

Understanding the determinants of substrate specificity in IMP family metallo- β -lactamases: The importance of residue 262

Kevin M. Pegg,¹ Eleanor M. Liu,² Alex C. George,¹ Alecander E. LaCuran,² Christopher R. Bethel,³ Robert A. Bonomo,^{3,4} and Peter Oelschlaeger^{2*}

¹Biological Sciences Department, College of Science, California State Polytechnic University, Pomona, California

²Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, Pomona, California

³Research Service, Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, Ohio

⁴Departments of Medicine, Pharmacology, Biochemistry, and Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, Ohio

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Abstract: In Gram-negative bacteria, resistance to β -lactam antibacterials is largely due to β -lactamases and is a growing public health threat. One of the most concerning β -lactamases to evolve in bacteria are the Class B enzymes, the metallo- β -lactamases (MBLs). To date, penams and cepems resistant to hydrolysis by MBLs have not yet been found. As a result of this broad substrate specificity, a better understanding of the role of catalytically important amino acids in MBLs is necessary to design novel β -lactams and inhibitors. Two MBLs, the wild type IMP-1 with serine at position 262, and an engineered variant with valine at the same position (IMP-1-S262V), were previously found to exhibit very different substrate spectra. These findings compelled us to investigate the impact of a threonine at position 262 (IMP-1-S262T) on the substrate spectrum. Here, we explore MBL sequence-structure-activity relationships by predicting and experimentally validating the effect of the S262T substitution in IMP-1. Using site-directed mutagenesis, threonine was introduced at position 262, and the IMP-1-S262T enzyme, as well as the other two enzymes IMP-1 and IMP-1-S262V, were purified and kinetic constants were determined against a range of β -lactam antibacterials. Catalytic efficiencies (k_{cat}/K_M) obtained with IMP-1-S262T and minimum inhibitory concentrations (MICs) observed with bacterial cells expressing the protein were intermediate or comparable to the corresponding values with IMP-1 and IMP-1-S262V, validating the role of this residue in catalysis. Our results reveal the important role of IMP residue 262 in β -lactam turnover and support this approach to predict activities of certain novel MBL variants.

Keywords: metallo- β -lactamase; point mutation; antibiotic resistance; enzyme evolution; IMP-1 antibody

Abbreviations: BSA, bovine serum albumin; ESI-MS, electrospray ionization mass spectrometry; IPTG, isopropyl β -D-1-thiogalactopyranoside; MBL, metallo- β -lactamase; MIC, minimum inhibitory concentration; MOPS, 3-(N-morpholino)propanesulfonic acid; PAR, 4-(2-pyridylazo)resorcinol; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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*Correspondence to: Peter Oelschlaeger, PhD, Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, 309 E. Second St., Pomona, CA 91766-1854. E-mail: poelschlaeger@westernu.edu

Introduction

Metallo- β -lactamases (MBLs) are bacterial resistance determinants that are becoming widely dispersed in Gram-negative bacteria. MBLs are grouped as class B β -lactamases¹ (B1-B3 subclasses)^{2,3} and contain one or two Zn(II) ions in the active site.^{4,5} MBLs threaten the current status of β -lactam antibacterial chemotherapy, because they inactivate a broad range of penicillins, cephalosporins, and carbapenems via cleavage of the amide bond in the β -lactam ring.⁴ Currently, three major MBL families are responsible for the crisis in antimicrobial chemotherapy (NDM, VIM, and IMP family MBLs).

IMP-type MBLs belong to subclass B1,^{2,3} in which Zn1 is coordinated by H116, H118, and H196 (3H site) and Zn2 by D120, C221, and H263 (DCH site). IMP-type enzymes are clinically important due to their broad substrate spectra, insensitivity to currently used β -lactamase inhibitors (clavulanate, sulbactam, and tazobactam), occurrence throughout the world, and the ability of their encoding genes to spread rapidly in bacteria.⁵⁻¹⁰ In addition, IMP MBLs are a very diverse enzyme family with currently 48 variants found in clinical isolates over the course of about two decades (www.lahey.org/Studies/other.asp#table1). Many of these IMP enzymes cluster around IMP-1¹¹ and IMP-2,¹² which are separated by 15% sequence divergence.^{8,9}

An important long-term goal is to understand the evolution of substrate specificity in MBLs, so that inhibitors that restore the efficacy of β -lactam antibacterials as well as MBL-resistant β -lactams may be developed. Here, as an initial step toward this goal, we explore the role of a single amino acid substitution at the critical position 262 in the IMP-1 MBL. Residue 262 is located near the active site, proximal to the H263 Zn(II) ligand (Fig. 1). A previous study of wild types IMP-1 and IMP-6 (IMP-1-S262G), as well as variants with other amino acids at position 262, exhibited a wide array of substrate profiles toward different β -lactam antibiotics with the V262 variant being most diverse from IMP-1.¹³ A “domino effect” helps explain the role of residue 262 and its effect on catalytic efficiencies toward “Type I” and “Type II” substrates (Fig. 2).¹⁴ Type I substrates are defined as those containing neutral R_2 groups with atoms with high electron density (e.g., nitrocefin, cephalothin, and cefotaxime), while Type II substrates contain axial methyl groups (penicillins) or positively charged R_2 groups (e.g., ceftazidime and imipenem) (Fig. 2). Previously IMP-1 (S262) was shown to inactivate Type II substrates better than IMP-1-S262V (referred to as IMP-6-G262V in the original report, because IMP-6 was the starting point for that mutational study¹³), while the latter has a more pronounced hydrolytic preference toward Type I substrates and poorly converts Type II substrates.¹³ In this study, we explore the role of

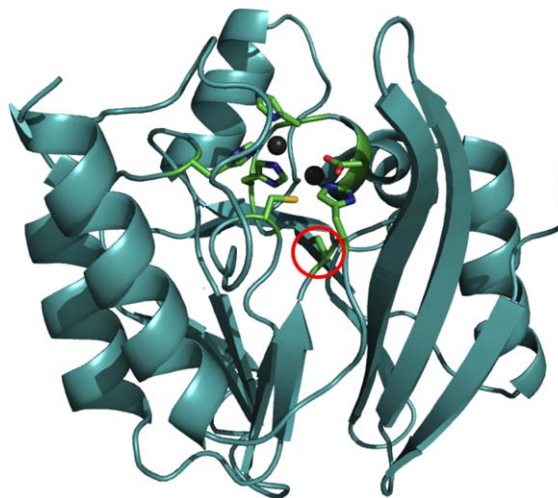


Figure 1. IMP-1 active site with Zn(II) ligands and residue 262. Serine 262 is highlighted with a red circle. The six remaining residues are Zn(II) ligands coordinating their respective Zn(II) ion (depicted as gray spheres). The enzyme backbone is represented as a cyan cartoon and residues as sticks with atoms colored in green (carbon), red (oxygen), blue (nitrogen), and yellow (sulfur). The top left Zn(II) ion is Zn1 in the 3H site and the bottom right one Zn2 in the DCH site. The image was created using PyMOL (www.pymol.org/) and PDB entry 1DD6 (Ref. 21).

residue 262 and attempt to qualitatively predict catalytic efficiencies of the novel variant IMP-1-S262T. Threonine was selected as an amino acid that has features of both serine (hydroxyl group) and valine (branched) (Fig. 3). Our hypothesis is that, based on this “intermediate” structure of threonine, a variant harboring this residue at position 262 would exhibit catalytic efficiencies that are intermediate to those of the enzymes harboring serine (IMP-1) and valine (IMP-1-S262V) at this position. Validation of this hypothesis will allow us to predict the evolution of natural variants at position 262 and anticipate strategies to design effective β -lactams and inhibitors.

Results

Expression and biophysical characteristics

The *bla* genes encoding IMP-1-S262T and IMP-1-S262V were generated by polymerase chain reaction (PCR)-based site-directed mutagenesis, expressed under control of the T7 promoter in *Escherichia coli* cells, and gene products were purified as described previously.¹⁵ Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and protein quantification indicated homogeneous proteins of >95% purity and overall yields of 1.1 mg and 1.3 mg per liter culture for IMP-1-S262T and IMP-1-S262V, respectively (Table I). The percentages of protein expressed in the soluble form, determined by comparing soluble and insoluble fractions after cell lysis via SDS-PAGE, were estimated

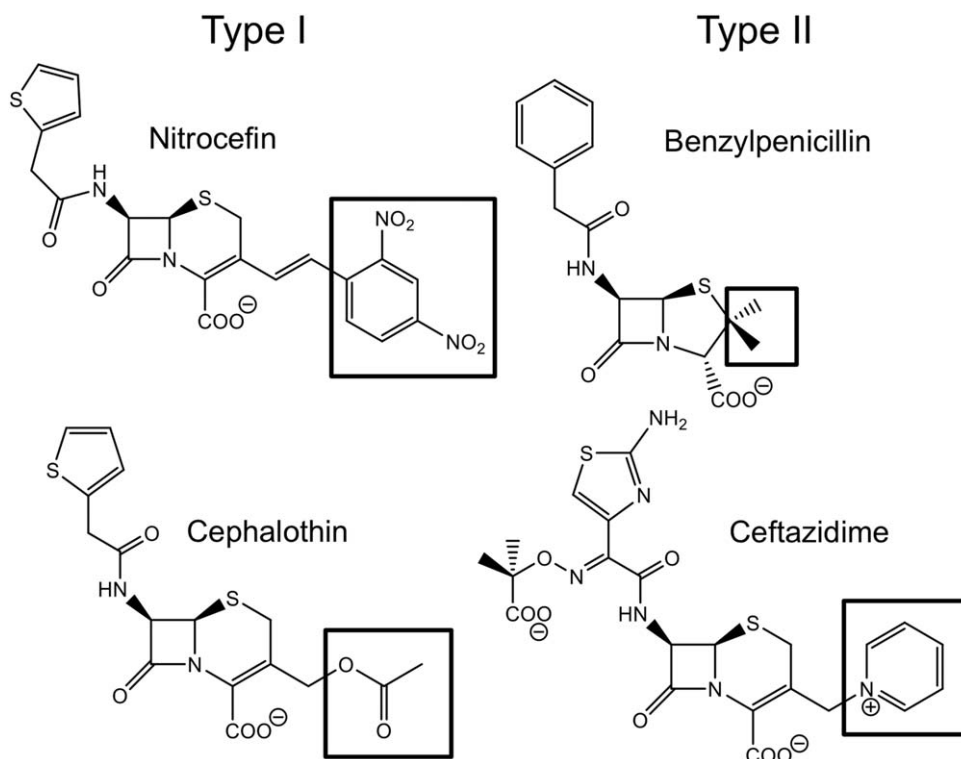


Figure 2. Structures of selected β -lactam substrates. Type I substrates (nitrocefin, cephalothin, and cefotaxime) possess R_2 groups with atoms of high electron density (indicated by black boxes); Type II substrates (ceftazidime, benzylpenicillin, ampicillin, imipenem, and meropenem) have axial methyl groups or positively charged and/or bulky R_2 groups.

to be $\sim 70\%$ and $\sim 50\%$ for IMP-1-S262T and IMP-1-S262V, respectively. These values are consistent with those of IMP-1 expressed and purified in a previous study¹⁵ and used for comparison in the present study, which yielded more ($\sim 90\%$) recombinant protein in the soluble fraction and more (2.0 mg) purified protein per liter culture.

The molecular masses of IMP-1-S262T and IMP-1-S262V determined by electrospray ionization mass spectrometry (ESI-MS) are consistent with the theoretical masses (Table I). Circular dichroism scans of IMP-1-S262T and IMP-1-S262V are superimposable with that of wild-type IMP-1, indicating a conserved secondary structure composed of α helices and β sheets (Fig. 4), typical for the $\alpha\beta\alpha$ fold of the zinc metallohydrolase family of the β -lactamase fold.^{16,17} The differences in the amplitude of the ellipticity are

most likely the result of uncertainty in the determination of protein concentration, which can be in the range of 10–15% rather than an indication of an actual change in the secondary structure. The 4-(2-pyridylazo)resorcinol (PAR) assay¹⁸ indicated both enzymes bound approximately 2 Zn(II) ions per molecule enzyme, just like IMP-1 (Table I).

Kinetic profiles

Kinetic parameters of IMP-1 were determined previously,¹⁵ and IMP-1-S262T and IMP-1-S262V were tested against nine antibiotics: nitrocefin, cephalothin, cefotaxime, ceftazidime, benzylpenicillin, ampicillin, imipenem, meropenem, and aztreonam. All antibiotics were hydrolyzed by these MBLs except for aztreonam. The kinetic constants for IMP-1-S262V and IMP-1 hydrolysis (Table II) are

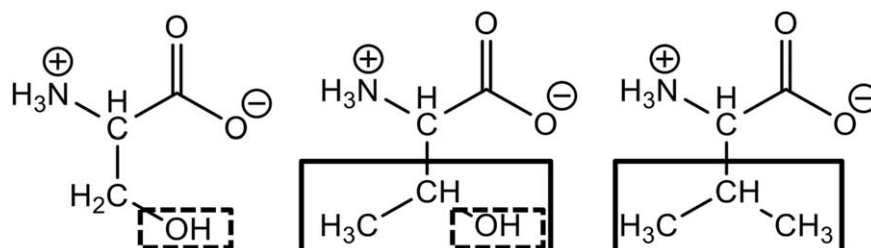


Figure 3. Structural characteristics of amino acids at position 262. The amino acid structures of serine (left), threonine (middle), and valine (right) are depicted in the ionized form with shared functional groups highlighted in boxes. Hydroxyl groups are highlighted in dashed boxes and branched (bulky) groups in solid boxes.

Table I. Expression Data and Biophysical Characteristics of IMP-1, IMP-1-S262T, and IMP-1-S262V

Property	IMP-1 ^a	IMP-1-S262T	IMP-1-S262V
% Soluble	~90	~70	~50
Yield of purified protein per liter of culture in mg	2.0	1.1	1.3
Molecular mass measured (calculated) in Da	25,128 (25,127)	25,125 (25,125)	25,112 (25,113)
Zn(II) ions/molecule enzyme	1.8 ± 0.2	1.8 ± 0.1	1.7 ± 0.1

^a Values reported by Liu *et al.*¹⁵

comparable to those in a previous study.¹³ Interestingly, when analyzing the values of IMP-1-S262T in comparison to those of IMP-1 and IMP-1-S262V, the k_{cat}/K_M values toward all but one substrate (cephalothin) are in the range between these other two proteins, as hypothesized (bolded numbers in Table I). Even the cephalothin value is only 24% outside that range, which could be attributed to experimental uncertainty in protein determination and pipetting errors.

Using equation $\Delta\Delta G_{enz} = -RT \ln((k_{cat}/K_M \text{ IMP-1-S262T})/(k_{cat}/K_M \text{ IMP-1-S262V}))$ to understand the energetics, this difference in k_{cat}/K_M corresponds to a negligible decrease of 0.13 kcal/mol in activation free energy.¹⁹ When analyzing k_{cat} and K_M values separately, the values for IMP-1-S262T are in the range between those of IMP-1 and IMP-1-S262V in three and four out of eight cases, respectively. For two Type I substrates, nitrocefin and cefotaxime, all three values (k_{cat} , K_M , and k_{cat}/K_M) of IMP-1-S262T lie between the values of the other two enzymes.

Susceptibility profiles

For the Type II substrates ceftazidime, benzylpenicillin, ampicillin, imipenem, and meropenem, the order of the MIC values for the three enzymes corresponds to that of the k_{cat}/K_M values (IMP-1=IMP-1-S262T>>IMP-1-S262V for ceftazidime and IMP-1>IMP-1-S262T>>IMP-1-S262V for the others). Thus, the predicted trends in catalytic efficiencies directly translate to trends in bacterial resistance levels. Actually, IMP-1-S262V does not provide *E. coli* DH10B cells with resistance to these Type II substrates according to Clinical and Laboratory Standards Institute standards²⁰ (except for ceftazidime, MICs are indistinguishable from the negative controls), while IMP-1 and IMP-1-S262T do for the most part (cells expressing IMP-1-S262T are not resistant to imipenem, but intermediate). Apart from lower k_{cat}/K_M values, this can be explained with a lower expression level of IMP-1-S262V in DH10B cells as shown by western blot (below). This is also apparent for the Type I substrates cephalothin and cefotaxime, as cells expressing IMP-1-S262V show lower MIC values than IMP-1 and IMP-1-S262T, although the purified enzyme exhibits higher or comparable k_{cat}/K_M values. This observation is in agreement with a previous study,¹³ where

purified IMP-1-S262V demonstrated high k_{cat}/K_M values toward cephalothin and cefotaxime, but cells expressing the enzyme resulted in relatively low MIC values. We attributed this difference to a lower level of functionally expressed MBL. In that respect, the previous report is consistent with the present results, although the cells (*E. coli* BL21 (DE3)) and expression vectors (pET30a) used were different. *E. coli* BL21 (DE3) cells and the pET30a expression vector were designed for overexpression of MBL in the cytoplasm and are not an ideal system for MIC assays, while *E. coli* DH10B cells and the pBC SK(+) expression vector used here are designed to mimic natural expression levels of MBL in the periplasm.

Western blot analysis

An IMP-1-specific antibody was produced, characterized, and tested for nonspecific binding to other MBLs (will be published elsewhere). The western blot analysis indicated that IMP-1 and IMP-1-S262T were expressed at comparable levels, while the expression level of functional IMP-1-S262V was about a tenth of that (Fig. 5). Although enzyme for purification was overexpressed in the cytoplasm vs. the periplasm, a similar trend was observed here: the amount of soluble protein of IMP-1-S262V was ~50% in this study versus ~70% for IMP-1-S262T and ~90% for IMP-1. Also in our previous study (cytoplasmic expression in *E. coli* BL21 (DE3) cells) less of IMP-1-S262V (~10%) was found in the

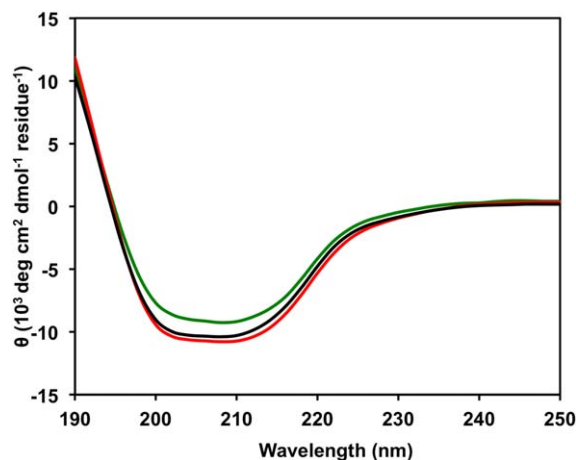


Figure 4. Circular dichroism scans of IMP-1 (black line), IMP-1-S262T (green line), and IMP-1-S262V (red line).

Table II. Kinetic Constants of IMP-1, IMP-1-S262T, and IMP-1-S262V with Nine β -Lactam Antibiotics

β -Lactam Antibiotic	IMP-1 ^a			IMP-1-S262T			IMP-1-S262V		
	k_{cat} (s ⁻¹)	K_M (μM)	k_{cat}/K_M ($\mu\text{M}^{-1} \text{s}^{-1}$)	k_{cat} (s ⁻¹)	K_M (μM)	k_{cat}/K_M ($\mu\text{M}^{-1} \text{s}^{-1}$)	k_{cat} (s ⁻¹)	K_M (μM)	k_{cat}/K_M ($\mu\text{M}^{-1} \text{s}^{-1}$)
Nitrocefin	181 \pm 8	9 \pm 1	20 \pm 2	394 \pm 8	8 \pm 1	48 \pm 5	562 \pm 5	5.9 \pm 0.4	96 \pm 6
Cephalothin	50 \pm 1	4.5 \pm 0.2	11.1 \pm 0.2	240 \pm 20	9 \pm 1	26 \pm 1	160 \pm 20	7.4 \pm 0.7	21 \pm 1
Cefotaxime	16.4 \pm 0.4	5.1 \pm 0.8	3.2 \pm 0.5	70 \pm 3	23 \pm 2	4.0 \pm 0.1	118 \pm 5	23 \pm 2	5.2 \pm 0.3
Ceftazidime	14 \pm 1	55 \pm 7	0.25 \pm 0.02	39 \pm 6	160 \pm 20	0.25 \pm 0.01	2.4 \pm 0.7	90 \pm 30	0.028 \pm 0.003
Benzylpenicillin	2000 \pm 100	530 \pm 50	3.8 \pm 0.1	1000 \pm 200	700 \pm 200	1.4 \pm 0.1	14 \pm 1	400 \pm 70	0.035 \pm 0.004
Ampicillin	260 \pm 30	210 \pm 30	1.2 \pm 0.1	450 \pm 40	700 \pm 100	0.62 \pm 0.04	3 \pm 2	400 \pm 300	0.009 \pm 0.003
Imipenem	105 \pm 5	35 \pm 4	3.0 \pm 0.2	340 \pm 30	150 \pm 20	2.3 \pm 0.1	11 \pm 3	230 \pm 60	0.06 \pm 0.01
Meropenem	22 \pm 1	2.9 \pm 0.2	7.7 \pm 0.4	57 \pm 6	11 \pm 3	4.6 \pm 0.7	21.0 \pm 0.3	34 \pm 1	0.61 \pm 0.01
Aztreonam	ND ^b	ND	ND	ND	ND	ND	ND	ND	ND

Experiments were performed in triplicate and values are reported as average \pm standard deviation. Values for IMP-1-S262T that are within the range between IMP-1 and IMP-1-S262V are highlighted in bold.

^a Values reported by Liu *et al.*¹⁵

^b Not detectable.

soluble fraction than of IMP-1 (~20%).¹³ In summary, the lower expression level of functional IMP-1-S262V serves as an explanation for the relatively low MIC values for IMP-1-S262V compared with its k_{cat}/K_M values.

Discussion

Catalytic efficiencies

IMP-1-S262T exhibited intermediate or comparable catalytic efficiencies to IMP-1 and IMP-1-S262V (Table II) toward the eight tested substrates, validating our hypothesis, while aztreonam was not a substrate for any of the enzymes. IMP-1 is the most effective out of all three tested enzymes in the hydrolysis of Type II β -lactams as explained by the “domino effect”.¹⁴ IMP-1-S262V is a relatively poor enzyme for the hydrolysis of Type II substrates, possibly due to its 1-methyl-ethyl side chain (Fig. 3) that could cause unfavorable steric interactions with H263, which itself could interact unfavorably with R₂ groups of Type II substrates, destabilizing its metal ligation of Zn²⁺ (Fig. 1) and the stability of the enzyme-substrate transition state complex. This scenario has been described previously.¹³

Overall, the “hybrid” side chain of threonine results in catalytic efficiencies toward Type II substrates comparable to or lower than S262, but the extent depends on the substrate: for ceftazidime and carbapenems (charged and/or bulky R₂ side chains) they are the same to about two thirds; for the penicillins they are about half to a third. T262 in IMP-1-S262T contains a hydroxyl group like S262 in IMP-1, which may have a favorable interaction with P68 (labeled P32 in the crystal structure²¹ and in our previous study¹⁴) and account for a portion of the “domino effect” with certain substrates, while the methyl group may interact unfavorably with H263, as in V262. A portion of the favorable effect of S262 in IMP-1 was missing with the corresponding resi-

due missing the hydroxyl group (A262 in IMP-1-S262A=IMP-6-G262A), resulting in slightly lower k_{cat}/K_M values of this variant toward Type II substrates than IMP-1 (S262), but higher than IMP-6 (G262).¹³ The difference in k_{cat}/K_M values toward Type II substrates obtained here with IMP-1-S262T versus IMP-1-S262V is similar to but bigger than those of IMP-1 versus IMP-1-S262A, suggesting that replacing the hydroxyl group with a methyl group (T \rightarrow V) has a more detrimental effect than simply removing it (S \rightarrow A).

IMP-1 is very efficient in hydrolyzing Type I substrates, but there is an increase in k_{cat}/K_M values

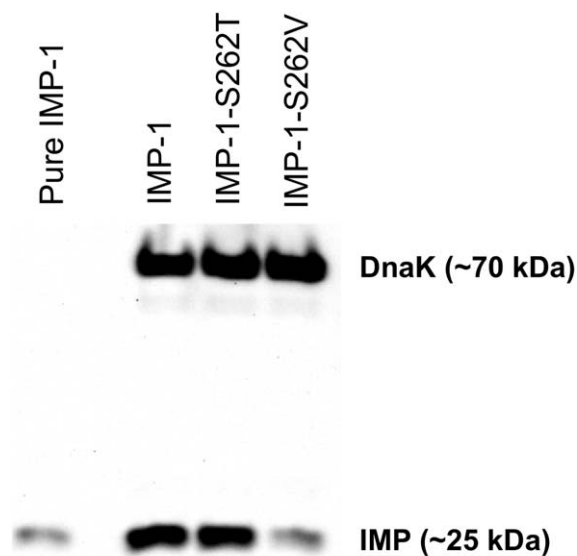


Figure 5. Image of a representative western blot of whole cells (*E. coli* DH10B) expressing IMP-1, IMP-1-S262T, and IMP-1-S262V. DnaK at ~70 kDa served as a constitutively expressed control. The band at ~25 kDa corresponds to the MBLs. Fifty ng of purified IMP-1 were loaded as positive control in the left lane. Expression levels of IMP-1 and IMP-1-S262T are comparable and that of IMP-1-S262V is about 10% of that.

Table III. Antibiotic Susceptibility Assay of *E. coli* DH10B Cells Harboring pBC SK(+)*bla*_{IMP} Plasmids

β -Lactam	MIC (μ g/ml)				
	pBC SK(+)			pBC SK(+) ^a	No Plasmid ^a
	<i>bla</i> _{IMP-1} ^a	<i>bla</i> _{IMP-1-S262T}	<i>bla</i> _{IMP-1-S262V}		
Cephalothin	512	512	128	16	16
Cefotaxime	256	128	8	0.125	0.25
Ceftazidime	256	256	1	0.5	0.5
Benzylpenicillin	256	128	32	32	32
Ampicillin	256	128	8	4	8
Imipenem	4	2	0.25	0.25	0.25
Meropenem	16	4	0.0625	<0.0625	0.0625
Aztreonam	0.25	0.25	0.25	0.25	0.25

MIC values were determined using the median value of six measurements.

Values for IMP-1-S262T that are within the range between IMP-1 and IMP-1-S262V are highlighted in bold.

^a Values reported by Liu *et al.*¹⁵

for IMP-1-S262T and IMP-1-S262V (Table II). As suggested previously,¹³ increased steric interaction of the 1-hydroxyl-ethyl side chain in IMP-1-S262T and the 1-methyl-ethyl side chain in IMP-1-S262V with H263 may not be detrimental or even favorable with these substrates, as their R₂ groups 2,4-dinitrostyryl (nitrocefin) and acetoxymethyl (cephalothin and cefotaxime) are kept from interacting with H263 by engaging in electrostatic interactions with neighboring residues in the binding pocket, such as N233.¹⁴

In this study meropenem behaved like a Type II substrate, although the [5-(dimethylcarbamoyl)pyrrolidin-3-yl]sulfanyl R₂ group with a pK_a of 7.4 (AstraZeneca, Mississauga, Ontario, Canada) could be neutral.¹⁵ However, the bulkiness of the R₂ group and the axial methyl group at C₁ may account for its Type II substrate-like character. Also, it is possible that this moiety is protonated due to the microenvironment in the active site of certain MBLs, but not others.

To our knowledge, this is the first analysis that predicts and explores the activity of an MBL variant across a broad spectrum of β -lactam antibacterials. Other sequence-structure-activity studies of the IMP-type β -lactamases have focused mostly on determining residues critical for activity,^{22–24} responsible for an altered substrate profile,²⁵ or important for the hydrolysis of specific antibiotics.²⁶ Interestingly, in the IMP-1 β -lactamase two residues that tolerate mutations, even though they are highly conserved, have been heretofore identified: N233,²⁷ which has previously been assigned a role in forming an oxyanion hole,^{21,28} and C221,²⁹ which is a functionally important Zn(II) ligand.^{21,22} Attempts have been made to predict enzyme activity of IMP variants using molecular dynamics simulations¹³ and computational protein design.³⁰ These studies did not yield accurate predictions, that is, either not all variants predicted to be more active were more active, or, if they were, it only applied to some of the

substrates. In the present study, we have successfully predicted activity of one variant against a broad spectrum of substrates using the available information of variants that have very closely related amino acids at the mutated position.

MIC values and western blot analysis

MIC values are not only representative of the catalytic efficiencies, but also other resistance mechanisms, such as reduced membrane permeability and efflux pumps.³¹ In the tested ElectroMAX *E. coli* DH10B cells carrying pBC SK(+) plasmids these factors that contribute to resistance were eliminated. IMP-1 conferred greater MIC values (Table III) of all tested β -lactams except for cephalothin and ceftazidime, whose MIC values were just as high for IMP-1-S262T. The MIC values of aztreonam were equivalent and as low as those of the controls, indicating susceptibility for cells expressing the three different enzymes. Nitrocefin was not tested in the MIC assays, because it is not clinically used and quite unstable. Overall, there is a good correlation between k_{cat}/K_M values and MIC values with the exception of IMP-1-S262V, which conferred lower resistance levels than expected, in fact none at all (susceptible) for the Type II substrates. This effect can be linked to a lower expression level of functional enzyme for this variant as shown by western blot analysis. Using an IMP-1-specific polyclonal antibody, as done here, has some advantages over using tagged protein and a tag-specific antibody,²⁷ since it cannot be excluded that a tag will have some impact on expression or other properties of recombinant protein. Therefore, it has been recommended that β -lactamases be expressed and studied without any artificially added tag.³²

Conclusions

The data presented herein demonstrate readily how a single point mutation can alter substrate

specificity and how sequence-structure-activity relationships can help predict hydrolytic preferences in IMP-1-S262T. Apart from one outlier (cephalothin), which can be considered within the error margin, IMP-1-S262T exhibited catalytic efficiencies in the range between those of IMP-1 and IMP-1-S262V, as predicted, and these translate into intermediate MIC values. Defining and testing hypotheses regarding the evolution of substrate specificity, as done in this report, is the ultimate test of our understanding of MBL function and its evolution under selective pressure.

We anticipate that the T262 mutant will be found in nature, as its encoding gene can be derived from the *bla*_{IMP-6} gene through a single nucleotide change and it would confer increased MICs toward ceftazidime, penicillins, and imipenem (a selective advantage in the clinical setting).^{15,25} However, the evolutionary advantage of expressing IMP-1-S262T versus other IMP variants will need to be defined by the clinical context in which this variant is recovered.

The approach presented here could be used to predict the phenotypes of other variants that are predicted to evolve. For instance, IMP-10 differs from IMP-1 by a V67F mutation, which leads to decreased MICs of penicillins.³³ Other variants (IMP-9, IMP-31, IMP-35, and IMP-45) possess isoleucine at the same position. Therefore, an IMP-1-V67I mutant may be expected to evolve or already exist. One may expect MICs of penicillins for cells expressing this variant to be similar to IMP-1 due to isoleucine being very similar (bulky) to valine or lower due to isoleucine being larger in size than valine, just like phenylalanine in IMP-10, but to a lesser degree. We are currently investigating the IMP-1-V67I variant and have found the MIC of ampicillin to be the same as for IMP-1 and that of benzylpenicillin to be between those for IMP-1 and IMP-10, consistent with what would be expected (unpublished results). Further studies must be undertaken as novel β -lactam compounds and inhibitors of MBLs are developed, so that a deeper understanding is achieved.

Materials and Methods

Preparation of plasmids for overexpression and minimum inhibitory concentration (MIC) assays. To mutate the *bla*_{IMP-1} gene cloned into a pET30a vector under control of the T7 promoter for cytoplasmic overexpression (pET30a-*bla*_{IMP-1}, kindly provided by Merck, Sharp & Dohme Corp, Rahway, NJ) to *bla*_{IMP-1-S262T}, the forward primer 5' – G GCA AAA CTG GTT GTT CCA **ACT** CAC AGT GAA GTT GGA GAC GC – 3' (bold letters indicating the mutated codon and bold underlined the mutated nucleotide) and the complementary reverse primer were designed and purchased from Integrated DNA Tech-

nologies (Coralville, IA). Polymerase chain reaction (PCR)-based site-directed mutagenesis was carried out in an Eppendorf Mastercycler Pro (Eppendorf, Hamburg, Germany) with pET30a-*bla*_{IMP-1} as a template using 30 cycles of denaturation at 95°C for 45 seconds, annealing at 51.3°C for 1 minute, and extension at 70°C for 2 minutes with additional final heating at 70°C for 10 minutes. *Escherichia coli* OverExpress C43 (DE3) cells (Lucigen, Middleton, WI) were transformed with *Dpn*I (Promega Corporation, Madison, WI)-digested and deionized PCR product by electroporation according to the manufacturer's instructions. Colonies were selected on lysogeny broth (LB) agar plates with 50 μ g/ml kanamycin, and plasmids were isolated from overnight cultures using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). The correct mutation was verified by DNA sequencing (Eurofins MWG Operon, Huntsville, AL). The vector pET30a-*bla*_{IMP-1-S262V} was taken from a previous study.¹³ The *bla*_{IMP-1} gene with its native leader sequence for periplasmic expression was excised from the pET26b vector (kindly provided by Dr. James Spencer, University of Bristol, UK) and inserted into the pBC SK(+) vector (Stratagene, Santa Clara, CA) resulting in pBC SK(+)-*bla*_{IMP-1}.¹⁵ To obtain pBC SK(+)-*bla*_{IMP-1-S262T}, PCR-based site-directed mutagenesis was employed using the same primers and cycling parameters as for pET30a-*bla*_{IMP-1-S262T}. To obtain pBC SK(+)-*bla*_{IMP-1-S262V}, pBC SK(+)-*bla*_{IMP-6} from our previous study¹⁵ served as a template and the forward primer 5' – G GCA AAA CTG GTT GTT CCA **GTT** CAC AGT GAA GTT GGA GAC GC – 3' and the complementary reverse primer were used. The cycling parameters were 16 cycles of denaturation at 96°C for 1.5 minutes, annealing at 60°C for 1 minute, and extension at 72°C for 22 minutes with additional final heating at 72°C for 10 minutes. *E. coli* TG1 electrocompetent cells (Lucigen, Middleton, WI) were transformed with *Dpn*I-digested and deionized PCR product, plasmids were isolated from selected cultures, and the *bla*_{IMP} mutant genes were sequenced as described above. ElectroMAX *E. coli* DH10B cells (Invitrogen, Grand Island, NY) were transformed with the confirmed pBC SK(+)-*bla*_{IMP} mutant plasmids and used for MIC assays.

β -Lactamase expression and purification

E. coli OverExpress C43 (DE3) cells harboring pET30a-*bla*_{IMP-1}, pET30a-*bla*_{IMP-1-S262T}, and pET30a-*bla*_{IMP-1-S262V} were grown in 1 liter of LB with 50 μ g/ml kanamycin at 37°C. Protein expression was induced in the log phase by isopropyl β -D-1-thiogalactopyranoside (IPTG, Fisher Scientific, Fair Lawn, NJ) at a final concentration of 0.5 mM at 21°C for an additional four hours. Cells were then harvested by centrifugation at 3,220 *g* at 4°C for 45 min. Pellets were resuspended in 50 mM 3-(N-

morpholino)propanesulfonic acid (MOPS) (pH 7.0) with 100 μM ZnSO_4 (MOPS buffer) and lysed by sonication (Model 120 Sonic Dismembrator; Fisher Scientific, Pittsburgh, PA) for a total of 3 min of pulsing at 70% intensity in 20-s periods interrupted by 20-s pauses. Soluble and insoluble fractions were separated by centrifugation at 38,900 g at 4°C for 35 min. The soluble fraction was syringe filtered (0.22 μm) and injected into two connected HiTrap CM FF ion-exchange columns (GE Healthcare, Piscataway, NJ) on an ÄKTA Purifier UPC 100 (GE Healthcare, Piscataway, NJ), washed with MOPS buffer until baseline absorbance at 280 nm was obtained and eluted with a 0–0.5 M NaCl gradient in MOPS buffer. Fractions containing MBL as judged by nitrocefin hydrolysis were pooled and injected into a HiPrep 26/60 Sephacryl S-100 HR column (GE Healthcare, Piscataway, NJ). Gel filtration fractions were eluted in MOPS buffer containing 100 mM NaCl and pooled for centrifugal concentration using an Amicon Ultra-15 Centrifugal Filter Unit (molecular weight cut-off of 10 kDa) (Millipore, Billerica, MA) at 3,220 g at 4°C for 20 min. Percentages of MBL in the soluble and insoluble fractions after cell lysis as well as homogeneity of purified protein (>95%) were determined via SDS-PAGE using standard protocols³⁴ at the 25 kDa marker.

Biophysical characterization

Protein concentration was determined in triplicate using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard (Bio-Rad, Richmond, CA). The molecular weight of pure protein (IMP-1-S262T and IMP-1-S262V) was determined by electrospray ionization mass spectrometry (ESI-MS) at the Protein/Peptide Microanalytical Laboratory at the California Institute of Technology (Pasadena, CA). For circular dichroism (CD) experiments, all three purified enzymes were dialyzed 1:2,000 in 5 mM phosphate buffer (pH 7.00) for 24 hours and the protein concentration was determined afterwards as described above. CD scans were done using an Olis DSM 20 Spectrophotometer (Olis, Bogart, GA). Three scans from 250–190 nm were averaged and converted to molar ellipticity to analyze secondary structure. Zn(II) ion content of the MBLs was determined using the 4-(2-pyridylazo)resorcinol (PAR) (Tokyo Chemical Industry, Tokyo, Japan) assay,¹⁸ except with the use of zinc-free 50 mM MOPS buffer (pH 7.0). Zn(II) concentrations were calculated by linearly plotting detected Zn(II) released from different concentrations of MBL (determined via Bio-Rad Protein Assay) upon denaturation in comparison to a series of different Zn(II) concentrations in the same buffer as a standard.

Kinetic assays

Steady-state kinetic parameters were determined using an SQ-2802 UV/Vis Spectrophotometer

(UNICO, Dayton, NJ). To protect diluted enzymes from denaturation, a final concentration of 10 $\mu\text{g}/\text{mL}$ BSA was added. Substrate and enzyme were pre-incubated separately for 5 min at 30°C in MOPS buffer before the reaction was initiated by rapid mixing. The appearance of product (nitrocefin) or disappearance of substrate (all other β -lactam antibiotics) was monitored at the specific wavelengths and converted to initial velocities using the specific extinction coefficients used previously.^{13,15,35} The initial velocity of each substrate was measured at 7–8 different concentrations spread out over a range chosen based on the previously published K_M values of IMP-1. Three series of initial velocities were determined and analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Nonlinear fitting of these series to the Michaelis-Menten equation under consideration of the enzyme concentration used yielded three k_{cat} , K_M , and k_{cat}/K_M values, respectively. The averages of these values \pm standard deviations are reported.

Susceptibility assays

Antibiotic susceptibility was determined by minimum inhibitory concentration (MIC) assays using a broth dilution method according to the guidelines from the Clinical and Laboratory Standards Institute.³⁶ An inoculum of 10^4 *E. coli* ElectroMAX DH10B cells harboring the *bla*_{IMP-1}, *bla*_{IMP-1-S262T}, and *bla*_{IMP-1-S262V} genes in the pBC SK (+) plasmid were grown in Mueller-Hinton II broth (MHB) pH 7.3 at 37°C for 20 h in the presence of one of eight β -lactam antibiotics supplied in serial dilutions in a 96-well plate assay format. Cells without the plasmid and with the plasmid without the MBL gene served as controls. Six measurements were carried out, and the median was determined as the MIC value.

Western blot analysis

Western blotting was employed to analyze expression levels of wild type and artificial variants of IMP-1 under conditions comparable with those in the susceptibility assay. Totally, 5 mL cultures of *E. coli* DH10B cells containing pBC SK(+) plasmids harboring the various *bla*_{IMP} genes in MHB containing 20 $\mu\text{g}/\text{mL}$ of chloramphenicol were grown at 37°C to an OD₆₀₀ of 0.8. A total of 50 μL aliquots of the cultures were pelleted and frozen overnight. Pellets were resuspended in 20 μL loading dye, separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Novex, Life Technologies, Carlsbad, CA) by electroblotting. Fifty ng of purified IMP-1 were used as a positive control. After blocking for 1 h with 5% nonfat dry milk, MBL presence on the blot was detected by incubation in 1 $\mu\text{g}/\text{mL}$ anti-IMP-1 polyclonal antibody (Ab) (raised in rabbits injected with purified IMP-1 and

purified on Protein G columns as described previously³⁷) and 1/10,000 dilution of mouse anti-DnaK monoclonal Ab (Enzo Life Sciences) overnight at 4°C. The membrane was washed four times, 15 min each, in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and subsequently incubated with 1/10,000 dilution of both horeseradish peroxidase (HRP)-Protein G conjugate (Bio-Rad) and HRP-goat anti-mouse Ab conjugate (Santa Cruz Biotechnology). All Ab/protein G solutions were prepared with 5% nonfat dry milk. After four additional washes, membranes were processed for exposure using the ECL kit (GE Healthcare) and FOTO/Analyst[®] FX (Fotodyne).

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