Supporting Information

Live-cell Profiling of Penicillin-Binding Protein Inhibitors in *Escherichia coli* MG1655

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Experimental Methods and Materials

Strains and Growth Media. *Escherichia coli* MG1655 ATCC 700926 was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia) and Luria-Bertani (Lennox, #L3022) was purchased from Sigma-Aldrich.

Antibiotics and reagents. Piperacillin sodium salt (#P8396), cephalexin hydrate (XC4895), cefaclor (#C6895), cefuroxime sodium salt (C4417), ceftriaxone disodium salt hemi(heptahydrate) (#C5893), Polymyxin B-nonapeptide HCl (PMBN; #P2076), ethylenediaminetetraacetic acid (EDTA), Triton-X-100, and lysozyme from chicken egg white were purchased from Sigma-Aldrich. Cefepime hydrochloride (#1097636) was purchased from USP Reference Standards. Avibactam sodium salt (HY-14879A) was purchased from MedChemExpress. Aztreonam (150415) was purchased from MPBiomedicals. Methicillin sodium salt (#21007) was purchased from Cayman Chemical Company. Penicillin G sodium salt (#P1770), meropenem trihydrate (#M2279) and *N*-phenyl-1-napthylamine (NPN; P0197) were purchased from Tokyo Chemical Industry. Faropenem sodium hemipentahydrate (#41111) was purchased from Astatech. Bocillin-FL sodium salt (#B13233) and propidium iodide (P1304MP) were purchased from ThermoFisher Scientific.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) gels were handcast in-house using Biorad 1.5 mm thick mini-protean gel cassettes with 15-well comb. Gels were composed of 10% resolving gel [10.5 mL of 1.5 M Tris-HCl buffer pH 8.8, 10.5 mL of acrylamide:bis-acrylamide 29:1 (40% solution, Bio-Rad)], 21 mL of H_2O, 140 µL of aqueous 10% ammonium persulfate (APS), 15 µL of tetramethylethylenediamine (TEMED, Bio-Rad) and 4.5% stacking gel [2.5 mL of 0.5 M Tris-HCl buffer pH 6.8, 1.125 mL of acrylamide:bis-acrylamide 29:1 (40% solution, Bio-Rad)], 6.375 mL of H_2O, 30 µL of aqueous 10% APS, 10 µL of TEMED. Amounts listed here are for four gels. Resolving gel was poured first (~2/3-3/4 full) and covered with 100% ethanol and allowed to polymerize. Ethanol was then rinsed away, excess water removed, and stacking gel poured to fill gel. Combs were added directly after pouring stacking gel and allowed to polymerize at room temperature (RT). Gels were stored in 1X Tris-glycine SDS (14 g/L glycine, 3 g/L Tris, 1 g/L SDS) running buffer at 4 °C for up to 2 weeks.

Growth Curve Analysis of *E. coli* MG1655

Overnight and fresh cultures of *E. coli* MG1655 were prepared as described. Growth curve analyses were carried out in biological triplicate. For each sample, 1 mL fresh culture grown to OD_{600} ~ 0.4 – 0.5 was harvested. Cells were washed with 1 mL PBS and centrifuged at 18,000 x *g* for 2 min at RT. The supernatant was removed, and cells were resuspended in 50 µL of respective solutions: 1) Tris 2) Tris-EDTA 3) Tris-PMBN 4) Tris-Colistin. Samples were incubated for 30 min at RT. Cells were washed with 1 mL PBS and centrifuged at 18,000 x *g* for 2 min at RT. The supernatant was removed, and the cells were resuspended in 0.3 mL of LB broth. To respective wells in a clear, flat bottom 96-well plate (Corning, Costar #3596) 15 µL of cells were added to 135 µL LB broth in the well (1:10 dilution). Using a Tecan Spark plate reader, the cells were grown with the instrument set to 37 °C, continuous shaking at 216 rpm, and absorbance measurements at 600 nm were taken every 20 min over an 18 hour period. Averages from triplicate data sets were plotted for each time-point with standard deviations represented as error bars.

SDS-PAGE Analysis

Following protein measurements, 30 µL of sample were aliquoted into new Eppendorf tubes. Ten microliters of 4X SDS Laemmli buffer (LB) were added and samples were vortexed. Samples were boiled at 95 °C for 5 min and vortexed. A total of 250 µg of protein were loaded onto a 10% SDS-PAGE gel for each sample (volumes varied based on the protein concentration). Proteins were separated by running gels in 1X Tris-glycine SDS running buffer for 1 ½ hours, or until the 4X SDS LB had run off the foot of the gel (180 V, 60 W, 40 mA/gel). Gels were removed from their cassettes, rinsed with DI water, and analyzed using a GE Typhoon FLA 9500 gel scanner. The following settings were used to scan the gels: 473 nm laser excitation with LPB filter (≥510 nm) using 600 volts for the PMT. Following fluorescence scanning, gels were stained with Brilliant Blue Coomassie to determine the protein loading in each lane. Following overnight staining with Coomassie, gels were destained overnight using a destaining solution (50% water, 40% MeOH, 10% AcOH). Coomassie staining was imaged using the same gel scanner as above with the following settings: 635 nm laser excitation with LPR filter (≥665 nm) using 800 volts for the PMT.

ImageJ Analysis for Inhibitor Titrations

Each gel was analyzed using ImageJ software (National Institutes of Health). For each PBP band in the gel, a box was drawn around the fluorescent band as tightly as possible. The integrated density was measured across the gel, moving from the negative control to the 10 mM treatment lane. For each lane, a new box was drawn around the respective fluorescent-PBP band, due to nonuniform bands across the gel. Once each fluorescent-PBP band was measured, a box was drawn around the negative control band and the gel background was measured directly above or below each respective fluorescent band. This procedure was used through the entire gel for each PBP. Background-subtracted fluorescent integrated density values were corrected to protein values, which were calculated by the total sum for the integrated density values for 4-5 protein bands from the Coomassie stain (**Fig. S2**). This was done to account for differences in protein loading between the lanes. Relative percent Bocillin-FL values were then calculated by correcting each inhibitor protein-corrected fluorescent intensity to the protein-corrected fluorescent intensity for the negative control. This was done uniformly for each PBP in the gel. The values from biological triplicate, or duplicate where indicated, experiments were then plotted against inhibitor concentration using non-linear regression analysis in GraphPad Prism (8.4.3) ([Inhibitor] vs response (three-parameter)) to determine the mean IC_{50}^{app} and standard deviations.

One-way ANOVA analysis for Bocillin-FL Labeling of the PBPs

Mean protein-corrected fluorescent values were determined from biological triplicate data sets. The mean values for each treatment condition were compared against each other for each of the 8 PBPs detected using one-way ANOVA with a post-hoc multiple comparisons analysis. Within each row (respective PBPs), column (respective treatment condition) means were compared. One family per row was used. A Tukey Test was used to correct for multiple comparisons using statistical hypothesis testing. Multiplicity adjusted p-values for each comparison were reported and the family-wise significance ($\alpha = 0.05$) and a confidence level was set at 95%. Adjusted p-values were output using the GraphPad style [0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****)].

One-way ANOVA analysis for NPN and PI Uptake

The mean fluorescent values were determined from biological triplicate data sets using the equation described in the NPN and PI assay methods section. The mean value for each treatment condition were compared against each other using a one-way ANOVA multiple comparisons analysis in GraphPad Prism 8.4.3. No matching or pairing was done, and equal standard deviations were assumed. Multiple comparisons were done by comparing the mean of each column to the mean of every other column (columns were respective treatments). A correction for multiple comparisons using statistical hypothesis testing was done using the Dunnett Test. Multiplicity adjusted p-values for each comparison were reported and the family-wise significance ($\alpha = 0.05$) and a confidence level was set at 95%. Adjusted p-values were output using the GraphPad style [0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****)].

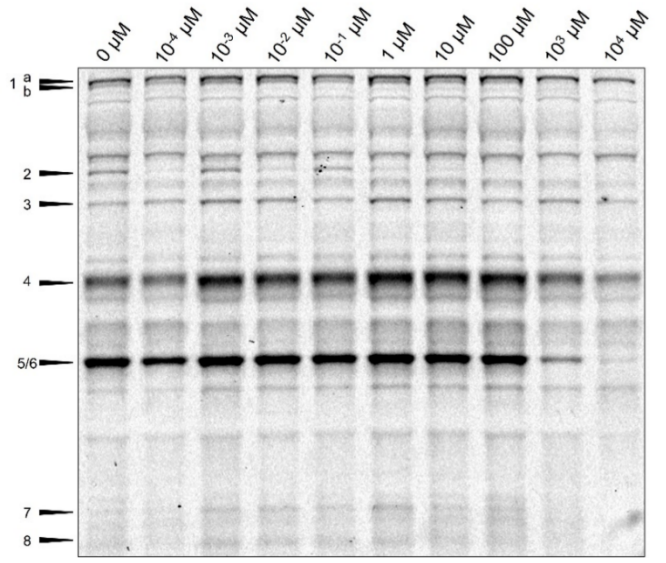
Titration of meropenem in *E. coli* MG1655 cell lysates

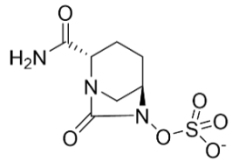
E. coli MG1655 was cultured as described and 0.5 mL of cultures at OD_{600} = 0.5 were harvested in respective eppi tubes. Cell pellets were washed with 1 mL PBS and then resuspended in 50 mM Tris pH 7.8 supplemented with 1 mM EDTA and 10 mg/mL lysozyme. Samples were incubated for 20 min at 37 °C. Cells were lysed on a vial tweeter and the membrane fractions were isolated as previously described. The supernatants were removed and the membrane fractions were resuspended in 50 µL 50 mM Tris pH 7.8 containing 0 – 10^4 µM meropenem and incubated at RT for 30 min. The membranes were pelleted at 21,000 x *g* for 10 min, supernatant was removed, and the pellets were washed with 1 mL PBS. The membrane fractions were resuspended in 50 µL 50 mM Tris pH 7.8 containing 25 µM Bocillin-FL and incubated for 30 min at RT, in the dark. The membranes were pelleted and washed with 1 mL PBS. Pellets were resuspended in 50 mM Tris pH 7.8 – 0.5% SDS and protein measurements were taken as previously described. The same procedure for preparing and loading samples onto 10% SDS-PAGE gels as described were used.

Microscopy Studies of *E. coli* MG1655 Treatments

E. coli MG1655 was inoculated from a –80 °C glycerol stock into 5 mL fresh LB. Five subsequent 1:10 serial dilutions were completed into 5 mL of LB. The tubes were incubated overnight at 37 °C, 220 rpm. After 14 hours, the 6^{th} aliquot was subcultured 1:10 (600 µL, in 5400 µL LB) and incubated to an OD_{600} of 0.5 at 37 °C, 220 rpm. Aliquots of 1 mL were centrifuged at

18,000 x *g* for 2 min. The supernatant was discarded, and pellets were washed with 1 mL 1X PBS. Pellets were resuspended in 50 µL of 50 mM Tris pH 7.8, Tris-200 µM EDTA, Tris-200 µM PMBN, or Tris-200 µM colistin and incubated at room temperature for 30 min. After incubation, samples were centrifuged at 18,000 x *g* for 2 min. The supernatant was discarded, and pellets were washed in 1 mL 1X PBS. Pellets were resuspended in 50 mM Tris pH 7.8 with 50% glycerol. Each sample (3 µL) was placed on a 1.5% agarose pad on a glass slide. Agarose pads were prepared as follows: Two microscope slides were covered with 3 layers of lab tape. A microscope slide was placed between the two slides covered with tape. Molten agarose (130 µL of 1.5%) was placed on the slide. A fourth slide was placed on the agarose, perpendicular to the bottom slides. The agarose cooled for 10 min. The top slide was removed and 3 µL of sample was placed on the agarose pad. A cover slip was placed on the samples, and they were imaged using phase-contrast on an Olympus IX-73 microscope and oil immersion with a UPlanSApo 100x phase-contrast (NA 1.40 Oil Ph3/0.17/FN26.5) objective. Micrographs were captured with a Hamamatsu Orca Flash 4.0 LT C11440 – 42U30 CMOS camera. Images were analyzed using CellSens Dimension Ver 2.3 by Olympus Corporation.





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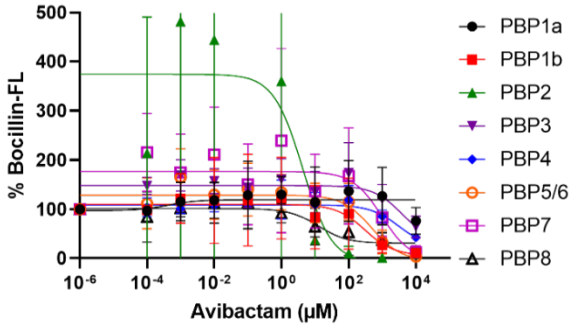

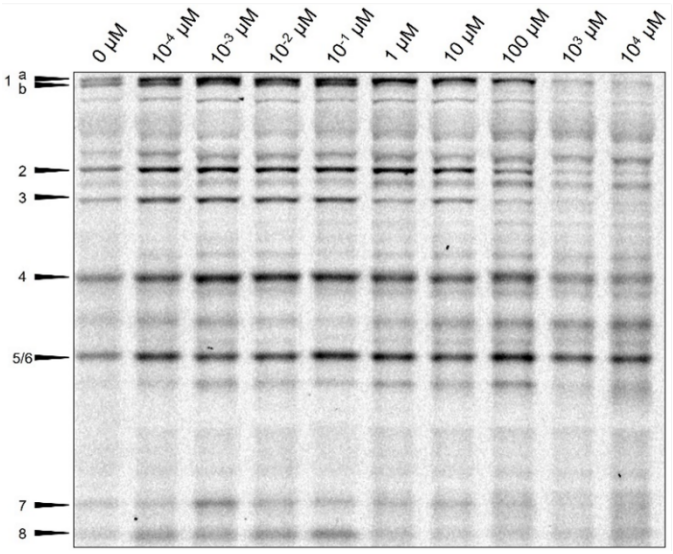

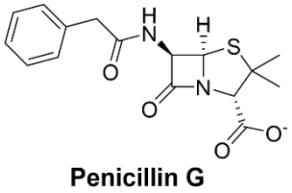

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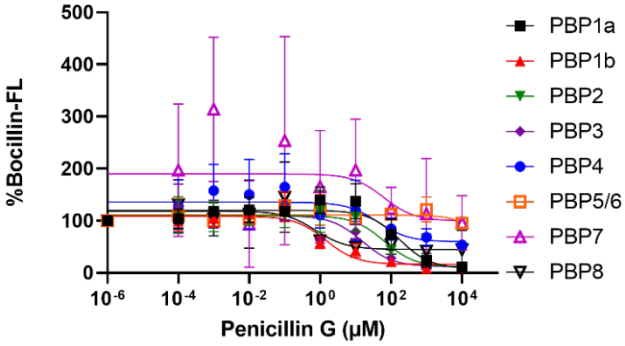

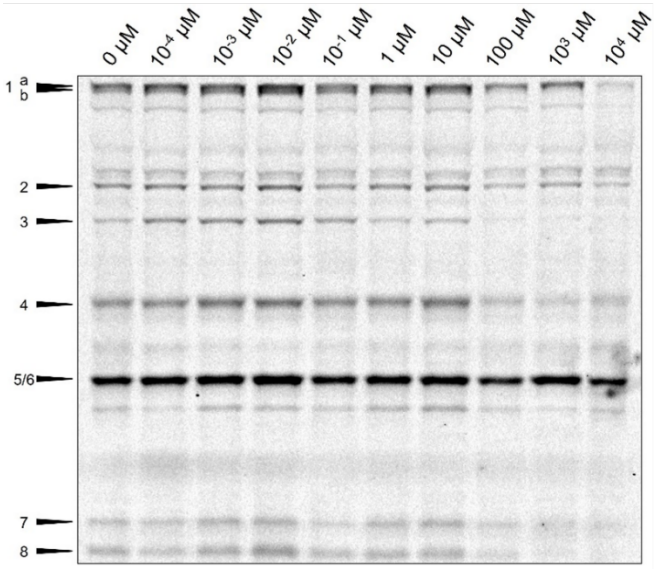

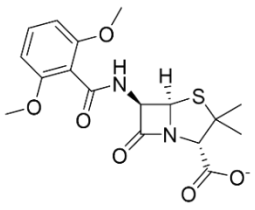

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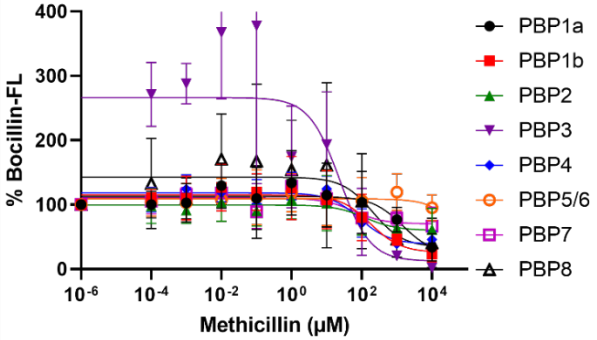

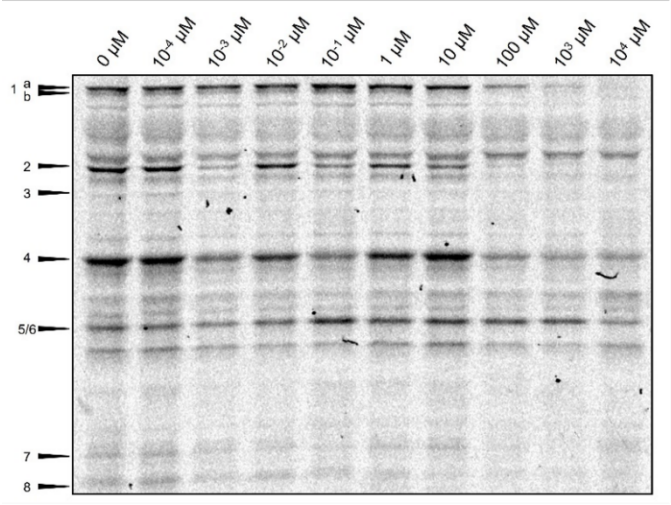

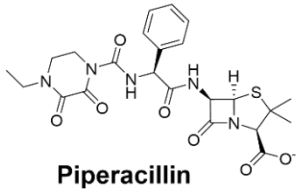

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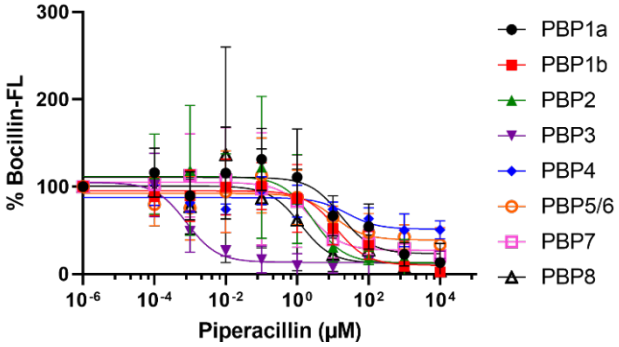
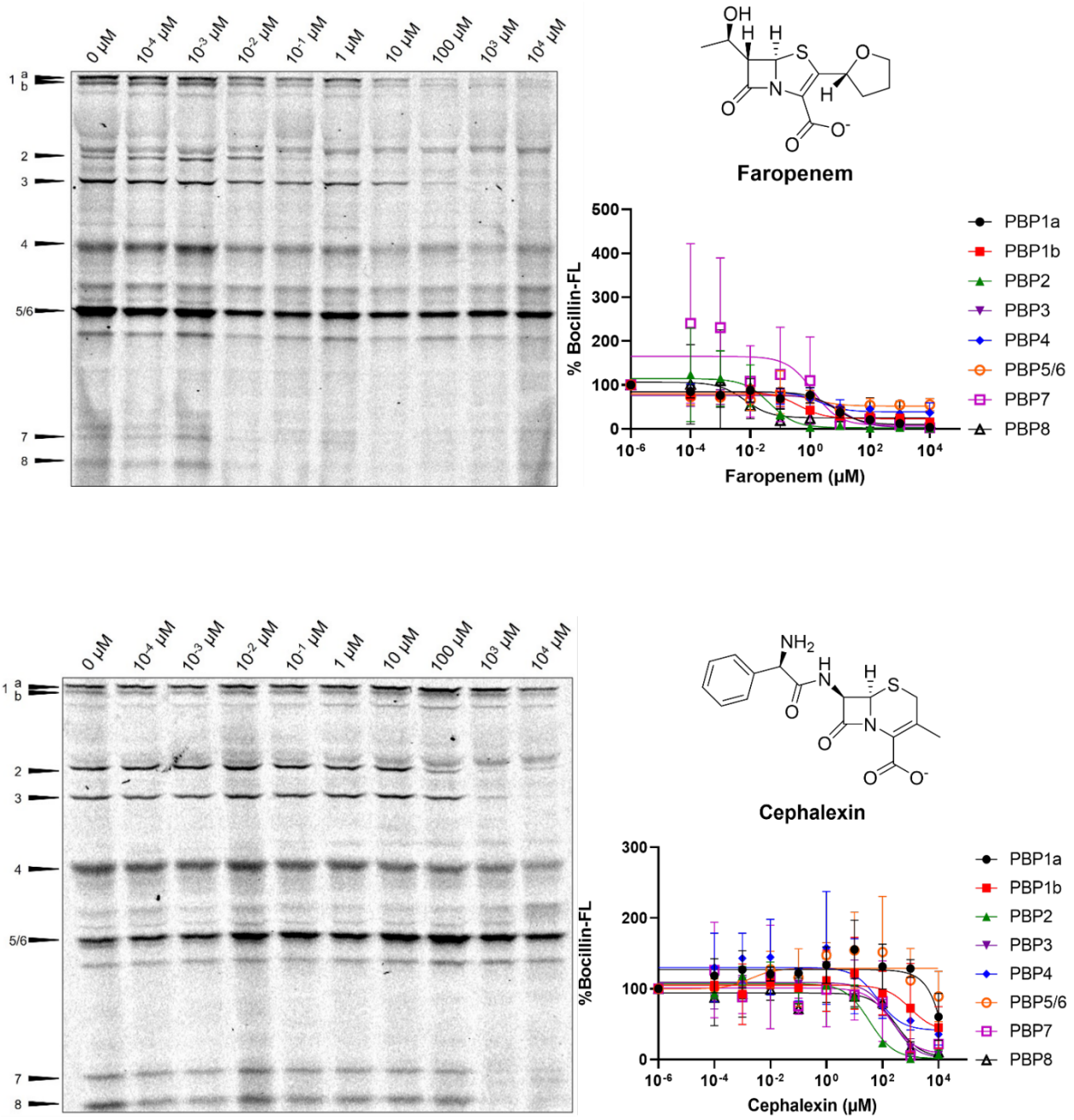
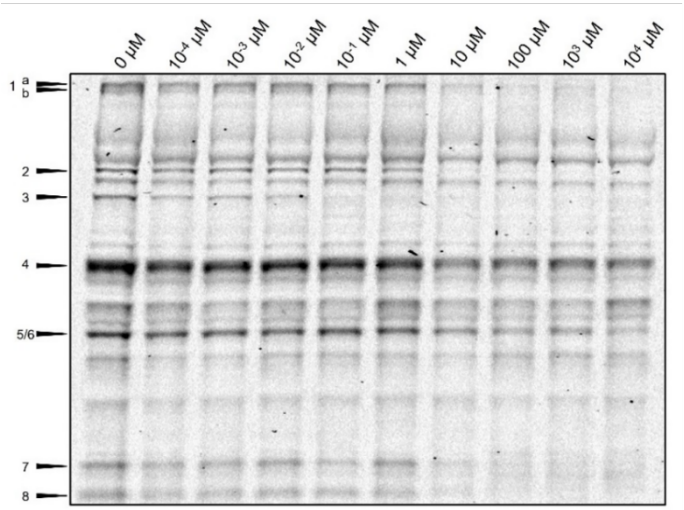
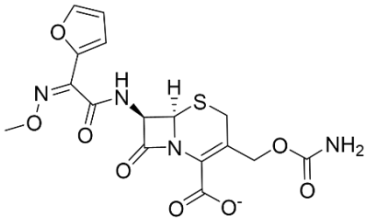



0 µM
10-4 µM
10-3 µM
10-2 µM
10-1 µM
1 µM
10 µM
100 µM
103 µM
104 µM
1 a b
2
3
4
5/6
7
8
Faropenem
% Bocillin-FL
Faropenem (µM)
PBP1a
PBP1b
PBP2
PBP3
PBP4
PBP5/6
PBP7
PBP8
Cephalexin
%Bocillin-FL
Cephalexin (µM)





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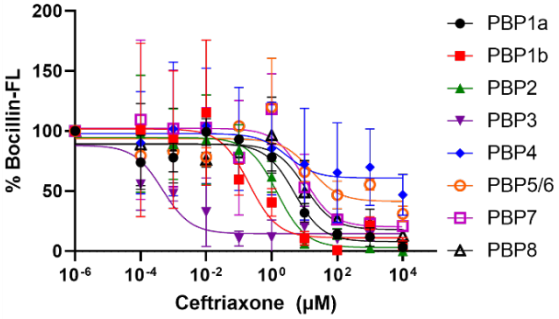

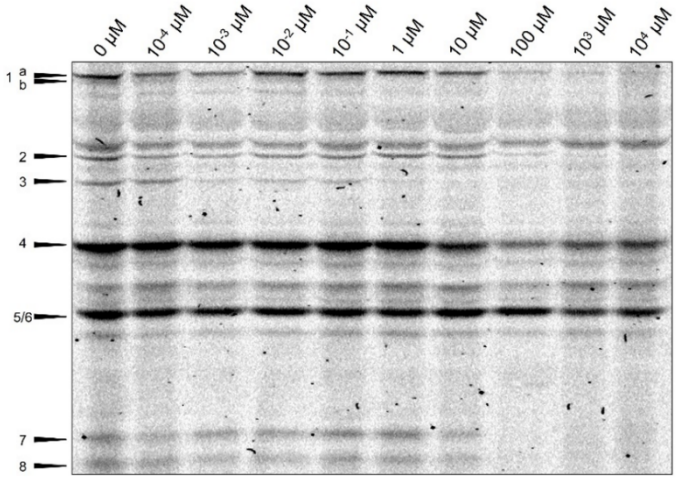

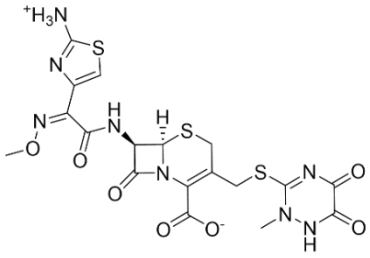

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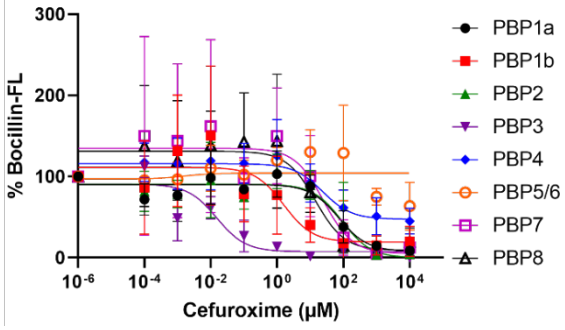

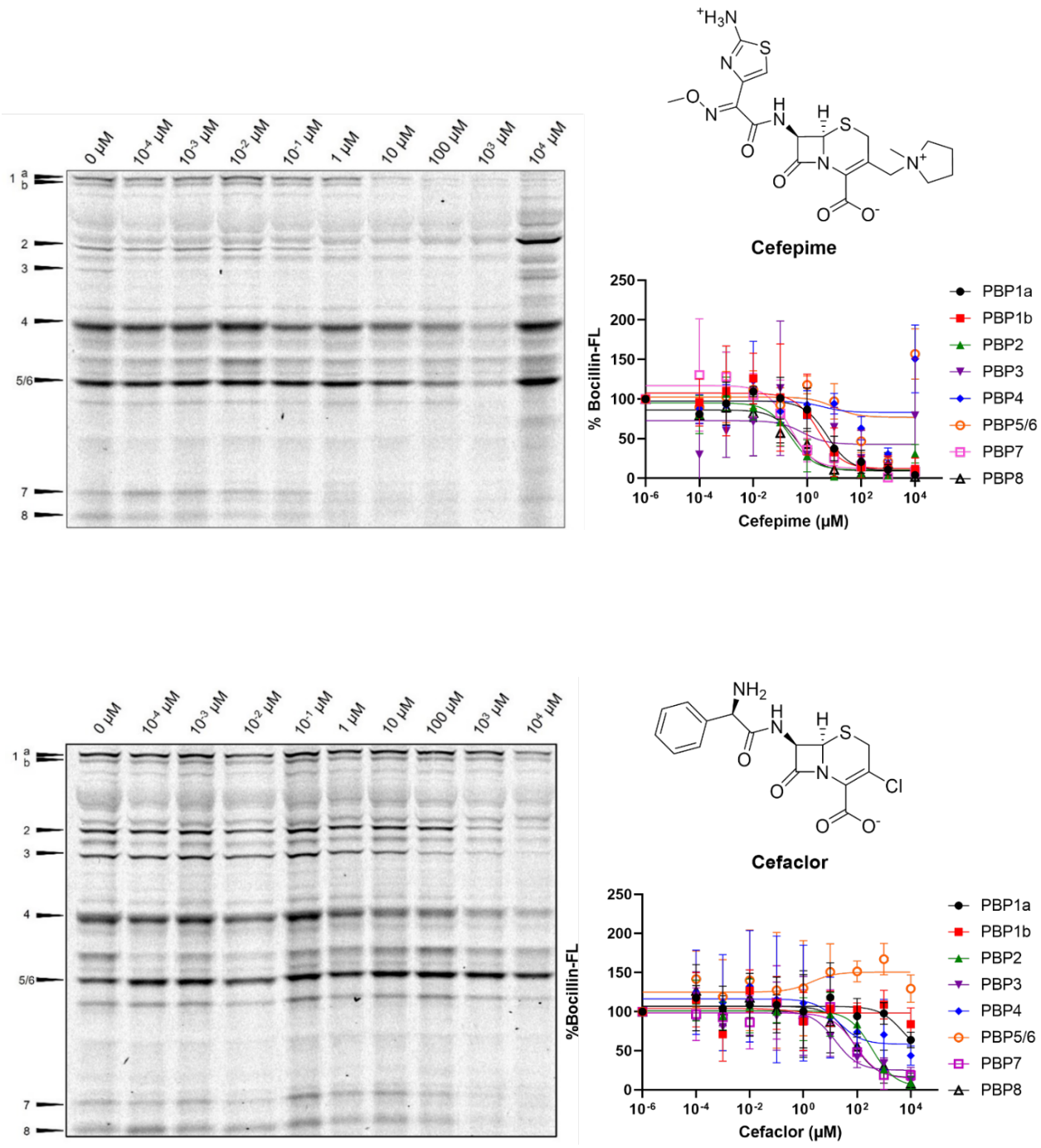

Figure S1. Representative SDS-PAGE analyses of one replicate from biological triplicate titrations of inhibitors against PBPs in *E. coli* MG1655 and the corresponding IC_{50} plots with mean values and standard deviations plotted from biological triplicates.

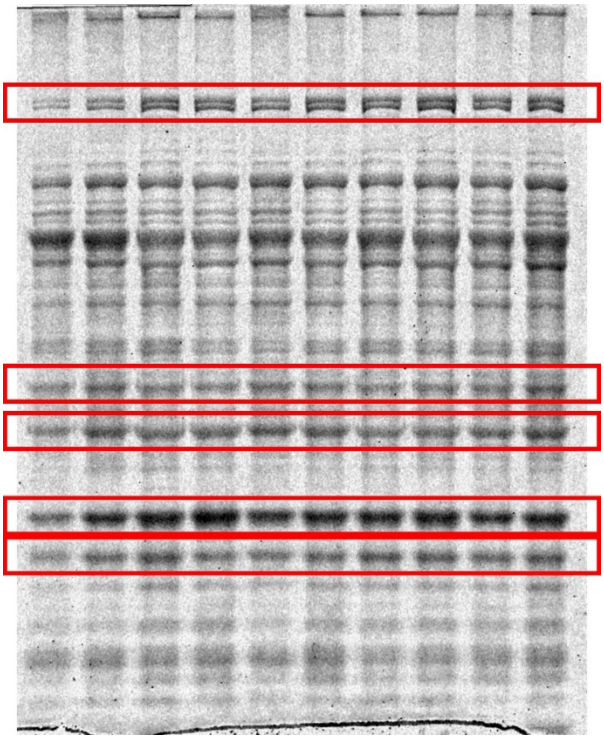

Figure S2. Representative Coomassie stain from a titration with Penicillin G. The red boxes indicate the protein bands that were used to measure protein integrated density. The sum of these integrated densities was used to correct the background-subtracted Bocillin-FL signals for each PBP in respective lanes. This procedure was used for all gel-based experiments.

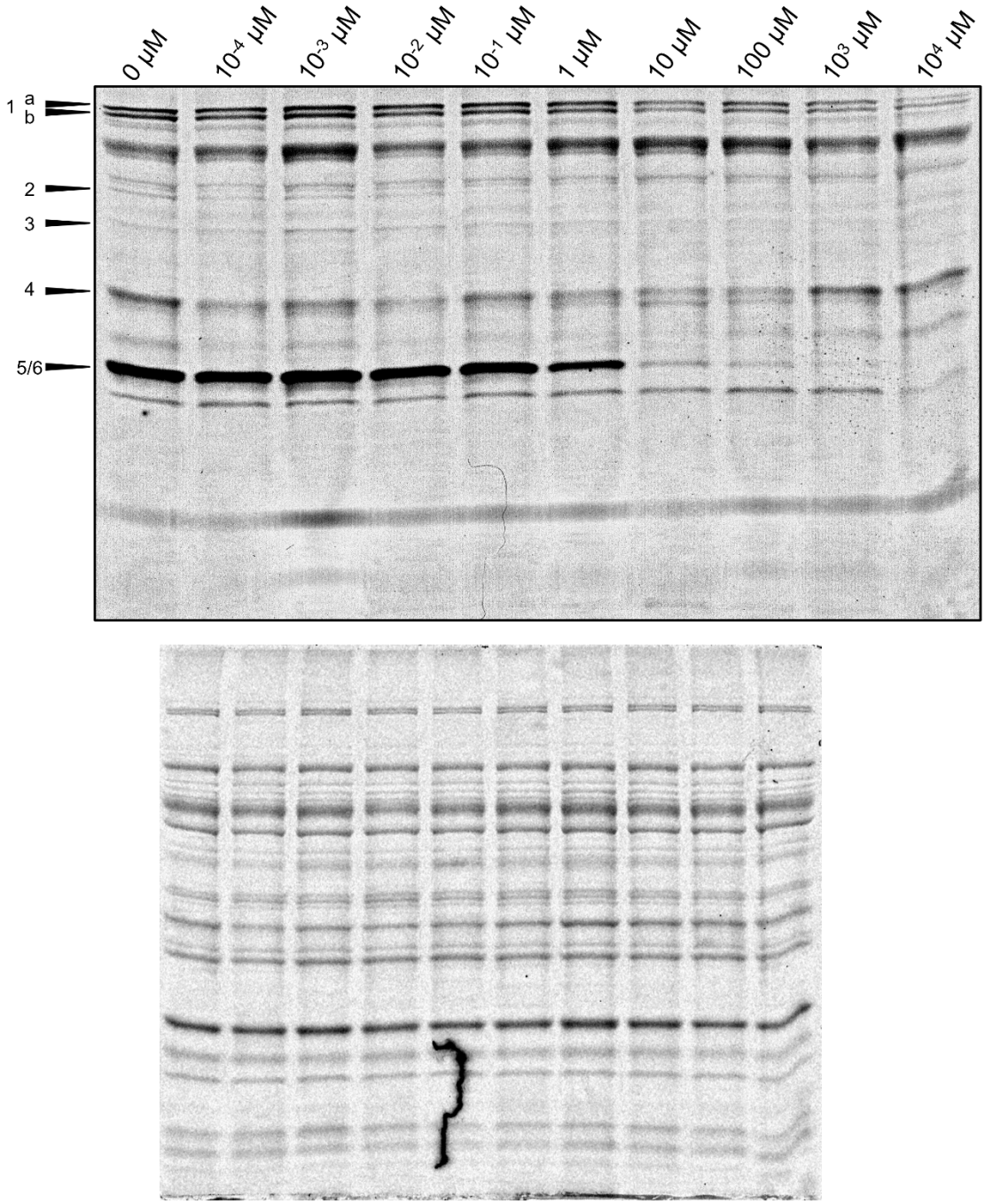

Figure S3. Titration of meropenem in *E. coli* MG1655 cell lysates. (Top) Membrane fractions were isolated from *E. coli* MG1655 following cell lysis and incubated with increasing concentrations of meropenem in Tris. Following a 30 min incubation, membranes were washed and treated with 25 µM Bocillin-FL for 30 min. SDS-PAGE analysis shows a lack of PBP7 and PBP8 being detected. A lack of PBP4 inhibition aligns with the *in vivo* titration data in Fig. 8 of the manuscript. (Bottom) Imaging of the Coomassie stained gel from above.

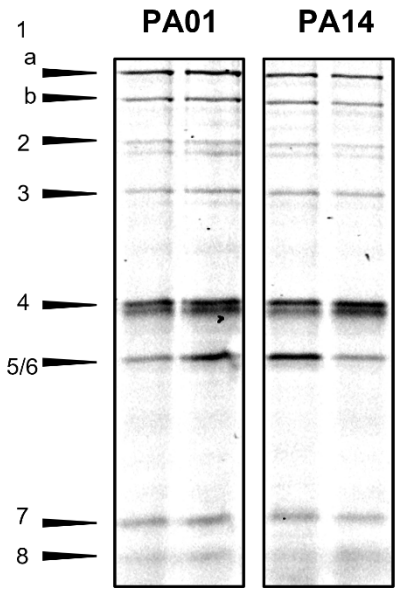

Figure S4. Bocillin-FL labeling in *P. aeruginosa*. Live *P. aeruginosa* was treated with 50 μM Bocillin-FL in 50 mM Tris pH 7.8 for 30 minutes, followed by cell lysis, and SDS-PAGE analysis, following the same procedures used for *E. coli* MG1655. Permeabilization reagents were not used.

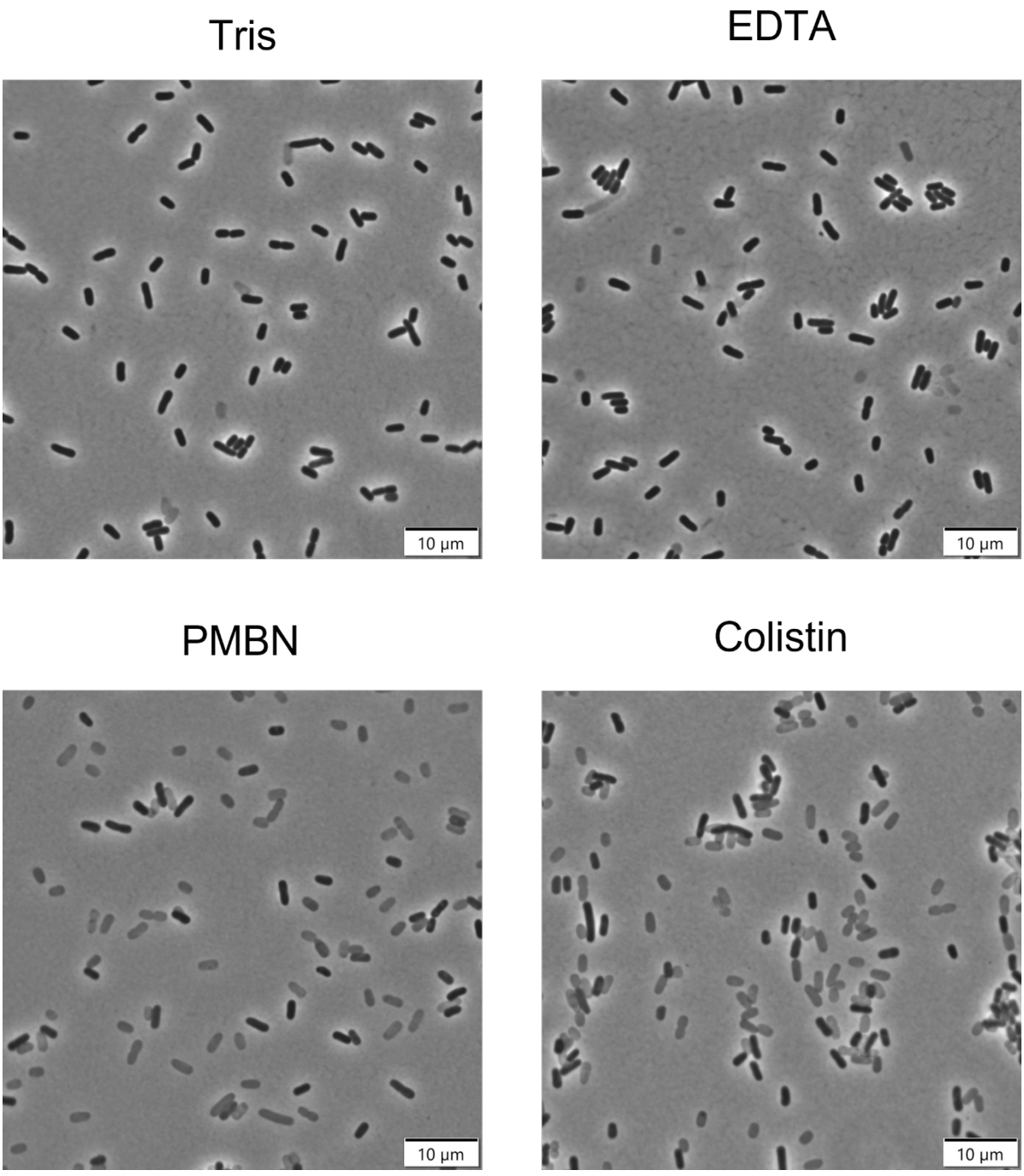

Figure S5. Phase-contrast microscopy of permeabilized *E. coli* MG1655 cells. Cells were treated with respective solutions for 30 minutes, followed by washing, slide mounting, and imaging. PMBN and colistin caused the most cell damage, as indicated by the numerous light-grey cells (likely empty sacculi). EDTA treatment yielded minimal damaged cells, consistent with the amount of membrane damage observed in the NPN and PI assays (**Fig. 6**). 100x magnification, scale bar is 10 µm.

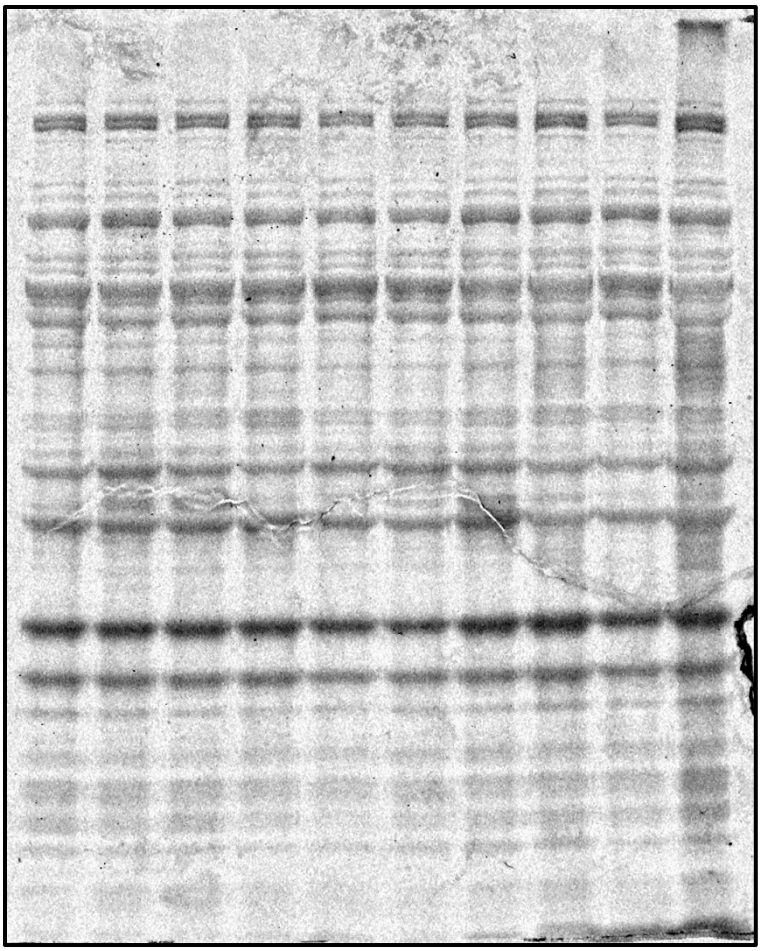

Figure S6. Proteome loading for aztreonam titration. Coomassie staining of the fluorescent gel in **Figure 7** for aztreonam titration demonstrates similar levels of proteome in all lanes. These results suggest the increase in Bocillin-FL labeling in the 10 mM titration (far right lane) is not due to increased proteome loading.