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Molecular Characterization of TEM-59 (IRT-17), a Novel Inhibitor-Resistant TEM-Derived β-Lactamase in a Clinical Isolate of *Klebsiella oxytoca*

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A clinical isolate of *Klebsiella oxytoca* (Kox 443) was found to have a low-level resistance to broad-spectrum penicillins (MICs of amoxicillin and ticarcillin, 256 and 32 μg/ml, respectively), without substantial potentiation by 2 μg of clavulanic acid per ml (amoxicillin- and ticarcillin-clavulanate, 128 and 8 μg/ml, respectively), while being fully susceptible to cephalosporins and other β-lactam antibiotics. These resistances were carried by a ca. 50-kb conjugative plasmid that encodes a single β-lactamase with a pI of 5.6. Compared to TEM-2, this enzyme exhibited a 3- to 30-fold higher K_m and a decreased maximal hydrolysis rate for β-lactams; higher concentrations of suicide inactivators (5- to 500-fold higher concentrations giving a 50% reduction in hydrolysis) were required for inhibition. Nucleotide sequence analysis revealed identity between the bla_{TEM} gene of Kox 443 and the bla_{TEM-2} gene, except for a single A-to-G change at position 590, leading to the amino acid change from Ser-130 Gly. This mutation has not been reported previously in the TEM type β-lactamases produced by clinical strains, and the novel enzyme was called TEM-59 (alternative name IRT-17). This is the first description of an inhibitor-resistant TEM-derived enzyme in the species *K. oxytoca*.

Klebsiella oxytoca is an opportunistic pathogen, commonly found in the environment and in the gut. During the last decade, this organism has been increasingly isolated in patients with various pathological processes (2). In a recent study, this species accounted for 26% of all klebsiellae collected in European intensive care units (32). *K. oxytoca* is intrinsically low level resistant to penicillins, due to the production of small amounts of a chromosomal, constitutive, and clavulanate-susceptible penicillinase called K1 or KOXY (2). Two forms of this enzyme, OXY-1 and OXY-2, have been differentiated on the basis of their isoelectric points and gene sequences (16). In addition, about 10% of *K. oxytoca* strains (32, 36) are resistant or have a decreased susceptibility to all β-lactams except for cephamycins and carbapenems (2, 17). These resistances are related to the overproduction of the chromosomal β-lactamase, caused by point mutations in the promoter sequence of the bla_{OXY} genes (17). Extended-spectrum β-lactamases such as TEM-3, TEM-24 (13), or the transposon-encoded TEM-12 (21) have been only occasionally reported in this species. Finally, as other enterobacteria, *K. oxytoca* can produce common plasmid- and/or transposon-mediated β-lactamases; TEM-1 is the most frequently identified (13, 36). Very recently, a *K. oxytoca* strain harboring an inhibitor-resistant OXY-2-derived β-lactamase was isolated (37). However, until now, no inhibitor-resistant TEM-derived enzyme (IRT) has been described in this species. We report here the molecular characterization of a novel IRT in a clinical isolate of *K. oxytoca*.

MATERIALS AND METHODS

Bacterial strains. The strain of *K. oxytoca* Kox 443 was isolated together with a methicillin-resistant strain of *Staphylococcus aureus* from the foot of a diabetic and arteritic 82-year-old woman, hospitalized in a vascular surgery unit of a university hospital of Bordeaux, France. This patient had been empirically treated with amoxicillin-clavulanic acid (3 g/24 h) over the previous 16 days for a febrile abdominal syndrome. The isolate was identified by the API 20E system and also by the Biotype 100-carbon source strips (bioMérieux, Marcy-L'Étoile, France) in order to avoid any confusion with *Klebsiella planticola*, the other indole-positive species of klebsiella (2, 16). A spontaneous mutant of *Escherichia coli* K-12 resistant to nalidixic acid and rifampin (*E. coli* Rif^r Nal^r) was used as the recipient in a transfer experiment and as the source of TEM-2 encoded by plasmid RP4.

Antimicrobial susceptibility testing. Antibiotic susceptibility patterns of the *K. oxytoca* Kox 443 and its transconjugant (Tc 443) were determined by a disk diffusion method (15) on Mueller-Hinton agar. MICs of various β-lactams, alone or in combination with β-lactamase inhibitors used at fixed concentrations (2 μg/ml for clavulanic acid, 8 μg/ml for sulbactam, and 4 μg/ml for tazobactam), were determined by an agar dilution method (15) on Mueller-Hinton medium, with an inoculum of 10^4 to 10^5 CFU per spot. The following antibiotics were provided as standard powders by the indicated laboratory suppliers: ampicillin, Bristol-Myers Squibb Laboratories; amoxicillin, ticarcillin, and clavulanic acid, SmithKline Beecham Pharmaceuticals; cephalothin, Lilly France SA; piperacillin and tazobactam, Wyeth Lederle Laboratories; and sulbactam, Pfizer Inc. Results were interpreted according to French national guidelines (14).

Isoelectric focusing. Analytical isoelectric focusing was performed as previously described (34) in polyacrylamide gels containing 0.8% ampholines with a pH range of 3.5 to 10.0. β-Lactamase activities were detected by an iodine-starch procedure in agar gel, using benzylpenicillin (75 μg/ml) as the substrate. The pIs of the β-lactamases produced by Kox 443 and its transconjugant were determined by comparison with the pI values of standard β-lactamases: TEM-1 (pI 5.4), TEM-2 (pI 5.6), TEM-30/IRT-2 (pI 5.2), and TEM-3 (pI 6.3).

Kinetic studies. The β-lactamases produced by the transconjugant Tc 443 and TEM-2 were partially purified from crude extracts by ion-exchange chromatography using AGMP-1 resin (Bio-Rad) (29). The resin was first treated with 0.1 M ammonia in water and then washed extensively with distilled water. After absorption of the extracts, elution was performed with a 0.1 M NaCl solution. The active fractions were pooled, dialyzed, and lyophilized. The Michaelis constant (K_m) and the maximal hydrolysis rate (V_{max}) were determined by a computerized microacidimetry assay (29), at pH 7 and 37°C in distilled water containing 85 mM NaCl. V_{max} values were expressed in comparison with those of benzylpenicillin, the latter being taken as 100%. The concentrations of the inhibitors giving a 50% reduction in hydrolysis (IC_{50}) of benzylpenicillin at 1 mM were measured after 10 min of preincubation of the enzymes with the inhibitors. The inhibition constant (K_i) was determined by a competition procedure with benzylpenicillin.

Transfer experiment. Conjugation between Kox 443 and *E. coli* Rif^r Nal^r was carried out by a broth mating procedure (15) in brain heart medium. Transconjugants were selected on Mueller-Hinton agar plates containing rifampin (100 μg/ml) and ampicillin (100 μg/ml).

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TABLE 1. MICs of β-lactam antibiotics for the clinical isolate Kox 443, its transconjugant Tc 443, and other *E. coli* strains

Strain	Plasmid-mediated enzyme	MIC (μg/ml) of[a]:								
		AM	AM + SUL8	AMX	AMX + CA2	TIC	TIC + CA2	PIP	PIP + TZ4	CF
K. oxytoca 443	TEM-59/IRT-17	64	16	256	128	32	8	64	8	4
E. coli Tc 443	TEM-59/IRT-17	128	64	512	512	64	64	128	16	8
E. coli K-12	TEM-2	>1,024	1,024	>1,024	64	>1,024	128	256	1	32
E. coli K-12	None	4	2	4	4	4	2	1	1	8

[a] AM, ampicillin; AMX, amoxicillin; TIC, ticarcillin; PIP, piperacillin; CF, cephalothin; SUL8, sulbactam at 8 μg/ml; CA2, clavulanic acid (2 μg/ml); TZ4, tazobactam (4 μg/ml).

Plasmid DNA analysis. Plasmid DNA from Kox 443 and its resulting transconjugant was extracted and purified with the protocol and reagents of a commercial kit (Qiagen plasmid midi kit), was then analyzed by electrophoresis in a 0.9% (wt/vol) agarose gel, and was visualized with ethidium bromide under UV light. The size of the plasmid was estimated after restriction by the endonuclease *Eco*RI.

Sequencing of DNA amplified by PCR. The amplification was performed with 5 ng of purified DNA plasmid from Kox 443 in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg of gelatin per ml, 200 μM (each) deoxynucleoside triphosphate, 0.5 μM primer, and 0.25 U of *Taq* polymerase (Oncor-Appligène). Each sample was first subjected to a cycle of denaturation at 94°C for 5 min and then was subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, elongation at 72°C for 1 min and a final elongation step at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1.5% (wt/vol) agarose gel. Primers used for amplification of the complete bla_{TEM} gene were as follows: forward, A (5′-GTATCCGCTCATGAGACAATA-3′), and reverse, B (5′-TCTAAAGTATATATGAGTAAACTTGGTCTG-3′), starting, respectively, at base 148 and 1103 of the bla_{TEM-1} gene according to the numbering of Sutcliffe (41). The PCR products were purified for sequencing the Microspin 400 purification system (Pharmacia LKB), then they were directly sequenced on both strands by automated fluorescent sequencing, the dye terminator method (Perkin-Elmer), and six primers, including (forward) A, C (5′-GGGCAAGAGCAACTCGG-3′; 5′ position: 461), and D (5′-CAGCAATGGCAACAACGTTG-3′; 5′ position: 753) and (reverse) B, F (5′-CAACGTTGTTGCCATTGCTGCAG-3′; 5′ position: 772), and G (5′-ACCGAGTTGCTCTTGCCC-3′; 5′ position: 478).

RESULTS

Antibiotic resistance pattern. By the disk diffusion method, Kox 443 was resistant to amoxicillin and amoxicillin-clavulanate; exhibited an intermediate susceptibility to ticarcillin; and was susceptible to piperacillin-tazobactam, cephalothin, cefoxitin, cefuroxime, cefotaxime, ceftazidime, latamoxef, aztreonam, and imipenem. In addition, strain Kox 443 was resistant to most aminoglycosides (gentamicin, tobramycin, dibekacin, and netilmicin), chloramphenicol, sulfonamides, trimethoprim, nalidixic acid and ofloxacin. The *E. coli* transconjugant Tc 443 exhibited the same antibiotic resistance pattern except for ofloxacin. Results of MICs are listed in Table 1. At the fixed concentrations chosen, the β-lactamase inhibitors lowered the MICs of penicillins by twofold dilutions or less, except for piperacillin-tazobactam (MICs decreased by 3 dilutions). Tc 443 was slightly more resistant to β-lactams than Kox 443, and clavulanate did not exhibit synergy with amoxicillin or ticarcillin.

β-Lactamase characterization. Analytical isoelectric focusing of crude β-lactamase extracts of Kox 443 and its transconjugant gave an identical band which comigrated with the reference enzyme TEM-2, at pI 5.6. In addition, Kox 443 expressed a β-lactamase of pI 6.3.

Kinetic parameters (K_m and V_{max}) of the enzyme produced by Tc 443 compared to those of TEM-2 are given in Table 2. The Tc 443 enzyme exhibited much higher K_m than TEM-2 for all β-lactams tested: the K_m values were 3 to 30 times more elevated for benzylpenicillin, ampicillin, amoxicillin, and piperacillin and were even too high to be accurately and reproducibly determined for ticarcillin and the cephalosporins. Moreover, the enzyme produced by Tc 443 hydrolyzed most penicillins somewhat more slowly than TEM-2 (2- to 7-fold decrease of the relative V_{max}, except for piperacillin, which was hydrolyzed two times faster) and cephalosporins (30-fold decrease of the relative V_{max} for cefoperazone): V_{max} values for cephalothin and cephaloridine were inferior or equal to the lowest rates that could be measured under the assay conditions used.

Inhibition parameters (K_i and IC_{50}) for the enzyme of Tc 443 and TEM-2 are compared in Table 3. The β-lactamase of Tc 443 exhibited a dramatic loss of affinity for the β-lactamase inhibitors: the K_i values were increased by a factor of 150 for sulbactam and by a factor of 1,700 to 1,800 for tazobactam and clavulanic acid. Larger quantities of inhibitors were needed for inhibiting the Tc 443 enzyme than TEM-2: the IC_{50}s were 5-fold higher for sulbactam, 35-fold higher for tazobactam, and 500-fold higher for clavulanic acid; however, tazobactam retained significant inhibitor potency (IC_{50}, 7 μM), 5 to 14 times more than sulbactam (35 μM) or clavulanic acid (100 μM).

Gene characterization. Upon mating *K. oxytoca* 443 with *E. coli* Rif^r Nal^r, amoxicillin-resistant transconjugants were selected at a frequency of 10^{-5}. The strain Kox 443 and its transconjugant contained a single plasmid of about 50 kb.

PCR amplification specific for the bla_{TEM} genes on both the *K. oxytoca* strain and its transconjugant gave a fragment of the expected size, 956 bp. Direct sequencing on both strands of the complete gene revealed that it was identical to bla_{TEM-2} except for a single point mutation (Table 4). This mutation consisted of the nucleotide change A to G (A→G) at position 590, (according to Sutcliffes numbering [41]), which leads to the amino acid substitution Ser to Gly (Ser→Gly) at position 130 (according to Ambler's numbering) (1). This substitution has not been previously described in TEM-type enzymes produced by clinical strains. Consequently, the enzyme synthesized by Kox 443 was designated TEM-59 (26), according to the TEM nomenclature (10), or IRT-17 in the IRT series.

TABLE 2. Kinetic parameters of TEM-2 and TEM-59/IRT-17

Antibiotic	TEM-2		TEM-59/IRT-17	
	K_m (μM)	Rel V_{max} (%)[a]	K_m (μM)	Rel V_{max} (%)
Benzylpenicillin	18	100	140	100
Ampicillin	20	93	580	54
Amoxicillin	25	80	520	55
Ticarcillin	12	13	—[b]	2
Piperacillin	40	85	120	183
Cephalothin	350	20	—	<1
Cephaloridine	800	55	—	<1
Cefoperazone	230	47	—	1.5

[a] V_{max} expressed relative to benzylpenicillin (100%).
[b] —, K_m value too high to be accurately determined.

TABLE 3. Inhibition parameters of TEM-2 and TEM-59/IRT-17

β-Lactamase	Clavulanic acid		Sulbactam		Tazobactam	
	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)
TEM-2	0.1	0.2	0.8	7	0.01	0.2
TEM-59/IRT-17	180	100	120	35	17	7

Nucleotide sequence accession number. The nucleotide sequence data reported here have been submitted to GenBank and have been assigned accession no. AF062386.

DISCUSSION

Inhibitor-resistant β-lactamases mainly derive from TEM-1 or TEM-2 enzymes by point mutations in the active site vicinity that confer a lower substrate affinity. Currently, 15 IRT enzymes have been identified (3, 5–7, 9, 11, 19, 38, 39, 40, 43, 44), excluding TEM-41/IRT-12 which was inadvertantly placed on the list (40). These β-lactamases have been found almost exclusively in *E. coli*, where they are present in up to 5% of the European clinical strains (12, 20); only rare isolates producing such enzymes have been reported in *Klebsiella pneumoniae* (4, 30) or *Proteus mirabilis* (7) responsible for human infections and in *Citrobacter freundii* isolated from calf feces (22). Recently, an inhibitor-resistant β-lactamase has been described in the SHV family, SHV-10 (35).

The clinical isolate of *K. oxytoca* Kox 443 had an antibiogram (resistance to amoxicillin-clavulanate and full susceptibility to cephalothin) suggesting the presence of an inhibitor-resistant β-lactamase. Indeed, the determination of the MICs confirmed that Kox 443 showed low-level resistance to penicillins and susceptibility to cephalothin and that clavulanic acid did not substantially potentiate penicillin activities. However, Tc 443 exhibited a lower resistance to amoxicillin than strains of *E. coli* producing most other IRT-type enzymes, as reported in the literature (MICs, >512 μg/ml), and a higher resistance to piperacillin alone or combined with tazobactam (MICs, <32 and <4 μg/ml, respectively); clavulanate at 2 μg/ml is usually more efficient, lowering the MICs of amoxicillin and ticarcillin by one or two dilutions (5–7, 9, 11, 19, 39, 40, 43, 44). MICs of penicillins, slightly lower for *K. oxytoca* 443 than for its *E. coli* transconjugant, could be related to a lower plasmid copy number in the original host or differential permeability or differential binding to penicillin-binding proteins.

The transferable β-lactamase produced by Kox 443 had an unexpected pI of 5.6. Indeed, all IRT-type β-lactamases reported so far have had a pI of either 5.2 or 5.4 (5–7, 9, 11, 19, 39, 40, 43, 44). The additional β-lactamase produced by Kox 443, which was nontransferable and had a pI of 6.3, likely was the chromosomal enzyme of the species, probably of an OXY-2 type (pIs 5.2 to 6.8) (13, 16, 36).

Kinetic studies demonstrated that the Tc 443 enzyme behaved like the IRT-type enzymes, i.e., reached saturation with β-lactams at considerably higher substrate concentrations (particularly for ticarcillin) and showed a reduction of hydrolysis (particularly for the cephalosporins). However, the increase of the K_m values of the Tc 443 enzyme compared to those of TEM-2 was higher for amoxicillin and lower for piperacillin than that of other IRTs (5- to 10-fold and 6- to 24-fold increase, respectively) (6, 9, 11, 43, 44); there was a decrease of the V_{max} values for amoxicillin and an increase for piperacillin in contrast with other IRTs (7, 43, 44). Inhibition data indicated that the Tc 443 enzyme had a weaker affinity than TEM-2 for β-lactamase inhibitors and that larger concentrations of suicide inhibitors were required for inhibition, with tazobactam remaining the most efficient compound. The increase of the IC_{50} values was in the same range as that of other IRTs: higher for clavulanate (40- to 1,200-fold) than for sulbactam (5- to 110-fold) and tazobactam (2- to 180-fold). Because tazobactam is almost as efficient as clavulanic acid in inhibiting TEM-1 or TEM-2 enzymes, i.e., about 100 times more efficient than sulbactam (31), such differential loss of affinity for IRTs left tazobactam the best inhibitor of these mutant enzymes (5, 6, 9, 11, 43).

Clavulanate resistance of Kox 443, together with resistance to aminoglycosides [likely due to an AAC(3)-II], chloramphenicol, sulfonamides, and trimethoprim, was easily transferred by conjugation to *E. coli* K-12. Both Kox 443 and its transconjugant harbored a single plasmid of ca. 50 kb. The genes encoding IRT β-lactamases are usually located on conjugative plasmids of variable size (33 to >180 kb) (4, 5, 11, 30, 44) that also code for such additional resistances (7, 9, 11, 43).

Specific PCR amplification showed that Kox 443 and its transconjugant carried a bla_{TEM} gene. Nucleotide sequence analysis revealed almost complete identity with bla_{TEM-2}, notably at the seven positions of the coding region which discriminate this gene from bla_{TEM-1A} and bla_{TEM-1B} (18, 41), in contrast with the molecular diversity previously found in the bla_{IRT} genes (3, 6, 11, 12, 30). The fact that the bla_{TEM} gene of Kox 443 arose from a TEM-2 ancestor is somewhat surprising, since all previously described IRT enzymes were TEM-1 derivatives, except for TEM-44/IRT-13 found in *P. mirabilis* (7), and since TEM-1 is much more frequent than TEM-2 in *K. oxytoca* (13, 36). Actually, the bla_{IRT} gene from Kox 443, named bla_{TEM-59}, differed from the bla_{TEM-2} gene by a single point mutation, A→G at position 590, leading to the amino acid modification Ser→Gly at position 130. This substitution agrees well with the pI of 5.6 of the enzyme: it remained that of TEM-2, since serine, which contains an uncharged group, was replaced by glycine, another uncharged amino acid.

IRT enzymes found in clinical strains differ from TEM-1 or TEM-2 by one to three amino acid substitutions, involving at least one of the three following residues: Met-69, Arg-244, or

TABLE 4. Nucleotide differences between *bla* genes

Nucleotide no.[a]	Nucleotide (amino acid)[b]			
	bla_{TEM-1A}[c]	bla_{TEM-1B}[c]	bla_{TEM-2}[c]	$bla_{TEM-59/IRT-17}$
226	C	T	C	C
317	C (Gln-39)	C	A (Lys-39)	A
346	A	A	G	G
436	C	T	T	T
590	A (Ser-130)	A	A	G (Gly-130)
604	G	T	G	G
682	T	T	C	C
925	G	G	A	A

[a] Nucleotide numbering is according to Sutcliffe (41).
[b] Amino acid numbering is according to Ambler et al. (1).
[c] Per reference 18.

Asn-276 (28). However, crystallographic data, kinetic study of mutant enzymes obtained by site-directed mutagenesis, and molecular modelling analysis have provided evidence that the highly conserved residue Ser-130 plays a crucial role in the structure and function of class A β-lactamases (25, 27, 33), particularly in the inactivation process by suicide inhibitors (8, 23, 24). Very recently, Vakulenko et al. (42) have demonstrated by PCR mutagenesis of TEM-1 that only four mutations were sufficient by themselves to confer β-lactam inhibitor resistance, including the three previously described mutations and Ser→Gly-130. The replacement of Ser-130 by amino acids other than glycine led to almost inactive enzymes. The Ser→Gly-130 mutant exhibited a profound decline in ampicillin resistance (the MIC declined from 16,000 to 1,000 μg/ml), with the relatively high residual resistance probably being related to the use of a high-copy-number plasmid.

The substitution Ser→Gly-130 was also found to be responsible for suicide inhibitor resistance in SHV-10, the only SHV-derived inhibitor-resistant β-lactamase reported at present (35). This enzyme, derived from the extended-spectrum β-lactamase SHV-9, an SHV-5 variant, partially retained its ability to hydrolyze penicillins but had a drastically reduced activity against cephalosporins (35). SHV-10 contained not only Ser→Gly-130 but also two additional amino acid substitutions, thus complicating the analysis of the effect of the Ser→Gly-130 replacement alone. Finally, the inhibitor-resistant OXY-2-derived β-lactamase (IRKO-1) produced by a strain of *K. oxytoca* was found to differ from the parental enzyme by four amino acid substitutions, including Ser→Gly-130 (37).

The molecular characterization of TEM-59/IRT-17 emphasizes the key role of Ser-130 in conferring susceptibility to β-lactamase inhibitors and provides further insight into understanding the catalytic process of class A β-lactamases. The discovery of an IRT enzyme in *K. oxytoca* confirms the spread of these enzymes in the *Enterobacteriaceae* family. However, the good susceptibility of the TEM-59-producing strain to all cephalosporins may limit the spread of the gene encoding this type of β-lactamase.

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