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Characterization of In77, a Class 1 Plasmid-located Integron Carrying the Novel Metallo-beta-lactamase Gene *bla*$_{GIM-1}$

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SENTRY ANTIMICROBIAL SURVEILLANCE

ABSTRACT

Objective: GIM-1 was recently described as the fourth type of mobile metallo-ß-lactamase (MßL). Primary studies showed that *bla*$_{GIM-1}$ was embedded in a class 1 integron with unusual size and structure. The aim of this study was to characterize the genetic environment of *bla*$_{GIM-1}$.

Methods: The integron structure was revealed with a walking sequencing strategy, using custome primers. Sequencing of the fragments was performed in both strands using DuPont Automated systems and the results analyzed using DNAstar. The plasmid obtained from the isolate harboring *bla*$_{GIM-1}$ was electroporated into *Escherichia coli* DH5α and a rifampin resistant mutant of *Pseudomonas aeruginosa* PA01 (RifR). Selection was performed in nutrient agar plates containing 4 mg/L of ceftazidime. Restriction profiles of the plasmid were carried out with different restriction enzymes, to obtain the plasmid size.

Results: In77 is a 6 kb integron showing all the key genetic components commonly found in a class 1 integron (the *intI1* integrase gene with its own promoter regions, an *attI1* recombination site and in the 3'-CS, the fused structure *qacEΔ1/sul1*). This integron harbored the recently described *bla*$_{GIM-1}$ in the first position. Downstream of the MßL gene was found an *aacA4*, followed by an *aadA1*. However, this second aminoglycoside resistance gene was interrupted by the insertion sequence, IS1394, previously described in a *Pseudomonas alcaligenes* isolate. This integron also carried an ESBL gene, *bla*$_{OXA-2}$ in the last position. Two frame shifts were found in this integron, one in the integrase gene and the second in the IS1394. Further characterization of the In77, revealed that this integron is likely to be located in a non-transferable 22 Kb plasmid.

Conclusions: This work describes a novel integron, In77, carrying the lately mobile MßL gene described, *bla*$_{GIM-1}$. Distinct features, such as the *addA1* interrupted by the IS1394 and the frame shifts that make the integrase and the insertion sequence stationary were also found in In77.

INTRODUCTION

During the last decade four types of acquired metallo-ß-lactamase (MßL) genes have been detected in Gram-negative pathogens, namely *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM}$ and *bla*$_{GIM}$. The first mobile MßL gene (*bla*$_{IMP-1}$) was found in *Pseudomonas aeruginosa* in Japan, where carbapenems dominate the parenteral ß-lactam market. For many years, the occurrence of *bla*$_{IMP-1}$ was confined to Japan. More recently, however, *bla*$_{IMP-1}$ and *bla*$_{IMP}$-variants have been reported from many other countries and across four continents. Two other types of clinically relevant MßLs have been described: the VIM family, which has also been detected in many countries, and the SPM, which so far appears to be restricted to Brazil. The fourth type of mobile MßL gene, *bla*$_{GIM-1}$, was recently discovered in five clonal, clinical isolates of *P. aeruginosa* submitted to The SENTRY Antimicrobial Surveillance Program. All five isolates were recovered from respiratory tract specimens in a medical site located in Dusseldorf, Germany. PFGE analysis showed that these isolates were identical and different from susceptible *P. aeruginosa* isolates recovered from the same medical site at the same period.

The integron-based recombination system is a powerful mechanism of discrete genetic rearrangement that operates in prokaryote genomes and that plays a major role in the spread of resistance determinants. Recent reports have shown that many mobile MßL genes are found in gene cassettes carried on class 1 integrons. This is the most common class of integrons encountered carrying antimicrobial resistance genes. The class 1 integrons possess a 5'-conserved sequence (5'-CS), which contains an *intI1* gene encoding an integrase, a recombination site *attI1* and a promoter, that is used to express adjacent genes acquired by gene cassette integration, and a 3'-CS. In the 3'-CS, usually lies a truncated genetic structure that confers resistance to quaternary ammonium compounds and sulfonamides, *qacEΔ1/sul1*.

In the present study we characterized a new class 1 integron structure, In77, carrying the novel MßL gene *bla*$_{GIM-1}$ in the first position and other unusual features.

MATERIALS AND METHODS

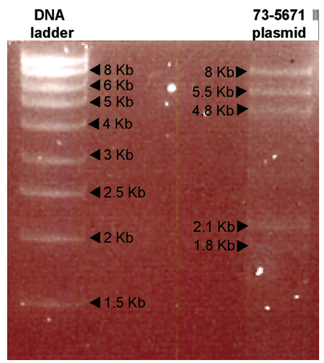
PCR experiments. The integron structure was revealed with a walking sequencing strategy. PCR reactions were carried out in 20 µL final volume using 10 µL of ABgene Expanded Hi-fidelity Master Mix (ABgene House, Surrey, UK). Primers were used at 10 ρM concentration and 1 µL of overnight bacterial culture at OD of 1 at 600 nm was used to provide the template. The cycling parameters were: 95ºC for 5 minutes followed by 30 cycles of denaturation at 95ºC for 1 minute, annealing at 45ºC for 1 minute and extension at 68ºC from 1 to 4 minutes, depending on the length of the sequence to be amplified, and ending with 5 minutes of incubation at 68ºC.

DNA sequencing and computer analysis. The PCR fragments obtained were sequenced on both strands using DuPont Automated System. The nucleotide sequences were analyzed using Lasergene software package (DNASTAR, Madison, WI). Obtained sequences were compared to sequences available over the internet (http://www.ebi.ac.uk/fasta33/).

Plasmid content, conjugation and electroporation. Plasmid extraction from *P. aeruginosa* 73-5671 was undertaken with QIAprep Spin Mini prep kit (Quiagen, West Sussex, UK). The plasmid obtained was electroporated into *E. coli* DH5α and a rifampin resistant mutant of *P. aeruginosa* PA01. Electroporation parameters were 2.5 kV, 25 µF and 400 Ω using the Bio-Rad Gene Pulser apparatus (BioRad, Richmond, CA). Selection for transformants was on performed in nutrient agar plates containing ceftazidime 4 mg/L. Conjugation experiments were performed in liquid medium with rifampin-resistant derivatives of *E. coli* K-12 and *P. aeruginosa* PA01 as recipients. Transconjugant selection was performed on nutrient agar with ceftazidime (4 mg/L) and rifampin (200 mg/L).

Restriction Profile of *bla*$_{GIM-1}$-carrying plasmid. Restriction endonuclease analyses of the plasmid were carried out with seven different restriction enzymes, namely *Bam*HI, *Eco*RI, *Hind*III, *Hinc*II, *Spe*I, *Sma*I, *Xba*I (Invitrogen, Carlsbad, CA) singly and in pairs, to determine the size of the plasmid. Reactions were performed as indicated by the manufacturer, at 25 or 37ºC with overnight incubation.

FIGURE 1. Results of agarose gel electrophoresis of the plasmid DNA preparation from the *P. aeruginosa* clinical isolate 73-5671 digested with *Hinc*II and *Sma*I. DNA size standards and approximated values for the plasmid fragments are reported on the sides.



RESULTS

- *bla*$_{GIM-1}$ is located in the first gene cassette position of this 6 kb class 1 integron, which was named In77. This integron revealed all key genetic components found in class 1 integrons.
- Two putative promoter regions (P1 and P2) precede the *bla*$_{GIM-1}$ start codon (ATG). The primary promoter (P1) has all the features of an intermediate strength promoter (-35 box TGGACA and the -10 box TAAACT being separated by 17 bp).
- The plasmid obtained from strain 73-5671 was estimated to be approximately 22 kb in size, as determined from DNA fragment profiles generated with the combination *Hinc*II and *Sma*I (Figure 1).
- Transfer of the ß-lactam resistance by electroporation and conjugation could not be demonstrated. However, PCR products with *bla*$_{GIM-1}$ and *bla*$_{OXA-2}$ primer sets were obtained in tests with plasmid preparations.
- The second cassette in In77 accommodates the *aacA4* gene that encodes an AAC(6')-Ib aminoglycoside acetyltransferase. This gene is likely to be fused with the MßL gene since there is no obvious 59-be between the two gene cassettes (Figure 2).
- The *aacA4* is followed by a second aminoglycoside resistant determinant, *aadA1* interrupted at nucleotide position 135 by a copy of IS1394 (GenBank accession number U37284) previously described in a *Pseudomonas alcaligenes* isolate.
- Following the IS1394 is the remainder of the *aadA1* gene cassette and another ß-lactamase gene cassette accommodating *bla*$_{OXA-2}$. This is followed by the fused gene cassette *qacEΔ1/sul1*.
- Two frame shifts were found in this integron, one in the integrase gene and the second in the IS1394. These frame shifts probably make the integrase and the insertion sequence stationary.

FIGURE 2. Schematic representation of integron In77 carrying *bla*$_{GIM-1}$ (the arrows in the gene boxes indicating the direction of transcription). The black dots indicate 59-be. In the third cassette position the *aadA1* is interrupted by a copy of the IS1394. Block arrows beneath the gene map indicate the positions of the primers used for PCR reactions and sequence analyses.

CONCLUSIONS

- The fact that the *bla*$_{GIM-1}$ is located in a distinct integron structure shows the plasticity of the integron-based recombination system and a great potential for dissemination.
- Of the four sub-classes of mobile MßL genes identified to date, two (*bla*$_{vim}$ and *bla*$_{gim}$) were originally found in Europe, perhaps signaling future difficulties for empirical treatment with carbapenems in this region.
- It is clearly desirable that the incidence of MßL-producing strains among key Gram-negative species, such as *P. aeruginosa*, *Enterobacter cloacae*, *Serratia marcescens* and *Klebsiella pneumoniae,* be carefully monitored as a guide to empiric therapy.

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