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In Vitro Prediction of the Evolution of the GES-1 β-Lactamase Hydrolytic Activity

Séverine Bontron,[1] Laurent Poirel[1,2*], and Patrice Nordmann[1,2,3]

[1]Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland, [2]INSERM U914 «Emerging Resistance to Antibiotics», Faculté de Médecine et Université Paris Sud, K.-Bicêtre, France, and [3]HFR-Hôpital Cantonal de Fribourg, Fribourg, Switzerland

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*Corresponding author. Mailing address: Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, rue Albert Gockel 3, CH-1700 Fribourg, Switzerland. Phone: 41-26-300-9582. E-mail: laurent.poirel@unifr.ch

Abstract

Resistance to ß-lactams is constantly increasing, due to the emergence of totally new enzymes, but also to the evolution of pre-existing ß-lactamases. GES-1 is a clinically-relevant extended-spectrum β-lactamase (ESBL) hydrolyzing penicillins and broad-spectrum cephalosporins, but sparing monobactams and carbapenems. However, several GES-1 variants (i.e. GES-2 and GES-5) previously identified among clinical isolates display an extended spectrum of activity toward carbapenems. To study the evolution potential of the GES-1 β-lactamase, this enzyme was submitted to in-vitro directed evolution, with selection on increasing concentrations of the cephalosporin cefotaxime, the monobactam aztreonam, or the carbapenem imipenem. The highest resistance levels were conferred by the combination of up to four substitutions. The A6T, E104K, G243A variant selected on cefotaxime, and the A6T, E104K, T237A, G243A variant selected on aztreonam, conferred high resistance to cefotaxime, ceftazidime, and aztreonam. Conversely, the A6T, G170S variant selected on imipenem conferred high resistance to imipenem and cefoxitin. Noteworthy, the A6T substitution involved in higher MICs for all ß-lactams is located in the leader peptide of the GES enzyme, therefore not present in the mature protein. Acquired cross resistance was not

observed since selection with CTX or ATM did not select for resistance to IPM and vice versa. Here we demonstrated that β-lactamase GES-1 exhibits peculiar properties with a significant potential to gain activity toward broad-spectrum cephalosporins, monobactams, and carbapenems.

INTRODUCTION

The main mechanism of resistance to ß-lactams in Gram negatives is the production of ß-lactamases, which are classified into four molecular classes, namely A, B, C, and D based on protein sequence analysis (1). Enzymes belonging to class A, C, and D, are serine enzymes, while those belonging to class B are metallo-enzymes requiring zinc ions for activity (2, 3). Over the past 70 years, since the introduction of penicillins and cephalosporins, the massive use of broad-spectrum ß-lactams has been at the origin of the selection of ß-lactamases with broadened hydrolytic activities. Emergence of those broad-spectrum ß-lactamases may correspond to three main phenomena; *i)* the evolution of pre-existing narrow-spectrum ß-lactamases with an extension of their hydrolytic profile due to key amino-acid substitutions, as observed for TEM and SHV β-lactamases, *ii)* the acquisition of enzymes possessing an intrinsic broad-spectrum hydrolytic activity, as observed for all CTX-M-type extended-spectrum β-lactamases (ESBLs) (4, 5), and *iii)* the evolution of broad-spectrum enzymes to expand or increase their hydrolytic activity to carbapenems.

GES-1 is an ESBL firstly identified in a *Klebsiella pneumoniae* (6), and then extensively reported from clinical isolates in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (7-10), but also frequently from the environmental (11-13). Similarly to other ESBLs, GES-1

hydrolyses penicillins and broad-spectrum cephalosporins, spares carbapenems, and is susceptible to the activity of clavulanic acid as inhibitor. However, by contrast to most ESBLs, GES-1 has a low activity toward the broad-spectrum cephalosporin cefotaxime (CTX), and does not hydrolyze monobactams (6). The GES family comprises 24 variants (GES-1 to GES-24) identified from clinical isolates, some of them having amino acid substitutions conferring peculiar hydrolytic properties (Table 1). The G170N and G170S substitutions (Ambler numbering [1]), located in the omega-loop of the enzyme and first described in GES-2 and GES-5, confer extended activity against carbapenems and cefoxitin (FOX) (this latter only for G170S), decreased hydrolysis of broad-spectrum cephalosporins, and decreased susceptibility to ß-lactam inhibitors (Table 1) (9, 14-16). On the other hand, the E104K, G243A, and G243S substitutions, identified in several GES variants, have been shown to confer higher activity toward broad-spectrum cephalosporins and the monobactam aztreonam (ATM), together with an increased susceptibility to ß-lactam inhibitors (Table 1).

Given its ability to evolve, GES-1 was chosen as a model enzyme for testing the diversification potential of ESBLs. Therefore, the GES-1 enzyme was subjected to directed evolution; this method consists of iterative rounds of random mutagenesis and selection, and is commonly used for altering or optimizing protein function (17, 18). GES-1 was submitted

to three different ß-lactam-based selective pressures, namely the broad-spectrum cephalosporin CTX, ATM, or the carbapenem imipenem (IPM). A series of variants with increased and/or broadened specificity was selected, from which the causality between the increased hydrolytic activity and the mutations could be inferred, and future evolutionary trajectories predicted.

MATERIALS AND METHODS

Construction and selection of mutagenized GES-1 libraries. The pBSKSII-kanR-GES-1 plasmid was used as the reference plasmid coding for GES-1. Plasmid pBSKSII-kanR is derived from pBluescriptII (a high copy number plasmid, 500 copies per cell), and encodes resistance to kanamycin. The entire bla_{GES-1} coding region (6) was amplified, the amplicon including 31 bp upstream of the ATG start, with primers No50 (GES-1-*HindIII*-F, 5'-gatgatAAGCTTACAAAGATAATTTCCATCTCAAGG-3') and No51 (GES-1-*NotI*-R, 5'-gatgatGCGGCCGCCTATTTGTCCGTGCTCAGGATG-3'), and cloned into the HindIII/NotI restriction sites of pBSKSII-kanR. The construct was verified by sequencing. Random mutagenesis was performed with the GenMorph II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), with primers No50 and No51, following the manufacturer

recommendations. The PCR amplified mutagenized product was purified, digested, and ligated into the HindIII/NotI restricion sites of pBSKSII-kanR. After purification, the ligation mixture was transformed into TOP10 electro-competent *E. coli* cells (Life Technologies, Zug, Switzerland). The library was plated on Luria broth plates supplemented with 25 μg/ml of kanamycin. At each round, the complexity of the library was at least 10^5 independent clones. The mean substitution rate was of 2 nucleotides per molecule, based on sequencing of 10 clones. This corresponds to at least $6x10^4$ distinct sequences, as calculated with the library statistics program PEDEL (19). For selection, the libraries were plated on increasing doses of the indicated antibiotic, with a 2-fold increment. Plasmids recovered from the clones obtained with the highest antibiotic concentrations (usually 100 to 300 clones) were isolated, re-transformed, and plated again on the same antibiotic concentration. At this step, at least 4 clones were analyzed by sequencing, and the rest of the clones was isolated as a pool, and used as a basis for the next round of random mutagenesis. Constructs with different combinations of mutations were made by sub-cloning, or with the Q5 Site-Directed Mutagenesis Kit (New England BioLabs, Ipswich, MA).

MICs, specific hydrolytic activities, and IC_{50}s measurements. MICs were measured by Etest (bioMérieux, Marcy l'Etoile, France). Specific hydrolytic activities were measured

from whole cell extracts of recombinant *E. coli* strains producing the different GES variants as described (6). Assays were performed in 500 µl total volume, using 150 µM (for benzylpenicillin [PEN], ATM, IPM, FOX) or 75 µM (for CTX and ceftazidime [CAZ]) of substrate. Hydrolysis measurements were performed at room temperature with a JENWAY spectrophotometer (Staffordshire, UK). The protein concentrations were measured with Bradford Reagent (Sigma-Aldrich, Buchs, Switzerland). The results were expressed in nmoles x min^{-1} x μg^{-1} extract. Experiments were made in triplicates from 3 independent cultures. Inhibitory concentrations inhibiting 50% of the hydrolysis activity (IC_{50}) were measured for clavulanic acid using PEN as substrate. A 3-min long preincubation step with clavulanic acid was used before adding PEN. Those experiments were performed in triplicates with three independent cultures.

RESULTS

Selection of GES variants conferring increased resistance to CTX, ATM, and IPM by directed evolution. GES mutants conferring increased resistance to CTX, ATM, or IPM were recovered after three (for ATM and IPM) or four (for CTX) rounds of mutagenesis and selection. Over the rounds the variants were successively selected with 1, 2, 4, and

16 μg/ml of CTX, with 1, 16, 128, and 256 μg/ml of ATM, or with 0.1, 0.125, and 0.25 μg/ml of IPM. Additional mutagenesis steps could not select variants with higher MICs. At the end of each round, at least four clones were sequenced. Mutations common to several clones were anticipated to be the phenotypically relevant ones, but additional substitutions, silent or not, did accumulate throughout the mutagenesis rounds. Therefore, some constructs were specifically generated to definitely correlate amino acid substitutions to resistance phenotypes (data not shown). The corresponding so-called cured variants (GES-C1 to -C5 for selection with CTX, GES-A1 to -A5 for selection with ATM, and GES-I1 to -I3 for selection with IPM) harboring the corresponding amino acid changes are depicted in Table 2A-C. The sequences of the originally isolated clones are listed in Table S1.

MICs of ß-lactams for the selected mutants. Upon selection with CTX, the A6T substitution (clone GES-C1), located in the signal peptide, was the only mutation selected after round 1 (Table 2A). At round 2, substitutions E104K or G243S were added to A6T (clones GES-C2 and GES-C3), and GES-C4 (A6T, G243A) was selected at round 3. Combination of substitutions A6T, E104K, G243A was selected at round 4 (clone GES-C5). MICs of CTX for these constructs gradually increased during the directed evolution, from 0.75 μg/ml for GES-1 to 48 μg/ml for GES-C5 (Table 2A). MICs of ATM and ceftazidime

(CAZ) increased concomitantly to those of CTX, while MICs of FOX, IPM, and the carbapenem ertapenem (ETP) remained unchanged (Table 2A). Four additional constructs were made to dissect the role of each amino acid change. Single E104K, G243S, or G243A substitutions (clones GES-C6, GES-C7, and GES-C8, respectively) conferred a lower resistance than A6T (clone GES-C1). Combination of E104K and G243A (clone GES-C9) was slightly more efficient than single mutations (Table 2A). Clone GES-C5 exhibiting substitutions A6T, E104K, and G243A combined three changes that individually conferred modest MIC increases, but when combined together resulted into a variant for which the MIC of CTX reached 48 μg/ml.

When selecting with ATM, single E104K (clone GES-A1) or G243A (clone GES-A2) mutations were selected in round 1, while a combination of those two was selected at round 2 (clone GES-A3). Ultimately, clones selected on ATM-128 additionally harbored a T237A substitution (E104K, T237A, G243A, clone GES-A4), and those selected on ATM-256 harbored the A6T substitution in addition to the three other changes (clone GES-A5) (Table 2B). MICs of ATM for these constructs increased during the directed evolution, from 0.25 μg/ml for GES-1 to >256 μg/ml for clones GES-A4 and GES-A5. In parallel MICs of CAZ increased sharply, while those of CTX more modestly. No change in MICs of FOX, IPM, or

ETP was observed (Table 2B). Each of the three E104K, T237A, or G243A substitutions (clones GES-C6, GES-A6, and GES-A2, respectively) had slightly increased MICs of ATM (from 1.5 to 3 μg/ml). Any dual combination resulted into higher MICs of ATM (from 12 or 32 μg/ml, clones GES-A3, -A7 and -A8), while the triple mutant had an MIC of ATM of >256 μg/ml (clone GES-A4) (Table 2B). Of note, while the T237A substitution correlated with increased MICs of ATM and CAZ, it was systematically deleterious for the MIC of CTX.

When selecting with IPM, substitution G170S was selected at round 1 (clone GES-I1). Then substitution c-1t lying 1 bp before the ATG start codon (clone GES-I2) and finally substitution A6T (clone GES-I3) were selected at rounds 2 and 3 (Table 2C). Overall, MICs of IPM increased from 0.25 μg/ml for wild-type GES-1 to 2 μg/ml for clone GES-I3. MICs of ETP and FOX increased concomitantly but conversely, MICs of ATM, CTX, and CAZ were lowered once the G170S mutation was selected (Table 2C).

In-vitro specific hydrolytic activities of the GES variants correlating with MICs values. In order to confirm that the higher MICs observed for the selected clones were indeed related to higher catalytic activities or higher amount of the different GES enzymes selected, and not to a non-enzymatic resistance mechanism, in-vitro specific hydrolytic activities of a

set of GES variants were determined. Overall, for all enzymes the increased hydrolytic activities toward CTX, CAZ, ATM, IPM, and FOX correlated with the higher MICs of the corresponding substrates (Tables 2A-C).

Differential inhibition of the GES selected variants by clavulanic acid. Determination of IC_{50}s of clavulanic acid was performed for a representative set of the GES variants. Clones GES-C1 and GES-C5 showed similar IC_{50} values of clavulanic acid compared to the wild-type GES-1 (Table 2A). However, clones GES-A4 and GES-A5 showed 10-fold lower IC_{50} values (0.6 μM) (Table 2B) likely due to the T237A substitution. Conversely clones GES-I3 and GES-I4 selected on IPM and harboring the G170S substitution showed significantly higher IC_{50} values (Table 2C). Overall, mutants selected on CTX showed similar susceptibility to clavulanic acid as GES-1, while clones selected on ATM showed increased, and clones selected on IPM lower susceptibility to clavulanic acid.

DISCUSSION

The directed evolution procedure used here combined the generation of mutagenized libraries with antibiotic selection, and allowed the selection of GES variants that may be categorized into two classes, being those selected on CTX or ATM conferring high resistance to CTX,

198 ATM, and CAZ on one hand, and those selected on IPM conferring high resistance to IPM
199 and FOX on the other hand.
200 Interestingly, the A6T substitution located into the signal sequence (18 amino-acid long for
201 GES-1) was selected through all three evolutionary routes. Noteworthy, signal sequences are
202 required for translocation to the periplasm (20). The A6T substitution in GES-1 systematically
203 conferred a 3- to 8-fold increase in the MICs of CTX, ATM, and IPM (Table 2A-C). In
204 accordance with our observation, a study including experimental mutagenesis of a consensus
205 signal sequence fused to a ß-lactamase gene increased ampicillin tolerance level of the host
206 cell up to 8-fold (21). To the best of our knowledge, it has never been clearly assessed that
207 mutations in the signal sequence of any ß-lactamase may confer increased resistance to ß-
208 lactams. The impact of such substitution should therefore be further investigated as a
209 mechanism leading to reduced susceptibility or even resistance to ß-lactams.
210 By selecting with IPM, the G170S mutation located in the Ω-loop of the catalytic site (amino
211 acids 159 to 182), a highly conserved motif among the β-lactamases, was recovered. This
212 substitution was previously shown to confer a 100-fold increased catalytic activity against
213 IPM when compared to GES-1 (14, 22, 23). The E104 residue is exposed near the entrance to
214 the binding site, and the E104K substitution is commonly found in the TEM family, where

this change participates to the expansion of the β-lactamase spectrum, more strikingly when associated with other substitutions such as R164S or G238S (24-26). Despite several studies, the mechanism of this synergism remains poorly understood (26). The G243A is not conserved among β-lactamases and this change might create subtle rearrangements in the disulfide bond. The T237 amino acid, together with the S70 residue, forms an oxyanion hole, which houses the ß-lactam carbonyl of the acyl-enzymes intermediate (27). Position 237, usually occupied by an Ala or Ser in most class A ß-lactamases, corresponds to a Thr residue in GES-1, but also in the PER-1 ESBL and in the class A KPC-2 carbapenemase. It was experimentally shown with KPC-2 that a T237A change resulted into lower hydrolysis of CTX (28). Conversely in TEM, the natural or experimentally generated A237T substitution confers an increased hydrolysis of CTX and a decreased hydrolysis of CAZ and ATM (22, 30). These observations correlate with the detrimental effect of the T237A substitution in GES for the hydrolysis of CTX, and the beneficial effect for the hydrolysis of CAZ and ATM. Substitutions E104K, G170S, T237A, G243S, and G243A selected through our study were previously described in GES alleles identified from clinical isolates (Table 1). Noticeably, substitution G170S, increasing carbapenem hydrolysis, was previously identified in the natural carbapenemase GES-5. Substitutions E104K, T237A, and G243A were also identified

in natural GES alleles, either alone (GES-9, G243A), associated to phenotypically uncharacterized mutations (GES-3, GES-7, GES-19, GES-22), or combined with other substitutions (GES-12 and GES-17) (Table 1). As inferred from our in-vitro directed evolution experiments, GES-5, GES-12 and GES-17 may therefore be prone to evolve into more active variants. On the other hand, some GES alleles recovered from clinical isolates combined the G170S mutation conferring higher activity toward carbapenems, together with another mutation conferring increased activity toward CTX, ATM, and CAZ (see GES-6, E104K, G170S, and GES-14, G243A, G170S). Comparative studies showed that the increased hydrolysis of CTX, CAZ, or ATM mediated by E104K or G243A was abolished by the additional presence of G170S (29, 31, 32). This precludes that the natural alleles GES-6 and GES-14 have been selected under successive distinct selective pressures. Similarly, detailed analysis of the CTX-M-type ESBL potential evolutionary trajectories showed that the diversification process of the CTX-M variants could only be explained by a selection with at least two antibiotics (33).

Our study showed that GES enzymes can evolve into two types of variants conferring higher resistance to CTX, ATM, or to IPM. Spontaneous evolution of antibiotic resistance is a multifactorial phenomenon, given the diversity of the genetic support of resistance genes, of

the bacterial strain (including potential changes of membrane permeability or of the penicillin-binding proteins), and of the nature and the concentration of the antibiotic. As a consequence, a higher number of possible mutations and evolutionary trajectories are possible, although constrained by intramolecular interactions (34). Nevertheless, a good correlation between in-vitro prediction inferred from the analysis of the selected variants, and those found in clinical isolates has been established here. Predictions regarding the occurrence of very efficient natural variants in term of catalytic efficiency using an in-vitro directed evolution was previously demonstrated for the TEM ß-lactamase (34-37). From our study we may speculate that selection with cephalosporins or monobactam might not select for GES variants possessing carbapenemase activity, and conversely selection with the carbapenem IPM might not select for GES variants possessing increased hydrolytic activity toward CTX, CAZ, or ATM. Interestingly, as previously noticed with CTX-M-type ESBLs possessing increased activity toward broad-spectrum cephalosporins (38), some antagonistic pleiotropy was observed, such as a decreased susceptibility to ß-lactamase inhibitors of some GES variants that exhibited increased catalytic activity toward carbapenems. Overall, such an approach sheds light on how clinical alleles have been selected, and might predict the future evolutionary trajectories of the ß-lactamases according to the antibiotic selection pressure.

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Table 1. GES variants isolated from clinical isolates, in relation to their hydrolysis profile

	Amino acid position[b]															Hydrolysis profile[c]					
Variant[a]	11	12	44	55	62	80	81	104	125	130	167	169	170	237	243	CTX	ATM	CAZ	IPM	FOX	References
GES-1	G	I	Q	I	M	V	F	E	A	S	P	M	G	T	G	+	-	++	-	-	(6)
GES-2													N			+/-	-	+	+	-	(9, 29)
GES-3					T			K								+	+	++	-	-	(39)
GES-4					T			K					S			+	+	++	+	+	(39)
GES-5													S			+/-	-	+	+	+	(29, 32, 40)
GES-6								K					S			+/-	-	++	+	+	(29)
GES-7								K	L							+	++	+++	-	-	(29, 41, 42)
GES-8									L							+	-	++	-	-	(42)
GES-9															S	++	+	+++	-	-	(8, 32)
GES-10		T			T					C											
GES-11															A	++	+	+++	-	-	(31, 32, 43)
GES-12														A	A	++	+	+++	-	-	(31, 32)
GES-13								K					N			+	+	++	-	-	(44)
GES-14													S		A	+/-	-	++	+	+	(31, 32)
GES-15											S		S								
GES-16			E										S								
GES-17								K							A						
GES-18						I							S			-	-	+	+	+	(40)
GES-19	A														A						
GES-20	A												S								
GES-21							L						S								
GES-22												L			A						
GES-23				L																	
GES-24					T								S								

[a] GES-1 to GES-24 clinical variants are listed according to http://lahey.org/studies/other.asp.

[b] Amino acid positions were assigned according to Ambler. Amino acid changes as compared to wild type GES-1 are indicated.

[c] When available, the hydrolysis profile of each variant was estimated from published MICs: + and -, hydrolysis and no hydrolysis, respectively, with dark grey related to an increased hydrolysis, and light grey to a decreased hydrolysis, as compared to wild type GES-1. For each variant, the amino acid substitution responsible for the change in the hydrolytic profile is framed. Of note, these comparisons are indicative, given the bacterial species and the genetic support differ according to the study.

Table 2. Selected GES variants, related MICs and in vitro specific hydrolytic activities of CTX, ATM, CAZ, IPM, ETP, FOX, and IC_{50} of clavulanic acid

Table 2A. Clones selected on CTX

		Amino acid position[b]			MIC[c] (μg/ml)						In vitro specific hydrolytic activity[d] (nmoles x min^{-1} x μg^{-1} extract)						IC_{50} clav. ac. (μM)
	Variant[a]	6	104	243	CTX	ATM	CAZ	IPM	ETP	FOX	PEN	CTX	ATM	CAZ	IPM	FOX	
Wild-type	GES-1	A	E	G	0.75	0.25	12	0.25	0.006	4	3.4±0.6	1.0±0.2	<0.1	<0.1	<0.1	<0.1	7.7±0.5
Round 1	GES-C1	T			4	1.5	128	0.38	0.016	6	9.1±0.6	2.8±0.5	<0.1	<0.1	<0.1	<0.1	10.3±3.8
Round 2	GES-C2	T	K		8	16	>256	0.38	0.016	6	-	-	-	-	-	-	-
	GES-C3	T		S	8	12	>256	0.38	0.016	3	-	-	-	-	-	-	-
Round 3	GES-C4	T		A	16	24	>256	0.38	0.032	3	5.2±0.9	12.1±1.0	1.8±0.7	1.5±0.3	<0.1	<0.1	-
Round 4	GES-C5	T	K	A	48	>256	>256	0.38	0.032	4	4.6±0.6	17.4±1.6	24.2±2.8	6.6±0.6	<0.1	<0.1	5.3±0.1
Conctructs	GES-C6		K		1.5	3	>256	0.25	0.008	4	-	-	-	-	-	-	-
	GES-C7			S	1.5	2	48	0.25	0.008	4	-	-	-	-	-	-	-
	GES-C8			A	3	3	128	0.38	0.012	3	-	-	-	-	-	-	-
	GES-C9		K	A	4	32	>256	0.25	0.012	4	-	-	-	-	-	-	-

Table 2B. Clones selected on ATM

	Variant[a]	Amino acid position[b]				MIC[c] (μg/ml)						In vitro specific hydrolytic activity[d] (nmoles x min^{-1} x μg^{-1} extract)						IC_{50} clav. ac. (μM)
		6	104	237	243	CTX	ATM	CAZ	IPM	ETP	FOX	PEN	CTX	ATM	CAZ	IPM	FOX	
Wild-type	GES-1	A	E	T	G	0.75	0.25	12	0.25	0.006	4	3.4±0.6	1.0±0.2	<0.1	<0.1	<0.1	<0.1	7.7±0.5
Round 1	GES-A1		K			1.5	3	>256	0.25	0.008	4	-	-	-	-	-	-	-
	GES-A2				A	3	3	128	0.38	0.012	3	-	-	-	-	-	-	-
Round 2	GES-A3		K		A	4	32	>256	0.25	0.012	4	1.3±0.2	4.1±0.6	4.7±0.5	1.4±0.3	<0.1	<0.1	
Round 3	GES-A4		K	A	A	2	>256	>256	0.38	0.025	4	8.7±2.3	4.5±1.1	14.2±3.0	4.0±0.6	<0.1	<0.1	0.6±0.07
Round 4	GES-A5	T	K	A	A	12	>256	>256	0.25	0.064	6	23.6±2.5	12.2±1.2	38.0±2.5	10.9±0.7	<0.1	<0.1	0.5±0.04
Conctructs	GES-A6			A		0.38	1.5	48	0.25	0.012	3	-	-	-	-	-	-	-
	GES-A7		K	A		0.75	32	>256	0.25	0.012	4	-	-	-	-	-	-	-
	GES-A8			A	A	1	12	>256	0.38	0.025	4	-	-	-	-	-	-	-

Table 2C. Clones selected on IPM

		Amino acid position[b]			MIC (μg/ml)[c]						In vitro specific hydrolytic activity (nmoles x min^{-1} x μg^{-1} extract)[d]						IC_{50} clav. ac. (μM)[e]
	Variant[a]	nt-1	6	170	CTX	ATM	CAZ	IPM	ETP	FOX	PEN	CTX	ATM	CAZ	IPM	FOX	
Wild-type	GES-1	c	A	G	0.75	0.25	12	0.25	0.006	4	3.4±0.6	1.0±0.2	<0.1	<0.1	<0.1	<0.1	7.7±0.5
Round 1	GES-I1			S	0.125	0.094	1.5	0.5	0.064	12	7.7±1.2	<0.1	<0.1	<0.1	0.15±0.0006	0.16±0.04	86±24
Round 2	GES-I2	t		S	0.19	0.125	2	0.75	0.094	24	12.4±3.2	<0.1	<0.1	<0.1	0.18±0.02	0.27±0.06	-
Round 3	GES-I3	t	T	S	1	0.19	12	2	0.25	>256	37.8±2.1	<0.1	<0.1	<0.1	0.64±0.04	0.97±0.13	150±12
Conctructs	GES-I4		T		4	1.5	128	0.38	0.016	6	-	-	-	-	-	-	-
	GES-I5		T	S	0.5	0.19	4	0.75	0.19	48	-	-	-	-	-	-	-

[a] Variants selected with CTX and derivative constructs are designated with a "C", those on ATM with an "A", and those on IPM with an "I". The round of directed evolution on which the variants were selected is indicated on the left. Antibiotic concentrations used for selection were the following: on CTX, round 1: 1μg/ml, round 2: 2μg/ml, round 3: 4μg/ml, round 4: 16μg/ml; on ATM, round 1: 1μg/ml, round 2: 16μg/ml, round 3: 128μg/ml, round 4: 256-μg/ml; on IPM, round 1: 0.1μg/ml, round 2: 0.125μg/ml, round 3: 0.25-μg/ml.

[b] Amino acid positions were assigned according to Ambler, except for mutations before the ATG start codon, where the nucleotide number relative to it is indicated. Amino acid changes as compared to wild type GES-1 are indicated.

[c] All the GES alleles are expressed in the highly susceptible *E. coli* TOP10.

[d] Specific activity values were measured by U.V. spectrophotometry from crude extracts of *E.coli* TOP10 producing the indicated variant, for each of the indicated antibiotics (PEN, CTX, CAZ, ATM, FOX, and IPM). The mean and the S.D. are indicated. (-) not determined.

[e] IC_{50}s of clavulanic acid were measured with PEN as a substrate. The mean and the S.D. are indicated.