Cell Reports

Supplemental Information

Bactericidal Antibiotics Induce Toxic Metabolic Perturbations that Lead to Cellular Damage

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Supplemental Experimental Procedures

Bacterial strain

MG1655 *E. coli* (ATCC# 700926) was used for all studies.

Metabolic profiling

Overnight cultures of MG1655 were inoculated in Luria/Lysogeny Broth (LB, Fisher) and grown at 37°C and 300 rpm in a humidity-controlled incubator shaker (Multitron II, ATR). On the day of the experiment, overnight cultures were diluted 1:500 in 100 mL LB and grown at 37°C and 300 rpm in 1 L baffled flasks to an optical density (OD_{600}) of approximately 0.3, before the addition of 3 μg/mL Amp, 7.5 μg/mL Kan, or 150 ng/mL Nor. Antibiotic concentrations were selected to achieve minimal cellular lysis at the first timepoint of collection and substantial cellular death at later timepoints. OD 600 nm was tracked to assure that no lysis resulted from treatment. For each replicate, 100 mL of bacteria was collected at t=0 (for the U.NT0 control) and at t=30 minutes, t=60 minutes, and t=90 minutes (for the antibiotic treatment conditions). The bacterial pellets were rapidly washed at 4°C in 1x PBS (pH 7.2, Fisher), and lysed and assayed by Metabolon Inc. (Durham, USA), as previously described (Shakoury-Elizeh et al., 2010). Metabolon provided relative quantification of concentration measurements (based on scaled peak intensities) for all identified metabolites.

Computational analysis

All computational analyses were performed in MATLAB (MATLAB 8.1, The MathWorks Inc., Natick, MA, 2013). The metabolic profiling data were normalized by BRADFORD protein concentration and scaled to set the median equal to one. Metabolites with three or more missing identifications (out of five replicates) in one or more conditions were excluded from the analysis due to insufficient coverage. The remaining missing entries were imputed using the k-nearest neighbors approach with the standardized Euclidean

distance metric. A Welch's two-sample t-test was used on log-transformed metabolite data to calculate p-values between conditions, and multiple hypothesis testing corrections were performed using the mafdr function in MATLAB to calculate the q-value. For the bar plots, fold changes were calculated for each treatment condition with respect to the untreated control, and the resulting values were log-transformed and plotted in MATLAB. Hierarchical clustering was performed using the clustergram function in MATLAB on log-transformed, standardized metabolite data for each timepoint. The biclustering analysis was performed using the correlation distance metric and average linkage metric. A hypergeometric test was performed using the hygecdf function in MATLAB to determine whether significantly increased or decreased metabolites (defined as having a fold change greater than 1.5, p-value less than 0.05, and q-value less than 0.1) for each condition were enriched for a particular pathway group. Principal component analysis was performed in MATLAB using the pca function with the SVD algorithm.

MG1655 collection and lysis for ELISA

Overnight cultures were diluted 1:250 in 25 mL LB, in 250 mL baffled flasks and grown to an OD_{600} of 0.2 to 0.3. Cells were then treated with antibiotics for one hour, at which time they were collected and spun down at 4000 rpm for 10 minutes in a benchtop swinging bucket centrifuge. Samples were then washed in PBS and pelleted at 13.2K rpm for five minutes in a benchtop microcentrifuge. Pellets were stored at -80°C. ~200 µL B-PER II with 100 µg/mL lysozyme and 5 U/mL DNase I (Thermo Scientific) were added to thawed sample pellets. Samples were then vortexed for 15 minutes at room temperature, spun down at 13.2k rpm for five min, and the supernatant was frozen at -80°C. A BCA assay (Pierce) was used to quantify protein concentration.

MDA ELISA

1 µg of sample proteins was diluted into 1.2 mL EIA buffer (1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA, and 0.1% sodium azide). 200 µL of each sample was then added to ELISA plates (Nunc MaxiSorp). The plate was covered with sealing tape and incubated at 4°C overnight. After the incubation, the plate was washed five times with 300 µL of EIA buffer per well. 250 µL of 5% bovine serum albumin in PBS (VWR) was added to each well and incubated at room temperature for two hours. Plates were again washed as above and 200 µL of 1:500 anti-malondialdehyde antibody (Abcam) was added to each well and incubated at 37°C for one hour. This was followed by another wash, after which a secondary goat anti-rabbit horse-radish peroxide (HRP) antibody was added at 1:10000 in 5% BSA for one hour at room temperature. After a final wash, 200 µL of Tetramethylbenzidine (TMB) reagent (Sigma Aldrich) was added to each well and incubated at room temperature for 5-20 minutes. 100 µL of 2 M H_2O_2 (Sigma) was added and plates were gently mixed. Absorbance was read at 450 nm using a SpectraMax M3 Microplate Reader spectrophotometer.

Protein carbonylation ELISA

Protein carbonylation was measured with a Protein Carbonyl ELISA kit (Enzo Life Sciences). The ELISA assay was performed according to the manufacturer's protocol. Briefly, lysed MG1655 cells were reacted with DNP conjugate and attached onto 96-well ELISA plates. This was probed with anti-DNP-biotin-antibody followed by streptavidin-linked HRP. TMB was added and absorbance was read at 450 nm. This value was compared to protein carbonyl standards samples treated in a similar fashion.

8-oxo-dG and 8-oxo-G quantitation

MG1655 bacteria pellets were obtained as described above. After washing in PBS, pellets were resuspended in 400 µL 1% SDS in dH_2O. The resuspension was added to

Lysing Matrix B tubes (MPBio) and vortexed three times for 45 seconds and put on ice between each vortex. RNA was extracted using a phenol chloroform prep and DNA was extracted using the QIAmp DNA Mini purification kit. 8-oxo-dG levels were quantified with an OxiSelect Oxidative DNA Damage ELISA kit (Cell Biolabs). 8-oxo-G levels were quantified with an OxiSelect Oxidative RNA Damage ELISA kit (Cell Biolabs). Samples were assayed in replicates of six.

GamGFP assay

An overnight LB culture of *E. coli* MG1655 tetGamGFP cells was diluted at 1:100 in 25 mL of LB broth in a 250 mL baffled flask. Cells were placed in a shaking incubator at 37°C and 200 rpm. After one hour, doxycycline was added at a final concentration of 20 ng/mL to induce GamGFP expression. Cells were incubated for another hour, then added to a 96-well plate, with or without drug. Cultures were incubated at 37°C and 900 rpm for a further two hours, at which point the cells were spun down and re-suspended in 2 µL/mL of DAPI/PBS and incubated at room temperature in the dark for 25 min. Cells were centrifuged again and then re-suspended in 150-300 µL of sterile PBS. To image cells, 2 µL of culture was placed atop a 1% agar pad mounted on a slide and sealed with a coverslip. Imaging was performed on a Zeiss Axio Observer Z1 microscope, using a 63x water lens and 2.5x optovar. The brightest z-plane was determined for each field of view, and fluorescence exposure times were optimized by automatic exposure on that plane; this was done because GFP intensity varied between treatments, and comparison of overall GFP intensities between samples is not required for intracellular puncta enumeration. Z-stacks were acquired at this fixed exposure time for both DAPI and GFP, and deconvolved using Huygens Proffessional software. Quantification of GFP foci was performed using Imaris software. Briefly, three-dimensional cell segmentation was performed based on the DAPI stain, and puncta localized within the DAPI-delimited

volume were detected using the vesicle detection tool. GFP aggregates not co-localized with the DAPI signal were excluded from the analysis. Z-stack sum projections were constructed using ImageJ software. Statistical significance of the difference between untreated control and each treatment was assessed using Sidak's multiple comparisons test: *, $p>0.05$; **, $p<0.01$; ***, $p<0.001$, where p-values are adjusted to account for multiple comparisons.

References

Shakoury-Elizeh, M., Protchenko, O., Berger, A., Cox, J., Gable, K., Dunn, T.M., Prinz, W.A., Bard, M., and Philpott, C.C. (2010). Metabolic response to iron deficiency in Saccharomyces cerevisiae. J. Biol. Chem. *285*, 14823–14833.

Supplemental Data Sets (Belenky et al.)

Bactericidal antibiotics induce toxic metabolic perturbations that lead to cellular damage

Figure S1 Metabolite Collection Kill Curves

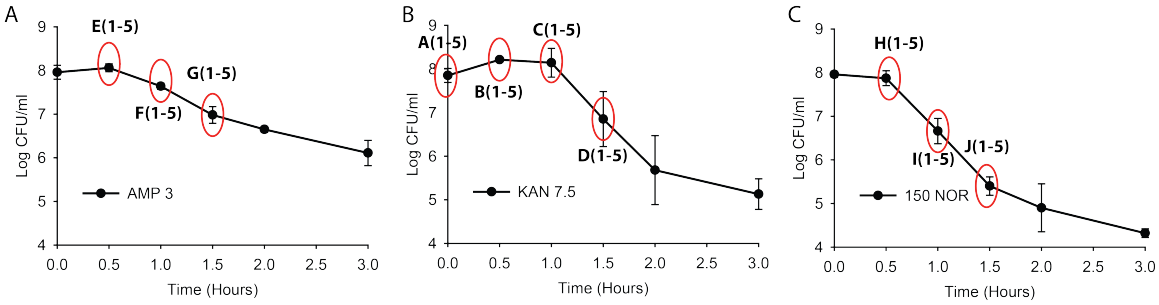

Figure S1: Metabolite Collection Kill Curves (Refers to Figure 1).
MG1655 cells at OD_{600} of ~ 0.3 were treated with antibiotics at the indicated concentration for 3 hours (Amp 3 µg/ml, Kan 7.5 µg/ml and Nor 150 ng/ml). The curves presented above represent five independent biological replicates. Bacteria for metabolomic analysis were collected at the points indicated by red ovals.

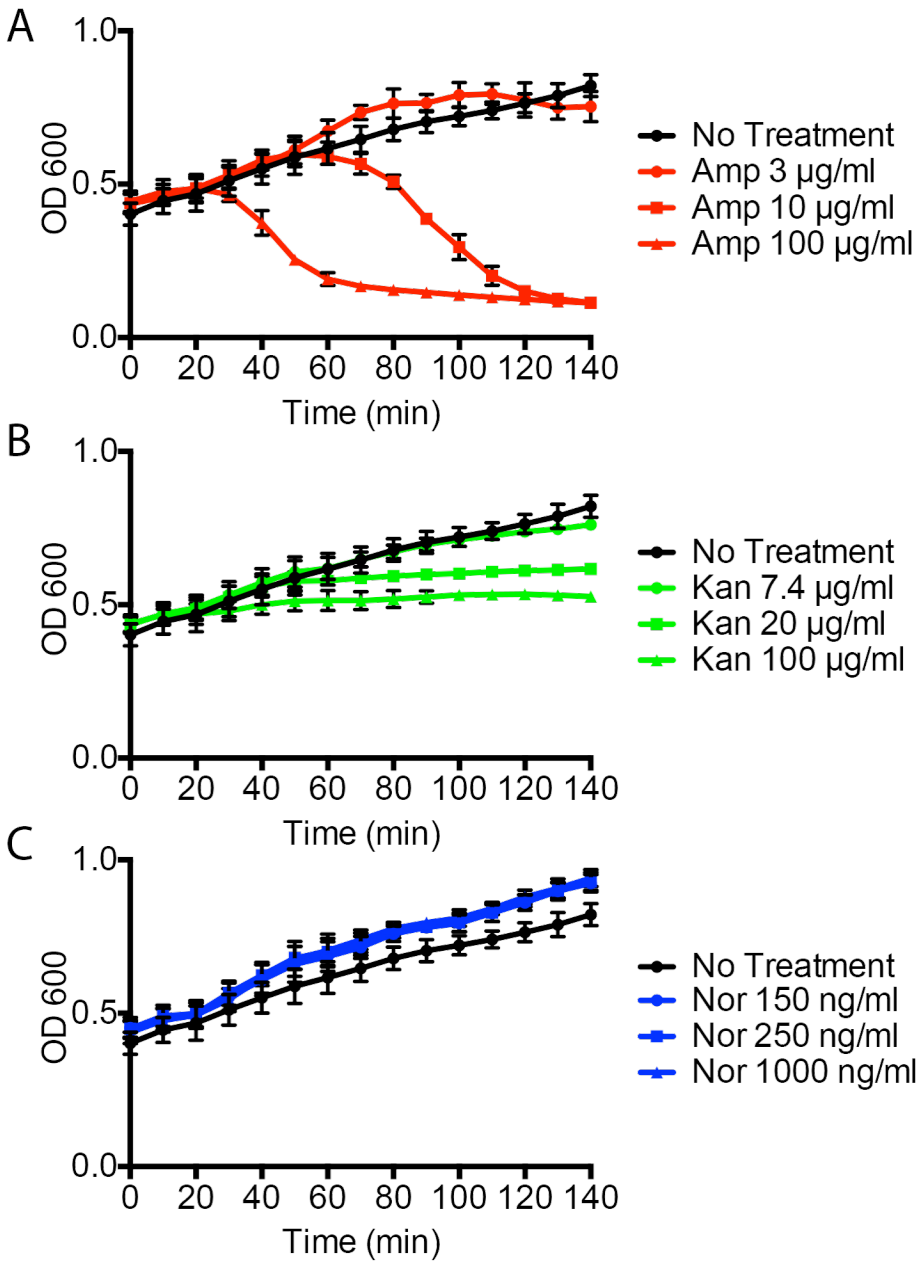

Figure S2: Selected Concentrations of Antibiotics Do Not Induce Lysis (Refers to Figure 2).
MG1655 cells at OD_{600} of ~ 0.3 were treated with antibiotics at the indicated concentration for 140 minutes. Amp induced lysis at Amp 10 and 100 µg/ml but not at 3 µg/ml. No lysis was observed with any concentrations of Kan or Nor. The curves presented above represent five independent biological replicates.

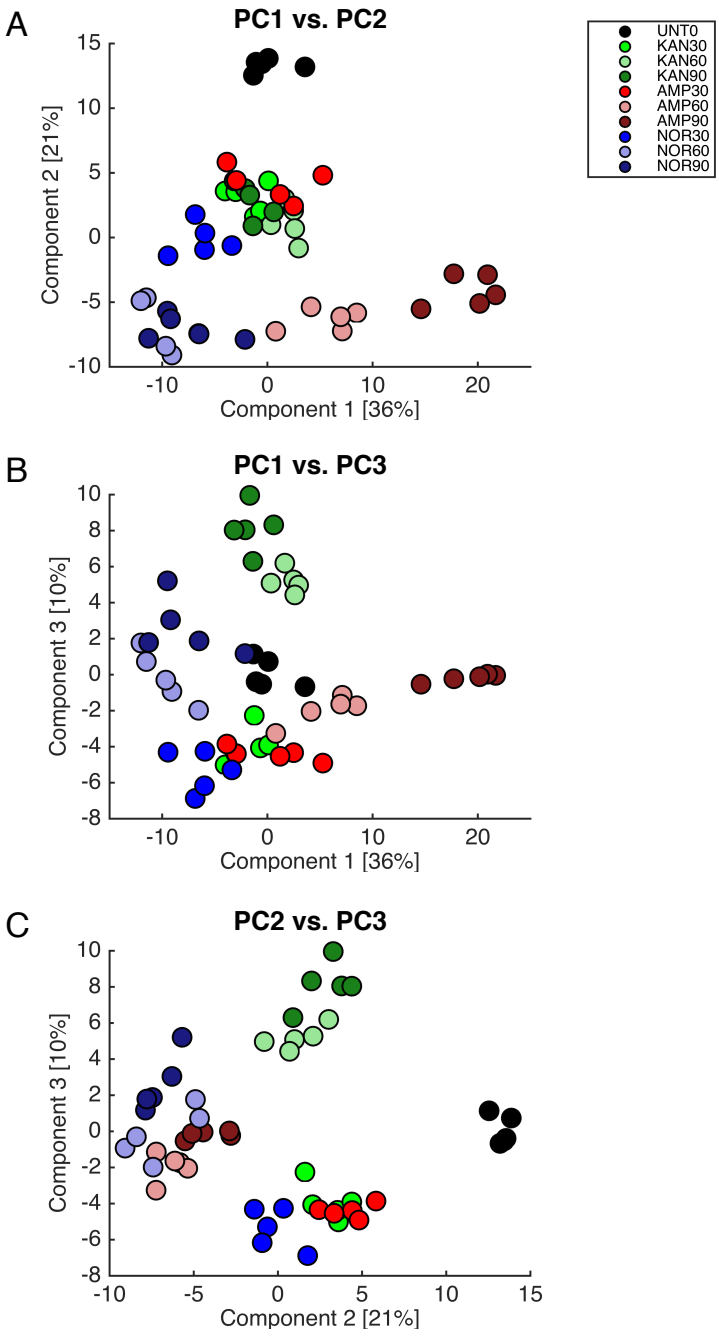

Figure S3: Principal Components Analysis of Metabolic Profiling Data
(Refers to Figure 1).
Principal components analysis was performed on the log-transformed and auto-scaled metabolomics data (all time points and treatment types) using the pca function in Matlab with the SVD algorithm.

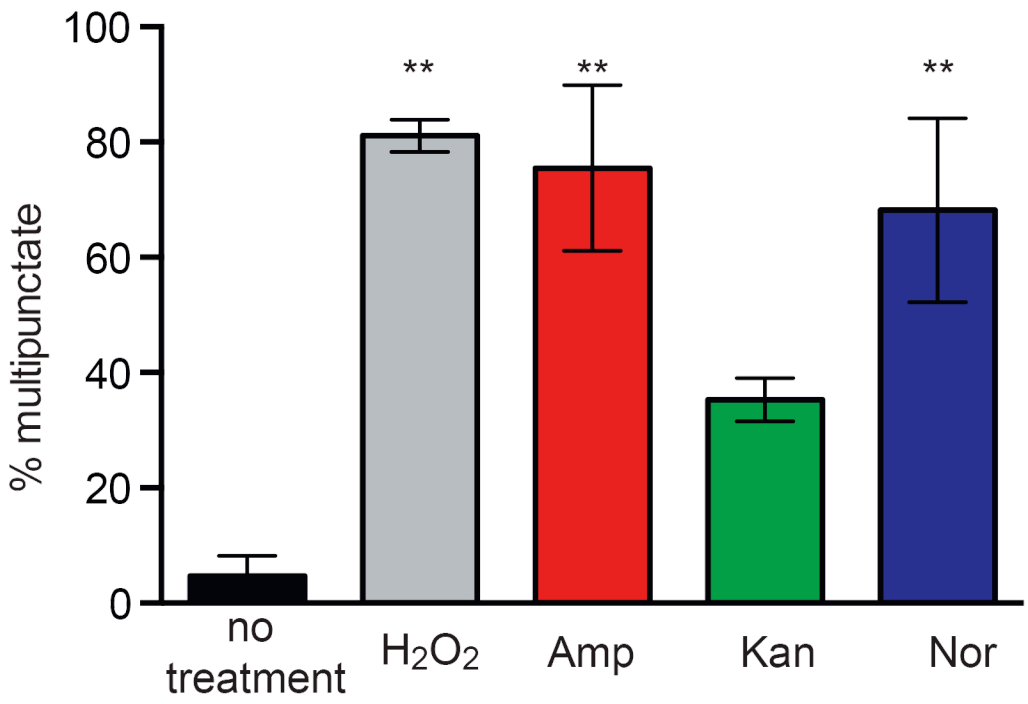

Figure S4: Bactericidal antibiotics induce double-strand breaks in E. coli (Refers to Figure 6).
Percent of cells with multiple GFP foci in the indicated treatment group. Bars represent the average of three independent experiments in which 50 to 150 cells were quantitated for each condition. Error bars represent SEM. Statistical significance of the difference between untreated control and each treatment was assessed using Sidak's multiple comparisons test: ** $p<0.01$, where p-values are adjusted to account for multiple comparisons.

Supplemental Data S1: Complete Set of Boxplots for Each Metabolite

(Refers to Figure 3).

See Supplemental Data S1