

Establishing Clonal Relationships between VIM-1-Like Metallo- β -Lactamase-Producing *Pseudomonas aeruginosa* Strains from Four European Countries by Multilocus Sequence Typing[∇]

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Ten multidrug-resistant *Pseudomonas aeruginosa* strains producing VIM-1-like acquired metallo- β -lactamases (MBLs), isolated from four European countries (Greece, Hungary, Italy, and Sweden), were analyzed for genetic relatedness by several methodologies, including *fliC* sequence analysis, macrorestriction profiling of genomic DNA by pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), and multilocus sequence typing (MLST). The four approaches yielded consistent results overall but showed different resolution powers in establishing relatedness between isolates (PFGE > RAPD > MLST > *fliC* typing) and could usefully complement each other to address issues in the molecular epidemiology of *P. aeruginosa* strains producing acquired MBLs. In particular, the recently developed MLST approach was useful in revealing clonal relatedness between isolates when this was not readily apparent using RAPD and PFGE, and it suggested a common ancestry for some of the VIM-1-like MBL-positive *P. aeruginosa* strains currently spreading in Europe. The MBL producers belonged in three clonal complexes/burst groups (BGs). Of these, one corresponded to the previously described BG4 and included serotype O12 strains from Hungary and Sweden, while the other two were novel and included serotype O11 or nonserotypable strains from Greece, Sweden, and/or Italy. Comparison of the integrons carrying *bla*_{VIM-1}-like cassettes of various isolates revealed a remarkable structural heterogeneity, suggesting the possibility that multiple independent events of acquisition of different *bla*_{VIM}-containing integrons had occurred in members of the same clonal lineage, although a contribution of integrase-mediated cassette shuffling or other recombination mechanisms during the evolution of similar strains could also have played a role in determining this variability.

The emergence of metallo- β -lactamases (MBLs) in major clinical pathogens was first described in the early 1990s in Japan and is now a problem of global magnitude (43). This is a matter of great concern because these enzymes can confer resistance to virtually all β -lactams and are not susceptible to the clinically available β -lactamase inhibitors (15).

Several types of these enzymes have been detected, namely, IMP, VIM, SPM, GIM, and SIM, mostly in *Pseudomonas aeruginosa* and other gram-negative nonfermenters, but also in

Enterobacteriaceae (19, 43). The IMP- and VIM-type enzymes, which are encoded by integron-borne genes, are currently the most widespread, being reported from several continents, and several allelic variants are known for each type (43). In Europe, where they were first detected (17, 27), the VIM-type enzymes are the most prevalent overall (8, 43). Although originally confined to the Mediterranean countries, VIM-producing *P. aeruginosa* strains have recently been detected in northern and eastern Europe. In 2003 and 2004, reports on the isolation of VIM-4 MBL-producing *P. aeruginosa* were published from Sweden and Hungary; in both cases, however, isolates were derived from Greek patients (11, 20). Furthermore, detection of both VIM-2 and VIM-4 in *P. aeruginosa* was reported from Poland (25, 42). A VIM-2-producing strain of *P. aeruginosa* was detected in Sweden in 2004, and in this case, the isolate was also derived from a Greek patient (C. G. Giske, unpublished data). In 2001, multidrug-resistant *P. aeruginosa* strains carrying *bla*_{VIM} genes were found to constitute approximately 20% of all *P. aeruginosa* isolates and 70% of the carbapenem-resistant isolates at a university hospital in northern Italy (16). This finding, together with previously published outbreaks in Greece and Italy (3, 10, 24, 28), underscores the

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TABLE 1. Comparison of findings obtained by various epidemiological typing methods

Isolate	Yr of isolation	Serotype	Place of isolation	VIM variant	Integron	PFGE type	RAPD type	<i>fliC</i> type	Allelic profile	ST	BG ^a	Cluster
VR143	1997	O11	Verona, Italy	VIM-1	In70.2	PA1	RA1	a1	38, 11, 3, 9, 1, 2, 4	227	BG11	I
PPV97	1997	O11	Pavia, Italy	VIM-1	In80	PA2	RA2	a1	38, 11, 3, 9, 1, 2, 4	227	BG11	I
105MG	2003	O11	Pavia, Italy	VIM-1	In107	PA3	RA3	a1	38, 11, 3, 9, 1, 2, 4	227	BG11	I
67MG	2003	NS ^b	Pavia, Italy	VIM-1	In110	PA4	RA4	a1	38, 11, 3, 9, 1, 2, 4	227	BG11	I
85MG	2003	O11	Pavia, Italy	VIM-1	In85	PB	RA5	a1	38, 11, 3, 9, 1, 2, 4	227	BG11	I
Ps100	2002	O11	Heraklion, Greece	VIM-4	In118	PC	RA6	a1	38, 11, 5, 13, 1, 2, 4	230	BG11	I
PA66	2001	O11	Stockholm, Sweden	VIM-4	In114	PD	RB	a1	38, 11, 5, 13, 1, 2, 4	230	BG11	I
PA396	2001	O12	Budapest, Hungary	VIM-4	In108	PE1	RC1	b	39, 5, 2, 4, 4, 4, 3	229	BG4	II
PA555	2002	O12	Pécs, Hungary	VIM-4	In112	PE2	RC2	b	39, 5, 2, 4, 4, 4, 3	229	BG4	II
AK5493	2004	O12	Stockholm, Sweden	VIM-2	In59	PF	RC3	b	39, 5, 2, 4, 4, 4, 3	229	BG4	II
134MG	2004	NS ^b	Pavia, Italy	VIM-1	In110	PG	RD	a2	28, 22, 5, 3, 1, 14, 19	228	BG12	

^a Corresponding to clonal complexes (five or more common alleles).

^b NS, not serotypable.

epidemiological significance of these multidrug-resistant pathogens in Europe.

In Italy, the first *P. aeruginosa* isolates producing the VIM-1 enzyme were found to belong to a single clonal lineage spreading in different hospitals since the late 1990s, which also included the VIM-1-producing index strain VR-143/97 (31). To date, *bla*_{VIM-1} alleles have not been detected in *P. aeruginosa* isolates from any other European country or elsewhere, although VIM-1 has been found in *Escherichia coli* and *Klebsiella pneumoniae* isolates from Greece and Spain (10, 35, 39). However, VIM-4, which differs from VIM-1 by a single amino acid substitution, has been detected in *P. aeruginosa* strains from other European countries (43), and a recent common ancestry for the two genes (and for strains carrying them) is not unlikely given the close similarity between the two variants (29).

Although several studies have explored the epidemiological relationships between isolates at a local level (8, 9, 18, 31), no international comparisons of MBL-producing *P. aeruginosa* strains have been undertaken so far to elucidate questions of continental epidemiology. One limitation has been the lack of a proper methodology for comparing isolates which are temporally and geographically distant and handled by different laboratories. Multilocus sequence typing (MLST) has been shown to be superior to methods such as macrorestriction profiling of genomic DNA by pulsed-field gel electrophoresis (PFGE) and random amplification of polymorphic DNA (RAPD) for addressing questions of regional or global epidemiology (6). This methodology was recently also developed for *P. aeruginosa* (5), although follow-up epidemiological studies have not yet been published.

In this study, we used several genotyping methods, including MLST, PFGE, RAPD, and *fliC* sequence analysis, to explore the relationships between VIM-1-producing *P. aeruginosa* strains spreading in Italy and representative VIM-4-producing isolates from Greece, Sweden, and Hungary. The resolution of different methods was evaluated in view of their applicability to understanding the international epidemiology of MBL-producing *P. aeruginosa* strains.

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MATERIALS AND METHODS

Bacterial strains and phenotypic and MBL determinant characterization. The *P. aeruginosa* strains investigated in this study are listed in Table 1. They included (i) two representatives of a VIM-1-producing dominant Italian clone spreading in different hospitals since 1997 (the index strain VR-143/97 and strain PPV97, which were epidemiologically unrelated to each other and slightly different by PFGE profiling and which carried *bla*_{VIM} integrons with different structures) (12, 31); (ii) four additional VIM-1-producing *P. aeruginosa* isolates from Italy (representative of those spreading more recently in a long-term care and rehabilitation facility in northern Italy [L. Pagani et al., unpublished results]); and (iii) four VIM-4-producing isolates from Greece ($n = 1$), Sweden ($n = 1$) (11), and Hungary ($n = 2$) (20). In addition, a VIM-2-producing isolate from Sweden (AK5493), obtained from a Greek national patient who had recently been hospitalized in Greece, was also included in the comparison. For all of the unpublished isolates, identification was confirmed by the API 20 NE test (bioMérieux, Marcy l'Etoile, France), the presence of *bla*_{VIM} genes was established by PCR, using consensus primers for *bla*_{VIM} (20), and the allelic variant of the MBL gene was determined by direct sequencing of both strands of PCR products (31). Antimicrobial susceptibility testing was performed with Etest (AB Biodisk, Solna, Sweden), using CLSI breakpoints for interpretation (2). Serotyping was carried out with monoclonal O antigen sera (Bio-Rad, Marnes-La-Coquette, France) as described in the manufacturer's instructions.

***fliC* sequence analysis.** A fragment of the coding region of the *fliC* gene from each strain was amplified by primers *fliA1* and *fliA2* (37). DNA was extracted by boiling a 100- μ l suspension of bacterial cells in sterile water. PCR products of 1,300 bp correspond to b-type *fliC*, while PCR products in the range of 1,164 to 1,185 bp correspond to a-type *fliC*. Sequencing of the PCR products was performed with the amplification primers and the sequencing primers *fliC*_{seqA} (5'-ATC GAC GAG ATG AGC GCA-3') and *fliC*_{seqB} (5'-CGG TCA GGT GAA GAA CAT C-3').

PFGE. PFGE typing was performed as described previously, using the SpeI restriction enzyme (32). DNA fragments were electrophoresed in 1% (wt/vol) agarose gels in 0.5 \times Tris-borate-EDTA buffer with the Gene Path system (Bio-Rad, Hercules, California) at 14°C and 6 V/cm for 20 h, with pulse times ranging from 0.5 to 20 s. Gel pictures were interpreted using GelCompar II software (Applied Maths, Kortrijk, Belgium). The PFGE patterns were scanned, and Dice analysis of peak positions was executed. The unweighted-pair group method using average linkages (UPGMA) was applied, and the bandwidth tolerance was set at 1.2%. Potential clonal relatedness was determined at an $\geq 80\%$ level of similarity, corresponding to a maximum six-band difference (13, 38). The statistical significance of the clusters was tested by cophenetic correlation analysis, performed in GelCompar II. The cutoff for acceptance of the dendrogram was set at a minimum cophenetic correlation coefficient (CCC) value of 70% (23).

RAPD. RAPD reactions were carried out in three different laboratories (A, B, and C) in order to evaluate the reproducibility of the method. Genomic DNA was purified by the phenol-chloroform method (34), with a High Pure PCR template preparation kit (Roche, Mannheim, Germany), and with a BioRobot M48 machine (QIAGEN, VWR International AB, Stockholm, Sweden), respectively. PCR was performed with the Expand High-Fidelity PCR system (laboratory A) (Roche, Mannheim, Germany) or with Invitrogen *Taq* polymerase (lab-

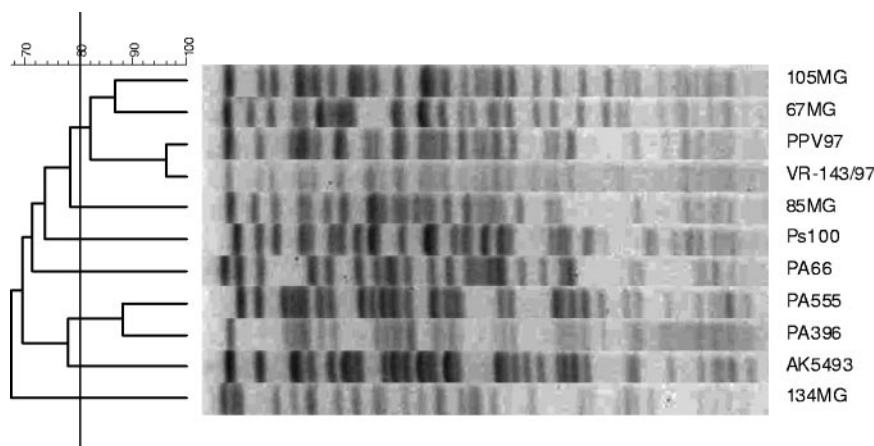


FIG. 1. Dendrogram generated by PFGE. The cutoff at the 80% level of genetic similarity is indicated by a vertical line. The CCC value for the dendrogram is 82%.

oratories B and C) (Invitrogen, Carlsbad, Calif.). For each RAPD PCR, 40 ng template DNA and primer 208 (5'-ACG GCC GAC C-3') were used as described earlier (22). PCRs were performed on a GeneAmp PCR System 2400 machine (Applied Biosystems, Foster City, Calif.), a Bio-Rad iCycler machine (Bio-Rad, Hercules, Calif.), and a GeneAmp PCR System 9700 machine (Applied Biosystems), respectively. PCR products were separated in 1.5% (wt/vol) agarose gels and analyzed by GelCompar II software as described for PFGE, using a cutoff of an $\geq 80\%$ level of similarity for potential clonal relatedness. The CCC values for the dendrograms were determined as described above.

MLST. MLST was performed according to the protocol published by Curran et al. (5). PCRs for the housekeeping genes *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *tpE* were performed using the same amplification mixture and reaction conditions as those described in the published protocol, with some modifications. The modifications included the addition of pure dimethyl sulfoxide at a concentration of 5 μ l per 100 μ l master mix and increasing the annealing temperature from 55 to 58°C. Nucleotide sequences were determined for both strands and compared to existing sequences in the MLST database (www.pubmlst.org) for assignment of allelic numbers, and sequence types (STs) were obtained. Isolates having five or more identical alleles were considered part of the same clonal complex (7). Following the terminology of Curran et al. (5), the clonal complexes were designated burst groups (BGs) in this work. The BURST algorithm was used for phylogenetic analysis, and PHYLIP software was used to generate a UPGMA dendrogram from the allelic profiles (www.pubmlst.org/analysis).

Structure of *bla*_{VIM}-containing integrons. For unpublished strains, the structure of the variable regions of the *bla*_{VIM}-containing integrons was determined by a PCR mapping and sequencing approach, as described previously (31). Sequences were analyzed and compared with those present in the EMBL/GenBank database, using the software tools available at the NCBI website (www.ncbi.nih.gov).

Nucleotide sequence accession numbers. Nucleotide sequences of unpublished integrons and *fliC* genes were submitted to the EMBL/GenBank database under the following accession numbers: DQ465011 (*fliC* type b), AJ865694 (*fliC* type a1), AJ865693 (*fliC* type a2), AJ634050 (In85), AJ786649 (In105), AY702100 (In112), AY866525 (In114), and AY460181 (In118).

RESULTS

Phenotypic traits. All VIM-1- and VIM-4-producing *P. aeruginosa* isolates were resistant to carbapenems (imipenem and meropenem), extended-spectrum cephalosporins (ceftazidime and cefepime), and piperacillin-tazobactam, and four of them were also resistant to aztreonam. The VIM-2-producing isolate was resistant to carbapenems but intermediately susceptible to extended-spectrum cephalosporins, piperacillin-tazobactam, and aztreonam. All isolates were resistant to tobramycin and ciprofloxacin. Only one isolate was gentamicin

susceptible, while three of the isolates were susceptible to amikacin. All isolates were colistin susceptible.

The serotypes of all isolates are listed in Table 1. All Italian isolates were found to be O11, except for two (67MG and 134MG) that were not serotypable. The Greek isolate (Ps100) and the Swedish isolate harboring *bla*_{VIM-4} (PA66) were also O11, while the two Hungarian isolates and the Swedish VIM-2 isolate were serotype O12.

***fliC* sequence typing.** The O12 isolates were found to be *fliC* type b without nucleotide polymorphisms, while all others were *fliC* type a (Table 1). Within the group of *fliC* type a, all isolates had an identical sequence (designated type a1), except for isolate 134MG (designated type a2).

PFGE and RAPD typing. By PFGE and RAPD, it was possible to identify two clusters of isolates (I and II) corresponding, overall, to their serotypes (O11 and O12) and one singleton nonserotypable isolate (134MG) (Fig. 1 and 2; Table 1).

Using PFGE, some clonally related isolates (Dice coefficient, $\geq 80\%$) could be identified within the two clusters (Fig. 1). In particular, all Italian isolates in cluster I were clonally related, except 85MG, for which the Dice coefficient was slightly lower than the 80% cutoff. PA66 and Ps100 showed more divergence, and thus a clonal relationship could not be demonstrated by PFGE either between them or with the Italian O11 isolates. Within cluster II, the two serotype O12 Hungarian isolates were clonally related, while AK5493 had a genetic similarity of about 76% with those isolates. The CCC of the UPGMA dendrogram was 82%.

By applying RAPD, the resolution within the clusters was somewhat lower. In particular, the Greek isolate Ps100 appeared to be clonally related to the Italian isolates of cluster I, while all three O12 isolates appeared to be clonally related (Fig. 2). Comparison of the dendrograms obtained in the three different laboratories revealed an overall good reproducibility of RAPD profiling in identifying different clusters (Fig. 2). However, the banding patterns observed for the individual isolates in the different laboratories showed substantial differences (Fig. 2), and when all RAPD profiles were combined and analyzed together, it became obvious that the same isolates did not always cluster (data not shown). This is in agreement with

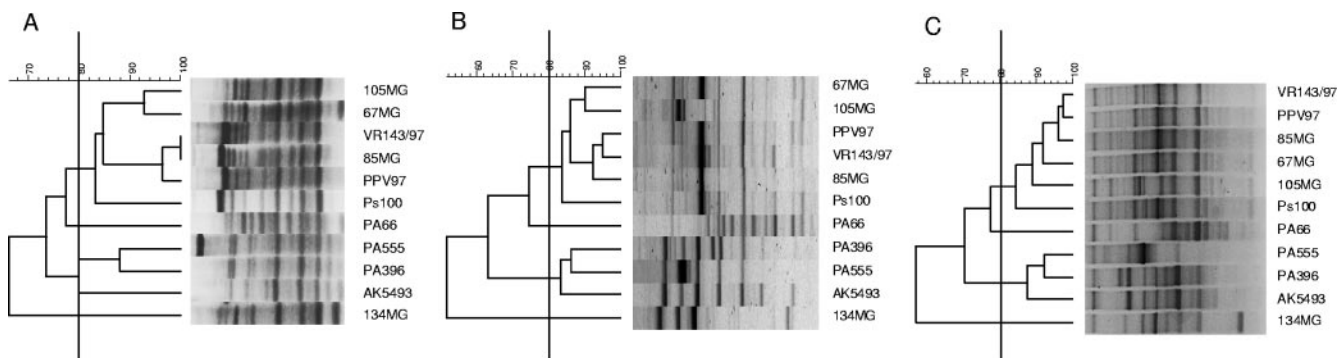


FIG. 2. Dendrograms generated by RAPD with primer 208 in three different laboratories (A to C). The cutoff at the 80% level of genetic similarity is indicated by vertical lines. The CCC values for the dendrograms are 89% (laboratory A), 94% (laboratory B), and 87% (laboratory C).

earlier observations reporting that most aspects of the PCR procedure, including small differences in the temperature profile and in the source and different batches of the *Taq* polymerase, may affect the reproducibility of the banding patterns (36). The CCCs of the three UPGMA dendrograms shown in Fig. 2A, B, and C were 89, 94, and 87% respectively. The average Dice coefficients (95% confidence intervals) calculated from the three data sets were 83.7% (81.9 to 85.6%) for isolates in cluster I and 83.6% (74.3 to 92.9%) for isolates in cluster II.

MLST. MLST showed that the isolates belonged to four different STs, all of which were original (www.pubmlst.org/paeruginosa/), and they were designated ST227-230. ST227 comprised all the Italian O11 isolates and one of the nonserotypable isolates (67MG), ST228 included the other Italian nonserotypable isolate (134MG), ST229 consisted of all O12 isolates, and ST230 was formed by the Greek and Swedish O11 isolates (Ps100 and PA66).

BURST analysis revealed that ST229 belongs to the previously described BG4 (5), while STs 227 and 230 form a novel BG, which we propose to designate BG11. No other isolates from the *P. aeruginosa* MLST database were found to belong to this novel clonal complex at the time of submission of the manuscript. ST228 belongs to a BG that has not been published previously but can be identified by BURST analysis, and we propose the name BG12 for this group (Table 1; Fig. 3). By applying commonly used cutoff values, PFGE and RAPD were unable to demonstrate clonal relationships between some of the isolates that belonged to the same ST or clonal complex by MLST due to the higher resolution of this analysis.

Integron structures. The structures of the variable regions of integrons carrying the *bla*_{VIM} gene cassette were determined for strains PA66, AK5493, Ps100, PA555, 67MG, 85MG, 105MG, and 134MG (for the other strains, it had previously been determined) (12, 20, 31).

Comparison of integron structures revealed a remarkable heterogeneity (Fig. 4). Only the two nonserotypable Italian strains, which were clonally unrelated according to RAPD, PFGE, and MLST, were found to carry identical integron structures, which corresponded to that of In110, an integron detected on a plasmid from a *Pseudomonas putida* strain isolated from a nearby area of northern Italy (21).

DISCUSSION

Acquired MBLs are emerging resistance determinants of increasing clinical importance, especially in *P. aeruginosa* (33). Understanding the epidemiology and mechanisms of dissemination of MBL determinants and MBL-producing strains is an essential step toward controlling this phenomenon.

In Europe, although infections caused by VIM-producing *P. aeruginosa* strains have been observed for almost a decade, little is yet known about the epidemiological relationships between isolates from different countries. Since the genes encoding enzymes of the VIM-1 lineage are usually chromosomally located (43), there is reason to believe that clonal dissemination could be of importance for the transmission of these isolates, although the mobilization of integrons facilitated by transposons represents another option (41). Transmission of isolates by human carriers has been suggested by some authors (20, 40), but no strong molecular epidemiological evidence has supported this hypothesis so far. One of the key challenges has been establishing a proper methodology for molecular epidemiological characterization and comparison of isolates with great temporal and geographic spread. The recently developed MLST scheme for *P. aeruginosa* (5) could emerge as an important tool for this purpose.

In this study, we compared a number of *P. aeruginosa* strains producing acquired MBLs of the VIM-1 lineage, representative of strains circulating in four different European countries where similar strains have been detected, by using several different typing methodologies, including the new MLST approach. Our results revealed different resolution powers for the different typing methodologies, with the highest resolution for PFGE, followed by those for RAPD, MLST, and *flaC* typing, which is in overall accordance with previous reports (30, 38). The lower resolution of RAPD than of PFGE (1) can be a disadvantage in cases of local outbreaks, but for our international collection of strains, RAPD, which earlier was proven to have good discriminatory power within the serotypes (14), could reproducibly assign the isolates to different clusters that also corresponded to the BGs identified by MLST. However, the lack of interlaboratory reproducibility of the banding patterns generated by RAPD under the experimental conditions applied indicated that RAPD results are not readily transportable and, as such, can only be used for intralaboratory com-

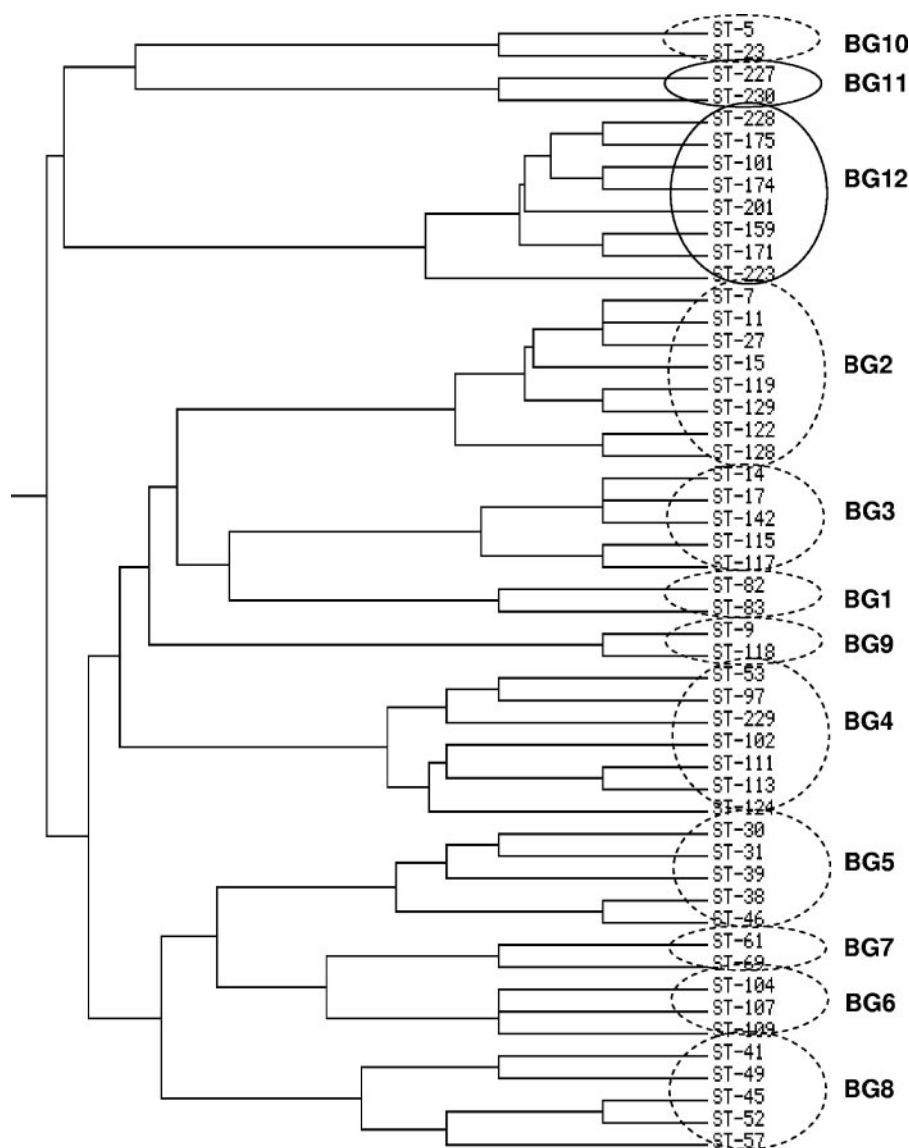


FIG. 3. Phylogenetic analysis (UPGMA dendrogram) based on findings with MLST. BG1 to BG10 are indicated by dotted circles, while the novel groups BG11, comprising ST227 (VR143/97, PPV97, 105MG, 67MG, and 85MG) and ST230 (Ps100 and PA66), and BG12, comprising ST228 (isolate 134MG) and other STs with at least five common alleles, are circled with continuous lines. ST229 (PA396, PA555, and AK5493) can be found in the previously described BG4.

parison of strain collections. By applying commonly used cutoff values, PFGE and RAPD were unable to demonstrate clonal relationships between some of the isolates that belonged to the same ST or clonal complex by MLST, which underscores the additional information that MLST can provide in addressing issues of molecular epidemiology for similar strains. In particular, MLST data pointed to a clonal relatedness for a number of serotype O11 or nonserotypable VIM-1-like enzyme-positive *P. aeruginosa* strains currently spreading in Europe and also revealed clonal relatedness between serotype O12 strains from different countries (which, in one case, also differed in the carriage of a *bla*_{VIM-2} rather than a *bla*_{VIM-1}-like allele).

The epidemiological significance of the STs described in this study is further supported by the observation that no isolates present in the *P. aeruginosa* MLST database at the time of

submission had identical allelic profiles to those of the isolates in this study and also by the fact that cluster I forms the novel clonal complex BG11 (comprising ST227 and ST230), with no single- or double-locus variants from the previously described 23 different STs of serotype O11 isolates (5). From the original and present MLST data, it appears that serotype O11 isolates are found in most BGs and, therefore, that this serotype does not closely correlate with particular STs or clonal complexes. On the other hand, most currently typed serotype O12 isolates belong to BG4, including ST229 strains, as determined for strains PA396, PA555, and AK5493. This is in accordance with the proposals of previous studies that serotype O12 strains, which form a tight cluster in terms of DNA relatedness, are particularly adept at acquiring or hosting the integrons that encode the MBL enzymes (4).

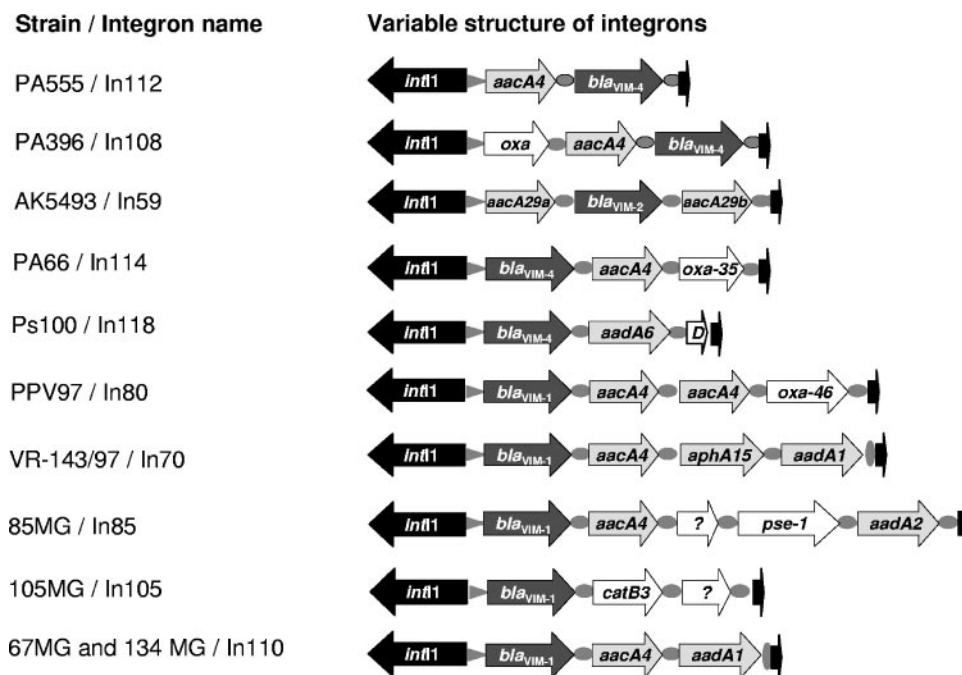


FIG. 4. Structures of variable regions of integrons carrying VIM determinants. Triangles indicate the *attI* primary insertion sites, ellipses indicate the *attC* recombination sites, and question marks indicate genes for which the full sequence was not determined.

It has been suggested by Pirnay et al. that *P. aeruginosa* has an epidemic population structure, implying that a limited number of clones would be widespread and that the clones would have emerged from a large number of unrelated genotypes (26). This hypothesis was further supported by the findings of Curran et al., who used the novel MLST scheme to type 143 clinical and environmental isolates (5). The findings in this study are also in accordance with the proposed epidemic population structure. Further analysis of VIM-producing O11 and O12 clinical isolates is needed to draw definite conclusions, but still our results indicate that at least one Mediterranean VIM-producing O11 clonal complex may be identified. The O11 strains from Italy and Greece all belong to the same clonal complex (BG11) according to MLST, and this observation is also supported by the absence of nucleotide polymorphisms in the hypervariable *fliC* gene. Also, our findings for the BG4 isolates, including two O12 strains isolated from Greek patients outside of Greece, could indicate the presence of an additional epidemic VIM-producing Mediterranean O12 clonal complex.

This study indicates an important role of serotype O11 and O12 *P. aeruginosa* strains in the appearance and/or dissemination of *bla*_{VIM} genes. With one exception, all characterized isolates belong to these two serotypes, similar to the case in a number of previous studies. From the current data, it is also possible to link the emerging VIM-harboring isolates in northern and eastern Europe to their proposed ancestor clones in the Mediterranean countries. Further analysis of isolates from the low-prevalence regions of Europe might unravel whether independent clones of VIM-producing *P. aeruginosa* may have emerged outside the Mediterranean region. The results presented in this work also indicate that MLST will likely emerge

as the key epidemiological tool for future studies of regional and global epidemiology of *P. aeruginosa*.

Finally, comparison of the integrons carrying *bla*_{VIM-1}-like cassettes in various isolates revealed a remarkable structural heterogeneity. This could reflect a scenario of multiple independent events of acquisition of different *bla*_{VIM}-containing integrons by members of various clonal complexes already circulating in nosocomial settings, although a contribution of integrase-mediated cassette shuffling or other recombination mechanisms during the evolution of similar strains could also have played a role in determining this variability.

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