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GES-13, a β-Lactamase Variant Possessing Lys-104 and Asn-170 in *Pseudomonas aeruginosa*▽

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GES-13 β-lactamase, a novel GES variant possessing Lys-104 and Asn-170, was identified in *Pseudomonas aeruginosa*. bla_{GES-13} was the single gene cassette of a class 1 integron probably located in the chromosome. GES-13 efficiently hydrolyzed broad-spectrum cephalosporins and aztreonam. Imipenem was a potent inhibitor of GES-13 but was not hydrolyzed at measurable rates.

GES-type β-lactamases are a distinct branch of class A enzymes of unknown origin. Ten GES variants (GES-1 to -9 and GES-11) differing by one to three amino acid residues have been described (7). bla_{GES} genes are commonly carried by integrons found in various species, predominantly *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (11). GES β-lactamases all exhibit extended-spectrum properties, hydrolyzing oxyimino-cephalosporins. However, differences in the substrate spectra have been observed. Variants with either Asn (GES-2) or Ser (GES-4, -5, and -6) at Ambler's position 170 are of special clinical interest since they exhibit increased carbapenemase activity compared to the remaining GES enzymes that possess Gly-170 (1, 12, 15, 16). Substitution of Lys for Glu-104 in various GES enzymes, such as GES-3 and GES-7, may enhance hydrolysis of ceftazidime (3, 14, 17). Notably, GES-4, an enzyme possessing Ser-170, Lys-104, and Thr-62, exhibits significant cefoxitin hydrolysis, in contrast to most GES variants that exhibit either weak activity or are virtually inactive against this substrate (16). Also, a Gly-to-Ser or -Ala substitution at position 243 in GES-9 (9) and GES-11 (6), respectively, confers increased activity against aztreonam. In this report, we describe GES-13, a novel GES variant from *P. aeruginosa* possessing both Lys-104 and Asn-170.

P. aeruginosa NL68 was a bla_{GES}-positive isolate identified during a screening for acquired carbapenemase genes in multidrug-resistant microorganisms collected during 2007 and 2008 at Evgenidion General Hospital in Athens, Greece. The isolate was considered responsible for a respiratory tract infection in a surgical patient in October 2008. *P. aeruginosa* NL68 was resistant to penicillins, penicillin-inhibitor combinations, ceftazidime, cefepime, aztreonam, imipenem, and meropenem, as determined by a microdilution technique (Table 1) (2). Disk diffusion tests showed that the isolate was also resistant to fluoroquinolones and aminoglycosides (data not shown).

A bla_{GES}-specific PCR, performed using genomic DNA prepared with the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) and the previously described primers GES-1A and GES-1B (9), produced an 864-bp amplicon that contained the entire bla_{GES} gene. The PCR product was cloned into a pCR2.1-TOPO vector (Invitrogen Life Science, Carlsbad, CA), and its sequence was determined in both strands. The gene studied differed from the prototype bla_{GES-1} gene by 3 nucleotides (nt), a G-to-A transition in codon 104 and a GG-to-AA transition in codon 170 (nt 900 and nt 1101 and 1102, respectively, in bla_{GES-1}; GenBank accession no. EU598463), resulting in a novel GES variant (GES-13) possessing Lys-104 and Asn-170. Isoelectric focusing of cell extracts of *P. aeruginosa* NL68 confirmed the production of an enzyme that was inhibited *in situ* by clavulanate and had an apparent isoelectric point (pI) of 6.9, comparable to that calculated for GES-13 (pI 6.98).

Attempts to transfer bla_{GES-13} by mixed broth and filter mating methods using as recipients *Escherichia coli* 26R793 (15) and a mutant of *P. aeruginosa* PAO1 highly resistant to rifampin and selection with rifampin plus ampicillin or ticarcillin, respectively, were unsuccessful. Analysis of plasmid DNA preparations, derived by either a Nucleobond BAC100 kit (Macherey-Nagel) or an alkaline lysis method (4), failed to identify plasmids in *P. aeruginosa* NL68. Also, electroporation experiments using *E. coli* DH5α competent cells, as well as *P. aeruginosa* PAO1 and plasmid preparations, did not produce β-lactam-resistant clones. A chromosomal origin of bla_{GES-13} is therefore possible. To determine the genetic environment of bla_{GES-13}, a PCR assay using primers INT-F, corresponding to an internal sequence of the IntI1 gene, and 3′CS, corresponding to a 3′ conserved segment of class 1 integrons, was performed as described previously (5). The PCR product was cloned into pBCSK(+) (Stratagene, La Jolla, CA), yielding plasmid pB-ges13. The insert comprised 1,641 bp with a typical class 1 integron structure including bla_{GES-13} as a single-gene cassette, a 59-base element consisting of 108 bases and homologous to that found in the GES-8-encoding integron (5), and a "weak" promoter sequence.

E. coli DH5α harboring pB-ges13 [*E. coli*(pB-ges13)] exhibited resistance to penicillins, older cephalosporins, and ceftazidime. Clavulanic acid and tazobactam partly restored susceptibility to penicillins and ceftazidime. Production of GES-13 also caused a significant increase in the MICs of cefotaxime,

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TABLE 1. Susceptibilities of GES-type β-lactamase-producing bacterial strains to β-lactam antibiotics

β-Lactam	MIC (μg/ml) for:			
	P. aeruginosa NL68 GES-13	*E. coli* DH5α (pB-ges13) GES-13	*E. coli* DH5α (pB-ges1) GES-1	*E. coli* DH5α
Amoxicillin	—[c]	≥512	≥512	2
Amoxicillin-CLA[a]	—	32	64	2
Ticarcillin	≥512	≥512	≥512	2
Ticarcillin-CLA	≥512	16	32	2
Piperacillin	256	64	64	1
Piperacillin-TZB[b]	128	2	16	0.5
Cefaclor	—	≥512	≥512	4
Cefuroxime	—	≥512	≥512	2
Cefoxitin	—	8	8	4
Cefotaxime	—	8	4	0.12
Ceftriaxone	—	16	16	0.12
Ceftazidime	≥512	≥512	256	0.5
Ceftazidime-CLA	≥512	16	16	0.5
Ceftazidime-TZB	256	4	16	0.5
Cefepime	64	0.5	1	≤0.06
Aztreonam	64	8	1	0.12
Imipenem	16	0.25	0.25	0.12
Imipenem-TZB	16	—	—	—
Meropenem	32	≤0.06	≤0.06	≤0.06

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml.
[b] TZB, tazobactam at a fixed concentration of 4 μg/ml.
[c] —, not tested.

ceftriaxone, cefepime, and aztreonam. Susceptibilities to cefoxitin and imipenem were slightly affected (MICs increased by 1 doubling dilution). Meropenem was equally active against *E. coli*(pB-ges13) and *E. coli* DH5α (Table 1).

E. coli(pB-ges13) was used for GES-13 purification. β-Lactamases were released from bacterial cells suspended in Tris buffer (20 mM, pH 8.0) by sonication. Extracts were loaded onto a Q-Sepharose column (Bio-Rad Laboratories, Hercules, CA), and proteins were eluted by a 0 to 1 M NaCl gradient. The fractions displaying β-lactamase activity (as tested by a nitrocefin assay) were pooled, dialyzed overnight against 20 mM morpholineethanesulfonic acid (pH 6.0), and loaded onto an S-Sepharose column (Bio-Rad Laboratories). The bound β-lactamase was eluted with 400 to 500 mM NaCl. The purity of the final preparation was >95%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The hydrolysis of penicillin G, nitrocefin, cephalothin, cefotaxime, ceftazidime, aztreonam, and imipenem was studied by spectrophotometry. Wavelengths and extinction coefficients for β-lactam substrates have been reported elsewhere (10, 13). Kinetic parameters confirmed the extended-spectrum properties of GES-13. The enzyme was able to hydrolyze cefotaxime and ceftazidime at measurable rates, the former substrate being more efficiently hydrolyzed due to higher k_{cat} and lower K_m values. GES-13 also hydrolyzed penicillin G, cephalothin, and nitrocefin with comparable efficiencies. Notably, the enzyme was able to hydrolyze aztreonam at rates similar to that observed for ceftazidime, but the K_m for the former substrate was higher. GES-13 also exhibited a weak activity against imipenem, but the respective hydrolysis rate was too low to be measured accurately (Table 2). Inhibitory activities of clavulanic acid, tazobactam, sulbactam, and imipenem against GES-13 were studied using cephalothin (100 μM) as a reporter substrate. The purified enzyme and various concentrations of inhibitors were preincubated at 30°C for 3 min. Results were expressed as 50% inhibitory concentrations (IC_{50}s). Tazobactam was a more potent inhibitor than clavulanic acid and sulbactam (the IC_{50}s were 0.06, 0.10, and 0.37 μM, respectively). GES-13 was also inhibited by low concentrations of imipenem (IC_{50} = 0.15 μM), reflecting the high affinity of this antibiotic for GES-type enzymes.

TABLE 2. Kinetic parameters of GES-13 for various β-lactam substrates[a]

β-Lactam	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Penicillin G	13.5 ± 1.7	139 ± 18	0.10
Nitrocefin	29.3 ± 1.2	197 ± 11	0.15
Cephalothin	10.1 ± 1.5	106 ± 6.0	0.10
Cefotaxime	69.0 ± 2.9	63 ± 6.0	1.09
Ceftazidime	30.4 ± 4.1	229 ± 26	0.13
Aztreonam	35.6 ± 5.9	405 ± 50	0.09
Imipenem	<0.01	—[b]	—

[a] k_{cat} and K_m values are means ± standard deviations of five independent measurements.
[b] —, not determinable.

To directly compare β-lactam resistance levels conferred by GES-13 and GES-1, a bla_{GES-1} integron similar to the one described here (GenBank accession no. EU598463) (8) was cloned into pBCSK(+), yielding recombinant plasmid pB-ges1, which was used to transform *E. coli* DH5α. Sequencing confirmed that bla_{GES-13}- and bla_{GES-1}-containing inserts differed by only 3 nt at bla_{GES} codons 104 and 170, leading to the respective amino acid changes. GES-13 and GES-1 conferred comparable levels of resistance to the β-lactam antibiotics tested, except for aztreonam, ceftazidime-tazobactam, and piperacillin-tazobactam combinations. In line with the results of the kinetic studies, production of GES-13 resulted in a significant increase in the MIC of aztreonam. *E. coli*(pB-ges13) was more susceptible to piperacillin-tazobactam than *E. coli*(pB-ges1) was, probably indicating a stronger inhibitory activity of tazobactam against GES-13 than GES-1.

P. aeruginosa NL68, apart from GES-13, also produced large amounts of the chromosomal cephalosporinase (data not shown). Operation of additional mechanisms such as decreased outer membrane permeability and efflux pumps cannot be excluded. Therefore, it was not possible to assess the contribution of GES-13 to the β-lactam resistance phenotype of this clinical strain. Nevertheless, the role of GES-13 in carbapenem resistance was probably not significant, as also indicated by the MICs of imipenem and imipenem-tazobactam for *P. aeruginosa* NL68 (Table 1). Indeed, GES-13 exhibited very weak activity against imipenem, despite the presence of Asn-170, which confers carbapenemase properties on GES-2, probably by facilitating the initial enzyme-substrate interaction (12, 14). Apparently, the effect of Lys-104 counters the improved hydrolytic efficiency against imipenem. On the other hand, the Glu-104-Lys mutation may be responsible for the increased hydrolysis of aztreonam by GES-13. The increased aztreonam MICs observed in *E. coli* laboratory strains producing GES-3 and GES-7 that also possess Lys-104 support this hypothesis (3, 17). The properties of GES-13 suggest that the Gly-170-Asn

and Glu-104-Lys mutations induce complex functional changes in GES β-lactamases.

Nucleotide sequence accession number. The nucleotide sequence of the bla_{GES-13}-containing integron has been assigned GenBank accession number GU169702.

REFERENCES

1. **Bae, I. K., Y. N. Lee, S. H. Jeong, S. G. Hong, J. H. Lee, S. H. Lee, H. J. Kim, and H. Youn.** 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.
2. **Clinical and Laboratory Standards Institute.** 2005. Performance standards for antimicrobial susceptibility testing: 15th informational supplement. Approved standard M100-S15. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Giakkoupi, P., L. S. Tzouvelekis, A. Tsakris, V. Loukova, D. Sofianou, and E. Tzelepi.** 2000. IBC-1, a novel integron-associated class A β-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. Antimicrob. Agents Chemother. **44:**2247–2253.
4. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
5. **Mavroidi, A., E. Tzelepi, A. Tsakris, V. Miriagou, D. Sofianou, and L. S. Tzouvelekis.** 2001. An integron-associated β-lactamase (IBC-2) from *Pseudomonas aeruginosa* is a variant of the extended-spectrum β-lactamase IBC-1. J. Antimicrob. Chemother. **48:**627–630.
6. **Moubareck, C., S. Brémont, M.-C. Conroy, P. Courvalin, and T. Lambert.** 2009. GES-11, a novel integron-associated GES variant in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **53:**3579–3581.
7. **Naas, T., L. Poirel, and P. Nordmann.** 2008. Minor extended-spectrum β-lactamases. Clin. Microbiol. Infect. **14**(Suppl. 1)**:**42–52.
8. **Papagiannitsis, C. C., L. S. Tzouvelekis, and V. Miriagou.** 2009. Relative strengths of the class 1 integron promoter hybrid 2 and the combinations of strong and hybrid 1 with an active P2 promoter. Antimicrob. Agents Chemother. **53:**277–280.
9. **Poirel, L., L. Brinas, N. Fortineau, and P. Nordmann.** 2005. Integron-encoded GES-type extended-spectrum β-lactamase with increased activity toward aztreonam in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **49:**3593–3597.
10. **Poirel, L., I. Le Thomas, T. Naas, A. Karim, and P. Nordmann.** 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In*52* from *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **44:**622–632.
11. **Poirel, L., T. Naas, and P. Nordmann.** 2008. Genetic support of extended-spectrum β-lactamases. Clin. Microbiol. Infect. **14**(Suppl. 1)**:**75–81.
12. **Poirel, L., G. F. Weldhagen, T. Naas, C. De Champs, M. G. Dove, and P. Nordmann.** 2001. GES-2, a class A β-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. Antimicrob. Agents Chemother. **45:**2598–2603.
13. **Power, P., M. Galleni, J. A. Ayala, and G. Gutkind.** 2006. Biochemical and molecular characterization of three new variants of AmpC β-lactamases from *Morganella morganii*. Antimicrob. Agents Chemother. **50:**962–967.
14. **Smith, C. A., M. Caccamo, K. A. Kantardjieff, and S. Vakulenko.** 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. D Biol. Crystallogr. **63:**982–992.
15. **Vourli, S., P. Giakkoupi, V. Miriagou, E. Tzelepi, A. C. Vatopoulos, and L. S. Tzouvelekis.** 2004. Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. FEMS Microbiol. Lett. **234:**209–213.
16. **Wachino, J., Y. Doi, K. Yamane, N. Shibata, T. Yagi, T. Kubota, and Y. Arakawa.** 2004. Molecular characterization of a cephamycin-hydrolyzing and inhibitor-resistant class A beta-lactamase, GES-4, possessing a single G170S substitution in the omega-loop. Antimicrob. Agents Chemother. **48:**2905–2910.
17. **Wachino, J., Y. Doi, K. Yamane, N. Shibata, T. Yagi, T. Kubota, H. Ito, and Y. Arakawa.** 2004. Nosocomial spread of ceftazidime-resistant *Klebsiella pneumoniae* strains producing a novel class A β-lactamase, GES-3, in a neonatal intensive care unit in Japan. Antimicrob. Agents Chemother. **48:**1960–1967.