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Multi-Resistance Genomic Islands May Be Present in Metallo-ß-Lactamase Producing *Pseudomonas aeruginosa* Isolates from Italian Hospitals: Report from the SENTRY Antimicrobial Surveillance Program

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SENTRY ANTIMICROBIAL SURVEILLANCE

ABSTRACT

Background: Previous work identified multiple Class 1 integrons harboured by all MßL containing isolates, as well as the insertion element IS*pa7*. In this report, we have identified sequences adjacent to the insertion site of IS*pa7* in Italian isolates.
Methods: Adjacent sequences to the IS*pa7* insertion elements were amplified by PCR. This method utilized nested primers anchored to the 5' IS*pa7* sequences and degenerate primers designed to randomly hybridize to upstream sequences. Sequencing was performed on both strands by usual methods.
Results: Our data has identified a number of fragments flanking the insertion site of IS*pa7* insertion elements in the genome of Italian strains. These fragments were of three different sizes, i.e. 0.8kb, 2.8kb and 4.5kb. Strains 85-2394, 86-10088 and 75-3634 produced two different DNA fragments indicating at least two IS*pa7* insertion elements in these strains, whereas strains 85-2966, 75-3600 and 86-10079 produced only one DNA fragment each. Sequence analysis from the ends of these fragments indicated a number of different insertion sites, but all insertion sites were within antibiotic resistance loci. Interestingly, sequencing of the opposite ends of these PCR fragments also displayed high identities to antibiotic resistant genes including bla_{PSE-1}, the small multi-drug resistance gene *smr*, as well as *aac*A4 and integron associated sequences.
Conclusions: These results suggest that a multi-resistance genomic island including many different resistance genes are present in the Italian strains. The discovery of multiple copies of the insertion element IS*pa7* in many of the Italian isolates may also confer mobility to this genomic island by producing a composite transposon and enhance the spread of *bla*MßL genes.

INTRODUCTION

Pseudomonas aeruginosa is a leading cause of nosocomial-acquired infections and is intrinsically resistant to many antimicrobials. Carbapenems, such as imipenem and meropenem, are highly active against *P. aeruginosa* and represent excellent therapeutic options for the treatment of infections caused by this pathogen. However, more recently, carbapenem resistance has been increasing worldwide. Low-level carbapenem resistance is often mediated by outer-membrane alterations in conjunction with the increased expression of one or more efflux pumps or native enzymes. High-level resistance to carbapenems (>32 µg/ml) is still uncommon in *P. aeruginosa* and is usually indicative of metallo-ß-lactamase (MßL) production.

MßLs are Ambler class B enzymes and constitute a heterogeneous family. However, they share four main characteristics: (i) activity against carbapenems; (ii) no obvious hydrolysis of monobactams; (iii) inhibition by chelating agents, such as EDTA and dipicolinic acid; and (iv) requirement of Zn^{+2} ions. Whilst some environmental bacteria possess MßL genes, recent attention has largely focused on those that are transferable.

As part of the SENTRY Antimicrobial Surveillance Program, MßL producing isolates were identified in three Italian medical centers (Genoa, Rome and Catania in Sicily). Genetic analysis of the isolates harbouring MßL gene cassettes indicated that many strains contained multiple integrons (Figure 1) and identified novel integrons in strains isolated in Genoa and Catania. Furthermore, the insertion element IS*pa7* was detected in all Italian MßL producing strains in an identical position. In this report, we characterize the sequences adjacent to the insertion site of IS*pa7* in these Italian MßL-producing strains.

MATERIALS AND METHODS

Bacterial isolates: A total of 383 non-replicative, randomly collected *P. aeruginosa* isolates were studied from the period 2000-2002. These strains were isolated in three hospitals located in distinct regions of Italy: Genoa (North), Rome (Central) and Sicily (South). Initial susceptibility testing results (MIC) identified 31 multi-drug resistant strains. These strains showed resistance to imipenem (MIC, ≥ 16 µg/ml), meropenem (MIC, ≥ 16 µg/ml) and ceftazidime (MIC, ≥ 32 µg/ml) and a positive MßL screening test (Etest). Of these, 25 isolates produced either VIM-1 or IMP-13, as detected by PCR. Individual isolates 85-4744, 85-2394, 86-10088, 86-10079, 86-10117, 75-3600 and 75-3634 harbouring unique integrons and representing all three medical centers were further analyzed to identify sequences upstream of the insertion site of the IS*pa7* insertion sequence by a degenerate flanking PCR approach.

Random flanking primer 2 step PCR: DNA sequences adjacent to the insertion site of IS*pa7* in Italian *P. aeruginosa* strains containing bla_{VIM-1} were amplified by a random primer PCR approach (Figure 2). This consisted of two nested primers designed within the IS*pa7* sequence approximately 20bp (IS*pa7*UP) and 100bp (IS*pa7*BT) downstream from the inverted repeat marking the left hand side of the IS*pa7* insertion sequence. The primer at position 100bp was biotinylated. Four different random primers were then designed with a novel sequence tag of 24bp followed by seven randomly assigned bases, four bases of two G and C residues in different combinations and a final T residue as shown below.

TGAGGCCTGTTCTACGACTC	IS*pa7*BT
CCGGAAGTTGATGGTCATCG	IS*pa7*UP
CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNGGCCT	Random 1
CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNGCGCT	Random 2
CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNCCGGT	Random 3
CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNCGCGT	Random 4

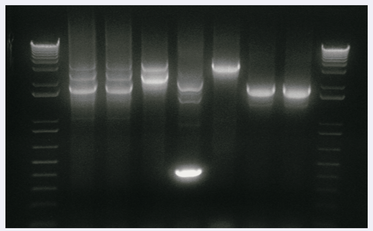
Step 1 included four PCR reactions with the biotinylated primer IS*pa7*BT at position 200bp and one of each of the random primers. The products of the PCR reaction were then incubated with Dynal streptavidin linked beads, washed and then denatured with 0.1M NaOH. The beads were then separated using a magnetic rack and the supernatant neutralised with 0.2M HCl and diluted prior to use as a template in the second step PCR.

Step 2 PCR consisted of amplification using a primer complimentary to the sequence tag of the random primer and anchored within the integron sequence with the nested primer at position 100bp. Individual products from Step 2 PCR were isolated from gels after electrophoresis and sequenced with the primer at position 100bp.

RESULTS

- PCR amplification of DNA flanking the insertion site of IS*pa7* in the genome of Italian strains identified fragments of three different sizes, i.e. 0.8kb, 2.8kb and 4.5kb.
- Strains 85-2394, 86-10088 and 75-3634 produced two different DNA fragments indicating at least two IS*pa7* insertion elements in these strains, whereas strains 85-2966, 75-3600 and 86-10079 produced only one DNA fragment each (Figure 3).
- Gene sequence analysis of the insertion site of IS*pa7* in these *P. aeruginosa* isolates identified IS*pa7* insertion into different antimicrobial resistance genes including bla_{PSE-1} and a gene cassette coding for a hypothetical protein from *Gleobacter violaceus* (Figure 4).

Figure 1. Agarose gel of PCR products from *P. aeruginosa* MßL containing strains amplified with primers designed against Class 1 integron 5' and 3' conserved sequences.



Legend: Lanes 1 and 9 represent 1kb plus DNA ladder, Lane 2 and 3 represent PCR products generated using template DNA of isolates 75-3600 and 75-3634 respectively. Lanes 4-8 represent PCR products generated by using DNA templates of strains 85-4744, 85-2394, 86-10088, 86-10117 and 86-10079, respectively.

Figure 2. Strategy for amplification of unknown DNA upstream of the insertion site of the insertion sequence IS*pa7*- see methods.

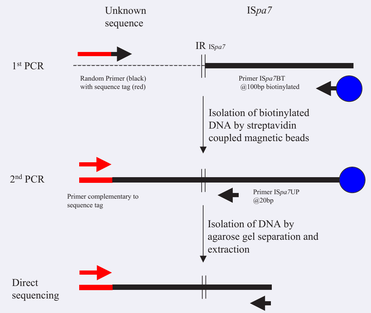
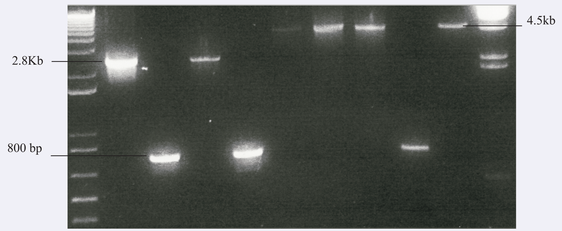
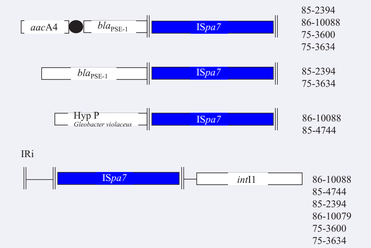


Figure 3. Agarose gel of PCR products produced using primers anchored to IS*pa7* and random primers designed to anneal to upstream sequences.



Legend: Lanes 1 and 11 represent DNA ladders, Lane 2 represents PCR products generated by using template DNA of isolates 85-4744, Lanes 3 and 4 represent PCR products generated by using template DNA from strain 85-2394, Lanes 5 and 6 represent PCR products generated from template 86-10088. Lane 7 represents PCR product generated from template 75-3600. Lanes 8 and 9 represent PCR products generated from template 75-3634. Lane 10 represents PCR products generated from template 86-10079.

Figure 4. Schematic illustrating different insertion sites for IS*pa7* in Italian *P. aeruginosa* isolates.



Legend: Genes are represented by open blocks, 59 base element by a filled circle and inverted repeats are indicated by parallel vertical lines. Two different insertion sites were found in the bla_{PSE-1} gene, which were ~500bp distant from each other as well as one insertion into a gene displaying 57% identity to a similar gene coding for an hypothetical protein from *Gleobacter violaceus*. All isolates were previously shown to have an additional insertion of IS*pa7* into a Class 1 integron immediately downstream of the integrase gene.

CONCLUSIONS

- Gene sequence analysis indicates multiple insertions of IS*pa7* in some Italian *P. aeruginosa* strains.
- Insertions are within antimicrobic genes or integron-associated genes and may suggest that IS*pa7* has a bias for these type of genes.
- Multiple copies of the insertion element IS*pa7* suggests that antibiotic resistance genes may move as part of a composite transposon.
- The association of multiple integrons, insertion elements and CR elements (isolates 85-4744 and 85-2394 also contain CR elements; see Poster #C2-1350) is reminiscent of the genomic island SGI1 from *Salmonella enterica* serovar Typhimurium and may indicate a similar structure in some of these isolates.

SELECTED REFERENCES

Jones RN, Biedenbach DJ, Sader HS, Fritsche TR, Toleman MA, Walsh TR. (2004). Emerging epidemic of a metallo-ß-lactamase-mediated resistances. *Diagnostic Microbiology and Infectious Diseases* [in press].

Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, Rossolini GM. (1999). Cloning and characterization of *bla*VIM, a new integron-borne metallo-beta-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. *Antimicrobial Agents and Chemotherapy* 43: 1584-1590.

National Committee for Clinical Laboratory Standards. (2003). *Approved standard M7-A6: Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. Sixth edition.* Wayne, PA:NCCLS.

Toleman MA, Biedenbach DJ, Bennett D, Jones RN, Walsh TR. (2004). Italian metallo-ß-lactamases, a national problem? *Journal of Antimicrobial Chemotherapy* [in press].

Toleman MA, Biedenbach D, Bennett D, Jones RN, Walsh TR. (2003). Genetic characterization of a novel metallo-beta-lactamase gene, bla_{IMP-13}, harboured by a novel Tn*5051*-type transposon disseminating carbapenemase genes in Europe: Report from the SENTRY worldwide antimicrobial surveillance programme. *Journal of Antimicrobial Chemotherapy* 52:583-590.