

Evolution of Drug Resistance: Insight on TEM β -Lactamases Structure and Activity and β -Lactam Antibiotics

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Abstract: Since the discovery of the first penicillin bacterial resistance to β -lactam antibiotics has spread and evolved promoting new resistances to pathogens. The most common mechanism of resistance is the production of β -lactamases that have spread thorough nature and evolve to complex phenotypes like CMT type enzymes. New antibiotics have been introduced in clinical practice, and therefore it becomes necessary a concise summary about their molecular targets, specific use and other properties. β -lactamases are still a major medical concern and they have been extensively studied and described in the scientific literature. Several authors agree that Glu166 should be the general base and Ser70 should perform the nucleophilic attack to the carbon of the carbonyl group of the β -lactam ring. Nevertheless there still is controversy on their catalytic mechanism. TEMs evolve at incredible pace presenting more complex phenotypes due to their tolerance to mutations. These mutations lead to an increasing need of novel, stronger and more specific and stable antibiotics. The present review summarizes key structural, molecular and functional aspects of ESBL, IRT and CMT TEM β -lactamases properties and up to date diagrams of the TEM variants with defined phenotype.

The activity and structural characteristics of several available TEMs in the NCBI-PDB are presented, as well as the relation of the various mutated residues and their specific properties and some previously proposed catalytic mechanisms.

Keywords: Antibiotics, β -lactamases, CMT, ESBL, IRT, TEM.

1. EVOLUTION OF RESISTANCES - β -LACTAMASES AND β -LACTAM ANTIBIOTICS

Currently, infectious diseases are accepted as one of the most important and relevant clinical conditions and, many times, fatal. With the inclusion of antibiotics in the clinical practice, mankind has taken this problem as a ceased problem in the history of human diseases. The first β -lactam antibiotic to be used in clinical practice was penicillin which was discovered and described by Alexander Fleming in 1928 and was used with success in medical practice in 1941, after the work of Howard Walter Florey and Ernst Chain [1]. β -lactams target the PBPs with success as this type of antibiotics mimics the D-Ala-D-Ala dipeptide in the peptidoglycan and form a very stable acyl-enzyme complex inhibiting PBPs from synthesizing peptidoglycan [2]. These antibiotics always feature a very reactive cyclic amid- β -lactam ring - which is a part of the bicyclic structure that forms the "core" of the antibiotic. A few common ring rearrangements can be appointed: penam, penem, carbapenem, cefem and monobactam.

Even though several β -lactams have been developed for use in clinical practice, the infectious agents have the ability to acquire and development mechanisms to resist the antibiotics action and the majority of the hospitals isolates are microorganisms highly resistant to antibiotics [3, 4]. Among such mechanisms the emergence of resistances can be associated with multiple causes, being the genetic mutations that lead to the production of a mutated protein, one of the most common.

Some of these mutant proteins may act as enzymes that increase the catalytic activity towards antibiotics or increase the resistance to the binding of specific inhibitors. Additionally, membrane transporters that export antibiotics prior to their binding to the target may be also mutated. The microorganisms that do not develop any mutation conferring resistance to the antibiotics may obtain these types of genes through the acquisition of mobile genetic elements such as integrons and transposons in plasmids (conjugation), in naked DNA from the environment (transformation) and in phage DNA (transfection) [3, 5, 6]. These mechanisms are considered as primary mechanisms of resistance (resistance occur at the primary level of metabolism). Mechanisms of resistance affecting secondary metabolism have also been described (biosynthesis of modified β -lactams that are antagonist to the modified proteins) [7].

Specific primary mechanisms of resistance to β -lactams can be associated with Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, acquisition of resistance is achieved through the production of β -lactamases (inactivation of the antibiotic) and production of mutated PBPs [7, 8]. Several mechanisms of resistance can be obtained with the mutation of these targets. It is possible that a less sensitive PBP is produced either by mutation of an endogenous protein or by acquisition of a new PBP. It is also possible to up regulate the production of PBPs allowing the pathogen to produce peptidoglycan [9].

The most common mechanism in Gram-negative bacteria is the production of β -lactamases but these organisms can also present mutation of outer membrane proteins such as porins. This allows them to avoid one of the mechanisms for the crossing of the antibiotic through the outer membrane to reach the active site (PBPs) and the production of efflux pumps capable of remove the antibiotics from the periplasma and transporting them out of the bacteria [7, 8].

Nowadays, it is accepted that the increase of antibiotic resistance is due to the negligent use of antibiotics or the lack of these to perform a proper medical treatment – World Health Organization (WHO). However, the emergent resistance of β -lactams was detected even before penicillin was ever used in medical practice [3, 4]. The first β -lactamase was described, in 1940, by Chain, E. et al. in *E. coli*, as a penicillinase but soon this resistance appeared in others species, particularly in staphylococci [3, 10]. It is believed that β -lactamases have PBPs as their ancestors and that, by the acquisition of new mutations, evolved and gain the capability to acylate and deacylate β -lactams [11]. Therefore, it becomes clear why PBPs and β -lactamases are both active-serine enzymes with similar active sites – in both exists a lysine near the catalytic serine. However, in PBPs the lysine is the initial base while in β -lactamases its role is not yet fully understood. Another major dissimilarity between the active site of these two types of proteins is the presence of a new glutamate residue in the Q-loop in the β -lactamases [12].

The most common isolated β -lactamases are TEMs and SHVs, as well as ESBL. Other examples are OXAs and SHO [3, 13, 14]. β -lactamases are primarily divided in four molecular classes, from A to D. ESBLs represent a major concern for public health [15] due to their ability to hydrolyze up to fourth generation cephalosporins, penicillins and aztreonam. Carbapenemases are another group of β -lactamases representing an emergent clinical concern [16, 17]. The main β -lactamases with these phenotypes are TEM, SHV CTX-M and VEB as ESBL representatives and KPC and GES as carbapenemases representatives [18-21]. After the discovery of TEM-1(1963), its prevalence increased and, in a 20 year times spawn, various variants of TEM-1 were detected with altered kinetic properties such as the ability to hydrolyze extended spectrum β -lactam antibiotics- ESBL and to resist inhibition- IRT [4]. Even though, the first CTX-M was discovered in 1989, the wide dissemination and fast evolution of these β -lactamases only occurred after the year 2000 when these were isolated in very important and prevalent pathogens [15, 22]. The alignment of the enzymes amino acidic sequences determined that unlike other ESBLs, these enzymes formed heterogeneous groups [18]. According with the alignment it was possible to classify them within five different clusters which names correspond to the first enzyme isolated from each group: CTX-M-1 [23], CTX-M-2 [24], CTX-M-8 [25], CTX-M-9 [26] and CTX-M-25 [27]. New CTX-M clusters have been reported [28, 29] showing that the number of clusters might increase in the future. The fast evolution and worldwide spread has been referred to as “CTX-M pandemic” [22].

At the moment no clinical isolates presenting inhibitor resistant CTX-M enzymes have been reported but mutants obtained in laboratory experiments capable of resist to treatment with β -lactam antibiotics/clavulanic acid were reported [30, 31]. With the increasing capability of microbes to resist antimicrobial chemotherapy, carbapenems are often used as last option to treat Gram-negative organisms' infections [32-34]. Unfortunately, enzymes capable of hydrolyzing these compounds have emerged – KPC β -lactamases [35, 36]. These serine carbapenemases belong to group 2f (Bush classification scheme [37, 38]) and belong to the Ambler molecular class A [39] as the EBLs that evolved from TEM-1. The first KPC (KPC-1) was isolated in North Carolina in 1996 [40] but rapidly spread worldwide [41-45] leading to the current existence of nine different KPC enzymes (KPC-1 to KPC-10) [35] since a correction of blaKPC-1 shows that KPC-1 and KPC-2 are the same enzyme [40]. KPC β -lactamases present a broad spectrum of activity and are capable of hydrolyzing most β -lactam antibiotics [21]. An additional concern on the resistances conferred by KPC β -lactamases is their capability to resist inhibition by β -lactamase inhibitors such as clavulanic acid and Tazobactam [40]. This family of enzymes is plasmid mediated and often isolated from *K. pneumoniae*, which has been identified as the responsible organism for multiple epidemics [46-51] due to the ability of these microorganisms to present multiple resistance mechanisms as well as their ability to transfer these. As a result, the mortality rates of infections with *K. pneumoniae* are very high [50].

1.1. TEM-1 Characterization

Upon antibiotics binding to the TEM-1, the small molecules hydrolyze the amide group of the β -lactam ring by a serine residue in the active binding site, which allows the classification of this enzyme as class A β -lactamase [52]. Although the classification is still used, it does not allow to clearly and efficiently differentiating some β -lactamases with distinct properties. To overcome this situation a more complex system of classification was developed by Bush [37]. This system is based on several characteristics of the enzyme such as molecular structure, biochemical properties and genetic sequence [37].

1.1.1. TEM-1 Structure

β -lactamase TEM-1 (Fig. 1) is organized in three domains Sandwich-like (α -Helices/ β -sheets/ α -Helices) and the binding site is located between the β -sheets domain and the domain that includes the α 2-Helix [52, 53]. The protein backbone is highly ordered and stable and the variability is only observed in the loops due to the lack of a well-defined secondary structure [54]. The high variability of the position of loop Q, may influence the proximity of water molecules near the active site, more precisely near the Glu166 residue. Fig. (1) illustrates the proximity of the residues that do not catalyze the reaction (highlighted in orange) to the catalytic residues. Their proximity may influence the ability of this enzyme to hydrolyze substrates (leading to a different orientation of the catalytic residues, enlargement of the

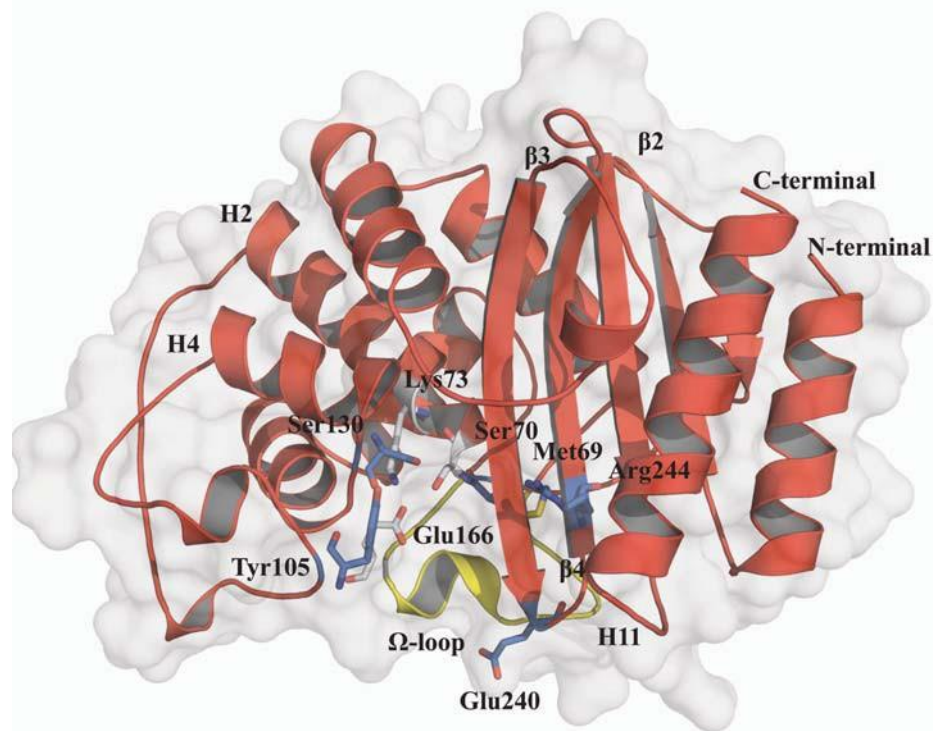


Fig. (1). Structural representation of TEM-1 (PDB ID: 1ZG4[60]) with the catalytic residues in a white stick representation and the residues near the catalytic pocket (the ones that can affect the enzymatic catalyzes) highlighted in blue. Some key secondary structures are identified.

catalytic pocket or displacement of water molecules in the catalytic pocket). However, there is a tradeoff: the destabilization of the enzymatic structure. The Ser130 residue has an important role in the catalysis, mainly due to its proximity to the residue Ser70. As this residue is irreversibly inhibited, it is crucial for the reaction with inhibitors of β -lactamases [3, 52, 54-58]. Kollman et al. [59] have shown that Ser130 can be responsible for the β -lactam ring opening due to the donation of an hydrogen, after the nucleophilic attack by Ser70.

1.1.2. TEM-1's Activity

Resistance to β -lactam antibiotics by TEMs can be explained by two main processes: acylation and deacylation [52]. In the acylation process, the Ser70 residue is deprotonated in order to perform the nucleophilic attack to the carbon of the carbonyl group in the β -lactam ring. The deprotonation process is achieved through the interaction of a water molecule with Glu166, the general base of the reaction [55, 61]. After accessing the importance of the Lys73 in the process, other theories refer the possibility of this residue acting as a base in the enzymatic process. However, it can also be important to stabilize the ligand in the hydrolysis

reaction. Thus, even now the mechanism is not yet fully

understood and many theories have been described (Scheme 1 demonstrates the most prevalent proposed mechanisms in the literature) [52, 55, 59, 61]. The main difference between the hypotheses presented in Scheme 1 is the activation of Ser70 to perform the nucleophilic attack. In Scheme 1-A Glu166 abstracts a hydrogen atom from Ser70 through a water molecule that is hold in place by hydrogen bonding with

both Glu166 and Ser70. Consequently, Ser70 performs the nucleophilic attack of the carbon atom of the carbonyl group of the β -lactam ring. In Scheme 1-B Glu166 abstracts a hydrogen atom from Ser70 through a hydrogen transport network involving Lys73. It allows Ser70 to perform the nucleophilic attack as in Scheme 1-A. In both mechanisms Ser130 acts as a catalytic acid donating a hydrogen atom that helps with the ring opening. The deacylation process of the formed intermediary is similar in both mechanisms and occurs with an activated water molecule bound by hydrogen bridges to Glu166, which releases Ser70 from the -lactamic ring in its protonated form [52].

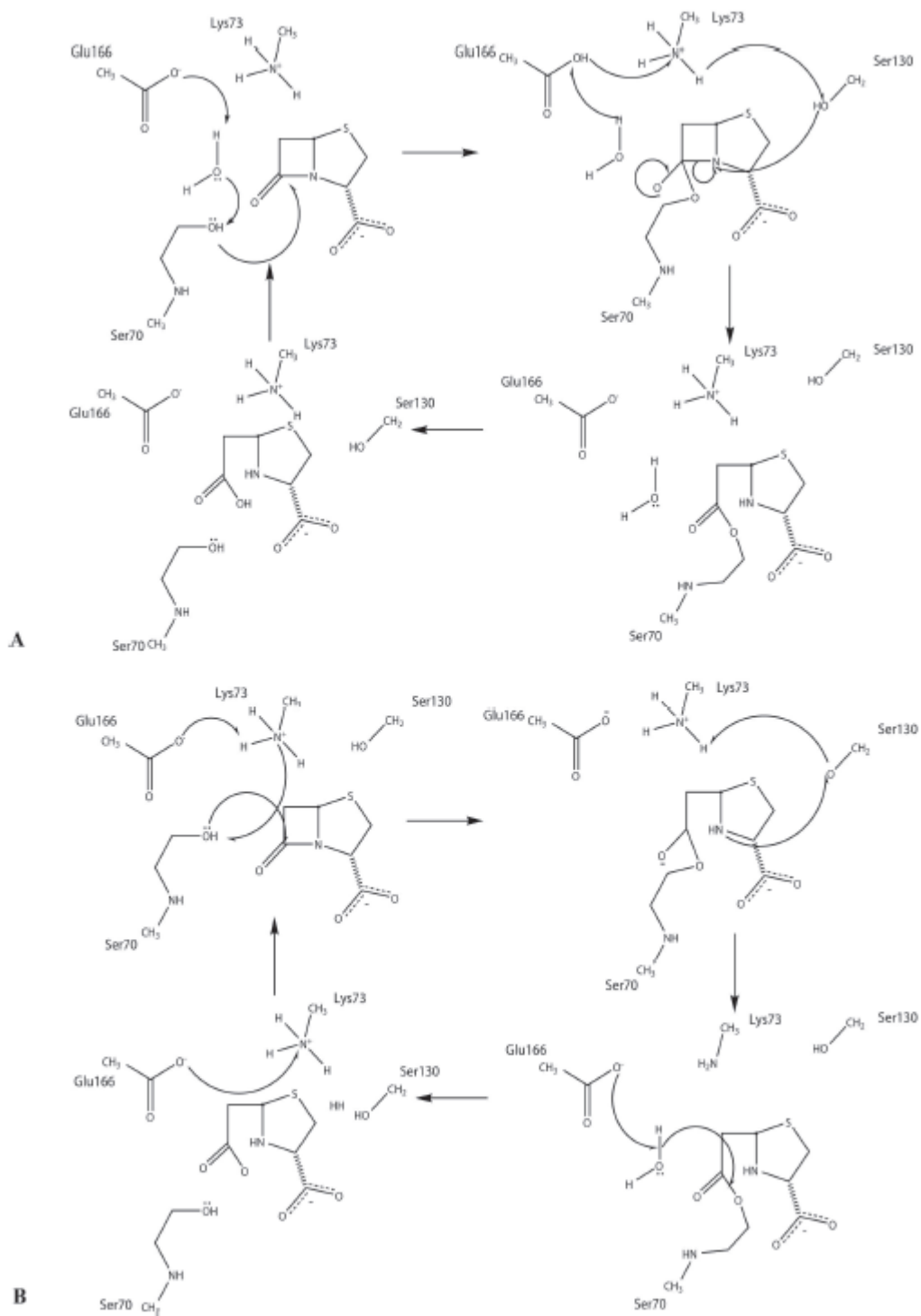
1.1.3. TEM Extended Spectrum M-lactamases – TEM ESBL

For decades new antibiotics were developed for clinical use. The usage of new antibiotics induced a selective pressure over microorganisms, which led to a co-development of the latter with production of enzymes capable of antibiotic degradation [5, 14].

TEM-1 degrades efficiently some penicillins but, despite its poorly filled out active site, it does not possess the needed space to accommodate big lateral chains like the ones in

ceftazidime and cefotaxime (3 generation cephalosporins)

[4, 14, 62]. With the introduction of this type of compounds, these enzymes have evolved. Up to 2001 [3] mutations were observed only on a small number of residues. After Bradford et al. compile the mutations on the existent ESBLs, not only several new enzymes have been identified but mutations on several different residues can now be observed. TEM ESBL started to be identified and isolated since the 70s, and nowadays more than 100 are known [3, 63].



Scheme (1). Catalytic mechanism of TEM enzymes. In A Glu166 act as general base activating a water molecule [59] and in B, Glu166 activates Ser70 through a proton transfer network with Lys73 [52].

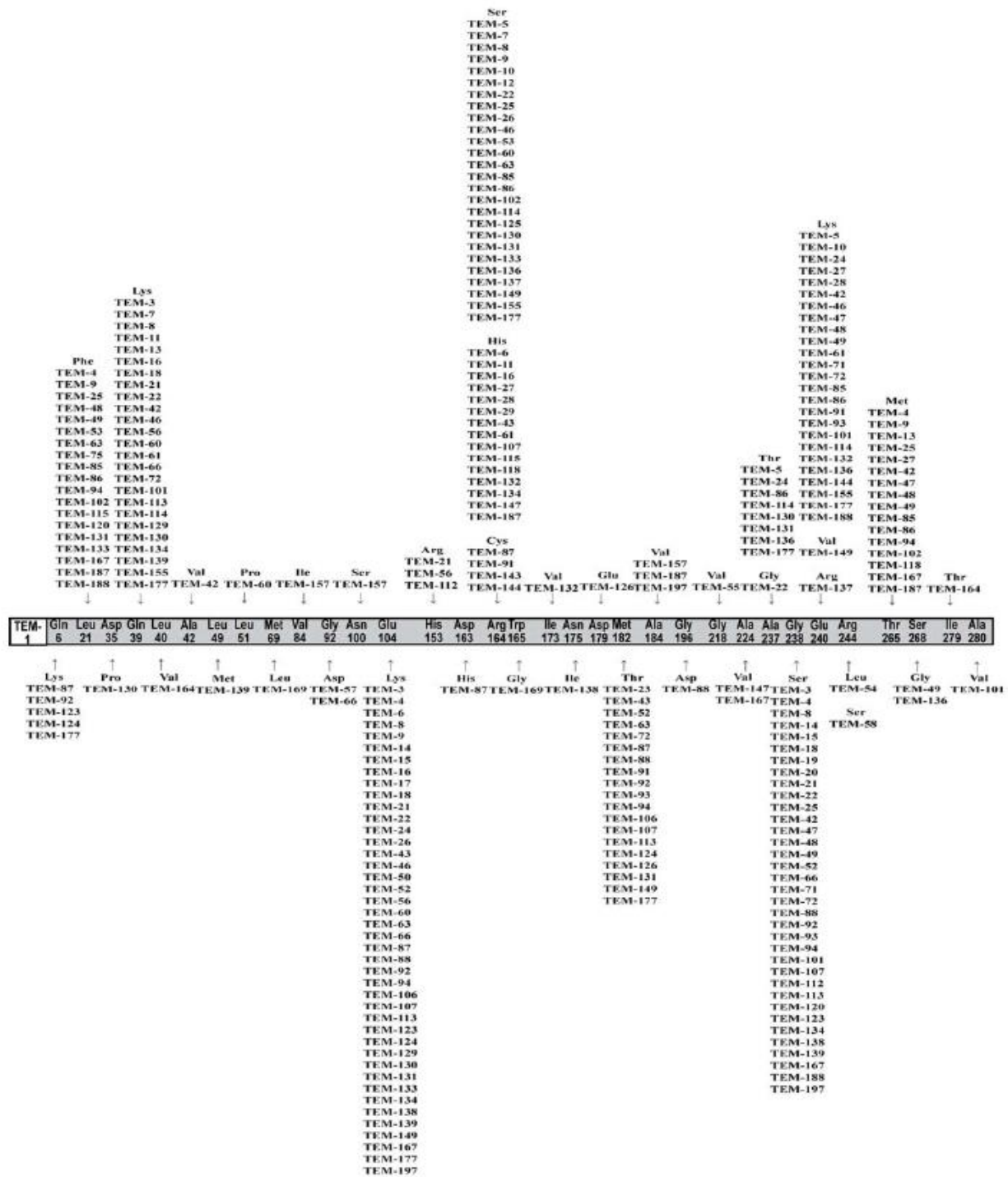


Fig. (2). Diagram of the mutations of TEM ESBL enzymes, compared to the TEM-1 sequence. Scheme based on available sequences and phenotype in Jacoby database [63].

The unusual mutations observed in this phenotype (even not in the active site) increased the plasticity of the active site, which allowed its enlargement. This enlargement made

them to be able to acylate larger β -lactam antibiotics, inactivating them. ESBL have shown to degrade extended spectrum β -lactams (around 100 times more efficient) but

with decreased catalytic activity for penicillins (100 fold less efficient) [64]. The enlargement and increase of plasticity of the active site do occur but with a decreased enzymatic stability [65]. The enlargement of the active site can be the result of the loss of H-bonds upon mutation that may lead to the loss of a defined secondary structures near the mutated residue as described for TEM-69 [14]. Having this in consideration, it is understandable that enzymes that present an additional factor that leads to higher enzymatic stability are naturally selected. This is mainly accomplished by evolutionary pressure, by the use of antibiotics with bigger lateral chains [4, 62]. In several ESBL, one factor was identified: the Met182Thr mutation. This mutation does not lead to any effect on the catalytic activity of the enzyme. Therefore, by itself does not bring any evolutionary advantage, which might be the reason why this mutation is not present by itself in any known enzyme [4, 5, 56, 66]. Wang X. et al. have demonstrated that bacteria that produce enzymes with the referred mutation are less susceptible to 3rd

generation cephalosporins, when compared with the wild

type. Effect that is observed in a higher degree with the increase of the temperature [14]. The TEM ESBL producing bacteria are capable of resisting the action of β -lactam antibiotics due to its degradation before they reach the target (PBP). These observations may be explained by the increase of the protein stability but it is still possible that it promotes the correct folding of the TEM variants [62].

A small amount of amino acids mutations with a high importance for the yield of extended spectrum enzymes have been described. Regarding such amino acid mutations Leu21Phe, Gln39Lys, Glu104Lys, Arg164His or Arg164Ser, Gly238Ser and Glu240Lys are among the most commonly described [3, 4, 63, 67]. Fig. (2) illustrates the various mutations, concerning TEM-1 sequence, of the ESBLs β -lactamases with sequence available in reference [63] at the time of this review.

1.1.4. TEM Inhibitors Resistant β -Lactamases – TEM IRT

The β -lactamases with the ability to resist the inhibition by clavulanic acid were discovered in the early 90s [64]. They were identified mainly from samples of *E. coli* but also from samples of other lineages, like *K. mirabilis* and *C. freundii* [3].

These types of enzymes confer resistance to clavulanic acid and sulbactam. Hence, microorganisms that produces

them are resistant to Clavamox treatment (amoxicillin-

clavulanic acid), ampicillin-sulbactam and other inhibitor combinations [4, 13, 67-69]. IRTs remain susceptible to Tazobactam, and this treatment may be performed in combination with piperacillin [69]. Mutations in the residues Met69, Ser130, Trp165, Arg244, Arg275 and Arg276 appear to be relevant in promoting this phenotype and its identification is more common in clinical isolates [3, 4, 58, 64, 70]. Substitutions in the Met69, Ser130, Arg244 and Arg276

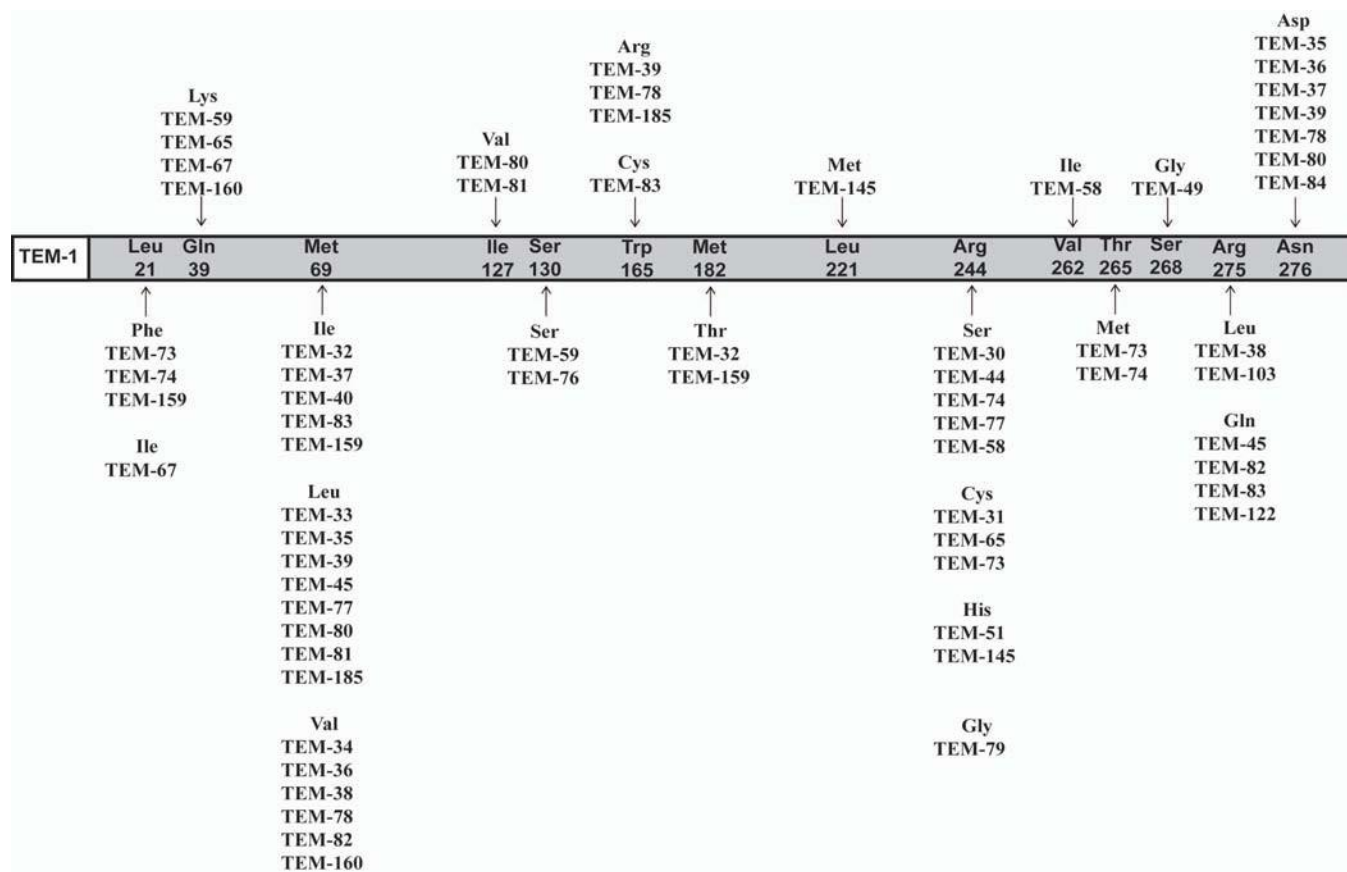


Fig. (3). Diagram of the mutations of the TEM IRT enzymes, compared to the TEM-1 sequence. Scheme based on available sequences and phenotype in Jacoby database [63].

residues were identified as resistance promoters ampicillin - clavulanic acid combinations and other studies have shown that mutations in Met69, Trp165 and Arg276 promote resistance to ceftazidime-clavulanic acid treatment [3, 57, 70, 71]. Mutations on the Arg275 residue do not promote considerable resistance to clavulanic acid inhibition. Nevertheless, mutations for Leu and Gln have been identified to increase cefotaxime resistance, suggesting that Arg275 mutations may promote resistance to inhibitors and β -lactam antibiotics [4, 62]. Fig. (3) illustrates the known mutations, relative to TEM-1, of the IRTs β -lactamases with sequence available in reference [63] at the time of this review.

The inherent mechanism of the inhibition is identical for all inhibitors and, unlike the mechanism of β -lactam hydrolysis, is already well known [64]. It comprehends 8-10 different intermediates in the inactivation pathway and derives of conventional hydrolysis intermediates [57]. The inhibitors form a covalent bond between residues Ser70 and Ser130 leading to the irreversible inactivation of the enzyme due to deprotonation of the Ser130 residue [58, 64]. Despite the hydrolytic pathway being 100 times faster than the inactivation pathway, the latter is irreversible, which leads to the inhibition of all the enzymes in the microorganism and loss of their activity [58]. Clearly, Ser130 is essential to the inactivation via and its role is very important for the formation of the IRT phenotype.

Other described mutations do not present direct interaction with the inhibitor nor with the proteic neighborhood of the active site. However, it is possible that these mutations cause torsion of Ser130 side chain, deflecting it from the active site, which leads to resistance to inhibitors [4, 64]. IRT

TEMs are less capable of hydrolyzing β -lactam antibiotics. As they are resistant to inhibitors, they can overcome this aspect and therefore, be resistant to the various treatments [58]. Mutants that present mutations in residues that are typically responsible for the EBSL and IRT phenotype, normally present just one of the phenotypes. However, some mutants (i.e. TEM-50 or CMT-1) confer resistance to clavulanic acid and present the ability to hydrolyze large spectrum cephalosporins [13, 63]. Several others have also been described as CMT or CMT-type enzymes. Although CMT variants present resistance to β -lactamase inhibitors, some can still be inhibited by the clavulanic acid at a higher rate than IRT variants. These are fundamental evidences that β -lactamases with more complex phenotypes and higher potential to promote resistance to treatments are evolving [72]. Fig. (4) is a diagram of the mutations described until now regarding the CMT/CMT-type phenotypes in comparison with the TEM-1 sequence [63].

1.1.5. Previously Described TEM Variants

For several years, researchers have study this family of enzymes. Such searching for knowledge concerning TEM enzymes, include the comprehension of their enzymatic mechanisms and shed light into the way they are evolving for better predicting their biological effect (antibiotic resistance). In the medical context, the comprehension of the genetics and biochemistry of the various β -lactamases will allow the development of better therapeutic approaches towards the infection control caused by pathogens that produce TEM enzymes. Table 1 lists some of the most important and well described variants. It becomes clear the relevance of some residues for their structure and activity.

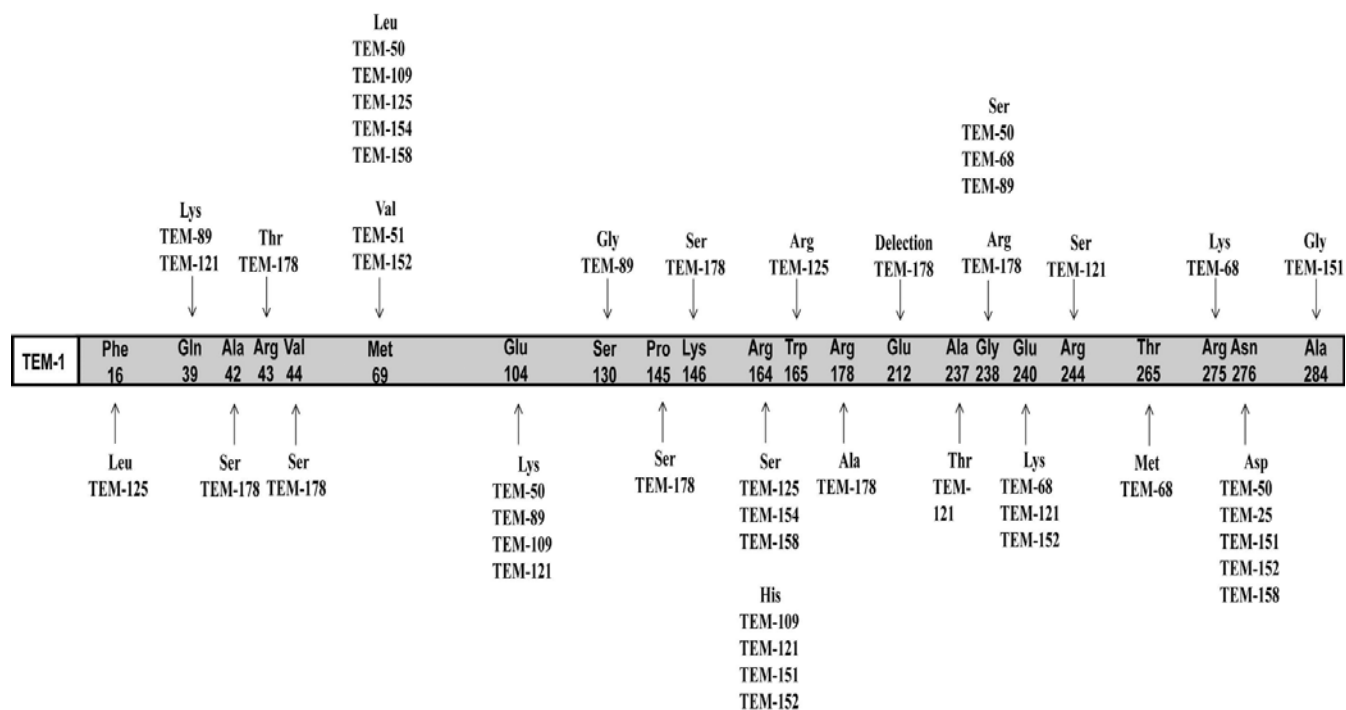


Fig. (4). Diagram of the mutations of the TEM CMT/CMT-type enzymes, compared to the TEM-1 sequence. Scheme based on available sequences and phenotype in Jacoby database [63].

Table 1. Biological and structural effect of the mutation of key residues of several TEM enzymes.

Variant	Mutation	Biological Effect	Structural Characteristics	PDB ID
Wild Type (WT)	-	Responsible for β -lactam antibiotics resistance (cephalosporins are moderate resistant). They open the β -lactam ring deactivating the molecule.	-	1ZG4 [52]
-	S70G	Less active than WT deactivating β -lactam antibiotics. It can still be active as Ser130 might perform the nucleophilic attack to the β -lactam ring or due the presence of an additional water molecule close to Gly70.	Lack of nucleophilic properties at residue 70 side-chain. The overall structure is very similar to WT except conformation of Ser130 and backbone near this residue.	1ZG6 [52, 58]
TEM-76	S130G	Less active than WT deactivating β -lactam antibiotics. Less prone to inhibition by clavulanate and tazobactam ($K_1 > K_1^{WT}$ for pre-acylation complexes) More stable at T_m than WT.	Distance between Ser70 and Gly130 is greater than in WT (7,3Å and 6,7Å in WT). Rotation of Lys234 (N shifts 1,5Å). H-Bond between Lys73 and Ser70 does not exist (N of Lys73 moves 0,5Å). New water molecule near Ser130 is responsible for its activity.	1YT4 [58]
Frequent in many TEM mutants	M182T	Similar activity than WT. Increased stability (stabilizes ESBL). Differences between TEM with Met182Thr increases with the increase of the temperature.	Met182 is 14,5Å from the active site and therefore, it does not affect β -lactamase activity.	1JWP/ 1M40 [14, 55]
TEM-69	E104K R164S M182T	ESBL	Loss of H-Bond between Arg164 and Asp179 and between Arg164 and Glu171. Loss of secondary structure near residue 170 (α -Helix) increases the catalytic pocket.	1JWP [14]
TEM-32	M69I M182T	More stable than TEM-40 (Met69Ile) and less stable than Met182Thr mutants. Resistance against β -lactamase inhibitors – IRT (Ser130 moves). Catalytic activity is reduced.	RMSD C_{α} = 0,41Å Ser70 distortion causes a 64° x1 of Ser130 (no longer makes H-Bonds with Ser70 and Lys73) Ser130 O_y - Ser70 O_y = 5,5Å (3,2Å in WT) Ser130 O_y - Lys73 N_{ζ} = 5,4Å (3,8Å in WT). New water molecule near Ser130	1LI0 [64]
TEM-34	M69V	More stable than WT. Resistant to inhibition (new H-Bond Ser130- Lys234: reduced ability to perform a nucleophilic attack)	RMSD C_{α} = 0,30Å Ser70 distortion causes a 27° x1 of Ser130. H-Bonds between Ser130 with Ser70, Lys73 and Lys234 (Ser130 O_y - Lys234 N_{ζ} = 2,8Å; Ser130 O_y - Lys73 N_{ζ} = 3,2Å). Two lone pairs are used to bond with the two Lys when in WT only one of these was verified. The rate of cross-linking between the two Ser is reduced, which leads to a lower inactivation.	1LI9 [64]
TEM-64	N276D	IRT phenotype K_{inact} 35% inferior than in WT with clavulanic acid. Small or nonexistent inhibition with Tazobactam and Sulbactam.	Overall structure similar to WT. Residues 272-290 (β -Sheet surface/ α - Helix H11), 26-40 (N-Terminal α - Helix H1) and 219-224 (α -Helix H10) move. RMSD=0,52Å RMSD (Asp276)=0,75Å. New interaction with Arg244 (Asp276 $O_{\delta 2}$ - Arg244 N_{Y2} =3,3Å). Nonexistent water molecule in the active site (less prone to inhibition).	1CK3 [70]
TEM-30	R244S	IRT phenotype due to water displacement.	Water displacement leads to lower affinity to inhibitor (Schiff's Base from inhibitor goes away from Ser130).	1LHY [64]

Legend: WT: Wild Type.

1.1.6. Relevant Amino Acids

Table 1 stresses out the importance of some residues for the structure and activity of the various TEM variants. Their

importance is related to their position in the protein as well as to specific pair interactions with residues in their microenvironment. Here we will characterize them:

1) Met69

The side chain of Met69 lies behind P3 forming a wall of the oxyanion pocket. By means of steric interactions it influences the position of Ala237 and Gly238. Due to the proximity with Ser70, it has an important role influencing the structural position of this residue and also of Ser130 (Fig. 5) [3, 64, 73].

2) Glu104

This glutamic acid residue is located in a conserved loop, highlighted in blue (101-111) and its hydrophilic side chain is exposed at the entrance of the binding site. Due to its direct contact with Asn132 (part of the conserved SDN loop,

highlighted in green (130-132)), if mutated it might affect the entry of bulky ligands (Fig. 5-A) [3, 67].

3) Ser130

Serine 130 is part of a complex hydrogen bonding network that contributes for the stability of the active site and the deprotonation process of Ser70. This residue is highly conserved. However, even though with consequences in the enzyme activity mutations or displacement of the side chain are tolerated (Fig. 5-A) [3, 57, 58, 69].

4) Arg164

This arginine residue is underneath the binding site in the Q-loop (162-179). The side chain is linked with Glu171 and

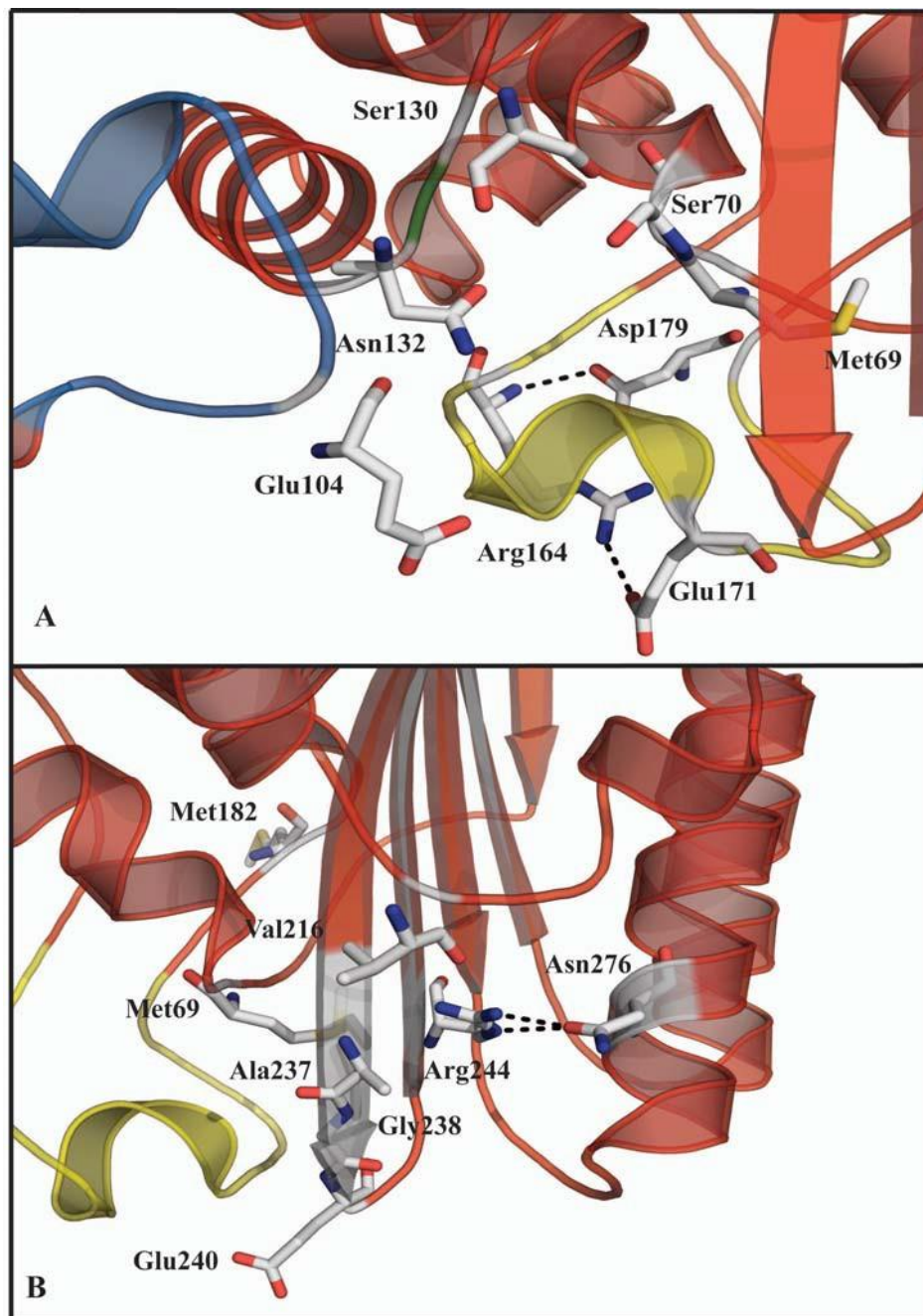


Fig. (5). Structural representation of key residues of TEM-1 (PDB ID: 1ZG4 [60]) and their main interactions.

Asp179 by salt bridge interactions and hydrogen bonds. An alteration in this residue might lead to an altered stability and conformation of the loop, and therefore, changes in the active site and the position of the catalytic residues (Fig. 5-A) [3, 67, 74].

5) Met182

Methionine 182 is distant from the active site but it has contact with the secondary structure in which Met69 is located. Mutations in this residue have been detected on clinical isolates but its role is not fully understood (Fig. 5-B) [3, 64, 66, 67].

6) Ala237

The side chain of alanine 237 is on the exposed side of β_3 , which faces the catalytic pocket. Therefore, the position of this residue influences the stability of β -lactam antibiotics binding by hydrogen bonding with them (groups NH and CO of the backbone) (Fig. 5-B) [3, 56, 67, 75].

7) Gly238

The side chain of glycine 238 is on the internal and more protected side of β_3 , close to the residue Met69. It has been demonstrated the importance of this residue both for the position of the β_3 , as well as the position of the Q-loop (steric conflict model). This affects the active site volume (Fig. 5-B) [56, 63, 67, 76].

8) Glu240

This residue is located at the end of β_3 and its side chain can interact with the substituents of bulky β -lactam antibiotics (i.e. cephalosporins). Mutations in this residue alone would not modify significantly the catalytic properties of the enzyme but it might compensate the destabilization of other mutations (i.e. Arg164Ser or Gly238Ser) (Fig. 5-B) [3, 4, 77-79].

9) Arg244

Arg244 is located in the β_4 and its long side chain reaches over to the adjacent β -strand (β_3) to the edge of the binding site. It is kept in place by two hydrogen bonds with Asn276. Arg244 together with Val216 can be responsible for holding a water molecule in place, which is thought to be important for the enzyme inactivation process (Fig. 5-B) [3, 67, 74, 80].

10) Asn276

This residue is located on the C-terminal α -helix (H11) and its carbonyl group interacts with Arg244 by two hydrogen bonds (Asn276 carbonyl group with guanidinium group of Arg244). A mutation in residue 276 will not affect the position of the surrounding residues (i.e. Arg244). However, the rotamer of the mutant will be substantially different for TEM—1 and consequently, a1 and a10 can be displaced (Fig. 5-B) [3, 67, 68, 70, 74].

2. CONCLUSIONS

With the introduction of antibiotics, selective pressure led to the survival of the fittest pathogens (with one or more mechanism to resist antibiotics). β -lactamases are the most common enzymes identified in clinical isolates of resistant

pathogens. Class A β -lactamases can present many distinct phenotypes (i.e. ESBL, IRT and CMT). TEMs evolved, by point mutations, from simple phenotypes (i.e. TEM-1) to highly complex ones (CMT/CMT-type). In this work we characterized in detail the structure of TEM-1 in order to better understand the possible effects of mutations as shown in Table 1. The effect of mutations supersedes the microenvironment of the mutated residues, which justifies the effect on the enzymatic catalysis of residues that are not near the catalytic pocket (i.e. Glu104Lys and Arg244Ser can affect protein stability in the Met182Thr mutant). The structure and activity of TEM β -lactamases is maintained by a very complex network of H-bridges between residues and between residues and specific water molecules. Examples are: (i) the water molecule stabilized by Glu166 and Ser70, which is the catalytic water and (ii) the water molecule stabilized by Arg244 and Val216 (proposed as an intervenient in the enzyme inhibition). With the current knowledge of the structural aspects of these enzymes and the relation between molecular structure and activity spectrum, the understanding of newly isolated TEM variants gains new insights.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

Declared none.

ABBREVIATIONS

ESBL	=	Extended Spectrum β -lactamase
CMT	=	Complex Mutant TEM IRT- Inhibitor Resistant
KPC	=	Klebsiella pneumoniae Carbapenemase
MRSA	=	Methicillin-resistant Staphylococcus aureus
PBP	=	Penicillin Binding Proteins
RMSD	=	Root Mean Square Deviation

SUPPLEMENTARY MATERIALS

Supplementary material is available on the publisher's web site along with the published article.

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