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Contribution of Oxidative Damage to Antimicrobial Lethality[▽]

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A potential pathway linking hydroxyl radicals to antimicrobial lethality was examined by using mutational and chemical perturbations of *Escherichia coli*. Deficiencies of *sodA* or *sodB* had no effect on norfloxacin lethality; however, the absence of both genes together reduced lethal activity, consistent with rapid conversion of excessive superoxide to hydrogen peroxide contributing to quinolone lethality. Norfloxacin was more lethal with a mutant deficient in *katG* than with its isogenic parent, suggesting that detoxification of peroxide to water normally reduces quinolone lethality. An iron chelator (bipyridyl) and a hydroxyl radical scavenger (thiourea) reduced the lethal activity of norfloxacin, indicating that norfloxacin-stimulated accumulation of peroxide affects lethal activity via hydroxyl radicals generated through the Fenton reaction. Ampicillin and kanamycin, antibacterials unrelated to fluoroquinolones, displayed behavior similar to that of norfloxacin except that these two agents showed hyperlethality with an *ahpC* (alkyl hydroperoxide reductase) mutant rather than with a *katG* mutant. Collectively, these data are consistent with antimicrobial stress increasing the production of superoxide, which then undergoes dismutation to peroxide, from which a highly toxic hydroxyl radical is generated. Hydroxyl radicals then enhance antimicrobial lethality, as suggested by earlier work. Such findings indicate that oxidative stress networks may provide targets for antimicrobial potentiation.

Antimicrobials that actively kill pathogens are expected to cure disease more rapidly and restrict the emergence of resistance better than agents that are largely bacteriostatic. Consequently, considerable effort has been devoted to understanding mechanisms of lethal action. In general, antimicrobials are thought to kill microbes through interaction with specific intracellular targets, followed by corruption of particular cellular processes (22). However, Collins and associates recently proposed that many bactericidal antimicrobials also share a common lethal pathway that involves the generation/accumulation of hydroxyl radicals (13, 23). This hypothesis is supported by elevated hydroxyl radical levels associated with lethal antimicrobial treatment (23). How this occurs is largely unknown.

Three major reactive oxygen species, superoxide, hydrogen peroxide, and hydroxyl radical, are generated as by-products of normal aerobic respiration (17, 20). All three are cytotoxic, but they display different kinetics and levels of severity. For example, the effects of superoxide and hydrogen peroxide are probably less acute than those of hydroxyl radicals, since both superoxide and hydrogen peroxide can be detoxified by induced scavenging enzymes. In contrast, no enzyme can detoxify hydroxyl radicals, making them extremely toxic and acutely lethal. Hydroxyl radicals derive from hydrogen peroxide through the Fenton reaction (14), which makes the regulation of the intracellular peroxide concentration a starting point for exploring genetic pathways that affect hydroxyl radical-mediated killing. Peroxide is generated mainly from superoxide through the action of superoxide dismutase (SOD) (16). Dismutation of superoxide can also occur spontaneously; however, the rate is about 10^5 M^{-1} s^{-1}, which is significant but about 4 orders of magnitude lower than enzymatic dismutation (15, 25). Consequently, disruption of SOD-mediated generation of peroxide is expected to reduce the accumulation of peroxide and thus the generation of hydroxyl radicals. Detoxification of hydrogen peroxide by conversion to water occurs via catalases/peroxidases at a rate of about 10^6 M^{-1} s^{-1}, faster than spontaneous superoxide dismutation but significantly slower than SOD-mediated dismutation (9, 10, 29). Disruption of catalase-peroxidase genes is expected to raise peroxide levels, which in turn may raise hydroxyl radical levels and lower cell survival associated with antimicrobial treatment. Consequently, we were surprised by the report that a superoxide dismutase (*sodB*) mutation increased lethal susceptibility to norfloxacin (13) rather than lowering it or having no effect due to the presence of a second superoxide dismutase (*sodA*).

We began the present study by examining norfloxacin action with the *sodB* mutant used previously (13). In our hands, this mutant exhibited the same susceptibility as the wild-type strain, as was true with either a *sodA* or a *sodB* mutant in another genetic background. In contrast, a *sodA sodB* double mutant showed reduced rather than increased lethal susceptibility to norfloxacin treatment. Genetic inactivation of catalase-peroxidase increased quinolone susceptibility. Together, these results solidified a role for peroxide in quinolone lethality. Both an iron chelator (an inhibitor of the Fenton reaction) and a hydroxyl radical scavenger lowered norfloxacin lethality, consistent with conversion of peroxide to hydroxyl radical, contributing to cell death. Similar conclusions were reached with other antimicrobial classes, represented by kanamycin and am-

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TABLE 1. Bacterial strains used in this study

Strain no.	Relevant genotype	Source/reference[a]
3140	BW25113 katE::kan	CGSC# 9453 (4)
3144	BW25113 ΔsodA	This work, by antibiotic marker excision from CGSC# 10798
3145	BW25113 ΔsodB	This work, by antibiotic marker excision from CGSC# 9402
3149	BW25113 sodA::kan ΔsodB	This work, by P1-medicated transduction from CGSC# 10798 to 3145
3156	BW25113 ΔsodA ΔsodB	This work, by antibiotic marker excision from 3149
3157	BW25113 ΔkatG	This work, by antibiotic marker excision from CGSC# 10827
3163	BW25113 ΔkatG katE::kan	This work, by P1-medicated transduction from 3140 to 3157
3200	BW25113 ΔahpC	This work, by antibiotic marker excision from CGSC# 8713
3201	BW25113 ΔkatG ΔkatE	This work, by antibiotic marker excision from 3163
3202	BW25113 ΔkatE	This work, by antibiotic marker excision from 3140
BW25113	Wild type	CGSC# 7636 (4)
CSH7	Wild type	Richard D'Ari (26)
CSH57A	Wild type	Richard D'Ari (26)
DKsodB	BW25113 ΔsodB	James Collins (13)
GC4468	Wild type	Richard D'Ari (7)
QC772	GC4468F sodA::Cm	Richard D'Ari (7)
QC773	GC4468F sodB::Kan	Richard D'Ari (7)
QC774	GC4468F sodA::Cm sodB::Kan	Richard D'Ari (7)
UM1	CSH7 katE1 katG14[b]	Richard D'Ari (26)
UM2	CSH57A katE2 katG15[b]	Richard D'Ari (26)

[a] CGSC#, Coli Genetic Stock Center strain number. All CGSC strains were obtained from Yale University Coli Genetic Stock Center.
[b] katG14 encodes a protein that is deficient in catalase but proficient in peroxidase activity, while katG15 encodes a protein deficient in both enzymatic activities.

picillin. These results help define an oxidative stress pathway that contributes to antimicrobial lethality.

MATERIALS AND METHODS

Bacterial strains, plasmid, and growth conditions. Strains of *Escherichia coli* K-12 used in the work are listed in Table 1. *E. coli* was grown in LB broth or on LB agar (28) supplemented with the indicated concentrations of antimicrobials. Construction of mutant strains was performed by sequential kanamycin marker excision (11) and bacteriophage P1-mediated transduction (33). Plasmid DNA (pCP20) (8) was introduced into host strains by bacterial transformation for antibiotic marker excision as reported previously (11).

Chemicals and reagents. Oxolinic acid, norfloxacin, rifampin (rifampicin), 2,2′-bipyridyl, and thiourea were purchased from Sigma Chemical Co. (St. Louis, MO). Ciprofloxacin was from Bayer AG (Wuppertal, Germany). Chloramphenicol and tetracycline were purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Kanamycin was from Fisher Scientific (Pittsburgh, PA). Ampicillin was purchased from Roche Diagnostics (Indianapolis, IN).

Antimicrobial susceptibility assays. Growth inhibition (MIC) was determined by broth dilution with visual inspection of a series of tubes each containing about 10^5 bacteria in 1 ml of LB medium supplemented with concentrations of drug increasing by 50% increments. Following an overnight incubation at 37°C, the lowest concentration that prevented visible growth was defined as the MIC.

Lethal activity was measured in two ways. Slow killing was expressed by the minimal bactericidal concentration (MBC) in which survival was reduced by 1,000-fold following an 18-h incubation of a series of 1-ml broth cultures each containing about 10^6 CFU bacteria and twofold incremental concentrations of antimicrobial. For rapid killing, overnight cultures were grown at 37°C with shaking at 250 rpm, diluted by 200-fold into fresh medium, and regrown to early- to mid-exponential phase (approximately 5×10^8 CFU/ml) before exposure to various concentrations of drug for a fixed time or to various times for a fixed drug concentration. Before and after treatment, cells were serially diluted and applied to drug-free agar plates for determination of viable counts. After 16 to 24 h of incubation at 37°C, bacterial colonies were counted and the percent survival was calculated relative to values taken at the time of antimicrobial addition. Rapid killing measurements with stationary- or lag-phase cultures were made as with exponentially growing cultures except that overnight cultures were either used directly (stationary phase) or diluted by 50-fold (lag phase) before being treated with the antimicrobials.

Effect of iron chelator and hydroxyl radical scavenger on antimicrobial lethality. Subinhibitory concentrations of 2,2′-bipyridyl (250 μM; 50% MIC) and thiourea (100 mM; 50% MIC) were added to bacterial cultures 10 min prior to initiation of antimicrobial treatment. The cultures were then processed as for the antimicrobial susceptibility assays described above.

RESULTS

Involvement of superoxide dismutase in rapid killing by norfloxacin. When we examined the ability of norfloxacin to block growth of an *E. coli* strain deficient in *sodB* (strain DKsodB), little effect was observed for the norfloxacin MIC

TABLE 2. Bacteriostatic activities of compounds investigated

Bacterial strain	Relevant genotype	MIC of compound[a]				
		Thiourea (mM)	Bipyridyl (μM)	Norfloxacin	Ampicillin	Kanamycin
BW25113	Wild type	200	500	0.04	15	0.6
DKsodB	ΔsodB	200	500	0.04	15	0.6
3144	ΔsodA	200	500	0.04	15	0.6
3145	ΔsodB	200	500	0.04	15	0.6
3156	ΔsodA ΔsodB	200	500	0.04	15	0.6
3157	ΔkatG	200	500	0.04	15	0.6
3200	ΔahpC	150	500	0.04	15	2.4
3201	ΔkatG ΔkatE	200	500	0.08	30	1.2
3202	ΔkatE	200	500	0.04	15	0.6

[a] Expressed in μg/ml unless otherwise indicated.
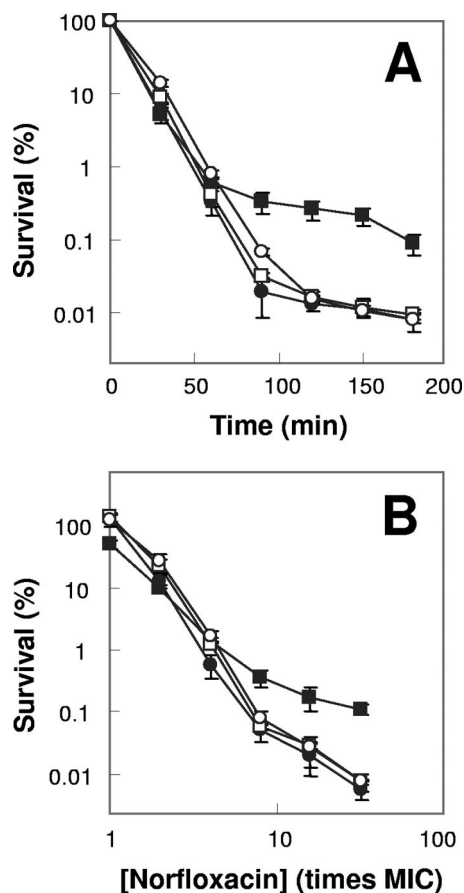
FIG. 1. Effects of superoxide dismutase deficiency on norfloxacin lethality. Exponentially growing *E. coli* cells were treated with 0.4 μg/ml (10 times the MIC) norfloxacin for various times (A) or with the indicated concentrations of norfloxacin (B) for 2 h. Symbols: filled circles, wild type; filled squares, *sodA sodB* mutant; open circles, *sodA* mutant; open squares, *sodB* mutant. Error bars indicate standard deviations for replicate samples. At least three replicate experiments were performed, and each had results similar to those shown.

(Table 2). Likewise, little effect was observed on lethal activities measured at various incubation times and norfloxacin concentrations (Fig. 1). The *sodB* mutation also had little effect on either the MIC or the lethal action of oxolinic acid or ciprofloxacin measured at various times and concentrations (not shown). When we examined a *sodA* mutant and another *sodB* mutant constructed with the same genetic background (strains 3144 and 3145), we also observed little effect on the norfloxacin MIC or on the ability of the drug to kill *E. coli*, measured at various times and norfloxacin concentrations (Table 2 and Fig. 1; also not shown). Similar data were obtained for oxolinic acid and ciprofloxacin (not shown). Deficiencies of *sodA* (strain QC772) or *sodB* (strain QC773) in a different genetic background also showed little effect on the MICs or lethal susceptibilities for the three quinolones tested (not shown). Thus, we conclude that deficiencies of either *sodA* or *sodB* have little or no effect on quinolone lethality.

Since the two *sod* gene products may have redundant activities, we examined the effect of mutations in both *sod* genes (strain 3156). No effect was observed on the MIC (Table 2).

However, norfloxacin lethality, measured either at various incubation times or at various drug concentrations, was 10-fold lower with the double mutant (Fig. 1A and B). The protective effect seen with the *sod* double mutant occurred only with treatment times of >50 min and norfloxacin concentrations of >4 times the MIC. Cells recovered from treatment in which protection was evident (e.g., 10 times the MIC, 120-min incubation with the *sodA sodB* double mutant) were not enriched for norfloxacin-resistant mutants (not shown). A 10- to 100-fold protective effect was also observed with a *sodA sodB* double mutant in a different genetic background (strain QC774), either when growing exponentially or when grown overnight, diluted by 50-fold, and then treated with norfloxacin or ciprofloxacin (not shown). Thus, a deficiency of superoxide dismutase activity partially protects from quinolone-mediated cell death.

Involvement of catalase in rapid killing by norfloxacin. If the protective effect of a *sodA sodB* deficiency is due to restricted conversion of superoxide to peroxide, then catalase/peroxidase-deficient mutants should cause an increase in peroxide accumulation and render cells more easily killed. We first measured bacteriostatic activity. Deficiencies of *katG* (strain 3157) and *katE* (strain 3202) had little effect on the norfloxacin MIC; the MIC of a *katG katE* double mutant (strain 3201) was only twice that of wild-type cells (Table 2). MICs showed a similar behavior with two other quinolones, oxolinic acid and ciprofloxacin (not shown). Thus, bacteriostatic activity is insensitive to catalase/peroxidase deficiencies.

In contrast to bacteriostatic activity being unaffected by catalase/peroxidase deficiency, lethal action of norfloxacin was increased 10- to 100-fold by a *katG* single mutation and by a *katG katE* double mutation when measured at various incubation times or at various drug concentrations as a function of the MIC (Fig. 2). The enhancement of killing by catalase/peroxidase deficiency was more pronounced at shorter incubation times (≤60) min and lower norfloxacin concentrations (e.g., two times the MIC gave the largest difference, while 32 times the MIC showed the smallest difference). Increased lethality was also observed for oxolinic acid and ciprofloxacin (not shown); similar results were obtained with a different genetic background (strains UM2 and CSH57A [not shown]). A *katE* single mutant failed to exhibit hyperlethality, and the *katG katE* double mutant showed the same lethal susceptibility as the *katG* single mutant (Fig. 2). Since KatG possesses both catalase and peroxidase activity, a *katG* mutant (strain UM1) deficient in catalase but proficient in peroxidase activity and a *katG* mutant (strain UM2) deficient in both enzymatic activities were also tested. The catalase deficiency alone (UM1) failed to cause the hyperlethal phenotype, while a *katG* mutant (UM2) lacking both catalase and peroxidase activity, showed hyperlethality (not shown). Collectively, these data allow us to conclude that catalase-peroxidase activity behaves in an opposite way to that of superoxide dismutase and that type I hydroperoxidase (KatG), not type II hydroperoxidase (KatE), is primarily responsible for removing excess hydrogen peroxide accumulated upon lethal quinolone exposure.

Effect of thiourea and ferrous chelator on norfloxacin lethality. We next examined the possibility that the increased lethality of norfloxacin associated with peroxide was due, at least in part, to an increase in the hydroxyl radical concentra-
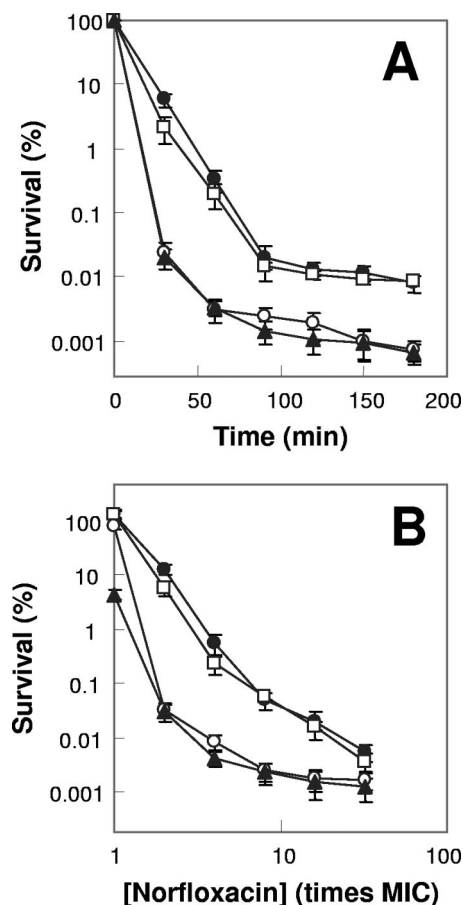
FIG. 2. Effects of catalase/peroxidase deficiency on norfloxacin lethality. Exponentially growing *E. coli* cells were treated with 10 times the MIC of norfloxacin (0.8 μg/ml for the *katG katE* double mutant and 0.4 μg/ml for other strains) for various times (A) or with the indicated concentrations of norfloxacin (B) for 2 h as in Fig. 1. Symbols: filled circles, wild type; open circles, *katG* mutant; open squares, *katE* mutant; triangles, *katG katE* mutant. Error bars indicate standard deviations for replicate samples. At least three replicate experiments were performed, and each had results similar to those shown.

tion by treating wild-type cells with an iron chelator (bipyridyl) that interferes with the Fenton reaction or with thiourea, a hydroxyl radical scavenger. Cotreatment with subinhibitory concentrations (e.g., 50% MIC) of either bipyridyl or thiourea failed to affect the norfloxacin MIC (not shown). However, the same cotreatments reduced norfloxacin-mediated killing by 10- to 100-fold when either the treatment time (Fig. 3A and C) or the norfloxacin concentration (Fig. 3B and D) was varied.

We also measured the effects of thiourea and bipyridyl separately on the *sodA sodB* (strain 3156) and *katG* (strain 3157) mutants. Both thiourea and bipyridyl had less protective effect with the *sodA sodB* strain than with wild-type cells (not shown), but they conferred similar overall protective effects with the *katG* mutant and wild-type *E. coli* (Fig. 3). The lesser protection with the *sodA sodB* mutant may derive from slower/less accumulation of peroxide and hydroxyl radical in the mutant than in the wild-type strain. The failure of thiourea and bipyridyl to restore killing of a *katG* mutant to wild-type levels is probably due to our use of subinhibitory scavenger/chelator

concentrations that were unable to completely eliminate hydroxyl radical accumulation. Cotreatment with bipyridyl, which inhibits generation of hydroxyl radicals, was slightly more protective than scavenging of hydroxyl radicals with thiourea when each was present at 50% MIC. Such a difference is possibly because hydroxyl radicals, once generated, are too reactive to allow thiourea enough time to scavenge them before they damage nearby macromolecules.

Involvement of superoxide dismutase and catalase-peroxidase in ampicillin- and kanamycin-mediated cell death. To determine whether peroxide-hydroxyl radical aspects of quinolone-mediated cell death also apply to the lethal action of β-lactams and aminoglycosides, we examined ampicillin- and kanamycin-mediated killing with *sod* and *kat* mutants. A deficiency of superoxide dismutatase (*sodA sodB*; strain 3156) conferred protection (Fig. 4 and 5); thiourea and bipyridyl were also protective (not shown), consistent with hydroxyl radical formation contributing to ampicillin and kanamycin lethality. However, a deficiency of catalase-peroxidase (*katG*; strain 3157) conferred wild-type susceptibility to ampicillin and kanamycin (Fig. 4 and 5), unlike the situation observed with fluoroquinolones.

We next considered the possibility that failure to observe hyperlethality in the *katG* mutant arose from ampicillin/kanamycin-mediated production/accumulation of hydrogen peroxide being below the K_m of KatG. To test this idea, we examined alkyl hydroperoxide reductase (encoded by *ahpCF*), which is thought to act as the primary scavenger of peroxides at low substrate concentrations due to a higher affinity (lower K_m) than KatG (31). When alkyl hydroperoxide reductase was deficient (Δ*ahpC*; strain 3200), *E. coli* was hypersensitive to ampicillin and kanamycin (Fig. 4 and 5). These data are consistent with ampicillin and kanamycin causing a lower accumulation of peroxide than norfloxacin, which results in AhpCF being the primary scavenger for hydrogen peroxide. A *katG ahpC* double mutant was not tested, since it grows very poorly under aerobic conditions and since the antimicrobials investigated have poor lethal activity with nongrowing cells.

Absence of involvement of catalase-peroxidase in activities of bacteriostatic antibacterials. To test the suggestion that the effects of hydroxyl radical formation are restricted to bactericidal agents (23), we examined the effects of *katG*, *ahpC*, and *katG katE* mutations on the activity of chloramphenicol, a bacteriostatic agent with *E. coli*. Mutant and wild-type MICs were identical (not shown), and even at 100 times the MIC (200 μg/ml), chloramphenicol failed to kill *E. coli*. No enhancement of killing was observed with *katG*, *katG katE*, and *ahpC* mutants (not shown). A similar conclusion was reached with another static agent, rifampin (not shown). These data support the assertion (23) that enhancement of hydroxyl radical action occurs only with bactericidal agents.

Effect of superoxide dismutase and catalase-peroxidase deficiencies on antimicrobial lethalities under various growth and treatment conditions. The phenomena described above were observed with rapid killing of exponentially growing cells. To test whether slow killing is also affected by oxidative stress pathway mutations, MBCs were measured with the three compound classes using the same set of isogenic strains (Table 3). Little difference in MBCs was observed among wild-type and mutant strains except for the *ahpC* mutant and the *sodA sodB*
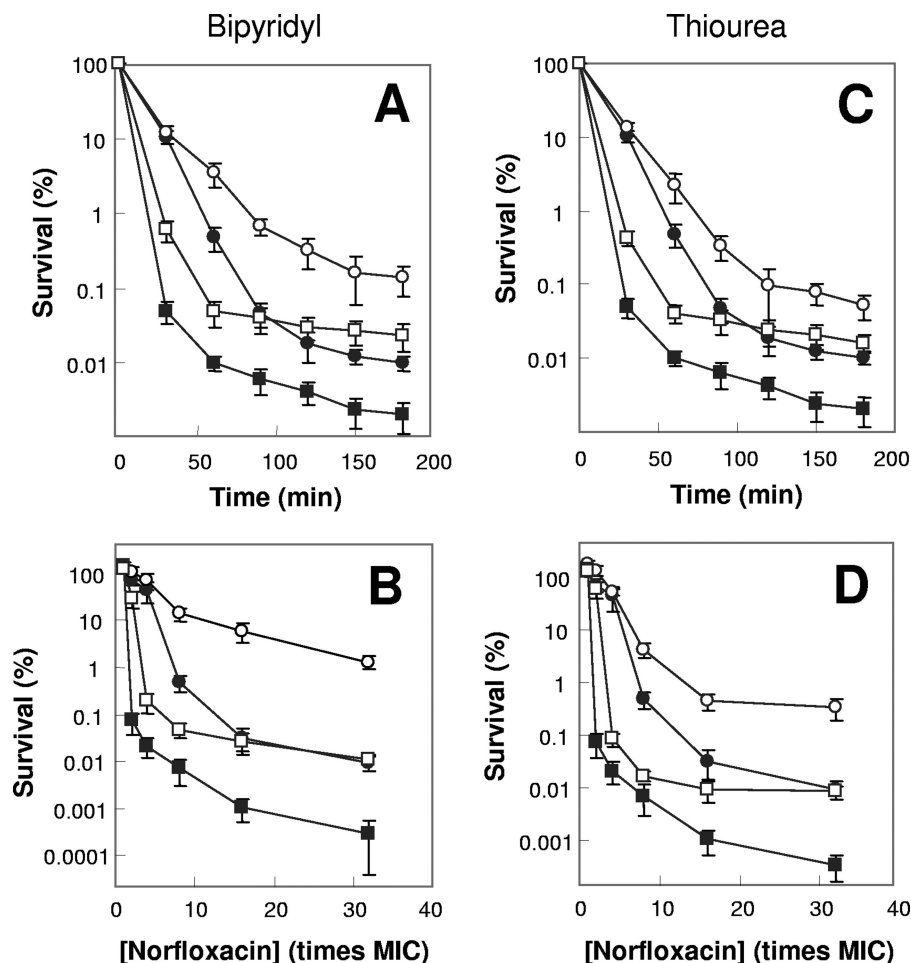
FIG. 3. Effects of a ferrous chelator and a hydroxyl radical scavenger on norfloxacin lethality. Exponentially growing *E. coli* cells were preincubated with 250 μM bipyridyl (A and B) or 100 mM thiourea (C and D) for 10 min before they were treated with 10 times the MIC of norfloxacin (0.4 μg/ml) for various times (A and C) or with the indicated concentrations of norfloxacin (B and D) for 2 h as in Fig. 1. Symbols: filled circles, wild type; open circles, wild type plus bipyridyl or thiourea; filled squares, *katG* mutant; open squares, *katG* mutant plus bipyridyl or thiourea. Error bars indicate standard deviations for replicate samples. At least three replicate experiments were performed, and each had results similar to those shown.

double mutant (both exhibited a two- to fourfold reduction in the MBC [strains 3200 and 3156; Table 3]). The slight decrease in the MBC seen with the *sodA sodB* mutant seems contradictory to the protective effect exhibited for this mutant in rapid killing assays. This discrepancy may derive from superoxides being chronically toxic, so that long-term exposure to elevated levels of superoxide is detrimental to cells, which would override short-term protective effects conferred by deficiencies in SodA and SodB. Such a possibility is currently under investigation. Rapid killing with lag-phase cells (stationary-phase culture diluted by 50-fold) by kanamycin and norfloxacin gave results similar to those seen with exponentially growing cells except for diminished protection from a *sodA sodB* deficiency for kanamycin (not shown). No strain tested was killed by ampicillin when in lag phase. Likewise, no killing was observed with wild-type and mutant strains when stationary cultures were directly treated with any of the three compounds tested (not shown). Thus, oxidative stress-stimulated antimicrobial lethality is absent in stationary-phase cells, but it is present at

similar levels during the exponential and lag phases of growth when lethality is observed.

DISCUSSION

Antimicrobial-mediated killing of some bacteria appears to involve oxidative stress, as suggested by the accumulation of superoxide anions following exposure to at least three antimicrobial classes (1, 5) and by reactive oxygen species being associated with ciprofloxacin susceptibility (19). The possibility was recently raised that hydroxyl radical overproduction may serve as a common path to antimicrobial-mediated cell death (13, 23). The present work provides support for a possible lethal pathway from antimicrobial treatment to hydroxyl radical accumulation via superoxides and peroxides (Fig. 6). A deficiency in superoxide dismutase (*sodA sodB* double mutation) reduced the lethal action of norfloxacin (Fig. 1), ampicillin (Fig. 4), and kanamycin (Fig. 5). Single mutations were insufficient, as indicated by experiments with norfloxacin (Fig.
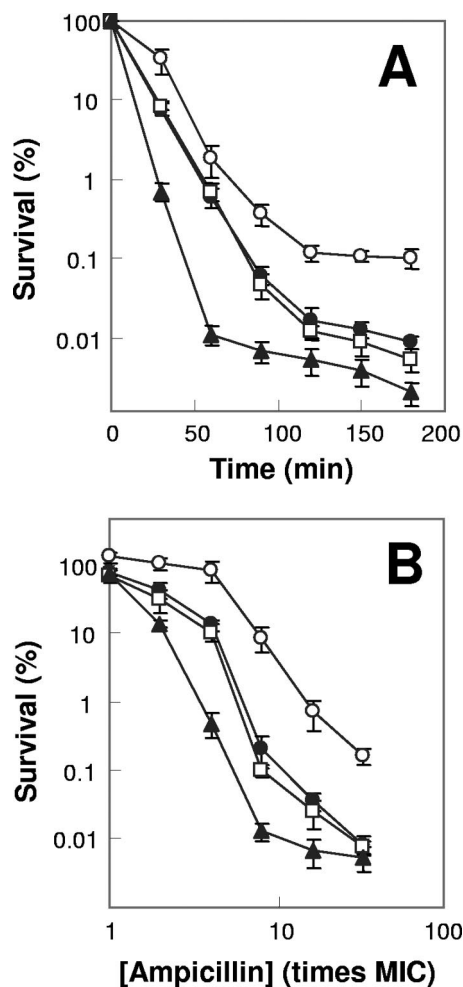
FIG. 4. Effects of *katG*, *sodA sodB*, and *ahpC* deficiencies on ampicillin lethality. Exponentially growing *E. coli* cells were treated with five times the MIC of ampicillin (150 μg/ml for the *katG katE* mutant and 75 μg/ml for other strains) for various times (A) or with the indicated concentrations of ampicillin (B) for 90 min. Symbols: filled circles, wild type; squares, *katG* mutant; open circles, *sodA sodB* mutant; triangles, *ahpC* mutant. Error bars indicate standard deviations for replicate samples. At least three replicate experiments were performed, and each had results similar to those shown.
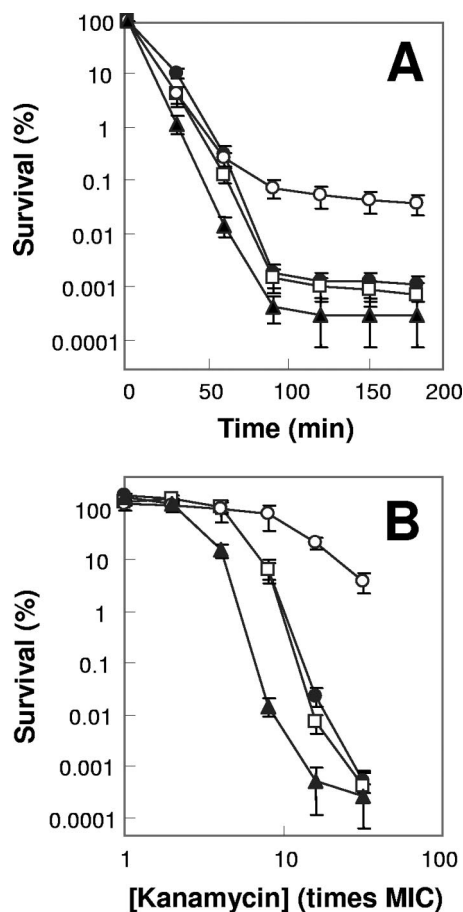

FIG. 5. Effects of *katG*, *sodA sodB*, and *ahpC* deficiencies on kanamycin lethality. Exponentially growing *E. coli* cells were treated with five times the MIC of kanamycin (12 μg/ml for the *ahpC* mutant and 3 μg/ml for other strains) for various times (A) or with the indicated concentrations of kanamycin (B) for 45 min. Symbols: filled circles, wild type; squares, *katG* mutant; open circles, *sodA sodB* mutant; triangles, *ahpC* mutant. Error bars indicate standard deviations for replicate samples. At least three replicate experiments were performed, and each had results similar to those shown.

1). Superoxide dismutases are expected to greatly accelerate the conversion of superoxide to peroxide (15, 25), which serves as a substrate for generation of highly toxic hydroxyl radicals through the Fenton reaction (14). Although spontaneous dismutation of superoxide to hydrogen peroxide can occur, the rate is 4 orders of magnitude lower than that of enzymatic dismutation (15, 25). Thus, in a *sodA sodB* mutant, catalase/peroxidase is expected to detoxify enough hydrogen peroxide to explain protection from norfloxacin-mediated killing. A deficiency of KatG catalase/peroxidase, which is expected to allow accumulation of peroxide, rendered cells hypersusceptible to the lethal action of quinolones (Fig. 2). A deficiency of alkyl hydroperoxide reductase (AhpCF) played that role for ampicillin and kanamycin (Fig. 4 and 5). An iron chelator expected to block the generation of hydroxyl radicals and a hydroxyl radical scavenger both reduced antimicrobial-mediated cell

death (Fig. 3 and data not shown). Thus, part of the lethal activity for members of at least three antimicrobial classes involves a surge in hydroxyl radical accumulation (13, 23) mediated by enzymes of superoxide and peroxide metabolism (Fig. 6).

TABLE 3. MBCs of compounds investigated

Bacterial strain	Relevant genotype	MBC of compound (μg/ml)		
		Norfloxacin	Ampicillin	Kanamycin
BW25113	Wild type	0.32	80	20
DKsodB	ΔsodB	0.32	80	20
3144	ΔsodA	0.32	80	20
3145	ΔsodB	0.32	80	20
3156	ΔsodA ΔsodB	0.16	20	10/20[a]
3157	ΔkatG	0.16/0.32[a]	80	20
3200	ΔahpC	0.16	40	10
3201	ΔkatG ΔkatE	0.32	80	20
3202	ΔkatE	0.16/0.32[a]	80	20

[a] Different values were obtained in replicate assays; in all other cases, replicate values were identical.
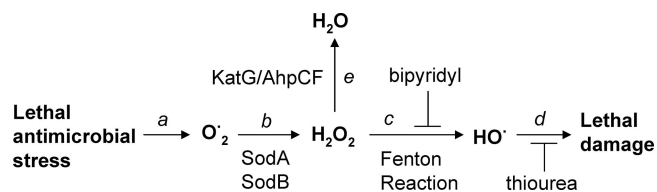
FIG. 6. Scheme depicting pathway by which bactericidal antimicrobial stress modulates lethal oxidative damage. Lethal stress from antimicrobial treatment causes an undefined redox imbalance, such that intracellular superoxides accumulate (step a). Upon conversion to hydrogen peroxide by dismutases (step b), hydroxyl radicals are generated from elevated levels of hydrogen peroxide via the Fenton reaction (step c). Hydroxyl radical species cause cell death (step d). Hydrogen peroxide is normally decomposed/detoxified by catalase/peroxidase (step e). Suppression of the generation of peroxide by a deficiency of both $sodA$ and $sodB$ protects cells from antimicrobial lethality, while inhibition of detoxification of peroxide via deficiencies in $katG$ (in the case of norfloxacin) or $ahpC$ (in the case of ampicillin and kanamycin) enhances cell death. Both an iron chelator (bipyridyl), which inhibits the Fenton reaction, and thiourea, a potent hydroxyl radical scavenger, protect cells from death.

Deficiencies in sod and kat conferred distinct phenotypes that were most pronounced at different antimicrobial exposures. For example, the protective effect of a $sodA$ $sodB$ double mutation was evident only at long treatment times and high antimicrobial concentrations (Fig. 1, 4, and 5). In contrast, the $katG$ defect increased lethality and the increase was most obvious at short treatment times and low drug concentrations (Fig. 2). These observations are consistent with the kinetic properties of the enzymes. Long incubation at a high drug concentration is expected to substantially elevate the superoxide concentration, which may not be acutely toxic, since the superoxide half-life in aqueous phase is long and since superoxide can be scavenged by both SODs and many types of antioxidant. However, if superoxide is enzymatically converted to H_2O_2 at a rate of $>10^9$ M^{-1} s^{-1}, as expected with wild-type cells (15, 25), catalase/peroxidase may become saturated, since its rate constant is only about 10^6 M^{-1} s^{-1} (29). The buildup of peroxide would then lead to production of hydroxyl radicals via the Fenton reaction and result in cell death. But when SOD is absent, the spontaneous rate (about 10^5 M^{-1} s^{-1}) (15, 25) may be insufficient to challenge the ability of catalase to detoxify H_2O_2. Then, little hydroxyl radical would be generated for enhancement of antimicrobial lethality, resulting in lower norfloxacin-mediated killing with a $sodA$ $sodB$ double-deficient strain (Fig. 1). An alternative explanation for the $sodA$ $sodB$-mediated protection is that spontaneous elevation of the intracellular superoxide concentration in the $sodA$ $sodB$ double mutant stimulates generation of "persisters"—a subpopulation of cells that tolerate a variety of lethal stresses (24). Such a possibility is currently under investigation.

At mild antimicrobial exposure, superoxide levels would not generate enough H_2O_2 to overwhelm catalase activity, regardless of how superoxide undergoes dismutation to H_2O_2. Thus, the absence/presence of SOD would have little effect on killing under mild antimicrobial conditions (Fig. 1). When $katG$ is deficient, even low levels of hydrogen peroxide generated by mild antimicrobial exposure cannot be detoxified, thereby increasing lethality (Fig. 2). In this situation, the additional removal of SOD ($sodA$ $sodB$ $katG$ triple mutant) would confer

protection (compared with results for the $katG$ single mutant) at mild antimicrobial exposures, as observed (not shown). We speculate that the reduced hyperlethality of the $katG$ mutant relative to results for the wild type at high drug exposures (Fig. 2) is due to the net effect of two factors: saturation of hydroxyl radical-mediated killing in the $katG$ mutant and enhanced hydroxyl radical-mediated killing in wild-type cells when H_2O_2 accumulation exceeds the capacity of KatG-mediated detoxification.

The scheme presented in Fig. 6 and the protective effects seen in the $sodA$ $sodB$ double mutant (Fig. 1, 4, and 5) are consistent with several other reports. For example, detrimental rather than protective effects are observed with SOD overexpression in $E.$ $coli$ cells treated with a superoxide generator, paraquat (6, 30). Since paraquat treatment increases superoxide levels (21), as does antimicrobial treatment (1, 5), rapid conversion of excessive superoxide to H_2O_2 by overexpressed SOD is likely to trigger lethal hydroxyl radical generation. Similar toxic effects have been observed when $E.$ $coli$ cells are treated with SOD mimetics (27), which leads to elevated levels of superoxide and H_2O_2 (27). Moreover, in cultured mouse epidermal cells, SOD overexpression causes sensitization to rather than protection against oxidants; such sensitization is corrected by concurrent overexpression of catalase or glutathione peroxidase (2, 3). Thus, the balance between SOD and catalase/peroxidase activity may determine how much and how rapidly intracellular H_2O_2 is generated, which in turn determines hydroxyl radical production.

Although the present work supports the general conclusion that antimicrobial lethality is tied to intracellular hydroxyl radical accumulation (13, 23), two aspects of recent studies merit attention. First, a $sodB$ mutant was reported to be more readily killed by the fluoroquinolones than wild-type cells (13, 23). We were unable to confirm that observation for norfloxacin (Fig. 1) and several other fluoroquinolones with several $E.$ $coli$ strains (not shown). Instead, a $sodA$ $sodB$ double mutant exhibited reduced lethal susceptibility to norfloxacin (Fig. 1), the result opposite to that expected from previous work (13). Second, the protective activities of thiourea and bipyridyl were previously noted using these two agents at concentrations that inhibit $E.$ $coli$ growth. Since growth inhibition blocks quinolone and ampicillin lethality (12, 18, 32), the protective effect of thiourea and bipyridyl could have been unrelated to hydroxyl radical accumulation. To reduce this possibility, we showed that subinhibitory concentrations of hydroxyl radical scavenger or iron chelator lowered norfloxacin lethality (Fig. 3). Subinhibitory scavenger and chelator treatments also reduced norfloxacin-mediated killing with a $katG$ mutant. However, bacterial survival was not restored to levels observed with the wild type plus a scavenger/chelator or even with the wild type alone (Fig. 3). Incomplete protection is probably due to our use of subinhibitory concentrations of the scavenger and chelator being insufficient to completely prevent hydroxyl radical accumulation in mutants.

While the scheme shown in Fig. 6 applies to β-lactam and aminoglycoside action, kanamycin- and ampicillin-mediated killing was insensitive to a $katG$ deficiency. Instead, it was enhanced by $ahpC$ depletion. These data are consistent with peroxide accumulation being stimulated by kanamycin or ampicillin at lower levels than by a quinolone, since the lower

levels would allow AhpCF, an enzyme having a greater affinity for hydrogen peroxide but a lower scavenging capacity (31), to serve as the primary peroxide scavenger after treatment with ampicillin and kanamycin.

In summary, oxidative stress pathways are involved in fluoroquinolone, β-lactam, and aminoglycoside lethality. Characterization of these pathways may provide new targets for small-molecule potentiators of antimicrobial lethality. The next step is to determine how these stress pathways distinguish between bacteriostatic and bactericidal events.

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