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# TEM-89 β-Lactamase Produced by a *Proteus mirabilis* Clinical Isolate: New Complex Mutant (CMT 3) with Mutations in both TEM-59 (IRT-17) and TEM-3

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**TEM-89 (CMT-3) is the first complex mutant β-lactamase produced by a clinical strain of *Proteus mirabilis* (strain Pm 631). This new enzyme, which has a pI of 6.28, is derived from TEM-3 and has a single amino acid substitution also encountered in TEM-59 (inhibitor-resistant TEM β-lactamase IRT-17): Ser-130 to Gly. TEM-89 hydrolyzed penicillins to the same extent that TEM-3 did but lost almost all hydrolytic activity for cephalosporins and, like TEM-59, was highly resistant to inhibitors.**

Inhibitor-resistant TEM (IRT) β-lactamases are mostly produced by strains of *Escherichia coli* (12, 21), but some of them have been described in *Klebsiella* spp. and *Proteus mirabilis* clinical strains (2, 3, 6, 15). Until now, 23 enzymes of this type have been reported (G. Jacoby and K. Bush, Amino acid sequences for TEM, SHV, and OXA extended-spectrum β-lactamases, http/www.lahey.org//studies/webt.htm, 2000), and they are widely described in strains responsible for community-acquired infections (11). The chimera enzymes which associate two types of mutations, one set of which is encountered in extended-spectrum β-lactamases (ESBLs) and the other set of which is encountered in inhibitor-resistant β-lactamases, are more unusual. Two such so-called complex mutants have been reported in the TEM family: TEM-50 (CMT-1) in a strain of *E. coli* and TEM-68 (CMT-2) in *Klebsiella pneumoniae* (8, 22). Complex mutant SHV-10 has been isolated from a strain of *K. pneumoniae* (19). In the present work we describe a novel one: TEM-89 produced by a clinical isolate of *P. mirabilis*.

Two strains of *P. mirabilis* were isolated from the same blood culture of a paraplegic and diabetic patient who had chronically infected wounds and who was hospitalized in the Nephrology Unit of the University Hospital in Dijon, France. The first one, strain Pm 631, was resistant to amoxicillin-clavulanate, whereas the second one, strain Pm 631SE, was susceptible to amoxicillin-clavulanate. The production of an ESBL was easily detected in Pm 631SE by a double-disk synergy test (13), but Pm 631was negative by this test. Analysis of chromosomal DNA by pulsed-field gel electrophoresis after digestion with *Sma*I as described previously (17) revealed that the two strains were identical (data not shown). Analytical isoelectric focusing (14) revealed that Pm 631 produced a β-lactamase of pI 6.28 and that Pm 631SE produced a β-lactamase of pI 6.3 (data not shown). A plasmid of about 25 kb was extracted from both strains by the method of Birnboim and Doly (4).

Conjugation experiments were performed with *E. coli* K-12 C600, which is resistant to sodium azide, and transconjugants were selected at a frequency of $10^{-2}$ on agar containing sodium azide (128 mg/liter) and netilmicin (8 mg/liter). The restriction patterns obtained after digestion of the plasmid with *Eco*RI were very similar in Pm 631, Pm 631SE, and the transconjugants. The MICs of the β-lactams tested for the clinical strains and their transconjugants (Tc), *E. coli* Tc 443 producing TEM-59 (IRT-17), and *E. coli* K-12 C600, which is resistant to sodium azide, are reported in Table 1. Pm 631 was more resistant to piperacillin than to ticarcillin, which is somewhat unusual for IRT β-lactamase-producing strains, but inhibitors were more efficient when they were combined with piperacillin (lowering the MICs by 3 or more dilutions). The synergy was much weaker in *E. coli* Tc 631SE. Permeability and binding to the beta-lactam targets were perhaps different in the original host and the transconjugants; this could explain the differences in the MICs.

PCR analysis was performed as described previously with plasmid DNA extracted from strain Pm 631, strain Pm 631SE, and their transconjugants with primers GOU1 (forward primer; 5′-ATAAAATTCTTGAAGACGAAA-3′), SIE2 (reverse primer; 5′-AAAACTCTCAAGGATCTTACC-3′), J (forward primer; 5′-CTTATTCCCTTTTTTGCGGC-3′), and E (reverse primer; 5′-GGTCTGACAGTTACCAATGC-3′) (7) at positions −5, 380, 236, and 1079, respectively, of the $bla_{\mathrm{TEM}}$ gene, according to Sutcliffe (25). Both strands of the PCR products were sequenced with an Applied Biosystems 373A sequencer according to the manufacturer's instruction. The coding region of the $bla_{\mathrm{TEM\text{-}89}}$ gene differed from that of the $bla_{\mathrm{TEM\text{-}2}}$ gene (10) by three mutations: a G-to-A change at position 512, an A-to-G change at position 590, and a G-to-A change at position 914. Thus, the $bla_{\mathrm{TEM\text{-}89}}$ gene differed from the $bla_{\mathrm{TEM\text{-}3}}$ gene by a single mutation at position 590. The amino acid sequences of the enzymes deduced as described by

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TABLE 1. MICs for *P. mirabilis* clinical isolates, *E. coli* K-12 C600, and the transconjugants producing TEM-89, TEM-3, and TEM-59

| Strain (β-lactamase) | MIC (μg/ml)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | AMZ | TIC | PIP | CEF | CTX | CAZ | ATM |
| Pm 631 (TEM-89) | 256 (128, 128, 32) | 64 (32, 16, 8) | >128 (16, 32, 2) | 16 (16, 16, 8) | 0.06 (0.06, 0.06, ≤0.03) | 0.12 (0.12, 0.12, 0.06) | 0.06 (0.06, 0.06, 0.06) |
| *E. coli* Tc 631 (TEM-89) | 512 (512, 256, 128) | 256 (256, 256, 128) | 64 (32, 16, 8) | 8 (8, 8, 4) | 0.06 (0.06, ≤0.03, ≤0.03) | 0.25 (0.25, 0.12, 0.12) | 0.25 (0.25, 0.25, 0.12) |
| Pm 631SE (TEM-3) | >512 (8, 4, 4) | >512 (8, 2, 2) | 64 (1, 1, 1) | 64 (8, 8, 8) | 4 (0.06, ≤0.03, ≤0.03) | 2 (0.12, 0.12, 0.12) | 0.12 (0.06, 0.03, 0.03) |
| *E. coli* Tc 631SE (TEM-3) | >512 (8, 4, 2) | >512 (16, 8, 8) | 32 (1, 1, 1) | 32 (8, 4, 4) | 1 (0.06, 0.06, ≤0.03) | 2 (0.25, 0.25, 0.12) | 1 (0.25, 0.25, 0.25) |
| *E. coli* Tc 443 (TEM-59) | >512 (512, 64, 128) | 32 (32, 32, 8) | >128 (>128, 16, >128) | 32 (16, 8, 4) | 0.06 (0.06, 0.06, ≤0.03) | 0.25 (0.25, 0.25, 0.12) | 0.25 (0.25, 0.25, 0.12) |
| *E. coli* K-12 C600 | 4 | 2 | 2 | 4 | 0.06 | 0.25 | 0.25 |

[a] AMZ, amoxicillin; TIC, ticarcillin; PIP, piperacillin; CEF, cephalothin; CTX, cefotaxime, CAZ, ceftazidime; ATM, aztreonam. The values in parentheses are MICs in the presence of 2 μg of clavulanic acid per ml, 4 μg of tazobactam per ml, and 8 μg of sulbactam per ml, respectively.

Ambler et al. (1) revealed that Pm 631SE produced TEM-3, whereas Pm 631 produced the new enzyme, TEM-89 (Lys-39, Lys-104, Gly-130, and Ser-238). TEM-89 represents a new variant of complex mutant β-lactamase which combines mutations encountered in the TEM-3 ESBL (Lys-39, Lys-104, Ser-238) with mutations encountered in TEM-59 (Lys-39, Gly-130), which belongs to the IRT β-lactamase family (3, 20, 24). The promoter sequence of $bla_{\text{TEM-89}}$ was identical to that of $bla_{\text{TEM-2}}$ except at position 147, where there is a T residue for $bla_{\text{TEM-2}}$ and an A residue for $bla_{\text{TEM-89}}$.

The TEM-2, TEM-3, TEM-59, and TEM-89 β-lactamases were purified to homogeneity by previously described methods (3). After precipitation with ammonium sulfate the enzyme was submitted to gel filtration chromatography on Toyopearl HW-50 resin (fractionation range, 500 to 80,000; Sigma). Then, the active fractions were pooled, dialyzed, concentrated, and purified by ion-exchange chromatography (fast-performance liquid chromatography) with MonoQ HR 5/5 resin (Pharmacia) (8, 14, 16). The purified enzymes were homogeneous, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (purity, ≥97%). Kinetic constants (Table 2) were determined by computerized microacidimetry (14) at pH 7 and 37°C in 0.1 M NaCl solution.

TEM-89 was characterized by low $k_{\text{cat}}$ values for all antibiotics, and these values were comparable to those of TEM-3 for penicillins. $K_m$ values for penicillins remained low. TEM-89 had low levels of hydrolytic activity for cephalothin and cephaloridine and had lost all detectable hydrolytic activity for cefuroxime, cefotaxime, ceftriaxone, and ceftazidime. In terms of $k_{\text{cat}}/K_m$, TEM-89 had an efficiency comparable to that of TEM-59. The concentrations of β-lactamase inhibitors required to attain 50% enzyme inhibition ($IC_{50}$s) were determined after incubation of the enzyme and the inhibitor for 10 min at 37°C. The reporter substrate was benzylpenicillin, which was used at a concentration of 1 mM. The values determined for TEM-89 were very high and were very similar to those for TEM-59, whereas TEM-3 was extremely susceptible (Table 3).

Two enzymes which bear the amino acid substitutions of an ESBL associated with substitutions of an IRT β-lactamase have been described previously. The first one, TEM-50 (CMT-1), associates mutations from the TEM-17 ESBL (Lys-104) plus those from TEM-35, IRT-4 (Leu-69, Asp-276) (22). The second one is TEM-68, which combines mutations from the TEM-47 ESBL (Ser-238, Lys-240, Met-265) and an IRT (Leu-275) (9). Surprisingly, TEM-50 and TEM-68 have conserved a capacity to hydrolyze cephalosporins associated with decreased inhibitor efficacy. In the case of TEM-89, the enzyme lost almost all activity against cephalosporins and was highly resistant to inhibitors, as was the case for TEM-59. From this point of view, TEM-89 can be compared with SHV-10, the first inhibitor-resistant ESBL and the single complex mutant from the SHV family (19). SHV-10 bears the mutations of the SHV-9 ESBL associated with the Ser-to-Gly change at position 130. A similar situation was also observed for IRKO-1, an inhibitor-resistant OXY-2-derived β-lactamase produced by *Klebsiella oxytoca*, which also had Gly-130 (23). SHV-10, IRKO-1, and TEM-89 have kept their abilities to hydrolyze penicillins but have lost nearly all activity against cephalosporins compared with the activities of their parental β-lactamases (SHV-9, OXY-2, and TEM-3, respectively). It therefore ap-

TABLE 2. Kinetics parameters of TEM-89 β-lactamase compared with those of TEM-2, TEM-3, and TEM-59 β-lactamases:[a]

| Substrate | TEM-2 | | | TEM-3 | | | TEM-89 | | | TEM-59 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu M^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu M^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu M^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) |
| Benzylpenicillin | 1,500 | 18 | 83 | 45 | 6 | 7.5 | 50 | 8.0 | 6.25 | 200 | 140 | 1.4 |
| Amoxicillin | 1,200 | 20 | 60 | 31 | 5 | 6.2 | 50 | 28 | 1.79 | 110 | 520 | 0.2 |
| Ticarcillin | 195 | 12 | 16.2 | 9 | 2 | 4.5 | 6 | 24 | 0.25 | 4 | ND[b] | <0.01 |
| Piperacillin | 1,275 | 40 | 31.8 | 72 | 12 | 6 | 55 | 56 | 0.89 | 360 | 120 | 3.0 |
| Cephalothin | 300 | 350 | 0.86 | 48 | 23 | 2.1 | 5 | 79 | 0.06 | <1 | ND | <0.01 |
| Cephaloridine | 825 | 800 | 1.0 | 43 | 30 | 1.4 | 5 | 160 | 0.03 | <1 | ND | <0.01 |
| Cefuroxime | 3 | 1,000 | <0.01 | 28 | 90 | 0.31 | <1 | ND | <0.01 | <1 | ND | <0.01 |
| Ceftriaxone | <1 | ND | <0.01 | 81 | 80 | 1 | <1 | ND | <0.01 | <1 | ND | <0.01 |
| Cefotaxime | 1.5 | ND | <0.01 | 202 | 100 | 2 | <1 | ND | <0.01 | <1 | ND | <0.01 |
| Ceftazidime | <1 | ND | <0.01 | 18 | 200 | 0.1 | <1 | ND | <0.01 | <1 | ND | <0.01 |

[a] The values given for $k_{cat}$ and $K_m$ were computed on the basis of at least three independent experiments. Standard deviations for $k_{cat}$ were 10% for values higher than 100 $s^{-1}$; otherwise, they were 20%. Standard deviations for $K_m$ were 15% for values between 10 and 100 μM, 30% for values higher than 100 μM, and close to 50% for values lower than 10 μM.

[b] ND, not detected (high $K_m$ or $K_i$ values).

pears that the mutation Ser-130 to Gly in class A β-lactamases can confer a high level of resistance to β-lactamase inhibitors and can lead to a reduction in the activity of the enzyme.

A broad diversity of TEM mutants has been described in *P. mirabilis* species, but the frequency of occurrence of TEM-2 is high (5). The only two IRT β-lactamases which have been characterized until now in *P. mirabilis* (TEM-44 [IRT-12] and TEM-65 [IRT-16]) were derived from TEM-2 (18). Our results are consistent with these observations: TEM-89 is also derived from TEM-2. This new enzyme has probably evolved from TEM-3 by a single amino acid substitution: Ser-130 to Gly.

The patient from whom strain Pm 631 was isolated received amoxicillin-clavulanate 2 weeks before he developed septicemia and had received cefotaxime 2 months earlier. The concentrations of the antibiotics in the large, chronically infected wounds were probably too low, thereby allowing the selection of Pm 631SE producing TEM-3 and of Pm 631 producing TEM-89.

TEM-89 conferred resistance to β-lactamase inhibitors, but the TEM-89-producing strain remained susceptible to the broad-spectrum cephalosporins widely used in our hospital. That could explain why the strain did not spread. Nevertheless, such complex mutants of clinical strains could reflect a new direction in the evolution of the enzyme. The benefit for the TEM-89-producing strains remains unclear, but the phenomenon should be taken into account.

**Nucleotide sequence accession number.** The GenBank accession number for $bla_{TEM-89}$ is AY039040.

TABLE 3. $IC_{50}$s of β-lactamase inhibitors

| β-Lactamase | $IC_{50}$ (μM)[a] | | |
|---|---|---|---|
| | Clavulanic acid | Sulbactam | Tazobactam |
| TEM-2 | 0.16 | 8 | 0.1 |
| TEM-3 | 0.01 | 0.4 | 0.01 |
| TEM-89 | 90 | 40 | 8 |
| TEM-59 | 100 | 35 | 7 |

[a] The standard deviations for these determinations were ±20%.

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