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 (astromycin) 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. The AAC(3)-I enzymes confer resistance to gentamicin and sisomicin.

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 In182, 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-τ [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 In182 (Fig. 1).

The first cassette of In182 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 In56, In58, In59, In105, and In106 (14, 15, 22).

The second cassette contains an ORF encoding a protein...
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\textsubscript{GES-1/IBC-1} cassettes (Fig. 3). Interestingly, this type of recombination site is also related to that found in the aadA1 cassette of In2, 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. YA115475). 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 In82 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'-GATGATCTCTACTCAAACC) and AAC-r (5'-TTAGGCCAGCA GGTGGAG) [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. aeruginosa* host carrying an empty vector is also shown for comparison.

**TABLE 1. MICs of various aminoglycosides for *P. aeruginosa* VA-182/00 and *E. coli* DH5a (pMLR36/02), carrying the cloned aac(3)-Ic gene**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> VA-182/00</td>
<td><em>E. coli</em> DH5a (pMLR36/02)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Neomycin</td>
<td>64</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Isepamicin</td>
<td>16</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Amikacin</td>
<td>32</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Sisomicin</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

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

CmlA5 (12) and CmlA6 (1), from which it differs by single amino acid residues. Therefore, this allele was named cmlA7.

**FIG. 1. Structure of the variable region of In82 from *P. aeruginosa* VA-182/00, which contains a bla\textsubscript{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).
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 unaltered that the blaGES-1 cassette of integron In60 (4) (identical to that of the blaGES-1 cassette [5], those of the aac(3)-Ia cassettes from integron In4 (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 In2 (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.

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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