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Biochemical Characterization of IMP-16, a Novel IMP Variant Harbored in a Class I Integron of a *Pseudomonas aeruginosa* Clinical Isolate from Brazil

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SENTRY ANTIMICROBIAL SURVEILLANCE

AMENDED ABSTRACT

Objectives. Several variants of IMP-type enzymes have been identified and they are from 1 to 17% amino acid residues divergent from IMP-1. In some cases, they also present with different kinetic properties. Here we report the biochemical characterization of a new IMP variant, IMP-16, that was found in *Pseudomonas aeruginosa* index strain (101-4704) clinical isolate from Brasilia, Brazil.
Methods. Primers targeting the 5'CS and 3'CS regions of class 1 integron were used to amplify the bla_{IMP-16} containing integron. These primers yielded PCR products, which were sequenced on both strands using DuPont Automated systems. After integron sequence analysis, the bla_{IMP-16} gene was subcloned into pPCRScriptCam SK+ expression vector and overexpressed in *Escherichia coli* DH5α. The IMP-16 protein was purified by Fast Performance Liquid Chromatography. Kinetic properties were determined with several ß-lactam substrates measuring hydrolytic activity under initial rate conditions.
Results. The N-terminus of the IMP-16 showed typical features of bacterial signal peptides that target proteins to the periplasmic space and the most likely cleavage site was located between the alanine at positions 18 and glycine at position 19. This produced a mature protein of 25,266 Da with a theoretical pI of 6.5. IMP-16 is a new IMP variant that differed from IMP-1 by 15% amino acid residues, being one of the most divergent variants so far identified. It was more similar to IMP-8 and IMP-11 (89.5 and 90.3%, respectively) at the level of mature protein. *E. coli* DH5α harboring the bla_{IMP-16} recombinant plasmid showed a similar ß-lactam resistance profile when compared with the index strain (101-4704), being resistant to nearly all ß-lactams tested, apart from aztreonam and carbapenems. The IMP-16 enzyme was overproduced in *E. coli* and purified (>95%). Kinetic analysis revealed K_m values of 72, 365, 7805 and 115 µM, and k_{cat} values of 23, 133, 800 and 1166 s^{-1} for meropenem, imipenem, penicillin and nitrocefin, respectively.
Conclusions. IMP-16 was a new highly divergent IMP variant detected in *P. aeruginosa*. Kinetic parameters showed the great structural and functional plasticity among this group of clinical important enzymes.

INTRODUCTION

IMP-type metallo-ß-lactamases are included in the Ambler molecular class B and in the group 3a of the Bush-Jacoby-Medeiros functional classification. Their clinical importance is based in the fact that they hydrolyze carbapenems, important therapeutic options for serious infections due to multi-drug resistant Gram-negative strains. IMP-type enzymes appear to be more prevalent in eastern Asian countries, such as Japan, but they have also been reported in several other geographic regions recently, indicating worldwide distribution.

Since the first report of IMP-1 producing clinical strains in 1999, the bla_{IMP} has been found in an integron-like structure. The IMP variants described to date present 1 to 17% amino acid residue variation from IMP-1 and these amino acid substitutions are reflected in functional differences. We report here the biochemical characterization of a new IMP variant, IMP-16, which was found in a *Pseudomonas aeruginosa* clinical strain (101-4704) isolated in Brasilia, Brazil through the SENTRY Antimicrobial Surveillance Program.

MATERIALS AND METHODS

Bacterial strain and plasmid. Bacterial strain and plasmid used in this study are described in Table 1. The IMP-16 – producing *P. aeruginosa* strain (101-4704) was isolated from a patient hospitalized in Brasilia, Brazil (2002).

Susceptibility testing. The strains used in this study were susceptibility tested by reference microdilution methodology according to the NCCLS. Antimicrobial agents were obtained from the respective manufacturers or purchased from a commercial chemical source (Sigma-Aldrich, Poole, UK). Quality control was performed by concurrent testing of *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212.

Recombinant DNA methodology. bla_{IMP-16} was amplified by PCR using the primers set Int1-1F – aacA30R and subsequently cloned into pPCRScriptCam SK+ in order to produce the recombinant plasmid pREM-4. The ribosome binding-site and the stop codon were included in order to allow gene expression. The recombinant plasmid was transferred into *E. coli* DH5α and selected by plating onto nutrient agar plates containing chloramphenicol (30 mg/L). The recombinant strains were subsequently screened by PCR using primer set M13F – M13R and their insert and orientation confirmed by sequencing.

ß-lactamase purification. A single colony of the recombinant *E. coli* pREM-4 was grown overnight in 10 ml of nutrient broth containing ceftazidime (10 mg/L) and chloramphenicol (30 mg/L) at 37°C. The cells were harvested by centrifugation (4,000 x *g* for 10 min at 4°C) and then added into 4 liters of terrific broth for aerobic growing in orbital shaking for 24 hours at 37°C. The cells were again harvested, as describe above, and resuspended in 100 ml of 10mM HEPES buffer (pH 7.5). Periplasmic preparation was obtained by addition of 400 µl of lysozyme solution (20 mg/ml) (Sigma-Aldrich) and 400 µl of calcium chloride (147 mg/ml). Cells debris was removed by centrifugation (9,000 x *g* for 20 min at 4°C) and the supernatant loaded onto a Q-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden) and proteins eluted with a linear NaCl gradient (0–1 M), at a flow rate of 2 ml/min. Fractions containing ß-lactamase activity were pooled, concentrated and then injected onto a Superdex 75 HR 10/30 column (Amersham–Pharmacia Biotech) previously equilibrated with HEPES buffer containing 50 µM $ZnCl_2$ and 0.2 M NaCl. Proteins were eluted at a flow rate of 0.7 ml/min. During the purification procedure the presence of ß-lactamase activity was monitored with nitrocefin solution.

Protein eletrophoretic technique. The yielded enzyme was submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the purity with a Mini-Protein II apparatus (Bio-Rad).

Kinetic measurements. Reactions were performed at 20ºC with 20µl of enzyme in 1 ml of HEPES buffer containing 50 µM $ZnCl_2$ and 0.2 M NaCl (pH 7.0). Hydrolysis was ascertained by observing the changes in absorbance for a variety of substrates at a range of concentrations in a Lambda 35 spectrophotometer (Perkin-Elmer, Cambridge, UK). The steady-state kinetic parameters K_m (µM) and k_{cat} ($seconds^{-1}$) were deduced from the initial rates of hydrolysis by using the Hanes-Woolf plot.

Computer sequence analysis. The nucleotide sequences comparisons were deduced using software available over the internet (http://www.ebi.ac.uk/fasta33/). Nucleotides sequence and the deduced protein product, alignments and phylogenetic relationships were determined using the Lasergene software package (DNASTAR, Madison, WI, USA).

RESULTS

bla_{IMP-16} sequence analysis and deduced protein sequence.

- The N-terminus of the IMP-16 protein showed typical features of bacterial signal peptides that target proteins to the periplasmic space. The most likely cleavage site was identified between the alanine at positions 18 and glycine at position 19.
- IMP-16 differed from IMP-1 by 15% of the amino acid residues and showed highest identity to IMP-8 and IMP-11 (89.5 and 90.3%, respectively).
- IMP-16 showed six unique amino acid differences when compared to others IMP variant; namely E50D, V74F, T88A, T122S, V183L and K252R (see Figure 1).

Antimicrobial resistance pattern of *E. coli* DH5α harboring the recombinant plasmid pREM-4.

- The *E. coli* DH5α harboring the recombinant plasmid pREM-4 showed an antimicrobial resistance profile consistent with those observed for the *P. aeruginosa* index strain 101-4704. The *E. coli* DH5α (pREM-4) showed decreased susceptibility to cefepime, piperacillin, piperacillin/tazobactan, imipenem and meropenem, and high level resistance to the others ß-lactams tested (Table 2).
- The index strain, as well as, the *E. coli* DH5α (pREM-4) remained highly susceptible to the monobactam, aztreonam (Table 2).

IMP-16 purification and kinetic parameters.

- The IMP-16 enzyme was overproduced and purified from *E. coli* pREM-4 throughout cation-exchange chromatography followed by a gel permeation chromatography step. The IMP-16 purified protein preparation appeared to contain a single band just under the 28.8 kDa and it was estimated to be >95% pure (Figure 2).
- IMP-16 was capable of hydrolyzing several ß-lactams, including penicillins, narrow- to expanded-spectrum cephalosporins and carbapenems. No hydrolysis of cefoxitin or aztreonam was observed (Table 3).
- IMP-16 kinetic parameters showed that cephalosporins and carbapenems were the best substrates (k_{cat}/K_m ratios $\geq 0.15\mu M^{-1} \cdot s^{-1}$), while penicillins were uniformly poorer substrates (k_{cat}/K_m ratios $\leq 0.13\mu M^{-1} \cdot s^{-1}$) (Table 3).

Table 1. Bacterial strain and plasmid used in this study.

Strain	Genotype/phenotype
P. aeruginosa 101-4704	Carbapenem-hydrolyzing clinical isolate
E. coli DH5α	*SupE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*
Plasmid	
pPCRScriptCam SK+	Chloramphenicol^r
pREM-4	1817-bp PCR product from bla_{IMP-16} and *aac(6')-30* cloned into pPCRScriptCam SK+

RESULTS

Table 2. Antimicrobial susceptibility profile of the IMP-16 – producing *P. aeruginosa* 101-4704 clinical isolate, the *E. coli* DH5α harboring recombinant plasmid pREM-4 and the recipient strain *E. coli* DH5α.

	MIC (mg/L)		
Antimicrobial Agents	*P. aeruginosa* 101-4704	*E. coli* DH5α (pREM-4)	*E. coli* DH5α
Ampicillin	>16	>16	2
Amoxacillin /Clavulanate	>16	>16	4
Cefazolin	>16	>16	≤2
Cefuroxime	>16	>16	4
Aztreonam	1	≤0.12	≤0.12
Ceftazidime	>16	>16	≤1
Ceftriaxone	>32	16	≤0.25
Cefepime	>16	4	≤0.12
Imipenem	>8	2	≤0.5
Meropenem	>8	1	≤0.06
Piperacillin	32	4	≤1
Piperacillin /Tazobactam	4	4	≤0.5
Ticarcillin	128	128	≤16
Ticarcillin /Clavulanate	128	128	≤16

Table 3. Kinetic parameters of the purified IMP-1, IMP-2, IMP-12 and IMP-16.

	IMP-1			IMP-2			IMP-12			IMP-16		
Antibiotic	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Penicillin	320	520	0.62	[b]	[b]	[b]	[b]	[b]	[b]	800	7805	0.10
Ampicillin	950	200	4.8	23	110	0.21	18	1500	0.012	137	1065	0.13
Carbenicillin	ND[a]	ND[a]	0.02	252	700	0.36	3.7	175	0.021	433	3331	0.13
Cloxacillin	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	33	380	0.09
Piperacillin	ND[a]	ND[c]	0.72	[b]	[b]	[b]	ND[c]	ND[a]	0.023	250	2804	0.09
Nitrocefin	63	27	2.3	275	95	2.9	570	72	7.9	1166	115	10.2
Cephalothin	48	21	2.4	[b]	[b]	[b]	118	16	7.4	77	42	1.8
Cefuroxime	8	37	0.22	[b]	[b]	[b]	61	7	8.7	52	49	1.06
Cefoxitin	16	8[c]	2	7	7	1.0	[b]	[b]	[b]	NH[d]	ND[a]	ND[a]
Ceftazidime	8	44	0.18	21	111	0.19	6.7	15	0.45	13	87	0.15
Cefotaxime	1.3	4[c]	0.35	[b]	[b]	[b]	56	22	2.5	35	36	0.97
Cefepime	7	11[c]	0.66	4	7	0.57	15	26	0.58	20	88	0.23
Imipenem	46	39	1.2	22	24	0.92	240	920	0.26	133	365	0.36
Meropenem	50	10	0.12	1	0.3	3.3	9.5	7.2	1.3	23	72	0.32
Aztreonam	<0.01	>1000	$<1x10^{-5}$	NH[d]	ND[a]	ND[a]	NH[d]	ND[a]	ND[a]	NH[d]	ND[a]	ND[a]

a. ND. Data could not be determined.
b. Data not available.
c. K_m was obtained as the K_i value.
d. NH. No hydrolysis detected.

Figure 1. Amino acid alignment of the IMP-16 protein with those of IMP-types enzymes. Differences in the amino acid sequences are noted by insertion of a single letter representing the amino acid change within that particular sequence. Stars under the IMP-16 sequence represent amino acids involved in the coordination of zinc ions, and residues known to not tolerate substitutions are underline. Numbering is according to the BBL scheme.

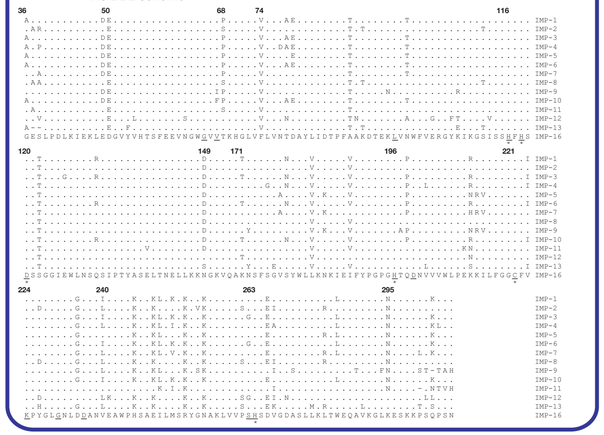
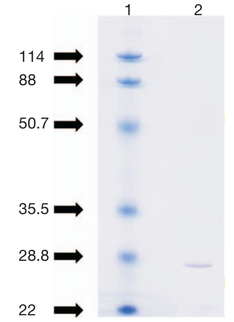


Figure 2. SDS-PAGE of the IMP-16 purified protein preparation. Lane 1, molecular weight markers, in kilodaltons, are indicated by arrows (Prestained SDS-PAGE Standards, Low Range Molecular Weight, Bio-Rad); Lane 2, the purified protein eluted from the gel filtration column.



CONCLUSIONS

- IMP-16 is a new highly divergent IMP-variant detected in a *P. aeruginosa* clinical strain isolated in Brazil. It represents the first IMP allelic variant detected in Latin America which underscores the diversity and wide dissemination of IMP-like enzymes.
- Residues important for the structure and function of IMP enzymes (His116, His118, Asp120, His196, Cys221, Lys224 and His263) were conserved in the IMP-16, as well as other residues that do not tolerate substitutions (Figure 1).
- In general, IMP-16 hydrolyzed ß-lactams less efficiently than IMP-1. However, it showed common features, such as an overall preference for cephalosporins and carbapenems rather than penicillins or monobactams.
- The main functional difference observed among IMP-16 kinetic parameters was a lack of cefoxitin hydrolysis. Although there were no amino acid changes in the active site of IMP-16, the substitution V74F, was located seven residues after the IMP loop and may affect its kinetics.

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