Introduction

Most bacteria resistant to β-lactam antibiotics produce a serine active-site β-lactamase. β-Lactamases are classified, based on their structure, into three classes, A, C, and D. From a clinical point of view, class A is the most important as it contains enzymes that confer resistance to penicillins and to first- and second-generation cephalosporins as well as to the main broad-spectrum β-lactam antibiotics such as third-generation cephalosporins, carbapenems and monobactams.

Together with Enterobacter spp. and Serratia spp., Klebsiella oxytoca is responsible for 30% of nosocomial infections. Generally, K. oxytoca spp. produce a class A β-lactamase which hydrolyses penicillins, narrow-spectrum cephalosporins and to a lesser extent, third-generation cephalosporins such as cefoperazone and ceftriaxone, and the monobactam, aztreonam. Following the intensive use in the early 1980s of broad-spectrum β-lactam antibiotics in clinical practice, up to 10% of isolates became resistant to cefoperazone and aztreonam. This might be related to the observation that 8–10% of clinical isolates were found to hyperproduce the

Characterization and amino acid sequence of the OXY-2 group β-lactamase of pl 5.7 isolated from aztreonam-resistant Klebsiella oxytoca strain HB60

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Klebsiella oxytoca strain HB60 is highly resistant to cefoperazone and aztreonam (MICs = 128 mg/L). It produces a chromosomally encoded β-lactamase of pl 5.7 which was highly efficient against penicillins, first-generation cephalosporins and cefoperazone, a non-oxymino third-generation cephalosporin. Aztreonam and oxymino broad-spectrum cephalosporins were less good substrates. The β-lactamase activity was susceptible to inhibition by clavulanic acid (IC₅₀ = 1 μM). The enzyme purified to homogeneity had a specific activity towards benzylpenicillin of 3670 U/mg. The 263 amino acid residues of the protein were sequenced by Edman degradation of proteolytic peptides. The β-lactamase was shown to belong to the OXY-2 group as it had only one amino acid substitution (Asn for Asp at ABL position 197) compared with the β-lactamase (pl 5.2) from the aztreonam-susceptible K. oxytoca strain SL911 and two substitutions (Ala223 for Val and Asp255 for Asn) compared with the β-lactamase (pl 6.4) from the aztreonam-resistant K. oxytoca strain D488. These three OXY-2-group enzymes behave in the same way towards β-lactam antibiotics. The variability in the resistance of these K. oxytoca strains would thus seem to be due to variation in the level of production of the β-lactamases rather than to structural alteration of the enzymes.

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β-lactamase as the result of a point mutation in the consensus sequence of the promoter. These isolates were still susceptible to oxymino cephalosporins, although the MICs of cefotaxime and ceftriaxone were increased about 50-fold recently, a colony hybridization study of K. oxytoca strains of worldwide origin demonstrated that the chromosomally encoded β-lactamase genes could be divided into two groups, bla_{oxy-1} and bla_{oxy-2}. Within each group, four different variants could be distinguished based on pl values. One OXY-1 β-lactamase of pl 7.5 and two OXY-2 β-lactamases of pl 5.2 and pl 6.4 have been sequenced. In this paper we report the characterization and the amino acid sequence of the OXY-2 β-lactamase of pl 5.7 purified from the clinical strain K. oxytoca H60, isolated for its high resistance to aztreonam.

Materials and methods

Bacterial strain

K. oxytoca strain H60 was isolated at Hôpital Begin (Pr. Thabaut, Saint-M Mandé, France). It was identified using the API 20E system and Biotype 100-carbon source strips (bioMérieux SA, Marcy l’Etoile, France).

Susceptibility testing

MICs were determined by a two-fold microdilution method in Mueller-Hinton broth (Difco, Fisher Scientific, OSL, E languac, France). MICs were read after 18 h incubation of an inoculum of 5 × 10^8 cfu/mL at 37°C. The MIC of amoxicillin was measured alone or in combination with potassium clavulanate (2 mg/L).

Transfer of resistance phenotype and detection of plasmid DNA

The rifampicin-resistant Escherichia coli strain K-12 J53-2 was used as the receptor strain in conjugative transfer experiments. Agar plates containing rifampicin (100 mg/L) and amoxicillin (8 mg/L) were used for transconjugant selection. Plasmid DNA content of H60 strain was analysed by the procedure of Kado & Liu.

Purification of the β-lactamase

The methods used for bacterial cultures, cell disruption, ammonium sulphate fractionations, adsorption on to and elution from DE52 cellulose (Whatman, Maidstone, UK) and chromatography on Sephadex G-100 (Pharmacia Biotech, Orsay, France) were as described previously. Ion-exchange HPLC was carried out on a Mono Q HR 5/5 column (Pharmacia) equilibrated with 20 mM Tris-HCl buffer, pH 7.7. Samples (400 μL) containing 5 mg (3290 U) of protein mixture from exclusion chromatography, in the same buffer, pH 7.7, were applied and the proteins eluted with a 500 mM NaCl, 20 mM Tris-HCl buffer, pH 7.7. Fractions with β-lactamase activity (330 mM NaCl) were pooled and desalted by reversed phase HPLC through a 7 μm Nucleosil C18 500 Å (4.6 × 250 mm) column (Interchim, Montlucon, France) eluted with acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid. After evaporation of the organic solvent, the β-lactamase was freeze-dried. The purity of the preparation was checked by N-terminal sequence analyses.

Analytical isoelectrofocusing

The pl was determined by analytical isoelectrofocusing in sucrose density and ampholyte gradients (pH range from 4.5 to 8.5) with an LKB 8101 column.

β-L actamase assays

One unit of β-lactamase activity is defined as the amount of enzyme hydrolysing 1 μmol of benzylpenicillin at 37°C and pH 7. During the purification procedure and for the p-hydroxymercuribenzoate inhibition study, the enzymatic activity was detected spectrophotometrically at 235 nm in 50 mM sodium phosphate buffer, pH 7, with 800 μM benzylpenicillin as substrate. For inhibition by p-hydroxymercuribenzoate, inhibitor (0.1 mM) and β-lactamase (2 U) were incubated for 5 min before addition of the substrate. The β-lactamase from K. oxytoca D488, an enzyme of the OXY-2 group, and the OXY-like MEN-1 β-lactamase were incubated for 5 min before addition of the substrate. The remaining activity against benzylpenicillin was measured. The data were plotted against inhibitor concentration to determine IC_{50} and against the inhibitor:enzyme ratio to determine the turnover number from the extrapolated value for 100% inactivation.

Trypsin and chymotrypsin proteolysis and carboxymethylation of peptides

Three milligrams (103 nmol) of β-lactamase were suspended in 1.5 mL of 0.05 M Tris-HCl buffer, pH 8.3. The protein was digested with 20 μg of trypsin (type XII, Sigma, St Quentin Fallavier, France) or chymotrypsin (type VII, Sigma) for 2 h at 25°C. The peptide mixtures were carboxymethylated and then separated by reversed phase HPLC as described previously.

β-L actamase digestion and subfractionation of tryptic peptides T-18a and T-20 by endoproteinase A sp-N

One hundred and three nmol of β-lactamase, or 20 nmol of peptides, were dissolved in 400 μL of 8 M urea for 30 min.
β-Lactamase from aztreonam-resistant K. oxytoca

The protein, or peptides, were then digested for 6 h at 37°C by adding 6 μg of endoproteinase Asp-N in 1.2 mL of 75 mM potassium phosphate buffer, pH 8. The resulting proteolytic peptides were subjected to reversed phase HPLC separations as previously reported.18,19

Purification of proteolytic peptides

Proteolytic fragments were separated at pH 2 by reversed phase HPLC on a 5 μm Nucleosil C18 (4 × 250 mm) column (Interchim) eluted with a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid as described previously.18 Tryptic, chymotryptic and endoproteinase Asp-N peptides were designated T, C and D, respectively. Peptides were numbered in the order in which they eluted from reversed phase HPLC at pH 2. Peptides recovered from subsequent chromatography at pH 6 were indicated by Roman numerals whereas lower case letters indicate peptides separated from a final chromatography under isocratic elution at pH 2.

Amino acid composition and sequence analyses

The methods for hydrolysis (1 nmol), amino acid composition analysis, 4-N,N-dimethylaminoazobenze-4'-isothiocyanate/phenylisothiocyanate double coupling Edman degradation (2–10 nmol) of peptides, as well as the method for quantitative determination of N-terminal amino acid derivatives by HPLC, have been described previously.16,18

Amino acid sequence accession number

The amino acid sequence of the β-lactamase from K. oxytoca strain HB60 has been submitted to the EMBL database and assigned the accession number P23954.

Results

Antibiotic susceptibility

The K. oxytoca strain HB60 was highly resistant to penicillins (MICs of amoxycillin, ticarcillin and piperacillin > 1024 mg/L), narrow-spectrum cephalosporins (MIC of cephalothin = 256 mg/L), aztreonam (MIC = 128 mg/L) and susceptible to cefoxitin (MIC = 2 mg/L), imipenem (MIC = 0.12 mg/L) and third-generation cephalosporins (MICs of cefotaxime and ceftazidime = 2 mg/L) with the exception of cefoperazone (MIC = 128 mg/L). Clavulanic acid lowered the susceptibility to penicillins although the strain was still resistant to the combination of amoxycillin and clavulane (MIC = 16 mg/L).

Location of β-lactamase determinant

No E. coli K12 J53-2 transconjugants were selected after overnight mixed incubation of K. oxytoca HB60 and the recipient strains. Moreover, analysis of DNA extracts from the K. oxytoca strain by electrophoresis in a 0.7% agarose gel did not reveal any plasmid DNA band. These results all suggested that the bla gene was encoded chromosomally rather than by a plasmid.

β-Lactamase purification

The β-lactamase was purified by a five-step procedure as summarized in Table I. The purified enzyme was adsorbed tightly on dialysis membrane. Therefore, the active fractions recovered from anion-exchange HPLC were desalted by reversed phase HPLC. The β-lactamase was purified 400-fold from crude extract of 9 U/mg specific activity. Reversed phase analysis of the Edman derivative of the N-terminal amino acid indicated a protein purity of 96%. By analytical isoelectric focusing in a sucrose density gradient the pI was found to be 5.7.

Kinetic parameters

The substrate profile was determined with a partially purified protein (658 U/mg). The kcat values were calculated from activities determined with highly purified β-lactamase and a molecular mass deduced from the amino acid sequence of the enzyme. The data for Kcat, Km and catalytic efficiency (kcat/Km) are shown in Table II. As expected from antibiotic susceptibilities, penicillins and narrow-spectrum cephalosporins were the best substrates for the β-lactamase of K. oxytoca HB60. With high Km values, methoxyimino third-generation cephalosporins were hydrolysed to a much lesser extent. For example, the catalytic efficiency for cefotaxime was only 1.5% of that for amoxycillin. Conversely, the catalytic efficiency for ceftazidime, a non-oximino third-generation cephalosporin, was identical to that for cephapirin. That was the result of a low Km value since, of the substrates, cefoperazone presented the lowest kcat. The β-lactamase hydrolysed aztreonam significantly with a kcat/Km value as high as that of broad-spectrum cephalosporins although the strain was 64-fold more susceptible to cefotaxime than to the mono-bactam. Cephemycins and imipenem were not affected by the enzyme. The β-lactamase was inhibited by clavulanate with a IC50 of 1 μM. The inactivation was progressive with a turnover number of 560 and a K1 of 0.12 μM. A solution of 0.1 mM p-hydroxymercuribenzoic acid inhibited 93% of the β-lactamase activity against benzylpenicillin. In the same conditions we found 97% inhibition of the β-lactamase from K. oxytoca D 488 and 80% inhibition of the plasmid-mediated M E N-1 β-lactamase.

Amino acid sequence determination

The strategy used to establish the sequence of the β-lactamase from Edman degradation analyses of intact protein and proteolytic peptides is shown in the Figure. An initial
reversed phase HPLC at pH 2 of the tryptic digest separated 22 peaks from which 29 peptides were recovered by additional chromatography at pH 2 or 6. After amino acid composition analysis, 19 peptides were subjected to Edman degradation. Except for T-18a and T-20, the complete amino acid sequences of the 19 tryptic peptides were determined (Figure). In most cases, lysine residues were not identified as Edman derivatives but were deduced from amino acid composition analyses. Peptide T-2 was placed N-terminally by comparison with the sequence of the intact protein whereas T-8, which ended with a leucine residue, was located at the C-terminal. Three fragments, at positions 16–19, 37–40 and 247–249, were not recovered. The corresponding peptides eluted from the HPLC column with the buffer peak. The primary structure of the 36-residue long peptide T-20 was fully determined by analysis of peptide T-20 D-4 resulting from fractionation of the parent peptide by Asp-N endoproteinase. In the same way, Edman degradation of peptide T-20 D-4 extended the amino acid sequence determination of peptide T-20 up to residue 243, which corresponds to position 270 according to the standard numbering scheme for class A β-lactamases (ABL). The overlaps between tryptic peptides and the identification of the domains remaining unidentified were carried out by analyses of peptides obtained from chymotryptic and endoproteinase Asp-N digests as indicated in the Figure. The β-lactamase of K. oxytoca HB60 consisted of 263 residues with a molecular mass of 28,287 Da.

Discussion

K. oxytoca exhibits a resistance phenotype which may be restricted to penicillins or extended to first-generation cephalosporins as well as to broad-spectrum β-lactam antibiotics such as aztreonam and cefopazone. Whatever their susceptibilities to β-lactam antibiotics K. oxytoca strains produce one of a set of chromosomally encoded β-lactamases characterized by eight pI values and belonging to two structurally different groups, OXY-1 and OXY-2. Two enzymes of the OXY-2 group have been sequenced. The amino acid sequence of β-lactamase of pI 5.2, isolated from the aztreonam-susceptible (MIC 0.12 mg/L) strain SL911, differed from that of the pI 6.4 β-lactamase from an aztreonam-resistant (MIC 32 mg/L) strain D488 by only three amino acid substitutions: Asp for Asn at ABL 197, Ala for Val at ABL-223 and Asp for Asn at ABL-255. Recently, the amino acid sequence of the β-lactamase RbiA produced by the aztreonam-resistant (MIC 128 mg/L) K. oxytoca strain SB23 has been reported. It was claimed that the protein differed from that of strain D488 by three substitutions: Asp for Asn at ABL-191, A la for Val at ABL-223 and A sp for A sn at

### Table I. Purification of the β-lactamase from K. oxytoca HB60

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonication</td>
<td>75,190</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>DE 52 cellulose</td>
<td>48,870</td>
<td>36</td>
<td>65</td>
</tr>
<tr>
<td>Ammonium sulphate fractionations</td>
<td>39,100</td>
<td>110</td>
<td>52</td>
</tr>
<tr>
<td>Gel filtration on Sephadex G-100</td>
<td>31,580</td>
<td>658</td>
<td>42</td>
</tr>
<tr>
<td>A non-exchange HPLC + desalting</td>
<td>13,540</td>
<td>3670</td>
<td>18</td>
</tr>
</tbody>
</table>

aData obtained from 50 g of wet bacteria.

### Table II. Kinetic parameters of the β-lactamase from K. oxytoca HB60

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>(k_{cat}) (1/s)</th>
<th>(K_m) (µM)</th>
<th>(k_{cat}/K_m) (1/mM s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylpenicillin</td>
<td>1730</td>
<td>55</td>
<td>31,500</td>
</tr>
<tr>
<td>A moxycillin</td>
<td>1600</td>
<td>90</td>
<td>17,800</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>330</td>
<td>85</td>
<td>3900</td>
</tr>
<tr>
<td>Pipercillin</td>
<td>575</td>
<td>25</td>
<td>2300</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>2190</td>
<td>480</td>
<td>4500</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>1480</td>
<td>170</td>
<td>8700</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>120</td>
<td>150</td>
<td>800</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>&lt;0.2</td>
<td>155</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>&lt;0.2</td>
<td>530</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>35</td>
<td>4</td>
<td>8750</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>145</td>
<td>525</td>
<td>280</td>
</tr>
<tr>
<td>Ceftriazone</td>
<td>350</td>
<td>350</td>
<td>1000</td>
</tr>
<tr>
<td>Cefpirome</td>
<td>105</td>
<td>1500</td>
<td>100</td>
</tr>
<tr>
<td>Cefodizime</td>
<td>120</td>
<td>480</td>
<td>250</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&lt;0.2</td>
<td>785</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>155</td>
<td>385</td>
<td>400</td>
</tr>
<tr>
<td>Imipenem</td>
<td>&lt;0.2</td>
<td>65</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

\(\text{Standard deviations} \leq 10\%\).

\(\text{Twenty micrograms of β-lactamase gave no detectable hydrolysis} (-0.01 \text{ mol/min}). The} \ K_m \ \text{was determined as} \ K_{i} \ \text{by substrate competition with benzylpenicillin.} \)
Lactamase from aztreonam-resistant *K. oxytoca* ABL-254. Actually, the ABL numbering scheme was partially misinterpreted and the three substitutions occurred at positions 197, 223, and 255, respectively. Finally, the sequences of the $\beta$-lactamase from strain SL911 and the RbiA enzyme are identical.

The aztreonam-resistant *K. oxytoca* HB60 strain produced a $\beta$-lactamase that has been purified to protein homogeneity. For the last step of the purification procedure, fractions with $\beta$-lactamase activity were desalted by reversed phase HPLC, as the purified enzyme adsorbed to dialysis membrane. The same feature has previously been observed during the purification of the $\beta$-lactamase from *K. oxytoca* D488 and the OXY-like MEN-1 enzyme.\textsuperscript{16}

**Figure.** Amino acid sequence of the $\beta$-lactamase from *K. oxytoca* HB60 strain. The ABL numbering scheme is shown in square brackets. Peptide nomenclature is given in Materials and methods. Residues identified by Edman degradation are underlined with solid lines. Dashed lines indicate residues checked only by amino acid composition analyses. Only the endoproteinase Asp-N and chymotryptic peptides useful for overlaps are shown. Arrows indicate residues of the catalytic cavity of class A $\beta$-lactamases implicated in the $\beta$-lactam acylation mechanism of the active-site serine at position 70. Stars indicate positions of the two amino acid substitutions Ala\textsubscript{223} for Val and Asp\textsubscript{255} for Asn with the sequence of the $\beta$-lactamase from *K. oxytoca* D488 strain. The single substitution Asn\textsubscript{197} for Asp compared with the enzyme from *K. oxytoca* SL911 strain is indicated by the plus sign.
The amino acid sequence of the \( \beta \)-lactamase from \( K. \) \( \text{oxytoca} \) HB 60 exhibits one substitution (Asn for Asp at ABL 197) compared with the OXY-2 group protein (pl 5.2) from strains SL 911\(^{12}\) or SB 23,\(^{10}\) and two substitutions (Ala for Val at ABL 223 and Asp for Asn at ABL 255) compared with the protein (pl 6.4) from strain D 488\(^{9}\) (see Figure). Therefore, the protein of pl 5.7 of \( K. \) \( \text{oxytoca} \) HB 60 is one of the four enzymes which constitute the OXY-2 group of \( \beta \)-lactamases.\(^{12}\) As has been suggested, the proteins of this group differ by only a few amino acid substitutions,\(^{12}\) whereas there are 24 amino acid substitutions between the sequence of OXY-1 \( \beta \)-lactamase from strain E 23004\(^{4}\) and that of OXY-2 \( \beta \)-lactamase from strain H B 60.\(^{21}\)

Kinetic constant values of the \( \beta \)-lactamase of \( K. \) \( \text{oxytoca} \) HB 60 (Table II) were rather similar to those reported for other chromosomal enzymes recovered from \( K. \) \( \text{oxytoca} \),\(^{5,7,10,21}\) Penicillins and narrow-spectrum cephalosporins were highly hydrolysed, while aztreonam and methoxyimino third-generation cephalosporins were less good substrates but were hydrolyzed significantly. Of the broad-spectrum cephalosporins, cefoperazone was the best substrate.\(^{5,8}\) Clavulanate inhibited \( \beta \)-lactam hydrolysis with IC\(_{50}\) and \( K_{i} \) values similar to those encountered within class A \( \beta \)-lactamases susceptible to this inhibitor.\(^{4,22,23}\) The \( \beta \)-lactamase was as strongly inhibited by p-hydroxymercuribenzoate as was the OXY-2 group \( \beta \)-lactamase from strain D 488 or the M E N-1 enzyme. O wing to the presence of a cysteine residue at position 69, close to the active-site serine, that behaviour was expected although it has been claimed that the inhibitor does not alter the activity of the enzyme from \( K. \) \( \text{oxytoca} \) E 23004 strain.\(^{5}\)

In terms of catalytic efficiencies, the three sequenced \( \beta \)-lactamases of the OXY-2 group were kinetically identical (Table III). The enzymes of that group belong to Ambler’s class A. The catalytic cavity of these enzymes is mainly made of nine residues at positions Ser70, Lys73, Ser130, A sn132, Glu166, A sn170, Lys(A rg)234, Thr(Ser)235 and A la237 (A BL numbering). A dense hydrogen bond network is established between these residues, two water molecules and the substrate; this correctly positions the carboxyl moiety of the \( \beta \)-lactam ring which acylates the active site serine at position 70.\(^{24}\) All amino acid sub-

<table>
<thead>
<tr>
<th>Cephaloridine</th>
<th>100</th>
<th>100</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>A moxycillin</td>
<td>390</td>
<td>363</td>
<td>160(^{d})</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>190</td>
<td>135</td>
<td>260</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>6</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>9</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

\( ^{a}\)Data are given as percentage of the cephaloridine value.

\( ^{b}\)Data from reference 9.

\( ^{c}\)Data from reference 10. R bIA is identical to the \( \beta \)-lactamase from \( K. \) \( \text{oxytoca} \) SL 911.\(^{12}\)

\( ^{d}\)Determined with ampicillin.

| Cefotaxime | 6   | 2   | 3   |
| Aztreonam  | 9   | 7   | 8   |

\( ^{a}\)Data are given as percentage of the cephaloridine value.

\( ^{b}\)Data from reference 9.

\( ^{c}\)Data from reference 10. R bIA is identical to the \( \beta \)-lactamase from \( K. \) \( \text{oxytoca} \) SL 911.\(^{12}\)

\( ^{d}\)Determined with ampicillin.

### References


β-Lactamase from aztreonam-resistant K. oxytoca

Escherichia coli K 12 has a different evolutionary origin from that of β-lactamases of the penicillinase type. Proceedings of the National Academy of Sciences of the USA 78, 4897–901.


