Supplementary Materials for

Killing by Bactericidal Antibiotics
Does Not Depend on Reactive Oxygen Species

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Correction: Two panels of fig. S2 were duplicated, and there was duplication of traces between figs. S2 and S6. Corrections were also made to the legend of fig. S5.
Title: Killing by Bactericidal Antibiotics Does not Depend on Reactive Oxygen Species

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Supplementary Materials:

Material and methods
Bacterial Strains and growth conditions. *E. coli* K12 strains BW25113 and MG1655 were cultured with aeration in LB broth or LB agar at 37°C. In anaerobic conditions the LB was supplemented with 100 mM potassium nitrate (Sigma-Aldrich, St. Louis, MS) unless otherwise noted. Anaerobic cultures were handled in an anaerobic chamber (Coy Laboratories, Township, MI). All reagents were equilibrated in the anaerobic chamber for at least 24 hrs.

Killing experiments. (I) Aerobic conditions. Overnight cultures were diluted 1:500 into 4.5 ml of LB broth in 30 ml inkwell bottles (Fisher Scientific, Boston, MA) and grown for 2.5 hrs at 37°C in a shaking incubator (300 rpm). 0.5 ml of either LB or LB+150 mM thiourea (Sigma-Aldrich, St. Louis, MS) was added to each inkwell. After addition of antibiotic, the cultures were returned to the shaker for the designated time. Before antibiotic addition (0) a sample was removed, serially diluted and plated on LB agar plates. The plates were incubated at 37°C, and cell survival was determined by colony count. (II) Anaerobic conditions. An overnight aerobic culture was diluted 1:1000 and cultured in the anaerobic chamber overnight. The culture was then diluted 1:500 into 4.5 ml of LB broth in inkwells (100 mM potassium nitrate was added in some experiments) which were sealed, placed in a shaking incubator (300 rpm) at 37°C for 2.5 hours. To confirm that the removed inkwells are sealed the oxygen levels were tested in the cultures that were removed from the anaerobic chamber and were found to be at the same levels as cultures that were not removed from the chamber. We used a dissolved oxygen probe with an Orion Star DO meter (Thermo Scientific, Beverly MA). For sample removal, inkwells were returned to the anaerobic chamber. Cell survival was determined by colony count in the anaerobic chamber.

Single cell analysis of Hydroxyphenyl fluorescein (HPF) fluorescence. HPF (Sigma-Aldrich, St. Louis, MS) was added at 5 µM after dilution of an overnight culture, which was grown as described for killing experiments. At designated time points samples were removed and analyzed by a FACS Aria II (BD Bioscience, San Jose, CA) cell sorter, with excitation at 488 nm and 515-520 nm emission filter. 10,000 cells were measured for each sample. The data was analyzed using FlowJo (Ashland, OR).

We calculated the shift in fluorescence as the difference between the average fluorescence of the population after treatment minus the average at time zero (before treatment, see Fig. S2). For correlating killing vs. shift (Fig. 1E) we tabulated the maximal shift registered. For norfloxacin and kanamycin the maximal shift occurred at 3 hours after addition of drug. For ampicillin the largest shift with a high concentration occurred at 30 minutes, and with a low concentration the maximal shift was at 60 minutes (at later time points the cells lyse).
Cell sorting. Overnight cultures were diluted 1:500 into 5 ml LB with 5 µM HPF and grown with shaking for 2.5 hours. Single cells were sorted onto a 384 point on LB agar to establish the fidelity of sorting. Norfloxacin was added at 0.25 µg/ml to each inkwell and the cultures were incubated for another 3 hours. Ampicillin was added at 25 µg/ml to each inkwell and the cultures were incubated for either 30 or 60 minutes. After antibiotic treatment 50 cells (norfloxacin) or 20 cells (ampicillin) were sorted onto each point of the 384 point array, for a total of 19,200 or 7680 cells respectively. Survival was estimated by the number of colonies that grew on the plate after 24 hours of incubation at 37°C.

MIC and MBC determination. MIC was determined with broth microdilution in accordance with NCCLS recommendations (21). Briefly, drugs were serially diluted in LB medium 2-fold in a 96 well plate (Costar, Corning Incorporated, Corning NY) in a final volume of 100 µl. Aerobic or anaerobic overnight cultures were diluted 1:10 into LB or LB+100 mM nitrate and cultured with aeration for 1 hour, either aerobically or anaerobically. Cells were then diluted 1:1000 and 100 µl were added to each well of a 96 well plate containing drugs. The plates were incubated overnight at 37°C. MIC was determined as the lowest concentration of an antibiotic where no visible growth was present in a well. After MIC determination, 100 µl of culture from wells corresponding to 1xMIC to 4xMIC was spread on an LB agar plate. After 24 hours, colonies were counted. MBC (Minimal Bactericidal Concentration) was the lowest drug concentration that killed ≥99.9% of the cells.
Fig. S1. Killing by ofloxacin, ampicillin and kanamycin with and without thiourea. E. coli BW25113 was grown to mid-exponential phase and treated with antibiotics for three hours. After treatment a sample was washed, diluted and plated for colony count. A. Ofloxacin (blue, no thiourea; red, with thiourea). B. Ampicillin (blue, no thiourea; red, with thiourea). C. Kanamycin (blue, no thiourea; red, with thiourea). Plasma concentrations of ampicillin can reach 8.2 $\mu$g/ml with oral administration and 67.7-149.9 $\mu$g/ml with intravenous administration. Ofloxacin plasma concentrations range between 1.33-5.64 $\mu$g/ml depending on the dose. Kanamycin plasma concentrations are between 20-25 $\mu$g/ml(24).
**Fig. S2. Quenching of HPF fluorescence by thiourea.** *E. coli* BW25113 were cultured to mid-exponential phase and treated with norfloxacin for three hours. After treatment a sample was analyzed for single cell HPF fluorescence with a FACS Aria II flow cytometer. A. The fluorescence histograms of the samples before (0) and after antibiotic treatment. Each column represents an independent biological experiment. TU indicates thiourea added to the culture at 150 mM at time zero. B. The shift in fluorescence was calculated as the difference between the average fluorescence of the sample before and after antibiotic treatment.
Fig. S3. Killing and HPF fluorescence in *E. coli* MG1655. *E. coli* was cultured to mid-exponential phase and treated with norfloxacin for three hours. One sample was washed, diluted and plated for colony count, and another one was analyzed for single cell HPF fluorescence. A. Colony count before (0) and after antibiotic treatment (blue, no thiourea; red, with thiourea). Thiourea was added to the culture at 150 mM. B. Example of fluorescence levels after three hours of treatment with different concentrations of norfloxacin. C. The maximal fluorescence signal is observed at low concentrations of norfloxacin.
Fig. S4. Sorting of high and low ROS populations after ampicillin treatment. *E. coli* BW25113 were cultured to mid-exponential phase and treated with 25 µg/ml of ampicillin for either 30 or 60 minutes. Before antibiotic treatment 384 cells were sorted, 1 cell/spot. After antibiotic treatment 7680 cells were sorted, 20 cells/spot. The surviving cells were counted after 24 hrs of incubation at 37°C. Low ROS fraction (blue bars) and high ROS fraction (red bars).
Fig. S5. The effect of thiourea on killing by antibiotics under anaerobic conditions. *E. coli* BW25113 was cultured anaerobically overnight, diluted 1:500, grown anaerobically to mid-exponential phase, and treated with antibiotics for three hours. After treatment a sample was washed, diluted and plated for colony count. Thiourea was added at 150 mM where indicated together with antibiotic. A. Ofloxacin (blue, no thiourea; red, with thiourea). B. Ampicillin (blue, no thiourea; red, with thiourea). C. Kanamycin (blue, no thiourea; red, with thiourea).
**Fig. S6. HPF fluorescence under anaerobic conditions.** *E. coli* BW25113 was cultured anaerobically overnight. The overnight cultures were diluted 1:500, grown anaerobically to mid-exponential phase and treated with norfloxacin for three hours. TU, thiourea added at 150 mM together with antibiotic. Single cell fluorescence was determined using a FACS Aria II flow cytometer. Each column represents an independent biological experiment.
References


