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Characterization of Class 1 Integrons from *Pseudomonas aeruginosa* That Contain the bla_{VIM-2} Carbapenem-Hydrolyzing β-Lactamase Gene and of Two Novel Aminoglycoside Resistance Gene Cassettes

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Two clonally unrelated *Pseudomonas aeruginosa* clinical strains, RON-1 and RON-2, were isolated in 1997 and 1998 from patients hospitalized in a suburb of Paris, France. Both isolates expressed the class B carbapenem-hydrolyzing β-lactamase VIM-2 previously identified in Marseilles in the French Riviera. In both isolates, the bla_{VIM-2} cassette was part of a class 1 integron that also encoded aminoglycoside-modifying enzymes. In one case, two novel aminoglycoside resistance gene cassettes, *aacA29a* and *aacA29b*, were located at the 5′ and 3′ end of the bla_{VIM-2} gene cassette, respectively. The *aacA29a* and *aacA29b* gene cassettes were fused upstream with a 101-bp part of the 5′ end of the *qacE* cassette. The deduced amino acid sequence AAC(6′)-29a protein shared 96% identity with AAC(6′)-29b but only 34% identity with the *aacA7*-encoded AAC(6′)-I1, the closest relative of the AAC(6′)-I family enzymes. These aminoglycoside acetyltransferases had amino acid sequences much shorter (131 amino acids) than the other AAC(6′)-I enzymes (144 to 153 amino acids). They conferred resistance to amikacin, isepamicin, kanamycin, and tobramycin but not to gentamicin, netilmicin, and sisomicin.

Among the expanded-spectrum β-lactamases in *Pseudomonas aeruginosa*, a few Ambler class B carbapenem-hydrolyzing β-lactamases have been characterized, including IMP-1, IMP-3, VIM-1, and VIM-2 (1, 8, 11, 12, 13, 16). IMP-1-like enzymes have spread among several gram-negative rods in Japan and are found in 1.3% of the *P. aeruginosa* isolates there, according to a national survey conducted from 1996 to 1997 (7; H. Kurokawa, T. Yagi, N. Shibata, K. Shibayama, and Y. Arakawa, letter, Lancet **354:**955, 1999). In the northern part of Italy (Verona) and in Greece, *P. aeruginosa* isolates have been identified that express VIM-1, which has 28% amino acid identity with IMP-1 (11, 24; G. Cornaglia, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1482, 1999). Recently the metallo-β-lactamase IMP-2, which possesses 90% amino acid identity with IMP-1, was identified from *Acinetobacter baumannii*, also in Verona (19). VIM-2, recently identified from *P. aeruginosa* COL-1 isolated in Marseilles (France) in 1996, shares 90% amino acid identity with VIM-1 (16). The VIM and IMP enzymes have a broad spectrum of hydrolysis of β-lactams that includes oxyiminocephalosporins and carbapenems.

Mobile cassettes contain genes most often mediating antibiotic resistance and a recombination site, designated 59-be (17, 18, 22). The 59-be sites vary in length (57 to 141 bp) and structure, but they are all bounded by a core site (GTTRRRY) at the recombinant crossover point and an inverse core site (RYYYAAC) at the 3′ end of the inserted gene (17, 18).

The four metalloenzyme genes that encode the VIM and IMP β-lactamases are each part of a gene cassette that is located in class 1 integrons (additionally in the class 3 integron for the bla_{IMP-1} gene cassette) (1, 11–13, 19). Integrons are genetic elements capable of integrating or mobilizing individual gene cassettes by a site-specific recombination mechanism that involves a DNA integrase IntI and two types of recombination sites, *attI* and 59-be (4, 6, 22). The 5′ conserved segment (5′-CS) of the integron structure contains the integrase gene (*intI*) and the recombination site *attI1* (17, 18). The 3′-CS of class 1 integrons carries the antiseptic-resistance *qacEΔ1* gene, an open reading frame of unknown function (*orf5*) and the *sul1* gene which confers resistance to sulfonamides (17, 18).

In the course of screening for carbapenem-hydrolyzing *P. aeruginosa* isolates, two *P. aeruginosa* clinical isolates were positive for bla_{VIM}-like genes in preliminary PCR-based analyses. Both isolates, RON-1 and RON-2, were compared to the *P. aeruginosa* COL-1 isolate and analyzed for their β-lactamase and integron contents. In addition to the bla_{VIM-2} and previously described aminoglycoside resistance gene cassettes, two cassette-integrated genes encoding novel aminoglycoside-modifying enzymes have been characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids, and susceptibility testing. The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* RON-1 and RON-2 were isolated in 1998 and 1997, respectively, at the hospital Raymond Poincaré located in a suburb of Paris. The antibiotic susceptibilities of the *P. aeruginosa* isolates and of the *Escherichia coli* recombinant strains were first

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
Strains		
E. coli DH10B	*araD139* Δ(*ara leu*)*7697 deoR endA1 galK1 galU nupG recA1 rpsL* F′-*mcrA* Δ(*mrr-hsdRMS-mrcBC*) ϕ80 *lacZ*ΔM15 Δ*lacX74*	Gibco BRL, Paris, France
E. coli JM109	*endA1 gyrA96 hsdR17* Δ(*lac proA*) *relA recA1 supE44 thi* F′ (*lacI*^{q} *lacZ*ΔM15 *proAB*$^+$ *traD36*)	15
In vitro-obtained rifampin-resistant *E. coli* JM109	Rifampin^r	15
In vitro-obtained rifampin-resistant *P. aeruginosa* PU21	*ilv*/*leu*, streptomycin^r, rifampin^r	15
P. aeruginosa COL-1	bla_{VIM-2} expressing clinical isolate	16
P. aeruginosa RON-1	Carbapenem-hydrolyzing clinical isolate	This study
P. aeruginosa RON-2	Carbapenem-hydrolyzing clinical isolate	This study
Plasmids		
pBK-CMV	Neomycin^r, kanamycin^r	Stratagene Inc., Ozyme, Amsterdam, The Netherlands
pPCRScript-Cam (SK+)	Chloramphenicol^r	Stratagene Inc.
pNOR-2002	5,648-bp *Bam*HI-fragment from *P. aeruginosa* RON-1 DNA in pBK-CMV	This study
pNOR-2003	5,061-bp *Bam*HI-fragment from *P. aeruginosa* RON-2 DNA in pBK-CMV	This study
pLO-1	1,541-bp PCR fragment containing *aacA29a* from pNOR-2003 in pPCRScript-Cam	This study
pLO-2	1,655-bp PCR fragment containing *aacA29b* from pNOR-2003 in pPCRScript-Cam	This study

[a] A superscript, "r" indicates resistance.

determined by the disk diffusion method on Mueller-Hinton (MH) agar (Sanofi-Diagnostics Pasteur, Marnes-La-Coquette, France). The MICs of selected β-lactams and aminoglycosides were then determined by an agar dilution technique on MH agar plates with an inoculum of 10^4 CFU per spot (15). The activities of 2′- and 6′-*N*-ethylnetilmicin were studied by diffusion on MH agar at 37°C with disks containing 100 μg of antibiotic.

Plasmid content, conjugation, and electroporation. Plasmid DNAs of *P. aeruginosa* RON-1 and RON-2 were extracted, analyzed, and tentatively electroporated as described previously (15). Transfer of β-lactam resistance markers from *P. aeruginosa* RON-1 and RON-2 into in vitro-obtained rifampin-resistant *E. coli* JM109 or rifampin-resistant *P. aeruginosa* PU21 was performed as described before (15, 16) with transconjugant selection on Trypticase soy (TS) agar plates containing either ceftazidime (4 μg/ml) or cefotaxime (0.5 μg/ml) and rifampin (200 μg/ml).

Cloning and DNA sequencing. Whole-cell DNAs from *P. aeruginosa* RON-1 and RON-2 were extracted as described previously (16). PCR experiments were performed first with these DNAs as a template and primers VIMB and VIMF designed to hybridize at the 5′ and 3′ ends of the bla_{VIM-1} and bla_{VIM-2} sequences (positions 2080 to 2099 and 2671 to 2689, respectively [16]) followed by DNA sequencing on both strands. Then, fragments of whole-cell DNAs from RON-1 and RON-2 digested with *Bam*HI (Amersham Pharmacia Biotech, Orsay, France) were ligated into the *Bam*HI site of pBK-CMV as previously described (14). *E. coli* DH10B harboring recombinant plasmid DNAs were selected on kanamycin (30 μg/ml) and ceftazidime (2 μg/ml) containing TS plates and were analyzed as already described (14). The cloned *Bam*HI fragments were sequenced on both strands with an Applied Biosystems sequencer (model ABI 373). Subsequently, aminoglycoside resistance genes from recombinant plasmid pNOR-2003 (see below) were amplified by a PCR technique (20) using primers hybridizing to the upstream region of the *attI1* site (5′-CS [5′-GGCATCCAAG CAGCAAG-3′], positions 1929 to 1949; see Fig. 2) and bla_{VIM-2} (VIM-2B [5′-CTACTCAACGACTGAGCG-3′] hybridizing at positions 2712 to 2729; see Fig. 2) or to bla_{VIM-2} (VIM-2A [5′-ATGTTCAAA CTTTTGAGTAAG-3′] at positions 2029 to 2049; see Fig. 2) and to the 3′-CS (QAC-EXT [5′-AATGCG GA TGTTGCGATTAC-3′] at positions 4151 to 4170; see Fig. 2). These genes were cloned into pPCRScriptCam SK+ (Stratagene), giving recombinant plasmids pLO-1 and pLO-2, respectively. The nucleotide and the deduced protein sequences were analyzed using softwares available over the Internet (http://www.fmi.ch/biology/research_tools.htlm; http://www.ncbi.nlm.nih.gov.; and http://genome.cbs.dtu.dk/services.SignalP/). Multiple nucleotide and protein sequence alignments were carried out online using the program ClustalW (http://www2.cbi.ac.uk/clustalW).

PFGE. Plugs were prepared according to the instructions of Bio-Rad. Whole-cell DNAs from *P. aeruginosa* COL-1, RON-1, and RON-2 isolates were digested with *Xba*I at 37°C overnight. Electrophoresis through a 1% agarose gel in 0.5× Tris-borate-EDTA buffer was performed using a CHEF DRII apparatus (Bio-Rad). Chromosomal fingerprints were compared by eye and assigned to pulsed-field gel electrophoresis (PFGE) types and subtypes (23).

β-Lactamase assays. Cultures of *P. aeruginosa* RON-1 and RON-2 were grown overnight in 10 ml of TS broth, and β-lactamase extracts were obtained and suspended in 0.5 ml of sodium phosphate buffer (0.1 M [pH 7.0]) (16). Hydrolysis of imipenem (100 μM) was determined quantitatively in a Pharmacia ULTROSPEC 2000 spectrophotometer as described previously (16). The protein content was measured using the Bio-Rad DC Protein assay.

Nucleotide sequence accession number. The nucleotide sequence data reported in this work have been assigned to the EMBL/GenBank nucleotide sequence database under accession no. AF263519 and AF263520.

RESULTS

Characterization of the carbapenem-hydrolyzing β-lactamase of *P. aeruginosa* RON-1 and RON-2 and their antibiotic resistance patterns. *P. aeruginosa* RON-1 was a clinical isolate from recurrent urinary tract infections of a tetraplegic patient who had recurrent renal lithiasis. He had been treated by several courses of antibiotics including aztreonam and fosfomycin. His past clinical history also reported urinary infections due to various enterobacterial isolates that were treated with ciprofloxacin or amikacin but not with carbapenems. *P. aeruginosa* RON-2 was isolated from a urinary tract infection of a hospitalized patient and, like *P. aeruginosa* COL-1, had been isolated prior to the isolation date (February 1997) of the bla_{VIM-1}-containing *P. aeruginosa* VR-143/97 in Verona, Italy (12). Patients infected with *P. aeruginosa* RON-1 or RON-2 did not have a history of travel to or hospitalization in Italy or Marseilles, where bla_{VIM-1} and bla_{VIM-2}, respectively, had been first identified. The presence of a carbapenem-hydrolyzing β-lactamase was suspected in *P. aeruginosa* RON-1 and RON-2 as a result of routine antibiotic susceptibility testing that showed that both strains were resistant to ceftazidime and imipenem but remained susceptible to the monobactam aztreonam. Determination of the MICs of β-lactams for these *P.*

TABLE 2. MICs of β-lactams for VIM-2-possessing *P. aeruginosa* clinical strains, *E. coli* DH10B harboring recombinant plasmids pNOR-2002 and pNOR-2003, and reference strain *E. coli* DH10B

β-Lactam (s)[a]	MIC (μg/ml)				
	P. aeruginosa RON-1	*P. aeruginosa* RON-2	*P. aeruginosa* COL-1[b]	*E. coli* DH10B (pNOR-2002 or pNOR-2003)[c]	*E. coli* DH10B
Amoxicillin	>512	>512	>512	>512	4
Amoxicillin + CLA	>512	>512	>512	>512	4
Ticarcillin	512	>512	>512	>512	4
Ticarcillin + CLA	>512	>512	>512	>512	4
Piperacillin	64	32	64	4	1
Piperacillin + TZB	128	64	16	4	1
Cephalothin	>512	>512	>512	128	2
Cefoxitin	>512	>512	>512	64	1
Ceftazidime	64	128	256	16	0.5
Cefotaxime	256	256	>512	8	0.06
Cefepime	64	32	64	0.25	0.03
Cefsulodin	512	512	512	512	0.25
Aztreonam	2	2	0.25	0.12	0.12
Imipenem	32	64	128	2	0.12
Meropenem	4	8	128	1	0.06

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.
[b] MICs of β-lactams for *P. aeruginosa* COL-1 have been reported previously (16).
[c] Recombinant plasmids pNOR-2002 and pNOR-2003 possess the bla_{VIM-2} gene from *P. aeruginosa* RON-1 and RON-2, respectively.

aeruginosa isolates confirmed these results (Table 2). No other *P. aeruginosa* isolate was identified harboring a similar β-lactam resistance profile in the same hospital from January 1997 to May 2000. Additionally, *P. aeruginosa* RON-1 and RON-2 were resistant to multiple aminoglycosides, tetracycline, chloramphenicol, fosfomycin, and fluoroquinolones and were of intermediate susceptibility to rifampin.

As assessed by their ability to hydrolyze imipenem, *P. aeruginosa* RON-1 and RON-2 produced a carbapenem-hydrolyzing β-lactamase (specific activity of 22 and 45 mU per mg of proteins, respectively). PCRs performed with whole-cell DNAs of *P. aeruginosa* RON-1 and RON-2 as templates followed by DNA sequencing revealed that both isolates possessed the same bla_{VIM-2} gene.

PFGE analysis showed that *P. aeruginosa* COL-1, RON-1, and RON-2 had distinguishable profiles (data not shown), although RON-1 and RON-2 were isolated from the same hospital.

The β-lactam resistance markers were not transferred by conjugation from *P. aeruginosa* RON-1 or RON-2 either to rifampin-resistant *E. coli* JM109 or to rifampin-resistant *P. aeruginosa* PU21. Analysis of the plasmid DNAs of *P. aeruginosa* RON-1 and RON-2 did not reveal evidence for any plasmid; electroporation experiments also failed. The bla_{VIM-2} gene was therefore likely chromosomally located in these isolates.

Structure of the bla_{VIM-2} cassette-integrated class 1 integron In58. A recombinant plasmid pNOR-2002 was retained as a result of cloning of RON-1 DNA. *E. coli* DH10B harboring pNOR-2002 gave the same β-lactam resistance profile as observed after cloning of the bla_{VIM-2} gene from *P. aeruginosa* COL-1 and its expression in *E. coli* (Table 2) (16). As reported, the carbapenem resistance was not expressed at a high level in *E. coli* (Table 2) (12, 16). *E. coli* JM109 (pNOR-2002) was resistant to amikacin, kanamycin, tobramycin, and sulfonamides while *E. coli* JM109 (pBK-CMV) was resistant to kanamycin and neomycin (data not shown).

Sequence analysis of the 5,648-bp *Bam*HI insert in pNOR-2002 revealed the structure of a class 1 integron, designated In58, with 5′-CS and 3′-CS ends (Fig. 1). The 5′-CS contained the integrase gene *intI1* and the *attI1* recombination site. Within the integrase gene, a weak promoter P_c (−35 [TGGA CA]; −10 [TAAGCT]) was identified (3). At the 3′-CS end, the *qacEΔ1* disinfectant determinant gene and the *suI1* sulfonamide resistance gene were identified as in most class 1 integrons (18). Between its 5′-CS and 3′-CS ends, In58 contained four gene cassettes containing antibiotic resistance genes (Fig. 1). Just downstream of the 5′-CS, an *aacA7* gene cassette encoding an AAC(6′)-I1 aminoglycoside acetyltransferase was identified as in *Enterobacter aerogenes* (Fig. 2) (2). Its 59-be differed by only three nucleotide substitutions out of 112 (GenBank accession no. U13880). The bla_{VIM-2} gene cassette was inserted as the second position and was identical to that inserted in In56 in *P. aeruginosa* COL-1 (16). The third cassette contained an *aacC1* gene encoding a 3-*N*-aminoglycoside acetyltransferase AAC(3)-I (9). This gene differed by 3 nucleotide changes out of 465 from the gene from *Serratia marcescens*. Only one mutation altered the amino acid sequence with a substitution of a proline for an alanine (GenBank accession no. S68049). The 59-be differed by only two mismatchs out of 108 bp (GenBank accession no. S68049). The fourth cassette contained an *aacA4* gene cassette identical to that reported from *Pseudomonas fluorescens* (GenBank accession no. AAA25685 [10]). It encodes an aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)-Ib′] that confers resistance to gentamicin, netilmicin, and tobramycin but does not modify amikacin.

Novel aminoglycoside resistance genes and structure of the bla_{VIM-2} cassette-integrated class 1 integron In59. A recombinant plasmid pNOR-2003 was retained as a result of cloning RON-2 DNA. *E. coli* DH10B (pNOR-2003) gave the same β-lactam resistance profile as observed for *E. coli* DH10B (pNOR-2002) (Table 2).

Sequence analysis of the cloned 5,061-bp *Bam*HI fragment

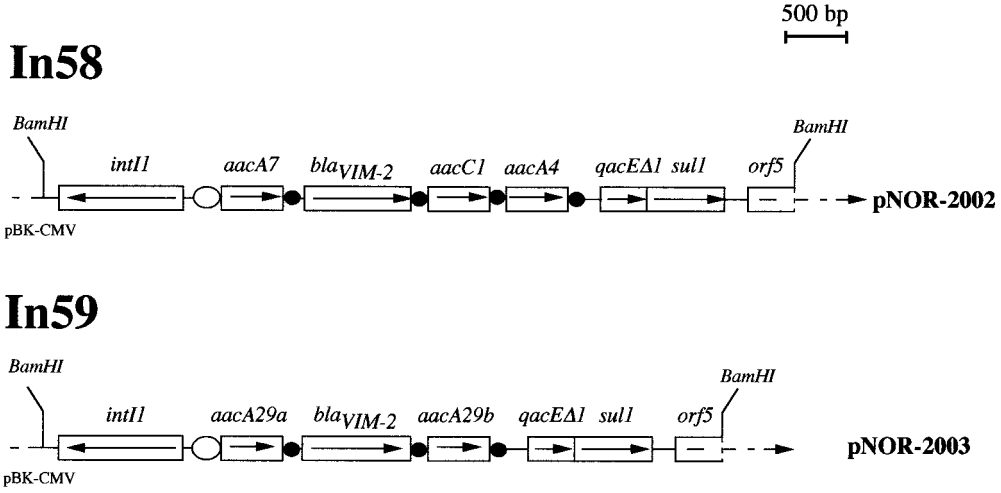


FIG. 1. Comparative structures of the class 1 integrons In58 and In59 that contain the bla_{VIM-2} gene cassette from *P. aeruginosa* RON-1 and RON-2 clinical isolates, respectively. The *intI1* integrase gene, which encodes the integrase, is contained in the 5′-CS, and the 3′-CS found downstream of the integrated gene cassette includes the sulfonamide resistance gene *sul1* and the disinfectant resistance determinant *qacEΔ1*. Inserted genes are indicated by boxes, and the arrows indicate their transcriptional orientation. The 59-be's are represented by black circles and the *attI1* recombination sites by white circles.

of pNOR-2003 showed another class 1 integron, designated In59. It contained 5′-CS and 3′-CS structures with the same P_c promoter as in In58 located downstream from the integrase gene, *intI* (Fig. 1 and 2). The bla_{VIM-2} gene cassette was identical to those found in In56 and In58.

The bla_{VIM-2} gene cassette was flanked by two novel aminoglycoside acetyltransferase cassette-associated genes, named *aacA29a* and *aacA29b*. AAC(6′)-29a shared 96% amino acid identity with AAC(6′)-29b, differing in only four amino acids located near the center of the protein (Fig. 3). AAC(6′)-29a and AAC(6′)-29b shared 35 and 34% identity with the most closely related 6′-*N*-aminoglycoside acetyltransferase *aacA7*-encoded AAC(6′)-I1, respectively. Recombinant plasmids that contained either *aacA29a* (pLO-1) or *aacA29b* (pLO-2) genes were used to transform *E. coli* JM109. *E. coli* JM109 harboring pLO-1 or pLO-2 had the same resistance profile, including resistance or a decreased susceptibility to amikacin, dibekacin, isepamicin, tobramycin, and kanamycin and susceptibility to gentamicin, netilmicin, and sisomicin (Table 3). *E. coli* JM109 (pNOR-2003) expressing *aacA29a* and *aacA29b* genes conferred a level of resistance to aminoglycosides similar to or higher than that observed for *E. coli* JM109 (pLO-1) or *E. coli* JM109 (pLO-2) (Table 3).

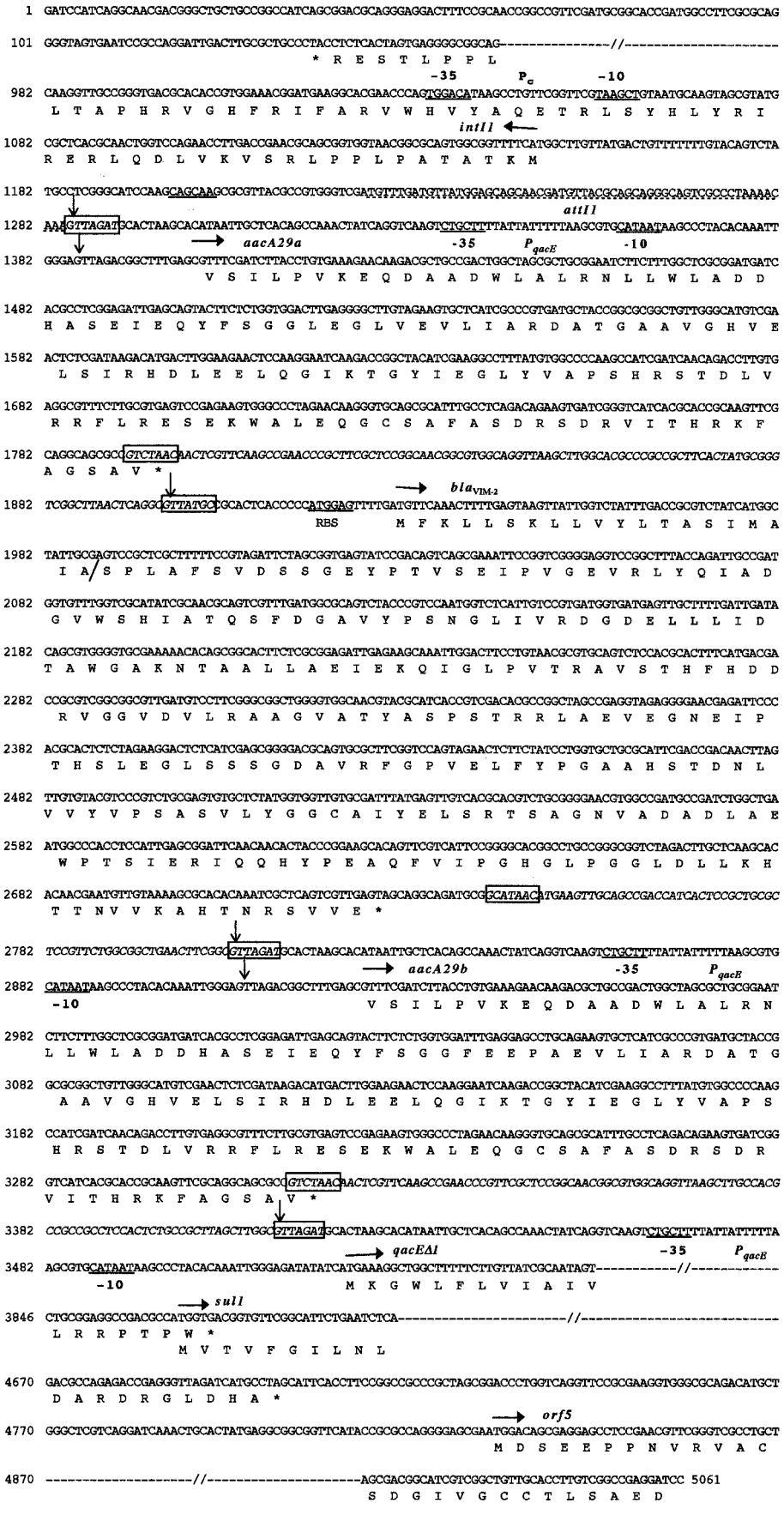
Disk susceptibility tests indicated that both transformants had a 6′-*N*-acetyltransferase of type I [AAC(6′)-I] resistance phenotype. Since 2′- and 6′-*N*-ethylnetilmicin exhibit similar levels of potency against aminoglycoside-susceptible strains, a significant decrease of 2′-*N*-ethylnetilmicin activity compared with that of 6′-*N*-ethylnetilmicin results in protection at the modifying site and can be taken as evidence for production of a 6′-*N*-acetyltransferase (21). The resistance to amikacin and susceptibility to gentamicin is characteristic of the AAC(6′)-I type. The *aacA29* aminoglycoside resistance genes accounted for part of the broad-spectrum aminoglycoside resistance observed for *P. aeruginosa* RON-2 (Table 3).

These *aacA29a* and *aacA29b* acetyltransferase gene cassettes possessed similar 59-be's made of 112 and 105 bp, respectively, that varied from one to the other by 17 bp. The *aacA29a* and *aacA29b* gene cassettes consisted of the region extending from position 1387 to 1898 and from position 2909 to 3413, respectively (Fig. 2). Interestingly, both the 59-be of the *aacA29a* gene cassette and that of the *aacA29b* gene cassette were related to the 111-bp-long 59-be of the *aacA7* cassette, differing by 31 and 36 bp, respectively (2). A fusion of the first 101 bp of the *qacE* cassette (5) to the upstream part of the *aacA29a* and *aacA29b* gene cassettes generated two novel cassettes extending from positions 1286 to 1898 and from 2808 to 3413, respectively (Fig. 2).

TABLE 3. MICs of various aminoglycosides for *P. aeruginosa* RON-2, *E. coli* JM109 harboring recombinant plasmids pLO-1 and pLO-2 containing *aacA29a* and *aacA29b* genes, respectively, and reference strain *E. coli* JM109

Strain	MIC (μg/ml)[a]							
	AMK	DIB	GEN	ISE	NET	TOB	KAN	SIS
P. aeruginosa RON-2	256	256	4	256	8	256	>256	16
E. coli JM109 (pLO-1)	2	2	<0.12	1	<0.12	8	32	0.25
E. coli JM109 (pLO-2)	4	4	<0.12	1	<0.12	4	16	0.25
E. coli JM109 (pNOR-2003)	32	32	<0.12	16	0.25	16	>256	1
E. coli JM109	<0.12	0.25	<0.12	<0.12	<0.12	<0.12	0.5	<0.12

[a] AMK, amikacin; DIB, dibekacin; GEN, gentamicin; ISE, isepamicin; NET, netilmicin; TOB, tobramycin; KAN, kanamycin; SIS, sisomicin.



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AAC(6')-Ic        MIVICDHDNLDAWLALRTALWPSGSPEDHRAEMREILAS--PHHTA-FMARGLDGAFVAFAEVALRYDYVNGCES 72
AAC(6')-Id        MIEACHSVECPGWLQLRFLLWPQDSADEHLAEMAIFVAE--PNRFAQFIAYDEANKPLGFVEAALRSDYVNGTNS 73
AAC(6')-If            MDEASLSMWVGLRSQLWPDHSYEDHILDSQHILSC--PDKYVSFLAINNQSQAIAFADAAVRHDYVNGCES 69
AAC(6')-Ig       MNIKPASEASLKDWLELRNKLWSDS-EASHLQEMHQLLAE---KYALQLLAYSD-HQAIAMLEASIRFEYVNGTET 71
AAC(6')-Ih       MNIMPISESQLSDWLALRCLLWPDH-EDVHLQEMRQLITQ---AHRLQLLAYTDTQQAIAMLEASIRYEYVNGTQT 72
AAC(6')-Ij       MNIMPVSESLMADWLGLRKLLWPDH-DEAHLQEMQRLLQQ---TQSLQLLAYSDTQQAIAMLEASIRYEYVNGTQT 72
AAC(6')-Ik       MNIKPASEASLKDWLKLRIKLWNDL-EESHLQEMHQLLAE---KHALQLLVYSD-DHAVGMLEASIRYEYVNGTET 71
AAC(6')-Il  MDSSPLVRPVETTDSASWLSMRCELWPDGTCQEHQSEIAEFLSGKVARPAAVLIAVAPDGEALGFAELSIR-PYAEECYS 79
AAC(6')-Ir       MKIMPVSEPFLADWLQLRILLWPDHEEDAHLLEMRQLLEQ---PHTLQLLSYNDQQQAVAMLEASIRYEYVNGTQS 73
AAC(6')-Is       MNIMPISESQLSDWLALRSLLWPDH-EDAHLLEMRHVLKQ---TDTLQLLVYSETQLAIAMLEASIRHEYVNGTQT 72
AAC(6')-It       MHIMPITESQLSDWLVLRCLLWPDI-EDADLQEMRQLITQ---AHCLQLLAYTNTQKAIGMLEASIRYEYVNGTQT 72
AAC(6')-Iu       MNILPISESQLSDWLALRSLLWPDH-EEAHLQEMRQLLKQ---TDTLQLLAYSETQHAIAMLEASIRHEYVNGTQT 72
AAC(6')-Iv       MKIMPISESQLSDWLVLRCLLWPDH-EEQHLQEMRQLITQ---AHCLQLLAYTDTQQAIAMLEASIRYEYVNGTQT 72
AAC(6')-Iw       MKIMPISEALLADWLQLRILLWPDH-EDAHLLEMRQLLTR---TDSLQLLAYSETQQPIAMLEASIRHEYVNGTQT 72
AAC(6')-Ix       MNIMPISESQLSDWLALRSLLWPDH-DDAHLLEMHQLLKQ---TDTLQLLAYTDSQQAVAMLEASIRHEYVNGTQT 72
AAC(6')-Iy       MDIRQMNKTHLEHWRGLRKQLWPGHPDDAHLADGEEILQA---DHLASFIAMAD-GVAIGFADASIRHDYVNGCDS 72
AAC(6')-Iz  MIASAPTIRQATPADAAAWAQLRLGLWPDA-DDP-LLELTQSLAD---AEGAVFLACAADGETVGFAEVRLRHDYVNGTES 76
AAC(6')-29a      MSILPVKEQDAADWLALRNLLWLADDHASEIEQYFSGGLE---GLVEVLIARDATGAAVGHVELSIRHD-LEELQG 72
AAC(6')-29b      MSILPVKEQDAADWLALRNLLWLADDHASEIEQYFSGGFE---EPAEVLIARDATGAAVGHVELSIRHD-LEELQG 72


AAC(6')-Ic   SPVAFLEGIYTAERARRQGWAARLIAQVQEWAKQQGCSELASDTDIANLDSQRLHAALGFAETERVVFYRKTLG      146
AAC(6')-Id   SPVAFLEGVYVLPEARRRGIAHALVGAVEIWARNRACTEFASDASTDNPESHRFHQSLGFKETERVVYFRKMLAPE    149
AAC(6')-If   SPVVYLEGIFVIPEQRGHGVAKLLVAAVQDWGVAKGCTEMASDAALDNHISYQMHQALGFEETERVVFFRKRIAG     144
AAC(6')-Ig   SPVGFLEGIYVLPAHRRSGVATMLIRQAEVWAKQFSCTEFASDAALDNVISHAMHRSLGFQETEKVVYFSKKID      145
AAC(6')-Ih   SPVAFLEGIFVLPEYRRSGIATGLVQQVEIWAKQFACTEFASDAALDNQISHAMHQALGFHETERVVYFKKNIG      146
AAC(6')-Ij   SPVAFLEGIYVLPDYRRSGIATHLVQQVEAWAKPFGCIEFASDAALDNRISHAMHQALGFHETERVVYFKKHIG      146
AAC(6')-Ik   SPVAFLEGIYVLPEYRRLGVATLLVRQVEAWAKQFSCTEFASDAALDNVISHAMHRALGFQETERVVYFSKKID      145
AAC(6')-Il   GNVAFLEGWYVVPSARRQGVGVALVKAAEHWARGRGCTEFASDTQLTNSASTSAHLAAGFTEVAQVRCFRKPL       152
AAC(6')-Ir   SPVAFLEGIYVLPEYRRLGVASTLVQQVEHWAKQFACTEFASDAALDNTISHAMHRALGFQETECVVYFKKNIS      147
AAC(6')-Is   SPVAFLEGIYVLPEYRRSGFATQLVQCVEEWAKQFACTEFASDAALENTISHAMHRALGFHETERVVYFKKNIG      146
AAC(6')-It   SPVAFLEGIYVLPEYRRSGIATGLVQHVEIWAKQFACTEFASDATLDNQISHAMHRALGFHETERVVYFKKNIG      146
AAC(6')-Iu   SPVAFLEGIYVLPEYRRSGIATQLVQCVEEWAKQFACTEFASDAALDNTISHAMHRALGFHETERVVYFKKNIS      146
AAC(6')-Iv   SPVAFLEGIYVLPEYRRSGIATGLVQHVEIWAKQFSCTEFASDAALDNQITHAMHQALGFQETERVVYFKKNIG      146
AAC(6')-Iw   SPVAFLEGIYVLPEHRRSGIATQLVQQVEQWAKQYACTEFASDAAIDNTISHAMHQALGFHETERVVYFKKNIS      146
AAC(6')-Ix   SPVAFLEGIYILPEYRRSGIATQLVQYVEEWAKQFACTEFASDAAIDNTISHAMHRALGFHETERVVYFKKNIG      146
AAC(6')-Iy   SPVVFLEGIFVLPSFRQRGVAKQLIAAVQRWGTNKGCREMASDTSPENTISQKVHQALGFEETERVIFYRKRC       145
AAC(6')-Iz   SPVGFLEGWYVQPQWQGSGVGRALLAAVQAWTRDAGCRELASDSRVEDVQAHAAHRACGFEETERVVYFRMPLEPSA  153
AAC(6')-29a  IKTGYIEGLYVAPSHRSTDLVRRFLRESEKWALEQGCSAFASDRS-DRVITHRKFAGSAV                    131
AAC(6')-29b  IKTGYIEGLYVAPSHRSTDLVRRFLRESEKWALEQGCSAFASDRS-DRVITHRKFAGSAV                    131
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FIG. 3. Comparisons of the deduced amino acid sequences of AAC(6′)-29a and AAC(6′)-29b proteins with those of the most closely related aminoglycoside acetyltransferases. Amino acid differences between AAC(6′)-29a and AAC(6′)-29b appear in grey. Identical amino acids in at least 17 sequences are indicated by asterisks; conserved amino acid substitutions are indicated by dots according to the following exchange groups: A, G, P, S, and T; H, K, and R; F, W, and Y; D, E, N, and Q; and I, L, M, and V. Boxed motifs at the carboxy terminal end of the proteins are conserved in most of the enzymes and are absent in AAC(6′)-29 proteins.

DISCUSSION

P. aeruginosa RON-1 and RON-2 were the second and third *P. aeruginosa* unrelated isolates in France that produced a carbapenem-hydrolyzing β-lactamase. As identified previously in *P. aeruginosa* COL-1 isolated from another French region, an identical bla_{VIM-2} gene was found. However, the plasmid location of bla_{VIM-2} found in *P. aeruginosa* COL-1 (as for the bla_{VIM-1} location in *P. aeruginosa* isolate VR-143/97 [12]) was not detected in *P. aeruginosa* RON-1 and RON-2. In all cases the bla_{VIM-2} gene cassettes were identical. Thus, spread of the bla_{VIM-2} gene cassette has already occurred in several class 1 integrons in *P. aeruginosa* in France. This spread may have occurred also in other gram-negative species (*Enterobacteriaceae*) in which carbapenem resistance is not expressed at a high level (8, 12, 16). Additionally, the origin of bla_{VIM-1} and bla_{VIM-2} remains unknown since these genes are not related to any known naturally occurring class B carbapenem-hydrolyzing β-lactamase gene.

Contrary to In56 from *P. aeruginosa* COL-1 that contains a

FIG. 2. Nucleotide sequence of a 5,061-bp *Bam*HI fragment of pNOR-2003 containing the VIM-2 coding sequence and part of integron In59. The start codons of the ORFs are indicated by horizontal arrows, and the deduced amino acid sequences are reported below the nucleotide sequence. Stop codons for each ORF are indicated by asterisks. Dashes in the nucleotide sequence indicate where the reported sequence was identical to published sequences. The −35 and −10 sequences of promoters P_c and putative *qacE*/*qacE*Δ*1* are indicated. The conserved core and inverse core sites located at each cassette boundary are boxed, and the composite 59-be's are italicized. The cassette boundaries are indicated by vertical arrows as well as the putative fusion points of the 5′ end of part of the *qacE* cassette to the *aacA29* gene cassettes. The *attI1* site is underlined with a dashed line.

single bla_{VIM-2} gene cassette, In58 and In59 from *P. aeruginosa* RON-1 and RON-2 contain the same bla_{VIM-2} gene cassette and additional gene cassettes containing multiple aminoglycoside *N*-acetyltransferase genes. Characterization of In59 revealed interesting features. It included two novel *aacA29* aminoglycoside resistance genes showing a G+C content of 55.6%, a value suggesting that they may not have originated from *P. aeruginosa*, thus further underlining the mobility of gene cassettes. The presence of a 101-bp sequence of a *qacE* cassette upstream of each *aacA29* cassette may have resulted from recombination at the sequence GATATAT of the *qacE* cassette and the core site of the ancestral *aacA29* cassette. The fact that this event took place between two nonhomologous recombining sites suggests a RecA-independent process such as an integrase-mediated process (6). The sequence located upstream of the *aacA29* genes that contain a weak promoter sequence for transcription of *qacE* and *sul1* genes (5) may also direct the transcription of the *aacA29* genes.

Comparison of AAC(6′)-29a and AAC(6′)-29b with related members of the 6′-*N*-aminoglycoside acetyltransferases revealed the presence of a large number of completely conserved residues, but an obvious truncation of their carboxyl termini, resulting in shorter proteins of 131 amino acid residues, as opposed to the 144 to 153 residues of all other members (Fig. 3). The AAC(6′)-29 sequences did not contain the highly conserved motif ETERVVYF found in most members of the 6′-*N*-aminoglycoside acetyltransferase family (Fig. 3). Since *E. coli* JM109 expressing each of the AAC(6′)-29 proteins was resistant to amikacin, dibekacin, isepamicin, kanamycin, and tobramycin and remained susceptible to gentamicin, netilmicin, and sisomicin, the AAC(6′)-29 proteins conferred a modified AAC(6′)-I phenotype. Further experiments need to be performed to establish if the truncation of the carboxyl termini is involved in alteration of the substrate specificity of AAC(6′)-29 proteins.

Finally, the simultaneous presence of broad-spectrum β-lactamase and multiple aminoglycoside acetyltransferase gene cassettes in class 1 integrons raises the question of whether the clinical use of either broad-spectrum β-lactams or broad-spectrum aminoglycosides may increase a selective pressure for such multiply resistant isolates and for episomal transfer of these integrons into a susceptible host. Future cure of *P. aeruginosa* infections may fail, as exemplified for infected patients hospitalized in some intensive care units in Japan, and now in Europe (7, 12, 16).

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