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

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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 H\textsubscript{2}O\textsubscript{2} 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 H\textsubscript{2}O\textsubscript{2}. 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.

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 disruption of target-specific processes, ultimately inducing oxidative stress responses 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.


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.

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).

*This Direct Submission article had a prearranged editor.

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

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 supplemented with new reagents/analytic tools; D.J.D., P.A.B., J.H.Y., I.C.M., J.D.M., C.T.Y.C., D.B., E.G.S., I.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.

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reactive oxygen species | DNA repair | mutagenesis

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E100–E2109 | PNAS | Published online May 6, 2014

www.pnas.org/cgi/doi/10.1073/pnas.1401876111
Antibiotics
Target-Specific Processes
Cellular Damage
Altered Metabolism, Respiration and Iron Homeostasis
ROS and Other Damaging Molecules
Bacterial Cell Death

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 μg/mL), gentamicin (Gent, 5 μg/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-Flour 2 (PF2). (C) Treatment of a quinolone-resistant strain ( gyrA17) with norfloxacin does not produce detectable ROS. Data shown reflect mean ± 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 ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

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′,4′-(p-hydroxyphenyl) fluorescein (HPF), which has high report 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), W41FAP, which is naturally active in the cytoplasm and exhibits HRP-like kinetics (56). In vitro experiments detailing APX H₂O₂...
specification are described in SI Appendix. The H$_2$O$_2$ 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$_2$O$_2$-dependent conversion of Amplex Red into a readily detectable fluorescent product. This method improves on the common, indirect method for measuring H$_2$O$_2$ in biological systems in which Amplex Red is oxidized extracellularly by exogenous HRP and H$_2$O$_2$ in the culture medium (49, 57). The extracellular assay assumes that the external concentration of H$_2$O$_2$ in the culture supernatant reflects the intracellular H$_2$O$_2$ concentration because of rapid diffusion. This assumption, however, is problematic because of biological constraints on H$_2$O$_2$ diffusion (58), scavenging compartmentation (59–61), and rapid Fenton chemistry destruction of intracellular H$_2$O$_2$ (62, 63). Importantly, intracellular APX uses the same dye as extracellular HRP to report on H$_2$O$_2$, and APX expression has no discernable effect on the rate of cell growth.

We found that untreated cells expressing APX exhibited stable baseline levels of Amplex Red fluorescence (Fig. 2A and B). In 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 μM H$_2$O$_2$ spike-in control and was significantly smaller than the supraphysiological levels of H$_2$O$_2$ 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$_2$O$_2$ by HRP when an E. coli strain lacking the three best-characterized H$_2$O$_2$-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$_2$O$_2$ would be readily detectable over the naturally elevated steady-state concentration found in this strain (49, 60) and that the absence of H$_2$O$_2$ detection in the supernatant implied that H$_2$O$_2$ was not generated following antibiotic treatment (49, 60). The failure of this extracellular assay to detect antibiotic-induced H$_2$O$_2$ 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$_2$O$_2$-scavenging activity by the catalase and peroxidase deletions in the Hpx− strain. Moreover, intracellular H$_2$O$_2$ could be destroyed by Fenton chemistry before diffusing into the medium. Second, H$_2$O$_2$’s dipole moment, similar to that of water (58), may prevent extracellular H$_2$O$_2$ from equilibrating with intracellular H$_2$O$_2$ by limiting H$_2$O$_2$ diffusion across the membrane. Indeed, such limitations in H$_2$O$_2$ permeability rationalized the initial observation that H$_2$O$_2$ 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 regulators. To test this hypothesis, we constructed GFP-based promoter reporter systems that report on oxidant stress and coregulated metabolic response activation (Fig. 2C 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$_2$O$_2$ 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. 2C 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$_2$O$_2$ (Fig. 2D). We quantified GFP expression induction over a range of H$_2$O$_2$ concentrations spanning six orders of magnitude (1 mM–1 nM), which encompasses the minimum levels of H$_2$O$_2$ reported to fully oxidize OxyR both in vivo (5 μM) 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 μM H$_2$O$_2$ as compared with untreated cells; this finding is consistent with reports of OxyR activation by submicromolar H$_2$O$_2$ (66, 67). GFP expression was increased by two orders of magnitude over that in untreated cells in response to exogenous H$_2$O$_2$ applied at concentrations approaching lethality (100 μM and 1 mM) (67), whereas ampicillin and norfloxacin induced GFP expression most comparable to 10 μM exogenous H$_2$O$_2$, similar to measurements by our enzymatic Amplex Red assay (Fig. 2A).
Because submicromolar H$_2$O$_2$ 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, supraphysiologic H$_2$O$_2$ 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 μM H$_2$O$_2$, we compared microarray gene-expression profiles from untreated wild-type cells with those from cells treated with ampicillin, gentamicin, norfloxacin, or 10 μM H$_2$O$_2$. 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 μM H$_2$O$_2$, suggesting soxS activation by antibiotic-induced superoxide but not by H$_2$O$_2$. Nonetheless, the expression of many genes in both the OxyR and SoxRS regulons was induced similarly by bactericidal antibiotics and 10 μM H$_2$O$_2$ (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$_2$O$_2$-scavenger genes would be activated similarly by 10 μM H$_2$O$_2$. Significant induction of ahpC and katG by supraphysiologic H$_2$O$_2$ doses was reported previously (49); however, we found that neither 10 μM H$_2$O$_2$ 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 μM H$_2$O$_2$ 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 μM H$_2$O$_2$. 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-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$_2$O$_2$ 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 and thioreua 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 compared to chelation by EDTA or nitrito-triacetate (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.
levels, we first tested the hy-
katG pretreatment directly indicates that antibiotic-induced (Fig. 5) 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 H₂O₂ levels, we first tested the hypothesis that increased dosage of H₂O₂-scavenging enzymes would reduce killing by bactericidal antibiotics. We found that increased dosage of bifunctional hydroperoxidase I, KatG [the primary scavenger of H₂O₂ 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 H₂O₂ 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 H₂O₂ at higher concentrations would lead to the induction of endogenous oxidative stress defense mechanisms and also would exert protection against antibiotic treatment. Pretreating cells with 1 mM H₂O₂, 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 H₂O₂ (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 H₂O₂ 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.
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 \( \beta \)-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 the 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 contributors to but not the sole arbiters of antibiotic-mediated killing. This effect also was observed with other clinically relevant \( \beta \)-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 reoxy 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 instance, treating on the specific drug on the strain, 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 perturbation 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 norfl Roxin 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 MG1655spp strain (7). Hpx– MG1655 (ahpCF::kanR, katG, KatE) was constructed for this study. MiC+ was determined as described below. For killing and protection experiments, concentrations used were 1–5× MiC+ 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 MiC+ 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 assembler 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 MG1655spp 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 pLtetO promoter for gene of interest expression control. The APX reporter gene (58) and alleles of katG and ahpCF 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 (58) using previously described primers. E Cell files for the resulting expression profiles were background adjusted and normalized using Robust Multiarray Averageing (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 determined as statistically significant. Regulation for gene sets was determined by regroups 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 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 RNAprotect (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 ST arrays were performed. Additional details are given in SI Appendix.

Antioxidant Pretreatment. The effect of pretreatment with the antioxidants l-glutathione (Sigma) or 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 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, norfl Roxin, 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 RNAprotect (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 ST arrays were performed. Additional details are given in SI Appendix.
incubator shaker to an OD\textsubscript{600} of \textasciitilde{}0.3 before 10-min pretreatment with antibiotics. 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 are in SI Appendix.

H\textsubscript{2}O\textsubscript{2} Pretreatment. The effect of pretreatment with H\textsubscript{2}O\textsubscript{2} (Acros) on antibiotic killing was assayed in MG1655 cells. Cells were grown in 25 mL LB in 250-mL baffled Erlenmeyer flasks at 37 °C and 200 rpm in a light-protected, humidity-controlled incubator shaker to an OD\textsubscript{600} of \textasciitilde{}0.3 before 15-min pretreatment with H\textsubscript{2}O\textsubscript{2} 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. Two 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 \textasciitilde{}1 ppm.


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