Metallo-β-Lactamase gene *bla*$_{SPM-1}$: Evaluation of its vicinities in unrelated *Pseudomonas aeruginosa* strains isolated from distinct Brazilian Hospitals

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Amended Abstract

Objective: To reveal the genetic environment around *bla*$_{SPM-1}$ in unrelated *Pseudomonas aeruginosa* (PSA) strains and the possible role of the common region 4 (CR4) in the *bla*$_{SPM-1}$ mobilization. Although common regions (CR) have been located near resistance genes, the role and function of these genetic elements has not been well established. A new allele of CR element, CR4, was recently described upstream of *bla*$_{SPM-1}$. CR4 was located downstream of *groEL*, a chaperonin encoding gene. **Methods:** 24 clonally unrelated PSA strains (distinct ribotype and PFGE patterns) harboring *bla*$_{SPM-1}$ isolated from 7 Brazilian cities were evaluated. Primers designed for detection of *bla*$_{SPM-1}$ were used with primers targeting the CR and *groEL* to determine the presence of these elements in the vicinity of *bla*$_{SPM-1}$. In addition, degenerated primers were designed against CR elements and used to amplify target strains. The amplicons had been sequenced in both strands and the DNA sequencing results analyzed. **Results:** Amplicons of expected size (800 pb) with CR primers were detected in all 24 isolates. PCR performed anchoring CR primers to *bla*$_{SPM-1}$ produced amplicons of 1.5 kb. Sequencing showed that CR4 was located straight upstream of *bla*$_{SPM-1}$ in all evaluated isolates. The presence of *groEL* was also detected in the 24 isolates. DNA sequencing results demonstrated the same features in all 2 kb amplicons obtained by PCR reaction using *groEL* primers. **Conclusion:** *groEL* followed by CR4 were found upstream of *bla*$_{SPM-1}$ in all unrelated PSA isolated from distinct Brazilian geographic regions. The same arrangement of these genes, without any insertions or deletions, was recovered from all 24 PSA isolates, showing a very conserved structure. These findings indicate that CR4 and *groEL* have been mobilized along with *bla*$_{SPM-1}$ and that CR4 may not be responsible for *bla*$_{SPM-1}$ dissemination among SPM-producing PSA isolated in Brazil. Although the mobilization of the plasmid carrying *bla*$_{SPM-1}$ is difficult due to its size (around 100Kb), based on our results, it seems more likely that this element may be responsible for the mobilization of *bla*$_{SPM-1}$.

Introduction

Acquired metallo-β-lactamases (MβLs) belong to five types: IMP, VIM, SPM-1, GIM-1 and the recently described SIM-1. SPM-1 was first described from a carbapenem-resistant *P. aeruginosa* strain responsible for causing urinary tract infection in a patient hospitalized at the Hospital São Paulo complex, located in São Paulo, Brazil. This metallo-enzyme is very distinct from the other MβLs, sharing 20 to 33% of similarity with members of IMP and VIM-families, GIM-1 and SIM-1. The gene encoding SPM-1 has been reported only in *P. aeruginosa* isolated in Brazil; however SPM-1-producing isolates has been observed in several distinct Brazilian regions.

Differently of most MβL-encoding genes that are carried as mobile gene cassettes on class 1 integrons, *bla*$_{SPM-1}$ was not embedded in an integron structure. This gene was located in a high molecular weight plasmid and was flanked upstream by a 495 bp open reading frame (ORF) encoding a putative recombinase. This element shows high similarity with other ORFs, that are found in the vicinities of a diversity of antimicrobial resistance genes. These genetic structures have been recently named common regions (CRs). Orf495, so-called CR4, was located downstream of a chaperonin encoding gene, *groEL*. A truncated version of *groEL* was also found downstream of *bla*$_{SPM-1}$.

In this study, we evaluated 24 unrelated *P. aeruginosa* isolates from different Brazilian regions to comparatively analyze the vicinities of the *bla*$_{SPM-1}$ gene.

Material & Methods

Bacterial Isolates. A total of 24 epidemiologically unrelated SPM-1-producing *P. aeruginosa* clinical isolates were studied. Twenty isolates were collected from seven different medical institutions throughout Brazil, during 2002 and 2003. The five remaining strains were isolated in the Hospital São Paulo during 1997, 2000 and 2001, the same medical institution were the first SPM-1-producing isolate was recovered.

PCR experiments. Amplification was carried out in 20μL final volume using ABgene Taq DNA Polimerase (ABgene House, Surrey, United Kingdom). Primers were used at 10ρM concentration and 1μL of overnight bacterial culture was used as a template. The cycling parameters were: 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, annealing at 45°C for 1 minute and extension 68°C ranging from 1 to 4 minutes and ending with 5 minute incubation at 68°C. Customers primers (Table 2) were designed based on the DNA sequence available in GenBank under the nucleotide accession numbers AY341249 and AJ492820. Degenerated primers (Table 2) were designed against four different described CR elements (*orfA*, *orf2*, *orf495* and *orf513*) and used to amplify target strains.

Material & Methods

DNA Sequencing and Sequence Analysis. Amplicons obtained were sequenced on both strands by dideoxychain terminator method with a 377 Applied Biosystems DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences and deduced amino acid sequences were analyzed using Lasergene software package (DNASTAR, Madison, WI, USA) and compared with the sequences available through the internet using BLAST and/or FASTA (http://www.ncbi.nlm.nih.gov/blast/ and http://www.ebi.ac.uk/fasta33/).

Automated ribotyping. MβL producing isolates were ribotyped using the Riboprinter Microbial Characterization System® (Qualicon, Wilmington, DE, USA). In brief, this automated process includes bacterial cell lysis, cleavage of DNA using the restriction enzime Pvu II, size separation using gel electrophoresis and modified Southern blotting. Results were analyzed by the Riboprinter and isolates were considered to have the same ribotype if the similarity coefficient was ≥ 0.93.

Primer	Target	Sequence (5'-3')	Accession number
SPM1F	*bla*$_{SPM-1}$	CCTACAATCTAACGGCGACC	AJ492820
SPM1R	*bla*$_{SPM-1}$	TCGCCGTGTCCAGGTATAAC	AJ492820
SPM1FF	*bla*$_{SPM-1}$	CCGTTGGCGAAATGAGAC	AJ515707
SPM1FR	*bla*$_{SPM-1}$	AATGGCGCTCCCGC	AJ515707
groEL1F	*groEL* (upstream *bla*$_{SPM-1}$)	AGGCGCATTACCCGGAC	AY341249
groEL1R	*groEL* (upstream *bla*$_{SPM-1}$)	GTTTGCAGCGTGACAACC	AY341249
groEL1FF	*groEL* (upstream *bla*$_{SPM-1}$)	GGGCGGCATGGATTTCTG	AY341249
CRF	*orf4*, *orf2*, *orf495* and *orf513*	CACTWCCACATGCTGTKKC	AF231986, AB115497, U42226, AY341249
CRR	*orf4*, *orf2*, *orf495* and *orf513*	CGCTTGAGSCGTTGCRYCC	AF231986, AB115497, U42226, AY341249
CR4FR	*orf495*	GTTCGCGGCCATTTCCC	AY341249
groEL2R	*groEL* (downstream *bla*$_{SPM-1}$)	GGTTCAGCACCACCGAG	AY341249

Table 1. Oligonucleotides used as primers for PCR amplification and sequencing.

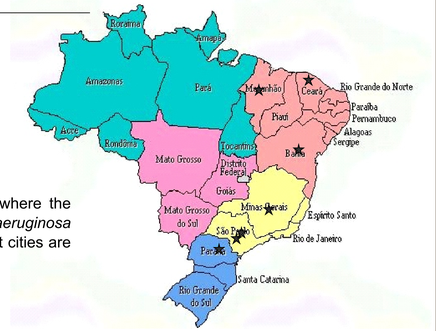

Figure 1. Location of the medical institutions where the epidemiologically unrelated SPM-1-producing *P. aeruginosa* strains were initially isolated. The seven different cities are marked with a black star.

Comments

- All these 24 strains belonged showed to distinct ribotypes and were considered genetically unrelated. (Table 2).
- The epidemiologically distinct *P. aeruginosa* isolates were recovered in 8 medical institutions located in 7 different cities, from 6 Brazilian states (Table 2 and Figure 1).
- PCR reactions with CR degenerated primers showed amplicons of expected size (800 pb) in all 24 isolates analyzed. DNA sequencing analysis of the amplicons showed that *orf495* (CR4) was present in all strains.
- PCR experiments performed by anchoring CR primers to *bla*$_{SPM-1}$ produced amplicons of 1.5 Kb indicating that CR4 is located immediately upstream of SPM-1. These DNA fragments were sequenced and exhibited identical sequences for all evaluated isolates. Sequences were identical to previously described *bla*$_{SPM-1}$ genetic context.
- PCR products obtained by amplification with primers for the *groEL* in combination with primers for CR4 and *bla*$_{SPM-1}$ were sequenced and revealed that *groEL* is present in the 24 isolates.
- Sequencing analysis of the 3 Kb fragment obtained with different primers annealing in CR4, *groEL* and *bla*$_{SPM-1}$ showed the same genetic arrangement previously reported upstream of the SPM-1 encoding gene (Figure 2).

Isolate Number	City	State	Ribotype Profile	Body Site
5	Santo André	São Paulo	77-7	Gastrointestinal
6	Londrina	Paraná	77-2	Urine
10	Belo Horizonte	Minas Gerais	105-3	Skin
12	Belo Horizonte	Minas Gerais	105-4	Catheter
13	Belo Horizonte	Minas Gerais	97-7	Respiratory
14	Belo Horizonte	Minas Gerais	105-1	Respiratory
17	São Paulo	São Paulo	88-1	Blood
21	Londrina	Paraná	77-1	Skin
43	Belo Horizonte	Minas Gerais	105-5	Blood
44	Londrina	Paraná	78-4	Skin
58	Santo André	São Paulo	77-8	Gastrointestinal
73	Salvador	Bahia	79-8	Skin
75	Belo Horizonte	Minas Gerais	130-1	Unknown
76	São Luis	Maranhão	129-4	Skin
77	Fortaleza	Ceará	82-5	Catheter
81	Santo André	São Paulo	77-5	Gastrointestinal
107	Belo Horizonte	Minas Gerais	105-7	Skin
109	São Luis	Maranhao	127-4	Urine
193	São Paulo	São Paulo	103-4	Blood
194	São Paulo	São Paulo	89-5	Blood
195	São Paulo	São Paulo	97-7	Blood
196	Salvador	Bahia	71-5	Skin
197	São Paulo	São Paulo	103-1	Blood

Table 2. Epidemiological data of the genetically unrelated SPM-1 producing *P. aeruginosa* strains evaluated.

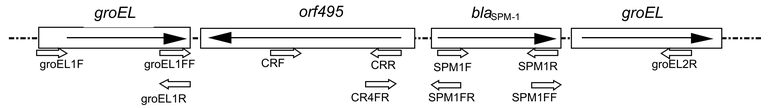

Figure 2. Schematic representation on genetic context harbouring *bla*$_{SPM-1}$ (the arrows in the gene boxes indicating the direction of transcription). Block arrows beneath the gene map indicate the positions of the primers used for PCR reactions and sequence analyses.

Conclusions

- The gene encoding SPM-1 was flanked upstream by *groEL* and CR4 in 24 epidemiologically unrelated *P. aeruginosa* isolates. These findings suggest that these elements have been transferred to genetically distinct strains along with the MβL encoding gene.
- The results of the present study indicate that CR4 could be either a part of a large system of transposition or a recognition site for mobilization event.
- Further studies are necessary to determine the exact role of CR4 in *bla*$_{SPM-1}$ mobilization as well as to evaluate the mechanism of *bla*$_{SPM-1}$ mobilization in genetically distinct *P. aeruginosa* isolates.

References

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