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Novel 3-*N*-Aminoglycoside Acetyltransferase Gene, *aac*(*3*)-*Ic*, from a *Pseudomonas aeruginosa* Integron

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A novel gene, *aac*(*3*)-*Ic*, encoding an AAC(3)-I aminoglycoside 3-*N*-acetyltransferase, was identified on a gene cassette inserted into a *Pseudomonas aeruginosa* integron that also carries a bla_{VIM-2} and a *cmlA7* gene cassette. The *aac*(*3*)-*Ic* gene product is 59 and 57% identical to AAC(3)-Ia and AAC(3)-Ib, respectively, and confers resistance to gentamicin and sisomicin.

Acetylation of aminoglycosides by acetyltransferases is one of the major mechanisms of acquired resistance to these compounds (2, 11). Acetylation, by a large number of different enzymes, may occur at 1-, 3-, 6′-, and 2′-amino groups of aminoglycosides and can involve virtually all the medically useful compounds (11). The 3-*N*-aminoglycoside acetyltransferases [AAC(3) enzymes] and the 6′-*N*-aminoglycoside acetyltransferases [AAC(6′) enzymes] are among the modifying enzymes most commonly encountered in clinical isolates (2, 10).

For the AAC(3) enzymes, several different proteins with different substrate specificities have been identified (18). The AAC(3)-I enzymes confer resistance to gentamicin, sisomicin, and fortimicin (astromicin) and are widespread among *Enterobacteriaceae* and nonfastidious gram-negative nonfermenters (9, 18). Two members of this group, AAC(3)-Ia and AAC(3)-Ib, divergent by approximately 30% of amino acid residues, have been identified (17, 21), with minor variants of each lineage (4, 7–9). The *aac*(*3*)-*I* alleles are found on mobile gene cassettes inserted into integrons (17, 18), a location that evidently facilitates their spread among different replicons and eventually among different strains and that likely accounts for the diffusion of these genes in the clinical setting.

In this work we report on the identification and characterization of a novel AAC(3)-I determinant, named *aac*(*3*)-*Ic*, that represents a third evolutionary lineage in this group of resistance genes.

Clinical isolate. *Pseudomonas aeruginosa* VA-182/00 is a multidrug-resistant strain isolated in the year 2000 from an inpatient at the Varese University Hospital in northern Italy. Two acquired β-lactamases (the VIM-2 metalloenzyme and the PER-1 extended-spectrum serine enzyme) that confer an exceedingly broad profile of resistance to β-lactams have already been characterized in this isolate (3). In vitro susceptibility to antimicrobial agents was determined by a macrodilution broth method (13) using cation-adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.). *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used for quality control of susceptibility testing. Aminoglycoside compounds were from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Netilmicin was from Essex (Munich, Germany); isepamicin was from Schering-Plough (Kenilworth, N.J.). *P. aeruginosa* VA-182/00 exhibited resistance to most aminoglycosides (Table 1).

Characterization of the variable region of In*182*, containing a novel AAC(3) determinant. The bla_{VIM-2} metallo-β-lactamase gene of VA-182/00 was shown to be carried on the chromosome (3), but the structure of the cognate integron has not been investigated. A PCR mapping and sequencing approach was adopted to investigate this point. PCR was carried out as described previously, as was sequencing of amplification products and of cloned fragments (16). Both strands were sequenced.

A bla_{VIM-2} specific primer (VIM1/2-for, 5′-TCTGGCTGA ATGGCCCACCTC) was used in combination with the INT/3CS primer (16). This yielded a 2.5-kb amplification product which contained the right-hand moiety of the variable region of the bla_{VIM-2}-containing integron, spanning the 3′ end of the bla_{VIM-2} cassette, and two additional gene cassettes (Fig. 1). The left-hand moiety of this integron was then amplified using the INT/5CS primer (16) and a primer designed on the basis of the sequence of the second gene cassette (AAC-r [see below]). This resulted in a 2.1-kb amplification product, partially overlapping the previous one, spanning the bla_{VIM-2} cassette and most of the second cassette (Fig. 1). Assembly of sequence data revealed an original array of three gene cassettes inserted into the recombination site of a type 1 integron with a 3′-conserved segment (3′-CS) containing a *qacEΔ1* allele; this integron was named In*182* (Fig. 1).

The first cassette of In*182* contains a bla_{VIM-2} gene which differs from other bla_{VIM-2} genes only by a silent G-to-T mutation at position 485 (the first nucleotide of the bla_{VIM-2} open reading frame [ORF] is taken as position 1) (15). The *attC* recombination site (59-base element) of this cassette is identical to that of bla_{VIM-2} cassettes found in other integrons including In*56*, In*58*, In*59*, In*105*, and In*106* (14, 15, 22).

The second cassette contains an ORF encoding a protein

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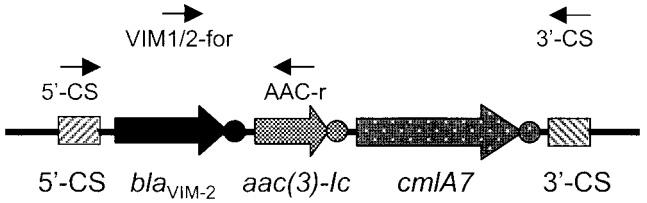

FIG. 1. Structure of the variable region of In*182* from *P. aeruginosa* VA-182/00, which contains a bla_{VIM-2} cassette, the new *aac*(*3*)-*Ic* cassette, and a *cmlA7* cassette. Hatched rectangles, 5′- and 3′-CS regions; filled arrows, cassette-borne resistance genes; circles, their *attC* recombination sites. The locations of primers INT/5CS, INT/3CS, VIM1/2-for, and AAC-r, used for PCR mapping of this region, are also shown (see text for more details).

TABLE 1. MICs of various aminoglycosides for *P. aeruginosa* VA-182/00 and *E. coli* DH5α(pMLR-36/02), carrying the cloned *aac*(*3*)-*Ic* gene[a]

Antibiotic	MIC (μg/ml)		
	P. aeruginosa VA-182/00	*E. coli* DH5α (pMLR36/02)	*E. coli* DH5α (pGEM-T-easy)
Streptomycin	>64	2	2
Kanamycin	>64	1	1
Neomycin	64	2	2
Spectinomycin	>64	16	16
Isepamycin	16	2	2
Tobramycin	>64	1	0.5
Amikacin	32	2	1
Netilmicin	>64	0.5	0.5
Gentamicin	>64	16	0.5
Sisomicin	>64	16	0.5

[a] The susceptibility of the *E. coli* host carrying an empty vector is also shown for comparison.

that exhibits 56.4 to 59.6% identity to known AAC(3)-I enzymes and is notably divergent from both AAC(3)-Ia and AAC(3)-Ib (Fig. 2). This determinant, therefore, represents a third evolutionary lineage of *aac*(*3*)-*I* genes and was named *aac*(*3*)-*Ic*. The *attC* recombination site of the *aac*(*3*)-*Ic* cassette is quite different from those found in gene cassettes carrying *aac*(*3*)-*Ia* (57% nucleotide identity) and shows greater similarity (76% nucleotide identity) to that of the $bla_{GES-1/IBC-1}$ cassettes (Fig. 3). Interestingly, this type of recombination site is also related to that found in the *aadA1* cassette of In*2*, which is almost identical in the regions overlapping the internal 2L and 2R core sites and differs from the former by a large deletion (52 bp) in the central region (Fig. 3).

The third cassette is identical to that found in a plasmid-borne type 1 integron from an uncultured environmental bacterium (A. Schlueter, GenBank accession no. AY115475). It contains an allelic variant of the *cmlA* gene, known to be responsible for nonenzymatic resistance to chloramphenicol (5). The product of the *cmlA* allele of In*182* is different from other known CmlA proteins and is most closely related to CmlA5 (12) and CmlA6 (1), from which it differs by single amino acid residues. Therefore, this allele was named *cmlA7*.

Cloning and expression of the *aac*(*3*)-*Ic* gene in *Escherichia coli*. The *aac*(*3*)-*Ic* gene was amplified from the genomic DNA of *P. aeruginosa* VA-182/00 by using primers AAC-f (5′-GAT GATCTCTACTCAAACC) and AAC-r (5′-TTAGGCAGCA GGTTGAGG) [nucleotides corresponding to the start and stop codons of *aac*(*3*)-*Ic* are underlined]. The 472-bp amplification product was cloned in the plasmid vector pGEM-T-Easy by using the pGEM-T-Easy Vector System I (Promega Corp., Madison, Wis.). One of the recombinants (pMLR36/02), which contained the cloned *aac*(*3*)-*Ic* gene in the same orientation as the P_{lac} promoter flanking the plasmid polylinker, and in which the authenticity of the cloned fragment had been confirmed by sequencing, was used for expression experiments with *E. coli* DH5α. In pMLR36/02 the *aac*(*3*)-*Ic* ORF was expected to be expressed, since the start codon overlaps the termination

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                          1                                                  80
AAC(3)-Ia          In4    --MGIIRTCRLGPDQVKSMRAALDLFGREFGDVATYSQHQPDSDYLGNLLRSKTFIALAAFDQEAVVGALAAYVLPRFEQ
                   pUO901 --MGIIRTCRLGPDQVKSMRAALDLFGREFGDVATYSQHQPDSDYLGNLLRSKTFIALAAFDQEAVVGALAAYVLPKFEQ
                   AC-54/97 --MGIIRTCRLGPDQVKSMRAALDLFGREFGDVATYSQHQPDSDYLGNLLRSKTFIALAAFDQEAVVGALAAYVLPKFEQ
                   InAB1  --MGIIRTCRLGPDQVQSMRAALDLFGREFGDVATYSQHQPDSDYLGNLLRSRTFIALAAFEQEAVVGALAAYVLPKFEQ

AAC(3)-Ib          Stone130 --MSIIATVKIGPDEISAMRAVLDLFGKEFEDIPTYSDRQPTNEYLANLLHSETFIALAAFDRGTAIGGLAAYVLPKFEQ
                   In60   --MSIIATVKIGPDEISAMRAVLDLFGKEFEDIPTYSDRQPTNEYLANLLHSETFIALAAFDRGTAIGGLAAYVLPKFEQ

AAC(3)-Ic          In182  MISTQTKITRLNSQDVGVMRAMLGMFGEAFEDAENYCRAQPSDSYLQDLLCGSGFIAIAALQGQEVIGGLAAYVLPKFEQ


                         81                                                 154
AAC(3)-Ia          In4    PRSEIYIYDLAVSGEHRRQGIATALINLLKHEANALGAYVIYVQADYGDDPAVALYTKLGIREEVMHFDIDPSTAT
                   pUO901 PRSEIYIYDLAVSGEHRRQGIATALINLLKHEANALGAYVIYVQADYGDDPAVALYTKLGIREEVMHFDIDPSTAT
                   AC-54/97 ARSEIYIYDLAVSGEHRRQGIATALINLLKHEANALGAYVIYVQADYGDDPAVALYTKLGIREEVMHFDIDPSTAT
                   InAB1  ARSEIYIYDLAVSGEHRRQGIATALINLLKHEANALGAYVIYVQADYGDDPAVALYTKLGIREDVMHFDIDPSSAT

AAC(3)-Ib          Stone130 ARSEIYIYDLAVASSHRRLGVATALISHLKRVAVELGAYVIYVQADYGDDPAVALYTKLGVREDVMHFDIDPRTAT
                   In60   ARSEIYIYDLAVASSHRRLGVATALISHLKRVAVELGAYVIYVQADYGDDPAVALYTKLGVREDVMHFDIDPLTNS

AAC(3)-Ic          In182  QRKEIYIYDLGVQGAYRRRGIATALINELQRIAHDIGAYVIFVQADYGDDPAVALYTKLGIREDVMHFDIEPQPAA
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FIG. 2. Comparison of the AAC(3)-Ic protein encoded by the second gene cassette of integron In*182* with other AAC(3)-I proteins including AAC(3)-Ia from integron In*4* (21), the AAC(3)-Ia variant from plasmid pUO901 (9), the AAC(3)-Ia variant from *Acinetobacter baumannii* AC-54/97 (8), the AAC(3)-Ia variant from integron InAB1 (7), AAC(3)-Ib from *P. aeruginosa* Stone 130 (17), and the AAC(3)-Ib variant from integron In*60* (4). Amino acid residues conserved in at least two different lineages of AAC(3)-I enzymes are shaded. Residues that differ within each lineage are italicized.

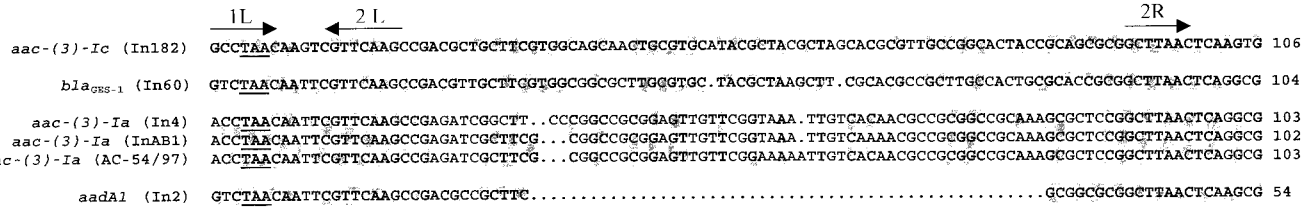

FIG. 3. Nucleotide sequence comparison of the *attC* recombination site of the *aac*(*3*)-*Ic* cassette from integron In*182* with other *attC* recombination sites including that of the bla_{GES-1} cassette of integron In*60* (4) (identical to that of the bla_{IBC-1} cassette [6]), those of the *aac*(*3*)-*Ia* cassettes from integron In*4* (21) [identical to that of the *aac*(*3*)-*Ia* cassette from pUO901 (9)], integron InAB1 (7), and an integron of *Acinetobacter baumannii* AC-54/97 (8), and that of the *aadA1* cassette of In*2* (20). The termination codon of each resistance gene is underlined. The locations of the 1L, 2L, and 2R core sites of the recombination elements (19) are indicated by arrows. Residues that are conserved in other sequences, in comparison with the *attC* recombination site of the *aac*(*3*)-*Ic* cassette, are shaded.

codon of the LacZ α-peptide-encoding sequence, creating an artificial operon. Compared to *E. coli* DH5α, DH5α(pMLR36/02) showed a notable reduction in susceptibilities to gentamicin and sisomicin and a slight reduction in susceptibilities to amikacin and tobramycin. Susceptibilities to streptomycin, kanamycin, neomycin, isepamycin, and netilmicin were unaffected (Table 1). These results confirmed that the *aac*(*3*)-*Ic* gene could contribute to aminoglycoside resistance with a pattern typical of AAC(3)-I enzymes (18). However, this enzyme was responsible only in part for the aminoglycoside resistance phenotype of VA-182/00.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL sequence database and assigned accession no. AJ511268.

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