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REVIEW

Evolution of extended-spectrum β-lactamases by mutation

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ABSTRACT

Antimicrobial resistance genes in pathogenic bacteria belong to the most rapidly evolving DNA sequences, which results in an enormous structural diversity of resistance effectors. Structural modifications of resistance genes by mutation and recombination, together with a multitude of events that stimulate their mobility and expression, allow microorganisms to survive in environments saturated with antimicrobial agents of various types and generations. Genes coding for β-lactamases in Gram-negative bacteria are a fascinating example of this multifocal and multidirectional evolution, with the extended-spectrum β-lactamases (ESBLs) being one of the most spectacular 'achievements'. Some of the ESBLs known today are 'ready-to-use' enzymes in their natural producers but these are often of low pathogenic potential, or none at all. The problem appears upon mobilisation of a gene encoding such an ESBL, and its acquisition and sufficient expression by a more virulent organism. Many ESBLs are generated by mutations in genes coding for broad-spectrum enzymes, which have been mobile since at least the 1960s and which have disseminated very widely in populations of pathogenic bacteria. Strong selection pressure exerted by antimicrobial use, especially with newer-generation β-lactam antibiotics, efficiently promotes these two modes of ESBL emergence and subsequent spread. It also stimulates further evolution of ESBLs by accumulation of other mutations with an astonishing variety of effects on β-lactamase structure and activity. Remarkably, more than 300 natural ESBL variants have been identified since the mid-1980s but in-vitro studies suggest that ESBL evolution has certainly not come to an end; they may also help in predicting future developments. The aim of this review is to briefly overview the role of various mutations in ESBL evolution.

Keywords β-lactamase, ESBL, evolution, mutation, review

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INTRODUCTION

Evolution at the molecular level involves the gradual accumulation of mutations (and other changes) in DNA sequences under the selective constraints of the environment [1]. Once a mutation affects the structure of a gene product, or gene expression, it may cause loss of function, cause gain of function, or be neutral. The spectrum of possible mutational effects may be wide, and a loss-of-function mutation may lead to the full or partial reduction of a gene product activity, whereas a gain-of-function mutation may confer either a new activity or enhance an existing one. Moreover, a single mutation may cause both a loss and gain of function, due to a structure-based 'trade-off' between the two functions, or between stability and activity of the gene product [2].

The rate of evolution of DNA sequences is highly variable [1]. Among the DNA sequences that evolve most rapidly are the bacterial genes responsible for antimicrobial resistance. Resistance often evolves rapidly after a new antibiotic enters into clinical practice, with this rapidity reflecting several characteristics of microorganisms, such as their large population sizes, short generation times, and the expansion of resistance by transmission of mobile genes. Other key factors include the strong selective pressure of antimicrobial use in humans, animals, and agriculture [3], the presence of mutational hot-spots

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in resistance genes, and the high structural flexibility of many resistance effectors [4].

β-Lactamases, especially those of Gram-negative bacteria, provide an excellent example of the evolution of resistance mechanisms. Their history has been extensively analysed on various time scales. Long-term analyses, based on protein sequences, have resulted in views of genealogy of the fundamental lineages of β-lactamase classes A, B, C and D [5] and their evolutionary relationship to penicillin-binding proteins [6], or of β-lactamase families [7]. Shorter-term studies aim to reveal the evolution of variants, often via single sequence changes (microevolution) [8], sometimes in specific geographical regions [9,10]. These analyses are carried out on naturally occurring β-lactamase variants by protein or DNA sequence comparisons. In the former case, the studies reveal only the evolutionary trends within β-lactamase families; in the latter case, the studies precisely reconstruct the history of particular enzyme lineages, considering also silent mutations and genetic context. Studies on laboratory mutants, obtained by in-vitro mutagenesis or via directed evolution, contribute to our understanding of the evolutionary past, and allow future predictions [11].

The largest structural/evolutionary group of β-lactamases is Ambler class A [12], which includes the vast majority of the Bush, Jacoby and Medeiros group 2 with 'penicillinases, cephalosporinases and broad-spectrum β-lactamases that are generally inhibited by active site-directed β-lactamase inhibitors' [5]. Intensive evolution of class A β-lactamases has resulted in great structural diversity, with numerous families and subfamilies, as well as recent modifications distinguishing multiple variants. Minor structural diversity can confer major differences in biochemical properties, affecting substrate spectra, inhibition profiles and, consequently, the phenotypes of resistant pathogens. Class A includes species-specific and/or acquired β-lactamases that are expressed constitutively or inducibly [13].

TEM AND SHV β-LACTAMASES: CONVERTING A BROAD-SPECTRUM ENZYME INTO AN EXTENDED-SPECTRUM β-LACTAMASE (ESBL)

TEM and SHV β-lactamases are the most studied class A enzymes. Both families have had a relatively long period of evolution in the 'clinical' era, resulting in the observation of high numbers of structural modifications, many of them affecting biochemical properties [14,15]. Moreover, owing to the very high prevalence of TEM and SHV β-lactamases in Gram-negative bacteria (mostly the Enterobacteriaceae), the enzymes have had a strong impact on the clinical context and the epidemiology of bacterial infections all over the world. The TEM and SHV families, as we now observe them, may be traced back to a few parental enzymes, namely TEM-1, TEM-2, SHV-1 and SHV-11. The deeper origins of TEM β-lactamases remain unrevealed, with all TEM variants being identified as acquired enzymes, encoded by mobile genes [13,15]. On the other hand, the parental SHV β-lactamases are specific for *Klebsiella pneumoniae*, and the mobile bla_{SHV} genes are the descendants of several escapes from the chromosome of this species [16]. The microevolution of TEM and SHV β-lactamases has been extremely intensive in recent years, as demonstrated by the almost exponential growth in numbers of variants identified since the mid-1980s. The number of recognised polymorphic sites in amino-acid sequences has rapidly increased as well, especially in the case of SHV enzymes, with *c.* 160 TEM and 100 SHV amino-acid sequences submitted to the http://www.lahey.org/studies/webt.asp website. These differ from each other in *c.* 50 positions. This number seems likely to increase, since, in a saturation mutagenesis study, 220 out of 263 amino-acid positions of the mature TEM-1 protein tolerate mutations, with the enzyme retaining good hydrolytic activity against ampicillin [17]. Among both the TEM and SHV families, three major types of activity have been identified: those of the broad-spectrum β-lactamases, the ESBLs, and the inhibitor-resistant β-lactamases.

ESBLs are the most 'spectacular' result of this microevolution [5,13,15,18–21]. These enzymes have an expanded substrate spectrum towards oxyimino-β-lactams (oxyiminocephalosporins and aztreonam). The responsible mutations, therefore, result in gain of function, but because the parent broad-spectrum enzymes still have trace activity against oxyimino-compounds, the ESBL activity should be seen as an improved activity rather than as something entirely new. This gain is almost always accompanied by partial loss of activity against penicillins [22].

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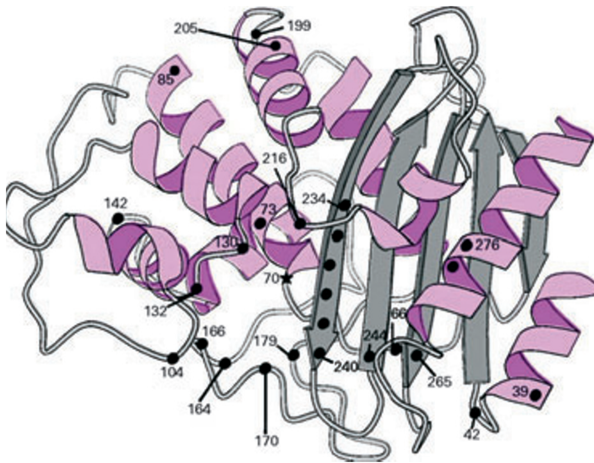

Fig. 1. Tertiary structure of a class A β-lactamase with positions important for activity indicated. Reproduced with permission from Matagne *et al.*, *Biochem J* 1998; **330**: 581–598. © the Biochemical Society.

The first natural ESBLs were identified in the mid-1980s [23,24] but soon they became widely disseminated in Gram-negative populations worldwide. The major factor responsible for this accumulation is the strong selective pressure of oxyimino-β-lactam use. TEM and SHV ESBLs have been extensively studied in terms of biochemistry, structural biology, molecular biology and clinical microbiology [13,15,18–21].

Among the many substitutions observed in TEM and SHV ESBLs, several are more or less directly associated with ESBL activity. These include mutations at positions Glu104 (TEM), Ala146 (SHV), Gly156 (SHV), Arg164 (TEM), Leu169 (SHV), Asp179 (SHV and TEM), Arg205 (SHV), Ala237 (TEM), Gly238 (TEM and SHV), and Glu240 (TEM and SHV) [14,22,25–27]. Most of these positions are in, or very close to, the oxyanion pocket that contains the enzyme's active site, and which itself is located at the interface between the two major domains of a class A β-lactamase molecule (Fig. 1). Specifically, most substitutions affect amino-acid residues within the B3 β-strand or Ω-loop [11,14,22]. Substitutions at positions 238, 164 and 179 seem to be especially critical for ESBL activity, and occur in the vast majority of TEM- and SHV-type ESBLs (http://www.lahey.org/studies/webt.asp). Single mutants, e.g., TEM-12, TEM-19, SHV-2, SHV-8 and SHV-24, have been identified in clinical isolates with an ESBL phenotype [24,28–32] but, except for SHV-2 [9,33–37], such enzymes are relatively rare.

Glycine at position 238 (B3 β-strand) is usually replaced by serine in ESBLs (32 TEMs and 22 SHVs) (http://www.lahey.org/studies/webt.asp), but alanine was observed in three natural SHV variants (SHV-13, -18 and -29) [38–40]. The characteristics of the sole TEM enzyme with Asp238, TEM-111 (GenBank AF468003), have not yet been published. The replacement of arginine at position 164 (Ω-loop) has so far been observed only in TEMs, either by serine, histidine or cysteine (with 23, 16 and three variants, respectively). Until recently, mutations of Asp 179 (Ω-loop) had been identified only in natural SHV variants, with replacements by glycine, alanine or asparagine (one variant each) (http://www.lahey.org/studies/webt.asp). Now TEM-126 has been found with a corresponding aspartate-to-glutamate substitution [41], while Asp179Gly/-Tyr mutants of TEM were selected only in directed evolution studies [42]. From the numbers of variants with mutations at positions 238, 164 and 179, and the sequence diversity of their corresponding genes, it is clear that each of the mutations must have been selected on multiple independent occasions.

Much effort has been applied to understanding the effect of common ESBL-type mutations on β-lactamase structure, biochemical properties, and the phenotypes of a producer organism. In these analyses, carried out on a variety of artificial single mutants, both the nature (e.g., hydrogen-bonding potential) and the side chain volume of the new residue are considered. In the case of the Gly238 mutations, various models are proposed, including improvement of substrate affinity by formation of extra hydrogen bonds to oxyimino-β-lactams (Gly238Ser in TEM) or by conformational changes leading to the expansion of the active site cavity, by displacement of the B3 β-strand or Ω-loop due to formation of novel intramolecular hydrogen bonds and/or steric conflicts caused by a larger-volume side chain (Gly238Ser and Gly238Ala) [14,22,43]. Recent crystallographic studies on TEM-52 [11], TEM-64 [44] and SHV-2 (which has only a single substitution) [45] confirm the concept of the significant B3 β-strand displacement, with new intramolecular hydrogen bonds in the case of the Gly238Ser mutation. This conformation change greatly enlarges the oxyanion cavity. Residues Arg164 and Asp179 strongly interact with each other, forming an ionic bond across the 'neck' of

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the Ω-loop, which is critical for conformation and stability. Mutations Arg164Ser/His/Cys and Asp179Gly/Ala/Asn disrupt this salt bridge, probably causing complete reorganisation of hydrogen bonds in the region, along with a significant conformational change at the entrance to the active site [14,41]. It is interesting that Arg164 mutations have been observed only in TEM enzymes so far, whereas those at Asp179 were, for a long time, found only in SHV enzymes. For the TEM-126 enzyme with Asp179Glu and Met182Thr mutations, subtle conformational changes due to modified hydrogen bonds at the Ω-loop's neck have been postulated [41].

Table 1 shows catalytic efficiencies, k_{cat}/K_m, or relative rates of hydrolysis, V_{rel}, against selected substrates as evaluated for a set of single mutants at positions 238, 164 and 179, and for the parent enzymes TEM-1 and SHV-1. Several artificial mutants corresponded to the natural β-lactamases TEM-19 (Gly238Ser), TEM-12 (Arg164Ser) and SHV-2 (Gly238Ser), whereas SHV-24 (Asp179Gly) itself was a natural variant [32,43,46,47]. The single TEM Gly238Ala mutant has not been identified in nature so far. All these variants had significantly increased activity against oxyimino-β-lactams. With the TEM-12 Arg164Ser mutant, the increase mostly affected ceftazidime (130-fold increase of k_{cat}/K_m) [46], whereas the TEM-19 Gly238Ser mutant demonstrated comparable increases in activity against both ceftazidime and cefotaxime (51-fold and 52-fold increase of k_{cat}/K_m, respectively) [43]. In general, these increases are rather small, and, in the case of the TEM-12 enzyme, for example, the rates of ceftazidime and cefotaxime hydrolysis are below 10% that of benzylpenicillin, which is considered as a breakpoint for identification of an enzyme as an ESBL [5]. At the same time, significant reductions of hydrolytic activity toward penicillins occurred, especially profound in the case of TEM-12 (93-fold decrease of k_{cat}/K_m against ampicillin) [46] and TEM-19 (benzylpenicillin, 50-fold; ampicillin, 25.5-fold) [43]. A variety of effects were observed with narrow-spectrum cephalosporins, depending on the enzyme, mutation type, and the substrate. MICs of representative β-lactams against *Escherichia coli* recombinant strains producing various single ESBL mutants are presented in Table 2. In accordance with the enzyme activity changes, MICs of oxyimino-β-

Table 1. Catalytic efficiency or relative rate of hydrolysis for TEM-1, SHV-1 and selected single-mutant extended-spectrum β-lactamase derivatives

Substrate	Catalytic efficiency, k_{cat}/K_m ($mM^{-1}s^{-1}$)							Relative rate of hydrolysis, V_{rel} (%)	
	TEM-1[43][a,d]	TEM Gly238Ser (TEM-19) [43][a,d]	TEM-1[46][b,d]	TEM Arg164Ser (TEM-12) [46][b,d]	SHV-1[47][c,d]	SHV Gly238Ser (SHV-2) [47][c,d]	SHV Gly238Ala [47][c,d]	SHV-1[e]	SHV-24 (Asp179Gly) [32][f]
PEN	17 100	341	13 000	910	35 000	2500	11 000	NA	–
AMP	28 600	1120	39 000	420	20 000	3000	5800	312.5	84.4
LOR	1360	1310	1200	92	3000	10 000	2300	100	100
CTX	2.77	144	0.78	1.1	ND	600	400	–	–
CAZ	0.055	3.04	0.04	5.2	–	–	–	0.04	1.8

PEN, benzylpenicillin; AMP, ampicillin; LOR, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime, NA, not analysed; ND, not determinable.
[a]This study was carried out with artificial mutants; TEM Gly238Ser corresponds to TEM-19.
[b]This study was carried out with artificial mutants; TEM Arg164Ser corresponds to TEM-12.
[c]This study was carried out with artificial mutants; SHV Gly238Ser corresponds to SHV-2; the SHV Gly238Ala single mutant has never been identified in nature.
[d]k_{cat}/K_m values were recalculated to $mM^{-1}s^{-1}$ for comparison purposes.
[e]Data for SHV-1 are from Ref. [5] and were recalculated with the hydrolysis rate against cephaloridine taken as 100%.
[f]This study was carried out with natural SHV-24 (Asp179Gly) enzyme; V_{rel} values were calculated from the original V_{max} values, with the V_{max} against cephaloridine taken as 100%.

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Table 2. Susceptibility of *Escherichia coli* laboratory strains producing TEM-1, SHV-1 and their selected single-mutant extended-spectrum β-lactamase derivatives

β-Lactam	MIC (mg/L)								
	TEM-1 [43][a]	TEM Gly238Ser (TEM-19) [43][a]	TEM-1 [48][b]	TEM Arg164Ser (TEM-12) [48][b]	SHV-1 [47][c]	SHV Gly238Ser (SHV-2) [47][c]	SHV Gly238Ala [47][c]	SHV-1 [49][d]	SHV Asp179Asn (SHV-8) [49][d]
AMP/AMX	4096	1024	>2048	>2048	16 000	8200	8200	NA	–
LOT	128	64	–	–	32	64	512	8	3
LOR	128	64	32	16	128	16	256	–	–
CTX	0.06	0.5	0.03	0.12	0.06	8	8	≤0.016	0.032
CAZ	0.25	0.5	0.12	4	1	8	8	0.094	2

AMP, ampicillin, AMX, amoxycillin; LOT, cephalothin; LOR, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime; NA, not analysed.
[a]This study was carried out with *E. coli* XL1-Blue producing TEM-1 and artificial TEM mutants; TEM Gly238Ser corresponds to TEM-19.
[b]This study was carried out with *E. coli* RYC1000 (OmpF[+]) producing TEM-1 and artificial TEM mutants; TEM Arg164Ser corresponds to TEM-12.
[c]This study was carried out with *E. coli* DH10B producing SHV-1 and artificial SHV mutants encoded by a high-copy vector; SHV Gly238Ser corresponds to SHV-2.
[d]This study was carried out with *E. coli* DH5α producing SHV-1 and artificial SHV mutants expressed by a weak promoter; SHV Asp179Asn corresponds to SHV-8.

lactams were increased when compared with TEM-1 or SHV-1 producers, but usually only to relatively low levels [43,47–49]. Significant quantitative differences could be seen between particular experimental models, reflecting differences among the various plasmid vectors and *E. coli* host strains used. For producers of enzymes with Arg164 and Asp179 mutations, MICs of ceftazidime were much higher than those of cefotaxime [48,49]. The MICs of penicillins were lower than those for TEM-1 and SHV-1 producers, although high-level resistance was usually retained.

The decrease in activity against penicillins accompanying major ESBL-type mutations is a good example of the trade-off between different protein functions. The conformational changes that result from these mutations lead to structural defects that affect protein folding or stability [50,51]. The TEM Gly238Ser mutant (TEM-19) had lower thermal stability, was more prone to trypsin proteolysis and equilibrium denaturation by guanidinium hydrochloride [52] and showed increased aggregation as compared with TEM-1 [53]. The ESBL-type mutations observed in nature are not the only ones to confer ESBL activity. Other substitutions do so in in-vitro studies, but some of these are associated with a much greater misbalance between the two activities and a greater decrease in activity against penicillins [42, 54, 55]. A good example is the TEM mutant Asp179Gly, which has never been found in nature. The amoxycillin MIC for the producer was only 64 mg/L, limiting the chance of selection in clinical environments, where penicillins are still in use [42]. The only natural TEM Asp179 mutant, TEM-126, has glutamic acid, which resembles the original aspartic acid, at this position; moreover, TEM-126 also carries the stabilising mutation Met182Thr, discussed in detail below [41]. A similar situation arises with substitution of arginine for leucine at position 169. A TEM Leu169Arg mutant was obtained in an in-vitro evolution study, and its producer had a significant increase in ceftazidime MIC when compared to the parent TEM-1 producer (4 vs. 0.25 mg/L) and a major decrease in amoxycillin MIC (8 vs. >1024 mg/L) [42]. A natural SHV ESBL variant carrying the single Leu169Arg mutation was identified and designated SHV-57. The enzyme showed a 100 000-fold decrease of k_{cat}/K_m against benzylpenicillin,

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but the ampicillin MIC for the producer was >32 mg/L. It is proposed that mutation at position 169 induces a significant conformational change of Asn170, one of the critical residues in the Ω-loop [26]. The negative effects of the ESBL mutations may concern not only structure and stability but also expression. Hujer *et al.* [47] have found that SHV Gly238 mutants are expressed at lower levels than SHV-1, owing to decreased translation. This phenomenon has not been observed for TEM ESBLs with single mutations—another significant difference between TEMs and SHVs.

As already mentioned, the levels of resistance to oxyimino-β-lactams are low in *E. coli* recombinant strains with single ESBL mutants, and this is also true for many clinical isolates that appear to be susceptible at CLSI breakpoints. There are several ways for bacteria to 'improve' their resistance. One way is by acquiring another resistance mechanism, often by lowering permeability of the outer-membrane [31,56–58]. Blázquez *et al.* analysed the influence of outer-membrane permeability on the resistance level conferred by single-mutant ESBLs by comparing the MICs for *E. coli* strains with or without porin OmpF, and producing either TEM-12 or TEM-19, expressed from the same plasmid vector. The results, shown in Table 3, demonstrated the very significant effect of porin alteration on the resistance to oxyimino-β-lactams [48]. Another way of increasing resistance is by raising the ESBL expression by promoter mutations or replacements (e.g., those delivered by insertion sequences, IS) or by ESBL gene copy amplification [59–63]. Randegger *et al.* investigated the effect of weak vs. strong promoters, located in front of various bla_{SHV} genes, on resistance levels of isogenic producer *E. coli* strains. A strong promoter was better able to increase resistance to oxyimino-β-lactams in strains expressing the single mutants SHV-2 Gly238Ser and SHV-8 Asp179Asn (Table 3) [49]. Various combinations of multiple factors must be responsible for the wide ranges of β-lactam MICs observed in clinical isolates producing single-mutant ESBLs [28,31,32]. In a study by Juteršek *et al.* [37], MICs of cefotaxime for a group of SHV-2-producing *K. pneumoniae* isolates from a single hospital in Slovenia ranged from 1 to 32 mg/L, and those of ceftazidime from 0.5 to 16 mg/L.

Table 3. Susceptibility of *Escherichia coli* laboratory derivatives producing selected single-mutant extended-spectrum β-lactamase derivatives of TEM-1 and SHV-1

	MIC (mg/L)							
β-Lactam	**TEM Gly238Ser (TEM-19) OmpF+[48][a]**	**TEM Gly238Ser (TEM-19) OmpF−[48][a]**	**TEM Arg164Ser (TEM-12) OmpF+[48][a]**	**TEM Arg164Ser (TEM-12) OmpF−[48][a]**	**SHV Gly238Ser (SHV-2) weak promoter [49][b]**	**SHV Gly238Ser (SHV-2) strong promoter [49][b]**	**SHV Asp179Asn (SHV-8) weak promoter [49][b]**	**SHV Asp179Asn (SHV-8) strong promoter [49][b]**
AMX	2048	2048	>2048	>2048	NA	–	–	–
LOT	–	–	–	–	>256	>256	3	6
LOR	32	64	16	64	–	–	–	–
CTX	0.06	1	0.12	0.25	4	12	0.032	1
CAZ	0.25	1	4	32	0.5	1	2	16

AMX, amoxycillin; LOT, cephalothin; LOR, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime; NA, not analysed.
[a]This study was carried out with *E. coli* RYC1000 (OmpF+) and MH621 (OmpF−) producing TEM-1 and artificial TEM mutants; TEM Gly238Ser and TEM Arg164Ser correspond to TEM-19 and TEM-12, respectively.
[b]This study was carried out with *E. coli* DH5α producing SHV-1 and artificial SHV mutants; SHV Gly238Ser and SHV Asp179Asn correspond to SHV-2 and SHV-8, respectively.

MULTIPLE MUTATIONS IN TEM AND SHV ESBLS

The third way of increasing ESBL-related resistance is the further evolution of the β-lactamases themselves, often by further mutations at Glu240 and Glu104. Glu240 is very often replaced in natural TEM (n = 27) and SHV (n = 17) enzymes, and is almost always changed to a basic amino acid, lysine (http://www.lahey.org/studies/webt.asp) or, rarely, arginine (TEM-137 [64] and SHV-86 (GenBank DQ328802)). TEM-149 (GenBank DQ105529) is the only enzyme with valine in this position. Except for SHV-31 [65] and SHV-97 (GenBank EF373973), the Glu240Lys/Arg mutation has always been identified in enzymes carrying one of the major ESBL-type mutations at position 238 or 164 (http://www.lahey.org/studies/webt.asp). The role of the mutation, however, has been well-studied with in-vitro mutants, including single TEM Glu240Lys mutants [66,67]. Position 240 is located at the end of the B3 β-strand, and the replacement of glutamic acid by a basic residue results in the formation of an electrostatic bond with the carboxyl group of the oxyimino substituent in ceftazidime and aztreonam. Most probably, the mutation does not have much effect on the enzyme structure [14]. Since several pairs of natural ESBLs show a difference only in the presence of Glu240Lys, e.g., TEM-12 and TEM-10 [68], TEM-25 and TEM-48 [69], and SHV-2 and SHV-5 [70], it follows that the mutation probably appeared independently several times in the evolution of TEM and SHV ESBLs.

Mutation at position 104 has been found only in the TEM family so far, occurring in as many as 41 variants and coexisting with mutations of Gly238 or Arg164 in 36 of these. In all cases, the glutamic acid residue is replaced by the basic residue lysine (http://www.lahey.org/studies/webt.asp). The single Glu104Lys mutant of TEM-1 identified in nature is TEM-17 [71] and that of TEM-2 is TEM-18 [72], whereas the consequences were studied in detail for a laboratory mutant [66]. Owing to protein folding, position 104 is located close to the entrance to the active site, and thus, as for position 240, Lys104 most probably interacts with carboxyl groups of ceftazidime and aztreonam [14]. It is also postulated that Glu104Lys stabilises the cavity structure affected by the mutation Gly238-Ser [22], and this was confirmed by crystallographic study of the TEM-52 enzyme [11]. In several pairs, the TEM enzymes differ only by the presence of the mutation Glu104Lys, e.g., TEM-1 vs. TEM-17 [71], TEM-2 vs. TEM-18 [72], or TEM-25 vs. TEM-4 [10,29], suggesting several independent selections in nature.

In the study by Sowek *et al.* [66], mutations Glu240Lys and Glu104Lys behaved similarly to each other (Table 4). Both single artificial mutants, TEM Glu240Lys and TEM-17 Glu104Lys, were more active against ceftazidime than TEM-1 (30-fold and 22.5-fold increase in k_{cat}/K_m, respectively), although the difference was much less profound than for TEM-12 Arg164Ser (650-fold). On the other hand, Venkatachalam *et al.* [67] studied Glu240Lys in the context of the Gly238Ser mutation, and observed an increase of k_{cat}/K_m for the single Glu240Lys mutant against ceftazidime as compared with TEM-1 (85-fold). This change was comparable to that of TEM-19 Gly238Ser (80-fold increase in k_{cat}/K_m); however, the Glu240Lys mutant was much less active against cefotaxime than TEM-19 (2.2-fold and 45.5-fold increases in k_{cat}/K_m, respectively). Both studies demonstrated a dramatic increase in catalytic efficiency against ceftazidime in the three double mutants, i.e., Arg164Ser/Glu240Lys, Glu104Lys/Arg164Ser and Gly238Ser/Glu240Lys, measured as the 4450-fold, 19 000-fold and 2855-fold increase in k_{cat}/K_m, respectively, as compared with TEM-1. These double mutants, corresponding to TEM-10, TEM-26 and TEM-71 [73–75], respectively, were much more active against ceftazidime (and aztreonam) than the single-mutant ESBLs. Mutations Glu240Lys and Glu104Lys do not significantly affect hydrolysis of penicillins or narrow-spectrum cephalosporins [66,67].

The role of Glu240Lys and Glu104Lys mutations is illustrated in Table 5, which shows MIC data from several studies on *E. coli* recombinants and clinical isolates producing various β-lactamases with mutations at positions 240, 104, 238 and 164. The MICs of oxyimino-β-lactams with single or double mutations at Glu240Lys and Gly238Ser produced by *E. coli* recombinants strictly followed the differences in catalytic efficiency [67]. Similar observations were made in isogenic *E. coli* strains producing TEM-2, TEM-18 or TEM-3 (the Glu104Lys/Gly238Ser variant of TEM-2), with slightly raised MICs of ceftazidime and cefotaxime for the TEM-18 producer and dramatically increased MICs of both compounds

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Table 4. Catalytic efficiency of hydrolysis by TEM-1 and its selected mutant derivatives

Substrate	Catalytic efficiency, k_{cat}/K_m ($mM^{-1}s^{-1}$) TEM-1 [66][a]	TEM Arg164Ser (TEM-12) [66][a]	TEM Glu240Lys [66][a,c]	TEM Arg164Ser/ Glu240Lys (TEM-10) [66][a]	TEM Glu104Lys (TEM-17) [66][a]	TEM Glu104Lys/ Arg164Ser (TEM-26) [66][a]	TEM-1 [67][b,d]	TEM Gly238Ser (TEM-19) [67][b,d]	TEM Glu240Lys [67][b,d]	TEM Gly238Ser/ Glu240Lys (TEM-71) [67][b,d]
PEN	20 000	3400	13 000	3000	19 000	5500	NA	–	–	–
AMP	–	–	–	–	–	–	20 400	1100	14 500	1290
LOR	1100	460	1300	210	1900	330	1370	804	1010	410
CTX	0.56	10	4.7	12	5.3	24	3.9	178	8.5	468
CAZ	0.02	13	0.61	89	0.45	380	0.02	1.6	1.66	57.1

PEN, benzylpenicillin; AMP, ampicillin; LOR, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime; NA, not analysed.
[a]This study was carried out with artificial mutants; TEM Arg164Ser corresponds to TEM-12, TEM Arg164Ser/Glu240Lys to TEM-10, TEM Glu104Lys to TEM-17, and TEM Glu104Lys/Arg164Ser to TEM-26.
[b]This study was carried out with artificial mutants; TEM Gly238Ser corresponds to TEM-19, and TEM Gly238Ser/Glu240Lys to TEM-71.
[c]The TEM Glu240Lys single mutant has never been observed in nature.
[d]k_{cat}/K_m values were recalculated to $mM^{-1}s^{-1}$ for comparison purposes.

Table 5. Susceptibility of organisms producing selected artificial or natural mutant extended-spectrum β-lactamase derivatives of TEM-1 and TEM-2

β-Lactam	MIC (mg/L) TEM-1 [67][a]	TEM Gly238Ser (TEM-19) [67][a]	TEM Glu240Lys [67][a]	TEM Glu238Ser/ Glu240Lys (TEM-71) [67][a]	TEM-2 (Glu39Lys) [72][b]	TEM-18 (Glu39Lys/ Glu104Lys) [72][b]	TEM-3 (Glu39Lys/ Glu104Lys/ Gly238Ser) [72][b]	No TEM [71][c]	TEM-17 (Glu104Lys) [71][c]
AMP/AMX	1024	512	2048	1024	4096	8192	8192	0.125	512
LOT	NA	–	–	–	32	16	128	–	–
CTX	0.03	0.25	0.03	1	≤0.06	0.25	64	0.015	16
CAZ	0.125	0.5	0.5	16	0.5	4	32	0.03	64

AMP, ampicillin; AMX, amoxycillin; LOT, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; NA, not analysed.
[a]This study was carried out with *Escherichia coli* XL1-Blue producing artificial TEM-1 derivatives; TEM Gly238Ser corresponds to TEM-19, and TEM Gly238Ser/Glu240Lys to TEM-71; TEM Glu240Lys has not been identified in nature so far.
[b]This study was carried out with *E. coli* recombinants producing TEM-2 Gln39Lys, TEM-18 Gln39Lys/Glu104Lys and TEM-3 Gln39Lys/Glu104Lys/Gly238Ser encoded by natural genes.
[c]This study was carried out with a *Capnocytophaga ochracea* clinical isolate producing TEM-17 Glu104Lys and its laboratory derivative without this or any other TEM enzyme.

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Table 6. Susceptibility of clinical isolates or *Escherichia coli* transconjugants producing selected natural double-mutant TEM and SHV extended-spectrum β-lactamases with mutations Glu240Lys or Glu104Lys

	MIC (mg/L)					
β-Lactam	**TEM-10 (Arg164Ser/ Glu240Lys) [73][a]**	**TEM-28 (Arg164His/ Glu240Lys) [78][b]**	**TEM-26 (Glu104Lys/ Arg164Ser) [76][c]**	**TEM-6 (Glu104Lys/ Arg164His) [77][d]**	**TEM-71 (Gly238Ser/ Glu240Lys) [75][e]**	**SHV-5 (Gly238Ser/ Glu240Lys) [37][f]**
AMP/AMX	>256	NA	–	2000	>64	>512
CTX	0.5	8	1	1	8	16
CAZ	64	>32	256	128	32	256

AMP, ampicillin; AMX, amoxycillin; CTX, cefotaxime; CAZ, ceftazidime; NA, not analysed.
[a]This study was carried out with *Klebsiella pneumoniae* clinical isolates producing TEM-10 (Arg164Ser/Glu240Lys).
[b]This study was carried out with an *E. coli* clinical isolate producing TEM-28 (Arg164His/Glu240Lys).
[c]This study was carried out with the *E. coli* J53-2 transconjugant producing TEM-26 (YOU-1) (Glu104Lys/Arg164Ser).
[d]This study was carried out with the *E. coli* C600 transconjugant producing TEM-6 (Glu104Lys/Arg164His).
[e]This study was carried out with a *K. pneumoniae* clinical isolate producing TEM-71 (Gly238Ser/Glu240Lys).
[f]This study was carried out with *K. pneumoniae* clinical isolates producing SHV-5 (Gly238Ser/Glu240Lys).

for the TEM-3 producer [72]. Clinical isolates producing various double-mutant ESBLs at positions 240 or 104 and 238 or 164 are usually characterised by clear resistance to oxyimino-β-lactams, especially in combinations with mutations at position 164 for ceftazidime (Table 6) [37,73,75–78]. Some such enzymes, e.g., TEM-3, TEM-10, TEM-26, SHV-5 and SHV-12 (the Gly238-Ser/Glu240Lys mutants of SHV-1 and SHV-11, respectively), are among the most widespread ESBL variants [36,74,76,79–82]. Interestingly, a remarkable effect on resistance of the Glu104Lys mutation alone was observed in a study in which a TEM-17-producing isolate of *Capnocytophaga ochracea* was compared with its ESBL-negative derivative, obtained by plasmid curing. The MICs of both cefotaxime and ceftazidime were much reduced by plasmid loss (Table 5) [71]. Similarly, production of SHV-31, which is a Glu240Lys mutant of SHV-11, increased the MICs of oxyimino-β-lactams, especially ceftazidime and aztreonam, significantly for an *E. coli* recombinant [65].

There are fewer data concerning the ESBL mutations Gly156Asp and Arg205Leu. The first of these has been identified alone in SHV-27 [25], and together with other mutations in SHV-32 [83], SHV-45 (GenBank AF547625) and SHV-93 (GenBank EF373969). The SHV-27-producing *K. pneumoniae* isolate was clearly resistant to cefotaxime (MIC 96 mg/L) and ceftazidime (MIC 8 mg/L) [25]. Arg205Leu was found alone in SHV-44 [84], and occurs also in SHV-3 and SHV-4, both of which also possess major ESBL-type substitutions [85,86]. The MICs of oxyimino-β-lactams for *E. coli* recombinant with SHV-44 were not increased significantly [84], although it has been proposed that the mutation may cause a conformational change that allows such substrates better access to the active site [14].

Apart from the mutations that increase oxyimino-β-lactam-hydrolysing activity directly, other significant mutations occur in ESBLs. One such mutation occurs at position 237 and has so far been observed exclusively in the TEM family, occurring in nine variants. In all but one of these, Ala237 is replaced by threonine; the exception is TEM-22, with glycine at this position [87]. These Ala237 mutations have been observed only in ESBLs, and almost always in those with the Arg164Ser ESBL-type mutation (http://www.lahey.org/studies/webt.asp). Ala237Thr was probably selected in several independent events, as variants in several pairs differ from each other only by this substitution, e.g., TEM-10 and TEM-5 [88], TEM-46 and TEM-24 [89], or TEM-85 and TEM-86 [10]. Position 237 is located in the B3 β-strand, and the Ala237Thr replacement has been postulated to lead to new hydrogen bond formation with some substrates (e.g., cefotaxime) [14].

In the study by Healey *et al.* [90], the single laboratory mutant Ala237Thr showed four-fold higher activity vs. cephalosporin C and cephalothin and ten-fold lower activity against penicillins than TEM-1. In a comparative analysis of TEM-46 (Gln39Lys/Glu104Lys/Arg164Ser/Glu240Lys)

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Table 7. Susceptibility of *Escherichia coli* recombinants producing selected artificial mutant TEM β-lactamases with mutations Ala237Thr or Met182Thr

β-Lactam	MIC (mg/L) TEM-1 [88][a]	TEM Ala237Thr [88][a]	TEM Arg164Ser/ Glu240Lys (TEM-10) [88][a]	TEM Arg164Ser/ Ala237Thr/ Glu240Lys (TEM-5) [88][a]	TEM-1 [50][b]	TEM Met182Thr (TEM-135) [50][b]	TEM Leu76Asn [50][b]	TEM Leu76Asn/ Met182Thr [50][b]
AMP	>2048	2048	>2048	1024	3000	2000	100	2000
LOT	32	32	16	128	NA	–	–	–
CTX	0.03	0.03	0.5	4	–	–	–	–
CAZ	0.12	0.12	128	16	–	–	–	–

AMP, ampicillin, LOT, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; NA, not analysed.
[a]This study was carried out with *E. coli* RYC1000 transformants producing TEM-1 or its laboratory mutants; TEM Arg164Ser/Glu240Lys corresponds to TEM-10, and TEM Arg164Ser/Ala237Thr/Glu240Lys to TEM-5; the TEM Ala237Thr mutant has not been observed in nature.
[b]This study was carried out with *E. coli* XL1-Blue transformants producing TEM-1 or its artificial mutants; only the Met182Thr single mutant has been identified in nature, TEM-135 (http://www.lahey.org/studies/webt.asp), but no data on it have been published as yet.

and TEM-24 (Gln39Lys/Glu104Lys/Arg164Ser/Ala237Thr/Glu240Lys), the Ala237Thr mutation increased catalytic efficiency against cephalothin and cefotaxime, and decreased that against penicillins [89]. These results led to the conclusion that the mutation stimulates hydrolysis of cephems and decreases that of penams. However, comparison of TEM-10 (Arg164Ser/Glu240Lys) and TEM-5 (Arg164Ser/Ala237Thr/Glu240Lys) [88], and TEM-85 (Leu21Phe/Arg164Ser/Glu240-Lys/Thr265Met) and TEM-86 (Leu21Phe/-Arg164Ser/Ala237Thr/Glu240Lys/Thr265Met) [10], revealed a more complex picture. A clear increase in cephalothin and cefotaxime MICs was accompanied by a decrease in the MICs of amoxycillin, piperacillin, ceftazidime and aztreonam; therefore, the mutation was described as modulating activity against particular substrates (Table 7). It may confer a selective advantage in clinical environments where a variety of β-lactams are in use [88].

As with position 237, mutation at position 182 has been identified only in natural TEM mutants so far. Found in 20 variants, it always entails replacement of methionine by threonine (http://www.lahey.org/studies/webt.asp). Except in TEM-135 (http://www.lahey.org/studies/webt.asp), the Met182Thr mutation has always been observed in ESBLs (18 variants) or inhibitor-resistant β-lactamases (one variant). Some of the enzymes with this substitution are characterised by the unusual combinations of ESBL-type mutations, as with TEM-106 (Glu104Lys/Met182Thr) (GenBank AY101578), TEM-124 (Gln6Lys/Glu104Lys/-Met182Thr) (GenBank AY327540), and TEM-126 (Asp179Glu/Met182Thr) [41]. The Met182Thr mutation was probably selected repeatedly on several independent occasions, in the evolution of, e.g., TEM-43 from TEM-6 [91], TEM-52 from TEM-15 [92], or TEM-94 from TEM-4 [10]. Interestingly, position 182 is relatively far away from the active site; however, it is located in the important hinge region between the two major class A β-lactamase domains. Crystallographic study of TEM-52 showed that Thr182 formed new intramolecular hydrogen bonds with two residues of one of the domains, Glu63 and Glu64, which may stabilise the active site topology reorganised by other mutations [11].

Huang and Palzkill showed that mutation Met182Thr increased the activity of laboratory TEM variants with severe structure and/or stability defects caused by other mutations. This was especially profound in the case of mutations at Leu76 (never observed in nature), which caused *E. coli* producers to have 12- to 30-fold lower ampicillin MICs than the TEM-1 producer. Strains producing the double-mutant enzymes, TEM with Leu76Asn/Ser and Met182Thr, by contrast, were characterised by ampicillin MICs only 1.5- to two-fold lower than those for the TEM-1 producer (Table 7). In another laboratory-selected inhibitor-

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Table 8. Catalytic efficiency and inhibition profiles of selected complex mutant TEM (CMT) β-lactamases, compared with their extended-spectrum β-lactamase and inhibitor-resistant TEM (IRT) counterparts

Compound	TEM-3 (Glu39Lys/ Glu104Lys/ Gly238Ser) [101][a]	TEM-59 (Glu39Lys/ Ser130Gly) [101][a]	TEM-89 (Glu39Lys/ Glu104Lys/ Ser130Gly/ Gly238Ser) [101][a]	TEM-6 (Glu104Lys/ Arg164His) [102][b]	TEM-33 (Met69Leu) [102][b]	TEM-109 (Met69Leu/ Glu104Lys/ Arg164His) [102][b]
Catalytic efficiency, k_{cat}/K_m ($mM^{-1}s^{-1}$)						
AMX	6.2	0.2	1.79	4.0	5.0	4.0
LOT	2.1	<0.01	0.06	0.3	0.01	0.1
CTX	2.0	<0.01	<0.01	0.1	ND	0.1
CAZ	0.1	<0.01	<0.01	0.4	ND	0.2
IC_{50} for inhibition (μM)						
CLA	0.01	100	90	0.01	1.9	0.13
TAZ	0.01	7	8	0.06	2.3	0.27

AMX, amoxycillin; LOT, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; CLA, clavulanic acid; TAZ, tazobactam; ND, not determinable.
[a]This study was carried out with natural TEM variants, TEM-3 (Gln39Lys/Glu104Lys/Gly238Ser), TEM-59/IRT-17 (Gln39Lys/Ser130Gly) and TEM-89/CMT-3 (Gln39Lys/Glu104Lys/Ser130Gly/Gly238Ser).
[b]This study was carried out with natural TEM variants, TEM-6 (Glu104Lys/Arg164His), TEM-33/IRT-5 (Met69Leu) and TEM-109/CMT-5 (Met69Leu/Glu104Lys/Arg164His).

resistant enzyme with mutations Met69Ile and Met182Thr (equivalent to natural TEM-32), mutation Met182Thr improved thermal stability compared with the single mutation Met69Ile. These authors concluded that the mutation acts as a global suppressor of the defects in β-lactamase structure or stability caused by the ESBL or inhibitor resistance (IR) mutations that alter the active site region [50]. In another study undertaken by the same group, Met182Thr was shown to reduce aggregation of the TEM Gly238Ser mutant enzyme [53]. Similarly, an enzyme-stabilising role was proposed for mutations Ala42Val, observed naturally in TEM-42 [11,93], and Leu51-Pro, identified in TEM-60 [94].

While some mutations confer ESBL activity, others diminish susceptibility to site-directed inhibitors (IR-type mutations). These occur at positions 69, 130, 187, 244, 275 and 276 of TEM and SHV enzymes, and have been extensively studied and reviewed [14,95,96]. Since IR mutations do not directly affect β-lactam hydrolysis, they will not be discussed in detail here. However, these mutations usually reduce the activity of TEM-1 against penicillins and, especially, narrow-spectrum cephalosporins [50,97,98], and may affect significantly any ESBL activity. From the mid-1990s, scientists have asked: 'what will happen when ESBL and IR mutations occur together in a single enzyme?' This intriguing question has been addressed by several laboratories working on artificial or natural variants dubbed 'complex mutant enzymes'. At least nine natural TEM or SHV β-lactamases with both ESBL- and IR-type mutations have been identified, comprising TEM-50, TEM-68, TEM-89, TEM-109, TEM-121, TEM-125, TEM-151, TEM-152 and SHV-10 [99–106]. As compared with their ESBL and IR β-lactamase counterparts (with only one mutation type) or with classic ESBLs, a compromise in one or both properties was usually observed (Table 8). TEM-89 and SHV-10 have no significant ESBL activity but are clearly inhibitor-resistant [101,105], and TEM-125 is a relatively weak ESBL, but has greatly increased resistance to inhibitors [104]. TEM-68, TEM-109 and TEM-121 are efficient ESBLs, at least for some oxyimino-β-lactams, but have only modestly reduced susceptibility to inhibitors [100,102,103]. Finally, TEM-50 combines both properties at moderate levels [99]. Similar observations were made in studies on laboratory mutants [107–109].

AN SHV 'CARBAPENEMASE'

Mutation at position 146 deserves a separate discussion. The substitution of valine for alanine at this site was identified as a single change in enzyme SHV-38, encoded by a chromosomal gene, most probably a direct derivative of the natural *K. pneu-*

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moniae bla_{SHV-1}. Although SHV-38 was not originally defined as an ESBL, the mutation was responsible for a marked increase in catalytic efficiency against cefotaxime and ceftazidime. At the same time, a decrease of activity against amoxycillin and cephalothin was observed. However, the most interesting factor is that the enzyme hydrolysed imipenem at a significant rate. The *E. coli* recombinant was clearly resistant to ceftazidime and showed an almost ten-fold increase in the imipenem MIC, as compared with the SHV-1 producer, even though it remained susceptible to the compound. It has been hypothesised that the mutation may be a starting point in the evolution of SHV-type enzymes that would combine the ESBL and carbapenemase activities, with potential 'improvement' via accumulation of further mutations [27]. Recently, three other variants with mutation Ala146Val (SHV-71, GenBank AM176546; SHV-72, AM176547; SHV-73, AM176548), and, interestingly, one with mutation Ala146Thr (SHV-80, GenBank AM176555), were identified; however, no data concerning these enzymes have been published as yet.

ACCUMULATION OF MUTATIONS IN TEM AND SHV ESBLS

In-vivo evolution of ESBLs is observed only very rarely. One occasion, however, was documented by Rasheed *et al.* [31], who analysed a series of *E. coli* isolates collected over a 3-month course of infection in a patient who had experienced several bacteraemic episodes. Comparison of the isolates revealed a complex pattern of linear and radiative evolution from a single initial strain, clearly reflecting the antimicrobial treatment profile and sequence. Among a variety of events observed was the acquisition of a point mutation by the bla_{SHV-1} gene, giving rise to bla_{SHV-8} (the Asp179Asn substitution in the protein sequence), encoding an ESBL. In another study, Bradford *et al.* [68] identified two ESBL genes, differing by one nucleotide, in a single *K. pneumoniae* isolate. The bla_{TEM-10} gene most probably emerged as a result of duplication of bla_{TEM-12}, followed by an additional mutation, determining the Glu240Lys substitution.

The vast majority of TEM- and SHV-type ESBLs identified worldwide in clinical isolates possess more than one or two mutations, as compared with TEM-1, TEM-2, SHV-1 and SHV-11. Review of both the non-synonymous (entailing amino-acid substitutions) and silent mutations in ESBL genes allows reconstruction of their evolutionary history, although often only partially. One such study considered ten TEM ESBL variants identified in Poland [10], six of which were probably related to each other and might originate from the TEM-25 enzyme reported in France [29] (Fig. 2). TEM-25 is characterised by amino-acid substitutions Leu21Phe, Gly238Ser and Thr265Met, and the 'Polish' variants acquired numerous further step-by-step mutations, mostly with a clear functional role, e.g., Glu104Lys, Glu240Lys, Met182Thr or Arg275Leu.

In some circumstances, combinations of mutations may have an effect when the individual mutations do not, as was the case of Met182Thr for example (discussed above) [50]. Two mutations with strictly defined functions may counteract each other, rather than having an additive effect. Several examples have already been presented from among the complex mutant β-lactamases possessing both ESBL- and IR-type substitutions [101,105]. Interestingly, the combination of the Arg164Ser and Gly238Ser major ESBL-type mutations also appears to be detrimental for enzymic activity against various substrates, especially cefotaxime and cephalothin [110]. Nevertheless, it has been observed once in nature, in TEM-8, a strong ESBL that, however, also has the Glu104Lys mutation [111].

The accumulation of mutations by bla_{TEM-1} under the selective pressure of cefotaxime, and

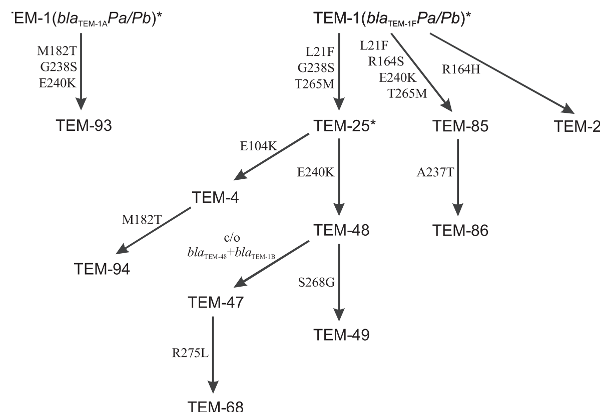

Fig. 2. Possible pathways of evolution of TEM-type extended-spectrum β-lactamases in Poland. Reproduced with permission from Baraniak *et al.*, *Antimicrob Agents Chemother* 2005; **49**: 1872–1880. © American Society for Microbiology.

their reciprocal effects on each other, were examined in the directed evolution study by Stemmer [112]. This study, performed with the in-vitro DNA shuffling approach, revealed that a TEM mutant bearing three amino acid substitutions, Glu104Lys, Met182Thr and Gly238Ser, conferred a 500-fold greater resistance to cefotaxime than TEM-1. The additional presence of Ala42Gly, Gly92Ser and Arg241His substitutions, and a promoter up mutation, G4205A, raised the cefotaxime resistance level 32 000-fold. Two subsequent studies, using the DNA analogue technique [113] or in-vivo DNA shuffling in a hypermutator *E. coli* strain [11], demonstrated again the selection of the triple Glu104Lys/Met182Thr/Gly238-Ser mutant, which had, in the meantime, been identified as the natural variant TEM-52 [92]. It has been hypothesised that the TEM-52-specific set of mutations might be a result of a preferred evolutionary pathway of accumulation of ESBL-type mutations, and that the 'supereffective' Stemmer's mutant with six changes might represent a 'new-generation' ESBL soon to appear under natural conditions [11]. However, Hall described the likelihood of the future selection of this set of six amino-acid substitutions as being rather low. In his site-directed mutagenesis study, Hall demonstrated a negative effect of mutations Gly92Ser and Arg241His on the quadruple mutant Ala42Gly/Glu104Lys/Met182Thr/Gly238Ser, which was already 85 333 times more effective than TEM-1. This quadruple mutant ESBL might arise from the acquisition of an Ala42Gly mutation by the natural TEM-52 enzyme [114]. Another mutation at position 42 (Ala42Val) has already been observed in nature, although in another sequence context (TEM-42) [93].

Recently, Weinreich *et al.* [115] analysed the pathways of accumulation of the Ala42Gly, Glu104Lys, Gly238Ser and Met182Thr substitutions, and the promoter mutation, G4205A, which raises the expression of TEM β-lactamases *c.* 2.5-fold. The striking conclusion was that, among 120 possible trajectories of mutation accumulation, only 18 were accessible for natural selection with cefotaxime, and the most favourable sequence was Gly238Ser → Glu104Lys → Ala42Gly → Met182Thr → G4205A. Many other possible intermediate combinations of the mutations had either negative or negligible effects on cefotaxime resistance of *E. coli* producing the enzymes, and thus seem unlikely [115].

OTHER CLASS A ESBLS

The evolution of ESBLs by mutation is not limited to the TEM and SHV families. During the last two decades, numerous other class A enzymes with ESBL activity have been identified, namely the CTX-M [116], PER [117], VEB [118], GES [119], BES [7], TLA [120], SFO [121] and BEL [122] families. The most diversified are the CTX-M and GES families, both of which demonstrated interesting examples of activity modifications due to amino-acid substitutions. The CTX-M family has become very prevalent recently in many countries [8,123,124]. In contrast to TEM and SHV enzymes, the members of this family have intrinsic ESBL activity, apparent also in their natural homologues in the genus *Kluyvera* [125–129], from which the $bla_{\text{CTX-M}}$ genes probably emerged through several mobilisation events [8]. These gene pools have undergone evolution, both in *Kluyvera* and since their mobilisation. The first phase of this process led to their diversification into five subfamilies, designated CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25, with inter-subfamily amino-acid identity of ≤90% [8]. The second phase has been microevolution within the subfamilies, probably both in *Kluyvera* and, for the mobile $bla_{\text{CTX-M}}$ genes, elsewhere. This has produced more than 60 variants so far, with intra-subfamily amino acid identities of >94% [8] (http://www.lahey.org/studies/webt.asp). The very rapid microevolution of CTX-M enzymes over the last decade is difficult to explain, as is their enormous 'epidemiological success'. Recent crystallographic studies reveal that, in contrast to TEM- and SHV-type ESBLs, the active site cavity volume of CTX-M enzymes is not enlarged and resembles those of TEM-1 and SHV-1 [130,131]. Activity against oxyimino-β-lactam substrates probably depends on specific interactions with side chains of the compounds, particularly via Ser237 and Asn104 [130–132].

One of the most distinctive features of CTX-M β-lactamases, recognised since their first descriptions, is their substrate preference for cefotaxime and ceftriaxone and negligible activity against ceftazidime. The most 'spectacular' aspect of the microevolution of CTX-M enzymes therefore concerns significant increases in their ceftazidime-hydrolysing activity. Mutations at two amino-acid positions have been found to be mainly responsible for these effects, both in nature and in

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Table 9. Catalytic efficiency of hydrolysis for selected CTX-M β-lactamases

	Catalytic efficiency, k_{cat}/K_m ($mM^{-1}s^{-1}$)			
Substrate	**CTX-M-3 [140][a,c]**	**CTX-M-15 (Asp240Gly) [140][a,c]**	**CTX-M-14 [138][b]**	**CTX-M-19 (Pro167Ser) [138][b]**
PEN	110 000	4000	1000	300
AMX	1000	500	100	10
LOR	500	1500	30	250
CTX	3500	3000	370	55
CAZ	ND	1	ND	0.1
FEP	1	10	40	ND

PEN, benzylpenicillin; AMX, amoxycillin; LOR, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ND, not determinable.
[a]This study was carried out with CTX-M variants; CTX-M-15 differs from CTX-M-3 only by the Asp240Gly mutation.
[b]This study was carried out with CTX-M variants, CTX-M-19 differs from CTX-M-14 (CTX-M-18) only by the Pro167Ser mutation.
[c]k_{cat}/K_m values were recalculated to $mM^{-1}s^{-1}$ for comparison purposes.

directed evolution studies. Among natural variants, the replacement of Asp240 by glycine (Asp240Gly) characterises CTX-M-15, CTX-M-16, CTX-M-25, CTX-M-27 and CTX-M-32—all with increased ceftazidimase activity—when compared with CTX-M-3, CTX-M-9, CTX-M-26-like, CTX-M-14 and CTX-M-1 enzymes, respectively [133–137]. On the other hand, substitution of Pro167 was observed in CTX-M-19 (for serine; Pro167Ser) and CTX-M-23 (for threonine; Pro167Thr); these are derivatives of CTX-M-14- and CTX-M-1-like enzymes, respectively [138,139]. Both positions are located in the active site regions, Asp240 in the B3 β-strand and Pro167 in the Ω-loop. The effect of Asp240Gly was studied at the crystal structure level (CTX-M-27) and shown not to affect the oxyanion cavity conformation but, rather, to enhance mobility of the B3 β-strand and thus, possibly, the protein flexibility. The same study showed that mutation Asp240Gly in CTX-M enzymes causes a structural defect that has a negative effect on thermal stability, as in ESBL- or IR-type TEM mutants. This observation again exemplifies the trade-off between activity and stability [131].

Comparison of CTX-M-15 vs. CTX-M-3 (mutation Asp240Gly), and of CTX-M-19 vs. CTX-M-14 (mutation Pro167Ser), shows significant increases in catalytic efficiency against ceftazidime (Table 9). Both mutations also increased activity against cephaloridine and decreased that against penicillins, like ESBL-type mutations in the TEM and SHV families. On the other hand, Asp240Gly and Pro167Ser differed greatly from each other with respect to their effects on cefotaxime- and cefepime-hydrolysing activities, and on the pattern of reductions in activity against penicillins [138,140]. The kinetic data generally correlated well with the results on susceptibility. Significant increases in ceftazidime MICs were observed, especially for producers of enzymes with the Pro167 substitutions, which were even more resistant to ceftazidime than to cefotaxime (Table 10). Despite the diminished enzyme activity, producers remained highly resistant to penicillins [136,138,139].

When *E. coli* producing CTX-M-2 was challenged with ceftazidime, only Pro167Ser mutants were obtained, with a high selection frequency [141]. In a similar study, both Asp240Gly and Pro167Ser mutants of CTX-M-9 were selected efficiently in each of three rounds of the experiment [142]. The substitution Pro167Ser was also identified in a variant of the *Klebsiella oxytoca* chromosomal β-lactamase, OXY-2-5 (which has *c.* 75% homology to CTX-M types [116,125]), leading to significantly increased activity against ceftazidime [143]. A variety of other substitutions, conferring or enhancing ceftazidime-hydrolysing activity, have been found in CTX-M derivatives during directed evolution studies, some of them at positions, or of types, known to be responsible for ESBL activity in the TEM or SHV families, i.e., Arg164His, Asp179Gly and Leu169Gln. It is possible that such CTX-M mutants will appear naturally in the future [142].

One of the newest families of class A ESBLs are GES enzymes, previously also called IBC types

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Table 10. Susceptibility of *Escherichia coli* recombinant strains producing selected CTX-M β-lactamases

	MIC (mg/L)			
β-Lactam	CTX-M-14 [136][a]	CTX-M-27 (Asp240Gly) [136][a]	CTX-M-14 [138][b]	CTX-M-19 (Pro167Ser) [138][b]
AMX	>2048	>2048	>512	>512
LOT	512	1024	>512	>512
CTX	16	16	64	4
CAZ	1	8	2	128
FEP	0.5	1	16	4

AMX, amoxycillin; LOT, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime.
[a]This study was carried out with *E. coli* DH5α transformants producing CTX-M variants, with CTX-M-27 differing from CTX-M-14 only by the Asp240Gly mutation.
[b]The data concern *E. coli* JM109 transformants producing sole CTX-M-14 (CTX-M-18) or CTX-M-19, differing from each other only by the Pro167Ser mutation.

Table 11. Catalytic efficiency of hydrolysis for selected GES β-lactamases against selected β-lactam compounds

	Catalytic efficiency, k_{cat}/K_m ($mM^{-1}s^{-1}$)			
Substrate	GES-1 [146][a]	GES-2 (Gly170Asn) [146][a]	GES-3 [149][b,c]	GES-4 (Gly170Ser) [149][b,c]
PEN	70	96	450	780
AMP/AMX	65	26	190	310
LOR	26	65	120	230
CTX	15	2.5	110	24
CAZ	188	ND	23	1.7
FOX	33	NH	NH	110
IPM	0.07	9	NH	81

PEN, benzylpenicillin; AMP, ampicillin; AMX, amoxycillin; LOR, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; IPM, imipenem; ND, not determinable; NH, not hydrolysed.
[a]This study was carried out with natural β-lactamase variants; GES-2 differs from GES-1 only by the Gly170Asn mutation.
[b]This study was carried out with natural β-lactamase variants; GES-4 differs from GES-3 only by the Gly170Ser mutation.
[c]k_{cat}/K_m values were recalculated to $mM^{-1}s^{-1}$ for comparison purposes.

[144]. The first producer isolate was identified in 1998 in France but originated from Guiana [119], and this was soon followed by reports from several countries throughout the world [145–148]. So far, nine variants have been reported; all are acquired enzymes with an unknown natural ancestor. GES β-lactamases are encoded by gene cassettes present in integrons, observed in *Pseudomonas aeruginosa* and several enterobacterial species. Despite relatively low affinity, these enzymes often confer clear resistance to various β-lactams in clinical isolates [119].

Among several point mutations that distinguish GES variants, substitutions for glycine at position 170 in the Ω-loop are especially interesting. Gly170 is replaced by asparagine (Gly170Asn) in the GES-2 enzyme as compared with GES-1 [146], and by serine (Gly170Ser) in GES-4 as compared with GES-3 [149]. Ser170 is also observed in variants GES-5 and GES-6 [150]. As shown in Table 11, the Gly170 replacements in GES-2 and GES-4 greatly reduced their activities against oxyimino-β-lactams, especially that of GES-2 toward ceftazidime. At the same time, however, these two enzymes exhibited significantly increased, although still low-level, hydrolytic activity against imipenem. Contradictory observations have been made regarding cefoxitin, for which GES enzymes mostly have relatively high affinity as compared with other class A β-lactamases, which some types can slowly hydrolyse. Whereas GES-2 showed a decrease in catalytic efficiency against cefoxitin when compared with GES-1, GES-4 was more active than GES-3 [146,149].

The heterologous expression of the two pairs of GES variants reveals the clear impact of the Gly170 mutations on β-lactam resistance pheno-

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Table 12. Susceptibility of organisms producing GES β-lactamases to β-lactams

	MIC (mg/L)					
β-Lactam	***Escherichia coli* DH10B (GES-1) [146][a]**	***E. coli* DH10B (GES-2) [146][a]**	***Pseudomonas aeruginosa* PU21 (GES-2) [146][a]**	***E. coli* XL1-Blue (GES-3) [149][b]**	***E. coli* XL1-Blue (GES-4) [149][b]**	***Klebsiella pneumoniae* cl. isol. (GES-4) [149][b]**
AMX	>512	>512	>512	>128	>128	>128
AMX–CLA	>128	16	>512	32	>128	>128
PIP	64	8	8	16	64	128
PIP–TAZ	8	8	16	0.5	16	64
CTX	4	1	128	2	1	16
CAZ	128	8	16	128	64	1024
FOX	8	4	>512	8	>128	>128
IPM	0.06	0.25	16	0.13	0.25	8

AMX, amoxycillin; CLA, clavulanic acid; PIP, piperacillin; TAZ, tazobactam; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; IPM, imipenem.
[a]The data presented concern *E. coli* DH10B transformants and a *P. aeruginosa* transconjugant producing GES-1 or GES-2, which differ only by the Gly170Asn mutation.
[b]The data presented concern *E. coli* XL1-Blue transformants and a *K. pneumoniae* clinical isolate producing GES-3 or GES-4, with GES-4 differing from GES-3 only by the Gly170Ser mutation.

types (Table 12). *E. coli* recombinants with GES-2 and GES-4 showed slightly increased imipenem MICs, and this effect was much augmented for GES-2 when expressed by a *P. aeruginosa* transconjugant or for GES-4 in a clinical *K. pneumoniae* isolate. Greater resistance in these latter species reflected the contribution of lower outer membrane permeability. An *E. coli* recombinant with the GES-2 enzyme showed clearly reduced MICs of ceftazidime and cefotaxime, compared to one with GES-1, but this effect was not as marked for the GES-4 producer, as compared to that with GES-3 [146,149]. On the other hand, the recombinant expressing GES-4 was highly resistant to cefoxitin and also had decreased susceptibility to β-lactamase inhibitor combinations. It should be emphasised that the original *K. pneumoniae* with the GES-4 enzyme was highly resistant to penicillins, ceftazidime, cefoxitin and β-lactam inhibitor combinations, with decreased susceptibility also to cefotaxime and imipenem. This phenotype, broader than the classical ESBL-mediated phenotype, is one of the most advanced β-lactam resistance phenotypes ever observed in Enterobacteriaceae [149].

CONCLUSION

This review aims to describe mutation during the evolution of class A ESBLs and its consequences for antimicrobial susceptibility. The production of β-lactamases is the most thrilling and intriguing antimicrobial resistance mechanism in Gram-negative bacteria. The high structural and functional plasticity of these enzymes creates a large playground for evolution, which has already produced an amazing variety of β-lactamase types, collectively compromising the vast majority of β-lactam antibiotics available for use. ESBLs represent a prime example of the factors responsible, in large part, for the global crisis in the treatment of infections due to Gram-negative bacteria. Their evolution and facile dissemination will continue to plague us in the future, with risks being exacerbated by the recently discovered over-representation of hypermutable *E. coli* strains among ESBL producers [151].

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