In70 of Plasmid pAX22, a bla\textsuperscript{VIM-1}\textsuperscript{*}-Containing Integron Carrying a New Aminoglycoside Phosphotransferase Gene Cassette

MARIA LETIZIA RICCIO, LUCIA PALLECCILI, ROBERTA FONTANA, AND GIAN MARIA ROSSOLINI 1*

Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, I-53100 Siena, 1 and Dipartimento di Patologia, Sezione di Microbiologia, Università di Verona, I-37134 Verona, Italy

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An Achromobacter xylosoxydans strain showing broad-spectrum resistance to β-lactams (including carbapenems) and aminoglycosides was isolated at the University Hospital of Verona (Verona, Italy). This strain was found to produce metallo-β-lactamase activity and to harbor a 30-kb nonconjugative plasmid, named pAX22, carrying a bla\textsuperscript{VIM-1} determinant inserted into a class 1 integron. Characterization of this integron, named In70, revealed an original array of four gene cassettes containing, respectively, the bla\textsuperscript{VIM-1} gene and three different aminoglycoside resistance determinants, including an aac\textsubscript{C}\textsuperscript{4} allele, a new aph-like gene named aph\textsuperscript{A15}, and an aad\textsuperscript{A1} allele. The aph\textsuperscript{A15} gene is the first example of an aph-like gene carried on a mobile gene cassette, and its product exhibits close similarity to the APH(3')-IIa aminoglycoside phosphotransferase encoded by Tn\textsubscript{5} (36% amino acid identity) and to an APH(3')-Ib enzyme from Pseudomonas aeruginosa (38% amino acid identity). Expression of the cloned aph\textsuperscript{A15} gene in Escherichia coli reduced the susceptibility to kanamycin and neomycin as well as (slightly) to amikacin, netilmicin, and streptomycin. Characterization of the 5' and 3' conserved segments of In70 and of their flanking regions showed that In70 belongs to the group of class 1 integrons associated with defective transposon derivatives originating from Tn402-like elements. The structure of the 3' conserved segment indicates that the closest ancestry with members of the In0-In2 lineage. In70, with its array of cassette-borne resistance genes, can mediate broad-spectrum resistance to most β-lactams and aminoglycosides.

VIM-1 and VIM-2 are new metallo-β-lactamases, 90% identical to each other at the sequence level, that have recently been identified in carbenapenem-resistant Pseudomonas aeruginosa nosocomial isolates from the Mediterranean area (12, 17, 21, 27). These enzymes exhibit a very broad substrate specificity (including carbenapenems and most other β-lactams) and were found to be encoded by determinants carried on mobile gene cassettes inserted into integrons (12, 17). Integrons and gene cassettes are elements that participate in a powerful site-specific recombination system, operating in procaryotic genomes, that plays a major role in spreading of antibiotic resistance genes in the clinical setting (see references 8 and 19 for reviews). For the above reasons, the bla\textsuperscript{VIM-1} and bla\textsuperscript{VIM-2} genes could become relevant resistance determinants in the clinical setting.

The bla\textsuperscript{VIM-1} gene was originally cloned from a P. aeruginosa strain that was isolated at the University Hospital of Verona (Verona, Italy), having caused an outbreak in the Intensive Care Unit of that hospital (14). In that strain, the bla\textsuperscript{VIM-1} gene was found to be part of a gene cassette carried on a class 1 integron that was only partially characterized (12) and was located on the bacterial chromosome.

Here we report on the finding of a plasmid-borne bla\textsuperscript{VIM-1} determinant in an Achromobacter xylosoxydans isolate from the same hospital and on the characterization of the bla\textsuperscript{VIM-1}\textsuperscript{*} containing integron carried by that plasmid.

**MATERIALS AND METHODS**

**Bacterial strains and genetic vectors.** A. xylosoxydans subsp. denitrificans AX-22 was isolated in 1998 from a urine specimen of an inpatient at the University Hospital of Verona, Verona, Italy, and identified according to standard procedures (23). Escherichia coli DH\textsubscript{55} (GIBCO-BRL, Gaithersburg, Md.) was used as the host for natural and recombinant plasmids. E. coli MKD-135 (argH rpoB18 rpoB19 recA rpsL) (kindly provided by G. Kholodii, Institute for Molecul Genetics, Russian Academy of Sciences, Moscow, Russia) and P. aeruginosa 10145/3 (an rpoB his derivative of strain ATCC 10145\textsuperscript{T}) were used as recipients in conjugation experiments. Bacteria were always grown aerobically at 37°C unless otherwise specified. The plasmids pBC-SK and pBluescript KS (Stratagene, Inc., La Jolla, Calif.) were used as cloning vectors.

**In vitro susceptibility testing.** Antibiotics were from commercial sources. MICs were determined by a macrodilution broth method (15), using Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, Mich.) and a bacterial inoculum of 5 × 10\textsuperscript{8} CFU per tube. Results were recorded after incubation for 18 h at 37°C and interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (16).

**β-lactamase assays.** Carbapenemase activity in crude cell extracts was assayed spectrophotometrically as described previously (12), using 150 μM imipenem as the substrate. One unit was defined as the amount of activity hydrolyzing 1 nmol of substrate per min under the assay conditions. Inhibition of the carbapenemase activity by EDTA was assayed as described previously (12). Protein concentration in solution was assayed by the method of Bradford with a commercial kit (Bio-Rad, Richmond, Calif.), with bovine serum albumin used as a standard. Analytical isoelectric focusing for detection of β-lactamases was performed as described previously (12).

**DNA analysis methodology.** Basic procedures for DNA extraction, analysis, and manipulation were performed as described by Sambrook et al. (22). Hybridization experiments were carried out using nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) and a probe labeled with \( ^{32} \)P by the random priming technique (22). The probe was a PCR-generated amplicon comprising the entire bla\textsuperscript{VIM-1} open reading frame (ORF) (12). P. aeruginosa VR-143/97 (12) and...
TABLE 1. MICs of various antimicrobial agents for *A. xylosoxydans* AX22, *E. coli* DH5α(pAX22), and *E. coli* DH5α(pMLR-aph70)*

| Antimicrobial agent | MIC (μg/ml) for strain: | AX22 | DH5α(pAX22) | DH5α(pMLR-aph70) | DH5α#
|---------------------|------------------------|------|-------------|-----------------|-------
| Ampicillin >256     | >256                   | —    | —           | 1               | 
| Mezlocillin >256    | >256                   | —    | —           | 1               | 
| Piperacillin >256   | 256                    | —    | 0.5         | 0.5             |      
| Cefotaxime ≥256     | 128                    | 0.12 | ≤0.12       |                 |      
| Ceftazidime ≥256    | 256                    | 0.12 | ≤0.12       |                 |      
| Cefepime ≥256       | 32                     | ≤0.12| —           |                 |      
| Imipenem ≥256       | 4                      | —    | 0.5         | 0.5             |      
| Meropenem ≥128      | 2                      | —    | ≤0.12       |                 |      
| Aztreonam ≥64       | 0.25                   | —    | 0.25        | 0.5             |      
| Gentamicin ≥256     | —                      | 0.5  | 0.5         | 0.5             |      
| Tobramycin ≥256     | —                      | 0.5  | 0.5         | 0.5             |      
| Netilmicin ≥256     | —                      | 0.5  | 0.25        |                 |      
| Amikacin ≥64        | —                      | 1    | 0.5         |                 |      
| Kanamycin ≥128      | —                      | 16   | —           |                 |      
| Streptomycin        | —                      | —    | 4           | 2               |      
| Neomycin ≥256       | —                      | 4    | 0           | 0.5             |      
| Spectinomycin       | —                      | —    | 8           | 8               |      

* The susceptibility of *E. coli* DH5α is also shown for comparison.
* MICS of aminoglycosides for *E. coli* DH5α(pBluescript KS) were identical to those for DH5α.
* —, not assayed.

P. aeruginosa ATCC27853 were included as positive and negative hybridization controls, respectively, in colony blot hybridization. Conjugation experiments were performed on MH agar plates. The initial donor/recipient ratio was 0.1. Mating plates were incubated at 30°C for 14 h. *E. coli* transconjugants were selected on MH agar containing kanamycin (25 μg/ml) plus rifampin (400 μg/ml). *P. aeruginosa* transconjugants were selected on MH agar containing tobramycin (16 μg/ml) plus rifampin (400 μg/ml). The detection sensitivity of the assay was ≥1 · 10⁶ transconjugants/recipient. Elektroporation of *E. coli* was performed with a Gene-Pulser apparatus (Bio-Rad) according to the manufacturer’s instructions. DNA sequences were determined on both strands of plasmid templates as described previously (12). Similarity searches against sequence databases were performed using an updated version of the BLAST program at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/). Computer analysis of the sequence data and multiple-sequence alignment were performed using the Wisconsin Package (version 8.1; Genetics Computer Group Inc., Madison, Wis.) and the Clustal W program at the server of the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/).

**RESULTS AND DISCUSSION**

Identification of *A. xylosoxydans* clinical isolate producing a plasmid-encoded VIM-like metallo-β-lactamase. *A. xylosoxydans* AX22 exhibited broad-spectrum resistance to β-lactams and aminoglycosides (Table 1). The β-lactam resistance pattern (including piperacillin, ceftazidime, and carbapenem resistance) was unusual for this species (5, 25), and the high-level carbapenem resistance suggested the production of an acquired carbapenemase. In fact, carbapenemase activity was detected in a crude extract of AX22 (specific activity, 184 ± 12 U/mg of protein), and this activity was reduced (≥80%) after incubation of the crude extract with 2 mM EDTA, suggesting the presence of a metallo-β-lactamase determinant.

A colony blot hybridization showed that DNA from AX22 was recognized by a bla_VIM1 probe (data not shown). Agarose gel electrophoresis of the total DNA extracted from AX22 revealed the presence of plasmid DNA which was recognized by the bla_VIM1 probe in a Southern blot hybridization (Fig. 1). The bla_VIM-containing plasmid, named pAX22, was purified and estimated to be approximately 30 kb in size, based on restriction analysis with various enzymes (Fig. 1). Electroporation of *E. coli* DH5α with the purified plasmid preparation yielded ampicillin-resistant transfectants which contained a plasmid apparently identical to pAX22 (data not shown). DH5α (pAX22) produced carbapenemase activity (specific activity of crude extract, 202 ± 14 U/mg of protein) and, compared to DH5α, exhibited a decreased susceptibility to several β-lactams (Table 1). An isoelectric focusing analysis of a crude extract of DH5α(pAX22) revealed the presence of a single band of β-lactamase activity with an isoelectric pH of 5.1 (data not shown), suggesting that the VIM-like enzyme was the only β-lactamase encoded by pAX22. In fact, the pattern of β-lactam susceptibility shown by DH5α(pAX22) was consistent with that previously observed for the same *E. coli* host carrying a cloned copy of the bla_VIM1 gene on a multicopy plasmid vector (12).

The potential for conjugal transfer of pAX22 was examined in diparental matings using either *E. coli* MKD-135 or *P. aeruginosa* 10145/3 as the recipient, but in neither case was conjugal transfer detected. A similar behavior was also observed with other medium-sized plasmids containing integrons carrying cassette-borne metallo-β-lactamase genes (11, 17). It would be interesting to further investigate the biology of these plasmids and their potential role in the dissemination of resistance determinants.

**Structure of bla_VIM-containing integrion carried by plasmid pAX22.** The 8.6-kb *EcoRI* fragment of pAX22 containing the bla_VIM1 determinant (Fig. 1) was subcloned into plasmid pBSCSK to obtain the recombinant plasmid pLUP-86R (Fig. 2). Restriction mapping and partial sequence analysis of this fragment revealed that the bla_VIM1 determinant was inserted into a class 1 integrin which carries an original array of four gene cassettes and was named In70 (Fig. 2).

The 5' conserved segment (5'-CS) of In70 contains an intI1 allele typical of class 1 integrons (19) and is identical to that of In0 (2) except for a single G → T transversion in the −35 hexamer of the P1 promoter (formerly Pm). Therefore, the configuration of the P1 promoter of In70 is hybrid, with a TTG
ACA −35 hexamer typical of the strong P1 promoter and a TAAGCT −10 hexamer typical of the weak P1 promoter (13), and is identical to that of the promoter previously found in the integron from plasmid pBWH301 (4) but different from the configurations of the promoters found in most other integrons (reference 13 and references therein, as well as results of a BLAST search on updated sequence databases). Compared to the bla<sub>VIM-1</sub>-containing integron cloned from <i>P. aeruginosa</i> VR-143/97 (12), the 5'-CS of In70 exhibits the same G→T transversion in the −35 hexamer that differentiates the P1 promoter of In70 from that of In0 (see above), as well as a G→C transversion in the region between the −35 and −10 hexamers of the same promoter. A similar finding may indicate cassette exchange rather than integron transfer in the dissemination process of the <i>bla</i><sub>VIM-1</sub> cassette.

At the left-hand end, the 5'-CS of In70 is bounded by a 25-bp IRi sequence identical to that associated with other integrons of the same family (9, 18). The nucleotide sequence flanking IRi was found to be identical to that flanking IRi of In5, of In18, and of In31 but different from that flanking the IRi of other integrons (Fig. 3A).

The 3'-conserved segment (3'-CS) of In70 closely resembles that of In0 and In2 (3) but directly merges with a truncated tni module typical of Tn402-like elements, with no insertion sequences present at the 3'-CS-tniBΔ junction (Fig. 2). Compared to In0 and In2 (3), the 3'-CS-tniBΔ junction of In70 exhibits a small deletion, apparently involving both sides (Fig. 3B), which could have been generated following an imprecise excision of IS1326. For this reason, the truncated tniB allele present in In70 was named tniBΔ3.

Altogether, these findings indicate that In70 is another member of the group of class 1 integrons associated with defective transposon derivatives originating from Tn402-like elements. Among them, In70 apparently shares the closest ancestry with members of the In0-In2 lineage (2, 3). However, the finding of an IRi-flanking region which is different from that of either In0 or In2 but identical to those of integrons of the In5-In31 lineage (11) raises the question of the mobility of the defective transposon carrying the integron.

**Gene cassettes in In70.** In70 contains four gene cassettes, each including a resistance gene. The first cassette carries a <i>bla</i><sub>VIM-1</sub> determinant (Fig. 2) and is identical to the <i>bla</i><sub>VIM-1</sub> cassette from <i>P. aeruginosa</i> VR-143/97, the VIM-1 index strain previously isolated in the same hospital (12). This finding suggests a common origin for the two determinants, although the original source

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**FIG. 2.** Physical map of the 8.6-kb EcoRI insert of plasmid pLUP-86R. The thick line represents the DNA insert; the thin lines represent vector sequences. Abbreviations: S, SalI; RI, EcoRI; B, BamHI; Bg, BglII; C, CiaI; K, KpnI; N, NcoI; P, PstI; Sa, Sau3A; X, XhoI. The horizontal dotted lines above the map indicate the sequenced regions. The structure of In70 and the locations of the various integron components are shown below the map. ORFs are indicated by arrows (truncated when not complete), the 59-be's of the gene cassettes are indicated by ovals, and the cassette boundaries are indicated by vertical bars. The 3'-CS has been sequenced only at its junctions, while the structure of the internal region has been deduced by comparison of the restriction map with that of the 3'-CS of other integrons (9).

**FIG. 3.** (A) Comparison of the IRi (boldfaced) and flanking region of In70 with those of other integrons of the same family. Identical flanking sequences are underlined. The sequences of In1, In2, In3, In4, and In5 (9), of In13, In16, and In18 (18); and of In31 (11) have all been reported previously. (B) Nucleotide sequence at the junction between the 3'-CS (boldfaced) of In70 and the truncated tni module, and comparison with the corresponding regions of In0 and In2 (3). The sites of insertion of IS1326 in In0 and in In2 and that of IS1353 in In2 are indicated.
remains unknown. It also indicates that \( \text{bla}\text{VIM-1} \), similar to \( \text{bla}\text{VIM-2} \) (17), can even be found on plasmids.

The second cassette of In70 contains an \( \text{aacA4} \) allele (Fig. 2) encoding an \( \text{AAC(6}^{\text{R}} \text{)}-\text{II aminoglycoside acetyltransferase} \) identical to that encoded by the \( \text{aacA4} \) allele from plasmid \( \text{pIP1855} \) of \( \text{Pseudomonas fluorescens} \) BM2687 (10).

The third cassette of In70 is original and contains a 795-bp ORF encoding a protein which exhibits the closest sequence similarities with an \( \text{APH(3}^{\text{R}} \text{)}-\text{IIb aminoglycoside phospho-} 

transferase from \( \text{P. aeruginosa} \) (7) (38% identity) and with the \( \text{APH(3}^{\text{R}} \text{)}-\text{IIa} \) enzyme encoded by Tn5 (1) (36% identity). The similarity to the above proteins is evident over the entire sequence and includes the three highly conserved motifs and the invariant or highly conserved residues typical of these enzymes (24) are shaded.

![FIG. 4. Amino acid sequence comparison among the putative \( \text{APH(3}^{\text{R}} \text{)} \) enzyme encoded by the third cassette of In70 [\( \text{APH(3}^{\text{R}} \text{)-In70} \)] and its closest relatives, the \( \text{APH(3}^{\text{R}} \text{)}-\text{IIa} \) enzyme encoded by Tn5 [\( \text{APH(3}^{\text{R}} \text{)-IIa} \)] (1) and the \( \text{APH(3}^{\text{R}} \text{)}-\text{IIb} \) enzyme from \( \text{P. aeruginosa} \) [\( \text{APH(3}^{\text{R}} \text{)-IIb} \)] (7). Identical residues are each indicated by an asterisk; conservative amino acid substitutions are each indicated by a dot. The three highly conserved motifs that are known to be involved in catalytic activity of the \( \text{APH} \) enzymes are overlined, and the invariant or highly conserved residues typical of these enzymes (24) are shaded.]

![FIG. 5. (A) Comparison of the 5’-be of the \( \text{aphA15} \) cassette of In70 (\( \text{aphA15} \)) with that of the \( \text{aacC1} \) cassette of In4 from Tn1696 (19). Identical residues are indicated by vertical bars. (B) Nucleotide sequence at the junction between the \( \text{aadA1} \) cassette and the the 3’-CS internal boundary of In70, showing the structure of the deleted 5’-be of the \( \text{aadA1} \) cassette. The termination codon of the \( \text{aadA1} \) coding sequence is italicized, and the conserved recombination core site located at the 3’-CS internal boundary is boxed. The 1L internal core site of the 5’-be (26) is overlined by an arrow. The deleted region, in comparison with the 5’-be of the \( \text{aadA1} \) cassette of In2 (19), is indicated below the sequence and the 2L and 2R internal core sites (26) are underlined by arrows.]

aphA15 GTCTAGAATTCCTCAAGCGGAGATC-GCTTCGCGCCGCGGGATCTTCTCTGAAATTTTCATAAAACGGCGCGCGCCACAGGCTCCTCAGCGCTTAACTGGGC

aacC1 ACCCTAGAATTCCTCAAGCGGAGATC-GCTTCGCGCCGCGGGATCTTCTCTGAAATTTTCATAAAACGGCGCGCGCCACAGGCTCCTCAGCGCTTAACTGGGC

A

aphA15

\text{aadA1 ORF} . . TAA . gtcctataa . .

\text{attgttcaagcgg} . . 16 \text{ bp} . . \text{ctgttaac}

B

\text{1L}

\text{2R}

\text{3’-CS}

\text{2L}

\text{2R}

\text{3’-CS}

\text{2L}

\text{2R}
vector pBluescript KS to obtain recombinant plasmid pMLR-aph70, in which the aphA15 determinant was located downstream of the Pfac promoter flanking the vector polylinker. E. coli DH5α(pMLR-aph70) showed a significant reduction of kanamycin and neomycin susceptibility, while the MICs of other aminoglycosides, including gentamicin, tobramycin, netilmicin, amikacin, streptomycin, and spectinomycin, were apparently unaffected or only slightly increased (Table 1), revealing a pattern consistent with that of APH(3′) enzymes (24). The 59-base element (59-be) was nearly identical to that of In70 was found to be nearly identical to that of the aminoglycoside resistance gene, catB3, in an integron in pWH1301. Anti microb. Agents Chemother. 39:686–693.

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