RESEARCH ARTICLE

Sublethal Exposure to Commercial Formulations of the Herbicides Dicamba, 2,4-Dichlorophenoxyacetic Acid, and Glyphosate Cause Changes in Antibiotic Susceptibility in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium

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ABSTRACT **Biocides, such as herbicides, are routinely tested for toxicity but not for sublethal effects on microbes. Many biocides are known to induce an adaptive multiple-antibiotic resistance phenotype. This can be due to either an increase in the expression of efflux pumps, a reduced synthesis of outer membrane porins, or both. Exposures of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to commercial formulations of three herbicides—dicamba (Kamba), 2,4-dichlorophenoxyacetic acid (2,4-D), and glyphosate (Roundup)—were found to induce a changed response to antibiotics. Killing curves in the presence and absence of sublethal herbicide concentrations showed that the directions and the magnitudes of responses varied by herbicide, antibiotic, and species. When induced, MICs of antibiotics of five different classes changed up to 6-fold. In some cases the MIC increased, and in others it decreased. Herbicide concentrations needed to invoke the maximal response were above current food maximum residue levels but within application levels for all herbicides. Compounds that could cause induction had additive effects in combination. The role of *soxS*, an inducer of the AcrAB efflux pump, was tested in β-galactosidase assays with *soxS-lacZ* fusion strains of *E. coli*. Dicamba was a moderate inducer of the *sox* regulon. Growth assays with Phe-Arg β-naphtylamide (PAβN), an efflux pump inhibitor, confirmed a significant role of efflux in the increased tolerance of *E. coli* to chloramphenicol in the presence of dicamba and to kanamycin in the presence of glyphosate. Pathways of exposure with relevance to the health of humans, domestic animals, and critical insects are discussed.**

IMPORTANCE **Increasingly common chemicals used in agriculture, domestic gardens, and public places can induce a multiple-antibiotic resistance phenotype in potential pathogens. The effect occurs upon simultaneous exposure to antibiotics and is faster than the lethal effect of antibiotics. The magnitude of the induced response may undermine antibiotic therapy and substantially increase the probability of spontaneous mutation to higher levels of resistance. The combination of high use of both herbicides and antibiotics in proximity to farm animals and important insects, such as honeybees, might also compromise their therapeutic effects and drive greater use of antibiotics. To address the crisis of antibiotic resistance requires broadening our view of environmental contributors to the evolution of resistance.**

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A biocide is a compound that is lethal to an organism. Biocides that are developed specifically to control bacteria include disinfectants, desiccants, and antimicrobial agents (e.g., antibiotics). The end of the antibiotic era has been forecast for decades. In the mid-1990s, two major American magazines, *Time* and *Newsweek*, ran cover stories on the dual threat of antibiotic resistance and new levels of pathogen virulence. In the last year, both the World Health Organization (1) and the U.S. Centers for Disease Control and Prevention (2) issued stern reports on the continuing and growing problem of antibiotic resistance. The latter estimates that in the United States alone, "more than two million people are sickened every year with antibiotic-resistant infections, with at least 23,000 dying as a result. The estimates are based on conservative assumptions and are likely minimum estimates." The emergence of antibiotic resistance in species that cause disease in humans and domestic animals is the result of human use (3). Most antimicrobial agents, including antibiotics, predate by billions of years the extensive application of antibiotics to humans, and the resistance of human pathogens has appeared in force only since the middle of the last century, corresponding to the time of their commercial use in medicine and agriculture.

All pharmacokinetic and pharmacodynamic models that are

applied to determining therapeutic doses and the frequency of administration consider *in vivo* susceptibility to the antibiotic (4). The therapeutic dose and frequency of administration are designed to keep concentrations above a certain minimum. Those models are in turn informed by the MIC measured *in vitro* (5). In a patient, drug concentrations will differ with location and mode of administration. Places in a patient where the concentration of the drug is near subtherapeutic levels are called "gray zones." Pathogens may persist in these zones to emerge later once the patient is off therapy (6). The higher the baseline MIC, the larger the gray zone. Even small increases in the MIC increase the chances of treatment failure (see, e.g., references 7 and 8). The aptly named MIC creep (9) can result from mutations that confer incrementally increased tolerance to an antibiotic, for example by decreasing permeability or increasing low-level secondary activities of deactivating enzymes (10). The change may also be physiological and transient (3, 11). This form of adaptive resistance is usually a species-uniform response that occurs only in environments that induce relevant changes in gene expression (12). Full susceptibility to the antibiotic is often restored when the inducing signal is removed (9, 11). This can confuse clinical assessments of patient isolates because, during subsequent culturing, the bacteria appear susceptible to the antibiotic. Depending on the environment, however, the phenotype may persist and mimic genetic inheritance (10).

Exposure to a compound not intended to be an antimicrobial agent can induce a cross-resistance. With this in mind, we tested whether exposures to commercial formulations of three herbicides—dicamba (3,6-dichloro-2-methoxybenzoic acid; Kamba), 2,4-dichlorophenoxyacetic acid (2,4-D), and glyphosate [*N*-(phosphonomethyl)glycine; Roundup]—may induce a changed response to antibiotics. While there is knowledge of the toxicity of these herbicides (the German Federal Institute for Risk Assessment evaluated nearly 300 studies for their recent draft report for the reevaluation of glyphosate [http://www.bfr.bund.de/en/the_bfr_has_finalised_its_draft_report_for_the_re_evaluation_of_glyphosate-188632.html]), very little is known about the effects, especially sublethal effects, that they or their most common breakdown products have on microbes in the environment or on the human microbiome.

Antibiotics from five different classes were chosen for this study: ampicillin (Amp; β-lactams), ciprofloxacin (Cip; fluoroquinolones), chloramphenicol (Cam), kanamycin (Kan; aminoglycosides), and tetracycline (Tet). Responses to these antibiotics have already been shown to be influenced by the presence of salicylic acid in our model organisms *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (see, e.g., references 13 to 17). The first two of the herbicides have some structural similarity to salicylic acid, but the third does not. We found a variety of responses consistent with induction of efflux pumps and reversible permeability changes.

RESULTS

MICs of herbicides. Although selected for use against plants, active ingredients or other components of commercial herbicide formulations can be toxic to microbes. Lethal exposures of the three commercial formulations were determined for strains of *E. coli* and *S.* Typhimurium (Table 1). The maximum herbicide concentrations used in all our experiments (≤1,950 ppm Kamba, ≤1,950 ppm 2,4-D, and ≤1,240 ppm Roundup) were below the MIC for each herbicide formulation. However, for some assays, the microbe's growth rate was decreased in the presence of the herbicide and antibiotic at and even below these concentrations. Therefore, plates were examined regularly for up to 4 days.

TABLE 1 MICs of herbicide formulations

Herbicide	MIC (ppm ae):	
	E. coli JB578	*S.* Typhimurium SL3770
Kamba^{500}	13,883	14,485
2,4-D	4648	5780
Roundup	7400	6190

Herbicide-induced antibiotic response alters the lethal dose. The efficiency of plating (EOP; the titer of a culture on treatment plates divided by the titer of the same culture on nontreatment plates) has long been a means of identifying nonlethal combinatorial effects of chemicals on bacteria. It reveals when exposures to sublethal concentrations of potential toxins alters survival after exposure to another toxin at otherwise-lethal concentrations. Previously, salicylic acid was shown to increase or decrease the EOP of *E. coli* on various antibiotics (13, 16). We performed a similar test using herbicides instead. Killing curves were generated for exposure to each of the antibiotics in the presence and absence of each of the herbicides. Bacteria were grown in petri plates on a medium with increasing concentrations of each antibiotic separately, and the EOP was compared to the EOP on a medium with a set concentration of herbicide and the same range of antibiotic concentrations (Fig. 1). The EOP remained high at low antibiotic concentrations independently of exposure to the herbicide, and the EOP was, as expected, low as the antibiotic concentration approached the MIC in the absence of a second agent. The limit of detection for the EOP was determined by the size of the population of bacteria that could practically be observed. However, the detection range is up to 8 orders of magnitude (1 to ~10^{-8}).

Exposure to sublethal concentrations of herbicide often raised the EOP at higher concentrations of antibiotic. For example, when *S.* Typhimurium was exposed simultaneously to Roundup and Kan, the EOP was about 0.78 at 16 μg/ml Kan. At this concentration, the EOP on medium with Kan but lacking herbicide was already below the detection limit (Fig. 1). In effect, the herbicide raised the concentration of antibiotic necessary to achieve a lethal dose. In some cases, the herbicide accentuated susceptibility to the antibiotic, as revealed through a lower EOP. For example, in *E. coli*, the EOP on 10 μg/ml Cam and Roundup was reduced to 3×10^{-5} compared to an EOP of 0.09 on Cam alone. The response varied by species, antibiotic, and herbicide. *S.* Typhimurium but not *E. coli* EOPs increased upon exposure to both Kamba and Amp. Roundup increased the EOP of both *E. coli* and *S.* Typhimurium exposed to Kan, but Kamba decreased it. 2,4-D exposure raised the EOP of *E. coli* on plates supplemented with Amp, but Kamba did not. One significant exception was a higher tolerance to Cip, which was seen after exposure in both species to any herbicide. The results of these experiments were confirmed in a blind fashion by one of us (G. C. Ferguson) at a different research institution. The researcher was not aware of the nature of any of the compounds or the identities of the strains that she used. The same

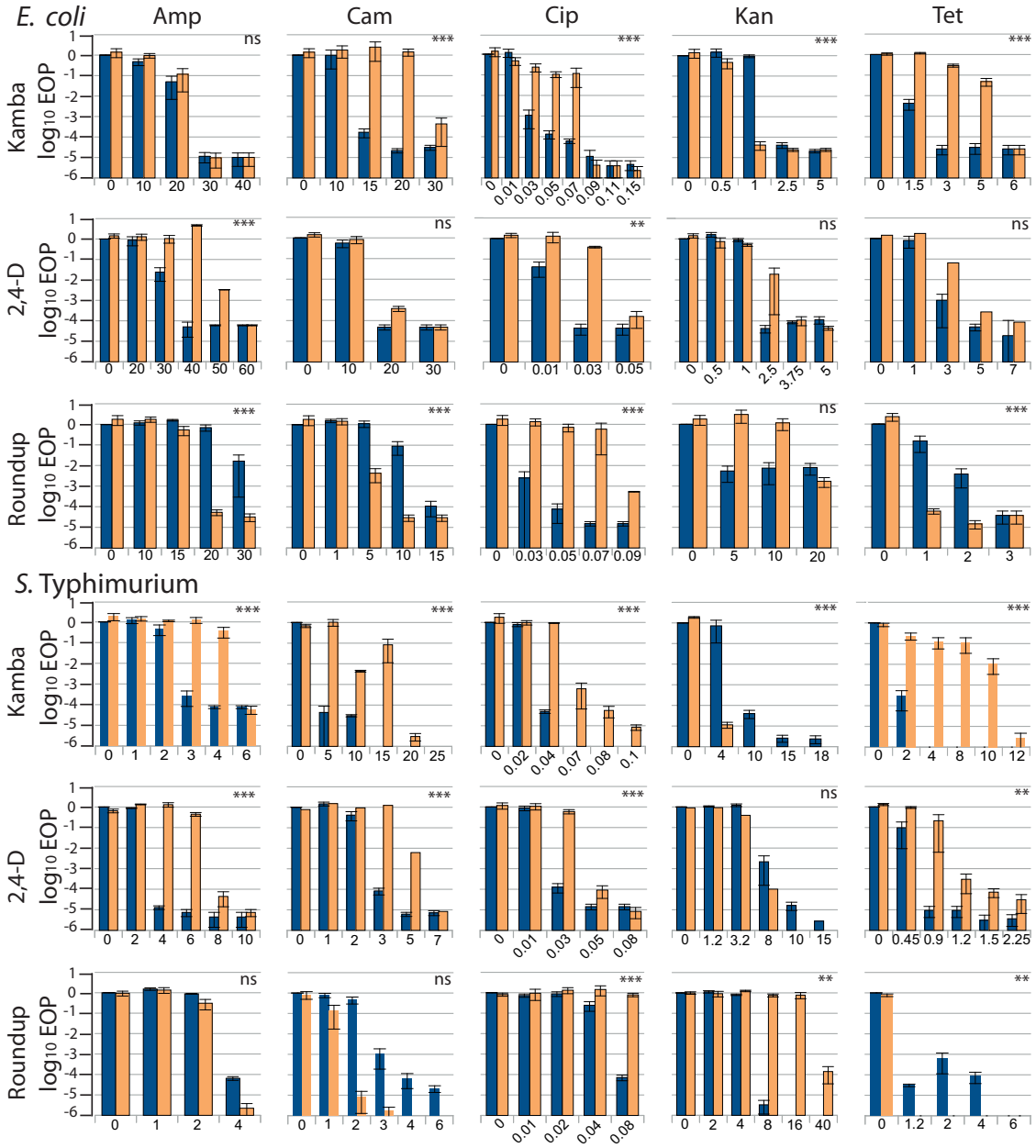

FIG 1 Killing curves. On the *x* axis, antibiotic concentrations (μg/ml) are plotted. Blue bars, no herbicide; orange bars, herbicide present. Herbicide concentrations used were as follows: for Kamba, 1,830 ppm/1,950 ppm (*E. coli*/*S.* Typhimurium); for 2,4-D: 1,830 ppm/1,950 ppm; and for Roundup, 1,240 ppm/1,240 ppm. Error bars are standard errors of the means (SEM). Asterisks indicate *P* values (see Materials and Methods for details). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

trends were observed for all combinations of bacteria, antibiotics, and herbicides (see Fig. S1 in the supplemental material).

We determined the antibiotic concentrations at which the EOP with herbicide changed by at least 1,000-fold from its level in medium not supplemented with herbicide (Table 2). The difference in antibiotic concentration is the change in susceptibility caused by the herbicide. Antibiotic concentrations at which the change in EOP was less than 1,000-fold might still be different enough from the MIC to have biological or clinical implications. However, in defining the sublethal effects of herbicides on bacterial responses to antibiotics for the first time, we have conservatively restricted our analysis to the biggest observed changes. In most cases, the herbicide increased or decreased the antibiotic concentration necessary for a 1,000-fold change in EOP by a factor of about 1.2 to 3.3. There were exceptions. *S.* Typhimurium's response to one antibiotic-herbicide combination (Amp plus Roundup) and *E. coli*'s response to four combinations (Amp plus Kamba, Amp plus Roundup, Cam plus 2,4-D, and Kan plus Roundup) never resulted in an EOP on herbicide medium that was 1,000-fold different from that on medium not supplemented with herbicide. However, some responses were even larger. The tolerated antibiotic concentrations varied 5-fold when

TABLE 2 Fold changes in antibiotic concentration to cause a 1,000-fold change in the EOP in the presence of a herbicide

Antibiotic	Herbicide	Fold change in antibiotic concn with:	
		E. coli[a]	S. Typhimurium
Amp	Kamba	0	2.33
	2,4-D	1.5	2
	Roundup	NA	0
Cam	Kamba	2	2.2
	2,4-D	0	2.33
	Roundup	1.5	2.5
Cip	Kamba	1.66	2.66
	2,4-D	1.66	1.66
	Roundup	1.8	5.8
Kan	Kamba	2.5	2.5
	2,4-D	1.5	1.2
	Roundup	NA	5
Tet	Kamba	2	3.33
	2,4-D	1.66	2.5
	Roundup	3	1.66

[a] NA, not applicable (the EOP did not drop below 0.001 at the highest tested concentration).

S. Typhimurium was exposed to two combinations (Cip plus Roundup [5.8-fold increase] and Kan plus Roundup [5-fold increase]). Strikingly, the tolerated Kan concentration rose from 6.9 μg/ml to 40 μg/ml during exposure to Roundup. This concentration is routinely used to culture genotypically Kan-resistant strains.

The range of the responses. To determine the minimum amount of herbicide needed to induce changes in the EOP and the maximum change in the EOP, dose-response curves were generated. Cultures were grown on a series of plates containing medium with a constant amount of antibiotic (near the MIC) and increasing amounts of herbicide. The "EOP ratio" in the presence or absence of herbicide [$EOP_{(H)}/EOP_{(-H)}$] for each herbicide concentration was plotted (Fig. 2) to create an easy visual of the magnitude of change. A ratio of >1 indicates that the herbicide reduces susceptibility to the antibiotic. The chosen antibiotic concentrations were predetermined through generating growth curves at a variety of combinations of concentrations of both the antibiotic and the herbicide (data not shown). Some combina-

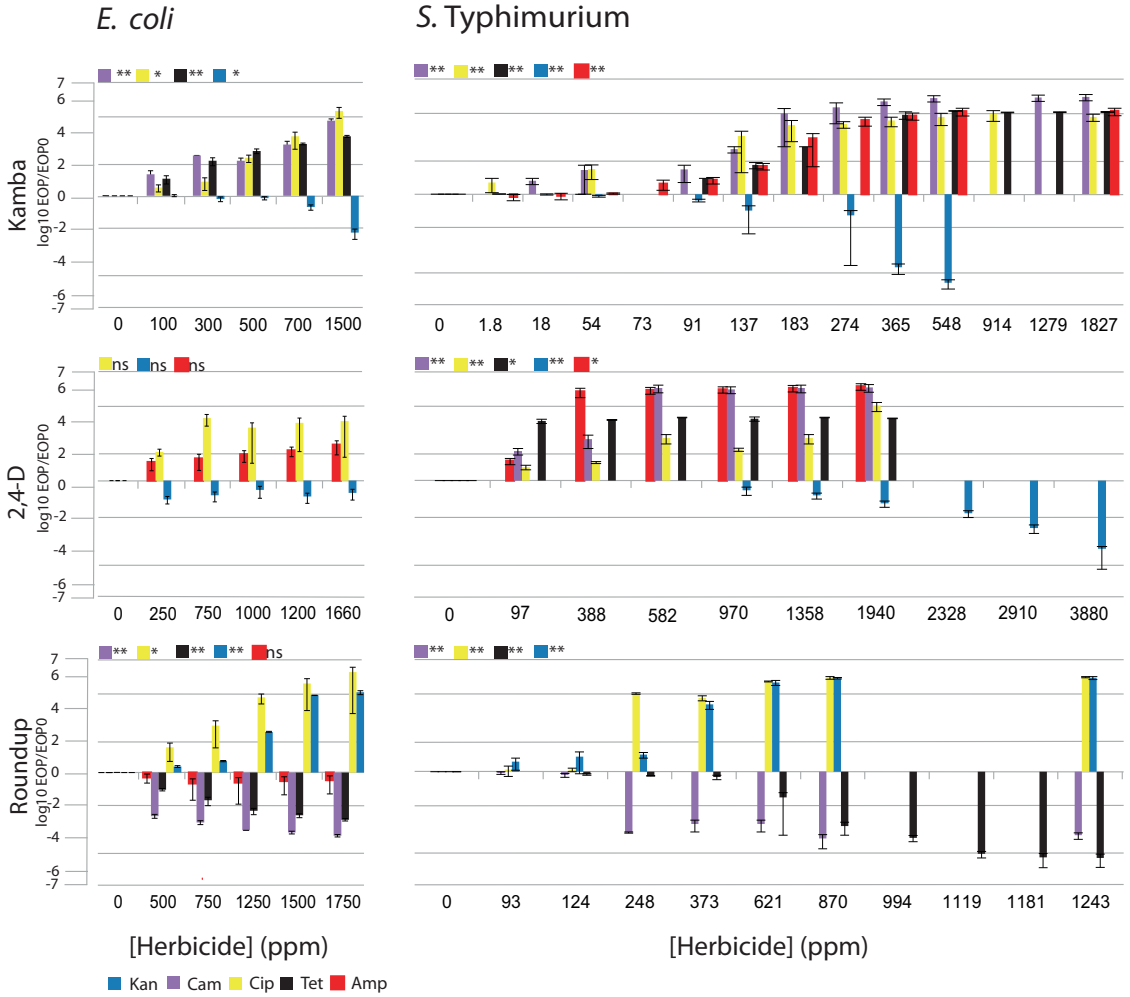

FIG 2 Dose-response curves. Antibiotic concentrations used (in μg/ml) were as follows: with 2,4-D, Amp at 4/40 (*S.* Typhimurium/*E. coli*), Cip at 0.003/0.003, Cam at 4.4/-, Kan at 6/2.5, and Tet at 0.75/-; with Kamba, Amp at 2/0, Cip at 0.03/0.05, Cam at 4/20, Kan at 2/1, and Tet at 0.75/3; and with Roundup, Amp at -/20, Cip at 0.05/0.07, Cam at 2/10, Kan at 12/10, and Tet at 0.45/1. Error bars are SEM, and asterisks indicate *P* values (see Materials and Methods for details). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant; -, not tested.

tions did not invoke a change, and these were consequently not pursued.

At the chosen antibiotic concentrations, an herbicide-induced antibiotic response was observed during culture of *S.* Typhimurium on medium with increasing concentrations of 2,4-D. The EOP ratio of 5.2×10^{-5} seen when the herbicide was combined with Kan shows significantly enhanced susceptibility. In contrast, the EOP ratios were significantly higher for the combinations of 2,4-D and Amp (8.1×10^5), Cam (6.7×10^5), Cip (4.9×10^4), and Tet (7.9×10^3). *E. coli* exposed to 2,4-D responded with smaller and not statistically significant changes in EOPs for Amp, Cip, and Kan. However, the trend of the observed effects for these antibiotics was the same as demonstrated for *S.* Typhimurium.

Kamba exposures caused the same pattern of responses as seen for 2,4-D. The EOP ratios for *S.* Typhimurium and *E. coli* decreased on medium containing Kan to 2.3×10^{-7} and 4.8×10^{-3}, respectively. Kamba increased survival when combined with Cip (1.2×10^5 and 2.1×10^5), Cam (1.4×10^6 and 5.2×10^3), Tet (1.6×10^5 and 5.5×10^3), or Amp (1.8×10^5). An exception was seen for *E. coli* plated on Amp, with which no response was observed.

Exposures to Roundup resulted in increases in the EOP ratios for Cip by 8.2×10^5 and 1.8×10^6 for *S.* Typhimurium and *E. coli*, respectively. However, Roundup induced greater susceptibility to Cam (1.0×10^{-4} and 1.1×10^{-4}) and Tet (4.0×10^{-6} and 1.1×10^{-3}). Tolerance to Kan increased (7.3×10^5 and 1.0×10^5). Roundup had no significant effect on the response to Amp by either species at the chosen antibiotic concentration.

We also tested whether the effects of different compounds could be additive. *E. coli* was exposed to either Kamba or salicylic acid at 250 ppm or to mixtures of the two compounds at various relative concentrations up to a total of 250 ppm. We found that there was no difference in the responses to the different combinations ($P < 0.001$) and that accounting for the proportion of Kamba did not significantly improve the model ($P = 0.306$). This suggests that the effects are indeed additive. As expected, exposures to less than 250 ppm of these two compounds resulted in a reduced response.

Efflux pumps and induction of the *soxRS* regulon account for the change in susceptibility in *E. coli*. Increased phenotypic tolerance to antibiotics has been attributed to changes in the synthesis of membrane proteins, leading to decreased antibiotic accumulation. In *E. coli* and *S.* Typhimurium, this can be due to either an increase in the expression of efflux pumps (see reference 18 and references therein and reference 19), a reduced synthesis of outer membrane porins (reviewed in reference 15), or both. The contribution of efflux pumps can be diagnosed using 25 mM Phe-Arg β-naphtylamide (PAβN), a known broad-spectrum efflux pump inhibitor (20, 21). Two combinations of antibiotic and herbicide were chosen for a PAβN assay with *E. coli*. These combinations, Kamba plus Cam and Roundup plus Kan, had caused an increase in the EOP. PAβN was added to exponentially growing cultures, which were then plated on medium containing PAβN plus antibiotic, herbicide, both, or neither. At 25 mM, PAβN alone does not change the EOP of the culture (Table 3). EOPs were compared to those of cultures grown without PAβN. The antibiotic concentration was just below the MIC and chosen to decrease the EOP without completely preventing growth (see the footnotes of Table 3). This enabled us to differentiate between the effects of PAβN with and without the herbicide. If active efflux contributes to the herbicide-induced antibiotic response, a decrease in the EOP is expected in the presence of PAβN.

TABLE 3 Influence of PAβN exposure on Kamba- and Roundup-induced tolerances in *E. coli*[a]

Test condition	EOP without PAβN	EOP with PAβN
LB	1.00	1.093 (0.093)
Kamba	1.42 (0.49)	0.292 (7.000×10^{-3})
Cam	2.28×10^{-3} (1.66×10^{-3})	$<10^{-7}$[b]
Kamba + Cam	1.01 (0.17)	$<10^{-7}$[b]
Roundup	0.88 (0.44)	$<10^{-7}$[b]
Kan	8.69×10^{-5} (4.07×10^{-5})	0.052 (0.034)
Roundup + Kan	1.44 (0.67)	$<10^{-7}$[b]

[a] Values are means of results from 3 independent experiments (SEM). Cam was at 10 μg/ml, Kan was at 5 μg/ml, Kamba was at 1,380 ppm, Roundup was at 1,250 ppm, and PAβN was at 25 mM.
[b] Below the detection limit.

Without PAβN, the EOPs conformed to the expected pattern. Exposure to the antibiotic led to a fall in the EOP by several orders of magnitude. This decrease was prevented by the herbicide. Neither Kamba nor Roundup alone changed the EOPs of the cultures. Combined with PAβN, Kamba was slightly more toxic, indicating that efflux pumps play a role in *E. coli*'s tolerance to the herbicide. PAβN significantly increased the toxicity of Cam, reducing the EOP to below the detection limit. Adding Kamba back did not rescue the cultures. The EOP remained below the detection limit, suggesting that efflux is part of an adaptive response to Kamba.

A different pattern was observed for the combination of Roundup and Kan. The presence of PAβN reduced the EOPs of cultures exposed to Roundup alone to below the detection limit, while it remained around 1 without PAβN. This suggests that efflux plays a large role in the adaptive response to Roundup. Exposure to Kan alone led to a 10,000-fold decrease in the EOP. However, the addition of PAβN increased the EOP to 0.05. The EOP in the presence of Roundup and Kan was undiminished at around 1. However, the presence of PAβN reduced survival to below the detection limit.

The overexpression of efflux pumps and a reduction in porins has been linked to induced tolerance to antibiotics shown by various organisms, such as *E. coli*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Streptococcus pneumoniae*, and *S.* Typhimurium (reviewed in reference 19). Tolerance can be due to mutations in repressor genes or to an activation by global regulators, such as the *marRAB* and *soxRS* regulons in *E. coli* (22). Both of these regulons are activated in response to environmental stressors and antimicrobial compounds (23–25).

SoxR is an activator of *soxS*, which in turn can upregulate the expression of efflux pumps. To determine whether the herbicide-induced antibiotic response was at least in part controlled by the *soxRS* regulon, we tested the induction of *soxS* using two *lacZ* fusion constructs in the Δ*soxRS* strain DJ901. The fusion alleles were identical, but one construct carried a copy of *soxR* (26) (Table 4). A *soxRS*-inducing agent should result in increased β-galactosidase activity in strain TN521 and cause no change in strain TN531, because the latter lacks *soxR*. Paraquat, a known inducer of the *soxRS* regulon, was used as a positive control. At 50 μM paraquat, β-galactosidase activity increased 8-fold in TN521 compared to its activities in the untreated control and TN531. Neither paraquat nor Kamba elicited a response in TN531. In the presence of 200 ppm dicamba, *soxS* expression was increased 3.3-fold in TN521

TABLE 4 Strains of *E. coli* and *S. enterica* serovar Typhimurium used

Strain	Description	Source or reference
E. coli		
JB578	HfrH Su^+ *thi gal* $r^- m^+$ Rif^r	Laboratory collection
TN521	DJ901(Δ*lac-4169*) Φ(*soxR*$^+$ *soxS′::lacZ*)	26
TN531	DJ901(Δ*lac-4169*) Φ(Δ*soxR* *soxS′::lacZ*)	26
S. Typhimurium		
SL3770	LT2 *pyr*$^+$ *rfa*$^+$	55

compared to that in the untreated control. At 400 ppm, 800 ppm, and 2,000 ppm Kamba, the corresponding increases were 3.7-fold, 4.5-fold, and 7-fold, respectively, compared to its expression in the untreated control.

DISCUSSION

The inappropriate use of a large number of antibiotics and the associated selective pressure on bacteria are compromising the long-term efficacy of antibiotic therapy (27). In addition, a much larger number of biocidal compounds are used regardless of their antimicrobial properties or without knowledge of them (28). When tested on microbes at all, products are tested only for lethal effects. However, as the problems of antibiotic resistance and chemotherapy failure worsen, more attention is being given to the effects of biocides at sublethal concentrations, which might create greater tolerances to antibiotics (29, 30).

We demonstrated for the first time that the susceptibility of bacteria to antibiotics can be changed upon simultaneous exposure to herbicides. Herbicides are used globally, may be nearly ubiquitous in the food supply, and are detected in humans (31), the atmosphere (32), pets (33), and homes (34, 35). When used as intended, these biocides are directed against plants. However, their application to, for example, urban lawns (36) or agricultural crops to improve preharvest desiccation (37) increases the chances that potential pathogens of humans or domestic animals will be exposed to them. They also may be applied more frequently during the year, such as onto crops genetically engineered to tolerate direct applications, which increases the exposure of beneficial insects, such as bees, and their microbiota (38).

The observed responses to the different herbicides varied by exposed species. *S.* Typhimurium exposure to sublethal concentrations of Kamba and 2,4-D resulted in significant increases in tolerance to Amp, Cam, Cip, and Tet and greater susceptibility to Kan. Exposure to Roundup significantly increased the tolerance to Kan and Cip but had no effect on or reduced the susceptibility to Amp, Cam, and Tet. Similar trends were observed when *E. coli* was exposed to Kamba and 2,4-D, except that no increase in tolerance to Amp was observed. Exposure to Roundup also increased tolerance to Kan and Cip but had no effect on the response, or increased susceptibility, to Amp, Cam, and Tet. Species-variable responses are in agreement with results published by others who reported significant differences in responses to various biocides even at the strain level (see, e.g., references 13 and 39).

Strikingly, the full effect of the herbicides was achieved by concomitant exposure to the herbicide and antibiotic. No priming through preexposure to the herbicide was required. By whatever means the herbicide increases or decreases susceptibility to an antibiotic, it does so before the antibiotic interferes with the herbicide-induced response. Since we used antibiotics that prevent *de novo* gene expression (i.e., Cam, Tet, Kan, and Cip), the effect of the herbicide either must be biophysical or must be able to induce gene expression faster than the antibiotic is able to reach its intracellular targets.

The pattern and rapidity of responses are suggestive of a change in target exposure to the antibiotic. This may be achieved by either an increase/decrease in efflux, permeability, or both. For example, activation of the AcrAB-TolC efflux pump in *E. coli* and *S.* Typhimurium has been shown to result in reduced susceptibility to fluoroquinolones, β-lactams, tetracycline, and chloramphenicol (25, 40). Increased susceptibility to aminoglycoside antibiotics was observed after exposure to salicylic acid (16), a known inducer of AcrAB-TolC (17). Similarly, we observed increased kanamycin susceptibility following exposure to Kamba or 2,4-D. In contrast, the *E. coli* AcrAD-TolC pump confers reduced susceptibility to aminoglycosides (40), which we see following exposure to Roundup.

Also consistent with the contribution of efflux or decreased permeability was the finding that a dicamba-based herbicide induced expression of a *soxS* fusion gene. Moreover, the efflux inhibitor PAβN restored *E. coli*'s susceptibility to Cam or Kan when it was combined with Kamba or Roundup, respectively.

The magnitudes of the herbicide-induced antibiotic responses varied and reached more than 3 times the measured MIC in the absence of the herbicide. Differences of this magnitude can have significant consequences for the treatment of bacterial infections (41, 42). For example, a study of hospitalized patients given ciprofloxacin found that a majority failed to reach efficacious doses for *E. coli*, *Klebsiella* spp., *Enterobacteriaceae*, *Proteus* spp., and *Pseudomonas aeruginosa* infections. In each case, the infection involved a ciprofloxacin-susceptible strain. A 2-fold change in the MIC of infecting strains, from ≤0.125 μg/ml to ≤0.25 μg/ml, was enough to cause 21% of patients to get a lower-than-target dose of the antibiotic, and when the MIC reached a 4-fold increase (to ≤0.5 μg/ml), 75% of patients failed to receive the target dose (43).

There are important outcomes of medical and environmental relevance for either an increase or a decrease in the MIC due to sublethal biocide exposure. Adaptive low-level resistance from increases in the MIC can have clinical implications by compromising therapy. In addition, a transient nonheritable increase in the MIC significantly increases the probability of spontaneous mutations that lead to heritable incremental or high-level changes in susceptibility in populations exposed to both biocide and antibiotics (44, 45). Adaptive low-level increases in susceptibility may also not be beneficial or even neutral. It is certain that the epidemic of antibiotic resistance is due largely to selection of resistant strains in environments with sufficient quantities of antibiotics to provide those strains with a competitive advantage (3). The concentration at which that advantage is achieved is directly related to the MICs for susceptible strains. Biocides that uniformly lower the MIC of an antibiotic make the antibiotic a relevant selective force at lower concentrations than it would be otherwise. In some environments contaminated with normally sublethal concentrations of antibiotics, the addition of a biocide may make those concentrations high enough to select for resistant strains.

The active ingredients of the three herbicides that we tested are

used in common commercial formulations worldwide. Drawing from the latest available U.S. statistics (46), glyphosate (number 1) and 2,4-D (number 5) were in the top 5 agricultural herbicides used in 2007. 2,4-D (number 1), glyphosate (number 2), and dicamba (number 5) were among the top five herbicides used in the home and garden sector. 2,4-D (number 1) and glyphosate (number 2) were the top two herbicides used by industry and government. Actual usage ranged from 8,200 to 8,400 metric tons of glyphosate in agriculture to 500 metric tons of dicamba in homes and gardens. Moreover, recent regulatory approval was issued for the use of dicamba-based and 2,4-D-based formulations to join existing glyphosate-based herbicides for use on the next generation of genetically modified crops in the United States, which is expected to significantly increase the use of dicamba and 2,4-D (47). The intent is to spray crops with one or more herbicides more frequently during the year and to directly spray crops as they grow. The effect may be more-concentrated exposures for farm animals through spray drift or the feed chain and more exposures for insects, soil, and plant microflora.

Even though home and garden use is only about 10% of agricultural use, it is if anything less regulated, with human and pet exposures more likely to be at or near application rates rather than maximum residue limits (MRLs).

We found that the concentration of herbicide needed to induce the maximum response to an antibiotic was above the maximum residue limits allowed under international trading laws (48). Thus, provided that food (and animal feed) contains less than the maximum residue limits, residue on food should not on its own induce a change in gut microbiota. However, we also found that the herbicide-induced antibiotic response was additive when chemicals that cause similar phenotypic changes were combined, e.g., Kamba and salicylic acid. This enlarges the range of potentially relevant human health or environmental exposures.

We also found that the concentration of herbicide needed to induce a detectable antibiotic response was lower than the label-specified herbicide application rate. Relevant environmental exposures then occur in urban and agricultural settings, as well as potentially in waterways. Insects or small mammals may be exposed to inducing concentrations as herbicides are applied. Where insects are also being exposed to antibiotics, e.g., honeybee hives treated with antibiotics either prophylactically or to eliminate diseases (49) or by means of the dung of antibiotic-treated farm animals or pets (50), all the conditions necessary for induced tolerance might be met. Antibiotics (51) and herbicides are detected in waterways, potentially creating conditions that maintain bacteria in the herbicide-induced antibiotic response.

The rise of antibiotic-resistant pathogens of humans and our domestic animals is attributed to the imprudent use of antibiotics in medicine and the environment. Nevertheless, not all treatment failures result from bacteria with full resistance genotypes. Transient, induced, higher MICs may contribute to these failures. Moreover, the pace of resistance evolution through MIC creep might be much slower if it were not for coupling adaptive resistance with the selection of incremental genotypic resistance made possible through an increase in the survival of populations exposed to normally lethal concentrations of a biocide. This increasingly chemically potent world necessitates a rethinking of how we measure and regulate exposures to common products. Testing each compound in isolation and only for severe effects on microbes, as is done during risk evaluations of herbicides, may underestimate its role in the emergence of antibiotic resistance phenotypes.

MATERIALS AND METHODS

Bacteria used in this study are detailed in Table 4. Liquid cultures were grown in LB (Invitrogen) at 37°C in a rotary incubator. Cultures on LB agar plates were incubated at 37°C for up to 4 days.

Antibiotics used were Kan (Gibco), Tet (Sigma), Amp (AppliChem), Cam (Sigma), and Cip (Pentex). Stock solutions were stored at −20°C. PAβN (Phe-Arg β-naphtylamide) was purchased from MP Biochemicals (Auckland, New Zealand). Except in the *soxS* assay, herbicides were the commercial formulations Kamba^{500} (Nufarm, Otahuhu, New Zealand), containing 500 g/liter dimethyl salt of dicamba; Roundup weed killer (Monsanto, Australia), containing 360 g/liter isopropylamine salt of glyphosate; and 2,4-D amine 800 WSG (Agpro, Auckland, New Zealand), containing 800 g/kg of the dimethylamine salt of 2,4-D. *soxS* assays were performed using Herbamba (HELM, Naucalpan, Mexico), containing 480 g/liter dimethyl salt of dicamba.

Dose-response assays, killing curves, and MIC determinations. Bacteria were grown in LB to saturation, harvested, and washed with LB to remove herbicides (when present). Dilutions were plated in duplicate on LB supplemented with antibiotics and/or herbicides. Plates were examined daily for up to 4 days, at which time no new colonies emerged. Results are averages from at least three independent experiments. Both species of bacteria were tested for their responses separately to all herbicides and antibiotics. The MIC is defined as the minimum concentration of agent in an agar plate at which no growth was observed when approximately 10^8 CFUs was applied to the plate surface.

Experiments were initially conducted using materials calculated in millimolar concentrations of the salt of the active ingredient as the unit. To allow comparisons with other herbicide formulations, all herbicide concentrations were converted to ppm acid equivalents (ae) of the active ingredients, rounded to the closest 10 ppm, and are listed as such in the main text (see Table S1 in the supplemental material).

To account for day-to-day differences in the densities of the culture used, results were normalized by determining the efficiency of plating (EOP), which is defined as the ratio of the titer of a culture (CFU/ml) on treatment plates to the titer of the same culture on unsupplemented LB plates $[(\mathrm{CFU/ml})_{\mathrm{treatment}}/(\mathrm{CFU/ml})_{\mathrm{LB}}]$. Observable values range from slightly above 1 (when cultures grow better on treatment plates than on LB) to as low as $\sim 10^{-8}$. The lower limits varied between experiments because EOP is a function of the titers of untreated cultures, which vary slightly between experiments. For the determination of the minimum inducing concentration, a ratio of the EOP determined with and without the addition of herbicide was calculated for each herbicide concentration.

Induction of SoxS. Saturated cultures of TN521 and TN531 were diluted 1:100 in fresh LB and incubated for 3 h (35°C and aerated by rotation), and then the following agents were added: paraquat (a known inducer) at 50 μM or dicamba to final concentrations of 200 ppm, 400 ppm, 800 ppm, and 2,000 ppm. Incubation was continued for a further 30 min before β-galactosidase activity was measured as described by Miller (52). Enzyme Miller units were calculated by considering the optical density (OD) of the yellow product, the cell density, volume, and incubation time. Results are averages from three independent experiments.

PAβN assays. Parallel cultures of *E. coli* were grown to an OD at 600 nm (OD_{600}) of ≈0.9. Twenty-five micrograms of PAβN per milliliter was added to one culture for the last 30 min. Cultures were then washed, and dilutions were plated on plates containing antibiotic, herbicide, or both in the presence and absence of 25 μg/ml PAβN. Plates were incubated at 37°C for up to 4 days, and EOPs were determined.

Statistical analysis. R was used for all statistical analyses (53). For killing curves, we tested for synergistic effects of herbicides and antibiotics on the log-transformed EOP scores. This was done using a multifactor analysis of variance (ANOVA) by evaluating the significance of an (antibiotic by herbicide) interaction term (the *P* value reported in Fig. 1). Antibiotic concentrations were treated as separate categories in the

ANOVA. Plots of residuals were used to test for violations of assumptions. To identify the antibiotic concentrations with significant differences among herbicide treatments, contrasts across herbicide concentrations when antibiotic levels were fixed were evaluated. This was done using the testInteractions function in the phia package in R (54). A Bonferroni correction was used within each experiment for this procedure.

When the dose-response curves were determined, many data points were near or below the detection limit, and as a result, the residuals from a standard ANOVA were not normally distributed. Hence, the equivalent nonparametric test, a Kruskal-Wallis one-way ANOVA, was used to test for differences in log-transformed EOP scores among herbicide concentrations (Fig. 2). We present a *P* value for a comparison of a null model where EOP is the same across all herbicide concentrations versus an alternative model where EOP differs among some herbicide concentrations.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00009-15/-/DCSupplemental.

Figure S1, PDF file, 0.5 MB.

Table S1, DOCX file, 0.04 MB.

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