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The Stringent Response Controls Catalases in *Pseudomonas aeruginosa* and Is Required for Hydrogen Peroxide and Antibiotic Tolerance

Malika Khakimova,[a] Heather G. Ahlgren,[b] Joe J. Harrison,[c]* Ann M. English,[d] Dao Nguyen[a,b,e]

Department of Microbiology and Immunology, McGill University, Quebec, Canada[a]; Department of Experimental Medicine, McGill University, Quebec, Canada[b]; Department of Microbiology, University of Washington, Seattle, Washington, USA[c]; Department of Chemistry and Biochemistry, Concordia University, Montreal, Quebec, Canada[d]; Department of Medicine, McGill University, Quebec, Canada[e]

***Pseudomonas aeruginosa*, a human opportunistic pathogen, possesses a number of antioxidant defense enzymes under the control of multiple regulatory systems. We recently reported that inactivation of the *P. aeruginosa* stringent response (SR), a starvation stress response controlled by the alarmone (p)ppGpp, caused impaired antioxidant defenses and antibiotic tolerance. Since catalases are key antioxidant enzymes in *P. aeruginosa*, we compared the levels of H_2O_2 susceptibility and catalase activity in *P. aeruginosa* wild-type and Δ*relA* Δ*spoT* (ΔSR) mutant cells. We found that the SR was required for optimal catalase activity and mediated H_2O_2 tolerance during both planktonic and biofilm growth. Upon amino acid starvation, induction of the SR upregulated catalase activity. Full expression of *katA* and *katB* also required the SR, and this regulation occurred through both RpoS-independent and RpoS-dependent mechanisms. Furthermore, overexpression of *katA* was sufficient to restore H_2O_2 tolerance and to partially rescue the antibiotic tolerance of ΔSR cells. All together, these results suggest that the SR regulates catalases and that this is an important mechanism in protecting nutrient-starved and biofilm bacteria from H_2O_2- and antibiotic-mediated killing.**

Reactive oxygen species (ROS) are spontaneously produced during aerobic respiration. During infections, bacteria are also challenged by ROS produced by host phagocytic cells. Given that ROS can readily damage membranes, DNA, and proteins (1, 2), bacteria possess multiple antioxidant defenses to survive during aerobic growth and *in vivo*. While it is expected that oxidative stress induces antioxidant defenses, interestingly, other stresses such as nutrient limitation also elicit antioxidant responses. For example, carbon and nitrogen starvation increases resistance to H_2O_2 in different bacterial species, but the mechanism remains poorly understood (3–6). Stationary-phase and biofilm bacteria are also nutrient limited and exhibit high oxidant tolerance (7–9). Does nutrient limitation induce starvation responses that also confer protection against oxidative stress? To investigate the contribution of starvation responses in inducing antioxidant defenses, we examined the role of the stringent response (SR).

The SR, which is controlled by the alarmone (p)ppGpp, is a conserved regulatory mechanism that coordinates physiological adaptations to nutrient starvation and other stresses. In Gram-negative bacteria, synthesis of (p)ppGpp is catalyzed by the RelA and SpoT proteins (10, 11). This alarmone primarily modulates gene transcription to shut down biosynthesis of macromolecules and cell replication while inducing mechanisms required for stress survival (12, 13). Although there are several reports that nutrient starvation confers oxidative stress tolerance (3–6), only a few studies have explored the link between the SR and control of oxidative-stress pathways (14, 15).

Pseudomonas aeruginosa is an opportunistic human pathogen that causes lethal acute infections and chronic biofilm infections in the airways of cystic fibrosis patients. *P. aeruginosa* has several enzymes that can detoxify H_2O_2, including two monofunctional catalases, KatA and KatB. Catalases are key components of antioxidant defenses: they are enzymatic H_2O_2 scavengers that can be highly induced (2, 16). Constitutively expressed, KatA is the dominant catalase during exponential- and stationary-phase growth. It is also required for H_2O_2 resistance and *in vivo* virulence (17, 18). In contrast, *katB* is induced by exogenous H_2O_2 stress and likely contributes to acquired H_2O_2 resistance (19). The *P. aeruginosa* *katE* (*katC*) and *katN* genes also encode putative catalases, but their functional roles remain unclear (17, 20).

Antioxidant defense genes in bacteria are regulated by several overlapping systems. These systems have been extensively studied in *Escherichia coli* and, to a lesser extent, in *P. aeruginosa* (1, 2). For example, in *P. aeruginosa*, OxyR is a H_2O_2-responsive transcriptional regulator that activates expression of a subset of genes involved in antioxidant defense (*katA*, *katB*, *ahpB*, and *ahpCF*) as well as in iron homeostasis (21–25). Additional global regulators also control expression of antioxidant enzymes, including the Las and Rhl quorum-sensing systems (26, 27), RpoS (28–30), and the iron uptake regulator Fur (31). Because *katA* expression is under quorum-sensing control, its expression increases rapidly upon entry into the stationary phase (26, 32). These overlapping regulatory networks allow *P. aeruginosa* to adapt its oxidative defense systems in response to different environmental conditions such as the growth phase and iron availability.

We have recently reported that SR inactivation in *P. aeruginosa* dramatically decreases the antibiotic tolerance of nutrient-starved cells and biofilms for multiple classes of antibiotics (33). Since

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Address correspondence to Dao Nguyen, dao.nguyen@mcgill.ca.
* Present address: Joe J. Harrison, Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada.
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recent studies suggest that oxidative-stress pathways contribute to bacterial cell death caused by bactericidal antibiotics (34–38), we hypothesized that robust antioxidant defenses are required for antibiotic tolerance. Our initial studies showed that the ΔSR (Δ*relA* Δ*spoT*) mutant had decreased superoxide dismutase and catalase activities and was more susceptible to oxidants than wild-type *P. aeruginosa* (33). In this study, we investigated how the SR mediates H_2O_2 tolerance and regulates catalases, which are highly efficient H_2O_2 scavengers. We explored the role of the stationary-phase alternative sigma factor RpoS as an intermediary mediator of the SR and looked at the contribution of catalases to antibiotic tolerance.

MATERIALS AND METHODS

Growth conditions. All bacterial strains were grown in lysogeny broth (LB) medium for all experiments, except when serine starvation was required to induce the SR. In order to induce the SR, planktonic bacteria were grown in M9 minimal medium (1 mM $MgSO_4$, 47 mM $Na_2HPO_4{\cdot}2H_2O$, 22 mM KH_2PO_4, 9 mM NaCl, 18 mM NH_4Cl, 10 mM glucose) at 37°C with shaking at 250 rpm. Subsequently, 500 μM serine hydroxamate (SHX; Sigma-Aldrich) was added to mid-log-phase cells (optical density at 600 nm [OD_{600}], 0.5) to cause serine starvation (39). Antibiotics were used at the following concentrations for selection: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, carbenicillin at 250 μg/ml, gentamicin at 10 μg/ml (*E. coli*) or 50 μg/ml (*P. aeruginosa*), and tetracycline at 10 μg/ml (*E. coli*) or 90 μg/ml (*P. aeruginosa*). When required, the inducers L-(+)-arabinose (L-ara; Sigma) and isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) were added to cultures at concentrations of 1% (wt/vol) and 4 mM, respectively. Stationary-phase planktonic bacteria were grown in LB medium at 37°C with shaking at 250 rpm for 16 to 18 h. Bacteria were grown in colony biofilms as previously described (40) with minor modifications. Here, 5×10^5 CFU from an overnight culture grown in LB was inoculated onto polycarbonate membrane filters (GE Water and Process Technologies) and placed on LB agar for 24 h at 37°C unless otherwise specified.

Bacterial strains. All strains and plasmids used in this study are listed in Table 1. The *P. aeruginosa* Δ*relA* Δ*spoT* (ΔSR) strain and complemented mutant (+SR) strain were obtained from a previous study (33). The *katA*, *katB*, and *rpoS* transposon mutants were obtained from the PAO1 two-allele transposon mutant library (41). To create the ΔSR *rpoS* triple mutant, the *rpoS*-B03 ISlacZ/hah allele was moved from strain PW7151 into the ΔSR mutant by transformation of the electrocompetent ΔSR mutant with PW7151 genomic DNA as previously described (42). The *rpoS* mutation was selected for using the tetracycline (Tc) resistance marker. All transposon mutations were confirmed by PCR. Integration of miniTn7 vectors into the *P. aeruginosa* chromosome was carried out by electroporation with the helper plasmid pTNS2 (47). Antibiotic selection and confirmation of miniTn7 insertions by PCR were carried out according to established protocols (47). Transformation of *P. aeruginosa* with replicating plasmids was done by standard electroporation techniques.

Plasmids and vector construction. Molecular biology procedures were carried out by standard methods, and all primers used in this work are listed in Table S1 in the supplemental material. Newly created plasmid constructs were confirmed by sequencing using M13 or other insert-specific primers (see Table S1 in the supplemental material). An IPTG-inducible *rpoS* construct and its parent plasmid were obtained from a previous study (43). A *relA* overexpression construct was created by PCR amplification of a 2.7-kb fragment containing the *P. aeruginosa* PAO1 *relA* open reading frame (ORF) and upstream promoter region as previously described (44). This PCR fragment was then inserted by HindIII and SpeI restriction and ligation into pBBR1-MCS5, creating the vector pDN53 (Table 1).

To overexpress *katA*, we generated a chromosomally integrated miniTn7 construct using Multisite Gateway technology (Invitrogen). The PAO1 *katA* ORF was amplified by PCR (using primers katA-F and katA-R), and this was recombined into pDONR221P5P2 using BP clonase (Invitrogen), generating the entry vector pJJH173. Next, *araC-pBAD* was amplified by PCR from pUT-miniTn5Pro (45) (using primers araC-pBAD-F and araC-pBAD-R) and recombined into pDONR221P1P5r, creating pJJH187. Subsequently, the entry vectors pJJH173 and pJJH187 were recombined with the destination vector pUC18-miniTn7T-Gm-GW (46) using LR Clonase II Plus (Invitrogen) to generate an arabinose-inducible *katA* overexpression construct (pMK297). Lastly, this miniTn7 construct was integrated into the PAO1 wild-type and ΔSR strains, creating MK298 and MK300, respectively. As a vector control, the miniTn7 from pUC18-miniTn7T-Gm was integrated into the PAO1 wild-type and ΔSR strains, creating MK318 and MK320, respectively.

We constructed transcriptional promoter-reporter fusions for *katA* and *katB* using MultiSite Gateway technology. The PAO1 *katA* and *katB* promoter regions were amplified by PCR (using primer pair pkatA-F and pkatA-R and primer pair pkatB-F and pkatB-R, respectively), and these were recombined with BP Clonase II into pDONR221P1P5r, creating pJJH167 and pJJH168, respectively. Next, a DNA fragment containing *lacZ* along with the adjacent RNase III and ribosomal binding site was amplified by PCR from mini-CTX-lacZ (47) (using primers lacZ-F and lacZ-R), and this was recombined into pDONR221P5P2 using BP clonase II (Invitrogen), generating the entry vector pMK314. Subsequently, each of the vectors pJJH167 and pJJH168 was recombined with pMK314 and the destination vector pUC18-miniTn7T-Gm-GW using LR Clonase II Plus (Invitrogen), generating the promoter-reporter constructs pMK365 and pMK361, respectively. The miniTn7 from pMK365 was integrated into the PAO1 wild-type and ΔSR strains, creating MK325 and MK327, respectively. Repeating this procedure with pMK361 generated strains MK361 and MK363, respectively.

A promoterless *lacZ* miniTn7 vector was also created as a control for promoter-reporter measurements. Here, a 100-bp sequence containing a multiple-cloning site was amplified by PCR from mini-CTX2 (48) (using primers PC-F and PC-R), and this was recombined with pDONRP1P5r using BP Clonase II, generating pMK71. Subsequently, pMK71 and pMK314 were recombined with pUC18-miniTn7T-Gm-GW to generate the promoterless *lacZ* fusion pMK339. Finally, the miniTn7 from pMK339 was integrated into the PAO1 wild-type and ΔSR strains, creating MK331 and MK335, respectively.

H_2O_2 challenge. For H_2O_2 killing of planktonic bacteria, 2.5×10^6/ml stationary-phase planktonic cells were incubated with 3 mM H_2O_2 for 2 h in LB medium at 37°C with shaking at 250 rpm. To induce *katB-lacZ* expression in planktonic bacteria, cells were grown to mid-log phase (OD_{600} of 0.5) and stimulated with 0.2 mM H_2O_2 pulses every 10 min for 60 min at 37°C (for a final added concentration of 1.2 mM H_2O_2). This sublethal stimulation effectively induces *katB* expression without significant bacterial killing (data not shown). For H_2O_2 killing of biofilm bacteria, colony biofilms were grown on 1.5% agar plates with dilute LB medium (25% or 5 g/liter). After 24 h of growth at 37°C, biofilms were statically immersed in 2 ml of 25% LB medium and challenged with 150 mM H_2O_2 pulses every 10 min for 30 min at 37°C (for a final added concentration of 450 mM H_2O_2). To induce *katB-lacZ* expression in biofilm bacteria, cells were grown on 1.5% agar plates with dilute LB medium (25% or 5 g/liter). After 24 h of growth at 37°C, biofilms were statically immersed in 1.5 ml of 25% LB medium and challenged with 5 mM H_2O_2 pulses every 10 min for 60 min at 37°C (for a final added concentration of 30 mM H_2O_2). Repeated pulses of H_2O_2 were used to approximate a continuous challenge. To neutralize any remaining H_2O_2, 0.2% sodium thiosulfate was added at the end of all H_2O_2 killing assays. Biofilm bacteria were resuspended in phosphate-buffered saline (PBS) using a vortex mixer. Viable cell counting was carried out using standard microdilution techniques for both planktonic and biofilm bacteria. The "CFU reduction" was defined as the difference in viable counts between untreated and treated cultures.

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Escherichia coli strains		
DH5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* φ80*lacZ*ΔM15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
ccdB Survival 2 T1R	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG fhuA*::IS2, Str^r	Invitrogen
EC100D pir-116	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) F80*lacZ*ΔM15 *lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK endA1 nupG* λ-*rpsL pir-116*, Str^r	Epicenter Biotechnologies
Pseudomonas aeruginosa strains		
PAO1	Wild type	Laboratory archive (33)
ΔSR	PAO1 Δ*relA* Δ*spoT*	33
+*SR*	ΔSR attCTX::*relA* attTn7::*spoT*	33
PW8191	PAO1 *katA*-B07 ISphoA/hah, Tc^r	41
PW8770	PAO1 *katB*-F08 ISphoA/hah, Tc^r	41
PW7151	PAO1 *rpoS*-B03 ISlacZ/hah, Tc^r	41
DN707	ΔSR *rpoS*-B03 ISlacZ/hah, Tc^r	This study
MK298	PAO1 attTn7::miniTn7-Gm-GW-*araC-pBAD*::*katA*, Gm^r	This study
MK300	ΔSR attTn7::miniTn7-Gm-GW-*araC-pBAD*::*katA*, Gm^r	This study
MK318	PAO1 attTn7::miniTn7-Gm, Gm^r	This study
MK320	ΔSR attTn7::miniTn7-Gm, Gm^r	This study
MK325	PAO1 attTn7::miniTn7-Gm-GW-*pkatA*::*lacZ*, Gm^r	This study
MK327	ΔSR attTn7::miniTn7-Gm-GW-*pkatA-lacZ*, Gm^r	This study
MK361	PAO1 attTn7::miniTn7-Gm-GW-*pkatB-lacZ*, Gm^r	This study
MK363	ΔSR attTn7::miniTn7-Gm-GW-*pkatB-lacZ*, Gm^r	This study
MK331	PAO1 attTn7::miniTn7-Gm-GW-promoterless-*lacZ*, Gm^r	This study
MK335	ΔSR attTn7::miniTn7-Gm-GW-promoterless-*lacZ*, Gm^r	This study
Plasmids		
pDONR221P1P5r	Multisite Gateway donor vector with attP1 and attP5r recombination sites, Cm^r Km^r	Invitrogen
pDONR221P5P2	Multisite Gateway donor vector with attP5 and attP2 recombination sites, Cm^r Km^r	Invitrogen
pUC18-miniTn7T-Gm	For gene insertion in Gm^s bacteria (*aacC1* on mini-Tn7T), Ap^r Gm^r	46
pUC18-miniTn7T-Gm-GW	pUC18-miniTn7T-Gm with a Gateway destination cloning site Ap^r Gm^r Cm^r	46
pTNS2	T7 transposase expression vector, Ap^r	46
mini-CTX2	Source template for a multiple cloning site (MCS), Tc^r	48
mini-CTX-lacZ	Source template for *lacZ*, Tc^r	47
pUT-miniTn5Pro	Source template for *araC-pBAD*, Ap^r Gm^r	45
pJJH167	pDONR221P1P5r with an *attL*-flanked, 658-bp fragment encoding the *P. aeruginosa* PAO1 *katA* promoter region, Km^r	This study
pJJH168	pDONR221P1P5r with an *attL*-flanked, 298-bp fragment encoding the *P. aeruginosa* PAO1 *katB* promoter region, Km^r	This study
pJJH173	pDONR221P5P2 with an *attL* flanked,-1,463-bp fragment encoding a 14-bp synthetic ribosomal binding site and the 1,449-bp *katA* ORF from *P. aeruginosa* PAO1, Km^r	This study
pJJH187	pDONR221P1P5r with an *attL*-flanked, 1,192-bp fragment encoding the *araC* repressor and the *pBAD* promoter, Km^r	This study
pMK71	pDONR221P1P5r with an *attL*-flanked, 100-bp fragment encoding the multiple cloning site from mini-CTX2, Km^r	This study
pMK314	pDONR221P5P2 with an *attL*-flanked, 3,276-bp fragment encoding an RNase III site and a ribosomal binding site flanking a *lacZ* ORF, Km^r	This study
pMK365	pUC18miniTn7T-Gm-GW containing a *pkatA*::*lacZ* promoter-reporter construct, Ap^r Gm^r	This study
pMK361	pUC18miniTn7T-Gm-GW containing a *pkatB*::*lacZ* promoter-reporter construct, Ap^r Gm^r	This study
pMK297	pUC18miniTn7T-Gm-GW containing *araC-pBAD*:: *katA*, Ap^r Gm^r	This study
pMK339	pUC18miniTn7T-Gm-GW containing a promoterless *lacZ*, Ap^r Gm^r	This study
pMMB67HE	Broad-host expression vector containing *lacI*^{q} and an ORF-less *ptac* promoter, Ap^r	43
pMMB-rpoS1	pMMB67HE *ptac*::*rpoS*, Ap^r	43
pBBR1-MCS5	Broad-host cloning vector, Gm^r	78
pDN53	pBBR1-MCS5 with a 2.7-kb fragment carrying *P. aeruginosa* PAO1 *relA* and its native promoter, Gm^r	This study

[a] Abbreviations used for genetic markers: Str, streptomycin; Tc, tetracycline; Km, kanamycin; Gm, gentamicin; Ap, ampicillin.

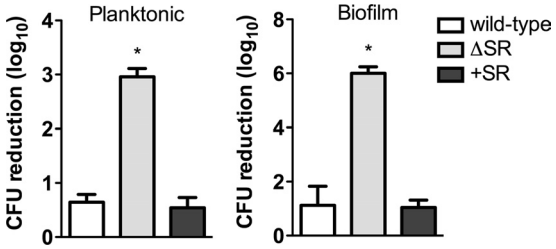

FIG 1 The ΔSR mutant is more susceptible to H_2O_2 killing than the wild-type strain in planktonic and biofilm cultures. Levels of susceptibility to H_2O_2 killing in the wild-type strain, ΔSR mutant, and +SR mutant (complemented strain) are presented as "CFU reduction," which represents the difference in viable counts between untreated and treated cultures. Stationary-phase planktonic cells grown in LB medium for 16 h were diluted to 2.5×10^6/ml and treated with 3 mM H_2O_2 for 2 h at 37°C. Biofilm cells grown on 25% LB agar were treated with three pulses of 150 mM H_2O_2 every 10 min for 30 min at 37°C. Error bars represent standard deviations (SD). *, $P \leq 0.0001$ (versus wild type) ($n \geq 6$).

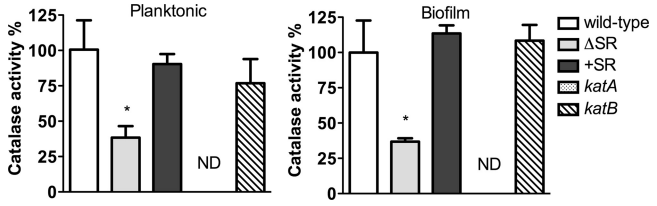

FIG 2 Catalase activity is reduced in the ΔSR mutant. The relative catalase activities of wild-type, ΔSR, +SR (complemented strain), *katA*, and *katB* mutant strains were measured in planktonic and biofilm cultures grown in LB medium to the stationary phase (16 h for planktonic, 24 h for biofilm). Catalase activity in the mutants was normalized to wild-type levels under the same growth conditions. ND, not detected. Error bars represent SD. *, $P \leq 0.01$ (versus wild type) ($n \geq 3$).

Antibiotic challenge. Colony biofilms were grown as described above on LB agar plates with 1% L-arabinose for 24 h and then transferred onto LB agar plates with 1% L-arabinose and colistin at 300 μg/ml (24 h of incubation) or ofloxacin at 20 μg/ml (4 h of incubation) for incubation at 37°C. Control LB agar plates contained 1% L-arabinose without antibiotics. To enumerate viable cells after antibiotic challenge, biofilm bacteria were resuspended in PBS using a vortex mixer and then plated for viable cell counting as described above.

Catalase activity assays. Catalase activity was measured using a catalase activity kit (Sigma) with minor modifications according to the supplier (49). Briefly, cell pellets from biofilms or planktonic cultures were washed three times in cold 50 mM potassium phosphate buffer (pH 7.0). Cells were lysed on ice by sonication (six 30-s pulses; amplitude, 20%). The total protein in the lysates was determined by the Bradford assay (Bio-Rad) using a bovine serum albumin standard curve. Catalase activity was measured spectrophotometrically at 240 nm. Aliquots (100 μl) of cell lysate containing 10 μg of protein were added to the assay solution. One unit of catalase activity was defined as the decomposition of 1 μmol · min^{-1} of H_2O_2. In order to pool results from biological replicates, assay results were calculated as relative catalase activity normalized to the wild-type strain.

Measurement of intracellular ROS levels. Intracellular levels of ROS were measured using 2′,7′-dichlorodihydrofluorescein ($DCFH_2$) or hydroxyphenyl fluorescein (HPF) (Molecular Probes-Invitrogen). Cells were grown in biofilms or to stationary phase in planktonic cultures as described above, washed, and resuspended in 1 ml of PBS by vortex mixing. HPF (5 μM) or $DCFH_2$ (10 μM) was added, and the cells were incubated for 20 min at 37°C in the dark. Cells were then washed and resuspended in PBS, and fluorescence was measured at 485-nm excitation and 535-nm emission (PerkinElmer Victor 2030 Explorer Multilabel Reader). The fluorescence values were normalized to the OD_{600} of each sample. In order to pool results from biological replicates, assay results were calculated as relative fluorescence normalized to the wild-type strain.

β-Galactosidase assays. β-Galactosidase (LacZ) activity was assayed as described by Zhang and Bremer (50). The background activity for promoter-reporter fusions was corrected by measuring the β-galactosidase activity in strains with a promoterless control construct. This baseline activity was subtracted from all readings, and the corrected β-galactosidase activities were expressed as Miller units.

Statistical analysis. Statistical differences between two means (with equal variance values) were determined using Student *t* tests. A Bonferroni correction was used to determine statistical significance when multiple comparisons were performed.

RESULTS

Inactivation of the stringent response (SR) decreases tolerance to hydrogen peroxide in planktonic and biofilm bacteria. In planktonic culture, the ΔSR mutant was significantly more susceptible to H_2O_2 killing than wild-type bacteria. H_2O_2 caused a 3 log_{10} reduction in viable ΔSR mutant bacteria compared to ~0.5 log_{10} in the wild type (Fig. 1). We also measured the susceptibility of ΔSR mutant biofilms to H_2O_2 killing. Since biofilm bacteria are characterized by a high tolerance of oxidants, we used a 150-fold-higher H_2O_2 concentration to challenge biofilm bacteria (three 150 mM pulses for biofilm cells versus a single 3 mM treatment for planktonic cells), and this caused a reduction in wild-type biofilm viable counts of only 1 log_{10}. Inactivation of the SR led to a massive increase in oxidant killing, with a 6 log_{10} reduction in viable counts (Fig. 1), suggesting that the SR is critical to biofilm H_2O_2 tolerance. Complementation of the *relA* and *spoT* genes restored oxidant tolerance in both planktonic and biofilm cultures, confirming that the increased oxidant susceptibility was due to deletion of these genes (Fig. 1).

The SR is required for full catalase activity in planktonic and biofilm growth. Catalases dismutate H_2O_2 to water and O_2 and are potent scavengers of hydrogen peroxide. Given that the ΔSR mutant was highly susceptible to H_2O_2, we next investigated whether it had impaired catalase activity. As shown in Fig. 2 and in Fig S1 in the supplemental material, both planktonic and biofilm stationary-phase ΔSR cells exhibited only ~35% catalase activity compared to the wild-type strain. Complementation of the *relA* and *spoT* genes also rescued catalase activity. As expected, the catalase activity was undetectable in the *katA* mutant, as KatA is the primary constitutive catalase expressed in the absence of H_2O_2 induction (19, 26). On the other hand, the *katB* mutant retained wild-type levels of catalase activity under planktonic and biofilm growth conditions. This was not surprising, since *katB* expression is known to be induced only under conditions of exogenous H_2O_2 stress. Of note, neither *katA* nor *katB* mutants had reduced viability in stationary-phase planktonic cultures or biofilm (see Fig. S1 in the supplemental material).

Starvation induces catalase activity through (p)ppGpp signaling. Since SR inactivation led to a decrease in catalase activity, we hypothesized that SR activation would have the opposite effect. In order to induce the SR in the wild type, we added serine hydroxamate (SHX). This serine analog causes serine starvation in bacteria grown in minimal medium, resulting in rapid accumula-

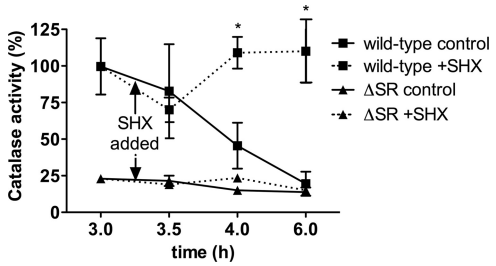

FIG 3 The SR and (p)ppGpp signaling upregulate catalase activity. Relative levels of catalase activity were measured during exponential- and early stationary-phase planktonic growth in M9 minimal medium in wild-type (■) and ΔSR (▲) bacteria without SHX treatment (solid line) or with 500 μM SHX (dashed line). Time t = 3 h corresponds to an OD_{600} of 0.5 (mid-exponential phase). Catalase activity was normalized to wild-type levels at t = 3 h under the control conditions. Error bars represent SD. *, $P \leq 0.05$ (versus wild-type control) ($n = 3$).

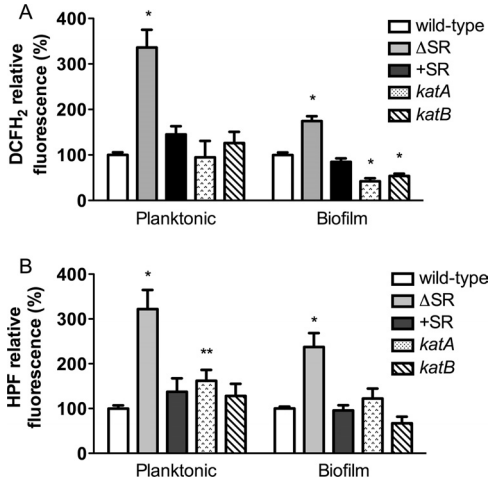

FIG 4 SR inactivation increases endogenous ROS formation. Relative intracellular levels of ROS were measured in stationary-phase planktonic (16 h) and biofilm (24 h) bacteria grown in LB medium. Cells were stained with $DCFH_2$ (A) and HPF (B). The relative levels of fluorescence (excitation, 485 nm; emission, 535 nm) were normalized to wild-type levels under the same conditions. The data of at least three independent experiments were pooled, and means are shown. Error bars represent standard errors of the means (SEM). *, $P \leq 0.001$; **, $P \leq 0.01$ (versus wild type).

tion of (p)ppGpp and growth arrest (39). It is worth noting that SHX treatment causes similar levels of growth arrest in both wild-type and ΔSR mutant strains but (p)ppGpp accumulation occurs only in the wild-type strain (33). Following addition of SHX to log-phase planktonic bacteria, catalase levels increased in the wild-type strain but not in the ΔSR mutant (Fig. 3). Since wild-type cells, but not ΔSR cells, synthesize (p)ppGpp in response to SHX, this suggests that SR induction upregulated catalase activity and that this requires (p)ppGpp signaling. Furthermore, catalase levels were also low in the ΔSR mutant not treated with SHX, indicating that (p)ppGpp is required for basal catalase activity. Surprisingly, catalase activity decreased in untreated wild-type bacteria upon entry into the stationary phase in minimal medium (Fig. 3; see also Fig. S2 in the supplemental material) but not in LB medium (see Fig. S2 in the supplemental material), a finding previously reported in *E. coli* (51, 52). The reason for the decreased catalase activity in M9 medium remains unclear.

Inactivation of the SR causes increased endogenous ROS. During aerobic growth, ROS are generated within cells through autoxidation of flavoenzymes and redox-active metabolites, such as phenazines, and from the partial reduction of O_2 during aerobic respiration. ROS scavengers, including catalases, are required to prevent excessive accumulation of these potentially toxic molecules. Given that catalase activity is reduced in the ΔSR mutant, we asked whether this was associated with increased endogenous intracellular ROS. Using $DCFH_2$, a nonspecific probe that detects H_2O_2 in the presence of redox-active iron (53), and HPF, a probe with relative specificity for hydroxyl radicals (54), we found that SR inactivation significantly increased ROS levels in both planktonic stationary-phase and biofilm cells (Fig. 4).

The role of RpoS in hydrogen peroxide tolerance. Previous studies have reported that the stationary-phase alternative sigma factor RpoS is required for H_2O_2 resistance in *P. aeruginosa* (28, 30). Since the SR is required for full RpoS expression (44) and can indirectly control gene expression by modulating the affinity of sigma factors (55), we explored the contribution of RpoS as an intermediary regulator of catalase activity and H_2O_2 tolerance. Both the ΔSR and *rpoS* mutants exhibited only 35% of wild-type catalase activity (Fig. 5A), and this was further reduced to 15% in the ΔSR *rpoS* triple mutant. Although the levels of H_2O_2 killing were comparable in the ΔSR and *rpoS* mutants, they were also further increased in the ΔSR *rpoS* triple mutant (Fig. 5B). Together, these results suggest that the SR exerts its effect partially through RpoS but that RpoS-independent mechanisms are also involved. Interestingly, overexpression of RpoS did not restore catalase activity in the ΔSR mutant (see Fig. S3A in the supplemental material), and neither did overexpression of RelA in the *rpoS* mutant (see Fig. S3B in the supplemental material). This supports the notion that the SR and RpoS are highly interdependent, as neither factor alone is sufficient to overcome a defect in the other.

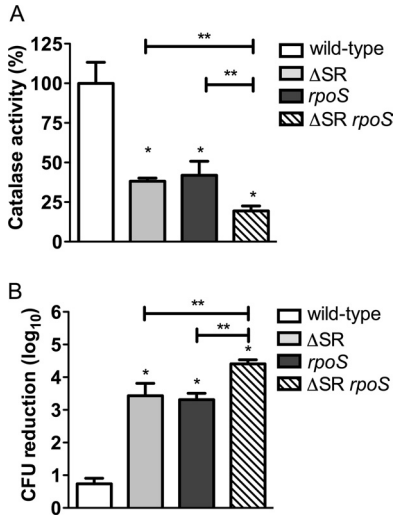

FIG 5 Catalase activity and H_2O_2 killing in *rpoS* mutants. (A) Relative catalase activities of wild-type, ΔSR, *rpoS*, and ΔSR *rpoS* triple-mutant strains grown planktonically in LB medium to the stationary phase (16 h). Error bars represent SD. *, $P \leq 0.0005$ (versus wild type); **, $P \leq 0.005$ (versus the ΔSR *rpoS* strain) ($n \geq 3$). (B) Susceptibility of wild-type, ΔSR, *rpoS*, and ΔSR *rpoS* triple-mutant strains to H_2O_2 killing. Cells were grown as stationary-phase planktonic cells, diluted to 2.5×10^6/ml, and then challenged with 3 mM H_2O_2 for 2 h in LB medium at 37°C. Error bars represent SD. *, $P \leq 0.001$ (versus wild type); **, $P \leq 0.01$ (versus ΔSR *rpoS*) ($n \geq 3$).

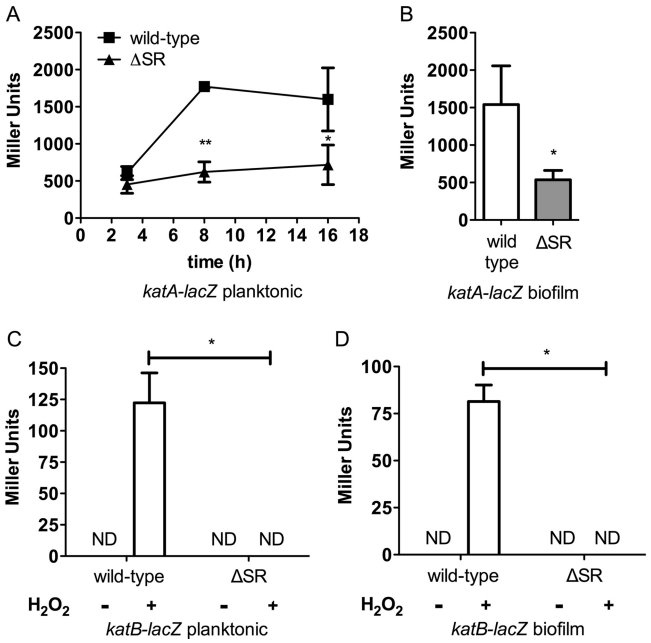

FIG 6 The SR regulates *katA* and *katB* gene expression levels. (A) β-Galactosidase activity of *katA-lacZ* reporter in wild-type (■) and ΔSR mutant (▲) cells during planktonic growth. Cultures were started at $t = 0$ h by diluting an overnight culture to an OD_{600} of 0.01. β-Galactosidase activity was measured at $t = 3$ h (mid-exponential phase), $t = 8$ h (early stationary phase), and $t = 16$ h (late stationary phase) during growth in LB medium at 37°C. Error bars represent SD. ND, not detected. *, $P \leq 0.05$; **, $P \leq 0.001$ (versus wild type) ($n \geq 3$). (B) β-Galactosidase activity of *katA-lacZ* reporter in wild-type and ΔSR mutant cells during biofilm growth. β-Galactosidase activity was measured after 24 h of growth on LB agar at 37°C. *, $P \leq 0.001$ (versus wild type) ($n \geq 6$). (C) β-Galactosidase activity of *katB-lacZ* reporter in wild-type and ΔSR planktonic cells with and without H_2O_2 challenge. Cells grown to mid-log phase ($OD_{600} = 0.5$) in LB medium were challenged with 0.2 mM H_2O_2 pulses every 10 min for 60 min at 37°C. β-Galactosidase activity was measured after H_2O_2 challenge or at the same time in the untreated samples. Error bars represent SD. *, $P \leq 0.0001$ (versus untreated wild type) ($n \geq 3$). (D) β-Galactosidase activity of *katB-lacZ* reporter in wild-type and ΔSR biofilm cells with and without H_2O_2 challenge. Biofilm cells grown on 25% LB medium for 24 h were challenged with 5 mM H_2O_2 pulses every 10 min for 60 min at 37°C (for a final added concentration of 30 mM H_2O_2). β-Galactosidase activity was measured after H_2O_2 challenge or at the same time in the untreated samples. Error bars represent SD. *, $P \leq 0.001$ (versus untreated wild type) ($n \geq 3$).

The SR regulates *katA* and *katB* gene expression levels. We fused the *katA* and *katB* promoters to *lacZ* to measure transcription of the *katA* and *katB* genes. Consistent with previous studies (18, 26), *katA*-LacZ activity was detected at 3, 8, and 16 h of planktonic growth, with the highest levels during early stationary phase (8 h). In contrast, *katA*-LacZ activity in the ΔSR mutant minimally increased during the stationary phase and was lower than wild-type levels at both 8 h and 16 h (Fig. 6A). *katA* expression was also reduced in biofilms of the ΔSR mutant (Fig. 6B). Since *katB* expression is OxyR dependent and is induced only by H_2O_2 stress (24), wild-type *katB*-LacZ activity was undetectable in the absence of exogenous H_2O_2 and increased after H_2O_2 challenge in both planktonic and biofilm bacteria (Fig. 6C and D). In contrast, both the noninduced and H_2O_2-induced *katB*-LacZ activities of the ΔSR mutant remained undetectable. These results thus revealed that the SR is involved in the transcriptional control of both growth-phase-dependent *katA* expression and H_2O_2-inducible *katB* expression.

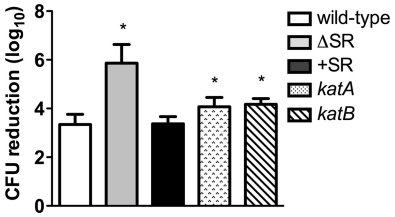

FIG 7 The ΔSR mutant is more susceptible to ofloxacin killing. Bacteria were grown as biofilms on LB agar for 24 h and then challenged with 20 μg/ml ofloxacin for 4 h at 37°C. CFU reduction values represent differences in viable counts between treated and untreated biofilms. Error bars represent SD. *, $P \leq 0.0001$ (versus wild type) ($n \geq 6$).

KatA overexpression restores full H_2O_2 tolerance and partial antibiotic tolerance in the ΔSR mutant. In a recent study, we hypothesized that impaired oxidative defenses in the ΔSR mutant led to reduced antibiotic tolerance, since antibiotic killing may be ROS mediated (33). To determine the contribution of catalases to antibiotic tolerance in *P. aeruginosa*, we first measured the ofloxacin tolerance of catalase mutants. The *katA* and *katB* single mutants showed a modest increase in susceptibility to ofloxacin compared to the wild type (Fig. 7), suggesting that each catalase provides only a small contribution to antibiotic tolerance. As previously observed, the ΔSR mutant was highly susceptible to ofloxacin. Next, we asked whether KatA overexpression could protect the ΔSR mutant against antibiotic killing. We generated a *pBAD-katA* construct with arabinose-inducible *katA* expression. Overexpression of KatA increased catalase activity to similar levels in the wild-type and ΔSR mutant strains (Fig. 8A) and fully restored H_2O_2 tolerance to the ΔSR mutant (Fig. 8B). Furthermore, KatA overexpression reduced killing of ΔSR mutant biofilms by colistin (3 versus 7 $\log_{10}$ CFU reduction) and ofloxacin (4 versus 6 $\log_{10}$ CFU reduction) (Fig. 8C and D) but no significant difference was observed in the wild-type background. Thus, our results indicate that wild-type catalase activity plays a vital role in protecting bacteria against antibiotic killing and KatA overexpression partially restores antibiotic tolerance in the ΔSR mutant.

DISCUSSION

Bacteria experience nutrient limitation during the stationary phase, in biofilm growth, or within the host during infections. While nutrient limitation can confer oxidative stress tolerance, thus protecting bacteria against host-generated or other exogenous ROS, the underlying mechanisms remain poorly defined (3–6). The SR is a global regulatory response induced by stress and starvation. It can reorganize cellular processes to shut down growth and induce protective mechanisms for stress survival (11, 12). Although the SR is a central stress response, only a few studies have examined its role in oxidative stress. For example, the *relA* mutant of *Enterococcus faecalis* is more susceptible to H_2O_2 challenge than the wild type (14, 15), while aerobic growth of the *relA* mutant of *Geobacter sulfurreducens* is impaired due to oxidative stress (56). Additionally, we and others have recently shown that SR inactivation in *P. aeruginosa* led to increased oxidant susceptibility (33, 57). Conversely, (p)ppGpp accumulation has been associated with oxidative stress tolerance in *Streptococcus mutans* (58), and transcriptomic studies of (p)ppGpp-regulated genes in *Rhizobium etli* identified several genes involved in oxidative stress resistance (59).

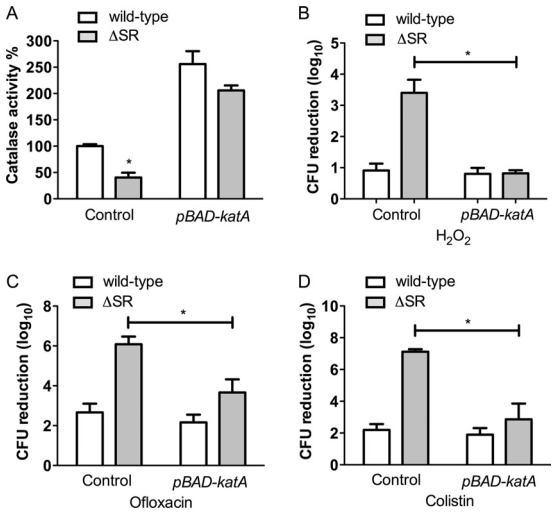

FIG 8 KatA overexpression rescues H_2O_2 and antibiotic tolerance in the ΔSR mutant. (A) Catalase activity in wild-type and ΔSR cells containing a *pBAD-katA* construct or vector control. Cells were grown planktonically to the stationary phase (16 h) in LB medium with 1% L-arabinose. Error bars represent SD. *, $P \leq 0.005$ (versus wild type) ($n = 3$). (B) Susceptibility to H_2O_2 killing in wild-type and ΔSR cells containing a *pBAD-katA* construct or vector control. Cells were grown planktonically to the stationary phase in LB medium with 1% L-arabinose for 16 h and then challenged with 3 mM H_2O_2 for 2 h. Error bars represent SD. *, $P \leq 0.0001$ (versus ΔSR vector control) ($n = 6$). (C and D) Antibiotic tolerance of wild-type and ΔSR cells containing a *pBAD-katA* construct or vector control. Cells were grown as biofilm cells on LB agar with 1% L-arabinose for 24 h and were then challenged with 20 μg/ml ofloxacin for 4 h (C) or 300 μg/ml colistin for 24 h (D) at 37°C. Error bars represent SD. *, $P \leq 0.0001$ (versus ΔSR vector control) ($n \geq 6$).

The increased susceptibility of the ΔSR mutant to H_2O_2 challenge led us to suspect that the SR regulates catalases. It is well established that *katA* expression is continuous and increases upon entry into the stationary phase (18, 26). Our *katA*-LacZ studies showed that *katA* expression was reduced during the stationary phase in the ΔSR mutant, paralleled by a reduction in catalase activity. The SR is therefore required for full *katA* expression and catalase activity under normal physiological conditions (including stationary phase), and this control occurs at the transcriptional level.

Since *katA* is positively regulated by RpoS and quorum sensing (26, 28), it is possible that the SR controls catalases through these intermediary regulators. Notably, the SR controls *rpoS* expression in *P. aeruginosa*: RpoS levels are reduced in a *relA* mutant and induced upon overexpression of RelA (44, 60). In *E. coli*, *rpoS* and RpoS-dependent promoters require (p)ppGpp for full activation (61, 62), and (p)ppGpp prevents RpoS proteolysis via IraP control (63). Here we found that catalase activity and H_2O_2 tolerance were equally reduced in the ΔSR and *rpoS* mutants. Supporting the idea that RpoS-dependent promoters require (p)ppGpp, overexpression of RpoS was sufficient to restore catalase levels in the *rpoS* mutant but not the ΔSR mutant strain. Furthermore, overexpression of *relA* was also not sufficient to restore catalase levels in the *rpoS* mutant, and thus increasing (p)ppGpp alone cannot overcome the RpoS defect. The ΔSR *rpoS* triple mutant showed further reductions in both catalase activity and H_2O_2 tolerance, suggesting that the SR likely controlled catalase activity through RpoS-independent as well as RpoS-dependent pathways. For example, the Las and Rhl quorum-sensing systems control *katA* expression, and the SR has been linked to quorum-sensing activation (44). These quorum-sensing systems may therefore mediate SR control of catalases, but this question is further complicated by the cross-talk between the quorum-sensing systems and RpoS (64). Further studies are required to elucidate this complex regulatory network.

Consistent with previous reports (17, 19, 22), we found that KatA is the dominant catalase expressed in the absence of exogenous H_2O_2 stress and is critical to H_2O_2 tolerance in biofilms (65). Reduced *katA* expression is therefore likely responsible for the H_2O_2 susceptibility of ΔSR mutant biofilms. In contrast, *katB* is tightly controlled by OxyR and is expressed only upon exogenous H_2O_2 challenge (Fig. 5B). OxyR is a redox-sensitive LysR-type transcriptional activator that is regulated at the posttranslational level by oxidation of its conserved cysteine residues (66). In its oxidized form, OxyR activates *katB* expression (and that of other *P. aeruginosa* genes, including *ahpB*, *ahpC*, and *katA*) by binding to their −35 OxyR promoter binding site. Since *katB* gene expression is also abrogated in the ΔSR mutant, the SR may be involved in OxyR-mediated control of *katB* expression either directly by affecting transcription or indirectly by modifying the redox state or expression of OxyR.

Since SR inactivation reduced catalase activity, we hypothesized that SR activation would upregulate this activity. Upon (p)ppGpp accumulation caused by SHX-mediated serine starvation, catalase activity increased in the wild-type strain but not in the ΔSR mutant. Thus, nutrient starvation can induce antioxidant defenses via (p)ppGpp signaling. SHX-mediated induction of the SR requires the use of minimal medium. Curiously, we also observed that the catalase activity of the wild type decreased in M9 minimal medium in the absence of SHX but not in LB rich medium. KatA and KatB are both heme-containing catalases requiring iron for activity (67). As iron limitation can significantly reduce catalase activity (68), this may explain the decreased wild-type catalase levels in M9 minimal medium. Furthermore, it has also been reported that catalase activity in *E. coli* decreases during growth in M9 medium, possibly due to glucose-mediated catabolite repression (51, 52). Whether catabolite repression of catalases also occurs in *P. aeruginosa* remains unknown. The mechanism underlying the reduced catalase activity in M9 minimal medium is likely multifactorial, and the exact contribution of iron limitation requires further investigation.

Recent studies suggest that bactericidal antibiotics kill bacteria through oxidative-stress-induced damage, in particular through the formation of highly reactive hydroxyl radicals (34–36, 69). Consistent with this model, the *E. coli oxyR* mutant is more susceptible to aminoglycoside killing (70). Inactivation of *katG*, *katE*, and *ahpCF*, responsible for H_2O_2 scavenging in *E. coli*, also significantly increases ciprofloxacin killing (69). As shown in our recent report as well as this current study, inactivation of the SR causes increased endogenous ROS (33). We hypothesized that this excessive oxidative burden was due to impaired antioxidant defenses (including catalases) and increased levels of pro-oxidant 4-hydroxyl-2-alkylquinolone (HAQ) molecules and showed that the increased endogenous oxidative burden enhanced antibiotic killing in a dose-dependent manner (33). As catalases are the primary H_2O_2 scavengers, we expected intracellular levels of H_2O_2 and possibly of hydroxyl radicals (generated through the Fenton reaction) to be elevated in the *katA* or *katB* mutants. Although the HPF signal was modestly increased in the Δ*katA* mutant during plank-

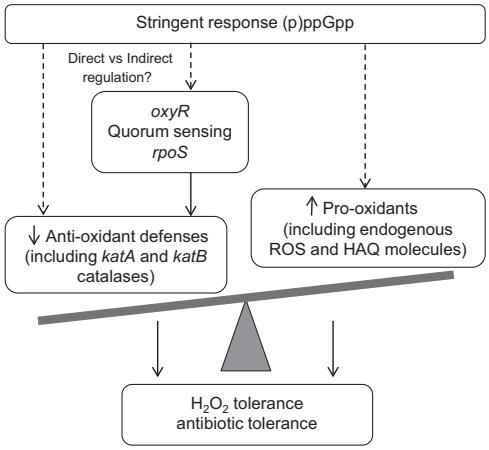

FIG 9 Proposed model for the role of the stringent response in H_2O_2 and antibiotic tolerance. The stringent response mediates antibiotic tolerance by coordinating a balance of antioxidant defenses and pro-oxidant metabolites. These mechanisms include direct or indirect regulation of catalases as well as 4-hydroxy-2-alkylquinoline molecules (HAQ).

tonic growth, the $DCFH_2$ fluorescence was not increased in either mutant but in fact slightly decreased. This may have been due to compensatory mechanisms, such as H_2O_2 scavenging by other catalases or alkyl hydroperoxidase reductases (24). For example, AhpCF has catalase-like activity and is required for optimal protection against H_2O_2 in *P. aeruginosa*, particularly in biofilms (25). Taken together, these results suggest that there are multiple defects in the ΔSR mutant leading to increased endogenous ROS, as the antioxidant defenses are highly redundant.

We also recently demonstrated that SHX-mediated starvation conferred ofloxacin tolerance to the wild-type but not to the ΔSR mutant (33) and now show that it induced catalase activity. Extrapolating from this model, we thus predicted that increasing antioxidant defenses would confer antibiotic tolerance. KatA overexpression led to increased catalase activity and restored wild-type H_2O_2 tolerance to the ΔSR mutant. Most interesting, KatA overexpression was also sufficient to significantly rescue the ΔSR mutant from killing by ofloxacin and colistin, two bactericidal antibiotics with different targets. While disrupting a single catalase gene caused only a modest impairment in ofloxacin tolerance, catalases are sufficient to protect cells against ROS-mediated killing upon both H_2O_2 and bactericidal challenges.

In conclusion, our work revealed that the SR regulates catalases, likely through a complex interplay of regulators, including RpoS. As shown in Fig. 9, our results fit within a proposed model where H_2O_2 and antibiotic tolerance result from a balance of prooxidant stress and antioxidant defenses. Through the regulation of catalases as well as other mechanisms, the SR modulates this equilibrium. Interestingly, nutrient starvation is associated with increased antioxidant defenses in several eukaryotic species, such as yeast, fish, and plants (71–74). Although cellular responses to nutrient limitation are clearly very different between eukaryotic and prokaryotic organisms, starvation may be a universal stress signal that triggers protective antioxidant defense mechanisms necessary for long-term survival. Antioxidant resistance is required for bacterial virulence and persistence *in vivo* (17, 75–77) as well as for survival of antibiotic treatments. While the notion of targeting bacterial catalases directly may be intriguing, this may be challenging given the redundancy of ROS scavenging systems. On the other hand, a broader targeting of antioxidant defenses or the SR may be a promising approach to compromising key mechanisms of bacterial pathogenicity (78).

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