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Inhibitor-Resistant OXY-2-Derived β-Lactamase Produced by *Klebsiella oxytoca*

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Klebsiella oxytoca **strains are generally moderately resistant to amoxicillin and ticarcillin due to the activities of the chromosomally encoded OXY-1 and OXY-2 class A β-lactamase families. These enzymes have the ability to hydrolyze not only penicillins but also cephalosporins, including cefuroxime, ceftriaxone, and aztreonam, and are inhibited by clavulanic acid. A *Klebsiella oxytoca* strain was isolated from a culture of blood from a patient who had been treated with amoxicillin-clavulanate (3 g/day) for 10 days 1 month earlier. This strain harbored an unusual phenotype characterized by resistance to amoxicillin-clavulanate. It produced an OXY-2-type β-lactamase (pI 6.3), as confirmed by PCR amplification with primers specific for the OXY-2-encoding gene. Gene sequencing revealed a point mutation (A→G) corresponding to the amino acid substitution Ser→Gly at position 130. This mutant enzyme was poorly inhibited by inhibitors, and its kinetic constants compared to those of the parent enzyme were characterized by an increased K_m value for ticarcillin, with a drastically reduced activity against cephalosporins, as is observed with inhibitor-resistant TEM enzymes. The substitution Ser→Gly-130 was previously described in the inhibitor-resistant β-lactamase SHV-10 derived from an SHV-5 variant, but this is the first report of such a mutant in OXY enzymes from *K. oxytoca*.**

Klebsiella oxytoca, like *Klebsiella* spp., carries a chromosomally encoded β-lactamase belonging to class A. Recently, the β-lactamase genes of *K. oxytoca* were divided into two main groups: $bla_{\text{oxy-1}}$ and $bla_{\text{oxy-2}}$ (9). These chromosomal enzymes preferentially inactivate penicillins, conferring resistance on amino- and carboxypenicillins. 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 from a modified promoter (5, 6, 8). The synergistic effects of the three β-lactamase inhibitors clavulanate, sulbactam, and tazobactam were generally poor and varied according to the type of OXY enzymes, the combination of drugs examined, and the type of inhibitor (7). Clavulanate was effective against the overproducing strains, even though it had a relatively high 50% inhibitory concentration (IC_{50}).

We report here on a clinical strain of *K. oxytoca* harboring an unusual resistance phenotype characterized by high-level resistance to amoxicillin, amoxicillin-clavulanate, piperacillin, piperacillin-tazobactam, and, to a lesser extent, ticarcillin and ticarcillin-clavulanate; these resistances were associated with susceptibility to all cephalosporins including cephalothin and aztreonam. This resistance phenotype was very similar to that observed when an inhibitor-resistant TEM (IRT) enzyme was produced in *Escherichia coli* (19).

MATERIALS AND METHODS

Bacterial strains. *K. oxytoca* KER producing a mutant of OXY-2 β-lactamase was isolated from the blood of a patient hospitalized in a gastroenterology unit of the hospital of Vannes, France. Seventeen clinical *K. oxytoca* strains were used for comparison of MICs: 14 *K. oxytoca* strains that produced an OXY-1 or an OXY-2 β-lactamase and that harbored the usual resistance phenotype of wild strains of this species and 3 β-lactamase-overproducing *K. oxytoca* strains that had high levels of resistance to penicillins, cephalothin, cefuroxime, and aztreonam but that were susceptible to cefoxitin, cefotaxime, ceftazidime, and imipenem.

K. oxytoca HB60 (OXY-2-2 producing) was used for comparison of kinetic constants.

Susceptibility to β-lactams. The MICs of amoxicillin, amoxicillin-clavulanate, ticarcillin, ticarcillin-clavulanate, piperacillin, piperacillin-clavulanate, piperacillin-sulbactam, piperacillin-tazobactam, cephalothin, cefotaxime, ceftazidime, and aztreonam were determined by dilution in Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) at an inoculum of 10^4 CFU per spot. Antibiotics were provided as powders by SmithKline Beecham, Paris, France (amoxicillin, ticarcillin, and clavulanate); Lederlé, Paris, France (piperacillin and tazobactam); Hoechst-Roussel, Paris, France (cefotaxime); Glaxo Wellcome, Paris, France (ceftazidime); Bristol-Myers-Squibb, Paris, France (aztreonam); Eli Lilly, Paris, France (cephalothin); and Pfizer, Paris, France (sulbactam).

The β-lactamase inhibitors clavulanate, sulbactam, and tazobactam were used at fixed concentrations of 2, 8, and 4 μg/ml, respectively.

Isoelectric focusing. Isoelectric focusing was performed with polyacrylamide gels as described previously (18). β-Lactamases with known pIs (TEM-1 [pI 5.4], TEM-2 [pI 5.6], OXY-2-2 [pI 5.7], and TEM-3 [pI 6.3]) were used as standards.

Determination of β-lactamase kinetic constants K_m and relative V_{max}. The enzyme extracts were purified by ion-exchange chromatography with AGMP-1 resin (Bio-Rad). The resin, in the chloride form, was treated with 0.1 M ammonia in water and was then washed extensively with water. After absorption of the extracts, elution was performed with a 0.1 M NaCl solution. The active fractions were pooled, dialyzed extensively, and lyophilized. The affinity (K_m) and relative V_{max} values were determined with purified extracts by using a computerized microacidimetric method (15). The K_m and relative V_{max} values of the OXY-2 β-lactamase mutant (strain KER) were compared with those of the β-lactamase OXY-2-2 (strain HB60) for penicillins and cephalosporins.

Studies of inhibition of the OXY-2-2 enzyme and of the mutant enzyme with clavulanate, sulbactam, and tazobactam were performed. The IC_{50} was determined after incubation of the inhibitor and the enzyme for 10 min (complete inactivation) at 37°C before measurement of the remaining enzymatic activity. The IC_{50} is defined as the inhibitor concentration causing 50% inhibition of benzylpenicillin hydrolysis by the enzyme.

DNA amplification. DNA amplification with a crude extract of *K. oxytoca* KER as the template was performed by PCR (18) in a DNA thermal cycler (Perkin-Elmer Cetus Instruments) with consensus primers OXY-A, 5′-d(GCC GCC GCC GTT CCG CTG)-3′, at position 347 and OXY-B, 5′-d (AAG CCC TTC GGT GAC GAT)-3′, at position 1180 on the lagging strand of the nucleotide sequence of the *bla* OXY-2 region (9).

The primers used to amplify the β-lactamase promoter were primer Q, 5′-d (TTC ACA AAG CGC TCG GCA AT)-3′, at position 43 and primer R, 5′-d (CTT TAC TGG TGC TGC ACA TG)-3′, at position 537 on the lagging strand (6).

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TABLE 1. MICs of β-lactams for clinical isolate *K. oxytoca* KER, wild OXY-producing *K. oxytoca* strains, and mutant strains of *K. oxytoca* overproducing OXY enzymes

Strain (enzyme)	MIC (μg/ml)[a]											
	Amoxicillin		Ticarcillin		Piperacillin				Cephalothin	Cefotaxime	Ceftazidime	Aztreonam
	Alone	With CA	Alone	With CA	Alone	With CA	With SUL	With TAZ				
K. oxytoca KER (IRKO-1)	512	256	16	8	128	128	128	64	0.5	0.01	0.03	0.03
K. oxytoca producing OXY enzymes (n = 14)	32–128	1–2	32–128	1–2	2–8	1–2	0.25–2	1–2	4[b]	0.03[b]	0.12[b]	0.12[b]
K. oxytoca overproducing OXY enzymes (n = 3)	>4,096	64–256	4,096	32–128	128–1,024	16–32	128–512	128–512	512–1,024	0.5–1	0.25	8–32

[a] CA, clavulanic acid (2 μg/ml); SUL, sulbactam (8 μg/ml); TAZ, tazobactam (4 μg/ml).
[b] Mode MICs.

Sequencing. The sequences of the PCR products were determined directly on an automatic sequencer (ABI 377) with AmpliTaq DNA polymerase FS (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.). Complete sequencing of the gene and of its promoter region was obtained with primers Q, OXY-A, and OXY-B.

RESULTS

Resistance phenotype of *K. oxytoca* KER. *K. oxytoca* KER expressed an unusual β-lactam resistance phenotype characterized by high levels of resistance to amoxicillin (MIC, 512 μg/ml) and piperacillin (MIC, 128 μg/ml) and a moderate level of resistance to ticarcillin (MIC, 16 μg/ml) (Table 1). Only a weak synergy was observed between clavulanate and amoxicillin (MIC, 256 μg/ml) or ticarcillin (MIC, 8 μg/ml). No synergy was observed between piperacillin and the three inhibitors (clavulanate, sulbactam, and tazobactam). This resistance to penicillin-inhibitor combinations was associated with high levels of susceptibility to cephalothin, cefotaxime, ceftazidime, and aztreonam (MICs, ≤0.5 μg/ml).

This resistance pattern was compared to those of 17 *K. oxytoca* strains belonging either to pattern 1 (n = 14), which includes the susceptible strains of *K. oxytoca*, or to pattern 3 (n = 3), which includes the overproducers of chromosomal β-lactamase (7). Strains of pattern 1 were moderately resistant to amoxicillin or ticarcillin (MICs, 32 to 128 μg/ml) and were highly susceptible to piperacillin (MIC, 2 to 8 μg/ml) and to the cephalosporins tested (MICs, ≤4 μg/ml). Strong synergy was observed between clavulanate and amoxicillin or ticarcillin (MICs, 1 to 2 μg/ml). Strains of pattern 3 were highly resistant to amoxicillin, ticarcillin (MICs, ≥4,096 μg/ml), and piperacillin (MICs, 128 to 1,024 μg/ml). Strains were resistant to combinations of amoxicillin and ticarcillin-clavulanate; however, synergy between clavulanate and amoxicillin or ticarcillin was observed since the MICs ranged from 64 to 256 and from 32 to 128 μg/ml, respectively. When we compared the MICs of piperacillin in combination with the three inhibitors (clavulanate, sulbactam, and tazobactam), a synergistic effect was observed only with clavulanate (MICs, 16 to 32 μg/ml). These strains were highly resistant to cephalothin (MICs, 512 to 1,024 μg/ml), moderately resistant to aztreonam (MICs, 8 to 32 μg/ml), and susceptible to cefotaxime and ceftazidime (MICs, ≤1 μg/ml).

Isoelectric focusing. By isoelectric focusing, one band of pI 6.3 was observed in *K. oxytoca* KER. The pIs of the β-lactamase in 17 crude extracts of *K. oxytoca* strains were between 5.2 and 8.8.

Enzymatic and kinetic parameters. The kinetic parameters of the new mutant enzyme IRKO-1 (pI 6.3) with regard to penicillins and cephalosporins were compared with those of the OXY-2-2 enzyme of pI 5.7 (strain HB60) (Table 2). The specific activity of the purified IRKO-1 protein is 100 U/mg, which is approximately 10-fold lower than that of the OXY-2-2 enzyme. The affinity (K_m) of mutant IRKO-1 for benzylpenicillin and amoxicillin was slightly lower than that of OXY-2-2, while the affinity of IRKO-1 for ticarcillin (K_m, 975 μM) was markedly (10-fold) lower than that of the OXY-2-2 enzyme (K_m, 85 μM). Small K_m values (≤25 μM) for piperacillin and cefoperazone were found for these two enzymes, with the V_{max} values of the mutant IRKO-1 enzyme being 2.5-fold higher than that of the parent enzyme. The hydrolytic activity of this mutant against cephalothin was drastically reduced (>100-fold), and that against cephaloridine was reduced to a lesser extent (7-fold). No hydrolytic activity of the IRKO-1 mutant against cefotaxime or aztreonam was detected.

Inhibition studies. The IC_{50}s of the three β-lactamase inhibitors clavulanate, sulbactam, and tazobactam for the new mutant and OXY-2-2 enzyme were compared (Table 3). The IC_{50}s of the three inhibitors for the IRKO-1 mutant were at least 200-fold higher than those for the OXY-2-2 enzyme.

Nucleotide sequencing. As indicated in Table 4, for *K. oxytoca* KER, which produced the first inhibitor-resistant *K. oxytoca* enzyme designated IRKO-1, nucleotide sequencing revealed a bla_{oxy} gene which differed by four mutations from the $bla_{oxy-2-1}$ gene reported previously (9). Three mutations consisted of the nucleotide change G→A, which leads to the amino acid substitution Gly→Ser at position 20 (1) and Asp→Asn at positions 197 and 255, following the numbering scheme for class A

TABLE 2. Kinetic constants for IRKO-1 enzyme (strain KER) and OXY-2-2 enzyme (strain HB60)

β-Lactam	Kinetic constants for the following β-lactamases[a]:			
	IRKO-1 (pI 6.3)		OXY-2-2 (pI 5.7)	
	K_m (μM)	Relative V_{max} (%)[b]	K_m (μM)	Relative V_{max} (%)[b]
Benzylpenicillin	110	100	55	100
Amoxicillin	120	75	90	92
Ticarcillin	970	12	85	19
Piperacillin	21	84	25	33
Cephalothin	730	0.5	170	85
Cephaloridine	2050	18	480	126
Cefoperazone	22	5	4	2.0
Cefotaxime	—[c]	<0.1	525	8.4
Aztreonam	—	<0.1	600	12

[a] Standard deviations are less than 10% for V_{max} values and less than 20% for K_m values.
[b] Values are relative to those for benzylpenicillin (taken as 100%).
[c] —, affinity was poor and no significant values could be determined by competition procedures (high K_i).

TABLE 3. IC_{50}s of clavulanic acid, sulbactam, and tazobactam for IRKO-1 enzyme (strain KER) and OXY-2-2 enzyme (strain HB60)

Enzyme	IC_{50} (μM)		
	Clavulanic acid	Sulbactam	Tazobactam
IRKO-1	50	>200	50
OXY-2-2	0.2	1	0.2

β-lactamases of Ambler et al. (1). A fourth mutation consisted of the nucleotide change A→G, which leads to the amino acid substitution Ser→Gly at position 130. Promoter sequencing revealed a G-to-A transition of the fifth base in the −10 consensus sequence (GATAGT).

DISCUSSION

Different forms of the chromosomal K1 β-lactamase of *K. oxytoca* have been reported (2, 9–11, 16). Chromosomal β-lactamase genes were recently divided into two main groups, bla_{oxy-1} and bla_{oxy-2} (9), each of which includes β-lactamases with four different pIs. The genes for these β-lactamases are being cloned and sequenced (9, 10).

K. oxytoca enzymes are penicillinases of class A that can hydrolyze cefoperazone effectively and other broad-spectrum cephems weakly (2). The OXY-2 β-lactamase group hydrolyzes several β-lactams including carbenicillin, cephalothin, some extended-spectrum cephalosporins (ceftriaxone), and aztreonam better than the OXY-1 β-lactamase group does (10). Fournier et al. (7), in a susceptibility analysis of 167 *K. oxytoca* strains, clearly individualized four susceptibility pattern clusters: pattern 1 includes the susceptible strains of *K. oxytoca*, pattern 2 includes strains producing plasmid-mediated TEM types, pattern 3 includes the overproducer of chromosomal β-lactamase, and pattern 4 includes strains producing an extended-spectrum β-lactamase.

The antibiotype of *K. oxytoca* KER differed from those of the strains with the four different patterns by its resistance to amoxicillin-clavulanate in association with a high degree of susceptibility to cephalosporins. This resistance pattern is similar to that observed when an IRT enzyme is produced (19). However, there is no TEM derivative in strain KER, which, on the basis of the pI value (pI 6.3) and promoter and gene sequence results, can owe its resistance to β-lactams only to the overproduction of a chromosomal OXY type β-lactamase. As reported previously (5, 7) OXY-2 β-lactamase overproduction, which is due to a mutated promoter (6, 8), increased the level of resistance to penicillins, some cephalosporins, and aztreonam. In addition, combinations of a β-lactam with clavulanate seem to be effective, sulbactam alone is not effective, and the ability of tazobactam to overcome the action of the *K. oxytoca* β-lactamase when it is overproduced in unpredictable (7). We failed to observe the expected increase in the level of resistance to penicillins, cephalosporins, and aztreonam in this mutant strain, which instead exhibited increased levels of resistance to combinations of penicillins and inhibitors.

Determination of the nucleotide sequence suggested the existence of a gene encoding a novel OXY-2-type enzyme since the enzyme included combinations of amino acid substitutions at positions 35, 197, 223, and 255 different from those observed in the OXY-2 variants reported previously. This novel variant, harboring the substitutions Asp-Asn at positions 197 and 255, differed from OXY-2-2 at position 255, from OXY-2-3 at position 223, and from OXY-2-4 at position 35. In addition, it harbored two amino acid substitutions at positions 20 and 130. The substitution Gly→Ser 20 that occurred in the signal sequence did not affect the catalytic properties of the enzyme. However, the amino acid substitution Ser→Gly at position 130 is very important in this mutant enzyme because the Ser-130 residue is highly conserved in class A β-lactamases and has a complex role. This residue participates in the geometry of the active site through its hydrogen bond between its side chain oxygen and the terminal nitrogen of Lys-234. It is also in the close vicinity of Ser-70 and Lys-73. Ser-130 participates in the binding of substrates interacting with the carboxylates of substrates and inhibitors and participates in the catalytic process in facilitating proton transfer to the nitrogen of β-lactam during its opening (12, 14).

Brown and coworkers (3) showed that Ser-130 is involved in the final step of TEM-2 β-lactamase inactivation by clavulanic acid. When Ser-70 is acylated with clavulanic acid, a rapid decarboxylation of clavulanic acid is expected on the basis of mass spectroscopic evidence. Chen and Herzberg (4) have previously shown by crystallography that a decarboxylation of clavulanic acid occurred during acylation of *Staphylococcus aureus* PC1 β-lactamase. Brown et al. (3) observed additional adducts, two of which are linked to Ser-70 by an ester bond; the first one is an aldehyde, whereas the second one is a hydrated aldehyde. Another adduct is linked to Ser-130 in the form of a β-substituted acrylic acid. A fourth adduct is an acrylic acid which is linked to Ser-70 and Ser-130 by an ester bond and an ether bond, respectively.

Thus, it is obvious that substitution of Ser-130 by a glycine yields a clavulanate-resistant β-lactamase, but surprisingly, the IRKO-1 β-lactamase remained an efficient enzyme. Previous observations with the natural mutant SHV-10 (17) and those obtained by directed mutagenesis of the ROB-1 β-lactamase (14) and the *Streptomyces albus* G β-lactamase (12) suggested that alteration of Ser-130 would yield enzymes with dramatically decreased catalytic properties for most, if not all, substrates. Molecular modeling performed with the TEM-1 β-lactamase by Jelsch et al. (13) in 1992 suggests that if Ser-130 is substituted by a glycine, a novel water molecule could take the

TABLE 4. Amino acid substitutions in the OXY-2 β-lactamase group

Enzyme	Strain	pI	Amino acid at the following position[a]:					
			20	35	**130**	197	223	255
OXY-2-1	SL911	5.2	(Gly)[b]	Asp	**Ser**	Asp	Ala	Asp
OXY-2-2	K1764	5.7				Asn		
OXY-2-3	D488	6.4				Asn	Val	Asn
OXY-2-4	11V	6.8		Ala		Asn		Asn
IRKO-1	KER	6.3	(Ser)		**Gly**	Asn		Asn

[a] Numbering is according to that given by Ambler et al. (1). Boldface indicates the amino acid substitution responsible for clavulanate resistance.
[b] Parentheses indicate signal peptide sequence.

place in the resulting cavity at a position very close to that of the oxygen of the previous serine. This water molecule could make a hydrogen bond with Lys-234 and moreover could participate in the binding of substrates and inhibitors, facilitating proton transfer to the nitrogen of a β-lactam during its opening.

Our study is the first to report on a clinical inhibitor-resistant mutant from a chromosomal enzyme of *K. oxytoca*. Therefore, we suggest that this mutant be designated IRKO-1. The use of amoxicillin-clavulanate (3 g/day) for 10 days in the treatment of our patient 1 month before the isolation of the strain from the patient's blood was probably responsible for the emergence of the mutant. However, the good susceptibility of the mutant to all cephalosporins may limit the spread of β-lactam resistance in hospitals.

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