

Kinetic and Crystallographic Studies of Extended-Spectrum GES-11, GES-12, and GES-14 β -Lactamases

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GES-1 is a class A extended-spectrum β -lactamase conferring resistance to penicillins, narrow- and expanded-spectrum cephalosporins, and ceftazidime. However, GES-1 poorly hydrolyzes aztreonam and cephamycins and exhibits very low k_{cat} values for carbapenems. Twenty-two GES variants have been discovered thus far, differing from each other by 1 to 3 amino acid substitutions that affect substrate specificity. GES-11 possesses a Gly243Ala substitution which seems to confer to this variant an increased activity against aztreonam and ceftazidime. GES-12 differs from GES-11 by a single Thr237Ala substitution, while GES-14 differs from GES-11 by the Gly170Ser mutation, which is known to confer increased carbapenemase activity. GES-11 and GES-12 were kinetically characterized and compared to GES-1 and GES-14. Purified GES-11 and GES-12 showed strong activities against most tested β -lactams, with the exception of temocillin, cefoxitin, and carbapenems. Both variants showed a significantly increased rate of hydrolysis of cefotaxime, ceftazidime, and aztreonam. On the other hand, GES-11 and GES-12 (and GES-14) variants all containing Ala243 exhibited increased susceptibility to classical inhibitors. The crystallographic structures of the GES-11 and GES-14 β -lactamases were solved. The overall structures of GES-11 and GES-14 are similar to that of GES-1. The Gly243Ala substitution caused only subtle local rearrangements, notably in the typical carbapenemase disulfide bond. The active sites of GES-14 and GES-11 are very similar, with the Gly170Ser substitution leading only to the formation of additional hydrogen bonds of the Ser residue with hydrolytic water and the Glu166 residue.

Extended-spectrum β -lactamases (ESBLs) of the GES type are increasingly reported among a large variety of Gram-negative bacterial species, including *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* (20). GES-type β -lactamases form a distinct branch of class A enzymes, but their origin is unknown. The first GES β -lactamase, GES-1, was described in 2000 (24). This enzyme confers resistance to penicillins, narrow- and expanded-spectrum cephalosporins, and ceftazidime. However, unlike most ESBLs, GES-1 poorly hydrolyzes monobactams. Moreover, although it contains the disulfide bond between Cys69 and Cys238 (using the standard numbering for class A β -lactamases [1]) characteristic of class A carbapenemases, GES-1 hydrolyzes carbapenems very slowly and is even inhibited by low concentrations of imipenem (25, 28). To date, 22 GES variants differing by one to three amino acid residues have been reported (<http://lahey.org/studies/other.asp>), and differences in the substrate spectra have been observed. The most alarming characteristic of the GES family of enzymes that distinguishes them from other ESBLs of the TEM and SHV superfamilies is their apparent ability to evolve into carbapenemases. GES-2, which differs from GES-1 by a single Gly170Asn substitution located inside the Ω -loop of the catalytic site, hydrolyzes carbapenems, but still at a low level (25). Variants with Ser at position 170 (GES-4, GES-5, GES-6, and GES-14) are of special clinical interest, since they exhibit increased carbapenemase activity (2–4, 30, 31). On the other hand, the Gly243Ser and Gly243Ala substitutions in GES-9 and GES-11, respectively, confer increased activity against aztreonam and ceftazidime (3, 18, 26).

Recently, three GES ESBLs (GES-11, GES-12, and GES-14) were observed in *A. baumannii* clinical isolates in Belgium. GES-12 and GES-14 differ from GES-11 by a single amino acid

substitution, Thr237Ala and Gly170Ser, respectively (3) (Table 1). This paper details the kinetic characterization of the GES-11 and GES-12 variants, compares them to the GES-1 and GES-14 enzymes (4, 24), and describes the first crystallographic structures of the GES-11 and GES-14 variants.

MATERIALS AND METHODS

Cloning, expression, and purification of GES-11, GES-12, and GES-14 β -lactamases. To overproduce GES-11, GES-12, and GES-14 β -lactamases in *Escherichia coli*, the corresponding *bla*_{GES} genes were amplified by using primers 5'-CCATGGCAGCGTTCATTCACGCACTATTACTG-3' and 5'-GAATTCCTATTTGTCCGTGCTCAGGATGAGTTG-3' containing NcoI and EcoRI restriction sites (underlined). The PCR products were then cloned into pET-28a (Novagen, Madison, WI) in the corresponding insertion sites. The expression vectors, named pET28/GES11, pET28/GES12, and pET28/GES14, respectively, were introduced into *E. coli* BL21(DE3) (New England BioLabs, Ipswich, MA). Bacteria were grown overnight at 37°C with shaking in 50 ml LB medium supplemented with 50 μ g/ml kanamycin. The bacterial suspension was diluted 100-fold into a total of 2 liters of fresh LB medium supplemented with kanamycin (50 μ g/ml), and the expression of the corresponding GES β -lactamase was induced with isopropyl- β -D-thiogalactopyranoside (final concentra-

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TABLE 1 Residues in positions 80, 170, 237, and 243 in GES β -lactamases

β -Lactamase	Residue at position:			
	80	170	237	243
GES-1	Val	Gly	Thr	Gly
GES-5	— ^a	Ser	—	—
GES-11	—	—	—	Ala
GES-12	—	—	Ala	Ala
GES-14	—	Ser	—	Ala
GES-18	Ile	Ser	—	—

^a —, the residue is the same as that in GES-1.

tion, 1 mM) when the culture reached an A_{600} of 0.6. The induced culture was incubated overnight at 28°C with shaking.

The GES β -lactamases were purified by ion-exchange chromatography and gel filtration. Briefly, the bacterial suspension was pelleted, resuspended in 60 ml of buffer A (20 mM Tris-HCl, pH 7.5), disrupted by sonication, and cleared by ultracentrifugation. The supernatant was then dialyzed against buffer A and loaded onto a Q-Sepharose FF column equilibrated with the same buffer. The proteins were eluted by a linear NaCl gradient (0 to 0.5 M). Fractions were analyzed by SDS-PAGE and by monitoring the hydrolysis of nitrocefin. The fractions containing the β -lactamase activity were pooled and dialyzed overnight against buffer A at 4°C. This partially purified enzyme was loaded onto a MonoQ column (Pharmacia Biotech) equilibrated with the same buffer. The proteins were eluted by a linear gradient of NaCl (0 to 0.5 M). The fractions containing the β -lactamase activity were pooled and concentrated to 2 ml by ultrafiltration using an Amicon Ultra 10 K. Size-exclusion chromatography was performed on a Hiload 16/60 Superdex 75 Prep-grade column (Pharmacia Biotech) equilibrated with buffer A supplemented with NaCl (250 mM). Finally, fractions containing the highest β -lactamase activities were pooled and subsequently dialyzed overnight at 4°C against 100 mM sodium phosphate buffer (pH 7.0). The enzymes were more than 95% pure as judged by SDS-PAGE and mass spectrometry.

Determination of kinetic parameters. All experiments were carried out at 30°C in 100 mM sodium phosphate buffer (pH 7.0). Imipenem was obtained from Merck Sharp and Dohme Research Laboratories (Rahway, NJ), nitrocefin was from ProGenosis (Liège, Belgium), and temocillin (Negaban) was from Eumedica Pharmaceuticals (Brussels, Belgium); all other antibiotics and inhibitors were from Sigma-Aldrich (St. Louis, MO). The hydrolysis of the antibiotics was monitored by following the change of absorbance resulting from the opening of the β -lactam ring using a Uvikon 943 spectrophotometer equipped with thermostatically controlled cells. The k_{cat} and K_m parameters were determined from initial rates of reactions, using both Hanes' linearization of the Henri-Michaelis-Menten equation and a direct nonlinear regression with the hyperbolic equation. Low and very high K_m values were determined as K_i values using nitrocefin as the reporter substrate. For low K_m values, the k_{cat} values were derived from the initial hydrolysis rates measured at saturating substrate concentrations, whereas for high K_m values, k_{cat} was derived directly from the k_{cat}/K_m ratio.

Specific β -lactamase activities ($\text{nmol min}^{-1} \text{mg}^{-1}$) were obtained using 100 μM each aztreonam, ceftazidime, cefotaxime, cefoxitin, ertapenem, meropenem, and imipenem as the substrates.

Fifty percent inhibitory concentrations (IC_{50} s) were determined using clavulanic acid and tazobactam as inhibitors as previously described (24, 25, 31).

GES-11 and GES-14 crystallization. Initial crystallization trials with the GES-11 (10 mg/ml) and GES-14 (8 mg/ml) enzymes were set up using Crystal Screens I and II and a PEG/Ion screen (Hampton Research) (resulting in 146 different initial conditions). Both β -lactamases were crystallized at 21°C from 0.05 M NaI and 20 to 25% (wt/vol) polyethylene glycol 3350, using the sitting-drop method with crystallization plates de-

signed by Taorad GmbH (Aachen, Germany). A volume of 0.5 μl of 20% (vol/vol) glycerol was added to the mixture of reservoir solution (1 μl) and protein solution (1 μl) before being left to equilibrate against the solution reservoir. Typically, prismatic crystals of GES-11 and GES-14 grew within a few days to dimensions of 200 by 80 by 80 μm and belong to space group P2₂1₂1 (for GES-11, $a = 76.680 \text{ \AA}$, $b = 85.240 \text{ \AA}$, and $c = 85.120 \text{ \AA}$; for GES-14, $a = 70.745 \text{ \AA}$, $b = 84.098 \text{ \AA}$, and $c = 85.026 \text{ \AA}$).

Data collection and processing. For data collection, crystals were transferred to a cryoprotectant solution containing reservoir solution supplemented with 30% (vol/vol) glycerol. The mounted crystals were flash-frozen in a liquid nitrogen stream. Near-complete X-ray data sets were collected using a Bruker FR591 rotating anode X-ray generator and a Mar345dtb detector. Diffraction data were processed with XDS (11). The scaling was done by XDS (11) for GES-11 and by SCALA from the CCP4 suite for GES-14 (5).

Structure determination and refinement. The structures of GES-11 and GES-14 were solved using a molecular replacement approach with Phaser (17) and the structure of GES-1 (Protein Data Bank [PDB] code 2QPN) as the starting model. The resulting models were rebuilt with ARP/wARP (23). The structures were refined in a cyclic process including manual inspection of the electron density with Coot (6) and refinement with Refmac (19). The refinement to convergence was carried out with isotropic B values and with the TLS parameter (21). The positions of the iodide ions (coming from the crystallization condition) were verified by calculating anomalous maps, and the occupancies of the ions were determined by the disappearance of differences in electron densities. Alternative conformations were modeled for a number of side chains, and occupancies were adjusted to yield similar B values for the disordered conformations.

PDB accession codes. Coordinates and structure factors were deposited in the Protein Data Bank (PDB) under accession codes 3V3R (GES-11) and 3TSG (GES-14).

RESULTS

Production and purification. After production of the three enzymes in *E. coli* BL21(DE3) and their purification to homogeneity, a yield of 20 to 30 mg of β -lactamase per liter of culture was typically obtained. The mass of the different proteins was verified by electrospray ionization-mass spectrometry (ESI-MS). Within experimental errors, the variants were found to exhibit the expected masses (29,230 versus 29,231 Da for GES-11, 29,200 versus 29,201 Da for GES-12, and 29,260 versus 29,261 Da for GES-14).

Comparison of hydrolytic activities. We compared the activities of GES-1, GES-11, GES-12, GES-14, and GES-5 against compounds representing the four major families of β -lactam antibiotics, namely, penicillins, cephalosporins, carbapenems, and monobactams. The kinetic data and the specific activities are reported in Tables 2 and 3, respectively.

GES-11 and GES-12 showed better affinity than GES-1 for all tested penicillins, as reflected by lower K_m values. However, the K_m values of GES-12 were somewhat higher than those of GES-11 (from 1.6- to 4.8-fold). GES-11 exhibited lower k_{cat} values than GES-1, with the exceptions of those for piperacillin and temocillin (6- α -methoxy-ticarillin). For these two penicillins, GES-12 also exhibited largely higher k_{cat} values than GES-1. GES-14 exhibited higher K_m values than GES-11, with the exceptions of ticarcillin and temocillin. Its k_{cat} values were higher than or similar to those of GES-11, except for ticarcillin. Temocillin was a very poor substrate of the five enzymes, with the lowest k_{cat}/K_m ratios among all of the β -lactams tested.

As already observed for GES-1 (24), GES-11 and GES-12 showed poor imipenem hydrolysis. The three enzymes exhibited strong affinity for imipenem (K_m values were ~ 1.5 , 1, and 1 μM

TABLE 2 Kinetic parameters for GES-14, GES-12, GES-11, GES-1, and GES-5 β -lactamases^a

Antibiotic	Kinetic parameters for:														
	GES-14			GES-12			GES-11			GES-1			GES-5		
	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
Benzylpenicillin	23	300	77	16	60	270	4	37	110	30	120	250	110	430	255
Amoxicillin	7	130	54	16	73	220	7	37	190	13	180	72	13	50	260
Ticarcillin	0.2	32	6	4.6	214	21	2.6	37	70	7	1,000	7	5	1,600	3
Piperacillin	60	750	80	96	300	320	27	120	225	8	900	9	160	1,000	160
Temocillin	0.02	400	0.05	0.16	1,000	0.16	0.015	550	0.03	0.002	4,000	0.0005	0.003	4,500	0.0007
Cephalothin	1,100	6,000	180	80	5,300	15	740	4,000	185	900	5,300	170	900	9,000	100
Cephaloridine	150	4,400	34	75	640	120	455	3,600	130	540	4,600	120	725	5,000	145
Nitrocefin	56	135	415	72	215	330	320	530	600	380	340	1,100	260	550	470
Cefoxitin	0.35	220	1.6	0.03	10	3	0.02	13	1.5	0.08	60	1.3	4.5	860	5
Cefotaxime	14	7,900	1.8	586	9,800	60	1,600	5,500	290	370	9,000	40	36	25,000	1.4
Ceftazidime	2.5	20,000	0.125	264	5,350	50	990	38,000	26	110	40,000	27.5	7	50,000	0.14
Cefepime	0.4	600	0.7	8	1,300	6	9	930	10	25	30,000	0.8	8	90,000	0.09
Aztreonam	3.5	5,000	0.7	25	740	34	53	3,200	17	4.8	4,000	1.2	0.06	1,800	0.03
Imipenem	0.09	2.9	31	0.02	1	20	0.015	1	15	0.007	1.5	5	0.7	4.6	150
Ertapenem	0.01	0.3	33	0.007	0.07	100	0.002	0.2	10	0.003	0.4	7.5	0.09	1.5	60
Meropenem	0.01	0.2	50	0.008	0.06	130	0.0035	0.1	35	0.0007	0.1	7	0.06	1	60

^a Data are the means from three independent experiments. Standard deviations were within 10% of the means. Values for GES-1 and GES-5 are from Bebrone et al. (unpublished).

for GES-1, GES-11, and GES-12, respectively) without efficient hydrolysis (k_{cat} values were only 0.007, 0.015, and 0.02 s⁻¹, respectively). On the other hand, for GES-14, which differs from GES-11 by the Gly170Ser substitution, the K_m value was in the μ M range (2.9 μ M) but the k_{cat} value (0.09 s⁻¹) was approximately 13-fold higher than that for GES-1. GES-14, which differs from GES-5 by the presence of the Gly243Ala mutation, exhibited a k_{cat} value for imipenem approximately 8-fold lower than that of the latter.

Although GES-1, GES-11, and GES-12 showed low k_{cat} values for carbapenems, their k_{cat}/K_m ratios were rather large owing to the very high affinity of these enzymes for this family of β -lactams. Furthermore, for all carbapenems tested, the k_{cat}/K_m ratios of GES-12 were similar to or even higher than those of GES-14 (Table 2). However, as shown in Table 3, the carbapenem concentration (100 μ M) was much higher than the K_m values; thus, each variant hydrolyzed these substrates at V_{max} . GES-14 exhibited higher k_{cat} values and thus higher specific activities than GES-1 and GES-11 for the three carbapenems. On the other hand, the k_{cat} values and specific activities of GES-12 were almost similar to those of GES-14 for meropenem and ertapenem. GES-5 exhibited the largest k_{cat} values and thus the highest specific activities for the three carbapenems (Tables 2 and 3).

As observed for GES-1 (24), GES-11 and GES-12 showed poor activities against cefoxitin. The K_m values determined as K_i values,

with nitrocefin as the reporter substrate, were \sim 60, 13, and 10 μ M for GES-1, GES-11, and GES-12, respectively. The k_{cat} values were only 0.08, 0.02, and 0.03 s⁻¹, respectively. GES-14 showed higher K_m and k_{cat} values than GES-1 and the GES-11 and GES-12 variants. On the other hand, GES-14 showed lower K_m and k_{cat} values than GES-5.

GES-11 showed the highest k_{cat} value for aztreonam (11-fold higher than GES-1) but a K_m value similar to that of GES-1. GES-12 also showed a significantly increased level of hydrolysis of aztreonam compared to that of GES-1. The better efficiency of GES-12 was due to a 5.4-fold decreased K_m value combined with a 5-fold increased k_{cat} value compared to GES-1 (Table 2). At 100 μ M aztreonam, the specific activities of GES-11 and GES-12 were approximately 14- and 27-fold higher than that of GES-1, respectively. GES-14 hydrolyzed aztreonam more efficiently than GES-5 (Tables 2 and 3). However, GES-14 showed a large decrease in aztreonam hydrolysis compared to GES-11 (Tables 2 and 3).

GES-11 also showed the highest k_{cat} value for ceftazidime (9-fold increase compared to GES-1), with a K_m value similar to that of GES-1. GES-12 showed a significantly increased level of hydrolysis of ceftazidime. The better efficiency of GES-12 was due to a 7.5-fold decreased K_m value combined with a 2.4-fold increased k_{cat} value compared to GES-1 (Table 2). At 100 μ M ceftazidime, the specific activities of GES-11 and GES-12 were approximately 9.5- and 18-fold higher than that of GES-1, respectively (Table 3). A similar observation was made for cefotaxime (Tables 2 and 3). As observed for aztreonam, the presence of the Gly170Ser substitution in GES-14 was detrimental to the hydrolysis of ceftazidime and cefotaxime. At 100 μ M substrate, the specific activities of GES-14 were 223- and 125-fold lower than those of GES-11 for ceftazidime and cefotaxime, respectively (Table 3). All three GES-11, GES-12, and GES-14 variants showed largely better affinities but lower k_{cat} values for cefepime than GES-1 (Table 2).

Comparison of susceptibilities to inhibitors. We tested two commercial β -lactamase inhibitors, and we found that tazobactam was the most active against all five GES variants. Lower IC₅₀s for both inhibitors were found with GES-11 and GES-12, which contain the Gly243Ala substitution, with GES-12 being the most

TABLE 3 Specific activity with 100 μ M substrate^a

Antibiotic	Sp act (nmol min ⁻¹ mg ⁻¹) of β -lactamase:				
	GES-14	GES-12	GES-11	GES-1	GES-5
Imipenem	193	42	31	15	1,330
Meropenem	22	17	7	1.5	120
Ertapenem	23	14	5	7	180
Cefoxitin	228	62	45	103	1,400
Cefotaxime	468	12,200	58,900	7,660	290
Ceftazidime	24	9,968	5,345	562	27
Aztreonam	141	6,220	3,248	234	7

^a Standard deviations were within 10% of the means. Values for GES-1 and GES-5 are from Bebrone et al. (unpublished).

TABLE 4 IC_{50} s of the commercial inhibitors clavulanic acid and tazobactam for GES-14, GES-12, GES-11, GES-1, and GES-5^a

Antibiotic	IC_{50} (μ M)				
	GES-14	GES-12	GES-11	GES-1	GES-5
Clavulanic acid	7	0.25	1	5	20
Tazobactam	4	0.35	0.5	3	10

^a Data are the means from three independent experiments. Standard deviations were within 10% of the means. Values for GES-1 and GES-5 are from Bebrone et al. (unpublished).

susceptible. In GES-14, the higher level of resistance to inhibitors conferred by the Gly170Ser mutation (8) already observed in GES-5 (13, 31) disappeared because of the presence of the single Gly243Ala mutation (Table 4).

Crystallographic structures. The crystal structures of the GES-11 and GES-14 variants were solved by molecular replacement using the GES-1 structure (PDB accession number 2QPN) as a model. After refinement, almost all residues lay in the allowed or favored region in the Ramachandran plot. Stereochemical parameters were calculated by PROCHECK (15) and were in the range expected for structures with similar resolutions. The crystallographic and model statistics for GES-11 and GES-14 structures are summarized in Table 5. Attempts to

TABLE 5 X-ray data collection and structure refinement for GES-11 and GES-14^a

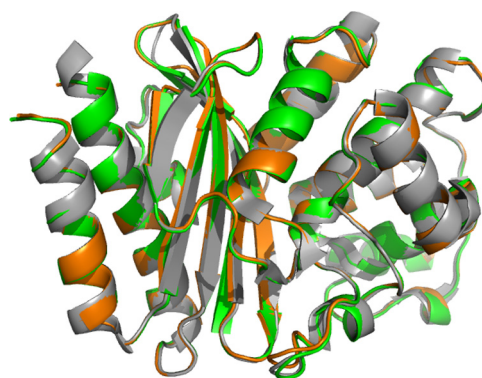
Parameter	Value for:	
	GES-11	GES-14
Data collection statistics		
Unit cell dimensions (\AA)		
<i>a</i>	70.7	70.7
<i>b</i>	85.2	84.1
<i>c</i>	85.1	85.0
Space group	P22 ₁ 2 ₁	P22 ₁ 2 ₁
No. of molecules/asymmetric unit	2	2
Resolution range (\AA)	1.88–20	1.87–20
No. of unique reflections	40,335	40,567
Overall completeness (%)	94.1/87.2	99.9/97.2
<i>I</i> / σ (<i>I</i>)	8.98/2.35	11.6/3.6
Multiplicity	4.0/3.3	7.1/7.0
R_{sym}^b	0.114/0.523	0.124/0.608
Twin fraction	0.07	
Refinement statistics		
Resolution range (\AA)	1.9–20	1.9–20
No. of reflections	37,055	38,560
No. of atoms (non-H)	4,453	4,461
R_{work}^c	0.19	0.16
R_{free}^d	0.23	0.20
RMSD from ideal		
Bond length (\AA)	0.010	0.009
Bond angle ($^\circ$)	1.393	1.220

^a *I* is the intensity of a reflection, and $\langle I \rangle$ is the average intensity. For values separated by a slash, the first number corresponds to the average value for shells of all resolutions while the second number corresponds to the value for the highest-resolution shell.

^b $R_{\text{sym}} = \sum |I_j - \langle I \rangle| / \sum \langle I \rangle$, where I_j is the intensity for reflection *j* and $\langle I \rangle$ is the mean intensity.

^c $R_{\text{work}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

^d R_{free} was similarly calculated, with approximately 10% of the data excluded from the calculation of R_{work} .

**FIG 1** Superposition of the overall crystallographic structures of GES-1 (in gray), GES-11 (in green), and GES-14 (in orange).

generate good-quality GES-12 crystals have been unsuccessful thus far.

GES-11. The crystal structure of GES-11 was refined in a resolution range of 1.9 to 20 \AA , yielding an R_{work} of 0.1939 and R_{free} of 0.2317 (Table 5). The crystals adopted a P22₁2₁ space group, with two molecules of GES-11 in the asymmetric unit. The final crystal structure contains 265 residues for molecule A (Ser24-Ala291) and 264 residues for molecule B (Ser24-Leu290). Furthermore, the structure contains 264 water molecules, 23 iodide ions, and 1 sodium ion from the crystallization buffer. Both molecules in the asymmetric unit are related by a 2-fold noncrystallographic symmetry. The interface (935 \AA^2) is dominated by hydrogen bonds and bridging water molecules. Superimposition of the individually refined models showed that they are essentially identical in structure, with a root mean square deviation (RMSD) value for the C α position of 0.283 \AA .

The Gly243Ala substitution has no effect on the overall structure of the GES β -lactamase. GES-11, like GES-1, shows the typical class A serine β -lactamase fold composed of two domains, one α/β and the other all α (Fig. 1). The methyl side chain (C β) of the Ala243 residue is oriented in the direction of the disulfide bond (Cys69-Cys238). This disulfide, which is characteristic of the carbapenemases, links the N terminus of helix H2 with the C terminus of β -strand S3. In GES-11, the Cys238 residue adopts a different conformation from that of GES-1 (SG is shifted from 1.7 \AA) (Fig. 2a). The hydrolytic water molecule and the Glu166 general base are also slightly affected (Fig. 2b) (the water molecule is shifted 0.96 \AA from its position in GES-1, while the carboxylate group of Glu166 is displaced by 0.6 \AA). The distance between the hydroxyl group of the Ser70 side chain (OG) and the oxygen (OE2) of the carboxyl group of Glu166 is now 3.33 \AA , while it is 3.96 \AA in GES-1. Due to the displacement of the hydrolytic water molecule, the distances between the latter and Ser70 and the latter and Glu166 are slightly larger in GES-11 than in GES-1 (Fig. 2c).

GES-14. The crystal structure of GES-14 was refined in a resolution range of 1.9 to 20 \AA , yielding an R_{work} of 0.163 and R_{free} of 0.205 (Table 5). The crystals adopted a P22₁2₁ space group, with two molecules of GES-14 in the asymmetric unit. The final crystal structure contains 265 residues for molecule A and molecule B (Ser24-Ser291). Furthermore, the structure contains 281 water molecules. Additionally, we observed 55 iodide ions from the crystallization buffer and 11 glycerol mol-

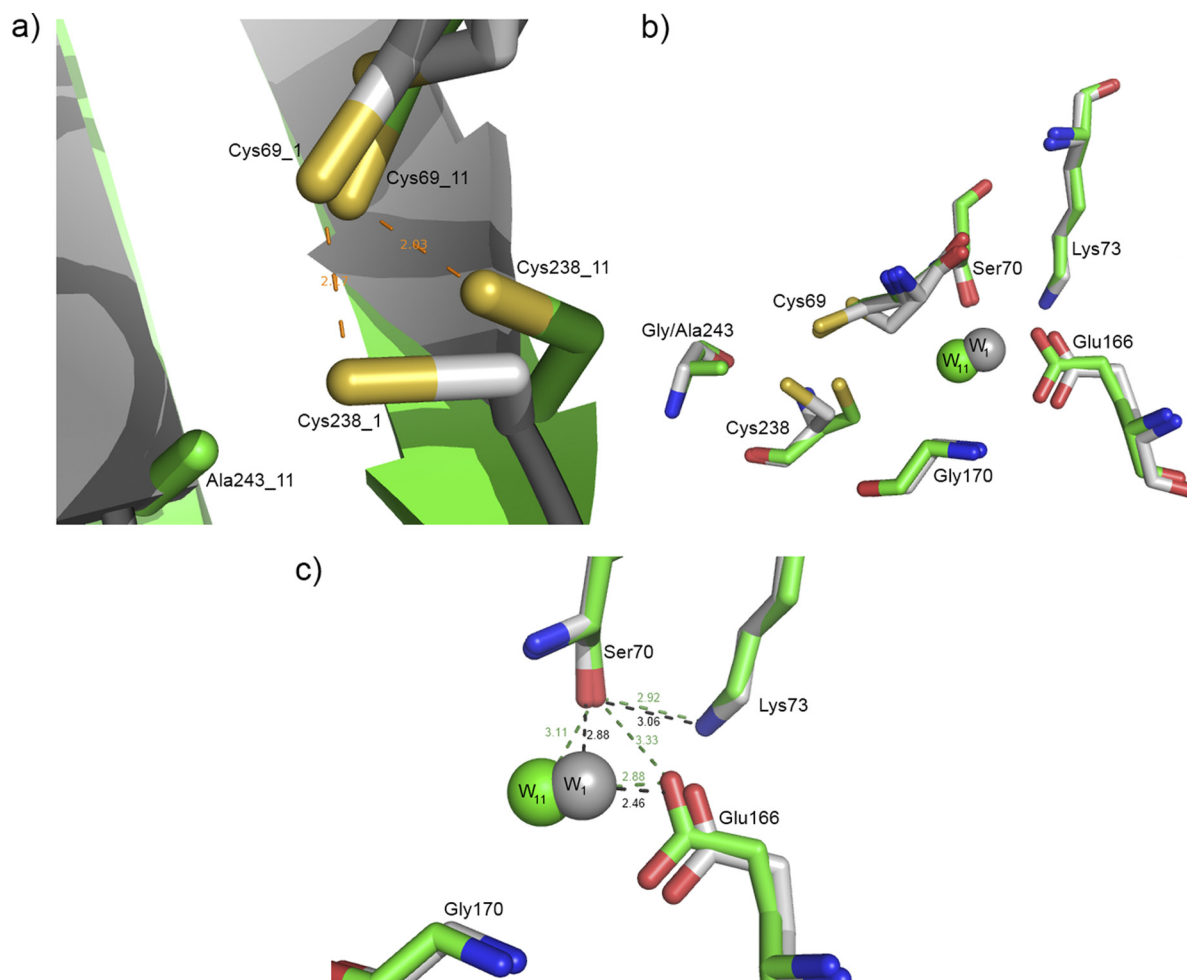


FIG 2 (a) Conformation of the Cys238 residue is slightly modified in GES-11 (Cys238_11) compared to GES-1 (Cys238_1). (b) Superposition of the active sites of GES-11 (in green) and GES-1 (in gray). The hydrolytic water molecule and the Glu166 general base are slightly displaced. (c) The distances between Ser70 and the hydrolytic water molecule and between Glu166 and the hydrolytic water molecule are slightly larger in GES-11 than in GES-1. In GES-11, Glu166 forms an additional hydrogen bond with Ser70.

ecules from the cryoprotecting solution. Both molecules in the asymmetric unit are related by a 2-fold noncrystallographic symmetry. The interface (943 Å²) is dominated by hydrogen bonds and by bridging water molecules. Superimposition of the individually refined models showed that they are essentially identical in structure, with an root mean square deviation (RMSD) value for the C α position of 0.277 Å.

GES-14 and GES-11 structures show the same dimer formation and are mostly identical (the RMSD value is 0.337 Å for the superposition of the respective dimers). They are also identical to the dimer formation in the other GES structures, GES-1 (27), GES-2 (7), and GES-18 (PDB code 3V3S).

Compared to GES-1 and GES-11, the side chain of the Ser170 residue forms an additional hydrogen bond with the hydrolytic water. A new hydrogen bond is also formed by the side chain oxygen of Ser170 to the carboxyl group of Glu166 (3.02 Å) (Fig. 3a). This was already observed for GES-18 (PDB code 3V3S) (C. Bebrone, P. Bogaerts, H. Delbrück, S. Bennink, M. B. Kupper, R. Rezende de Castro, Y. Glupczynski, and K. M. Hoffmann, unpublished data). If one excepts these two new hydrogen bonds due to the Gly170Ser substitution, the active site of

GES-14 does not differ from that of GES-11. The hydrolytic water molecule occupies similar positions in the two active sites (Fig. 3b). On the other hand, the hydrolytic water molecule of GES-14 (Gly170Ser-Gly243Ala) is shifted by 1.5 Å from its counterpart position in the GES-18 (Val80Ile-Gly170Ser) variant and is closer to that of GES-1. The Cys238 residue also adopts a different conformation from that of GES-18 (SG is shifted from 2.3 Å) (Fig. 3c).

DISCUSSION

The kinetic parameters measured for both GES-11 and GES-12 variants showed that they exhibited strong activity against most β -lactams tested, with the exception of temocillin, cefoxitin, and carbapenems. Furthermore, temocillin was the poorest substrate for all of the GES variants tested.

The Gly243 residue present in GES-1 is not conserved in other class A β -lactamases, where it is mainly a threonine (e.g., in NMCA, SME-1, KPC-2, or BLIC or in the β -lactamase from *Proteus vulgaris* K1) or a serine (e.g., in TEM-1). GES-9, with a Gly243Ser substitution, hydrolyzes aztreonam and ceftazidime despite high K_m values (26). As already suggested by measure-

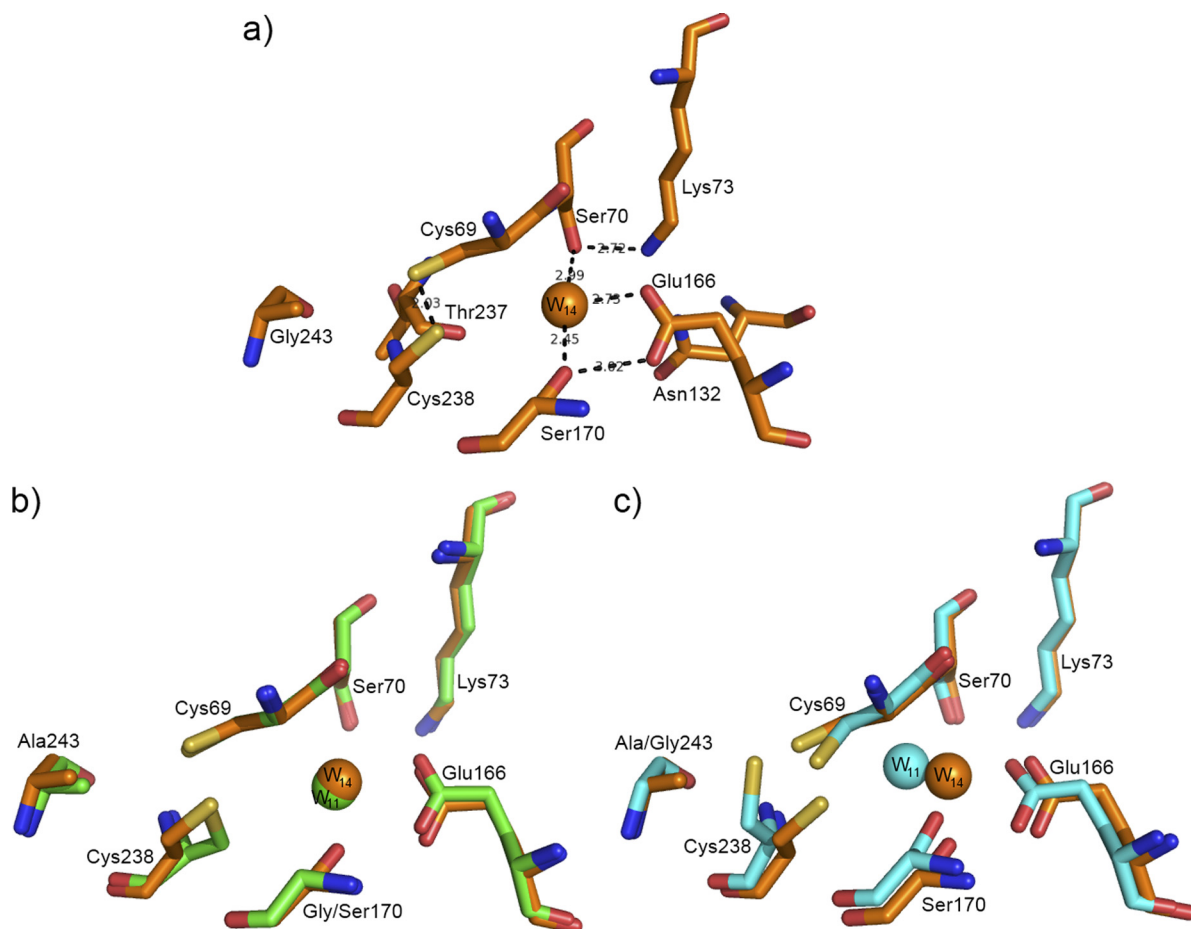


FIG 3 (a) Active site of GES-14. (b) Superimposition of the active sites of GES-14 (in orange) and GES-11 (in green). (c) Superimposition of the active sites of GES-14 (in orange) and GES-18 (in cyan).

ments of crude extracts (3), detailed kinetic measurements with the purified enzyme demonstrate that GES-11, bearing a Gly243Ala mutation (located on the β -strand B4), displays a higher hydrolytic activity than GES-1 against oxyimino- β -lactams such as cefotaxime, ceftazidime, and aztreonam. For these three substrates, the better efficiency of GES-11 is mainly due to higher turnover rates. The K_m values remain very high. On the other hand, GES-11 presents better affinities for penicillins than GES-1. As observed for GES-9 (26), the substitution at position 243 does not allow GES-11 to efficiently hydrolyze cefoxitin or carbapenems. Also, the Gly243Ala substitution increases the sensitivity of the enzyme to β -lactam inhibitors such as clavulanic acid and tazobactam.

From a structural point of view, the Gly243Ala mutation has no effect on the global structure of the enzyme. This position is not directly in contact with the catalytic active residues but is situated behind the typical Cys69-Cys238 disulfide bond. The conformation of the Cys238 residue seems affected by the substitution. However, a superposition of all of the known structures of GES variants and molecules from the asymmetric unit showed a large variety of different conformations for this residue.

GES-12 differs from GES-11 by a single Thr237Ala substitution. The Thr237 in GES-type enzymes is atypical, with this residue commonly being alanine in class A β -lactamases. A serine

residue at position 237 is conserved in the CTX-M group and also in other ESBLs (27). The side chain of residue 237 is on the outer, exposed side of the B3 β -strand that forms the right edge of the binding site. It is already known that residue 237 plays a role in substrate selectivity in class A β -lactamases (10, 12). In the TEM, KOXY-2, and SME-1 enzymes, the replacement of the typical Ala237 residue with a hydrogen bond acceptor such as threonine enhances the catalytic efficiency against cefotaxime (12, 16, 32). Accordingly, KPC-2, CTX-M-4, the β -lactamase from *P. vulgaris* K1, and Toho-1 show reduced catalytic efficiencies against oxyiminocephalosporins when Ser/Thr237 is replaced by Ala (9, 22, 27, 29). On the basis of the crystallographic observation of hydrogen bonding of cefotaxime to the side chain OH group of the Thr237 residue in the related D-Ala-D-Ala peptidase (14), it is likely that the ESBL variants exhibiting the Ala237Ser mutation utilize the side chain OH group of Ser237 rather than the backbone CO group for hydrogen bonding to rigid expanded-spectrum cephalosporins (12, 29). Here, the introduction of the Thr237Ala mutation in GES-11, yielding GES-12, led to a 2-fold better efficiency against aztreonam and ceftazidime, even if the k_{cat} values were decreased, due to a strong positive effect on the affinities of the enzyme for both antibiotics. On the other hand, GES-11 was the most potent enzyme against cefotaxime and cefepime. This different effect could be explained by the fact that

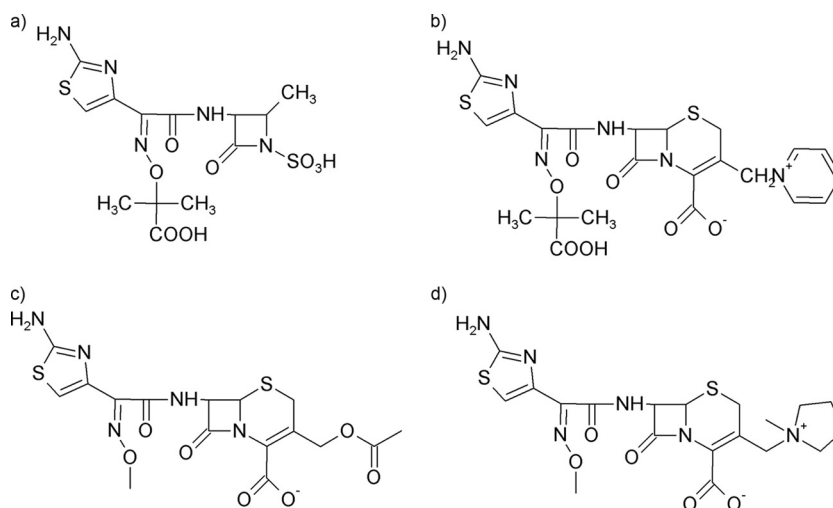


FIG 4 Some of the antibiotics used in this study. (a) Aztreonam; (b) ceftazidime; (c) cefotaxime; and (d) cefepime.

these two antibiotics contain a less complex imino side chain (OCH_3) than ceftazidime and aztreonam (containing two methyl groups and a carboxylic acid group) (Fig. 4). Also, as already observed in Toho-1 (27) and CTX-M-4 (9), the Ser237Ala mutation resulted in increased K_m and k_{cat} values for penicillins. For meropenem and ertapenem, the k_{cat} values of GES-12 were somewhat similar to those of GES-14.

GES-14, bearing both Gly243Ala and Gly170Ser mutations, was compared to GES-11 (Gly243Ala) and to GES-5 (Gly170Ser) to evaluate the individual effect of each mutation on the activity. The presence of the Gly243Ala mutation in GES-14 decreased the catalytic efficiency against imipenem, other tested carbapenems, and cefoxitin compared to that of GES-5, while the efficiency against ceftazidime, aztreonam, or cefotaxime was similar to or increased compared to that of GES-5 (Tables 2 and 3). On the other hand, compared to GES-11, due to the presence of the Gly170Ser mutation, GES-14 showed an increased hydrolytic activity against imipenem, other tested carbapenems, and cefoxitin together with a decrease in activity toward ceftazidime, aztreonam, and cefotaxime. Thanks to the presence of these two mutations and their opposite effects, GES-14 displays a broad-spectrum activity against most β -lactams, including, in particular, oxyiminocephalosporins, monobactams, carbapenems, and cefoxitin. Cefoxitin usually is not hydrolyzed by class A ESBLs. As already observed for GES-18 (PDB code 3V3S; Bebrone et al., unpublished), the change from Gly to Ser in position 170 was connected to some subtle local rearrangements in the active site. Ser170 is now forming a hydrogen bridge with Glu166, and the hydrolytic water molecule presents an additional H-bond with Ser170.

It is interesting that both Gly170Ser and Gly243Ala mutations have effects (decreased or increased hydrolysis, respectively) that are identical for the aztreonam monobactam and the ceftazidime third-generation cephalosporin. This phenomenon could be attributed to the fact that these two antibiotics are similar, having the same (C3/C7) side chain, even if the latter is attached to a different nucleus.

All variants showed high K_m values for cephalosporins and even very high K_m values for cefepime and ceftazidime, maybe due to their similar C3 side chains (Fig. 4).

Unfortunately, the solved structures of the GES-11 and GES-14 apoenzymes do not explain the differences in substrate specificity or in sensitivity to inhibitors induced by the Gly243Ala substitution, and these challenges should be addressed by solving the crystal structures of enzyme complexes with oxyiminocephalosporin and carbapenem substrates and clavulanic acid or tazobactam inhibitors.

Conclusion. In extended-spectrum GES β -lactamases, the replacement of the Gly243 residue by an alanine leads to an approximately 10-fold increase of enzyme efficiency toward aztreonam and ceftazidime. On the other hand, this substitution has a detrimental effect on the resistance to inhibitors. It has a negligible or modest effect on the rate of imipenem hydrolysis. This effect can become more significant for ertapenem and meropenem in the presence of an additional Thr237Ala (GES-12) or Gly170Ser (GES-14) substitution.

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