Supplemental Material:

Suppression of β-Lactam Resistance by Aspergillomarasmine A is Influenced by Both the Metallo-β-Lactamase Target and the Antibiotic Partner

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Running Title: β-Lactam Partner Impacts Aspergillomarasmine A Potency

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TABLE S1 Minimum inhibitory concentration (MIC) values of different β-lactam antibiotics against MBL-producing *E. coli* BW25113. All 19 MBL genes were cloned into the pGDP2 vector. *E. coli* BW25113 containing the pGDP2 plasmid, but no MBL gene was used as a control. All MIC assays were conducted in duplicate.

MBL	Minimum Inhibitory Concentration (μg/mL)					
	Meropenem	**Doripenem**	**Ertapenem**	**Imipenem**	**Cefotaxime**	**Ampicillin**
None	0.06	0.03	0.03	0.25–0.50	0.06	4
NDM-1	32	32	> 64	16	> 64	> 64
NDM-4	64	64	> 64	> 64	> 64	> 64
NDM-5	64	64	> 64	32	> 64	> 64
NDM-6	64	32	> 64	64	> 64	> 64
NDM-7	64	32	> 64	32	> 64	> 64
VIM-1	32	8–16	16	8–16	> 64	> 64
VIM-2	16	8	16–32	16	> 64	> 64
VIM-7	32	8	64	64	64	> 64
CAM-1	8	16	16	32	> 64	> 64
DIM-1	16	16	32	4	> 64	> 64
IND-1	16	8–16	32	64	> 64	> 64
GIM-1	16	8	64	4	> 64	> 64
IMP-1	16	8	32	16–32	64	> 64
IMP-7	16	8	32	4	> 64	> 64
IMP-27	32	32	64	4	> 64	16
SPM-1	32	16	64	16–32	> 64	> 64
CphA2	16–32	16–32	> 64	8–16	≤ 0.50	4–8
L1	16	16	16–32	4–8	1	> 64
AIM-1	32	16	> 64	64	64	> 64

TABLE S2 Minimum inhibitory concentration (MIC) values of different β-lactam antibiotics against MBL-producing *K. pneumoniae* ATCC 33495. All 8 MBL genes were cloned into the pGDP2 vector. *K. pneumoniae* ATCC 33495 containing the pGDP2 plasmid, but no MBL gene was used as a control. All MIC assays were conducted in duplicate.

MBL	Minimum Inhibitory Concentration (μg/mL)					
	Meropenem	**Doripenem**	**Ertapenem**	**Imipenem**	**Cefotaxime**	**Ampicillin**
None	0.06	0.06–0.13	0.06	1	0.13	64
NDM-1	> 64	> 64	> 64	64	> 64	> 64
NDM-4	> 64	> 64	> 64	> 64	> 64	> 64
NDM-5	> 64	> 64	> 64	32	> 64	> 64
NDM-6	> 64	> 64	> 64	64	> 64	> 64
VIM-2	64	32	> 64	>64	> 64	> 64
IMP-7	32	32	32–64	8–16	> 64	> 64
CphA2	> 64	> 64	> 64	> 64	≤ 0.50	64
AIM-1	> 64	64	> 64	> 64	> 64	> 64

TABLE S3 Minimum inhibitory concentration (MIC) values of different β-lactam antibiotics against MBL-producing *E. coli* BW25113 Δ*bamB*Δ*tolC*. All 8 MBL genes were cloned into the pGDP2 vector. *E. coli* BW25113 Δ*bamB*Δ*tolC* containing the pGDP2 plasmid, but no MBL gene was used as a control. All MIC assays were conducted in duplicate.

MBL	Minimum Inhibitory Concentration (μg/mL)		
	Meropenem	**Doripenem**	**Ampicillin**
None	0.06	0.06	0.50
NDM-1	32	64	> 64
NDM-4	64	> 64	> 64
NDM-5	64	32	> 64
NDM-6	64	64	> 64
VIM-2	16	8	> 64
IMP-7	16	16	> 64
CphA2	32	16	2
AIM-1	64	32	> 64

TABLE S4 Concentration of AMA needed to restore the activity of different β-lactam antibiotics to their EUCAST susceptibility breakpoint concentration in MBL-producing *E. coli* BW25113. All 19 MBL genes were cloned into the pGDP2 vector. All bioassays were conducted in duplicate. This table shows the results from replicate 2.

MBL	[AMA] at the Susceptibility Breakpoint of the Antibiotic (μg/mL) [a]					
	Meropenem	**Doripenem**	**Ertapenem**	**Imipenem**	**Cefotaxime**	**Ampicillin**
NDM-1	8	12	16	12	64	64
NDM-4	16	16	64	16	> 64	> 64
NDM-5	12	24	64	16	> 64	> 64
NDM-6	16	16	32	12	64	> 64
NDM-7	16	24	64	24	> 64	> 64
VIM-1	8	8	12	12	24	64
VIM-2	8	8	8	8	12	16
VIM-7	8	8	8	8	8	12
CAM-1	4	12	8	8	24	> 64
DIM-1	12	12	16	4	24	64
IND-1	8	8	12	12	24	> 64
GIM-1	12	8	32	8	> 64	> 64
IMP-1	16	12	64	24	> 64	64
IMP-7	24	12	> 64	16	> 64	32
IMP-27	32	24	> 64	≤ 0.5	> 64	12
SPM-1	12	24	64	12	> 64	> 64
CphA2	> 64	64	> 64	> 64	≤ 0.5	≤ 0.5
L1	12	12	24	8	≤ 0.5	> 64
AIM-1	64	64	> 64	24	> 64	> 64

[a] The EUCAST susceptibility breakpoint concentrations for meropenem, doripenem, ertapenem, imipenem, cefotaxime and ampicillin are 2, 1, 0.5, 2, 1 and 8 μg/mL, respectively.

TABLE S5 Concentration of AMA needed to restore the activity of different β-lactam antibiotics to their EUCAST susceptibility breakpoint concentration in MBL-producing *K. pneumoniae* ATCC 33495. All 8 MBL genes were cloned into the pGDP2 vector. All bioassays were conducted in duplicate. This table shows the results from replicate 2.

MBL	[AMA] at the Susceptibility Breakpoint of the Antibiotic (μg/mL) [a]					
	Meropenem	**Doripenem**	**Ertapenem**	**Imipenem**	**Cefotaxime**	**Ampicillin**
NDM-1	12	16	24	12	64	> 64
NDM-4	16	24	64	24	> 64	> 64
NDM-5	16	24	64	24	> 64	> 64
NDM-6	12	16	64	24	> 64	> 64
VIM-2	8	8	8	12	16	> 64
IMP-7	32	24	> 64	64	> 64	> 64
CphA2	> 64	> 64	> 64	> 64	≤ 0.5	> 64
AIM-1	64	64	> 64	> 64	> 64	> 64

[a] The EUCAST susceptibility breakpoint concentrations for meropenem, doripenem, ertapenem, imipenem, cefotaxime and ampicillin are 2, 1, 0.5, 2, 1 and 8 μg/mL, respectively.

TABLE S6 Concentration of AMA needed to restore the activity of different β-lactam antibiotics to their EUCAST susceptibility breakpoint concentration in MBL-producing *E. coli* BW25113 Δ*bamB*Δ*tolC*. All 8 MBL genes were cloned into the pGDP2 vector. All bioassays were conducted in duplicate. This table shows the results from replicate 2.

MBL	[AMA] at the Susceptibility Breakpoint of the Antibiotic (μg/mL) [a]		
	Meropenem	**Doripenem**	**Ampicillin**
NDM-1	8	12	32
NDM-4	16	24	> 64
NDM-5	16	16	> 64
NDM-6	16	24	> 64
VIM-2	8	8	24
IMP-7	24	24	> 64
CphA2	> 64	> 64	≤ 0.5
AIM-1	64	64	> 64

[a] The EUCAST susceptibility breakpoint concentrations for meropenem, doripenem and ampicillin are 2, 1 and 8 μg/mL, respectively.

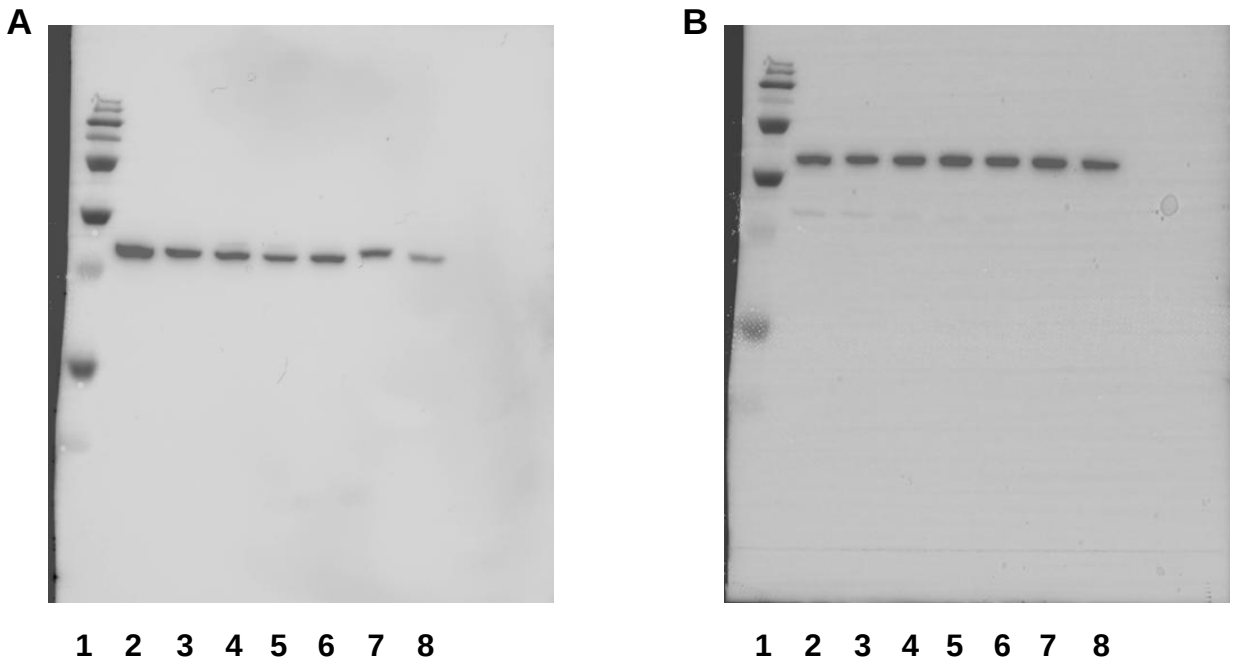

FIG S1 Protein expression levels of MBL enzymes in *E. coli* BW25113. The Western blot was conducted as described in the Materials and Methods section. The antibodies used to probe the Western blot were a) mouse-derived anti-FLAG antibodies conjugated to HRP, and b) mouse-derived anti-RpoA antibodies and anti-mouse IgG antibodies conjugated to HRP. Lane 1: PageRuler Prestained Protein Ladder, lane 2: NDM-1, lane 3: NDM-4, lane 4: NDM-5, lane 5: NDM-6, lane 6: NDM-7, lane 7: VIM-2, lane 8: IMP-7.

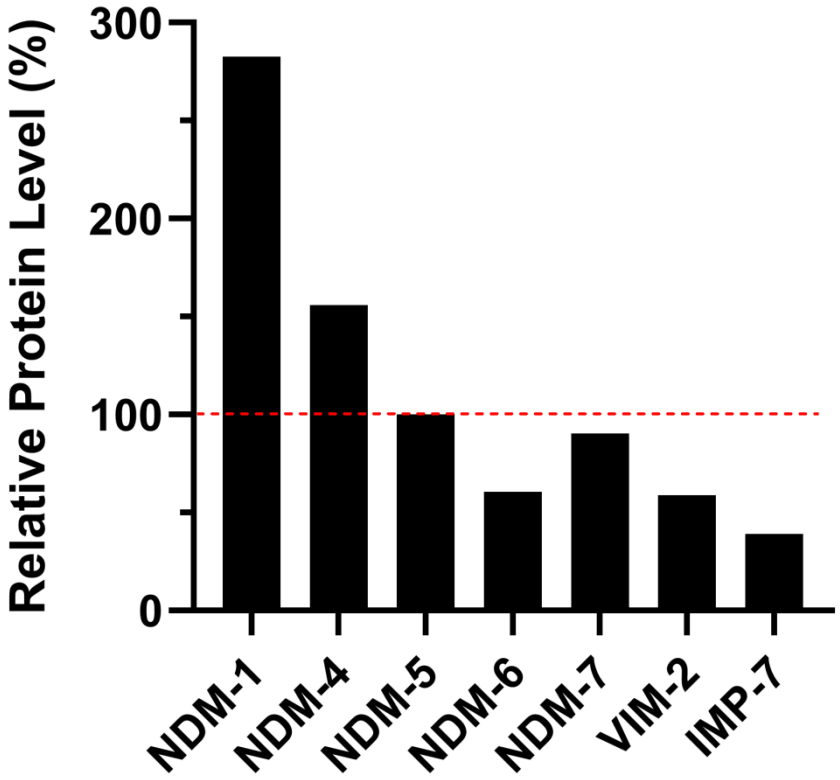

FIG S2 Relative protein expression levels of MBL enzymes in *E. coli* BW25113. Proteins expression levels were detected using a Western blot probed with mouse-derived anti-FLAG antibodies conjugated to HRP. The Western blot was conducted as described in the Materials and Methods section. Antibodies targeting RpoA served as loading controls. Protein band intensities were quantified using Image Lab. These results were then plotted and analyzed using GraphPad Prism 8. The protein expression levels of the MBL enzymes are shown in relation to NDM-5.

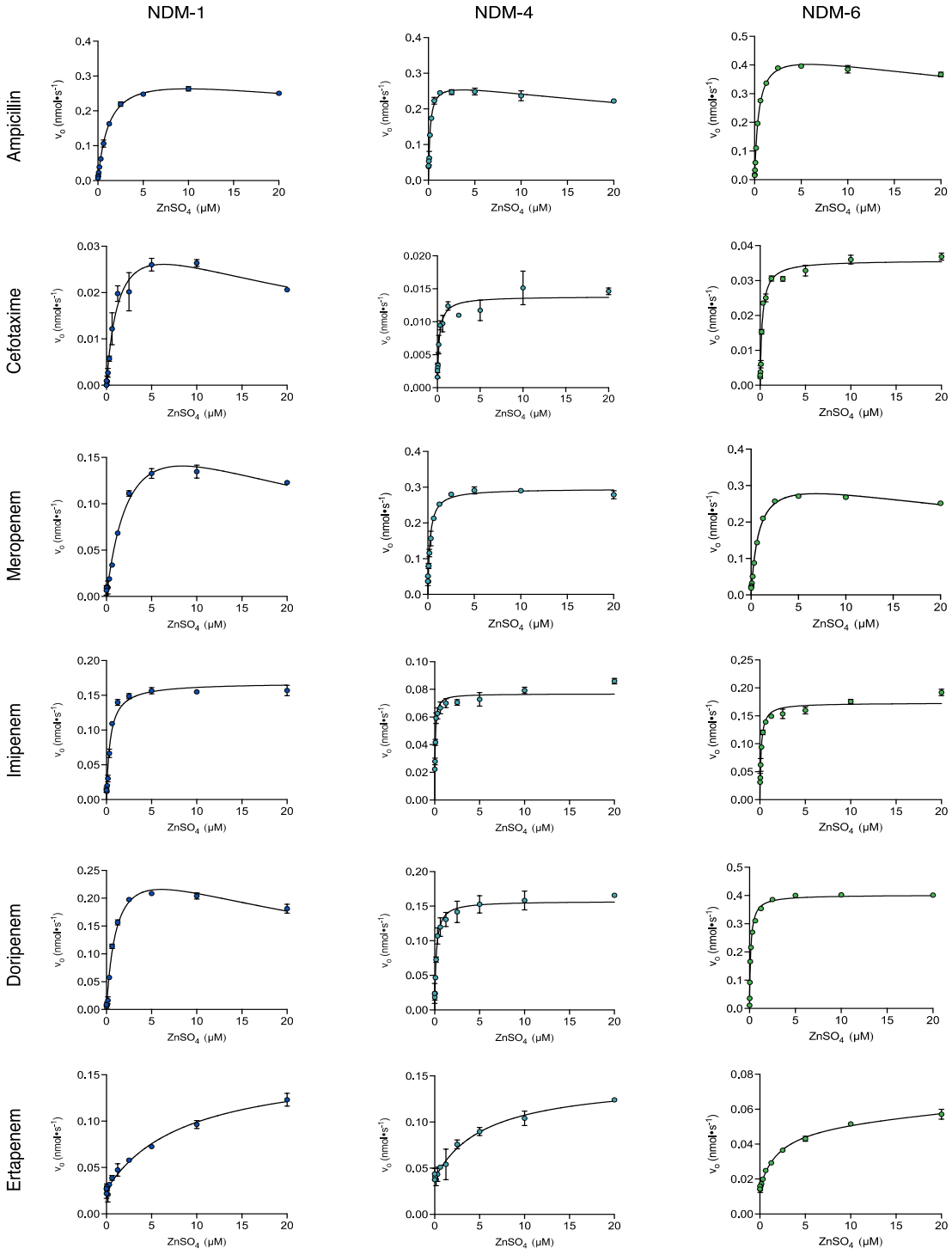

FIG S3 Zinc-dependence of MBL-catalyzed hydrolysis of β-lactam antibiotics. Kinetic analysis of each MBL and substrate used the initial rates of reaction of enzyme (4–10 nM) in Chelex-treated 25 mM HEPES:NaOH buffer (pH 7.5) with 100 nM AMA at 25°C. Reaction mixtures contained saturating substrate concentrations (0.25–0.5 mM) and included varying amounts of $ZnSO_4$ (0.0007, 0.0015, 0.03, 0.15, 0.31, 0.62, 1.2, 2.5, 5, 10, 20 μM). Nonlinear regression analysis was performed using GraphPad Prism 8.

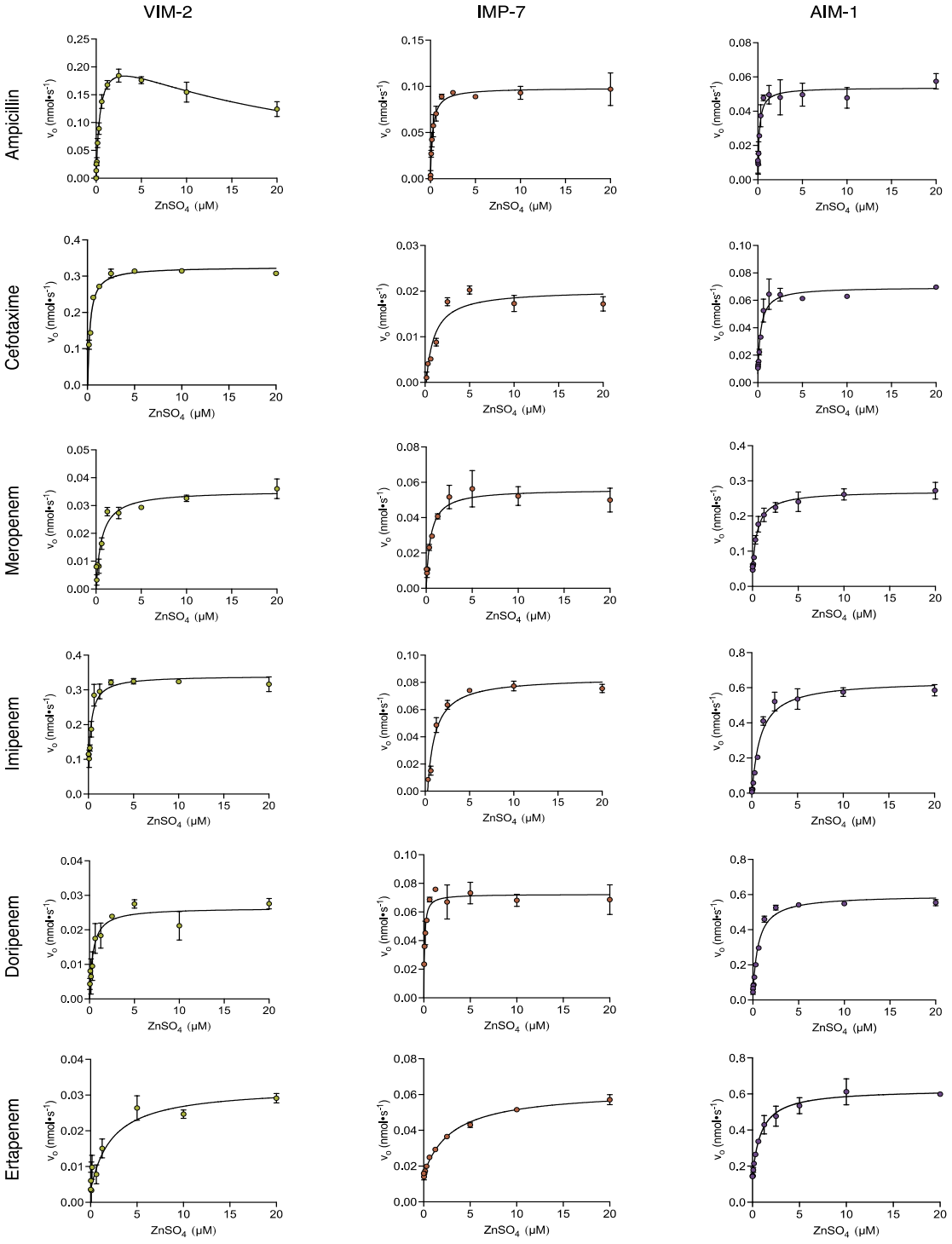

FIG S3 Continued.

TABLE S7 Sequences of the primers used to create the NDM variants by site-directed mutagenesis. The changed nucleotide is indicated in red in the primer sequence. The restriction enzyme recognition sites are underlined in the primer sequence.

Primer Name	Primer Sequence
NDM FWD	5′–TACCCTCATATGGAATTGCCCAATATTATGCACC–3′
NDM REV	5′–TACCCTAAGCTTTCAGCGCAGCTTGTCGGC–3′
V88L FWD	5′– CCGCGTGCTGTTGGTCGATACCGCCTG –3′
V88L FWD	5′– TATCGACCAACAGCACGCGGCCGCCATC–3′
D130N FWD	5′–GCGGTATGAACGCGCTGCATGCG–3′
D130N REV	5′–GCAGCGCGTTCATACCGCCCATCTTG–3′
M154L FWD	5′–AAGAGGGGCTGGTTGCGCCGCAAC–3′
M154L REV	5′–GCAACCAGCCCCTCTTGCGGGGCAAG–3′
A233V FWD	5′–CGCGTCAGTGCGCGCGTTTGGTG–3′
A233V REV	5′–ACGCGCGCACTGACGCGGCGTAG–3′

TABLE S8 Sequences of the primers used to create the overexpression constructs and the FLAG-tagged MBL genes. The forward primers for the overexpression constructs were used to remove the signal peptide of the MBL genes to allow for cytoplasmic expression. The FLAG reverse primers were used to remove the stop codon from the MBL genes to allow the insertion of the C-terminal FLAG-tag. The primers created for NDM-1 could also be used for NDM-4, NDM-5, NDM-6 and NDM-7 since all of these NDM genes share identical sequences at the binding site for the forward and reverse primers. The restriction enzyme recognition sites are underlined in the primer sequence.

Primer Name	Sequence of Primer
NDM-1 FWD	5′–TACACGTGGTCTCCAGGTATGGGTGAAATCCGCCCGAC–3′
NDM-1 REV	5′–TACCCTAAGCTTTCAGCGCAGCTTGTCGGC–3′
VIM-2 FWD	5′–ATTAACATATGGTAGATTCTAGCGGTGAGTATCCGACAGT–3′
VIM-2 REV	5′–TATATGCTAGCCTACTCAACGACTGAGCGATTTGTGTG–3′
IMP-7 FWD	5′–TATATGCTAGCATGGAGGCTTTGCCAGATTTAAAAATTG–3′
IMP-7 REV	5′–TTATTCTCGAGTTAGTTACTTGGTTTTGATAGCTTTTTACT–3′
AIM-1 FWD	5′–TATAGACATATGTCCGATGCACCAGCGAGTCGTG–3′
AIM-1 REV	5′–TATAGACTCGAGTCAAGGGCGCGCACCAGATG–3′
NDM-1 FLAG FWD	5′–GATGACGACAAGTGAAAGCTTGCGGCCGCA–3′
NDM-1 FLAG REV	5′–GTCTTTGTAGTCGCGCAGCTTGTCGGCCAT–3′
VIM-2 FLAG FWD	5′–GATGACGACAAGTAACTCGAGCACCACCAC–3′
VIM-2 FLAG REV	5′–GTCTTTGTAGTCCTCAACGACTGAGCGATT–3′
IMP-7 FLAG FWD	5′–GATGACGACAAGTAACTCGAGCACCACCAC–3′
IMP-7 FLAG REV	5′–GTCTTTGTAGTCGTTACTTGGTTTTGATAG–3′

TABLE S9 Retention time, collision energies and parent-daughter ion transitions for AMA and the different β-lactam antibiotics.

β-Lactam Antibiotic	Retention Time (min)	Parent $[M+H]^+$	Daughter $[M+H]^+$	Collision Energy (eV)
Bn-AMA [a]	2.85	412.1351	192.0655	24
Meropenem	2.82	384.1581	68.0511	40
Doripenem	2.27	421.1213	274.0671	20
Imipenem	1.09	300.1028	98.0068	40
Ertapenem	3.21	476.1498	432.1594	10
Cefotaxime	3.30	456.0657	125.0064	40
Ampicillin	3.11	350.1173	106.0827	10

[a] For AMA detection, samples were derivatized with benzoyl chloride to produce N-benzoyl AMA.