Active Starvation Responses Mediate Antibiotic Tolerance in Biofilms and Nutrient-Limited Bacteria

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studies have implicated nucleosomes as being important for regulation through either their physical location on the genome relative to regulatory sites or their covalent modification to specify docking of regulatory complexes. We extend these examples by describing an extensive interface between a regulatory factor and the core histones of the nucleosome, thereby showing how the nucleosome can be a direct component of regulation.

It is instructive to note how covalent modification of histones affects formation of this complex. Both acetylation of H4K16 and methylation of H3K79 are expected to disrupt several interactions that contribute to the BAH_{Sir3}-NCP interface. Acetylation of K16 is the more important of these modifications in vivo and would disrupt a larger number of molecular interactions based on the structure. Thus, with this complex, covalent modification of histones does not create a docking interface but rather has the potential to disrupt contacts and thereby cause a substantial change in the energetics of interaction.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/334/6058/977/DC1
Materials and Methods
SOM Text
Figs. S1 to S5
Table S1
References (*54*–*68*)

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Active Starvation Responses Mediate Antibiotic Tolerance in Biofilms and Nutrient-Limited Bacteria

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Bacteria become highly tolerant to antibiotics when nutrients are limited. The inactivity of antibiotic targets caused by starvation-induced growth arrest is thought to be a key mechanism producing tolerance. Here we show that the antibiotic tolerance of nutrient-limited and biofilm *Pseudomonas aeruginosa* is mediated by active responses to starvation, rather than by the passive effects of growth arrest. The protective mechanism is controlled by the starvation-signaling stringent response (SR), and our experiments link SR-mediated tolerance to reduced levels of oxidant stress in bacterial cells. Furthermore, inactivating this protective mechanism sensitized biofilms by several orders of magnitude to four different classes of antibiotics and markedly enhanced the efficacy of antibiotic treatment in experimental infections.

In the laboratory, marked antibiotic tolerance can be produced by starving bacteria for nutrients (*1*). Starvation also contributes to tolerance during infection, as nutrients become limited when they are sequestered by host defenses and consumed by proliferating bacteria (*2*, *3*). One of the most important causes of starvation-induced tolerance in vivo is biofilm growth, which occurs in many chronic infections (*4*–*6*). Starvation in biofilms is due to nutrient consumption by cells located on the periphery of biofilm clusters and by reduced diffusion of substrates through the biofilm (*7*). Biofilm bacteria show extreme tolerance to almost all antibiotic classes, and supplying limiting substrates can restore sensitivity (*8*).

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How does starvation produce such pronounced antibiotic tolerance? A leading hypothesis implicates the inactivity of antibiotic targets in growth-arrested cells as a central mechanism (*9*). Target inactivity could block antibiotic action because bactericidal agents subvert their targets to produce toxic products. Thus, if targets are inactive, quinolones will likely generate fewer DNA breaks, aminoglycosides will produce less protein mistranslation, and β-lactams will cause lower levels of peptidoglycan accumulation that trigger cell lysis.

However, growth arrest during starvation occurs in the context of pervasive physiological changes induced by starvation responses. This fact raises the possibilities that tolerance depends on these adaptive responses and that growth arrest and target inactivity per se are not sufficient. Identifying tolerance mechanisms is important to devising new therapeutic strategies. For example, if tolerance is inseparably linked to target inactivity, sensitizing cells could require stimulating bacterial growth, a worrisome approach during infection. Alternatively, if physiological adaptations are critical, disrupting starvation response mechanisms could enhance bacterial killing.

To investigate the relative contributions of growth arrest and starvation physiology to tolerance, we sought experimental conditions in which nutrient-limited cells could be studied in the presence and absence of starvation responses. Many bacterial species sense and respond to nutrient limitation using a regulatory mechanism known as the stringent response (SR). Carbon, amino acid, and iron starvation activate the SR by inducing the *relA* and *spoT* gene products to synthesize the alarmone (p)ppGpp. This signal regulates the expression of many genes and is also involved in virulence (*10*–*12*).

We inactivated the SR by disrupting *relA* and *spoT* in *Pseudomonas aeruginosa,* which causes lethal acute and chronic infections and is a model organism for studying biofilms. SR inactivation eliminated (p)ppGpp production stimulated by the starvation-inducing serine analog, serine hydroxamate (SHX) (Fig. 1A) (*13*). Note that SHX-induced starvation produced a nearly identical pattern of growth arrest in the wild type and Δ*relA spoT* mutant (Fig. 1B). This allowed us to compare antibiotic tolerance in starvation-arrested cells with and without SR-activated responses. In wild-type bacteria, serine starvation reduced the number of bacteria killed by ofloxacin; the difference was ~2300-fold (Fig. 1C). In contrast, serine starvation reduced killing by only ~34-fold in the Δ*relA spoT* mutant (Fig. 1C), despite the fact that growth was arrested in both strains (Fig. 1B).

SHX treatment may not replicate typical starvation physiology, thus we studied stationary-phase cultures and biofilms where nutrient limitation occurs spontaneously (*7*). Whereas stationary-phase growth of wild-type *P. aeruginosa* produced ~10^6 ofloxacin-tolerant bacteria, the Δ*relA spoT* mutant produced <10^4 (Fig. 1D). In biofilms, inactivation of the SR reduced the number of ofloxacin-tolerant cells by a factor of 10^3 (Fig. 1E). The susceptibility of the mutant in stationary phase and biofilms was restored by complementation with wild-type copies of *relA* and *spoT* (Fig. 1, D and E).

A possible explanation for the marked tolerance of wild-type biofilms was that the SR restrained growth and the activity of antibiotic targets under the conditions we tested. However, growth curves of stationary-phase cultures and biofilms revealed that both the wild-type and Δ*relA spoT* mutant strains had ceased growing before antibiotics were added (fig. S1). We also directly measured the activity of functions targeted

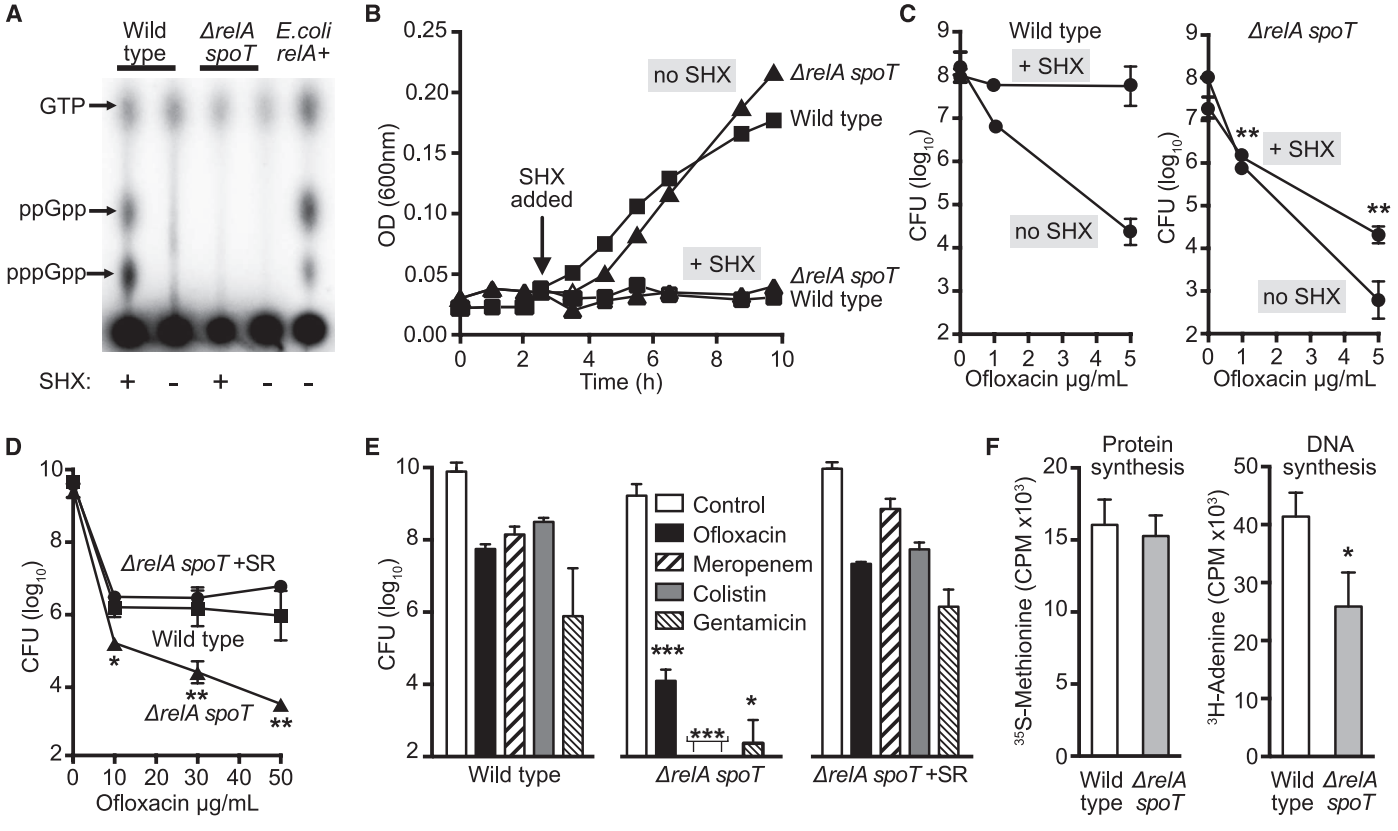


Fig. 1. SR inactivation impairs starvation-induced, stationary-phase, and biofilm antibiotic tolerance. (**A**) Detection of (p)ppGpp by thin-layer chromatography. The *E. coli relA*+ strain expresses an inducible *relA*. (**B**) Growth curves of wild-type and Δ*relA spoT* strains, with and without SHX treatment. OD, optical density (absorbance) at 600 nm. (**C**) Ofloxacin tolerance of log-phase bacteria after SHX-induced starvation. CFU, colony-forming units. Error bars, SD. $^{**}P \leq 0.001$ versus wild type. (**D**) Ofloxacin tolerance of stationary-phase wild-type, Δ*relA spoT*, and Δ*relA spoT* +SR strains. Error bars, SD. $^{*}P \leq 0.05$ or $^{**}P \leq 0.001$ versus wild type. (**E**) Antibiotic killing of biofilms treated with ofloxacin (30 μg/ml), meropenem (300 μg/ml), colistin (300 μg/ml), and gentamicin (50 μg/ml). Error bars, SD. $^{*}P \leq 0.05$ or $^{***}P \leq 0.0005$ versus wild type. (**F**) Rates of protein and DNA synthesis in biofilms measured by [^{35}S]methionine and [^{3}H]adenine incorporation. Error bars, SD. $^{*}P \leq 0.05$ versus wild type.

by several antibiotics at the time of drug treatment. Despite being more sensitive to killing, biofilms formed by the Δ*relA spoT* mutant showed similar rates of protein and RNA synthesis (Fig. 1F and fig. S2) and lower rates of DNA synthesis compared with the wild-type strain (Fig. 1F). These data indicate that reduced drug target activity or growth arrest per se are not responsible for the tolerance of stationary-phase and biofilm bacteria, and that active SR-mediated responses are required.

We decided to focus subsequent work on biofilms because their extreme antibiotic tolerance contributes to the persistence of chronic infections (*4*). The sensitizing effect of SR inactivation was seen with extended treatment times (fig. S3) and in biofilms grown for longer periods (fig. S4). Although SR inactivation sensitized biofilms grown in microtiter wells (fig. S5) and on filters on agar plates (Fig. 1E), we did not see an effect in a reactor system in which medium

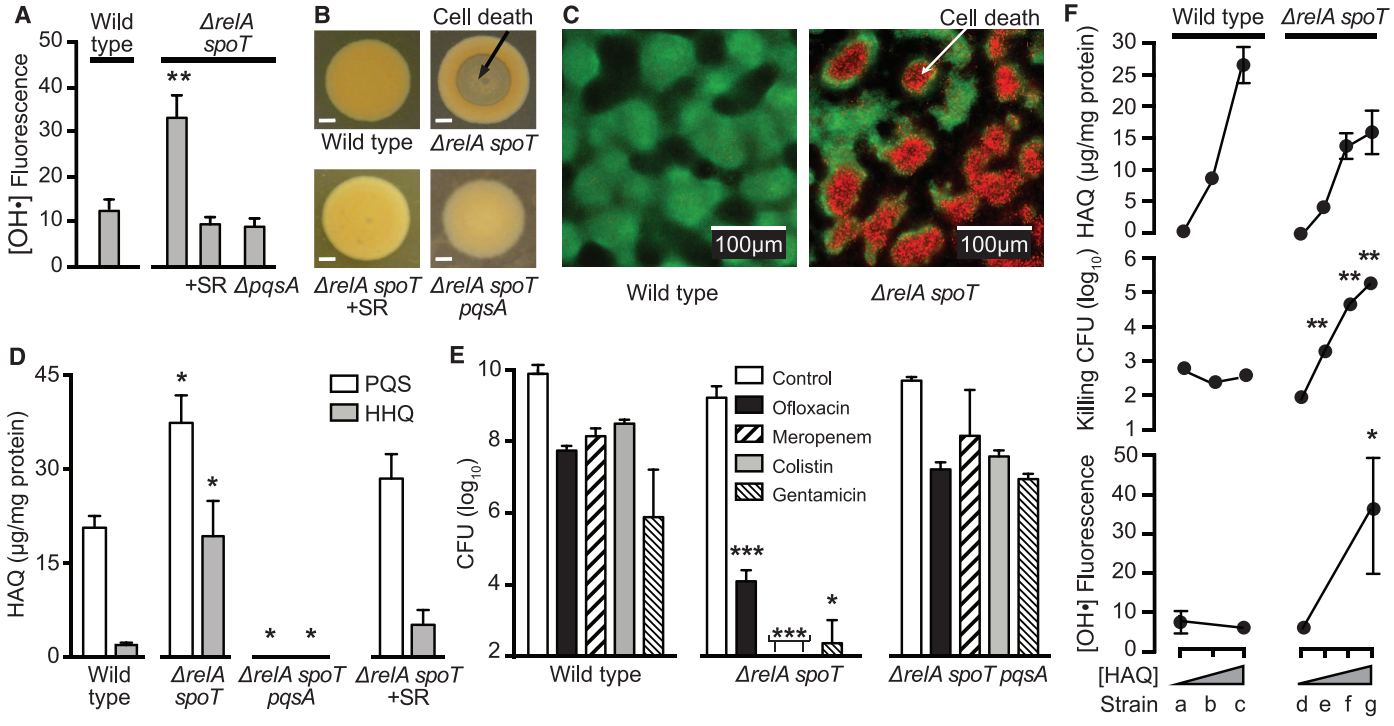
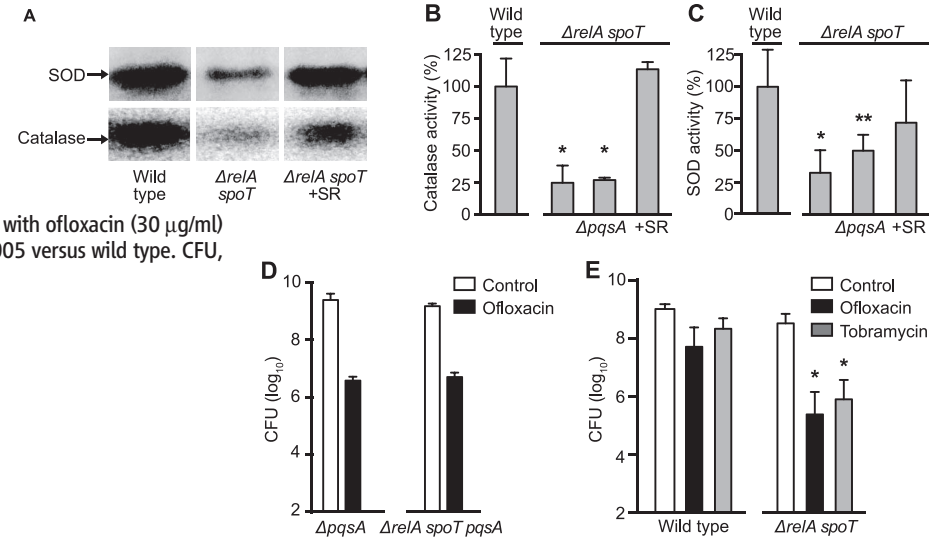


Fig. 2. HAQs mediate antibiotic susceptibility in the Δ*relA spoT* mutant. (**A**) Endogenous levels of hydroxyl radicals (OH•) in biofilms. OH• was measured using the probe HPF (3′-*p*-hydroxyphenyl fluorescein). Error bars, SD. $^{**}P \leq 0.005$ versus wild type. (**B**) Autolysis occurs in the Δ*relA spoT* mutant after prolonged growth on agar (arrow). Scale bar, 2.5 mm. (**C**) Spontaneous cell death in Δ*relA spoT* biofilms detected by viability staining (live cells are green and dead cells red). Images were acquired with the same microscope settings. (**D**) HAQ measurements by LC-MS. Error bars, SD. $^{*}P \leq 0.01$ versus wild type. (**E**) Antibiotic killing of biofilms treated with ofloxacin (30 μg/ml), meropenem (300 μg/ml), colistin (300 μg/ml), and gentamicin (50 μg/ml). CFU, colony-forming units. Error bars, SD. $^{*}P \leq 0.05$ or $^{***}P \leq 0.0005$ versus wild type. (**F**) Relation between HAQ levels, ofloxacin tolerance, and [OH•] in wild-type and Δ*relA spoT* biofilms. Strains producing graded HAQ expression in the wild type include (a) Δ*pqsA* control, (b) wild-type control, and (c) Δ*pqsA pqsA-E+*. Strains producing graded HAQ expression in Δ*relA spoT* include (d) Δ*relA spoT pqsA* control, (e) Δ*relA spoT pqsA pqsA-C+*, (f) Δ*relA spoT* control, and (g) Δ*relA spoT pqsA pqsA-E+*. Error bars, SD. Biofilm killing $^{**}P \leq 0.001$ versus Δ*pqsA* control; OH• levels $^{*}P \leq 0.05$ versus Δ*pqsA* control.

Fig. 3. SR inactivation impairs oxidative defenses. (**A**, **B**, and **C**) SOD and catalase activity in biofilms as measured by native protein activity gel staining (A) and biochemical assays (B and C). Error bars, SD. An image of intact gels from (A) is shown in fig. S11. $^{*}P \leq 0.05$ or $^{**}P \leq 0.001$ versus wild type. (**D**) Biofilms lacking HAQs show similar ofloxacin tolerance with or without an intact SR. Error bars, SD. (**E**) Antibiotic tolerance in *E. coli* biofilms treated with ofloxacin (30 μg/ml) and tobramycin (50 μg/ml). Error bars, SD. $^{**}P \leq 0.005$ versus wild type. CFU, colony-forming units.



flowed continuously (fig. S6). We also measured sensitivity to antibiotics with four different mechanisms of action and found that SR inactivation increased the number of bacteria killed by a factor of 10^2 to 10^5 in both the laboratory strain and clinical isolates (Fig. 1E and fig. S7).

Our finding that the SR mediated resistance to drugs that interact with different cellular targets suggested that it disrupts a killing mechanism common to diverse agents. Recent work indicates that, regardless of their primary targets, bactericidal antibiotics induce hydroxyl radical (OH•) production and kill cells by oxidative damage (*14*–*16*). This finding led us to hypothesize that SR inactivation might sensitize biofilms by increasing endogenous oxidative stress. We found that SR inactivation raised OH• levels in biofilms (Fig. 2A) and increased biofilm killing by the oxidants paraquat and phenazine methosulfate (fig. S8), which is also consistent with increased endogenous oxidant production.

What could account for the increased endogenous oxidative stress in the Δ*relA spoT* mutant? A clue about the mechanism emerged when we noted spontaneous cell death in the central areas of Δ*relA spoT* colonies (Fig. 2B) and biofilm clusters (Fig. 2C). Previous work linked this autolysis phenotype to the overproduction of 4-hydroxy-2-alkylquinoline molecules (HAQs) by *P. aeruginosa* (*17*).

HAQs function in intercellular signaling and iron chelation (*18*–*20*). HAQs also have prooxidant effects, and overexpressing HAQs in wild-type *P. aeruginosa* modestly increased susceptibility to antibiotics [~25% more killing by ciprofloxacin (*21*)]. Liquid chromatography–mass spectrometry (LC-MS) analysis confirmed that the Δ*relA spoT* mutant produced higher levels of HAQs than the wild-type strain (Fig. 2D). Of note, the Δ*relA spoT* mutant was deficient in production of prooxidant phenazines (*22*) (fig. S9), which made it unlikely that these molecules caused oxidative stress in the Δ*relA spoT* mutant.

To investigate whether HAQ overproduction mediated the antibiotic sensitivity of Δ*relA spoT* mutant biofilms, we inactivated *pqsA* and thus eliminated HAQ biosynthesis in this strain (Fig. 2D). Notably, wild-type levels of tolerance to ofloxacin, colistin, gentamicin, and meropenem were restored (Fig. 2E). Disrupting *pqsA* in the Δ*relA spoT* mutant also abolished autolysis of colonies (Fig. 2B) and restored wild-type OH• levels in biofilms (Fig. 2A). We used gene expression constructs that generated varying amounts of HAQs (*23*) to determine whether a dose-response relation existed between HAQs and antibiotic susceptibility. As shown in Fig. 2F, modest increases in HAQ levels substantially enhanced antibiotic sensitivity in Δ*relA spoT* biofilms. HAQ expression also increased OH• levels in Δ*relA spoT* biofilms (Fig. 2F).

The SR has pleiotropic effects on bacterial physiology. Thus, we considered the possibility that antibiotic sensitivity depends on other defects produced by SR inactivation, in addition to elevating HAQs. To test this, we expressed the HAQ gene constructs described above in wild-type *P. aeruginosa*. In contrast to the sensitivity produced in the Δ*relA spoT* mutant, progressive increases in HAQ levels had minimal effects on antibiotic susceptibility in bacteria with an intact SR, even though higher HAQ levels were achieved (Fig. 2F and fig. S10). Expressing HAQs in wild-type biofilms also failed to increase OH• levels (Fig. 2F)

The different responses of wild-type and Δ*relA spoT* biofilms to high HAQ levels led us to hypothesize that the mutant had impaired antioxidant defenses, as this defect could sensitize cells to the prooxidant effect of HAQs. We measured catalase and superoxide dismutase (SOD) activity in biofilms and found that SR inactivation significantly decreased both (Fig. 3, A to C, and fig. S11). SOD and catalase levels were also low in the Δ*relA spoT pqsA* triple mutant (Fig. 3, B and C), thus impaired oxidant defenses were independent of HAQ overproduction. These findings suggest that both impaired antioxidant defenses and HAQ overproduction are required for antibiotic sensitivity.

To test this idea further, we compared the antibiotic susceptibility of Δ*pqsA* and Δ*relA spoT pqsA* mutant biofilms and found no difference (Fig. 3D). This comparison was informative as neither strain expressed HAQs, but Δ*pqsA* biofilms produce SOD and catalase at near wild-type levels (fig. S12), whereas SOD and catalase are low in Δ*relA spoT pqsA* biofilms (Fig. 3, B and C). These data show that isolated increases in HAQ levels or decreases in SOD and/or catalase activity fail to change antibiotic susceptibility in the biofilm conditions we tested. Taken together, the data are consistent with a model in which the SR mediates the antibiotic tolerance of *P. aeruginosa* biofilms by both curtailing HAQ production and inducing antioxidant defenses (fig. S13).

Although the SR is conserved in almost all Gram-positive and Gram-negative bacteria, HAQ biosynthetic genes are not. This led us to investigate whether the SR mediated tolerance in species that do not produce HAQs. Inactivation of *relA spoT* in *Escherichia coli* decreased the number of antibiotic-tolerant bacteria by over 65-fold (Fig. 3E). The *E. coli* Δ*relA spoT* mutant biofilms also had reduced catalase and elevated OH• levels (fig. S14). These results show that the SR mediates biofilm tolerance in another Gram-negative pathogen, in addition to *P. aeruginosa*, and raises

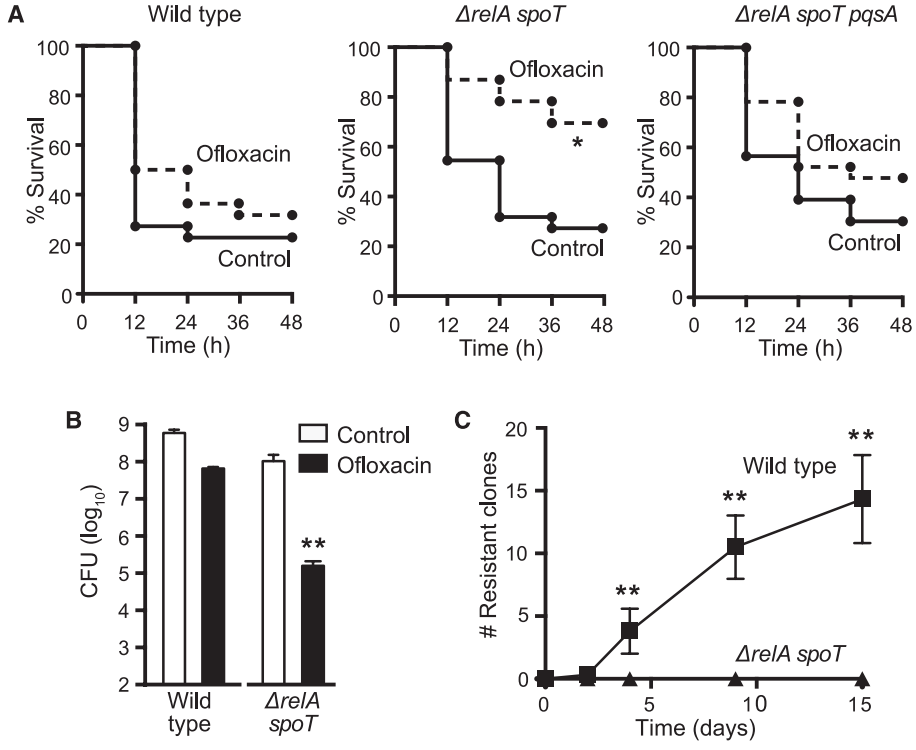


Fig. 4. SR inactivation improves antibiotic efficacy in murine infections and blocks the emergence of resistant mutants. (**A**) Ofloxacin treatment is more effective against lethal infections produced by the Δ*relA spoT* strain than in infections caused by wild-type or Δ*relA spoT pqsA P. aeruginosa*. Graphs represent pooled data from three independent experiments, with at least 15 mice per group. $^{**}P \leq 0.005$ versus treated wild-type infections. (**B**) Ofloxacin treatment is more effective in subcutaneous biofilm infections if the SR is inactivated. CFU, colony-forming units. Graphs represent pooled data from two independent experiments, with at least six mice per group. Error bars, SEM. $^{**}P \leq 0.001$ versus treated wild-type infections. (**C**) Resistant mutants emerge after prolonged exposure to ofloxacin in the wild type but not the Δ*relA spoT* strain. $^{**}P \leq 0.005$ versus wild type.

the possibility that the control of oxidant stress may be a common mechanism.

To investigate the effect of targeting the SR to increase antibiotic activity in lethal infections, we infected mice with stationary-phase *P. aeruginosa*. Whereas ofloxacin failed to increase the survival of mice infected with wild-type bacteria, it was highly effective against the Δ*relA spoT* strain (Fig. 4A). Furthermore, eliminating HAQ biosynthesis abolished the susceptibility of the mutant in vivo (Fig. 4A), as was seen in vitro (Fig. 2E). Inactivation of the SR also increased antibiotic activity in a murine bioflm model (Fig. 4B). Finally, because tolerance allows bacteria to survive sustained drug exposure, tolerant subpopulations are thought to be an important source of genetic antibiotic-resistant mutants (*9*, *24*). As shown in Fig. 4C, SR inactivation eliminated the emergence of ofloxacin-resistant clones in conditions promoting adaptive resistance.

Whether cells recognize it or not, starvation will eventually stop growth and the activity of antibiotic targets. However, the capacity to sense and respond to starvation allows bacteria to arrest growth in a regulated manner that maximizes chances for long-term survival. Our data show that interfering with this orderly process sensitizes experimentally starved, stationary-phase, and biofilm bacteria to antibiotics, without stimulating their growth. Furthermore, our experiments suggest that starvation responses protect by curtailing the production of prooxidant metabolites and increasing antioxidant defenses. Thus, antibiotic-tolerant states may depend on physiological adaptations without direct connections to antibiotic target activity or to drug uptake, efflux, or inactivation. Identifying these adaptations, and targeting them to enhance the activity of existing drugs, is a promising approach to mitigate the public health crisis caused by the scarcity of new antibiotics.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S14
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H_2S: A Universal Defense Against Antibiotics in Bacteria

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Many prokaryotic species generate hydrogen sulfide (H_2S) in their natural environments. However, the biochemistry and physiological role of this gas in nonsulfur bacteria remain largely unknown. Here we demonstrate that inactivation of putative cystathionine β-synthase, cystathionine γ-lyase, or 3-mercaptopyruvate sulfurtransferase in *Bacillus anthracis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* suppresses H_2S production, rendering these pathogens highly sensitive to a multitude of antibiotics. Exogenous H_2S suppresses this effect. Moreover, in bacteria that normally produce H_2S and nitric oxide, these two gases act synergistically to sustain growth. The mechanism of gas-mediated antibiotic resistance relies on mitigation of oxidative stress imposed by antibiotics.

Until recently H_2S has been known merely as a toxic gas. It is now associated with beneficial functions in mammals from vasorelaxation, cardioprotection, and neurotransmission to anti-inflammatory action in the gastrointestinal tract (*1*–*3*). The ability of H_2S to function as a signaling molecule parallels the action of another established gasotransmitter, nitric oxide (NO) (*3*–*5*). Like NO, H_2S is produced enzymatically in various tissues (*1*–*3*). Three H_2S-generating enzymes have been characterized in mammals: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST). CBS and CSE produce H_2S predominantly from L-cyst(e)ine (Cys). 3MST does so via the intermediate synthesis of 3-mercaptopyruvate produced by cysteine aminotranferase (CAT), which is inhibited by aspartate (Asp) competition for Cys on CAT (*1*) (fig. S1).

In contrast to mammal-derived H_2S, bacteria-derived H_2S has been known for centuries but was considered to be only a byproduct of sulfur metabolism, with no particular physiological function in nonsulfur microorganisms. Likewise, little is known about the metabolic pathways involving H_2S in mesophilic bacteria. However, analysis of bacterial genomes has revealed that most, if not all, have orthologs of mammalian CBS, CSE, or 3MST (figs. S1 and S2), which suggested an important cellular function(s) that preserved these genes throughout bacterial evolution. We became interested in the role of these enzymes after establishing that endogenous NO protects certain Gram-positive bacteria against antibiotics and oxidative stress (*6*–*8*). Considering some functional similarities between mammalian gasotransmitters (*1*–*3*), we hypothesized that bacterial H_2S may, similarly, be cytoprotective.

To determine whether CBS, CSE, or 3MST produces H_2S in bacteria, we inactivated each enzyme genetically or chemically in four clinically relevant and evolutionarily distant pathogenic species: *Bacillus anthracis* (Sterne), *Pseudomonas aeruginosa* (PA14), *Staphylococcus aureus* (MSSA RN4220 and MRSA MW2), and *Escherichia coli* (MG1655). The first three species have the CBS/CSE operon, but not 3MST, whereas *E. coli* carries 3MST, but not CBS/CSE. The chromosomal organization of H_2S genes (fig. S3) and the strategy we used for their replacement prevented any polar effects. We monitored H_2S production in wild-type (wt) and mutant cells using lead acetate [$Pb(Ac)_2$], which reacts

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