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Molecular and Biochemical Characterization of a Novel Class B ß-lactamase, GIM-1: a New Subclass of Metallo-ß-Lactamase: Report from the SENTRY Antimicrobial Surveillance Program

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AMENDED ABSTRACT

Background: There are three major types of mobile metallo-ß-lactamase (MßL) genes; bla_{IMP}, bla_{VIM} and bla_{SPM}. As part of the SENTRY Program, numerous *P. aeruginosa* (PSA) have been screened for resistance (R) to carbapenems (imipenem [IMP] and meropenem [MER]) and ceftazidime (CTZ). Five PSA isolates from Dusseldorf were detected, the index strain from a 37 yo male ICU patient with nosocomial pneumonia.

Methods: MßL production was confirmed by the Etest® MßL strip and was further studied by biochemical assay techniques. Isolates that were PCR-negative for bla_{IMP}, bla_{VIM} and bla_{SPM}, were screened by amplifying the region between the integrase and *aacA4* genes. Amplicons (> 500 bp) were sequenced. Hydrolysis rates were measured for 10 ß-lactams and inhibition analysis evaluated with aztreonam (ATM), clavulanic acid (CA) and EDTA.

Results: Sequence analysis revealed a novel MßL gene, designated bla_{GIM-1}. The gene lies between a Class 1 integron and *aacA4* within a 6.0kb integron. bla_{GIM-1} is carried on a large plasmid. GIM-1 comprises 250 amino acids (MW, 25.5 Kda) and a pI value of 5.4. GIM-1 differs from the IMP, VIM and SPM-1 enzymes by 39 - 43%, 28 - 31% and 28%, respectively. GIM-1 also possesses a unique main active site, HSHEDR. GIM-1 actively hydrolyzes IMP, MER, penicillins and cephalosporins, where IMP and cefotaxime are the preferred substrates. ATM and CA showed very unusual kinetic properties. These features indicate a new type of MßL.

Conclusions: GIM-1 is a unique MßL and represents the fourth type of mobile MßL to be characterized. bla_{GIM} is associated with a Class 1 integron and comprises part of a large gene cassette including *aacA4.* These data indicate that antimicrobial therapy for PSA infections in Europe may be further compromised by the dissemination of this and previously documented MßL genes.

INTRODUCTION

Since the early 1990s when IMP-1 was first described from a clinical isolate in Japan, reports of metallo-ß-lactamase (MßL)-producing strains have been increasing all over the world. IMP-type, VIM-type and SPM-1 enzymes have disseminated among clinically important pathogens, such as *Pseudomonas* spp, *Acinetobacter* spp and members of the family Enterobacteriacea.

MßL genes are important resistant determinants and most are carried in mobile gene cassettes on class 1 integrons, which have great potential to spread. The class 1 integrons are the most common class that carries antimicrobial resistant genes. These integrons contain a 5'-conserved segment (CS), an *intI1* gene encoding an integrase, a recombination site *attI1*, a promoter, and a 3'-CS. In the 3'-CS, usually lies a truncated genetic structure that confers resistance to quaternary ammonium compounds and sulfonamides, *qacE∆1/sul1*. Integrons are able to capture genes via a site-specific recombination between two sites, one in the integron and one in the cassette. Both of these recombination sites confer mobility due to their recognition by the integrase that catalyzes the integration of the gene cassette between the *attI1* in the integron and the 59-be of the gene cassette.

We report the biochemical and genetic characterization of GIM, a novel subclass of the Ambler class B enzymes. GIM-1 was found on a plasmid harboring a new integron. The carbapenem-resistant *P. aeruginosa* clinical isolates that produce GIM-1 were detected by the SENTRY Antimicrobial Surveillance Program in Germany.

MATERIALS AND METHODS

Bacterial Strains. The SENTRY Program collects clinical isolates from medical centers located in North America, Latin America, Europe, Asia, Africa and Australia. Among other selected pathogens, *P. aeruginosa* strains resistant to imipenem (MIC, ≥ 16 μg/ml), meropenem (MIC, ≥ 16 μg/ml), and ceftazidime (MIC, ≥ 32 μg/ml) have been routinely screened for MßL genes since 2001.

Phenotypic detection of metallo-ß-lactamases. MßL Etest® strips (AB BIODISK, Solna, Sweden) and disk approximation tests were used to screen for class B ß-lactamase producing strains. Carbapenemase activities of cell sonicates from overnight broth cultures were determined using a Lambda 35 spectrophotometer (Perkin-Elmer, Cambridge, UK). Reactions were performed with imipenem and meropenem in assay buffer (50 mM Cacodylate, 100 mM sodium chloride, 100 mM zinc chloride, pH 7.0) at a total volume of 2.05 ml. In addition, tests with and without EDTA were carried out to confirm the inhibition with chelating agents.

PCR experiments. Screening for bla_{IMP}, bla_{VIM} and bla_{SPM} was performed by PCR amplification with primers based on the internal sequences of the MßL genes and known structures of the class 1 integrons. The cycling parameters were: 95ºC for 5 minutes followed by 30 cycles of 95ºC for 1 minute, annealing at 45ºC for 1 minute and extension 68ºC for 1 to 4 minutes and ending with 5 minute incubation at 68ºC.

DNA sequencing and protein analyzes. PCR fragments were sequenced in both strands using DuPont Automated systems. The nucleotide and deduced aminoacid sequences were analyzed using Lasergene software package (DNASTAR, Madison, WI) and compared to sequences available on internet websites.

Analytical IEF. The ß-lactamase extracts from *P. aeruginosa* isolates were obtained by cell lyses with BugBuster (Novagen, Nottingham, UK) and the experiment was performed with NOVEX (Invitrogen, Carlsbad, CA) apparatus. The focused ß-lactamases were detected by overlaying the gel with nitrocefin solution (BD Microbiology Systems, Cockeysville, MD). Isoelectric points were estimated by linear regression analysis by comparison to reference proteins and using a pI 4.5 to 9.5 Standard IEF Marker (Bio-Rad, Watford, United Kingdom).

MATERIALS AND METHODS (Continued)

Kinetic measurements. Purified ß-lactamase was used to determine the kinetic parameters at 25ºC in 2 ml of Assay buffer. Hydrolysis was measured in a Lambda 35 spectrophotometer (Perkin-Elmer, Cambridge, UK) by observing the changes in absorption as a result of the opening ß-lactam ring in the specific wavelengths for each of 20 antimicrobial agents evaluated (Table 1).

COMMENTS

- Initial MßL screening experiments showed carbapenemase activity and positive EDTA inhibition in five *P. aeruginosa* isolates from a medical center located in Dusseldorf, Germany. The index isolate was recovered from a 37 yo male ICU patient with nosocomial pneumonia in February 2002. The additional isolates were collected from February to July 2002 in the same hospital.
- All five GIM-1 producing isolates showed similar antimicrobial susceptibility patterns. They were highly resistant to 28 of the 29 antimicrobial agents tested (including all ß-lactams, aminoglycosides and quinolones). The isolates were only susceptible to polymyxin B.
- Preliminary PCR amplification experiments failed to detect bla_{VIM}, bla_{IMP} and bla_{SPM}-like genes. The analysis of the sequenced amplicons of elements found in the class 1 integron carrying antimicrobial resistance (*intI1* and *aacA4*) showed a 900bp fragment encoding a new MßL, named bla_{GIM-1} (German imipenemase).
- GIM-1 showed low identity with SPM-1 (28.0%) and most of the IMP and VIM-variants. The GIM-1 nucleotide sequence displayed greatest identities with the IMP-variants IMP-1 and IMP-6 (43.15 and 43.5%, respectively) and with VIM-variants (ranging from 31.2% with VIM-7 to 28.8% with VIM-1, VIM-4 and VIM-5).
- The nucleotide sequence of bla_{GIM-1} presented a GC content of 42.15% and encoded a mature protein displaying 232 aminoacids (25,501.70 Da), and a theoretical pI of 5.34.
- The putative protein sequence demonstrated that GIM-1 possesses the motifs involved in the catalytic activity conserved in MßL enzymes: **HXHXD** (residues 95 to 99), Hys156, Cys175 and Hys218.
- Additional analysis of the genetic environment of bla_{GIM-1}, revealed that this MßL gene is located in the first position of a class 1 integron with all key genetic components commonly found in this structure (*intI1*, *att1* in the 5'-CS side and *qacE∆1/sul1* in the 3'-CS side).
- Downstream of bla_{GIM-1} lies an *aacA4* that appears to be truncated with the MßL gene, since there is no 59-be between the two genes. Upstream of the 3'-CS side of this integron lies an *oxa-2* gene cassette.
- Analytical IEF experiments revealed two ß-lactamases, one with a pI of 5.6, that corresponds to GIM-1. The second ß-lactamase exhibited a pI of 7.7 and it is probably an OXA-2.
- The kinetic parameters of GIM-1 showed that this new enzyme hydrolyzes all the ß-lactam compounds tested, except aztreonam, azlocillin and the serine ß-lactamase inhibitors, clavulanic acid and tazobactam.
- The highest k_{cat}/K_m ratios were found with cephalothin, cefuroxime and ceftazidime, and the lowest ratios were demonstrated with moxalactam, ticarcillin and carbenicillin.
- When compared to IMP-1, VIM-1, VIM-2 and SPM-1 against imipenem, GIM-1 showed 1.) higher affinity; 2.) a similar hydrolysis rate (k_{cat}); and 3.) a lower k_{cat}/K_m ratio.

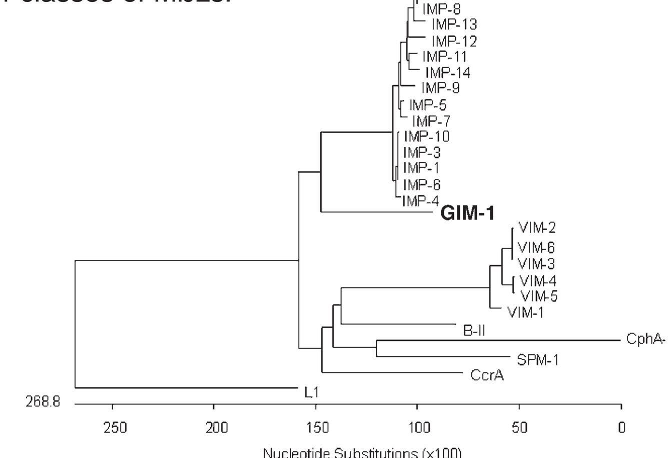

Figure 1. Phylogenetic tree for the major classes of MßLs.

RESULTS

Table 1. Steady-state kinetic parameters of the purified GIM-1 in comparison with those of IMP-1, VIM-1, VIM-2 and SPM-1.

	GIM-1			IMP-1			VIM-1			VIM-2			SPM-1		
Antimicrobial	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 ($\mu M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Penicillin	6.63	46.62	0.142	320	520	0.62	29	841	0.034	55.8	49	1.14	108	38	2.8
Ampicillin	3.28	20.81	0.157	950	200	4.8	37	917	0.04	-[b]	-[b]	-[b]	117	72	1.6
Carbenicillin	4.13	170.57	0.024	ND[a]	ND[a]	0.02	167	75	2.2	-[b]	-[b]	-[b]	74	814	0.09
Azlocillin	ND[a]	ND[a]	ND[a]	-[b]	-[b]	-[b]	1525	123	12	-[b]	-[b]	-[b]	53	147	0.35
Piperacillin	6.91	69.73	0.099	ND[a]	ND[a]	0.72	1860	3500	0.53	32.7	72	0.45	117	59	2
Ticarcillin	2.28	57.6	0.039	1.1	740	0.0015	452	1117	0.41	31.7	46	0.69	ND[a]	<0.35	ND[a]
Nitrocefin	5.81	12.37	0.469	63	27	2.3	95	17	5.6	-[b]	-[b]	-[b]	0.53	4	0.12
Cephalothin	15.85	22.05	0.718	48	21	2.4	281	53	5.3	56.2	44	1.28	43	4	11.7
Cefuroxime	5.86	7.3	0.802	8	37	0.22	324	42	7.7	12.1	22	0.55	37	4	8.8
Cefoxitin	8.31	206.23	0.040	16	8[c]	2	26	131	0.2	3	24	0.12	8	2	4
Ceftazidime	18.24	31.56	0.577	8	44	0.18	60	794	0.076	89	98	0.90	28	46	0.6
Cefotaxime	1.08	4.57	0.236	1.3	4[c]	0.35	169	247	0.68	27.5	32	0.86	16	9	1.9
Cefepime	17.2	431.8	0.039	7	11[c]	0.66	549	145	3.8	4.7	184	0.03	18	18	1
Imipenem	27.14	287.43	0.094	46	39	1.2	2.0	1.5	1.3	9.9	10	0.99	33	37	1
Meropenem	2.69	25.37	0.106	50	10	0.12	13	48	0.27	1.4	5	0.28	63	281	0.22
Moxalactam	14.46	1035.05	0.014	88	10[c]	8.8	-[b]	-[b]	-[b]	14.8	80	0.18	13	97	0.13
Aztreonam	ND[a]	ND[a]	ND[a]	>0.01	>1000	$<1x10^{-5}$	<0.01	>1000	$<1x10^{-5}$	<0.5	ND[a]	ND[a]	ND[a]	<0.3	ND[a]
Clavulanic acid	ND[a]	ND[a]	ND[a]	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	ND[a]	>0.1	ND[a]
Tazobactam	ND[a]	ND[a]	ND[a]	>1000	>3.98	0.0039	5.3	337	0.016	-[b]	-[b]	-[b]	0.6	3	0.2

a. ND = parameters could not be determined. b. Data not available. c. K_m was obtained as the K_i value.

Figure 2. Alignment of amino acid sequence of GIM-1 with the three leading representatives of the MßL groups, IMP-1, VIM-1 and SPM-1.The putative active sites (HXHXD-residues 95 to 99, Hys156, Cys175 and Hys218 were numbered according to GIM-1) are highlighted.

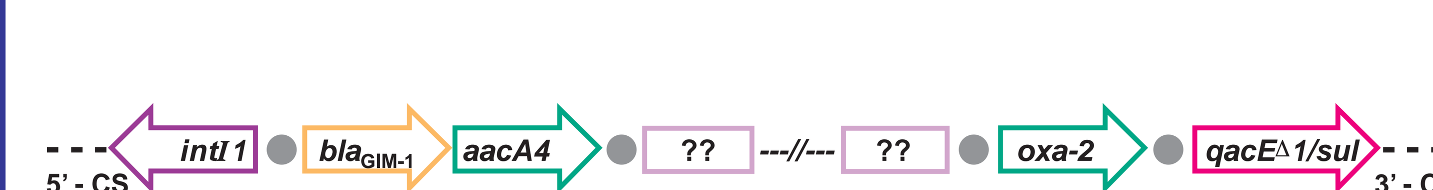

Figure 3. Schematic representation of the integron carrying bla_{GIM-1} (arrows indicate its translational orientation). The dots indicate the 59-be, which were not observed between bla_{GIM-1} and the following gene cassette *aacA4*, indicating that both genes were fused. Further sequence analysis will be required to determine the contents of the region between *aacA4* and *oxa-2* gene cassettes.

CONCLUSIONS

- Our results indicate that bla_{GIM-1} is a novel metallo-ß-lactamase gene that is significantly different from the IMP, VIM or SPM groups of enzymes.
- As with many other ß-lactamase genes, bla_{GIM-1} presented a GC content (42%) indicating a non-*P. aeruginosa* origin.
- The analysis of the integron that harbors bla_{GIM-1} indicated that it is a unique integron. It differs from the other class 1 integrons in length and structure.
- The purified GIM-1 demonstrated a broad substrate profile, no clear preferences for any of the ß-lactams tested and was slightly more active against imipenem than meropenem.
- In general, the k_{cat}/K_m ratios were lower than those of IMP-1, VIM-1, VIM-2 and SPM-1.
- *P. aeruginosa* isolates with bla_{GIM-1} have disseminated among five patients in the medical center where the index strain was initially detected.

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