ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2005, p. 3593–3597 Vol. 49, No. 8
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.8.3593–3597.2005

Integron-Encoded GES-Type Extended-Spectrum β-Lactamase with Increased Activity toward Aztreonam in *Pseudomonas aeruginosa*

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Received 15 February 2005/Returned for modification 22 March 2005/Accepted 4 May 2005

A *Pseudomonas aeruginosa* strain expresses an extended-spectrum β-lactamase, GES-9, which differs from GES-1 by a Gly243Ser substitution, is inhibited by clavulanic acid and imipenem, and hydrolyzes aztreonam. The bla_{GES-9} gene was located inside a class 1 integron structure containing two copies of a novel insertion sequence belonging to the IS*1111* family.

Extended-spectrum β-lactamases (ESBLs) are reported increasingly for *Pseudomonas aeruginosa* (29), including TEM and SHV variants, PER-1, VEB-1-like (mostly from southeast Asia) (9), and GES/IBC-type enzymes (29) that have been identified in *P. aeruginosa* in France, South Africa, Greece, and Brazil (4, 6, 15, 23, 24). Recently, a nomenclature update has been proposed for GES-like enzymes (14), such as GES-5 and GES-6 from Japan (27, 28), IBC-1, GES-3, and GES-4 from Greece (8, 26). The bla_{GES} genes are part of class 1 integrons, with the exception of a bla_{GES-1} gene from a *Klebsiella pneumoniae* strain from Portugal which was embedded in a class 3 integron (5).

P. aeruginosa DEJ was isolated from a rectal swab of a patient hospitalized at the hospital Bicêtre in March 2004 for a stroke who had not been hospitalized before and did not travel abroad. *P. aeruginosa* DEJ was resistant to all β-lactams except imipenem, piperacillin (Table 1), colistin, and fosfomycin (17). A synergy between aztreonam- and clavulanic acid-containing disks suggested the production of an ESBL (22). PCR experiments performed with primers specific for ESBL-encoding genes (9) revealed that *P. aeruginosa* DEJ possessed a bla_{GES}-type gene. No transfer of resistance markers to *Escherichia coli* or to *P. aeruginosa* reference strains was obtained by conjugation and transformation (20). Plasmid extraction (10) did not identify plasmids in *P. aeruginosa* DEJ, suggesting a chromosomal location of the bla_{GES}-like gene.

Cloning experiments, performed as described previously (19), gave rise to recombinant strains with an ESBL phenotype. *E. coli* DH10B (pDEJ-1) was resistant to amino- and ureido-penicillins and to narrow- and extended-spectrum cephalosporins and was susceptible to cephamycins and carbapenems (Table 1). In addition, resistance to aztreonam reached a higher level than that observed for a GES-1-producing *E. coli* recombinant strain (Table 1) (21).

A bla_{GES-9} gene was identified in the 5,466-bp insert of recombinant plasmid pDEJ-1. GES-9 differed from GES-1 by only a Gly-to-Ser change at Ambler position 243 (Fig. 1) (1). This substitution is located near Ambler position 240, known to be a key amino acid residue for extension of the hydrolysis profile of CTX-M-type β-lactamases (2).

E. coli DH10B (pDEJ-1) produced a β-lactamase with a pI value of 5.8 according to isoelectric focusing results. Purification of β-lactamase GES-9 was performed as established for GES-1 (21). The specific activity of purified β-lactamase GES-9 against benzylpenicillin was 140 U/mg of protein, and its purification factor was 40-fold with an estimated purity of >95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). β-Lactamase GES-9 had a broad-spectrum hydrolysis profile (Table 2). The catalytic activity (k_{cat}/K_m) of GES-9 for aztreonam was high (60 $mM^{-1} \cdot s^{-1}$), despite a low affinity for this substrate. Although GES-2, GES-3, and GES-4 hydrolyze imipenem as a result of a Gly170Ser substitution, GES-9 that possesses a Gly170 residue spared carbapenems. Activity inhibition measurements showed

TABLE 1. MICs of β-lactams for *P. aeruginosa* DEJ, *E. coli* DH10B harboring recombinant plasmid pDEJ-1 from *P. aeruginosa* DEJ, *E. coli* DH10B harboring recombinant plasmid pC1, and the *E. coli* DH10B reference strain

β-Lactam(s)[a]	MIC (μg/ml) for:			
	P. aeruginosa DEJ	*E. coli* DH10B (pDEJ-1) (GES-9)	*E. coli* DH10B (pC1) (GES-1)[b]	*E. coli* DH10B
Amoxicillin	>512	>512	>512	2
Amoxicillin + CLA	>512	128	>128	2
Ticarcillin	>512	>512	>512	1
Ticarcillin + CLA	512	128	64	1
Piperacillin	16	64	64	1
Piperacillin + TZB	16	8	8	1
Cephalothin	>512	256	256	4
Cefuroxime	>512	512	256	2
Cefoxitin	>512	4	8	2
Ceftazidime	>512	512	128	0.06
Cefotaxime	>512	32	4	0.06
Cefepime	512	1	0.5	0.06
Moxalactam	>512	0.12	0.12	0.06
Aztreonam	512	16	1	0.12
Imipenem	0.25	0.25	0.25	0.12

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml. TZB, tazobactam at a fixed concentration of 4 μg/ml.
[b] Data in this column are from reference 23.

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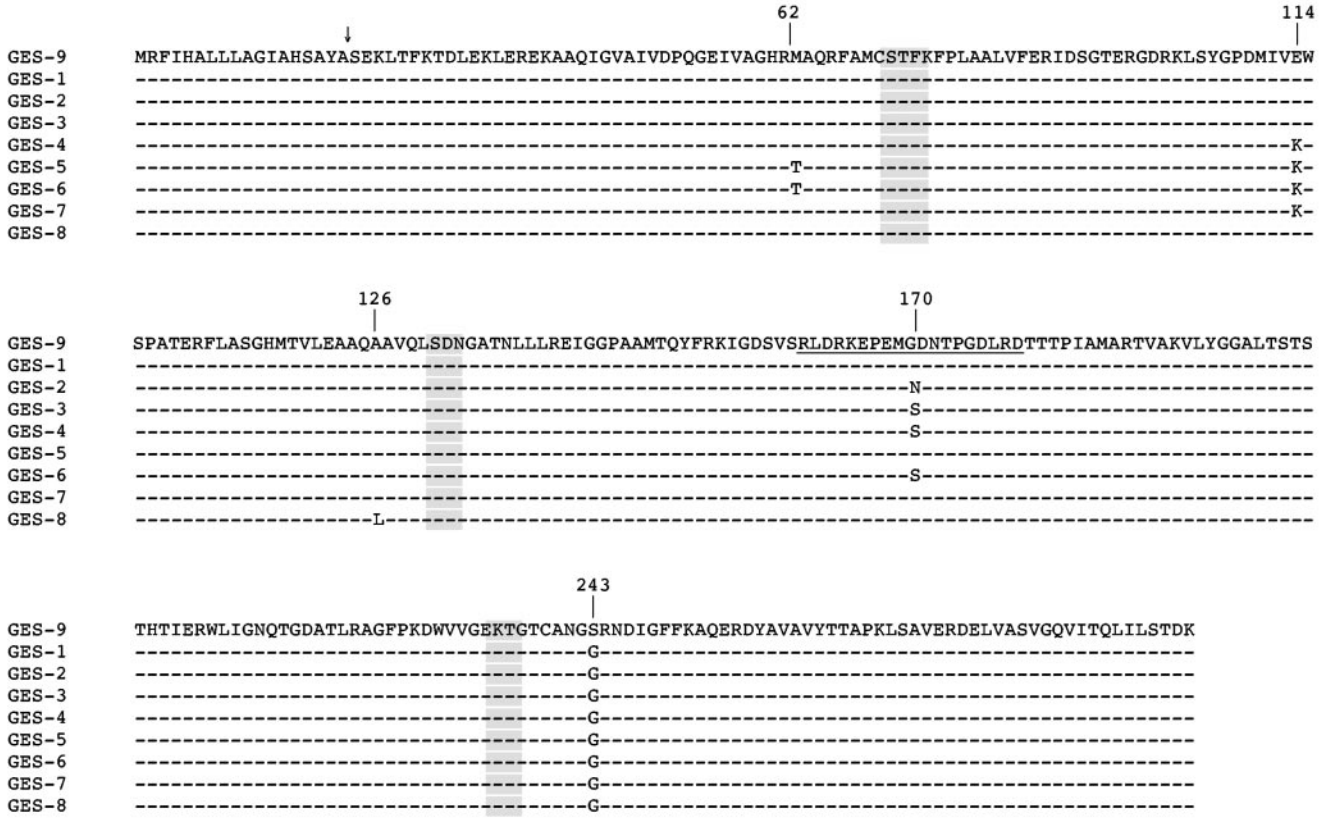

FIG. 1. Comparison of the amino acid sequence of β-lactamase GES-9 of *P. aeruginosa* DEJ to that of other GES enzymes. Names correspond to an update of the GES nomenclature, with GES-1 (21), GES-2 (24), GES-3 (26), GES-4 (26), GES-5 (27), and GES-6 (28), and include name changes for IBC-1 and IBC-2, named here GES-7 (8) and GES-8 (15), respectively. Numbering of β-lactamases is according to Ambler et al. (1). The vertical arrow indicates the putative cleavage site of the leader peptide of the mature β-lactamases. The amino acid residues that are part of the omega loop of Ambler class A β-lactamases are underlined.

that GES-9 was inhibited by clavulanic acid (0.45 μM), tazobactam (0.5 μM), and sulbactam (0.5 μM) and very strongly inhibited by imipenem (50% inhibitory concentration [IC_{50}], 10 nM). In addition, as observed for GES-1 and VEB-1 (21, 22), cefoxitin was an inhibitor of GES-9 activity (IC_{50}, 1 μM).

Sequence analysis of the 5.4-kb insert of plasmid pDEJ-1 revealed that the bla_{GES-9} gene was part of a cassette, preceded by an *intI1* integrase gene of a class 1 integron that was named In109. The 59-base-pair element (59-be) sequence of the bla_{GES-9} gene cassette was interrupted by a novel insertion sequence (IS) element, IS*Pa21* (Fig. 2A). The IS*Pa21* element was 1,374-bp long and possessed 13-bp-long perfect inverted repeats (IRs). Its transposase shared 35% amino acid identity with that of IS*Pa11* identified in *P. aeruginosa* and 32% with

TABLE 2. Kinetic parameters of purified β-lactamase GES-9 compared to those of GES-1 and GES-2

Substrate	GES-9[a]			GES-1[b] k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	GES-2[c] k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)		
Benzylpenicillin	8	12	670	70	96
Amoxicillin	20	50	400	65	26
Piperacillin	35	140	250	13	23
Cephalothin	250	850	300	52	112
Cefotaxime	>10	>1,500	nd[d]	15	2.5
Ceftazidime	>50	>1,000	nd	188	ND[e]
Cefepime	25	1,000	25	1.6	0.6
Aztreonam	>300	>5,000	nd	ND	ND
Imipenem	<0.01	ND	ND	0.07	9

[a] Data in this column are means from three independant experiments. Standard deviations were within 10% of the geometric means.
[b] Data in this column are from reference 23.
[c] Data in this column are from reference 26.
[d] nd, not determinable due to too-high K_m value.
[e] ND, no detectable hydrolysis (<0.01 s^{-1}).

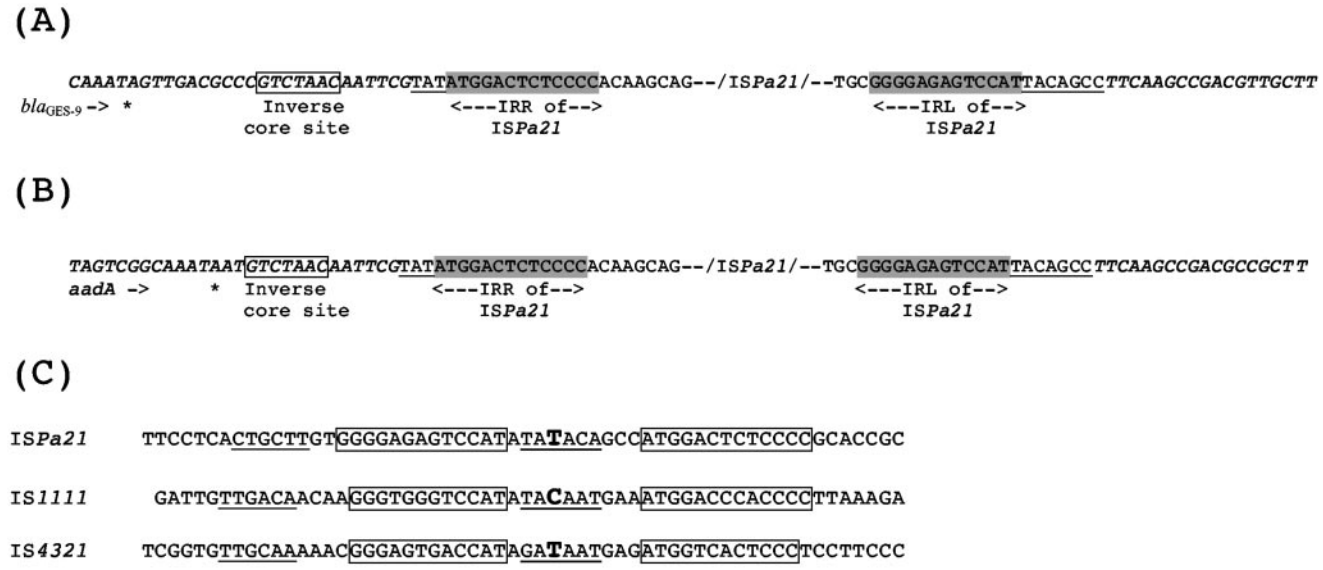

FIG. 2. Features of insertion sequence IS*Pa21*. Analysis of the sites of insertion (A) of the IS*Pa21* element in the 59-be of bla_{GES-9} and (B) of the *aadA* gene cassette. The 59-be sequences are indicated in italics. The stop codons of the bla_{GES-9} and *aacA4* genes are indicated by an asterisk. The inverted repeats left (IRL) and right (IRR) of IS*Pa21* are shaded in gray, and the nucleotides that belong to IS*Pa21* but that are not part of the IRs are underlined. (C) Putative promoter sequences driving the expression of the transposase gene of IS*Pa21* in its circular form compared to those of IS*1111* and IS*4321* belonging to the same family. The base that may originate from either the left or the right end of the IS is shown in boldface, according to the observations made by Partridge et al. (18). In this configuration, the −35 and −10 promoter sequences are separated by 16 bp.

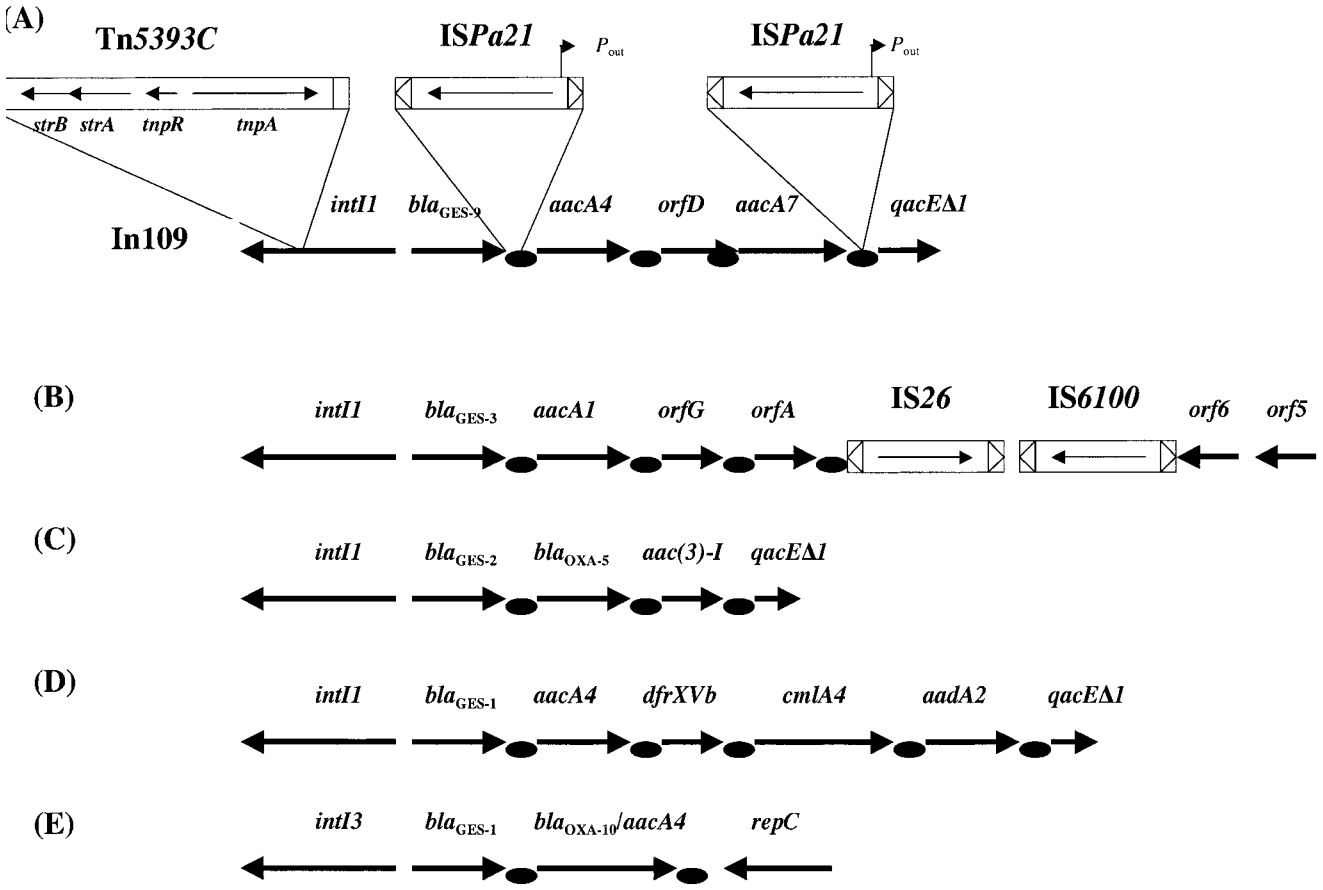

FIG. 3. Schematic map representing integron structures identified in association with bla_{GES}-like genes. The 59-be's are indicated by black circles. The horizontal arrows indicate the transcription orientations. The integron In109 structure identified in this study is indicated (A), as well as (B) the bla_{GES-5}-containing class 1 integron identified on pKGB525 (27), (C) the bla_{GES-2}-containing class 1 integron identified on pLAP-1 (24), (D) the bla_{GES-1}-containing class 1 integron identified on pTK-1 (21), and (E) the bla_{GES-1}-containing class 3 integron identified on p22K9 (5).

that of IS*1111* (18), indicating that IS*Pa21* belonged to the IS*1111* family. Uncommonly for IS elements, the IRs of IS*Pa21* were not located at its termini, and no target site duplication was identified on each side of IS*Pa21* (Fig. 2A). In the IS*Pa21* sequence, 7 bp separate the IR from the left-hand end of the element (IRL) and 3 bp separate the IR from the right-hand end (IRR). A similar observation has been made for IS*5075* (7) and IS*4321* (25), other members of the IS*1111* family, and also for IS*1383* from *Pseudomonas putida* (13, 16). Sequence analysis of the IS*Pa21* element revealed that its transposase-encoding gene was likely expressed under the control of a promoter consisting of a −35 region located inside the right-hand end of the IS and a −10 region created by the fusion of the right- and left-hand terminal sequences of the IS in its circular form (Fig. 2C) as observed for other elements of the IS*1111* family (18).

Downstream of the bla_{GES-9} gene cassette, part of the *aacA4* gene cassette encoding an AAC(6′)-Ib aminoglycoside acetyltransferase was identified in plasmid pDEJ-1 (Fig. 3). PCR mapping identified the complete *aacA4* gene cassette and its downstream-located sequences (Fig. 3). Detailed analysis revealed that IS*Pa21* may provide promoter sequences for the expression of the *aacA4* gene. Indeed, a −35 (TTGGCC) motif and a −10 (TTTCAT) motif separated by 17 bp were able to constitute an efficient promoter (Fig. 3).

The third cassette contained the *orfD* gene encoding a putative protein of unknown function previously identified in class 1 integrons in *Aeromonas salmonicida* and in *Enterobacter aerogenes* (3, 12). The fourth and last cassette was the *aadB* gene cassette that encodes an aminoglycoside 2′-*O*-adenylyltransferase conferring resistance to gentamicin (3). Surprisingly, a second copy of the IS*Pa21* element was identified inside its 59-be (Fig. 1). Again, no duplication of the target site of the IS*Pa21* insertion was noticed (Fig. 2B). Analysis of the insertion sites of IS*Pa21* in In109 revealed that it targeted identical nucleotide motifs in the 59-be of bla_{GES-9} and of the *aadB* gene cassettes (Fig. 2B).

Analysis of the sequences located upstream of bla_{GES-9} revealed that the integrase gene was truncated by the insertion of transposon Tn*5393C* (Fig. 3). Sequencing of pDEJ-1 identified the 78-bp long inverted IRL and part of the *tnpA* transposase gene of this transposon previously identified in the R plasmid pRAS2 from the fish pathogen *Aeromonas salmonicida* (11). PCR mapping revealed that Tn*5393C* was entire in *P. aeruginosa* DEJ, including the *strA* and *strB* streptomycin resistance genes. In addition, the 3′ end of the integrase gene was present at the right-hand end of Tn*5393C*, suggesting that its insertion occurred independently inside the *intI1* gene.

Conclusion. This study emphasizes the spread of GES-type ESBLs in *P. aeruginosa* with further identification in France. A novel GES-type β-lactamase with a broad spectrum hydrolysis profile extended to aztreonam was identified, its gene being located in a class 1 integron different from the other GES-positive integrons (Fig. 3).

Nucleotide sequence accession number. The nucleotide and protein sequences of the In109 integron content have been registered in GenBank under accession no. AY920928.

This work was funded by a grant from the Ministère de l'Education Nationale et de la Recherche (UPRES-EA3539), Université Paris XI, France, and by the European Community (6th PCRD, LSHM-CT-2003-503-335). L.P. is a researcher for the INSERM, Paris, France. L.B. was a recipient of the Sociedad Espanola de Enfermedades Infecciosas y Microbiologia Clinica (SEIMC).

We thank C. Torres for constant support.

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