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Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria

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Abstract

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 (1). 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 (2). Starvation also contributes to tolerance during infection, as nutrients become limited when they are sequestered by host defenses and consumed by proliferating bacteria (3, 4). One of the most important causes of starvation-induced tolerance *in vivo* is biofilm growth, which occurs in many chronic infections (5–7). Starvation in biofilms is due to

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nutrient consumption by cells located on the periphery of biofilm clusters, and reduced diffusion of substrates through the biofilm (8). Biofilm bacteria show extreme tolerance to almost all antibiotic classes, and supplying limiting substrates can restore sensitivity (9).

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. 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 triggers cell lysis.

However, growth arrest during starvation occurs in the context of pervasive physiological changes induced by starvation responses. This fact raises the possibility that tolerance depends upon 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). Importantly, 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 by ~2,300 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 (8). Whereas stationary phase growth of wild-type *P. aeruginosa* produced ~ 10^6 ofloxacin-tolerant bacteria, the Δ*relA spoT* mutant produced less than 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 1D and 1E).

A possible explanation for the marked tolerance of wild type biofilms was that the SR restrained growth, or the activity of antibiotic targets in 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 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 S2), and lower rates of DNA synthesis compared to the wild-type strain (Fig 1F). These data indicated 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 (5). The sensitizing effect of SR inactivation was seen with extended treatment times (Fig S3) and in biofilms grown for longer periods (Fig S4). While SR inactivation sensitized biofilms grown in microtiter wells (Fig S5), we did not see an effect in a reactor system employing continuously flowing media (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 10^2–10^5 in both the laboratory strain and clinical isolates (Figs 1E and S7).

The fact that the SR mediates 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-alkylquinolines molecules (HAQs) by *P. aeruginosa* (17). HAQs function in intercellular signaling and iron chelation (18–20). HAQs also have pro-oxidant effects, and overexpressing HAQs in wild-type *P. aeruginosa* modestly increased susceptibility to antibiotics (e.g. ~25% more killing by ciprofloxacin) (21). Liquid chromatography-mass spectrometry 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 pro-oxidant phenazines (22) (Fig S9), making 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*, thus eliminating HAQs biosynthesis in the Δ*relA spoT* strain (Fig 2D). Remarkably, 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 if a dose-response relationship 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 HAQs 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 S10). Interestingly, 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 HAQs levels led us to hypothesize that the mutant had impaired anti-oxidant defenses, as this defect could sensitize cells to the pro-oxidant effect of HAQs. We measured catalase and superoxide dismutase (SOD) activity in biofilms, and found that SR inactivation significantly decreased both (Fig 3A–C and S11). SOD and catalase levels were also low in the Δ*relA spoT pqsA* triple mutant (Fig 3B–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 3B–C). These data show that isolated increases in HAQ levels, or decreases in SOD/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 anti-oxidant defenses (Fig S13).

While 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 Δ*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 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 (1, 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 pro-oxidant metabolites and increasing anti-oxidant defenses. Thus, antibiotic-tolerant states may depend upon physiological adaptations without direct connections to antibiotic target activity; or 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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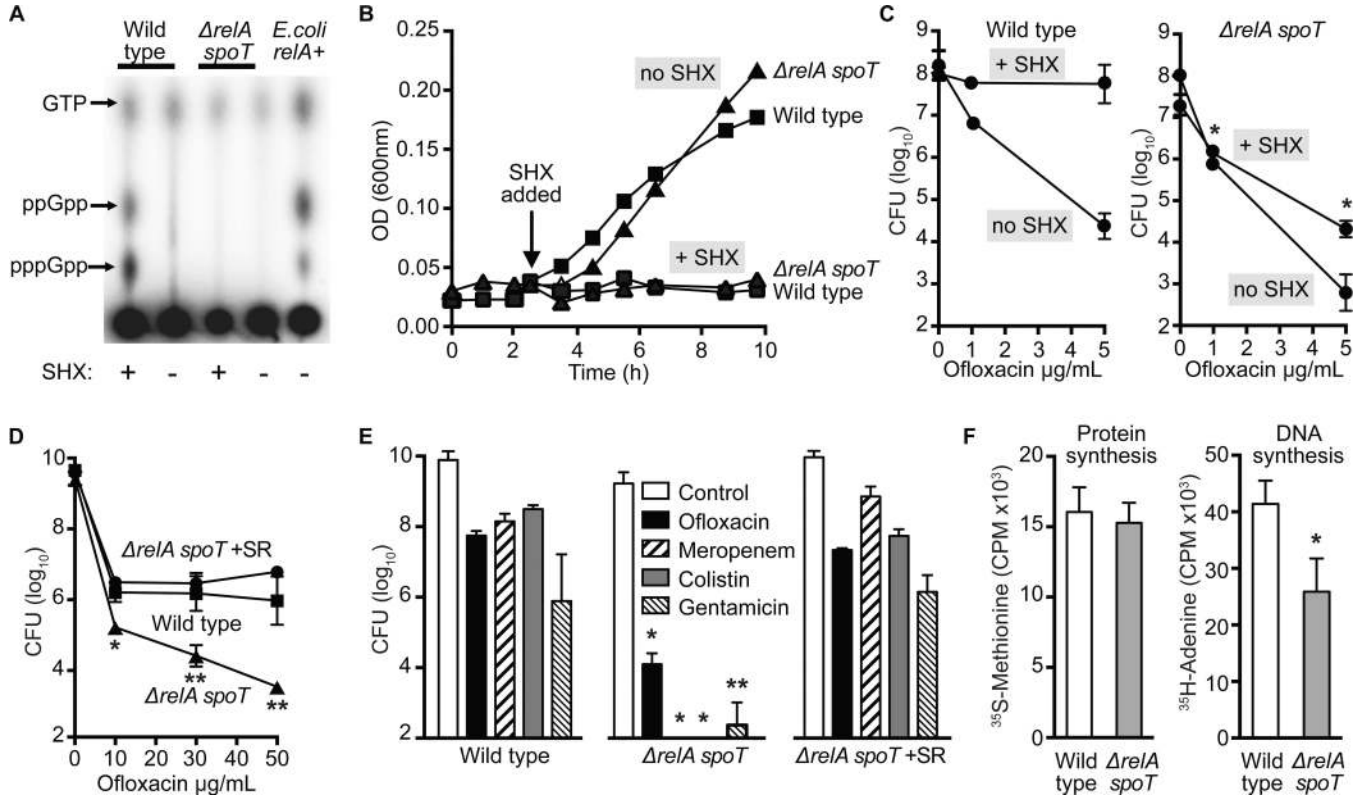

Figure 1. SR inactivation impairs starvation-induced, stationary phase, and biofilm antibiotic tolerance

A. Detection of (p)ppGpp by thin layer chromatography. The *E.coli relA*+ strain expresses an inducible *relA*.

B. Growth curves of wild type (⑤) and Δ*relA spoT* (⑩) strains, with and without SHX treatment.

C. Ofloxacin tolerance of log-phase bacteria following SHX-induced starvation. Error bars indicate SD. P ≤0.001 (*) versus wild type.

D. Ofloxacin tolerance of stationary-phase wild-type (⑤), Δ*relA spoT* (⑩) and Δ*relA spoT* +SR (③) strains. Error bars indicate SD. P ≤0.05 (*) or ≤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 indicate SD. P ≤0.0005 (*) or ≤0.05 (**) versus wild type.

F. Rates of protein and DNA synthesis in biofilms measured by S^{35}-methionine and H^3-adenine incorporation. Error bars indicate SD. P ≤0.05 (*) versus wild type.

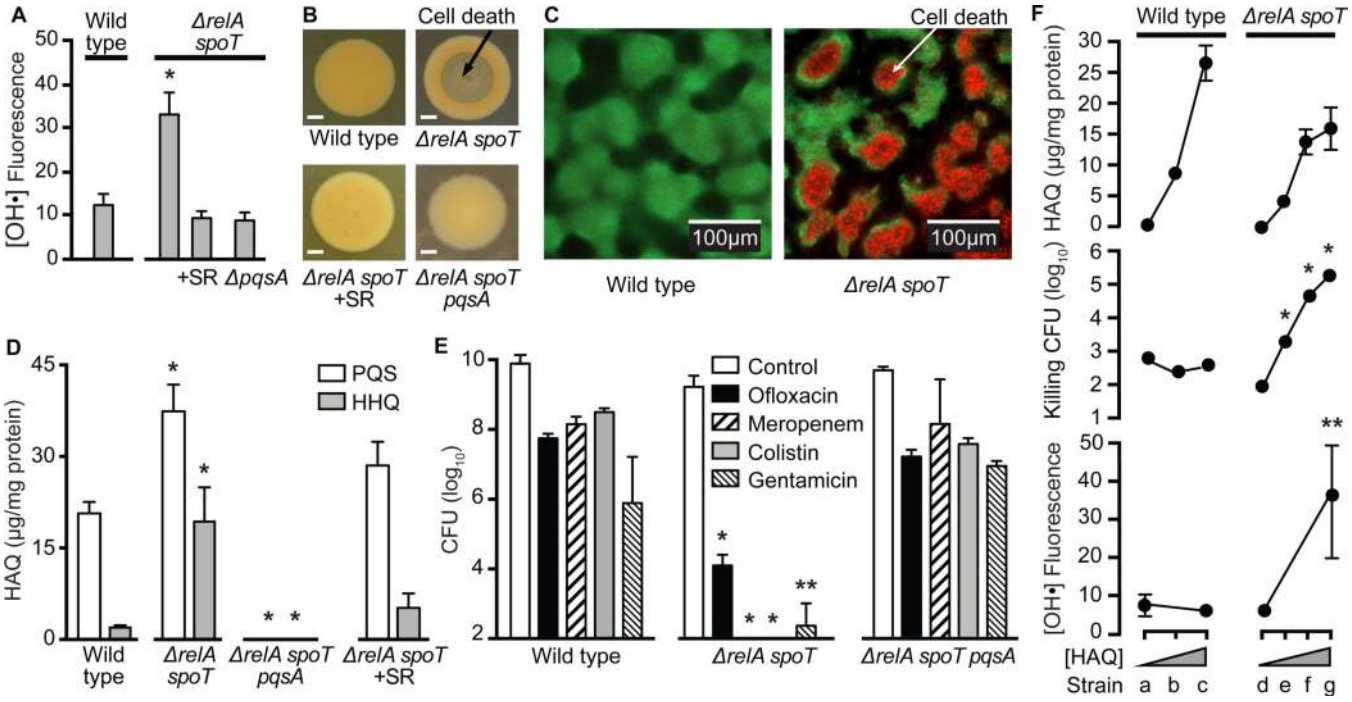

Figure 2. HAQs mediate antibiotic susceptibility in the Δ*relA spoT* mutant

A. Endogenous levels of hydroxyl radicals (OH•) in biofilms. OH• was measured using the probe HPF (3'-p-hydroxyphenyl fluorescein). Error bars indicate SD. P ≤0.005 (*) versus wild type.

B. Autolysis occurs in the Δ*relA spoT* mutant after prolonged growth on agar (arrow). Scale bar indicates 2.5 mm.

C. Spontaneous cell death in Δ*relA spoT* biofilms detected by viability staining (live cells are green, dead cells red). Images were acquired with the same microscope settings.

D. HAQ measurements by LC/MS. Error bars indicate SD. P ≤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). Error bars indicate SD. P ≤0.0005 (*) or ≤0.05 (**) versus wild type.

F. Relationship 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* ctrl, b) wild type ctrl, c) Δ*pqsA pqsA-E*+. Strains producing graded HAQ expression in Δ*relA spoT* include: d) Δ*relA spoT pqsA* ctrl, e) Δ*relA spoT pqsA pqsA-C*+, f) Δ*relA spoT* ctrl, g) Δ*relA spoT pqsA pqsA-E*+. Error bars indicate SD. Biofilm killing P ≤0.001 (*) versus Δ*pqsA* ctrl; OH• levels P ≤0.05 (**) versus Δ*pqsA* ctrl.

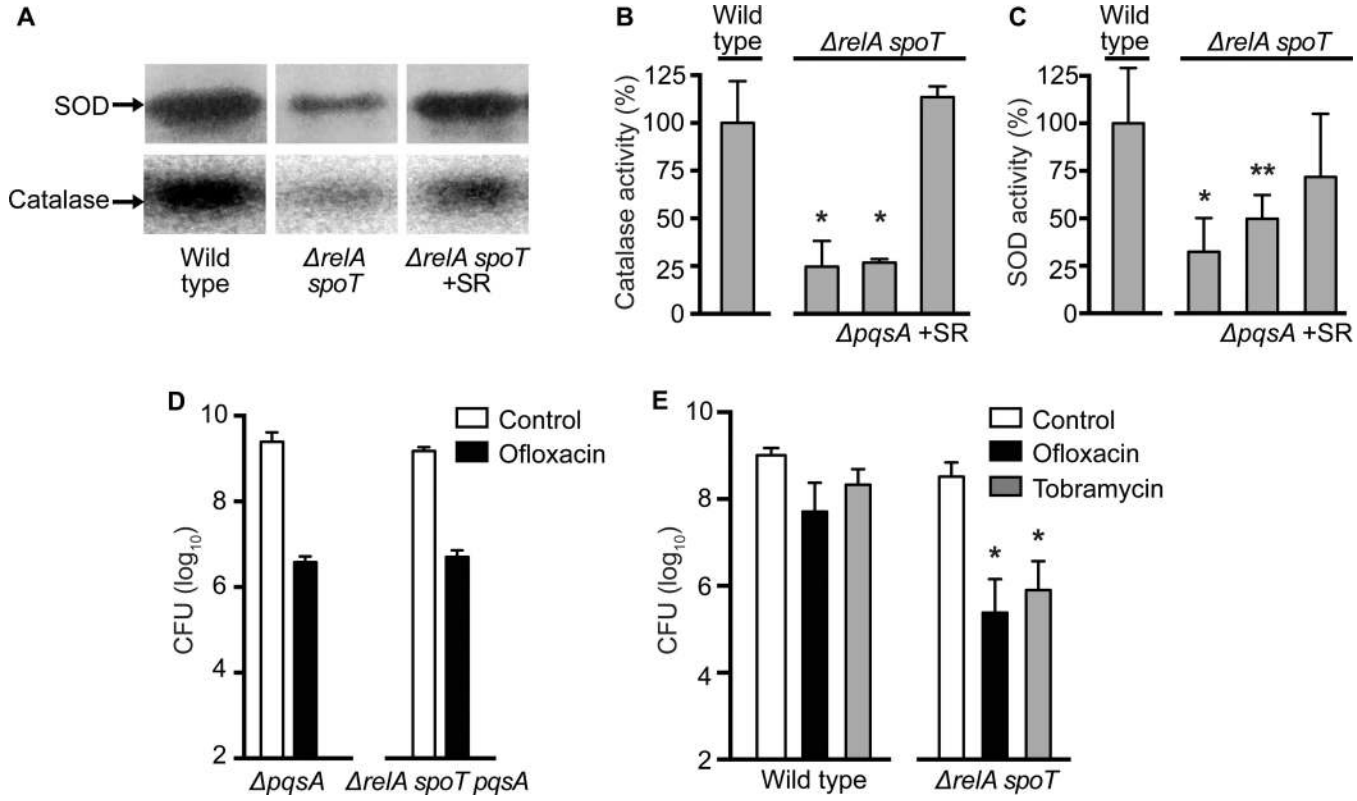

Figure 3. SR inactivation impairs oxidative defenses

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 indicate SD. An image of intact gels from (A) are shown in Fig S11. P ≤0.001 (*), P ≤0.05 (**) versus the wild type.

D. Biofilms lacking HAQs show similar ofloxacin tolerance with or without an intact SR. Error bars indicate SD. P ≤0.001 (*) versus Δ*pqsA*.

E. Antibiotic tolerance in *E.coli* biofilms treated with ofloxacin (30 μg/mL) and tobramycin (50 μg/mL). Error bars indicate SD. P ≤0.005 (*) versus wild type.

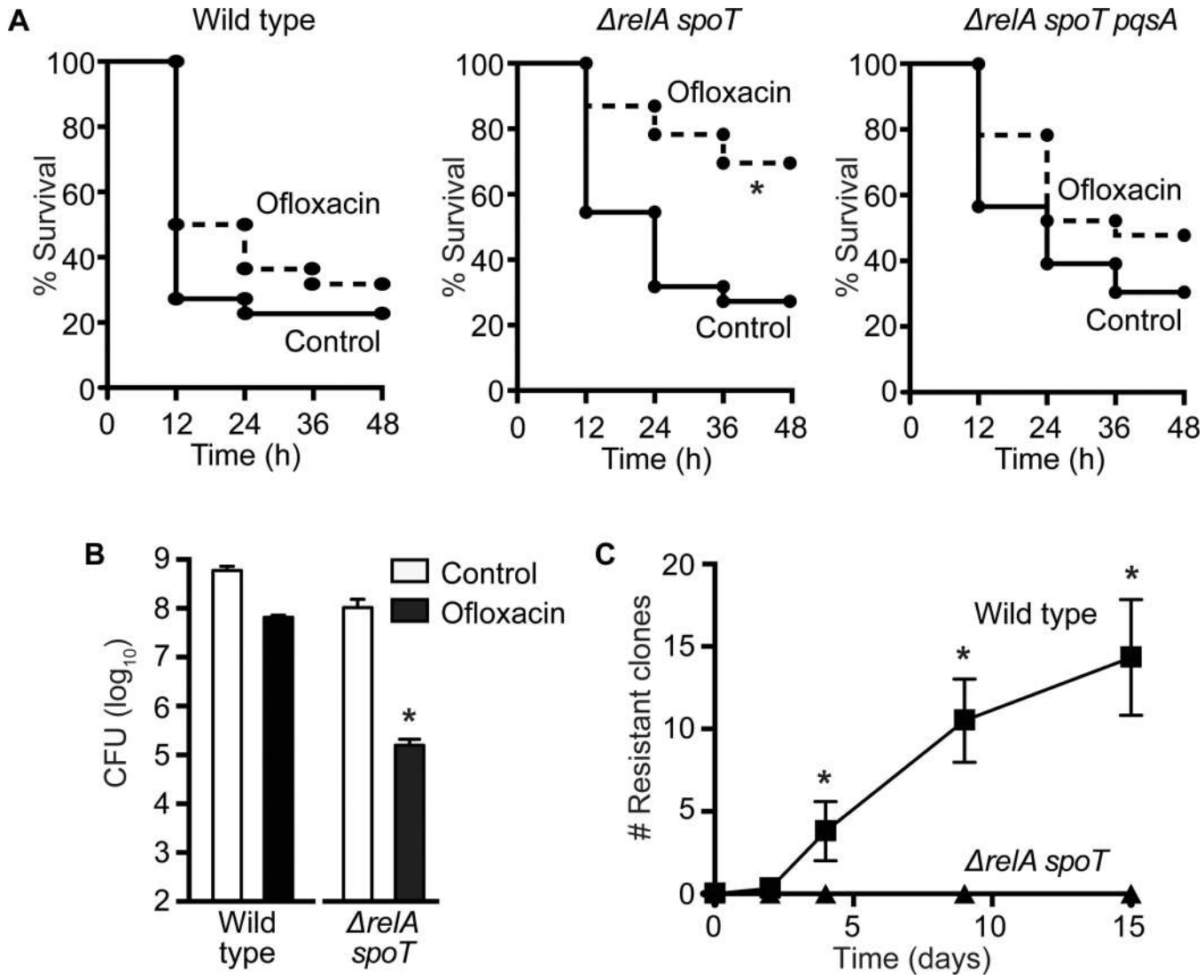

Figure 4. SR inactivation improves antibiotic efficacy in murine infections, and blocks the emergence of resistant mutants

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 ≤0.005 (*) versus treated wild-type infections.

B. Ofloxacin treatment is more effective in subcutaneous biofilm infections if the SR is inactivated. Graphs represent pooled data from two independent experiments, with at least six mice per group. Error bars indicate SEM. P ≤0.001 (*) versus treated wild-type infections.

C. Resistant mutants emerge after prolonged exposure to ofloxacin in the wild-type (⑤), but not the Δ*relA spoT* strain (⑩). P ≤0.005 (*) versus wild type.