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

LAURENT POIREL,1 THIERRY LAMBERT,2 SALIH TÜRKOGLU,1,3 ESTHEL RONCO,4 JEAN-LOUIS GAILLARD,3 AND PATRICE NORDMANN1*

Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris-Sud, 94275 Le Kremlin-Bicêtre, 1 Centre d’Etudes Pharmaceutiques, 92296 Châtenay-Malabry, 2 and Service de Microbiologie, Hôpital Raymond Poincaré, Assistance Publique Hôpitaux de Paris, Faculté de Médecine Paris-Ouest, 92380 Garches, France, and Department of Virology and Immunology, Istanbul Faculty of Medicine, 34390 Capa Istanbul, Turkey

Received 19 May 2000/Returned for modification 24 August 2000/Accepted 17 November 2000

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 express the class B carbapenem-hydrolyzing β-lactamase VIM-2 previously identified in Marseilles in the French Riviera. In both isolates, the blaVIM-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 blaVIM-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’)-I, the closest relative of the AAC(6’)-I family enzymes. These aminoglycoside acetyltansferrases 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 enzymes have spread among several gram-negative rods in Japan and are found in 1.3% of the P. aeruginosa isolates (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 identifying antibiotic resistance and a recombinant site, designated 59-be (17, 18). 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 blaIMP-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 IntI1 and two types of recombination sites, attI and 59-be (4, 6, 22). The 5′-CS of the integron structure contains the integrase gene (intI) and the recombinase site attI1 (17, 18). The 3′-CS of class 1 integrons carries the antiseptic-resistance qacEΔI gene, an open reading frame of unknown function (orf5) and the sull gene which confers resistance to sulfonamides (17, 18).

In the course of screening for carbapenem-hydrolyzing Pseudomonas aeruginosa isolates, two P. aeruginosa clinical isolates were positive for blaVIM-type 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 blaVIM-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
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 kanamycin (30 µg/ml) containing TS plates and RON-1 or RON-2 into in vitro-obtained rifampin-resistant E. coli JM109 or rifampin-resistant P. aeruginosa PU21 as transferred as described previously (16). Transfer of RON-1 and RON-2 were extracted, analyzed, and tentatively elec-
troporated as described previously (15). 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-lactama-
tase 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 fosfo-
mycin. 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. aerugi-
nosa 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 isolate date (February 1997) of the *bla*<sub>VIM-2</sub>-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*<sub>VIM-1</sub> and *bla*<sub>VIM-2</sub>, 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 az-
treomycin. Determination of the MICs of β-lactams for these *P.
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 *aacE1* 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′)-II aminoglycoside acetyltransferase was identified as in *Enterobacter aerogenes* (Fig. 2) (2). Its 59-bp differed by only three nucleotide substitutions out of 112 (GenBank accession no. U13880). The *bla*$_{VNIM-2}$ 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 *aacA1* 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-bp differed by only two mismatches 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*$_{VNIM-2}$ 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\textsubscript{VIM-2} gene cassette from 
\textit{P. aeruginosa} RON-1 and RON-2 clinical isolates, respectively. The \textit{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 \textit{qacE}A1. Inserted genes are indicated by boxes, and the arrows indicate their transcriptional orientation. The 59-be’s are represented by black circles and the \textit{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 \textit{Pc}
 promoter as in In58 located downstream from the integrase gene, \textit{intI1} (Fig. 1 and 2). The \textit{blavim-2} gene cassette was identical to those found in In56 and In58.

The \textit{blavim-2} gene cassette was flanked by two novel aminoglycoside acetyltransferase cassette-associated genes, named \textit{aacA29a} and \textit{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 \textit{aacA7}-encoded AAC(6′)-I1, respectively. Recombinant plasmids that contained either \textit{aacA29a} (pLO-1) or \textit{aacA29b} (pLO-2) genes were used to transform \textit{E. coli} JM109. \textit{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). \textit{E. coli} JM109 (pNOR-2003) expressing \textit{aacA29a} and \textit{aacA29b} genes conferred a level of resistance to aminoglycosides similar to or higher than that observed for \textit{E. coli} JM109 (pLO-1) or \textit{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 \textit{aacA29} aminoglycoside resistance genes accounted for part of the broad-spectrum aminoglycoside resistance observed for \textit{P. aeruginosa} RON-2 (Table 3).

These \textit{aacA29a} and \textit{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 \textit{aacA29a} and \textit{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 \textit{aacA29a} gene cassette and that of the \textit{aacA29b} gene cassette were related to the 111-bp-long 59-be of the \textit{aacA7} cassette, differing by 31 and 36 bp, respectively (2). A fusion of the first 101 bp of the \textit{qacE} cassette (5) to the upstream part of the \textit{aacA29a} and \textit{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 \textit{P. aeruginosa} RON-2, \textit{E. coli} JM109 harboring recombinant plasmids pLO-1 and pLO-2 containing \textit{aacA29a} and \textit{aacA29b} genes, respectively, and reference strain \textit{E. coli} JM109

<table>
<thead>
<tr>
<th>Strain</th>
<th>AMK</th>
<th>DIB</th>
<th>GEN</th>
<th>ISE</th>
<th>NET</th>
<th>TOB</th>
<th>KAN</th>
<th>SIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{P. aeruginosa} RON-2</td>
<td>256</td>
<td>256</td>
<td>4</td>
<td>256</td>
<td>8</td>
<td>256</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>\textit{E. coli} JM109 (pLO-1)</td>
<td>2</td>
<td>2</td>
<td>0.12</td>
<td>1</td>
<td>0.12</td>
<td>8</td>
<td>32</td>
<td>0.25</td>
</tr>
<tr>
<td>\textit{E. coli} JM109 (pLO-2)</td>
<td>4</td>
<td>4</td>
<td>&lt;0.12</td>
<td>1</td>
<td>&lt;0.12</td>
<td>4</td>
<td>16</td>
<td>0.25</td>
</tr>
<tr>
<td>\textit{E. coli} JM109 (pNOR-2003)</td>
<td>32</td>
<td>32</td>
<td>&lt;0.12</td>
<td>16</td>
<td>0.25</td>
<td>16</td>
<td>&gt;256</td>
<td>1</td>
</tr>
<tr>
<td>\textit{E. coli} JM109</td>
<td>&lt;0.12</td>
<td>0.25</td>
<td>&lt;0.12</td>
<td>&lt;0.12</td>
<td>&lt;0.12</td>
<td>&lt;0.12</td>
<td>0.5</td>
<td>&lt;0.12</td>
</tr>
</tbody>
</table>

a AMK, amikacin; DIB, dibekacin; GEN, gentamicin; ISE, isepamicin; NET, netilmicin; TOB, tobramycin; KAN, kanamycin; SIS, sisomicin.
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 β-lactamase gene was found. However, the plasmid location of β-lactamase found in P. aeruginosa COL-1 (as for the β-lactamase location in P. aeruginosa isolate VR-143/97 [12]) was not detected in P. aeruginosa RON-1 and RON-2. In all cases the β-lactamase gene cassettes were identical. Thus, spread of the β-lactamase 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 β-lactamase 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. 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.
single blaVIM-2 gene cassette, In8 and In59 from P. aeruginosa RON-1 and RON-2 contain the same blaVIM-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 acetyltransferase 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 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 ETTERVYF 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).

ACKNOWLEDGMENTS

This work was funded by the Ministère de l’Education Nationale et de la Recherche (grant UPRES-JE 2227), Université Paris XI, Paris, France.

We thank T. Naas and J. Blanchard for helpful discussions.

REFERENCES


ANTIMICROB. AGENTS CHEMOTHER.