REVIEW ARTICLE

Natural evolution of TEM-1 β-lactamase: experimental reconstruction and clinical relevance

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Abstract

TEM-1 β-lactamase is one of the most well-known antibiotic resistance determinants around. It confers resistance to penicillins and early cephalosporins and has shown an astonishing functional plasticity in response to the introduction of novel drugs derived from these antibiotics. Since its discovery in the 1960s, over 170 variants of TEM-1 – with different amino acid sequences and often resistance phenotypes – have been isolated in hospitals and clinics worldwide. Next to this well-documented 'natural' evolution, the *in vitro* evolution of TEM-1 has been the focus of attention of many experimental studies. In this review, we compare the natural and laboratory evolution of TEM-1 in order to address the question to what extent the evolution of antibiotic resistance can be repeated, and hence might have been predicted, under laboratory conditions. We also use the comparison to gain an insight into the adaptive relevance of hitherto uncharacterized substitutions present in clinical isolates and to predict substitutions not yet observed in nature. Based on new structural insights, we review what is known about substitutions in TEM-1 that contribute to the extension of its resistance phenotype. Finally, we address the clinical relevance of TEM alleles during the past decade, which has been dominated by the emergence of another β-lactamase, CTX-M.

Introduction

The TEM β-lactamases are among the best-studied antibiotic resistance enzymes around. They act by hydrolysing the β-lactam ring of penicillins, cephalosporins and related antibiotics and are found at high frequencies in hospitals and clinics around the world (Medeiros, 1997; Matagne *et al.*, 1998). TEM-1, the first TEM allele identified, was isolated from penicillin-resistant bacteria in 1963 (Datta & Kontomichalou, 1965). Around the beginning of the 1980s, many new antibiotics were introduced onto the market in an effort to cope with the growing problems of β-lactamase-producing bacteria. This initiated the so-called 'β-lactamase cycle', where the introduction of novel β-lactam antibiotics was consistently followed by the appearance of new or modified β-lactamases conferring resistance to these antibiotics (Sykes & Bush, 1982). For TEM-1, over 170 of such modified variants have been described by now and new alleles continue to appear. All of these TEM variants differ in amino acid sequence (in general by one to five substitutions) and many of them differ in resistance phenotype (the degree of resistance they confer to different antibiotics). While TEM-1 only confers resistance to penicillins and early cephalosporins, the resistance spectrum of its descendants has fanned out to second-, third- and fourth-generation cephalosporins, monobactams and β-lactamase inhibitors.

The detailed description of the natural evolution of TEM alleles, combined with the experimental ease of selecting for increased antibiotic resistance, has made TEM-1 a frequently used tool for exploring the potential of novel protein engineering techniques (Stemmer, 1994; Zaccolo & Gherardi, 1999; Long-McGie *et al.*, 2000; Camps *et al.*, 2003; Fujii *et al.*, 2004; Kopsidas *et al.*, 2007). Soon, the potential of using *in vitro* evolution to repeat or even predict the natural evolution of TEM-1 was recognized (Vakulenko *et al.*, 1998), and, by adjusting the mutational spectrum and enzyme expression levels, *in vitro* evolution protocols were further fine tuned in order to mimic the natural evolutionary process (Barlow & Hall, 2002).

Here, we compare the amino acid substitutions found in all known clinical TEM isolates with those found in 25 laboratory studies that have looked at the directed (*in vitro*) or experimental (*in vivo*) evolution of TEM-1. The aim of this comparison is twofold. First, despite the recognition that laboratory evolution can play an important role in predicting resistance to antibiotics before their clinical debut (Martinez *et al.*, 2007), the accuracy with which laboratory evolution can repeat the natural evolution of antibiotic resistance genes has been addressed only marginally (Orencia *et al.*, 2001). Second, the function of many substitutions present in clinical TEM alleles is not known, i.e. it is not known whether or not they contribute to the extension of the resistance phenotype. By looking at the parallel occurrence of substitutions in clinical and laboratory studies, adaptive effects are derived. We also list substitutions that have been identified in several experimental studies, but not (yet) in clinical isolates. Based on this new information, we discuss substitutions that play a role in extension of the resistance spectrum of TEM-1, with a special emphasis on the latest insights into the structural effects of the most important substitutions. Finally, we discuss TEMs' clinical relevance in the past decade.

Comparison of the natural and laboratory evolution of TEM-1

Because most clinically used antibiotics belong to a limited number of structurally related groups, an antibiotic resistance gene that confers resistance to a certain antibiotic often has low-level activity against related antibiotics (Martinez *et al.*, 2007). Starting from this promiscuous activity, point mutations in the antibiotic resistance gene can increase its resistance to the related antibiotics. The evolution of TEM-1 β-lactamase is a classical illustration of this phenomenon. After its discovery in 1963, TEM-1 rapidly spread to many species of the human bacterial flora, but no reports on TEM variants with altered kinetic properties appeared for the next 20 years (Medeiros, 1997). However, coinciding with the introduction of a series of novel β-lactam antibiotics around the beginning of the 1980s, novel TEM variants began to appear. These variants typically contained one to three amino acid substitutions that caused an extension of the resistance spectrum to one or more of the new β-lactams. The TEM alleles known today include variants that confer resistance to most of the novel β-lactams that have been introduced in the past three decades. The natural evolution of TEM-1 illustrates the importance of predicting the evolution of antibiotic resistance along with the development of novel antibiotics. An important factor in this is analysing the evolvability of antibiotic resistance genes, i.e. their ability to acquire novel substrate specificities as a result of changes in their genotype (Martinez *et al.*, 2007). Here, we study the evolvability of TEM-1 challenged with novel β-lactams and inhibitors in a comparative analysis of the substitutions present in 25 laboratory evolution experiments on TEM-1 with those present in the ~170 clinical TEM alleles that have been described to date.

An overview of the experimental studies included can be found in Table 1, while Fig. 1 provides an overview of the frequency of substitutions found at different positions within the TEM-1 amino acid sequence of clinical and laboratory isolates. How the data were gathered and which data have been included is discussed in the Supporting Information. In total, 80 different substitutions were recorded at 58 different amino acid residues in clinical isolates (see Table S1). Of course, not all of these substitutions are necessarily adaptive, i.e. confer increased resistance to β-lactam antibiotics. Because our primary interest is in substitutions for which an adaptive effect has been demonstrated, we first made an inventory of these substitutions, which occur at only 18 residues (Table 2; the location of these residues in the three-dimensional structure of TEM-1 is shown in Fig. 2a). Based on the type of effect, we have divided this group into three subgroups of residues harbouring (1) substitutions with an effect on β-lactam resistance (10 residues), (2) substitutions with an effect on resistance to β-lactamase inhibitors (six residues) and (3) substitutions with an effect on both (two residues).

Substitutions with a known effect on the resistance phenotype

Substitutions involved in extension of the β-lactam resistance spectrum

Almost all substitutions that have been found with a high frequency in clinical isolates and that have been shown to be involved in increasing resistance to more modern β-lactam antibiotics (i.e. E104K, R164C, R164H, R164S, A237T, G238S and E240K) were identified in multiple experimental studies at a high frequency (see Table 2). The only exception is Q39K, which has been identified frequently in clinical isolates, but not in laboratory studies. This substitution has long been thought to be an example of neutral variation present in the TEM-1 background, although it was later shown to slightly, but consistently increase the minimal inhibitory concentration (MIC) for cephaloridine, ceftazidime and aztreonam (Blazquez *et al.*, 1995), while it slightly increases the catalytic efficiency (MICs not measured) to a series of related antibiotics (Chaibi *et al.*, 1996). Q39K is present in 32 clinical isolates and in two experimental isolates identified after selection for increased resistance to ceftazidime (Fujii *et al.*, 2006) and cefotaxime (M.L.M. Salverda & J.A.G.M. de Visser, unpublished data), while an

Table 1. Overview of experimental and directed evolution studies on TEM-1

Reference	No. of alleles*	Selective agent(s)	Mutations in starting allele	Experimental technique
Antibiotic resistance				
Hall & Knowles (1976)	1	CepC		DE; chemical mutagenesis
Stemmer (1994)	1	Ctx		DE; DNA shuffling
Huang & Palzkill (1997)	6	Amp	L76N	EE; mutator *E. coli*
Zaccolo & Gherardi (1999)	7	Ctx		DE; high-frequency random mutagenesis
Long-McGie *et al*. (2000)	2	Ctx		EE; phagemid in hypermutator *E. coli* strains
Blazquez *et al*. (2000)	18	Caz, Amx		EE; *E. coli*
Orencia *et al*. (2001)	1	Ctx		DE; hypermutator *E. coli*, DNA shuffling
Barlow & Hall (2002)	9	Caz, Ctx, Cxm, Atm†		DE; error-prone PCR
Barlow & Hall (2003b)	8	Fep		DE; error-prone PCR
Camps *et al*. (2003)	2	Atm		EE; *in vivo* error-prone DNA replication
Fujii *et al*. (2004)	3	Caz	V84I/A184V	DE; error-prone rolling circle amplification
Driffield *et al*. (2006)	1	Caz	V84I/A184V	EE; mutator *E. coli*
Fujii *et al*. (2006)	1	Caz		DE; gene shuffling
Holloway *et al*. (2007)	4	Amp, Ctx		EE; *E. coli*
Kopsidas *et al*. (2007)	1	Ctx		DE; error-prone RNA replication
Bershtein & Tawfik (2008)	3	Ctx	V84I/A184V	DE; error-prone PCR
M.L.M. Salverda & J.A.G.M. de Visser (unpublished data)	74	Ctx	Various	DE; error-prone PCR
Antibiotic-inhibitor resistance				
Vakulenko *et al*. (1998)	1	Amp, clavulanate‡		DE; error-prone PCR
Vakulenko & Golemi (2002)	1	Caz, clavulanate‡		DE; error-prone PCR, DNA shuffling
Baldwin *et al*. (2008)	3	Caz, clavulanate‡		DE; trinucleotide exchange
Retention and regain of resistance				
Bershtein *et al*. (2008)	2	Amp	V84I/A184V, L76N	DE; error-prone PCR
Goldsmith & Tawfik (2009)	1	Amp	V84I/A184V	EE/DE; RNA polymerase mutant, error-prone PCR
Stabilizing mutations				
Osuna *et al*. (2002)	2	Amp	Circularized TEM-1	DE; DNA shuffling
Hecky & Müller (2005)	1	Amp	Truncated TEM-1, V84I/A184V	DE; DNA shuffling, error-prone PCR
Kather *et al*. (2008)	6	Amp		DE; error-prone PCR

*Number of independent alleles present in the respective study (see text).
†In addition to resistance to these drugs, mutated alleles were submitted to selection for retention of resistance to ampicillin, pipericillin and cephalotin.
‡β-Lactam inhibitor.
Amx, amoxicillin; Atm, aztreonam; Caz, ceftazidime; CepC, cephalosporin C; Ctx, cefotaxime; Cxm, cefuroxime; Fep, cefepime; DE, directed evolution; EE, experimental evolution.

alternative substitution at this position (Q39R) was recorded in a stabilization study (Bershtein *et al*., 2008) and in a study selecting for increased cefotaxime resistance (M.L.M. Salverda & J.A.G.M. de Visser, unpublished data). The low frequency of TEM-2 (which differs from TEM-1 by the presence of Q39K only) in clinical *Escherichia coli* isolates compared with the frequent presence of Q39K in extended-spectrum TEM-alleles has led to the suggestion that Q39K might provide host strains with better survival at low concentrations of certain cephalosporins or monobactams, thus facilitating the acquisition of new substitutions that further increase resistance (Blazquez *et al*., 1995). This effect might explain the low frequency of the occurrence of this substitution in experimental studies where, due to more stringent selective conditions and higher mutation rates (when directed evolution is involved), substitutions with large effects dominate. An interesting side observation is that the net charge of the 14–18 residue region of mature proteins (in case of TEM-1 starting at residue 26) affects translocation across the cytoplasmic membrane in gram-negative bacteria (Kajava *et al*., 2000). Both Q39K and Q39R (found in two experimental isolates) result in a charge alteration of this region and may, in this way, affect β-lactamase translocation and thus the effective concentration of enzyme in the periplasmic space. Increases in the effective enzyme concentration can result in subtle effects on catalytic parameters. Given the huge distance of residue 39 to the binding pocket (see Fig. 2a), such an indirect effect on catalytic activity seems more likely.

There are four amino acid substitutions that have been shown to be involved in extending the resistance spectrum of TEM-1, but that nevertheless occur at a low frequency in

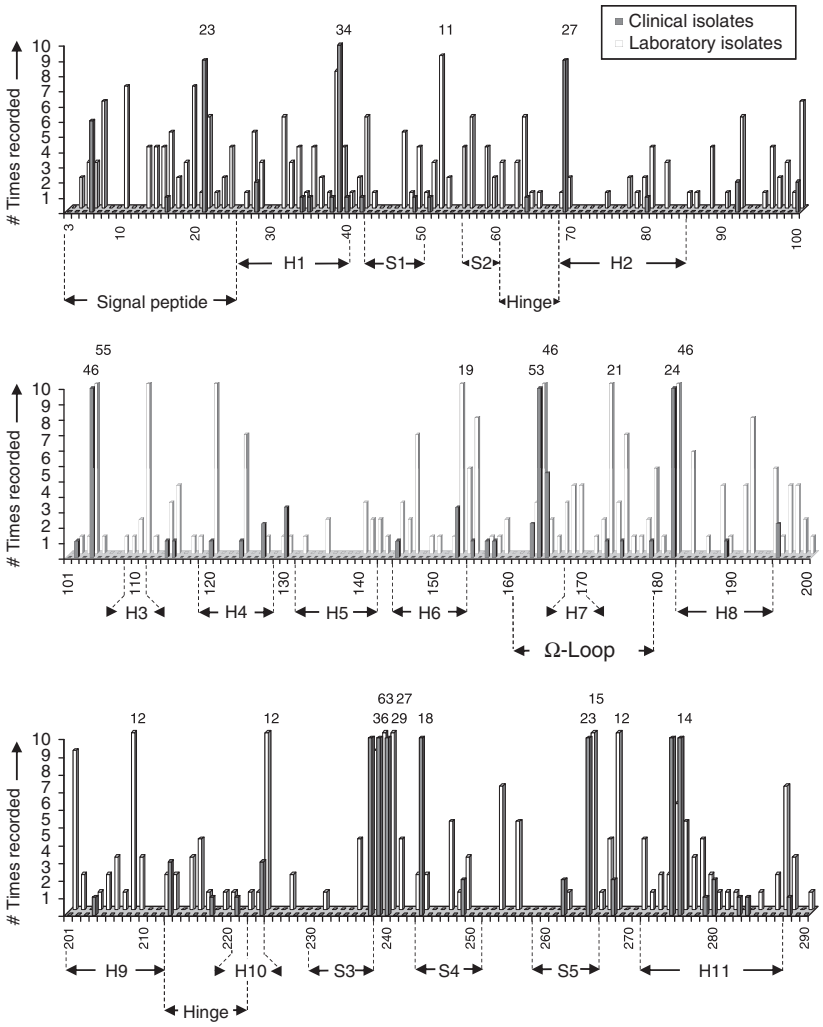

Fig. 1. Occurrence of substitutions in clinical and laboratory isolates of TEM β-lactamase. The amino acid numbering according to Ambler *et al.* (1991) is on the *x*-axis (note that residues 1, 2, 239 and 253 do not exist in this numbering scheme). Structural features of the enzyme according to Jelsch *et al.* (1993) are shown below these numbers (H, α-helix; S, β-sheet). Substitutions occurring > 10 times in either of the two datasets are shown as occurring 10 times here (the exact number of occurrences is shown above the respective columns).

clinical isolates. Reversion of substitution L51P in the genetic background of the only clinical isolate it occurs in (TEM-60) was found to decrease both activity towards certain antibiotics and enzyme stability, which led to the conclusion that the substitution plays a role in both the activity and the stability of the enzyme (Caporale *et al.*, 2004). Alternative substitutions L51F and L51I have been found in experiments twice (Osuna *et al.*, 2002; M.L.M. Salverda & J.A.G.M. de Visser, unpublished data) and once (Osuna *et al.*, 2002), respectively. It is interesting that two of the three alternative substitutions found at this position in experiments were identified in one of the structural perturbation studies, where they persisted in the clones characterized after multiple rounds of mutagenesis and selection (Osuna *et al.*, 2002). This suggests that L51F and L51I enhance enzyme stability and are thus functionally synonymous to L51P. A wide variety of substitutions have been found at the adjacent residue 52 and, judging from the nature of these studies, it seems that these substitutions are also involved in increasing enzyme stability (Hecky & Müller, 2005; Bershtein *et al.*, 2008).

Substitution I173V was found in two *in vitro* evolution studies (Barlow & Hall, 2002, 2003b) before the report of the first and only clinical isolate carrying this substitution (Zarnayova *et al.*, 2005). The substitution was later identified in three more experimental studies (Fujii *et al.*, 2004; Bershtein & Tawfik, 2008; M.L.M. Salverda & J.A.G.M. de Visser, unpublished data), resulting in a total of 13 independent observations in laboratory studies. Alternative substitutions I173L (Bershtein & Tawfik, 2008), I173M (Bershtein & Tawfik, 2008), I173R (Osuna *et al.*, 2002; Bershtein & Tawfik, 2008) and I173T (Barlow & Hall, 2002; Driffield *et al.*, 2006; M.L.M. Salverda & J.A.G.M. de Visser, unpublished data) were also identified in experiments, suggesting that a wide variety of substitutions at this positions can result in functional effects. The effect of I173V on resistance phenotype was extensively tested after its identification in laboratory experiments, and although a number of β-lactam antibiotics were screened, the

Table 2. The 18 amino acid residues at which substitutions with a demonstrated effect on resistance phenotype have been recorded in clinical isolates (substitutions with functional effects are shown in bold and references to these effects can be found in the footnotes)

Amino acid residue	Substitution	No. of clinical isolates	No. of laboratory isolates	Confers resistance against
39	**Q39K**	34	2	β-Lactams*,†
	Q39R	0	2	
51	L51I	0	1	
	L51F	0	2	
	L51P	1	0	β-Lactams¶
69	**M69I**	4	1	Inhibitors‖
	M69L	14	1	Inhibitors‖
	M69V	9	0	Inhibitors‖
104	E104A	0	3	
	E104G	0	2	
	E104K	46	46	β-Lactams‡‡
	E104Q	0	1	
	E104V	0	3	
130	**S130G**	3	1	Inhibitors§§
164	**R164C**	4	3	β-Lactams‖‖
	R164G	0	3	
	R164H	19	17	β-Lactams‖‖
	R164N	0	1	
	R164S	30	21	β-Lactams‖‖
	R164Y	0	1	
165	W165C	1	0	
	W165G	1	2	
	W165R	3	0	Inhibitors***
173	I173L	0	1	
	I173M	0	2	
	I173R	0	2	
	I173T	0	3	
	I173V	1	13	β-Lactams†††
175	N175D	0	5	
	N175I	1	0	β-Lactams§§§
	N175S	0	1	
	N175Y	0	1	
179	**D179E**	1	0	β-Lactams‖‖‖
	D179G	0	4	β-Lactams****
	D179Y	0	1	β-Lactams****
182	M182K	0	1	
	M182L	0	1	
	M182R	0	1	
	M182T	24	41	β-Lactams/ Inhibitors‡,§
	M182V	0	2	
237	**A237G**	1	0	β-Lactams**
	A237S	0	2	
	A237T	9	7	β-Lactams††
238	G238A	0	3	
	G238D	1	0	
	G238N	1	0	
	G238S	34	60	β-Lactams‡
240	E240D	0	1	
	E240G	0	5	β-Lactams¶¶
	E240H	0	2	
	E240K	27	17	β-Lactams††
	E240Q	0	1	
	E240R	1	0	
	E240V	1	1	
244	**R244C**	4	1	Inhibitors***
	R244G	1	0	
	R244H	3	0	Inhibitors***
	R244L	1	0	
	R244S	9	1	Inhibitors***
268	**S268G**	2	10	β-Lactams/ Inhibitors‡‡
	S268N	0	1	
	S268T	0	1	
275	**R275L**	3	3	Inhibitors***,‡‡‡
	R275Q	5	3	
276	**N276D**	12	3	Inhibitors¶¶¶
	N276K	1	0	
	N276S	1	1	
	N276T	0	1	

*Blazquez *et al*. (1995).
†Chaibi *et al*. (1996).
‡Hall (2002).
§Huang & Palzkill (1997).
¶Caporale *et al*. (2004).
‖Chaibi *et al*. (1998).
**Arlet *et al*. (1993).
††Blazquez *et al*. (1998).
‡‡Vakulenko & Golemi (2002).
§§Thomas *et al*. (2005).
¶¶Zaccolo & Gherardi (1999).
‖‖Vakulenko *et al*. (1999).
***Chaibi *et al*. (1999).
†††Barlow & Hall (2003b).
‡‡‡Kather *et al*. (2008).
§§§Chouchani *et al*. (2006).
¶¶¶Saves *et al*. (1995b).
‖‖‖Delmas *et al*. (2005).
****Vakulenko *et al*. (1995).

substitution was only found to increase resistance to cefuroxime and cefotaxime (Barlow & Hall, 2003b). However, the substitution was later identified in a laboratory study that used ceftazidime as the sole selective agent (Fujii *et al*., 2004), indicating a broader phenotypic effect.

Despite the presence of N175I in a single clinical isolate only (TEM-138), the substitution is thought to contribute to increased ceftazidime resistance (Chouchani *et al*., 2006). In experimental studies, alternative substitutions N175Y and N175D were found once (M.L.M. Salverda & J.A.G.M. de Visser, unpublished data) and five times (Barlow & Hall, 2003b; Bershtein *et al*., 2008; M.L.M. Salverda & J.A.G.M. de Visser, unpublished data), respectively. Although the exact mechanism remains obscure, the high frequency at which N175D occurs strongly suggests that this substitution is involved in increasing resistance to cefotaxime (M.L.M. Salverda & J.A.G.M. de Visser, unpublished data) and

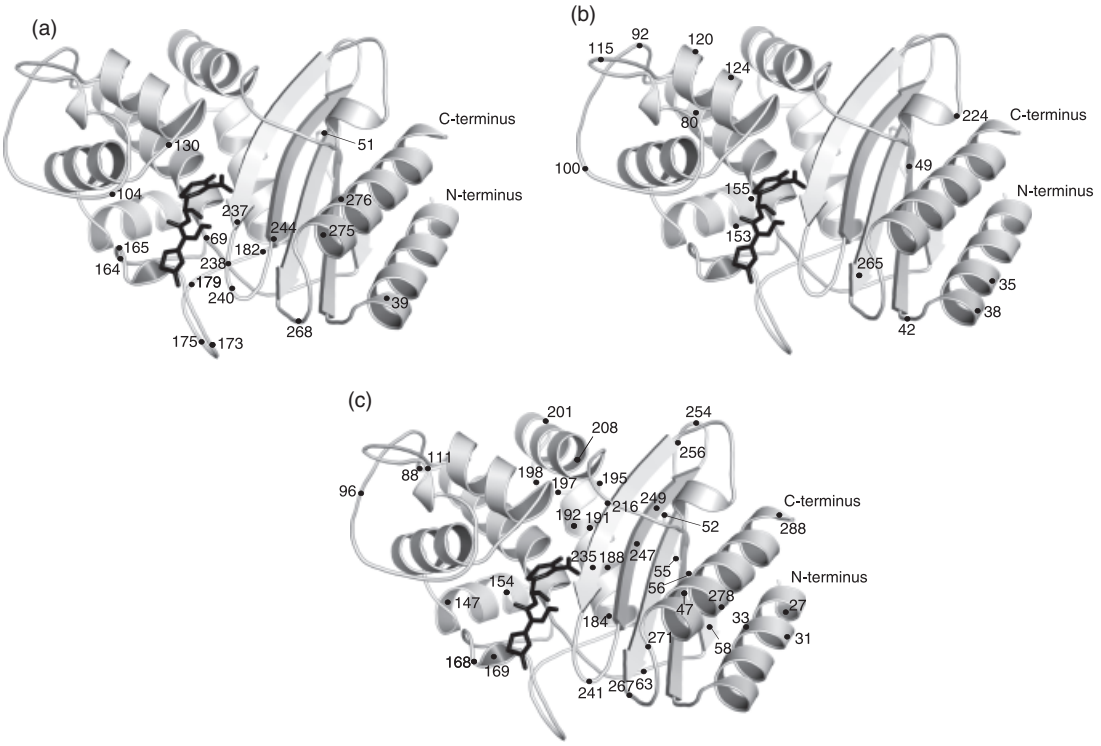

Fig. 2. Three-dimensional structure of TEM-1 β-lactamase (PDB code 1BTL, cefotaxime molecule obtained from a crystal structure of TOHO-1 β-lactamase, PDB code 1IYO). (a) Amino acid residues at which substitutions with a demonstrated effect on resistance phenotype have been found (listed in Table 2). (b) Amino acid residues at which substitutions with an unknown effect on resistance phenotype have been found in clinical isolates and for which data from laboratory evolution indicate the existence of substitutions with adaptive effects (the residues in bold in Tables 3 and 4). (c) Amino acid residues at which substitutions are found with a high frequency in experimental isolates, but not in clinical isolates (see Table 5).

cefepime (Barlow & Hall, 2003b). The Ω-loop where N175D resides is involved in the formation of the active site pocket (Jelsch *et al.*, 1993) and numerous substitutions within this loop are known to alter the resistance phenotype.

Substitution D179E, reported in a single clinical isolate (TEM-126), was found to improve the catalytic efficiency against ceftazidime (Delmas *et al.*, 2005). Although D179E was not found in experiments, alternative substitutions D179G (Blazquez *et al.*, 2000; Fujii *et al.*, 2004; M.L.M. Salverda & J.A.G.M. de Visser, unpublished data) and D179Y (Blazquez *et al.*, 2000) were found at this position four times and once, respectively. Because of its anchoring function in the Ω-loop, substitutions of residue 179 have been studied extensively and both D179G and D179Y increase the MIC for ceftazidime 16-fold (Vakulenko *et al.*, 1995). In agreement with this, D179G and D179Y were recorded in laboratory studies that use ceftazidime as a selective agent (Blazquez *et al.*, 2000; Fujii *et al.*, 2004). Interestingly, although no effect on cefotaxime resistance could be measured for a mutant generated *in vitro* (Vakulenko *et al.*, 1999), substitution D179G was isolated twice in an experiment that used cefotaxime as a single selective agent (M.L.M. Salverda & J.A.G.M. de Visser, unpublished data), indicating that the effect on resistance phenotype might be broader than assumed previously. A detailed study of the effect of all possible amino acid substitutions at residue 179 on resistance phenotype showed that although nearly all substitutions increased ceftazidime resistance, at the same time they almost all drastically reduced resistance to ampicillin (Vakulenko *et al.*, 1995). In a laboratory evolution experiment on fluctuating β-lactam pressure, substitutions D179G and D179Y were only found in the absence of fluctuating selection pressure (Blazquez *et al.*, 2000). Pleiotropic effects of substitutions at this residue might thus have prevented their appearance in clinical isolates so far. It was recently shown that synchronic or diachronic exposure to two different antibiotics has most probably contributed to the allelic diversification of close relatives of TEMs, the CTX-M alleles (Novais *et al.*, 2010).

Substitutions involved in inhibitor resistance

Substitutions at residues 69, 130, 165, 244, 275 and 276 are all thought to play an exclusive role in inhibitor resistance (Chaibi *et al.*, 1999; Yang *et al.*, 1999), and most of them are found at a high frequency in the dataset of clinical isolates.

Substitutions at residues 69, 130, 244 and 276 were also identified in an experimental study selecting for increased resistance to the β-lactam–inhibitor combination ampicillin–clavulanate (Vakulenko *et al.*, 1998). The two other experimental studies on inhibitor resistance used a combination of ceftazidime–clavulanate and identified the most frequently found substitution at residue 276 (Vakulenko & Golemi, 2002) as well as substitutions at residues 69 and 165 (Baldwin *et al.*, 2008). There is evidence for the involvement of substitutions at the remaining residue (275) in increasing the resistance to inactivation by clavulanate, although the effects are small (Chaibi *et al.*, 1999; Fiett *et al.*, 2000). This might explain why substitutions at this residue have not been found in the experimental studies that have used clavulanate as a selective agent. Surprisingly, substitutions at residue 275 have been found in other experiments that used selection regimes without inhibitors: R275L and R275Q were both found in studies selecting for stabilizing mutations (Osuna *et al.*, 2002; Kather *et al.*, 2008) and cefotaxime resistance (M.L.M. Salverda & J.A.G.M. de Visser, unpublished data). This suggests that substitutions at this residue are able to increase resistance to novel β-lactams and inhibitors at the same time. Substitutions at residue 275 may in fact be functionally similar to the so-called 'global suppressor' mutations (Huang & Palzkill, 1997) described below, in terms of their location far away from the binding pocket. This conclusion is supported by the recent observation that R275L has a strongly stabilizing effect (Kather *et al.*, 2008).

Substitutions involved in both β-lactam and inhibitor resistance

Substitutions M182T and S268G have been shown to increase resistance to both β-lactams and β-lactam–inhibitor combinations (Huang & Palzkill, 1997; Vakulenko & Golemi, 2002). Typical for these modulating substitutions is that their effect is only apparent in the background of other substitutions that affect resistance phenotype. Substitution M182T, present in 21 clinical isolates, was found in the majority of the experimental studies. Substitution S268G is far less common among clinical isolates (present only in TEM-49 and TEM-136). This substitution was found in five different experimental studies: four using cefotaxime as a selective agent (Holloway *et al.*, 2007; Kopsidas *et al.*, 2007; Bershtein & Tawfik, 2008; M.L.M. Salverda & J.A.G.M. de Visser, unpublished data) and one using a combination of ceftazidime and clavulanate (Vakulenko & Golemi, 2002). In agreement with evidence from TEM variants with and without this substitution (Vakulenko & Golemi, 2002), this indicates that the substitution plays a role in increased resistance to both β-lactams and inhibitors and may very well have a similar mode of action as described for M182T (Huang & Palzkill, 1997; Sideraki *et al.*, 2001; Wang *et al.*, 2002a).

Summarizing, we can conclude that laboratory evolution has been highly successful in identifying adaptive substitutions found in clinical TEM alleles. The somewhat lower capture of substitutions involved in inhibitor resistance can be explained by the scarcity of experimental studies in this area.

Substitutions with an unknown effect on resistance phenotype

There are 40 residues at which substitutions have been identified in clinical isolates that have either not been tested for their effect on resistance phenotype or that failed to yield any measurable results when tested. For 14 of these residues, substitutions were identified in multiple clinical isolates (Table 3), while for the remaining 26 residues, a substitution was only found in a single clinical isolate (Table 4). We analyse to what extent clinical and laboratory data overlap for both groups in order to identify to what extent laboratory data support the adaptive effects of substitutions found at these residues (based on their frequency of occurrence). An overview of amino acid residues where laboratory evolution suggests such adaptive effects can be found in Fig. 2b.

Substitutions found in multiple clinical isolates

At 14 amino acid residues, multiple substitutions without a known effect on resistance phenotype have been identified in clinical isolates. The frequent identification of G92D, H153R, A224V and T265M in laboratory experiments suggests adaptive effects for these four substitutions. All of these substitutions have been encountered in studies on increased cefotaxime resistance. It was demonstrated recently that both H153R and A224V increase enzyme stability (Bershtein *et al.*, 2008; Kather *et al.*, 2008). Note that we have not scored these substitutions (as well as R120G, E147G, L201P and I208M mentioned further on) as 'adaptive' because it has not (yet) been shown that they contribute to increased resistance to β-lactams, even though effects on enzyme stability have been described. Considering the fact that both other substitutions (G92D and T265M) are distant from the binding pocket, are always observed in the background of other substitutions, and can be found in studies that select for stabilizing or compensatory mutations, they most probably have stabilizing effects as well.

The high frequency of occurrence in laboratory studies suggests that substitutions at residues 21 and 100 also affect resistance phenotype, although the evidence is less straightforward than for the substitutions mentioned above (see Table 3). Although no clear effect on resistance phenotype has been found for substitution Q6K, it was hypothesized that because the positively charged amino-terminal region of the signal

Table 3. The 14 amino acid residues at which substitutions with an unknown effect on resistance phenotype have been found in multiple clinical isolates

Amino acid residue	Substitution	No. of clinical isolates	No. of laboratory isolates
6	Q6K	6	1
	Q6R	0	2
21	L21F	22	1
	L21H	0	1
	L21I	1	1
	L21P	0	3
28	E28A	0	1
	E28D	1	0
	E28G	0	1
	E28K	1	0
	E28M	0	1
92	G92D	2	4
	G92S	0	2
100	N100D	0	1
	N100G	0	1
	N100H	0	1
	N100K	0	2
	N100S	2	1
	N100T	0	1
127	I127V	2	1
153	H153D	0	1
	H153L	0	2
	H153D	0	1
	H153Q	0	2
	H153R	3	10
	H153S	0	1
	H153Y	0	2
163	D163E	0	1
	D163G	1	1
	D163H	1	0
	D163V	0	1
196	G196D	1	0
	G196S	1	1
213	A213E	0	1
	A213P	0	1
	A213V	3	0
224	A224T	0	1
	A224V	3	11
262	V262A	0	1
	V262I	2	0
265	T265A	0	2
	T265M	23	13
280	A280T	0	1
	A280V	2	0

Substitutions found at these residues in clinical and laboratory isolates are shown. Residue numbers for which laboratory evolution indicates the presence of adaptive substitutions are in bold.

peptide plays an important role in efficient protein secretion across the cytoplasmic membrane (Inouye *et al.*, 1982), substitution of the polar glutamine (Q) residue at position 6 for a positively charged lysine (K) residue could result in an effect on resistance phenotype (Perilli *et al.*, 2002).

Table 4. The 26 amino acid residues with an unknown effect on resistance phenotype, at which a substitution has been found in a single clinical isolate only

Amino acid residue	Substitution	No. of clinical isolates	No. of laboratory isolates
16	F16C	0	1
	F16L	1	4
34	K34E	1	0
	K34R	0	1
35	D35A	0	2
	D35G	0	1
	D35N	0	1
	D35P	1	0
38	D38A	0	1
	D38E	0	1
	D38G	0	1
	D38N	1	2
	D38V	0	2
	D38Y	0	1
40	L40V	1	0
	L40W	0	1
42	A42G	0	6
	A42V	1	0
49	L49M	1	3
	L49V	0	1
64	E64K	1	1
80	V80E	1	0
	V80G	0	1
	V80I	0	3
102	L102M	0	1
	L102V	1	0
114	T114A	0	2
	T114M	0	1
	T114P	1	0
115	D115A	0	1
	D115E	0	2
	D115G	1	0
	D115N	0	1
120	R120E	0	1
	R120G	1	5
	R120K	0	1
	R120S	0	3
124	S124G	0	2
	S124N	1	3
	S124R	0	2
145	P145A	1	1
	P145L	0	1
	P145Q	0	1
155	M155I	1	4
	M155L	0	2
	M155T	0	1
	M155V	0	1
157	D157E	1	0
	D157S	0	1
158	H158N	1	0
	H158Y	0	1
189	T189A	0	1
	T189K	1	0
204	R204Q	1	1

Table 4. Continued.

Amino acid residue	Substitution	No. of clinical isolates	No. of laboratory isolates
218	G218E	1	0
221	L221M	1	0
279	I279L	0	1
	I279M	0	1
	I279T	1	0
283	G283C	1	0
284	A284G	1	0
289	H289L	1	0
	H289Q	0	1
	H289Y	0	2

Substitutions found at these residues in laboratory isolates are also shown. Residue numbers for which laboratory evolution indicates the presence of adaptive substitutions are in bold.

Interestingly, the experimentally encountered substitution to arginine (R) also confers a positive charge, and indeed, it has been shown recently that this substitution increases enzyme expression (Goldsmith & Tawfik, 2009). This suggests that Q6K can indeed have subtle functional effects. For the remaining positions (28, 127, 163, 196, 213, 262 and 280), we find no indications of the adaptive effects of substitutions.

Substitutions found in a single clinical isolate

There are 26 amino acid residues that have been altered in a single clinical isolate only and for which no phenotypic effect is known (see Table 4). For nine of these residues (16, 35, 38, 49, 80, 115, 120, 124 and 155), substitutions have been found at high frequencies in experiments, suggesting adaptive effects. Additionally, substitution A42G, known to play a role in cefotaxime resistance (Stemmer, 1994; Hall, 2002), was identified frequently in laboratory experiments and this substitution may be functionally similar to the A42V substitution found in TEM-42.

Predictions and recommendations

Prediction of substitutions that may be encountered in future clinical isolates

Amino acid residues at which no substitutions have been found in clinical isolates, but at which substitutions have repeatedly been recorded in laboratory experiments are of special interest, because these substitutions may be expected in future clinical isolates. In order to make a conservative estimate of this group, we consider a particular substitution to have arisen by selection whenever it was found three times or more in independent laboratory isolates, and we consider an amino acid residue to be under selection whenever four or more substitutions of this residue have been found (in which case the exact substitutions may vary). The results of this analysis are shown in Table 5, and the selective background in which these substitutions were identified is shown in Table 6 (data on substitutions of unknown effect that have been found in clinical isolates and for which laboratory evolution indicates adaptive effects have been added to this table for completeness). An overview of the location of all these amino acid residues in the three-dimensional structure of TEM-1 is shown in Fig. 2c.

A substantial number of apparently adaptive substitutions can be found in the signal peptide (Table 5). It has been demonstrated recently that mutations in this region can increase periplasmic expression levels (Goldsmith & Tawfik, 2009). Only four substitutions have been found at three amino acid positions in the signal peptide in clinical isolates. We have shown here that at least two of these (Q6K and F16L) most probably have adaptive effects. Furthermore, laboratory studies suggest that adaptive substitutions are possible at several other residues in the signal peptide (see Table 5).

Concerning the mature protein, laboratory studies have identified numerous amino acid substitutions that can clearly affect the resistance phenotype, but that have nevertheless not been found in clinical isolates so far. Predicted substitutions can be observed in the binding pocket, but also far away from it (see Fig. 2c). Many of the substitutions that are distant to this pocket are probably compensatory mutations, which can (partly) neutralize the destabilizing effect of mutations that increase enzyme activity. Their frequent identification in studies selecting for stabilizing substitutions after a perturbation points in the same direction (see Table 6). In fact, such stabilizing effects have been recorded recently for R120G, E147G, L201P and I208M (Bershtein *et al.*, 2008; Kather *et al.*, 2008; Marciano *et al.*, 2008). The recent observation that different stabilizing substitutions in TEM-1 can compensate different destabilizing effects (Marciano *et al.*, 2008) emphasizes the importance of characterizing stabilizing substitutions. Substitutions in parts of the enzyme that contribute to the formation of the binding pocket are also observed, and such substitutions may have a more direct effect on resistance phenotype by affecting enzyme activity, as has been observed for I173V (Barlow & Hall, 2003b) and D179G (Vakulenko *et al.*, 1995).

Several amino acid substitutions were identified in laboratory studies before their identification in clinical isolates. Substitution I173V was identified in laboratory studies on TEM-1 in 2002 (Barlow & Hall, 2002), while it was not reported in clinical isolates until 2005 (Zarnayova *et al.*, 2005). The effect of this substitution on resistance to various antibiotics was extensively described before this first clinical report, and co-occurrence with one of the two other substitutions present in the clinical isolate (R164H) was detected in earlier laboratory studies as well (Barlow & Hall, 2003b). Another substitution, R120G, was described as early as 2002 (Osuna *et al.*, 2002), while it was reported for the first time in a clinical

Table 5. Amino acid substitutions found at a high frequency in laboratory isolates that have not been found in clinical isolates

Structure*	AA†	Substitution	No. of laboratory isolates
SP	7	H7P	1
		H7Q	2
		H7R	2
		H7Y	2
	10	V10A	1
		V10I	7
	13	I13M	1
		I13T	2
		I13V	1
	14	P14A	1
		P14L	1
		P14S	1
		P14T	1
	15	F15C	1
		F15L	2
		F15V	1
	19	F19C	2
		F19I	1
		F19L	2
		F19S	1
		F19V	2
	24	F24C	1
		F24L	2
		F24S	1
H1	27	P27L	1
		P27S	4
	31	V31A	4
		V31E	1
		V31G	1
	33	V33A	1
		V33I	2
		V33L	1
S1	47	I47V	5
Turn S1/S2	52	N52D	3
		N52H	1
		N52K	1
		N52S	3
		N52T	2
		N52Y	1
	55	K55E	1
		K55Q	1
		K55R	2
S2	56	I56T	3
		I56V	3
	58	E58G	3
		E58K	1
Hinge I	63	E63A	1
		E63D	1
		E63G	2
		E63K	2
H2	88	Q88E	1
		Q88H	1
		Q88L	1
		Q88R	1
S4	247	I247L	1
		I247V	4
	249	A249V	3
Turn S4/S5	254	D254A	1
		D254G	3
		D254H	1
		D254N	2
	256	K256N	1
		K256Q	2
		K256R	1
		K256T	1
Loop	96	H96D	1
		H96R	1
		H96Y	2
H3/loop	111	K111E	1
		K111M	2
		K111Q	2
		K111R	3
		K111T	2
H6	147	E147A	2
		E147G	3
		E147K	2
	154	N154D	2
		N154S	2
		N154Y	1
Ω-Loop	168	E168A	1
		E168G	1
		E168K	1
		E168V	1
	169	L169P	2
		L169R	2
H8	184	A184T	1
		A184V	5‡
	188	T188I	2
		T188K	1
		T188R	1
	191	R191H	4
	192	K192E	2
		K192N	2
		K192R	2
		K192T	2
	195	T195A	1
		T195F	1
		T195I	1
		T195S	2
Turn H8/H9	197	E197D	1
		E197G	1
		E197K	2
	198	L198I	1
		L198P	1
		L198R	1
		L198V	1
H9	201	L201I	1
		L201P	5
		L201Q	1
		L201R	1
	208	I208K	2

Table 5. Continued.

Structure*	AA†	Substitution	No. of laboratory isolates
		I208L	1
		I208M	6
		I208T	1
		I208V	2
Hinge II	216	V216A	1
		V216I	3
S3	235	S235T	4
Turn S3/S4	241	R241H	4
Turn S5/H11	267	G267A	1
		G267R	2
		G267V	1
	271	T271A	1
		T271I	2
		T271P	1
H11	278	Q278E	1
		Q278L	1
		Q278R	2
	288	K288E	3
		K288Q	1
		K288R	2
		K288T	1

*Structure of the part of TEM-1 the respective amino acid residue is localized according to Jelsch *et al*. (1993).
†Amino acid residue number.
‡Also present in three clinical isolates, but together with V84I and therefore not the result of natural selection (see text).
H, α-helix; S, β-sheet; SP, signal peptide.

allele in 2008 (GenBank number FJ197316). Substitution M155I, found in the laboratory in 2003 (Barlow & Hall, 2003b), was not reported in clinical isolates until 2009 (GenBank number AM941159), while A224V was first reported in the laboratory in 2002 (Osuna *et al*., 2002), but not identified in clinical isolates until 2005 (GenBank number DQ279850).

Although deletions have not been reported in clinical TEM isolates, it has been shown that several single amino acid deletions can increase ceftazidime resistance considerably (Simm *et al*., 2007). The introduction of multiple deletions has been shown to result in a similar effect (Osuna *et al*., 2004). The promoter region of TEM is most often not part of experiments, but we also address it briefly here. In clinical isolates of TEM, four different promoters (*P3*, *P4*, *P5* and *Pa*/*Pb*) are found (Lartigue *et al*., 2002). *Pa*/*Pb* is actually a pair of overlapping promoters, located further upstream of TEM-1 than the three other promoters. Promoters *P4* and *P5* are similar to *P3*, but differ from it by a single mutation in the −10 and −35 region, respectively. It was shown that the expression of two different TEM alleles (TEM-1 and TEM-30) under control of the four different promoters can result in a huge variation in MIC levels for several β-lactam antibiotics

Table 6. Selective backgrounds (in laboratory studies) of substitutions encountered frequently in laboratory studies that have not been found in clinical isolates

Position	Selective regime* (no. of alleles identified)	No. of occurrences in stabilization studies†
7	Ctx (2)	5
10	Ctx (4); Fep (2)	2
13	Ctx (1)	3
14	Ctx (2)	2
15		4
16	Ctx (3)	2
19	Ctx (3)	5
21	Ctx (5)	1
24	Ctx (2)	2
27	Ctx (2); Fep (1)	2
31	Ctx (2)	4
33	Ctx (3)	1
35	Ctx (2)	2
38	Ctx (5); Fep (1)	3
42	Ctx (3); Amp+Cla (1); Caz+Cla (1)	1
47	Ctx (2); Caz+Cla (1)	2
49	Ctx (4)	
52	Amp+Cla (1)	10
55		4
56	Ctx (3)	3
58	Ctx (1)	3
63	Caz (1)	5
80	Fep (1)	3
88	Ctx (2)	2
92	Ctx (4)	2
96	Fep (1)	3
100	Ctx (2)	5
111	Ctx (2); Amp+Cla (1)	7
115	Ctx (2)	2
120	Ctx (3)	7
124	Ctx (5)	2
147	Fep (1)	6
153	Ctx (12); Amp+Cla (1)	6
154		5
155	Ctx (6); Fep (1)	1
168	Atm (1)	3
169	Caz (1); Ctx (1); Caz+Cla (1)	
184	Ctx (5); Caz+Cla (1)	
188	Ctx (4)	
191	Ctx (4)	
192	Ctx (6); Amp+Cla (1)	1
195	Ctx (1); Amp+Cla (1)	3
197	Ctx (3)	1
198	Ctx (3)	1
201	Ctx (3); Amp+Cla (1)	4
208	Ctx (5)	7
216	Ctx (3)	1
224	Ctx (7); Fep (1)	4
235	Ctx (3); Cxm (1)	
241	Ctx (4)	
247	Ctx (2)	3
249	Ctx (3)	
254	Ctx (4); Caz (1); Cxm (1)	1

Table 6. Continued.

Position	Selective regime* (no. of alleles identified)	No. of occurrences in stabilization studies†
256	Ctx (2)	3
265	Ctx (12); Cxm (1)	2
267	Ctx (2); Caz (1); Atm (1)	
271	Ctx (2)	2
278	Ctx (2)	2
288	Caz+Cla (1)	6

For the residues in bold substitutions with a previously unknown effect on resistance phenotype *have* been found in clinical isolates, and laboratory evolution indicates adaptive effects.

*See Table 1 for abbreviations. The number of independent laboratory alleles containing substitutions at this position is in parentheses.

†Number of independent occurrences in the lower five studies of Table 1.

(Lartigue *et al.*, 2002). In experiments, a mutation identified between the −35 and −10 sites of the *P3* promoter after selection for increased cefotaxime resistance was shown to increase expression levels two- to threefold (Stemmer, 1994). This mutation is different from the ones that characterize the different promoters and has not been recorded in clinical TEM isolates so far.

Practical recommendations for the predictive use of *in vitro* evolution

What is the best way to test how antibiotic resistance genes will respond to novel antibiotics? The first question to address is which antibiotic resistance enzymes can be expected to evolve increased activity to a new drug. The comparison of the *in vitro* evolution of cefepime resistance starting from the β-lactamases TEM-1 and CMY-2 shows that simply focusing at enzymes with the highest initial activity can be misleading. It was shown that CMY-2 is more active than TEM-1 against cefepime and that it confers clinical resistance to ceftazidime, an antibiotic considerably related to cefepime, while TEM-1 does not. After several rounds of *in vitro* evolution, however, TEM variants were isolated that conferred higher cefepime resistance than isolated CMY-2 variants that underwent a similar treatment (Barlow & Hall, 2003a, b).

Once relevant enzymes have been selected, *in vitro* evolution, followed by *in vivo* selection using the novel drug should yield quick and detailed information about the evolutionary potential of the resistance enzymes. For this purpose, error-prone PCR is an easy and straightforward *in vitro* evolution technique that offers the advantage of controllable mutation rates and mutation spectra. Although high mutation rates can potentially grant access to parts of sequence space that are not accessible by *in vivo* evolution (e.g. when a combination of several substitutions confers high resistance, while the individual substitutions do not increase resistance or reduce it), the *in vitro* evolution of TEM-1 using a mutation rate of $\sim$30 mutations kb^{-1} resulted only in the identification of the clinically observed TEM-52 (Zaccolo & Gherardi, 1999).

Finally, in order to gain an insight into structure–function relationships, it is important to distinguish between substitutions that alter the kinetic properties of the respective enzyme and substitutions that increase enzyme stability. In general, substitutions that alter the activity of an enzyme tend to reduce its stability (DePristo *et al.*, 2005), which accounts for TEM-1 as well (Raquet *et al.*, 1995; Wang *et al.*, 2002a). Substitutions that counteract such effects by restabilizing the enzyme are frequently observed as secondary mutations. Studying the *in vitro* evolution of destabilized variants of antibiotic resistance enzymes can quickly result in the identification of such substitutions and help to make the important distinction between substitutions that are directly involved in substrate binding and turnover and ones that are not. Another important observation in this respect is that most stabilizing substitutions are in fact reversions back to the family consensus and inferred ancestor (Bershtein *et al.*, 2008). Comparison with these sequences can therefore clarify the specific role of a substitution as well.

How to make predictions about the evolution of antibiotic resistance enzymes that have started to diversify relatively recently? Phylogenetic analysis of existing alleles can identify 'ancestral' and 'derived' sequences, and when resistance phenotypes are known, this can provide indications about the selective landscape. Such an analysis was recently undertaken for the CTX-M-1 cluster of CTX-M alleles (Novais *et al.*, 2010). Phylogenetic analysis of the existing alleles within this cluster, combined with knowledge of their resistance phenotype, indicated that selection for cefotaxime and ceftazidime resistance may have been the driving selective force underlying the diversification of this cluster. A subsequent test identified five substitutions under positive selection, while another possible adaptive substitution was identified based on the phylogeny and resistance phenotypes. Reconstruction of all combinations of these substitutions and analysis of their resistance phenotype showed that indeed the combination of cefotaxime and ceftazidime was the main selective force responsible for the diversification of this cluster, while at the same, it identified the possible adaptive trajectories involved. It was argued that such knowledge of the selective landscape and mutational trajectories can help to determine the right conditions for *in vitro* evolution and in this way further improve its use as a predictive tool (Novais *et al.*, 2010).

Structural insight into the functional effects of substitutions

In order to understand how a particular amino acid substitution can result in alteration of the catalytic

parameters and the resistance spectrum of TEM-1, structural information is needed. Over the years, the tertiary structures of many TEM variants have been determined by X-ray crystallography and some excellent reviews on a structural insight into the functional effects of frequently encountered substitutions in TEM-1 have been published (Knox, 1995; Matagne *et al.*, 1998; Yang *et al.*, 1999; Gniadkowski, 2008; Page, 2008). We extend these reviews with recent structural information in order to provide a detailed overview of what is known about the structural aspects of substitutions that alter TEM's resistance profile.

M69

Substitutions Ile-69, Leu-69 and Val-69 confer resistance to the β-lactamase inhibitors clavulanic acid, sulbactam and tazobactam. Like β-lactams, these inhibitors initially undergo a nucleophilic attack by the β-lactamase, but eventually they contribute to the formation of a cross-link between Ser-70 and Ser-130, which in turn leads to irreversible inactivation of the β-lactamase. Based on molecular modelling, it was previously believed that a combination of hydrophobicity and steric constraints affecting the active site Ser-70 could explain the kinetic behaviour of Ile-69 and Val-69 (Chaibi *et al.*, 1998). Structural analysis of TEM-32 (M69I/M182T) and TEM-34 (M69V) has shown that both Ile-69 and Val-69 indeed lead to distortion of Ser-70, but that in both cases, this results in a conformational change of Ser-130, causing weakening of the cross-link between Ser-70 and Ser-130 and diminished inhibition (Wang *et al.*, 2002b) (see Fig. 3a). Molecular modelling of Leu-69 showed no major conformational changes, leading to the suggestion that in this case, hydrophobicity could be the cause of the kinetic changes (Chaibi *et al.*, 1998), but unfortunately no crystal structure is available for this mutant. In the closely related SHV enzymes (68% similarity in amino acid sequence), substitutions M69I and M69V have been shown to increase inhibitor resistance as well (Giakkoupi *et al.*, 1998; Dubois *et al.*, 2004).

E104

The glutamic acid at residue 104 is located in the strongly conserved loop 101–111 (Jelsch *et al.*, 1993; Petit *et al.*, 1995). Substitution E104K, which is frequently observed in clinical isolates, does not drastically alter the resistance spectrum as is observed for R164S or G238S (Petit *et al.*, 1995). However, E104K does have a drastic effect when present in the background of other substitutions such as R164H, R164S and G238S (Petit *et al.*, 1995), increasing the activity against third-generation cephalosporins and aztreonam (Sowek *et al.*, 1991; Barlow & Hall, 2002). Despite early expectation that structural analysis might soon clarify this synergistic interaction (Petit *et al.*, 1995), it is still poorly understood. It has been suggested that the long lysine side chain could interact directly with the carboxylic acid group present in ceftazidime and aztreonam (Knox, 1995). Alternatively, it was pointed out that because Glu-104 is in direct contact with residue 132, which is part of the conserved SDN loop (130–132) that is involved in substrate binding, perturbation of this contact might result in direct effects on the entry of cephalosporins into the active site (Petit *et al.*, 1995). The crystal structure of TEM-64 (E104K/R164S/M182T) has not shed any further light on this situation (Wang *et al.*, 2002a). It is interesting to note, however, that substitutions E104K and E240K have comparable effects and seem to be neutral in each other's background (Sowek *et al.*, 1991). In both cases, the same functional ammonium group can be modelled in a similar position relative to cefotaxime (Raquet *et al.*, 1994), while electrostatic repulsion would probably not allow the two ammonium groups to lie in neighbouring positions in the double mutant (Sowek *et al.*, 1991). This observation hints towards a more direct mode of action of E104K, as opposed to perturbation of the contact with the SDN loop. Additionally, it has been suggested that E104K might act as a stabilizing mutation that can compensate for the destabilizing effect of substitutions at residues 164 and 238 (Raquet *et al.*, 1995), although this effect was not found in a related study (Wang *et al.*, 2002a).

S130

Substitution S130G, present in three clinical isolates, confers increased resistance to all three inhibitors (Thomas *et al.*, 2005). This effect is straightforward, on the one hand, because S130G prevents the irreversible cross-linking of Ser-70 and Ser-130 (see Fig. 3b) that causes inhibition (Fisher *et al.*, 1978; Knowles, 1985). On the other hand, this is puzzling, because Ser-130 is a strictly conserved catalytic residue that is part of a complex hydrogen bonding network that contributes to the stability of the active site (Matagne & Frere, 1995). Indeed, the substitution decreases the rate of catalysis for several β-lactams, but apparently the remaining activity to these β-lactams is sufficient to confer resistance *in vivo* (Thomas *et al.*, 2005). No other substitutions have been found at this site and site-directed random mutagenesis of this codon, followed by selection for growth on ampicillin–clavulanate, yielded only S130G (Vakulenko *et al.*, 1998), indicating that only this substitution is tolerated. It was demonstrated that S130G confers inhibitor resistance in SHV enzymes as well (Prinarakis *et al.*, 1997).

R164

The arginine at residue 164 forms two salt bridge interactions in the Ω-loop (to Glu-171 and Asp-179) and is as such important for the conformation and stability of this

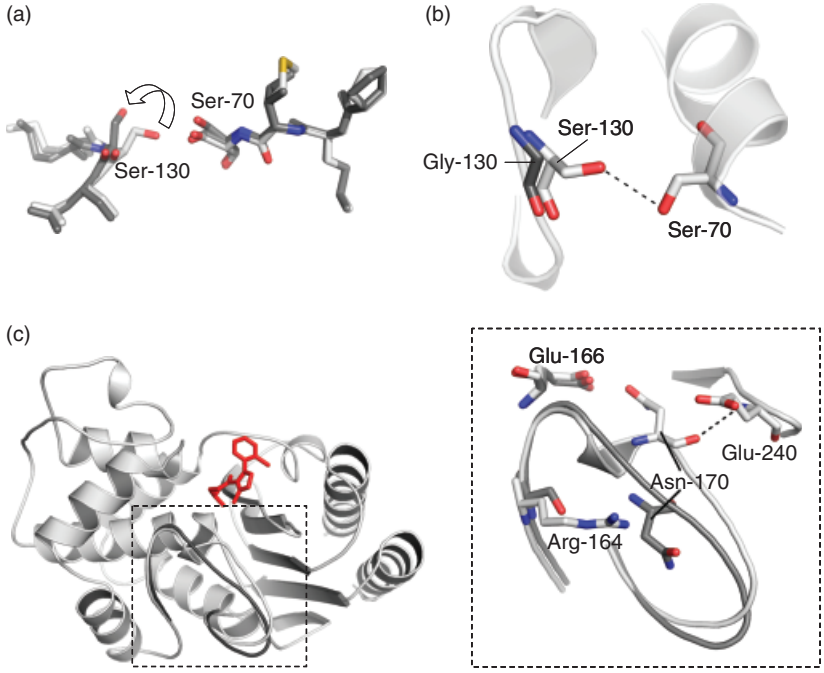

Fig. 3. Structural details of common substitutions in TEM-1. (a) Comparison of TEM-1 (white, PDB code 1FQG) and TEM-32 (M69I/M182T, dark grey, PDB code 1LI0). Side chains of residues 69, 70 and 130 are shown in colour. The rearrangement of Ser-130 in TEM-32 (indicated by arrow) is thought to prevent cross-linking of Ser-70 and Ser-130 by β-lactam inhibitors. (b) Formation of the cross-link between Ser-70 and Ser-130 (dashed line) made by β-lactam inhibitors in TEM-1 (white, PDB code 1BTL) is impossible in TEM-76 (of which only residue Gly-130 is shown in dark grey, PDB code 1YT4). (c) Left: TEM-1 β-lactamase (white, PDB code 1BTL) with a boronic acid inhibitor modelled in the binding pocket (red, obtained from PDB code 1JWZ). The Ω-loop is located in the boxed area and the alternative confirmation of this loop in TEM-64 (E104K/R164S/M182T, PDB code 1JWZ) is shown in dark grey. Right: enlargement of the boxed area on the left, showing the positioning of the side chains of residues 164, 166 and 170 in both TEM-1 (white) and TEM-64 (grey). Outside of the loop, residue 240 and its hydrogen bond to Asn-170 (dashed line) are also shown. Replacement of the large Arg-164 by the much smaller Ser results in collapse of the Ω-loop and dramatic repositioning of the loop. Figures 3 and 4 were created using PyMOL (DeLano, 2002).

loop (and hence the active site topology) and for the correct positioning of the catalytically important Glu-166 (Jelsch *et al.*, 1993; Maveyraud *et al.*, 1996). Substitutions of this residue are linked to increased resistance to third-generation cephalosporins, in particular cefotaxime and ceftazidime (Vakulenko *et al.*, 1999). In clinical isolates, His-164 and Ser-164 are most frequently encountered. The latter substitution has received particular attention from biochemists. Initially, it was proposed that the movement of the Ω-loop due to Arg-164Ser could increase the flexibility of the Ω-loop (Knox, 1995) or displace the 85–142 region (Maveyraud *et al.*, 1996), which would in turn result in better access for cephalosporins with large oxyimino side chains. A later hypothesis presumed that abolishment of the interaction between Arg-164 and Asp-179 due to the smaller Ser side chain would result in a collapse of the top of the Ω-loop to fill the void created in the middle of the loop, in turn resulting in an active site with higher accessibility for β-lactams with larger side chains (Taibi-Tronche *et al.*, 1996). This hypothesis was more recently strengthened by a study of the crystal structure of TEM-64 (E104K/R164S/M182T) in which such a collapse of the Ω-loop was indeed observed (Wang *et al.*, 2002a) (see Fig. 3c).

M182

Substitution M182T is aberrant in the sense that it is located far away from the active site and does not seem to have much effect on enzyme kinetics (Wang *et al.*, 2002a). It does, however, increase the thermodynamic stability of the enzyme and acts as a global suppressor of missense mutations that reduce enzyme stability (Huang & Palzkill, 1997; Bloom *et al.*, 2005; Kather *et al.*, 2008). Despite the general consensus that M182T is an intriguing substitution, the structural explanation for its effect has changed over the years. Initial molecular modelling suggested that the Thr-182 hydroxyl group forms a new hydrogen bond to Glu-64, strengthening the dense hydrogen bond network that stabilizes both the active site and the interface between the enzyme's α- and αβ-domain (Farzaneh *et al.*, 1996). This model seemed to fit to a 2.4 Å structure of a TEM variant containing M182T (Orencia *et al.*,

2001). However, more recent studies based on high-resolution structures have shown that Thr-182 acts as an N-cap residue for the 183–195 helix, forming an additional H-bond to Ala-185 (Minasov *et al.*, 2002; Wang *et al.*, 2002a; Kather *et al.*, 2008) (see Fig. 4a). A possible alternative or additional explanation for the mode of action of Thr-182 is that it suppresses aggregation and misfolding induced by other mutations (Sideraki *et al.*, 2001). In any case, as predicted by its global effect, the substitution appears both in clinically isolated TEM alleles that confer resistance to extended-spectrum cephalosporins and in alleles that confer resistance to β-lactam inhibitors.

A237

Substitution A237T was identified as early as in 1976 in the very first laboratory evolution experiment on TEM, an elegant study by Hall & Knowles (1976) on increased cephalosporin C resistance after chemical mutagenesis. It enhances activity towards cephalosporins by introducing another hydrogen bond with the carbonyl group of the β-lactam ring (Knox, 1995; Kuzin *et al.*, 1995). In addition, A237G has been shown to increase resistance to aztreonam (Arlet *et al.*, 1993; Cantu *et al.*, 1996).

G238

Substitution of the glycine at residue 238 for serine provides resistance to third-generation cephalosporins such as cefotaxime and ceftazidime (Huang *et al.*, 1994; Venkatachalam *et al.*, 1994; Giakkoupi *et al.*, 2000). Two models have been proposed to explain the effect of this substitution: the hydrogen bond model and the steric conflict model. The former model speculated that the formation of a hydrogen bond between the serine residue and the oxime oxygen of extended-spectrum antibiotics increases affinity to the antibiotic (Raquet *et al.*, 1994). However, detailed analysis of several G238 mutants has shown that this is not the case (Cantu & Palzkill, 1998). In the steric conflict model, the introduction of the larger Ser side chain is believed to lead to expansion of the active site cavity, either by repositioning of the B3 β-strand on which residue 238 resides (Knox, 1995) or by repositioning of the Ω-loop (Saves *et al.*, 1995a; Cantu & Palzkill, 1998). Confusingly, there is structural support for both hypotheses. Structural analysis of TEM-52 (E104K/M182T/G238S) has shown that Ser-238 indeed causes a repositioning of the loop 238–243, causing a large rotation of the backbone carbonyl of Glu-240 (see Fig. 4b), which in turn is believed to create extra space for β-lactams with more bulky side chains (Orencia *et al.*, 2001). On the other hand, the crystal structure of a G238A mutant shows that Ala-238 can cause a steric clash with the main chain oxygen of Asn-170, resulting in a slight repositioning of the Ω-loop and enlargement of the active site cavity in the region where the oxyimino group of third-generation cephalosporins would be expected to bind (Wang *et al.*, 2002a). It has been hypothesized that Ser-238 could have similar effects (Saves *et al.*, 1995a; Cantu & Palzkill, 1998). Both G238S and

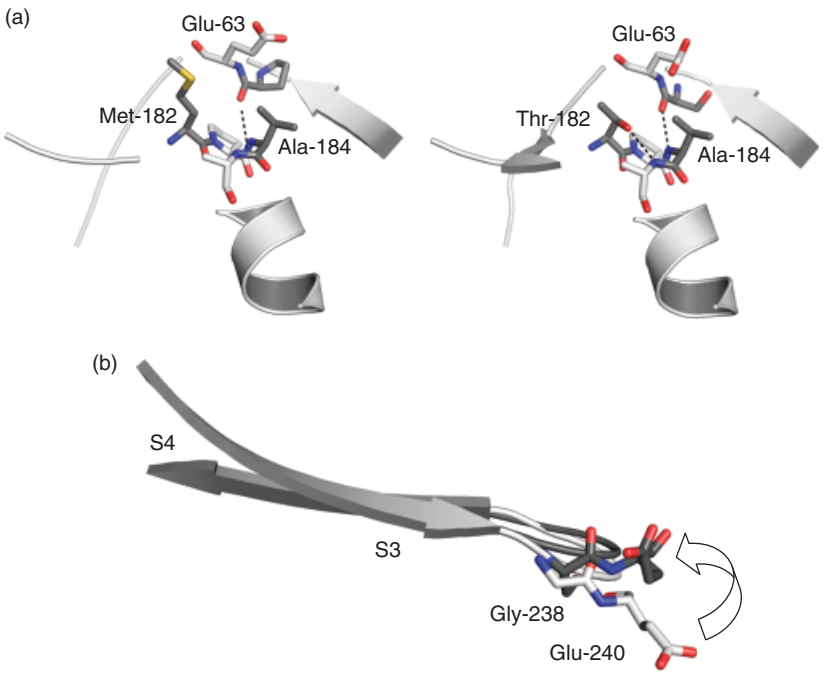

Fig. 4. Structural details of common substitutions in TEM-1. Organization of amino acids 62–63 and 182–185 in (a) TEM-1 (PDB code 1BTL) and (b) the M182T-containing mutant described by Kather *et al.* (2008) (PDB code 3DTM), after which the picture was drawn. Amino acids 62, 182 and 184 are in grey, while 63, 183 and 185 are in white. Substitution of Met-182 by Thr creates a new hydrogen bond between Thr-182 and Ala-185, which contributes to the formation of a more favourable N-cap of the 183–195 helix. (c) Repositioning of residues in the loop connecting β-sheets 3 and 4 due to substitution G238S. Both sheets and their connecting loop are shown in white (PDB code 1BTL), the alternative conformation of the connecting loop in TEM-52 (E104K/M182T/G238S, PDB code 1HTZ) is shown in grey. Side chains for residues at position 238 and 240 are also shown; the arrow indicates the repositioning of Glu-240 in TEM-52.

G238A are also frequently observed in SHV alleles where they contribute to increased cefotaxime resistance (Podbielski *et al.*, 1991; Yuan *et al.*, 2000).

E240

Although common, substitution E240K is exclusively found in the background of substitutions at residue 164 or 238. By itself, this substitution confers small changes in the catalytic efficiencies for ceftazidime and aztreonam (Labia *et al.*, 1988; Venkatachalam *et al.*, 1994; Blazquez *et al.*, 1998). It was suggested that this can be explained by the formation of an electrostatic bond between Lys-240 with the carboxylic acid group on these oxyimino β-lactams (Knox, 1995). The effect of E240K is more broad and pronounced in the background of R164S (Sowek *et al.*, 1991; Bradford & Sanders, 1995; Blazquez *et al.*, 1998) and G238S (Huang *et al.*, 1994; Venkatachalam *et al.*, 1994; Cantu *et al.*, 1996); resistance to ceftazidime and aztreonam is increased drastically in these double mutants, as is resistance to cefotaxime. In this respect, it has been suggested that substitutions at residues 164 and 238 might ameliorate the effect of introducing a larger residue at position 240 (Page, 2008). An alternative explanation is that E240K might be a stabilizing substitution that compensates for the destabilizing consequences of R164S or G238S (Raquet *et al.*, 1995). However, in this case, one might expect the substitution to appear in *in vitro* studies that select for stabilizing mutations, which is not the case (Osuna *et al.*, 2002; Hecky & Müller, 2005; Bershtein *et al.*, 2008; Kather *et al.*, 2008; Goldsmith & Tawfik, 2009). Interestingly, both E240G (Hecky & Müller, 2005; Bershtein *et al.*, 2008) and E240H (Kather *et al.*, 2008) have been found in such studies. In clinical SHV isolates, E240K is frequently observed, while D240G is common in CTX-Ms.

R244

The long side chain of Arg-244 reaches over β-strand B3 to the edge of the binding site, and is kept in place by two hydrogen bonds to Asn-276 (Jelsch *et al.*, 1993). In clinical isolates, changes to the smaller leucine, threonine, serine, histidine or cysteine are observed. The latter three substitutions are more frequently observed and result in reduced resistance to penicillin, but increased resistance to all three inhibitors (Delaire *et al.*, 1992; Bret *et al.*, 1997). Structure-based analysis of the inhibition mechanism of clavulanic acid indicates that together with the main chain carbonyl group of Val-216, the guanidinium of Arg-244 can serve as an 'electrostatic anchor' for a conserved water molecule that is thought to be a proton source essential for the inactivation mechanism (Imtiaz *et al.*, 1993, 1994). It has been argued that replacing Arg-244 with an amino acid with shorter side chains would result in failure to activate this water (Imtiaz *et al.*, 1993). These ideas were confirmed more recently when the crystal structure of TEM-30 (R244S) became available, in which the water molecule is indeed absent, which is thought to reduce the binding affinity of inhibitors (Wang *et al.*, 2002b).

N276

It has been shown that N276D confers clavulanic acid resistance (Saves *et al.*, 1995b). The carbonyl group of Asn-276 accepts two hydrogen bonds from Arg-244, orienting the guanidinium group of Arg-244 (Delaire *et al.*, 1992; Jelsch *et al.*, 1993). Molecular modelling predicted the formation of a salt bridge between Arg-244 and Asp-276 in the N276D mutant, but it incorrectly assumed a shift in the orientation of the Arg-244 side chain (Saves *et al.*, 1995b; Canica *et al.*, 1998). Structural analysis has now revealed that N276D indeed results in the formation of a salt bridge to Arg-244 and in significant movement of Asp-276 (Swaren *et al.*, 1999). However, this does not result in displacement of Arg-244, but rather in displacement of the entire helix H11, in turn resulting in a displacement of H1 and H10. Two different phenomena observed in this crystal structure could explain the decreased affinities observed. First, it has been suggested that Arg-244 can act as a counterion for the carboxylates of β-lactams and inhibitors on binding to the active site (Zafaralla *et al.*, 1992). The enhanced interactions between Arg-244 and Asp-276 significantly decrease the positive electrostatic potential generated by Arg-244 by neutralizing its guanidinium group, which could in turn explain the decreased affinity for β-lactams and inhibitors (Swaren *et al.*, 1999). Second, the occupancy of the water molecule present at hydrogen bond distance from Val-216 and Arg-244 is much reduced. The molecule, although clearly present in the wild-type structure, seems only transiently present in the N276D mutant (Swaren *et al.*, 1999), which could explain the mutants' reduced affinity for inhibitors (see the section on R244 for details on this mechanism). Additionally, we would like to point out that N276D was one of the dominant substitutions identified in a study selecting for stabilizing substitutions (Osuna *et al.*, 2002), suggesting a stabilizing effect.

Clinical relevance of TEMs

The TEM β-lactamases have been one of the most commonly encountered groups of resistance genes among clinical isolates of gram-negative bacteria since the 1960s (Datta & Kontomichalou, 1965; Datta & Richmond, 1966). They are broadly distributed among the *Enterobacteriaceae* (Matthew, 1979), but have also been found in *Neisseriaceae* (Elwell *et al.*, 1977), *Pseudomonas* (Labia *et al.*, 1975) and *Haemophilus* (Medeiros & Obrien, 1975) species. TEMs

often co-occur with other chromosomal (AmpC) or plasmidic (SHV, OXA, CTX-M) β-lactamases (Park *et al*., 2005; Barlow *et al*., 2009) and they are common in commensal populations inhabiting the human gut (Sommer *et al*., 2009). However, in recent years, the CTX-Ms (a recently discovered family of β-lactamases named after their common ability to confer cefotaxime resistance) have started spreading rapidly among populations of clinical, gram-negative bacteria (Shahid *et al*., 2009). There have been multiple predictions that, at their current rate of spread, the CTX-Ms are the most common resistance determinant in clinical gram-negative populations (Livermore *et al*., 2007; Rossolini *et al*., 2008). Indeed, this shift has already been reported in most surveillance studies, where CTX-Ms were found in the majority of strains. However, the abundance of CTX-Ms has not necessarily decreased the occurrence of TEMs, although it has significantly decreased the attention that TEMs are given. In many strains, TEMs and CTX-Ms co-occur and provide complementary contributions to the overall resistance of the strain.

TEMs are frequently encountered in clinical isolates expressing CTX-Ms

In a surveillance study conducted in a Swedish hospital and associated facilities from 2001 to 2006 (Fang *et al*., 2008), CTX-Ms were detected in 92% of isolates, with TEMs present in 63% of isolates. In 62% of the strains, CTX-Ms and TEMs co-occurred. Similar results have been found elsewhere (Mulvey *et al*., 2004; Hernandez *et al*., 2005; Brasme *et al*., 2007; Machado *et al*., 2007; Romero *et al*., 2007; Kjerulf *et al*., 2008). In a nationwide study conducted in France in 2006 (Arpin *et al*., 2009), 73% of extended-spectrum β-lactamase (ESBL) producers expressed a TEM β-lactamase, whereas 65% expressed a CTX-M β-lactamase. Thirty percent of the ESBL producers collected in this study expressed both TEM and CTX-M β-lactamases.

Mroczkowska & Barlow (2008a, b) gave a possible reason for the frequent co-occurrence of TEM and CTX-M β-lactamases. They found that recombination occurs between alleles with similar sequences when those alleles co-occur in a single strain. This occurred even if the resistance phenotypes conferred by the alleles were complementary. They also found a statistically significant overabundance of clinical strains coexpressing CTX-Ms and TEMs and an underrepresentation of strains expressing two TEM alleles if a random distribution of alleles is assumed (Barlow *et al*., 2009), which could be caused by recombination or by transposon immunity, i.e. the reduced uptake of β-lactamase by a plasmid already carrying a similar β-lactamase (Robinson *et al*., 1977). CTX-Ms and TEMs are likely to co-occur with each other, because the acquisition of one does not seem to hinder the acquisition of the other. This suggests that the TEMs are likely to persist in bacterial populations even as CTX-Ms sweep through them.

New TEMs continue to evolve and confer important phenotypes

Despite there being at least 174 TEMs that differ in amino acid sequence (see http://www.lahey.org/Studies/temtable.asp), new TEMs continue to arise. There are at least 10 that have been assigned names and that are awaiting published reports and descriptions of the phenotypes they confer. Phenotypic data were released in 2007 for three novel complex mutant TEMs (Robin *et al*., 2007a, b). Historically, the abilities to confer resistance to extended-spectrum β-lactams and penicillin/β-lactamase inhibitor combinations have been mutually exclusive. However, a handful of alleles that simultaneously confer resistance to both types of antibiotics have emerged among the TEMs (Neuwirth *et al*., 2001; Robin *et al*., 2005, 2006). The emergence of those alleles along with the *in vitro* evolution results described in this paper suggests that the evolutionary potential of the TEMs has not been fully explored in clinically relevant bacteria. We anticipate that TEMs are likely to continue to contribute to the failure of β-lactam treatment in patients. The close match between substitutions found in clinical and laboratory TEM isolates suggests that *in vitro* studies are important to clinicians because they may reveal novel phenotypes that will arise in the future. This will facilitate the proactive design of susceptibility tests to detect those phenotypes as well as help determine what back-up treatments are best.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Setup of the comparison of the natural and laboratory evolution.
Table S1. Amino acid substitutions in clinically and experimentally isolated TEM alleles.

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