Molecular characterization of SPM-1, a novel metallo-β-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme

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The gene encoding the metallo-β-lactamase SPM-1 was cloned from a genomic library of Pseudomonas aeruginosa strain 48-1997A. The insert carrying spm-1 possessed a GC content of 47%, indicating that it is of non-Pseudomonas origin. Upstream of spm-1 there is a small open reading frame (ORF), which is homologous to the LysR family of proteins (69% identity to the LysR protein from Salmonella enterica serovar Typhimurium). Downstream of spm-1 there is the start of an ORF, the product of which shows close homology with the GroEL-type proteins from Xanthomonas campestris. No transmissible element could be identified upstream or downstream of spm-1. The spm-1 gene is carried on a plasmid that can transform both Escherichia coli and P. aeruginosa to ceftazidime resistance. SPM-1 contains the classic metallo-β-lactamase zinc-binding motif HXHXD and shows the highest identity (35.5%) to IMP-1. SPM-1 is a distinctly different metallo-β-lactamase from VIM and IMP and, accordingly, represents a new subfamily of mobile metallo-β-lactamases. The predicted molecular weight of the protein was 27515 Da, significantly higher than that of IMP (25041 Da) or VIM (25322 Da). SPM-1 possesses a unique loop of 23 residues that accounts for the higher molecular mass.

Keywords: metallo-β-lactamase, SENTRY, Pseudomonas aeruginosa

Introduction

The advent of carbapenems into clinical practice heralded a new treatment option for irradiating serious bacterial infections caused by cephalosporin- and penicillin-resistant bacteria.1 However, carbapenem resistance has now been observed in Enterobacteriaceae and in non-fermenter species such as Pseudomonas aeruginosa and Acinetobacter spp. The common form of resistance is through either lack of drug penetration (i.e. porin mutations and efflux pumps) and/or carbapenem-hydrolysing β-lactamases. Based on molecular studies, two classes of carbapenem-hydrolysing enzymes have been described: serine enzymes possessing a serine moiety at the active site; and metallo-β-lactamas (class B), requiring divalent cations, usually zinc, as metal cofactors for enzyme activity.2,3

In 1991, a report of a new plasmid-mediated metallo-β-lactamase, IMP-1, in a P. aeruginosa isolate caused great concern due to the potential risk of IMP being disseminated widely to other bacterial species.4 A 1996–1997 survey of IMP-1-producing Gram-negative bacteria in Japan showed that 144 of 3222 (4.4%) Serratia marcescens strains produced IMP-1 through the acquisition of plasmids carrying the imp gene.5 For many years, the detection of IMP-1-producing isolates was restricted to Japan, but recently the appearance of other IMP-type enzymes has been reported throughout South-East Asia, including Hong Kong and Singapore.5,7 An imp allelic variant, which encodes IMP-2, was detected in an

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Acinetobacter baumannii strain isolated in Italy, the first European example. A novel family of class B metallo-β-lactamases, the VIM family (VIM-1, VIM-2 and VIM-3 enzymes), was described in P. aeruginosa and Acinetobacter spp. in Europe. The vim gene, like the imp gene, is carried on mobile gene cassettes inserted into a class 1 integron. Although an IMP variant has been reported from Canada, to date there are no reports of mobile metallo-β-lactamases being reported from the USA or Latin America. In 1999, a 4-year-old female patient was diagnosed with acute lymphoblastic leukaemia and was subsequently admitted to hospital in January 2001 (she also suffered a relapse in November 2000) after her third cycle of chemotherapy at the Instituto de Oncology Pediatricia (Sao Paulo, Brazil). She became neutropenic and presented with episodes of high fever. She was initially treated with ceftriaxone (50 mg/kg/day) and amikacin (15 mg/kg/day) for an ethmoid sinus infection. In early February she had a consolidation of the lower right lobe, and ceftriaxone was replaced by ceftazidime (50 mg/kg/day) for the treatment of suspected pneumonia. Later that month, a urine culture showed a pure growth (>10⁵ cfu/mL) of a P. aeruginosa (designated 48-1997A) susceptible only to polymyxin B, and ceftazidime was replaced by polymyxin B. Five days later, an identical P. aeruginosa isolate exhibiting the same antimicrobial susceptibility profile was isolated from a blood culture. The patient died the following 24 h incubation at 37°C, the MIC was determined as the lowest concentration of antibiotic that inhibited the growth in those dilutions that, when inoculated on to nutrient agar containing no antibiotic, gave rise to single colonies.

Determination of MICs

Mid-log phase grown cultures were diluted to 10⁻³ in water. Ten microlitres from each dilution was spotted on to dried nutrient Iso-Sensitest agar, containing serial dilutions of the appropriate antibiotic, using a multipoint inoculator. After 24 h incubation at 37°C, the MIC was determined as the lowest concentration of antibiotic that inhibited the growth in those dilutions that, when inoculated on to nutrient agar containing no antibiotic, gave rise to single colonies.

Metallo-β-lactamase expression in P. aeruginosa 48-1997A and from recombinant DNA in E. coli

Cellular extracts of P. aeruginosa 48-1997A pre-incubated with EDTA (20 mM) or a serine β-lactamase inhibitor (BRL42715 at 5 µM) had hydrolytic activity determined for cloxacillin, oxacillin, penicillin, ampicillin, imipenem, meropenem, cefalotin, ceftazidime and nitrocefin. The assays were carried out as described previously by measuring the breakdown of the substrate at a specific wavelength for that β-lactam, except for nitrocefin, where the accumulation of product was measured at 482 nm. Specific activity was measured as nanomoles of substrate hydrolysed/min/mg of protein.

Isoelectric focusing

Isoelectric focusing (IEF) was carried out as described previously. Isolates from P. aeruginosa 48-1997A and recombinant clones expressed in E. coli were visualized by staining the IEF gels with 100 µM nitrocefin. Confirmation of the metallo-β-lactamase pI value was performed by pre-incubating the crude cell extract with either 20 mM EDTA or 5 µM BRL42715 and repeating the IEF.

PCR screening for vim and imp metallo-β-lactamase genes

For amplification using primers based on the conserved regions of the imp and vim genes, PCR analysis was carried out using AB-gene Expand Hi-fidelity master mix containing a mix of Pfu/noriprooofreading Taq polymerases and dNTPs. Primers were used at 10 pM concentrations, and 1 µL of bacterial culture (OD₆₀₀ = 1) was used as a template. Cycling parameters were 95°C for 5 min followed by 30 cycles of 95°C for 1 min, annealing at 40°C for 1 min and extension 68°C for 1 min and ending with a 5 min incubation at 68°C. PCR products were visualized by electrophoresis on 0.8% agarose gels in Tris boric acid/EDTA buffer (pH 7.0) and stained with 1% ethidium bromide. The following primers were used for vim and imp PCR screening (reading 5’→3’) and were based on consensus sequences for each of the genes. The sequences are as follows: vim forward,
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GTCTATTTGACCGCGTC; *imp* reverse, CTACTCAACGACTGAGCG; *vim* forward, ATGACGAAGTTATCTGTTATTC; and *imp* reverse, GTGCGCAAGCTGTTGAG.

Recombinant DNA methodology

Genomic DNA was isolated from *P. aeruginosa* strain 48-1997A by the cetyl-tri-ammonium bromide method. Plasmids were purified by the alkaline lysis method using the Qiagen miniprep kit. For construction of the genomic library, size fractionated Sau3AI fragments (>1 kb) were purified after gel electrophoresis using a Qiagen gel purification kit. Five micrograms of purified genomic fragments were ligated after gel electrophoresis using a Qiagen gel purification kit. Plasmids were purified by the alkaline lysis method using the Plasmid Plus kit. DNA sequencing and sequence analysis

Sequencing was carried out on both strands by the dideoxy-chain termination method with a Perkin Elmer Biosystems 377 DNA sequencer. Sequence analysis was carried out using the Lasergene DNASTAR software package. Sequence alignments were done using Clustal W and PAM 250 matrix. The pI value of the protein was determined using DNAstar phylogenetic tree analysis software. The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree measures the distance between sequences (DNAstar, London, UK).

Results

Phenotypic expression of a metallo-β-lactamase from *P. aeruginosa* 48-1997A

*P. aeruginosa* strain 48-1997A was resistant to all β-lactams except for aztreonam, which had an MIC of 4 mg/L. More often than not, the level of resistance was very high (>256 mg/L). Hydrolytic activities of cellular extracts pre-incubated with and without BRL42715 are shown in Table 1. The addition of EDTA and BRL42715 to cellular extracts inhibited the hydrolysis of meropenem by 93% and 1.4%, respectively. These data indicate that isolate 48-1997A possesses a broad-spectrum metallo-β-lactamase displaying penicillinase, cephalosporinase and carbapenemase activities.

IEF counterstained with nitrocefin was used to determine the pI value of strain 48-1997A enzymes. This index strain possessed two enzymes: a serine enzyme (pI, 6.9) inhibited by EDTA and resistant to BRL42715; and a second enzyme (pI, 7.5) that is sensitive to EDTA and resistant to BRL42715 inhibition.

Screening for known mobile metallo-β-lactamase genes

To determine whether this strain was an *imp* or *vim* derivative, primers based on conserved regions (aligning the sequences of *vim* 1–3 and *imp* 1–9) of these genes were used in a low stringent PCR screen (annealing at 40°C). Positive controls were *P. aeruginosa* containing *imp* or *vim*. Screening with primers based on *imp* and *vim* conserved regions was negative, whereas the *imp* and *vim* controls were positive, implying that the metallo-β-lactamases gene from *P. aeruginosa* 48-1997A was not a close derivative of *imp* or *vim*.

Cloning of the metallo-β-lactamase gene *spm-1* from *P. aeruginosa* 48-1997A

The gene encoding the metallo-β-lactamase was isolated from a genomic library of *P. aeruginosa* strain 48-1997A constructed in the plasmid vector pK18 and transformed into the *E. coli* host DH5α by electroporation using a Bio-Rad Gene Pulser. Plating of the library on to X-gal (30 mg/L) and kanamycin (25 mg/L) plates yielded in excess of 500,000 recombinants per 500 ng of recombinant DNA.

Table 1. Hydrolytic activities (nmol/min/mg of protein) of *P. aeruginosa* 48-1997A with and without the serine-β-lactamase, BRL42715, and *E. coli* (24-S) expressing SPM-1 (figures in brackets are percentage values taken as imipenem having a relative value of 100)

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th>48-1997A with BRL42715</th>
<th><em>E. coli</em> (24-S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meropenem</td>
<td>468 (600)</td>
<td>460 (708)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>78 (100)</td>
<td>65 (100)</td>
</tr>
<tr>
<td>Penicillin</td>
<td>444 (569)</td>
<td>336 (517)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>660 (846)</td>
<td>221 (340)</td>
</tr>
<tr>
<td>Cefaloridine</td>
<td>710 (910)</td>
<td>288 (443)</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>55 (71)</td>
<td>20 (37)</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>18 (24)</td>
<td>9.5 (15)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>21 (26)</td>
<td>8.4 (13)</td>
</tr>
<tr>
<td>Nitrocefin</td>
<td>98 (116)</td>
<td>2.4 (3.7)</td>
</tr>
</tbody>
</table>

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Biochemical analysis of recombinant SPM-1

Cellular extracts from *E. coli* 24-S (expressing SPM-1) are shown in Table 1. These values, when compared with the crude cell extracts from strain 48-1997A (in the presence of BRL42715 (5 µM)), show almost identical ratios of hydrolysis for the different β-lactams, indicating that the enzyme expressed from the cloned gene is responsible for the activity seen in strain 48-1997A when pre-incubated with BRL42715 (inhibiting serine β-lactamase activity). The pl value of SPM-1 expressed in *E. coli* was 7.5 (data not shown), confirming the IEF analysis from the crude cell extracts on *P. aeruginosa* 48-1997A.

Sequence analysis of *spm-1* and its genetic context

The DNA insert carried by 24-S possessed a GC content of 47%, indicating that it is of non-*Pseudomonas* origin, which in comparison is ∼66% (http://www.sanger.ac.uk/Projects/P_fluorescens/). 24-S contained an open reading frame (ORF) encoding a putative protein of 264 amino acids displaying homology with previously cloned metallo-β-lactamases: namely, IMP-type proteins. The sequence of *spm-1* has been deposited in the EMBL database with the accession number AJ492820. The N-terminus of the predicted protein shows typical features of bacterial signal peptides: namely, a hydrophobic portion of ∼13 amino acids, preceded by five highly charged molecules that target protein secretion to the periplasm. Sequencing of the N-terminus of the mature protein identified the cleavage site of the signal peptide between serines 18 and 19. The predicted molecular weight of the protein was 27515 Da, which is significantly lower than that of IMP (25041 Da) or VIM (25322 Da).8,18 The theoretical pl value of SPM-1 (not including the leader sequence) is calculated to be 8.91, which is significantly different from the actual value of 7.5 measured by IEF. This phenomenon has been reported for other β-lactamases.17

Upstream of *spm-1* there is a small ORF, designated ORF2, which is homologous to the LysR family of proteins (69% identity to the LysR protein from *Salmonella enterica* serovar *Typhimurium*) (Figure 1). The spacing between the ORFs is 150 bp. ORF2 is predicted to be transcribed in the opposite direction from that of *spm-1*; however, the protein appears to be truncated and therefore non-functional. Downstream of *spm-1* there is the start of an ORF, designated ORF3. ORF3 is predicted to be transcribed in the same direction as *spm-1*, the product of which shows close homology with the GroEL-type proteins from *Xanthomonas campestris* and *S. enterica* with 73% and 65% identity, respectively.19 Within the cloned insert, no sequence showing any degree of homology with integrons was found. The insert carried by 24-S was confirmed by PCR to be that of strain 48-1997A.

Alignment and homology with other class B enzymes

The protein sequence of SPM-1 contains the metallo-β-lactamase zinc-binding motif HFHLD, as well as other residues that have been implicated in binding two zinc ions (Figure 2). The mature protein of SPM-1 showed the highest identities to the following metallo-β-lactamases: 35.5% to IMP-1, 32.2% to ImiS, 32.1% to CphA, 30% to BCII and 27% to CcrA.20-24 When aligned with the other class B β-lactamases, SPM-1, similarly to the *Aeromonas* metallo-β-lactamases and L1 from *Stenotrophomonas maltophilia*, has a loop that is not present in the other class B enzymes (Figure 2).21,26 In SPM-1 this loop comprises 23 amino acids, whereas in the *Aeromonas* spp. class B enzymes and L1 it is 17 and 10 amino acids, respectively. Although the highest identity is seen with IMP-1, SPM-1 shares more identity with the C-terminals of IMP-1 than with the N-terminus. From the N-terminus to the start of (but not including) the loop, SPM-1 exhibits 31% identity to IMP-1, whereas, from (but not including) the loop to the C-terminus, SPM-1 exhibits 47% identity to IMP-1.4 As judged by the sequence homology alone, SPM-1 should be classified with the group 3a β-lactamases.

The phylogenetic tree (Figure 3) shows SPM-1 aligning with the IMP-type class B enzymes rather than with the others. However, the clustal weighting clearly shows that SPM-1 is different and only distantly related to the IMP-type enzymes. Although the next highest identity was seen with the *Aeromonas* spp. class B enzymes ImiS and CphA,21,22 the phylogenetics indicate that SPM-1 is distantly related to these enzymes.

![Figure 1](https://academic.oup.com/jac/article-abstract/50/5/673/754356/56561761)
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Discussion

E. coli carrying recombinant clones of spm-1 could be selected for on media containing ceftazidime at high concentrations. However, like vim and imp, spm-1 when expressed in E. coli does not confer resistance to the carbapenems (imipenem and meropenem MICs of 1 and 0.25 mg/L, respectively) or aztreonam (1 mg/L) but does confer resistance to the penicillins and all cephalosporins.

Given the low GC content (47%) of the insert carrying spm-1, there is little doubt that the gene has been imported into Pseudomonas, despite the fact that attempts to move it across into either an E. coli or Pseudomonas recipient failed (data not shown). However, we have determined that spm-1 is present on a large plasmid and that this can be used to transform both E. coli and P. aeruginosa to ceftazidime resistance. Both vim- and imp-type genes have been shown to be associated with class 1 type integrons. However, immediately upstream or downstream of spm-1 there were no sequences homologous with transmissible elements. One hundred and fifty base pairs upstream of spm-1 there is a gene encoding a LysR-type protein. The gene context of spm-1 is similar to the qac/LysR locus associated with the β-lactamase PSE-1 on a multidrug-resistance island found in S. enterica serovar Typhimurium DT104 (Figure 1).19

Sequence analysis of SPM-1 shows it to be most similar to IMP-1 but that it contains a loop of 23 residues that explains the higher molecular mass. Interestingly, SPM-1 shows more homology for IMP-1 towards the C-terminus of the protein than to the N-terminus, and this demarcation seems to occur immediately before and after the loop. Although it is mere speculation, it is interesting to raise the possibility that SPM-1 could be a hybrid protein of two ancestral class B type enzymes, one being more IMP-1-like than the other. The alignment of SPM-1 with the other class B enzymes clearly shows that SPM-1 contains the classic HXHXD motif as well as key histidine (positions 165 and 221) and cysteine (position 184) residues that are capable of coordinating two zinc molecules per molecule of enzyme.

The data indicate that spm-1 is a novel metallo-β-lactamase gene that is significantly different from the IMP or VIM groups of enzymes. Although we could not identify any transmissible element adjacent to spm-1, the gene is likely to be mobile. Crude enzyme kinetics indicate that SPM-1 can hydrolyse all classes of β-lactam antibiotics. Rather like IMP

Figure 3. Phylogenetic tree showing clustal weighting and relatedness of SPM-1 to other metallo-β-lactamases. SPM-1 is clustally aligned with the IMP-type β-lactamases rather than with the class B enzymes.
and VIM, SPM-1 poses a significant threat to metallo-β-lactam regimens that may be used to treat systemic infections, notwithstanding the fact that there is no clinically available metallo-β-lactamase inhibitor.

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