Clonal Relatedness and Conserved Integron Structures in Epidemiologically Unrelated \textit{Pseudomonas aeruginosa} Strains Producing the VIM-1 Metallo-\(\beta\)-Lactamase from Different Italian Hospitals

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Three epidemiologically independent \textit{Pseudomonas aeruginosa} isolates, representative of the first VIM-1 metallo-\(\beta\)-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 \textit{bla}\textsubscript{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 \textit{bla}\textsubscript{VIM-1}-containing integrons from \textit{Achromobacter xylosoxidans} and \textit{Pseudomonas putida} isolates, respectively, from the same geographic area (northern Italy) and were found to be inserted close to the \textit{res} site of a \textit{Tn5051}-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 \textit{bla}\textsubscript{VIM-1}-containing integrons.

During the last decade, acquired metallo-\(\beta\)-lactamases (MBLs) have started to emerge among \textit{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 \(\beta\)-lactamase inhibitors (5, 13, 29, 34), enables the microbial host to be resistant to virtually all \(\beta\)-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-\(\beta\)-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 \textit{P. aeruginosa} isolates from Brazil (46), and a fourth type, GIM-1, has been described in \textit{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 \(\beta\)-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 \textit{bla}\textsubscript{IMP} genes, the \textit{bla}\textsubscript{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 \textit{P. aeruginosa}, representative of the first VIM-1 producers isolated at three different Italian hospitals, and analyzed the genetic contexts of their \textit{bla}\textsubscript{VIM-1} determinants. The results suggested that the three

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isolates were members of the same clonal complex and revealed conserved integron structures, pointing to a common ancestry of the \( \text{bla}_{\text{VIM-1}}\)-containing integrons carried by those isolates. (These results were presented in part at the 43rd International Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 14 to 17 September 2003 [M. L. Riccio, L. Pallecchi, J. D. Doquier, S. Cresti, R. Fontana, L. Pagani, C. Lagatolla, and G. M. Rossolini, Abstr. 43rd Intersci Conf. Antimicrob. Agents Chemother., abstr. C2-2003]).

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 \( \beta \)-lactamase on the basis of hybridization assays (39); the nature of their VIM determinants was confirmed as \( \text{bla}_{\text{VIM-1}}\) 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, Marne-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 \( 5 \times 10^{6} \) 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 (15), 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 Biosciences) for the separation 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, Marne-la-Coquette, France).

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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 \( \beta \)-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 \( \mu \)g/ml for VR-143/97 and TS-832035 and \( > 256 \) \( \mu \)g/ml for PPV-108. Fosfomycin MICs were 16 \( \mu \)g/ml for VR-143/97 and PPV-108 and \( > 256 \) \( \mu \)g/ml for TS-832035. Azithromycin MICs were \( > 128 \) \( \mu \)g/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 dehydroxylation activity, unlike the majority of *P. aeruginosa* strains.

Analytical IEF analysis of cell extracts for detection of \( \beta \)-lactamase activity showed the presence of \( \beta \)-lactamases of PI 8.4 and 5.2 in all three isolates and the presence of an additional \( \beta \)-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.
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′/H11032-CS of the integron, and some of the 5′/H11032-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 an *Achromobacter xylosoxidans* strain isolated in 1998 from the same hospital (38) (Fig. 1).

This cassette array of this integron is also identical to that of an integron recently described from an *A. xylosoxidans* 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 IRi sequence typical of Tn402-like elements (36). In this case, however, a 1,669-bp insertion sequence (IS), named ISPa7, is present between the end of the integrase gene and IRi (Fig. 1). ISPa7 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). ISPa7 is very similar to an IS present in a gene island from an *Achromobacter xylosoxidans* strain (96% amino acid identity between the two putative transposases) and also to an IS present in the chromosome of *P. aeruginosa* PAO1 (85% identity between the two putative transposases). The inverted repeats of the latter ISs are shorter than those of ISPa7 (9 and 11 bp, respectively) but are otherwise identical in the overlapping part. ISPa7 is inserted between the i3 and i4 19-bp repeats that are present in the region internal to IRi (36) (Fig. 3). Compared to In70, the 5′-CS of this integron differs in the presence of ISPa7 and two point mutations: a T3G transition in the −35 hexamer of the Pc promoter (17) and a C→G transversion in the region between the −35 and the −10 hexamers of the Pc promoter.
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, associated with In70.2 is different from that flanking the 25-bp IR, associated with In70, while it exhibits remarkable similarity to a region flanking the res site of the mercury resistance transposons Tn5051 (27), Tn501 (1), and Tn21 (18) and of Tn5051 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 Tn5051 (90%) than with Tn21 (78%) (Fig. 3). The region of similarity includes the C-terminal part of ure2-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 Tn5051-like element of VR-143/97, the site of insertion of the Tn402-like element carrying bla_{VIM-2} or the bla_{IMP-13}-containing integron in the Tn5051 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, 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 res 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, 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 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 (at least up to an XhoI site located approximately 12 kb upstream of the IR boundary).
sults also indicate that the bla\textsubscript{VIM}\textsuperscript{-}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 \textit{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 \textit{P. aeruginosa} (33). The bla\textsubscript{VIM}-\textsuperscript{-}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 \textit{Pc} 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\textsubscript{402}\textsuperscript{-}derivative inserted in proximity of the res\textsubscript{i} site; however, the insertion sites of the Tn\textsubscript{402}\textsuperscript{-}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\textsubscript{VIM}-\textsuperscript{-}positive \textit{P. aeruginosa} isolates that emerged in northern Italy in the late 1990s are probably derived from insertional events of Tn\textsubscript{402}\textsuperscript{-}like elements associated with In70-like integrons in a conserved Tn\textsubscript{5051}\textsuperscript{-}like backbone present in the chromosomes of...
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 insertion event, while PPV-108 probably results from a different insertion event that occurred in a member of the same clonal complex. The presence of a Tn5051 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 Tn402-like elements carrying In70.2 and In70.3 occurred after the Tn5051 backbone had undergone the recombinational 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 Tn5051-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\(\text{VIM-1}\)-containing integrons carried on plasmids from gram-negative nonfastidious nonmotile enteric species 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 Tn402-like elements associated with In70.2 and In70.3 could be represented by plasmids circulating in the gram-negative nonfastidious nonmotile enteric microbiota from that area, from which the elements could have transposed to the res site of the Tn5051-like transposon backbone present in the P. aeruginosa chromosome. In fact, Tn402-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 that the site of insertion is ISP7, which is a rather unusual feature. The site of insertion of ISP7 is between the i3 and i4 repeats, located close to the i end of Tn402-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 ISP7 insertion and the potential functional implications of the presence of ISP7 in that position remain to be clarified.

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