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Association of the *bla*$_{VIM-1}$ Metallo-ß-lactamase Gene, the Small Multi-drug Resistance Gene *smr*, the *bla*$_{PSE-1}$ Gene in a Unique Class 1 Integron Found in *Pseudomonas aeruginosa* Isolates from Sicily: Report from the SENTRY Antimicrobial Surveillance Program

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AMENDED ABSTRACT

Background: Two *Pseudomonas aeruginosa* isolates, 85-4744 and 85-2966, were obtained from bloodstream infections in a Sicilian hospital. Both strains harboured the *bla*$_{VIM-1}$ metallo-beta-lactamase gene carried on an unusual integron which was sequenced in full.

Methods: Isolates were screened by amplifying the region between the 5'and 3'CS of Class 1 integrons. PCR was also used to identify any *Tn*21-like transposons, ISpa7 elements and "common regions" (CR) that may be associated with the MßL gene. Amplicons (> 500bp) were sequenced using a DuPont Automated system. Sequence analysis was carried out using DNAstar.

Results: The *bla*$_{VIM-1}$ gene was found in the first gene cassette position in an integron also containing a number of gene cassettes in the following order: the small multi-drug resistance conferring gene cassette *smr*; a gene coding for a hypothetical protein from *Gleobacter violaceus*; an *aac*A4 gene cassette and a *bla*$_{PSE-1}$ gene cassette. The integron was unusual in that the integrase was truncated and the 5' sequence missing. These strains also harboured the insertion element IS*Pa7*, the *Tn*5051 *tnp*R sequence and also a CR region recently associated with the MßL gene *bla*$_{SPM-1}$.

Conclusions: This Class 1 integron is unique in many ways: 1) The gene cassettes *smr* and *bla*$_{PSE-1}$ have not been associated with MßL gene cassettes before; 2) The integron harboured an unidentified gene related to sequences from *Gleobacter violaceus*; 3) The integrase gene is truncated towards the 3' end. The strains were also unusual in containing a CR region, an element which has been associated with horizontal movement of antibiotic resistance genes.

INTRODUCTION

The genes encoding metallo-ß-lactamase (MßLs) can be plasmid or chromosomally mediated and are important resistant determinants considering that most are carried as mobile gene cassettes on class 1 integrons. The class 1 integrons possess a 5'-conserved segment (5'-CS) on one side, which contains an *intI1* gene encoding an integrase, a recombination site *attI1*, a promoter and a 3'-CS in the opposite side. Within the 3'-CS, usually lies a truncated genetic structure that confers resistance to quaternary ammonium compounds and sulfonamides, *qacEΔ1/sul1*. Integrons are able to capture genes *via* a site-specific recombination event between two sites, one in the integron and one in the cassette. Both of these recombination sites confer mobility due to their recognition by the integrase that catalyze the integration of the gene cassette between the *attI1* in the integron and the 59-base element (be) in the gene cassette. It has now been established that some of the Class 1 integrons carrying MßL genes from Europe are located in a transposition locus (*tnp* region) termed *Tn*5051-like.

Recently, there have been many reports of VIM and IMP-type MßLs across Europe particularly from the Mediterranean area. The MßL genes reported from Italy include *bla*$_{VIM-1}$, *bla*$_{VIM-2}$, *bla*$_{VIM-4}$, *bla*$_{IMP-1}$, *bla*$_{IMP-2}$, *bla*$_{IMP-12}$, and *bla*$_{IMP-13}$. This study describes in detail the genetic context of the MßL genes from Sicily and including the organization and structure of their novel integrons. The carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates from Italy were submitted to the SENTRY Antimicrobial Surveillance Program in 1999-2002.

MATERIALS AND METHODS

Bacterial Strains. A diverse collection of clinical isolates (383 strains) from medical centers located in Genoa, Rome and Catania were submitted to the SENTRY Antimicrobial Surveillance Program in 1999 - 2002. Among other selected pathogens, *P. aeruginosa* strains resistant to imipenem (MIC, ≥ 16 µg/ml), meropenem (MIC, ≥ 16 µg/ml), and ceftazidime (MIC, ≥ 32 µg/ml) have been routinely screened for MßL genes. Strains fitting this criteria and used in this study are listed in Table 1.

Susceptibility testing and phenotypic detection of MßL. All isolates collected in the SENTRY Program were tested for susceptibility using the reference broth microdilution method described by the National Committee for Clinical Laboratory Standards (NCCLS). MßL Etest® strips (AB BIODISK, Solna, Sweden) were used to screen Class B ß-lactamase production on Mueller Hinton Agar (OXOID, Basingstoke, UK). Tests were performed and interpreted according to the manufacturer's instructions.

PCR experiments. The presence of Class 1 integrons in each strain was assessed using the Class 1 specific primers Int1F and QacR designed to anneal to the 5' and 3' conserved sequences, respectively. The genetic structure of each MßL containing integron was deduced by amplifying the 5' and 3' sections of each integron. Combinations of primers for VimMF and QacR and VimMR and Int1F were used in the PCR experiments.

DNA sequencing and analysis. PCR fragments obtained with integron primers were sequenced on both strands using Perkin Elmer systems 377 DNA Sequencer. The deduced amino acid sequences were determined using Lasergene software package (DNASTAR, Madison, WI) and compared to sequences available over the internet (http://www.ebi.ac.uk/fasta33/).

Pulsed Field Gel Electrophoresis (PFGE). Genomic DNA was prepared in agarose blocks and digested with the restriction enzyme SpeI (Invitrogen, Carlsbad, CA). Electrophoresis was performed on the CHEF-DR III (BioRad, Richmond, CA), with the time ramped from 5 to 90 seconds. Isolates with identical profiles were assigned the same type. Isolates that differed by one to six bands were assigned as a subtype. Strains were considered different by PFGE if more than six bands were different.

RESULTS

- 383 *P. aeruginosa* clinical isolates were submitted to the SENTRY Antimicrobial Surveillance Program from the three Italian SENTRY sites located in Genoa (North Italy), Rome and Catania (Sicily) in the years 1999-2002 and 31 thirty-one isolates that were MßL positive by Etest were further investigated. Four of the 31 originated from Catania (Table 1).
- Isolates 85-2394 and 85-14297 were identical in that two PCR products of size 2.5kb and 3.5kb were produced, whereas PCR using isolates 85-2966 and 85-4744 produced three products of size 0.4kb, 1.8kb and 2kb for both isolates (Figure 1).
- The respective PCR products of Class 1 integrons from 85-4744 and 85-2394 were sequenced and joined as described above to produce the full-length sequence of the integrons depicted in Figure 2.
- The Class 1 integron of isolate 85-4744 possessed a unique structure. The second gene cassette in this integron adjacent to the *bla*$_{VIM-1}$ gene cassette had a GC% of 61% and encodes a putative protein of 105 amino acids. This protein shares 100% identity with a putative small multi-drug resistance protein *smr-2*, which is encoded by an identical gene cassette from integron In111 found on the self-transferable plasmid pAK33.
- *smr-2* gene shares 97% identity to *orf0* previously described in a Class 1 integron from a *Serratia marcescens* clinical isolate SCH88050909. This gene encodes a protein that exhibited high identity with the Qac transporters. These proteins are highly similar to QacF from In40 and possess the typical motifs of the small multi-drug resistance proteins mediating efflux of lipophilic drugs.
- The third gene cassette contained an *orf* encoding a protein of 183 amino acids that displayed highest identities to hypothetical proteins from *Gleobacter violaceus* (57% ID, GenBank acc AP006572) and *Bradyrhizobium japonicum* (51% ID GenBanK acc AP005961) of undetermined function. This gene cassette had a GC content of 47% and included a 59be of 75bp. The fourth gene cassette of this integron was an *aac*A4 gene cassette identical to numerous other *aac*A4 cassettes listed (GenBank).
- The final gene cassette in the integron from *P. aeruginosa* 85-4744 encoded the ß-lactamase PSE-1 that mediates resistance to ampicillin. This gene cassette was 100% identical to the PSE-1 gene cassettes of In28 and the antibiotic resistance gene cluster of the Salmonella genomic island of *Salmonella typhimurium* DT104. The PSE-1 gene had a GC% of 41%, characteristic of a horizontally transferred/imported gene.

Table 1. MIC results for ß-lactams and aminoglycosides for Italian MßL-producing isolates.

Strain	IMP	MER	CAZ	CPM	AZT	GEN	TOB	AMK	Ribotype	Plamid/ chromosome	Genotype
85-2394	>256	>8	>16	>16	>16	>8	>16	8	252.45.6	chromosomal	*vim-1*
85-2966	>256	>8	>16	>16	>16	>8	>16	8	105.1034.2	chromosomal	*vim-1*
85-14297	>256	>8	>16	>16	>16	>8	>16	16	258.60.2	chromosomal	*vim-1*
85-4744	>256	>8	>16	>16	>16	>8	>16	8	105.1034.2	chromosomal	*vim-1*

Abbreviations: IMP = imipenem; MER = meropenem; CAZ = ceftazidime; CPM = cefepime; AZT = aztreonam; GEN = gentamicin; TOB = tobramycin; AMK = amikacin.

Figure 1. Variable regions of class 1 integrons amplified with intI1F and QacR primers. Left-Right: 1kb plus DNA marker; lanes 2-8: Genoese 1, Genoese 2, Rome 1, 85-4744, 85-2394, Rome 3 and Rome 4; 1kb plus marker. Variable regions of Class 1 integrons amplified with IntI1F and QacR primers.

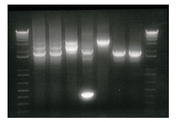


Figure 2. Schematic representation of MßL containing integrons found in Catania. Genes are represented as open rectangles with arrows indicating the direction of their transcription. Solid circles represent 59be recombination sites. Open elipses represent attI1 sites and double vertical lines indicate the inverted repeats IRi at the left-hand ends of the various integrons. The insertion element IS*pa7* is indicated as an open rectangle with their inverted repeats as solid rectangles at either end.

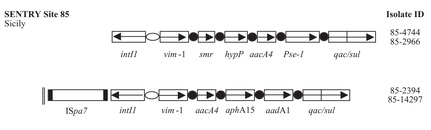
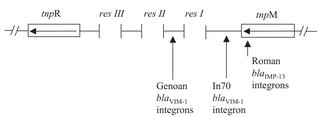


Table 2. Primers used for PCR amplification and sequencing of Italian isolate integrons and mobile elements.

Primer name	Target	Sequence (5'-3')	Expected Product size	Nucleotide numbers	Source (accession number)
VimF	*attI1*	TTATGGAGCAGCAACGATGT	621bp	-81 - -61	*vim1-3*
VimR	*vim1-3*	CGAATGCGCAGCACCAGG	-	519-539	*vim1-3*
intI1F	*intI1*	GCCTGTTCGGTTCGTAAGCT	-	86-106	Class 1 integrase
QacR	*qacEΔ1*	CGGATGTTGCGATTACTTCG	-	57-37	qacEΔ1
VimMF	*vim-1*	TGCGCATTCGACCGACAATC	-	3675-3695	Y18050
VimMR	*vim-1*	GTCGAATGCGCAGCACCAGG	-	3767-3747	Y18050
aacA4F	*aacA4*	TGCGATGCTCTATGAGTGGC	455bp	59	Alignment
aacA4R	*aacA4*	ATGTACACGGCTGGACCATC	-	514	Alignment
aacA29F	*aacA29*	AAGAACAAGACGCTGCCGAC	357bp	32	Alignment
aacA29R	*aacA29*	AACTTGCGGTGCGTGATGAC	-	389	Alignment
aphA15F	*aphA15*	CCTCGACGAAGTATCTGAAC	670bp	12	Alignment
aphA15R	*aphA15*	TTTCTCGATGCAAGCGCCAG	-	682	Alignment
smrEXT	*smr*	TTCTGCTATGGCTGGCTCAG	-	1316-1336	This study
Glob1	*glob*	ATGTCACAAGAGGAACGGCG	-	1882-1902	This study
Glob2	*glob*	GGTCGCGAGAATGATGTAGC	-	1990-1970	This study
PSEFF-r	*PSE-1*	CTTGCAAAAACCACGGATGG	-	2871-1851	This study
PSERF-r	*PSE-1*	CTCTGCCATTGAAGCCTGTG	-	3618-3598	This study
PSEMR	*PSE-1*	GAAGCACGCATCATCGAGTG	-	3174-3154	This study
PSERF	*PSE-1*	CACAGGCTTCAATGGCAGAG	-	3598-3618	This study
PSEMR-r	*PSE-1*	CACTCGATGATGCGTGCTTC	-	3154-3174	This study

Figure 3. Schematic representation of the position of insertion of the various MßL integrons into the *tnp* region of *Tn*5051-like transposons or partial transposon sequences, together with the sequence immediately upstream of the inverted IRi terminal inverted repeat of the particular integron. Sequences in bold represent IRi sequences marking the end of the various integrons.



GAAGGCAACTCTATTCTGACGATTT**TGTCGTTTTCAGAAGACGGCTGCAC Genoa site 75**
CGCAGCAACTGGTGGTGGTCGATC**TGTCGTTTTCAGAAGACGGCTGCAC Roman site 86**
CTCGACGATTTCCGCGCCTTCCGGG**TGTCGTTTTCAGAAGACGGCTGCAC In70 N. Italy**

CONCLUSIONS

- In this study, MßL-producing isolates accounted for 6.5% of all Italian SENTRY *P. aeruginosa* strains and for 39.1% of all imipenem resistant strains tested. MßL-producers accounted for 10.6%, 6.0% and 2.7% of all *P. aeruginosa* strains from the SENTRY sites in Genoa (Northern Italy), Rome and Catania (Sicily) respectively, over this period.
- Many MßL-producing isolates from this study and all the isolates from Genoa harboured multiple integrons, at least three being harboured by the isolates from Genoa and perhaps as many as four being harboured by isolates 85-4744 and 85-2966 from Sicily.
- PCR analysis of all strains for *Tn*5051 sequences detected sequences for *tnp*R of *Tn*5051 in all strains but only *tnp*A in strains harbouring *bla*$_{IMP-13}$ (Figure 3).
- In addition to the In70 integron harboured by isolates 85-2394 and 85-14297, two other isolates 85-4744 and 85-2966 from Sicily also harboured *bla*$_{VIM-1}$ containing integrons, which contained an *aac*A4 cassette, the small multi-drug resistance gene cassette *smr-2*, a *bla*$_{PSE-1}$ gene cassette and a cassette encoding a hypothetical protein similar to *orf* from *Gleobacter violaceus*.
- The mobility of these alleles, both genetically and geographically, coupled with the difficulty of eradicating isolates harbouring them from the hospital environment indicates that they are a clear and present threat to current antimicrobial chemotherapy and highlights the importance of surveillance programs such as SENTRY.

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