GES-18, a New Carbapenem-Hydrolyzing GES-Type β-Lactamase from *Pseudomonas aeruginosa* That Contains Ile80 and Ser170 Residues

Carine Bebrone,[a] Pierre Bogaerts,[b] Heinrich Delbrück,[a] Sandra Bennink,[a] Michaël B. Kupper,[a] Roberta Rezende de Castro,[b] Youri Glupczynski,[b] Kurt M. Hoffmann[a]

Institute of Molecular Biotechnology, RWTH-Aachen University, c/o Fraunhofer IME, Aachen, Germany[a]; Reference Laboratory for Monitoring of Antimicrobial Resistance in Gram-Negative Bacteria, Centre Hospitalier Universitaire de Mont-Godinne, Université Catholique de Louvain, Yvoir, Belgium[b]

A clinical isolate of *Pseudomonas aeruginosa* recovered from the lower respiratory tract of an 81-year-old patient hospitalized in Belgium was sent to the national reference center to determine its resistance mechanism. PCR sequencing identified a new GES variant, GES-18, which differs from the carbapenem-hydrolyzing enzyme GES-5 by a single amino acid substitution (Val80Ile, in the numbering according to Ambler) and from GES-1 by two substitutions (Val80Ile and Gly170Ser). Detailed kinetic characterization showed that GES-18 and GES-5 hydrolyze imipenem and cefoxitin with similar kinetic parameters and that GES-18 was less susceptible than GES-1 to classical β-lactamase inhibitors such as clavulanate and tazobactam. The overall structure of GES-18 is similar to the solved structures of GES-1 and GES-2, the Val80Ile and Gly170Ser substitutions causing only subtle local rearrangements. Notably, the hydrolytic water molecule and the Glu166 residue were slightly displaced compared to their counterparts in GES-1. Our kinetic and crystallographic data for GES-18 highlight the pivotal role of the Gly170Ser substitution which distinguishes GES-5 and GES-18 from GES-1.

GES-type extended-spectrum β-lactamases (ESBLs) have been discovered in several Gram-negative bacteria, including *Enterobacteriaceae* and nonfermenters (1–4). *Pseudomonas aeruginosa* isolates that express GES-type β-lactamases have been reported from South America (5, 6), South Africa (7), and China (8). The nosocomial spread of isolates expressing GES-1 and GES-5 in Spain was reported in 2009 (9), and a new GES-13 variant was recently found in Greece (10).

GES-type β-lactamases form a distinct branch of the Ambler class A enzymes, but their origin is unknown. The first GES β-lactamase, GES-1, was reported in a *Klebsiella pneumoniae* isolate from France in 2000 and was found to be encoded by a plasmid-borne integron (11). Twenty-two GES variants have been discovered thus far, differing from each other by 1 to 3 amino acid substitutions (http://lahey.org/studies/other.asp) that affect substrate specificity. The GES-1 β-lactamase exhibits extended-spectrum characteristics, hydrolyzing oximinocephalosporins. However, unlike most ESBLs, GES-1 does not hydrolyze aztreonam. Moreover, although containing the disulfide bond characteristic of class A carbapenemases (Cys69-Cys238, using the standard numbering for class A β-lactamases [12]), GES-1 lacks the ability to significantly hydrolyze carbapenems and is even inhibited by low concentrations of imipenem due to its very strong affinity for this substrate (11, 13). Carbapenemase activity has been demonstrated in certain variants with a substitution (Asn or Ser) at the Gly170 residue, including GES-2, GES-4, GES-5, GES-6, and GES-14 (1, 2, 14–17). The residue at position 170 is located in the Ω-loop, which forms the bottom of the active site, and Asn is found at this position in most class A β-lactamases. In contrast to most class A β-lactamases, GES-4, GES-5, GES-6, and GES-14 also hydrolyze cefoxitin (1, 2, 14, 16, 17).

The *bla*GES-5 gene was detected for the first time in 2004 in an *Escherichia coli* isolate (*E. coli* 365-02) from a patient in Greece as part of a gene cassette located on a class 1 integron carried by a plasmid (16). The *bla*GES-5 gene was subsequently found to be carried by *K. pneumoniae*, *Enterobacter cloacae*, and *P. aeruginosa* in many parts of the world (4).

Here we report the detection of a new variant (GES-18), in a clinical isolate of *P. aeruginosa* collected from a patient hospitalized in Belgium in 2010. The *bla*GES-18 gene was cloned in *E. coli*, and we characterized the kinetic properties and crystallographic structure of the purified GES-18 protein. This variant differs from GES-5 by a single substitution (Val80Ile) and from GES-1 by two substitutions (Val80Ile and Gly170Ser).

MATERIALS AND METHODS

Antibiotics. Imipenem was obtained from Merck Sharp and Dohme Research Laboratories (Rahway, NJ), nitrocefin was obtained from ProGenosis (Liège, Belgium), and temocillin (Negaban) was obtained from Eumedica Pharmaceuticals (Brussels, Belgium); all other antibiotics and inhibitors were from Sigma-Aldrich (St. Louis, MO).

Bacterial strains and antimicrobial susceptibility. *P. aeruginosa* PA11122 is a *bla*GES-positive isolate identified during a screen for acquired carbapenemase resistance genes in multidrug-resistant *P. aeruginosa* and *Acinetobacter baumannii* isolates collected in 2010 in the setting of a national resistance survey. Bacterial species identification was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) with a Microflex LT mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany). *P. aeruginosa* reference strain PAO1 (18), *P. aeruginosa* PU21 (19), and *E. coli* TOP10 (Invitrogen, Merelbeke, Belgium) and BL21 were used for plasmid transfer and/or cloning experiments. Antimicrobial susceptibility was determined by the disc diffusion method and by Etest (bioMérieux, Brussels, Belgium) on Mueller-Hinton

(MH) agar plates (Bio-Rad, Nazareth, Belgium) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (20) in the presence of 2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) when testing pET28a/GES clones. Meropenem-dipicolinic acid (DPA) discs (Neo-Sensitabs KPC-MBL confirmation kit; Rosco Diagnostica, Taastrup, Denmark) were used to screen for metallo-β-lactamase production.

PCR screening, characterization of the genetic environment of bla_{GES-18}, and plasmid extraction. Detection of carbapenemases (bla_{VIM}, bla_{IMP}, bla_{NDM-1}, bla_{KPC}) was performed by DNA microarray (Check-MDR CT102; Check-Points, Wageningen, Netherlands) according to a previously described protocol (21). Genes encoding class A ESBL enzymes (BEL, GES, VEB, and PER) were sought by PCR as previously described (1). The genetic context of bla_{GES-18} was determined by PCR sequencing of the integron variable region with primers matching the 5′ and 3′ conserved segments of class 1 integrons (22) using an external sequencing service (Macrogen, Seoul, South Korea) and a primer walking strategy. Nucleotide sequences were analyzed with the NCBI BLASTN algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Plasmid DNA was extracted using the Kieser method (23) and the QIAfilter plasmid midikit (Qiagen, Venlo, Netherlands) according to the manufacturer′s instructions.

Cloning, expression, and purification of GES-1, GES-5, and GES-18 β-lactamases. The genes encoding GES-1, GES-5, and GES-18 were cloned into pET28a using the NcoI and EcoRI restriction sites. The different vectors were then introduced into the *E. coli* BL21(DE3) strain (New England BioLabs, Ipswich, MA). Overexpression and purification of these three proteins were performed as described for the GES-11, GES-12, and GES-14 enzymes (24).

Determination of kinetic parameters. All experiments were carried out at 30°C in 100 mM sodium phosphate buffer (pH 7.0). Kinetic measurements of purified GES β-lactamases were performed as described previously (24). Low and very high K_m values were determined as K_i values using 100 μM 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 (25). Despite its low value, the K_m parameter for imipenem was determined by initial rates method [since, according to the work of Frase et al., K_m is $> K_{s(i)}$ (26)].

Fifty percent inhibitory concentrations (IC$_{50}$s) were determined using 100 μM benzylpenicillin as the substrate and clavulanic acid and tazobactam as inhibitors, as previously described (11, 15, 17).

GES-18 crystallization. The GES-18 β-lactamase (13 mg/ml) was crystallized at 21°C from a 20 mM citric acid–80 mM Bis-Tris propane (pH 8.8)–10% (wt/vol) polyethylene glycol (PEG) 3350 solution, using the sitting drop method with crystallization plates from Taorad GmbH (Aachen, Germany). The reservoir solution (1 μl) was mixed with the protein solution (1 μl), and the mixture was left to equilibrate against the solution reservoir. Typically, prismatic GES-18 crystals grew within a few days to dimensions of 80 by 80 by 250 μm and represented space group P2$_1$2$_1$2$_1$ (a = 74.184 Å, b = 80.506 Å, and c = 87.748 Å).

Data collection and processing. For data collection, crystals were gradually transferred to a cryoprotectant solution comprising reservoir solution supplemented with 30% (vol/vol) glycerol. The mounted crystals were flash-frozen at 100 K 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 (27) and scaled with SCALA from the CCP4 suite (28).

Structure determination and refinement. The structure of GES-18 was solved by molecular replacement using Phaser (29) and the structure of GES-1 (PDB file 2QPN) as a search model. The resulting model was rebuilt using ARP/wARP (30). The structures were iteratively refined by manual inspection of the electron density with Coot (31) and refinement with Refmac5 (32). Refinement to convergence was carried out with isotropic B values and using TLS parameters (33). Alternative conformations were modeled for a number of side chains, and occupancies were adjusted to yield similar B values for the alternative conformations.

TABLE 1 MICs of antibiotics for *P. aeruginosa* strain PA11122 expressing GES-18 and for *E. coli* BL21 carrying plasmid pET28a-GES-18, -GES-5, or -GES-1^a

Antibiotic	MIC (μg/ml)				
	P. aeruginosa PA11122	*E. coli* carrying plasmid:			
		pET28a	pET28a-GES-1	pET28a-GES-5	pET28a-GES-18
Ampicillin	ND	1.5	>256	>256	>256
Amoxicillin-clavulanic acid	ND	1.5	32	64	128
Ticarcillin-clavulanic acid	>256	ND	ND	ND	ND
Piperacillin	>256	0.75	>256	>256	>256
Piperacillin-tazobactam	>256	0.75	1.5	32	32
Cefuroxime	ND	0.064	128	8	16
Ceftazidime	32	0.064	16	1	1.5
Cefotaxime	ND	0.064	4	0.38	0.38
Cefepime	96	0.064	0.5	0.094	0.094
Aztreonam	8	<0.016	0.25	0.023	0.047
Imipenem	>32	0.25	0.25	0.75	0.75
Meropenem	>32	0.032	0.047	0.125	0.125
Cefoxitin	ND	0.125	0.25	8	8

^{a} MICs were determined by Etest. ND, not determined.

Accession numbers. The nucleotide sequence data in this article have been deposited in the EMBL/GenBank nucleotide sequence database under accession number JQ028729. Coordinates and structure factors have been deposited in the Protein Data Bank (PDB) under accession number 3V3S.

RESULTS

Clinical isolate and resistance traits. *P. aeruginosa* PA11122 was isolated from an endotracheal aspirate obtained from an 81-year-old patient hospitalized in Antwerp, Belgium. This patient developed a ventilator-associated pneumonia while hospitalized in the intensive care unit. Isolate PA11122 was resistant to most β-lactams, including imipenem and meropenem (Table 1), and it was also resistant to aminoglycosides (gentamicin, tobramycin, and amikacin) and to ciprofloxacin. Phenotypic screening tests for the presence of acquired extended-spectrum β-lactamases and of carbapenemases failed to demonstrate any synergistic effect in the presence of different inhibitors, including clavulanic acid and dipicolinic acid (DPA) or EDTA. PCR screening was negative for metallo-β-lactamases as well as for class D oxacillin-hydrolyzing β-lactamases, but it yielded positive results for bla_{GES} ESBL only. PCR sequencing confirmed that bla_{GES} was a new allele, with an 864-bp open reading frame encoding a 287-amino-acid preprotein (including signal peptide) designated GES-18. This variant differed from GES-5 by a single substitution (Val80Ile) and from GES-1 by two substitutions (Val80Ile and Gly170Ser).

Genetic context. The bla_{GES-18} gene was shown to be part of a cassette inserted between two copies of *aacA4* located upstream of bla_{PSE-1} and *aadA2* on a new class 1 integron, assigned number In762 in the integron database INTEGRALL (34). More than 4,550 bp of the integron was sequenced as shown schematically in Fig. 1 (accession number JQ028729).

Repeated plasmid extractions failed to visualize any plasmid by agarose gel electrophoresis. The nucleic acid extracts were nevertheless used for electroporation, but no transformants of the wild-type *P. aeruginosa* strain PAO1 were obtained, suggesting that
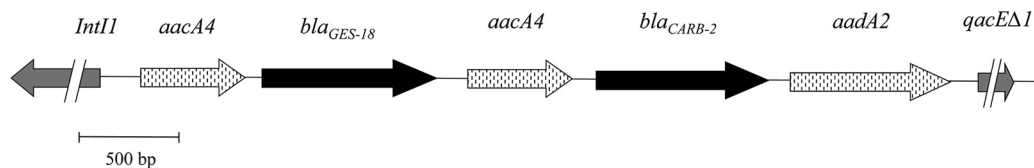
FIG 1 Schematic representation of In762 (accession number JQ028729) containing the *bla*$_{GES-18}$ gene. Double slashes indicate the limit of sequencing, including a partial sequence of the integrase gene *intI1* (5′-CS) and *qacEΔ1* (3′-CS). Arrows indicate the direction of transcription of the coding regions.

bla$_{GES-18}$ was possibly located on the chromosome in this isolate. Mating-out experiments between the clinical strain PA11122 as the donor and the rifampin-resistant *P. aeruginosa* strain PU21 as the recipient also failed to yield transconjugants.

Cloning of GES-18. Compared to *E. coli* BL21 clone pET28a/GES-1, *E. coli* BL21 pET28a/GES-18 was more susceptible to oxyiminocephalosporins but more resistant to cefoxitin and to meropenem as well as to penicillin–β-lactamase inhibitor combinations such as amoxicillin-clavulanic acid and piperacillin-tazobactam (Table 1).

Production and purification. After production of the GES-1, GES-5, and GES-18 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 masses of the different proteins were verified by electrospray ionization-mass spectrometry (ESI-MS). Within experimental errors, the variants were found to exhibit the expected masses (29,216 versus 29,217 Da for GES-1, 29,256 versus 29,257 Da for GES-5, and 29,260 versus 29,261 for GES-18).

Comparison of hydrolytic activities. We compared the activities of GES-18 (Val80Ile, Gly170Ser), GES-5 (Gly170Ser), and GES-1 against compounds representing the four major families of β-lactam antibiotics, namely, penicillins, cephalosporins, carbapenems, and monobactams (Table 2).

The kinetic parameters of GES-18 and of GES-5 were similar for several substrates (e.g., ticarcillin, temocillin, cefotaxime, ceftazidime, aztreonam, cefoxitin, and carbapenems), indicating the conservation of many enzymatic properties (Table 2). However, the k_{cat} values of GES-18 were about half of those of GES-5 for

some penicillins (amoxicillin and piperacillin) and cephalosporins (cephalothin, nitrocefin, and cephaloridine). On the other hand, the K_m values of GES-5 and GES-18 were almost in the same range for all β-lactam agents tested.

The carbapenemase activity of GES-18 was similar to that of GES-5 but higher than that of GES-1. The Gly170Ser mutation in GES-18 led to approximately 22-, 5-, and 6-fold increases in activity (k_{cat}/K_m) against imipenem, ertapenem, and meropenem, respectively (30-, 8-, and 8.5-fold increases for GES-5). This predominantly reflected the substantial increase in k_{cat} values (approximately 60-, 20-, and 60-fold higher for GES-18 and approximately 100-, 30-, and 86-fold higher for GES-5, against imipenem, ertapenem, and meropenem, respectively) as well as the moderate increase in K_m values (approximately 2-, 3-, and 10-fold higher for GES-18 and approximately 3-, 3.7-, and 10-fold higher for GES-5, respectively).

GES-18 and GES-5 were also active against cefoxitin (Table 2). The k_{cat} values were approximately 42- and 56-fold higher than GES-1, but the K_m values also increased (approximately 17- and 14-fold, respectively), resulting in k_{cat}/K_m ratios only 2.5- and 4-fold higher, respectively.

GES-18 and GES-5 had relatively poor efficiencies against ceftazidime (the k_{cat}/K_m ratios were 22- and 19-fold lower than that of GES-1). The kinetic parameters for cefotaxime hydrolysis indicated that GES-1 was the most efficient enzyme against this substrate (Table 2). Aztreonam was also a poor substrate for GES-5 and GES-18, with very low k_{cat} values and relatively high K_m values (Table 2).

Comparison of susceptibilities to inhibitors. We tested two

TABLE 2 Kinetic parameters for GES-18, GES-5, and GES-1 β-lactamases[a]

Antibiotic	GES-18			GES-5			GES-1		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Benzylpenicillin	70	400	175	110	430	255	30	120	250
Amoxicillin	7	40	175	13	50	260	13	180	72
Ticarcillin	5	1,000	5	5	1,600	3	7	1,000	7
Piperacillin	75	700	100	160	1,000	160	8	900	9
Temocillin	0.004	3,500	0.001	0.003	4,500	0.0007	0.002	4,000	0.0005
Cephalothin	500	6,000	80	900	9,000	100	900	5,300	170
Cephaloridine	360	4,600	70	725	5,000	150	540	4,600	120
Nitrocefin	100	330	300	260	550	470	380	340	1100
Cefoxitin	3.4	1,000	3	4.5	860	5	0.08	60	1.3
Cefotaxime	>20	>20,000	1	>28	>20,000	1.4	370	9,000	40
Ceftazidime	>2.5	>20,000	0.125	>3	>20,000	0.15	>56	>20,000	2.8
Cefepime	>1	>20,000	0.05	>2	>20,000	0.1	>16	>20,000	0.8
Aztreonam	0.08	3,000	0.02	0.06	1,800	0.03	4.8	4,000	1.2
Imipenem	0.4	3.5	110	0.7	4.6	150	0.007	1.5	5
Ertapenem	0.06	1.3	40	0.09	1.5	60	0.003	0.4	7.5
Meropenem	0.04	1	40	0.06	1	60	0.0007	0.1	7

[a] Data are the means of three independent experiments. Standard deviations were within 10% of the means (with the exception of imipenem, for which they were 30%).

TABLE 3 X-ray data collection and structure refinement

Parameter	GES-18
Data collection statistics	
Unit cell parameters (Å)	
a	74.184
b	80.506
c	87.748
Space group	P2₁2₁2₁
Molecules/asymmetric unit	2
Resolution range (Å)	25–1.87
No. of observations	336,536/45,819
No. of unique reflections	43,240/5,966
Overall completeness (%)	96.2/90.7
$I/\sigma I$	32.8/5.9
Multiplicity	7.8/4.3
R_{sym}	0.051/0.267
Refinement statistics	
Resolution range (Å)	25–1.9
No. of reflections	41,653
No. of atoms (non-H)	4,741
R_{work}	0.1576
R_{free}	0.1955
RMSD from ideal	
Bond (Å)	0.009
Angle (°)	1.418

commercial β-lactamase inhibitors and found that tazobactam was the most active against all three GES β-lactamase variants. GES-1 was the most susceptible to inhibition by clavulanic acid and tazobactam (the IC₅₀s of GES-1 were 5 and 3 μM, respectively). Higher apparent IC₅₀s for both inhibitors were seen with the Ser170-containing GES-5 and GES-18 variants. GES-18 exhibited IC₅₀s of 25 and 10 μM for clavulanic acid and tazobactam, respectively. For GES-5, these values were 20 and 10 μM, respectively.

Structure determination. To gain insight into the differences in substrate specificity between GES-1 and the GES-18 variant with the Ser170 substitution, the crystal structure of GES-18 was solved by molecular replacement using the structure of GES-1 (PDB code 2QPN) as a model. After refinement, almost all residues lay in the allowed or favored regions in the Ramachandran plot. Stereochemical parameters were calculated using PROCHECK (35) and fell within the range expected for structures with similar resolutions. The crystallographic and model statistics for the structure are summarized in Table 3. Attempts to generate good-quality GES-5 crystals have been unsuccessful so far.

The crystal structure of GES-18 was refined to a resolution of 1.9 Å, yielding an R_{work} value of 0.1576 and an R_{free} value of 0.1955. The crystals belong to the P2₁2₁2₁ space group, with two molecules of GES-18 in the asymmetric unit.

The final atomic resolution featured two independent structural models, model A comprising 269 amino acids (residues Ser24 to Lys295) and model B comprising 268 amino acids (residues Ser24 to Asp294). Furthermore, 440 water molecules were identified in the electron density map. Both models are related by a 2-fold noncrystallographic rotation axis and present an interface of approximately 890 Å² dominated by hydrogen bonds and bridging water molecules. Superimposition of the individually refined models showed that they are essentially identical in structure, with a Cα root mean square deviation (RMSD) of 0.313 Å.

The structures of the GES-18 monomers show the typical class A serine β-lactamase fold comprising two domains, one α/β and the other all α (Fig. 2). The GES-18 dimer is essentially identical to the dimer in the P2₁ asymmetric unit of the GES-1 and GES-2 structures (13, 36). A superposition yields RMSD values of 0.454 Å and 0.336 Å compared to GES-1 and GES-2, respectively.

Residue 80 is located in a hydrophobic core within helix H2, which bears the catalytic Ser70 of the class A signature motif SXXK at its N terminus (Fig. 2). This residue is partially oriented to the protein surface. In GES-18, Val80 is replaced by Ile, which has a larger side chain (Fig. 2). Residues 140 to 145 of the neighboring region are situated at a typical distance for hydrophobic contacts from Ile80 and are marginally displaced in the GES-18 model compared to GES-1. The distance between Ile80:CD and Ile143:CG2 is 3.87 Å (Fig. 2b). The substitution with Ile possibly allows the protein to close a small hydrophobic cavity and thus reduces its hydrophobic surface area, perhaps influencing the stability and also the flexibility of the protein structure. Due to its location on
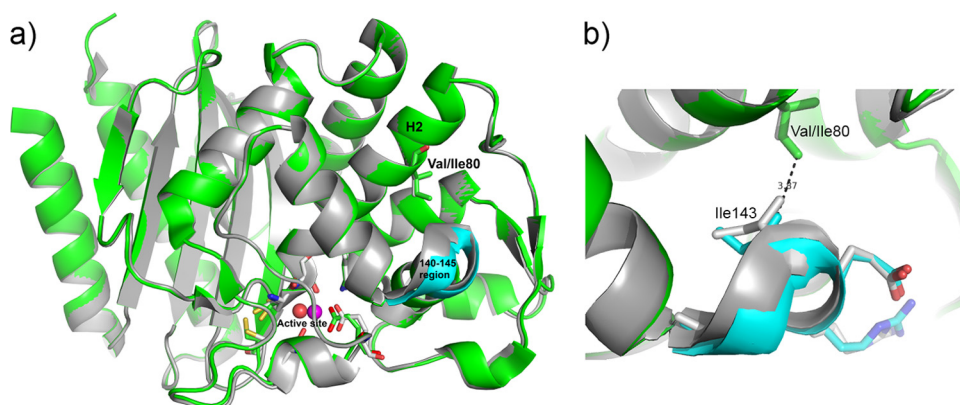

FIG 2 Superimposition of the crystallographic structures of GES-18 (in green) and GES-1 (in gray). (a) The active sites and residues (Val/Ile) at position 80 are represented by sticks. The respective hydrolytic water molecules are represented by spheres (in red for GES-18 and in magenta for GES-1). The 140-to-145 region which is slightly perturbed by the Val80Ile mutation is colored in cyan for GES-18. (b) Residues at position 80, Val or Ile, are represented by sticks (in green for GES-18 and in gray for GES-1). The 140-to-145 region which is slightly perturbed by the Val80Ile mutation is colored in cyan for GES-18. The residues 140 to 145 are also represented by sticks (cyan for GES-18 and gray for GES-1).
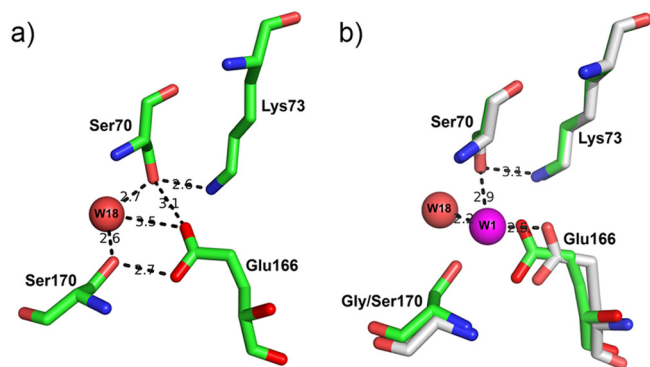
a)

b)

FIG 3 (a) Representation of the active site of GES-18. The network of hydrogen bonds is represented. (b) Superimposition of the respective active sites of GES-1 (in gray) and GES-18 (in green). The hydrolytic water molecule is represented in magenta for GES-1 (W1) and in red for GES-18 (W18). W18 is shifted by 2.15 Å compared to its position in GES-1. The hydrogen bond distances between W1 and Ser70, Lys73, and Glu166 are also represented.

the catalytically important SXXK-bearing helix H2, the mutation might indirectly influence the catalytic behavior.

Hydrogen bonding between Glu166, Lys73, and Ser70 is crucial in the activation of the Ser70 hydroxyl group for the nucleophilic attack of the β-lactam ring (acylation) and that of the hydrolytic water for the hydrolysis of the acyl enzyme intermediate (deacylation) (37–40). In GES-18, the presence of the Ser170 residue modifies the network of hydrogen bonds in the active site. In particular, the side chain of the Ser170 residue can form a hydrogen bond with the hydrolytic water molecule and may therefore stabilize the water molecule in the active site (Fig. 3a). In GES-2, the hydrolytic water molecule is also hydrogen bonded to the side chain of Asn170 (PDB code 3NI9) (36). Moreover, in GES-18, the hydrolytic water molecule is shifted by 2.15 Å compared to its position in GES-1 (Fig. 3b) and by 2.67 Å compared to its position in GES-2. The Ser170 side chain therefore potentially stabilizes the network of three catalytic residues (Ser70, Lys73, and Glu166) by forming an additional hydrogen bond with the carboxylate group of Glu166 (Fig. 3a). Furthermore, the distance between Ser70 and Glu166 is shorter in GES-18 (3.06 Å) than in GES-1 (3.96 Å) and in GES-2 (4.38 Å). Consequently, the hydroxyl moiety of Ser70 can form a direct hydrogen bond with the Glu166 carboxyl group (Fig. 3a), an interaction that could enhance the nucleophilicity of the Ser70 hydroxyl group for the initial attack on the carbonyl C atom of the β-lactam ring.

DISCUSSION

We have characterized GES-18, a novel carbapenem-hydrolyzing GES-type β-lactamase from *P. aeruginosa*. GES-18 differs from GES-5 by a single Val80Ile substitution located on helix H2 in a hydrophobic core distant from the catalytic center. The Val80 residue in CTX-M-1 is known to be involved in cefotaxime hydrolysis, and a Val80Ala substitution may affect the dynamics and flexibility of this enzyme (41). We have shown that the Val80Ile substitution in GES-18 does not significantly modify the substrate profile in comparison to GES-5, but the k_{cat} value was modified for some substrates.

As previously shown for GES-5 (39), the presence of Ser170 increased the carbapenemase and cephamycinase activities of the enzyme compared to those of GES-1. Cefoxitin is a poor substrate for most class A β-lactamases, and cephamycinase activity is typically associated with class C β-lactamases (42). The ability of GES variants

containing Ser170 to use cefoxitin and imipenem as the substrates may be associated with the different positioning of the 7α-methoxy group or the equivalent 6α-hydroxyethyl substituent of the substrate (39). Furthermore, the Gly170Ser substitution markedly reduces the activity of the enzyme against ceftazidime and aztreonam. These antibiotics share the same (C-3/C-7) side chain even though the latter is attached to a different nucleus. In agreement with a recent study (43), we also found that the Gly170Ser substitution affected resistance to β-lactamase inhibitors. From a structural perspective, the Gly170Ser substitution induces subtle local rearrangements in the active site (Fig. 3). Ser70 and Ser170 form hydrogen bonds with Glu166, and the hydrolytic water molecule presents an additional H bond with Ser170, yielding a more rigid structure. The flexibility of the protein backbone is also reduced through the replacement of a glycine by a serine residue. Molecular dynamic simulations of GES apoenzymes have also demonstrated that in GES-5, the Ω loop and the catalytic Ser70, Lys73, and Glu166 residues are less mobile than the corresponding regions of GES-1 (39). Furthermore, our structure demonstrates that the hydrolytic water molecule and Glu166 were displaced compared to their counterparts in GES-1, potentially achieving positioning that is catalytically more favorable for the hydrolysis of carbapenems or cephamycins.

Although the solved structure of the GES-18 apoenzyme provides insight into the differences in substrate specificity induced by the Gly170Ser substitution, it does not explain the strong positive effect on the acylation and deacylation rates for imipenem (e.g., GES-5 acylates imipenem 5,000 times faster than does GES-1 and deacylates this substrate 15 times faster [26, 39]). This challenge can be addressed by solving the crystal structures of enzyme complexes with carbapenem substrates.

In conclusion, the Val80Ile substitution does not significantly alter the substrate profile of the enzyme compared to that of GES-5. The Gly170Ser substitution in GES-18 affects not only cephamycin and carbapenem hydrolysis but also inhibitor resistance. The crystallographic structure of GES-18 is similar to other solved GES structures (GES-1 and GES-2), the Val80Ile and Gly170Ser substitution inducing only minor local rearrangements, but these included the displacements of the hydrolytic water molecule and the catalytically important Glu166 residue, which altogether could provide the structural basis for partially explaining differences in substrate specificity and performance.

ACKNOWLEDGMENTS

REFERENCES

1. Bogaerts P, Naas T, El Garch F, Cuzon G, Deplano A, Delaire T, Huang TD, Lissoir B, Nordmann P, Glupczynski Y. 2010. GES extended-spectrum β-lactamases in *Acinetobacter baumannii* isolates in Belgium. Antimicrob. Agents Chemother. 54:4872–4878.
2. Bonnin RA, Nordmann P, Potron A, Lecuyer H, Zahar JR, Poirel L. 2011.

Carbapenem-hydrolyzing GES-type extended spectrum β-lactamase in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **55**:349–354.

3. **Moubareck C, Brémont S, Conroy MC, Courvalin P, Lambert T.** 2009. GES-11, a novel integron-associated GES variant in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **53**:3579–3581.

4. **Naas T, Poirel L, Nordmann P.** 2008. Minor extended-spectrum β-lactamases. Clin. Microbiol. Infect. **14**:42–52.

5. **Pasteran F, Faccone D, Petroni A, Rapoport M, Galas M, Vázquez M, Procopio A.** 2005. Novel variant (bla(VIM-11)) of the metallo-β-lactamase bla(VIM) family in a GES-1 extended-spectrum-β-lactamase-producing *Pseudomonas aeruginosa* clinical isolate in Argentina. Antimicrob. Agents Chemother. **49**:474–475.

6. **Picão RC, Poirel L, Gales AC, Nordmann P.** 2009. Diversity of β-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolates causing bloodstream infections in Brazil. Antimicrob. Agents Chemother. **53**:3908–3913.

7. **Poirel L, Weldhagen GF, De Champs C, Nordmann P.** 2002. A nosocomial outbreak of *Pseudomonas aeruginosa* isolates expressing the extended-spectrum β-lactamase GES-2 in South Africa. J. Antimicrob. Chemother. **49**:561–565.

8. **Wang J, Zhou JY, Qu TT, Shen P, Wei ZQ, Yu YS, Li LJ.** 2010. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Chinese hospitals. Int. J. Antimicrob. Agents **35**:486–491.

9. **Viedma E, Juan C, Acosta J, Zamorano L, Otero JR, Sanz F, Chaves F, Oliver A.** 2009. Nosocomial spread of colistin-only-sensitive sequence type 235 *Pseudomonas aeruginosa* isolates producing the extended-spectrum β-lactamases GES-1 and GES-5 in Spain. Antimicrob. Agents Chemother. **53**:4930–4933.

10. **Kotsakis SD, Papagiannitsis CC, Tzelepi E, Legakis NJ, Miriagou V, Tzouvelekis LS.** 2010. GES-13, a β-lactamase variant possessing Lys-104 and Asn-170 in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **54**:1331–1333.

11. **Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P.** 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **44**:622–632.

12. **Ambler RP, Coulson AF, Frère JM, Ghuysen JM, Joris B, Forsman M, Levesque RC, Tiraby G, Waley SG.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276**:269–270.

13. **Smith CA, Caccamo M, Kantardjieff KA, Vakulenko S.** 2007. Structure of GES-1 at atomic resolution: insights into the evolution of carbapenemase activity in the class A extended-spectrum β-lactamases. Acta Crystallogr. Sect. D **63**:982–992.

14. **Bae IK, Lee YN, Jeong SH, Hong SG, Lee JH, Lee SH, Kim HJ, Youn H.** 2007. Genetic and biochemical characterization of GES-5, an extended-spectrum class A β-lactamase from *Klebsiella pneumoniae*. Diagn. Microbiol. Infect. Dis. **58**:465–468.

15. **Poirel L, Weldhagen GF, Naas T, De Champs C, Dove MG, Nordmann P.** 2001. GES-2, a class A β-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. Antimicrob. Agents Chemother. **45**:2598–2603.

16. **Vourli S, Giakkoupi P, Miriagou V, Tzelepi E, Vatopoulos AC, Tzouvelekis LS.** 2004. Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. FEMS Microbiol. Lett. **234**:209–213.

17. **Wachino J, Doi Y, Yamane K, Shibata N, Yagi T, Kubota T, Arakawa Y.** 2004. Molecular characterization of a cephamycin-hydrolyzing and inhibitor-resistant class A β-lactamase, GES-4, possessing a single G170S substitution in the omega-loop. Antimicrob. Agents Chemother. **48**:2905–2910.

18. **Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Riezer J, Saier MH, Hancock RE, Lory S, Olson MV.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406**:959–964.

19. **Jacoby GA.** 1974. Properties of R plasmids determining gentamicin resistance by acetylation in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **6**:239–252.

20. **Clinical and Laboratory Standards Institute.** 2012. Performance standards for antimicrobial susceptibility testing. CLSI M100-S22. Clinical and Laboratory Standards Institute, Wayne, PA.

21. **Naas T, Cuzon G, Bogaerts P, Glupczynski Y, Nordmann P.** 2011. Evaluation of a DNA microarray (Check-MDR CT102) for rapid detection of TEM, SHV, and CTX-M extended-spectrum β-lactamases and of KPC, OXA-48, VIM, IMP, and NDM-1 carbapenemases. J. Clin. Microbiol. **49**:1608–1613.

22. **Lévesque C, Piché L, Larose C, Roy PH.** 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. **39**:185–191.

23. **Kieser T.** 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12**:19–36.

24. **Delbrück H, Bogaerts P, Kupper MB, Rezende de Castro R, Bennink S, Glupczynski Y, Galleni M, Hoffmann KM, Bebrone C.** 2012. Kinetic and crystallographic studies of extended spectrum GES-11, GES-12 and GES-14 β-lactamases. Antimicrob. Agents Chemother. **56**:5618–5625.

25. **Cornish-Bowden A.** 2001. Fundamentals of enzyme kinetics. Portland Press Ltd., London, United Kingdom.

26. **Frase H, Shi Q, Testero SA, Mobashery S, Vakulenko SB.** 2009. Mechanistic basis for the emergence of catalytic competence against carbapenem antibiotics by the GES family of β-lactamases. J. Biol. Chem. **284**:29509–29513.

27. **Kabsch W.** 1993. Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. J. Appl. Crystallogr. **26**:795–800.

28. **Collaborative Computational Project Number 4.** 1994. CCP4 suite: programs for protein crystallography. Acta Crystallogr. Sect. D **50**:760–763.

29. **McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ.** 2007. Phaser crystallographic software. J. Appl. Crystallogr. **40**:658–674.

30. **Perrakis A, Morris R, Lamzin VS.** 1999. Automated protein model building combined with iterative structure refinement. Nat. Struct. Biol. **6**:458–463.

31. **Emsley P, Cowtan K.** 2004. Coot: model-building tools for molecular graphics. Acta Crystallogr. Sect. D **60**:2126–2132.

32. **Murshudov GN, Vagin AA, Dodson EJ.** 1997. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. Sect. D **53**:240–255.

33. **Painter J, Merritt EA.** 2006. Optimal description of a protein structure in terms of multiple groups undergoing TLS motion. Acta Crystallogr. Sect. D **62**:439–450.

34. **Moura A, Soares M, Pereira C, Leitao N, Henriques I, Correia A.** 2009. INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. Bioinformatics **25**:1096–1098.

35. **Laskowski RA, MacArthur MW, Moss DS, Thornton JM.** 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. **26**:283–291.

36. **Frase H, Smith CA, Toth M, Champion MM, Mobashery S, Vakulenko SB.** 2011. Identification of products of inhibition of GES-2 β-lactamase by tazobactam by X-ray crystallography and spectrometry. J. Biol. Chem. **286**:14396–14409.

37. **Drawz SM, Bonomo RA.** 2010. Three decades of beta-lactamase inhibitors. Clin. Microbiol. Rev. **23**:160–201.

38. **Golemi-Kotra D, Meroueh SO, Kim C, Vakulenko SB, Bulychev A, Stemmler AJ, Stemmler TL, Mobashery S.** 2004. The importance of a critical protonation state and the fate of the catalytic steps in class A β-lactamases and penicillin-binding proteins. J. Biol. Chem. **279**:34665–34673.

39. **Kotsakis SD, Miriagou V, Tzelepi E, Tzouvelekis LS.** 2010. Comparative biochemical and computational study of the role of naturally occurring mutations at Ambler positions 104 and 170 in GES β-lactamases. Antimicrob. Agents Chemother. **54**:4864–4871.

40. **Meroueh SO, Fisher JF, Schlegel HB, Mobashery S.** 2005. Ab initio QM/MM study of class A β-lactamase acylation: dual participation of Glu166 and Lys73 in a concerted base promotion of Ser70. J. Am. Chem. Soc. **127**:15397–15407.

41. **Pérez-Llarena FJ, Kerff F, Abián O, Mallo S, Fernández MC, Galleni M, Sancho J, Bou G.** 2011. Distant and new mutations in CTX-M-1 β-lactamase affect cefotaxime hydrolysis. Antimicrob. Agents Chemother. **55**:4361–4368.

42. **Philippon A, Arlet G, Jacoby GA.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46**:1–11.

43. **Frase H, Toth M, Champion MM, Antunes NT, Vakulenko SB.** 2011. Importance of position 170 in the inhibition of GES-type β-lactamases by clavulanic acid. Antimicrob. Agents Chemother. **55**:1556–1562.