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MECHANISMS OF RESISTANCE

Laboratory Variants GES^{G170L}, GES^{G170K}, and GES^{G170H} Increase Carbapenem Hydrolysis and Confer Resistance to Clavulanic Acid

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ABSTRACT The Guiana extended-spectrum (GES) β-lactamase GES^{G170H}, GES^{G170L}, and GES^{G170K} mutants showed k_{cat}, K_m, and k_{cat}/K_m values very dissimilar to those of GES-1 and GES-5. The enhancement of the hydrolytic activity against carbapenems is potentially due to a shift of the substrate in the active site that provides better positioning of the deacylating water molecule caused by the presence of the imidazole ring of H170 and of the long side chain of K170 and L170.

KEYWORDS β-lactamases, GES, enzyme kinetics, *in silico* molecular modeling

Guiana extended-spectrum (GES) β-lactamases have been discovered in a large variety of Gram-negative bacterial species (1, 2). At the time of writing, 42 different variants of GES-1 have been identified (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/GES), and some of them have been described as carbapenemases (i.e., GES-2, GES-4, GES-5, GES-6, GES-14, GES-16, GES-18, and GES-20) (3, 4). The first GES β-lactamase, GES-1, has a glycine at position 170, whereas GES variants, classified as carbapenemases, have a residue of serine (G170S) or asparagine (G170N). Several studies demonstrated that a detectable carbapenemase activity is correlated with the role of residue 170 (Ambler numbering scheme), which belongs to the Ω-loop (5–7). Nevertheless, there are some GES variants, such as GES-13, which, despite having the natural mutation G170N, showed a weak activity against carbapenems (8). Most of the GES variants are susceptible to classical β-lactamase inhibitors, such as tazobactam and clavulanic acid (9). In the most common class A β-lactamases, position 170 is occupied by asparagine (i.e., TEM, SHV, KPC, CTX-M, SME, NMCA, and some GES types), glycine/serine (some GES types), or histidine (PER and VEB). The aim of the present study was to investigate the role of residue 170 in GES-1, when glycine is replaced by histidine (H), leucine (L), and lysine (K). In particular, our intent was to evaluate the evolution of carbapenemase activity in these variants. In this context, *in silico* modeling simulations have been performed to evaluate the interactions of these residues with imipenem.

bla$_{GES}^{G170H}$, *bla*$_{GES}^{G170L}$, and *bla*$_{GES}^{G170K}$ mutants were generated by site-saturation mutagenesis using pET-24-GES-1 plasmid as the template (10). Mutated *bla* genes, including signal peptide, were cloned into pET-24a(+) vector, and the recombinant plasmids were transferred in *Escherichia coli* BL21(DE3) for protein expression. Recombinant clones were grown in the presence of cefotaxime (4 mg/liter). Among the selected clones, only the crude extracts of three of them (*E. coli*/pET-24-GES^{G170H}, *E. coli*/pET-24-GES^{G170L}, and *E. coli*/pET-24-GES^{G170K}) showed a significant activity against carbapenems. The three GES-1 mutants were purified as previously described (10). Antimicrobial susceptibilities were determined by conventional broth microdilution procedures, as suggested by the Clinical and Laboratory

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TABLE 1 β-Lactam susceptibility of *E. coli* BL21(DE3) strains carrying bla_{GES-1}, bla_{GES-5}, bla_{GES}^{G170H}, bla_{GES}^{G170L}, and bla_{GES}^{G170K} genes

	MIC (mg/liter) for *E. coli* BL21(DE3) strain carrying plasmid[b]:					
β-Lactam(s)[a]	pET-24/GES-1	pET-24/GES-5	pET-24/GES-1^{G170H}	pET-24/GES-1^{G170L}	pET-24/GES-1^{G170K}	pET-24
Piperacillin	>512	>512	512	>512	>512	1
Piperacillin-tazobactam	8	8	16	2	2	1
Amoxicillin	>512	>512	128	64	64	0.5
Amoxicillin-clavulanic acid	16	16	128	1	1	0.5
Imipenem	1	4	4	1	0.25	0.0625
Ertapenem	0.0625	0.5	1	1	0.5	0.0625
Meropenem	0.0625	1	2	2	1	0.0625
Cefazolin	>128	>128	8	16	8	0.125
Cefoxitin	2	16	4	8	2	0.125
Cefotaxime	16	4	8	8	8	0.0625

[a]Tazobactam was used at fixed concentration of 4 mg/liter, and the amoxicillin-clavulanic acid combination was used at a ratio of 2:1.
[b]MICs were determined on microdilution plates using an inoculum of 5×10^5 CFU/ml as reported by CLSI (11). The experiments were performed in triplicate. The analysis of the obtained data was carried out as suggested by CLSI (19).

Standard Institute (CLSI), using a bacterial inoculum of 5×10^5 CFU/ml (11). IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the cation-adjusted Mueller-Hinton broth (CAMHB), used for the MIC assay, at a concentration of 0.4 mM. The MICs for *E. coli*/pET-24-GES^{G170H}, *E. coli*/pET-24-GES^{G170L}, and *E. coli*/pET-24-GES^{G170K} were compared with those of *E. coli*/pET-24-GES-1 and *E. coli*/pET-24-GES-5. The MIC results are shown in Table 1. The combination piperacillin-tazobactam was able to reduce MIC values of piperacillin in all strains (MICs ranging from 2 mg/liter to 16 mg/liter) to a susceptible level. Clavulanic acid brought a decrease in MIC values for amoxicillin to an intermediate level in *E. coli*/pET-24-GES-1 and *E. coli*/pET-24-GES-5 and to a susceptible level in *E. coli*/pET-24-GES^{G170L} and *E. coli*/pET-24-GES^{G170K}, while in *E. coli*/pET-24-GES^{G170H}, the MIC value of 128 mg/liter was unchanged. GES-1, GES^{G170H}, GES^{G170L}, and GES^{G170K} conferred a susceptible phenotypic profile to *E. coli* BL21 (DE3) against cefoxitin, with MICs ranging from 2 mg/liter to 8 mg/liter, while GES-5 showed an intermediate value of 16 mg/liter. All recombinant strains were resistant to cefotaxime, with MIC values of $\geq$4 mg/liter. *E. coli*/pET-24-GES-1 and *E. coli*/pET-24-GES-5 were resistant to cefazolin (MIC of >128 mg/liter), whereas the mutant strains showed susceptibility to cefazolin. Intermediate MIC values for meropenem and ertapenem were determined for *E. coli*/pET-24-GES^{G170H} and *E. coli*/pET-24-GES^{G170L}, and susceptible values were found for *E. coli*/pET-24-GES^{G170K}, as for *E. coli* harboring GES-1 and GES-5.

Steady-state kinetic experiments were carried out at 25°C in 20 mM sodium phosphate buffer (pH 7.0), containing 0.2 M KCl to prevent enzyme instability. The substrate hydrolysis was monitored by using a Perkin-Elmer Lambda 25 spectrophotometer (Perkin-Elmer Italia, Monza, Italy). Each kinetic value is the mean of three different measurements; the error was below 10%. Kinetic parameters were determined under initial-rate conditions using Origin Pro 8.5.1 to generate Michaelis-Menten curves. For K_m values lower than 25 μM and higher than 1 mM, K_m was determined as the inhibitor constant (K_i), using nitrocefin as the reporter substrate (12). Inhibition experiments with clavulanic acid and tazobactam were monitored directly by using nitrocefin as the reporter substrate. Competitive inhibition assays were performed using the equation $v_0/v_i = 1 + [(K_m \times I)/(K_m + S) \times K_i]$, where v_i and v_0 represent the initial rates of hydrolysis of nitrocefin with and without inhibitor, respectively, I is the concentration of inhibitor or poor substrate, K_i is the inhibition constant, K_m is the Michaelis constant, and S is the concentration of the reporter substrate. The plot v_0/v_i versus I yielded a straight line of slope: $K_m/(K_m + S) \times K_i$ (12, 13).

In compounds behaving as transient inhibitors, accumulation and slow hydrolysis of *EC** were studied on the basis of the model

$$E + C \overset{K_d}{\rightleftarrows} E \cdot C \overset{k_{+2}}{\rightarrow} EC^* \overset{k_{+3}}{\rightarrow} E + P$$

where E is enzyme, C is substrate, $E \cdot C$ is the Henri-Michaelis complex, *EC** is the acyl-enzyme complex, and P is the hydrolysis product. k_{+2} and k_{+3} are the first-order

acylation and deacylation constants, respectively. K_d is the dissociation constant of Henry-Michaelis complex. In the case of poor substrates, the values of k_i (the first-order rate constant characterizing the *EC** accumulation) were obtained by time course hydrolysis of nitrocefin according to equations 1 and 2, as previously reported (12):

$$(v_t - v_{ss})/(v_0 - v_{ss}) = e^{-k_i t} \quad (1)$$

v_0, v_t, and v_{ss}, respectively, are the rate transformation of substrate at time zero, time t, and steady state:

$$k_i = k_{+3} + \frac{k_{+2}\,[C]}{[C] + K\left(1 + [S]/K_m\,S\right)} \quad (2)$$

Steady-state kinetic constants (K_m and k_{cat}) were calculated for GES^{G170H}, GES^{G170L}, and GES^{G170K} and compared to those of GES-1 and GES-5 toward selected β-lactams (Table 2).

Imipenem. Surprisingly, GES^{G170H} exhibited a k_{cat} value for imipenem of 3 s^{-1}, approximately 4- and 500-fold higher than those of GES-5 and GES-1, respectively. The k_{cat} for imipenem calculated for GES^{G170L} was slightly less than that of GES-5 but 3-fold higher than that of GES-1. Instead, the k_{cat} for GES^{G170K} was comparable to that of GES-1. K_m values for GES^{G170H} and GES^{G170L} were 24 and 25 μM, respectively, and unlike other GES enzymes, were determined by following direct hydrolysis of substrate. The k_{cat}/K_m values calculated for the three mutants were comparable to that of GES-1 and lower than that of GES-5.

Meropenem. Meropenem was well hydrolyzed by GES^{G170H} and GES^{G170L}, with k_{cat} values of 10 s^{-1} and 6 s^{-1}, respectively. Instead, GES^{G170K} exhibited a lower k_{cat} value (0.35 s^{-1}), similar to that calculated for GES-5 but at least 50-fold higher than that for GES-1. With the exception of GES^{G170H}, GES^{G170L} and GES^{G170K} showed the same K_m value as GES-1 (0.08 μM). GES^{G170H} exhibited a K_m value of 40 μM, which was measured by direct hydrolysis of meropenem. The catalytic efficiency values calculated for the three mutants were higher than those for GES-1 and, with the exception of GES^{G170H}, also higher than those for GES-5. In particular, GES^{G170L} exhibited k_{cat}/K_m values 150- and 862-fold higher than those for GES-5 and GES-1, respectively.

Ertapenem. GES^{G170H}, GES^{G170L}, and GES^{G170K} hydrolyzed ertapenem better than GES-1 and GES-5, with a behavior similar to that found for meropenem. GES^{G170H} hydrolyzed ertapenem more efficiently than GES^{G170L} and GES^{G170K}. However, due to the higher K_m value (10 μM), GES^{G170H} showed a k_{cat}/K_m value lower than those of GES^{G170L} and GES^{G170K}. In GES^{G170H}, the K_m value is probably affected by the presence of a bulky and partially ionized imidazole ring. The histidine 170 was also found in PER variants, and the β-lactam kinetic profiles of PER-7 and PER-8 showed that PER enzymes, unlike the main ESBLs, are able to efficiently hydrolyze imipenem (14).

Penicillins. For benzylpenicillin, K_m values for GES^{G170H}, GES^{G170K}, and GES^{G170L} were 31, 6, and 0.07 μM, respectively. Compared to GES-1 and GES-5, the three mutants showed a drastic decrease in k_{cat} values. In detail, GES^{G170H} and GES^{G170L} exhibited k_{cat} values approximately 20- and 292-fold lower than that of GES-1 and 42- and 635-fold lower than that of GES-5, respectively. Benzylpenicillin behaved as a poor substrate for the GES^{G170K} mutant, showing a low K_m value (6 μM) calculated as K_i. However, kinetic inhibition experiments to ascertain whether benzylpenicillin behaved as a transient inactivator were performed using kinetic models and equations previously reported (12, 13). The variation of k_i versus benzylpenicillin concentration was linear. We could then compute the values of the deacylation constant ($k_{+3} = 0.014$ s^{-1}) and the second-order acylation constant ($k_{+2}/K_d = 0.6 \times 10^{-3}$ μM^{-1} s^{-1}). Because of the lower K_m value, GES^{G170L} showed k_{cat}/K_m ratios 10- and 7- fold higher than those of GES-1 and GES-5, respectively. In comparison to GES-5 and GES-1, the three mutants showed very low K_m values, calculated, as for the other penicillins, using nitrocefin as a reporter substrate. The hydrolytic activity was drastically reduced in the three mutants. GES^{G170H} and GES^{G170L} showed k_{cat}/K_m values higher than those of both GES-1 and GES-5, essentially due to low K_m values. A drastic decrease in K_m and k_{cat} values was also observed for GES^{G170H}, GES^{G170K}, and GES^{G170L} compared to GES-1 and GES-5. However, among the

TABLE 2 Determination of kinetic constants of GES^{G170H}, GES^{G170L}, and GES^{G170K} compared to GES-1 and GES-5[a]

Substrate	Variant[b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat}/K_m ratio of:	
					GES_{mut}/GES-5	GES_{mut}/GES-1
Imipenem	GES-1	0.8 ± 0.1	0.006 ± 0.001	0.007 ± 0.002	0.004	1
	GES-5	0.4 ± 0.1	0.7 ± 0.1	1.75 ± 0.7	1	250
	GES^{G170H}	24 ± 2	3 ± 0.5	0.12 ± 0.03	0.07	17
	GES^{G170L}	25 ± 3	0.2 ± 0.02	0.008 ± 0.0017	0.004	1.1
	GES^{G170K}	0.009 ± 0.001	0.008 ± 0.001	0.89 ± 0.21	0.05	127
Meropenem	GES-1	0.08 ± 0.01	0.007 ± 0.001	0.087 ± 0.023	0.17	1
	GES-5	0.3 ± 0.02	0.15 ± 0.01	0.50 ± 0.067	1	5.7
	GES^{G170H}	40 ± 3	10 ± 0.5	0.25 ± 0.031	0.5	2.9
	GES^{G170L}	0.08 ± 0.01	6 ± 0.5	75.00 ± 15.6	**150**	**862**
	GES^{G170K}	0.08 ± 0.01	0.35 ± 0.02	4.37 ± 0.79	8.7	50
Ertapenem	GES-1	0.25 ± 0.02	0.003 ± 0.0005	0.01 ± 0.025	0.025	1
	GES-5	0.26 ± 0.01	0.1 ± 0.01	0.38 ± 0.053	1	38
	GES^{G170H}	10 ± 1	6 ± 0.2	0.60 ± 0.08	1.6	60
	GES^{G170L}	0.31 ± 0.1	2.5 ± 0.05	8.1 ± 2.77	**21**	**810**
	GES^{G170K}	0.11 ± 0.01	0.2 ± 0.02	1.8 ± 0.34	4.7	180
Benzylpenicillin	GES-1	210 ± 15	117 ± 5	0.56 ± 0.064	0.7	1
	GES-5	310 ± 20	254 ± 2	0.82 ± 0.059	1	1.5
	GES^{G170H}	31 ± 2[c]	6 ± 0.2	0.19 ± 0.018	0.23	0.34
	GES^{G170L}	0.07 ± 0.01	0.4 ± 0.02	5.71 ± 1.1	**7**	**10**
	GES^{G170K}	6 ± 0.5[c]	0.014 ± 0.001[d]	0.0006 ± 0.0009[e]	0.0007	0.001
Ampicillin	GES-1	260 ± 20	263 ± 3	1.01 ± 0.089	3	1
	GES-5	148 ± 12	53 ± 1	0.36 ± 0.036	1	0.36
	GES^{G170H}	1 ± 0.05[c]	4 ± 0.1	4.00 ± 0.3	11.1	4
	GES^{G170L}	0.4 ± 0.03[c]	0.8 ± 0.02	2.00 ± 0.2	**5.5**	**2**
	GES^{G170K}	4 ± 0.5[c]	1 ± 0.05	0.25 ± 0.044	0.7	0.25
Piperacillin	GES-1	1,400 ± 150	184 ± 2	0.13 ± 0.015	6.5	1
	GES-5	>3,000	>58	ND[f]	—	—
	GES^{G170H}	8 ± 0.5[c]	23 ± 1	2.87 ± 0.3	143	22
	GES^{G170L}	0.4 ± 0.05[c]	1 ± 0.05	2.50 ± 0.44	**125**	**19**
	GES^{G170K}	15 ± 1.2[c]	0.5 ± 0.05	0.03 ± 0.054	1.5	0.23
Carbenicillin	GES-1	1,250 ± 35	41 ± 2	0.03 ± 0.002	6	1
	GES-5	>3,000	>16	ND	—[h]	—
	GES^{G170H}	485 ± 15	8 ± 1	0.02 ± 0.003	—	0.67
	GES^{G170L}	2 ± 0.5[c]	10 ± 1	5.00 ± 1.75	—	**167**
	GES^{G170K}	23 ± 1[c]	1 ± 0.05	0.50 ± 0.047	—	16.7
Cefazolin	GES-1	1,440 ± 35	375 ± 3	0.26 ± 0.008	0.15	1
	GES-5	500 ± 20	844 ± 2	1.69 ± 0.072	1	6.5
	GES^{G170H}	35 ± 3	9 ± 1	0.26 ± 0.05	0.15	1
	GES^{G170L}	117 ± 2	58 ± 2	0.49 ± 0.025	0.29	1.9
	GES^{G170K}	86 ± 8	5 ± 0.2	0.06 ± 0.008	0.035	0.23
Cefoxitin	GES-1	50 ± 5	NH[g]	ND	—	—
	GES-5	380 ± 20	30 ± 0.2	0.08 ± 0.006	1	—
	GES^{G170H}	22 ± 2	0.7 ± 0.1	0.03 ± 0.007	0.375	—
	GES^{G170L}	3 ± 0.5[c]	8 ± 1	2.67 ± 0.78	**33**	—
	GES^{G170K}	2 ± 0.6[c]	NH	ND	—	—
Cefotaxime	GES-1	12,000 ± 150[c]	>119	ND	—	—
	GES-5	9,600 ± 220[c]	>10	ND	—	—
	GES^{G170H}	910 ± 80	14 ± 1	0.015 ± 0.002	—	—
	GES^{G170L}	102 ± 8	5 ± 0.1	4.17 ± 0.41	—	—
	GES^{G170K}	5 ± 0.4[c]	1 ± 0.03	0.20 ± 0.02	—	—

(Continued on next page)

TABLE 2 (Continued)

Substrate	Variant[b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat}/K_m ratio of:	
					GES_{mut}/GES-5	GES_{mut}/GES-1
Nitrocefin	GES-1	330 ± 15	168 ± 2	0.51 ± 0.029	0.11	1
	GES-5	170 ± 12	769 ± 3	4.52 ± 0.34	1	8.9
	GES^{G170H}	127 ± 6	9 ± 0.2	0.07 ± 0.005	0.015	0.14
	GES^{G170L}	91 ± 7	8 ± 0.6	0.09 ± 0.014	0.005	0.18
	GES^{G170K}	171 ± 10	1 ± 0.05	0.006 ± 0.0006	0.001	0.01

[a]Each kinetic value is the mean of three different measurements; the error was below 10%. Kinetic parameters were determined under initial-rate conditions using Origin Pro 8.5.1 to generate Michaelis-Menten curves. For K_m values lower than 25 μM and higher than 1 mM, K_m was determined as k_i using nitrocefin as the reporter substrate.
[b]For each substrate shown, data for GES-1 and GES-5 are from Piccirilli et al. (10).
[c]K_m was calculated as K_i, using nitrocefin as the reporter substrate (12).
[d]Calculated as k_{+3} (K_i values as a function of substrate concentration) (12).
[e]Calculated as k_{+2}/K (K_i values as a function of substrate concentration) (12).
[f]ND, not determined.
[g]NH, no hydrolysis.
[h]—, not possible to calculate.

three mutants, GES^{G170H} hydrolyzed piperacillin better than GES^{G170K} and GES^{G170L}, even if its k_{cat}/K_m value is similar to that calculated for GES^{G170L}. The three GES mutants have K_m and k_{cat} values lower than those calculated for GES-1 and GES-5. Unlike GES^{G170L} and GES^{G170K}, GES^{G170H} showed a K_m value of 485 μM, which was calculated by measuring direct hydrolysis of substrate. With the exception of GES^{G170H}, GES mutants showed k_{cat}/K_m values higher than those for GES-1.

Cephalosporins. GES^{G170H}, GES^{G170L}, and GES^{G170K} mutants showed a decrease in k_{cat} and K_m values toward cefazolin, cefotaxime, and cefoxitin. Cefoxitin was a poor substrate for GES^{G170H}, which showed a k_{cat} of 0.7 s^{-1} and was not hydrolyzed by GES^{G170K}. Unlike GES-1, GES-5, and GES^{G170K}, K_m values for GES^{G170H} and GES^{G170L} were calculated by direct hydrolysis of cefotaxime.

In the current study, position 170 has also been studied in relation to interaction with inhibitors. Indeed, the residue 170 in GES enzymes plays an important role in the inhibition by clavulanic acid (9). Inhibition experiments with clavulanic acid and tazobactam were monitored directly by using nitrocefin as the reporter substrate (12). Tazobactam and clavulanic acid behaved as competitive inhibitors for GES-1, GES-5, GES^{G170K}, GES^{G170H}, and GES^{G170L}. Tazobactam and clavulanic acid were able to inhibit efficiently GES^{G170L} and GES^{G170K}, with K_i and 50% inhibitory concentration (IC_{50}) values lower than those calculated for GES-1 and GES-5 (Table 3). On the contrary, GES^{G170H} exhibited an increase in K_i values for both tazobactam and clavulanic acid. In particular, a very high K_i value was calculated for clavulanic acid (>200 μM). The same behavior has been reported for GES-4, where the IC_{50}s for clavulanic acid and tazobactam were similar to those calculated for classical inhibitor-resistant TEM β-lactamases (IRT) (15).

In order to explain the effect of G170L, G170K, and G170H substitutions on substrate hydrolysis, molecular modeling was performed using the 1.1-Å resolution X-ray structure of GES-1 (Protein Data Bank code 2QPN) as the template (16). Initial attempts

TABLE 3 Determination of K_i and IC_{50} for GES^{G170H}, GES^{G170L}, and GES^{G170K} against clavulanic acid and tazobactam[a]

Enzymes	Clavulanic acid		Tazobactam	
	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)
GES-1	21 ± 1	38 ± 2	2.0 ± 0.5	3.5 ± 0.5
GES-5	17 ± 1	15 ± 1	1.7 ± 0.2	2.0 ± 0.5
GES^{G170L}	8 ± 1	15 ± 1	0.048 ± 0.01	0.06 ± 0.01
GES^{G170K}	7 ± 1	13 ± 1	0.25 ± 0.05	0.3 ± 0.05
GES^{G170H}	>200	>200	10 ± 1	18 ± 1

[a]K_i and IC_{50} were determined using nitrocefin as the reporter substrate at the following concentrations: 200, 100, 90, 80, and 100 μM for GES-1, GES-5, GES^{G170H}, GES^{G170L}, and GES^{G170K}, respectively.

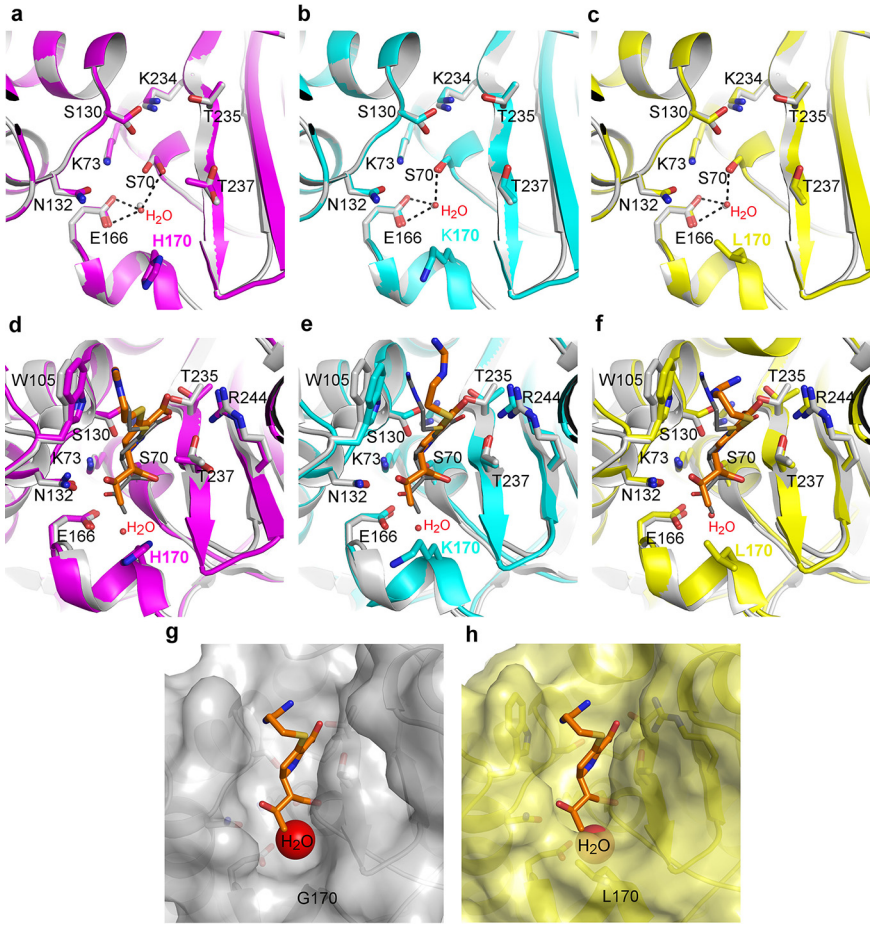

FIG 1 Models of the GES^{G170H}, GES^{G170K}, and GES^{G170L} mutants. (a) Superimposition of the GES-1 structure (PDB code 2QPN) in white and the GES^{G170H} model in magenta represented as a cartoon. Important active site residues are represented as sticks. The catalytic water molecule is shown as a small red sphere, and black dashed lines highlight its polar contacts. (b) Same as panel a for the GES^{G170K} model in cyan. (c) Same as panel a for the GES^{G170L} model in yellow. (d) Superimposition of the GES-1 structure in complex with imipenem (PDB code 4GOG) in white (imipenem as gray sticks) and the GES^{G170H} corresponding model in magenta (imipenem as orange sticks) represented as a cartoon. Important active site residues are represented as sticks and the catalytic water molecule by a small red sphere. (e) Same as panel d for the GES^{G170K} model in cyan. (f) Same as panel d for the GES^{G170L} model in yellow. (g) GES-1 structure represented as transparent surface surrounding the mixed cartoon and stick representation of panel a; the catalytic water molecule is shown as a red sphere, and the imipenem molecule from the superposed GES^{G170L} model is represented as orange sticks. (The GES-1:imipenem structure could not be used because of the absence of a properly positioned catalytic molecule.) (h) Same as panel g. For the GES^{G170L} model, the catalytic water molecule is shielded by L170.

to obtain the three models using mutations and rotamer selection in PyMOL (The PyMOL Molecular Graphics System, Version 2.3.2, Schrödinger, LLC.) followed by the energy minimization procedure implemented in YASARA (version 17.1.28; YASARA Biosciences GmbH) (17) produced an unrealistic displacement of the β-strand containing the third conserved motif that significantly enlarged the active site, as revealed by the model of the GES^{G170S} mutant reminiscent of GES-5, for which a high-resolution crystallographic structure is also available. The use of the homology modeling procedure of the YASARA software still using 2QPN as the starting model (with the catalytic water molecule as part of the template) yielded models of the GES^{G170H}, GES^{G170L}, and GES^{G170K} mutants with no unrealistic region detected. As shown in Fig. 1a to c, the three mutations did not induce any significant alteration of the position of the catalytic site residues compared to GES-1, which is also very similar to GES-5. The root mean square deviation (RMSD) for all Cα carbons is below 0.1 Å compared to GES-1. When considering all atoms of the conserved catalytic residues (SxxK, SxN, KTG, and E166),

the RMSD is below 0.52 Å, further highlighting the lack of significant modification to the structure, including in the Ω-loop that contains the mutations (Fig. 1a to c). In addition to the low RMSD values compared to 2QPN, the overall Z-scores for the GES^{G170H}, GES^{G170L}, and GES^{G170K} models were, respectively, 0.684, 0.635, and 0.659 (the positive values indicating the X-ray-like quality of the model), indicating that the quality present in the starting modeled was conserved. To evaluate the influence of a substrate on these models, we superimposed the GES-1:imipenem acyl-enzyme structure (PDB code 4GOG) on the models, copied the coordinates of the imipenem molecule in the acyl-enzyme form into the pdb files of the models (with the catalytic water molecule remaining part of the model), created a covalent bond between imipenem and S70, and used YASARA to perform an energy minimization with each of the three acyl-enzymes generated. These models of the mutants in the acyl-enzyme form with imipenem point to a systematic displacement of imipenem (between 0.9 and 1.4 Å of the hydroxyethyl group compared to its position in the GES-1:imipenem acyl-enzyme structure) away from the 170 position and concomitantly slightly away from E166 (Fig. 1d to f). The hydroxyethyl group in the α orientation on the C-6 carbon of the carbapenem tested, instead of the β conformation characteristic of the other substituent at this position in the penicillin and cephalosporin parts of this study, is known to interfere with the catalytic water molecule activated by E166 during deacylation (7). In GES-1, with a glycine at position 170, this water molecule is less constrained in its catalytic position during the acylation because a path toward the solvent remains accessible, while in the mutants tested in this study, this path is obstructed (Fig. 1g and h). It is therefore possible that the displacement of the substrate combined with the fencing by residue 170 restores a better positioning of the water molecule activated by E166 during hydrolysis, at least partially explaining the significant improvement observed for the k_{cat} values of the three mutants toward carbapenems. In the GES^{G170H}:imipenem model, an additional shift of the E166 side chain is observed. More recent publications have demonstrated that substitutions at position 170 seem to modify the distance between E166 and S70 residues by changing the position of water molecule involved in the deacylation step (6, 7, 18). This was not observed in our models, with only a 0.1-Å decrease (3.9-Å) of the E166-S70 distance in the GES^{G170L} and GES^{G170K} models compared to GES-1 and a 0.9-Å increase (4.9-Å) in the GES^{G170H} model due to a slight rotation of the S70 hydroxyl. These effects do not preclude an additional role for a potential modification of the Ω-loop dynamic like the P174E substitutions (10). The improved k_{cat} values of the three mutants for the carbapenems remain, however, significantly lower than the one reached by GES-1 against most penicillins and cephalosporins. In conclusion, in this study we have showed how a single mutation at position 170 in GES enzymes (i.e., G170H substitution) could develop the ability to efficiently inactivate most of the β-lactams, including carbapenems, and to resist to clavulanic acid. The combination of resistance to expanded-spectrum β-lactams, carbapenems, and inhibitors makes some GES-producing bacteria very dangerous in clinical settings.

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