Genetic Variability among \textit{ampC} Genes from \textit{Acinetobacter} Genomic Species 3\textsuperscript{V}

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As a part of a nationwide study in Spain, 15 clinical isolates of \textit{Acinetobacter} genomic species 3 (AG3) were analyzed. The main objective of the study was to characterize the \textit{ampC} genes from these isolates and to determine their involvement in \beta-lactam resistance in AG3. The 15 AG3 isolates showed different profiles of resistance to ampicillin (range of MICs, 12 to $>256$ \textmu g/ml). Nucleotide sequencing of the 15 \textit{ampC} genes yielded 12 new \textit{AmpC} enzymes (ADC-12 to ADC-23). The 12 AG3 enzymes showed 93.7 to 96.1% amino acid identity with respect to the \textit{AmpC} enzyme from \textit{Acinetobacter baumannii} (ADC-1 enzyme). Eight out of fifteen \textit{ampC} genes were expressed in \textit{Escherichia coli} cells under the control of a common promoter, and with the exception of one isolate (isolate 65, which showed lower \beta-lactam MICs), significant differences in overall \beta-lactam MICs for \textit{E. coli} cells expressing AG3 \textit{ampC} genes were not revealed. Significant differences in \textit{ampC} gene expression in AG3 clinical isolates were revealed by reverse transcription-PCR analysis. A detailed analysis of the 12 \textit{AmpC} protein sequences revealed that amino acid replacements (in comparison with those of \textit{ADC-1}) occurred mainly in the same positions, although none were located in important functional domains such as the \Omega-loop or conserved \beta-lactamase motifs. Kinetic experiments performed with three representative \textit{AmpC} enzymes (ADC-14, -16, and -18) in some cases revealed dramatic changes in $K_m$ and $k_{cat}$ values for \beta-lactams. No \textit{ISAb1} was detected upstream of the \textit{ampC} genes. Our results reveal 12 new \textit{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 \textit{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 \textit{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 \textit{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 \textit{Acinetobacter} genomic species 3 (AG3) (1, 10, 25, 29, 35). With regard to \beta-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 \textit{Acinetobacter} sp. isolates (226 \textit{A. baumannii}, 15 AG3, and 3 unidentified isolates), we aimed to determine the molecular basis of \beta-lactam resistance and specifically ampicillin resistance in 15 AG3 clinical isolates. For this purpose, the \textit{ampC} genes from all isolates were sequenced and further characterized to assess their activities and specificities toward \beta-lactams. Overall, 12 new \textit{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 \textit{A. baumannii} isolates from clinical samples were assembled from 28 hospitals in Spain. A total of 244 isolates of \textit{Acinetobacter} sp. were collected: 226 \textit{A. baumannii}, 15 AG3, and 3 unidentified \textit{Acinetobacter} sp isolates. The 15 AG3 isolates used for further studies were isolates 14, 21, 20, 52, 56, 60, 65, 67, 69, 90, 103, 109, 128, 195, and 243, which were all isolated from different hospitals. \textit{Escherichia coli} DH5\textalpha [F\textsuperscript{φ80lacZAM15 ΔlacZYA-argF[U169 deoR recA1 endA1 hsdR17(rk-km\textsuperscript{A} m15\textsuperscript{A}) phoA supE44 thi-1 gyrA96 relA1 tk]M15 lacZAM15 ΔlacZYA-argF[U169 deoR recA1 endA1 hsdR17(rk-km\textsuperscript{A} m15\textsuperscript{A}) phoA supE44 thi-1 gyrA96 relA1] and \textit{E. coli} BL21 [F\textsuperscript{ampF hsdS(pK\textsubscript{F} m15\textsuperscript{A}) gld dcm}] were used for determining antibiotic MICs and for analysis of expression and purification of proteins, respectively.

Bacterial strains were frozen in \textit{Brucella} glycerol broth (10%) (BBL Microbiology Systems, Cockeysville, MD) and were maintained at $-80^\circ$C until necessary. LB medium was supplemented with ampicillin (20 \textmu g/ml) or kanamycin (50 \textmu g/ml) (Sigma-Genoys 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, cefalothin, cefotaxime, cefoxitin, ceftazidime, and cefotaxime. Cefepime was obtained from Sigma-
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 pl values were determined as previously reported with commercial isoelectric focusing (IEF) gels (pH 3.5 to 9.5; Pharmacia LKB, Piscatway, NJ) by using a PhastSystem electrophoresis system (Pharmacia). Sonicated extracts of microorganisms expressing β-lactamases of known pl 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, 32, 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 BamH I and HindIII sites in pBG518 (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 pBG518 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 electro- porated 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://info.biogeni.fr) was used to align the multiple protein sequences (28).

**Detection of IS*abalu* in AG3 isolates.** *Isabalu*-like sequences were previously identified immediately upstream of the *bla*<sub>ampC</sub> gene in ceftazidime-resistant *A. baumannii* isolates, where the strong promoter of the *ISabalu* insertion increased the expression of the *bla*<sub>ampC</sub> gene (16). To find out whether the *ISabalu* 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 *Isabalu*, and primer pair P8/P10 was used to detect IS*abalu* 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 purified mature proteins lacking the GST fusion protein appeared on sodium dodecyl sulfate-poly- acrylamide gels as a band of 43 kDa (≥95% purity). Kinetic experiments were performed at 25°C using a Nicolette 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*	extsubscript{cat}, *K*	extsubscript{m}, and *k*	extsubscript{cat}/*K*	extsubscript{m} were studied for the antibiotics ampicillin, cephalothin, cefoxitin, cefotaxime, cefotaxime, cefazidime, aztreonam, and imipenem. The *K*	extsubscript{m} values were calculated as *K*	extsubscript{m} 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 *I*	extsubscript{max} was calculated by considering an antibiotic concentration six times the *K*	extsubscript{m}. As the results of the Michaelis-Menten equation, as previously described (26). Studies of the 50% inhibitory concentration (*IC*	extsubscript{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).

**Semi-quantitative 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 baumannii* 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 amplification were designed using specific *ampC* sequences present in the AG3 isolates. The primers are listed in Table 1. The primers P3 and P4 were used to detect the *ampC* gene from AG3. The *ampC* gene from *A. baumannii* strain DSM 25308 (this study) was used as an internal control. The RT-PCR was performed in a SmartCycler (Cepheid, CA) using 50 ng of total RNA. The conditions were as follows: 30 min at 50°C for reverse transcription, 90°C for 2 min, followed by 40 cycles of 15 s at 94°C, 1 min at 55°C, and 1 min at 72°C. The amplified fragment of the *ampC* gene was detected using the amplification protocol of the Smart Cycler and the Smart Analizer (Cepheid). The specificity of the amplified fragment was confirmed by agarose gel electrophoresis.

### Table 1. Oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Description&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference/source</th>
</tr>
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<tbody>
<tr>
<td>P1 ARDRA F</td>
<td>5′-TGGCTCAGATTTACGCTGGGCGC-3′</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>P2 ARDRA R</td>
<td>5′-TACCTGTTACGACTTACCACCC-3′</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>P3 REP F</td>
<td>5′-TTTGGCCGCGTCATACGGC-3′</td>
<td>5, 33</td>
<td></td>
</tr>
<tr>
<td>P4 REP R</td>
<td>5′-AGCTTCTATTACGGCTAC-3′</td>
<td>5, 33</td>
<td></td>
</tr>
<tr>
<td>P5 ampC F</td>
<td>5′-aaggttcatATGGATTTGTTGTC-3′</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>P6 ampC R</td>
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</tr>
<tr>
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<td>5′-aaggttcatATGGATTTGTTGTC-3′</td>
<td>6</td>
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<tr>
<td>P8 ISabalu F</td>
<td>5′-aaggttcatATGGATTTGTTGTC-3′</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>P9 ISabalu R</td>
<td>5′-aaggttcatATGGATTTGTTGTC-3′</td>
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<td>P10 amp Int R</td>
<td>5′-GCCGACTTTAGAGAAGTAAGA-3′</td>
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<td>This study</td>
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<td>This study</td>
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</tr>
<tr>
<td>P13 ampC F</td>
<td>5′-aaggttcatATGGATTTGTTGTC-3′</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>P14 ampC R</td>
<td>5′-aaggttcatATGGATTTGTTGTC-3′</td>
<td>This study</td>
<td></td>
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<tr>
<td>P15 RT-PCR ampC F</td>
<td>5′-AGTCTTTAATTTTTCTGAGAC-3′</td>
<td>GenBank accession no. AY2104699</td>
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<tr>
<td>P16 RT-PCR ampC R</td>
<td>5′-AGTCTTTAATTTTTCTGAGAC-3′</td>
<td>GenBank accession no. AY2104699</td>
<td></td>
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<tr>
<td>P17 RT-PCR control gyr A F</td>
<td>5′-AACATCGCCGTGCTTGTT-3′</td>
<td>GenBank accession no. AY2104699</td>
<td></td>
</tr>
<tr>
<td>P18 RT-PCR control gyr A R</td>
<td>5′-GCCATACCTACGGCGC-3′</td>
<td>GenBank accession no. AY2104699</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Lowercase letters represent restriction sites and tail nucleotides.

<sup>b</sup> F, forward oligonucleotide; R, reverse oligonucleotide.
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 *groEL* 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 *groEL* 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 pl isolation 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 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 (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 (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 (OMPs) were performed as previously described (13, 14) with bacterial AG3 isolates 20 and 103 (with clear ampicillin resistance).

**Determination of antibiotic MICs.** MICs were determined by Etest for the 15 AG3 clinical isolates included in the study. High MICs of cephalothin 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.

**RESULTS**

**IEF analysis and antimicrobial susceptibility pattern.** IEF was performed with sonicated extracts obtained from 15 AG3 isolates. A single pl 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 cefotaxime were observed with all AG3 clinical isolates. Although most of the AG3 isolates were resistant to ampicillin, 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. baumanii* 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-
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 \( \beta \)-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 \( \leq 256 \) \( \mu \)g/ml) and those from clinical isolates 14, 56, 65, 69, 109, and 195 (ampicillin MIC ranges of 24 to 48 \( \mu \)g/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 host, and the \( \beta \)-lactam MICs were determined (Table 3). The MICs of different \( \beta \)-lactams, including ampicillin and meropenem, were determined for all E. coli transformants except the transformant that expresses the \( \beta \)-lactamase AmpC of AG3 isolate 65 (ADC-14), for which the MICs were slightly lower.

Kinetic experiments. To confirm that changes in AmpC amino acid sequence are related to differences in the catalytic efficiency of AmpC enzymes toward \( \beta \)-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\(_{50}\). However, there was a moderate degree of sulbactam inhibition, and the IC\(_{50}\) 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.
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 a protein band in each isolate, which corresponded to the polyclonal rabbit antiserum against ADC-7 enzyme revealed. For this, Western blot analysis and RT-PCR were carried out. The presence of the previously sequenced PCR-based experiment 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, 1.6, 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 ampCICs) were observed by semiquantitative RT-PCR (with the exception of positive control isolate 92). Overall, AG3 clinical strains 20 and 103 (with high ampCICs) 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 were observed, 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 minor.

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

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th>MIC (µg/ml) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> DH5α</td>
</tr>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>3</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>0.38</td>
</tr>
<tr>
<td>Cephalothin</td>
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<tr>
<td>Cefoxitin</td>
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</tr>
<tr>
<td>Cefuroxime</td>
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<tr>
<td>Cefazidime</td>
<td>0.19</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.064</td>
</tr>
<tr>
<td>Cefepime</td>
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<tr>
<td>Imipenem</td>
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<tr>
<td>Meropenem</td>
<td>0.008</td>
</tr>
</tbody>
</table>

* Identical MICs were obtained with three different transformants.

**TABLE 4. Kinetic experiments performed with AmpC enzymes from the indicated AG3 isolates**

<table>
<thead>
<tr>
<th>Drug</th>
<th>AmpC from isolate (ADC type)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65 (ADC-14)</td>
</tr>
<tr>
<td></td>
<td>Mean <em>K</em>m (µM) (SD)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.12 (±0.04)</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>6.75 (±2.3)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>0.02 (±0.01)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>0.02 (±0.01)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.08 (±0.02)</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>26.09 (±4.49)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>6.01 (±2.26)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2.29 (±0.60)</td>
</tr>
</tbody>
</table>

* The *IC*50 values of clavulanate were 235.52 µM (± 0.71), 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 11.63 µM (± 2.03) for isolates 65, 103, and 195, respectively.

* ND, not done; NC, not calculated.
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 ISAbal 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 ISAbal-like element may be inserted in the upstream region of the ampC gene, thus hypervexpressing 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**

<table>
<thead>
<tr>
<th>AG3 isolate</th>
<th>Negative control in the presence of:</th>
<th>Cloxacillin in the presence of:</th>
<th>PAN in the presence of:</th>
<th>CCCP in the presence of:</th>
<th>Cloxacillin + PAN in the presence of:</th>
<th>Cloxacillin + CCCP in the presence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMP CE MEM</td>
<td>AMP CE MEM</td>
<td>AMP CE MEM</td>
<td>AMP CE MEM</td>
<td>AMP CE MEM</td>
<td>AMP CE MEM</td>
</tr>
<tr>
<td>AMP</td>
<td>CE</td>
<td>MEM</td>
<td>AMP CE MEM</td>
<td>AMP CE MEM</td>
<td>AMP CE MEM</td>
<td>AMP CE MEM</td>
</tr>
<tr>
<td>14</td>
<td>32 &gt;256 0.5</td>
<td>4</td>
<td>128 0.25</td>
<td>32 &gt;256 0.25</td>
<td>24 &gt;256 0.25</td>
<td>16 128 0.38</td>
</tr>
<tr>
<td>20</td>
<td>256 &gt;256 6</td>
<td>128 &gt;256 3</td>
<td>96 &gt;256 3</td>
<td>192 &gt;256 4</td>
<td>128 &gt;256 3</td>
<td>64 24 1.5</td>
</tr>
<tr>
<td>21</td>
<td>24 &gt;256 1</td>
<td>8</td>
<td>&gt;256 0.5</td>
<td>12 &gt;256 0.47</td>
<td>12 &gt;256 0.5</td>
<td>4 192 0.64</td>
</tr>
<tr>
<td>52</td>
<td>48 &gt;256 0.5</td>
<td>8</td>
<td>&gt;256 0.19</td>
<td>16 &gt;256 0.38</td>
<td>16 &gt;256 0.38</td>
<td>6 12 0.094</td>
</tr>
<tr>
<td>56</td>
<td>24 192 0.38</td>
<td>6</td>
<td>&gt;256 0.094</td>
<td>24 96 0.25</td>
<td>8 &gt;256 0.38</td>
<td>6 16 0.125</td>
</tr>
<tr>
<td>60</td>
<td>32 &gt;256 0.75</td>
<td>16</td>
<td>&gt;256 0.75</td>
<td>32 &gt;256 0.19</td>
<td>32 &gt;256 0.5</td>
<td>8 28 0.25</td>
</tr>
<tr>
<td>65</td>
<td>32 &gt;256 0.5</td>
<td>16</td>
<td>&gt;256 0.5</td>
<td>32 &gt;256 0.5</td>
<td>12 &gt;256 0.75</td>
<td>8 28 0.25</td>
</tr>
<tr>
<td>67</td>
<td>48 &gt;256 0.5</td>
<td>8</td>
<td>&gt;256 0.25</td>
<td>32 &gt;256 0.25</td>
<td>32 &gt;256 0.25</td>
<td>8 28 0.19</td>
</tr>
<tr>
<td>69</td>
<td>32 &gt;256 0.25</td>
<td>8</td>
<td>&gt;256 0.19</td>
<td>32 &gt;256 0.25</td>
<td>32 &gt;256 0.25</td>
<td>8 28 0.19</td>
</tr>
<tr>
<td>90</td>
<td>12 &gt;256 0.5</td>
<td>7</td>
<td>192 1</td>
<td>12 &gt;256 0.38</td>
<td>8 &gt;256 1.5</td>
<td>6 64 0.25</td>
</tr>
<tr>
<td>103</td>
<td>&gt;256 &gt;256 3</td>
<td>64</td>
<td>192 1.5</td>
<td>64 &gt;256 1</td>
<td>12 192 0.75</td>
<td>16 24 1</td>
</tr>
<tr>
<td>109</td>
<td>48 &gt;256 0.38</td>
<td>6</td>
<td>128 0.25</td>
<td>48 &gt;256 0.25</td>
<td>8 &gt;256 0.38</td>
<td>4 24 0.19</td>
</tr>
<tr>
<td>128</td>
<td>24 &gt;256 0.19</td>
<td>8</td>
<td>48 0.25</td>
<td>24 &gt;256 0.125</td>
<td>8 &gt;256 0.38</td>
<td>4 32 0.094</td>
</tr>
<tr>
<td>195</td>
<td>32 &gt;256 1</td>
<td>16</td>
<td>&gt;256 1</td>
<td>16 &gt;256 1</td>
<td>16 96 0.38</td>
<td>400 400</td>
</tr>
<tr>
<td>243</td>
<td>12 &gt;256 0.25</td>
<td>4</td>
<td>12 &gt;256 0.19</td>
<td>8 &gt;256 0.25</td>
<td>4 192 0.19</td>
<td>2 12 0.094</td>
</tr>
</tbody>
</table>

*a* AMP, ampicillin; CE, cephalothin; MEM, meropenem; negative control, no inhibitor added.

*b* Only inhibitor added.
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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REFERENCES


