SUPPLEMENTAL DATA

Experimental Procedures

Cloning of the Genes for Expression of GES Enzymes—The template (pET:GES-1) was a plasmid containing a synthetic gene for GES-1, cloned between the NdeI and HindIII sites of the plasmid pET24a(+) (Novagen), which has been described (1). GES-2 is a variant of GES-1 with a G170N substitution and was generated using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer's instructions. The following oligonucleotide primers, oHF003 (5' CGTAAAGAACCGGAAATGAACGACAACACGCCAGG 3'; codon for Asn underlined) and oHF004 (5' CCTGGCGTGTTGTCGTTCATTTCCGGTTCTTTACG 3') were used with pET:GES-1 as the template. Similarly, GES-5 is a variant of GES-1 with a G170S substitution and was generated with the oligonuclotide primers oHF001 (5' CGTAAAGAACCGGAAATGAGCGACAACACGCCAGG 3'; codon for Ser underlined) and oHF002 (5' CCTGGCGTGTTGTCGCTCATTTCCGGTTCTTTACG 3'), again using pET:GES-1 as the template. The sequences for the desired genes in the resultant plasmids pET:GES-2 (GES-2) and pET:GES-5 (GES-5) were verified by DNA sequencing.

Cloning into a Constitutive Expression Vector for Minimal Inhibitory Concentration (MIC) Determinations—A vector for evaluating the MIC of different enzymes under an identical promoter was constructed. This vector, pHF016, contains the pBR origin of replication, a kanamycin antibiotic selection marker, and a constitutive expression promoter. The pBR origin of replication was PCR-amplified from pET24a(+) using the oligonucleotide primers oHF014 (5' GCGGATATCCCCGTAGAAAAGATCAAAGG 3'; EcoRV site underlined) and oHF016 (5' GTATCTGCAGAGCGCTGGCATTGACCCTG 3'; PstI site underlined). The gene for the kanamycin antibiotic selection marker was PCR-amplified from pET24a(+) using the primers oHF017 (5' GTTTCTGCAGTCAGGTGGCACTTTTCG 3'; PstI site underlined) and oHF018 (5' GGGAGATATCTCATGAACAATAAAACTGTC 3'; EcoRV site underlined). These two fragments were digested with EcoRV and PstI and ligated together to generate the plasmid pHF007. A DNA fragment containing two PstI sites, at both the 5' and 3' ends, and encoding the constitutive promoter of the D-amino acid aminotransferase gene of *Geobacillus toebii* (2), a multicloning site, and the termination signal from the T2 element of the *Escherichia coli* rrnB gene was custom synthesized (Celtek Genes). This synthetic DNA fragment was digested with EcoRV and recloned into the unique EcoRV site of pHF007, resulting in the plasmid pHF016. The sequence of the entire vector was verified by DNA sequencing.

The plasmids pET:GES-1, pET:GES-2, and pET:GES-5 (described above) were digested with NdeI and HindIII to liberate the GES-1, -2, and -5 genes, respectively, and cloned into the unique NdeI and HindIII restriction sties of pHF016 to generate the plasmids pHF:GES-1, pHF:GES-2, and pHF:GES-5. These plasmids were subsequently used in determining the MIC for each enzyme.

Expression and Purification of the GES Enzymes—To express GES-1, *E. coli* BL21 (DE3) was transformed with pET:GES-1 and cells containing the construct selected on LB agar supplemented with 60 μg/mL kanamycin. Selected cells were grown in M9 minimal medium supplemented with 60 μg/mL kanamycin at 37 °C, 220 rpm until the OD_{600} = 0.4. Isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM and the cells were further grown at 25°C, 220 rpm for 24 h. The cells were pelleted by centrifugation at 20000 × *g*, 4 °C and the media were concentrated by centrifugal filtration at 3000 × *g*, 4 °C using a Centricon Plus 70 (Millipore) concentrator with a 10 kDa molecular weight cut off filter. The concentrated medium was then dialyzed against Buffer A (20 mM Tris, pH 7.5) and fractionated on a DEAE (BioRad) column (2.5 x 22 cm) using a linear gradient of NaCl (0 – 0.3 M) in Buffer A. The fractions

containing GES-1 were pooled and dialyzed against 20 mM HEPES (pH 7.6) containing 25% glycerol and stored at -80 °C. The enzyme concentration was determined spectrophotometrically using the BCA (bicinchoninic acid) protein assay kit (Pierce), using BSA as a standard. SDS-PAGE showed the enzyme purity to be greater than 95%. GES-2 and -5 were expressed and purified in the same manner, using the plasmids pET:GES-2 and pET:GES-5, respectively.

Synthesis of the Product of Imipenem Hydrolysis—A solution of imipenem (3 mg) in 0.05 M aqueous H_2SO_4 (3.0 mL) was stirred at room temperature for 1 h. The acidic solution was brought to pH 7.0 by the gradual addition of solid barium hydroxide. The resultant precipitate of barium sulfate was removed by filtration. The product was subsequently purified by C_{18} silica gel column using a gradient solvent system (water/AcCN=100/0 to 95/5 to 90/10). The product was an equal mixture of rapidly interconverting Δ^1- and Δ^2-tautomers, as determined from the ^{1}H-NMR spectrum. The spectroscopic characteristics of the product mixture were identical to those described by Ratcliffe *et al* (3).

Molecular Dynamics Simulation of the GES-1 Enzyme—The initial geometry of the enzyme was extracted from the crystal structure (PDB ID: 2QPN) using Sybyl8.0 (21). The program xleap in Amber 9 (22) was used to integrate all atomic parameters to the topology file, and geometrical parameters to the coordinate file. A total of 10,402 TIP3P water molecules were added, surrounding the enzyme, which formed an 86.1 Å × 75.3 Å × 68.6 Å box. The entire complex was initially energy minimized in a total of 5,000 steps followed by the equilibration, by methodology reported earlier (23). After the equilibration, samplings were made in molecular dynamics simulation at 300 K for 6 ns. A total of 4,000 conformations were sampled. The 400 conformations in the last 0.1 ns were averaged and subsequently optimized upon 50,000-step energy minimization, by the methods that have been published (24).

Tables

TABLE 1
Kinetic Parameters for Hydrolysis of Nitrocefin by GES-1, -2 and -5
These parameters were used in determination of the dissociation and deacylation rate constants.

Parameter	GES-1	GES-2	GES-5
k_{cat} (s^{-1})	213 ± 12	7.2 ± 0.4	282 ± 13
K_m (μM)	158 ± 20	5 ± 1	117 ± 15

TABLE 2
MICs of selected β-lactam antibiotics for *E. coli* JM83 producing various GES enzymes
All MIC data is reported in μg/mL

Antibiotic	Control[a]	GES-1	GES-2	GES-5
benzylpenicillin	16	2048	1024	2048
amoxicillin	4	> 2048	2048	> 2048
ticarcillin	4	1024	2048	512
piperacillin	2	256	128	512
oxacillin	128	4096	1024	1024
cephalothin	8	1024	512	2048
cefuroxime	4	256	1024	32
ceftazidime	0.25	128	32	8
cefotaxime	0.063	8	16	0.5
ceftriaxone	0.063	16	32	4
cefepime	0.016	1	1	0.125
cefoxitin	2	4	8	128
cefmetazole	1	4	2	32
moxalactam	0.125	1	1	2
aztreonam	0.063	1	2	0.125

[a]Parental *E. coli* JM83 strain harboring the vector pHF016 containing no β-lactamase gene

Figures

Figure 1

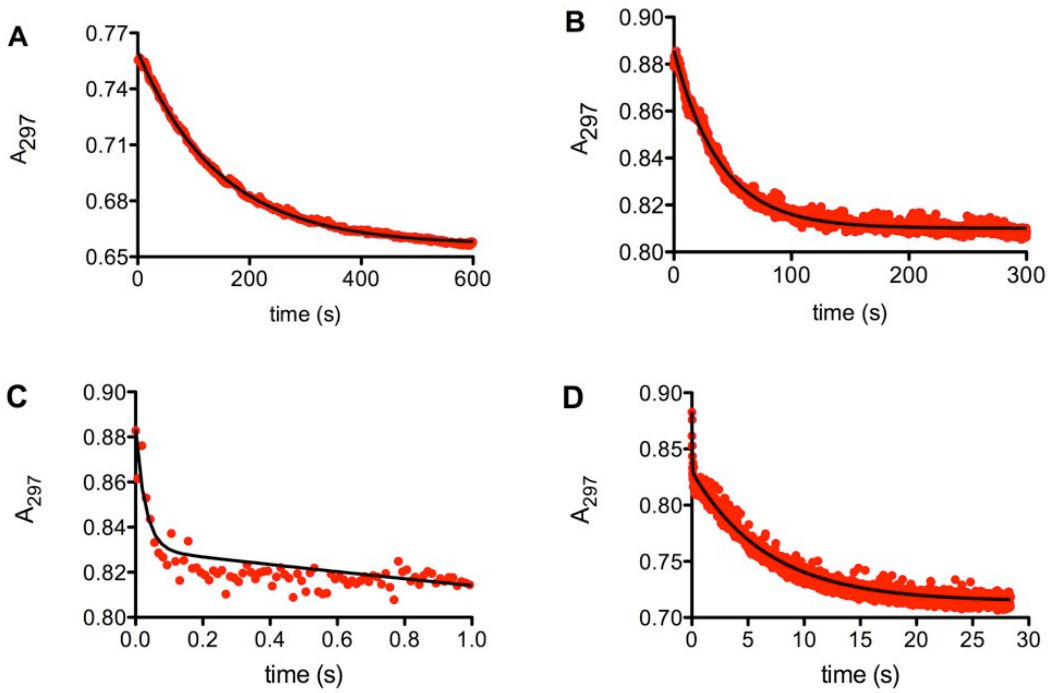

FIGURE 1. **The hydrolysis of imipenem by GES-1, -2, and -5 under single-turnover conditions.** The time courses for acylation of GES-1 (A), GES-2 (B), and the rapid (C) and slow (D) phases of the acylation event for GES-5 are shown under single-turnover conditions for imipenem, with the lines representing the best fit for Equation 2 (A and B) or 3 (C and D). Reactions containing 15 μM imipenem were initiated by the addition of the GES enzyme (to a final concentration of 75 μM) and the acylation event monitored at 297 nm.

Figure 2

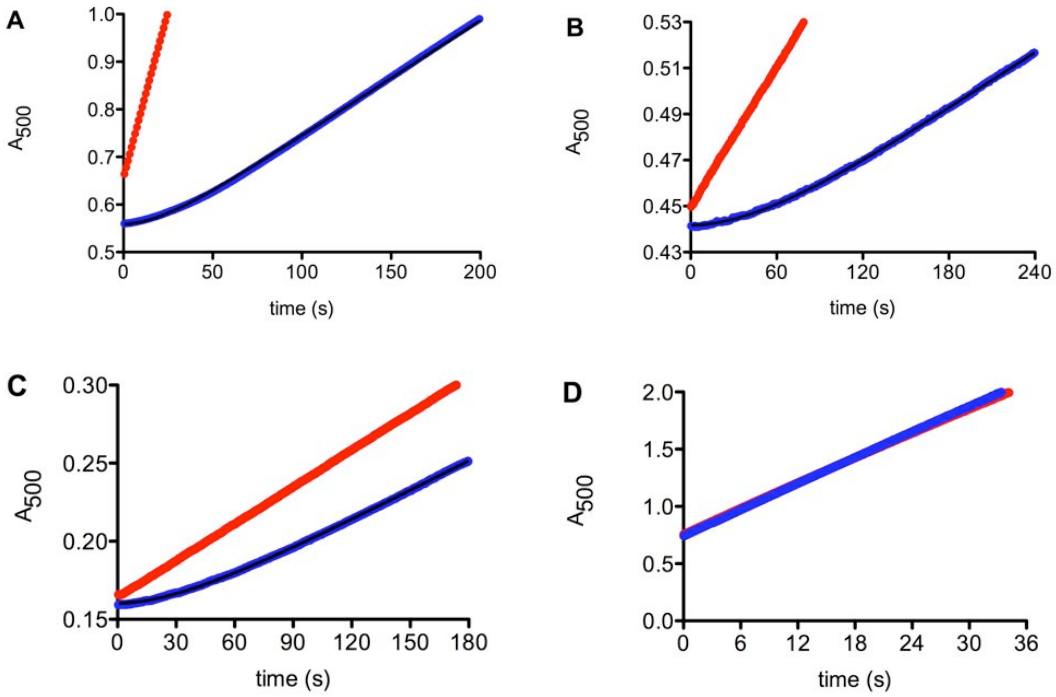

FIGURE 2. **The recovery of GES-1, -2, and -5 activity after incubation with imipenem.** Representative time courses for the recovery of activity when a given enzyme was pre-incubated for 2 min in the presence (blue) or in the absence (red) of a saturating concentration of imipenem (10 x K_m), followed by 1:100 dilution (panel A with GES-1, panel C with GES-2, and panel D with GES-5) or 1:1000 (panel B with GES-1) into an excess of nitrocefin (800 μM in the case of GES-1, 50 μM in the case of GES-2, or 600 μM in the case of GES-5). The gradual first-order recovery of activity and the attendant increase in the steady-state hydrolysis of nitrocefin by GES-1 (panels A and B) and GES-2 (panel C) was monitored at 500 nm. Due to high affinity of GES-1 for imipenem (K_s = 10 ± 2 nM), full recovery of enzymatic activity could not be observed with either a 100- or 1000-fold dilution as the final concentration of imipenem still exceeded the value for K_s. The absence of the recovery phase is documented for GES-5 (panel D). The experimental time courses for GES-1 (panels A and B) and GES-2 (panel C) are superimposed with a black line representing the best fit of the data for Equation 4.

References

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2. Poo, H., Song, J. J., Hong, S. P., Choi, Y. H., Yun, S. W., Kim, J. H., Lee, S. C., Lee, S. G., and Sung, M. H. (2002) *Biotechnol. Lett.* **24**, 1185-1189
3. Ratcliffe, R. W., Wildonger, K. J., Dimichele, L., Douglas, A. W., Hajdu, R., Goegelman, R. T., Springer, J. P., and Hirshfield, J. (1989) *J. Org. Chem.* **54**, 653-660