Supporting Information

Development of a Whole-Cell Biosensor for β-Lactamase Inhibitor Discovery

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Methodology

Reagents

Imipenem, meropenem, amoxicillin, tazobactam, and clavulanic acid were purchased from Glentham Life Sciences. Avibactam was purchased from Medkoo Biosciences. Dipicolinic acid and nitrilotriacetic acid were purchased from Fisher Scientific. Captopril was purchased from VWR. EDTA was purchased from Thermo Fisher Scientific. Embelin was purchased from Toronto Research Chemicals Inc. 2TY media components (tryptone, yeast extract, sodium chloride) and cation-adjusted Mueller Hinton Broth (CAMHB; Becton Dickinson) were purchased from Fisher Scientific.

Bacterial culturing

All *Escherichia coli* cultures used for cloning experiments were grown in autoclaved 2TY media (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) supplemented with the appropriate selection antibiotic, where required. Liquid cultures were incubated at 37 °C and 200 rpm. Overnight plate cultures were grown on 2TY agar plates supplemented with the appropriate selection antibiotic, where required.

Biosensor development

The *ampR* gene and *ampC* promoter were amplified from the genomic DNA of *P. aeruginosa* 2_1_26 (BEI Resources) by polymerase chain reaction (PCR). The *luxCDABE* cassette was amplified from the pAKgfplux1 plasmid (Addgene # 14083) by PCR.[1] pUC19 vector (New England BioLabs) was digested with SacI (New England BioLabs) and purified by gel extraction following agarose gel electrophoresis. The PCR-amplified *ampR/*P*ampC* and *luxCDABE* sequences were cloned into pUC19 by HiFi assembly (New England BioLabs). Next, the ampicillin resistance marker in this plasmid was disrupted by restriction digest with ScaI (New England Biolabs), and the kanamycin resistance gene from pHSG298 (National BioResource Project, NBRP) was cloned into this site using HiFi assembly to generate the plasmid pAMPLUX. The sequence of

pAMPLUX was confirmed by whole plasmid sequencing (Plasmidsaurus). Chemically competent *E. coli* BW25113 (NBRP) cells were transformed with pAMPLUX and subsequently used for biosensor assays.

Preparation of β-lactamase-producing strains

The TEM-116 gene and promoter were amplified from pUC19 by PCR. Genes and native promoters for NDM-1 (*Klebsiella pneumoniae* AR0041), IMP-1 (*Pseudomonas aeruginosa* AR0103), VIM-2 (*P. aeruginosa* AR0100), KPC-2 (*P. aeruginosa* AR0098), and OXA-48 (*Enterobacter aerogenes* AR0074) were amplified from the genomic DNA of their respective organisms by PCR. All β-lactamase genes (along with their native promoters) were cloned into the HindIII site of pACYC184 (NBRP) by HiFi assembly. The sequences of the recombinant plasmids were confirmed by Sanger sequencing (The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, ON) and whole plasmid sequencing (Plasmidsaurus).

Minimum inhibitory concentration (MIC) testing

All MIC testing was conducted according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[2] *E. coli* BW25113 cells (untransformed, transformed with pAMPLUX, or transformed with a pACYC184 plasmid encoding a β-lactamase) were cultured overnight at 37 ˚C on 2TY agar plates supplemented with the appropriate antibiotic, where required. Antibiotic and inhibitor stocks were prepared in sterile water and serially diluted in CAMHB in clear 96-well non-treated flat-bottom microplates (Falcon). Cell suspensions were prepared by resuspending colonies in CAMHB growth media to OD_{600} = 0.1 (approx. 1.5 x 10^8 CFU/mL). These suspensions were diluted in CAMHB to approx. 5 x 10^6 CFU/mL, and 20 µL of the suspension was added to 180 µL of each antibiotic/inhibitor treatment, giving a final inoculum of 5 x 10^5 CFU/mL. Plates were incubated for 16 - 18 hours at 37 ˚C without shaking, and OD_{600} readings were taken using a Synergy LX plate reader (Agilent BioTek).

Biosensor bioactivity testing

Biosensor cells (*E. coli* BW25113 transformed with pAMPLUX) were grown overnight at 37 ˚C on 2TY agar plates supplemented with 50 μg/mL kanamycin. Imipenem and amoxicillin stocks were prepared in sterile water, and 180 μL of each antibiotic was added to a white LUMITRAC 200 96-well plate (Greiner Bio-One) in quadruplicate. Biosensor cell suspensions were prepared to OD_{600} = 0.2 by suspending colonies in 2TY growth media, and 50 μL of suspended cells were added to each imipenem and amoxicillin treatment. Luminescence readings were taken every hour for 3 hours using a Spectramax ID3 plate reader (Molecular Devices); between readings, the plate was incubated at 37 ˚C.

Biosensor β-lactamase inhibition assays

E. coli BW25113 cells transformed with the pACYC184 plasmids carrying β-lactamase genes were grown overnight at 37 ˚C on 2TY agar plates supplemented with 25 μg/mL chloramphenicol. Biosensor cells were grown overnight at 37 ˚C on 2TY agar plates supplemented with 50 μg/mL kanamycin. All β-lactam antibiotics and β-lactamase inhibitors were dissolved in sterile water. Serial dilutions of these solutions were prepared in 2TY, and 180 μL of each treatment was added to white LUMITRAC 200 96-well plates (Greiner Bio-One). Cell suspensions of β-lactamase-producing *E. coli* were prepared by suspending colonies in 2TY growth media to an OD_{600} of 0.2, and 20 μL of each cell suspension was added to each well containing β-lactam antibiotics or β-lactam/β-lactamase inhibitor combinations. Plates were incubated for 1.5 hours at 37 ˚C without shaking. Biosensor cell suspensions were prepared to OD_{600} = 0.2 by suspending colonies in 2TY growth media, and 50 μL of cells were added to the microplate wells containing β-lactamase-producing cells and β-lactam antibiotics and/or β-lactamase inhibitors. The resulting mixtures were incubated for 2 hours at 37 ˚C. Luminescence readings were acquired using a Spectramax ID3 multimode plate reader (Molecular Devices). Luminescence values were normalized to the sample which gave the highest luminescence output and expressed as a percentage. The level of β-lactamase

activity under the assay conditions was expressed as the % luminescence value subtracted from 100%. IC_{50} values for each inhibitor were determined using the "non-linear curve fit [inhibitor] vs. response" function in GraphPad Prism v9.5.

Growth-based assays for measuring β-lactamase inhibition

E. coli BW25113 cells transformed with pACYC184 plasmids carrying β-lactamase genes were cultured overnight at 37 ˚C on 2TY agar plates supplemented with 25 μg/mL chloramphenicol. Antibiotic and inhibitor stocks were prepared in sterile water and serially diluted in 2TY growth media in clear 96-well non-treated flat-bottom microplates (Falcon). Cell suspensions were prepared by suspending colonies in 2TY growth media to OD_{600} = 0.2, and 20 μL of this suspension was added to each treatment well containing antibiotics alone or in combination with β-lactamase inhibitors. Plates were incubated for 16 - 18 hours at 37 ˚C without shaking, and OD_{600} readings were taken using a Synergy LX plate reader (Agilent BioTek).

Supplementary Data: Tables and Figures

Table S1. Strains used in this study.

***E. coli* Strain**	**Characteristics**	**Source**
NEB10β	Wild-type	New England BioLabs
BW25113	Wild-type	NBRP
BW25113 Δ*ompF*	*ompF* gene disrupted with kan^R marker	Keio Collection[3] (NBRP, Japan)
BW25113-Biosensor	Transformed with pAMPLUX	This study
BW25113-TEM-116	Transformed with pACYC184 $bla_{TEM-116}$	This study
BW25113Δ*ompF*-TEM-116	*ompF* gene disrupted with kan^R marker, transformed with pACYC184 $bla_{TEM-116}$	This study
BW25113-KPC-2	Transformed with pACYC184 bla_{KPC2}	This study
BW25113-OXA-48	Transformed with pACYC184 bla_{OXA-48}	This study
BW25113-NDM-1	Transformed with pACYC184 bla_{NDM-1}	This study
BW25113-IMP-1	Transformed with pACYC184 bla_{IMP-1}	This study
BW25113-VIM-2	Transformed with pACYC184 bla_{VIM-2}	This study

Table S2. Minimum inhibitory concentration (MIC) values for amoxicillin and meropenem against the strains used in this study.

***E. coli* Strain**	**MIC (Meropenem, µg/mL)**	**MIC (Amoxicillin, µg/mL)**
BW25113	0.06	4
BW25113-Biosensor	0.06	4
BW25113-TEM-116	0.06	> 256
BW25113-KPC-2	>32	> 256
BW25113-OXA-48	2	> 256
BW25113-NDM-1	>32	> 256
BW25113-IMP-1	>32	> 256
BW25113-VIM-2	4	> 256

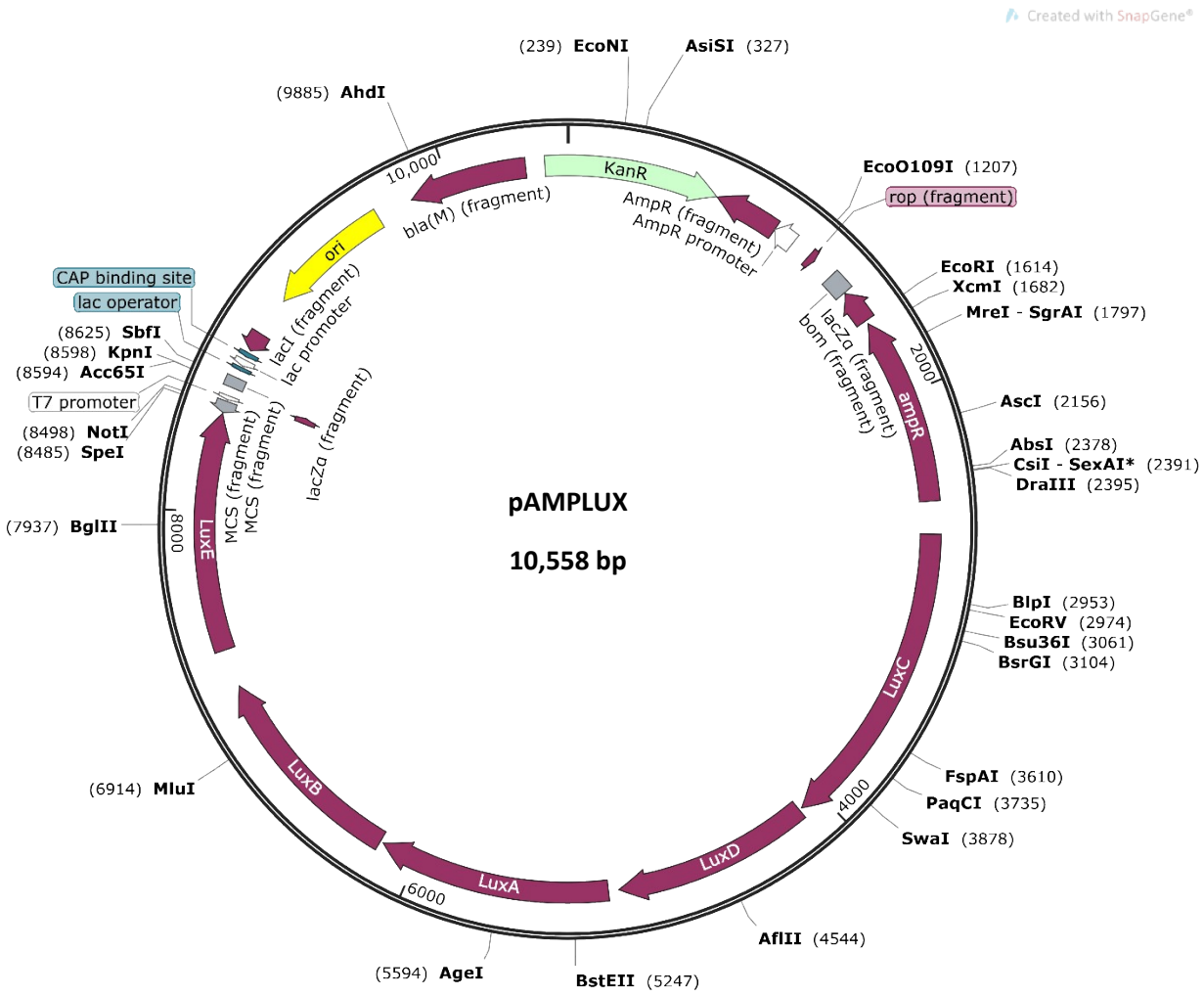

Figure S1. Plasmid map for pAMPLUX. The *ampR*-P*ampC* and *luxCDABE* fragments were cloned into pUC19 at the SacI restriction site in the multiple cloning site (MCS). The ampicillin resistance marker (*bla*) in the resulting vector was disrupted by digestion with ScaI, and the kanamycin resistance marker (KanR) was cloned into this site. The plasmid map was prepared by Plasmidsaurus.

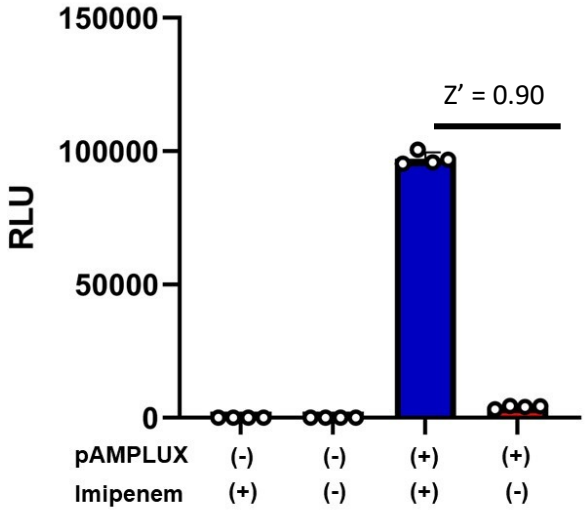

Figure S2. Verifying specificity of luminescent signal produced by *E. coli* transformed with pAMPLUX in response to β-lactam exposure. Wild-type *E. coli* BW25113 cells (- pAMPLUX) and cells transformed with the pAMPLUX plasmid (+ pAMPLUX) were incubated in 2TY media supplemented with imipenem (1.6 µM), or 2TY media alone. Luminescence readings were taken after two hours. *E. coli* BW25113 cells that were not transformed with pAMPLUX did not produce luminescence, regardless of whether or not they were exposed to imipenem. *E. coli* cells transformed with pAMPLUX produced luminescence when treated with imipenem, while minimal background luminescence was observed following media treatment alone. To assess the assay performance, the Z-factor (Z') was determined based on the RLU values obtained from the biosensor cells following treatment with imipenem or media alone. Note that a Z' value greater than 0.5 indicates good separation between positive and negative signals.[4]

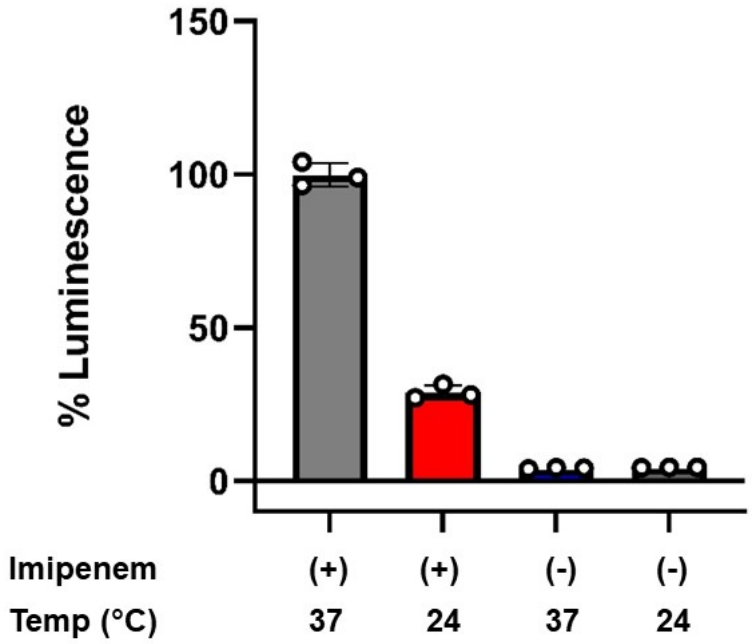

Figure S3. Performance of the biosensor at different incubation temperatures. Biosensor cells were treated with either 2TY media supplemented with imipenem (1.6 µM) or 2TY media alone. These cell suspensions were incubated for two hours either at 37 ˚C or at ambient room temperature (approximately 24 ˚C), and luminescence was measured. Based on these results, 37 ˚C was chosen as the temperature for subsequent assays.

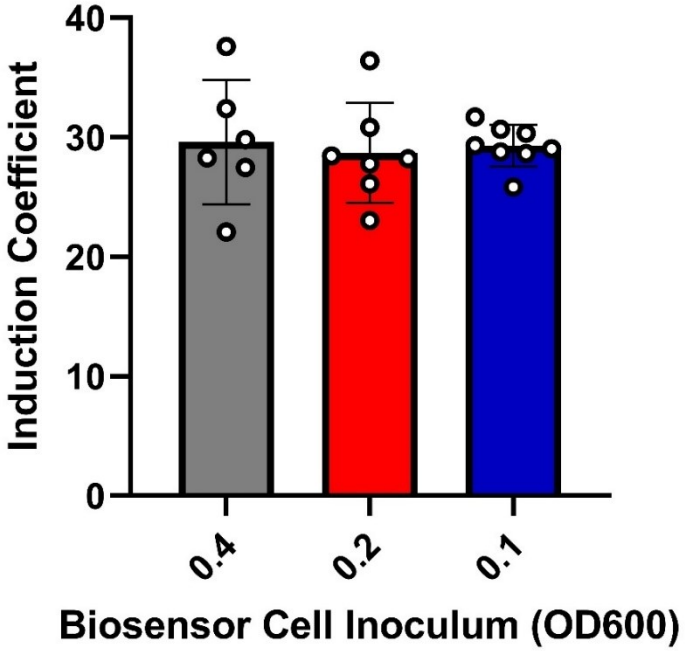

Figure S4. Impact of biosensor cell density on induction coefficient. Biosensor cell suspensions were prepared in 2TY media to OD_{600} = 0.4, 0.2, or 0.1, then diluted 10-fold in 2TY supplemented with imipenem (1.6 µM, final concentration). These mixtures were incubated at 37 °C for two hours, and luminescence readings were taken. The induction coefficient was determined by dividing the RLU values obtained from samples treated with imipenem with the RLU values obtained from samples that were treated with 2TY media alone. There was no apparent difference in the induction coefficients determined from the three different biosensor cell suspensions.

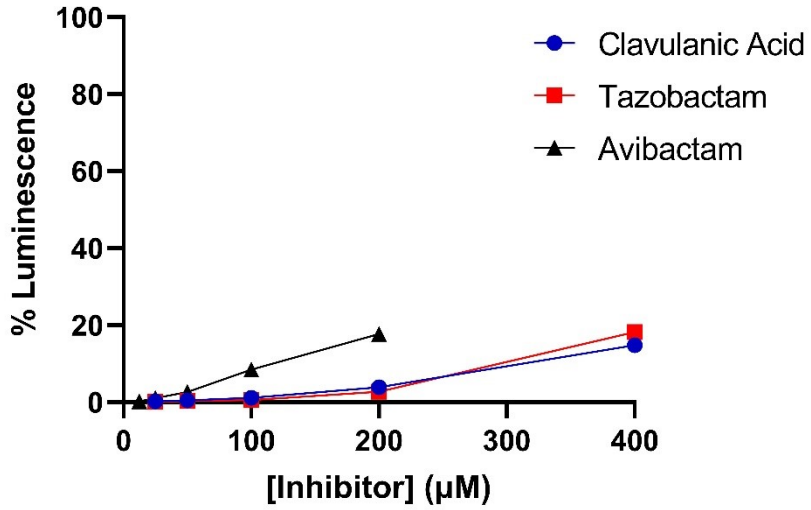

Figure S5. Induction of biosensor luminescence by SBL inhibitors in the absence of a β-lactam antibiotic. Biosensor cells were treated with serial dilutions of clavulanic acid, tazobactam or avibactam prepared in 2TY. Mixtures were incubated for two hours, and luminescence readings were taken. The luminescence values were normalized to the luminescence obtained when biosensor cells were treated with amoxicillin (20 µM). n=4, error bars indicate S.D.

Table S3. IC_{50} values for inhibitors against β-lactamase-producing cells and against purified β-lactamases.

Enzyme	Inhibitor	Inhibitor concentration needed to reduce MIC to susceptible range[a,b]	IC50 (μM, Cell)[c]	IC50 (μM, Enzyme)[d]
TEM-116	Tazobactam[e]	200	118 ± 32.2	0.13[5 j]
	Clavulanic acid[e]	100	64.2 ± 18.7	0.08[5]
	Avibactam[e]	30	13.2 ± 2.1	0.008[6]
KPC-2	Tazobactam[e]	N.I.[m]	N.I.[m]	N.I.[m]
	Clavulanic acid[e]	N.I.[m]	N.I.[m]	N.I.[m]
	Avibactam[e]	60	29.2 ± 10.1	0.17[6]
OXA-48	Tazobactam[e]	N.I.[m]	N.I.[m]	N.I.[m]
	Clavulanic acid[e]	N.I.[m]	N.I.[m]	N.I.[m]
	Avibactam[e]	60	35.5 ± 4.6	0.18[7]
NDM-1	Dipicolinic acid[f]	300	238.5 ± 4.5	3.8[8]
	Nitrilotriacetic acid[f]	600	383.7 ± 6.3	1.3[8]
	Ethylenediaminetetraacetic acid[f]	40	11.4 ± 0.6	0.052[9]
	Captopril[f]	600	461 ± 47	20.1[10]
	Embelin[f]	300	146 ± 13	2.1[11]
IMP-1	Dipicolinic acid[f]	300	243.8 ± 4.2	N.R.[n]
	Nitrilotriacetic acid[f]	N.I.[m]	N.I.[m]	N.R.[n]
	Ethylenediaminetetraacetic acid[f]	200	51.8 ± 5.6	55[12]
	Captopril[f]	600	299 ± 19	7.1[10]
	Embelin[f]	N.I.[m]	N.I.[m]	100[11]
VIM-2	Dipicolinic acid[g]	N.T.[h,o]	210.3 ± 10.2	2.9[8]
	Nitrilotriacetic acid[g]	N.T.[h,o]	331 ± 46	2.4[8]
	Ethylenediaminetetraacetic acid[g]	N.T.[h,o]	68.2 ± 22.5	9.3[k]-200[13, 14]
	Captopril[g]	N.T.[h,o]	145 [i]	0.07[10]
	Embelin[g]	N.I.[h]	N.I.[m]	200[11 l]

[a] The susceptibility breakpoints for meropenem and amoxicillin are 1 μg/mL and 4 μg/mL, respectively; [b] measured based on cell growth after 18 hours using the same conditions as biosensor assays;[c] Cell-based IC_{50} values determined in this study using the biosensor. All inhibitors were tested against *E. coli* BW25113 cells producing the β-lactamase indicated; [d] Inhibitor IC_{50} values reported in literature, references are indicated with superscript numbers; [e] administered with 20 μM amoxicillin; [f] administered with 2.5 μM meropenem; [g] administered with 650 nM meropenem; [h] did not test with breakpoint concentration of meropenem due to poor VIM-2 expression in *E. coli*, resulting from inefficient periplasmic processing of

the enzyme[15]; [i] could not be accurately determined due to the shape of the dose response curve; [j] reported IC_{50}s are for TEM-1, not TEM-116; [k] IC_{50} for EDTA reported for VIM-1; [l] IC50 for embelin was only reported for VIM-1, not VIM-2. [m]N.I. = not inhibited; [n]N.R. = not reported; [o]N.T. = not tested.

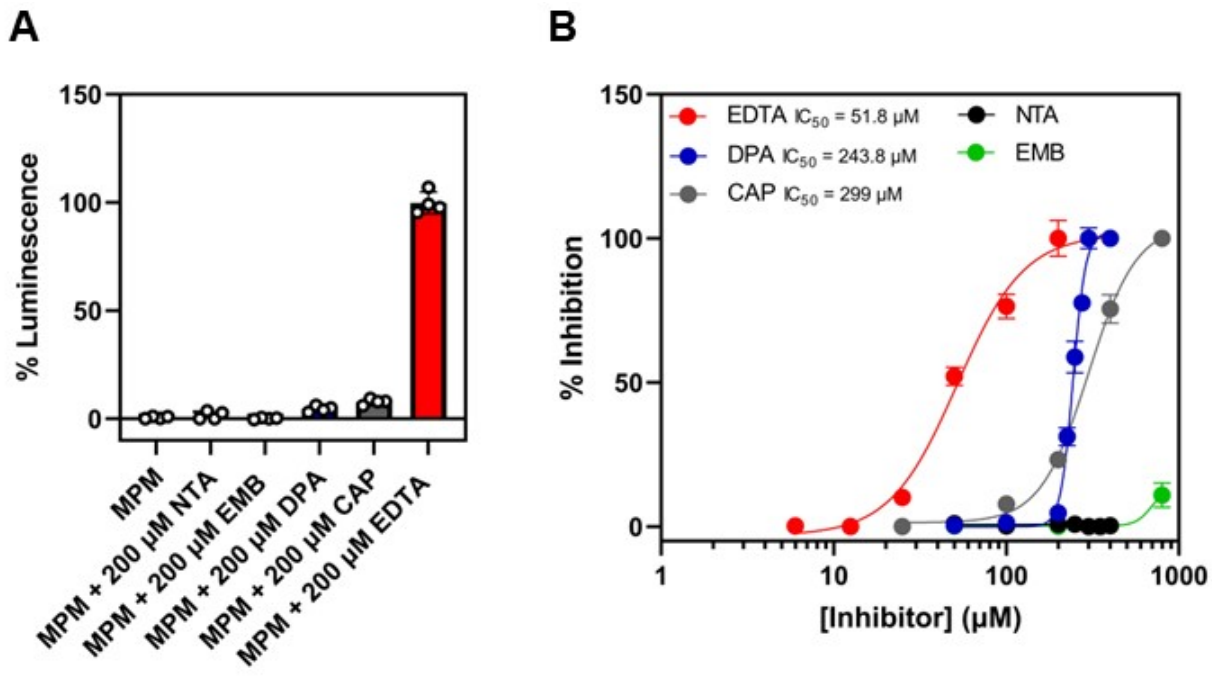

Figure S6. IMP-1 inhibition assays. (A) Single point and (B) dose response analysis of IMP-1 inhibition. IMP-1-producing *E. coli* were treated with meropenem (MPM; 2.5 µM) in combination with ethylenediaminetetraacetic acid (EDTA), dipicolinic acid (DPA), nitrilotriacetic acid (NTA), captopril (CAP) or embelin (EMB) at the concentrations indicated. Luminescence readings were normalized to the sample with the greatest luminescence to determine % inhibition. n=4, error bars indicate S.D.

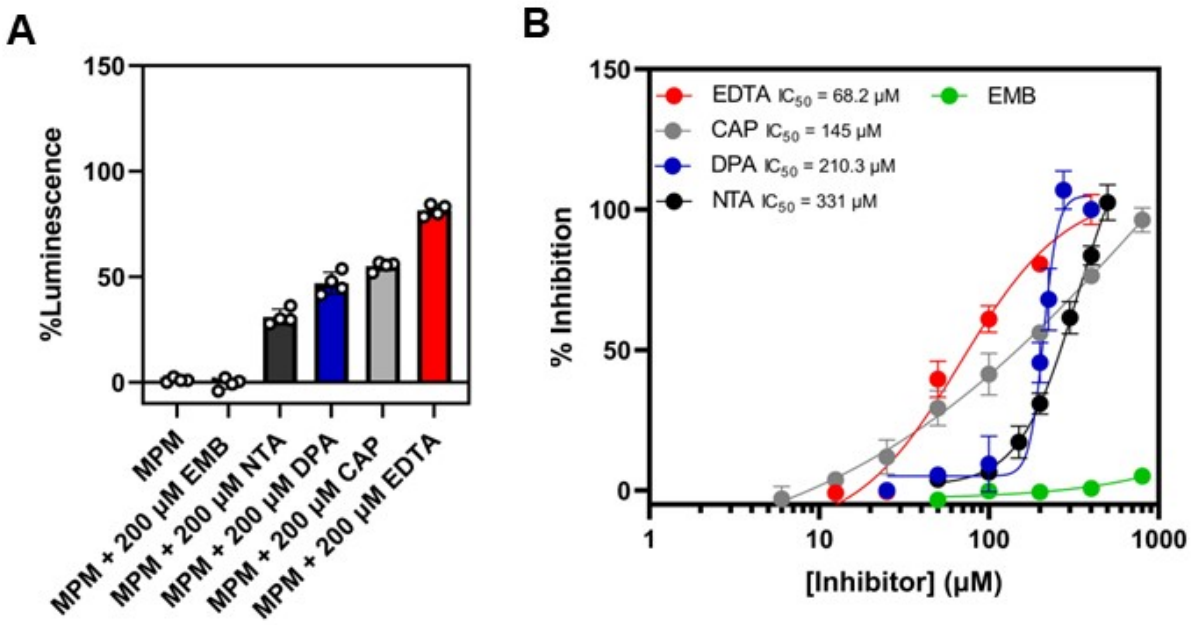

Figure S7. VIM-2 inhibition assays. (A) Single point and (B) dose response analysis of VIM-2 inhibition. VIM-2-producing *E. coli* were treated with meropenem (MPM; 650 nM) in combination with ethylenediaminetetraacetic acid (EDTA), dipicolinic acid (DPA), nitrilotriacetic acid (NTA), captopril (CAP) or embelin (EMB) at the concentrations indicated. Luminescence readings were normalized to the sample with the greatest luminescence to determine % inhibition. n=4, error bars indicate S.D.

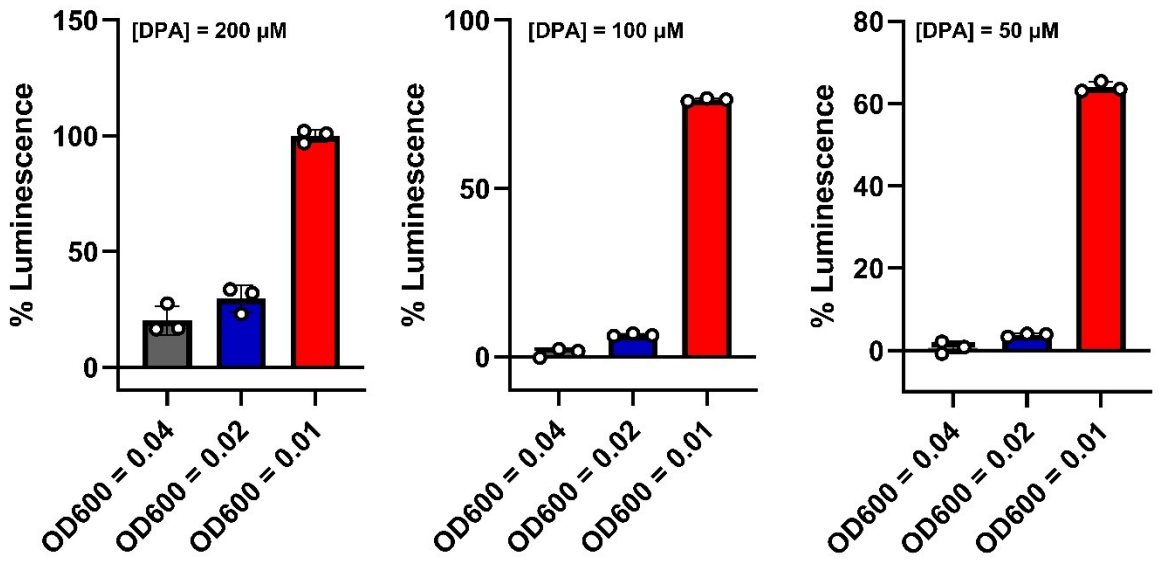

Figure S8. Effects of β-lactamase culture inoculum on apparent potency of dipicolinic acid (DPA) against NDM-1 in *E. coli* cells. Suspensions of NDM-1-producing *E. coli* were prepared to OD600 = 0.4, 0.2 or 0.1, then diluted ten-fold in 2TY supplemented with meropenem (final concentration 2.5 µM) and DPA. Luminescence readings were normalized to the sample with the greatest luminescence. n=4, error bars indicate S.D.

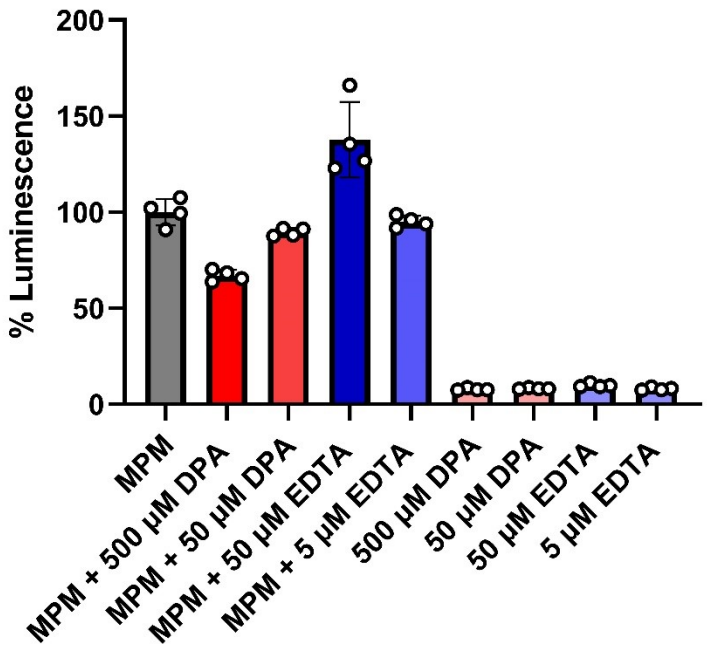

Figure S9. Impact of EDTA and DPA on biosensor induction. Biosensor cells were treated with meropenem (MPM; 2.5 µM) alone or in combination with several concentrations of ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid (DPA). Elevated luminescence readings for MPM + 50 µM EDTA indicate increased antibiotic entry due to permeabilization of the outer membrane by EDTA. Lower luminescence readings observed for MPM + 500 µM DPA is indicative of biosensor cell death due to permeabilization of the outer membrane by DPA. Luminescence readings were normalized to the meropenem only treatment. n=4, error bars indicate S.D.

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