

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

Daniel J. Dwyer^{a,b,c,1}, Peter A. Belenky^{a,b,c,1}, Jason H. Yang^{a,b,c,1}, I. Cody MacDonald^{a,b,c}, Jeffrey D. Martell^d, Noriko Takahashi^e, Clement T. Y. Chan^{a,b,c}, Michael A. Lobritz^{a,b,c,f,g}, Dana Braff^{a,b,c}, Eric G. Schwarz^{a,b,c}, Jonathan D. Ye^{a,b,c}, Mekhala Pati^h, Maarten Verduyck^e, Paul S. Ralifo^h, Kyle R. Allisonⁱ, Ahmad S. Khalil^{b,c,f}, Alice Y. Ting^d, Graham C. Walker^e, and James J. Collins^{a,b,c,f,j,2}

^aHoward Hughes Medical Institute, Departments of ^bBiomedical Engineering and ^cChemistry, and ^dCenter of Synthetic Biology, Boston University, Boston, MA 02215; Departments of ^eChemistry and ^fBiology, Massachusetts Institute of Technology, Cambridge, MA 02139; ^gWyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115; ^hDivision of Infectious Diseases, Massachusetts General Hospital, Boston, MA 02114; ⁱDepartment of Systems Biology, Columbia University, New York, NY 10032; and ^jDepartment of Medicine, Boston University School of Medicine, Boston, MA 02118

Edited* by Charles R. Cantor, Sequenom, Inc., San Diego, CA, and approved April 9, 2014 (received for review January 30, 2014)

Deeper understanding of antibiotic-induced physiological responses is critical to identifying means for enhancing our current antibiotic arsenal. Bactericidal antibiotics with diverse targets have been hypothesized to kill bacteria, in part by inducing production of damaging reactive species. This notion has been supported by many groups but has been challenged recently. Here we robustly test the hypothesis using biochemical, enzymatic, and biophysical assays along with genetic and phenotypic experiments. We first used a novel intracellular H₂O₂ sensor, together with a chemically diverse panel of fluorescent dyes sensitive to an array of reactive species to demonstrate that antibiotics broadly induce redox stress. Subsequent gene-expression analyses reveal that complex antibiotic-induced oxidative stress responses are distinct from canonical responses generated by supraphysiological levels of H₂O₂. We next developed a method to quantify cellular respiration dynamically and found that bactericidal antibiotics elevate oxygen consumption, indicating significant alterations to bacterial redox physiology. We further show that overexpression of catalase or DNA mismatch repair enzyme, MutS, and antioxidant pretreatment limit antibiotic lethality, indicating that reactive oxygen species causatively contribute to antibiotic killing. Critically, the killing efficacy of antibiotics was diminished under strict anaerobic conditions but could be enhanced by exposure to molecular oxygen or by the addition of alternative electron acceptors, indicating that environmental factors play a role in killing cells physiologically primed for death. This work provides direct evidence that, downstream of their target-specific interactions, bactericidal antibiotics induce complex redox alterations that contribute to cellular damage and death, thus supporting an evolving, expanded model of antibiotic lethality.

reactive oxygen species | DNA repair | mutagenesis

The increasing incidence of antibiotic-resistant infections coupled with a declining antibiotic pipeline has created a global public health threat (1–6). Therefore there is a pressing need to expand our conceptual understanding of how antibiotics act and to use insights gained from such efforts to enhance our antibiotic arsenal. It has been proposed that different classes of bactericidal antibiotics, regardless of their drug–target interactions, generate varying levels of deleterious reactive oxygen species (ROS) that contribute to cell killing (7, 8). This unanticipated notion, built upon important prior work (9–11), has been extended and supported by multiple laboratories investigating wide-ranging drug classes (e.g., β -lactams, aminoglycosides, and fluoroquinolones) and bacterial species (e.g., *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Mycobacterium tuberculosis*, *Bacillus subtilis*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Burkholderia cepacia*, *Streptococcus pneumoniae*, *Enterococcus faecalis*) using independent lines of evidence (12–39). Importantly, these ongoing efforts have served to refine aspects of the initial model and show that antibiotic-induced ROS generation is a more complex process than originally suggested, likely involving additional means of production.

The redox stress component of antibiotic lethality is hypothesized to derive from alterations to multiple core aspects of cellular physiology and stress response activation. Specifically, this component includes alterations to central metabolism, cellular respiration, and iron metabolism initiated by drug-mediated disruptions of target-specific processes and resulting cellular damage (Fig. 1A). Important support for this hypothesis can be found in pathogenic clinical isolates whose drug tolerance involves mutations in oxidative stress response and defense genes and not exclusively in drug target mutagenesis (40–48).

Recent critiques of this evolving model have misinterpreted an essential aspect of the hypothesis. Specifically, these recent studies (49–51) are predicated on the notion that ROS are the sole arbiters of antibiotic lethality, thereby implying that the model suggests that antibiotics do not kill by disrupting their well-established, target-specific processes. However, the evolving model is completely consistent with the literature indicating that bactericidal antibiotics are capable of inducing lethal cellular damage via interference with target-specific processes, ultimately

Significance

Substantial knowledge exists about how antibiotics interfere with core bacterial processes by binding to specific targets. Recently it has become appreciated that blocking these functions alters cellular redox state, and these perturbations may contribute to the lethality of antibiotics. In this work we explore whether antibiotic treatment of bacteria affects cellular oxidative stress and the role of such stress in antibiotic-mediated killing. We find that antibiotics dynamically alter cellular respiration and induce lethal levels of intracellular hydrogen peroxide. Antioxidants, including oxidative stress defense proteins, significantly reduce the killing by antibiotics, which is highly sensitive to the presence of molecular oxygen. These findings underscore the complex nature of antibiotic action and suggest practical approaches to enhancing our current antibiotic arsenal.

Author contributions: D.J.D., P.A.B., J.H.Y., I.C.M., J.D.M., N.T., C.T.Y.C., A.Y.T., G.C.W., and J.J.C. designed research; D.J.D., P.A.B., J.H.Y., I.C.M., J.D.M., N.T., C.T.Y.C., D.B., E.G.S., J.D.Y., M.P., M.V., and P.S.R. performed research; D.J.D., P.A.B., J.D.M., A.Y.T., and J.J.C. contributed new reagents/analytic tools; D.J.D., P.A.B., J.H.Y., I.C.M., J.D.M., C.T.Y.C., M.A.L., D.B., E.G.S., M.P., K.R.A., A.S.K., A.Y.T., G.C.W., and J.J.C. analyzed data; and D.J.D., P.A.B., J.H.Y., J.D.M., M.A.L., G.C.W., and J.J.C. wrote the paper.

Conflict of interest statement: J.J.C. is a scientific cofounder and Scientific Advisory Board chair of EnBiotix, Inc., a start-up focused on antibiotic development.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database (accession no. [GSE56133](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56133)).

¹D.J.D., P.A.B., and J.H.Y. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: jcollins@bu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1401876111/-DCSupplemental.

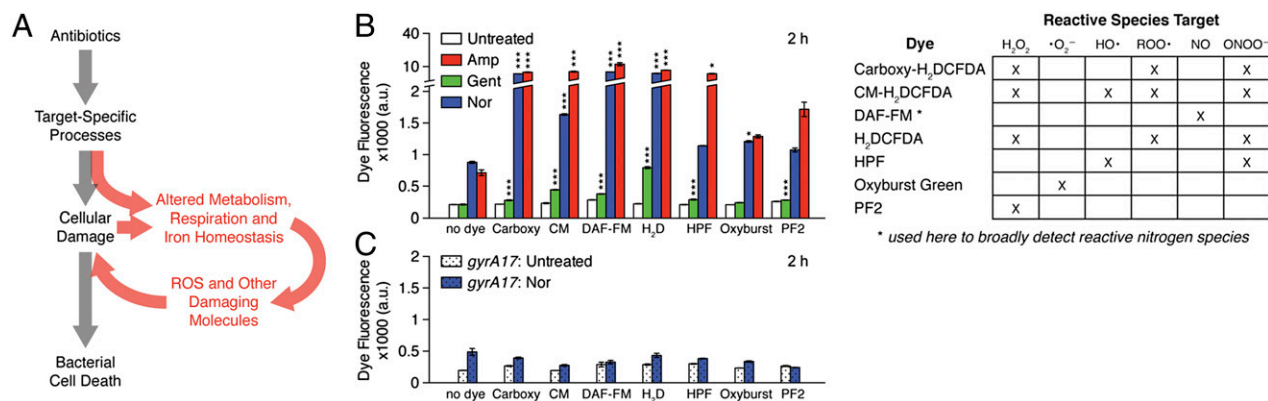


Fig. 1. Bactericidal antibiotics promote the generation of toxic reactive species. (A) Bactericidal antibiotics of different classes are capable of inducing cell death by interfering with their primary targets and corrupting target-specific processes, resulting in lethal cellular damage. Target-specific interactions trigger stress responses that induce redox-related physiological alterations resulting in the formation of toxic reactive species, including ROS, which further contribute to cellular damage and death. (B) Treatment of wild-type *E. coli* with ampicillin (Amp, 5 μ g/mL), gentamicin (Gent, 5 μ g/mL), or norfloxacin (Nor, 250 ng/mL) induces ROS, detectable by several chemically diverse fluorescent dyes with ranging specificity. One-way ANOVA was performed to determine statistical significance against the no-dye autofluorescence control. The dyes used were 5/6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H₂DCFDA); 5/6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA); 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM); 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA); 3'-(*p*-hydroxyphenyl) fluorescein (HPF); OxyBURST Green (Oxyburst); and Peroxy-Fluor 2 (PF2). (C) Treatment of a quinolone-resistant strain (*gyrA17*) with norfloxacin does not produce detectable ROS. Data shown reflect mean \pm SEM of three or more technical replicates. Where appropriate, statistical significance is shown and computed against the no-treatment control or no-dye control (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

resulting in cell death. Rather than refute this traditional view of antibiotic action, the hypothesis extends it by suggesting that an additional component of toxicity results from ROS, which are generated as a downstream physiological consequence of antibiotics interacting with their traditional targets. In this respect, reactive species are thought to contribute causatively to drug lethality.

However, an important gap exists in our general understanding of how bacteria respond physiologically to antibiotic-target interactions on a system-wide level, how these responses contribute to antibiotic killing, and how the extracellular environment protects or exacerbates the intracellular contributions to cell death. Here, we use a multidisciplinary set of biochemical, enzymatic, biophysical, and genetic assays to address these issues and expand our understanding of antibiotic-induced physiological responses and factors contributing to antibiotic lethality. Data from the present work indicate that antibiotic lethality is accompanied by ROS generation and that such reactive species causatively contribute to antibiotic lethality.

Results

Antibiotic Lethality Is Accompanied by ROS Generation. Using systems-level analyses, our previous results suggest that one consequence of the activation of the stress response and the physiological alterations induced by bactericidal antibiotics upon interference with target-specific cellular processes is the formation of an intracellular redox state conducive to the generation of deleterious reactive species (7, 8). Such reactive species, including ROS, may be generated by altered respiratory or enzymatic activity as well as by auto-oxidation or metal-catalyzed oxidation reactions. We previously have used 3'-(*p*-hydroxyphenyl) fluorescein (HPF), which has high reported in vitro selectivity for highly reactive species which include hydroxyl radicals but not superoxide or H₂O₂ (52), to assess antibiotic-induced ROS production in both Gram-negative and Gram-positive bacteria (7, 8).

To determine more robustly whether antibiotics induce reactive species generation in vivo, we first performed a high-throughput ROS quantification experiment using a diverse panel of fluorescent reporter dyes used extensively in the literature to detect ROS (Fig. 1B) (53). We cultured wild-type *E. coli* MG1655 in the presence of each dye and monitored the activation of these dyes following treatment with bactericidal antibiotics. The specificity of one dye in our panel, HPF, was questioned recently based on

the reversibility of HPF fluorescence in an in vitro enzymatic assay (49); using a traditional in vitro Fenton reaction-based assay, we found that Fenton-catalyzed HPF fluorescence is irreversible (SI Appendix, Fig. S1), as described in detail along with other in vitro results in SI Appendix. In light of this result, we used a panel of dyes to increase both the sensitivity of this biochemical assay to a much broader range of reactive species and the diversity of reaction chemistries involved—including direct oxidation, nucleophilic oxidant-mediated liberation of an inhibitory leaving group, or oxidant-mediated opening of a boronate “cage”—that activate fluorescence (53, 54). It is unlikely that activation of all dyes would result from nonspecific or confounding chemical events and oxidizing species. We found that for the majority of dyes tested, wild-type cells treated with ampicillin (a β -lactam), gentamicin (an aminoglycoside), or norfloxacin (a fluoroquinolone) exhibited statistically significant increases in fluorescence compared with controls for antibiotic treatment-related autofluorescence (55), in which no dye was added. Collectively, the broad activation of these dyes suggests several different reactive species that may damage biomolecules are produced in response to antibiotic treatment. Notably, treatment by ampicillin, gentamicin, and norfloxacin induced ROS to different extents, as measured by each dye tested. These results are consistent with the broader literature indicating antibiotics of different classes interfere with different targets, suggesting that ROS may be generated by multiple means.

As a critical test of the hypothesis that reactive species are generated as a downstream physiological consequence of an antibiotic interacting with its traditional target, exposure of *gyrA17* quinolone-resistant cells (in which the primary drug target is mutated) to norfloxacin did not induce significant changes in fluorescence for any dyes tested as compared with the autofluorescence control (Fig. 1C). This observation directly supports the hypothesis that reactive species are induced in response to antibiotic stress rather than as an off-target effect of the drug (e.g., redox cycling).

However, fluorescent dyes can provide only a coarse snapshot of reactive species in a cell. To determine directly and specifically if bactericidal antibiotics promote intracellular H₂O₂ generation, we developed a novel intracellular enzymatic assay using a recently engineered variant of ascorbate peroxidase (APX), W^{41F}AP, which is naturally active in the cytoplasm and exhibits HRP-like kinetics (56). In vitro experiments detailing APX H₂O₂

specificity are described in *SI Appendix*. The H_2O_2 measurement is made using Amplex Red, a fluorogenic dye that diffuses across cell membranes into the cytoplasm. Within the cell, APX catalyzes the rapid H_2O_2 -dependent conversion of Amplex Red into a readily detectable fluorescent product. This method improves on the common, indirect method for measuring H_2O_2 in biological systems in which Amplex Red is oxidized extracellularly by exogenous HRP and H_2O_2 in the culture medium (49, 57). The extracellular assay assumes that the external concentration of H_2O_2 in the culture supernatant reflects the intracellular H_2O_2 concentration because of rapid diffusion. This assumption, however, is problematic because of biological constraints on H_2O_2 diffusion (58), scavenging compartmentation (59–61), and rapid Fenton chemistry destruction of intracellular H_2O_2 (62, 63). Importantly, intracellular APX uses the same dye as extracellular HRP to report on H_2O_2 , and APX expression has no discernible effect on the rate of cell growth.

We found that untreated cells expressing APX exhibited stable baseline levels of Amplex Red fluorescence (Fig. 2*A* and *B*). In

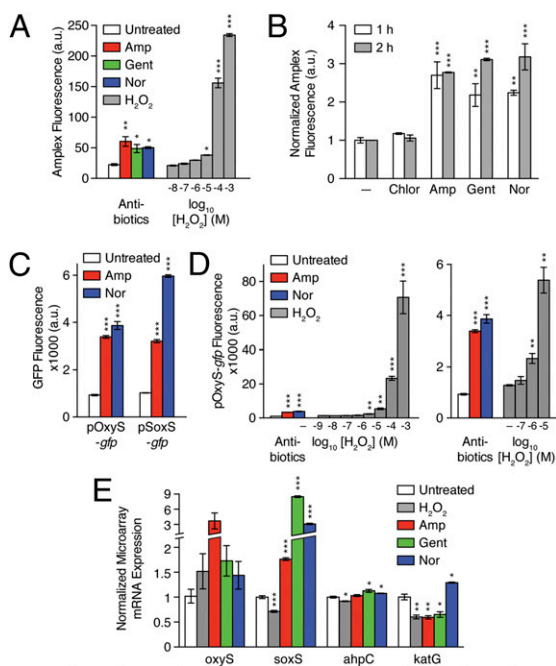


Fig. 2. Antibiotics trigger physiologically relevant generation of H_2O_2 . (*A*) Treatment of wild-type *E. coli* with ampicillin (Amp, 5 μ g/mL), gentamicin (Gent, 5 μ g/mL) or norfloxacin (Nor, 250 ng/mL) induces H_2O_2 production, detected by the intracellular enzymatic sensor APX, using Amplex Red fluorescence as an output. Antibiotic induction was compared with an exogenous H_2O_2 dose-range control. (*B*) Fold change of antibiotic-induced, H_2O_2 -mediated Amplex Red fluorescence at 1 h and 2 h posttreatment, compared with the no-treatment control with basal H_2O_2 production. (*C*) Antibiotic-induced ROS trigger endogenous oxidative stress responses. Treatment with ampicillin (5 μ g/mL) or norfloxacin (250 ng/mL) induces GFP expression from promoters regulated by H_2O_2 -sensitive OxyR (pOxyS-gfp) and superoxide-sensitive SoxR (pSoxS-gfp) in wild-type *E. coli*. Data from additional GFP promoter reporters are included in *SI Appendix*. (*D*) Antibiotics induce physiologically relevant levels of oxidative stress. Antibiotic induction of GFP expression from pOxyS was compared with an exogenous H_2O_2 dose-range control. (*E*) Antibiotic-induced redox stress is comparable to physiologically relevant oxidative stress perturbations at the transcriptional level. Microarrays were performed on cells treated with H_2O_2 (10 μ M), ampicillin (5 μ g/mL), gentamicin (5 μ g/mL), or norfloxacin (250 ng/mL) to quantify altered expression. Data shown reflect mean \pm SEM or three or more technical replicates. Where appropriate, statistical significance is shown ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$). In each instance, an untreated control was used for normalization and determination of statistical significance.

comparison, when APX-expressing cells were treated with ampicillin, gentamicin, or norfloxacin, we observed significant two- to threefold changes in the level of Amplex Red fluorescence in our assay in samples taken at 1 and 2 h posttreatment. Notably, the level of Amplex Red fluorescence induced by bactericidal antibiotics at 1 h posttreatment was comparable to our 10 μ M H_2O_2 spike-in control and was significantly smaller than the supraphysiological levels of H_2O_2 typically used to study oxidative stress responses (49). In contrast, treatment of APX-expressing cells with the bacteriostatic drug chloramphenicol yielded no effect on Amplex Red fluorescence.

These findings contrast with the recent failure to detect increased extracellular H_2O_2 by HRP when an *E. coli* strain lacking the three best-characterized H_2O_2 -scavenging enzymes (*ahpCF*, *katE*, and *katG*, referred to as “Hpx–”) was treated with bactericidal antibiotics. These earlier experiments assumed that drug-induced increases in extracellular H_2O_2 would be readily detectable over the naturally elevated steady-state concentration found in this strain (49, 60) and that the absence of H_2O_2 detection in the supernatant implied that H_2O_2 was not generated following antibiotic treatment (49, 60). The failure of this extracellular assay to detect antibiotic-induced H_2O_2 production may be caused by two confounding factors. First, recent work has shown that cytochrome bd oxidase displays high catalase activity (59), which may compensate for the loss of known H_2O_2 -scavenging activity by the catalase and peroxidase deletions in the Hpx– strain. Moreover, intracellular H_2O_2 could be destroyed by Fenton chemistry before diffusing into the medium. Second, H_2O_2 's dipole moment, similar to that of water (58), may prevent extracellular H_2O_2 from equilibrating with intracellular H_2O_2 by limiting H_2O_2 diffusion across the membrane. Indeed, such limitations in H_2O_2 permeability rationalized the initial observation that H_2O_2 scavenging is compartmented (60).

In addition to these biochemical and enzymatic approaches, we determined whether antibiotic-induced ROS could trigger redox stress responses in vivo by using a genetic reporter assay. We reasoned that if bactericidal antibiotic interference with target-specific processes results in ROS generation, then one would expect to observe activation of oxidative stress regulons. To test this hypothesis, we constructed GFP-based promoter reporter systems that report on oxidant stress and coregulated metabolic response activation (Fig. 2*C* and *SI Appendix*, Fig. S4); the full complement of reporter constructs is detailed in *SI Appendix*. In our assay, we focused on the activity of the promoters pOxyS and pSoxS, which are activated by the main regulators of the response to H_2O_2 and superoxide, OxyR and SoxR, respectively (64). We assessed whether ampicillin or norfloxacin induces expression from a diverse set of promoters; gentamicin was not tested because of the confounding effects of aminoglycosides on GFP reporter assays. We found that treatment with ampicillin or norfloxacin elicited significantly increased pOxyS and pSoxS activity, indicating bactericidal antibiotic-induced activation of OxyR and SoxR (Fig. 2*C* and *SI Appendix*, Fig. S4).

Because this assay was quantitative, we assessed the sensitivity of OxyR to antibiotic treatment by comparing antibiotic-induced pOxyS-gfp expression with that of a dose-range of H_2O_2 (Fig. 2*D*). We quantified GFP expression induction over a range of H_2O_2 concentrations spanning six orders of magnitude (1 mM–1 nM), which encompasses the minimum levels of H_2O_2 reported to fully oxidize OxyR both in vivo (5 μ M) and in vitro (50 nM, in the presence of antioxidants) (61, 65). Significant changes in pOxyS-gfp expression were detected at a threshold near 1 μ M H_2O_2 as compared with untreated cells; this finding is consistent with reports of OxyR activation by submicromolar H_2O_2 (66, 67). GFP expression was increased by two orders of magnitude over that in untreated cells in response to exogenous H_2O_2 applied at concentrations approaching lethality (100 μ M and 1 mM) (67), whereas ampicillin and norfloxacin induced GFP expression most comparable to 10 μ M exogenous H_2O_2 , similar to measurements by our enzymatic Amplex Red assay (Fig. 2*A*).

Because submicromolar H_2O_2 is sufficient to induce cytotoxicity, including significant DNA damage (68), these results suggest that the dynamic range for OxyR activation exceeds the true oxidative stress capacity of wild-type cells by orders of magnitude. Consequently, supraphysiological H_2O_2 perturbations (49) poorly simulate the cytotoxic oxidative stresses experienced by cells in culture and suggest that such perturbations are inappropriate controls for studying OxyR-regulated responses to antibiotic-induced oxidative stress.

To validate our estimates that bactericidal antibiotics induce oxidative stresses similar to exogenous treatment with $10\ \mu M\ H_2O_2$, we compared microarray gene-expression profiles from untreated wild-type cells with those from cells treated with ampicillin, gentamicin, norfloxacin, or $10\ \mu M\ H_2O_2$. Similar to our results using pOxyS-*gfp* (Fig. 2C), we found that all treatments increased *oxyS* expression in comparison with our untreated control (Fig. 2E). Interestingly, *soxS* expression was increased by bactericidal antibiotics but was decreased significantly by $10\ \mu M\ H_2O_2$, suggesting *soxS* activation by antibiotic-induced superoxide but not by H_2O_2 . Nonetheless, the expression of many genes in both the OxyR and SoxRS regulons was induced similarly by bactericidal antibiotics and $10\ \mu M\ H_2O_2$ (SI Appendix, Tables S1 and S2).

Given that bactericidal antibiotics induce substantial activation of the oxidative stress regulon, by extension it may be expected that H_2O_2 -scavenger genes would be activated similarly by $10\ \mu M\ H_2O_2$. Significant induction of *ahpC* and *katG* by supraphysiologic H_2O_2 doses was reported previously (49); however, we found that neither $10\ \mu M\ H_2O_2$ nor bactericidal antibiotics induced significant up-regulation of *ahpC* or *katG* expression (Fig. 2E and SI Appendix, Table S2). This observation highlights the complexity in the oxidative stress response and suggests that intrinsically induced stress responses, such as those arising from antibiotic treatments, may be similar but not necessarily equivalent to those canonically induced by exogenous oxidants. In particular, these data demonstrate that bactericidal antibiotics trigger oxidative stress responses similar to $10\ \mu M\ H_2O_2$ and emphasize the importance of using physiologically relevant controls for investigating cellular responses to antibiotic stress.

Collectively, our biochemical, enzymatic, genetic, and microarray experiments indicate that bactericidal antibiotics induce the formation of reactive species and trigger stress responses similar to those elicited by $10\ \mu M\ H_2O_2$. Possible sources for antibiotic-induced ROS are auto-oxidation reactions involving cofactor-bearing respiratory enzymes or electron leakage, both of which would be enhanced by elevated respiratory activity and increased respiratory enzyme titer, as well as by stress-induced uncoupling (69–72). We hypothesized that the antibiotic-induced increases in ROS would be accompanied by elevated respiratory activity. To test this hypothesis, we developed a flexible assay that dynamically measures bacterial oxygen consumption rate (OCR) using the Seahorse Extracellular Flux Analyzer platform. This assay permits continuous quantification of real-time oxygen consumption of multiple samples in parallel with high sensitivity and with detection limited only by the diffusion rate of molecular oxygen in the culture medium. Overall, we found that ampicillin and norfloxacin significantly elevated OCR, whereas bacteriostatic chloramphenicol led to a rapid reduction in oxygen consumption when cells were grown in defined minimal medium with a single carbon source ($10\ mM$ glucose) (Fig. 3 and SI Appendix, Fig. S3). These results support recent observations that antibiotic stress can trigger sharp increases in the ATP/ADP ratio (73). Relative to the untreated control, gentamicin induced an immediate but transient increase in OCR, whereas ampicillin and norfloxacin induced delayed but sustained OCR increases. The varied respiratory activities stimulated by these different antibiotics parallel the varied increases in reactive species dosage we detected biochemically with the fluorescent dyes (Fig. 1B), suggesting that differential respiratory behaviors may give rise to different levels of ROS. Together, these results are consistent with the hypothesis that bactericidal antibiotics induce redox-

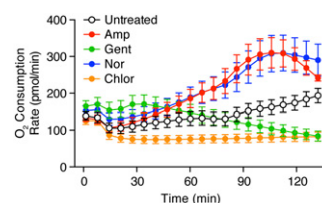


Fig. 3. Antibiotic stress induces redox-related alterations to cell physiology. Treatment of wild-type *E. coli* with bactericidal ampicillin (Amp, $5\ \mu g/mL$), gentamicin (Gent, $5\ \mu g/mL$), or norfloxacin (Nor, $250\ ng/mL$), but not bacteriostatic chloramphenicol (Chlor, $10\ \mu g/mL$), elevates respiratory activity as indicated by elevated OCR, measured by the Seahorse Extracellular Flux Analyzer. Data shown reflect mean \pm SEM of three or more technical replicates.

related alterations to cell physiology, and that these alterations are sensitive to respective primary target effects.

ROS Causatively Contribute To Antibiotic Lethality. If bactericidal antibiotics induce ROS-mediated cellular damage as part of their lethality, then one would anticipate oxidative damage to nucleic acids and their building blocks during drug treatment. Although superoxide and H_2O_2 do not cause oxidative damage to nucleotides, Fenton chemistry does, through the formation of either highly reactive hydroxyl radicals (62, 63, 74, 75) or iron-oxo intermediates (76). Accordingly, these and previous data point to hydroxyl radicals as agents of antibiotic mutagenesis, and this notion is supported by observations that anaerobic growth or thiourea addition reduces mutation rates to near normal levels (77). DNA polymerase IV critically mediates such mutagenesis by incorporating oxidized dNTPs as replicative substrates (78) under both sublethal (79) and lethal (25) antibiotic doses. Because overexpression of the mismatch repair enzyme MutS reduces antibiotic mutagenesis (79), we tested whether MutS overexpression would similarly protect against antibiotic lethality. We found that increases in MutS expression that do not discernibly affect growth rate strikingly reduce the killing by bactericidal antibiotics (Fig. 4). As controls, we tested mutations affecting the ability of MutS to recognize mismatches (F36A) (80) or its ATPase (K620A) (81) and observed reduced ability for MutS to suppress killing by all three classes of bactericidal antibiotics. These results suggest that the well-characterized roles of MutS in long-patch postreplicative mismatch repair account for much of the suppression, although our results do not exclude the possibility that MutS plays additional roles (82, 83). These observations, coupled to the protection elicited by MutS and MutT overexpression (25) and enhanced killing elicited by RecA deletion (8, 20), indicate a common DNA-damage component to killing by bactericidal antibiotics, including those whose targets are at the cell membrane (β -lactams) or the ribosome (aminoglycosides).

We hypothesize that such damage is best explained by nucleotide oxidation following ROS formation and that the subsequent incorporation of these nucleotides into DNA leads to double-strand breaks. We note that iron (II) chelation by nucleoside triphosphates, which is biologically significant (84), would favor the localized production of hydroxyl radicals by Fenton chemistry (85). In purine nucleotides, the C8 position is particularly close to the complexed Fe^{2+} (86) and hence would be favorably disposed to react with a newly generated hydroxyl radical (71) or with an iron-oxo radical (76). Moreover, chelation of Fe^{2+} by a nucleoside triphosphate increases the rate of the Fenton reaction comparably to chelation by EDTA or nitrilotriacetate (87). Minor amounts of nucleotide oxidation can be significant because they impart a gain of function to the target (88), and even trace amounts of 8-oxo-dG in dNTP pools have been shown to have significant biological effects (89). In addition to the elevated respiratory activity, we observed antibiotic-induced alterations to iron homeostasis that may further enhance nucleotide oxidation, as described in SI Appendix.

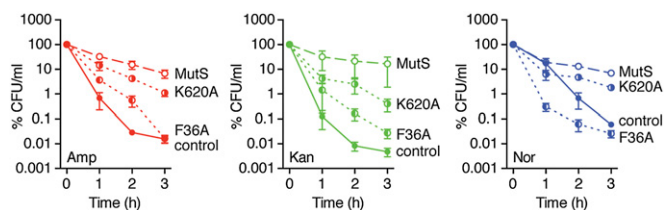


Fig. 4. Antibiotic-induced ROS damage DNA nucleotides. Overexpression of the DNA mismatch repair protein MutS inhibits killing by ampicillin (Amp, 5 μ g/mL), kanamycin (Kan, 5 μ g/mL), or norfloxacin (Nor, 250 ng/mL). Overexpression of recognition (F36A) or ATPase (K620A) mutants reduces MutS's ability to suppress killing, indicating oxidative nucleotide damage. Data shown reflect mean \pm SEM of three or more technical replicates for all data points. Where SEM is small, error bars are present but are inside symbols.

To determine if reactive species causatively contribute to antibiotic killing, we investigated drug lethality under conditions in which ROS accumulation is limited. With direct evidence that bactericidal drugs increase H_2O_2 levels, we first tested the hypothesis that increased dosage of H_2O_2 -scavenging enzymes would reduce killing by bactericidal antibiotics. We found that increased dosage of bifunctional hydroperoxidase I, KatG [the primary scavenger of H_2O_2 at high levels (66)] markedly decreased antibiotic killing at expression levels that did not discernibly affect growth rate (Fig. 5A). To distinguish between the reduction of toxicity being caused by increases in KatG catalytic activity or by an indirect effect of protein overproduction, we tested a KatG mutant with significantly reduced catalytic activity, H106Y (90); this mutant also possesses some peroxidase activity and, importantly, retains the ability to bind heme, thus conferring a similar pleiotropic effect on iron metabolism. The H106Y mutant KatG exhibited a reduced ability to suppress killing by ampicillin, gentamicin, and norfloxacin, implying that the combined catalase and peroxidase activity of KatG are responsible for much of this effect. Similar results were obtained when we tested native and mutant forms [AhpF C348S (29)] of the alkylhydroperoxide reductase, AhpCF, which is the primary scavenger of H_2O_2 at low concentrations (SI Appendix, Fig. S8A) (66). These results complement independent observations by others that deficiencies in KatG or AhpC increase killing by ampicillin, kanamycin, and norfloxacin (15).

Although treatment with ampicillin, gentamicin, and norfloxacin did not strongly induce these native scavenging enzymes, we hypothesized that pretreating cells with H_2O_2 at higher concentrations would induce these endogenous oxidative stress defense mechanisms and also would exert protection against antibiotic treatment. Pretreating cells with 1 mM H_2O_2 , which strongly induced OxyR activity (Fig. 2D) and exceeded the threshold required for strong induction of both *ahpC* and *katG* (49), we observed a transient 1-log protection from lethality by ampicillin, gentamicin, and norfloxacin (SI Appendix, Fig. S5D). This protection could be increased by pretreating cells with 5 mM H_2O_2 (Fig. 5B), indicating sensitivity to the magnitude of oxidative stress defenses induced. Moreover, these results strongly suggest that the significant protection against bactericidal antibiotics observed in catalase- and peroxidase-deficient *Hpx*⁻ cells under fully aerobic conditions (SI Appendix, Fig. S5A) is caused not by the lack of ROS formation (49) but instead by the compensatory induction of native oxidative stress defenses (SI Appendix, Fig. S5B) to manage the elevated oxidative stress experienced by these cells in aerobic growth and culture (60, 68). Indeed, such protection is absent when *Hpx*⁻ cells are cultured and treated under fully anaerobic conditions (SI Appendix, Fig. S5E), as described in SI Appendix. Together, the protection observed by KatG or AhpCF overexpression and by induction of native oxidative stress defenses with H_2O_2 pretreatment directly indicates that antibiotic-induced oxidative stress contributes to lethality.

As an independent test of the hypothesis that ROS contribute to killing by antibiotics, we asked whether antioxidants also could reduce drug lethality. We found that pretreatment with glutathione, a natural antioxidant known to protect *E. coli* from a variety of biological stresses (11, 91), provided at least 1-log of protection from cell death induced by ampicillin, gentamicin, and norfloxacin at 4 h posttreatment (Fig. 5C). Similar results were obtained when cultures were pretreated with ascorbic acid, another antioxidant that also reacts with a range of radical species (Fig. 5D). Importantly, pretreatment with glutathione or ascorbic acid did not introduce growth defects (SI Appendix, Fig. S8 B and C). Additional supportive in vitro results related to the scavenging capacity of antioxidants, including the recently challenged use of thiourea (49), are presented in SI Appendix.

Interestingly, the protection given by KatG or AhpCF overexpression or by glutathione or ascorbic acid pretreatment differed among the different classes of antibiotics administered. In most cases, the greatest protection was seen with ampicillin

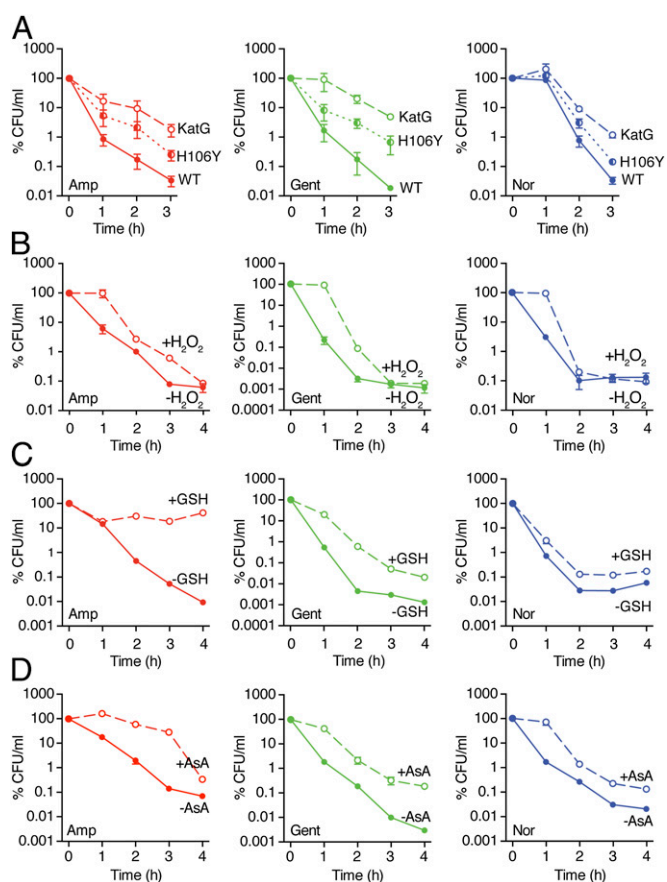


Fig. 5. ROS contribute to the lethality elicited by bactericidal antibiotics. (A) Overexpression of the bifunctional peroxidase/catalase KatG inhibits killing by ampicillin (Amp, 10 μ g/mL), gentamicin (Gent, 5 μ g/mL), or norfloxacin (Nor, 125 ng/mL). Overexpression of heme cofactor-bearing mutant (H106Y), with markedly decreased catalase activity, reduces KatG's ability to suppress killing. (B) Pretreatment with H_2O_2 (5 mM) for 15 min induces transient protection against killing by ampicillin (5 μ g/mL), gentamicin (5 μ g/mL), or norfloxacin (250 ng/mL). (C) Preincubation with glutathione (GSH, 50 mM), a natural antioxidant, inhibits killing by ampicillin (10 μ g/mL), gentamicin (5 μ g/mL), or norfloxacin (250 ng/mL). (D) Preincubation with ascorbic acid (AsA), another natural antioxidant, inhibits antibiotic killing by ampicillin (10 μ g/mL, 1 mM AsA), gentamicin (5 μ g/mL, 50 mM AsA), or Nor (250 ng/mL, 50 mM AsA). Transient protection of killing by ampicillin with 50 mM AsA pretreatment is depicted in SI Appendix, Fig. S8D. Data shown reflect mean \pm SEM of three or more technical replicates for all data points. Where SEM is small, error bars are present but are inside symbols.

treatment and the least with norfloxacin treatment, although our panel of ROS-sensitive fluorescent dyes detected similar amounts of ROS with ampicillin and norfloxacin treatment (Fig. 1B). Although ROS are known to damage many aspects of cell physiology, including proteins, lipids, and metabolites, these results highlight the lethal effects of nucleotide oxidation. DNA incorporation of oxidized nucleotides can lead to double-strand breaks (25, 32), which may be redundant with the primary damage generated by gyrase inhibition under quinolone treatment. Such redundancy may explain why ampicillin treatment confers the greatest protection, because damage by nucleotide oxidation would be least redundant with insults to the cell wall by β -lactams. Moreover, the significant increase in protection over that conferred by gentamicin, despite the low level of ROS detected, supports observations that very little ROS production is required to potentiate antibiotic killing (92). Because the level of ROS induced by antibiotics is small relative to the cell's normal capacity to handle oxidative stress (Fig. 2), these results also suggest that the lethality induced by such ROS is synergistic with the damage directly caused by interference with the primary target, because the contribution of ROS to killing is sensitive to the background state of cells already stressed by antibiotic treatment.

It was suggested recently (93) that, in studies attempting to correlate changes in HPF fluorescence with the extent of killing by norfloxacin (50), the fluorescent dye HPF may have acted as an antioxidant. This effect is plausible, given that fluorescent dyes used to detect ROS in vivo interact with reactive species as part of their chemistry, thereby quenching such species. Similar to our observations using glutathione or ascorbic acid, we found that HPF attenuated norfloxacin-induced cell death (SI Appendix, Fig. S8E), as is consistent with the notion that an antioxidant removes, prevents, or delays oxidative damage to biomolecular targets (74). These results confound recent attempts to correlate HPF fluorescence with antibiotic killing (50), because the dyes themselves may possess antioxidant activity and affect ROS-dependent lethality.

As a third independent test, we examined drug lethality under strict anaerobic conditions, predicting that the absence of environmental oxygen would limit the formation of reactive species and therefore would diminish antibiotic killing. To test this possibility, we compared antibiotic killing in conditions of strictly aerobic and strictly anaerobic growth. To ensure strict anaerobic growth conditions for the culture, treatment, sample dilution, and incubation of cells for quantifying cfus, we performed experiments in an anaerobic chamber (under nitrogen, with catalyst- and air-locked pass-through) and incubated survival assay samples in an anaerobic BD GasPak EZ container within the chamber. To ensure the most stringent anaerobic conditions achievable, we did not remove the culture vessels from the chamber during anaerobic drug incubations (50) or plate the bacteria aerobically before counting colonies (49). Previous observations using *mutT* mutants have noted equivalent mutation frequencies when grown either aerobically or anaerobically in undefined rich (but not in minimal) medium (94), indicating that undefined rich medium may promote confounding redox reactions under anaerobic conditions. Consequently, we chose Neidhardt fully defined complete medium, which contains only sulfate as a potential terminal electron acceptor (95), for anaerobic culturing. Using this defined medium, we maintained experimental consistency by treating aerobically and anaerobically grown cells at the same optical density at entrance to exponential phase, circumventing pitfalls associated with differentially treating anaerobic cultures at a lower density due to culture medium (49).

Compared with aerobically treated cells at concentrations based on the aerobic minimum inhibitory concentrations (MICs), we found that strict anaerobic conditions attenuated killing by bactericidal antibiotics at many drug concentrations (Fig. 6A). In each case, we observed 1- to 4-log increased survival compared with the killing achieved under aerobic conditions. In examining high antibiotic concentrations, we found drug lethality to be significantly reduced but not completely eliminated under strict anaerobic conditions (Fig. 6B), supporting the hypothesis that ROS are

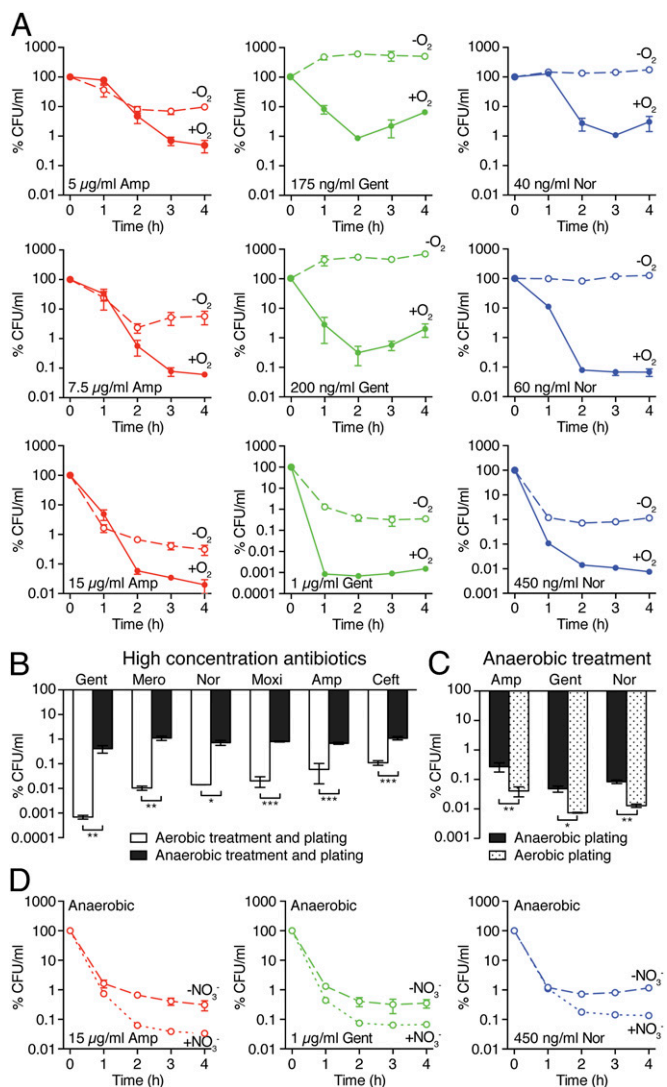


Fig. 6. Antibiotic killing efficacy is sensitive to the availability of molecular oxygen and to alternative electron acceptors. (A) Strict, fully anaerobic treatment and plating reduces killing by 1–4 log over fully aerobic treatment and plating. Survival upon treatment (at indicated concentrations) with ampicillin (Amp), gentamicin (Gent), or norfloxacin (Nor) was assessed in nitrate-free Neidhardt complete defined medium. (B) Strict anaerobic conditions significantly inhibit antibiotic killing for high-concentration treatments with ampicillin (15 μ g/mL), gentamicin (1 μ g/mL), norfloxacin (400 ng/mL), and clinically relevant β -lactams [meropenem (Mero, 900 ng/mL)], ceftriaxone (Ceft, 3.5 μ g/mL), or fluoroquinolones [moxifloxacin (Moxi, 275 ng/mL)]. (C) Exposure of anaerobically treated cells to environmental oxygen enhances antibiotic lethality. Cells were treated with ampicillin (25 μ g/mL), gentamicin (1.25 μ g/mL), or norfloxacin (650 ng/mL) under strict anaerobic conditions and then were diluted, plated, and incubated under either strict anaerobic or aerobic conditions. (D) Alternative electron acceptors enhance killing under strict anaerobic conditions by ampicillin (15 μ g/mL, 10 mM KNO_3), gentamicin (5 μ g/mL, 5 mM KNO_3), or norfloxacin (250 ng/mL, 5 mM KNO_3). Cells were supplemented with up to 10 mM nitrate, similar to concentrations found in LB (98). Data shown reflect mean \pm SEM of three or more technical replicates for all data points. Where SEM is small, error bars are present but are inside symbols. Where appropriate, statistical significance is shown (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

contributors to but not the sole arbiters of antibiotic-mediated killing. This effect also was observed with other clinically relevant β -lactams (meropenem and ceftriaxone) and fluoroquinolones (moxifloxacin) (Fig. 6B and SI Appendix, Fig. S9). These results clearly indicate that the availability of molecular oxygen plays

a significant role in the lethality of bactericidal antibiotics. These results, along with recent observations that kanamycin can damage DNA bases (26) and induce SOS response in strains with mutated 8-oxo-dG-processing genes (*mutM*, *mutY*, and *mutT*) (96), highlight a DNA-damaging component to aminoglycoside lethality, as is consistent with previous MutT results (25). More broadly, these findings suggest that suppressive alterations to proton motor force cannot wholly explain anaerobic protection against aminoglycoside lethality (49–51).

Collectively, these genetic, biochemical, and environmental perturbations indicate that reactive species contribute to antibiotic lethality. Given the redox-related metabolic alterations associated with ROS formation observed under aerobic antibiotic stress, we questioned whether antibiotic treatment under strict anaerobic conditions would prime cells for enhanced killing upon subsequent environmental oxygen exposure, thereby exacerbating cellular damage and augmenting drug lethality. When cells were cultured and treated under strict anaerobic conditions but then were diluted, plated, and incubated under aerobic conditions as previously described (49), we observed a greater than 1-log enhancement in killing by ampicillin, gentamicin, or norfloxacin (Fig. 6C). These results extend observations by others that anaerobic culture and treatment inhibits killing by quinolones (97).

If the protection incurred by the strict anaerobic conditions were caused by the inhibition of reactive species formation, we hypothesized that supplementation with an alternative electron acceptor that enhances respiratory electron flow, such as nitrate, also might enhance antibiotic killing. Relative to sulfate assimilation, denitrification involving nitrate as a redox acceptor is favored during anaerobic respiration. When Neidhardt medium was supplemented with up to 10 mM nitrate at the time of antibiotic treatment, cells treated under strict anaerobic conditions with high concentrations of ampicillin, gentamicin, or norfloxacin yielded ~1-log greater killing (Fig. 6D). Because LB contains ~10 mM nitrate (98), the use of LB under anaerobic conditions may confound interpretations regarding the impact of environmental oxygen on antibiotic lethality (49, 50). These measurements support our overall hypothesis that antibiotic-associated changes in redox physiology contribute to antibiotic toxicity and indicate a critical role for cellular respiration in the resulting antibiotic killing. These data also may support a potential role for reactive nitrogen species in cellular killing (99, 100). Considered together, these data broadly imply that, even under growth conditions that constrain maximum lethality, antibiotic-treated cells are well-poised for death caused by target-specific interference and are primed for extrinsic factors to trigger intrinsic contributions to lethality.

Discussion

In this work, we show that bactericidal antibiotics induce physiological alterations to the cellular redox state, promoting the formation of reactive species including ROS. Although numerous studies similarly have found that oxidative stress is associated with antibiotic treatment (12–17, 19–23, 25–36), some have suggested that ROS production is an epiphenomenon of the death process and not a causal contributor to drug lethality. Using a broad set of independent methodologies, we demonstrate that ROS generated by antibiotic treatment contribute directly to antibiotic lethality. Notably, we also find that redox-related alterations triggered by bactericidal antibiotic stress are sensitive to the class of antibiotics used, indicating a role for the target-specific effects of antibiotics on the extent of these alterations. Further, our results highlight the influence of environmental conditions tested on the intracellular contributions to cell death. For example, depending on the specific drug being assayed, we observe between 1–4 logs of killing, which can be perturbed by extrinsic factors such as the level of aeration and availability of terminal electron acceptors. Interesting recent work has illustrated that phenotypes such as cell death, mutagenesis, oxidative stress, and related activation of OxyR can all be reduced, depending on the method of culturing (101). These findings are consistent with

a recent commentary on the current ROS debate (93), which suggested that differences in experimental conditions may help explain the incongruent conclusions regarding the ROS hypothesis reached by others based on the absence or minimal extent of such phenotypes (49–51). Our related recent work involving metabolic modeling of ROS production predicted and experimentally confirmed that even small increases in ROS levels can enhance antibiotic lethality (92). It would be interesting to pursue studies that examine antibiotic lethality in strains in which basal ROS levels are increased genetically or chemically but experimental conditions constrain drug-induced ROS production.

In association with the understanding that bactericidal antibiotics alter cellular metabolism as part of their killing, our data provide a foundation for rationally designing strategies to improve therapeutic options against bacterial infections. Our findings broadly indicate that antibiotic-treated cells are well-poised for death by their target-specific effects and are primed for additional lethality by extrinsic factors. For instance, our observation that nitrate supplementation enhances antibiotic killing in anaerobic cultures not only supports the notion that cellular respiration is involved in antibiotic lethality but also implies that agents boosting respiratory activity or electron transport may improve antibiotic killing. This notion suggests extrinsic manipulation of bacterial cell metabolism may be exploited to enhance the killing efficacy of existing antibiotics. Indeed, recent work demonstrates that metabolic perturbations (102) or ROS elevation (92) can improve bactericidal lethality.

Observations of antibiotic-induced ROS generation in a diverse range of pathogens (12–17, 19–23, 25–36) suggest that this phenotype is highly conserved. It is interesting to hypothesize that ROS derived from antibiotic-induced physiological alterations may participate in a generalized stress response that augments death at lethal levels but promotes mutagenesis under sublethal doses. This differential activity may provide an explanation for an otherwise paradoxical aspect of the reactive species contribution to antibiotic lethality—that bacteria possess a complex conserved physiological response to lethal doses of antibiotic that contributes to their own demise and is influenced by the growth environment. In nature, bacteria are most likely to be exposed to sublethal concentrations of antibiotics (103, 104), which have been shown to induce beneficial antibiotic mutagenesis capably (79). Consequently, antibiotic-induced and metabolism-fueled ROS may provide a mechanism for acquiring beneficial mutations when stresses are small (77) but induce lethality when stresses are large (25). Dual-function stress responses may facilitate these processes by initiating protective defense mechanisms under low stresses and inducing programmed cell death pathways under large stresses (105). Of note, the differences between sublethal and lethal antibiotic doses are quite small relative to prescribed concentrations, thereby conferring particular relevance to studies analyzing phenotypes at respective drug-dose thresholds. By extension, studies assessing the levels of ROS that are sufficient to enhance antibiotic lethality will be of similar importance in light of results suggesting that this threshold may be quite small in comparison with cytotoxic concentrations of directly applied exogenous oxidants (92).

The significant threat of antibiotic resistance requires that we expand our understanding of how antibiotics affect bacterial metabolism and achieve lethality. Our work exemplifies how systems-level analyses can help dissect the complexity involved in responses to drug–target interactions and clarify their downstream regulatory and biochemical contributions to cellular damage and death. Information from such studies can be leveraged for the development of novel treatment strategies for resistant and recalcitrant infections while providing a foundation for rationally designed strategies to improve current therapeutic options.

Materials and Methods

Bacterial Strains. In our study, we compared the physiological changes associated with treatment of wild-type MG1655 *E. coli* (ATCC no. 700926) with

ampicillin, gentamicin, or norfloxacin with observations of untreated cultures. Where indicated, we also studied the effects of kanamycin, meropenem, ceftriaxone, or moxifloxacin treatment. All antibiotics were obtained from Sigma and Acros Organics. For experiments requiring tightly controlled, plasmid-based protein expression, we used the previously described MG1655pro strain (7). Hpx- MG1655 (*ahpCF::kanR, katG, katE*) was constructed for this study. MIC₉₀s were determined as described below. For killing and protection experiments, concentrations used were 1–5× MIC₉₀ unless otherwise indicated. Additional details are given in *SI Appendix*.

Determination of MIC. Overnight cultures were diluted 1:10,000 in an appropriate medium (LB, M9, or Mops EZ Rich Minimal Medium) at MIC₉₀ for each drug and medium combination using the microbroth plate dilution method. Aerobic MICs were measured after 24 h of incubation in a light-protected, humidity-controlled shaker outfitted for microplates. Anaerobic MICs were measured after 24 h of incubation in a Coy Type B Anaerobic Chamber (Coy Lab Products) on a platform shaker.

Plasmid Construction. All plasmids were transformed into MG1655 or MG1655pro cells by standard molecular biology protocols. Most plasmids used in this study were constructed using the pZE21-mcs1 vector (106), with *kanR* gene for selection, and the TetR-regulated pL(tetO) promoter for gene of interest expression control. The APX reporter gene (56) and alleles of *katG* and *ahpCF* for dosage studies were subcloned into pZE21-mcs1. The *katG* and *ahpCF* genes were PCR amplified from MG1655. Mutant alleles of *katG* (H106Y) and *ahpF* (C345S) were constructed by standard site-directed mutagenesis [Q5 site-directed mutagenesis kit (New England Biolabs)] of the pZE21-*katG* and -*ahpCF* plasmids, respectively, and were verified by sequencing. The pCA24N::MutS plasmid for MutS dosage studies was obtained from the ASKA overexpression plasmid library (107). Mutant alleles of *mutS* (F36A, K620A) were constructed by standard site-directed mutagenesis of the pCA24N::MutS plasmid and were verified by sequencing. Additional details are given in *SI Appendix*.

Fluorescent Dye-Based ROS Detection. Fluorescent dyes were used to quantify ROS production in MG1655 or quinolone-resistant *gyrA17* (*E. coli* Genetic Stock Center no. 4366). The following dyes were used: carboxy-H₂DCFDA (mixed isomers), chloromethyl-H₂DCFDA (mixed isomers), DAF-FM diacetate, H₂DCFDA, HPF, OxyBURST Green, and Peroxy-Fluor 2 (PF2), or an equivalent volume of dH₂O (our no-dye control). PF2 was a generous gift of the Chang laboratory at the University of California, Berkeley; all other dyes were from Life Technologies. Cells were grown in 200 μL of Luria-Bertani medium (Fisher) in 96-well, 2-mL deep-well culture plates 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 OD₆₀₀ of ~0.2 before antibiotic treatment. OD₆₀₀ measurements were made using a SpectraMax M5 Microplate Reader spectrophotometer (Molecular Devices). For analysis, samples were diluted ~100-fold in 1× PBS (pH 7.2) into a 96-well microplate for fluorescence determination using a Fortessa flow cytometer (Becton Dickinson) outfitted with a microplate autosampler. Mean GFP fluorescence (FL1-A) was quantified using the following photomultiplier tube (PMT) voltages: forward scatter (FSC) 500, side scatter (SSC) 250, FL1-A 500. Acquisition was performed at the lowest flow rate (~2 μL/s), with thresholding on FSC at a value of 500. This method and statistical analysis are described further in *SI Appendix*.

Intracellular H₂O₂ Measurement. MG1655pro cells with the pZE21-APX plasmid were used to quantitate intracellular hydrogen peroxide. Cells were grown in 10 mL LB (with selection) in 250-mL baffled flasks to an OD₆₀₀ of ~0.2 at 37 °C and 300 rpm in a light-protected, humidity-controlled incubator shaker (Multitron II; ATR) before the addition of anhydrotetracycline (30 ng/mL) to induce APX expression. Cultures were grown to an OD₆₀₀ of ~1.2 before dilution to an OD₆₀₀ of 0.3 in LB containing indicated perturbations. H₂O₂ dose responsiveness was assessed using freshly prepared H₂O₂ dilutions made in dH₂O from a stabilized 10-M stock solution (Sigma). At designated time points, samples were concentrated 10-fold in 1× PBS containing 50 μM Amplex UltraRed (Life Technologies) and were transferred to black, opaque-bottomed 96-well microplates. Amplex UltraRed fluorescence then was measured using the SpectraMAX M5 Microplate Reader using the 568/591 filter combination. This method and statistical analysis are described further in *SI Appendix*.

GFP Promoter–Reporter Fusion. MG1655 cells expressing the pZE2-pOxyS-*gfp*, pSoxS-*gfp*, pL(FurO)-*gfp*, pHemH-*gfp*, pTrxC-*gfp*, or pL(MetO)-*gfp* reporter plasmids were used to quantitate promoter activity after the addition of antibiotics or H₂O₂. Cells were grown in 25 mL LB (with selection) in 250-mL baffled flasks at 37 °C and 300 rpm in a light-protected, humidity-controlled

incubator shaker to an OD₆₀₀ of ~0.3 before transfer to 24-well microplates containing indicated treatments. Microplates were grown at 37 °C and 900 rpm in a light-protected, humidity-controlled incubator shaker. H₂O₂ dose-response curves were prepared as above. For analysis, samples were diluted ~100-fold in 1× PBS (pH 7.2) into a 96-well microplate for fluorescence determination 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 1,000. This method and statistical analysis are described further in *SI Appendix*.

Genomewide Microarrays. Microarray analysis was performed on MG1655 cells treated with ampicillin, gentamicin, norfloxacin, kanamycin, or 10 μM H₂O₂. H₂O₂ was prepared as above. Cells were grown in 10 mL LB in 250-mL baffled flasks at 37 °C and 300 rpm in a light-protected, humidity-controlled incubator shaker to an OD₆₀₀ of ~0.3 before transfer to 14-mL polypropylene tubes and application of indicated treatments. Samples for total RNA collection were taken immediately before treatment (time 0) and at 1 h post-treatment. Total RNA was obtained using RNeasy Protect Bacteria Mini Kit (Qiagen), the RNeasy Protect Bacteria Mini Kit (Qiagen), and Turbo DNA-free (Life Technologies) DNase treatment according to respective manufacturers' instructions. cDNA preparation and hybridization to Affymetrix GeneChip *E. coli* Genome 2.0 microarrays were performed as previously described (7). CEL files for the resulting expression profiles were background adjusted and normalized using Robust Multiarray Averaging (108). Statistical significance was computed using Welch's *t* test. Triplicate (technical replicate) measurements from treated MG1655 samples were compared with the triplicate (technical replicate) untreated samples. For each set of comparisons, *P* values were corrected for false-discovery rate (FDR) (109). Genes with FDR-corrected *P* values ≤0.05 were deemed statistically significant. Regulation for genes in the OxyR and SoxRS regulons was identified as annotated in RegulonDB v8.2 (110). Microarray data collected in this study are available for download on the Gene Expression Omnibus (GEO), accession no. GSE56133. Detailed descriptions of the method and analysis are given in *SI Appendix*.

Bacterial Respiration. Bacterial respiration, expressed as OCR, was quantified using an XF⁹⁶ Extracellular Flux Analyzer (Seahorse Bioscience). Cells were grown in 25 mL M9 minimal medium (with 10 mM glucose carbon source; Fisher) in 250-mL baffled flasks at 37 °C and 300 rpm in a light-protected, humidity-controlled incubator shaker to an OD₆₀₀ of ~0.3 before dilution to an OD₆₀₀ of 0.02 in M9 glucose for measurements. For OCR measurements, cells were seeded onto poly-D-lysine-coated XF cell culture microplates, and cellular respiration was quantified. Basal OCR was measured for ~15 min before automated antibiotic injection to assure uniform cellular seeding and then was quantified every 6 min for the duration of the experiment post-treatment. Additional details are given in *SI Appendix*.

MutS Overexpression. The effect of increased expression of MutS, MutS (F36A), or MutS (K620A) on antibiotic killing was assayed in MG1655 cells. Cells were grown in 10 mL LB (with selection and 1 mM isopropyl β-D-1-thiogalactopyranoside inducer) in 250-mL baffled flasks at 37 °C and 300 rpm in a light-protected, humidity-controlled incubator shaker to an OD₆₀₀ of ~0.1 before indicated treatments. At designated time points, samples for survival measurements (change in colony cfu/mL) were serially diluted in 1× PBS. Samples were plated onto LB agar plates and incubated at 37 °C overnight before cfu determination. Log percent survival (% cfu/mL) was determined by calculating the change in cfu/mL at each time point compared with that at pretreatment (time 0). Mean survival and SEM were calculated across all experiments for each treatment from at least three independent technical replicates. Additional details are given in *SI Appendix*.

KatG Overexpression. The effect of increased expression of KatG or KatG (H106Y) on antibiotic killing was assayed in MG1655pro cells. Cells were grown in 10 mL LB (with selection and 5 ng/mL aTc for KatG expression) in 250-mL baffled flasks at 37 °C and 300 rpm in a light-protected, humidity-controlled incubator shaker to an OD₆₀₀ of ~0.3 before transfer to 24-well microplates containing indicated treatments. Microplates were grown at 37 °C and 900 rpm in the shaker described above. Log percent survival (% cfu/mL) was determined as described above for at least three independent technical replicates. Additional details are given in *SI Appendix*.

Antioxidant Pretreatment. The effect of pretreatment with the antioxidants L-glutathione (Sigma) or (+)-Sodium L-ascorbate (Sigma) on antibiotic killing was assayed in MG1655 cells. Cells were grown in 25 mL LB in 250-mL baffled flasks at 37 °C and 300 rpm in a light-protected, humidity-controlled

incubator shaker to an OD₆₀₀ of ~0.3 before 10-min pretreatment with antioxidants. Cultures then were transferred to 24-well microplates containing the indicated treatments and were grown as described above. Log percent survival (% cfu/mL) was determined as described above for at least three independent technical replicates. Additional details and the HPF experiment method are given in *SI Appendix*.

H₂O₂ Pretreatment. The effect of pretreatment with H₂O₂ (Acros) on antibiotic killing was assayed in MG1655 cells. Cells were grown in 25 mL LB in 250-mL baffled flasks at 37 °C and 300 rpm in a light-protected, humidity-controlled incubator shaker to an OD₆₀₀ of ~0.3 before 15-min pretreatment with H₂O₂ in 15-mL conical centrifuge tubes. Cultures then were pelleted and resuspended in fresh LB before they were transferred to 24-well microplates containing indicated treatments and grown as described above. Log percent survival (% cfu/mL) was determined as described above for at least three independent technical replicates. Additional details are in *SI Appendix*.

Strict Anaerobic Growth and Survival, With and Without Nitrate. For all anaerobic growth-related 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 as well as for the anaerobic equilibration of media, PBS, and survival assay plates. 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 ppm.

Cells were grown in 1 mL Neidhardt Complete Minimal Medium for Enterobacteria (95) with glucose (Mops EZ Rich Minimal Medium; Teknova) in 14-mL culture tubes on a platform shaker at ~300 rpm to an OD₆₀₀ at ~0.2 in the anaerobic chamber before treatment. OD₆₀₀ samples were taken in the chamber, and the 96-well sample microplate was transferred out of the chamber for SpectraMAX M5 microplate reader measurements. Analogous conditions were used for aerobic comparison. At designated time points, samples were serially diluted in 1× PBS and plated onto LB agar plates. Plates were incubated 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 h. To test the effects of molecular oxygen exposure on survival, samples were removed from the chamber and were diluted, plated, and incubated on LB agar plates under ambient conditions. To test the effects of nitrate addition on survival, up to 10 mM KNO₃ was added with antibiotic treatment, and samples were prepared and incubated under strict anaerobic conditions. For all assays, log percent survival (% cfu/mL) was determined as described above for at least three independent technical replicates. All methods are described in *SI Appendix*.

ACKNOWLEDGMENTS. This work was supported by the National Institutes of Health (NIH) Director's Pioneer Award Program (DP1 OD003644 to J.J.C.; DP1 OD003961 to A.Y.T.), Howard Hughes Medical Institute (J.J.C.), NIH (R01 CA021615 to G.C.W.), Wyss Institute (J.J.C.), and Institut Merieux (J.J.C.; A.S.K.). J.D.M. is a National Defense Science and Engineering Graduate Fellow. G.C.W. is an American Cancer Society Professor.

- Centers for Disease Control and Prevention (2013) *Antibiotic Resistance Threats in the United States* (CDC, Atlanta). Available at <http://www.cdc.gov/drugresistance/threat-report-2013>. Accessed on April 18, 2014.
- Wright GD (2007) The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5(3):175–186.
- Spellberg B, et al.; Infectious Diseases Society of America (2008) The epidemic of antibiotic-resistant infections: A call to action for the medical community from the Infectious Diseases Society of America. *Clin Infect Dis* 46(2):155–164.
- Fischbach MA, Walsh CT (2009) Antibiotics for emerging pathogens. *Science* 325(5944):1089–1093.
- Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 74(3):417–433.
- Piddock LJ (2012) The crisis of no new antibiotics—what is the way forward? *Lancet Infect Dis* 12(3):249–253.
- Dwyer DJ, Kohanski MA, Hayete B, Collins JJ (2007) Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. *Mol Syst Biol* 3(91):1–15.
- Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130(5):797–810.
- Becerra MC, Albesa I (2002) Oxidative stress induced by ciprofloxacin in *Staphylococcus aureus*. *Biochem Biophys Res Commun* 297(4):1003–1007.
- Albesa I, Becerra MC, Battán PC, Páez PL (2004) Oxidative stress involved in the antibacterial action of different antibiotics. *Biochem Biophys Res Commun* 317(2):605–609.
- Goswami M, Mangoli SH, Jawali N (2006) Involvement of reactive oxygen species in the action of ciprofloxacin against *Escherichia coli*. *Antimicrob Agents Chemother* 50(3):949–954.
- Kohanski MA, Dwyer DJ, Wierzbowski J, Cottarel G, Collins JJ (2008) Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. *Cell* 135(4):679–690.
- Breidenstein EB, Khaira BK, Wiegand I, Overhage J, Hancock RE (2008) Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrob Agents Chemother* 52(12):4486–4491.
- Davies BW, et al. (2009) Hydroxyurea induces hydroxyl radical-mediated cell death in *Escherichia coli*. *Mol Cell* 36(5):845–860.
- Wang X, Zhao X (2009) Contribution of oxidative damage to antimicrobial lethality. *Antimicrob Agents Chemother* 53(4):1395–1402.
- Gusarov I, Shatalin K, Starodubtseva M, Nudler E (2009) Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. *Science* 325(5946):1380–1384.
- Girgis HS, Hottes AK, Tavazoie S (2009) Genetic architecture of intrinsic antibiotic susceptibility. *PLoS ONE* 4(5):e5629.
- Bizzini A, Zhao C, Auffray Y, Hartke A (2009) The Enterococcus faecalis superoxide dismutase is essential for its tolerance to vancomycin and penicillin. *J Antimicrob Chemother* 64(6):1196–1202.
- Yeom J, Imlay JA, Park W (2010) Iron homeostasis affects antibiotic-mediated cell death in *Pseudomonas* species. *J Biol Chem* 285(29):22689–22695.
- Liu A, et al. (2010) Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: Generating an antibiotic bar code. *Antimicrob Agents Chemother* 54(4):1393–1403.
- Wang X, Zhao X, Malik M, Drlita K (2010) Contribution of reactive oxygen species to pathways of quinolone-mediated bacterial cell death. *J Antimicrob Chemother* 65(3):520–524.
- Shatalin K, Shatalina E, Mironov A, Nudler E (2011) H2S: A universal defense against antibiotics in bacteria. *Science* 334(6058):986–990.
- Nguyen D, et al. (2011) Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* 334(6058):982–986.
- Calhoun LN, Kwon YM (2011) The ferritin-like protein Dps protects *Salmonella enterica* serotype Enteritidis from the Fenton-mediated killing mechanism of bactericidal antibiotics. *Int J Antimicrob Agents* 37(3):261–265.
- Foti JJ, Devadoss B, Winkler JA, Collins JJ, Walker GC (2012) Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. *Science* 336(6079):315–319.
- Kang TM, et al. (2012) The aminoglycoside antibiotic kanamycin damages DNA bases in *Escherichia coli*: Caffeine potentiates the DNA-damaging effects of kanamycin while suppressing cell killing by ciprofloxacin in *Escherichia coli* and *Bacillus anthracis*. *Antimicrob Agents Chemother* 56(6):3216–3223.
- Grant SS, Kaufmann BB, Chand NS, Haseley N, Hung DT (2012) Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals. *Proc Natl Acad Sci USA* 109(30):12147–12152.
- Liu Y, et al. (2012) Inhibitors of reactive oxygen species accumulation delay and/or reduce the lethality of several antistaphylococcal agents. *Antimicrob Agents Chemother* 56(11):6048–6050.
- Ling J, et al. (2012) Protein aggregation caused by aminoglycoside action is prevented by a hydrogen peroxide scavenger. *Mol Cell* 48(5):713–722.
- Sampson TR, et al. (2012) Rapid killing of *Acinetobacter baumannii* by polymyxins is mediated by a hydroxyl radical death pathway. *Antimicrob Agents Chemother* 56(11):5642–5649.
- Luo Y, Helmann JD (2012) Analysis of the role of *Bacillus subtilis* σ (M) in β -lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol Microbiol* 83(3):623–639.
- Dwyer DJ, Camacho DM, Kohanski MA, Callura JM, Collins JJ (2012) Antibiotic-induced bacterial cell death exhibits physiological and biochemical hallmarks of apoptosis. *Mol Cell* 46(5):561–572.
- Van Acker H, et al. (2013) Biofilm-grown *Burkholderia cepacia* complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. *PLoS ONE* 8(3):e58943.
- Viłchžė C, Hartman T, Weinrick B, Jacobs WR, Jr. (2013) Mycobacterium tuberculosis is extraordinarily sensitive to killing by a vitamin C-induced Fenton reaction. *Nat Commun* 4(1881):1–10.
- Frawley ER, et al. (2013) Iron and citrate export by a major facilitator superfamily pump regulates metabolism and stress resistance in *Salmonella Typhimurium*. *Proc Natl Acad Sci USA* 110(29):12054–12059.
- Morones-Ramirez JR, Winkler JA, Spina CS, Collins JJ (2013) Silver enhances antibiotic activity against gram-negative bacteria. *Sci Transl Med* 5(190):90ra81.
- Khakimova M, Ahlgren HG, Harrison JJ, English AM, Nguyen D (2013) The stringent response controls catalases in *Pseudomonas aeruginosa* and is required for hydrogen peroxide and antibiotic tolerance. *J Bacteriol* 195(9):2011–2020.
- Jensen PO, et al. (2014) Formation of hydroxyl radicals contributes to the bactericidal activity of ciprofloxacin against *Pseudomonas aeruginosa* biofilms. *Pathog Dis* 70(3):440–443.
- Lupien A, et al. (2013) Genomic characterization of ciprofloxacin resistance in a laboratory-derived mutant and a clinical isolate of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 57(10):4911–4919.
- Kanafani H, Martin SE (1985) Catalase and superoxide dismutase activities in virulent and nonvirulent *Staphylococcus aureus* isolates. *J Clin Microbiol* 21(4):607–610.
- Sherman DR, et al. (1996) Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* 272(5268):1641–1643.

42. Kelley CL, Rouse DA, Morris SL (1997) Analysis of ahpC gene mutations in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 41(9):2057–2058.
43. McMurry LM, Oethinger M, Levy SB (1998) Overexpression of marA, soxS, or acrAB produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiol Lett* 166(2):305–309.
44. Oethinger M, Podglajen I, Kern WV, Levy SB (1998) Overexpression of the marA or soxS regulatory gene in clinical topoisomerase mutants of *Escherichia coli*. *Antimicrob Agents Chemother* 42(8):2089–2094.
45. Koutsolioutsou A, Martins EA, White DG, Levy SB, Demple B (2001) A soxRS-constitutive mutation contributing to antibiotic resistance in a clinical isolate of *Salmonella enterica* (Serovar typhimurium). *Antimicrob Agents Chemother* 45(1):38–43.
46. Koutsolioutsou A, Peña-Llopis S, Demple B (2005) Constitutive soxR mutations contribute to multiple-antibiotic resistance in clinical *Escherichia coli* isolates. *Antimicrob Agents Chemother* 49(7):2746–2752.
47. Chittesham Thomas V, et al. (2013) A dysfunctional tricarboxylic acid cycle enhances fitness of *Staphylococcus epidermidis* during β -lactam stress. *MBio* 4(4).
48. Páez PL, Becerra MC, Albesa I (2010) Antioxidative mechanisms protect resistant strains of *Staphylococcus aureus* against ciprofloxacin oxidative damage. *Fundam Clin Pharmacol* 24(6):771–776.
49. Liu Y, Imlay JA (2013) Cell death from antibiotics without the involvement of reactive oxygen species. *Science* 339(6124):1210–1213.
50. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K (2013) Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science* 339(6124):1213–1216.
51. Ezraty B, et al. (2013) Fe-S cluster biosynthesis controls uptake of aminoglycosides in a ROS-less death pathway. *Science* 340(6140):1583–1587.
52. Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T (2003) Development of novel fluorescence probes that can reliably detect reactive oxygen and distinguish specific species. *J Biol Chem* 278(5):3170–3175.
53. Wardman P (2007) Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: Progress, pitfalls, and prospects. *Free Radic Biol Med* 43(7):995–1022.
54. Lin VS, Dickinson BC, Chang CJ (2013) Boronate-based fluorescent probes: Imaging hydrogen peroxide in living systems. *Methods Enzymol* 526:19–43.
55. Renggli S, Keck W, Jenal U, Ritz D (2013) Role of autofluorescence in flow cytometric analysis of *Escherichia coli* treated with bactericidal antibiotics. *J Bacteriol* 195(18):4067–4073.
56. Martell JD, et al. (2012) Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. *Nat Biotechnol* 30(11):1143–1148.
57. Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP (1997) A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: Applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem* 253(2):162–168.
58. Bienert GP, et al. (2007) Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J Biol Chem* 282(2):1183–1192.
59. Borisov VB, et al. (2013) Cytochrome bd oxidase from *Escherichia coli* displays high catalase activity: An additional defense against oxidative stress. *FEBS Lett* 587(14):2214–2218.
60. Seaver LC, Imlay JA (2001) Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*. *J Bacteriol* 183(24):7182–7189.
61. Mishra S, Imlay J (2012) Why do bacteria use so many enzymes to scavenge hydrogen peroxide? *Arch Biochem Biophys* 525(2):145–160.
62. Imlay JA, Chin SM, Linn S (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* 240(4852):640–642.
63. Halliwell B, Gutteridge JM (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 219(1):1–14.
64. Chiang SM, Schellhorn HE (2012) Regulators of oxidative stress response genes in *Escherichia coli* and their functional conservation in bacteria. *Arch Biochem Biophys* 525(2):161–169.
65. Aslund F, Zheng M, Beckwith J, Storz G (1999) Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc Natl Acad Sci USA* 96(11):6161–6165.
66. González-Flecha B, Demple B (1997) Homeostatic regulation of intracellular hydrogen peroxide concentration in aerobically growing *Escherichia coli*. *J Bacteriol* 179(2):382–388.
67. Stone JR, Yang S (2006) Hydrogen peroxide: A signaling messenger. *Antioxid Redox Signal* 8(3–4):243–270.
68. Park S, You X, Imlay JA (2005) Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx- mutants of *Escherichia coli*. *Proc Natl Acad Sci USA* 102(26):9317–9322.
69. González-Flecha B, Demple B (1995) Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. *J Biol Chem* 270(23):13681–13687.
70. Cabisco E, Tamarit J, Ros J (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol* 3(1):3–8.
71. Imlay JA (2013) The molecular mechanisms and physiological consequences of oxidative stress: Lessons from a model bacterium. *Nat Rev Microbiol* 11(7):443–454.
72. Messner KR, Imlay JA (1999) The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. *J Biol Chem* 274(15):10119–10128.
73. Akhova AV, Tkachenko AG (2014) ATP/ADP alteration as a sign of the oxidative stress development in *Escherichia coli* cells under antibiotic treatment. *FEMS Microbiol Lett* 353(1):69–76.
74. Halliwell B, Gutteridge JMC (2007) *Free Radicals in Biology and Medicine* (Oxford Univ Press, New York, NY), 4th Ed.
75. Imlay JA (2003) Pathways of oxidative damage. *Annu Rev Microbiol* 57:395–418.
76. Yamamoto N, Koga N, Nagaoka M (2012) Ferryl-oxo species produced from Fenton's reagent via a two-step pathway: Minimum free-energy path analysis. *J Phys Chem B* 116(48):14178–14182.
77. Kohanski MA, DePristo MA, Collins JJ (2010) Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol Cell* 37(3):311–320.
78. Yamada M, et al. (2006) Involvement of Y-family DNA polymerases in mutagenesis caused by oxidized nucleotides in *Escherichia coli*. *J Bacteriol* 188(13):4992–4995.
79. Gutierrez A, et al. (2013) β -Lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. *Nat Commun* 4(1610):1–9.
80. Yamamoto A, Schofield MJ, Biswas I, Hsieh P (2000) Requirement for Phe36 for DNA binding and mismatch repair by *Escherichia coli* MutS protein. *Nucleic Acids Res* 28(18):3564–3569.
81. Haber LT, Walker GC (1991) Altering the conserved nucleotide binding motif in the *Salmonella typhimurium* MutS mismatch repair protein affects both its ATPase and mismatch binding activities. *EMBO J* 10(9):2707–2715.
82. Klocko AD, et al. (2011) Mismatch repair causes the dynamic release of an essential DNA polymerase from the replication fork. *Mol Microbiol* 82(3):648–663.
83. Wang G, Alamuri P, Humayun MZ, Taylor DE, Maier RJ (2005) The Helicobacter pylori MutS protein confers protection from oxidative DNA damage. *Mol Microbiol* 58(1):166–176.
84. Weaver J, Pollack S (1989) Low-Mr iron isolated from guinea pig reticulocytes as AMP-Fe and ATP-Fe complexes. *Biochem J* 261(3):787–792.
85. Floyd RA (1983) Direct demonstration that ferrous ion complexes of di- and triphosphate nucleotides catalyze hydroxyl free radical formation from hydrogen peroxide. *Arch Biochem Biophys* 225(1):263–270.
86. Richter Y, Fischer B (2003) Characterization and elucidation of coordination requirements of adenine nucleotides complexes with Fe(II) ions. *Nucleosides Nucleotides* 22(9):1757–1780.
87. Rush JD, Maskos Z, Koppenol WH (1990) Reactions of iron (II) nucleotide complexes with hydrogen peroxide. *FEBS Lett* 261(1):121–123.
88. Winterbourn CC (2008) Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol* 4(5):278–286.
89. Pursell ZF, McDonald JT, Mathews CK, Kunkel TA (2008) Trace amounts of 8-oxo-dGTP in mitochondrial dNTP pools reduce DNA polymerase gamma replication fidelity. *Nucleic Acids Res* 36(7):2174–2181.
90. Hillar A, et al. (2000) Modulation of the activities of catalase-peroxidase HPI of *Escherichia coli* by site-directed mutagenesis. *Biochemistry* 39(19):5868–5875.
91. Smirnova G, Muzyka N, Oktyabrsky O (2012) Transmembrane glutathione cycling in growing *Escherichia coli* cells. *Microbiol Res* 167(3):166–172.
92. Brynildsen MP, Winkler JA, Spina CS, MacDonalld IC, Collins JJ (2013) Potentiating antibacterial activity by predictably enhancing endogenous microbial ROS production. *Nat Biotechnol* 31(2):160–165.
93. Fang FC (2013) Antibiotic and ROS linkage questioned. *Nat Biotechnol* 31(5):415–416.
94. Fowler RG, Erickson JA, Isbell RJ (1994) Activity of the *Escherichia coli* mutT mutator allele in an anaerobic environment. *J Bacteriol* 176(24):7727–7729.
95. Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for enterobacteria. *J Bacteriol* 119(3):736–747.
96. Baharoglu Z, Krin E, Mazel D (2013) RpoS plays a central role in the SOS induction by sub-lethal aminoglycoside concentrations in *Vibrio cholerae*. *PLoS Genet* 9(4):e1003421.
97. Malik M, Hussain S, Drlica K (2007) Effect of anaerobic growth on quinolone lethality with *Escherichia coli*. *Antimicrob Agents Chemother* 51(1):28–34.
98. Xu J, Xu X, Verstraete W (2000) Adaptation of *E. coli* cell method for micro-scale nitrate measurement with the Griess reaction in culture media. *J Microbiol Methods* 41(1):23–33.
99. Burney S, Caulfield JL, Niles JC, Wishnok JS, Tannenbaum SR (1999) The chemistry of DNA damage from nitric oxide and peroxy nitrite. *Mutat Res* 424(1–2):37–49.
100. Fang FC (2004) Antimicrobial reactive oxygen and nitrogen species: Concepts and controversies. *Nat Rev Microbiol* 2(10):820–832.
101. Kram KE, Finkel SE (2013) Culture volume and vessel affect long-term survival, mutation frequency, and oxidative stress in *E. coli*. *Appl Environ Microbiol* 80(5):1732–1738.
102. Allison KR, Brynildsen MP, Collins JJ (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* 473(7346):216–220.
103. Davies J, Spiegelman GB, Yim G (2006) The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* 9(5):445–453.
104. Kümmerer K (2003) Significance of antibiotics in the environment. *J Antimicrob Chemother* 52(1):5–7.
105. Dorsey-Oresto A, et al. (2013) YihE kinase is a central regulator of programmed cell death in bacteria. *Cell Rep* 3(2):528–537.
106. Lutz R, Bujard H (1997) Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/11-2 regulatory elements. *Nucleic Acids Res* 25(6):1203–1210.
107. Kitagawa M, et al. (2005) Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): Unique resources for biological research. *DNA Res* 12(5):291–299.
108. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19(2):185–193.
109. Storey JD, Tibshirani R (2003) Statistical significance for genomewide studies. *Proc Natl Acad Sci USA* 100(16):9440–9445.
110. Salgado H, et al. (2013) RegulonDB v8.0: Omics data sets, evolutionary conservation, regulatory phrases, cross-validated gold standards and more. *Nucleic Acids Res* 41(Database issue):D203–D213.