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Abolition of Individual Antioxidant Enzymes Did Not Affect Antibiotic-mediated Killing of *Escherichia coli*

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Three classes of bactericidal antibiotics induce oxidative damages in addition to their interactions with specific intracellular targets. By utilizing knockout strains of *Escherichia coli* that lack antioxidant enzymes, we investigated whether deficiencies in the oxidant scavenging pathways would render the mutants more susceptible to ampicillin, norfloxacin and tobramycin. Although the antibiotics mediated detectable increase in oxidative stress, abolishing each of the antioxidant enzymes, manganese superoxide dismutase, hydroperoxidase I or alkyl hydroperoxide reductase did not increase the antibiotic susceptibilities of *E. coli*. We concluded each of the three enzymes by itself was not essential in the defense against antibiotic-mediated killing.

Bactericidal antibiotics can be categorized into classes based on their mode of action. Aminoglycosides inhibit protein production; quinolones inhibit DNA replication and repair; β-lactams inhibit cell wall synthesis. Recent work has shown that despite their specific modes these three classes of drugs may share a common mechanism of killing by inducing oxidative damages (1). Kohanski *et al*. proposed that bacteria subjected to the bactericidal antibiotics hyper activate electron transport chain, producing superoxide anion, which in turn destabilizes the iron-sulfur cluster redox-cycling system and releases ferrous iron into the cytoplasm. Accumulated ferrous iron reduces hydrogen peroxide, which is dismutated from superoxide, to hydroxyl radicals (1). The highly reactive oxygen species (ROS), superoxide and hydroxyl radicals, can damage DNA, proteins and lipids, resulting in cell death (Fig. 1) (2).

In response to oxidative stress, *Escherichia coli* expresses three classes of ROS scavenging enzymes: superoxide dismutases (SOD) which dismutate superoxide into hydrogen peroxide, hydroperoxidase I/II (HPI/HPII) and alkyl hydroperoxide reductase (Ahp) which scavenge hydrogen peroxide into oxygen and water (Fig. 1) (2, 3). *E. coli* has two cytoplasmic SODs: the iron SOD (FeSOD, encoded by *sodB*) is made constitutively whereas the manganese SOD (MnSOD, encoded by *sodA*) is made only in aerobic conditions and is strongly elevated when subjected to superoxide-generating agents. HPII (encoded by *katE*) and Ahp (encoded by *ahpC* and *ahpF*) are the main scavengers of hydrogen peroxide generated by aerobic respiration. HPI (encoded by *katG*) exhibits a high K_m and therefore can scavenge supernormal levels of hydrogen peroxide. Ahp and HPI are regulated by the OxyR regulon, a widespread system in bacteria to defend against oxidative stress (2).

This study investigated whether the abolition of MnSOD, HPI or Ahp in *E. coli* would increase its susceptibilities to the three classes of bactericidal antibiotics. Representative antibiotics for each group chosen were ampicillin, norfloxacin and tobramycin. We found that the mutants lacked one of each enzyme were not more susceptible. This might have been due to complex enzyme inter-regulation and redundancy.

MATERIALS AND METHODS

Bacterial strains and experimental conditions. The strains used in this study are listed in Table 1. To abolish the inducible antioxidant enzymes, single gene knockout clones JW3879-1 (referred to subsequently as the Δ*sodA* mutant) which lacks MnSOD, JW1721-1 (referred to subsequently as the Δ*katG* mutant) which lacks HPI, JW0598-2 (referred to subsequently as the Δ*ahpC* mutant) which lacks Ahp, JW3933-3 (referred to subsequently as the Δ*oxyR* mutant) which cannot induce either HPI and Ahp expression in the presence of hydrogen peroxide (4), and BW25113 (referred to subsequently as WT) were chosen for the study. All strains were obtained through the University of British Columbia MICB 421culture collection but were originally from the Keio collection (5).

Cultures were routinely grown at 37 °C in M9 salts minimal media (0.05% NaCl, 0.7% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.02% $MgSO_4{\cdot}7H_2O$, 0.3 M $CaCl_2$, and supplemented with 0.2% glycerol).Experiments conducted in 96 well plates were considered microaerophilic while cultures in shaking flasks were considered aerobic. Cultures were treated with 10 μg/ml ampicillin (Sigma), 2 μg/ml tobramycin (Sigma) or 0.24 μg/ml norfloxacin (Sigma) unless noted otherwise.

MIC assay. The method employed was adapted from a previous study (6). Overnight cultures of all strains were diluted to a cell density of 106 cells/ml and added to a 96 well plate containing serial 1/2 dilutions of ampicillin (16 μg/ml to 0.03 μg/ml), norfloxacin (0.24 μg/ml to 0.0005 μg/ml), or tobramycin (2 μg/ml to 0.0039 μg/ml). Positive controls were cells without antibiotics and negative controls were M9 medium alone. The plate was incubated overnight, and the MIC was established as the lowest concentration of antibiotic at which no visible growth was observed.

Growth assay with paraquat or hydrogen peroxide. Overnight cultures of indicated strains were diluted to an OD_{595} of 0.2 and added to a 96 well plate containing serial 1/2 dilutions of paraquat (100 μM to 0.2 μM, Sigma) or hydrogen peroxide (2000 μM to 3.9 μM, VWR). Positive controls were cells without treatments and negative controls were M9 medium alone. The plate was then incubated and turbidity was monitored for 3 hours following the treatments.

Growth assay with antibiotics. Overnight cultures of all strains were diluted to an OD_{595} of 0.2 and treated with ampicillin, tobramycin or norfloxacin. The cultures were incubated in the shaking flasks and turbidity was monitored for 3 hours following the treatments.

DCFH-DA staining. 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) is a non-fluorescent compound that becomes highly fluorescent when oxidized by intracellular

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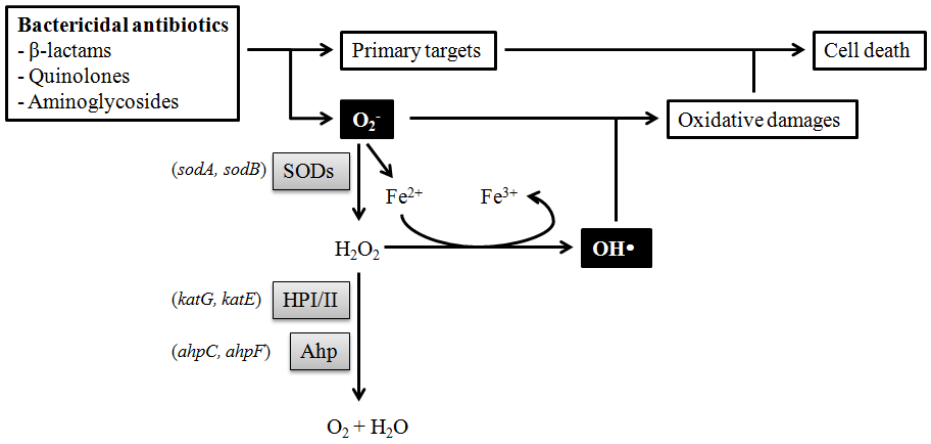

FIG 1 The antibiotic-mediated oxidative stress model. Kohanski *et al.* (1) proposed three classes of bactericidal antibiotics induce oxidative damages. Enzymes involved in the defense against superoxide and hydrogen peroxide potentially include superoxide dismutases (SODs), hydroperoxidase I/II (HPI/HPII), and alkyl hydroperoxide reductase (Ahp). Associated genes that encode the pathway enzymes are indicated in brackets.

oxidants, especially by hydrogen peroxide. Overnight cultures of all strains were diluted to an OD_{595} of 0.2 and incubated with 10 μM of DCFH-DA for 15 minutes. The dye-loaded cells were then centrifuged, washed with PBS and resuspended in fresh M9 medium. Cultures were treated with ampicillin, norfloxacin and tobramycin for 2 hours in dark. Fluorescence intensity readings were measured at 498 nm excitation and 522 nm emission wavelengths and were normalized to the corresponding turbidity at OD_{595}.

TBARS assay. Thiobarbituric acid reactive substances assay (TBARS, Cayman) quantifies lipid oxidation by measuring the amount of a by-product of the process, malondialdehyde (MDA). Overnight cultures of indicated strains were diluted to an OD_{595} of 0.2 and treated with 500 μM hydrogen peroxide or antibiotics for 30 minutes. Cultures were immediately centrifuged and washed once with PBS. Pellets were lysed in PBS buffer containing 1% Triton X-100 and 10 mM butylated hydroxytoluene to prevent further oxidation. Cells were sonicated in the buffer on ice. TBARS was conducted according to the manufacturer's protocol. Absorbance was measured at 530 nm.

RESULTS

Abolition of MnSOD impaired superoxide scavenging. Paraquat is a superoxide-generating agent and *E. coli* responds to it by inducing a number of enzymes including MnSOD, which dismutates superoxide (7). It has been shown that abolition of MnSOD in *E. coli* resulted in increased sensitivity to paraquat (8). We performed a similar experiment to characterize the Δ*sodA* mutant by challenging it with various doses of paraquat and monitoring growth, and the result was nearly identical (Fig. 2). The basal growth rate of the Δ*sodA* mutant was slightly lower than that of WT under microaerophilic conditions, as the Δ*sodA* mutant only grew 1.6 fold in 3 hours but WT grew 1.9 fold. A concentration of 25 μM paraquat, which had little effect on the growth of WT, completely inhibited the growth of the Δ*sodA* mutant, and a concentration as low as 0.2 μM was sufficient to halt its growth after 80 minutes. These results indicated that the Δ*sodA* mutant lacked MnSOD and was defective in superoxide scavenging.

TABLE 1 *E. coli* K-12 strains used in this study

Strain	Genotype	Phenotype
BW25113	WT	Parental strain
JW3879-1	Δ*sodA::kan*	Lacks MnSOD which scavenges O_2^-
JW1721-1	Δ*katG::kan*	Lacks HPI which scavenges H_2O_2
JW0598-2	Δ*ahpC::kan*	Lacks Ahp which scavenges H_2O_2
JW3933-3	Δ*oxyR::kan*	Lacks the regulon that induces HPI and Ahp expression when stressed

Abolition of HPI or Ahp altered hydrogen peroxide sensitivity. A previous study demonstrated that deletion of either HPI or Ahp in *E. coli* did not affect the capability to scavenge physiological amount of hydrogen peroxide (< 20 μM/s), but a simultaneous ablation of the two resulted in aerobic growth defects in minimal medium (9). To confirm that the Δ*katG*, Δ*ahpC* and Δ*oxyR* mutants obtained each possessed one of the three phenotype mentioned, namely, single-knockout of HPI or Ahp, and double-knockout of both enzymes, the mutants were challenged with serial dilutions of hydrogen peroxide and their growths were monitored (Fig. 3). Of the three mutants tested, only the Δ*oxyR* mutant displayed a reduced basal growth rate under microaerophilic conditions, the other two mutants had similar basal growth compared to WT. The hydrogen peroxide sensitivity was elevated in the Δ*katG* mutant, since only 500 μM was needed to completely inhibit its growth whereas 2000 μM was needed to do the same in WT. A concentration of 125 μM, which had virtually no effect on the growth of WT, suppressed the growth of the Δ*katG* mutant. Only when treated with a concentration of 30 μM or lower the Δ*katG* mutant grew the same as the control (data not shown). The Δ*ahpC* mutant was more resistant to the high doses of hydrogen peroxide but was more sensitive to the low doses compared to WT. The inhibitory dose for WT, 2000 μM, did not fully suppress the growth of the Δ*ahpC* mutant; yet

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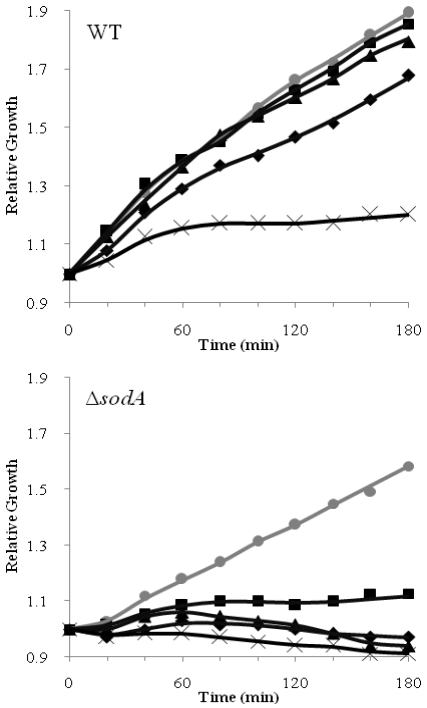

FIG 2 The Δ*sodA* mutant was defective in superoxide scavenging. Serial dilutions of paraquat were added to exponential cultures at zero time. Turbidity readings were normalized to the initial turbidity of each culture at zero time. ●, 0 μM; ■, 0.2 μM; ▲, 25 μM; ◆, 50 μM; ×, 100 μM.

a concentration of 125 μM that failed to affect WT growth, caused the grow rate to slow down in the Δ*ahpC* mutant after 80 minutes. The hydrogen peroxide sensitivity in the Δ*oxyR* mutant was puzzling, as the growth of the mutant was almost independent of the doses treated, suggesting it was more resistant than all the other strains. Considering the OxyR regulon contained at least 20 genes and half of them have not yet been assigned with a known function, the increased hydrogen peroxide resistance might have come from the abolition of those unknown proteins (10). Therefore, when subjected to supernormal levels of oxidative stress, the Δ*katG* mutant was more vulnerable but the Δ*ahpC* mutant was more resistant; however, when subject to physiological levels of oxidative stress, the Δ*ahpC* mutant was more susceptible.

Abolition of the antioxidant enzymes did not increase the antibiotic susceptibilities. Once the abilities of the mutants to scavenge ROS were characterized, antibiotic challenge experiments were performed to determine whether the antibiotic susceptibilities were increased in the mutants compared to WT. A MIC assay was first conducted (Table 2). Due to the poor growth of the Δ*oxyR* mutant in air, the culture did not become turbid overnight and therefore the mutant was excluded from the assay. WT had MICs of 2 μg/ml for ampicillin, 0.06 μg/ml for norfloxacin and 0.25 μg/ml for tobramycin. These figures were comparable to the MICs reported for the reference *E. coli* strains ATCC 25922; they were 4 μg/ml for ampicillin, 0.06 μg/ml for norfloxacin and 0.5 μg/ml tobramycin (6). None of the mutants had lower MICs; instead, the MICs of ampicillin for the Δ*sodA* mutant and the MICs of all three antibiotics for the Δ*ahpC* mutant were doubled compared to those of WT.

TABLE 2 Minimal inhibitory concentrations of ampicillin, norfloxacin and tobramycin for the tested strains.

Strains	Minimal inhibitory concentration (μg/ml)		
	Ampicillin	Norfloxacin	Tobramycin
WT	2	0.06	0.25
Δ*sodA*	4	0.06	0.25
Δ*katG*	2	0.06	0.25
Δ*ahpC*	4	0.12	0.50

The basal growths of mutants were not all the same in the aerobic growth assay (Fig. 4). The antibiotic concentrations used were capable of inducing oxidative stress in the previous study (1), and were around 2 to 10 fold higher than the MICs determined in this study. These concentrations were also the minimum doses required to kill an OD_{595} 0.2 culture incubated overnight under microaerophilic conditions (data not shown).The Δ*sodA* and Δ*ahpC* mutants displayed lower basal growth rates than WT under aerobic conditions; they grew 2.5 fold in 3 hours whereas WT grew 3 fold. These differences in basal growth rates were larger than those observed under microaerophilic conditions, where the Δ*sodA* mutant grew 1.6 fold but WT grew 1.9 fold (Fig. 2), and the Δ*ahpC* mutant grew 1.7 fold but WT grew 1.8 fold (Fig. 3). The basal growth rate for the Δ*oxyR* mutant was also lower under aerobic conditions than under microaerophilic conditions (Fig. 3). This was expected in a mutant that lacks both HPI and Ahp.

The growth assay results were consistent with the MIC assay results and further confirmed the observation that none of the mutants were more susceptible than WT (Fig. 4). The Δ*katG* mutant responded to the antibiotics in a similar fashion to WT, suggesting HPI was not essential in the defense against the antibiotics at the chosen concentrations. The Δ*sodA* mutant also responded similarly to tobramycin and norfloxacin, but it was more resistant to ampicillin compared to WT. Although the times required for ampicillin to function in the Δ*sodA* mutant and in WT were both 80 minutes, the rate of decrease in turbidity was lower in the Δ*sodA* mutant than in WT. At 3 hours, the Δ*sodA* mutant's turbidity was reduced 40% compared to its control, whereas WT was reduced 60%. All three antibiotics were less effective against the Δ*ahpC* mutant; the same doses of ampicillin and tobramycin that were bactericidal after 80 minutes in WT were only bacteriostatic in the Δ*ahpC* mutant, and the same dose of norfloxacin required 40 minutes longer to function when compared to WT. Finally, the Δ*oxyR* mutant did not respond to any of the three antibiotics, displaying marked increase in antibiotic resistance.

Antibiotic-induced oxidative stress was not elevated in the mutants. Since abolition of the antioxidant enzymes did not increase the antibiotic susceptibilities, we asked whether the doses chosen were sufficient to induce oxidative stress and whether the stress accumulated excessively in the Δ*sodA* and Δ*katG* mutants, the two

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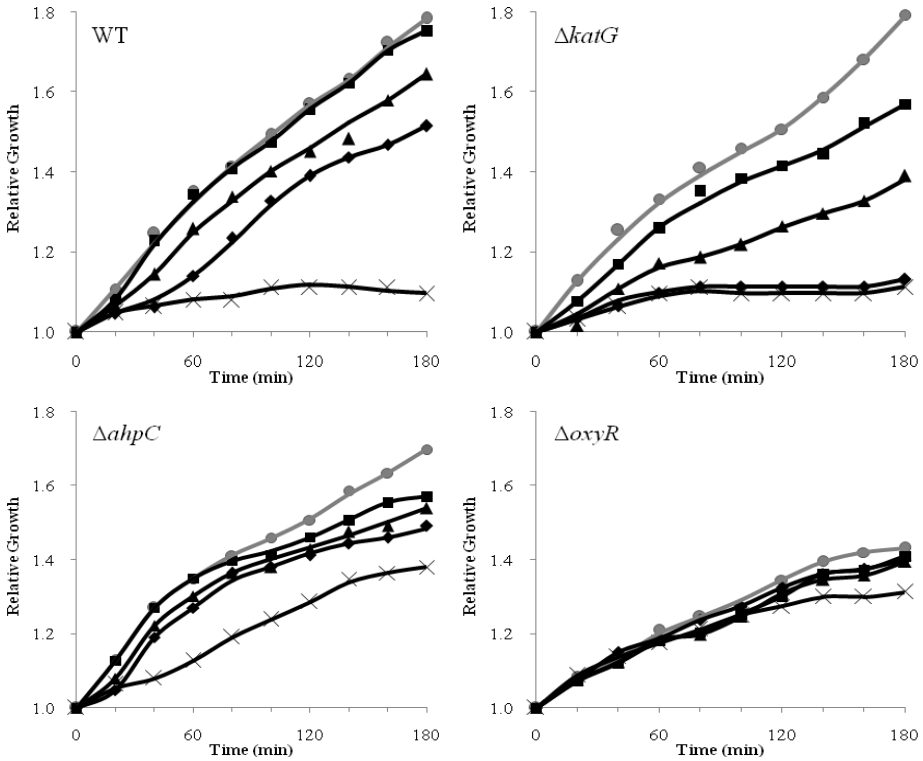

FIG 3 Hydrogen peroxide sensitivity was different amongst the ROS scavenging mutants. Serial dilutions of hydrogen peroxide were added to exponential cultures at zero time. Turbidity readings were normalized to the initial turbidity of each culture at zero time. ●, 0 μM; ■, 125 μM; ▲, 250 μM; ◆, 500 μM; ×, 2000 μM. Results are representative of 2 independent experiments.

mutants with confirmed scavenging defects. DCFH-DA staining was performed to evaluate intracellular oxidative stress (Fig. 5). In the absence of cells, the dye was directly oxidized to form the fluorescent product by 1 mM of hydrogen peroxide but was not oxidized by the three antibiotics (data not shown). The background oxidative stress in the Δ*sodA* mutant was comparable to that of WT, whereas in the Δ*katG* mutant the signal was twice as high. Ampicillin and tobramycin treatments consistently increased the endogenous oxidative stress in all three strains; however the increases were not larger in the mutants, suggesting only limited stress was induced by antibiotics and the missing enzymes were not essential in scavenging the induced oxidative stress. Norfloxacin treatment increased the stress only in WT but not in the mutants; this was expected in the Δ*sodA* mutant but not in the Δ*katG* mutant.

TBARS was also performed to assess the oxidative stress induced by antibiotics (Table 3). However, the assay failed to detect any significant change in output signals upon hydrogen peroxide or antibiotic challenges. The absorbance readings were close to the background values and therefore we concluded the particular assay kit obtained was not sensitive enough to be used in *E. coli*.

DISCUSSION

Several studies demonstrated that oxidative stress was important in the killing mediated by at least three classes of antibiotics (1, 11, 12, 13).The present work attempted to build on the observations and tested whether the antioxidant enzymes MnSOD, HPI or Ahp were essential in the defense against the antibiotics. Unexpectedly, knocking out each of the three enzymes in *E. coli* did not increase antibiotic susceptibilities even though a similar study reported different findings (14).

MnSOD is important in dismutating superoxide into the hydrogen peroxide, a process that can occur spontaneously but the reaction is 4 magnitudes slower than the enzymatic conversions (15). FeSOD has a lower efficiency than MnSOD and therefore is most suitable for scavenging physiological levels of superoxide (8), whereas MnSOD is part of the SoxRS regulon that responds to elevated levels of superoxide (16). The Δ*sodA* mutant displayed reduced growth in air (Fig. 4) and hyperlethality to paraquat (Fig. 1), suggesting even with FeSOD the mutant was defective in superoxide scavenging. Contrary to the original hypothesis, the Δ*sodA* mutant shown increased resistance to ampicillin, but not tobramycin or norfloxacin, as shown by the MIC results (Table 2) and the growth assay (Fig. 4). These results supported Kohanski's model (Fig. 1): since hydroxyl radicals were more harmful than superoxide anion then inhibiting the conversion would provide survival benefits. However, the oxidative stress profile of the Δ*sodA* mutant was not different compared to that of WT when treated with ampicillin (Fig. 5), suggesting the difference in susceptibility was not due to oxidative stress.

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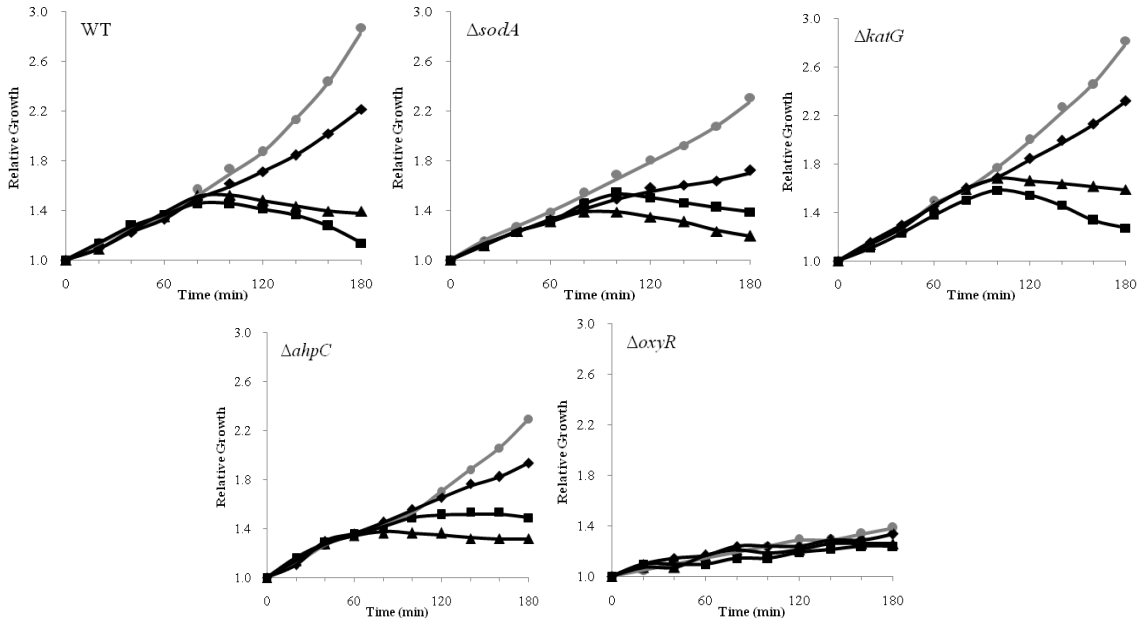

FIG 4 Effect of ampicillin, norfloxacin and tobramycin on the relative growth of the ROS scavenging mutants. Antibiotics were added to exponential cultures at zero time. Turbidity readings were normalized to the initial turbidity of each culture at zero time. ●, no treatment; ■, ampicillin 10 μg/ml; ◆, norfloxacin 0.24 μg/ml; ▲, tobramycin 2 μg/ml. Results are representative of 2 independent experiments.

Wang *et al*. reported that their *sodA sodB* double-knockout strain but not their *sodA* single-knockout strain was less susceptible to the killing mediated by all three classes of antibiotics, although the double-knockout strain had the same MICs as their WT(14).Resolving this apparent conflict will require characterization of the oxidative stress in the *sodAsodB* double-knockout strain upon antibiotic challenges.

The main scavengers of hydrogen peroxide in *E. coli* are Ahp and HPI. Although both enzymes are members of the OxyR regulon, Ahp has a higher substrate affinity and a lower Kmthan HPI, and therefore is the primary scavenger under physiological conditions whereas HPI are important in combating elevated stress (9). Abolition of HPI, although increased the bacterial sensitivity to supernormal levels of hydrogen peroxide, did not alterthe aerobic growth rate or the susceptibilities to the antibiotics (Fig. 3 and 4, Table 2). This effect was consistent with the findings of Wang *et al*. (14). Even though the Δ*katG* mutant had high background oxidative stress, the antibiotics failed to induce excessive accumulation of additional stress (Fig. 5). The results suggested HPI was not necessary for the defense against the antibiotics; the oxidative stress induced was effectively scavenged by Ahp and HPII.

Abolition of Ahp resulted in a complex phenotype. When challenged with hydrogen peroxide, the Δ*ahpC* mutant was more sensitive to the lower doses, but at the same time, more resistant to the higher doses (Fig. 4).In addition, the Δ*ahpC* mutant grew slower aerobically than microaerophilically (Fig. 3 and 4). These results suggested abolition of Ahp led to the accumulation of oxidative stress to an equilibrium level that was insufficient to fully induce HPI and yet exceeded the scavenging capacity of HPII, and therefore caused damages to the cells. On the other hand, one study also reported that abolition of Ahp resulted in 2-fold increase in scavenging efficiency when treated with supernormal level of hydrogen peroxide (150 μM) due to a full induction of HPI (17).We believe the observation that the Δ*ahpC* mutant had an elevated resistance to the antibiotics (Fig. 4, Table 2) could be explained in a similar fashion, such that the antibiotic-mediated stress reached a critical concentration so it was scavenged by HPI. To confirm this belief, a similar experiment should be conducted to check HPI expression. In contrast to our results, Wang *et al*. shown that their *ahpC* mutant was significantly more susceptible to ampicillin and kanamycin even at 50 times MICs (14), a dose that our results would predict to give the opposite

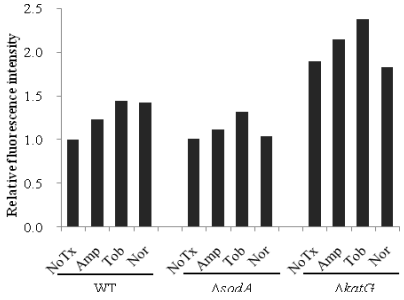

FIG 5 Effect of ampicillin, norfloxacin and tobramycin on the relative oxidative stress in the ROS scavenging mutants. Fluorescence measurements of DCFH-DA staining were taken 2 hours following antibiotic treatments. NoTx, no treatment; Amp, ampicillin 10 μg/ml; Tob, tobramycin 2 μg/ml; Nor, norfloxacin 0.24 μg/ml. All measurements were normalized to the non-treated WT culture.

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TABLE 3 Effect of norfloxacin and tobramycin on the lipid oxidation in the ROS scavenging mutants.

Strains	Treatment	TBARS ($\times 10^{-2} A_{530}/OD_{595}$)
WT	None	1.1
	H_2O_2	1.7
	Norfloxacin	1.5
	Tobramycin	1.6
Δ*katG*	None	1.3
	H_2O_2	1.9
	Norfloxacin	1.5
	Tobramycin	1.7
Δ*oxyR*	None	0.6
	H_2O_2	0.9
	Norfloxacin	0.8
	Tobramycin	0.8

response. However, their mutant had a MIC for kanamycin 4 times higher than that of their WT. The inconsistency in the discussed results requires further investigations.

The Δ*oxyR* mutant was originally chosen to represent an *ahpCkatG* double-knockout in order to eliminate enzyme redundancy. Indeed the two enzymes were not expressed as the mutant had severe aerobic growth defects, but it also displayed higher resistance to both the hydrogen peroxide and the antibiotics compared to WT (Fig. 3 and 4), suggesting that other unknown members on the OxyR regulon altered the scavenging ability of the mutant. Although outside of the scope of this work, this finding could potentially lead to novel mechanisms in peroxide scavenging.

Our TBARS results indicated the lipid peroxidation induced by hydrogen peroxide or the antibiotics was not significantly increased in the mutants than in WT. We were uncertain whether the differences in the value presented in Table 3 were due to measurement errors or actual increases in lipid peroxidation, since the average absorbance values recorded were close to the background values (0.04 and 0.035, respectively).Previous studies have reported significant increases in TBARS signals upon chemical or antimicrobial challenges in *E. coli*(18, 19, 20). We noticed the assay kits used in these studies had standard curves with 2.5 to 10 fold stronger signals when compared to ours; and even then, the amount of MDA detected was in range of nanomoles per sample. This was consistent with the fact that *E. coli* has low levels of polyunsaturated fatty acids, which is the required substrates for lipid peroxidation (21). Thus, we concluded that a more sensitive assay kit was needed to address our experimental question.

In summary, abolishing each of MnSOD, HPI or Ahp did not increase the antibiotic susceptibilities of *E. coli* to ampicillin, tobramycin and norfloxacin. However, we found that abolition of Ahp increased the bacterial resistance to both certain doses of hydrogen peroxide and the antibiotics, suggesting that ROS scavenging could potentially play a role antibiotic-mediated killing but the effect was probably masked by the complex enzyme inter-regulation and redundancy in the scavenging pathway. Future experiments focusing on eliminating the enzyme redundancy are needed to better test the hypothesis.

FUTURE DIRECTIONS

The two major challenges presented in the study were the complex enzyme redundancy in the antioxidant pathways and the insufficiency of assay sensitivities. To resolve the first challenge, a *sodAsodB* double-knockout and a *katGahpC* double-knockout strains should be used. Be aware that the double-knockouts cannot grow aerobically so only survivability assays, but not growth suppression assays, can be performed. Viable plate counts following a larger range of antibiotic treatments, instead of turbidity readings, would reflect more accurately on the degree of antibiotic-mediated killing. Finally, a more sensitive assay to assess oxidative stress should be utilized. One potential assay is the amplex red staining, which was shown capable of detecting as low as 0.1 μM hydrogen peroxide in *E. coli* (9).

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