Kinetic Properties of Four Plasmid-Mediated AmpC β-Lactamases

Cédric Bauvois,1 Akiko Shimizu Ibuka,2 Almeida Celso,1 Jimena Alba,3 Yoshikazu Ishii,3 Jean-Marie Frère,1 and Moreno Galleni1*

Centre d’Ingénierie des Protéines, Université de Liège, Sart Tilman, Belgium; Department of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan; and Department of Microbiology, Toho University School of Medicine, 5-21-16 Omorinishi, Ota-ku, Tokyo 1438540, Japan

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β-Lactams illustrate the growing phenomenon of bacterial resistance to antibiotics. Since the beginning of their utilization, over 50 years ago, the introduction of new compounds of the β-lactam ring (20). On the basis of their primary structures, β-lactamases are grouped into four classes (A, B, C, and D) (8), and the various enzymes exhibit specific activity spectra. Enzymes of classes A, C, and D are located either on transferable plasmids or on the bacterial chromosome (14). Initially, class C β-lactamases (AmpC) were described as chromosomal enzymes (6). However, over the last 20 years, more than 20 plasmid-encoded class C enzymes have been identified (18). These proteins may be classified into six subgroups based on amino acid sequence similarities. Each subgroup contains plasmid-borne class C enzymes and their closest chromosomal relatives (18). Clinical isolates harboring plasmidic AmpC exhibit high resistance toward β-lactam antibiotics, such as cephamycins and monobactams.

The present work was focused on an extensive characterization of four plasmid-borne class C β-lactamases; ACT-1 and MIR-1 (subgroup 2), CMY-2 (subgroup 1), and CMY-1 (subgroup 6), in order to determine if their kinetic properties were significantly different from those of the chromosome-encoded enzymes, as suggested before. As will be discussed below, this paper provides the first biochemical data showing that these enzymes have not yet significantly improved their catalytic mechanisms and thus do not deserve the appellation of extended-spectrum β-lactamases, as previously suggested by some authors (1, 7).

Materials and Methods

Antibiotics and other chemicals. Nitrocefin was from Oxoid (Basingstoke, Hants, United Kingdom). Ampicillin, benzylpenicillin, cephalothin, cephaloridine, oxacillin, ceftazidime, cefotaxime, and oxacillin were from Sigma (St. Louis, MO). Cephalexin and cefazolin were from Eli Lilly and Co. (Indianapolis, IN). Imipenem and kanamycin were from Merck Sharp Dohme (Brussels, Belgium). Aztreonam was from Bristol-Myers Squibb (Brussels, Belgium). The antibiotic extinction molar coefficients were described previously (10, 11). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were from ImmunoSource (Halle, Belgium) and ICN Biomedical Inc. (Aurora, OH), respectively.

Bacterial strains and vectors. The strains Escherichia coli SNO3 pMG232 (MIR-1 producer), E. coli XL1 pMG261 (ACT-1 producer), and E. coli XL1 pMG262 (CMY-2 producer) were gifts of G. A. Jacoby (Section of Infectious Disease, Lahey Clinic, Burlington, MA). The E. coli strain producing CMY-1 was a gift of Yunsop Chong (College of Medicine, Yonsei University, Seoul, Korea). E. coli DH5α ( Gibco-BRL-Life Technologies, Eragny, France) and E. coli BL21(DE3) (Novagen Inc., Madison, WI) was used to clone the PCR products and pET22b (+) (Novagen, Inc.) was used to produce the different plasmid-encoded class C β-lactamases. For all the cloning experiments, the E. coli strains carrying the different ampC genes were grown at 37°C in Luria-Bertani (LB) medium in the presence of 50 µg/ml ampicillin or 50 µg/ml kanamycin.

Construction of expression vectors. The plasmids pMG232, pMG261, and pMG262 were extracted by using the GFX Micro Plasmid Prep kit (Pharmacia, Uppsala, Sweden). The plasmid containing the blaCMY1 gene was isolated with the help of the Sepagene kit (Sanko Junyaku Co., Tokyo, Japan). The different β-lactamase genes were amplified by PCR. The different PCR primers were designed in order to introduce the NdeI and the HindIII restriction sites up- and downstream of the genes, respectively (Table 1). The PCR conditions were as follows: incubation for 1 min at 95°C, 30 cycles of 1 min at 95°C in 1 min at 60°C for blaCMY1, blaACT1, and blaMIR1 or 1 min at 58°C for blaCMY2 and 1 min at 72°C. The PCR were completed by incubating the reaction mixtures at 72°C for 5 min. Taq DNA polymerase (Eurogentec, Seraing, Belgium) was used for PCR. The amplified fragments were introduced into the polyT pGEM-T-easy vector. The pGEM vectors containing blaACT1, blaCMY1, blaCMY2, and blaMIR1 were called pBC-1, pBC-2, pBC-3, and pBC-4, respectively. They were used to transform E. coli DH5α competent cells. The genes were completely sequenced to

* Corresponding author. Mailing address: Centre d’Ingénierie des Protéines, B6 Sart Tilman, Université de Liège, B-4000 Liège, Belgium. Phone: 32-4-3663419. Fax: 32-4-3663396. E-mail: mgalleni@ulg.ac.be.
verify the presence of the new restriction sites and the absence of unwanted mutations. Read sequencing was performed using an automated laser fluorescent DNA sequencer (Amersham Pharmacia Biosciences, Uppsala, Sweden). The different pBC plasmids were digested by NdeI and HindIII restriction enzymes. The 1.5-kb DNA fragment containing the ampC genes was gel purified and ligated into the pET-28b(+) vector digested by the same restriction enzyme mentioned above. The corresponding plasmids pBCte, pBCe, pBCse, and pBCxe, allowing the overexpression of the ACT-1, CMY-1, CMY-2, and MIR-1 β-lactamases, respectively, were introduced into E. coli BL21(DE3).

Production and purification of ACT-1, MIR-1, CMY-1, and CMY-2. For all enzymes, a 50-ml preculture was grown overnight in LB medium at 37°C in the presence of kanamycin (50 μg/ml). Two liters of fresh medium (LB for CMY-1 and CMY-2, 2XYT for ACT-1 and MIR-1) was inoculated with the preculture. The cultures were incubated at 28°C for the production of ACT-1 and CMY-2 or at 37°C for the production of CMY-1 and MIR-1. At an A600 of 1, IPTG (0.1 mM final concentration) was added to the cultures. They were incubated for 4, 7, 4, and 5 additional hours for the production of ACT-1, CMY-2, CMY-1, and MIR-1, respectively.

The purification of ACT-1 was performed as follows. The bacteria were harvested by centrifugation at 5,000 × g for 10 min at 4°C. The cells were resuspended in 50 ml of 15 mM sodium phosphate buffer, pH 7 (buffer A), and disrupted with the help of a cellular disruptor (Constant System Ltd., United Kingdom). Cell debris was eliminated by centrifugation at 15,000 × g for 30 min at 4°C. The supernatant was recovered and dialyzed overnight against buffer A at 4°C. The sample was clarified by filtration through a 0.45-μm Durapore membrane filter (Millipore, County Cork, Ireland). The crude extract was then loaded at 5 ml/min on an SP Sepharose fast-flow column (30 cm × 1 cm, 1 ml) connected to an Intel Pentium III personal computer. The variations in absorbance were determined as competitive inhibition constants, KIC, in the presence of a good penicillin binding protein, the antibiotic concentration in the periplasmic cavity.

The theoretical MICs. As previously described (9), the external antibiotic concentration (Ip) necessary to obtain a periplasmic antibiotic concentration (Ip) is given by equation 1.

\[
I_p = I_e + \frac{k_eq \cdot E_p \cdot I_p}{P \cdot A \cdot (K_{eq} + I_p)}
\]

where P is the permeability of the outer membrane, A is the membrane area by unit weight, keq and Keq are the kinetic parameters of the β-lactamase, and Ip is its concentration by unit of cell weight. When Ip is sufficient to inactivate the essential penicillin binding proteins, the antibiotic concentration in the periplasmic space is lethal (Ip = Ip), and the external concentration is equal to the MIC (equation 2).
RESULTS AND DISCUSSION

Purification of the plasmid-encoded AmpCs. The goal of this work was to study the differences between the chromosomal and the ACT-1, MIR-1, CMY-1, and CMY-2 plasmid-encoded class C β-lactamases. Purification of these four enzymes was successfully performed using a method previously established for their chromosomal equivalents, confirming that they conserved similar physicochemical properties. The plasmid-encoded AmpCs exhibit relatively high isoelectric points (>8), allowing their purification at pH 6.5 or 7 with the help of cation-exchange chromatography. All the β-lactamases were purified in one or two chromatographic steps. In the cases of MIR-1 and CMY-1, the enzymes were eluted from an SP Sepharose column at NaCl concentrations of 40 and 75 mM, respectively. The purity of the enzyme preparations was higher than 95%. The purification yields of MIR-1 and CMY-1 were 20 and 60%, respectively. Purification of ACT-1 and CMY-2 was completed by affinity chromatography using 3-aminophenyl boronic acid as a ligand. This compound behaved as a competitive inhibitor with $K_I$ values of 86 and 314 μM for ACT-1 and CMY-2, respectively. The β-lactamases were eluted by washing the column with a solution of 0.5 M sodium borate, 0.5 M NaCl. This step allowed the recovery of pure ACT-1 and CMY-2. Unfortunately, all the enzymes bound to the column could not be recovered. Therefore, the purification yields of ACT-1 and CMY-2 were 22% and 60%, respectively. The low purification yields were also due to the poor stability of the proteins during the different purification steps. The CMY-1 and CMY-2 enzymes precipitated when the protein concentration was higher than 3 mg/ml. The best storage conditions for the plasmid-encoded AmpCs were to keep the enzymes frozen at −20 or −70°C in 50 mM MOPS, pH 7, or 50 mM 2-(cyclohexylamino)ethanesulfonic acid, pH 10, at a concentration higher than 1 mM.

The N-terminal sequences of the different β-lactamases (NH₂-PMSEKD, NH₂-AAKTEQ, NH₂-APMSEK, and NH₂-GEAPSV for ACT-1, CMY-2, MIR-1, and CMY-1, respectively) were in agreement with the predicted N-terminal sequences. All the enzymes were found to exhibit the expected masses (39,379 versus 39,780 for ACT-1, 39,855 versus 39,850 for CMY-1, 39,160 versus 39,160 for MIR-1, and 38,755 versus 38,756 for CMY-2). In the case of CMY-1, the various gene manipulations resulted in the addition of five residues (GSGNH) at the C terminus.

Enzymatic titration by aztreonam. The concentrations of active enzymes were checked by titration with a good inactivator, aztreonam. This experiment allowed us to verify that differences observed between the kinetic parameters of the plasmid-encoded and chromosomal class C enzymes were not due to under- or overestimations of the enzyme concentrations. Figure 1 shows the titration of ACT-1 (5 μM) by aztreonam. Residual activities were determined using cephalothin as a reporter substrate. As expected, in the presence of excess aztreonam (>5 μM), the residual activity was close to zero (~3%). In this case, the enzyme was inactivated when the [aztreonam]/[ACT-1] ratio was about 1.25. This result indicated an underestimation of the enzyme concentration, which could result in an overestimation of the $k_{cat}$ values. The presence of a small turnover of the ACT-1–aztreonam complex made it necessary to incubate the enzyme with a large excess of aztreonam to completely inactivate the β-lactamase. Similar results were obtained with the other plasmid-determined class C β-lactamases. [Enzyme] /[aztreonam] ratios of 0.9, 0.8, and 1.1 were found for CMY-2, CMY-1, and MIR-1, respectively. The kinetic data in Tables 2 and 3 were calculated accordingly using the active enzyme concentration, but these corrections did not significantly influence the results.

Kinetic parameters of plasmid-encoded class C β-lactamases. First, we decided to compare the kinetic behavior of the CMY-1 enzyme we produced to that of the wild-type form of CMY-1. The CMY-1 enzyme was purified from the original strain. Its kinetic parameters versus cephalothin and cefoxitin were comparable to those obtained for the modified CMY-1 enzyme. We therefore decided to perform all the other experiments with this β-lactamase.

Substrate inhibition of the plasmid-encoded class C β-lactamases was observed at high concentrations of nitrocefin. While

$$\text{MIC} = I_p + \frac{k_{cat} \cdot E_0 \cdot I_p}{P - A \cdot (K_m + I_p)}$$

On the basis of this equation, we computed the theoretical MICs of the different E. coli strains using $I_p$ values previously established by Frère (9), the kinetic parameters of Galleni et al. and Galleni and Frère (10, 11) for the E. coli AmpC, and our own kinetic parameters for MIR-1. Enzyme concentrations were measured, during the exponential phase, on cells growing in NB medium at 37°C. One milliliter of culture was disrupted by using a nonionic detergent, B-PER II. After a 5-min centrifugation at 20,000 $\times g$, the supernatant was recovered and the β-lactamase activity was determined as the initial rate of hydrolysis of the saturating concentration of cephalothin prepared in buffer M. It is worth noting that these equations are valid only at the steady state, i.e., when the external concentration of antibiotic is constant, the production of enzyme is constitutive, and the half-life of the acyl-enzyme is short compared to the generation time of the bacteria.
and their steady-state kinetic parameters, respectively. Data taken from references 10 and 11.

The Michaelis equation, those for CMY-1 can be fitted to a curve characterized by the following equation:

\[ v = \frac{V_{\text{max}} 	imes [S]}{[S] + ([S]^2/K_c) + K_m} \]  

where \( S, V_{\text{max}}, K_m, \) and \( K_c \) are the substrate concentration, the maximum rate, the Henri-Michaelis constant, and the inhibition constant, respectively. The \( K_{\text{cat}}, K_m, \) and \( K_c \) values were estimated to be 2,220 \pm 150 \text{ s}^{-1}, 230 \pm 30 \text{ mM}, and 660 \pm 80 \text{ mM}, respectively. Similar behavior was observed for the ACT-1 and CMY-2 \( \beta \)-lactamases, which indicated that the plasmid-encoded and the chromosomal AmpC \( \beta \)-lactamases have different behaviors toward nitrocefin.

A large number of studies have been performed on the in vitro susceptibility of \( E. \ coli \) strains producing plasmid-mediated class C \( \beta \)-lactamases (18). These investigations indicated that plasmid-encoded enzymes consistently conferred resistance to different \( \beta \)-lactams, such as penicillins, cephalosporins, cephemycins, and even monobactams. Nevertheless, it was not clear how the production of plasmid-derived enzymes modified the resistance patterns of the hosts. According to some authors, the acquisition of bacterial resistance was due to new catalytic properties of these class C \( \beta \)-lactamases (1). Some plasmid-encoded AmpCs received their names for their supposedly improved activities against one substrate or group of substrates. For example, CMY, FOX, and MOX are the acronyms for cephemycinase, cefoxitinase, and moxaalactama, respectively. By contrast, other authors indicated that plasmid-derived AmpCs are distinct from the chromosomal ones only in the localization of their genetic material (18). Table 2 shows the steady-state kinetic parameters of the studied plasmid-encoded and chromosomal class C \( \beta \)-lactamases for good substrates. In the case of the CMY-1 enzyme, we produced a \( \beta \)-lactamase containing five additional amino acids at the C-terminal end. In order to assess if the presence of extra amino acids modified the kinetic properties of the enzyme, we decided to produce and purify the CMY-1 \( \beta \)-lactamase (see above) from the \( E. \ coli \) strain obtained from Yunsop Chong. The steady-state kinetic constants were determined for cephalothin (\( k_{\text{cat}} = 500 \pm 50 \text{ s}^{-1}, K_m = 20 \pm 4 \text{ mM}, \) and \( k_{\text{cat}}/K_m = 25 \text{ M}^{-1}\text{s}^{-1}) \), cefoxitin (\( k_{\text{cat}} = 0.07 \pm 0.01 \text{ s}^{-1}, k_{\text{cat}}/K_m = 1.1 \text{ M}^{-1}\text{s}^{-1}, \) and \( K_m = 0.06 \pm 0.015 \text{ M} \)). The data indicated that the presence of five amino acids at the C terminus of CMY-1 did not affect the catalytic properties of the \( \beta \)-lactamase.

The turnover of plasmid-encoded enzymes was typically smaller for penicillin than for cephalosporins. No major differences were seen between the two types of enzymes. In contrast to what was suggested on the basis of the MICs, CMY-1, ACT-1, and MIR-1 have lower catalytic efficiencies toward ampicillin (two to six times smaller) than the other class C
enzymes. Nonetheless, some kinetic peculiarities were observed, and in particular, the $k_{cat}/K_m$ of cephaloridine for the ACT-1 and CMY-2 enzymes was somewhat smaller than for their chromosomal counterparts. Surprisingly, a biphasic kinetic progress curve was observed for the hydrolysis of cephaloridine by CMY-2, preventing analysis with a simple kinetic model. This behavior was previously observed for this substrate with the chromosomal AmpC of *Serratia marcescens* (10). Nevertheless, these results showed that the activity profiles toward cephaloridine for the tested plasmid-mediated and chromosomal class C β-lactamases are similar for the tested plasmid-mediated and chromosomal class C β-lactamases.

Kinetic parameters for poor substrates are included in Table 3. $k_{cat}$ and $K_m$ values for cefoxitin, cefuroxime, and cefotaxime could be directly obtained for ACT-1, CMY-1, CMY-2, and MIR-1. When determined, the $k_{+3}$ (rate constant of deacylation) values were similar to $k_{cat}$ and consequently, $k_{+3}$ was small compared to $k_{+2}$ (rate constant of acylation). Also, the rate-limiting step of the hydrolytic pathway corresponds to the deacylation step, i.e., the hydrolysis of the acyl-enzyme complex. The kinetic parameters of ACT-1 and CMY-1 did not present significant differences compared to the chromosomal AmpCs. Nevertheless, CMY-1 exhibited a low $K_m$ value and a relatively low rate of acylation by cefuroxime. The CMY-2 enzyme was characterized by relatively low $K_m$ values against all the tested poor substrates. With oxacillin, the $k_{cat}$ value (0.015 s$^{-1}$), which gives a minimum $k_{+3}$ value, obtained by initial rate measurements was threefold higher than the $k_{+3}$ value (0.005 s$^{-1}$) obtained by monitoring the hydrolysis of nitrocefin in the presence of increasing concentrations of oxacillin. Interestingly, the measured and the computed values of $K_m$ are in good agreement. At the present time, no clear explanation can be proposed to account for the differences in the $k_{+3}$ values.

The analysis of our kinetic data confirmed that the four plasmid-encoded β-lactamases exhibited the typical properties attributed to class C β-lactamases. The comparison of the chromosome-encoded enzyme parameters shows that most of the kinetic parameters of the plasmid-encoded enzymes lie between the extreme values obtained for the known chromosomal AmpCs. In particular, the CMY enzymes do not present increased activities toward cefoxitin. None of the four enzymes is less sensitive to aztreonam inactivation. On the contrary, CMY-2 presents the highest acylation rate observed so far for this substrate, and the deacylation constant is very low ($k_{+3} < 10^{-5}$ s$^{-1}$). Interestingly, a few $k_{cat}$ values showed a slight increase with some poor substrates. The catalytic efficiencies of the four plasmid-mediated enzymes toward cefuroxime were higher than those of chromosomal AmpCs. Moreover MIR-1 had a relatively high $k_{cat}$ against cefuroxime, cefotaxime, and oxacillin. However, as the $K_m$ values were also increased, its catalytic efficiency was not different from those of the chromosomal enzymes.

**MICs of E. coli DH5α strains and overproduction of the plasmid-encoded class C β-lactamases.** The strains producing

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**TABLE 3. Kinetic parameters of plasmid-encoded AmpC β-lactamases for poor substrates**

<table>
<thead>
<tr>
<th>β-Lactamase</th>
<th>Antibiotics</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (μM$^{-1}$ s$^{-1}$)</th>
<th>$k_{+3}$ (s$^{-1}$)</th>
<th>$K_m$ (μM$^{-1}$ s$^{-1}$)</th>
<th>$K_m$ calc (μM)</th>
<th>$K_m$ max (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT-1(P99)</td>
<td>Cefoxitin</td>
<td>0.37 (0.06)</td>
<td>0.74 (2.5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.5 (0.024)</td>
</tr>
<tr>
<td></td>
<td>Cefuroxime</td>
<td>0.12 (0.05)</td>
<td>6.3 (3.1)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.019 (0.016)</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>0.05 (0.015)</td>
<td>0.7 (1.5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.07 (0.01)</td>
</tr>
<tr>
<td></td>
<td>Imipenem</td>
<td>0.011 (0.003)</td>
<td>0.03</td>
<td>0.009 (0.002)</td>
<td>0.024 (0.06)</td>
<td>0.38 (0.04)</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Aztreonam</td>
<td>ND</td>
<td>0.0021 (0.0002)</td>
<td>0.18 (0.26)</td>
<td>0.012 (0.0012)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxacillin</td>
<td>0.044</td>
<td>0.5</td>
<td>ND</td>
<td>0.65</td>
<td>ND</td>
<td>0.09</td>
</tr>
<tr>
<td>MIR-1(P99)</td>
<td>Cefoxitin</td>
<td>0.64 (0.06)</td>
<td>0.7 (2.5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.75 (0.024)</td>
</tr>
<tr>
<td></td>
<td>Cefuroxime</td>
<td>3.4 (0.05)</td>
<td>5.7 (3.1)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.6 (0.016)</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>2.7 (0.015)</td>
<td>0.67 (1.5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4 (0.01)</td>
</tr>
<tr>
<td></td>
<td>Imipenem</td>
<td>0.012 (0.003)</td>
<td>0.08</td>
<td>0.007 (0.002)</td>
<td>0.05 (0.06)</td>
<td>0.14 (0.04)</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Aztreonam</td>
<td>ND</td>
<td>0.0016 (0.0002)</td>
<td>0.218 (0.26)</td>
<td>0.008 (0.0012)</td>
<td>ND</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Oxacillin</td>
<td>0.18</td>
<td>1.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.1</td>
</tr>
<tr>
<td>CMY-1(CAV-1)</td>
<td>Cefoxitin</td>
<td>0.05 (0.5)</td>
<td>0.9 (1.25)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.055 (0.4)</td>
</tr>
<tr>
<td></td>
<td>Cefuroxime</td>
<td>0.02</td>
<td>4 ± 0.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>0.01 (0.2)</td>
<td>0.67 (2)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.015 (0.1)</td>
</tr>
<tr>
<td></td>
<td>Imipenem</td>
<td>0.082</td>
<td>0.04</td>
<td>0.007</td>
<td>0.043</td>
<td>0.16</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Aztreonam</td>
<td>ND</td>
<td>&lt;0.008</td>
<td>0.36</td>
<td>&lt;0.002</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxacillin</td>
<td>0.009</td>
<td>0.45</td>
<td>0.016</td>
<td>0.33</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>CMY-2(Citrobacter freundii)</td>
<td>Cefoxitin</td>
<td>0.23 (0.32)</td>
<td>3.3 (1.3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.07 (0.026)</td>
</tr>
<tr>
<td></td>
<td>Cefuroxime</td>
<td>0.017 (50.04)</td>
<td>15 (2)</td>
<td>0.014</td>
<td>6.4</td>
<td>0.0022</td>
<td>0.0011 (0.02)</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>0.004 (0.016)</td>
<td>3.3 (3.4)</td>
<td>0.003</td>
<td>2.9</td>
<td>0.001</td>
<td>0.0012 (0.006)</td>
</tr>
<tr>
<td></td>
<td>Imipenem</td>
<td>0.033 (0.017)</td>
<td>ND</td>
<td>Biexponential kinetic</td>
<td>Biexponential kinetic</td>
<td>Biexponential kinetic</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Aztreonam</td>
<td>ND</td>
<td>&lt;0.0060 (0.0003)</td>
<td>2 (0.18)</td>
<td>&lt;0.003 (0.0014)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxacillin</td>
<td>0.015</td>
<td>25</td>
<td>0.005</td>
<td>6.5</td>
<td>0.0008</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

*Experiments were performed in 50 mM MOPS, pH 7, 50 mM NaCl. ND, not determined. Data taken from references 10 and 11. Names and numbers between parentheses correspond to the chromosomal class C β-lactamases and their steady-state kinetic parameters, respectively. Standard deviations are within 10%.*
plasmid-encoded class C β-lactamases are characterized by very high MIC values for the different β-lactam families (18). In agreement with our genetic study, these high resistance values cannot be explained simply by increased activities against these compounds. These data raised a question. Indeed, if the four plasmid-mediated β-lactamases are not more active than their chromosomal equivalents, how can one explain the high MIC values associated with their production? To determine if β-lactamase overproduction was not responsible for these resistance profiles, we compared the MIC values for *E. coli* DH5α producing either the plasmid-encoded ACT-1 and MIR-1 enzymes or the chromosomal *E. coli* AmpC encoded by a plasmid (pAD7). This experiment was performed in two media of low or high osmolarity, the nutrient and the Luria-Bertani broths, respectively. First, MIC values obtained for the *E. coli* AmpC were similar to those obtained with the MIR-1 and ACT-1 enzymes (Table 4) (suppression). Determination of the enzyme concentration showed that all the transformants produced nearly 10 times more β-lactamase than was produced by the overproducer TE18 previously described by Nikaido and Normark (16). Such production may, as previously shown (13, 15, 21), cause a strong increase in resistance, even toward poor substrates.

Second, a comparison was made between the observed and computed MIC values. As shown in Table 4, in the absence of β-lactamase production, the experimental values coincide with the calculated ones, indicating that our results agree with the calculated values (C). The *I*ₚ values were those previously established by Frère (9). The enzyme concentrations were 1.3 and 0.845 nmol/mg (dry weight) for the AmpC and MIR-1 producers, respectively. In the case of the *E. coli* DH5α strain, no β-lactamase activity was detected. ND, not determined.

Finally, considering the effect of the medium, for all substrates tested, higher MIC values were obtained in LB medium. This was especially striking in the case of aztreonam, for which the difference reached a factor of 16. This effect could be correlated with the levels of the OmpF and OmpC porins. Indeed, the production of these proteins is related to the osmolarity of the medium (2). In media of high osmolarity (like LB), the synthesis of the larger OmpF porin is repressed in *E. coli*. Since this protein enhances the entry of several β-lactam antibiotics (15), it is clear that MIC values must be influenced by the medium osmolarity. In agreement with this hypothesis, we observed that this phenomenon was especially marked for aztreonam. Indeed, its two negative charges retard penetration through the OmpC porin, which is more selective than OmpF (15).

**Conclusions.** Bacterial resistance to expanded-spectrum β-lactam antibiotics associated with the production of class C β-lactamases has taken two different paths. The first is represented by the production of the GC-1 enzyme. In this case, a tandem tripeptide insertion in the β-lactamase modifies the structure of the omega loop and induces a broadening of the β-lactamase activity (3, 17). The second path is well illustrated by the four enzymes studied in the present paper, showing that resistance could originate from overproduction of plasmid-mediated enzyme. Since their discovery, emergence of new plasmid-encoded β-lactamases has not ceased to occur. In addition, an increasing number of variants has been isolated. These facts enhance the probability of obtaining a true extended-spectrum enzyme. Consequently, these β-lactamases also deserve complete biochemical characterization.

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