT I CG CAG GGG G GGG G TTT F CAG CAG CAG X TCA S TAT Y AAT	F CAG Q CGA T AAA T AAA K TTC F TCT S ATT I GTT V GTT Q V AAA K AAT	P ATG GTT V CTA I TAT I TAT Y GTG V AAG GTC V CGT R GCA	A Q AAC P CCG P CAA Q CCG P CAA Q CCG P ATG M ATG M TTT F ATT I I ATA	L AAC P GAC D ACA T GTC V GCA A AAA K TAC Y ATT I CCA P AAG	G TAT Y GGC G M GAT D GGC G G CCT P CAT H CCT P CAT N AAA	L GCT A CCA P TTG A TTG A A T TA A A A A A A A A A A	753 801 849 945 993 1041 1083 1137 1188
P AAAA K TTT CTT CTT AGT S CAA Q ATG ATG AAA K ACT T GAA E GAG	F CAT H GGC G TTT F CGG R TAT Y ACT T GTG GGC G C AAT N CGC R	D AGC S TAT Y GCC A ATT I GCA A CAG Q TTA CAG Q TTA TCA S ATT I ATT I	Q TAT Y AACC N CCA P CAT H ATT I GCA A CAT A C CAT A C CAT A C CAT A C CAT A C CAT A C CAT A C CAT A C CAT A C CAT A C C C C	V GTA Q GCA A GCC A A AAT L GAC D ATT I AAC N TTA L GCA A	L AAT N GAA E TAC Y AAC N GAA E GGT G GGT G GGT G GGT G GTC V GCT A	E GTA V AAT N GGC G CTT L ACA T TGG W AAT N AAG K TTC F ATG M TAT Y	K CCT P CAG Q T CAT T CAT H GAA E GAA E GGA G GAA C TTA L GCT A	T AAG K CCG P AAA K CCA P CAA Q GAG E GAA E CCT P ACA T ACC T GTG V	I ACC T ATT I CG CAG GGG GGG GGG GGG GTTT F CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG	F CAG Q CGA T T AAA K TTC F TCT S ATT I GTT Q TC Q TG Q TG Q TG Q TG Q TG Q TG	P ATG GTT U CTA TAT I TAT Y GTG K GTG K GTC V CGT R GCA	A Q AAC P CCC P CCG P CAA Q CCG P ATG ATG M TTT F ATT I ATA	L AAC N CCC P GAC D ACA T GTC V GCA A AAA K TAC Y AAA I CCA P AAAG K	G TAT Y GGC G ATG GAT D GGC G CAT CCT P CAT H CCT P AAT N AAA K	L GCT A CCA P TTG L ATT A AAT A AAA K AAAA K GAA E TAA -	753 801 849 945 993 1041 1089 1137 1189 1233
P AAAA K TTT CTT CTT CTT AGT AGT AGAA ACT CAA ACT CAA ACT CAA CAA CAA CAA C	F CAT H GGC G GAT D TTT F CGG R TAT T GTG G G G G C G C G C C C C C C C C	D AGC S TAT Y GCC A ATT I GCA A CAG Q TTA CAG T T CAG S ATT I ATT I I TAG	Q TAT Y AAC CCA P CAT H ATT I GCA TTA ACC T GCC G G G C AAG K CCA	V GTA V CAA GCA A GCC A AAT N CTT GAC J AAT I GAA AAA	L AAT N GAA E TAC N GAA E GGT G GGT G GGT G GTC A ACA	E GTA V AAT N GGC G CTT L ACA T TGG W AAT AAG K TTC F ATG M TAT Y AAA	K CCT P CAG GTC V ACC T CAT H GAA E GAA E GGA G TTA L GCT A AGA	T AAAG K CCCG P AAAA K CCA P CAAA Q GAAG CCT P ACCA T ACCC T GTCG V Q GAAA	I ACC T ATT I CGG Q GGG G GGG G TTT F CAG CAG CAG CAG CAG CAG CAG C CAG C CAG C CAG C C CAG C C C C	F CAG Q CGA R ACA T C ACA T C T C T C T C T C T C T C	P ATG GTT CTA CTA TAT TAT TAT TAT TAT GTG GTG K GTC CGT R GCA A	A CAA Q AAC P CCCC P CCCG P CCAA Q CCCG M ATG M ATG M TTT F ATT I ATA	L AAC N CCC P GAC D ACA T GTC V GCA A AAA K TAC Y ATT CCA P AAG K	G TAT Y GGC G ATG GAT D GGC G ACG ACG C CT P CAT H CCT H CCT N AAA X K	L GCT A CCA P TTG L ATT A AT TTA A AAA K AAA K AAA K GAA E TAA CTT	753 801 849 945 993 1041 1085 1131 1185 1233

FIG. 1. Nucleotide sequence of the 1.3-kb fragment. The amino acid sequence deduced for AmpC G3  $\beta$ -lactamase is shown on the line

(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  $\beta$ -lactamases were analyzed by isoelectric focusing as described by Matthew et al. (18). The sonicated extract of strain AJC68081 contained a single  $\beta$ -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  $\mu$ l) 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<sub>2</sub> (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-B-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

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  $\beta$ -lactamase active site SVSK, the conserved triad KTG, and the typical class C motif YXN are shown in boldface type (12).

	% Identity with:										
β-Lactamase <sup>a</sup>	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			

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

<sup>a</sup> 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  $\beta$ -lactamases (Table 2). At a protein level, the highest similarities detected (98.69 to 97.65%) were with the AmpC  $\beta$ -lactamases AmpC ABAC-1, AmpC ABAC-2, and AmpC RYC52763 from A. baumannii, respectively (4, 17). Comparison of the amino acids in the AmpC  $\beta$ -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  $\beta$ -lactamase encoded by the 1.3-kb DNA insert, the insert was cloned in the pBGS18<sup>-</sup> plasmid (25) (which carries a kanamycin resistance gene), resulting in the pBE-1 plasmid. The  $\beta$ -lactam patterns of resistance to amoxicillin, cephalothin, cefuroxime, and cefoxitin and, to a lesser extent, amoxicillinclavulanic 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  $\beta$ -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  $\beta$ -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  $\beta$ -lactamase, measured as specific enzymatic activity (in micromoles of nitrocefin hydrolyzed per minute per microgram of protein) when the inducer was added (159  $\pm$  38  $\mu$ mol/min/ $\mu$ g of protein without the inducer, 145  $\pm$  15  $\mu$ mol/min/ $\mu$ g of protein in the presence of inducer). These experiments indicated that the AG3 AmpC  $\beta$ -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  $\beta$ -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 ( $\geq$ 95% purity) (data not shown). Kinetic experiments were performed as previously described (4). The specific activity of semipurified AmpC  $\beta$ -lactamase was 49,000 µmol of nitrocefin/min/µg of protein. The relative enzymatic efficiency  $(V_{\text{max}}/K_m)$  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  $\mu$ g/ml). The *ampC* gene was disseminated among

TABLE 3. Kinetic parameters of the Acinetobacter genon	ic species	3 AmpC	β-lactamase
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Antibiotic	$K_m (\mu M)$	Relative $V_{\max} (\%)^a$	$V_{\rm max}/K_m$	Relative $V_{\text{max}}/K_m \ (\%)^a$	Hydrolysis rate (µmol/min/µl) <sup>b</sup>	Relative hydrolysis rate $(\%)^a$	IC <sub>50</sub> (µM) <sup>c</sup>
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	$\mathrm{NH}^{e}$	
Clavulanic acid							>250
Sulbactam							13

<sup>a</sup> Normalized with respect to value for ampicillin (taken as 100%).

<sup>b</sup> Hydrolysis rates were determined by using 100 µM concentrations of the indicated substrates.

<sup>c</sup> IC<sub>50</sub>, 50% inhibitory concentration.

<sup>d</sup> ND, not done.

<sup>e</sup> NH, no hydrolysis detected.

all of the AG3 isolates (data not shown). This result strongly suggests that AmpC  $\beta$ -lactamase may play a role in the  $\beta$ -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  $\beta$ -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  $\beta$ -lactamase under study in resistance to  $\beta$ -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  $\beta$ -lactamases have been detected in clinical strain AJC68081.

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

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

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