Biochemical analysis of TEM-134, a new TEM-type extended-spectrum β-lactamase variant produced in a *Citrobacter koseri* clinical isolate from an Italian hospital

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**Objectives:** Kinetic characterization of TEM-134, a new TEM-type extended-spectrum β-lactamase variant isolated from *Citrobacter koseri* during an Italian nationwide survey. TEM-134 is a natural derivative of TEM-2 with the following substitutions: E104K, R164H and G238S.

**Methods:** Recombinant TEM-134 was purified from *Escherichia coli* HB101 (pMGP-134) by three chromatographic steps (cation-exchange chromatography, gel permeation and fast chromatofocusing). Steady-state kinetic parameters ($K_m$ and $k_{cat}$) were determined by measuring substrate hydrolysis under initial rate conditions using the Hanes linearization of the Michaelis–Menten equation. Modelling was carried out using the software Modeller (version 9.1).

**Results:** TEM-134 hydrolysed with variable efficiency ($k_{cat}/K_m$, ranging from $5 \times 10^3$ to $8.0 \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$) penicillins, narrow-spectrum cephalosporins, cefepime, ceftaxime, cefadroxime and aztreonam, which appeared to be the best substrate. Molecular modelling of the enzyme indicated that the R164H substitution may result in a compromised omega loop in TEM-134 and this may be responsible for its narrower spectrum of activity.

**Conclusions:** Kinetic data and molecular modelling suggested that R164H has a mild detrimental effect on the global activity of the enzyme.

Keywords: Enterobacteriaceae, antibiotic resistance, class A

**Introduction**

Bacterial resistance to expanded-spectrum β-lactam antibiotics represents an increasing risk in hospital and community infections worldwide. Among Enterobacteriaceae this resistance is often due to the emergence and dissemination of the plasmid-encoded extended-spectrum β-lactamases (ESBLs), as response to the overuse of oxyimino-cephalosporins in clinical therapy. ESBLs can efficiently hydrolyse many broad-spectrum β-lactams such as cefotaxime, cefadroxime, cefepime and aztreonam, and include several families of enzymes (CTX-M, PER, VEB, GES, TEM and SHV types). These ESBLs are also susceptible to β-lactamase inhibitors used in clinical practice (i.e. clavulanic acid and tazobactam).

Overall, the ESBLs derived from TEM-1/-2 and SHV-1 prototype enzymes by one or more mutations in selected positions of the *bla*TEM and *bla*SHV genes remain among the most common determinants of resistance to expanded-spectrum β-lactams. Since the first description of TEM-type ESBLs, a large number have been discovered (K. Bush and G. Jacoby, http://www.lahey.org/studies).

According to the published reports in Europe, ESBLs appear to have increased among enterobacteria over the period 1997–2002 and their prevalence differs from country to country.

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Italy, two nationwide surveys were carried out to evaluate the prevalence of Enterobacteriaceae producing ESBLs, in 1999 and in 2003, respectively. In the latter survey, we described the emergence of a new natural TEM-derived ESBL, TEM-134, produced by \textit{Citrobacter koseri} isolated from the urine of a patient admitted to the emergency room of the Hospital of Varese (Northern Italy). TEM-134 is a natural derivative of TEM-2 carrying a unique combination of amino acid substitutions: E104K, R164H and G238S.  

The purpose of this study was to purify and characterize from a biochemical stand point the TEM-134 enzyme.

\textbf{Materials and methods}

\textit{Purification and biochemical characterization of TEM-134 \( \beta \)-lactamase}

TEM-134 was purified from 6 L of a culture of \textit{Escherichia coli} HB101 (pMGP-134)\(^7\) after induction by 0.4 mM isopropyl-\( \beta \)-D-thiogalactopyranoside. Cells were harvested by centrifugation, washed twice with 100 mM Tris-\( \mathrm{HCl} \) buffer (pH 8.0) and disrupted by sonication (30 W for 30 s, five cycles). PMSF 100 \( \mu \)M was added to inhibit intracellular protease activity. The membrane debris was removed by high-speed centrifugation (105 000 \( g \) for 30 min) and the cleared lysate was loaded onto a Sepharose-\( Q \) fast-flow column (2.0 \( \times \) 20 cm; Ghealth-Biosciences, Milan, Italy) equilibrated with 100 mM Tris-\( \mathrm{HCl} \) buffer (pH 8.0) and the \( \beta \)-lactamase was eluted with a linear gradient of NaCl (0–1 M) in the same buffer. Fractions containing \( \beta \)-lactamase activity were pooled and loaded onto a Superdex 75 HR column (2.0 \( \times \) 160 cm; Ghealth-Biosciences, Milan, Italy) equilibrated with 50 mM sodium phosphate buffer, pH 7.0, supplemented with 0.1 M NaCl. The fractions containing \( \beta \)-lactamase activity were dialysed at 4 \( ^{\circ} \mathrm{C} \) against 25 mM Bis-Tris buffer (pH 7.0), and loaded onto a Mono P HR 5/20 column (Amersham Biosciences, Milan, Italy) equilibrated with the same buffer. The protein was eluted with 10-fold-diluted Polybuffer 74 (Amersham Biosciences). At the end of each purification step, \( \beta \)-lactamase activity was monitored spectrophotometrically by measuring the hydrolysis of 100 \( \mu \)M ceftazidime using 50 mM sodium phosphate buffer, pH 7.0.

Gel isoelectric focusing was performed in 5\% polyacrylamide gels containing ampholines (pH range, 3.5–9.5). The pl value was determined by focusing 20 \( \mu \)g of the purified enzymes and the \( \beta \)-lactamase activity was detected by zymogram technique using 100 \( \mu \)M nitrocefin.

Steady-state kinetic parameters \( (K_m \text{ and } k_{cat}) \) were determined by measuring substrate hydrolysis under initial rate conditions and by using the Hanes linearization of the Michaelis–Menten equation. Substrate hydrolysis was measured with a lambda 2 spectrophotometer (Applied BioSystem, Monza, Italy) at 30 \( ^{\circ} \mathrm{C} \) in 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M KCl to prevent enzyme instability. \( K_m \) values lower than 5 \( \mu \)M were determined as \( K_m \), with 100 \( \mu \)M nitrocefin as a reporter substrate. Each kinetic value is the mean of five different measurements. Inhibition by clavulanic acid and tazobactam was monitored with 100 \( \mu \)M nitrocefin as the reporter substrate.

The molecular modelling of TEM-134 enzyme was performed using the software Modeller version 9.1 (www.salilab.org).

\textbf{Results and discussion}

TEM-134 was purified from \textit{E. coli} HB101 (pMGP-134)\(^7\) by three chromatographic steps, and the molecular mass and the isoelectric point calculated for the purified enzyme were 28 600 and 5.2, respectively. The pl was in accordance with that calculated for crude extract previously.\(^7\)

As shown in Table 1, TEM-134 hydrolysed with moderate to good efficiency several \( \beta \)-lactams including penicillins, cefazolin, cefepime, cefotaxime, ceftazidime and aztreonam. The highest catalytic rate constants \( (k_{cat}) \) were observed for ceftazidime \( (k_{cat} = 9 \text{ s}^{-1}) \) and piperacillin \( (k_{cat} = 4.7 \text{ s}^{-1}) \). Overall, TEM-134 exhibited good hydrolytic activity against aztreonam, cefotaxime and ceftazidime, the \( k_{cat} \)/\( K_m \) value for aztreonam being \( 2\)-fold greater than that for cefotaxime and 4-fold greater than that for ceftazidime. The lowest \( k_{cat} \)/\( K_m \) Values were observed for cefazolin and cefepime (Table 1). The kinetic data

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Substrates} & \textbf{TEM-134} & \textbf{TEM-3}\(^8\) & \\
\hline
Ampicillin\(^a\) & 53 \( \pm \) 2 & 0.54 & 6 \\
Piperacillin & 67 \( \pm \) 3 & 4.70 & 23 \\
Benzyilpenicillin\(^a\) & 1 \( \pm \) 0.02 & 0.80 & NA \\
Cefazolin & 47 \( \pm \) 1.5 & 0.26 & 94 \\
Cefotaxime & 4 \( \pm \) 0.05 & 0.25 & 51 \\
Cefazidime & 238 \( \pm \) 12 & 9.00 & 175 \\
Cefepime & 48 \( \pm \) 2 & 0.28 & NA \\
Aztreonam & 4 \( \pm \) 0.03 & 0.60 & NA \\
Nitrocefin & 5 \( \pm \) 0.02 & 1.90 & 6 \\
\hline
\multicolumn{2}{|c|}{\textbf{$K_m$ (\( \mu \)M)}} & \textbf{$k_{cat}$ (s\(^{-1}\))} & \textbf{$k_{cat} \times K_m$ (\( \mu \)M\(^{-1}\) s\(^{-1}\))} & \\
\multicolumn{2}{|c|}{\textbf{TEM-134}} & \textbf{TEM-3}\(^8\) & \\
\hline
Ampicillin\(^a\) & 53 \( \pm \) 2 & 0.54 & 6 \\
& 0.01 & & \\
Piperacillin & 67 \( \pm \) 3 & 4.70 & 23 \\
& 0.07 & & \\
Benzyilpenicillin\(^a\) & 1 \( \pm \) 0.02 & 0.80 & NA \\
& 0.80 & & \\
Cefazolin & 47 \( \pm \) 1.5 & 0.26 & 94 \\
& 0.005 & & 0.54 \\
Cefotaxime & 4 \( \pm \) 0.05 & 0.25 & 51 \\
& 0.063 & & 0.040 \\
Cefazidime & 238 \( \pm \) 12 & 9.00 & 175 \\
& 0.28 & & 7 \\
Cefepime & 48 \( \pm \) 2 & 0.28 & NA \\
& 0.15 & & \\
Aztreonam & 4 \( \pm \) 0.03 & 0.60 & NA \\
& 0.2 & & 1 \\
Nitrocefin & 5 \( \pm \) 0.02 & 1.90 & 6 \\
& 0.39 & & 7.3 \\
\hline
\end{tabular}
\caption{Comparison of kinetic parameters between TEM-134 and TEM-3\(^8\) ESBLs}
\end{table}

\(^{a}\)\( K_{cat} = K_r \), using 100 \( \mu \)M nitrocefin as reporter substrate.

\(^{8}\)NA, not available.

Each kinetic value is the mean of five different measurements; error was below 5\%.

TEM-3 presents the following amino acid substitutions (with respect to TEM-2): E104K and G238S.

\( K_m \) values lower than 5 \( \mu \)M were determined as \( K_m \), with 100 \( \mu \)M nitrocefin as a reporter substrate.

\( k_{cat} \) is the mean of five different measurements. Inhibition by clavulanic acid and tazobactam was monitored with 100 \( \mu \)M nitrocefin as the reporter substrate.

\( K_r \) is the mean of five different measurements. Inhibition by clavulanic acid and tazobactam was monitored with 100 \( \mu \)M nitrocefin as the reporter substrate.

\( k_{cat} \)/\( K_m \) values lower than 5 \( \mu \)M were determined as \( K_m \), with 100 \( \mu \)M nitrocefin as a reporter substrate.

\( K_r \) is the mean of five different measurements. Inhibition by clavulanic acid and tazobactam was monitored with 100 \( \mu \)M nitrocefin as the reporter substrate.

\( k_{cat} \)/\( K_m \) values lower than 5 \( \mu \)M were determined as \( K_m \), with 100 \( \mu \)M nitrocefin as a reporter substrate.

\( K_r \) is the mean of five different measurements. Inhibition by clavulanic acid and tazobactam was monitored with 100 \( \mu \)M nitrocefin as the reporter substrate.

\( k_{cat} \)/\( K_m \) values lower than 5 \( \mu \)M were determined as \( K_m \), with 100 \( \mu \)M nitrocefin as a reporter substrate.
obtained for penicillins, cefotaxime, ceftazidime and aztreonam were in agreement with antimicrobial susceptibility data calculated for the same antibiotics using E. coli HB101 (pMG-134). Surprisingly the $k_{cat}/K_m$ values for ceftazolin and cefepime were similar, whereas the MIC values previously reported showed that E. coli HB101 (pMG-134) was resistant to ceftazolin (MIC > 64 mg/L) but susceptible to cefepime (MIC = 0.25 mg/L). This phenomenon might be due to conceivable differences in permeability towards those antibiotics and/or to differences in activity against penicillin binding proteins of E. coli HB101. All inhibitors tested inhibited the TEM-134 enzyme, with $K_a$ values of 0.055, 0.8 and 2.4 μM for tazobactam, sulbactam and clavulanic acid, respectively.

Comparing the kinetic parameters of TEM-134 with those reported for TEM-3 (which compared with TEM-134 has the same set of mutations except R164H), the $k_{cat}/K_m$ values observed for TEM-134 are overall lower (Table 1). In particular, the $k_{cat}/K_m$ values calculated for ceftazidime are similar for both enzymes while a notable reduction of catalytic efficiency (16-fold) was observed for TEM-134 versus cefotaxime. In all TEM-type enzymes, the side chain of the residue at position 238 is placed on the inner side of the B3 β-strand. The G238S mutation occurs in several TEM variants leading to increased activity against cefotaxime, as observed in the TEM-3 enzyme. The guanidinium side chain of Arg-164 is strongly linked by electrostatic attraction and hydrogen bonds to conserved Asp-179 across the neck of the omega loop. A single mutation at position 164 (e.g. serine in place of arginine) leads to a significant level of resistance to ceftazidime. A reduction of hydrogen bonds may weaken the omega loop allowing more flexibility to accommodate the bulky substituents of oxyimino cephalosporins. In addition, high-level resistance to ceftazidime, cefotaxime and aztreonam is often achieved with a combined set of mutations at positions 104, 164 and 238. The increasing affinity towards oxyimino-cephalosporins depends more on the concerted action of residues at positions 164 and 238 through an active site cavity expansion, by shifting the position of an active site omega loop that then allows more bulky extended-spectrum antibiotics to bind. However, the combination of R164S and G238S mutations in TEM-1 was shown to determine a detrimental effect on the activity against most β-lactam antibiotics.

Molecular modelling of TEM-134 was performed to determine the potential influence of these residues on the kinetic behaviour of the enzyme. As shown in Figure 1, molecular modelling of TEM-134 showed that His-164 might provide the necessary hydrogens to Asp-179 for hydrogen bond formation. The nitrogen of the secondary amino group of imidazole and the backbone nitrogen of His-164 are sufficiently close to the carboxylic group of Asp-179, respectively, 2.90 and 3.05 Å from oxygens, to allow the formation of two hydrogen bonds. However, because of the $pK_a$ of the imidazolic group ($\sim$6.0), His-164 might not provide the necessary positive charge for an electrostatic attraction, as observed with the guanidinium group of arginine ($pK_a$ $\sim$12.5). On the other hand, the pH of the medium or microenvironmental variation of pH around His-164 can modify the charge state of the imidazolic group. Thus, histidine at position 164 could result in a compromise between the constrained omega loop with arginine and the flexible loop with serine at the same position. On the basis of kinetic data reported in the present work, His- and Arg-164 seem to have the same effect on the catalytic profile of ceftazidime. However, although His-164 improves the catalytic activity for ceftazidime, it seems to have a mild detrimental effect on the global activity of the enzyme. This event might explain why the substitution R164H is fairly uncommon (18 TEM variants including the TEM-134 enzyme) compared with the R164S mutation (25 TEM variants), while the combination of His-164 and Ser-238 appears only in TEM-107 (AY101764) and TEM-134 natural variants.

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**Transparency declarations**

None to declare.

**References**


