ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2010, p. 4864–4871 Vol. 54, No. 11
0066-4804/10/$12.00 doi:10.1128/AAC.00771-10

Comparative Biochemical and Computational Study of the Role of Naturally Occurring Mutations at Ambler Positions 104 and 170 in GES β-Lactamases[▽]

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Received 7 June 2010/Returned for modification 17 July 2010/Accepted 1 August 2010

In GES-type β-lactamases, positions 104 and 170 are occupied by Glu or Lys and by Gly, Asn, or Ser, respectively. Previous studies have indicated an important role of these amino acids in the interaction with β-lactams, although their precise role, especially that of residue 104, remains uncertain. In this study, we constructed GES-1 (Glu104, Gly170), GES-2 (Glu104, Asn170), GES-5 (Glu104, Ser170), GES-6 (Lys104, Ser170), GES-7 (Lys104, Gly170), and GES-13 (Lys104, Asn170) by site-specific mutagenesis and compared their hydrolytic properties. Isogenic comparisons of β-lactam resistance levels conferred by these GES variants were also performed. Data indicated the following patterns: (i) Lys104-containing enzymes exhibited enhanced hydrolysis of oxyimino-cephalosporins and reduced efficiency against imipenem in relation to enzymes possessing Glu104, (ii) Asn170-containing enzymes showed reduced hydrolysis rates of penicillins and older cephalosporins, (iii) Ser170 enabled GES to hydrolyze cefoxitin efficiently, and (iv) Asn170 and Ser170 increased the carbapenemase character of GES enzymes but reduced their activity against ceftazidime. Molecular dynamic simulations of GES apoenzyme models, as well as construction of GES structures complexed with cefoxitin and an achiral ceftazidime-like boronic acid, provided insights into the catalytic behavior of the studied mutants. There were indications that an increased stability of the hydrogen bonding network of Glu166-Lys73-Ser70 and an altered positioning of Trp105 correlated with the substrate spectra, especially with acylation of GES by imipenem. Furthermore, likely effects of Ser170 on GES interactions with cefoxitin and of Lys104 on interactions with oxyimino-cephalosporins were revealed. Overall, the data unveiled the importance of residues 104 and 170 in the function of GES enzymes.

GES β-lactamases (GES-1 to GES-16) (www.lahey.org/studies/) comprise a distinct group of molecular class A enzymes with extended-spectrum properties differing by one to three amino acid residues. The respective genes, whose origin(s) remains unknown, occur exclusively in the form of cassettes carried by class 1 integrons identified in both chromosomes (mostly in *Pseudomonas aeruginosa*) and plasmids (mostly in *Klebsiella pneumoniae*) (18, 19). GES-producing microorganisms have emerged in many geographic areas. Nevertheless, their incidence so far remains relatively low, and therefore their clinical significance is limited. On the other hand, GES enzymes have caught considerable attention due to their ability to interact with carbapenems and, in the cases of GES-2, -4, -5, and -6, to hydrolyze imipenem at measurable rates (1, 18, 26, 27). The latter β-lactamases possess either Asn (GES-2) or Ser (GES-4, GES-5, and GES-6) at Ambler position 170, while in the remaining GES variants the respective residue is Gly. Another difference common among GES enzymes occurs at position 104, which is occupied by Lys in GES-3, GES-4, GES-6, GES-7, and GES-13, instead of Glu, which is found in the remaining variants (7, 10, 26, 27, 28). The presence of Lys-104 has been associated with increased activity against oxyimino-β-lactams, mainly ceftazidime and aztreonam (7, 10).

Determination of the crystal structure of GES-1 has provided useful clues to the configuration of the active site and the catalytic mechanism (23). Nevertheless, the functions of various amino acid residues implicated in β-lactam hydrolysis remain uncertain. As pointed out by Smith et al. (23), comparison of the published kinetic constants of the GES enzymes poses difficulties, partly due to differences in methodologies used. In this study, we compared the hydrolysis spectra, obtained under similar conditions, of six laboratory-constructed GES variants differing at positions 104 and/or 170 (Table 1). We also attempted to gain insights into the functional roles of the respective residues by molecular simulations.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5α [*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used as a host of recombinant plasmids. The previously described high-copy-number plasmid pBC-ges1, carrying a class 1 integron containing bla_{GES-1} as a single gene cassette in its variable region (GenBank accession no. EU598463) (16), was used as a source of the bla_{GES-1} gene during cloning experiments. The chloramphenicol- and tetracycline-resistant plasmid pACYC184 was utilized as a cloning vector.

Susceptibility to β-lactams. MICs of various β-lactam antibiotics, including ampicillin, amoxicillin-clavulanate, ticarcillin, ticarcillin-clavulanate, piperacillin, piperacillin-tazobactam, cefoxitin (FX), cefotaxime (CTX), ceftazidime, aztreonam, imipenem, and meropenem, were determined by the Etest method according to the recommendations of the manufacturer (AB bioMérieux, Solna, Sweden).

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▽ Published ahead of print on 9 August 2010.

TABLE 1. Studied GES variants differing from GES-1 by one or two residues at Ambler positions 104 and 170

β-Lactamase	Amino acid residue at position:	
	104	170
GES-1	Glu	Gly
GES-7[a]	Lys	Gly
GES-2	Glu	Asn
GES-5	Glu	Ser
GES-6	Lys	Ser
GES-13	Lys	Asn

[a] Initially reported as IBC-1 (7).

Production of bla_{GES} genes by site-specific mutagenesis. The class 1 integron containing bla_{GES-1}, obtained after BamHI/EcoRI digestion of the pBC-ges1 plasmid and treatment with the Klenow fragment of *E. coli* DNA polymerase, was cloned into the EcoRV site of pACYC184, yielding the recombinant plasmid pAC-ges1, used to transform *E. coli* DH5α. The bla_{GES-2}, bla_{GES-5}, bla_{GES-6}, bla_{GES-7}, and bla_{GES-13} genes were obtained by site-directed mutagenesis, using the pAC-ges1 and pBC-ges1 plasmids as templates, a QuikChange kit (Stratagene, La Jolla, CA), and the following mutagenic primers (with mutations shown in bold): G170N-F, 5′-CCGGAAAGAGCCGGAGATG**AAC**GACAACACACCTGGCGAC-3′; G170N-R, 5′-GTCGCCAGGTGTGTTGTC**GTT**CATCTCCGGCTCTTTCCGG-3′; G170S-F, 5′-CGGAAAGAGCCGGAGATG**AG**CGACAACACACCTGGCG-3′; G170S-R, 5′-CGCCAGGTGTGTTGTC**GCT**CATCTCCGGCTCTTTCCG-3′; E104K-F, 5′-GGGCCGGACATGATCGTC**A**AATGGTCTCCTGCCACGG-3′; and E104K-R, 5′-CCGTGGCAGGAGACCA**TTT**GACGATCATGTCCGGCCC-3′. The generated plasmids were used to transform *E. coli* DH5α, and the incorporation of the correct mutations was verified by sequencing (ABI 8000 sequencer; Applied Biosystems, Foster City, CA) in both directions, using the primers Int/F and 3′CS (16). Production of each GES β-lactamase by the clones carrying the pBC-ges and pAC-ges plasmids was examined by analytical isoelectric focusing of crude protein extracts, using nitrocefin staining. The Glu104 variants (GES-1, GES-2, and GES-5) were focused at pH 5.9, and the Lys104 variants (GES-6, GES-7, and GES-13) were focused at pH 6.9 (data not shown).

Purification of GES variants. β-Lactamases were released from the pBC-ges-harboring clones by mild sonication of bacterial cells suspended in Tris buffer (20 mM; pH 8.0). Extracts were loaded on a Q-Sepharose column (Bio-Rad Laboratories, Hercules, CA), and proteins were eluted with a 0 to 1 M NaCl gradient. Fractions displaying β-lactamase activity (as tested by a nitrocefin assay) were pooled, dialyzed overnight against 20 mM morpholineethanesulfonic acid (MES), pH 6.0 (Lys104 variants), or 20 mM MES, pH 5.5 (Glu104 variants), and loaded on an S-Sepharose column (Bio-Rad Laboratories). The bound β-lactamase molecules were eluted with 400 to 500 mM NaCl. Finally, preparations were dialyzed against 50 mM KP_i, pH 7.6, and concentrated using ultrafiltration (Amicon 10-kDa-cutoff filter; Millipore Corp., Billerica, MA). The purity of the final preparations was >95%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined by the Bradford method. Enzymes were stored at −20°C in 20% glycerol until further use.

Determination of kinetic parameters. Hydrolysis of β-lactam substrates used at various concentrations was carried out at 25°C in 50 mM NaCl, 50 mM morpholinepropanesulfonic acid (MOPS), pH 7.0. Absorbance was monitored using a Hitachi U-2001 UV/visible spectrophotometer. Wavelengths (λ) and extinction coefficients (Δε) for β-lactam substrates were as follows: for penicillin G, λ = 233 nm and Δε = 900 $M^{-1} \cdot cm^{-1}$; for cephalothin, λ = 262 nm and Δε = 7,660 $M^{-1} \cdot cm^{-1}$; for cefoxitin, λ = 260 nm and Δε = 7,700 $M^{-1} \cdot cm^{-1}$; for cefotaxime, λ = 266 nm and Δε = 6,700 $M^{-1} \cdot cm^{-1}$; for ceftazidime, λ = 260 nm and Δε = 8,660 $M^{-1} \cdot cm^{-1}$; for aztreonam, λ = 318 nm and Δε = 660 $M^{-1} \cdot cm^{-1}$; and for imipenem, λ = 300 nm and Δε = 9,000 $M^{-1} \cdot cm^{-1}$ (20). Steady-state kinetic constants were determined by nonlinear regression analysis, using Prism software (GraphPad, La Jolla, CA).

Inhibition studies. Activities of the mechanism-based inhibitors clavulanic acid, sulbactam, and tazobactam against GES-type β-lactamases were assessed by spectrophotometry, using cephalothin as a reporter substrate at a concentration equal to the respective K_m, and were expressed as the 50% inhibitory concentration (IC_{50}). Reactions were carried out at 25°C. Inhibitors were preincubated with each enzyme for 5 min before the addition of the substrate.

Computational studies. Three-dimensional models of GES-2, -5, -6, -7, and -13 were built by homology modeling, using the crystal structure of the GES-1 apoenzyme (Protein Data Bank [PDB] accession no. 1QPN; resolution, 1.1 Å) (23) as a template and the software MODELLER (5). Models exhibiting the best scores for MODELLER's probability density function were further evaluated in terms of energy and geometry, using the discrete optimized protein energy (DOPE) statistical potential of MODELLER (22) and PROCHECK (11), respectively. The crystal structure of GES-1 and the selected GES models were solvated in a TIP3P water model (a rigid 3-point model) in a cubic box, neutralized, and minimized by 50,000 steps of steepest descent. The temperature was increased slowly to 300 K, and the systems were simulated isotropically (pressure, 1 atm) for 100 ps, with protein atoms position restrained, using the software GROMACS (25) and Amber99 force-field ports (24). Minimization was then carried out by 50,000 steps of steepest descent, and the above procedure was repeated four times by relaxing position restraints of protein atoms gradually at each new cycle (21). For the stable phase of each simulation, an average structure was computed and minimized until convergence to the selected criterion (maximum force [F_{max}] < 100 J mol^{-1} nm^{-1}).

Three-dimensional covalent complexes of the GES enzymes with FX and a boronic acid analogue carrying the oxyimino side chain of ceftazidime (CB4) were constructed by superpositioning of the respective crystal structures of the CTX-M-9 complexes (PDB accession no. 1YMX and 1YLY, respectively) (3) onto the GES molecules, using the Cα atoms for the root mean square fit. The generated GES-FX and GES-CB4 complexes were relaxed by five cycles of energy minimization and 100-ps position restraint molecular dynamic simulations in an NPT explicit solvent ensemble, using GROMACS and Amber99 force-field software as described above. Finally, molecular dynamic trajectories were obtained by 6-ns simulations, and average structures were computed and minimized.

RESULTS AND DISCUSSION

Comparison of hydrolytic activities. Although differences in the k_{cat} and K_m values for penicillin G were observed between GES-1, GES-7, GES-5, and GES-6, the respective catalytic efficiencies were comparable. Significantly lower activity against penicillin G was seen for the remaining variants, GES-2 and GES-13, mainly due to lower k_{cat} values. The same pattern was also observed for cephalothin. While hydrolysis rates for the latter substrate were high with GES-1, GES-7, GES-5, and GES-6, the k_{cat} values for GES-2 and GES-13 were >10-fold lower than those for the former enzymes. Affinities for cephalothin were comparable for GES-1, GES-2, GES-5, and GES-6, as indicated by the respective K_m values. A lower K_m for cephalothin was observed with GES-7, which also exhibited a slightly higher k_{cat}, thus being the most effective of the enzymes tested against this substrate. A lower efficiency against cephalothin was observed for GES-2 due to a significantly lower k_{cat}. Similar to the case with GES-2, the efficiency of GES-13 against cephalothin was relatively low. The latter GES variant displayed both reduced affinity and relatively low rates of hydrolysis against cephalothin. In keeping with previous studies (1, 27), only GES-5 and GES-6 exhibited significant hydrolytic efficiencies against cefoxitin, while in the remaining GES variants hydrolysis of this substrate was not detectable. Of the former two enzymes, GES-6 was the more efficient against cefoxitin, exhibiting both higher hydrolysis rates and higher affinity. Measurable rates of hydrolysis of ceftazidime were observed for all six GES variants. The highest catalytic efficiency against ceftazidime was observed with GES-7. Significantly lower "ceftazidimase" activities were seen with GES-1, GES-6, and GES-13, due to lower k_{cat} values. For GES-2 and GES-5, while K_m values were comparable to those of the other variants, k_{cat} values were very low, resulting in relatively poor efficiencies against ceftazidime. Aztreonam was an unfavorable

TABLE 2. Kinetic parameters of GES variants for β-lactam substrates[a]

β-Lactam	GES-1 (E104, G170)			GES-7 (K104, G170)			GES-2 (E104, N170)		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Penicillin G	30.5	120	0.26	14.2	39.0	0.36	5.75	87.6	0.07
Cephalothin	218	87.8	2.48	313	23.7	13.2	2.94	53.9	0.05
Cefoxitin	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ceftazidime	10.5	137	0.077	132	69.2	1.90	0.21	98.2	0.002
Aztreonam	ND	ND	ND	45.5	241	0.19	ND	ND	ND
Cefotaxime	56.8	152	0.37	31.4	81.6	0.38	37.2	261	0.14
Imipenem	0.006	1.2	0.005	ND	ND	ND	0.016	0.87	0.02

[a] Values for k_{cat} and K_m are means for three independent measurements not differing by more than 10%, except in cases of very low hydrolysis rates (GES-1, -2, and -13 against imipenem), where differences ranged from 20 to 30%. ND, not determinable. Hydrolysis was either not detectable or too low to be measured reliably.

substrate for GES-1, GES-2, GES-5, and GES-6 (hydrolysis was either not detectable or too slow to be measured accurately). In contrast, GES-7 and GES-13 hydrolyzed aztreonam at high rates, although the K_m values were relatively high. GES-7 was more efficient than GES-13 against aztreonam. Kinetic parameters for cefotaxime hydrolysis indicated that GES-13 was the most effective variant, followed by GES-7, GES-1, GES-2, GES-6, and GES-5. The relatively lower efficiencies of the last two variants against cefotaxime were due to low k_{cat} values. Hydrolysis of imipenem was observed with GES-1, GES-2, GES-5, GES-6, and GES-13. For the remaining variant, GES-7, hydrolysis was not detectable. GES-5 exhibited the highest "carbapenemase" activity, followed by GES-6. Both enzymes hydrolyzed imipenem much faster than GES-1, GES-13, and the "carbapenemase" GES-2 (Table 2).

Comparison of susceptibilities to inhibitors. Of the three tested inhibitors, tazobactam was the most active against all six β-lactamase variants, followed by clavulanic acid, while the inhibitory activity of sulbactam was significantly lower. The GES variants that were most susceptible to inhibition by tazobactam and clavulanate were GES-1, GES-2, and GES-13. The higher apparent IC_{50} values for all three inhibitors tested were seen with the Ser170-possessing variants GES-5 and GES-6 (Table 3).

Comparison of β-lactam resistance levels. MICs of ampicillin and ticarcillin exceeded the concentrations used in susceptibility testing. Thus, conclusions cannot be drawn about any potential differences in the efficiencies of the GES variants against these drugs. Piperacillin was effective against the GES-producing clones, with the respective MICs being elevated slightly compared to that for *E. coli* DH5α. The highest piperacillin MICs were observed for the GES-6- and GES-7-producing clones. The above penicillins, however, were not tested as substrates. MICs of the remaining β-lactam antibiotics generally corroborated the results of the hydrolysis and inhibition experiments. The relatively high susceptibilities to inhibition by clavulanic acid of GES-1, GES-2, GES-5, and GES-13 were in line with the low MICs of amoxicillin- and ticarcillin-clavulanate combinations. The higher MICs of the latter combinations against the GES-6- and GES-7-producing clones reflected, at least partly, the decreased susceptibility of these variants to clavulanic acid. Tazobactam almost fully restored the activity of piperacillin for all GES-producing clones. The activity of cefoxitin was also in agreement with the hydrolysis data. The clones producing GES-5 and GES-6 were inhibited by significantly higher concentrations of the drug than the remaining GES-producing clones, for which cefoxitin MICs were just slightly elevated compared to that for *E. coli* DH5α. The higher hydrolytic efficiencies of GES-7 and GES-13 against ceftazidime were reflected in the high MICs of this drug against the respective strains. MICs of ceftazidime were also elevated for the GES-1 and GES-6 producers. The lowest ceftazidime MICs were observed for the GES-2 and GES-5 producers. A similar pattern of differences in resistance levels was also seen with aztreonam, although the MIC values were significantly lower than those of ceftazidime. An increase in the MICs of cefotaxime was observed for all GES producers compared to *E. coli* DH5α. This increase was more pronounced for the GES-13-, GES-7-, GES-1-, and GES-2-producing strains. Cefotaxime MICs for the GES-5 and GES-6 producers were just two doubling dilutions higher than that for *E. coli* DH5α. A relatively significant increase in the MIC of imipenem was observed for the clone producing GES-5. For the remaining GES-producing strains, the respective MICs were three (GES-2 and GES-6 producers) and one to two (GES-1, GES-7, and GES-13) doubling dilutions higher than the imipenem MIC for *E. coli* DH5α (Table 4).

TABLE 3. Activities of mechanism-based inhibitors against GES β-lactamase variants

Inhibitor	IC_{50} (μM)[a]					
	GES-1 (E104, G170)	GES-7 (K104, G170)	GES-2 (E104, N170)	GES-5 (E104, S170)	GES-6 (K104, S170)	GES-13 (K104, N170)
Clavulanic acid	0.1	0.8	0.08	0.5	1.7	0.09
Sulbactam	1.5	1.3	0.5	4.5	8.0	0.3
Tazobactam	0.03	0.06	0.03	0.5	0.5	0.04

[a] Values are means for three measurements differing by <10%.

GES–β-lactam interaction patterns. Kinetic constants, along with resistance levels, revealed some GES–β-lactam interaction patterns that may be indicative of the role of the amino acid residues at positions 104 and 170. (i) The presence of Asn170 instead of Gly170 (GES-2 and GES-13) reduced hydrolytic efficiencies against both penicillin G and cephalothin, two drugs that are commonly good substrates for most class A β-lactamases. This happened irrespective of the residue occupying position 104 (Glu or Lys). Although Asn170 in most class A β-lactamases may facilitate hydrolysis by interacting with

TABLE 2—*Continued*

GES-5 (E104, S170)			GES-6 (K104, S170)			GES-13 (K104, N170)		
k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
28.6	88.9	0.32	27.2	64.2	0.42	13.5	139	0.10
167	61.1	2.73	260	54.0	4.81	10.1	106	0.10
7.50	221	0.03	12.1	82.3	0.15	ND	ND	ND
0.24	135	0.002	9.30	149	0.063	30.4	229	0.13
ND	ND	ND	ND	ND	ND	35.6	405	0.09
3.55	133	0.03	5.10	95.6	0.05	69.0	63.4	1.09
0.332	1.27	0.26	0.166	3.92	0.042	0.009	3.10	0.003

one of the catalytic waters (4), it appears that in the case of GES enzymes, the role of this residue was rather detrimental regarding activity against penicillins and older cephalosporins. (ii) Ser170 instead of Gly170 enabled GES-5 and GES-6 to efficiently hydrolyze cefoxitin (a 7α-methoxy substituent-possessing substrate unfavorable for most class A enzymes), irrespective of the presence of Glu or Lys at position 104. The negligible activity of the remaining GES enzymes may resemble the "inhibitory" behavior of cefoxitin against CTX-M-9, an enzyme structurally similar to GES-1 (superposition of CTX-M-9 and GES-1 crystal structures indicated that the two enzymes share a nearly identical active site). The crystal structure of the CTX-M-9–cefoxitin acyl enzyme has indicated that the 7α-methoxy substituent may displace the deacylation water (3). (iii) Asn or Ser at position 170 increased efficiency against imipenem compared to that with the Gly170 variants. "Carbapenemase" activity was more pronounced for GES-5. Measurement of the microscopic rate constants for the individual catalytic steps of GES-1, GES-2, and GES-5 against imipenem (6) revealed that the rate-limiting step for the reaction of GES-1 is acylation. GES-2 displayed an increased acylation rate (k_2), by a factor of 3, compared to GES-1, while GES-5 exhibited both increased acylation and deacylation rates (5,000 and 15 times, respectively). Since imipenem carries a 6α-hydroxyethyl substituent equivalent to the 7α substitution of cefoxitin, it is likely that Ser170 affected GES-imipenem interaction in a similar fashion. (iv) Asn170 and Ser170 in the presence of Glu104 decreased activity against oxyimino-cephalosporins, especially ceftazidime. Interestingly, GES-2 retained high hydrolytic efficiency against cefotaxime. (v) Lys104 exhibited a clearly detrimental effect on "carbapenemase" activity conferred by either Asn170 or Ser170 (GES-6 and GES-13, respectively). Comparison of steady-state kinetic constants between GES-5 and GES-6 and between GES-2 and GES-13 indicated that in both cases, the above reduction was due to a lowering of k_{cat} and an increase of K_m, likely reflecting lower acylation rates. (vi) Lys104 in combination with Gly, Asn, or Ser at position 170 (GES-7, GES-13, and GES-6, respectively) increased activity against ceftazidime compared to that of Glu104-possessing variants. The most pronounced "ceftazidimase" effect was seen with GES-7. Although the reduction of the K_m conferred by the Glu104Lys substitution in the presence of Gly170 suggested an improvement of affinity, the significant increase of the k_{cat} of GES-13 over that of GES-2 and of the k_{cat} of GES-6 over that of GES-5 against ceftazidime (150- and 60-fold, respectively) may indicate that acylation and deacylation were both affected (15). Note that GES-7 and GES-13 were also more active against aztreonam, which has an oxyimino carboxy-dimethyl (R1) side chain in common with ceftazidime.

Simulations of GES apoenzymes. Molecular dynamic simulations of GES apoenzymes revealed several differences between position 104 and 170 variants. Of the six enzymes, GES-1 exhibited the most flexibility. During GES-1 simulation,

TABLE 4. Etest MICs of β-lactam antibiotics against *E. coli* DH5α clones producing GES variants under isogenic conditions

Inhibitor	MIC (μg/ml)						
	E. coli (GES-1 [E104, G170])	*E. coli* (GES-7 [K104, G170])	*E. coli* (GES-2 [E104, N170])	*E. coli* (GES-5 [E104, S170])	*E. coli* (GES-6 [K104, S170])	*E. coli* (GES-13 [K104, N170])	*E. coli* DH5α
Ampicillin	>256	>256	>256	>256	>256	>256	2
Amoxicillin-clavulanic acid[a]	8	32	3	16	32	3	2
Ticarcillin	>256	>256	>256	>256	>256	>256	4
Ticarcillin-clavulanic acid[b]	6	>256	1.5	16	>256	8	4
Piperacillin	8	12	4	8	12	8	2
Piperacillin-tazobactam[c]	1	1	1	2	2	1	1
Cefoxitin	3	3	3	16	48	3	2
Ceftazidime	8	>256	0.5	0.75	4	32	0.12
Aztreonam	0.094	2	0.032	0.032	0.094	2	0.032
Cefotaxime	1	1.5	0.75	0.125	0.125	3	0.032
Imipenem	0.25	0.125	0.50	1.5	0.50	0.25	0.064
Meropenem	0.016	0.016	0.032	0.094	0.047	0.023	0.008

[a] Penicillin/inhibitor ratio of 2:1.
[b] Inhibitor was present at a fixed concentration of 2 μg/ml.
[c] Inhibitor was present at a fixed concentration of 4 μg/ml.

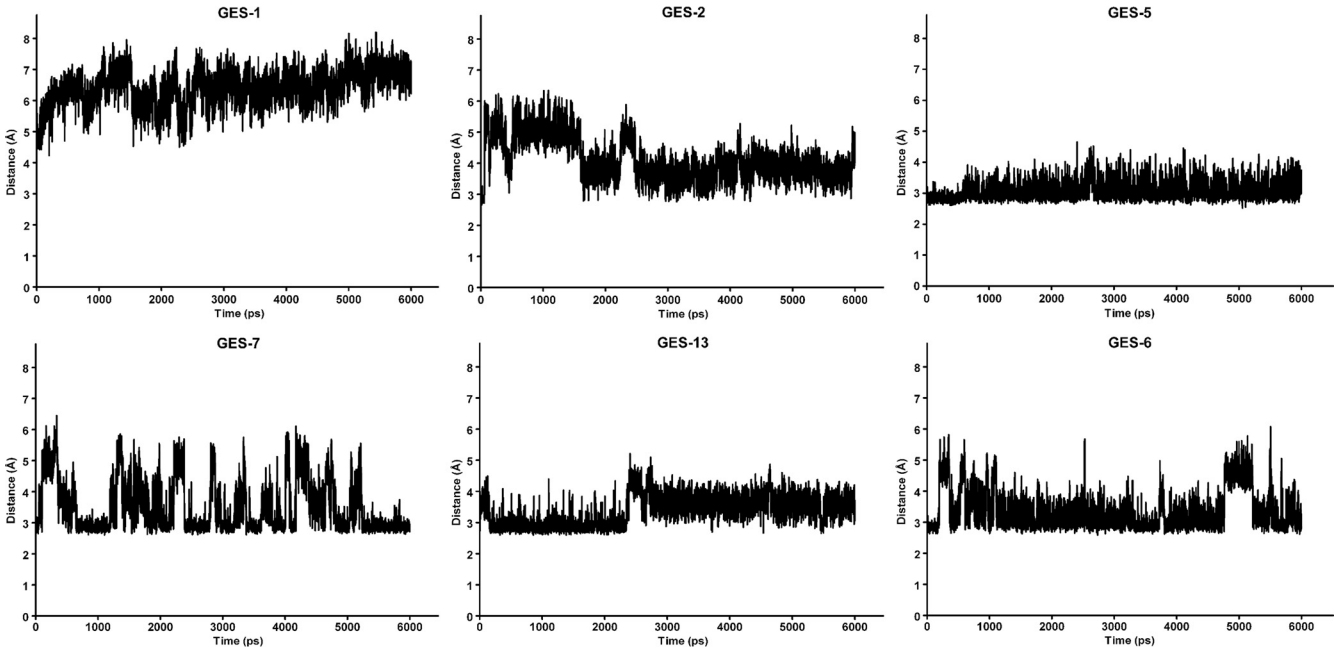

FIG. 1. Variation of the distance between Ser70 Oγ and Lys73 Nζ as a function of time during molecular dynamic simulations of GES apoenzymes. The stabilizing effect of Asn170 (GES-2 and GES-13) and Ser170 (GES-5 and GES-6) is apparent. Plots also indicate the better "fit" of Ser170 in the active site of GES enzymes than that of Asn170 and the diverged effects of the Glu104Lys substitution. The same picture was obtained by measuring distances between the Glu166 and Lys73 side chains.

the Ω loop and the catalytic residues Ser70, Lys73, and Glu166 displayed higher mobilities than the respective regions in the remaining variants. Increasing Ω loop motility may account for the fact that deacylation is not the rate-limiting step of imipenem hydrolysis by GES-1 (6) (in contrast to the case for most class A β-lactamases [14]). Atomic fluctuations of the above triad of catalytic residues seem to be affected mainly by residue 170 and to a lesser extent by residue 104. More motions were observed in the presence of Gly, followed by Asn and Ser, at position 170. The above observations could be illustrated by the change of the distance between Lys73 Nζ and Ser70 Oγ as a function of time (Fig. 1).

The side chains of Asn170 and Ser170 stabilized the above network of three interacting residues through a hydrogen bond with the carboxylate of Glu166. Hydrogen bonding between Glu166, Lys73, and Ser70 is crucial in determining the activation of the Ser70 hydroxyl for the nucleophilic attack of the β-lactam ring (acylation) and the activation of the hydrolytic water for the collapse of the acyl enzyme intermediate (deacylation) (4, 8, 13). Therefore, the observed differences in the

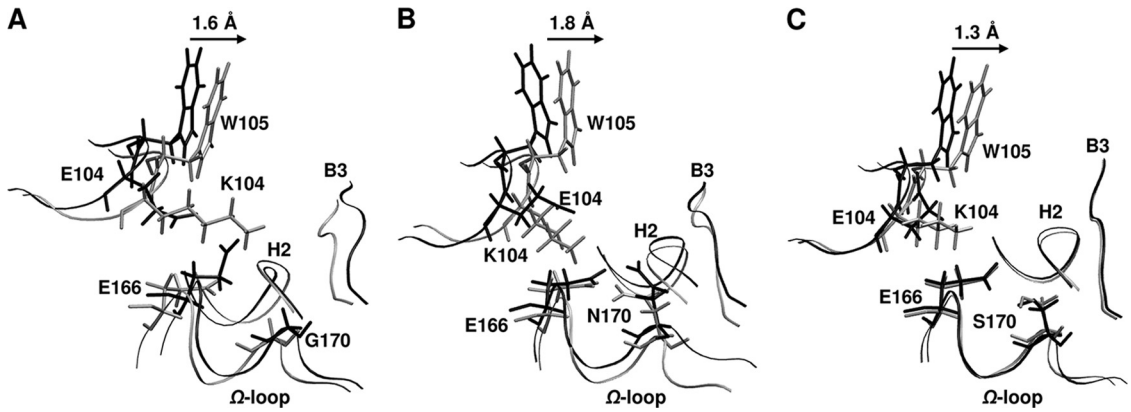

FIG. 2. Comparison of average apoenzyme structures of Glu104 and Lys104 variants. Comparison was carried out by superpositioning, using the Cα atoms for the root mean square fit. Some of the secondary structural elements that form the active site cavity are shown as ribbons, and residues of interest are shown as sticks. For each pair of enzymes (GES-1 and GES-7 [A], GES-2 and GES-13 [B], and GES-5 and GES-6 [C]), the Glu104 variant is colored black and the Lys104 variant gray. In all cases, the Glu104Lys substitution caused a movement of Trp105 to the interior of the active site. An estimation of the degree of Trp105 movement can be given by measuring the distance between the Trp105 Cβ atoms of Glu104 and Lys104 variants. The above effect of Lys104 could be explained by its interactions with the helix of the Ω loop that lies beneath. Displacements of the side chain of Glu166 in the case of GES-1 (A) and of the side chain of Asn170 in GES-2 (B) are also apparent.

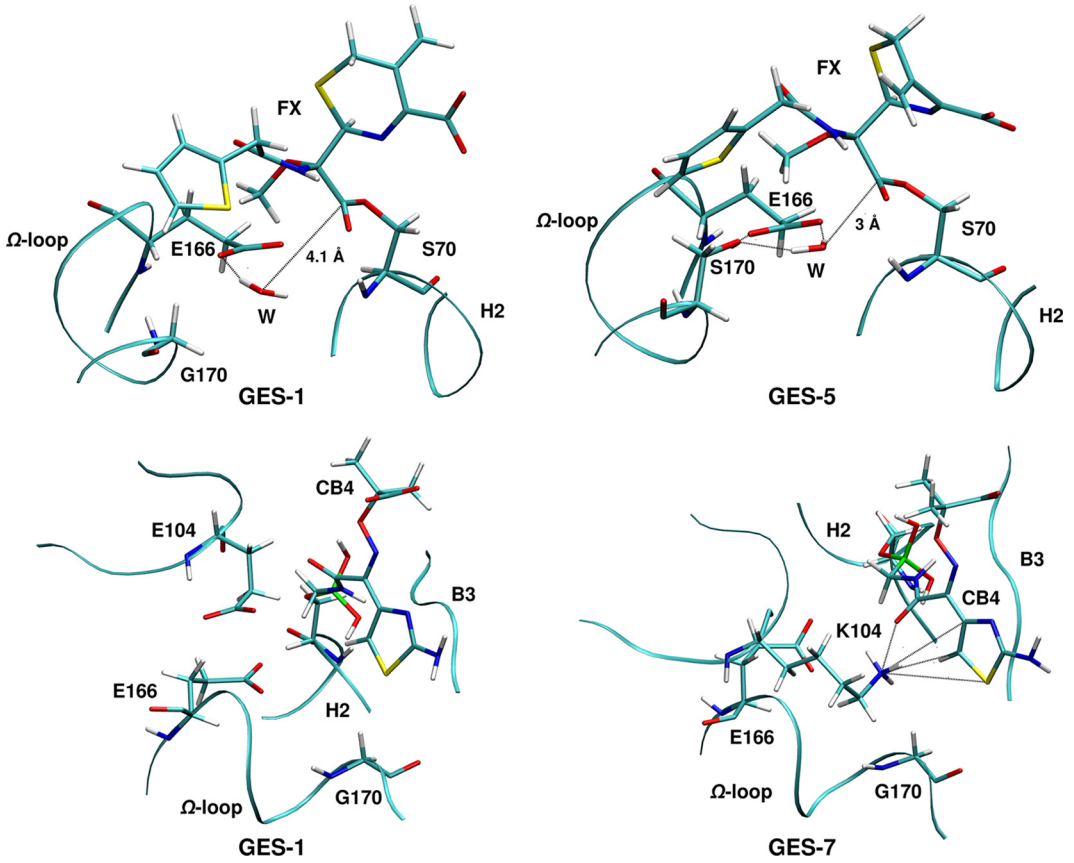

FIG. 3. Complexed structures of GES-1 and GES-5 with cefoxitin (top) and of GES-1 and GES-7 with the boronic acid analogue of ceftazidime (bottom). (Top) The Gly170Ser substitution seems to dramatically alter the conformation of cefoxitin at the acyl enzyme complex, especially the positioning of the 7α-methoxy group. The putative "deacylating" water, forming a hydrogen bond with Glu166 in the two complexes, is better positioned in the case of GES-5, where it is bound with an extra hydrogen bond with the hydroxyl group of Ser170, which interacts with the carboxylate of Glu166. The altered alignment of cefoxitin may account for the higher hydrolytic efficiencies of Ser170 variants against this substrate. (Bottom) Lys104 in the GES enzymes can be positioned at a distance close to the negatively charged atoms of the R1 side chain of ceftazidime (grey lines indicate distances of 3.0 to 4.5 Å), thus improving the affinity by providing an extra docking site for the side chains of expanded-spectrum cephalosporins.

catalytic properties of the six GES variants could be attributed to differences in positioning and mobility of important residues.

Comparisons of average minimized structures computed from the last 500 ps of Molecular Dynamics simulations showed again the diversification of the GES-1 apoenzyme from the rest of the variants regarding important alterations in the positioning of the B3 β-strand and the Ω loop. An interesting difference was observed between the Glu104 and Lys104 variants concerning the loop containing residues 96 to 105 and, especially, the positioning of Trp105 (Fig. 2). Irrespective of the residue occupying position 170, Lys104 caused a significant movement of Trp105 inward at the active site. Note that for the GES-5–GES-6 pair, the above difference appeared to be the only one between the active sites of the two simulated models (Fig. 2C).

Previous mutagenesis studies with SME-1 (12) and structural studies of KPC-2 (9) have indicated a putative role of position 105 in imipenem hydrolysis by class A carbapenemases. In flexible docking simulations of GES-1, GES-2, and GES-5 with imipenem, a comparable movement of Trp105 could be associated with the higher carbapenemase activity of the latter two variants (23). As calculated here, Trp105 in the GES-2 apoenzyme is positioned 1.1 Å up and out from the active site compared to that in GES-1, while Trp105 of GES-5 occurs at a position roughly similar to that in GES-1. Modeling data along with the hydrolytic properties of the GES variants may indicate a potential relationship between positioning of Trp105 and acylation of the GES active site by imipenem.

Simulations of GES complexes. Fully relaxed structures of the GES enzymes covalently complexed with a cefoxitin molecule (acyl enzyme) provided insights into the increased hydrolysis of this substrate by Ser170 variants. In GES-1, the 7α-methoxy group appeared to displace the "deacylating" water, at a distance of 4.1 Å, from the carbon of the ester bond (Fig. 3, top panels). On the other hand, in the Ser170 variants, it seemed that the rigid surface formed by the hydrogen-bonded side chains of Ser170 and Glu166 displaced the 7α-methoxy group in a way that permitted a catalytically more favorable positioning of the "deacylating" water.

Covalent complexes of the GES variants with the achiral boronic acid analogue of ceftazidime indicated that Lys104

may improve affinity for oxyimino-cephalosporins. The distances between the charged amine group of Lys104 and the partially negative charged atoms of the aminothiazole ring as well as the peptide bond oxygen indicated strong electrostatic interactions (Fig. 3). On the other hand, in the Glu104 variants, the negatively charged side chain of Glu appeared to face the solvent due to repulsion with the negative charges of the oxyimino side chain (Fig. 3). These findings indicated an improved affinity of the Lys104 variants not only against the side chains of oxyimino-cephalosporins but also against those of older cephalosporins and penicillins (which possess equivalent negative charges).

Residue 104 is not conserved among class A β-lactamases. In SHV and TEM β-lactamases, replacement of a negatively by a positively charged residue (e.g., replacement of either Asp or Glu by Lys) increases activity against expanded-spectrum cephalosporins, especially when combined with a hydroxyl-possessing residue in the B3 strand (e.g., Ser238) (2, 17). It can be argued that this "prerequisite" is fulfilled in GES enzymes, since position 237 is occupied by Thr. Kinetic and dynamic alterations induced by the Glu104Lys substitution in GES enzymes showed that Lys104, apart from providing an additional binding site, also provoked more complex effects on the interactions with oxyimino-cephalosporins and other β-lactams. In apoenzyme simulations, Lys104 was positioned in parallel with the helix region of the Ω loop interacting with Pro167 and, in this way, could affect the behavior of important residues such as residue 170 and Glu166 (Fig. 2).

Concluding remarks. The present data suggest that naturally occurring mutations at positions 104 and 170 induce complex effects on the substrate spectra of GES β-lactamases. Changing Glu104 to Lys improves extended-spectrum properties but lowers efficiency against imipenem, probably by affecting acylation. In contrast, replacement of Gly170 with either Asn or Ser, which are able to form an extra hydrogen bond with Glu166 and catalytic waters, enhances "carbapenemase" activity. Notably, GES variants possessing Asn170, an important catalytic residue for class A β-lactamases, exhibit diminished activity against penicillins and older cephalosporins, while the less bulky Ser170 residue restores efficiency against these substrates. We cannot provide a plausible explanation for the role of residue 170 in hydrolysis of penicillins and older cephalosporins.

Molecular dynamic simulations of GES apoenzymes allow, at least in some cases, a better understanding of the observed catalytic properties. Increased stability of the hydrogen bonding network of Glu166-Lys73-Ser70 seems to facilitate hydrolysis of imipenem. Yet elucidation of the potential roles of protein motions and the loop containing residues 96 to 105 in carbapenemase activity requires further work. Comparisons of covalent complexes of cefoxitin with the GES variants indicated that the activity of Ser170-containing enzymes against this substrate may be associated with different positioning of the 7α-methoxy group. Regarding interaction of the Lys104-possessing variants with oxyimino-β-lactams, an improved affinity could partly explain the increased catalytic efficiency.

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