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Clonal Relatedness and Conserved Integron Structures in Epidemiologically Unrelated *Pseudomonas aeruginosa* Strains Producing the VIM-1 Metallo-β-Lactamase from Different Italian Hospitals

Maria Letizia Riccio,[1] Lucia Pallecchi,[1] Jean-Denis Docquier,[1]† Stefania Cresti,[1] Maria Rosaria Catania,[2] Laura Pagani,[3] Cristina Lagatolla,[4] Giuseppe Cornaglia,[5] Roberta Fontana,[5] and Gian Maria Rossolini[1]*

Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, Siena,[1] Dipartimento di Medicina Sperimentale, Seconda Università di Napoli, Naples,[2] Dipartimento di Scienze Morfologiche Eidologiche e Cliniche, Sezione di Microbiologia, Università di Pavia, Pavia,[3] Dipartimento di Scienze Biomediche, Sezione di Microbiologia, Università di Trieste, Trieste,[4] and Dipartimento di Patologia, Sezione di Microbiologia, Università di Verona, Verona,[5] Italy

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Three epidemiologically independent *Pseudomonas aeruginosa* isolates, representative of the first VIM-1 metallo-β-lactamase producers detected at three different hospitals in northern Italy, were investigated to determine their genomic relatedness and to compare the structures of the genetic supports for the VIM-1 determinants. The three isolates, all of serotype O11, appeared to be clonally related according to the results of genotyping by macrorestriction analysis of genomic DNA by pulsed-field gel electrophoresis and random amplification of polymorphic DNA. Investigation of the genetic support for the bla_{VIM-1} determinant revealed that it was carried on identical or almost identical integrons (named In70.2 and In70.3) located within a conserved genomic context. The integrons were structurally related to In70 and In110, two plasmid-borne bla_{VIM-1}-containing integrons from *Achromobacter xylosoxidans* and *Pseudomonas putida* isolates, respectively, from the same geographic area (northern Italy) and were found to be inserted close to the *res* site of a Tn*5051*-like transposon, different from any of those described previously, that was apparently carried on the bacterial chromosome. The present findings suggest that the three VIM-1-producing isolates are members of the same clonal complex which have been spreading in hospitals in northern Italy since the late 1990s and point to a common ancestry of their bla_{VIM-1}-containing integrons.

During the last decade, acquired metallo-β-lactamases (MBLs) have started to emerge among *Pseudomonas aeruginosa* isolates and other gram-negative nosocomial pathogens (2, 19, 32). The production of these enzymes, which exhibit an exceedingly broad substrate specificity and which are not susceptible to conventional β-lactamase inhibitors (5, 13, 29, 34), enables the microbial host to be resistant to virtually all β-lactams (including carbapenems) and drastically reduces the repertoire of agents useful for antimicrobial chemotherapy. Moreover, acquired MBL genes are often clustered with other resistance determinants within the variable region of integrons (32). In fact, MBL-producing strains usually exhibit a multidrug-resistant phenotype that also includes non-β-lactam agents and may represent a therapeutic challenge (19).

Two major types of acquired MBLs, the IMP and VIM enzymes, have been identified in gram-negative nosocomial pathogens, with a number of allelic variants known for each type (http://www.lahey.org/Studies/). A third type of acquired MBL, SPM-1, has recently been described in *P. aeruginosa* isolates from Brazil (46), and a fourth type, GIM-1, has been described in *P. aeruginosa* isolates from Germany (M. Castanheira et al., Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-669, 2003; GenBank/EMBL nucleotide sequence accession no. AJ720678).

The VIM-type enzymes are among the β-lactamases with the broadest substrate specificities (4, 5, 34, 45). They were first detected in Europe (12, 15, 21, 34, 35, 48), where they are apparently the most common type of acquired MBL, but they have also been reported in the Far East and in the Americas (see references 16 and 32 and references therein; 41, 45), revealing a broad distribution. Similar to the bla_{IMP} genes, the bla_{VIM} determinants are also carried on integron-borne gene cassettes (32) and can exploit the integron recombination system for mobility.

VIM-1 was the first described allelic variant, and to date it has been reported only in Italy and Greece (15, 28, 38). In the study described in this paper we investigated three epidemiologically unrelated clinical isolates of *P. aeruginosa*, representative of the first VIM-1 producers isolated at three different Italian hospitals, and analyzed the genetic contexts of their bla_{VIM-1} determinants. The results suggested that the three

* Corresponding author. Mailing address: Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, Policlinico "Le Scotte," 53100 Siena, Italy. Phone: 39-0577-233134. Fax: 39-0577-233334. E-mail: rossolini@unisi.it.

† Present address: Centre d'Ingénierie des Protéines & Laboratoire d'Enzymologie, Université de Liège, Allée de la Chimie, B-4000 Liège, Belgium.

isolates were members of the same clonal complex and revealed conserved integron structures, pointing to a common ancestry of the bla_{VIM-1}-containing integrons carried by those isolates.

(These results were presented in part at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 14 to 17 September 2003 [M. L. Riccio, L. Pallecchi, J. D. Docquier, S. Cresti, R. Fontana, L. Pagani, C. Lagatolla, and G. M. Rossolini, Abstr. 43rd Intersci Conf. Antimicrob. Agents Chemother., abstr. C2-2020, 2003].)

MATERIALS AND METHODS

Bacterial isolates. *P. aeruginosa* VR-143/97, the VIM-1 index strain (15), was isolated in February 1997 from a surgical wound of an inpatient in the general intensive care unit of the University Hospital of Verona, where it caused an outbreak (3). *P. aeruginosa* PPV-108 was isolated in November 1998 from a decubitus ulcer of an inpatient in the Vascular Surgery Unit of the University Hospital of Pavia, and it was one of the first VIM-1 producers detected in that hospital (39). *P. aeruginosa* TS-832035 was isolated in February 1999 from the blood of an inpatient admitted to the general intensive care unit of the University Hospital of Trieste, and it was the first VIM-1 producer detected in that hospital (39). The last two isolates have already been reported to produce a VIM-type β-lactamase on the basis of hybridization assays (39); the nature of their VIM determinants was confirmed as bla_{VIM-1} in this work by sequencing the cognate integrons (see below). The three hospitals are located in three different regions of northern Italy and are hundreds of kilometers apart. A review of the patients' records did not reveal any epidemiological relationship among the three isolates. Identification of the isolates was confirmed with the API 20NE identification system (Bio-Mérieux, Rome, Italy). Serotyping of the isolates was carried out with antisera specific for different *P. aeruginosa* O serotypes (Bio-Rad, Marnes-la-Coquette, France).

In vitro susceptibility testing. MICs were determined by a broth macrodilution method (30) with cation-supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) and a bacterial inoculum of 5×10^5 CFU per tube. The results were recorded after incubation for 18 h at 37°C. The results of susceptibility testing were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (31). The sources of the antimicrobial agents were as described previously (13), unless otherwise specified. Ciprofloxacin was from Bayer (Leverkusen, Germany), levofloxacin and rifampin were from Aventis Pharma (Strasbourg, France), phosphomycin was from Crinos (Milan, Italy), and azithromycin was from Pfizer (Rome, Italy). Susceptibility to polymyxin B was tested by disk diffusion, and the results were interpreted according to the modified zone criteria proposed by Gales et al. (6). Polymyxin B disks were from Oxoid Ltd. (London, United Kingdom). *P. aeruginosa* ATCC 27853 was used as a control strain for susceptibility testing.

Analytical IEF. Analytical isoelectric focusing (IEF) was performed in precast 5% polyacrylamide gels containing ampholytes (pH range, 3.5 to 9.5; Ampholine PAGplate; Amersham Biosciences) by using the nitrocefin chromogenic substrate for zymographic detection of β-lactamases, as described previously (15).

Determination of outer membrane protein profiles. The outer membrane fraction was prepared essentially as described previously (25) and was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as reported by Laemmli (11). The acrylamide concentration in the resolving gel was 11% (wt/vol). After electrophoresis the protein bands were stained with Coomassie brilliant blue R250. *P. aeruginosa* PAO1 and TNP065 (Δ*oprC* Δ*oprD*) (51) were used as positive and negative controls for OprD production, respectively.

Genotyping techniques. Analysis of genomic DNA macrorestriction patterns by pulsed-field gel electrophoresis (PFGE) was carried out after digestion with SpeI, essentially as described previously (22). PFGE was carried out in 1% agarose gels in 0.5× TBE (Tris-borate-EDTA) buffer (40) with a CHEF-DR III apparatus (Bio-Rad) at 14°C and 6 V/cm for 18 h by using pulse times ranging from 0.5 to 20 s and a 120° switch angle. Bacteriophage lambda concatemers (Bio-Rad) were used as DNA size markers. Clonal relationships based on PFGE patterns were interpreted according to the criteria proposed by Tenover et al. (43). Genotyping by random amplification of polymorphic DNA (RAPD) was carried out with primers 208 (5′-ACGGCCGACC) and 272 (5′-AGCGGGCC AA), and the results were interpreted as described previously (23).

DNA analysis techniques. Basic recombinant DNA procedures were performed as described by Sambrook and Russel (40). Genomic DNA was extracted from *P. aeruginosa* as described previously (15). Extraction of plasmid DNA from *P. aeruginosa* was attempted by the alkaline lysis method (40). Plasmid pBC2AL, which contains part of the bla_{VIM-1} integron of strain VR-143/97 (15), was used to complete the sequence of the 5′ conserved segment (5′-CS) of the bla_{VIM-1}-containing integron of VR-143/97 and to determine the sequence flanking the integron-associated IR_i repeat. The authenticity of those sequence data was confirmed by PCR mapping and sequencing by using VR-143/97 genomic DNA as the template, as described for the other isolates (see below). PCR mapping for characterization of the variable region of the bla_{VIM-1}-containing integrons and of the 5′-CS and flanking sequences was carried out with primers UPSTR/f (5′-TTC GTA ATC GCG GCA TCC GTG) plus VIM-DIA/r (5′-AGG TGG GCC ATT CAG CCA GA) and VIM-DIA/f (5′-CAG ATT GCC GAT GGT GTT TGG) plus INT-3′CS/r (5′-CTC TCT AGA TTT TAA TGC GGA TG). The locations of the primer-specific sequences is shown in Fig. 1. PCR was always performed with the Expand High-Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany) in a 50-μl volume, as described previously (37). The DNA sequences of both strands of the PCR amplicons or the plasmid templates were determined with custom sequencing primers, as described previously (37). Searches for similarities of the sequences with those in sequence databases were performed with the BLAST program at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/). Southern blot analysis was carried out on dried gels, as described previously (49). A PCR-generated amplicon containing the entire bla_{VIM-1}-coding sequence and a 2.2-kb fragment external to the 5′-CS of the integron obtained by BamHI and HindIII digestion of plasmid pBC2AL (Fig. 1) were used as probes in the Southern blot experiments. DNA probes were labeled with ^{32}P by the random priming technique with a commercial kit (Rediprime II DNA Labeling system; Amersham Biosciences).

Nucleotide sequence accession numbers. The nucleotide sequences of the three VIM-1-producing *P. aeruginosa* isolates reported in this paper are presented in the EMBL/GenBank/DDBJ sequence databases under the accession numbers Y18050, AJ581664, and AJ581665, respectively.

RESULTS

Phenotypic features of the VIM-1-producing *P. aeruginosa* isolates. Three epidemiologically unrelated *P. aeruginosa* isolates representative of the first VIM-1 producers isolated at three different Italian hospitals were investigated in this study. All isolates exhibited a multidrug-resistant phenotype, including resistance to antipseudomonal β-lactams (carbenicillin, piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, imipenem, meropenem, and aztreonam), aminoglycosides (gentamicin, netilmicin, and tobramycin), and fluoroquinolones (ciprofloxacin and levofloxacin); but the isolates were susceptible to amikacin (except for VR-143/97, which was intermediate) and polymyxin B. Rifampin MICs were 16 μg/ml for VR-143/97 and TS-832035 and >256 μg/ml for PPV-108. Fosfomycin MICs were 16 μg/ml for VR-143/97 and PPV-108 and >256 μg/ml for TS-832035. Azithromycin MICs were >128 μg/ml for all three isolates.

All isolates belonged to serotype O11. The biochemical profiles of the three isolates, determined with the API 20NE system, were also identical by those tests that can show intraspecies variability. In particular, none of the three isolates produced arginine dehydrolase activity, unlike the majority of *P. aeruginosa* strains.

Analytical IEF analysis of cell extracts for detection of β-lactamase activity showed the presence of β-lactamases of pI 8.4 and 5.2 in all three isolates and the presence of an additional β-lactamase of pI 7.8 in TS-832035. The pI 5.2 band was consistent with VIM-1, while the most alkaline band was likely contributed by one or both of the resident enzymes (AmpC and OXA-50) of *P. aeruginosa* (7). The nature of the pI 7.8 enzyme detected in TS-832035 was not further investigated in this work.

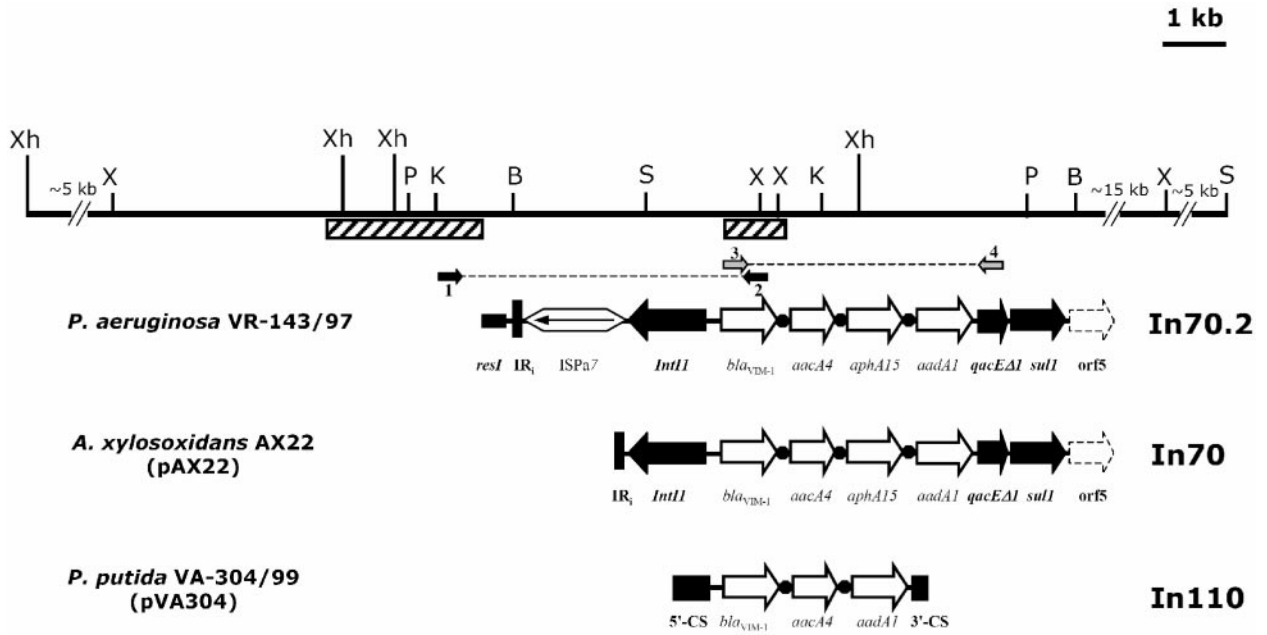

FIG. 1. Schematic representation of the structure of integron In70.2 and flanking regions from *P. aeruginosa* VR-143/97. Open reading frames are indicated by arrows; the *attC* recombination sites (59-base-element sequence) of gene cassettes are indicated by circles. A restriction map of the genomic region containing the integron, as determined by Southern blot analysis, is shown at the top. The locations of the primers used for PCR mapping (1, primer UPSTR/f; 2, primer VIM-DIA/r; 3, primer VIM-DIA/f; and 4, primer INT-3′-CS/r) are also shown at their corresponding positions. The regions corresponding to the probes (probes 2AL-UP and bla_{VIM-1}) used for the Southern blot experiments are indicated by hatched rectangles. The structures of In70 (38) and In110 (20) are also shown for comparison. Restriction endonuclease cleavage sites are indicated as follows: B, BamHI; K, KpnI; P, PstI; S, SpeI; X, XbaI; Xh, XhoI.

Analysis of the outer membrane proteins of the three isolates revealed that each of them apparently lacked OprD (data not shown).

Genomic relatedness of *P. aeruginosa* isolates. The genomic relatedness of the three isolates was investigated by comparing the macrorestriction profiles of SpeI-digested genomic DNA analyzed by PFGE. The PFGE profiles of the three isolates were not identical but differed from each other by less than five bands (Fig. 2), suggesting clonal relatedness. RAPD analysis yielded similar profiles for the three isolates (Fig. 2), also supporting their close genomic relatedness.

Structure of the bla_{VIM-1}-containing integron of VR-143/97. The structure of the bla_{VIM-1}-containing integron of VR-143/97 has been only partially characterized (15). In this work, sequencing was extended to include the entire cassette array, the 5′-CS of the integron, and some of the 5′-CS flanking region (Fig. 1).

The variable region contains four gene cassettes, including the bla_{VIM-1} cassette, an *aacA4* cassette, an *aphA15* cassette, and an *aadA1* cassette. The last cassette exhibits a partially deleted *attC* recombination site and is followed by a *qacEΔ1* allele, typical of the 3′ conserved segment (3′-CS) of *sul1*-associated class 1 integrons (8). The cassette array is identical, except for two point mutations in the *aacA4* gene cassette, to that of In70, a plasmid-borne integron from an *Achromobacter xylosoxidans* strain isolated in 1998 from the same hospital (38) (Fig. 1). It is also related to that of In110, a plasmid-borne integron from *Pseudomonas putida* VA-304/99 isolated in 1999 at the University Hospital of Varese (northern Italy) (20), which differs by the lack of the *aphA15* cassette (20) (Fig. 1).

The cassette array of this integron is also identical to that of an integron recently described from a *P. aeruginosa* isolate from Sicily (EMBL/GenBank accession no. AJ784804), while it is notably different from those of the bla_{VIM}-containing integrons from Greek isolates and from those of integrons containing other bla_{VIM}-type genes (http://www.ncbi.nlm.nih.gov).

The 5′-CS of the integron contains an *intI1* integrase gene and is bounded by a 25-bp IR_i sequence typical of Tn*402*-like elements (36). In this case, however, a 1,669-bp insertion sequence (IS), named ISPa*7*, is present between the end of the integrase gene and IR_i (Fig. 1). ISPa*7* has perfectly matched inverted terminal repeats of 17 bp flanked by direct repeats of 4 bp (Fig. 3). It includes an open reading frame that occupies most of its length and encodes a hypothetical protein of 476 amino acids which exhibits a DDE sequence motif typical of transposases (24). ISPa*7* is very similar to an IS present in a gene island from *P. aeruginosa* strain SG17 M (14) (96% amino acid identity between the two putative transposases) and also to an IS present in the chromosome of *P. aeruginosa* PAO1 (42) (85% identity between the two putative transposases). The inverted repeats of the latter ISs are shorter than those of ISPa*7* (9 and 11 bp, respectively) but are otherwise identical in the overlapping part. ISPa*7* is inserted between the i3 and i4 19-bp repeats that are present in the region internal to IR_i (36) (Fig. 3). Compared to In70, the 5′-CS of this integron differs in the presence of ISPa*7* and two point mutations: a T→G transversion in the −35 hexamer of the P_c promoter (17) and a C→G transversion in the region between the −35 and the −10 hexamers of the P_c promoter.

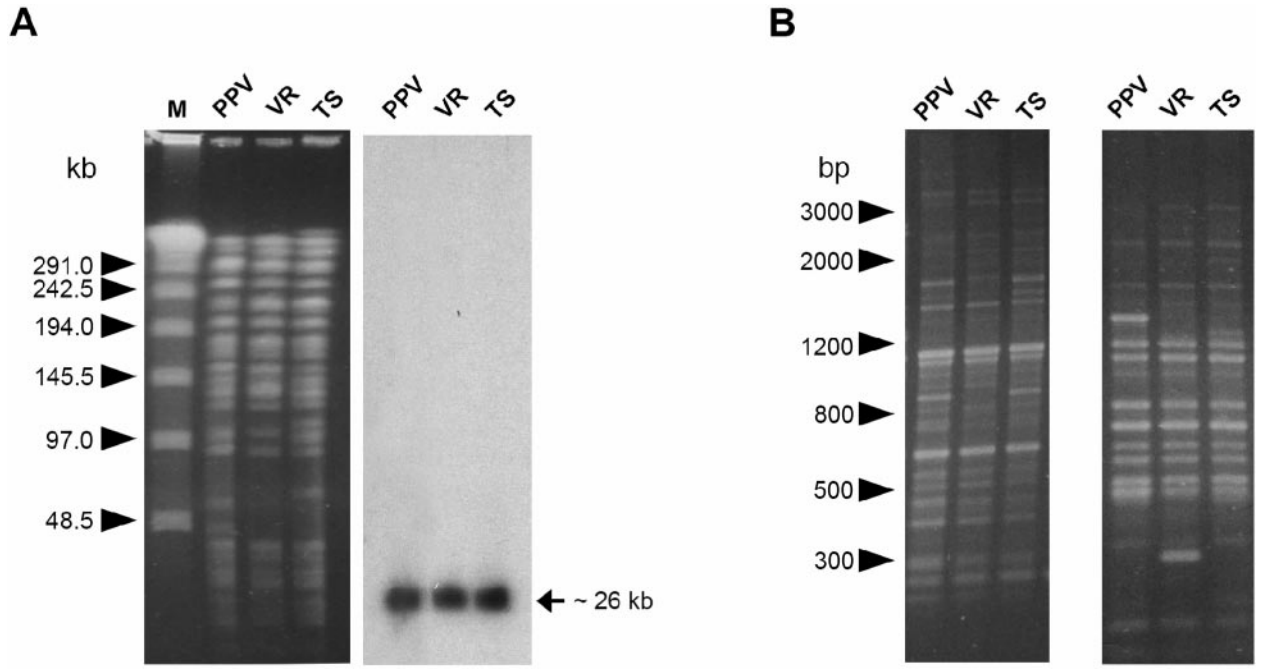

FIG. 2. Analysis of genomic relatedness among the three *P. aeruginosa* isolates. (A) PFGE profiles of SpeI-digested genomic DNAs (left side) and results of Southern blot hybridization obtained with the bla_{VIM-1}-specific probe (right side); (B) RAPD profiles obtained with primer 208 (left side) and primer 272 (right side). VR, isolate VR-143/97; PPV, isolate PPV-108; TS, isolate TS-832035. DNA size markers (lane M) are shown on the left.

The bla_{VIM-1}-containing integron of VR-143/97 was named In70.2 (after its similarity with In70).

The sequence flanking the 25-bp IR_i associated with In70.2 is different from that flanking the 25-bp IR_i associated with In70, while it exhibits remarkable similarity to a region flanking the *res* site of the mercury resistance transposons Tn*5051* (27), Tn*501* (1), and Tn*21* (18) and of Tn*5051* derivatives associated with integrons carrying the bla_{VIM-2} or the bla_{IMP-13} MBL gene recently found in *P. aeruginosa* clinical isolates from Europe (44, 50) (Fig. 3). The similarity was higher with Tn*5051* (90%) than with Tn*21* (78%) (Fig. 3). The region of similarity includes the C-terminal part of *urf2-tnpM* and part of the *res* site, terminating just beyond the *resI* subsite, which in VR-143/97 is not flanked by other *res* subsites, and a transposition module typical of this transposon family (Fig. 3 and data not shown). In the Tn*5051*-like element of VR-143/97, the site of insertion of the Tn*402*-like element carrying In70.2 is different from that of the element carrying In2 in Tn*21* and from that of the element carrying the bla_{VIM-2}- or bla_{IMP-13}-containing integron in the Tn*5051* derivatives from *P. aeruginosa* isolates 81-11963A and 86-14571A (Fig. 3).

Structure of the bla_{VIM-1}-containing integrons and 5′ flanking sequences of PPV-108 and TS-832035. The structures of the bla_{VIM-1}-containing integrons and 5′ flanking sequences of the other two isolates were determined by PCR mapping and direct sequencing, as detailed in the Materials and Methods section. The results showed that the cassette array and the 5′-CS region of the bla_{VIM-1}-containing integron of TS-832035 was identical to that of In70.2, while that of PPV-108 differed by a G→C transversion at position 95 of the *intI1* gene. The latter integron was named In70.3. Concerning the region flanking IR_i, in TS-832035 it was identical to that in VR-143/97, while in PPV-108 the homology started 148 bp upstream, in correspondence to the *resI* subsite (Fig. 3).

Genetic location and context of the bla_{VIM-1}-containing integrons. Plasmid DNA was not detectable in any of the three isolates by agarose gel electrophoresis of plasmid preparations. In all cases, a Southern blot of undigested genomic DNA separated by conventional agarose gel electrophoresis with a bla_{VIM-1}-specific probe showed a single hybridization signal located in correspondence to the band of chromosomal DNA (data not shown).

The environment of the bla_{VIM-1}-containing integrons was investigated by Southern blot analysis of genomic DNA with a bla_{VIM-1}-specific probe and a probe (probe 2AL-UP) corresponding to a region located upstream of the IR_i boundary in VR-143/97 (Fig. 1). The hybridization profiles obtained with the three isolates were apparently identical except for that for PPV-108, for which the KpnI, PstI, and XbaI bands recognized by the bla_{VIM}-specific probe and the XhoI band recognized by the 2AL-UP probe (Fig. 4) were slightly smaller, in agreement with the smaller size of the region between IR_i and the *res* site in isolate PPV-108 (see above). A Southern blot analysis of the macrorestriction bands separated by PFGE with a bla_{VIM-1}-specific probe yielded a single hybridization signal with an approximately 26-kb band for all isolates (Fig. 2). These results suggest that the genetic environment of the bla_{VIM-1}-containing integrons is overall conserved in all three isolates, both in the 3′-CS and the downstream region (at least up to a SpeI site located approximately 20 kb downstream of the 3′-CS) and in the upstream region of IR_i (at least up to an XhoI site located approximately 12 kb upstream of the IR_i boundary). The re-

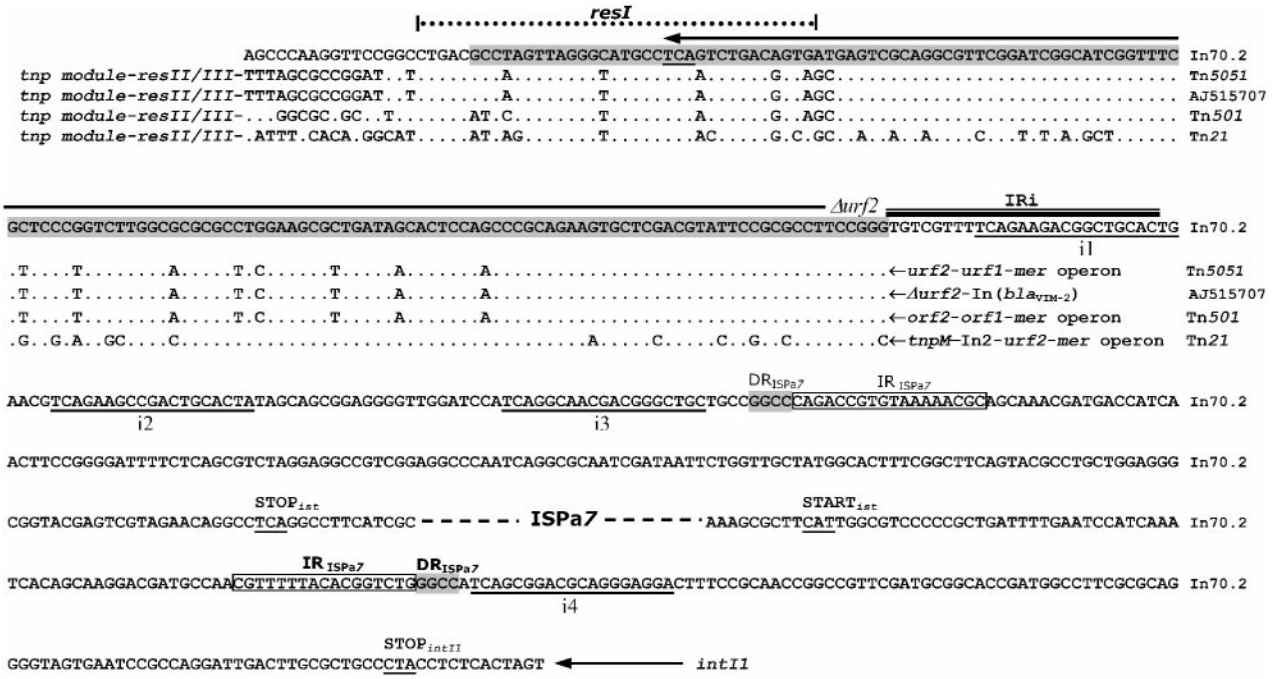

FIG. 3. Nucleotide sequence of part of the 5′-CS of In70.2, showing the insertion site of ISPa*7* and the region flanking IR_i. The stop codon of *intI1* and the start and stop codons of the transposase gene (*ist*) of ISPa*7* are underlined. The 17-bp inverted repeats of ISPa*7* are boxed, and the 4-bp direct repeats flanking ISPa*7* are shaded in gray. IR_i and the i1 to i4 inverted repeats typical of the extremities of Tn*402*-like elements are also indicated. The sequence upstream of IR_i is compared to those of Tn*5051*, the Tn*5051*-like derivative associated with bla_{VIM-2} or bla_{IMP-13} MBL genes, Tn*501*, and Tn*21* (see text for references). Identical nucleotide residues are indicated by dots. The stop codon of Δ*urf2 urf2 orf2 tnpM* and the *resI* site are also indicated. For *P. aeruginosa* PPV-108, which carries In70.3, the region upstream of IR_i shaded in gray is missing.

sults also indicate that the bla_{VIM}-containing integron is apparently present in a single copy in each isolate.

DISCUSSION

Acquired MBLs are emerging resistance determinants of increasing clinical importance (2, 19, 32), and understanding the mechanisms involved in the spread of acquired MBL genes is a relevant issue. To achieve some insight into this, we have subjected to a comparative characterization three epidemiologically unrelated *P. aeruginosa* isolates representative of the first VIM-1 producers detected in three hospitals located in different regions of northern Italy. The three isolates shared the same serotype and biotype; and although they were not identical, they appeared to be clonally related to each other by PFGE and RAPD genotyping, suggesting that the three isolates likely belong to an epidemic clonal complex circulating in northern Italy. In addition, their population structure was consistent with the present knowledge on the population structure displayed by *P. aeruginosa* (33). The bla_{VIM-1}-containing integrons carried by the three isolates had an identical set of gene cassettes and were either identical to each other (the integrons of VR-143/97 and TS-832035, named In70.2) or different by a single nucleotide at the level of the P_c integron promoter (the integron of PPV-108, named In70.3), suggesting that they share a common ancestry. Both In70.2 and In70.3 were associated with a Tn*402* derivative inserted in proximity of the *res* site of a Tn*5051*-like backbone located in a conserved genomic context; however, the insertion sites of the Tn*402* derivatives containing In70.2 and In70.3 in the cognate transposon were different, likely reflecting independent insertional events. Overall, these findings suggest that (i) the bla_{VIM-1}-positive *P. aeruginosa* isolates that emerged in northern Italy in the late 1990s are probably derived from insertional events of Tn*402*-like elements associated with In70-like integrons in a conserved Tn*5051*-like backbone present in the chromosomes of

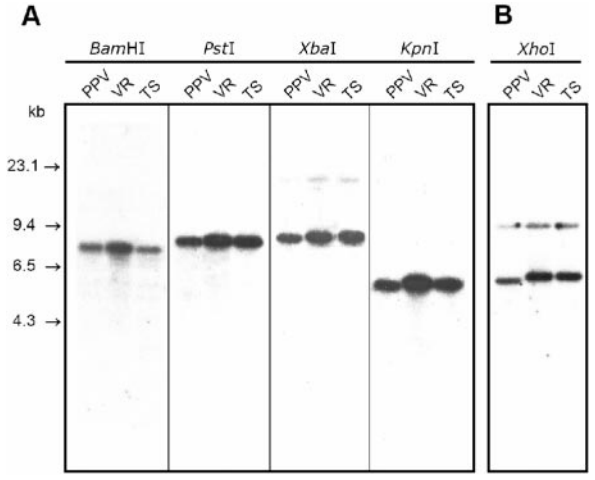

FIG. 4. Results of Southern blot analysis of genomic DNAs of the three *P. aeruginosa* isolates (abbreviations are as indicated in the legend to Fig. 2) obtained with the bla_{VIM-1}-specific probe (A) or the 2AL-UP probe (B). DNA size standards are indicated on the left.

members of an epidemic clonal complex circulating in that area and (ii) VR-143/97 and TS-832035 likely originated from the same ancestor after the occurrence of a similar insertional event, while PPV-108 probably results from a different insertional event that occurred in a member of the same clonal complex. The presence of a Tn*5051* backbone, which is conserved in all three isolates and which is apparently lacking the transposition module (probably due to a recombinational event that occurred at the *res* site, where recombinational events are known to be frequent [27]), supports the view that the insertional events of the Tn*402*-like elements carrying In70.2 and In70.3 occurred after the Tn*5051* backbone had undergone the recombination event at the *res* site, which eliminated the linkage with the transposition module and which probably caused chromosomal fixation. Alternative possibilities, including that of the generation of Tn*5051*-like elements containing In70.2 and In70.3 in different hosts and subsequent delivery to members of the *P. aeruginosa* epidemic clonal complex circulating in this setting, would seem less likely.

The relatedness of In70.2 and In70.3 with the bla_{VIM-1}-containing integrons carried on plasmids from gram-negative nonfastidious nontermenters from the same geographic area, such as In70 (38), points to a common evolutionary origin of these integrons and suggests that the source of the Tn*402*-like elements associated with In70.2 and In70.3 could be represented by plasmids circulating in the gram-negative nonfastidious nontermenter microbiota from that area, from which the elements could have transposed to the *res* site of the Tn*5051*-like transposon backbone present in the *P. aeruginosa* chromosome. In fact, Tn*402*-like elements are known to have a unique targeting mechanism with a strong preference for insertion into or close to *res* sites (9, 26). A major difference between In70 and In70.2 and In70.3 is represented by the presence of an insertion sequence in the 5′-CS of the In70.2 and In70.3 integrons, which is a quite unusual feature. The site of insertion of ISPa*7* is between the i3 and i4 repeats, located close to the i end of Tn*402*-like elements, which are not involved in transposase binding (10), in a region that could play a role in rescuing the interrupted *res* site by substituting for the lost portion (47). The significance of this region as a hotspot for ISPa*7* insertion and the potential functional implications of the presence of ISPa*7* in that position remain to be clarified.

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