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E240V Substitution Increases Catalytic Efficiency toward Ceftazidime in a New Natural TEM-Type Extended-Spectrum β-Lactamase, TEM-149, from *Enterobacter aerogenes* and *Serratia marcescens* Clinical Isolates▽

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The aim of this study was to characterize a novel extended-spectrum β-lactamase that belongs to the TEM family, the TEM-149 enzyme, and that was isolated from the urine of two hospitalized patients from different hospitals in southern Italy. The peculiarity of this enzyme was the finding of a valine residue at position 240. The array of amino acid substitutions found in TEM-149 was as follows: E104K, R164S, M182T, and E240V. A reversion of a threonine residue at position 182 was also performed to create a new mutant, TEM-149^{T182M}, in order to assess the contribution of this substitution on the kinetic profile and the stability of TEM-149. The *bla*$_{TEM-149}$ and *bla*$_{TEM-149/T182M}$ genes were cloned into pBC-SK, and the corresponding enzymes were purified from recombinant *Escherichia coli* HB101 by the same procedure. Both enzymes hydrolyzed all β-lactams tested, with a preference for ceftazidime, which was found to be the best substrate. By comparison of the kinetic parameters of the TEM-149 and the TEM-149^{T182M} enzymes, a reduction of the catalytic efficiency for the TEM-149^{T182M} mutant was observed against all substrates tested except benzylpenicillin, cefotaxime, and aztreonam. Tazobactam, clavulanic acid, and sulbactam were good inhibitors of the TEM-149 β-lactamase.

Bacterial resistance to β-lactam antibiotics is primarily mediated by the production of β-lactamases, a group of enzymes which are able to catalyze the hydrolysis of the amide bond in the β-lactam ring. The rapid spread of β-lactam resistance among different bacterial species is facilitated by the transmission of β-lactamase-encoding genes via mobile genetic elements, such as plasmids, transposons, and integron-borne mobile gene cassettes (7, 9).

The massive use of expanded-spectrum cephalosporins since the 1980s has selected for the emergence of β-lactamases that can hydrolyze these compounds (the so-called extended-spectrum β-lactamases [ESBLs]) in the clinical setting. Since the first detection of plasmid-mediated ESBLs, the SHV-2 and TEM-3 enzymes (22), several ESBL types, and a large number of allelic variants have been described, mostly in the family *Enterobacteriaceae* but also in other gram-negative pathogens; and their dissemination represents a worldwide problem in hospitalized and community patients (25). Classical ESBLs have evolved from the broad-spectrum TEM-1, TEM-2, and SHV-type enzymes by amino acid substitutions (2, 3, 16). Today the number of known TEM-type and SHV-type ESBL variants isolated from clinical strains is very high and continues to grow each year, which is indicative of the ongoing evolution of these enzymes (G. Jacoby and K. Bush, http://www.lahey.org/studies/webt.htm). Recently, several types of non-TEM and non-SHV ESBLs (e.g., CTX-M, PER, VEB, GES, TLA, BES, and BEL) have also emerged in gram-negative bacteria (2, 16).

The TEM-type variants remain among the most prevalent ESBLs (2, 16). They are derived from TEM-1 or TEM-2 enzymes by changes in the substrate specificity due to amino acid substitutions that occur at specific positions, such as positions 104, 164, 238, and 240. Specifically, the substitutions of a lysine for a glutamate at position 104, a serine (or a histidine or a cysteine) for arginine at position 164, a serine for a glycine at position 238, and a lysine for a glutamate at position 240, either alone or in various combinations, are able to increase the catalytic activity toward oxyimino-cephalosporins and monobactams (11, 15, 26).

In Italy, two nationwide surveys were carried out in 1999 and 2003 to evaluate the prevalence of ESBL production among clinical isolates of the *Enterobacteriaceae* (14, 17). In this work we describe the characterization of a new natural TEM-type derivative with ESBL activity, named TEM-149, which was detected in clinical isolates of *Enterobacter aerogenes* and *Serratia marcescens* collected during the most recent survey.

MATERIALS AND METHODS

Bacterial strains and genetic vectors. *Enterobacter aerogenes* SS-13 and *Serratia marcescens* CT-188 were isolated in 2003, during the second national Italian survey on ESBL production in the *Enterobacteriaceae*. They were isolated from the urine of inpatients at the Sassari and Catania teaching hospitals, respectively. *Escherichia coli* HB101 (*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) was used as the recipient of the ESBL-encoding recombinant plasmid. pBC-SK (Stratagene, Inc., La Jolla, CA) was used as the vector for the cloning experiments with the *bla*$_{TEM-149}$ and the *bla*$_{TEM-149/T182M}$ genes.

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Gene transfer experiments. Recombinant plasmids pTEM-149 and pTEM-149^{T182M} were inserted into *E. coli* HB101 by the electroporation technique, and the transformants were selected on Luria-Bertani agar plates supplemented with ceftazidime (16 μg/ml) and chloramphenicol (30 μg/ml). Electroporation of the large plasmid DNA preparation from *E. aerogenes* SS-13(pEA13) and *S. marcescens* CT-188(pSM188) into *E. coli* HB101 was carried out with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) with 2 μl (approximately 500 ng) of the plasmid DNA preparation and under the conditions recommended by the manufacturer. The selection of transformed cells was carried out with 16 μg/ml of ceftazidime.

Conjugation experiments were carried out in Mueller-Hinton broth by using *Escherichia coli* K-12 as the recipient and an initial donor/recipient ratio of 0.1. Transconjugants were selected on Mueller-Hinton agar containing ceftazidime (16 μg/ml) plus streptomycin (1,000 μg/ml) for selection.

Antibiotics. All β-lactam compounds except clavulanic acid, ceftazidime, piperacillin, and tazobactam were from Sigma Chemical Co. (St. Louis, MO); clavulanic acid and ceftazidime were from GlaxoSmithKline (Verona, Italy); and piperacillin and tazobactam were from Wyeth-Lederle (Catania, Italy).

In vitro susceptibility testing. The determination of the MICs was performed by the conventional broth macrodilution procedure with a bacterial inoculum of 5×10^5 CFU/ml, as recommended by the CLSI (6).

Recombinant DNA methodologies. Plasmids were extracted from *E. aerogenes* SS-13 and *S. marcescens* CT-118 by the alkaline lysis method (20) and were analyzed by agarose gel electrophoresis. PCR experiments were performed with 20 ng of plasmid DNA as the template, using primers TEM_for (5′-GGGGG**GGTACC**ATGAGTATTCAACATTTCCGT-3′) and TEM_rev (5′-GGGGG**GAATTC**TTACCAATGCTTAATCAGTGA-3′). The restriction sites were inserted to facilitate cloning (the KpnI and EcoRI sites are underlined and boldfaced in the two sequences, respectively). The reaction was carried out in a total volume of 100 μl, as described previously (17). Direct sequencing of the amplicons was performed on both strands derived from three independent PCRs according to the dideoxy chain termination method by using an ABI Prism 310 automatic sequencer (Applied Biosystems, Monza, Italy). For the cloning experiments, the purified amplicon, digested with KpnI and EcoRI, was cloned into the pBC-SK vector (Stratagene, Inc.) to produce the recombinant plasmid called pTEM-149.

Construction of the TEM-149^{T182M} mutant. The T182M mutation of the TEM-149 β-lactamase was generated by site-directed mutagenesis by use of the overlap extension method (24). The mutation involved the nucleotide at position 537, in which the cytosine residue was changed into thymine, resulting in a change of codon ACG, which encodes a threonine, into codon ATG, which encodes a methionine. Primers Met182_for (5′-TGACACCACGA**T**GCCTGCA G-3′) and Met182_rev (5′ CTGCAGGC**A**TCGTGGTGTCA 3′) (where the mutated nucleotide is shown underlined and boldfaced) were used in combination with the external primers TEM_for and TEM_rev to generate two partially overlapping DNA fragments, which were subsequently used in an overlap extension reaction coupled to amplification of the entire coding sequence with the external primers. The resulting amplicon was cloned in plasmid pBC-SK to obtain recombinant plasmid pTEM-149^{T182M}. The sequence of the plasmid insert was determined to confirm the authenticity and the introduction of a mutated nucleotide at position 537.

Production and purification of TEM-149 and TEM-149^{T182M} β-lactamases. An overnight culture of *E. coli* HB101(pTEM-149) and *E. coli* HB101(pTEM-149^{T182M}) grown in Luria-Bertani broth was diluted 10-fold with 6 liters of the same medium containing chloramphenicol (30 μg/ml). The crude extract was prepared as described previously (18). The first step of purification consisted of passage through a Sepharose-Q fast-flow column as described previously (18). Fractions containing β-lactamase activity were pooled, concentrated 20-fold with an Amicon concentrator (YM 10 membrane; Millipore, Bedford, MA), and loaded onto a Superose 12 column (GE Healthcare, Milan, Italy) preequilibrated with 50 mM Tris HCl buffer, pH 8.0, supplemented with 0.15 M NaCl. Elution was performed with the same buffer at a flow rate of 1.0 ml/min. The TEM-149 and TEM-149^{T182M} enzymes were purified by the same procedure. The total protein concentration was determined by the method of Bradford (1), with bovine serum albumin used as the standard. The β-lactamase activity was determined in 1 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl at 30°C by observing the hydrolysis rate of ceftazidime (100 μM). One unit of β-lactamase activity was defined as the amount of the enzyme which hydrolyzes 1 mmol of substrate per minute. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (13) with a fixed 12.5% (wt/vol) polyacrylamide gel in the presence of 0.1% SDS and with a Mini-Protean II apparatus (Bio-Rad Laboratories). Isoelectric focusing was performed as described previously (18).

TABLE 1. Pattern of β-lactam resistance mediated by TEM-149 and TEM-149^{T182M} ESBLs in *E. coli* HB101 in comparison with the patterns in *E. aerogenes* and *S. marcescens* clinical isolates

Antibiotic[a]	MIC (μg/ml)				
	E. aerogenes SS-13	*S. marcescens* CT-188	*E. coli* HB-101 (pTEM-149)	*E. coli* HB-101 (pTEM-149^{T182M})	*E.coli* HB-101 (pBC-SK)
PIP	>256	>256	>256	32	1
TZP[b]	>256	128	1	1	1
AMX	>64	>64	>64	>64	8
AMC	64	64	8/4	8/4	8
CFZ	>64	>64	32	4	1
CTX	>128	>128	1	4	<0.125
CAZ	>64	>64	>64	>64	0.5
FEP	>64	>64	8	1	<0.125
AZT	>64	>64	>64	64	<0.125

[a] Antibacterial agent abbreviations: PIP, piperacillin; TZP, piperacillin-tazobactam; AMX, amoxicillin; AMC, amoxicillin-clavulanate; CFZ, cefazolin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam.
[b] Tazobactam was used at fixed concentration of 4 μg/ml.

Determination of kinetic parameters. Steady-state kinetic parameters (K_m and k_{cat}) were determined by measuring substrate hydrolysis under initial rate conditions and by using the Hanes linearization of the Michaelis-Menten equation (21). Substrate hydrolysis was measured with a Lambda 2 spectrophotometer (Applied Biosystems) at 25°C in 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M KCl to prevent enzyme instability. When the K_m values were less than 5 μM, the K_m value was determined as K_i, with 100 μM nitrocefin used as the reporter substrate.

The thermal stability of the TEM-149 and TEM-149^{T182M} enzymes was determined by measuring the residual activity of the enzyme and by using 100 μM ceftazidime in 50 mM sodium phosphate buffer, pH 7.0, after incubation of the enzyme for various times at 25°C, 40°C, or 55°C. The residual activity relative to the activity of the corresponding enzyme, which was set at 100%, was calculated after incubation at 25°C for the same times.

Each kinetic value is the mean of five different measurements; the error was below 5%. Inhibition by clavulanic acid and tazobactam was monitored with 100 μM nitrocefin as the reporter substrate.

The molecular modeling of the TEM-149 enzyme was performed with the software Modeler (version 9.1; www.salilab.org).

Nucleotide sequence accession number. The nucleotide sequence of the $bla_{TEM-149}$ gene has been submitted to the EMBL-GenBank database and has been assigned accession number DQ 369751.

RESULTS

E. aerogenes SS-13 and *S. marcescens* CT-188 were resistant to amoxicillin, amoxicillin-clavulanate, piperacillin, piperacillin tazobactam, cefotaxime, ceftazidime, aztreonam, and cefepime (Table 1). In both isolates ESBL production was suspected by the susceptibility pattern and was confirmed by the positive results of a double-disk synergy test with clavulanate, ceftazidime, and cefepime (14). By colony blot hybridization analysis with probes specific for the bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{PER} genes, the isolates had tested positive only with the bla_{TEM} probe (14).

Isoelectric focusing analysis, performed with the crude extract of each isolate, showed two bands of β-lactamase activity with pIs of 5.8 and >9.0, respectively. The former was consistent with the production of a TEM-type enzyme, while the latter was most probably ascribed to the production of the AmpC enzyme resident in these species.

Plasmid analysis, performed with both isolates, revealed the presence of a large plasmid (>100 kb). These plasmids, named pEA13 (from *E. aerogenes* SS-13) and pSM188 (from *S. marcescens* CT-188), were electroporated into *E. coli* HB101, and

TABLE 2. Catalytic parameters of the TEM-149, TEM-149^{T182M} mutant, and TEM-10 enzymes with some β-lactam antibiotics[a]

Antibiotic	TEM-149			TEM-149^{T182M}			TEM-10 (19)		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Ampicillin	5[b] ± 0.5	5.4	1.08	19[b] ± 1	2.4	0.127	4	12.5	3.1
Piperacillin	1.4[b] ± 0.3	2.7	1.92	3.4[b] ± 0.5	5.0	1.47	NA[c]	NA	NA
Benzylpenicillin	0.6[b] ± 0.05	3.5	5.8	0.7[b] ± 0.05	10.5	15	2	51	25.5
Cefazolin	68 ± 5	2.2	0.032	35 ± 2	0.56	0.016	417	12.5	0.030
Cefotaxime	43 ± 2	0.07	0.0016	32 ± 3	0.41	0.013	30	0.3	0.010
Ceftazidime	19 ± 1	8.3	0.44	26 ± 2	8.3	0.32	607	17	0.028
Cefepime	16 ± 1	2.4	0.15	30 ± 2	0.43	0.014	NA	NA	NA
Aztreonam	51 ± 3	4.7	0.092	27 ± 1	3.6	0.13	28	2	0.07
Nitrocefin	20 ± 2	7.4	0.37	25 ± 2	26	1.04	47	59	1.25

[a] Each kinetic value is the mean of five different measurements; the error was below 5%.
[b] $K_m = K_i$ with 100 μM nitrocefin as the reporter substrate.
[c] NA, not available.

the transformants were grown in presence of ceftazidime (16 μg/ml). A Southern blot hybridization performed with whole genomic DNA revealed that the bla_{TEM}-related sequences were plasmid borne (data not shown).

No conjugational transfer of ceftazidime resistance was observed in repeated experiments (under the experimental conditions adopted, the detection sensitivity of the assay was $\geq 5 \times 10^{-7}$ transconjugants per recipient).

The sequences of the PCR products of the bla_{TEM} alleles carried by pEA13 and pSM188 were determined and turned out to be identical. Compared to the TEM-1 sequence, the encoded enzyme showed an original array of amino acid changes (E104K, R164S, M182T, and E240V). The enzyme, named TEM-149, showed an unusual valine residue at position 240, which was never found in natural TEM variants (G. Jacoby and K. Bush, http://www.lahey.org/studies/webt.htm). The PCR amplicon containing the $bla_{TEM-149}$ gene was cloned into the pBC-SK vector to obtain recombinant plasmid pTEM-149. A mutant of the TEM-149 β-lactamase, in which the T182M substitution was introduced, was also prepared by site-directed mutagenesis to assess the effect of this mutation in this new sequence context. The mutated TEM-149^{T182M} was cloned into the pBC-SK vector to obtain recombinant plasmid pTEM-149^{T182M}.

The in vitro susceptibilities of *E. coli* HB101(pTEM-149) and *E. coli* HB101(pTEM-149^{T182M}) to various β-lactams were investigated. In *E. coli*, the production of the TEM-149 ESBL was able to confer resistance to penicillins, cefazolin, ceftazidime, and aztreonam but not to cefepime or cefotaxime (although the MICs of the last two compounds were clearly increased compared to those for strain HB101 carrying an empty vector). The production of TEM149^{T182M} resulted in a similar behavior; but the impact on resistance to piperacillin, cefazolin, cefotaxime, and cefepime was lower overall (Table 1). This phenomenon is due to the different levels of expression of the two enzymes in *E. coli*. The specific activity measured for TEM-149 was five times higher than that measured for TEM-149^{T182M}. Tazobactam and clavulanic acid were able to efficiently restore the susceptibilities of both *E. coli* recombinants to piperacillin and amoxicillin, respectively (Table 1).

TEM-149 and TEM-149^{T182M} were purified by two chromatographic steps, which yielded enzymes more than 95% pure, as evaluated by SDS-PAGE analysis (data not shown). The molecular mass and the isoelectric point were 28,600 Da and 5.8, respectively, for both enzymes (data not shown).

Kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) were determined for TEM-149 and TEM-149^{T182M} (Table 2). Both enzymes hydrolyzed cefazolin, cefotaxime, ceftazidime, cefepime, aztreonam, and penicillins. Among the penicillins, benzylpenicillin was the best substrate for both TEM-149 and TEM-149^{T182M}. Among the oxyimino-cephalosporins, ceftazidime was the best substrate for both enzymes, while cefotaxime was an overall poor substrate and was hydrolyzed more efficiently by the TEM-149^{T182M} enzyme. TEM-149 hydrolyzed cefepime with a catalytic efficiency 10-fold higher than that of TEM-149^{T182M}. Finally, both enzymes had similar behaviors with aztreonam. Concerning the inhibitors, tazobactam, clavulanic acid, and sulbactam behaved as competitive inhibitors, with K_i values of 0.018, 0.26, and 0.21 μM, respectively, for TEM-149 and 0.026, 0.11, and 0.15 μM, respectively, for TEM-149^{T182M}.

The thermal stability of TEM-149 and TEM-149^{T182M} was determined by measuring the residual activity after incubation of the enzymes at three different temperatures: 25°C, 40°C, and 55°C. TEM-149 exhibited half-lives of 90 min at 25°C, 70 min at 40°C, and 30 min at 55°C, whereas TEM-149^{T182M} showed half-lives greater than 120 min at 25°C and 40°C and 45 min at 55°C. Compared to the thermal stability of TEM-149, the thermal stability of the TEM-149^{T182M} mutant was substantially increased.

DISCUSSION

TEM-149 is a natural TEM-1-derived ESBL with an unusual amino acid substitution at position 240, a valine for a glutamate. The other amino acid substitutions found in TEM-149 involved positions 104 (E104K), 164 (R164S), and 182 (M182T). On the basis of its amino acid substitutions, TEM-149 could be derived from other ceftazidimase ESBLs, such as TEM-46/CAZ-9 (E104K, R164S, and E240K), TEM-24/CAZ-6 (E104K, R164S, A237T, and E240K), TEM-64 (L21F, E104K, R164S, M182T), and TEM-10 (R164S and E240K). In the study described in this paper, we correlated the kinetic parameters of the TEM-149 and the TEM-149^{T182M} enzymes with those reported by Raquet et al. for the well-characterized TEM-10 enzyme (19). This is the first finding of a valine at position 240, which has never been described in other natural

variants or laboratory mutants belonging to the TEM, SHV, and CTX-M families (4, 5, 26).

In this study we also report on the purification and kinetic analysis of TEM-149^{T182M}, a TEM-149 mutant with a reversion of a residue at position 182. This mutant was made to evaluate the effect of the methionine at position 182 in combination with the pattern of amino acid substitution found in TEM-149.

The M182T substitution has been identified in both ESBLs and inhibitor-resistant enzymes (TEM-32) and can suppress the effect of deleterious substitutions by altering enzyme folding and stability (11, 24). It is interesting to note that M182T in combination with substitutions at residue R164 exerts a positive effect on TEM variants (26).

The M182T substitution has been reported to have no effect on enzyme activity but, rather, to have an effect on its stability (8). Thermal stability experiments performed with TEM-149 and its mutant showed that at different temperatures (25°C, 40°C, and 55°C) TEM-149^{T182M} is more stable than TEM-149. Nevertheless, if we compare the kinetic parameters of the TEM-149 and the TEM-149^{T182M} enzymes, a slight reduction in the catalytic efficiency of the TEM-149^{T182M} mutant against all substrates tested except benzylpenicillin, aztreonam, and cefotaxime was observed. Actually, in the TEM-149^{T182M} enzyme the k_{cat}/K_m value for cefotaxime was eightfold higher than that observed for the TEM-149 wild type. Cefotaxime behaves as a poor substrate for TEM-149, with a k_{cat}/K_m value sixfold less than that observed for TEM-10. In TEM-149, the threonine at position 182 might increase the catalytic activity of the enzyme by altering its folding. Generally, M182 could correct the stability defect that occurs in the enzyme as a result of amino acid substitution (10, 23). Comparison of the TEM-149 and TEM-149^{T182M} enzymes with the TEM-10 enzyme (R164S and E240K) (Table 2) showed k_{cat}/K_m values for ceftazidime that were 11- to 16-fold higher than the k_{cat}/K_m values observed for TEM-10 (19). Residue 240 is placed at the end of the B3 beta strand (12). The side chain of E240 can interact with the amino group of the amino-thiazolic substituent of cephalosporins. A lysine is observed at this position in several TEM mutants. The lysine side chain is able to form an electrostatic bond with the carboxylic acid group of the oximino moiety of ceftazidime. In this case, an increase in the k_{cat} value is observed (12). However, no modification of K_m has been observed. Comparing the K_m value for ceftazidime of TEM-149 (K_m = 19 μM) with that of TEM-64 (K_m = 393 μM) (27), we observed that the K_m value of TEM-149 is 20-fold lower than that of TEM-64. In this case the presence of a nonpolar residue, such as valine, at position 240 can facilitate the accommodation of the bulky oximino substituent of ceftazidime (Fig. 1). The result is an increase in affinity, although the catalytic efficiencies are about the same (0.44 $\mu M^{-1}\ s^{-1}$ for TEM-149 and 0.18 $\mu M^{-1}\ s^{-1}$ for TEM-64).

The enhanced catalytic efficiencies of TEM-149 and TEM-149^{T182M} for ceftazidime and, slightly, for aztreonam with respect to those of TEM-10 that were observed could also be attributed to the presence of a lysine at position 104. The side chain amino group of lysine 104 is able to form an ionic bond with the peculiar side chain of ceftazidime and aztreonam, whereas the acid side chain of the glutamic acid at position 104 induces unfavorable interactions (12). Thus, K104 improves the catalytic activities of ESBLs against ceftazidime and aztreonam and improves them less so against cefotaxime.

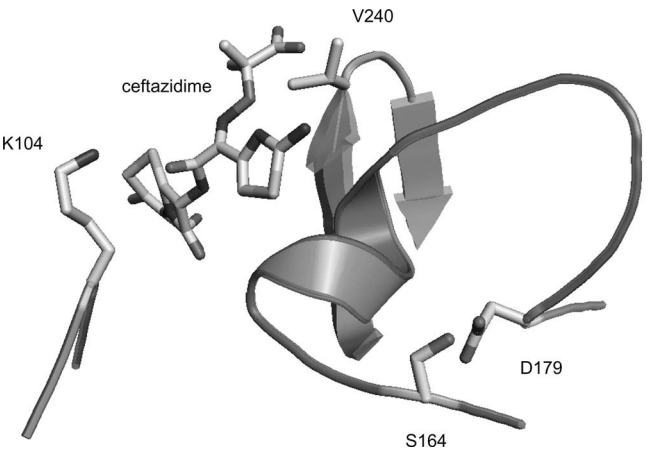

FIG. 1. Molecular modeling of the Michaelis complex of TEM-149 with ceftazidime, showing the residues discussed in the text. The hydroxy group of the serine at position 164 is sufficiently close to the carboxylic oxygens of the aspartate at position 179 to allow the formation of one hydrogen bond. The lysine at position 104 is able to form an ionic bond with the carbonilic oxygens of the oxyimino chain of ceftazidime. The valine at position 240 is placed on the omega loop at the end of the B3 beta strand.

The arginine at position 164 in TEM variants is located in the omega loop and usually makes a salt bond and a hydrogen bond with D179 (12). The replacement of arginine with the neutral amino acid serine makes the omega loop more flexible because of the elimination of the electrostatic attraction between residues 164 and D179. This allows the accommodation of bulky β-lactam substituents. In conclusion, the combination of mutations found in the TEM-149 enzyme contributes to a better orientation and a better accommodation of ceftazidime and aztreonam in the catalytic site of the enzyme.

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