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Genetic Variability among *ampC* Genes from *Acinetobacter* Genomic Species 3[▽]

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As a part of a nationwide study in Spain, 15 clinical isolates of *Acinetobacter* genomic species 3 (AG3) were analyzed. The main objective of the study was to characterize the *ampC* genes from these isolates and to determine their involvement in β-lactam resistance in AG3. The 15 AG3 isolates showed different profiles of resistance to ampicillin (range of MICs, 12 to >256 μg/ml). Nucleotide sequencing of the 15 *ampC* genes yielded 12 new AmpC enzymes (ADC-12 to ADC-23). The 12 AG3 enzymes showed 93.7 to 96.1% amino acid identity with respect to the AmpC enzyme from *Acinetobacter baumannii* (ADC-1 enzyme). Eight out of fifteen *ampC* genes were expressed in *Escherichia coli* cells under the control of a common promoter, and with the exception of one isolate (isolate 65, which showed lower β-lactam MICs), significant differences in overall β-lactam MICs for *E. coli* cells expressing AG3 *ampC* genes were not revealed. No significant differences in *ampC* gene expression in AG3 clinical isolates were revealed by reverse transcription-PCR analysis. A detailed analysis of the 12 AmpC protein sequences revealed that amino acid replacements (in comparison with those of ADC-1) occurred mainly in the same positions, although none were located in important functional domains such as the Ω- loop or conserved β-lactamase motifs. Kinetic experiments performed with three representative AmpC enzymes (ADC-14, -16, and -18) in some cases revealed dramatic changes in K_m and k_{cat} values for β-lactams. No IS*Aba1* was detected upstream of the *ampC* genes. Our results reveal 12 new *ampC* genes in AG3. The enzymes showed a moderate degree of variability, and they are tentatively named ADC-12 to ADC-23.

Species belonging to the genus *Acinetobacter* are widely distributed in nature (2, 3) and are reported to be the cause of ever-increasing numbers of nosocomial infections. Molecular methods based on DNA-DNA hybridization or sequencing of the 16S subunit of the ribosome have been described for up to 33 different groups (31). Groups 1, 2, 3, and 13 are phenotypically similar and traditionally known as the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. Except for those in group 1, these genomic species are important nosocomial pathogens that frequently cause outbreaks of infection in intensive care units and burn units (18, 20, 32). Although mechanisms of antibiotic resistance in *A. baumannii* have been described (4, 6, 13, 14, 19, 23, 24, 31, 34, 35), there are few descriptions of the mechanisms of resistance in *Acinetobacter* genomic species 3 (AG3) (1, 10, 25, 29, 35). With regard to β-lactam resistance in AG3, two metalloenzymes, VIM-2 and IMP-4, and a chromosomal cephalosporinase have been described for this species (1, 10, 34, 35).

As a part of a nationwide, multicenter study in Spain, which included analysis of 244 *Acinetobacter* sp. isolates (226 *A. baumannii*, 15 AG3, and 3 unidentified isolates), we aimed to determine the molecular basis of β-lactam resistance and specifically ampicillin resistance in 15 AG3 clinical isolates. For this purpose, the *ampC* genes from all isolates were sequenced and further characterized to assess their activities and specificities toward β-lactams. Overall, 12 new *ampC* genes were discovered in AG3. Following a classification that is currently under development, the genes were designated ADC-12 to ADC-23.

MATERIALS AND METHODS

Bacterial strains. In November 2000, all *A. baumannii* isolates from clinical samples were assembled from 28 hospitals in Spain. A total of 244 isolates of *Acinetobacter* spp. were collected: 226 *A. baumannii*, 15 AG3, and 3 unidentified *Acinetobacter* sp isolates. The 15 AG3 isolates used for further studies were isolates 14, 20, 21, 52, 56, 60, 65, 67, 69, 90, 103, 109, 128, 195, and 243, which were all isolated from different hospitals. *Escherichia coli* DH5α [F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*] and *E. coli* BL21 [F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm*] were used for determining antibiotic MICs and for analysis of expression and purification of proteins, respectively.

Bacterial strains were frozen in *Brucella* glycerol broth (10%) (BBL Microbiology Systems, Cockeysville, MD) and were maintained at −80°C until analysis. Strains of *E. coli* were grown at 37°C in Luria-Bertani (LB) medium. When necessary, LB medium was supplemented with ampicillin (20 μg/ml) or kanamycin (50 μg/ml) (Sigma-Genosys Ltd., United Kingdom).

Antimicrobial agents and determination of MICs. Antibiotic susceptibility profiles were determined by Etest according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). The following antibiotics were purchased from Sigma-Aldrich (Madrid, Spain): ampicillin, piperacillin, cephalothin, cefoxitin, cefuroxime, ceftazidime, and cefotaxime. Cefepime was obtained from Sigma-

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TABLE 1. Oligonucleotides used in the study

Oligonucleotide	Description[b]	Sequence[a]	Reference(s) or source
P1	ARDRA F	5′-TGGCTCAGATTGAACGCTGGCGGC-3′	30
P2	ARDRA R	5′-TACCTTGTTACGACTTCACCCC-3′	30
P3	REP F	5′-TTTGCGCCGTCATCAGGC-3′	5, 33
P4	REP R	5′-ACGTCTTATCAGGCCTAC-3′	5, 33
P5	*ampC* F	5′-aaggatccATGCGATTTAAAAAAATTTC-3′	6
P6	*ampC* R	5′-aaaagcttAGGATATGTTTGGTTC-3′	6
P7	*ampC* R	5′-aaaagcttTTATTTCTTTATTGCATTC-3′	6
P8	IS*Aba1* F	5′-aaaggatccCTCTGTACACGACAAATTTCAC	This study
P9	IS*Aba1* R	5′-aaagaattcCTCTGTACAGCATAAAAATAGAT	This study
P10	*amp* Int R	5′-GCCGACTTGATAGAA-3′	This study
P11	*ampC* F pGEX (isolate 65)	5′-aaggatccGGCAATACACCAAAAGACCAAG-3′	This study
P12	*ampC* F pGEX (isolate103)	5′-aaggatccGGTAATACACCAAAAGAGCAA-3′	This study
P13	*ampC* F pGEX (isolate 195)	5′aaggatccGGCAATACACCAAAAGAACAAG-3′	This study
P14	*ampC* R pGEX	5′-aagaattcTCTTTTTTATGTTTAGCTACGG-3′	This study
P15	RT-PCR *ampC* F	5′-AAGTTTTAACTTTTTTCAAAG-3′	This study
P16	RT-PCR *ampC* R	5′-AATTACTGTCTAATAAAGTTT-3′	This study
P17	RT-PCR control *gyrA* F	5′-AATCTGCCCGTGTCGTT-3′	GenBank accession no. AY204699
P18	RT-PCR control *gyrA* R	5′-GCCATACCTACGGCGA-3′	GenBank accession no. AY204699

[a] Lowercase letters represent restriction sites and tail nucleotides.
[b] F, forward oligonucleotide; R, reverse oligonucleotide.

Genosys Ltd. (United Kingdom), imipenem was obtained from Merck Sharp and Dohme (Madrid, Spain), and meropenem was obtained from AstraZeneca (Madrid, Spain).

ARDRA. The species were identified by amplified ribosomal DNA restriction analysis (ARDRA) (30). AG3 was also identified by sequencing of the 16S rRNA gene with oligonucleotides P1 and P2 (Table 1).

REP-PCR. Repetitive extragenic palindromic sequence (REP)-based PCR (REP-PCR) was used to evaluate the possible clonal relationship between the different isolates of AG3 used in the study. The REP-PCR sequence allows amplification of the localized regions between the REP zones. The primers used are described in Table 1 (primers P3 and P4). The amplification reaction was carried out as previously described (5). We consider that two isolates were epidemiologically unrelated when two or more different bands were detected in them (5, 33).

Cellular extract preparation and IEF. β-Lactamases were obtained by sonication of cultures of all isolates of AG3 grown overnight at 37°C in LB medium and centrifugation at 14,000 rpm (MiniSpin microcentrifuge; Eppendorf, Hamburg, Germany) for 10 min (22). The pI values were determined as previously reported with commercial isoelectric focusing (IEF) gels (pH 3.5 to 9.5; Pharmacia LKB, Piscataway, NJ) by using a PhastSystem electrophoresis system (Pharmacia). Sonicated extracts of microorganisms expressing β-lactamases of known pIs were used as controls.

Cloning experiments and recombinant plasmids. Total DNA was extracted from 15 AG3 clinical isolates with the MasterPure DNA purification kit (Epicentre, Madison, WI) according to the manufacturer's instructions. The *ampC* genes from the 15 AG3 clinical isolates described above were cloned by PCR by use of oligonucleotides P5 and P6 (Table 1) for isolates 21, 52, 60, 67, 90, 128, and 243. For the remaining AG3 clinical isolates, oligonucleotides P5 and P7 were used. The Expand High Fidelity PCR system (Roche Diagnostics, Indianapolis, IN) was used for the amplification procedure under the following experimental conditions: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min for a total of 28 cycles; an initial cycle of 2 min at 94°C; and a final cycle of 10 min at 72°C. The amplicons were purified with a High Pure PCR product purification kit (Roche Diagnostics, GmbH, Mannheim, Germany). In 8 out of 15 isolates (isolates 14, 20, 56, 65, 69, 109, and 195), and for expression and MIC studies, the amplified fragment was cloned into BamHI and HindIII sites in pBGS18 (harboring a kanamycin resistance marker) under the control of the strong promoter of the CTX-M-14 β-lactamase gene (positions 1501 to 1740 [GenBank accession number AF252622]) (13). With the remaining seven AG3 isolates, the amplified fragment was directly cloned into pBGS18 at the same restriction sites. In both cases, ligation was carried out with a Rapid DNA ligation kit (Roche Diagnostics, Indianapolis, IN). The DNA was electroporated in *E. coli* DH5α cells, and the clones were selected on LB plates with 20 μg/ml of ampicillin and 50 μg/ml of kanamycin. Plasmids from selected transformants were purified and examined to check the accuracy of the cloning procedure. Two clones from each gene transformation were selected, and nucleotide sequencing was carried out. Sequencing of nucleotides was performed by use of the *Taq* DyeDeoxiTerminator cycle sequencing kit before analysis using an automatic DNA sequencer (377 Abi-Prism; Perkin-Elmer). Each gene was sequenced on both strands. The ClustalW program (http://infobiogen.fr) was used to align the multiple protein sequences (28).

Detection of IS*Aba1* in AG3 isolates. IS*Aba1*-like sequences were previously identified immediately upstream of the bla_{AmpC} gene in ceftazidime-resistant *A. baumannii* isolates, where the strong promoter of the IS*Aba1* insertion increased the expression of the bla_{AmpC} gene (16). To find out whether the IS*Aba1* element was present in AG3, a PCR assay was performed with primer pairs for this element and the *ampC* gene from AG3 (primer pair P8/P9 was used to detect IS*Aba1*, and primer pair P8/P10 was used to detect IS*Aba1* upstream of the *ampC* gene).

β-Lactamase purification and kinetic experiments. Three representative AmpC enzymes from isolates 65, 103, and 195 were purified for kinetic experiments. For this, the *ampC* genes were cloned into vector pGEX-6P-1, which allows the production of a fusion protein from glutathione *S*-transferase (GST) and the AmpC enzyme. The primer pairs used for PCR amplification and cloning into pGEX-6P-1 were P11/P14 for isolate 65, P12/P14 for isolate 103, and P13/P14 for isolate 195 (Table 1). β-Lactamase was purified to homogeneity by use of the GST gene fusion system (Amersham Pharmacia Biotech, Europe, GmbH) according to the manufacturer's instructions. The mature purified proteins lacking the GST fusion protein appeared on sodium dodecyl sulfate-polyacrylamide gels as a band of 43 kDa (≥95% purity). Kinetic experiments were performed at 25°C using a Nicolete Evolution 300 spectrophotometer (Thermo Electron Corporation, Waltham, MA) with quartz cuvettes of optical path lengths of 1 and 0.2 cm. The tests were each repeated three times with phosphate-buffered saline (PBS) with 20 mg/liter bovine serum albumin. The kinetic parameters k_{cat}, K_m, and k_{cat}/K_m were studied for the antibiotics ampicillin, cephalothin, cefoxitin, cefuroxime, cefotaxime, ceftazidime, aztreonam, and imipenem. The K_m values were calculated as K_i values in competitive assays with Centa (Calbiochem, Merck, Darmstadt, Germany) as the substrate, as previously described for putative poor substrates such as imipenem and meropenem. The V_{max} was calculated by considering an antibiotic concentration six times the K_m and by use of the Michaelis-Menten equation, as previously described (26). Studies of the 50% inhibitory concentration (IC_{50}) were conducted by incubating the purified proteins (1 μg/ml) for 10 min in the presence of inhibitors of class A β-lactamases (clavulanic acid and sulbactam).

Semiquantitative RT-PCR. To detect *ampC* gene expression, reverse transcriptase PCR (RT-PCR) was carried out with 15 AG3 clinical isolates as well as with an *Acinetobacter* sp. isolate with a high level of *ampC* gene expression (as a positive control). Total RNA was extracted from cultures grown overnight in LB medium at 37°C with the Trizol Max bacterial RNA isolator kit (Invitrogen, Carlsbad, CA), and the RNA was then treated with DNase (Sigma-Genosys Ltd., United Kingdom). The Qiagen OneStep RT-PCR kit was used for RT-PCR analysis with a 200-ng sample of total RNA. The primers used for gene ampli-

TABLE 2. β-Lactam susceptibility profile of AG3 isolates included in the study

β-Lactam	MIC (μg/ml) for AG3 isolate:														
	14	20	21	52	56	60	65	67	69	90	103	109	128	195	243
Ampicillin	32	256	24	48	24	32	32	48	32	12	>256	48	24	32	12
Piperacillin	16	24	24	12	12	24	16	16	16	12	24	16	12	24	32
Cephalothin	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Cefoxitin	>256	>256	>256	>256	64	>256	128	>256	>256	128	192	>256	128	>256	48
Cefuroxime	32	48	48	32	16	64	24	48	32	16	48	32	32	64	32
Ceftazidime	3	4	6	3	1.5	3	4	3	4	6	4	3	3	4	4
Cefotaxime	24	16	24	12	4	24	12	16	16	12	16	16	12	24	16
Cefepime	3	4	12	2	2	4	3	4	3	16	3	2	2	6	3
Imipenem	0.25	1	0.38	0.25	0.19	0.25	0.19	0.19	0.25	0.38	0.75	0.19	0.12	0.19	0.19
Meropenem	0.50	6	1	0.50	0.38	0.75	0.50	0.50	0.25	0.75	3	0.38	0.19	1	0.25

fication were designed to hybridize in highly conserved fragments in all sequences of *ampC* genes (P15 and P16 in Table 1), which amplified an internal product of 470 bp. As an internal control for the RT-PCR, the *gyrA* gene from AG3 was used as a template with oligonucleotides P17 and P18 (Table 1), which amplified the 344 bp of this gene. The conditions of the RT-PCR were as follows: an initial cycle of reverse transcription at 50°C for 30 min, followed by amplification of the DNA with a initial cycle of 15 min at 95°C, 23 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min and a final cycle of 10 min at 72°C. Aliquots were removed during the amplification process at cycles 14, 18, and 22 (exponential phase of amplification). The bands were revealed in agarose gels, as described above. The intensity of *ampC* gene bands was compared with that of *gyrA* gene bands.

Western blot analysis. Western blot analysis was used to detect and assess AmpC expression in the AG3 isolates with polyclonal antibodies raised against ADC-7 (19). Bacterial extracts were obtained as described above for pI analysis and were loaded onto sodium dodecyl sulfate-polyacrylamide gels (12%) in a minigel apparatus (Bio-Rad, Hercules, CA). The proteins were transferred onto Immobilon-P membranes (Millipore, Billerica, MA). The membranes were blocked with 5% (wt/vol) blocking agent (skim milk) in PBS-Tween. After the membranes were washed with PBS-Tween, they were incubated with a mixture of the polyclonal rabbit anti-ADC-7 antiserum diluted 1:1,500. The membranes were then washed and incubated with secondary conjugated rabbit antiserum diluted 1:2,000 (ECL Western blotting reagent; Amersham Pharmacia Biotech, United Kingdom). All incubations were done for 1 h at 25°C. The Western blot was revealed by overlaying the membranes with luminol, a substrate of the horseradish peroxidase enzyme, ligated to secondary antibody.

OMP purification. Purification and analysis of outer membrane proteins (OMPs) were performed as previously described (13, 14) with bacterial AG3 isolates 20 and 103 (with clear ampicillin resistance) and isolates 21, 56, and 128 (ampicillin susceptible).

Determination of antibiotic MICs in the presence of cloxacillin and the efflux pump inhibitors PAN and CCCP. To assess the putative role of an efflux pump mechanism involved in β-lactam resistance in AG3, antibiotic MICs in the presence and absence of chemical efflux pump inhibitors were determined. Thus, MICs were determined in the presence of 150 μg/ml of cloxacillin as an inhibitor of cephalosporinase activity (7) and of efflux pump inhibitors, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) at 25 μM (5.1 μg/ml) (15) and Phe-Arg β-naphthylamide dihydrochloride (PAN; Sigma) at 25 μg/ml (8), alone and with antibiotics.

Theoretical modeling of the three-dimensional structure of AmpC enzymes. The theoretical three-dimensional structure of the ADC-12 enzyme was obtained by theoretical homology modeling with different computer software packages: BLAST (to align sequences), Deep View (to obtain the theoretical structure from previously aligned genes), and UCSF Chimera (protein model edition). For this, the ADC-12 sequence was compared with the previously published structure of CMY-10 (Protein Data Bank accession number 1ZKJ) (21), which showed the highest amino acid identity (45%). We used the Deep View program in combination with the Swiss-model server to generate a homology-based model of the enzyme. As part of the default pipeline of the ProModII modeling program (implemented in Swiss-model), a final step of structure "dumping" was performed, resulting in an unrefined, fast energy minimization process, mainly with the purpose of avoiding atomic clashes. No further minimization or molecular dynamic equilibration was executed.

Nucleotide sequence accession numbers. The nucleotide sequences of the ADC-type enzymes have been submitted to the GenBank database under accession numbers AM283529 (ADC-12), AM283528 (ADC-13), AM283527 (ADC-14), AM283526 (ADC-15), AM283525 (ADC-16), AM283524 (ADC-17), AM283523 (ADC-18), AM283522 (ADC-19), AM283521 (ADC-20), AM283520 (ADC-21), AM283519 (ADC-22), and AM283518 (ADC-23).

RESULTS

IEF analysis and antimicrobial susceptibility pattern. IEF was performed with sonicated extracts obtained from 15 AG3 isolates. A single pI of ca. 9 was detected in all strains, probably corresponding to a chromosomal cephalosporinase. The antibiotic MICs determined by Etest for the 15 AG3 clinical isolates are shown in Table 2. High MICs of cephalothin and cefoxitin and a moderate degree of resistance to cefuroxime and cefotaxime were observed with all AG3 clinical isolates. Although most of the AG3 isolates were resistant to ampicillin (as deduced from the Clinical and Laboratory Standards Institute breakpoints for the *Enterobacteriaceae* determined for ampicillin) (12), a wide range of MICs was obtained (12 to >256 μg/ml). With two of the isolates, isolates 20 and 103, high MICs of ampicillin were obtained (256 and >256 μg/ml, respectively). Interestingly, meropenem MICs were 6 and 3 μg/ml for the same two isolates, respectively. The MICs of meropenem for the remaining AG3 isolates were within the range of 0.19 to 1 μg/ml.

REP-PCR analysis. A different DNA band pattern was obtained for each AG3 isolate by REP-PCR, and the isolates were therefore assumed to be genetically unrelated (data not shown).

Amino acid sequence diversity of AmpCs of AG3. The *ampC* genes from AG3 clinical isolates were amplified and sequenced as described in Materials and Methods. The 15 *ampC* genes were composed of 1,152 nucleotides, which encode an open reading frame of 384 amino acids. The *ampC* genes of isolates 52, 56, 60, and 195 were identical; thus, there was a total of 12 different AmpC-encoding gene sequences (Fig. 1). These sequences differ from those of the previously reported ADC-type genes, and following the recently developed uniform numerical system for this family of AmpC β-lactamases, we tentatively named them ADC-12 to ADC-23. To explain whether or not differences in antibiotic MICs (mainly ampicillin) were due to differences in the amino acid compositions of AmpC enzymes, a detailed examination of the amino acid sequences of the AmpC enzymes was carried out and compared with that of AmpC from *A. baumannii* or ADC-1 (Fig. 1). Overall, the genes showed 93.7 to 96.1% identity with ADC-1. Although a moderate degree of genetic variability was observed, the pat-

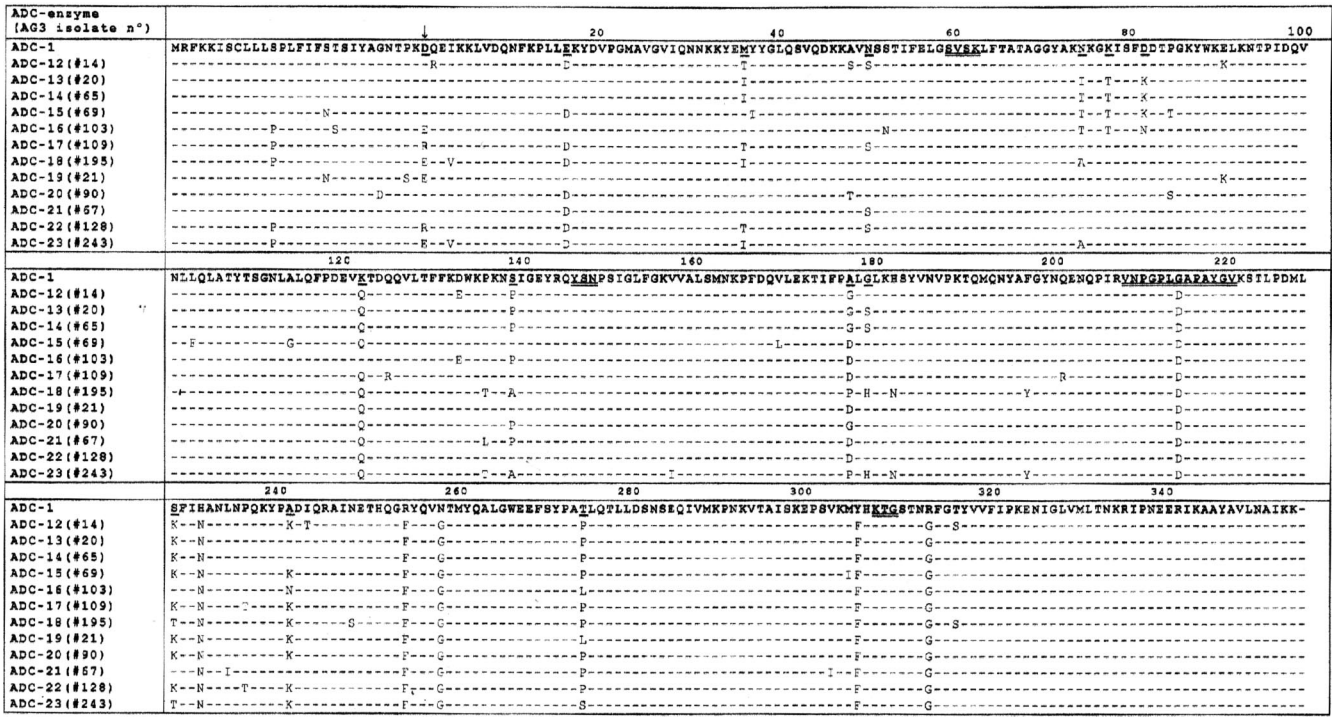

FIG. 1. Amino acid sequence alignment among the 12 AmpC β-lactamases from AG3 (ADC-12 to -23) with respect to that of *A. baumannii* (ADC-1). Amino acid differences are indicated. The typical β-lactamase domains (SVSK, YSN, and KTG) and the Ω-loop are double underlined. The amino acid replacements present in at least four or more proteins (relative to that of ADC-1) with more than one residue are underlined. The vertical arrow indicates the position of the +1 amino acid (after cleavage of signal peptide). ADC-1, AmpC from *A. baumannii* (see reference 6).

tern does not appear to follow a random profile, as some positions are more likely to be replaced than others. Indeed, some amino acid positions were replaced in at least four of the ADC-type enzymes analyzed with more than one residue (Fig. 1) and are shown in Fig. 3. Graphical analysis revealed no changes in the relationships with important domains or catalytic regions of the AmpC enzyme. Analysis of amino acid sequences of ADC-13 and ADC-16 (isolated from AG3 isolates 20 and 103, for which the highest ampicillin MICs were obtained) did not reveal any significant differences (in amino acid composition or position) with respect to the remaining ADC-type enzymes.

Cloning and expression of *ampC* genes in the *E. coli* host. To confirm whether or not amino acid changes have any effect on the phenotype of ampicillin or β-lactam resistance, we cloned and expressed several *ampC* genes in an *E. coli* host. For this, *ampC* genes from clinical isolates 20 and 103 (ampicillin MICs of 256 and >256 μg/ml) and those from clinical isolates 14, 56, 65, 69, 109, and 195 (ampicillin MIC ranges of 24 to 48 μg/ml) were cloned into pBGS18 under the control of a common external CTX-M-14 gene promoter and were then transformed into a common *E. coli* DH5α host, and the β-lactam MICs were determined (Table 3). The MICs of different β-lactams, including ampicillin and meropenem, were similar for all *E. coli* transformants except the transformant that expresses the β-lactamase AmpC of AG3 isolate 65 (ADC-14), for which the MICs were slightly lower.

Kinetic experiments. To confirm that differences in AmpC amino acid sequence are related to differences in the catalytic efficiency of AmpC enzymes toward β-lactams, three representative AmpC enzymes were chosen for further biochemical experiments. Those of AG3 isolates 65 (ADC-14), 103 (ADC-16), and 195 (ADC-18) were purified to homogeneity, and the kinetic parameters K_m, k_{cat}, and k_{cat}/K_m were determined (Table 4). The three AmpC enzymes showed almost identical catalytic efficiencies (k_{cat}/K_m) toward ampicillin and cephalothin, although ADC-14 showed important differences regarding the K_m and k_{cat} values, thus revealing differences in the biochemical behavior. Indeed, the k_{cat} for ampicillin of ADC-14 was between 13 and 8 times lower than the corresponding values for ADC-18 and -16, respectively, and also 25 and 24 times lower for cephalothin, respectively. Moreover, ADC-14 showed a lower catalytic efficiency for cefoxitin than ADC-18 and ADC-16 (2.1 and 1.9 times, respectively), cefuroxime (3.4 and 4.4 times, respectively), and cefotaxime (6.4 and 4.4 times, respectively), which are consistent with MICs obtained with *E. coli* harboring *ampC* genes (Table 3). With regard to imipenem, the three enzymes had similar K_m values, although the k_{cat}/K_m values for ADC-18 were almost seven times higher. No hydrolysis was detected with aztreonam.

Regarding the inhibition studies, IC_{50}s showed a typical class C profile, with high clavulanic acid IC_{50}s. However, there was a moderate degree of sulbactam inhibition, and the IC_{50}s for clavulanic acid and sulbactam with ADC-14 were lower, which indicates that differences in amino acid sequence (primary structure of the enzyme) are related to differences in the catalytic properties of the ADC-type enzymes.

TABLE 3. MICs of β-lactam antibiotics tested with *E. coli* DH5α carrying the *ampC* genes under the control of a common promoter[a]

β-Lactam	MIC (μg/ml) for:								
	E. coli DH5α	*E. coli* carrying *ampC* from AG3 isolate:							
		14	20	56	65	69	103	109	195
Ampicillin	3	128	96	96	32	96	128	64	96
Piperacillin	0.38	4	4	3	4	3	4	4	4
Cephalothin	3	>256	>256	>256	192	>256	>256	>256	>256
Cefoxitin	2	32	32	16	3	16	32	24	24
Cefuroxime	1.5	64	96	48	4	32	64	32	48
Ceftazidime	0.19	0.38	0.75	0.25	0.25	0.19	0.50	0.19	0.50
Cefotaxime	0.064	0.75	0.125	0.38	025	0.38	0.75	0.38	0.75
Cefepime	0.016	0.023	0.19	0.023	0.032	0.023	0.032	0.023	0.032
Imipenem	0.125	0.125	0.023	0.19	0.19	0.19	0.125	0.12	0.25
Meropenem	0.008	0.023	0.023	0.023	0.023	0.023	0.023	0.032	0.032

[a] Identical MICs were obtained with three different transformants.

Expression of the *ampC* gene in AG3. The next step was to assess whether or not differences in β-lactam MICs in AG3 clinical isolates may be due to differences in *ampC* gene expression. For this, Western blot analysis and RT-PCR were carried out.

Western blot analysis of the sonicated AG3 extracts with polyclonal rabbit antiserum against ADC-7 enzyme revealed a protein band in each isolate, which corresponded to the expected molecular mass (43 kDa) of the *Acinetobacter* sp. AmpC and which migrated at the same level of ADC-7 (Fig. 2). Furthermore, the presence of the previously sequenced IS*Aba1* (involved in high-level bla_{AmpC} expression in *A. baumannii*) (16) in the genome of the 15 AG3 isolates under study was discounted by the results of a series of PCR-based experiments (data not shown).

A band was observed at 470 bp by RT-PCR, which corresponded to the *ampC* gene in all 15 strains. A band of 344 bp of the *gyrA* gene (as an internal control) was observed in all AG3 isolates. The ratios of *ampC*/*gyrA* among AG3 isolates 20, 21, 28, 65, 103 and 243 and one *Acinetobacter* sp. isolate overexpressing the AmpC enzyme (isolate 92) were as follows: 1.9, 1.8, 1.7, 1.7, 1.6, 1.7, and 4.2, respectively. Therefore, no differences in the amounts of mRNA in the *ampC* genes of AG3 clinical isolates 20 and 103 or the remaining AG3 clinical isolates (with lower ampicillin MICs) were observed by semiquantitative RT-PCR (with the exception of positive control isolate 92). Overall, AG3 clinical strains 20 and 103 (with high ampicillin MICs) did not show higher *ampC* gene expression than the remaining AG3 isolates.

OMP analysis. To assess whether or not differences in OMP expression were associated with differences in susceptibility to β-lactams, OMP profiles were obtained from isolates 20 and 103 as well as from representative ampicillin-susceptible isolates 21, 56, and 128. No differences in OMPs at molecular masses of 22, 29, 33 to 36, 40, and 45 kDa were visualized among the bacterial isolates (data not shown), thereby ruling out the involvement of OMPs in ampicillin or β-lactam resistance in AG3 isolates.

Effect of cloxacillin and efflux pumps inhibitors on MICs. As no differences in either OMP or AmpC expression among AG3 isolates or catalytic efficiency against ampicillin were observed among AmpCs, we proposed that other mechanisms, such as an efflux pump, may also be operating in some AG3 clinical isolates and that this may account for differences in β-lactam MICs. The MICs of ampicillin, cephalothin, and meropenem were therefore determined in the presence of cloxacillin, CCCP, and PAN (Table 5). The antibiotic MICs for all AG3 clinical isolates were moderately lower in the presence of cloxacillin, although the effect was most apparent with ampicillin (between two and eight times lower). In the presence of the efflux pump inhibitors PAN or CCCP, the antibiotic MICs were slightly lower with some isolates, although the effect was

TABLE 4. Kinetic experiments performed with AmpC enzymes from the indicated AG3 isolates[a]

Drug	AmpC from isolate (ADC type)[b]:								
	65 (ADC-14)			103 (ADC-16)			195 (ADC-18)		
	Mean K_m (μM) (SD)	Mean k_{cat} (s^{-1}) (SD)	k_{cat}/K_m (s^{-1} mM^{-1})	Mean K_m (μM) (SD)	Mean k_{cat} (s^{-1}) (SD)	k_{cat}/K_m (s^{-1} mM^{-1})	Mean K_m (μM) (SD)	Mean k_{cat} (s^{-1}) (SD)	k_{cat}/K_m (s^{-1} mM^{-1})
Ampicillin	0.12 (±0.04)	0.30 (±0.08)	2,447.15	1.03 (±0.4)	2.6 (±0.66)	2,518.91	1.5 (±0.47)	4.05 (±1.06)	2,700.94
Cephalothin	6.73 (±2.3)	56.52 (±22.38)	8,398.16	139.24 (±16.72)	1,380.80 (± 490.13)	9,916.65	148.27 (±13.23)	1,418.36 (±318.51)	9,566.33
Cefoxitin	0.02 (±0.01)	0.02 (±0.004)	941.18	0.18 (±0.04)	0.33 (±0.02)	1,875.71	0.13 (±0.03)	0.27 (±0.004)	1,992.54
Cefuroxime	0.02 (±0.01)	0.01 (±0.002)	529.41	0.27 (±0.03)	0.62 (±0.20)	2,342.1	0.27 (±0.04)	0.48 (±0.17)	1,791.05
Cefotaxime	0.08 (±0.02)	0.004 (±0.002)	48.19	0.32 (±0.12)	0.07 (±0.01)	211.84	0.2 (±0.01)	0.06 (±0.004)	306.93
Ceftazidime	26.09 (±4.49)	0.01 (±0.001)	0.38	110.34 (±6.44)	0.02 (±0.003)	0.22	84.02 (±21.12)	0.02 (±0.006)	0.25
Aztreonam	6.01 (±1.26)	ND	NC	3.85 (±0.49)	ND	NC	4.54 (±0.41)	ND	NC
Imipenem	2.29 (±0.60)	0.003 (±0.001)	1.31	2.56 (±0.73)	0.004 (±0.0005)	1.56	2.51 (±0.86)	0.02 (±0.003)	9.57

[a] The IC_{50} values of clavulanate were 235.52 μM (± 01.71), 1,462.48 μM (±111.07), and 1,928.02 μM (±357.02) for isolates 65, 103, and 195, respectively; the IC_{50} values of sulbactam were 1.12 μM (±0.29), 7.75 μM (±1.20), and 11.65 μM (±2.03) for isolates 65, 103, and 195, respectively.

[b] ND, not done; NC, not calculated.

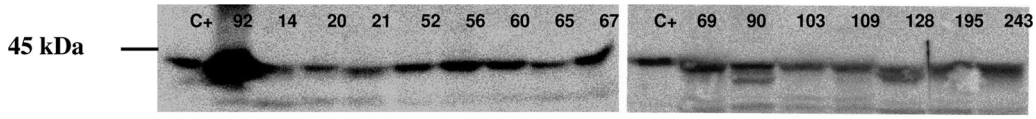

FIG. 2. Western blotting with anti-ADC-7 antibody and with protein extracts (10 μg) obtained from the 15 different AG3 isolates indicated above the gel. Purified ADC-7 enzyme (15 ng) (a gift from R. A. Bonomo) (C+) and protein extracts of an *Acinetobacter* sp. isolate overexpressing the AmpC enzyme (isolate 92) were included as positive controls.

most evident with AG3 isolate 103, for which the ampicillin MICs were at least 10.6 and 8 times lower with PAN and CCCP, respectively. The meropenem MICs were four and three times lower with the latter isolate, respectively. Cloxacillin and CCCP also exerted a synergistic effect on cephalothin MICs (>256 μg/ml without inhibitors compared with 12 to 96 μg/ml when both are added), which suggests the presence of an efflux operating at a constitutively low level in AG3. MICs of cloxacillin, PAN, and CCCP alone for AG3 isolates were higher than the concentration used in combination with antibiotics at 150 μg/ml, 25 μg/ml, and 25 μM (5.1 μg/ml), respectively. Therefore, MICs were the final effect of the antibiotic-inhibitor combination rather than of the inhibitor by itself (Table 5).

Theoretical modeling of the ADC enzymes. The theoretical overall folding three-dimensional structure of the ADC-12 enzyme is shown in Fig. 3. β-Lactamase domains (blue circles, with domain I in green) and the Ω-loop (turquoise) are shown in Fig. 3, as are the amino acid replacements (orange circles) in the ADC-type enzymes (relative to ADC-1) (Fig. 1). Amino acid replacements are randomly located along the protein sequence regardless of the phenotype of conferred ampicillin or β-lactam resistance, and none of them appear to affect the Ω-loop or any of β-lactamase domains (Fig. 3).

DISCUSSION

During the course of a multicenter study carried out in 2000, 244 *Acinetobacter* sp. isolates were analyzed. Fifteen of the isolates were identified as belonging to AG3 (6.1%); these microorganisms may represent an emergent genomic species of *Acinetobacter* (11, 29). Very few reports have been made regarding the role of β-lactamases in β-lactam resistance mechanisms in AG3 (1, 10, 35). In the present study, the range of ampicillin MICs for AG3 isolates was 12 to >256 μg/ml, with two isolates, isolates 20 and 103, showing high MICs (256 and >256 μg/ml, respectively).

A possible involvement of AmpC hyperexpression in clinical isolates was ruled out by RT-PCR analysis. Moreover, determination of MICs in the presence of cloxacillin revealed a slight decrease in β-lactam MICs. Therefore, although constitutively present, the role of AmpC in β-lactam resistance in AG3 appears to be moderate (which is consistent with the absence of IS*Aba1* upstream of the *ampC* gene); thus, the treatment of infections caused by AG3 isolates with β-lactams should be avoided, as it cannot be ruled out that in the near future, an IS*Aba1*-like element may be inserted in the upstream region of the *ampC* gene, thus hyperexpressing the protein.

The sequencing of nucleotides and the deduced amino acid sequence of the 13 *ampC* genes reported here showed some degree of genetic variability. Although 4 out of 15 AG3 clinical isolates harbored an identical *ampC* gene (for which any epidemiological relationship was clearly discounted), the AmpC enzymes under study revealed a set of amino acid replacements, which, for unknown reasons, are located in specific positions in the amino acid sequence (Fig. 1). A steric view of these mutations in the overall fold structure of the modeled ADC-12 β-lactamase (Fig. 3) revealed that these replacements

TABLE 5. MICs for AG3 isolates in the presence of the indicated inhibitors

AG3 isolate	MIC (μg/ml)[a]																				MIC of CCCP (μM)[b]
	Negative control in the presence of:			Cloxacillin in the presence of:			PAN in the presence of:			CCCP in the presence of:			Cloxacillin + PAN in the presence of:			Cloxacillin + CCCP in the presence of:			Cloxacillin[b]	PAN[b]	
	AMP	CE	MEM	AMP	CE	MEM	AMP	CE	MEM	AMP	CE	MEM	AMP	CE	MEM	AMP	CE	MEM			
14	32	>256	0.5	4	128	0.25	32	>256	0.25	24	>256	0.25	16	128	0.38	6	24	0.25	2,048	>400	400
20	256	>256	6	128	>256	3	96	>256	3	192	>256	4	128	>256	3	64	24	1.5	1,024	>400	400
21	24	>256	1	8	>256	0.5	12	>256	0.47	12	>256	0.5	4	192	0.64	6	48	0.25	512	>400	>400
52	48	>256	0.5	8	>256	0.19	48	>256	0.38	16	>256	0.38	8	>256	0.5	6	12	0.094	1,024	400	>400
56	24	192	0.38	6	>256	0.38	16	>256	0.094	24	96	0.25	8	>256	0.38	6	16	0.125	512	>400	>400
60	32	>256	0.75	16	>256	0.75	32	>256	0.19	32	>256	0.5	12	>256	0.75	8	28	0.25	1,024	400	>400
65	32	>256	0.5	16	128	0.19	32	>256	0.25	16	128	0.25	16	128	0.38	12	24	0.19	1,024	>400	>400
67	48	>256	0.5	8	128	0.25	48	>256	0.25	32	192	0.25	8	128	0.25	8	24	0.19	1,875	400	>400
69	32	>256	0.25	8	>256	0.19	32	>256	0.25	24	>256	0.25	8	>256	0.38	6	24	0.19	>2,048	400	>400
90	12	>256	0.75	6	192	1	12	>256	0.38	8	>256	1.5	6	64	0.25	2	24	0.38	512	>400	>400
103	>256	>256	3	64	192	1.5	48	>256	0.75	64	>256	1	12	192	0.75	16	24	1	1,024	400	>400
109	48	>256	0.38	6	128	0.25	48	>256	0.25	32	>256	0.25	8	128	0.38	4	24	0.19	2,048	400	>400
128	24	>256	0.19	8	48	0.25	24	>256	0.125	24	>256	0.19	8	48	0.19	4	32	0.094	2,048	400	>400
195	32	>256	1	16	>256	1	48	>256	1	32	>256	0.5	16	>256	1	16	96	0.38	1,024	400	>400
243	12	>256	0.25	4	192	0.125	12	>256	0.19	8	>256	0.25	4	192	0.19	2	12	0.094	2,048	>400	400

[a] AMP, ampicillin; CE, cephalothin; MEM, meropenem; negative control, no inhibitor added.
[b] Only inhibitor added.

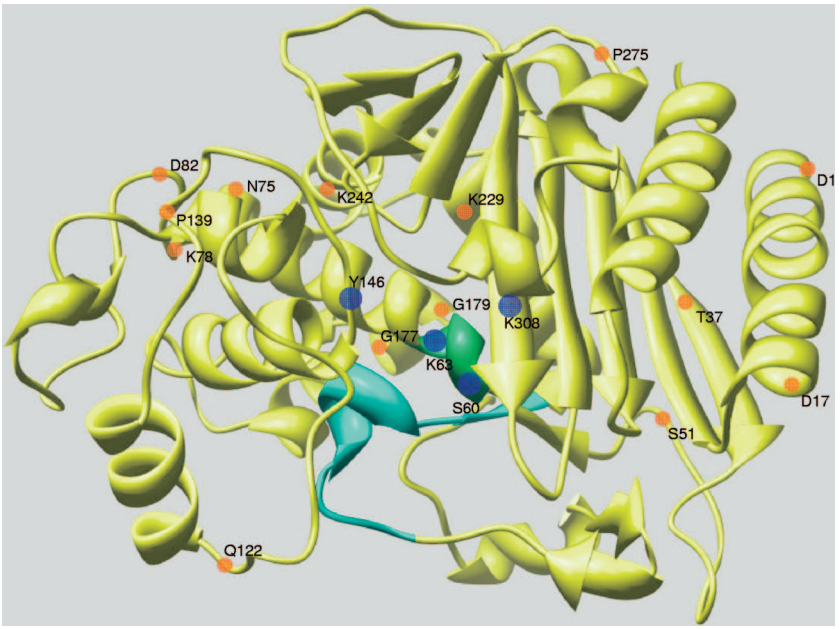

FIG. 3. Theoretical model of the crystallographic structure of the ADC-12 AmpC β-lactamase. Amino acids belonging to the main β-lactamase domains (SVSK, YSN, and KTG) are represented in the figure as blue circles (60-63SXXK, Y146, and K308). The SVSK domain is also shown in green. The Ω-loop is shown in turquoise. Residues at positions 1, 17, 37, 51, 75, 78, 82, 122, 139, 177, 229, 242, and 275 (Fig. 1), which were replaced in four or more ADC-type enzymes (relative to ADC-1), are represented by orange circles (starting at position +1 of the mature protein with D). The diagram was drawn with the USCF Chimera software package.

are located far from the active site of the enzyme. However, although there were no significant differences in MICs among different ADC-type enzymes, the biochemical analysis of three representative AmpC enzymes (ADC-14, -16, and -18) (one isolated from a highly ampicillin-resistant AG3 isolate and the other two isolated from ampicillin-susceptible AG3 strains) did reveal significant differences in some of the measured kinetic parameters (Table 4). This supports the idea that whereas some amino acid changes may be neutral, others are associated with dramatic changes in the catalytic efficiency or biochemical parameters of the AmpC enzymes (see K_m and/or k_{cat} values for ADC-14 and cephalosporins in comparison with ADC-16 and -18).

Other mechanisms such as a loss of porins, efflux pumps, and penicillin binding protein alterations may be involved in β-lactam resistance in *Acinetobacter* spp. (13, 14, 24). To study the involvement of non-AmpC-related mechanisms in some β-lactam MICs, we studied OMP and efflux pump expression.

Efflux pumps have been described for *A. baumannii* and AG3 (9, 17, 27) and have been detected in the recently revealed genome of *Acinetobacter baylyi* (www.genoscope.fr). Thus, experiments were carried out with the chemical inhibitors PAN and CCCP. The ampicillin MICs decreased (by at least 10 times) only for AG3 isolate 103 in the presence of either PAN or CCCP, thus revealing that efflux pumps may operate by pumping out β-lactams in some AG3 strains.

In summary, we report here the identification and analysis of 12 new *ampC* genes from AG3. We also report further information regarding β-lactam resistance in AG3. A uniform numerical system for the classification of cephalosporinase from *Acinetobacter* spp. is currently under development, and in accordance with this classification, we tentatively named our 12 new AmpC enzymes ADC-12 to ADC-23.

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