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Variability of Chromosomally Encoded β-Lactamases from *Klebsiella oxytoca*

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The β-lactamase genes of *Klebsiella oxytoca* were previously divided into two main groups: bla_{OXY-1} and bla_{OXY-2}. The two β-lactamase groups were each represented by β-lactamases with four different pIs. In each group, one form of β-lactamase is more frequent than the others combined. The β-lactamase gene of each representative β-lactamase with a different pI that was not yet sequenced (pIs 5.7, 6.8 [OXY-2], 7.1, 8.2, and 8.8 [OXY-1]) was cloned and sequenced. The susceptibility patterns as well as relative rates and kinetic parameters for β-lactam hydrolysis revealed that OXY-2 enzymes hydrolyzed several of the β-lactams that were examined (carbenicillin, cephalothin, cefamandole, ceftriaxone, and aztreonam) at a greater rate than the OXY-1 enzymes did. Comparison of *K. oxytoca* β-lactamases with plasmid-mediated extended-spectrum β-lactamases MEN-1 and TOHO-1 implied that the threonine at position 168 present in OXY-2 β-lactamase instead of the alanine in OXY-1 could be responsible for its modified substrate hydrolysis. In each group, the β-lactamase with a variant pI differs from the main form of β-lactamase by one to five amino acid substitutions. The substrate profile and the 50% inhibitory concentrations revealed that all substitutions differing from the main form of β-lactamase were neutral except one difference in the OXY-1 group. This substitution of an Ala to a Gly at position 237 increases the hydrolysis of some β-lactams, particularly aztreonam; decreases the hydrolysis of benzylpenicillin, cephaloridine, and cefamandole, and decreases the susceptibility to clavulanic acid (fivefold increase in the 50% inhibitory concentration).

β-Lactams are among the most used antibiotics. Resistance to β-lactams is most often due to the production of β-lactamases in gram-negative bacteria (35). *Klebsiella oxytoca*, like most enteric bacteria, carries a chromosomally encoded β-lactamase. Although for many enterobacteria the chromosomal β-lactamases belong to class C, for *Klebsiella* spp. such as *Klebsiella pneumoniae* and *K. oxytoca*, the β-lactamase produced is a class A enzyme (1). The chromosomal β-lactamase of *K. oxytoca* is synthesized at a low level and confers resistance to amino- and carboxypenicillins. Recently, the β-lactamase genes were divided into two main groups: bla_{OXY-1} and bla_{OXY-2} (12). A high degree of identity (89.7%) was observed between these two β-lactamases. These β-lactamases belong to the functional group 2be of the extended-spectrum β-lactamases (5). They hydrolyze penicillins, cephalosporins such as ceftriaxone, and aztreonam (2, 14, 26, 36). Each group of β-lactamase is represented by at least four different forms according to their pIs: for OXY-1, 7.1, 7.5, 8.2, and 8.8, with the form with a pI of 7.5 representing 88% of all OXY-1 enzymes, and for OXY-2, 5.2, 5.7, 6.4, and 6.8, with the form with a pI of 5.2 representing 59% of all OXY-2 enzymes (12). Three enzymes were previously sequenced: the β-lactamase of strain E23004 with a pI of 7.5 (OXY-1) (2) (the same enzyme from strains SL781 and SL7811 was sequenced later [11]), the β-lactamase of strain D488 (OXY-2) with a pI of 6.4 (31), and the β-lactamase of strain SL911 with a pI of 5.2 (OXY-2) (12).

The aim of this study was to characterize the sequences of the other β-lactamases of *K. oxytoca* and to examine the influence of the various residues on enzyme hydrolysis. A β-lactamase gene of each representative β-lactamase with a different pI that was not yet sequenced (pIs 5.7, 6.8 [OXY-2], 7.1, 8.2, and 8.8 [OXY-1]) was cloned and sequenced. The susceptibility patterns, substrate profiles, and kinetic parameters for β-lactam hydrolysis revealed that OXY-2 enzymes hydrolyze some β-lactams at a greater rate than OXY-1 enzymes. In the OXY-1 group, a Gly for Ala-237 substitution modifies the hydrolysis of several β-lactams, including some extended-spectrum β-lactams, and decreases the susceptibility to clavulanic acid.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. All *K. oxytoca* strains were previously identified and analyzed for their resistance phenotype as well as the pIs, the levels of production, and the type (OXY-1 or OXY-2) of their β-lactamases (9, 12).

Antibiotics. The following drugs were kindly provided by the companies listed: ceftriaxone, Hoffmann-La Roche; aztreonam, Bristol-Myers Squibb; clavulanic acid, SmithKline Beecham; and tazobactam, Lederle Cyanamid Canada. The other drugs were purchased from Sigma.

Determination of susceptibility pattern. The susceptibility pattern of each *K. oxytoca* strain was determined by the disk diffusion method with Mueller-Hinton agar (6).

Cloning of β-lactamase genes. The β-lactamase gene of each *K. oxytoca* strain was amplified by PCR with two primers: primer 383 (5′-d[GGG GAT CCA GCC GGG GCC AA]-3′) and primer S (5′-d[CGG GCC TGT TCC CGG GTT AA]-3′). Primer 383 (position 58, according to the coordinates used by Fournier et al. [12]) has a *Bam*HI site, and primer S (position 1200) has a *Sma*I site. PCR was performed with annealing at 60°C with *Taq* DNA polymerase. PCR products from *K. oxytoca* 756, 563, and 1879-77 were digested with *Bam*HI and *Sma*I and ligated into the *Bam*HI-*Sma*I sites of pBGS18+. Recombinant plasmids were introduced into *Escherichia coli* JM101. PCR products from *K. oxytoca* K1764 and 11V were digested with *Bam*HI and ligated in pTZ/PC (kindly provided by D. C. Tessier) that had been digested with *Bam*HI and *Xcm*I. Recombinant plasmids were introduced into *E. coli* NM522.

Sequencing and protein analysis. The cloned β-lactamase genes were sequenced with the T7 sequencing kit (Pharmacia). In order to determine that the sequenced mutations were not introduced by *Taq* polymerase, each mutation was verified by direct sequencing of a pool of three independent PCR products.

The Genetics Computer Group (GCG) program ISOELECTRIC was used to calculate the pIs of the β-lactamases. An alignment with truncated proteins was constructed as described previously (12). A phylogenetic tree was derived from

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Source or reference
Strains		
E. coli JM101	*supE thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	37
E. coli NM522	F′*lacI*^{q}(*lacZ*)*M15 proA*$^+$*B*$^+$/*supE thi* Δ(*lac-proAB*) Δ(*hsdMS-mcrB*)5(r_k^- m_k^- McrBC$^-$)	13
K. oxytoca SL781	Clinical isolate with OXY-1-1 β-lactamase (1978)	France, 8
K. oxytoca SL7811	In vitro overproducing mutant of *K. oxytoca* SL781	8
K. oxytoca 756	Clinical isolate with OXY-1-2 β-lactamase	France
K. oxytoca 563	Clinical isolate with OXY-1-3 β-lactamase	United Kingdom
K. oxytoca 1879-77	Clinical isolate with OXY-1-4 β-lactamase	United States
K. oxytoca SL911	Clinical isolate with OXY-2-1 β-lactamase (1991)	France, 12
K. oxytoca SL9111	In vitro overproducing mutant of *K. oxytoca* SL911	9
K. oxytoca K1764	Clinical isolate with OXY-2-2 β-lactamase	Germany
K. oxytoca 11V	Clinical isolate with OXY-2-4 β-lactamase	France
K. oxytoca SL9021	In vitro overproducing mutant of *K. oxytoca* SL902	9
Plasmids		
pBGS18+	Cloning vector, Km^r	32
pTZ/PC	Cloning vector, Ap^r	D. C. Tessier
pLQ950	1,150-bp *Bam*HI-*Sma*I fragment of PCR product from *K. oxytoca* 756 cloned in pBGS18+, Ap^r Km^r	This study
pLQ951	1,150-bp *Bam*HI-*Sma*I fragment of PCR product from *K. oxytoca* 563 cloned in pBGS18+, Ap^r Km^r	This study
pLQ952	1,150-bp *Bam*HI-*Sma*I fragment of PCR product from *K. oxytoca* 1879-77 cloned in pBGS18+, Ap^r Km^r	This study
pLQ953	1,150-bp *Bam*HI fragment of PCR product from *K. oxytoca* K1764 cloned in pTZ/PC, Ap^r	This study
pLQ954	1,150-bp *Bam*HI fragment of PCR product from *K. oxytoca* 11V cloned in pTZ/PC, Ap^r	This study

[a] Ap, ampicillin; Km, kanamycin.

this alignment by using the GCG program GROWTREE with Kimura's correction and by the neighbor-joining method (12).

β-Lactamase assays. Crude β-lactamase preparations were obtained from the different strains after centrifugation of sonicated cell extracts. Each extract contained a single β-lactamase activity, as determined by isoelectric focusing. In order to have sufficient amounts of enzyme, enzyme assays were done with overproducing mutants derived from susceptible strains (SL7811 for SL781 and SL9111 for SL911). For the susceptible strain 1879-77, assays were performed with *E. coli* NM522 carrying plasmid pLQ952. Strain D488, which carries the β-lactamase sequenced by Reynaud et al. (31), was replaced by *K. oxytoca* SL9021, whose β-lactamase has the same pI (pI 6.4). Values were means from two to three determinations. Variations were within ±20%.

β-Lactamase activity was determined spectrophotometrically at 25°C with a Beckman DU64 spectrophotometer. Optimal wavelengths were determined from difference spectra of hydrolyzed versus unhydrolyzed substrates as described previously (4). The relative rates of hydrolysis were determined with penicillins at 500 μM and cephalosporins as well as aztreonam at 100 μM. K_m and V_{max} values were calculated by linear regression analysis of Woolf-Augustinson-Hofstee plots for cephaloridine, cefamandole, and ceftriaxone. At least six different substrate concentrations were used. For benzylpenicillin and aztreonam, the kinetic parameters were calculated from complete time courses (22) because their K_ms were too low and too high, respectively.

The concentrations of β-lactamase inhibitors required to inhibit 50% of the β-lactamase activity (IC_{50}s) were measured after 10 min of preincubation at 37°C by using 100 μM cephaloridine as the substrate. The enzyme concentrations used in the assays were adjusted to produce 55% hydrolysis of cephaloridine in 10 min at 25°C.

Nucleotide sequence accession numbers. The nucleotide sequences of bla_{OXY-1} and bla_{OXY-2} genes have been given accession numbers Z30177 and Z49084, respectively, in the EMBL-GenBank-DDBJ data libraries.

RESULTS

Susceptibility pattern. The susceptibility patterns of 79 *K. oxytoca* strains were determined according to the phenotype of the strain (susceptible or overproducing strain) and to the β-lactamase type (OXY-1 or OXY-2) (Table 2). In the overproducing strains, the strains harboring the OXY-1 β-lactamase seemed to be more susceptible than those with the OXY-2 β-lactamase. The inhibition zone diameters with cephalothin, cefamandole, cefuroxime, cefotaxime, and aztreonam tended to be higher for the OXY-1 group. In the susceptible strains, similar results were observed: the inhibition zone diameters of cephalothin, cefamandole, and aztreonam tended to be higher for the OXY-1 group.

Cloning. The β-lactamase gene of a representative β-lactamase of each pI group was cloned. The β-lactamase genes of *K. oxytoca* 756, 563, and 1879-77 (OXY-1 group) were amplified by PCR and cloned into pBGS18+. The β-lactamase genes of *K. oxytoca* K1764 and 11V (OXY-2 group) were amplified by PCR and cloned into pTZ/PC that had been digested with *Xcm*I and *Bam*HI. The *Sma*I site of primer S could not be used because bla_{OXY-2} contains a *Sma*I site.

Sequencing and protein analysis. The β-lactamase genes were sequenced (Table 3). In order to simplify the description of the results, β-lactamases of pI 7.5 (strain SL781), pI 7.1 (756), pI 8.2 (563), and pI 8.8 (1879-77) were named OXY-1-1, OXY-1-2, OXY-1-3, and OXY-1-4, respectively. In the OXY-2 group, β-lactamases of pI 5.2 (strain SL911), pI 5.7 (K1764), pI 6.4 (D488), and pI 6.8 (11V) were named OXY-2-1, OXY-2-2, OXY-2-3, and OXY-2-4, respectively. Silent substitutions were observed in comparison with the sequence of $bla_{OXY-1-1}$ (EMBL-GenBank-DDBJ data library accession no. Z30177; $bla_{OXY-1-2}$ [C→T at position 900], $bla_{OXY-1-4}$ [C→A at position 804]) and in comparison with the sequence of $bla_{OXY-2-1}$ (EMBL-GenBank-DDBJ data library accession no. Z49084; $bla_{OXY-2-2}$ and $bla_{OXY-2-4}$ [A→G at position 887] and $bla_{OXY-2-2}$ [C→A at position 1013]). In comparison with the reference β-lactamases of SL781 (OXY-1-1) and SL911 (OXY-2-1), one to five amino acid differences were observed in the β-lactamase genes of the other *K. oxytoca* strains (Table 3). These amino acid differences were almost all present in the last third of the protein (from positions 197 to 289); three mutations, however, were present in the beginning of the protein (positions 26, 30, and 35).

For the OXY-1 group, the substitutions modifying the pIs were at different places. For the OXY-1-2 enzyme, two substitutions explained the decrease in the pI in comparison with the pI of the OXY-1-1 enzyme: replacement of the uncharged Gln

by the basic His and replacement of the uncharged Gly by the acidic Glu. Because histidine contains a weakly basic imidazolium function, glutamic acid, which possesses a net negative charge, more than compensated for the charge of His. For the OXY-1-3 enzyme, the substitution of the uncharged Gly by the basic Lys increased the pI. For OXY-1-4, the substitution of the acidic Glu by the basic Lys increased the pI more than the pI for OXY-1-3 because of the loss of the acidic Glu. For the OXY-2 group, the substitution of Asp to one or more uncharged residues at positions 35, 197, and 255 had an additive effect on the pI. The other mutations were substitutions of an uncharged amino acid by another one. The pIs were previously measured by analytical isoelectric focusing (12), and pI values were also calculated by computer. The computer-predicted pI values corresponded well with the measured values for many of the enzymes (data not shown).

For four substitutions, the new amino acid was that of the β-lactamase of the other group; for example, at position 230 of OXY-1-2, Ala (OXY-1-1) was replaced by Val. For the OXY-2 group, Val was present at this position for all β-lactamases. This indicated the close relationship between the two β-lactamase types.

A phylogenetic tree and a matrix were constructed with the different β-lactamases (Fig. 1). Analysis of this tree represents possible evolutionary relationships between the different β-lactamases.

An alignment of both groups of β-lactamases with the plasmid-mediated extended-spectrum β-lactamases MEN-1 and TOHO-1 is shown in Fig. 2. Several differences were observed in this alignment between the OXY-1 β-lactamase and the other β-lactamases: the amino acids that are common to OXY-2, MEN-1, and TOHO-1 were positioned at residues 55, 58, 72, 153, 168, 171, and 230.

Substrate hydrolysis determinations. The relative rates of hydrolysis were determined for the eight enzymes (Table 4). As shown in Table 4, the OXY β-lactamases strongly hydrolyzed most β-lactams tested. However, aztreonam and extended-spectrum cephalosporins were poorly hydrolyzed by both β-lactamase types. The OXY-2 β-lactamases hydrolyzed several β-lactams including carbenicillin, cephalothin, cefamandole, some extended-spectrum cephalosporins (ceftriaxone), and aztreonam better than the OXY-1 group. In the OXY-2 group, the relative rates of hydrolysis for the four β-lactamases were relatively homogeneous: less than a twofold difference was observed. Unlike the OXY-2 group, the rates of hydrolysis for the OXY-1 group were more variable. OXY-1-2 and OXY-1-3 enzymes hydrolyzed cefuroxime, extended-spectrum cephalosporins (cefoperazone), and aztreonam slightly better than the other two β-lactamases of this group (OXY-1-1 and OXY-1-4) did.

In confirmation of these findings, the kinetic parameters for hydrolysis of various β-lactams by the three representative enzymes are summarized in Table 5. The kinetic values of these three enzymes were variable. The three K_m values were low for benzylpenicillin, indicating a high affinity for this β-lactam. For the other β-lactams, except for cefamandole with the OXY-1-1 enzyme the K_ms were higher. The V_{max} values of cefamandole and aztreonam for the OXY-2-1 β-lactamase were sixfold higher than those for the OXY-1 β-lactamases. The relative V_{max}/K_m values confirmed that the OXY-2 β-lactamase better hydrolyzed some β-lactams tested, particularly ceftriaxone and aztreonam, than the OXY-1 group. In the OXY-1 group, the most notable kinetic effect was seen for cephaloridine: the V_{max} and K_m values for the OXY-1-1 β-lactamase were higher and lower, respectively, than those for the OXY-1-2 β-lactamase, resulting in an increase in the hydrolytic

TABLE 2. Susceptibility patterns, according to β-lactamase type and phenotype, of 79 *K. oxytoca* strains[a]

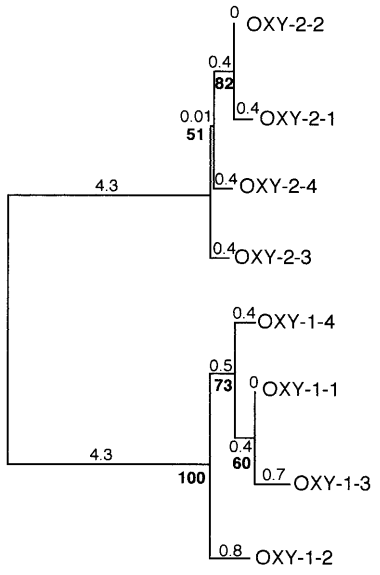
β-Lactamase type (no.)	Phenotype[b]	Inhibition zone diam (mm [mean ± SD])[c]											
		Amoxicillin (25 μg)	Amoxicillin (20 μg) + clavulanate (10 μg)	Ticarcillin (75 μg)	Ticarcillin (75 μg) + clavulanate (10 μg)	Piperacillin (75 μg)	Cephalothin (30 μg)	Cefamandole (30 μg)	Cefuroxime (30 μg)	Cefoperazone (30 μg)	Cefotaxime (30 μg)	Ceftazidime (30 μg)	Aztreonam (30 μg)
OXY-1 (20)	W	11.5 ± 3.9	29.0 ± 2.3	15.8 ± 4.8	30.5 ± 2.0	25.1 ± 3.1	26.8 ± 2.8	32.4 ± 2.5	28.8 ± 2.5	28.3 ± 3.1	35.8 ± 1.4	33.8 ± 1.7	36.5 ± 1.8
OXY-2 (19)	W	9.6 ± 2.4	27.4 ± 1.7	17.4 ± 2.7	29.7 ± 1.3	24.4 ± 2.0	21.9 ± 1.9	26.4 ± 1.9	26.6 ± 1.9	26.1 ± 1.6	35.5 ± 1.6	32.9 ± 1.4	34.3 ± 2.0
OXY-1 (20)	OV	6.6 ± 1.0	15.8 ± 2.6	6.6 ± 1.0	18.4 ± 3.1	6.9 ± 1.0	9.1 ± 2.3	15.1 ± 2.7	11.8 ± 4.5	7.6 ± 1.2	30.5 ± 2.7	30.9 ± 2.0	19.3 ± 5.2
OXY-2 (20)	OV	6.5 ± 0.8	14.0 ± 1.9	6.5 ± 0.8	17.6 ± 2.5	6.9 ± 1.1	6.5 ± 0.6	6.8 ± 0.8	7.0 ± 1.2	7.1 ± 1.4	26.7 ± 3.2	30.1 ± 2.8	13.2 ± 4.6

[a] Resistance was determined by a disk diffusion method.
[b] W, wild-type susceptible strain; OV, overproducing strain.
[c] The disk contents are specified in parentheses.

TABLE 3. Isoelectric points and modified codons and amino acids of the different variants in comparison with the β-lactamases OXY-1-1 and OXY-2-1

β-Lactamase and strain (β-lactamase)	pI[a]	Modification (amino acid) at the following codon[b]:									
		26	30	35	197	218	223	230	237	255	288	289
OXY-1												
SL781 (OXY-1-1)[c]	7.5	GCC (Ala)	CAG (Gln)	GAT (Asp)	AAT (Asn)	GGG (Gly)	GCA (Ala)	GCG (Ala)	GCC (Ala)	AAT (Asn)	GAA (Glu)	GGG (Gly)
756 (OXY-1-2)	7.1	GTC (Val)	CAC (His)			GAG (Glu)		GTG (Val)	GGC (Gly)			
563 (OXY-1-3)	8.2								GGC (Gly)			AGG (Arg)
1879-77 (OXY-1-4)	8.8							GTG (Val)			AAA (Lys)	
OXY-2												
SL911 (OXY-2-1)[c]	5.2	ACC (Thr)	CAT (His)	GAT (Asp)	GAT (Asp)	GGG (Gly)	GCG (Ala)	GTG (Val)	GCC (Ala)	GAT (Asp)	GAA (Glu)	GGG (Gly)
K1764 (OXY-2-2)	5.7				AAT (Asn)							
D488 (OXY-2-3)[c]	6.4				ND (Asn)		ND (Val)			ND (Asn)		
11V (OXY-2-4)	6.8			GCT (Ala)	AAT (Asn)					AAT (Asn)		

[a] pI values were determined by analytical isoelectric focusing (12).
[b] The corresponding amino acids of the modified codons are indicated in brackets. ND, the nucleotide sequences were not determined. The numbering of the amino acids is that of Ambler et al. (1).
[c] The β-lactamase sequences of *K. oxytoca* SL781 (11), SL911 (12), and D488 (31) were published previously.



ß-Lactamases	OXY-1-				OXY-2-		
	1	2	3	4	1	2	3
OXY-1-2	5						
OXY-1-3	2	5					
OXY-1-4	2	5	4				
OXY-2-1	31	31	33	31			
OXY-2-2	30	30	32	30	1		
OXY-2-3	30	30	32	30	3	2	
OXY-2-4	30	30	32	30	3	2	2

FIG. 1. Phylogenetic tree for the eight β-lactamases of *K. oxytoca* and matrix of amino acid substitutions. Concerning the phylogenetic tree, branch lengths are to scale, and the numbers along each branch are percent divergences. The percentages in boldface refer to the number of times that a grouping occurs in 100 sample trees. The table indicates the number of amino acid substitutions.

efficiency (3.5-fold) (Table 5). For cefamandole, the K_m value was low for the OXY-1-1 β-lactamase. Because the V_{max} value was similar to that for the OXY-1-2 enzyme, a significant increase in the hydrolytic efficiency was observed for the OXY-1-1 β-lactamase. The better hydrolysis of aztreonam by the OXY-1-2 enzyme was due to a lower K_m value, indicating a higher affinity for this β-lactam.

Inhibition studies. The IC_{50}s for all β-lactamases with two β-lactamase inhibitors are listed in Table 6. For clavulanic acid, IC_{50}s were relatively homogeneous for OXY-2 enzymes but not for those of the OXY-1 group. The same division found for the substrate profile was observed: IC_{50}s for the OXY-1-1 and OXY-1-4 enzymes were fivefold lower than those for the OXY-1-2 and OXY-1-3 enzymes. For tazobactam, less than a twofold difference in the IC_{50}s was observed for all β-lactamases.

DISCUSSION

To characterize the expression of the two groups of β-lactamases, OXY-1 and OXY-2, described previously (12), the susceptibility patterns and the substrate profiles were determined. The studies of the susceptibility patterns and substrate

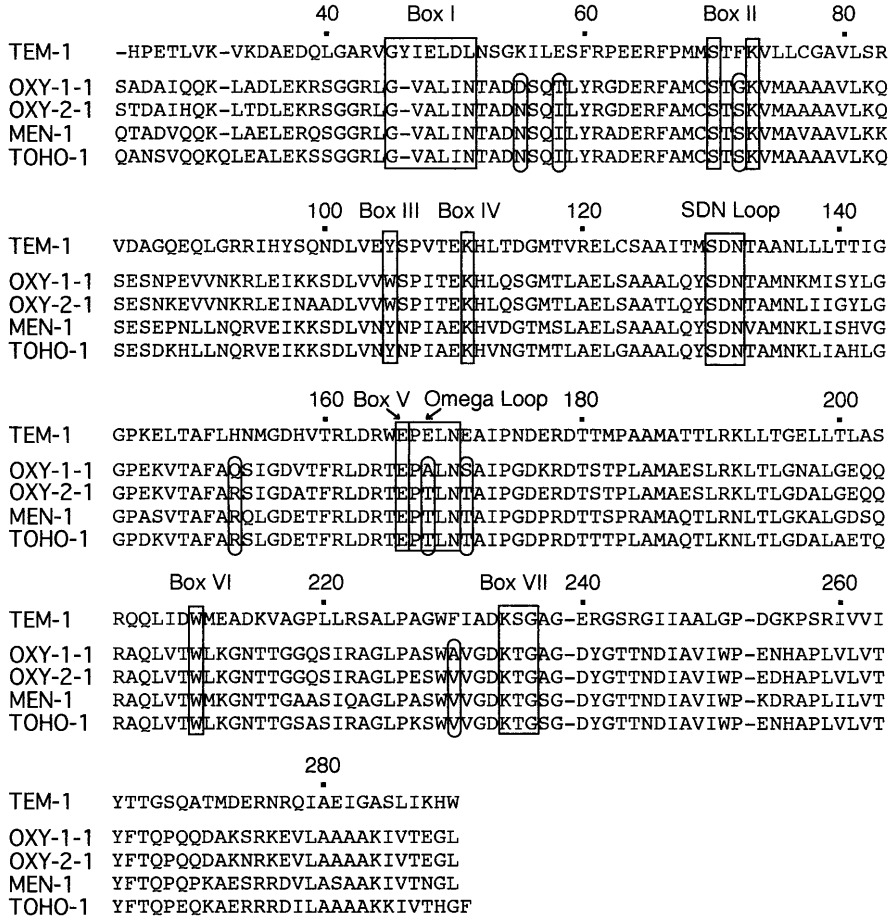


FIG. 2. Alignment of the mature amino acid sequences of both the OXY β-lactamases with those of the TEM-1 β-lactamase (34) and the extended-spectrum β-lactamases MEN-1 (3) and TOHO-1 (18). The numbering is that of Ambler et al. (1): the ABL (class A β-lactamase) consensus numbering scheme. Boxes I to VII (21), the SDN loop (19), and the omega loop (29) are boxed. Residues common to OXY-2, MEN-1, and TOHO-1 are circled.

profiles clearly separate the strains harboring the OXY-1 enzymes from the strains with the OXY-2 enzymes. A recent publication (23) described the β-lactamase OXY-2 sequenced previously (12) and renamed RBI; it is responsible for resistance to aztreonam and cefoperazone-sulbactam because of its ability to hydrolyze these β-lactams and to be resistant to sulbactam. However, the β-lactamase OXY-2 itself does not provide resistance to cefoperazone if it is not overproduced (Table 2) (10), and the difference in hydrolysis between OXY-1 and OXY-2 could not explain such a difference in the resistance phenotype. It seems more likely that the resistance of these strains is due to the overproduction of the β-lac-

TABLE 4. Relative rate of hydrolysis of β-lactams for the different *K. oxytoca* β-lactamases

β-Lactam	Hydrolysis rate (%) for the following β-lactamases[a]:							
	OXY-1				OXY-2			
	OXY-1-1	OXY-1-2	OXY-1-3	OXY-1-4	OXY-2-1	OXY-2-2	OXY-2-3	OXY-2-4
Benzylpenicillin	100	100	100	100	100	100	100	100
Ampicillin	97	93	85	82	100	96	106	99
Carbenicillin	8.1	8.7	7.3	5.8	25	25	25	22
Cephalothin	4.5	10	10	4.5	21	20	19	19
Cephaloridine	31	23	21	30	17	18	18	17
Cefuroxime	1.1	4.3	3.7	0.8	4.7	4.3	4.9	4.4
Cefamandole	14	8.0	8.3	14	31	41	41	28
Cefotaxime	0.4	1.0	0.9	0.5	0.8	1.1	1.2	0.9
Ceftriaxone	1.0	2.3	2.5	0.8	3.1	4.3	4.2	3.0
Cefoperazone	0.4	1.3	1.3	0.2	0.5	0.6	0.6	0.5
Aztreonam	0.5	1.8	1.9	0.5	2.0	2.1	2.0	1.7

[a] Hydrolysis rates of each antibiotic were normalized with respect to that of benzylpenicillin (100%).

TABLE 5. Kinetic parameters for the three representatives of *K. oxytoca* β-lactamases

Enzyme	Substrate	K_m (μM)	V_{max} (μM · min^{-1} · μg of $protein^{-1}$)	Hydrolytic efficiency (V_{max}/K_m) (10^{-3} · min^{-1} · μg of $protein^{-1}$)
OXY-1-1	Benzylpenicillin	38	56	1,470
	Cephaloridine	132	30	227
	Cefamandole	40	5.3	132
	Ceftriaxone	125	0.8	6.4
	Aztreonam	728	1.8	2.5
OXY-1-2	Benzylpenicillin	57	41	719
	Cephaloridine	292	18	62
	Cefamandole	173	4.3	25
	Ceftriaxone	222	2.0	9.0
	Aztreonam	209	1.5	7.2
OXY-2-1	Benzylpenicillin	74	37	500
	Cephaloridine	112	13	116
	Cefamandole	190	32	168
	Ceftriaxone	114	2.2	19
	Aztreonam	689	7.7	11

tamase. Unfortunately, the production of β-lactamase was not determined in their study (23), but the transition (G→A) in the fifth base of the −10 consensus sequence of the promoter was present in the β-lactamase gene bla_{RBI} and this observation confirms the overproduction theory. We have previously described this mutation as being responsible for the overproduction of the β-lactamase (9, 11).

The amino acid sequence identity between OXY-1-1 and OXY-2-1 β-lactamases is 89.7% (12); these β-lactamases differ from each other by about 30 residues (Fig. 1). The OXY-2 enzymes hydrolyze several β-lactams more effectively than the OXY-1 enzymes do (Table 5). One (or two) of the amino acid differences between the OXY-2 and OXY-1 β-lactamases probably affects the hydrolytic properties. The positions that modified activity to the plasmid-mediated β-lactamases are close to one of the boxes described by Joris et al. (21) or are in the SDN loop (19): positions Arg-43, Ser-70, Lys-73, Glu-104, Ser-130, Arg-164, Glu-166, Asp-179, Gln-205, Lys-234, Ala-237, Gly-238, Glu-240, and Gly-242 (TEM-1 β-lactamase) (Fig. 2) (20, 24). However, at these positions, OXY-1 and OXY-2 β-lactamases are identical. Therefore, the residue(s) responsible for the difference between OXY-1 and OXY-2 was elsewhere. In order to try to find this residue, these two groups of *K. oxytoca* β-lactamases were compared with two related plasmid-mediated extended-spectrum β-lactamases, MEN-1 and TOHO-1 (3, 18). The detailed comparison of the extended-spectrum β-lactamase TOHO-1 with other β-lactamases implied that replacement of Asn-276 by a basic residue, Arg or Lys, is an important modification in the extension of the substrate specificity (18). However, both β-lactamases OXY-1 and OXY-2 have the basic residue Lys at this position. This substitution could explain the extended-spectrum activities of *K. oxytoca* β-lactamases, but not the difference between OXY-1 and OXY-2 enzymes. In an alignment with these two extended-spectrum β-lactamases, the amino acids that are common to OXY-2, MEN-1, and TOHO-1 were positioned at residues 55, 58, 72, 153, 168, 171, and 230 (Fig. 2). The positions 55, 58, 153, 171, and 230 have not been described as having an important role in the substrate specificity and may not be involved in the increase in activity of the OXY-2 β-lactamase. Position 72 is within box II. However, a mutagenesis involving all the possible substitutions was applied to residues 72, 73, and 74 of the plasmid-mediated β-lactamase TEM-1 (29). No significant modification of the MICs of cefotaxime and ceftazidime was observed. It is unlikely that position 72 plays a role in the modification of activity. Residues 166 to 170, which form a significant portion of the substrate-binding pocket, are on the omega loop (29). Although the Glu-168 residue is on this loop, its side chain is not in a position to interact directly with the substrate in the structures of *Staphylococcus aureus*, *Bacillus licheniformis*, and TEM-1 β-lactamases (16, 25, 33). However, a glycine-for-glutamate substitution at position 168 can provide increased resistance to both cefotaxime and ceftazidime (MICs increase twofold) and allows for the retention of a high level of resistance to ampicillin. Probably, the modification of the residue at this position results in an alteration in the conformation of the loop (29). The other residues tested at this position (Val, Ala, and Asp) do not provide increased activity toward cefotaxime (30). By comparison with 23 other class A β-lactamases, only OXY-2, MEN-1, and TOHO-1 have a threonine at position 168; the other β-lactamases have a glutamate or an aspartate (data not shown). Glu and Asp do not provide increased activity toward extended-spectrum β-lactams (29). Therefore, residue Ala-168 of the OXY-1 β-lactamase could be responsible for its lower substrate hydrolysis. Nevertheless, because residue Thr-168 was not tested by mutagenesis, we could not exclude the possibility that the mutation(s) responsible for the increased activity of the OXY-2 β-lactamase is elsewhere.

The ability of the *K. oxytoca* β-lactamases to hydrolyze extended-spectrum β-lactams could confer resistance to these antibiotics when the β-lactamase is overproduced (8). One strategy to overcome the resistance due to the overproduced β-lactamase is use of the combination of a β-lactam with a β-lactamase inhibitor (27). A previous study indicated that combination of a β-lactam with clavulanic acid seems to be effective, but sulbactam is ineffective and tazobactam is unpredictable, in overcoming the actions of *K. oxytoca* β-lactamases (10). The inhibitory action of clavulanic acid on OXY-2 β-lactamases is relatively homogeneous (IC_{50}, 1.1 μM). In the OXY-1 group, the IC_{50}s of OXY-1-1 β-lactamases are relatively low (about 0.3 μM), as described previously (10), and those of the OXY-1-2 group are much higher (about 1.9 μM). However, by comparison with the TEM-1 β-lactamase (IC_{50}, 0.09 μM in assays with nitrocefin as the substrate) (5), these IC_{50}s are 12-fold higher. In addition to the substitution at position 276 that could affect the susceptibility of the enzyme to clavulanic acid, a substitution of Ser for the Arg-244 is responsible for resistance to clavulanic acid (24). The presence

TABLE 6. IC_{50}s of two β-lactamase inhibitors for the different *K. oxytoca* β-lactamases[a]

β-Lactamase inhibitor	IC_{50} (μM)							
	OXY-1				OXY-2			
	OXY-1-1	OXY-1-2	OXY-1-3	OXY-1-4	OXY-2-1	OXY-2-2	OXY-2-3	OXY-2-4
Clavulanic acid	0.3	1.6	2.1	0.4	1.3	1.2	1.0	0.9
Tazobactam	3.4	3.3	4.1	3.7	5.0	4.3	3.9	5.2

[a] Inhibitory concentrations were determined with 100 μM cephaloridine.

of a threonine at this position could be responsible for the relatively high IC_{50}s observed for the *K. oxytoca* β-lactamases (18).

In a previous study, we observed a great heterogeneity of the action of tazobactam: some strains were susceptible to the combination β-lactam–tazobactam, but others were resistant (10). As suspected previously (10), this heterogeneity is not due to the mutation(s) in the β-lactamase; it probably depends on the recipient cell itself.

Another interesting result is the difference between the OXY-1-1 group (containing OXY-1-1 and OXY-1-4 β-lactamases) and the OXY-1-2 group (containing OXY-1-2 and OXY-1-3 β-lactamases). The only substitution common to the latter is a replacement of Ala-237 in the OXY-1-1 group by Gly in the OXY-1-2 group (Table 3). It should be noted that a glycine at position 237 was observed in the β-lactamases of *Streptomyces cellulosae* (28), *Streptomyces fradiae*, and *Streptomyces lavandulae* (7). Two plasmid-mediated extended-spectrum β-lactamases, TEM-5 and TEM-24, contain threonine at this position. Mutagenesis experiments showed that asparagine as well as threonine substitutions increase catalytic efficiency on cephems over penems (15). The OXY-1-2 group exhibits a higher level of hydrolysis activity than the OXY-1-1 group, particularly against aztreonam (Table 5). The efficiency of hydrolysis of penicillins and narrow-spectrum cephalosporins for the OXY-1-2 group is lower than that for the OXY-1-1 group. In the β-lactamase TEM-1, the side chain at residue Ala-237 is on the outer, exposed side of the B3 β-strand that forms the right edge of the binding site (24). Residue 237 belongs to the oxyanion pocket. This pocket polarizes the β-lactam's carbonyl group, which is strongly attracted by the hydrogen bonding of backbone NH amide groups of amino acids at positions 70 and 237. Cefotaxime, with its branched oximino substituent, is unable to form a hydrogen bond to the alanine at position 237, but replacement of Ala-237 with a hydrogen bond acceptor such as threonine should enhance the binding of this cephalosporin (24). Because glycine is not a hydrogen bond acceptor, the mechanism of the extension of activity is probably different: perhaps the glycine with its expanded range of permissible angles modifies the conformation of the pocket and thereby increases the hydrolysis activity of the enzyme for cephalosporins.

The OXY-1-2 group appears more resistant to clavulanic acid than the OXY-1-1 group (Table 6). The substitution Gly for Ala-237 seems to decrease the susceptibility to clavulanic acid. Among mutations that render β-lactamase resistant to clavulanic acid, the residue at position 69 is important because it forms the back well of the oxyanion pocket. By steric contact, it influences the positioning of β-strand residues 237 and 238 (24). The possibility that the substitution at position 237, probably modifying the oxyanion pocket, changes the susceptibility of the β-lactamase to clavulanic acid could not be excluded. Surprisingly, no significant change in the IC_{50}s is observed for tazobactam, either between OXY-1 and OXY-2 β-lactamases or between OXY-1-1 and OXY-1-2 β-lactamases. In the mechanism of inhibition, it would appear that the hydrogen bonding of residue 237 for sulfones like sulbactam cannot be as significant as that for the clavulanate chemistry (17). This could explain the fact that substitution of Ala to Gly at position 237 modifies susceptibility to clavulanic acid, but not to tazobactam. However, because differences other than that at residue 237 are observed between β-lactamases from the OXY-1-1 group and the OXY-1-2 group, site-directed mutagenesis studies will be necessary to confirm that Gly at this position modifies hydrolysis of β-lactams and increases resistance to clavulanic acid.

In conclusion, the four OXY-2 β-lactamases whose relative rates of hydrolysis seem to be relatively homogeneous hydrolyzed β-lactams differently than the OXY-1 β-lactamases. Within the OXY-1 group, some differences in the catalytic efficiencies of several β-lactams were observed. These differences could be due to a modified residue at position 237: a glycine instead of an alanine.

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