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Acknowledgments: This work was made possible by NSF grant EF 0332882 (to A.P.M.W.). Partial support came from the Deutsche Forschungsgemeinschaft (DFG) (CRC TR1, IRTG 1525/1 and WE2231/7-1 to A.P.M.W., EXC 1028 to M.J.L. and A.P.M.W., CRC 680 to M.J.L., and a DFG Mercator Fellowship to G.S.). G.S. appreciates support from NSF (MCB 0925298) and the College of Arts and Sciences, Oklahoma State University (OSU). We thank M. Hanikenne and E. Koonin for helpful discussion, and A. Doust and P. Pelser for advice on phylogenetic analyses. We are grateful to B. Sears for introduction to and assistance with CsCl purification of bisbenzamide-treated nuclear DNA. Some of the computing for this project was performed at the OSU High Performance Computing Center. Sequence data have been deposited at DNA Data Bank of Japan, EMBL, and GenBank under accession ADNM00000000 (SRA012465). The version described in this paper is the first version, ADNM01000000.

Supplementary Materials

www.sciencemag.org/cgi/content/full/339/6124/1207/DC1
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18 October 2012; accepted 8 January 2013
10.1126/science.1231707

Cell Death from Antibiotics Without the Involvement of Reactive Oxygen Species

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Recent observations have suggested that classic antibiotics kill bacteria by stimulating the formation of reactive oxygen species (ROS). If true, this notion might guide new strategies to improve antibiotic efficacy. In this study, the model was directly tested. Contrary to the hypothesis, antibiotic treatment did not accelerate the formation of hydrogen peroxide in *Escherichia coli* and did not elevate intracellular free iron, an essential reactant for the production of lethal damage. Lethality persisted in the absence of oxygen, and DNA repair mutants were not hypersensitive, undermining the idea that toxicity arose from oxidative DNA lesions. We conclude that these antibiotic exposures did not produce ROS and that lethality more likely resulted from the direct inhibition of cell-wall assembly, protein synthesis, and DNA replication.

In recent decades, the growing number of antibiotic-resistant pathogens has spurred efforts to further understand and improve the efficacy of the basic antibiotic classes. Most clinically used antibiotics target cell-wall assembly, protein synthesis, or DNA replication. However, recent reports have raised the possibility that although these antibiotics block growth by directly inhibiting the targets mentioned above, they may owe their lethal effects to the indirect creation of reactive oxygen species (ROS) that then damage bacterial DNA (*1*–*10*).

The evidence supporting this proposal included the observation that cell-penetrating dyes were oxidized more quickly inside antibiotic-treated bacteria (*3*–*8*). Furthermore, iron chelators (*3*, *4*, *7*–*9*), which suppress hydroxyl-radical–generating Fenton chemistry, and thiourea (*4*, *6*, *8*–*10*), a potential scavenger of hydroxyl radicals, lessened toxicity. Mutations that diminish fluxes through the tricarboxylic acid cycle were protective (*3*–*5*), suggesting a key role for respiration, and DNA-repair mutants were somewhat sensitive (*4*, *8*). Systems analysis of aminoglycoside-treated *Escherichia coli* suggested a model that fits these data (*5*). It was postulated that interference with ribosome progression would release incomplete polypeptides, some of which are translocated to the cell membranes where they might trigger envelope stress. The Arc regulatory system is perturbed, potentially accelerating respiration and thereby increasing the flux of superoxide and hydrogen peroxide into the cell interior. These two oxidants have known sequelae that ultimately lead to DNA damage. Specifically, superoxide and hydrogen peroxide damage the iron-sulfur clusters of dehydratases (*11*, *12*), releasing iron atoms and elevating the pool of intracellular unincorporated iron (*13*, *14*). This iron can then react with hydrogen peroxide in the Fenton reaction, generating hydroxyl radicals that either directly damage DNA (*15*) or indirectly oxidize the deoxynucleotide pool, which is subsequently incorporated into DNA (*16*). This scenario could explain the observed oxidation of intracellular dyes, protection by scavengers and chelators, a requirement for respiration, and the sensitivity of DNA-repair mutants.

This model is plausible, so we devised experiments to directly test the molecular events that underpin it. The bacterial strain (*E. coli* MG1655), growth medium (LB), and antibiotic doses were chosen to match those of previous studies (*4*). Kanamycin was used to target translation, ampicillin to block cell-wall synthesis, and norfloxacin to disrupt DNA replication.

Superoxide and hydrogen peroxide are generated inside cells when flavoenzymes inadvertently transfer a fraction of their electron flux directly to molecular oxygen (*15*, *17*). Thus, neither of these ROS can be formed under anoxic

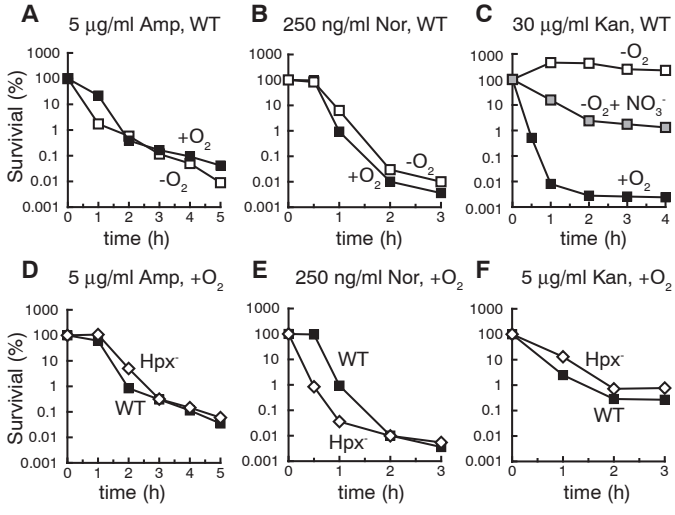

Fig. 1. Antibiotic efficacy does not require oxygen or H_2O_2. (**A** to **C**) Wild-type cells were treated with ampicillin (A), norfloxacin (B), or kanamycin (C) in the presence (solid squares) or absence (open squares) of oxygen. In (C), anoxic killing was also tested in the presence of 40 mM KNO_3 (gray squares). (**D** to **F**) Wild-type cells (solid squares, MG1655) and congenic Hpx^- cells (open diamonds, AL427) were treated with antibiotics ampicillin (D), norfloxacin (E), or kanamycin (F). Results are representative of at least three biological replicates.

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conditions. We found, however, that ampicillin and norfloxacin were as lethal to cells in an anaerobic chamber as to cells in air-saturated medium (Fig. 1, A to C). Anoxia sharply diminished the toxicity of kanamycin, but lethal activity was partially restored at high kanamycin doses when nitrate was provided as an alternative respiratory substrate. This pattern mirrors the effects of oxygen and nitrate upon kanamycin import, which is governed by the magnitude of the proton motive force (*18*, *19*). Thus, ROS were not required for the lethal actions of kanamycin, and they made no apparent contribution at all to the toxicity of ampicillin and norfloxacin.

Hydroxyl radicals are formed inside cells when ferrous iron transfers electrons to H_2O_2, so hydroxyl-radical stress is greatest when either H_2O_2 or ferrous concentrations are high. An *E. coli* mutant that lacks catalase and peroxidase activities (denoted Hpx^-) cannot scavenge H_2O_2 and more easily accumulates it to toxic levels; substantial damage to proteins and to DNA ensues (*12*, *20*, *21*). However, this mutant was not more sensitive to ampicillin or kanamycin than were wild-type cells (Fig. 1, D and F). It was more sensitive to norfloxacin, an effect that might result from the preexisting DNA damage in the scavenger mutant, coupled with the inhibitory effect of norfloxacin on DNA metabolism (*22*) (Fig. 1E).

We directly tested the prediction that antibiotic treatment would augment respiration and thereby increase superoxide and H_2O_2 formation. The respiratory chain is normally a minor source (<20%) of these oxidants (*17*), so a very large flux increase would be needed to substantially amplify total intracellular oxidant production. However, measurements of oxygen consumption with a Clark-type electrode showed that respiration slowly declined, rather than increased, when cells were treated with kanamycin (Fig. 2A). This trend persisted throughout the period during which the number of viable cells declined by >99%. Norfloxacin had little effect on respiration. Ampicillin transiently increased respiration, perhaps by causing envelope damage that dissipated the back-pressure of the proton motive force, before cell lysis ended metabolism. In no case did respiration accelerate enough to be a sufficient explanation for toxicity.

E. coli features a transcription factor, OxyR, that is activated by H_2O_2 whenever its levels approach the concentrations (0.5 to 1 μM) sufficient to damage enzymes or DNA (*23*). The OxyR regulon includes genes whose products assist in H_2O_2 scavenging (*katG* and *ahpCF*), free-iron control (*dps*, *fur*, and *yaaA*), and enzyme protection (*mntH* and *sufABCDE*) (*15*, *24*). When cells were treated with ampicillin and norfloxacin, they accumulated lethal damage within 1 hour; however, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis indicated that OxyR-controlled genes were not induced (Fig. 2B). These antibiotic-treated cells robustly activated *katG*, *ahpC*, and *yaaA* when exogenous H_2O_2 was added as a control. (After 1 hour, kanamycin-treated cells failed to respond even to authentic H_2O_2, perhaps because OxyR protein was depleted in these translationally blocked cells.) This result was confirmed by measurements of *katG::lacZ* expression (Fig. 2, C and D). Thus, neither ampicillin nor norfloxacin create enough H_2O_2 stress to trigger the natural H_2O_2 sensor within the cell.

The rate of intracellular H_2O_2 formation can be directly measured using catalase/peroxidase mutants. Endogenous H_2O_2 rapidly diffuses from these cells out into the growth medium, and the rate of its accumulation can be quantified by direct assay (fig. S1) (*17*). When cells were treated with lethal concentrations of kanamycin and ampicillin, H_2O_2 production did not increase (Fig. 2, E to H). In norfloxacin-treated cells H_2O_2 formation increased slightly (<2-fold) after 1 hour but not at all within the first 30 min when >99% of cells accumulated lethal damage. In contrast, when cells were treated with a nonlethal dose of the redox-cycling drug paraquat, H_2O_2 production was stimulated ~7-fold. We conclude that these classic bactericidal antibiotics did not promote H_2O_2 formation.

There is one other way that hydroxyl-radical formation might be accelerated: by increases in the amount of unincorporated iron. Iron can accumulate inside cells under conditions of superoxide stress, due to the destruction of labile enzymic iron-sulfur clusters (*13*, *14*), and this mechanism was postulated to occur during antibiotic exposure (*4*). We monitored the status of 6-phosphogluconate dehydratase, an iron-sulfur

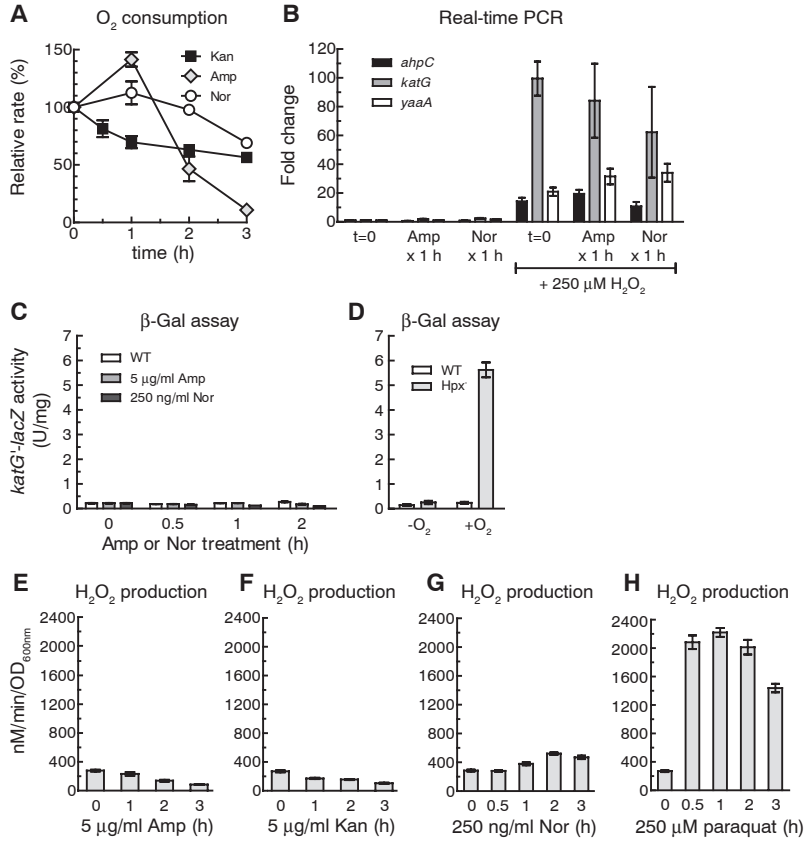

Fig. 2. Antibiotics do not create H_2O_2 stress. (**A**) O_2 consumption rates were not substantially elevated by antibiotic treatment. Oxygen consumption by wild-type cells was measured with a Clark-type electrode before and during exposure to 5 μg/ml kanamycin, 5 μg/ml ampicillin, or 250 ng/ml norfloxacin. Respiration rates were normalized to optical density. (**B**) The OxyR regulon was not induced by ampicillin (5 μg/ml) or norfloxacin (250 ng/ml). mRNA was collected from naïve and antibiotic-exposed wild-type cells, and the expression of OxyR-responsive genes was quantified by qRT-PCR. Signals were normalized to that of the housekeeping gene *gapA*, and fold inductions were calculated. Where indicated, cultures were additionally treated with exogenous H_2O_2 at room temperature for 10 min before mRNA collection. (**C** and **D**) Expression of a *katG'::lacZ* fusion was measured in exponential-phase wild-type cells (AL441) before and after antibiotic incubations (C). As a positive control, expression was measured in wild-type and Hpx^- cells (AL494) before ($-O_2$) and after ($+O_2$) 1-hour aeration (D). (**E** to **H**) Classic antibiotics—ampicillin (E), kanamycin (F), and norfloxacin (G)—did not promote H_2O_2 formation. Aerobic Hpx^- cells (AL427) were treated with antibiotic, and at designated time points samples were collected and the ongoing rate of H_2O_2 production was measured. The redox-cycling compound paraquat (H) was included as a positive control; this dose was insufficient to cause cell death (viable counts increased for >3 hours). Data in this figure are reported as the means and SEM from at least three independent experiments.

enzyme that quickly loses activity in superoxide-stressed cells. We observed that the overall enzyme activity diminished during hours of antibiotic treatment, but the fraction of enzymes with incomplete clusters did not significantly rise (Fig. 3, A to C). In contrast, in control mutants that lack superoxide dismutase, the entire enzyme population was inactive due to cluster damage.

Levels of intracellular unincorporated iron were directly imaged by whole-cell electron paramagnetic resonance (EPR) spectroscopy (*14*). These levels did not rise in kanamycin- or norfloxacin-treated cells (Fig. 3, D and E). (Levels could not be measured after ampicillin treatment, as lysing cells could not be concentrated for the measurement.) In contrast, free-iron levels were increased 4-fold in *fur* regulatory mutants that oversynthesize iron import systems (Fig. 3F). Notably, although these *fur* mutants were slightly more sensitive to ampicillin, they were actually more resistant to kanamycin and norfloxacin (fig. S2). This result, also observed by others (*3*), indicates that intracellular free-iron levels do not correlate with antibiotic sensitivity.

DNA is substantially damaged when hydroxyl radicals are rapidly formed inside cells, and mutants that are deficient in the excision or recombinational repair of oxidative DNA lesions are particularly vulnerable (*15*). The extreme sensitivity of *recA* and *polA* mutants to exogenous H_2O_2 is illustrated in fig. S3A. However, *recA* strains exhibited little or no hypersensitivity to killing doses of kanamycin or ampicillin, suggesting that these antibiotics have little effect upon the rate of DNA damage (Fig. 3, G and H). The *recA* mutants were hypersensitive to norfloxacin (Fig. 3I), as expected, because this antibiotic directly introduces DNA strand breaks by interrupting the catalytic cycle of topoisomerases. The *polA* mutants were actually more resistant to kanamycin and ampicillin than were wild-type strains (fig. S3B), a phenomenon that likely reflects the slower growth rate of this mutant. In sum, these data indicate that, unlike H_2O_2 stress, these antibiotics do not introduce lethal damage into the DNA of cells.

Other workers noted that lower doses (1 to 2 μg/ml) of ampicillin slowly kill *recA* mutants while being merely bacteriostatic to wild-type strains (*4*, *8*). We reproduced that result (fig. S4A). However, this behavior also occurred under anoxic conditions, when ROS are nonexistent (fig. S4B). The slight sensitivity of *recA* mutants may reflect defects in septation from their innate replication problems. At higher doses of ampicillin, i.e., at doses sufficient to kill wild-type bacteria, recombination proficiency had little or no impact on death rate.

Collectively, these data indicate that the known mechanisms of oxidative stress by ROS were not involved in causing the death of antibiotic-treated cells. Oxygen was dispensable for toxicity, and neither OxyR activity nor direct measurements of H_2O_2 formation indicated that ROS were formed at accelerated rates. Further, no customary markers of oxidative damage—enzyme damage, increases in free-iron levels, or hypersensitivity of DNA-repair mutants—were detected.

Fluorescein-based dyes such as hydroxyphenyl fluorescein are more quickly oxidized within antibiotic-treated cells than in control cells, and this fact has been regarded as evidence that hydroxyl radicals are present (*25*). Indeed, thiourea, a presumptive scavenger of hydroxyl radicals, diminished the antibiotic-augmented fluorescence. However, we observed that although thiourea efficiently blocked dye oxidation in a simple in vitro Fenton system, ethanol—another hydroxyl-radical scavenger of similar efficiency (*26*)—failed to do so, even at far higher doses (fig. S5, A and B). This result implies that the dye was actually oxidized by the high-valence iron (FeO^{2+}) initially formed by the Fenton reaction, rather than by the hydroxyl radical to which it decomposes; thiourea, a sulfur compound, may have re-reduced the metal before it oxidized the dye. Indeed, the dye was very rapidly oxidized by horseradish peroxidase (fig. S5C), which has an analogous high-valence metal intermediate but does not release hydroxyl radicals (*27*). The dye was even oxidized by ferricyanide, a ferric-iron chelate of moderate oxidizing potential (+0.44 V) (fig. S5E). Finally, the fluorescence of the oxidized dye

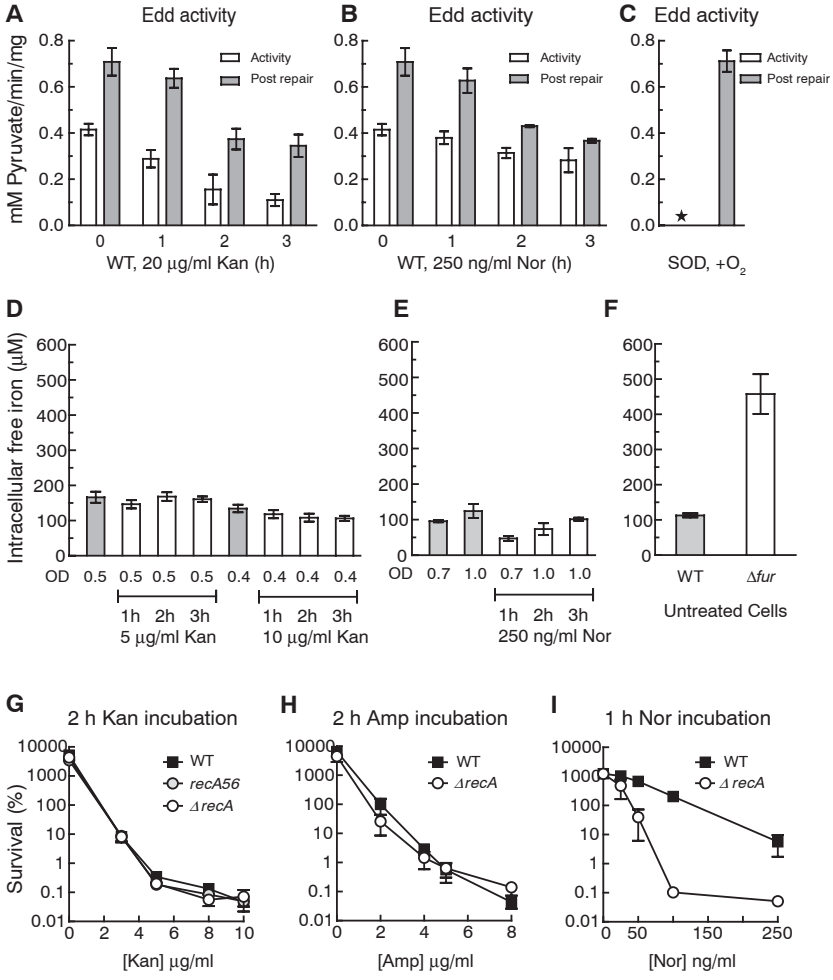

Fig. 3. Antibiotic lethality does not correlate with levels of unincorporated iron. (**A** to **C**) The superoxide-sensitive enzyme Edd was not damaged by cell exposure to kanamycin or norfloxacin. Wild-type cells growing in aerobic LB with 0.2% gluconate were incubated with lethal doses of kanamycin (A) (20 μg/ml) or norfloxacin (B) (250 ng/ml), and Edd activity was measured before (open bar) and after (closed bar) in vitro cluster repair with ferrous iron/dithiothreitol. (C) represents control data from a superoxide dismutase (SOD)–deficient strain (KI232). The star denotes <4% normal activity. (**D** to **F**) Kanamycin (D) and norfloxacin (E) did not increase intracellular iron levels. The levels of intracellular unincorporated iron in wild-type cells were determined by whole-cell EPR analysis before and during antibiotic exposure. Gray bars represent iron levels in untreated cells at similar optical densities. (F) Levels of unincorporated iron were measured in untreated congenic wild-type cells (MG1655) and Δ*fur* mutants (Jem913). (**G** to **I**) DNA-repair mutants are not hypersensitive to antibiotic doses that kill wild-type cells. Wild-type cells (solid squares) and *recA* mutants (open circles) were treated with the indicated doses of kanamycin (G) or ampicillin (H) for 2 hours, or norfloxacin (I) for 1 hour. Fig. S3A demonstrates the hypersensitivity of *recA* mutants to authentic H_2O_2. Data in this figure are reported as the means and SEM from at least three independent experiments.

product was itself quenched after the fact by addition of the same doses of thiourea that were used in antibiotic studies (fig. S5D); this effect cannot be due to hydroxyl-radical scavenging activity. Thus, it is plausible that fluorescein-based dyes are predominantly oxidized in vivo by oxidized enzymic metal centers, which may be more prominent in antibiotic-treated cells as metabolism fails. In any case, one must be cautious in interpreting the oxidation of these dyes inside cells.

Finally, cell protection by either thiourea or iron chelators cannot be regarded as diagnostic of lethal Fenton chemistry. Both agents suppressed antibiotic sensitivity even under anaerobic conditions (fig. S6), when death occurs through pathways that do not involve ROS. We conjecture that they did so by reducing growth and/or metabolic rates. In general, it is unlikely that exogenous agents like thiourea could substantially diminish the lifetimes of hydroxyl radicals in vivo, because these radicals already react at nearly diffusion-limited rates with intracellular biomolecules that are collectively at molar concentrations (*28*). Iron chelators have pleiotropic effects, including the repression of tricarboxylic acid–cycle and respiratory enzymes (*29*), and may thereby diminish the membrane potential that drives aminoglycoside uptake. Synthesis of these enzymes is also repressed when cells are fed glucose, and this treatment analogously diminished kanamycin sensitivity (fig. S7). Thus, it is advisable to follow up experiments that rely upon these agents with ones that employ more direct markers of oxidative stress.

We conclude that under our experimental conditions, these major classes of antibiotics did not exert their lethal actions through the known mechanisms of oxidative stress. Of course, this does not preclude the possibility that antibiotics trigger mechanisms of stress that do not involve the ROS that were tested here.

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Acknowledgments: We thank D. Hassett (University of Cincinnati) for helpful conversations about this topic, and J. Collins, G. Walker, and members of their laboratories for generously sharing ideas, unpublished data, and strains. We also thank M. Nilges of the Illinois EPR Research Center for assistance. This work was supported by GM49060 from the National Institutes of Health. The authors declare no conflict of interest.

Supplementary Materials

www.sciencemag.org/cgi/content/full/339/6124/1210/DC1
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Figs. S1 to S7
Table S1
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14 November 2012; accepted 24 January 2013
10.1126/science.1232751

Killing by Bactericidal Antibiotics Does Not Depend on Reactive Oxygen Species

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Bactericidal antibiotics kill by modulating their respective targets. This traditional view has been challenged by studies that propose an alternative, unified mechanism of killing, whereby toxic reactive oxygen species (ROS) are produced in the presence of antibiotics. We found no correlation between an individual cell's probability of survival in the presence of antibiotic and its level of ROS. An ROS quencher, thiourea, protected cells from antibiotics present at low concentrations, but the effect was observed under anaerobic conditions as well. There was essentially no difference in survival of bacteria treated with various antibiotics under aerobic or anaerobic conditions. This suggests that ROS do not play a role in killing of bacterial pathogens by antibiotics.

Bactericidal antibiotics are an essential component of our antimicrobial arsenal. However, even the best bactericidal antibiotics have limited efficacy against dormant persister cells, specialized survivors that are phenotypic variants of the wild type (*1*, *2*). Bactericidal antibiotics from each of the three main classes have unique mechanisms of killing. Fluoroquinolones convert DNA gyrase/topoisomerase into an endonuclease (*3*); aminoglycosides cause mistranslation, leading to production of toxic peptides (*4*, *5*); and β-lactams lead to autolysis (*6*, *7*). In dormant persisters, targets of these antibiotics have limited activity, resulting in tolerance (*2*). Understanding the detailed mechanism of killing is essential for developing better therapeutics. Recently, an alternative unified mechanism of antibiotic killing was proposed (*8*, *9*), according to which bactericidal compounds, irrespective of their mode of action, induce formation of reactive oxygen species (ROS) by activating the electron transport chain, which kills bacterial cells. The initial study reported that antibiotics induce ROS production and that their quenching by thiourea protects *Escherichia coli* cells from killing (*8*). Subsequent studies report ROS-dependent killing in different bacterial species and detail the mechanism leading to cell death (*10*–*15*). The ROS hypothesis of antibiotic killing became widely accepted but does not account for many observations. For example, mutants lacking ROS production have not been reported among drug resistant clinical isolates, and *Streptococcus pneumoniae*, which is highly susceptible to killing by bactericidal antibiotics, lacks an electron transport chain (*16*, *17*), the proposed source of ROS. Thus, we decided to reexamine the role of ROS in cell death and consequently found that killing by antibiotics is unrelated to ROS production.

Thiourea was reported to protect *E. coli* from killing by norfloxacin, a fluoroquinolone antibiotic (*8*). Norfloxacin was used at a fairly low concentration (0.25 μg/ml), only two to four times as high as the minimal inhibitory concentration (MIC). The peak plasma concentration of norfloxacin has been reported to range from 1.3 to 1.6 μg/ml, with a half-life of 3 to 7 hours (*18*). We therefore examined the effect of thiourea on killing at a range of antibiotic concentrations that included clinically achievable levels.

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