### **Supplemental Information for**

# Antibiotics induce redox-related physiological alterations as part of their lethality

Daniel J. Dwyer<sup>a,b,1</sup>, Peter Belenky<sup>a,b,1</sup>, Jason H. Yang<sup>a,b,1</sup>, I. Cody MacDonald<sup>a,b</sup> Jeffrey D. Martell<sup>c</sup>, Noriko Takahashi<sup>d</sup>, Clement T. Y. Chan<sup>a,b</sup>, Michael A. Lobritz<sup>a,b,e,f</sup> Dana Braff<sup>a,b</sup>, Eric G. Schwarz<sup>a,b</sup>, Jonathan D. Ye<sup>a,b</sup>, Mekhala Pati<sup>g</sup>, Maarten Vercruysse<sup>d</sup>, Paul S. Ralifo<sup>g</sup>, Kyle R. Allison<sup>h</sup>, Ahmad S. Khalil<sup>b</sup>, Alice Y. Ting<sup>c</sup>, Graham C. Walker<sup>d</sup>, and James J. Collins<sup>a,b,e,i,\*</sup>

- a Howard Hughes Medical Institute
- b Department of Biomedical Engineering and Center of Synthetic Biology, Boston University, 36 Cummington Mall, Boston, MA 02215
- c Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139
- d Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139
- e Wyss Institute for Biologically Inspired Engineering, Harvard University, 3 Blackfan Circle, Boston, MA 02115
- f Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit St., Boston, MA 02114
- g Department of Chemistry, Boston University, 24 Cummington Mall, Boston, MA 02215
- h Department of Systems Biology, Columbia University, 1130 St. Nicholas Ave., New York, NY 10032
- i Department of Medicine, Boston University School of Medicine, 72 E. Concord St., Boston, MA 02118
- 1 denotes equal contributions

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#### **Supplemental Materials and Methods**

#### Cell strains and knockout strain construction

In the present study, we compared the physiological changes associated with treatment of wildtype MG1655 *E. coli* (ATCC# 700926) with ampicillin (Amp), gentamicin (Gent) or norfloxacin (Norf) to untreated cells. Where indicated, we also studied the effects of chloramphenicol (Chlor), kanamycin (Kan), meropenem (Mero), ceftriaxone (Ceft) or moxifloxacin (Moxi) treatment. All antibiotics were obtained from Sigma and Acros Organics.

In addition to wildtype cells, we also studied the physiological changes associated with the treatment of quinolone-resistant gyrA17 cells (obtained from the *E. coli* Genetic Stock Center, CGSC# 4366), previously described MG1655 $\Delta fur$  (1) and the Hpx- MG1655 mutant (*ahpCF::kanR*, *katG*, *katE*) constructed for this study. Briefly, mutant alleles (*katG::kanR*, *katE::kanR*) were successively transferred via P1 phage transduction from the KEIO *E. coli* single-gene knockout library (2). The *ahpCF::kanR* allele was introduced by the Datsenko and Wanner method (3), involving the Lambda Red recombinase. Prior to each successive round of mutagenesis, the *kanR* gene was eliminated using the pCP20 plasmid encoded Flp recombinase. Removal was confirmed by colony PCR.

For experiments involving controlled, plasmid-based protein expression, we utilized the previously described MG1655pro strain (1). The "pro" cassette (also Z1), which encodes single copies of the SpecR (for selection) and *lacIq* genes, and tandem copies of the *tetR* gene, is chromosomally inserted at the *attB* site (4). The MG1655pro strain affords exceptionally tight transcriptional regulation and tunable expression of the gene of interest.

#### **Plasmid construction**

All plasmids were transformed into MG1655 or MG1655pro cells using standard protocols from molecular biology. Most plasmids used in this study were constructed using the pZE21-mcs1 plasmid backbone (4). This high-copy plasmid encodes the *kanR* gene for selection, and the TetR-regulated pL(tetO) promoter to control transcription of the gene of interest. The APX reporter gene (5), as well as respective native and mutant alleles of *katG* and *ahpCF*, were cloned into pZE21-mcs1, under the transcriptional control of the pL(tetO) promoter. The *katG* and *ahpCF* genes were PCR amplified from MG1655. Mutant alleles of *katG* (H106Y) and *ahpF* (C348S) were constructed by standard site-directed mutagenesis (Q5 site-directed mutagenesis kit [NEB]) of the pZE21-*katG* and *-ahpCF* plasmids, respectively, and verified by sequencing.

For green fluorescent protein (GFP, *gfpmut3B*) promoter-reporter experiments, we used the previously described pSoxS-*gfp* and pL(FurO)-*gfp* reporters (1), and built pOxyS-*gfp*, pHemH-*gfp*, pTrxC-*gfp* and pL(MetO)-*gfp* for this study. Briefly, the pOxyS, pHemH, pTrxC promoters were synthesized as gBlock gene fragments (IDT) based on sequences found on EcoCyc. The pL(MetO) engineered promoter design was based on the design principles of pL(TetO) and pL(FurO), and also synthesized as a gBlock gene fragment. Using previously described pZE21-*gfp* as a plasmid backbone, promoter gBlock fragments were cloned in place of the pL(TetO) promoter, upstream of the *gfp* gene.

The pCA24N::MutS plasmid for MutS dosage studies was obtained from the ASKA overexpression plasmid library (6). Mutant alleles of *mutS* (F36A, K620A) were constructed by standard site-directed mutagenesis of the pCA24N::MutS plasmid, and verified by sequencing.

#### Fluorescent dye-based ROS detection

Overnight cultures of MG1655 or quinolone-resistant gyrA17 cells were inoculated in LB, and grown at 37°C and 300 RPM in a humidity-controlled incubator shaker. On the day of each experiment, overnight cultures were diluted 1:500 in 100 mL LB, then distributed in 200  $\mu$ L aliquots to each 2 mL well of a 96-well, deep-well culture plate containing either 10  $\mu$ M of each fluorescent dye tested (carboxy-H<sub>2</sub>DCFDA [mixed isomers], chloromethal-H<sub>2</sub>DCFDA [mixed

isomers], DAF-FM diacetate, H<sub>2</sub>DCFDA, HPF, OxyBURST Green, and PF2. PF2 was a generous gift of the Chang Lab at UC Berkeley; all other dyes were from Life Technologies) or an equivalent volume of dH<sub>2</sub>O (our no dye control). 96-well plates were grown at 37°C and 900 RPM in a light-protected, humidity-controlled incubator shaker outfitted for microplate experiments (Multitron II, ATR). Cultures were grown to an optical density (OD<sub>600</sub>) of ~0.2 before antibiotic treatment. OD<sub>600</sub> measurements were made using a SpectraMax M5 Microplate Reader spectrophotometer (Molecular Devices). We assayed for the ability of 5  $\mu$ g/mL Amp, 5  $\mu$ g/mL Gent or 250 ng/mL Nor to induce reactive species-dependent fluorescent changes in MG1655 cells compared to respective antibiotic treated, no-dye controls. We also assayed the ability of 250 ng/mL Nor to induce reactive species-dependent fluorescent changes in *gyrA17* cells compared to our norfloxacin treated, no-dye control.

At each time point, samples were taken and diluted ~100-fold in 1X PBS (pH 7.2) into a 96-well microplate for analysis using a Fortessa flow cytometer (Becton Dickinson) outfitted with a microplate autosampler. Mean GFP fluorescence (FL1-A) was quantified using the following PMT voltages: FSC 500, SSC 250, FL1-A 500. Acquisition was performed at the lowest flow rate (~2  $\mu$ L/s), with thresholding on FSC at a value of 500.

Statistical significance was calculated for each set of technical replicate measurements using one-way ANOVA, making comparisons to the no-dye, autofluorescence control included in each replicate. Measurements were deemed statistically significant when the corresponding p-values were  $\leq 0.05$ .

#### HPF fluorescence quenching

Hydroxyl radicals were generated *in vitro* by addition of  $200 \ \mu M H_2O_2$  and  $100 \ \mu M$  iron (II) sulfate. HPF was added to the *in vitro* reaction after 30 s, at a final concentration of  $10 \ \mu M$ . Thiourea was added at the specified concentrations 60 s after HPF addition. The fluorescent signal was monitored using a SpectraMax M5 Microplate Reader spectrophotometer, using the 490/515 filter combination.

#### Fenton reaction quenching by electron paramagnetic resonance (EPR)

To detect hydroxyl radical production using electron paramagnetic resonance (EPR), we employed the radical spin trap, 5, 5-dimethylpyrroline-N-oxide (DMPO, Sigma). Hydroxyl radicals were generated *in vitro*, via the Fenton reaction, with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> and 100  $\mu$ M iron (II) sulfate (Fisher). DMPO (in dH<sub>2</sub>O) was added to the *in vitro* reaction after 60 s, at a final concentration of 100 mM.

To characterize the relative hydroxyl radical scavenging capacity of ethanol or thiourea, specified concentrations of these scavengers were added to the reactions 30 s prior to the addition of DMPO. EPR spectra were collected at room temperature using a flat-cell in Bruker X-band ELEXSYS E500 spectrometer. The experiments were made at 9.8 GHz (X-band), 20 mW microwave power, and 1 Gauss modulation amplitude.

#### Enzymatic intracellular H<sub>2</sub>O<sub>2</sub> measurement

For all experiments, MG1655pro cells with the pZE21-APX plasmid were grown in LB, at 37°C and 300 RPM in a humidity-controlled incubator shaker. Overnight cultures were inoculated in LB with Kan (30  $\mu$ g/mL) and spectinomycin (Spec, 10  $\mu$ g/mL) for plasmid and strain selection, respectively. On the day of each experiment, overnight cultures were diluted 1:500 in 10 mL LB, in 250 mL baffled flasks, with Kan (30  $\mu$ g/mL) for plasmid selection. Cultures were grown to an optical density (OD<sub>600</sub>) of ~0.2 before addition of anhydrotetracycline (aTc, 30 ng/mL) to induce APX expression. Cultures were then grown to an OD<sub>600</sub> of ~1.2 before dilution back down to an OD<sub>600</sub> of 0.3 in LB without additional selection or inducer. 2 mL aliquots of diluted culture were then dispensed into 14 mL polypropylene tubes (one tube/time

point) containing the appropriate perturbation. We assayed for  $H_2O_2$  generation in untreated cells compared to cells treated with 5 µg/mL Amp, 5 µg/mL Gent, 250 ng/mL Nor, or 10 µg/mL Chlor.

At each time point, 1 mL from each respective treatment was centrifuged at 7,000 RPM for 1 min in a benchtop microcentrifuge, then resuspended in 100  $\mu$ L of either LB or 1X PBS (pH 7.2, Fisher) containing 50  $\mu$ M Amplex UltraRed (Life Technologies). Samples were transferred to black, opaque-bottomed 96-well microplates and centrifuged at 3,000 RPM for 2 min in a benchtop swinging bucket centrifuge (Heraeus Multifuge X1R, Thermo). For H<sub>2</sub>O<sub>2</sub> spike-in experiments, untreated cells were aliquoted into wells containing the appropriate concentration of H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> dilutions were made in dH<sub>2</sub>O from a stabilized 10 M stock solution (Sigma) fresh immediately before addition. In order to quantify Amplex UltraRed fluorescence in high-throughput under peak excitation and emission, samples were measured using the SpectraMAX M5 Microplate Reader with the 568/591 filter set, with readings taken every 2 min for 30 min.

Statistical significance was calculated for each set of technical replicate measurements at 1 hr and 2 hrs using a one-way ANOVA, making comparisons to the untreated control. Measurements were deemed statistically significant when the corresponding p-values were  $\leq 0.05$ .

#### APX H<sub>2</sub>O<sub>2</sub> specificity

The W41F mutant of pea ascorbate peroxidase (APX) was expressed and purified from the BL21-DE3 strain of *E. coli* as described previously (5). Horseradish peroxidase, catalase, and cytochrome c were purchased from Sigma Aldrich as lyophilized powders and dissolved in 1X PBS (pH 7.5). HRP and cytochrome c concentrations were determined using  $\varepsilon_{405} = 100,000 \text{ M}^{-1} \cdot \text{ cm}^{-1}$  and  $\varepsilon_{550} = 10,000 \text{ M}^{-1} \cdot \text{ cm}^{-1}$ , respectively. Catalase was prepared as a 10 mg/mL solution. Iron (II) perchlorate hydrate and potassium superoxide were purchased from Sigma Aldrich. H<sub>2</sub>O<sub>2</sub> was purchased from EMD Millipore and determined using  $\varepsilon_{240} = 43.6 \text{ M}^{-1} \cdot \text{ cm}^{-1}$ . Amplex UltraRed was purchased from Life Technologies. Fluorescence measurements were performed in 96-well flat-bottom transparent plates on a Tecan Safire plate reader. Absorbance measurements for the cytochrome c reduction assay were performed on a Nanodrop 2000c UV-vis spectrophotometer (Thermo Scientific) using its cuvette reader.

To prepare superoxide stock solutions, potassium superoxide (7.7 mg) was mixed with anhydrous dimethyl sulfoxide (DMSO) from a freshly opened, single-use ampule (800  $\mu$ L) in a 1.5 mL Eppendorf tube. The resulting mixture was sonicated for 10 min, then briefly centrifuged to form a pellet of undissolved potassium superoxide. The supernatant was diluted 8-fold in anhydrous DMSO. The resulting solution was immediately utilized as a 100X stock in the Amplex UltraRed and cytochrome c reduction assays.

To estimate the superoxide concentration in these stocks, 400  $\mu$ L of oxidized cytochrome c (25  $\mu$ M) was added to a plastic cuvette and absorbance was determined against a 1X PBS blank. 4  $\mu$ L of 100X superoxide stock was added and quickly mixed using a pipette. The reduction of oxidized cytochrome c by superoxide was monitored using  $\epsilon_{550} = 19,500 \text{ M}^{-1} \cdot \text{cm}^{-1}$  for reduced cytochrome c - oxidized cytochrome c (7). Since superoxide rapidly dismutates in aqueous solutions, the concentration of reduced cytochrome c in this experiment represents a lower bound for the concentration of superoxide that was added initially. Absorbance at 550 nm was monitored until it stopped increasing, which was less than 10 s after the addition of superoxide. For catalase co-incubations, the same procedure was followed except that 4  $\mu$ L of 10 mg/mL catalase was added prior to superoxide addition. A<sub>550</sub> increased slightly upon addition of catalase. Separate measurements confirmed that this change was due to inherent absorbance of catalase at 550 nm, not from a catalase-induced change in the absorbance of cytochrome c. Following this protocol, the 100X stocks were determined to contain > 100  $\mu$ M superoxide.

To read the Amplex UltraRed samples, the various additives (APX, HRP, catalase,  $H_2O_2$ , superoxide, iron (II) perchlorate) were delivered as distinct 1  $\mu$ L drops of 100X stock solutions to

the bottom of a single well of a transparent, flat-bottom 96-well plate. The drops were segregated to prevent mixing. A freshly prepared 50  $\mu$ M solution of Amplex UltraRed in 1X PBS (pH 7.5), with 0.5% DMSO as a co-solvent, was added to each well to initiate each reaction. The final reaction volume in each well was 100  $\mu$ L. Amplex UltraRed was protected from light exposure as much as possible to avoid photooxidation (8). Reactions were allowed to proceed for 20 min at room temperature in the dark. End-point fluorescence measurements were recorded on a Tecan Safire plate reader using a 568/589 filter combination.

#### **GFP** promoter-reporter fusions

Overnight cultures of MG1655 cells with the pZE2-pOxyS-*gfp*, pSoxS-*gfp*, pL(FurO)*gfp*, pHemH-*gfp*, pTrxC-*gfp* or pL(MetO)-*gfp* reporter plasmids were inoculated in LB with Kan (30 µg/mL) selection, and grown at 37°C and 300 RPM in a humidity-controlled incubator shaker. On the day of each experiment, overnight cultures were diluted 1:200 in 25 mL LB, in 250 mL baffled flasks, with Kan selection. Cultures were grown to an OD<sub>600</sub> of ~0.3 before transfer in 500 µL aliquots to wells of a 24-well microplate containing appropriate treatments. 24well plates were grown at 37°C and 900 RPM in a humidity-controlled incubator shaker outfitted for microplate experiments. We assayed for the effect of treatment with 5 µg/mL Amp or 250 ng/mL Nor on GFP reporter expression from the promoter-reporter constructs listed above in comparison to untreated cells. We also assayed specifically for the effect of H<sub>2</sub>O<sub>2</sub> dose response treatment on GFP reporter expression from the pOxyS-*gfp* construct. H<sub>2</sub>O<sub>2</sub> dilutions were made in dH<sub>2</sub>O from a stabilized 10 M stock solution (Sigma) fresh immediately before addition.

After treatment for 90 min, samples were taken and diluted ~100-fold in 1X PBS (pH 7.2) for analysis using a FACS Aria II flow cytometer. Mean GFP fluorescence (FL1-A) was quantified using the following PMT voltages: FSC 200, SSC 200, FL1-A 325. Acquisition was performed at a low flow rate (~30 events/s), with thresholding on FSC at a value of 1000.

Statistical significance was calculated for each set of technical replicate measurements using a Student's t-test, making comparisons to the untreated control. Measurements were deemed statistically significant when the corresponding p-values were  $\leq 0.05$ .

#### Genome-wide microarrays

For all experiments, MG1655, MG1655 $\Delta fur$ , and MG1655 Hpx- (*ahpCF::kanR*, *katG*, *katE*) were grown in LB, at 37°C and 300 RPM in a humidity-controlled incubator shaker. Overnight cultures of MG1655 and MG1655 $\Delta fur$  were inoculated in LB, while MG1655 Hpxwas inoculated in LB with Kan (30 µg/mL) selection. On the day of experiment, overnight cultures were diluted 1:500 in 10 mL LB, in 250 mL baffled flasks. Cultures were grown to an OD<sub>600</sub> of ~0.3, then aliquoted in 2 mL volumes into 14 mL polypropylene tubes (one tube/time point) containing the appropriate perturbation. We assayed for differences in mRNA concentration among: untreated MG1655 cells or MG1655 treated with 5 µg/mL Amp, 5 µg/mL Gent, 250 ng/mL Nor, 5 µg/mL Kan or 10 µM H<sub>2</sub>O<sub>2</sub>, untreated MG1655 $\Delta fur$  cells or MG1655 $\Delta fur$  treated with 5 µg/mL Gent, and untreated MG1655 Hpx- cells.

Samples for total RNA collection and genomewide microarray analysis were taken immediately before treatment (time zero) and at 1 hr post-treatment. Total RNA was obtained using the RNeasy Protect Bacteria Mini Kit (Qiagen) according to manufacturer's instructions. RNAprotect (Qiagen) was added directly to culture samples, which were then vortexed and allowed to incubate at room temperature for 5 min, before being centrifuged at 3,000 RPM for 10 min in a swinging bucket centrifuge, and stored overnight at -80°C. Total RNA was then extracted using the RNeasy kit, and samples were DNase treated using Turbo DNA-*free* (Life Technologies). RNA concentration was estimated using the NanoDrop-1000 spectrophotometer (NanoDrop). cDNA preparation and hybridization to Affymetrix GeneChip *E. coli* Genome 2.0 microarrays were performed as described previously (1).

CEL files for the resulting expression profiles were background adjusted and normalized using Robust Multi-array Averaging (RMA) (9). Statistical significance was computed using Welch's t-test. Triplicate measurements from MG1655 samples treated with H<sub>2</sub>O<sub>2</sub>, Amp, Gent, Nor or Kan were compared with the triplicate untreated samples. Triplicate measurements from untreated MG1655 $\Delta fur$  and MG1655 Hpx- samples were also compared with untreated MG1655 samples. Triplicate measurements from MG1655 $\Delta fur$  samples treated with Gent were compared with untreated MG1655 $\Delta fur$  samples. For each set of comparisons, p-values were corrected for false discovery rate (FDR) (10). Genes with FDR-corrected p-values  $\leq 0.05$  were deemed statistically significant. Regulation for genes in the OxyR and SoxRS regulons were identified as annotated in RegulonDB v8.2 (11). Microarray data collected in this study are available for download on the Gene Expression Omnibus (GEO), Accession GSE56133.

#### Hpx- survival

Overnight cultures of MG1655 Hpx- cells were inoculated in LB with 30 µg/mL Kan selection, and grown at 37°C and 300 RPM in a light-protected and humidity-controlled incubator shaker. On the day of each experiment, overnight cultures were diluted 1:500 in 10 mL LB, in 250 mL baffled flasks, with Kan (30 µg/mL) selection. Cultures were grown to an OD<sub>600</sub> of ~0.3 before transfer in 500 µL aliquots to wells of a 24-well microplate containing appropriate treatments. 24-well plates were grown at 37°C and 900 RPM in a light-protected and humidity-controlled incubator shaker outfitted for microplate experiments. We assayed for the effect of the *ahpCF::kanR katG katE* triple deletion on the survival of cells treated with 5 µg/mL Amp, 5 µg/mL Gent, or 250 ng/mL Nor in comparison to wildtype MG1655 cells.

For colony forming unit (CFU/mL) measurements, 10  $\mu$ L of culture was collected at designated time points and serially diluted in 1X PBS. 5  $\mu$ L of each dilution was plated onto square Petri dishes containing 20 mL of LB-Agar (Fisher), and each incubated at 37°C overnight. When possible, only dilutions that yielded more than 10 colonies were counted, and CFU/mL values were calculated using the formula:

$$\frac{CFU}{mL} = \frac{\# Colonies \cdot Dilution Factor}{0.005 mL}$$

Log percent survival values were then calculated using the formula:

% Survival = 
$$100 \cdot \frac{(CFU/mL)_{t=x}}{(CFU/mL)_{t=0}}$$

Measurements were taken from at least three different experiments. Mean survival and standard error of the mean (SEM) were calculated across all experiments for each treatment.

#### **Bacterial respiration**

For all experiments, starter cultures of MG1655 cells were grown in LB, at 37°C and 300 RPM in a light-protected and humidity-controlled incubator shaker. Overnight cultures were diluted 1:200 in 25 mL of M9 minimal medium, in 250 mL baffled flasks. Cultures were grown to an  $OD_{600}$  of ~0.3, then diluted to  $OD_{600}$  of 0.02 in dH<sub>2</sub>O for measurements. Bacterial respiration, expressed as oxygen consumption rates (OCR), was quantified using an XF<sup>e</sup>96 Extracellular Flux Analyzer (Seahorse Bioscience).

90  $\mu$ L of diluted cells were transferred to wells of a Poly-D-lysine (PDL)-coated XF Cell Culture Microplate (Seahorse Bioscience). To coat plate wells, 15  $\mu$ L of 1 mg/mL PDF in 100 mM Tris-HCl (pH 8.4) was added to each well, followed by overnight drying, and two dH<sub>2</sub>O rinses. Cells were attached to the PDL-coated wells by 10 min centrifugation at 4,000 RPM in a benchtop swinging bucket centrifuge. After centrifugation, the volume was raised to 180  $\mu$ L in each well by gentle addition of 90  $\mu$ L 2X M9. Poly-lysine treatment has been shown to not significantly alter respiration in *E. coli* (12).

During the measurement cycle, the XF<sup>e</sup>96 Extracellular Flux Analyzer creates an analysis chamber of ~6  $\mu$ l and thus the effective cellular OD<sub>600</sub> in this chamber once the cells are attached to the bottom is ~0.3. Basal OCR was measured for ~15 min prior to the injection of antibiotics to assure uniform cellular seeding. Cells in M9 with 10 mM glucose had and initial OCR of ~135 pmol/min.

The effects of 5  $\mu$ g/mL Amp, 5  $\mu$ g/mL Gent or 250 ng/mL Nor treatment were compared to untreated cells or cells treated with 10  $\mu$ g/mL Chlor. After the addition of drug, OCR was quantitated every 5 to 6 min for the duration of the experiment. Data acquired after the total oxygen in the well dropped below ~80 mmHg were excluded from analysis.

OCR was also normalized to the number of viable cells quantified using the LIVE/DEAD® BacLightTM Bacterial Viability and Counting Kit - for Flow Cytometry (Life Technologies), according to kit instructions. For this assay, cells were cultured and treated on a parallel XF Cell Culture Microplate and assayed at 30 and 60 min. To prepare cells for measurement, 50  $\mu$ L of cell culture was added to 250  $\mu$ L assay mix (100  $\mu$ L fluorescent beads, 30  $\mu$ L SYBR green DNA stain, and 20  $\mu$ L propidium iodide in 20 mL assay medium [M9 medium, with phosphate replaced with NaCl to preserve ionic strength]) and incubated for 15 min before counting. Measurements were made with a FACS Aria II flow cytometer (Becton Dickinson). The following PMT voltages were used: FSC 200, SSC 200, FL1-A 325, FL2-A 390. Acquisition was performed at a low flow rate (~30 events/s), with thresholding on FSC at a value of 1000.

#### **MutS** overexpression

Overnight cultures of MG1655 cells with the pCA24N::MutS, pCA24N::MutS (F36A), or pCA24N::MutS (K620A) plasmid were inoculated in LB with 20  $\mu$ g/mL Chlor selection, and grown at 37°C and 200 RPM in a humidity-controlled incubator shaker. On the day of experiment, overnight cultures were diluted to an OD<sub>600</sub> of 0.01 in 10 mL LB, in 250 mL baffled flasks, with Chlor selection and 1 mM IPTG to induce MutS expression. Cultures were grown to an OD<sub>600</sub> of ~0.1 before treatment. We assayed for the effect of increased MutS (or either F36A/K620A mutant) dosage on the survival of cells treated with 5  $\mu$ g/mL Amp, 5  $\mu$ g/mL Kan, or 250 ng/mL Nor in comparison to MG1655 cells carrying an empty vector (pCA24N) (13). Samples were taken at designated time points. CFU/mL measurements were performed and log percent survival values calculated as described above.

#### KatG overexpression

Overnight cultures of MG1655pro cells with the pZE21-*katG* (or *katG* H106Y mutant) plasmid were inoculated in LB with 30 µg/mL Kan and 10 µg/mL Spec for plasmid and strain selection, respectively, and grown at 37°C and 300 RPM in a light-protected and humidity-controlled incubator shaker. On the day of experiment, overnight cultures were diluted 1:500 in 10 mL LB, in 250 mL baffled flasks, with Kan selection and 5 ng/mL aTc to induce KatG expression. The concentration of aTc used, which did not discernably affect growth, was empirically determined. Cultures were grown to an OD<sub>600</sub> of ~0.3 before transfer in 500 µL aliquots to wells of a 24-well microplate containing appropriate treatments. 24-well plates were grown at 37°C and 900 RPM in a light-protected and humidity-controlled incubator shaker outfitted for microplate experiments. We assayed for the effect of increased KatG (or H106Y mutants) dosage on the survival of cells treated with 10 µg/mL Amp, 5 µg/mL Gent, or 125 ng/mL Nor in comparison to uninduced cells. CFU/mL measurements were performed and log percent survival values calculated as described above.

#### **AhpCF** overexpression experiments

An AhpCF mutant with an AhpF C348S substitution was generated by site-directed mutagenesis and confirmed by sequencing. Overnight cultures of MG1655pro cells with the pZE21-*ahpCF* (or *ahpF* S348C mutant) plasmid were inoculated in LB with 30  $\mu$ g/mL Kan and 10  $\mu$ g/mL Spec for plasmid and strain selection, respectively, and grown at 37°C and 300 RPM in a light-protected and humidity-controlled incubator shaker. On the day of experiment, overnight cultures were diluted 1:500 in 10 mL LB, in 250 mL baffled flasks, with Kan selection and 5 ng/mL aTc to induce AhpCF expression. The concentration of aTc used, which did not discernably affect growth, was empirically determined. Cultures were grown to an OD<sub>600</sub> of ~0.3 before transfer in 500  $\mu$ L aliquots to wells of a 24-well microplate containing appropriate treatments. 24-well plates were grown at 37°C and 900 RPM in a light-protected and humidity-controlled incubator shaker outfitted for microplate experiments. We assayed for the effect of increased AhpCF (or C348S mutants) dosage on the survival of cells treated with 10  $\mu$ g/mL Amp, 5  $\mu$ g/mL Gent, or 125 ng/mL Nor in comparison to uninduced cultures. CFU/mL measurements were performed and log percent survival values calculated as described above.

#### Antioxidant pre-treatment

To test the effects of pre-treatment with glutathione or ascorbic acid, overnight cultures of MG1655 cells were inoculated in LB and grown at 37°C and 300 RPM in a light-protected and humidity-controlled incubator shaker. On the day of experiment, overnight cultures were diluted 1:200 in 25 mL LB, in 250 mL baffled flasks. Cultures were grown to an OD<sub>600</sub> of ~0.3 before addition of L-Glutathione (Sigma) or (+)-Sodium L-ascorbate (Sigma) for 10 min pre-treatment. A 1 M stock solution of L-Glutathione in reduced form was prepared in dH<sub>2</sub>O and titrated to pH 6.8 with 1 N NaOH (Fisher). A 1 M stock solution of (+)-Sodium L-ascorbate was prepared in dH<sub>2</sub>O and titrated to pH 6.8 with 12 N HCl Optima (Fisher).

After antioxidant pre-treatment, 500  $\mu$ L aliquots were transferred to wells of a 24-well microplate containing appropriate treatments. 24-well plates were grown at 37°C and 900 RPM in a humidity-controlled incubator shaker outfitted for microplate experiments. We assayed for the effect of antioxidant pre-treatment on the survival of cells treated with 10  $\mu$ g/mL Amp, 5  $\mu$ g/mL Gent, or 250 ng/mL Nor in comparison to similarly treated cells without antioxidant addition. Samples were taken at designated time points. CFU/mL measurements were performed and log percent survival values calculated as described above.

Protection by HPF was tested by first inoculating overnight cultures of MG1655 cells in LB and growing them at 37°C and 300 RPM in a humidity-controlled incubator shaker. On the day of experiment, overnight cultures were diluted 1:250 in 3 mL LB containing HPF (0, 5, 10, 15, 20 or 25  $\mu$ M), or an equivalent volume of dimethyl formamide (DMF, Fisher, 0.1% v/v), in 14 mL culture tubes. Cultures were grown to an OD<sub>600</sub> of ~0.3 before transfer in 500  $\mu$ L aliquots to wells of a 24-well microplate containing appropriate treatments. 24-well plates were grown at 37°C and 900 RPM in a light-protected and humidity-controlled incubator shaker outfitted for microplate experiments. We assayed for the ability of HPF to serve as an antioxidant and increase the survival of cells treated with 5  $\mu$ g/mL Amp, 5  $\mu$ g/mL Gent, or 125 ng/mL Nor in comparison to similarly treated cells without HPF. Samples were taken at designated time points. CFU/mL measurements were performed and log percent survival values calculated as described above.

#### H<sub>2</sub>O<sub>2</sub> pre-treatment

Overnight cultures of MG1655 cells were inoculated in LB and grown at 37°C and 300 RPM in a light-protected and humidity-controlled incubator shaker. On the day of experiment, overnight cultures were diluted 1:250 in 25 mL LB, in 250 mL baffled flasks. Cultures were grown to an  $OD_{600}$  of ~0.3 and 4 mL were transferred into 15 mL conical centrifuge tubes with a final concentration of H<sub>2</sub>O<sub>2</sub>. Cultures were incubated for 15 min and then pelleted at 5,000 RPM in a benchtop swinging bucket centrifuge and resuspended in 4 mL of fresh LB.

After  $H_2O_2$  pre-treatment, 500 µL aliquots were then transferred to wells of a 24-well microplate containing appropriate antibiotic treatments. 24-well plates were grown at 37°C and 900 RPM in a light-protected and humidity-controlled incubator shaker outfitted for microplate experiments. We assayed for the effect of  $H_2O_2$  pre-treatment on the survival of cells treated with ampicillin, gentamicin and norfloxacin in comparison to similarly treated cells without  $H_2O_2$  pre-treatment. Samples were taken at designated time points. CFU/mL measurements were performed and log percent survival values calculated as described above.

#### Iron homeostasis using EPR

Overnight cultures of MG1655 and MG1655 $\Delta fur$  cells were inoculated in Neidhardt minimal medium and grown at 37°C and 300 RPM in a light-protected and humidity-controlled incubator shaker. On the day of experiment, overnight cultures were diluted 1:500 in 20 mL Neidhardt minimal medium, in 250 mL baffled, sterile polypropylene disposable flasks. Cultures were grown to an OD<sub>600</sub> of ~0.25 before treatment with 5 µg/mL Amp, 150 ng/mL Gent or 60 µg/mL Nor. We assayed for the effect of the *fur* deletion, as well as bactericidal antibiotic treatment, on global iron homeostasis.

At 2 hrs post-treatment, the entire volume of culture was pelleted at 3,000 RPM in a benchtop swinging bucket centrifuge, washed twice with 1X PBS (pH 7.2), then resuspended in 500  $\mu$ L sterile dH<sub>2</sub>O. Samples were immediately transferred to EPR quartz tubes (Wilmad) and flash frozen with liquid nitrogen. EPR spectra were collected using a Bruker X-band ELEXSYS E500 spectrometer equipped with an ESR900 continuous flow liquid helium cryostat. The experiments were made at 9.4 GHz (X-band), 20 mW microwave power, and 10 Gauss modulation amplitude at 12 K.

#### Total acid-soluble iron estimation

Overnight cultures of MG1655 and MG1655 $\Delta fur$  cells were inoculated in Neidhardt Complete Minimal Medium for Enterobacteria (14) with glucose (MOPS EZ Rich Minimal Media, Teknova) and grown at 37°C and 300 RPM in a light-protected and humidity-controlled incubator shaker. On the day of experiment, overnight cultures were diluted 1:500 in 4 mL Neidhardt minimal medium, in 14 mL polypropylene culture tubes. Cultures were grown to an OD<sub>600</sub> of ~0.4 before sample collection. We assayed for the effect of the *fur* deletion on total iron content.

The entire volume of culture was pelleted at 3,000 RPM in a benchtop swinging bucket centrifuge, washed twice with 1X PBS (pH 7.2), then resuspended in 200  $\mu$ L dH<sub>2</sub>O. Cell concentration was determined by microscopy using this sample. 100  $\mu$ L of sample was mixed with 500  $\mu$ L 30% redistillation-purified nitric acid (Fisher), then incubated overnight at 95°C to solubilize all iron ions. In a 96-well microplate, 20  $\mu$ L of sample was mixed with 40  $\mu$ L 6.4 M ammonium acetate (Sigma), 20  $\mu$ L 0.2 M sodium ascorbate (Sigma), 20  $\mu$ L 10 mM bathophenanthroline disulfonate (BPS, Sigma), and 100  $\mu$ L of dH<sub>2</sub>O for a final volume of 200  $\mu$ L. Following a room temperature incubation for 5 min, absorbance (535 nm for the BPS-iron complex) was measured using a SpectraMAX M5 Microplate Reader. Iron content was determined by comparison to a standard curve with iron(II) sulfate at concentrations of 0, 50, 100, 200, 400 and 600  $\mu$ M.

Statistical significance was calculated for each set of triplicate measurements using a Student's t-test, making comparisons between measurements from wildtype MG1655 and MG1655 $\Delta fur$  cells. Measurements were deemed statistically significant when the corresponding p-values were  $\leq 0.05$ .

#### Strict anaerobic growth and survival, with and without nitrate

Aerobic growth and survival control assays were performed by first inoculating overnight cultures of MG1655 in Neidhardt Complete Minimal Medium for Enterobacteria (14) with glucose (MOPS EZ Rich Minimal Media, Teknova) and growing them at 37°C and 300 RPM in a humidity-controlled incubator shaker. On the day of experiment, overnight cultures were diluted 1:100 in 1 mL of Neidhardt medium, in 14 mL culture tubes. Cultures were grown to an OD<sub>600</sub> of ~0.2 before treatment. Appropriate concentrations of antibiotics were determined by approximating the MIC for each drug in Neidhardt medium by macrobroth dilution. Samples were taken at designated time points. CFU/mL measurements were performed and log percent survival values calculated as described above. When CFU/mL determination was below the limit of detection of the standard method, and counting the 10<sup>0</sup> dilution was necessary, 100  $\mu$ L of sample was collected, washed, resuspended in 100  $\mu$ L 1X PBS (pH 7.2), then plated separately. Data shown represent at least three independent experiments, with SEM depicted.

For the strict anaerobic growth and survival experiments, a Coy Type B Vinyl Anaerobic Chamber (Coy Lab Products) was used for the growth, treatment, dilution and incubation of MG1655 cells at 37°C. Twin palladium catalysts, a Coy Oxygen/Hydrogen Analyzer (Coy Lab Products) and 5% Hydrogen in Nitrogen gas mix (AirGas) were used to maintain the steady-state anaerobic environment at < 1 parts per million. Neidhardt minimal glucose medium and 1X PBS (pH 7.2) were made and sterile filtered aerobically, then transferred to the anaerobic chamber and allowed to equilibrate before use.

Overnight cultures of MG1655 cells were inoculated in Neidhardt medium, and grown aerobically overnight at 37°C and 300 RPM in a humidity-controlled incubator shaker. Overnight cultures were transferred to the anaerobic chamber, then diluted 1:100 in 1 mL of equilibrated Neidhardt medium, in 14 mL culture tubes. Cultures were grown on a platform shaker at 300 RPM to an OD<sub>600</sub> of ~0.2 before treatment (OD<sub>600</sub> samples were taken in the chamber and the 96-well sample microplate transferred out of the chamber for measurement with a SpectraMAX M5 microplate reader). At this OD<sub>600</sub>, MG1655 cells grown in Neidhardt medium were in early exponential phase for both aerobic and strict anaerobic conditions.

For CFU/mL measurements, 10  $\mu$ L of each culture was collected at designated time points and serially diluted in equilibrated 1X PBS. 5  $\mu$ L of each dilution was plated onto square Petri dishes containing 20 mL of LB-Agar (allowed to equilibrate in the chamber on the day of experiment). Spot-plated Petri dishes were placed within a GasPak EZ Standard Incubation Container (Becton Dickinson) along with a GasPak EZ Anaerobe Sachet with Indicator (Becton Dickinson), sealed and incubated within the anaerobic chamber at 37°C for 24 hrs. Log percent survival values were calculated as described above. Data shown represent at least three independent experiments, with SEM depicted. Where indicated, statistical significance was calculated for each set of triplicate measurements using a Student's t-test, making comparisons to the untreated control. Measurements were deemed statistically significant when the corresponding p-values were  $\leq 0.05$ .

#### Nitrate supplementation

To test the effect of nitrate supplementation on the survival of antibiotic-treated cultures grown under strict anaerobic conditions, potassium nitrate (KNO<sub>3</sub>, Fisher) was used as an adjuvant. This design was based on recently published methods that used LB, which naturally contain ~10 mM nitrate, as a growth medium (15, 16).

Overnight cultures of MG1655 cells were inoculated in Neidhardt medium, and grown aerobically overnight at 37°C and 300 RPM in a humidity-controlled incubator shaker. Overnight cultures were transferred to the anaerobic chamber, then diluted 1:100 in 1 mL of equilibrated Neidhardt medium, in 14 mL culture tubes. Cultures were grown at 300 RPM to an  $OD_{600}$  of ~0.2 before addition of up to 10 mM KNO<sub>3</sub> and the appropriate antibiotic. This concentration of KNO<sub>3</sub>

was determined to not have a discernable effect on growth. CFU/mL measurements were performed and log percent survival values calculated as described above.

#### **Supplemental Results**

#### Fenton reaction-mediated HPF fluorescence is irreversible

Among the dyes assessed in our high-throughput ROS detection assay was 3'-(*p*-hydroxyphenyl) fluorescein (HPF). We have previously utilized HPF, which has reported *in vitro* selectivity for highly reactive species, which include hydroxyl radicals and peroxynitrite (17), to assess antibiotic-induced ROS generation in both Gram-negative and Gram-positive bacteria (1, 18). Recently, the selectivity of HPF was questioned based on *in vitro* experiments utilizing an enzymatic system to generate reactive iron-oxo (ferryl-oxo) radicals from iron coordinated by a heme cofactor (15). In this system, 20 µg/ml horseradish peroxidase (HRP) plus 100 µM H<sub>2</sub>O<sub>2</sub> could rapidly induce HPF (2 µM) fluorescence and 100 mM thiourea could suppress fluorescence when added to the reaction. The observation that thiourea reversed HPF fluorescence led to the assertion that HPF may be non-specifically activated by iron-oxo radicals.

In vitro iron-oxo radical generation has been shown to be dependent on the source of iron, and sensitive to the concentration of  $H_2O_2$  utilized (19). Recent molecular dynamics simulations predict that iron-oxo radicals may be favored products over hydroxyl radicals in *in vitro* Fenton reactions (20). We questioned whether HPF fluorescence would also be reversible in a simplified hydroxyl radical generating reaction using Fenton's reagent (iron (II) +  $H_2O_2$ ). Using iron (II) sulfate and  $H_2O_2$ , we found activation of HPF fluorescence to be irreversible (Fig. S1*A*). More specifically, thiourea blocked the HPF signal if added prior to HPF oxidation in the Fenton reaction, but was unable to quench the signal of HPF that had already been oxidized (using over 3 orders of magnitude of thiourea, 1  $\mu$ M – 5 mM).

HPF fluorescence is activated when the phenolic leaving group (which blocks fluorescence) is liberated by hydroxylation, thereby "releasing" the fluorescent fluorescein with 1:1 stoichiometry (17). This makes the reported reversibility of HPF fluorescence by thiourea surprising. Moreover,  $H_2O_2$  is required for the formation of all known HPF activating species characterized *in vitro*, indicating that antibiotic-induced HPF fluorescence implicates upstream ROS formation. The broader implication of these *in vitro* results is that HPF may be activated by several oxidizing species, which are likely to damage a range of biomolecules *in vivo*.

## Differences in thiourea and ethanol quenching of Fenton reaction-generated radicals, revealed by electron paramagnetic resonance (EPR) and the radical spin trap DMPO

This recent work further proposed that thiourea does not effectively scavenge hydroxyl radical *in vivo* and *in vitro* (15). To reach this conclusion, the study compared the ability of thiourea and ethanol to quench the HPF fluorescence induced by an *in vitro* Fenton reaction and found less efficient quenching by ethanol. In that study, thiourea and ethanol were expected to possess similar efficiencies for scavenging hydroxyl radicals based on the 1967 work of Anbar and Neta (21). However, more recent work has shown that thiourea and ethanol scavenging rates for hydroxyl radicals may actually differ by at least one order of magnitude (22).

In light of our finding that thiourea could not reverse HPF fluorescence activated by a traditional *in vitro* Fenton reaction, we sought to assess the relative scavenging capacities of thiourea and ethanol by electron paramagnetic resonance (EPR), utilizing the spin trap 5,5-Dimethyl-1-pyrroline N-oxide (DMPO). We found that adducts formed by adding DMPO to the product of an *in vitro* Fenton reaction (200  $\mu$ M H<sub>2</sub>O<sub>2</sub> and 100  $\mu$ M iron (II) sulfate) yielded a four peak EPR spectrum, characteristic of the DMPO–OH spin adduct (Fig. S1*B*). The addition of 2 mM thiourea to the reaction 30 s prior to the addition of DMPO reduced the intensity of the DMPO–OH spin adduct peak, demonstrating robust scavenging of Fenton reaction products. In stark contrast, we found that addition of ethanol 30 s prior to the addition of DMPO was up to 100 times less effective at reducing the DMPO–OH peak, indicating that ethanol is less efficient than thiourea in scavenging Fenton reaction-generated radicals.

#### In vitro analysis of APX sensitivity and selectivity for hydrogen peroxide

Response of APX to  $H_2O_2$  in vitro

Our engineered variant of ascorbate peroxidase (APX [<sup>W41F</sup>APX]) (5), gave a robust fluorescence signal in response to  $H_2O_2$  in the presence of 50µM Amplex UltraRed (Fig. S2*A*). Addition of 1 nM APX with 1 µM  $H_2O_2$  caused a ~20-fold fluorescence increase relative to Amplex UltraRed alone. Addition of catalase, an enzyme that degrades  $H_2O_2$  efficiently and specifically, abolished the fluorescence, indicating that the signal resulted from the  $H_2O_2$ dependent oxidation of Amplex UltraRed. Notably, heavy excess of catalase relative to APX (~1,000-fold) was required in order to abolish the fluorescence, underscoring the extremely fast kinetics of the reaction between APX and  $H_2O_2$  (8.0  $\cdot$  10<sup>7</sup> M<sup>-1</sup>s<sup>-1</sup>, (23)). When APX was incubated with Amplex UltraRed without addition of  $H_2O_2$ , a 6-fold fluorescence response was observed relative to Amplex alone.

Co-incubation with catalase abolished the fluorescence, indicating that trace  $H_2O_2$  was present in the Amplex solution, and APX was able to utilize even this trace  $H_2O_2$  to generate signal. APX performed similarly *in vitro* to horseradish peroxidase (HRP), an enzyme that is well established for Amplex-based detection of extracellular  $H_2O_2$  (24). End-point fluorescence measurements were utilized *in vitro* in order to avoid photooxidation artifacts.

#### Specificity of <sup>W41F</sup>APX for H<sub>2</sub>O<sub>2</sub> over superoxide

We tested whether APX could utilize superoxide in place of  $H_2O_2$  for the oxidation of Amplex UltraRed. Superoxide was generated directly by addition of potassium superoxide, and its concentration determined by the cytochrome c reduction assay (Fig. S2B). We first determined that incubation of Amplex UltraRed with > 1  $\mu$ M superoxide, a supra-physiologic concentration (22, 25, 26), caused no increase in fluorescence signal, indicating that superoxide does not react with Amplex UltraRed inside cells. Next, we co-incubated APX with superoxide in an Amplex UltraRed-containing solution. A strong fluorescence response was observed, but co-addition of catalase (to consume H<sub>2</sub>O<sub>2</sub>) abolished the signal, suggesting that the fluorescence was related to spontaneous dismutation of superoxide to  $H_2O_2(27)$ , and subsequent utilization of  $H_2O_2$  by APX for catalysis. Superoxide associates reversibly with catalase (28), raising the concern that coaddition of catalase may have removed not only  $H_2O_2$ , but also superoxide, from the solution. Importantly, a separate cytochrome c reduction assay, performed under conditions matched to the Amplex UltraRed experiment, demonstrated that superoxide was not depleted from the solution by catalase. HRP, which is considered a specific reporter for  $H_2O_2$  over superoxide (24), performed similarly to APX in our assay. Therefore, we conclude that APX cannot utilize superoxide in place of  $H_2O_2$  to generate a fluorescence signal.

#### Non-reactivity of Amplex UltraRed toward Fenton intermediates

Again, it has recently been suggested (15) that phenol-containing fluorogenic dyes may react with intermediates of the Fenton reaction, which occurs between  $H_2O_2$  and  $Fe^{2+}$  inside cells to generate the highly toxic hydroxyl radical (22). Therefore, we tested the stability of Amplex UltraRed in the presence of  $H_2O_2$  and  $Fe^{2+}$  (Fig. S2*C*). At supra-physiological levels of both  $H_2O_2$  and  $Fe^{2+}$  (10  $\mu$ M and 100  $\mu$ M, respectively, with upper physiological limits being ~0.7  $\mu$ M (29) and ~80  $\mu$ M (30)), a slight increase in fluorescence (1.8-fold) was observed. However, this signal was much lower than the ~20-fold increase observed in the presence of 1  $\mu$ M  $H_2O_2$  and 1 nM APX. We conclude that intracellular reactions between Fenton intermediates and Amplex UltraRed are unlikely to generate substantial fluorescence signal compared to the APX /  $H_2O_2$ -derived signal. In addition, we determined that APX activity was not enhanced by the presence of Fenton intermediates. On the contrary, addition of  $Fe^{2+}$  in the presence of APX and  $H_2O_2$  decreased the fluorescence signal, most likely a result of  $Fe^{2+}$  competing with APX for  $H_2O_2$  binding.

We note that 0.5% DMSO (70 mM) was present in our experiments as a co-solvent for Amplex UltraRed. Since 75 mM DMSO has previously been used to quench hydroxyl radicals (31), it is likely that the hydroxyl radicals generated in our Fenton reactions were quenched before they could react with Amplex UltraRed. Therefore, we have not proven Amplex UltraRed to be nonreactive toward hydroxyl radicals. However, considering the extremely low steady-state concentration of hydroxyl radical and its ability to react with any biomolecule at rates approaching the diffusion limit (~10<sup>9</sup> M<sup>-1</sup>s<sup>-1</sup>) (32), it is unlikely that reactions between Amplex UltraRed and hydroxyl radicals contribute substantially to the fluorescence signal, considering that both APX and H<sub>2</sub>O<sub>2</sub> are present at considerably higher concentrations than hydroxyl radicals

#### Antibiotic treatment alters activity from OxyR, SoxR, Fur and MetJ responsive promoters

As described in the main text, we sought to determine if antibiotic-induced reactive species could trigger redox stress responses *in vivo* by using a biophysical assay. To address this, we constructed green fluorescent protein (GFP)-based promoter reporter systems that report on oxidant stress and co-regulated metabolic response activation (Figs. 2C and S3A). More specifically, we tested the activity of the native *E. coli* pOxyS, pHemH and pTrxC promoters, which are activated by the  $H_2O_2$  responsive regulator OxyR, and the native pSoxS (part of the bidirectional pSoxRS promoter), which is activated by the superoxide responsive regulator, SoxR. In addition, we tested the activity of two engineered promoters, pL(furO) and pL(metO), which are activated by derepression of the iron uptake/homeostasis gene repressor, Fur, and the methionine biosynthesis/transport gene repressor, MetJ, respectively.

In our assay, we assessed whether ampicillin or norfloxacin could induce expression from this diverse set of promoters; as noted in the main text, gentamicin was not tested because of the confounding effects of aminoglycosides on GFP reporter assays. Uniformly, we found that treatment with ampicillin or norfloxacin elicited significantly increased expression from all promoters tested. These data demonstrate that, in addition to pOxyS (Fig. 2*C*), pHemH and pTrxC activity is also increased, further indicating that bactericidal antibiotics induce activation of OxyR (Fig. S4). Additionally, our results demonstrate that bactericidal antibiotics induce derepression of Fur and MetJ. In particular, the pL(furO) results implies that iron homeostasis is perturbed by bactericidal antibiotics.

## Catalase and peroxidase knockout does not sensitize cells to antibiotics due to overwhelming compensatory responses

In *E. coli*,  $H_2O_2$  scavenging is accomplished by three enzymes: alkyl hydroperoxidase reductase (AhpCF), catalase G (KatG) and catalase E (KatE) (33). While AhpCF and KatG are strongly regulated by OxyR, KatE expression typically only occurs under stationary phase following RpoS induction. Others have previously shown that deficiencies in either KatG or AhpCF increase sensitivity to and killing by bactericidal antibiotics, including ampicillin, kanamycin and norfloxacin (34), supporting the hypothesis that ROS contribute to antibiotic lethality. As these represent the primary mechanism by which *E. coli* removes  $H_2O_2$ , one would therefore expect that an *E. coli* mutant lacking these critical ROS scavengers (Hpx-, *ahpCF katE katG*) would exhibit hypersusceptibility to bactericidal antibiotics due to ROS overload. Consistent with the genotype, anaerobically grown Hpx- mutants exhibit extreme growth defects when exposed to oxygen. These defects are associated with increased (~2 µM) steady-state  $H_2O_2$ levels (15, 35, 36). However, when this expectation was tested, antibiotic sensitivity surprisingly did not increase (15), leading to the conclusion that ROS do not contribute to lethality.

In the present study, we observed protection against treatment by bactericidal antibiotics associated with overexpression of either KatG (Fig. 5*A*) or AhpCF (Fig. S8*A*), consistent with the increase in sensitivity observed in either KatG or AhpCF deficient mutants (34). Consistent with the lack of antibiotic hypersensitivity reported by others (15), when we treated Hpx- cells with ampicillin, gentamicin or norfloxacin, we surprisingly observed significant protection (Fig. S5*A*).

To better understand how such protection in a triple deletion mutant may be reconciled with the hypersensitivity observed in single deletion mutants (34), we transcriptionally profiled exponential phase Hpx- cells and compared these profiles to those from wildtype cells.

Microarray analyses revealed substantial compensatory changes in gene expression in response to the elevated basal intracellular  $H_2O_2$  stress. First, we observed increases in basal expression of antioxidant defense and oxidative damage repair genes in Hpx- cells, including striking 10-30 fold increases in *suf* genes encoding *de novo* iron-sulfur cluster assembly (Fig. S5*B*, Table S4). Second, we also observed significantly elevated basal expression of genes in the multiple antibiotic resistant (*marRAB*) operon (Fig. S5*B*), which includes numerous transport genes. Interestingly, others have previously shown that MarRAB overexpression induces a similar antibiotic survival phenotype to what we have observed here in the Hpx- cells (37). These findings collectively highlight difficulties in interpreting negative-result phenotypes from genetic perturbations (15, 16, 38), as strong loss-of-function genotypes may provide sufficient pressure to select for strains possessing systemic compensatory changes, including changes in pathways seemingly unrelated to the genetic perturbation (39). Moreover, these findings support our overall conclusions that ROS causatively contribute to antibiotic lethality, as the Hpx- cells exhibit highly significant upregulation of oxidative damage repair genes (Fig. S5*B*).

To independently test the hypothesis that oxidative stress precondition may explain the protection observed in the Hpx- cells, we pre-treated wildtype cells with  $H_2O_2$  for 15 min and quantified killing following greatment with ampicillin, kanamycin and norfloxacin. As our microarrays from cells treated with either antibiotics or 10  $\mu$ M  $H_2O_2$  did not exhibit significant broad-scale induction of oxidative stress defense mechanisms (Fig. 2*E*), we chose to pre-treat cells with  $H_2O_2$  at concentrations that could much more significantly activate oxidative stress responses. Our present data (Fig. 2*D*) and work by others (15) indicate that treatment with  $\geq$  250  $\mu$ M  $H_2O_2$  can activate OxyR by at least 60-fold. We therefore chose to pre-treat cells with 1 mM  $H_2O_2$ , expecting that pre-conditioning with such a large oxidative stress would induce protective oxidative stress defenses.

First, we pre-treated cells with 1 or 5 mM  $H_2O_2$  for 15 min and measured growth of wildtype cells following washout. We found that a short pre-treatment at these concentrations did not induce any lethality or growth inhibition (Fig. S5*C*). When we followed these pre-treatments with either ampicillin, gentamicin or norfloxacin, we observed a transient ~1-log protection from antibiotic killing (Fig. S5*D*), supporting our hypothesis that oxidative stress pre-conditioning can induce protective changes in gene expression. Such protection is sensitive to the pre-conditioned stress, as increasing the pre-treatment concentration to 5 mM  $H_2O_2$  prolonged the protection against antibiotics (Fig. 5*B*). These results strongly suggest that the protection observed in Hpx-cells is most likely due to protective compensatory changes in gene expression in response to the elevated basal oxidative stress state of these cells.

Taken together, our findings broadly indicate that bactericidal antibiotics induce ROS accumulation, which trigger non-canonical oxidative stress responses. Such responses may offer some degree of protection against antibiotic lethality when sufficiently stimulated, though the present work suggests that the induction of these oxidative stress responses by bactericidal antibiotics may be small and below the threshold required for robust protection (Fig. 2, S3*A*). Indeed, future studies will be required to better understand such dual functionality in bacterial stress responses systems (40).

#### Antibiotics induce alterations in biomolecule-associated iron metabolism

If ROS production is increased by bactericidal antibiotic inhibition of target-specific processes, then alterations in iron homeostasis should be detectable due to the known interplay between ROS and iron (41-43). We observed an increase in GFP expression from the pL(furO) promoter indicating that ampicillin and norfloxacin induce derepession by the Fur iron uptake/homeostasis gene repressor protein (Fig. S4). To further test this hypothesis, we first used

a recent whole-cell electron paramagnetic resonance (EPR) spectroscopy method (44) to broadly assess the physiological impact of bactericidal antibiotics on the intracellular biomoleculeassociated iron landscape. While the common exogenous chelator-based EPR assay reports only on the abundance of "free", chelatable, unincorporated iron (15, 45), our chelation-free EPR assay also surveys ferric, non-heme iron in the cell, specifically including biomolecule-associated forms of iron, such as that found in Fe-S clusters or enzymes using iron as a prosthetic metal for catalysis. As iron in varied bound states yields different paramagnetic properties, differences in the chelator-free EPR spectra reflect global changes to iron metabolism (44, 46).

We used this chelator-free EPR assay to monitor global changes in iron homeostasis of wildtype cells resulting from antibiotic treatment. Treatment of wildtype cells with ampicillin, gentamicin or norfloxacin induced substantial changes in EPR spectra demonstrating that antibiotics induce complex changes to iron homeostasis, evident by the loss of spectral peaks in ampicillin and gentamicin treated cells and emergence of new spectral peaks in norfloxacin treated cells (Fig. S6*A*). The labile iron pool (LIP) exists during homeostasis in dynamic equilibrium to meet demands on iron metabolism while minimizing the generation of toxic ROS, and includes iron chelated by wide-ranging biomolecules (47). Redox stress alters this pool, with the net effect of increasing the levels of unregulated iron species that can be redox active (48, 49). As damaged Fe-S cluster enzymes may retain partial clusters under oxidizing conditions (still included in the LIP), and cluster repair can diminish non-liganded iron availability (30, 50), these and recent findings support the hypothesis that the biologically-relevant site of Fenton chemistry is likely to be iron-ligand complexes and not necessarily "free" iron (30, 50).

Fur is the master regulator of *E. coli* iron metabolism and behaves as a repressor when bound by ferrous iron, thus acting as a global iron sensor (51-53). To determine the effect that a *fur* deletion would have on EPR-detectable changes in iron homeostasis, we performed our chelator-free assay on untreated and antibiotic-treated  $\Delta fur$  cells. In stark contrast to our wildtype results, untreated  $\Delta fur$  cells lost nearly all EPR features, confirming that the paramagnetic species observed in wildtype cells involve iron. Moreover, antibiotic treatment of  $\Delta fur$  cells did not trigger the formation of new species as observed in wildtype cells. This led us to hypothesize that the lack of detectable EPR features in whole  $\Delta fur$  cells was related to reduced cellular iron content. Previous studies utilizing visible spectroscopy to monitor cellular iron content revealed that  $\Delta fur$  cells contain less (54, 55), or similar (30), levels of iron compared to wildtype cells, a proposed result of the *fur* deletion on iron-bearing protein concentration (55). We similarly found that untreated  $\Delta fur$  cells indeed contained reduced cellular levels of acid-soluble iron per cell relative to untreated wildtype cells (Fig. S6*C*), consistent with our EPR data.

We hypothesized that changes in expression to Fur-regulated genes might explain the reduced iron content in  $\Delta fur$  cells. To test this hypothesis, we compared steady-state mRNA levels between untreated, exponential phase wildtype and  $\Delta fur$  cells (Fig. S7A, Table S5). Recent work has demonstrated that stress-induced iron export, mediated by the TCA cycle intermediate and natural ferric iron chelator, citrate, can reduce antibiotic toxicity (56). We therefore further hypothesized that expression of requisite citrate export machinery components may be enhanced in  $\Delta fur$  cells over wildtype cells. Examining the microarray data, we found that  $\Delta fur$  cells exhibited significantly upregulated expression of genes required for iron-citrate export, supporting the notion that citrate may reduce cellular iron content during stress (56). Together, these findings suggest that decreased iron content related to Fur derepression reduces substrate availability for toxic Fenton chemistry, which may explain why  $\Delta fur$  cells exhibit decreased antibiotic killing (1, 15, 38).

#### Transcriptome analysis of cross-talk between iron regulatory and oxidative stress responses

To examine the influence of the Fur master iron regulator on antibiotic-induced alterations to other stress-related cellular processes, we first compared microarray data between untreated wildtype and  $\Delta fur$  cells. As bactericidal antibiotic killing is reduced in  $\Delta fur$  cells (1, 15,

38), we hypothesized that deletion of Fur might yield steady-state adaptive genetic responses that would protect against oxidative stress. Indeed, we observed statistically significant enhancement of *ahpCF* and *katE* expression in  $\Delta fur$  cells by microarray analysis (Fig. S7B and Table S5).

Because Fur is known to cross-regulate OxyR genes (57) and exhibits derepression following bactericidal antibiotic treatment, these results prompted us to investigate regulatory cross-talk between Fur and antibiotic-induced oxidative stress responses. We hypothesized that Fur may actively regulate oxidative stress responses by repressing oxidative stress defense and repair genes, highlighting complex overlapping feedback with iron metabolism. Revisiting the expression data from wildtype cells treated with bactericidal antibiotics and the 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> control, we observed a strong inverse correlation between *fur* expression and the expression of most genes with known cross-regulation by both Fur repressor and OxyR activator. This was best exemplified by gentamicin-treated cells, which exhibited the largest significant increase in *fur* expression and the largest significant decrease in many OxyR-activated genes.

To directly explore the magnitude of Fur regulatory cross-talk into antibiotic-induced oxidative stress responses, we performed additional microarrays comparing untreated and gentamicin-treated, wildtype and  $\Delta fur$  cells. First, we observed significant de-repression of most genes cross-regulated by Fur and OxyR in both untreated and gentamicin-treated  $\Delta fur$  cells, verifying active cross-talk and feedback between iron metabolism and oxidative stress-related genetic programs. Moreover, while gentamicin generally repressed these genes in wildtype cells, gentamicin-induced repression of these genes was absent in  $\Delta fur$  cells. These results highlight the regulatory complexity intrinsic to genetic overlap between iron metabolism and oxidative stress response programs, providing evidence that Fur can directly modify the magnitude of stress responses triggered by bactericidal antibiotics. Together, our EPR, iron content and microarray results offer insights into the observations that  $\Delta fur$  cells exhibit decreased antibiotic killing (1, 15, 38). Specifically, these data suggest that Fur deletion may reduce substrate availability for toxic Fenton chemistry by reducing intracellular iron content and that Fur cross-regulation into the oxidative stress response may reduce the overall magnitude of antioxidant buffering and stress response induction.

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### **Supplemental Figures**



Figure S1. *In vitro* comparison of Fenton reaction product scavenging by thiourea and ethanol. (A) Thiourea is unable to quench Fenton reaction-mediated HPF fluorescence *in vitro*. Addition of thiourea (1  $\mu$ M – 5 mM) to Fenton reaction (200  $\mu$ M H<sub>2</sub>O<sub>2</sub> and 100  $\mu$ M iron (II) sulfate)-induced HPF (10  $\mu$ M) did not alter HPF fluorescence. (B) EPR-based comparison of hydroxyl radical scavenging by thiourea and ethanol. Using EPR and the hydroxyl radical spin trap, DMPO, ethanol is shown to be roughly 100X less efficient than thiourea at reducing the DMPO-hydroxyl radical spin adduct. Data shown reflect mean ± SEM.



**Figure S2.** *In vitro* detection of ROS in *E. coli*. (A) Specificity determination for APX intracellular  $H_2O_2$  sensor (APX [<sup>W41F</sup>APX]). Purified APX detects  $H_2O_2$  *in vitro*, but not superoxide. A solution containing APX (1 nM) and 50 µM Amplex UltraRed produces a strong fluorescent signal in the presence of 1 µM  $H_2O_2$ . Co-incubation with catalase (10 mg/mL) abolishes this signal. Superoxide also produces signal, but not when catalase is present. APX substitution by 1 nM horseradish peroxidase (HRP) gave similar results. (B) Approximation of *in vitro* superoxide concentration derived from potassium superoxide by cytochrome c reduction. Cytochrome c reduction was not affected by the presence of catalase (10 mg/mL), indicating catalase did not remove superoxide from the solution. (C) APX and Amplex UltraRed do not give a fluorescence response to Fenton intermediates. A solution containing 50 µM Amplex UltraRed was allowed to incubate with various concentrations of  $H_2O_2$  and  $Fe^{2+}$  in the presence or absence of 1 nM APX. 0.5% DMSO (70 mM), a quencher of hydroxyl radicals, was present as a co-solvent for Amplex UltraRed under these conditions. Data shown reflect mean  $\pm$  SEM;  $n \ge 3$ .



**Figure S3.** *In vitro* detection of respiration in *E. coli*. Oxygen consumption rate (OCR) normalized by viable cell count. *E. coli* cells grown in M9 minimal media exhibit slow growth, detected with flow cytometry (*left*). When normalized to the number of viable cells, cells treated with ampicillin (Amp, 5 µg/mL), gentamicin (Gent, 5 µg/mL) or norfloxacin (Nor, 250 ng/mL) exhibit larger OCR than either untreated wildtype cells or cells treated with bacteriostatic chloramphenicol (Chlor, 10 µg/mL) (*right*). Data shown reflect mean  $\pm$  SEM; n  $\geq$  3. Where SEM is small, error bars are present but inside symbols.



**Figure S4. Bactericidal antibiotics trigger oxidative stress responses.** Bactericidal antibiotics alter the behavior of transcription factors regulating oxidative stress responses. Treatment with Amp (5 µg/mL) or Nor (250 ng/mL) induces GFP expression from promoters regulated by H<sub>2</sub>O<sub>2</sub>-sensitive OxyR [pOxyS-*gfp*; pHemH- *gfp*; pTrxC-*gfp*], superoxide-sensitive SoxR [pSoxS-*gfp*], iron-responsive Fur [pL(FurO-*gfp*)] and methionine-sensitive MetJ [pL(MetO)-*gfp*] in wildtype *E. coli*. Data shown reflect mean  $\pm$  SEM. n  $\geq$  3 technical replicates for all data points. Where SEM is small, error bars are present but inside symbols. Where appropriate, statistical significance is shown (\*: p  $\leq$  0.05; \*\*: p  $\leq$  0.01; \*\*\*: p  $\leq$  0.001).



**Figure S5. Endogenous oxidative stress responses protect against antibiotic treatment.** (A) Catalase and peroxidase deficient Hpx- (*ahpCF::kanR, katG, katE*) mutant *E. coli* exhibit protection from killing by ampicillin (Amp, 5 µg/mL), gentamicin (Gent, 5 µg/mL) or norfloxacin (Nor, 120 ng/mL). (B) Untreated Hpx- (*ahpCF, katG, katE*) mutant *E. coli* exhibit significantly enhanced expression of OxyR and MarR regulon genes, indicative of protective adaptations to elevated basal oxidative stress. (C) Pre-treatment with 1 mM or 5 mM H<sub>2</sub>O<sub>2</sub> does not inhibit growth. (D) Pre-treatment with 1 mM H<sub>2</sub>O<sub>2</sub> induces transient protection against Amp (5 µg/mL), Gent (5 µg/mL) or Nor (250 ng/mL). (E) Anaerobically grown and treated Hpx- cells do not exhibit significant protection from treatment by Amp (10 µg/mL), Gent (5 µg/mL) or Nor (450 ng/mL). Data shown reflect mean ± SEM. n ≥ 3 technical replicates for all data points. Where SEM is small, error bars are present but inside symbols. Where appropriate, statistical significance is shown (\*: p ≤ 0.05; \*\*: p ≤ 0.01; \*\*\*: p ≤ 0.001). In each instance, an untreated control was used for normalization and statistical significance determination.







Figure S7. Antibiotic-induced alterations to iron homeostasis can inhibit oxidative stress responses. (A) Untreated  $\Delta fur$  cells exhibit compensatory increases to gene expression of ferric citrate transporter subunits over wildtype cells, as detected by microarrays. (B) Untreated  $\Delta fur$ cells exhibit compensatory increases in  $H_2O_2$  scavenging genes (*ahpC*, *ahpF*, *katE*), indicating adaptive protection to oxidative stress. (C) Antibiotic-induced reactive species stimulate Fur activity. Treatment with ampicillin (Amp, 5 µg/mL) or norfloxacin (Nor, 250 ng/mL) induces GFP expression from the OxyR and SoxRS-sensitive Fur promoter (pL(FurO)-gfp). (D) OxyR genes cross-regulated by Fur are inversely correlated to *fur* expression in wildtype cells treated with bactericidal antibiotics, suggesting Fur repression of oxidative stress response genes. Microarrays were performed on cells treated with H<sub>2</sub>O<sub>2</sub> (10 µM), Amp (5 µg/mL), gentamicin (Gent, 5 µg/mL) or Nor (250 ng/mL) to quantify altered expression. (E) Deletion of fur derepresses OxyR regulated genes and abolishes antibiotic-induced alterations to oxidative stress response genes. Microarrays were performed on  $\Delta fur$  cells treated with Gent (5 µg/mL). Statistical significance was computed between untreated  $\Delta fur$  and wildtype cells, as well for untreated and Gent-treated wildtype and  $\Delta fur$  cells. Data shown reflect mean  $\pm$  SEM: n > 3. Where appropriate, statistical significance is shown (\*:  $p \le 0.05$ ; \*\*:  $p \le 0.01$ ; \*\*\*:  $p \le 0.001$ ).





(A) Relationship between increased dosage of the hydroperoxidase, AhpCF, and antibiotic killing by ampicillin (Amp, 10 µg/mL), gentamicin (Gent, 5 µg/mL) or norfloxacin (Nor, 125 ng/mL). Overexpression of a catalytically-inactive mutant (C348S) reduces the ability of AhpCF to suppress killing. (B) Pre-treatment with 50 mM glutathione (GSH) does not inhibit growth. (C) Pre-treatment with 1 mM or 50 mM ascorbic acid (AsA) does not inhibit growth. (D) Pre-incubation with 50 mM AsA may transiently protect against antibiotic killing by Amp (10 µg/mL). (E) Pre-incubation with the fluorescent ROS dye HPF (10 µM), affects antibiotic killing by Amp (5 µg/mL), Gent (5 µg/mL) or Nor (125 ng/mL), highlighting its ability to act as an antioxidant, particularly for those treatments that induce high signal (Fig. 1B). Data shown reflect mean  $\pm$  SEM. n  $\geq$  3 technical replicates for all data points. Where SEM is small, error bars are present but inside symbols.



Figure S9. Antibiotic killing efficacy is highly sensitive to the availability of molecular oxygen. Strict, fully anaerobic treatment and plating reduces killing by 1-4 log over fully aerobic treatment and plating. Survival upon treatment with ceftriaxone (Ceft), meropenem (Mero) or moxifloxacin (Moxi) was assessed in nitrate-free Neidhardt minimal medium. Data shown reflect mean  $\pm$  SEM.  $n \ge 3$  technical replicates for all data points. Where SEM is small, error bars are present but inside symbols.

Strain	Medium	Drug	<b>MIC</b> <sub>90</sub>
wildtype MG1655	LB	Amp	3 μg/ml
		Gent	3 µg/ml
		Kan	8 μg/ml
		Nor	160 ng/ml
		$H_2O_2$	1.25 mM
wildtype MG1655	LB + 50 mM GSH	Amp	8 μg/ml
		Gent	12.5 µg/ml
		Nor	160 ng/ml
wildtype MG1655	LB + 50 mM AsA	Amp	3 µg/ml
		Gent	12.5 μg/ml
		Nor	$25 \mu g/ml$
wildtype MG1655	$LB + 10 \mu M HPF$	Amp	6 μg/ml
51	·	Gent	12.5 μg/ml
		Nor	300 ng/ml
wildtype MG1655	M9 Minimal	Amp	2 µg/ml
51		Gent	$1 \mu g/ml$
		Nor	50  ng/ml
wildtype MG1655	Neidhardt (aerobic)	Amp	6.25 µg/ml
51		Ceft	130  ng/ml
		Gent	100 ng/ml
		Nor	80 ng/ml
		Mero	30 ng/ml
		Moxi	30 ng/ml
wildtype MG1655	Neidhardt (anaerobic)	Amp	12.5 µg/ml
J 1		Gent	100  ng/ml
		Nor	80 ng/ml
Hpx-	LB	Amp	5 μg/ml
1		Gent	$10 \mu g/ml$
		Nor	160  ng/ml
		$H_2O_2$	360 µM
MG1655pro	LB	Amp	3 µg/ml
1		Gent	$8 \mu g/ml$
		Nor	160 ng/ml
KatG	LB	Amp	3 µg/ml
		Gent	$3 \mu g/ml$
		Nor	100 ng/ml
AhpCF	LB	Amp	3 μg/ml
Ĩ		Gent	6 μg/ml
		Nor	130 ng/ml
pCA24N	LB	Amp	3 µg/ml
1		Gent	$10 \mu g/ml$
		Kan	$12 \mu g/ml$
		Nor	150 ng/ml
MutS	LB	Amp	3 μg/ml
		Gent	6 μg/ml
		Kan	12.5 μg/ml
		Nor	150 ng/ml

# Table S1 – Minimum Inhibitory Concentrations (MIC<sub>90</sub>) for strains and perturbations used in killing and protection assays

10 μ Fold		10 μM H Fold	$H_2O_2$	5 μg/ml / Fold	Атр	5 μg/ml ( Fold	Gent	5 μg/ml Fold	Kan	250 ng/ml Fold	l Nor
oxyR I	Regulon	Change	р	Change	р	Change	р	Change	р	Change	р
ahpC	b0605	0.917	0.023	1.032	0.269	1.126	0.027	1.186	0.006	1.076	0.048
ahpF	b0606	0.858	0.059	0.969	0.681	0.936	0.507	1.027	0.611	0.943	0.313
dps	b0812	0.674	0.001	0.725	0.002	1.192	0.027	0.932	0.201	0.678	0.001
dsbG	b0604	1.421	0.011	1.270	0.024	0.603	0.007	0.869	0.098	1.190	0.062
fhuF	b4367	2.016	0.130	1.725	0.184	0.258	0.022	0.906	0.778	2.183	0.105
flu	b2000	0.701	0.102	0.537	0.001	0.584	0.005	0.553	0.001	0.703	0.005
fur	b0683	1.424	0.001	1.553	0.004	1.752	0.000	1.817	0.000	1.238	0.017
gntP	b4321	0.731	0.003	0.612	0.001	4.357	0.001	1.411	0.017	0.811	0.026
gor	b3500	0.998	0.978	0.866	0.076	0.758	0.030	0.879	0.089	1.364	0.017
grxA	b0849	1.102	0.537	1.759	0.051	1.824	0.003	1.809	0.000	0.649	0.015
hcp	b0873	0.377	0.005	0.311	0.002	0.367	0.004	0.324	0.008	0.455	0.015
hcr	b0872	0.454	0.005	0.433	0.009	0.507	0.015	0.426	0.007	0.861	0.258
hemH	b0475	0.880	0.047	0.898	0.037	1.013	0.820	1.079	0.035	0.781	0.064
katG	b3942	0.601	0.004	0.594	0.003	0.646	0.020	0.637	0.010	1.291	0.041
mntH	b2392	1.253	0.059	1.353	0.036	1.958	0.003	1.677	0.005	1.277	0.051
nfsA	b0851	0.959	0.524	0.917	0.028	0.782	0.037	1.034	0.301	1.020	0.412
oxyR	b3961	0.844	0.019	0.880	0.039	1.894	0.001	1.365	0.004	0.913	0.105
oxyS	b4458	1.419	0.340	3.103	0.096	1.675	0.091	1.913	0.031	1.390	0.229
rimK	b0852	1.295	0.001	1.358	0.004	0.861	0.106	1.229	0.010	1.011	0.832
sufA	b1684	1.649	0.084	1.277	0.286	0.737	0.206	0.910	0.665	2.149	0.026
sufB	b1683	1.699	0.015	1.376	0.056	0.608	0.017	0.908	0.485	2.236	0.004
sufC	b1682	1.871	0.037	1.391	0.158	0.650	0.091	0.983	0.929	1.969	0.030
sufD	b1681	1.780	0.032	1.445	0.027	0.602	0.012	0.852	0.249	1.798	0.016
sufE	b1679	2.242	0.022	1.900	0.021	0.825	0.198	1.191	0.237	1.511	0.054
sufS	b1680	1.547	0.083	1.295	0.173	0.620	0.044	0.759	0.151	1.474	0.086
trxC	b2582	1.649	0.001	1.583	0.000	6.060	0.000	2.737	0.004	1.443	0.000
uxuA	b4322	0.698	0.036	0.608	0.000	4.084	0.000	1.584	0.007	1.392	0.000
uxuB	b4323	0.774	0.046	0.691	0.001	3.008	0.001	1.410	0.001	1.348	0.012
yaaA	b0006	1.026	0.613	0.935	0.286	1.196	0.052	1.087	0.193	1.255	0.030
ybjC	b0850	0.914	0.006	0.908	0.002	1.013	0.834	1.032	0.062	0.970	0.140
ybjN	b0853	1.274	0.053	1.460	0.001	0.985	0.801	1.056	0.175	1.404	0.008
yhjA	b3518	0.279	0.007	0.296	0.007	0.445	0.015	0.331	0.007	0.236	0.004

Table S2 – OxyR-regulated gene expression changes

Entries highlighted in red denote genes whose fold change was  $\geq 1.2X$  with  $p \leq 0.05$ . Entries highlighted in green denote genes whose fold change was  $\leq 0.8X$  with  $p \leq 0.05$ .

Table	62	CorD	6	lated	~~~~		· ····································	abaraaa
<b>I</b> able	33 -	SOXK	s-regu	lateu	gene	ехі	ression	changes
					8 .	- 1		

		10 μM H Fold	I <sub>2</sub> O <sub>2</sub>	5 μg/ml A Fold	Атр	5 μg/ml Gent Fold		5 μg/ml Kan Fold		250 ng/ml Nor Fold	
soxS R	legulon	Change	р	Change	р	Change	р	Change	р	Change	р
acnA	b1276	0.647	0.019	0.518	0.002	0.692	0.057	0.753	0.010	1.247	0.025
acrA	b0463	0.967	0.710	0.974	0.541	1.092	0.112	1.247	0.019	0.994	0.900
acrB	b0462	0.834	0.046	0.824	0.001	0.424	0.001	0.872	0.018	0.781	0.001
fldA	b0684	1.545	0.001	1.541	0.000	1.632	0.001	1.671	0.001	1.896	0.000
fldB	b2895	1.070	0.396	1.158	0.027	1.961	0.003	0.996	0.747	1.114	0.002
fpr	b3924	0.537	0.001	0.568	0.000	0.441	0.004	0.675	0.001	1.245	0.001
fumC	b1611	0.678	0.016	0.564	0.000	0.710	0.007	0.694	0.039	1.218	0.048
fur	b0683	1.424	0.001	1.553	0.004	1.752	0.000	1.817	0.000	1.238	0.017
inaA	b2237	1.284	0.026	1.591	0.008	1.553	0.006	1.682	0.002	0.785	0.032
marA	b1531	1.244	0.054	2.102	0.000	4.533	0.000	3.362	0.001	1.938	0.000
marB	b1532	1.194	0.096	2.905	0.001	7.213	0.000	4.096	0.002	2.464	0.003
marR	b1530	1.882	0.023	4.561	0.000	11.889	0.000	5.785	0.003	1.921	0.001
micF	b4439	1.870	0.189	4.448	0.001	1.646	0.019	4.897	0.000	2.237	0.032
nfo	b2159	0.894	0.068	0.898	0.069	0.807	0.137	0.983	0.725	1.634	0.000
nfsA	b0851	0.959	0.524	0.917	0.028	0.782	0.037	1.034	0.301	1.020	0.412
nfsB	b0578	0.945	0.318	1.031	0.502	0.898	0.156	1.145	0.049	0.782	0.012
pgi	b4025	0.779	0.052	0.729	0.000	0.478	0.039	0.708	0.001	0.914	0.033
poxB	b0871	0.560	0.055	0.596	0.009	0.657	0.028	0.530	0.006	2.063	0.006
pqiA	b0950	1.324	0.024	1.445	0.022	0.671	0.068	0.798	0.064	0.739	0.073
pqiB	b0951	1.240	0.003	1.183	0.006	0.458	0.005	0.733	0.019	0.655	0.009
ptsG	b1101	0.801	0.060	0.996	0.966	0.331	0.000	0.359	0.000	1.170	0.049
rfaZ	b3624	0.868	0.353	0.894	0.132	0.985	0.775	0.759	0.039	1.705	0.002
ribA	b1277	1.052	0.281	0.986	0.659	1.166	0.036	1.032	0.556	1.034	0.569
rimK	b0852	1.295	0.001	1.358	0.004	0.861	0.106	1.229	0.010	1.011	0.832
rob	b4396	0.638	0.019	0.546	0.000	1.169	0.006	0.817	0.008	0.742	0.000
sodA	b3908	1.095	0.045	1.105	0.022	0.767	0.004	1.008	0.803	1.527	0.001
soxS	b4062	0.714	0.001	1.764	0.000	8.482	0.000	4.262	0.000	3.085	0.000
tolC	b3035	0.970	0.421	0.918	0.096	0.793	0.140	1.093	0.000	1.036	0.253
waaY	b3625	0.926	0.539	0.963	0.281	1.023	0.770	0.843	0.042	1.751	0.000
ybjC	b0850	0.914	0.006	0.908	0.002	1.013	0.834	1.032	0.062	0.970	0.140
ybjN	b0853	1.274	0.053	1.460	0.001	0.985	0.801	1.056	0.175	1.404	0.008
ygiA	b3036	1.485	0.091	1.374	0.083	1.260	0.190	1.607	0.040	1.728	0.014
ygiB	b3037	0.855	0.130	0.814	0.001	1.036	0.194	0.942	0.055	0.802	0.021
ygiC	b3038	1.096	0.073	1.143	0.007	0.609	0.013	1.002	0.929	0.872	0.017
zinT	b1973	1.326	0.058	1.122	0.297	0.880	0.392	0.925	0.448	1.065	0.543
zwf	b1852	0.986	0.512	0.941	0.057	0.751	0.018	0.930	0.041	1.152	0.010

Entries highlighted in red denote genes whose fold change was  $\geq 1.2X$  with  $p \leq 0.05$ . Entries highlighted in green denote genes whose fold change was  $\leq 0.8X$  with  $p \leq 0.05$ . 

 Table S4 – Steady-state gene expression of OxyR-regulated genes in aerobically grown

 Hpx- (ahpCF::kanR, katG, katE) mutant cells

		Hpx- Unt WT Un	reated vs. treated
		Fold	
OXYR F	tegulon	Change	<u>p</u>
ahpC	60605	0.004	0.000
ahpF	60606	0.011	0.001
dps	60812	3.252	0.001
dsbG	b0604	1.132	0.210
fhur	64367	0.725	0.373
flu	62000	5.019	0.000
fur	b0683	2.535	0.000
gntP	b4321	0.770	0.010
gor	b3500	3.204	0.000
grxA	b0849	30.397	0.000
hcp	b0873	0.263	0.001
hcr	b0872	0.387	0.003
hemH	b0475	8.812	0.000
katG	b3942	0.005	0.000
mntH	b2392	11.665	0.000
nfsA	b0851	0.338	0.002
oxyR	b3961	0.654	0.002
oxyS	b4458	42.544	0.001
rimK	b0852	0.667	0.001
sufA	b1684	15.035	0.001
sufB	b1683	17.156	0.001
sufC	b1682	20.501	0.002
sufD	b1681	18.397	0.000
sufE	b1679	31.080	0.000
sufS	b1680	19.885	0.000
trxC	b2582	14.451	0.000
uxuA	b4322	0.793	0.075
uxuB	b4323	0.797	0.079
yaaA	b0006	8.739	0.000
ybjC	b0850	0.311	0.003
ybjN	b0853	1.345	0.001
yhjA	b3518	0.333	0.011

Entries highlighted in red denote genes whose fold change was  $\ge 1.2X$  with  $p \le 0.05$ . Entries highlighted in green denote genes whose fold change was  $\le 0.8X$  with  $p \le 0.05$ .

# Table S5 – Steady-state gene expression of OxyR-regulated genes in untreated and gentamicin-treated *fur* deletion mutant cells

		∆fur Untreated vs. WT Untreated		Δfur 5 μ Δfur	g/ml Gent vs. Untreated
		Fold		Fold	
oxyR	Regulon	Change	<u> </u>	Change	<u>p</u>
ahpC	b0605	1.260	0.005	1.094	0.006
ahpF	60606	1.293	0.018	0.972	0.219
dps	60812	1.612	0.001	0.765	0.002
dsbG	b0604	0.941	0.433	0.947	0.362
fhuF	b4367	5.553	0.026	1.036	0.191
flu	b2000	0.771	0.010	0.668	0.003
fur	b0683	0.004	0.000	1.085	0.779
gntP	b4321	0.680	0.002	1.330	0.005
gor	b3500	1.036	0.541	0.778	0.003
grxA	b0849	1.340	0.013	1.040	0.474
hcp	b0873	0.495	0.012	0.646	0.002
hcr	b0872	0.535	0.011	0.749	0.019
hemH	b0475	0.927	0.028	0.839	0.009
katG	b3942	0.901	0.200	0.875	0.000
mntH	b2392	4.566	0.001	1.061	0.158
nfsA	b0851	1.019	0.626	0.960	0.302
oxyR	b3961	0.840	0.024	1.109	0.006
oxyS	b4458	1.519	0.094	1.209	0.102
rimK	b0852	0.969	0.492	1.014	0.758
sufA	b1684	9.174	0.005	1.168	0.003
sufB	b1683	9.222	0.001	1.060	0.063
sufC	b1682	10.884	0.003	1.021	0.627
sufD	b1681	10.951	0.001	0.965	0.179
sufE	b1679	16.164	0.001	0.949	0.249
sufS	b1680	11.782	0.001	1.020	0.686
trxC	b2582	1.289	0.002	1.514	0.000
uxuA	b4322	0.919	0.067	1.091	0.074
uxuB	b4323	1.011	0.812	0.930	0.169
yaaA	b0006	1.019	0.721	0.956	0.478
ybjC	b0850	0.956	0.031	1.068	0.012
ybjN	b0853	0.817	0.005	1.149	0.011
yhjA	b3518	0.576	0.052	0.577	0.002

Entries highlighted in red denote genes whose fold change was  $\geq 1.2X$  with  $p \leq 0.05$ . Entries highlighted in green denote genes whose fold change was  $\leq 0.8X$  with  $p \leq 0.05$ .