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Dimethyl Sulfoxide Protects *Escherichia coli* from Rapid Antimicrobial-Mediated Killing

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The contribution of reactive oxygen species (ROS) to antimicrobial lethality was examined by treating *Escherichia coli* with dimethyl sulfoxide (DMSO), an antioxidant solvent frequently used in antimicrobial studies. DMSO inhibited killing by ampicillin, kanamycin, and two quinolones and had little effect on MICs. DMSO-mediated protection correlated with decreased ROS accumulation and provided evidence for ROS-mediated programmed cell death. These data support the contribution of ROS to antimicrobial lethality and suggest caution when using DMSO-dissolved antimicrobials for short-time killing assays.

One approach to help stem the emergence of new antimicrobial resistance is to kill bacterial pathogens rapidly, thereby quickly reducing bacterial burden and restricting effects of stress-induced mutagenesis (1, 2). Reactive oxygen species (ROS) have been proposed to be key factors in antimicrobial lethality (3–5), and substantial evidence supports this proposition (3–19). However, their role in lethality has been challenged (20, 21). If ROS are indeed integral to antimicrobial-mediated killing, compounds that act as antioxidants and radical scavengers should reduce antimicrobial lethality. We chose to examine this hypothesis using the radical scavenger dimethyl sulfoxide (DMSO) (22, 23), because it is also a popular solvent that is widely used in the pharmaceutical industry and in antimicrobial research due to its (i) low toxicity, (ii) ability to dissolve both organic and inorganic compounds, (iii) ability to remain in a liquid state over a broad temperature range (e.g., from 19°C to 189°C), (iv) ability to enhance cell membrane permeability, and (v) miscibility in water and a wide range of organic solvents. We report here that DMSO interferes with rapid killing of *Escherichia coli* and *Acinetobacter baumannii* by members of three antimicrobial classes.

E. coli K-12 strains BW25113 and ATCC 25922 and *A. baumannii* strain ATCC 17978 were grown in Luria-Bertani (LB) broth or on LB agar at 37°C. LB medium, ampicillin, and kanamycin were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Oxolinic acid, ciprofloxacin, and DMSO were acquired from Sigma-Aldrich Co. (St. Louis, MO). Meropenem (Sumitomo Dainippon Pharma Co. Ltd.) was obtained from Zhongshan Hospital Pharmacy. The fluorescent probe carboxy-H_2DCFDA [5(6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate] was purchased from Invitrogen (Grand Island, NY). All chemical stock solutions were dissolved in sterile water (except carboxy-H_2DCFDA, which was dissolved in DMSO) and stored at −80°C until use. MICs were assayed by broth dilution according to CLSI protocols (24); exponentially growing cultures were diluted to 10^5 CFU/ml for MIC determinations. To measure rapid bacterial killing, exponentially growing cultures at about 5×10^8 CFU/ml were treated with antimicrobials, after which they were serially diluted and plated on drug-free agar. Viable colony counts were determined after an overnight incubation at 37°C; percentage survival rates were calculated relative to viable counts of samples taken immediately before antimicrobial addition. To measure intracellular ROS accumulation, a fluorescence dye, carboxy-H_2DCFDA, was used. Carboxy-H_2DCFDA readily penetrates *E. coli* cells (25, 26); once it enters cells, the compound is converted by cellular esterases into a membrane-impermeable cognate that can be oxidized to a fluorescent form by superoxide, hydrogen peroxide, or hydroxyl radicals (25). The fluorescent signal can then be analyzed

TABLE 1 Effects of DMSO on antimicrobial-mediated growth inhibition

Bacterial strain	[DMSO] (% [vol/vol])[a]	MIC (μg/ml) for[a]: Oxo	Cip	Amp/Mer	Kan	DMSO (% [vol/vol]
BW25113	0	0.6	0.024	8/ND	6	15
	5	0.6	0.024	8/ND	3	ND
	7.5	0.6	0.024	8/ND	3	ND
ATCC 25922	0	0.15	0.018	8/ND	16	10
	5	0.15	0.009	8/ND	8	ND
ATCC 17978	0	ND	0.3	ND/0.4[b]	4	6
	3	ND	0.15	ND/0.2[b]	2	ND

[a] Oxo, oxolinic acid; Cip, ciprofloxacin; Amp, ampicillin; Mer, meropenem; Kan, kanamycin; ND, not determined.
[b] Meropenem rather than ampicillin was used for *A. baumannii* since the MIC of ampicillin is too high.

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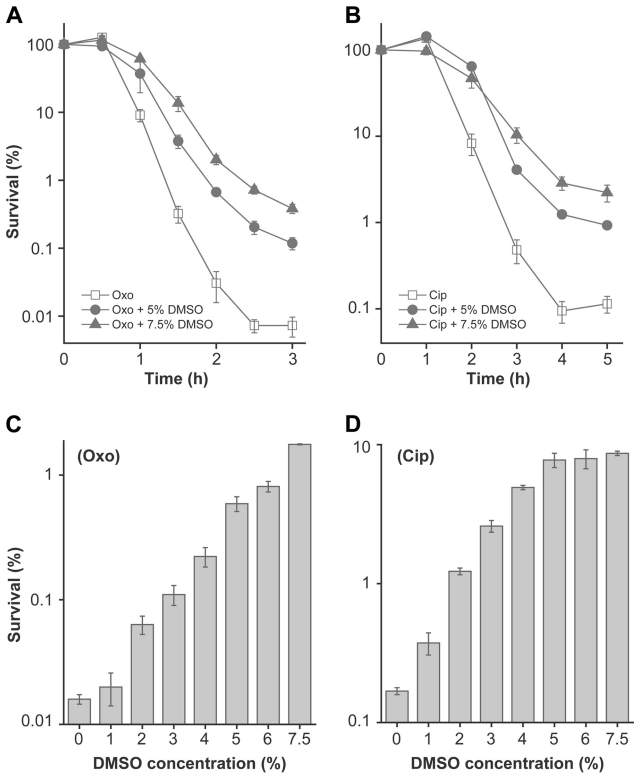

FIG 1 DMSO counteracts quinolone-mediated lethality. Exponentially growing *E. coli* BW25113 cells were treated with 9 μg/ml (15× MIC) oxolinic acid (Oxo) (A and C) or 0.048 μg/ml (2× MIC) ciprofloxacin (Cip) (B and D) for the indicated times (A and B) in the absence or presence of 5% or 7.5% DMSO. Cultures were also treated with 9 μg/ml (15× MIC) oxolinic acid for 2.5 h (C) or 0.048 μg/ml (2× MIC) ciprofloxacin for 4 h (D) in the presence of various concentrations of DMSO. Shown are the average values from experiments conducted at least three times. Error bars indicate deviations as standard errors of the mean.

by flow cytometry and fluorescence microscopy. *E. coli* cells were pretreated with 5 μM carboxy-H_2DCFDA (1,000-fold dilution from 5 mM stock; DMSO carryover, 0.1%) for 10 min, followed by DMSO pretreatment for another 10 min before the addition of oxolinic acid (15× MIC, 9 μg/ml). After 150 min of oxolinic acid treatment, cells were washed with 1× phosphate-buffered saline (PBS) to remove the antimicrobial and extracellular carboxy-H_2DCFDA, concentrated by centrifugation (17,000 × *g* for 1 min), and used to measure ROS levels by flow cytometry or fluorescence microscopy with a Beckman Coulter CyAn ADP analyzer or an Olympus BX43 microscope, respectively. Poststress programmed cell death was assessed by diluting exponentially growing cultures into LB liquid medium at 37°C to a cell density of 10^5 to 10^6 CFU/ml. Cells were then treated with 9 μg/ml oxolinic acid (15× MIC) for 90 min, and 200-μl samples were plated onto LB agar lacking or containing 7.5% (vol/vol) DMSO (one-half MIC). The ratio of the number of cells recovered from DMSO-containing agar to that of DMSO-free agar indicated the poststress programmed cell death.

We began by determining the MIC for DMSO, which was 15% (vol/vol). Subinhibitory DMSO concentrations (7.5% [one-half MIC] and 5% [one-third MIC]) had little effect on exponential growth of bacterial cultures (see Fig. S1 in the supplemental material) and no effect on the MIC of oxolinic acid, ciprofloxacin, or ampicillin; however, 5% and 7.5% DMSO each caused a 2-fold reduction in the kanamycin MIC (Table 1).

We next examined the effect of DMSO on rapid antimicrobial killing (minimal bactericidal concentration [MBC] was not measured, because it is insensitive to ROS [9, 11]). For quinolones (oxolinic acid and ciprofloxacin), coincubation with 5% or 7.5% DMSO suppressed lethality by 10- to 100-fold (Fig. 1A and B). The protective effect of DMSO was dose dependent in that decreasing the DMSO concentration also decreased the level of protection (Fig. 1C and D). Even at concentrations as low as 1%, a protective

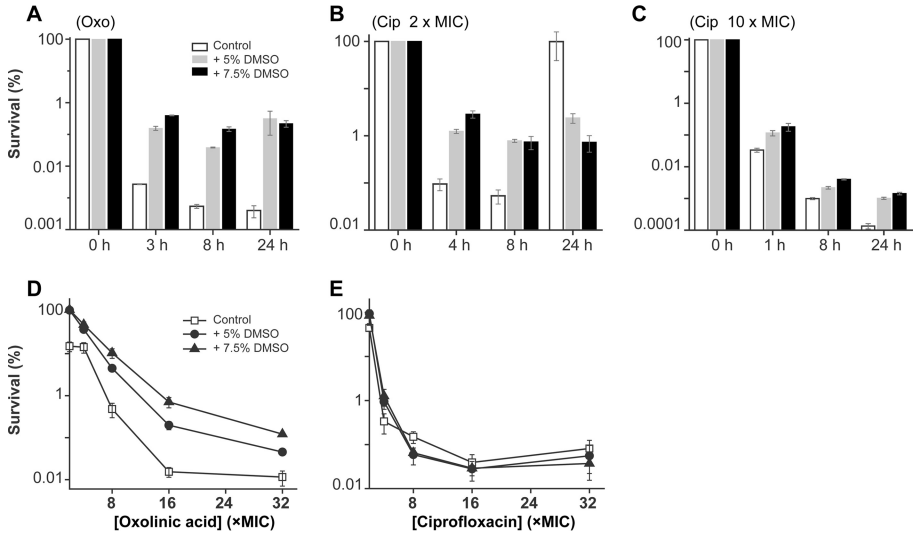

FIG 2 Increased incubation times and drug concentrations diminish DMSO-mediated protection from killing by ciprofloxacin but not by oxolinic acid. Exponentially growing cultures of *E. coli* strain BW25113 were treated with 9 μg/ml (15× MIC) oxolinic acid (Oxo) (A), 0.048 μg/ml (2× MIC) ciprofloxacin (B), or 0.24 μg/ml (10× MIC) ciprofloxacin (C) for the indicated times in the absence or presence of 5% or 7.5% DMSO. Cells were also treated with various concentrations of oxolinic acid for 2.5 h (D) or ciprofloxacin (E) for 75 min. The culture regrowth seen in the 24-h ciprofloxacin-only sample shown in panel B may have derived from selection of ciprofloxacin-resistant mutants at the low drug concentrations and long incubation times used. Shown are the average values from experiments conducted at least three times. Error bars indicate deviations as standard errors of the mean.

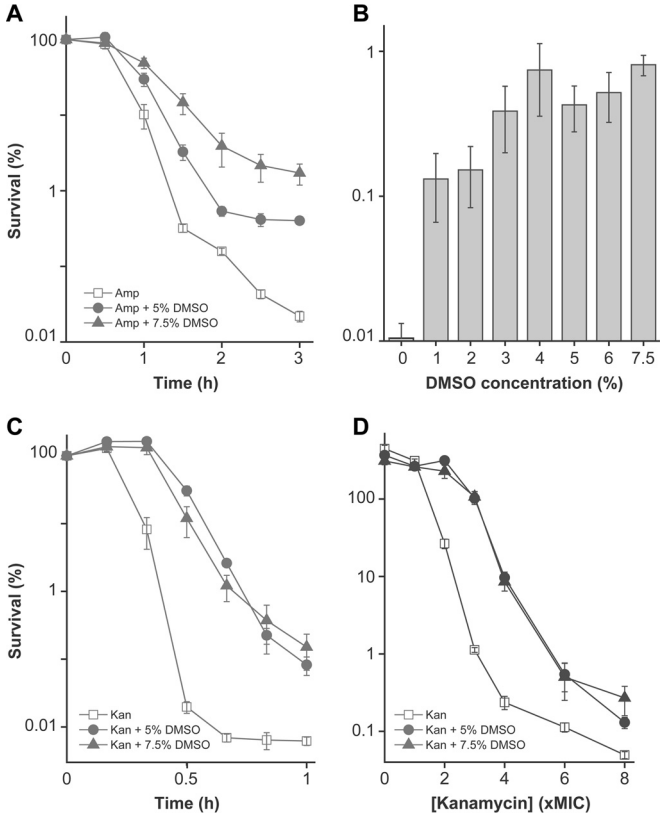

FIG 3 DMSO counteracts ampicillin- and kanamycin-mediated lethality. Exponentially growing cultures of *E. coli* BW25113 were treated with 16 μg/ml (2× MIC) ampicillin (Amp) for various times in the absence or presence of 5% or 7.5% of DMSO (A), 16 μg/ml (2× MIC) ampicillin for 3 h in the presence of various concentrations of DMSO (B), 4× MIC of kanamycin (Kan) in the absence (24 μg/ml kanamycin) or presence (12 μg/ml kanamycin) of 5% or 7.5% DMSO for the indicated times (C), and various concentrations of kanamycin (normalized to the MIC) for 30 min in the absence or presence of 5% or 7.5% DMSO (D). Shown are the average values from experiments conducted at least three times. Error bars indicate deviations as standard errors of the mean.

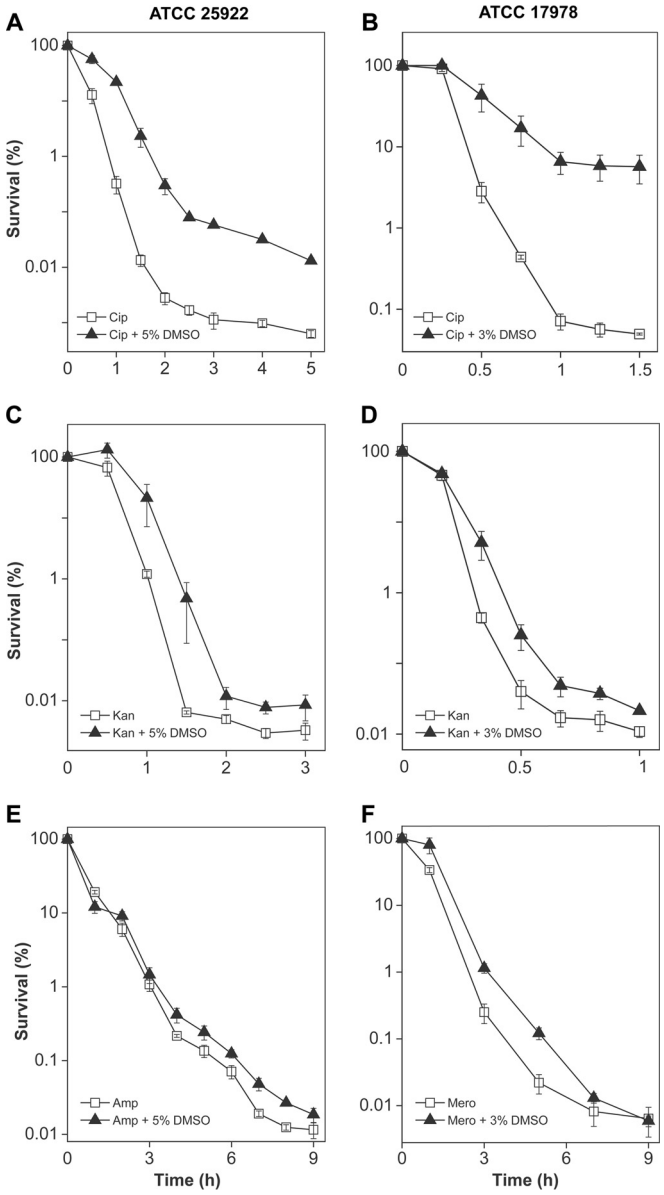

FIG 4 DMSO protects ATCC strains of *E. coli* and *A. baumannii* from antimicrobial-mediated lethality. Exponentially growing *E. coli* (ATCC 25922) (A, C, and E) or *A. baumannii* (ATCC 17978) (B, D, and F) cells were treated with 2× MIC of ciprofloxacin (Cip) (A and B), 2× MIC of kanamycin (Kan) (C and D), 20× MIC of ampicillin (Amp) (E), or 8× MIC of meropenem (Mero) (F) in the absence or presence of DMSO (one-half MIC, 5% for ATCC 25922 or 3% for ATCC 17978) for the indicated times. Shown are the average values from experiments conducted at least three times. Error bars indicate deviations as standard errors of the mean.

effect of DMSO was evident (Fig. 1C and D). As incubation time and drug concentration increased, the DMSO-mediated protective effect with oxolinic acid, a compound that depends mainly on ROS to kill bacteria (27), persisted, while that with ciprofloxacin, a compound that has both ROS-dependent and -independent modes of killing, gradually diminished (Fig. 2). At high concentrations of ciprofloxacin (Fig. 2E), DMSO showed no protective effect. These data support the idea that DMSO interferes with ROS-mediated lethality. In both cases, DMSO had no effect on MICs, which emphasizes the mechanistic difference between transient ROS-mediated killing and MICs.

A similar protective effect of DMSO was also observed with two other classes of antimicrobials, ampicillin and kanamycin. With ampicillin, 7.5% DMSO reduced killing by about 100-fold, while 5% DMSO reduced it by about 10-fold (Fig. 3A); at 1% DMSO, ampicillin-mediated killing was reduced by 10-fold after 3 h of incubation (Fig. 3B). Since DMSO lowered the kanamycin MIC, we normalized the absolute kanamycin concentration to its MIC for killing measurements to separate static from lethal effects (28). At 4× MIC of kanamycin, DMSO reduced killing by up to 1,000-fold when incubation was for 30 min (Fig. 3C). At various concentrations of kanamycin (normalized to MIC), reductions in lethality were 5- to 100-fold at both 5% and 7.5% DMSO (Fig. 3D). It appears that 5% DMSO is saturating, since 7.5% DMSO conferred no more protection than did 5%, possibly because kanamycin triggers less ROS-mediated killing than quinolones and ampicillin. As with quinolones, increasing the incubation time and drug concentration to 24 h and 10× MIC, respectively, reduced the DMSO-mediated protective effects for both ampicillin

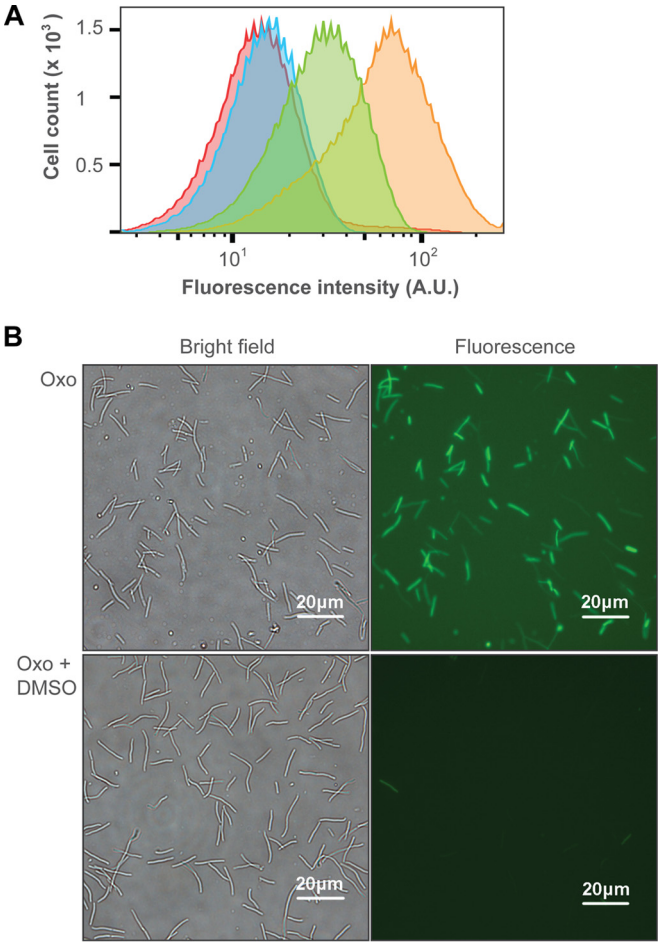

FIG 5 DMSO reduces oxolinic acid-mediated intracellular ROS accumulation. Exponentially growing *E. coli* (BW25113) cultures were pretreated with 5 μM carboxy-H_2DCFDA for 10 min, which was followed by DMSO pretreatment for another 10 min before cultures received oxolinic acid (15× MIC, 9 μg/ml) for 150 min. Samples taken immediately after and immediately before oxolinic acid treatment were washed once, resuspended in 1× phosphate-buffered saline, and subjected to flow cytometry (A) or microscopy (B). Red curve, untreated control; blue curve, DMSO pretreatment only; orange curve, oxolinic acid alone; green curve, oxolinic acid plus 7.5% DMSO. A.U., arbitrary units. Experiments in triplicate produced similar results.

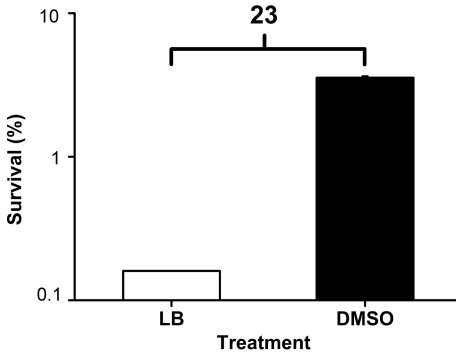

FIG 6 DMSO protects *E. coli* from stress-induced ROS-mediated programmed cell death. Exponentially growing cultures of *E. coli* BW25113 were serially diluted into prewarmed LB medium to a cell density of 10^5 to 10^6 CFU/ml. Cultures were then treated with 9 μg/ml oxolinic acid for 90 min, followed by immediate plating onto LB agar lacking or containing 7.5% (vol/vol) DMSO. The ratio of input cells (CFU) recovered from agar with DMSO to those without DMSO is indicated above paired columns. Shown are the average values from experiments conducted at least three times. Error bars indicate deviations as standard errors of the mean.

and kanamycin (see Fig. S2 in the supplemental material). ROS-mediated killing may be masked by the antimicrobials exerting more direct lethality at longer exposure times and higher drug concentrations.

To generalize our observations beyond applicability to a laboratory strain of *E. coli*, we next examined DMSO and antimicrobial lethality with two ATCC strains, ATCC 25922 (*E. coli*) and ATCC 17978 (*A. baumannii*). With these two strains, DMSO showed the greatest protection with quinolones, moderate protection with kanamycin, and little protection with β-lactams (Fig. 4). Thus, the DMSO-mediated protective effect does not appear to be limited to a specific bacterial strain or species.

Since ROS have been implicated in bacterial death arising from a variety of stressors (3–6, 11) and since DMSO is reported to be an antioxidant that can scavenge hydroxyl radicals (22, 23), we examined the hypothesis that DMSO reduces antimicrobial-stimulated ROS accumulation. Intracellular ROS levels were measured by flow cytometry (Fig. 5A) and microscopy (Fig. 5B); both assays showed that coincubation with 7.5% DMSO reduced oxolinic acid-induced ROS accumulation. Thus, the ROS scavenging activity of DMSO (22, 23) appears to be involved in reducing rapid antimicrobial-mediated killing.

We note that DMSO inhibits bacterial growth (MIC) through an ROS-unrelated mechanism, because (i) subinhibitory concentrations of hydrogen peroxide do not increase the DMSO MIC, and (ii) subinhibitory concentrations of other antioxidants (vitamin C and glutathione) do not reduce the DMSO MIC (data not shown). We speculate that DMSO inhibits growth via membrane perturbation; further work is required to establish the mechanism for growth inhibition.

We also examined DMSO for its effects on ROS-mediated antimicrobial-induced poststress programmed cell death (3). For this experiment, we treated an *E. coli* culture with 15× MIC oxolinic acid for 90 min and then plated the cells onto drug-free agar containing or lacking DMSO. DMSO in the agar reduced killing by 23-fold (Fig. 6), indicating that, at the time of plating, more than 95% of cells that would have been counted as dead on DMSO-free agar were still alive. After plating, these cells die from a poststress self-destructive process that involves ROS, since thiourea, another ROS scavenger, also protects bacteria from antimicrobial killing and antimicrobial-induced programmed cell death (3, 6, 9, 11). Collectively, these data are consistent with DMSO protecting from rapid antimicrobial-mediated killing through reduction of intracellular ROS levels.

The interpretation of the effects of DMSO and other antioxidants (3, 7, 9, 11) and inhibitors of ROS accumulation (3, 9, 15) on antimicrobial lethality (21) is complicated by possible off-target effects of these compounds. However, the argument for the involvement of ROS in antimicrobial action is bolstered by both the present results and those obtained using complementary genetic and molecular approaches (6, 11, 15). Moreover, scavenging/blocking ROS accumulation is the common feature shared by a variety of diverse compounds (thiourea [3, 9, 11], bipyridyl [3, 9, 11], glutathione [9], vitamin C [7], and DMSO) that protect from antimicrobial killing, while off-target growth inhibitory effects of

these compounds are less likely to derive from the same unspecified mechanisms. Another implication stemming from our study is that use of DMSO as a solvent for antimicrobials may need to be reconsidered because concentrations as low as 1% can reduce their efficacy, measured in the present case as rapid killing.

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