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Molecular Characterization of a Novel Class 1 Integron Containing bla_{GES-1} and a Fused Product of *aac(3)-Ib*/*aac(6′)-Ib′* Gene Cassettes in *Pseudomonas aeruginosa*

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As seen by the disk diffusion method, the clinical strain of *Pseudomonas aeruginosa* Pa695, resistant to all extended-spectrum cephalosporins and aminoglycosides, exhibited an unusual synergistic effect between ceftazidime and imipenem. This isolate produced an extended-spectrum β-lactamase (ESBL) with a pI of 5.8 that appeared to be chromosomally encoded. Cloning experiments revealed that this ESBL was encoded by bla_{GES-1}, previously described in an integron from *Klebsiella pneumoniae*. In *P. aeruginosa* Pa695, a higher level of resistance to ceftazidime than to ticarcillin was observed, and no synergy between the β-lactamase inhibitors and extended-spectrum cephalosporins was detected, in contrast to the resistance pattern observed in *K. pneumoniae*. Further sequence analysis demonstrated that the bla_{GES-1} gene cassette was located in a class 1 integron, which contained another sequence corresponding to the fused *aac(3)-Ib* and *aac(6′)-Ib′* gene cassettes. The fusion product was functional, as was the product of each gene cloned separately: AAC(3)-I, despite the deletion of the four last amino acids, and AAC(6′), which carried three amino acid changes compared with the most homologous sequence. The AAC(3)-I protein conferred an expected gentamicin and fortimicin resistance, and the AAC(6′), despite the Leu-119→Ser substitution, yielded resistance to kanamycin, tobramycin, and dibekacin, but slightly affected netilmicin and amikacin, and had no apparent effect on gentamicin. The fusion product conveyed a large profile of resistance, combining the AAC(6′) activity with a higher level of gentamicin resistance without accompanying fortimicin resistance.

Pseudomonas aeruginosa is intrinsically susceptible to a limited number of antimicrobial agents, mainly including β-lactams (especially ceftazidime and imipenem) and aminoglycosides (particularly tobramycin and amikacin). In addition, strains with an acquired resistance to these antibiotics are widespread. The major enzymatic mechanism of resistance to broad-spectrum cephalosporins in *P. aeruginosa* is the overproduction of the chromosomally encoded AmpC cephalosporinase (6). Alternatively, this resistance may result from the production of extended-spectrum β-lactamases (ESBLs), mainly belonging to Ambler's class D (4) and rarely to class A, like some TEM and SHV derivative β-lactamases (27). Moreover, uncommon types of class A enzymes have also been reported in this species, notably PER-1 (28) and VEB-1 (25), and a few class B carbapenem-hydrolyzing enzymes, including IMP-1 (18), VIM-1 (19), and VIM-2 (33). The two most-common mechanisms of aminoglycoside resistance in *P. aeruginosa* are impermeability and production of antibiotic-modifying enzymes, mostly 6′-*N*-aminoglycoside acetyltransferase of type II [AAC(6′)-II] (gentamicin, tobramycin, and netilmicin phenotype) and 2″-*O*-nucleotidyltransferase of type I [ANT(2″)-I] (gentamicin and tobramycin); AAC(3)-I (gentamicin) and AAC(6′)-I (tobramycin, netilmicin, and amikacin) are much less frequent (23).

Some genes encoding ESBLs and aminoglycoside-modifying enzymes are located in gene cassettes present in the variable region of integrons. These elements are characterized by the ability to integrate gene cassettes, usually antibiotic resistance genes, by site-specific recombination (7, 14, 34). Among the three major classes of integrons previously described, class 1 is the most frequently encountered. Class 1 integrons consist of a 5′-conserved segment (5′-CS) that contains an *intI1* gene coding for an integrase, a recombination site *attI1*, and generally, a 3′-CS carrying the *qacEΔ1* gene, the *sul1* gene, and an open reading frame (ORF) of unknown function (ORF 5) (21). Gene cassettes are composed of one coding sequence, and at its 3′ end, a so-called 59-base element (59-be), which varies considerably in length, and is bounded by a core site (GTT RRRY) at the recombinant crossover point and an inverse core site (RYYYAAC) at the 3′ end of the inserted gene (8, 38). The usual location of integrons on mobile genetic elements such as plasmids and transposons and their ability to integrate gene cassettes explain why they play a major role in the spread of antibiotic resistance (14).

In this work, we report the analysis of a *P. aeruginosa* strain exhibiting an extended-spectrum β-lactam resistance pattern. Cloning experiments revealed the presence of the bla_{GES-1} gene cassette within a novel class 1 integron. Further molecular characterization of this integron identified an *aac(3)-Ib* and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli DH10B	*araD139* Δ(*ara,leu*)*7697 deoR endA1 galK1 galU nupG recA1 rpsL* F′ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80 *lacZ*Δ*M15*Δ*lacX74* Sm^r	Gibco BRL
E. coli JM109	*endA1 gyrA96 hsdR17* Δ(*lac proA*) *relA recA1 supE44 thi* F′ (*lacI*^{q} *lacZ*Δ*M15 proAB*$^+$ *traD*36)	Promega
E. coli HB101	F-*mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20* (Sm^r) supE44 λ^-	Gibco BRL
In vitro-obtained rifampin-and nalidixic acid-resistant *E. coli* K-12	Rif^r Nal^r	This study
In vitro-obtained rifampin-resistant *P. aeruginosa* ATCC 27853	Rif^r	This study
P. aeruginosa Pa695	Extended-spectrum cephalosporin-and aminoglycoside-resistant clinical isolate	This study
Plasmids		
pBK-CMV phagemid	Kan^r Neo^r	Stratagene, Inc.
pGEM-T	Amp^r	Promega
pTK1	Natural plasmid from *K. pneumoniae* ORI-1 containing bla_{GES-1} and *aac(6′)-Ib′* genes	32
pC18	Recombinant pBK-CMV plasmid with a 2.86-kb genomic *Sau*3AI fragment containing bla_{GES-1}	This study
pC23	Recombinant pBK-CMV plasmid with a 18.5-kb genomic *Sau*3AI fragment containing bla_{GES-1}	This study
pI18	2,390-bp PCR fragment containing the pC18 insert in pGEM-T	This study
pA3A6	1,104-bp PCR fragment containing the *aac(3)-Ib/aac(6′)-Ib′* gene from pC18 in pGEM-T	This study
pA3	541-bp PCR fragment containing the *aac(3)-Ib* gene from pC18 in pGEM-T	This study
pA6	588-bp PCR fragment containing the *aac(6′)-Ib′* gene from pC18 in pGEM-T	This study
pA6In52	588-bp PCR fragment containing the *aac(6′)-Ib′* gene from pTK1 in pGEM-T	This study

aac(6′)-Ib′ fused gene cassette. The aminoglycoside-resistance pattern of the gene fusion product was analyzed.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1. The strain of *P. aeruginosa* Pa695 was isolated in 1999 from a respiratory sample of a woman hospitalized in an adult intensive care unit (ICU) at the Pellegrin Hospital in Bordeaux, France. This isolate was identified and serotyped by conventional methods (16). *P. aeruginosa* ATCC 27853 and *Escherichia coli* JM109 served as controls for MIC determination. A spontaneous rifampin- and nalidixic acid-resistant (Rif^r Nal^r) mutant of *E. coli* K-12 and a Rif^r mutant of *P. aeruginosa* ATCC 27853 were used as recipient strains in conjugation assays, and *E. coli* HB101 in transformation experiments. *E. coli* DH10B and *E. coli* JM109 were the host strains for cloning experiments. All bacterial strains were routinely cultured at 37°C on Mueller-Hinton (MH) agar medium (Sanofi-Diagnostics Pasteur, Marnes la Coquette, France), or grown in Luria broth (GibcoBRL, Cergy Pontoise, France) or Trypticase soy broth (Diagnostics Pasteur, Marnes la Coquette, France).

Antibiotic susceptibility testing. Antibiotic susceptibility of *P. aeruginosa* Pa695 and the *E. coli* recombinant strains was determined at first by the disk diffusion method on MH agar medium, according to French guidelines (http://www.sfm.asso.fr); disks of 2′- and 6′-*N*-ethylnetilmicin (100 μg), fortimicin (100 μg), 5-episisomicin (10 μg), and apramycin (100 μg) were kindly provided by Schering-Plough (Herouville Saint Clair, France). MICs of 12 β-lactams and six aminoglycosides were determined by a standard agar dilution method on MH agar plates, using an inoculum of 10^4 CFU per spot (http://www.sfm.asso.fr). β-Lactams were tested alone or in combination with a fixed concentration of clavulanic acid (2 mg/liter), sulbactam (4 mg/liter), tazobactam (2 mg/liter), or imipenem (0.01 mg/liter).

β-Lactamase extraction and IEF. β-Lactamases produced by *P. aeruginosa* Pa695 and *E. coli* DH10B harboring the recombinant plasmid pC18 were released by ultrasonic treatment, and their pIs were determined by isoelectric focusing (IEF) on an ampholin polyacrylamide gel (pH 3.5 to 10), as described by Matthew et al. (22). Enzyme activities were detected by the iodine procedure in gel, using benzylpenicillin (75 mg/liter) as the substrate.

Plasmid content analysis, conjugation, and transformation experiments. Transfer of resistance genes to Rif^r Nal^r *E. coli* K-12 and Rif^r *P. aeruginosa* ATCC 27853 was attempted by a filter mating technique. Plasmid DNA extraction was carried out for the clinical strain of *P. aeruginosa* using the three following methods: an alkaline-lysis method (1), a technique using alkaline sodium dodecyl sulfate at elevated temperatures (15), and the Qiagen (Courtaboeuf, France) plasmid DNA midi kit. The putative plasmid DNA extract from *P. aeruginosa* Pa695 was electroporated into *E. coli* HB101 with selection on ampicillin (100 mg/liter)-containing MH plates. Plasmid extraction for *E. coli* recombinant clones was performed with a simple boiling-lysis procedure (36).

PCR experiments. The detection of β-lactamase genes was performed under standard PCR conditions (36), using published or laboratory designed sets of primers (Table 2). The aminoglycoside resistance genes *aac(3)-Ib* from pC18 and *aac(6′)-Ib′* from pC18 or pTK1 were separately amplified with the primer sets 5′AAC3–3′AAC3 and 5′AAC6′-3′AAC6′, respectively (Table 2). The fused form of the aminoglycoside resistance genes *aac(3)-Ib/aac(6′)-Ib′* from pC18 was amplified with the primer set 5′AAC3–3′AAC6′. A ribosome binding site (AG GAGGT) was included in the forward primer to allow gene expression during the cloning experiments, and the reverse primer contained a stop codon. The amplicons were revealed by electrophoresis on a 1.5% agarose gel and a subsequent exposure to UV light in the presence of ethidium bromide.

Cloning experiments and recombinant plasmid analysis. Total DNA of *P. aeruginosa* Pa695 was extracted as previously described (33), partially restricted by *Sau*3AI, and ligated into the *Bam*HI-restricted pBK-CMV phagemid (Strat-

TABLE 2. Oligonucleotides used as primers for PCR amplification of β-lactam and aminoglycoside resistance genes

Gene(s)	Primer	Sequence (5′ to 3′)[a]	Reference
bla_{TEM-1}	TEM-A2	GTATCCGCTCATGAGACAATA	39
	TEM-ext	TCTAAAGTATATATGAGTAAAC	
bla_{SHV-1}	OS0	CTCGCCTTTATCGGCCCTCAC	2
	OS5	CGGCCACGCGGGTTAGCG	
$bla_{OXA-10, -11, -14, -16, -17}$	OPR1	GTCTTTCGAGTACGGCATTA	44
	OPR2	ATTTTCTTAGCGGCAACTTAC	
bla_{PER-1}	PER-A	ATGAATGTCATTATAAAAGC	28
	PER-1B	AATTTGGGCTTAGGGCAGAA	
bla_{VEB-1}	VEB-F	CGACTTCCATTTCCCGATGC	25
	VEB-B	GGACTCTGCAACAAATACGC	
bla_{GES-1}	GES-1A	ATGCGCTTCATTCACGCAC	32
	GES-1B	CTATTTGTCCGTGCTCAGG	
bla_{OXA-13}	OXA-13.1	GCCGCATATGTAATTACTGC	24
	OXA-13.2	ATTTTCTTAGCGGCAACTTAT	
bla_{OXA-18}	OXA-18F	ATTTCAACGGTTTGCCTTACG	30
	OXA-18B	TTGGCATCGGAAAGCGAACC	
aac(3)-Ib	5′ AAC3	CATCATAGGAGGTTGTTTGATGTTATGGAGCAG	This study
	3′ AAC3	TTATTATGGATCAATGTCGAAGTGCA	This study
aac(6′)-Ib′	5′ AAC6′	CATCATAGGAGGTGATCCAATGACCAACAGCAACGATTCCG	This study
	3′ AAC6′	CCTCGATGGAAGGGTTAGGC	This study

[a] The RBS and start and stop codons are underlined in the primers where they were included.

agene, La Jolla, Calif.). *E. coli* DH10B strains harboring the recombinant plasmids were selected on MH agar plates containing amoxicillin (30 mg/liter) and kanamycin (30 mg/liter). A double-restriction digestion analysis with *Hin*dIII and *Pst*I enzymes allowed precise mapping of recombinant plasmids by electrophoresis on a 0.8% agarose gel. The PCR products of the aminoglycoside resistance genes, either fused or separated, were ligated into the pGEM-T vector (Promega, Charbonnières, France). In order to analyze the expression of the fused gene within the integron environment, a PCR product corresponding to the insert in recombinant plasmid pC18 was also ligated into vector pGEM-T (pI18). *E. coli* JM109 strains carrying the recombinant plasmids were selected on MH agar plates containing tobramycin (4 mg/liter) for *aac(6′)-Ib′* and *aac(3)-Ib*/*aac(6′)-Ib′* genes. For the *aac(3)-Ib* gene, since the conferred gentamicin resistance was at a low level and the pGEM-T vector contained a bla_{TEM} gene, selection was done on ampicillin (100 mg/liter), and then plasmid analysis after *Pst*I and *Hin*dIII restriction allowed identification of the clone harboring the insert in the right sense.

DNA sequencing. The 2.86-kb cloned DNA fragment from recombinant plasmid pC18 was sequenced on both strands using the dideoxy-chain termination method with the D Rhodamine dye terminator kit (Perkin-Elmer, Courtaboeuf, France). Further sequence analysis was performed on PCR products using laboratory-designed sequencing primers and parts of a larger recombinant plasmid, pC23, containing an 18.5-kb genomic *Sau*3AI fragment. Sequences were analyzed with an automatic sequencer ABI 377 (Perkin Elmer), using the Sequencing Analysis software and compared to each other and to homologous sequences using the Sequence Navigator software. The nucleotide and the deduced protein sequences were analyzed using the software available over the Internet at the National Center of Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under the accession number AF355189.

RESULTS AND DISCUSSION

Clinical case. *P. aeruginosa* Pa695, of serotype P11, was isolated from the sputum of a 46-year-old woman hospitalized in 1999 at the Pellegrin Hospital in Bordeaux, France. This patient had been admitted in the adult ICU for vascular brain damage 1 month previously. Two days after her hospitalization she had a pulmonary infection due to *Streptococcus pneumoniae* and *Haemophilus influenzae*, but she recovered under antimicrobial therapy with intravenous amoxicillin (6 g)-clavulanic acid, lasting 17 days, and with pristinamycin (6 g), lasting 15 days. Four weeks later, a control sputum sample revealed the presence of *P. aeruginosa* Pa695 (10^7 CFU/ml) and a TEM-24 (as verified by sequencing)-producing strain of *Enterobacter aerogenes* (2×10^7 CFU/ml). In the absence of fever, the patient did not receive any antibiotic. She had not recently traveled to French Guiana or Greece, where GES-1 β-lactamase was previously found (11, 32). No patient coming from these countries was concomitantly hospitalized in the same ICU during the same period of time.

By the disk diffusion method, *P. aeruginosa* Pa695 was seen to be resistant to all potentially active β-lactam agents except for imipenem and aztreonam. Surprisingly, a synergistic effect was seen between imipenem and ceftazidime or cefsulodin as observed with OXA-13 in *P. aeruginosa* Pae391 (24), but not between clavulanic acid and ceftazidime. These results suggested the presence of an ESBL of an unusual type. No other *P. aeruginosa* or enterobacterial isolate with a similar ESBL resistance profile was isolated among patients of the same unit and hospital. Pa695 was additionally resistant to all aminoglycosides and all fluoroquinolones.

Characterization of the β-lactam resistance pattern, the *bla* gene, and its genetic environment. Preliminary PCR amplification experiments of known ESBLs in *P. aeruginosa* (TEM, SHV, OXA derivatives, PER-1, and VEB-1) failed to give positive results. Conjugation experiments between Pa695 and *E. coli* K-12 Rif^r Nal^r or *P. aeruginosa* ATCC 27853 Rif^r, did not yield any transconjugant. Despite repeated attempts, plasmid DNA analysis of *P. aeruginosa* Pa695 did not show any plasmid, and transformation by electroporation of plasmid DNA extract into *E. coli* HB101 was unsuccessful. Thus, the *bla* gene seemed to be chromosomally located in this strain. After cloning experiments two recombinant plasmids, pC18 (containing a 2.86 kb insert) and pC23 (containing an 18.5-kb insert), were selected for subsequent analysis and sequencing.

β-Lactam MICs for *P. aeruginosa* Pa695 (Table 3) showed an intermediate susceptibility to ticarcillin (32 mg/liter), cefepime (16 mg/liter), and ceftazidime (32 mg/liter) and a low-level

TABLE 3. MICs of β-lactams for the clinical strain of *P. aeruginosa* Pa695 and the reference strain of *P. aeruginosa* ATCC 27853

β-Lactam(s)[a]	MIC (mg/liter) for:	
	P. aeruginosa Pa695	*P. aeruginosa* ATCC 27853
Amoxicillin	>512	512
Amoxicillin + CLA	>512	512
Ticarcillin	32	8
Ticarcillin + CLA	16	8
Ticarcillin + SUL	16	8
Ticarcillin + TZB	16	8
Ticarcillin + IPM	16	8
Piperacillin	64	2
Piperacillin + TZB	8	2
Cephalothin	>512	>512
Cefuroxime	512	256
Cefoxitin	512	>512
Cefotaxime	64	16
Ceftazidime	32	2
Ceftazidime + CLA	32	2
Ceftazidime + SUL	32	2
Ceftazidime + TZB	32	2
Ceftazidime + IPM	16	2
Cefsulodin	64	2
Cefsulodin + IPM	32	2
Cefepime	16	1
Cefepime + CLA	8	1
Cefepime + TZB	8	1
Aztreonam	4	4
IPM	1	2

[a] Abbreviations: CLA, clavulanic acid (2 mg/liter); SUL, sulbactam (4 mg/liter); TZB, tazobactam (2 mg/liter); IPM, imipenem (0.01 mg/liter).

resistance to cefotaxime (64 mg/liter). In contrast, it was susceptible to aztreonam and imipenem. The addition of β-lactamase inhibitors (clavulanic acid, sulbactam, or tazobactam at 2, 8, or 4 mg/liter, respectively) or imipenem at 0.01 mg/liter did not modify or only slightly decreased the MICs of the tested penicillins and cephalosporins, except for piperacillin plus tazobactam (eightfold decrease in MIC). IEF analysis revealed that *P. aeruginosa* Pa695 and *E. coli* DH10B(pC18) produced a β-lactamase with a pI of 5.8.

Sequence analysis of a 4,808-bp DNA fragment was performed at first with the recombinant plasmid pC18 and then with PCR-amplified fragments from the longer insert of the recombinant plasmid pC23, using laboratory-designed primers. The nucleotide sequence of the ESBL-encoding gene differed by a single silent mutation at position 591 from bla_{GES-1}, recently described in *Klebsiella pneumoniae* (32), and its amino acid sequence differed by two substitutions from IBC-1, an ESBL reported in *Enterobacter cloacae* (11). GES-1 is known to inactivate most β-lactams except for aztreonam and imipenem and to be inhibited by clavulanic acid, sulbactam, and tazobactam and strongly inhibited by imipenem (32). These features were recognized in *E. coli* DH10B(pC18) (data not shown). By MIC determination, GES-1 in Pa695 seemed to affect ticarcillin less than ceftazidime, and the β-lactamase inhibitors such as imipenem had a very slight inhibitory effect (Table 3). Indeed, the ESBL inhibition might be masked by the expression of the chromosomally encoded cephalosporinase of Pa695, either partially derepressed or induced by clavulanate and imipenem. By the disk diffusion method the synergistic effect between cefsulodin and imipenem could lead to a confusion with the OXA-13 enzyme. However, the main difference is the ceftazidime resistance conveyed by GES-1 β-lactamase.

The bla_{GES-1} gene was found in a cassette located in a class 1 integron (Fig. 1). Indeed, the 5′-CS end contained the *intI1* integrase gene, the *attI1* recombination site, and the promoter region including the P1 promoter (also called Pc) (regions −35[TGGACA] and −10[TAAACT]) under a hybrid 1 form with a weak activity (20) and different from that present in In52, the bla_{GES-1} integron in *K. pneumoniae* (32). At the 3′-CS end, the quaternary ammonium compound-resistance gene *qacEΔ1*, and the sulfonamide resistance gene *sul1* were identified, as reported in most class 1 integrons (13, 34). The bla_{GES-1} gene cassette contained a core site (GTTAGAC), an inverse core site (GTCTAAC), and a 59-be site of 110 bp different from that of the truncated bla_{GES-1} gene cassette previously reported in In52 but identical to the 59-be in the bla_{IBC-1} gene cassette (Fig. 1).

The finding in *P. aeruginosa* of the bla_{GES-1} gene, previously described in *Enterobacteriaceae*, underlines the interspecies spread of this integron-located ESBL gene cassette. GES-1 is another Ambler class A β-lactamase, besides PER-1, VEB-1, TEM-4, TEM-24, TEM-42, and SHV-2a, which have been previously described in this species. Finally, the bla_{GES-1} gene in Pa695 was the first description of a nonimported case in France, suggesting the worldwide spread of this enzyme.

Aminoglycoside resistance conferred by the integron. Sequencing of pC18 and pC23 revealed that the integron contained another cassette made up of a 1,005-bp coding region which consisted of the *aac(3)-Ib* gene fused with the sequence of an *aac(6′)-Ib′* gene. The last 15 nucleotides of the *aac(3)-Ib* sequence were missing, and the sequence continued with a leucine codon instead of a valine at the beginning of the *aac(6′)-Ib′* sequence (Fig. 2). Cassette fusion may occur by deletion events with end points of the genes in two adjacent gene cassettes leading to the presence of one or two truncated genes or by partial or total loss of the 59-be of the first cassette (34). In the present study the fused cassette contained the truncated *aac(3)-Ib* gene and the complete *aac(6′)-Ib′* gene. The core site of the fused cassette was identical to that identified for the *aac(3)-Ib* cassette (37) and the 59-be located at the 3′ end of this ORF was identical to that found for some *aac(6′)-Ib′* cassettes (31, 32). Thus, the fused cassette had a core site (GTTAGGT) and an inverse core site (GCCTAAC) presenting a 1-bp mismatch (Fig. 1).

By the disk diffusion method, *E. coli* DH10B(pC18) exhibited an unusual high-level resistance to tobramycin, compared with a relative susceptibility to gentamicin, netilmicin, and amikacin. Aminoglycoside MICs (Table 4) were consistent with this observation. Since *E. coli* DH10B is streptomycin resistant due to a chromosomal mutation, and pBK-CMV harbors a kanamycin-neomycin resistance gene, the pC18 insert was cloned in the pGEM-T vector (pI18) (Fig. 3) and expressed in *E. coli* JM109. *E. coli* JM109(pI18) exhibited a similar resistance pattern compared with *E. coli* DH10B(pC18) except for streptomycin and neomycin susceptibility and a lower level of kanamycin resistance. To assess the functionality of the separate *aac(3)-Ib* and *aac(6′)-Ib′* genes, and of the fused *aac(3)-Ib/aac(6′)-Ib′* gene, the corresponding PCR products were cloned in the pGEM-T vector (Fig. 3) used to trans-

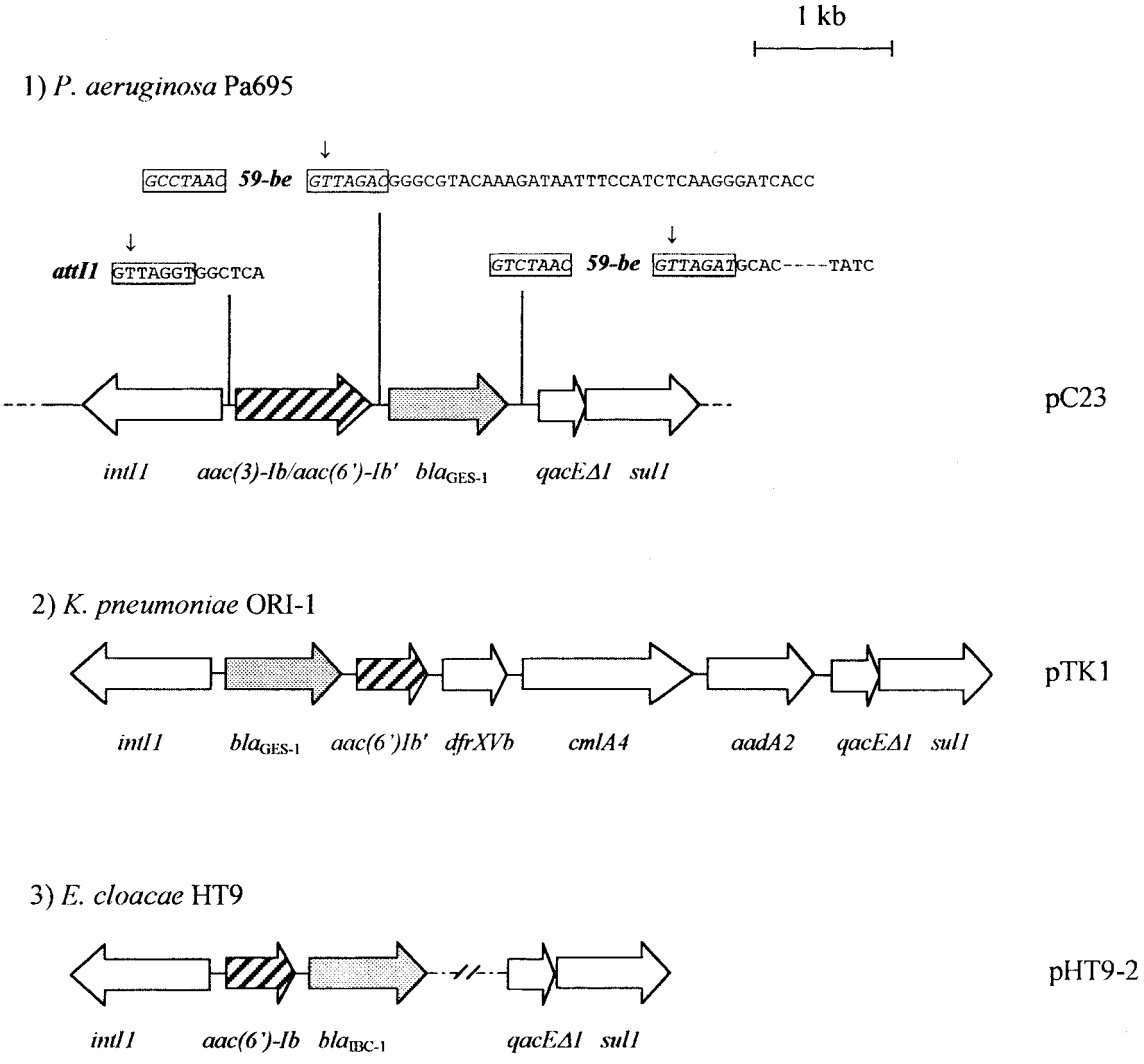

FIG. 1. Schematic representations of the different bla_{GES-1}- and bla_{IBC-1}-containing integrons. (1) Part of the structure of the recombinant plasmid pC23 encoding bla_{GES-1} in *P. aeruginosa* Pa695. The horizontal arrows indicate the translation orientation. The solid lines represent the sequenced fragment from *P. aeruginosa* Pa695 with the different genes boxed, and the dotted lines indicate the unanalyzed sequence. The conserved core and inverse core sites are boxed, and the cassette boundaries are represented by vertical arrows. Dashes are used to indicate where the reported sequence was identical to already-published sequences. (2) Structure of the bla_{GES-1} gene cassette-containing integron from *K. pneumoniae* ORI-1 (33). (3) Structure of the bla_{IBC-1} gene cassette-containing integron from *E. cloacae* HT9 (12).

form *E. coli* JM109. The three types of clones were obtained by the same method, and in all cases the pGEM-T promoter allowed the expression of the cloned gene.

The *aac(3)-Ib* gene encoding a 3-*N*-aminoglycoside acetyltransferase was identical to that conferring gentamicin and fortimicin resistance previously described in a strain of *P. aeruginosa* (37), except for a silent substitution (C→A) at position 207 of the gene, and the absence of the 15 last nucleotides. The *E. coli* JM109(pA3) strain, which expressed the truncated *aac(3)-Ib* gene, exhibited a decreased susceptibility to gentamicin as demonstrated by MIC determination. Moreover, by the disk diffusion method, a small diameter of 10 mm around the fortimicin disk was observed with *E. coli* JM109(pA3) instead of a diameter of 30 mm as observed with the host strain. Thus, despite the lack of the four last amino acids the *aac(3)-Ib* gene conferred resistance to fortimicin and a low-level resistance to gentamicin, similar to results described by Schwocho et al. (37).

The *aac(6′)-Ib′* sequence of the fused cassette encoded a 6′-*N*-aminoglycoside acetyltransferase that was characterized by a Leu-119→Ser substitution [numbering of the reference *aac(6′)-Ib* sequence (42)], and differed by three amino acid substitutions—Val-18→Leu, Leu-42→Val, and Ser-100→Gly—from the already-described *aac(6′)-Ib′* genes (5, 17, 31, 32, 41). The amino acid at position 119 has been found to be critical functionally in that a Leu-to-Ser switch at this position was responsible for the loss of amikacin resistance conferred by the *aac(6′)-Ib* gene and the acquisition of gentamicin resistance, conveyed by the *aac(6′)-Ib′* gene (17). These genes encoded a protein with an AAC(6′)-II specificity, i.e., kanamycin, tobramycin, netilmicin, and gentamicin resistance and amikacin susceptibility. However, *E. coli* JM109(pA6),

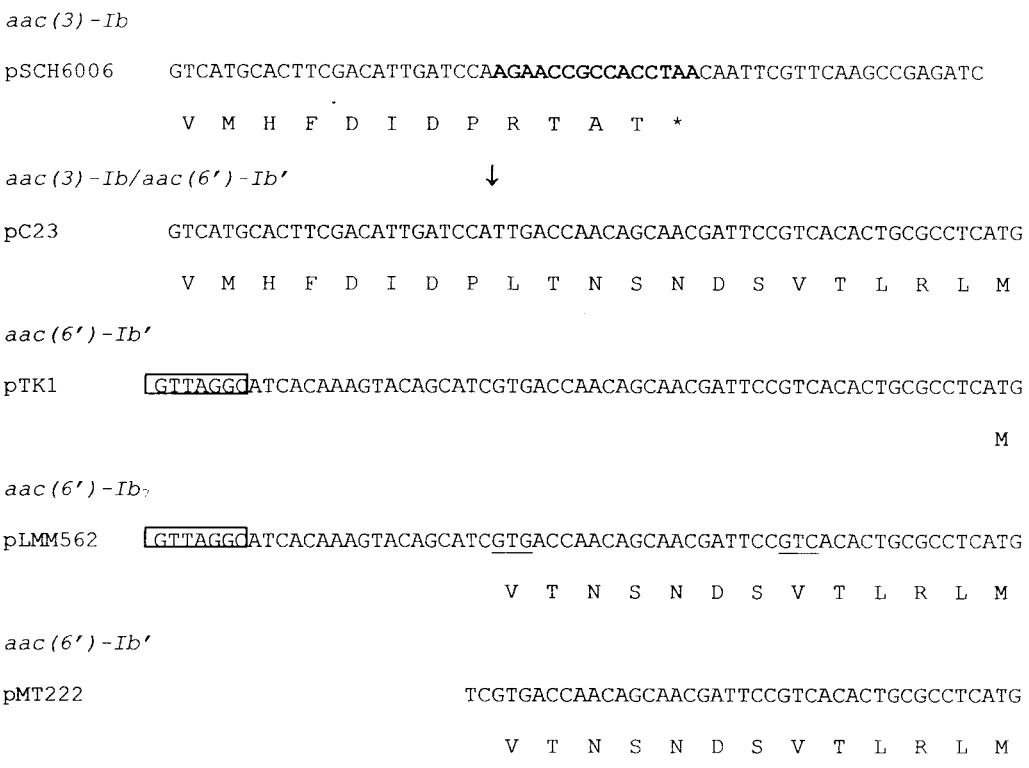

FIG. 2. Sequence comparison of the fusion point of the *aac(3)-Ib/aac(6′)-Ib′* fused cassette in Pa695 and the ends of the original *aac(3)-Ib* and *aac(6′)-Ib′* gene cassettes. The data were compiled from the sequences obtained from plasmids pSCH6006 (37), pTK1 (32), pLMM562 (5), pMT222 (41), and the one analyzed here. The possible start codons are underlined, and the stop codons are indicated by asterisks. The deleted region of the *aac(3)-Ib* gene is shown in boldface type. The conserved core sites are boxed, and the vertical arrow indicates the site of fusion between the *aac(3)-Ib* and *aac(6′)-Ib′* genes. The deduced amino acid sequence is designated in single-letter code below the nucleotide sequence.

which harbored the *aac(6′)-Ib′* gene, exhibited an unusual aminoglycoside resistance pattern, with kanamycin and tobramycin resistance, reduced netilmicin and amikacin susceptibility, and gentamicin susceptibility (Table 4). The natural plasmid pTK1 from *K. pneumoniae* ORI-1 also contains an *aac(6′)-Ib′* gene cassette beside the bla_{GES-1} gene cassette in In52 (32). In order to evaluate whether the mutations influenced the enzyme specificity, the recombinant plasmid pA6In52 was constructed by cloning the *aac(6′)-Ib′* gene of In52 in the pGEM-T vector and expressed in *E. coli* JM109. The MIC determination revealed slight differences between the two clones, *E. coli* JM109(pA6) and *E. coli* JM109(pA6In52) (Table 4). The recombinant plasmid pA6In52 conferred the expected resistance profile, i.e., reduced susceptibility to gentamicin and full susceptibility to amikacin, with MICs similar to those previously reported (5, 35). In contrast, the recombinant plasmid pA6 conferred a marked resistance to tobramycin, a reduced susceptibility to amikacin, and an increased susceptibility to gentamicin, suggesting a role of the mutations in the resistance pattern conferred by this enzyme. As shown in Fig. 2, several potential start codons have been proposed for the *aac(6′)-Ib* genes (5, 10). However, in our construction the compared genes necessarily started from the initiation codon introduced in the primer, validating their comparison.

TABLE 4. Aminoglycoside resistance patterns of various strains

Strain	MIC (mg/liter) of[a]:						Additional aminoglycoside resistance marker(s)
	STR	KAN	GEN	TOB	AMK	NET	
Pa695	32	512	512	256	32	>512	2′NET, EPI, DIB
E. coli DH10B(pC18)	>512	>512	2	16	8	16	2′NET, EPI, NEO, DIB
E. coli JM109(pI18)	2	64	2	16	4	8	2′NET, EPI, DIB
E. coli JM109(pA3A6)	2	128	2	32	4	8	2′NET, EPI, DIB
E. coli JM109(pA3)	2	2	2	0.5	1	0.5	FOR
E. coli JM109(pA6)	2	64	0.5	32	4	4	2′NET, EPI, DIB
E. coli JM109(pA6In52)	2	128	2	8	1	4	2′NET, EPI, DIB
E. coli JM109	2	2	0.2	0.5	1	0.5	

[a] Abbreviations: STR, streptomycin; KAN, kanamycin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; NET, netilmicin; 2′NET, 2′-*N*-ethylnetilmicin; EPI, 5-episisomicin; NEO, neomycin; DIB, dibekacin; FOR, fortimicin.

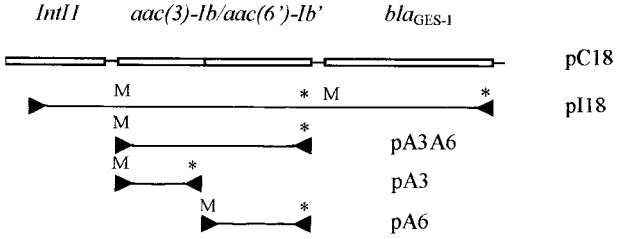

FIG. 3. Schematic representation of cloning experiments of the integron and its *aac(3)-Ib*, *aac(6′)-Ib′* and *aac(3)-Ib/aac(6′)-Ib′* genes performed in pGEM-T vector after PCR amplification. Arrowheads represent primer positions and their orientations, and M and * indicate the location of the start and the stop codon, respectively.

E. coli JM109(pA3A6), which expressed the product of the *aac(3)-Ib/aac(6′)-Ib′* gene fusion, had a resistance profile identical to *E. coli* JM109(pI18), indicating that this protein was functional and expressed in the same manner as in the integron. The MIC determination showed that the AAC(3)-I/AAC(6′) protein fusion had a broad activity, combining the effect of the protein encoded by the *aac(6′)-Ib′* gene, with a decreased gentamicin susceptibility. However, it did not yield fortimicin resistance, indicating the activity of a unique protein rather than the additive effect of two enzymes. Northern blot experiments should allow the demonstration of whether one or two RNAs are transcribed from the fused gene. A single enzyme frequently modifies several antibiotics, via the same modification mechanism of each substrate. The sole example of a bifunctional aminoglycoside resistance enzyme described at present is the AAC(6′)-APH(2″), found in strains of streptococci and staphylococci (9, 43). In the case of the fused gene product AAC(3)-I/AAC(6′), it is difficult to establish whether the enzyme is really bifunctional or the presence of the AAC(3)-I increases the activity of the AAC(6′) protein.

Although the AAC(6′) of type I is common in *Enterobacteriaceae*, this enzyme, along with AAC(3)-I, is infrequent in *P. aeruginosa* (23). The existence of a fused product of both genes in Pa695 raises the hypothesis, as does the finding of bla_{GES-1}, that the integron studied here may have originated from enterobacteria. Moreover, the 5′ sequences flanking the *aac(6′)-Ib* cassette junctions display considerable genetic plasticity (5), and some studies have reported translational fusion with the *aac(6′)-Ib* gene (3, 26, 40), suggesting that these sequences are favorable for expression of fused genes. On the other hand, the integron described here was bounded at the 5′ end by a 25-bp inverted repeat (IRi) (TGTCGTTTTCAGAA GACGGCTGCAC) identical to the IRi sequence identified at the boundary of several integrons (13, 18, 29), and the nucleotide sequence upstream of the IRi was from Tn*501,* suggesting that the integron was inserted into Tn*501* or a close relative transposon, which would itself be inserted in the chromosome.

In conclusion, we report here the characterization of a new class 1 integron found in *P. aeruginosa*. This integron contains the bla_{GES-1} gene previously reported in *Enterobacteriaceae*, showing the interspecies diffusion of this ESBL-encoding gene, as recently described for VEB-1 (12). Further molecular analysis led to the discovery of a functional fused gene encoding an AAC(3)-I/AAC(6′) protein. This enzyme conferred a specific resistance pattern, combining the activity of an unusual AAC(6′) with an increased effect on gentamicin. This work confirms the major role of integrons in the spread of resistance genes and gives an insight into the multiple and complex recombinations occurring in these genetic elements.

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