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Superoxide-Mediated Protection of *Escherichia coli* from Antimicrobials

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Antimicrobial lethality is promoted by reactive oxygen species (ROS), such as superoxide, peroxide, and hydroxyl radical. Pretreatment with subinhibitory concentrations of plumbagin or paraquat, metabolic generators of superoxide, paradoxically reduced killing for oxolinic acid, kanamycin, and ampicillin. These pretreatments also reduced an oxolinic acid-mediated ROS surge. Defects in SoxS MarA or AcrB eliminated plumbagin- and paraquat-mediated MIC increases but maintained protection from killing. Thus, superoxide has both protective and detrimental roles in response to antimicrobial stress.

Antimicrobial resistance has become a grave challenge for human health care. One countermeasure is to increase our ability to kill pathogens, since rapid, extensive killing should help restrict the emergence of resistance (1–3). A general vulnerability of bacteria to antimicrobial therapy may lie in their response to oxidative stress, and many different antimicrobial classes act, at least in part, through the generation of reactive oxygen species (ROS) (4–6). Thus, promoting a surge in ROS should enhance antimicrobial lethality. In particular, increased superoxide could lead to increased peroxide and, subsequently, increased hydroxyl radical and cell death (4, 5). Paradoxically, superoxide may also have a protective role, as suggested by the observation that subinhibitory concentrations of plumbagin, a metabolic generator of superoxide (7), reduce the killing of *Escherichia coli* by bleomycin, a lethal DNA-damaging agent (8). However, bleomycin-based studies are complex, because superoxide is also involved in bleomycin activation (9). Thus, it is uncertain whether plumbagin can protect bacteria from many different lethal antimicrobials, as required for superoxide to play a central role in the live-or-die decision made by bacteria when challenged with lethal stressors (10, 11).

In the present work, we treated strains of *Escherichia coli* K-12 (listed in Table 1) with subinhibitory concentrations of plumbagin or paraquat, another metabolic generator of superoxide (12, 13), to assess the effect of moderate superoxide levels on the lethal activity of several antimicrobials. *E. coli* was grown aerobically at 37°C in LB liquid medium and on LB agar (14). All antimicrobials, plumbagin, and paraquat were obtained from Sigma-Aldrich (St. Louis, MO). Antimicrobial susceptibility (defined by MIC) was measured by broth dilution according to the Clinical and Laboratory Standards Institute (CLSI) protocol (15). Lethal action was measured by growing cultures to mid-log phase, treating with an antimicrobial, and then plating on drug-free agar for determining the percentage of survival relative to the counts in aliquots taken immediately before addition of antimicrobial. Intracellular ROS levels were measured by flow cytometry (16), using carboxy-H2DCFDA [5-(and-6)-carboxy-2″,7″-dichlorodihydrofluorescein diacetate; Life Technologies, Grand Island, NY] as a fluorescent probe for intracellular ROS accumulation.

At subinhibitory concentrations (1/4 MIC), plumbagin and paraquat showed no effect on exponential growth rate, although they did cause 30- to 60-min delays in entering exponential growth phase following dilution of stationary-phase cultures (see Fig. S1 in the supplemental material). With wild-type cells, plumbagin and paraquat reduced the bacteriostatic activity of antimicrobials, as shown by increased MICs for oxolinic acid, ampicillin, and kanamycin (4-, 2-, and 2-fold, respectively) (Table 2). When overnight cultures were diluted 100-fold with fresh medium containing plumbagin at 1/4 MIC, grown to exponential phase, and then treated with antimicrobials, the lethal activity for oxolinic acid, kanamycin, and ampicillin decreased when measured at a fixed concentration of drug for various times (Fig. 1A, C, and E) or at various drug concentrations for a fixed time (Fig. 1B, D, and F). Paraquat also decreased the lethal action of these anti-

TABLE 1 Bacterial strains used

Strain	Relevant genotype	Source
3568	Wild type	CGSC no. 7636
3569	Δ*soxS*::*kan*	CGSC no. 10891
3570	Δ*soxS*::*kan*	This work, by P1-mediated transduction from 3569 into 3568
3571	Δ*soxS*	This work, by antibiotic marker excision (30) from 3570
3572	Δ*marA*::*kan*	CGSC no. 11269
3573	Δ*soxS* Δ*marA*::*kan*	This work, by P1-mediated transduction from 3572 into 3571
3574	Δ*soxS* Δ*marA*	This work, by antibiotic marker excision (30) from 3573
3705	Δ*acrB*::*kan*	CGSC no. 8609
3706	Δ*acrB*	This work, by antibiotic marker excision (30) from 3705

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TABLE 2 Bacteriostatic activity of antimicrobials[a]

Strain	MIC (μg/ml) of PL, PQ, or antimicrobial with or without PL or PQ[b]										
	PL	PQ	Oxo	Oxo + 1/4 PL	Oxo + 1/4 PQ	Amp	Amp + 1/4 PL	Amp + 1/4 PQ	Kan	Kan + 1/4 PL	Kan + 1/4 PQ
3568 (wild type)	25	103	0.8	3.2	3.2	24	48	48	2	4	3
3574 (Δ*soxS* Δ*marA*)	15	51.5	0.6	0.6	0.6	16	16	16	1.5	1.5	1.5
3706 (Δ*acrB*)	9	103	0.1	0.1	0.1	8	8	8	1	1	1

[a] Cells were grown to mid-log phase in the absence or presence of 1/4 MIC of plumbagin or paraquat, diluted 5,000-fold in LB liquid medium or medium containing plumbagin or paraquat, mixed with various antimicrobials at 2-fold increments with concentrations in two staggered dilution series, and incubated overnight at 37°C with shaking.
[b] Abbreviations: PL, plumbagin (1/4 PL = PL at 1/4 MIC); PQ, paraquat (1/4 PQ = PQ at 1/4 MIC); Oxo, oxolinic acid; Kan, kanamycin; Amp, ampicillin.

microbials (Fig. 2), but it did not affect killing by UV or heat (see Fig. S2). Nor did it convert chloramphenicol into a lethal agent (see Fig. S2). Thus, the two metabolic generators of superoxide protect *E. coli* from the lethal action of several different antimicrobial classes. Since the antimicrobial concentrations were normalized to the MICs, protection from killing was not due simply to the increase in MIC shifting the concentration-kill curve.

We next asked whether the superoxide-mediated protection correlated with the ROS levels associated with antimicrobial treatments. Wild-type cells were grown to mid-log phase in the pres-

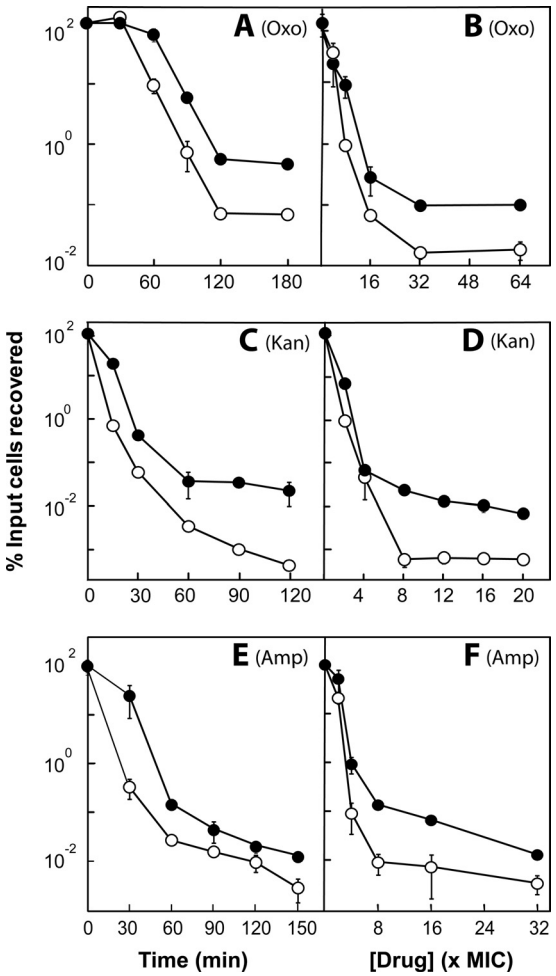

FIG 1 Effect of plumbagin on antimicrobial lethality. Wild-type *E. coli* (strain 3568) was grown to mid-log phase in the presence (filled circles) or absence (empty circles) of plumbagin (1/4 MIC [6.25 μg/ml]) and treated with antimicrobials either at a fixed concentration for the indicated times (A, C, E) or at various concentrations for a fixed time (B, D, F). (A) Oxolinic acid (Oxo) at 10× MIC. (B) Oxolinic acid for 120 min. (C) Kanamycin (Kan) at 3× MIC. (D) Kanamycin for 30 min. (E) Ampicillin (Amp) at 5× MIC. (F) Ampicillin for 75 min. At the end of treatment, aliquots were removed, serially diluted, and plated for determination of viable counts. Percentage of survival was determined as described in the text. Error bars indicate standard deviations.

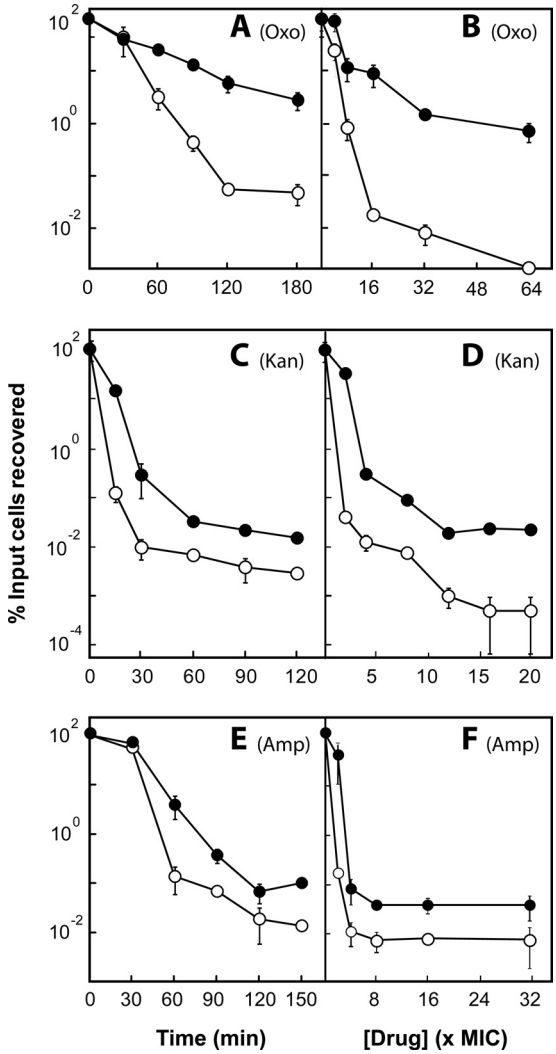

FIG 2 Effect of paraquat on antimicrobial lethality. Experiments were as described in the legend to Fig. 1 except that paraquat (1/4 MIC [25.7 μg/ml, 0.1 mM]) rather than plumbagin was used to pretreat cells before antimicrobial exposure.

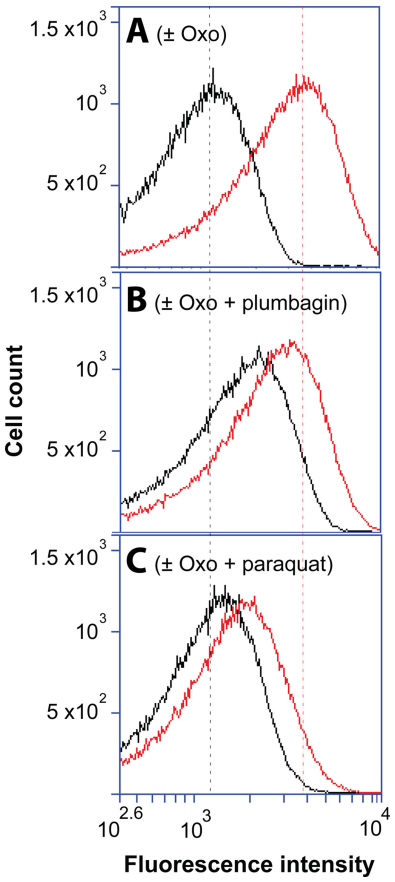

FIG 3 Effect of plumbagin and paraquat pretreatment on oxolinic acid-mediated ROS surge. Overnight cultures of wild-type *E. coli* (strain 3568) were diluted 100-fold and grown to mid-log phase in the presence or absence of 1/4 MIC of plumbagin or paraquat before they were treated with 10× MIC of oxolinic acid. At 90 min after oxolinic acid addition, aliquots taken from both quinolone-treated and untreated samples were pulse labeled with the fluorescent probe carboxy-H2DCFDA for 60 min before samples were subjected to flow cytometry analysis. A total of 100,000 cells were counted for each sample. (A) Population peak overlay of fluorescently labeled cells treated with (red curve) or without (black curve) oxolinic acid. (B) Same as described for panel A but cells were pretreated with 1/4 MIC of plumbagin before oxolinic acid treatment. (C) Same as described for panel A but cells were pretreated with 1/4 MIC of paraquat before oxolinic acid treatment. Dotted lines are drawn from the peak positions of oxolinic acid-treated (red) and untreated (black) curves in panel A for alignment with the respective curve peaks in panels B and C.

ence or absence of 1/4 MIC plumbagin or paraquat before they were treated with oxolinic acid. At 90 min after oxolinic acid addition, aliquots from both quinolone-treated and untreated samples were pulse labeled for 60 min with carboxy-H2DCFDA, a fluorescent probe that reacts with all three ROS (superoxide, peroxide, and hydroxyl radical). Then, aliquots were analyzed by flow cytometry. Treatment with oxolinic acid markedly increased the fluorescent signal (Fig. 3A). Treatment with plumbagin or paraquat alone moderately elevated the fluorescence (Fig. 3, compare black-curve peak positions among the 3 panels); however, these compounds, particularly paraquat, reduced the fluorescent signal elicited by oxolinic acid (Fig. 3, compare red-curve peak positions). The extent of ROS reduction paralleled the protection from killing: paraquat reduced the ROS signal more than plumbagin (Fig. 3, compare C and B), and it exhibited a greater protective effect (compare Fig. 1 and 2, especially panels A and B). Similar results were obtained with lower oxolinic acid concentrations (e.g., 8× MIC), longer exposure times (e.g., 120 min), and with other antimicrobials, such as ampicillin, kanamycin, and norfloxacin (data not shown). Thus, exposure to moderate levels of superoxide before antimicrobial stress interferes with subsequent antimicrobial-stimulated accumulation of ROS and cell death.

Since superoxide induces the SoxS and MarA transcription factors that activate protective repair pathways and efflux pumps (17–19), we next asked whether paraquat-mediated protection from antimicrobials is mediated by these two superoxide-activated regulators. A *soxS marA* double deletion eliminated the paraquat- and plumbagin-associated MIC increases seen with wild-type cells (Table 2), implicating both gene products in bacteriostatic effects (single deletions showed partial effects; data not shown). However, the double deletion failed to eliminate paraquat-mediated protection from killing (Fig. 4A, C, and E). Thus, superoxide-mediated protection from killing extends beyond the upregulation of efflux pumps.

Since the AcrAB-TolC efflux system is the major pump under Sox-MarA control, we also examined an *acrB*-deficient mutant for paraquat-mediated protection from killing by oxolinic acid, kanamycin, and ampicillin. The *acrB* mutation reduced the MICs by 8-, 3-, and 2-fold for oxolinic acid, ampicillin, and kanamycin, respectively (Table 2). Paraquat and plumbagin failed to increase the MICs in the *acrB* mutant (Table 2), indicating that the MIC increases induced by these agents in wild-type cells are due to increased AcrAB-TolC pump activity. But the *acrB* mutation allowed paraquat-mediated protection from killing to occur (Fig. 4B, D, and F). Thus, AcrA-TolC-mediated efflux is not required for superoxide-mediated protection from antimicrobial killing.

During the course of the work, pretreatment with paraquat or plumbagin was reported to increase the level of persister cells and confer tolerance to killing by fluoroquinolones but not by ampicillin or kanamycin (20). That work used a short paraquat/plumbagin pretreatment time (30 min), which might have prevented observation of a protective effect with ampicillin and kanamycin. Since plumbagin increased the MIC by 4-fold for quinolones but no more than 2-fold for ampicillin and kanamycin (Table 2), the greater effect on quinolone MIC could shift the concentration-kill curves more for quinolones and appear to decrease killing only for quinolones in that work (20). When we used a range of drug concentrations for concentration-kill curves and normalized the concentrations to the MICs for killing kinetics, pretreatment with subinhibitory concentrations of plumbagin or paraquat for 120 to 150 min reduced bacterial killing by all three drug classes tested (Fig. 1 and 2). Our results are consistent with persister cells usually being tolerant to a variety of stressors over a broad range of drug concentrations and exposure times (21).

The results described above establish that a moderate increase in superoxide can protect from a variety of lethal antimicrobials, possibly by activating ROS defense systems that help suppress the ROS surge triggered by subsequent antimicrobial treatment (Fig. 3). However, once superoxide exceeds a critical level, it becomes highly lethal (see Fig. S3 in the supplemental material). The protective effect of moderate levels of superoxide extends beyond the MIC change mediated by SoxS MarA and AcrAB-TolC, since protection from killing was also observed with *soxS marA* and *acrB* mutant cells that showed no plumbagin- or paraquat-mediated

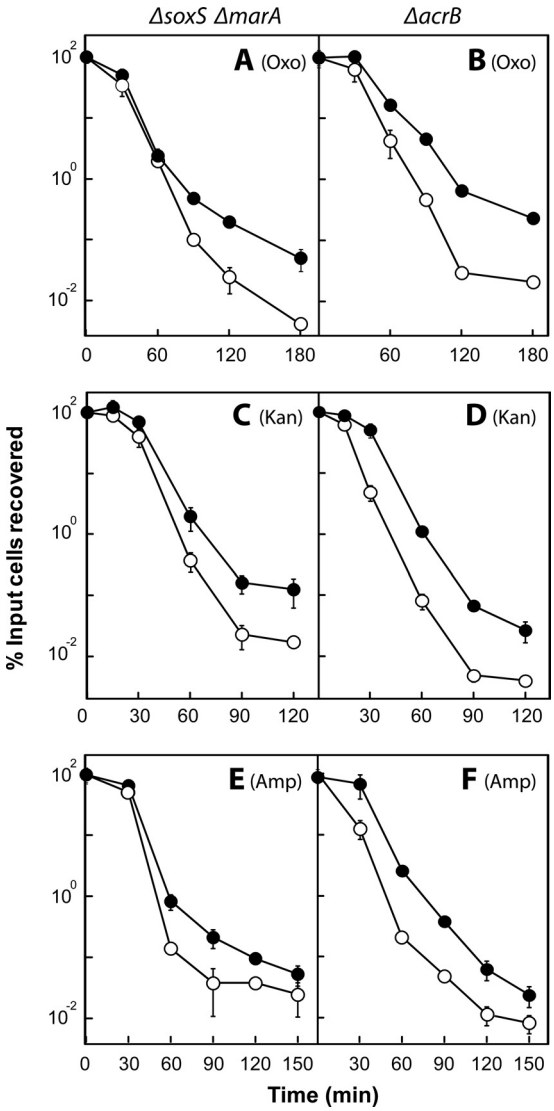

FIG 4 Effect of deficiency in *soxS marA* and *acrB* on paraquat-mediated protection from the lethal action of oxolinic acid, kanamycin, and ampicillin. *soxS marA*-deficient (strain 3574) (A, C, E), and *acrB*-deficient (strain 3706) (B, D, F) *E. coli* cells were grown to mid-log phase in the presence (filled symbols) or absence (empty symbols) of paraquat (1/4 MIC) and treated with 10× MIC of oxolinic acid (A and B), 3× MIC of kanamycin (C and D), or 5× MIC of ampicillin (E and F) for various times. At the end of treatment, aliquots were removed, serially diluted, and plated for determination of viable counts. Percentage of survival was determined as described in the text. Error bars indicate standard deviations.

MIC change. Moreover, an efflux inhibitor, verapamil, decreased the MIC of AZI-219 (an experimental antituberculosis agent) but showed no increase in killing if drug concentrations were normalized to the MIC (22). We conclude that superoxide-induced SoxS-MarA-mediated upregulation of drug efflux is not the only protective superoxide action, in contrast to a recent report (20). Since subinhibitory concentrations of plumbagin and paraquat affect the expression of hundreds of genes (23, 24), some of these gene products may have unknown functions besides facilitation of superoxide detoxification and induction of efflux pumps. Identifying the genes responsible for plumbagin- and paraquat-mediated protection from killing constitutes a new line of investigation that can profit from systems-based analyses.

The protective effect of superoxide generators (Fig. 1 and 2) and a *sodA sodB* double mutant (5) during lethal stress, combined with the destructive effect of ROS described previously (see Fig. S3 in the supplemental material) (4, 5), lead us to speculate that ROS have two opposing functions during lethal stress. A large surge in ROS ensures death (4, 6, 11, 25), while a moderate increase in superoxide triggers protective pathways against lethal stress (Fig. 1 to 4) (20). Whether ROS stimulate or mitigate stress-mediated cell death depends on the type and severity of the stress (10), which makes the choice of stressor and experimental conditions crucial for determining whether ROS are detrimental, protective, or of no effect (10, 16, 20, 26). These observations indicate that the ROS contribution to antimicrobial lethality may be more complex than originally envisioned (4), and these complexities indicate that challenges to the concept that ROS play an important role in antimicrobial lethality (27–29) require the examination of a broad set of stressor types and experimental conditions.

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