Salmonella typhimurium intercepts *Escherichia coli* signaling to enhance antibiotic tolerance

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Bacterial communication plays an important role in many population-based phenotypes and interspecies interactions, including those in host environments. These interspecies interactions may prove critical to some infectious diseases, and it follows that communication between pathogenic bacteria and commensal bacteria is a subject of growing interest. Recent studies have shown that *Escherichia coli* uses the signaling molecule indole to increase antibiotic tolerance throughout its population. Here, we show that the intestinal pathogen *Salmonella typhimurium* increases its antibiotic tolerance in response to indole, even though *S. typhimurium* does not natively produce indole. Increased antibiotic tolerance can be induced in *S. typhimurium* by both exogenous indole added to clonal *S. typhimurium* populations and indole produced by *E. coli* in mixed-microbial communities. Our data show that indole-induced tolerance in *S. typhimurium* is mediated primarily by the oxidative stress response and, to a lesser extent, by the phage shock response, which were previously shown to mediate indole-induced tolerance in *E. coli*. Further, we find that indole signaling by *E. coli* induces *S. typhimurium* antibiotic tolerance in a *Caenorhabditis elegans* model for gastrointestinal infection. These results suggest that the intestinal pathogen *S. typhimurium* can intercept indole signaling from the commensal bacterium *E. coli* to enhance its antibiotic tolerance in the host intestine.

Rather than acting autonomously, bacterial cells communicate with one another to coordinate their efforts and relay vital information. Interspecies and intraspecies bacterial communication has been implicated in many community-dependent behaviors including virulence (1), biofilm formation (2), and antibiotic tolerance (3). Communication may therefore allow control of heterogeneity, which is important in determining fitness of microbial populations (4). Recently, we reported that bacterial communication through indole signaling induces persister formation in *Escherichia coli* (3). Persistence is an antibiotic-tolerant phenotype in which a dormant subpopulation of cells (persisters) survives antibiotic treatment without having genetically encoded resistance factors (5, 6). In *E. coli*, we found that indole signaling induced oxidative stress response and phage shock response pathways, thereby increasing the persister frequency within the population. This work suggested that bacteria can use intraspecies signaling to modify the antibiotic tolerance of their population in response to environmental conditions.

Indole signaling is used by bacteria in the distal intestine of humans and other mammals (7). In this environment, alkaline and low nutrient conditions induce expression of the indole-producing tryptophanase (*tnaA*) enzyme in commensal *E. coli* and related bacteria (8). Indole concentrations in the mammalian intestine (~300 μM to 1 mM) (9, 10) can induce antibiotic tolerance in *E. coli* without adversely affecting growth (11). As the mammalian intestine contains a richly mixed microbial population (12), signaling molecules such as indole might be detected and used by both commensal and pathogenic bacteria. Although there is increasing interest in the roles that commensal bacteria play in mammalian health (13), the mechanisms by which commensal bacteria interact with invading pathogens are not yet well understood.

We hypothesized that pathogenic bacteria could use communication signals produced by commensal bacteria to sense and adjust their physiological state to the host environment. As indole induces antibiotic tolerance in *E. coli*, we hypothesized that it might also increase tolerance in related pathogens. *Salmonella typhimurium* is one such pathogen which, although it does not produce indole (14), has been shown to respond to signaling molecules produced by other bacteria (15). *S. typhimurium* is a common gastrointestinal pathogen and a major epidemiological threat, as it is a causative agent of gastroenteritis and sepsis. This pathogen can survive macrophage engulfment and persist within phagocytes, resulting in an asymptomatic but infectious carrier state (16) where antibiotic tolerance is a significant problem (17). We therefore sought to determine if indole signaling by *E. coli* might be exploited by *S. typhimurium*, leading to increased tolerance of the pathogen in a host intestinal environment.

Here we show that indole signaling can indeed increase the antibiotic tolerance of *S. typhimurium*. This tolerance can be induced by exogenous indole in *Salmonella*-only cultures or by indole produced by *E. coli* in a mixed-microbial population. Our data suggest that this tolerance is mediated, in part, by oxidative stress and phage shock response systems. Further, we find, using a *Caernohabditis elegans* infection model (18), that indole induces antibiotic tolerance of *S. typhimurium* in a mixed-microbial, intestinal environment.

Results

We first sought to test whether indole induced antibiotic tolerance in *S. typhimurium* (strain LT2). We treated exponential-phase *S. typhimurium* grown in tryptophan-free medium (M9CG

Significance

Bacterial communication plays an important role in many population-based phenotypes and interspecies interactions, including those in host environments. Social interaction within bacterial communities, and particularly communication between pathogenic and commensal bacteria, is a subject of growing interest with relevance to ecology and human health. In this study, we show a case of interspecies communication where the intestinal pathogen *Salmonella typhimurium* increases its antibiotic tolerance in response to the bacterial signaling molecule indole, even though *S. typhimurium* does not natively produce indole. These results suggest that this intestinal pathogen can benefit from indole signaling produced by *E. coli* and other commensal bacteria.

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The authors declare no conflict of interest.

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[M9 + 0.2% Casamino acids + 0.4% (wt/vol) glucose]; *Materials and Methods* and *SI Materials and Methods*) with a range of indole concentrations to determine the effect on tolerance to carbenicillin and ciprofloxacin, antibiotics that are used in clinical treatment of enteric *Salmonella* infections (19) (Fig. 1*A*). We found that exogenous indole increased tolerance to both antibiotics by over threefold, demonstrating that indole enhances antibiotic tolerance despite the inability of *S. typhimurium* to produce this signal (14). Interestingly, the protective range of indole was different for the two antibiotics. Carbenicillin tolerance peaked in the presence of 50 μM indole and declined at higher indole concentrations, whereas ciprofloxacin tolerance was enhanced by higher concentrations of indole, peaking at 125 μM indole. The reason for this difference is unclear, but it may suggest that the protective processes induced by indole play differing roles against different antibiotics.

As addition of exogenous indole increased tolerance of *S. typhimurium* cultures, we postulated that indole produced by *E. coli* in a mixed-microbial environment would also induce *S. typhimurium* tolerance. To test this, we mixed an exponential-phase culture of *S. typhimurium* with a stationary-phase culture of *E. coli* [*E. coli* K-12 wild-type strain (EMG2) Pro, Table S1] grown in the presence of tryptophan to enable indole production. After a 1-h incubation, the mixed culture was treated with ciprofloxacin, as fluoroquinolone antibiotics are known to retain bactericidal activity in dense, starved cultures (20) such as those used in these assays. Antibiotic-treated cultures were plated on selective media to quantify the induced antibiotic tolerance in *S. typhimurium* (*Materials and Methods* and *SI Materials and Methods*). As expected, baseline tolerance of *S. typhimurium* was higher under dense coculture conditions than in exponential-phase culture (Fig. 1*A*) (21). We found that coculture with wild-type *E. coli* increased the tolerance of *S. typhimurium* to ciprofloxacin when cultures were grown in the presence of tryptophan (Fig. 1*B* and Fig. S1). Interestingly, the relationship between tolerance and tryptophan concentration was not strictly monotonic, with the benefit of tryptophan peaking at 1 mM. By measuring the *E. coli*-produced indole in cultures, we determined that 1 mM tryptophan corresponded to 125–300 μM of produced indole (Fig. S2), consistent with tolerance levels observed when indole was exogenously added (Fig. 1*A*). The conferred advantage for *S. typhimurium* of the mixed-microbe environment required indole production, as coculture with *E. coli* unable to produce indole (Δ*tnaA* strain) did not induce tolerance (Fig. 1*B*). Plasmid-based expression of *tnaA*, but not a *gfp* control, restored the coculture tolerance phenotype to the Δ*tnaA* knockout strain (Fig. 1*C*), further demonstrating the specific effect of indole on induced antibiotic tolerance in *S. typhimurium*. These results indicate that *E. coli* indole signaling can induce antibiotic tolerance in *S. typhimurium*.

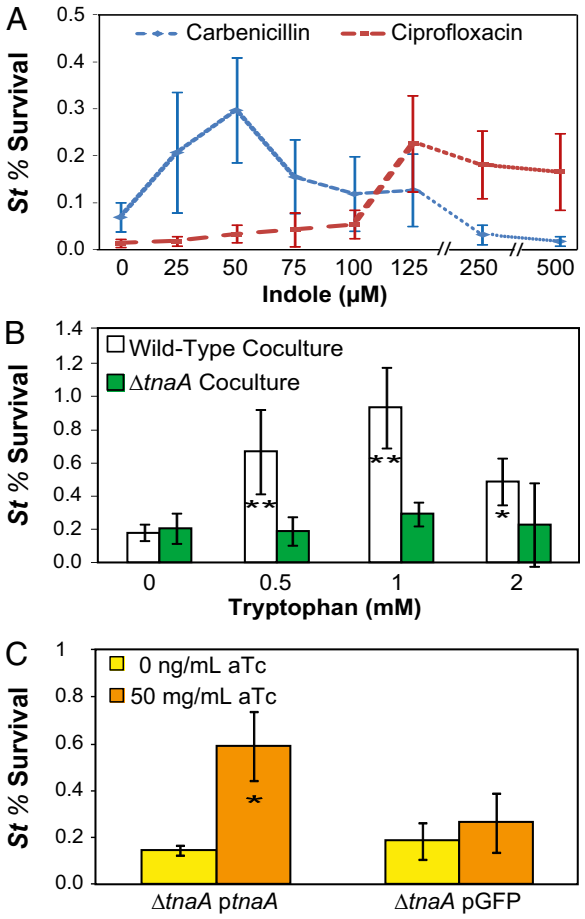

Fig. 1. *E. coli* indole signaling induces antibiotic tolerance in *Salmonella typhimurium*. (*A*) Exponential-phase cultures of *S. typhimurium* (*St*, $n \geq 3$) were incubated with indole for 1 h before treatment with carbenicillin (100 μg/mL) or ciprofloxacin (0.5 μg/mL). (*B*) Coculture of *S. typhimurium* with indole-producing cultures of *E. coli* ($n \geq 6$). *T* tests were performed vs. the 0 mM tryptophan condition. (*C*) Expression of plasmid-borne *tnaA* in *E. coli* for induction of *S. typhimurium* tolerance in coculture ($n \geq 3$). Cultures were grown in M9CG + 2 mM tryptophan with or without anhydrotetracycline (aTc) for induction of plasmid-borne genes. *T* tests were performed vs. the uninduced condition (0 ng/mL aTc). Assays in *B* and *C* were performed using ciprofloxacin (0.5 μg/mL). Error bars represent mean ± SD of biological replicates, and stars indicate significance level of two-sided two-sample *t* tests assuming unequal variance (*$P \leq 0.05$; **$P \leq 0.01$).

We previously determined that indole-induced tolerance in *E. coli* was mediated by the oxidative stress response (*oxyR*) and phage shock response (*psp*) (3). As these processes are largely conserved in *S. typhimurium*, with some differences in effectors and regulation (22, 23), we reasoned that indole-induced tolerance might function through a similar mechanism in *S. typhimurium.* To test this hypothesis, we performed qPCR on mRNA transcripts from several genes in these pathways from *S. typhimurium* cultures treated for 30 min with 50 μM and 125 μM indole, the concentrations that induced high carbenicillin and high ciprofloxacin tolerance, respectively. Our qPCR results (Fig. 2*A*) indicated that, as in *E. coli*, expression of the OxyR and phage shock regulons is induced in *S. typhimurium* by indole. We found that 125 μM indole strongly induced the expression of genes in the OxyR regulon [*dps* and hydroxyperoxidase I (*katG*)] and an effector of the phage shock pathway (*pspE*), although 50 μM indole produced weak induction of OxyR regulon genes and did not have a detectable effect on *pspE* (Fig. 2*A*). These results demonstrate that, as in *E. coli*, indole can stimulate in *S. typhimurium* expression of genes in the oxidative stress and phage shock pathways. Previous reports have indicated that higher levels of indole induced expression of genes involved in virulence and drug efflux pumps in *S. typhimurium* (24). We did not observe induction of these genes (presented here as "Other," Fig. 2*A*) when cultures were treated with indole concentrations that induce increased antibiotic tolerance. Interestingly, indole treatment produced a small but consistent decrease in transcript abundance of a transcriptional regulator associated with multiple antibiotic resistance (*ramA*); this gene product has been shown to control expression of multidrug efflux pumps in *S. typhimurium* (24), suggesting that indole treatment (at the levels considered here) may actually decrease efflux in this system.

We next used genetic knockouts to determine whether the OxyR and phage shock pathways are involved in indole-induced antibiotic tolerance in *S. typhimurium*. The Δ*oxyR* and Δ*pspBC* mutants were constructed to allow inactivation of the OxyR and phage shock responses, respectively (*SI Materials and Methods*). We

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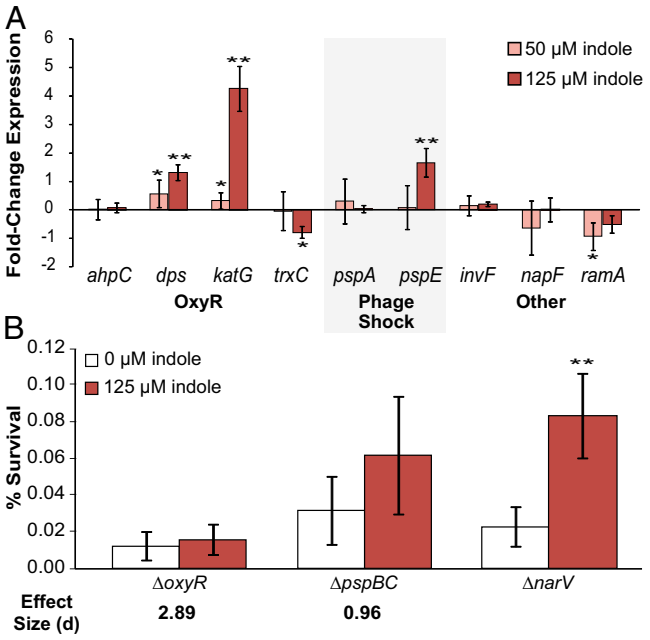

Fig. 2. Response of OxyR and phage shock pathways to indole in *S. typhimurium*. (*A*) Transcriptional response of *S. typhimurium* to indole treatment as determined by qPCR. *S. typhimurium* cultures were treated with 0, 50 (pink bars) or 125 (red bars) μM indole (*SI Materials and Methods*). Results are shown as fold-change expression ($-\Delta\Delta C_t$) of indole-treated vs. untreated cultures. Error bars represent mean ± SD of three to four biological replicates. (*B*) Exponential-phase cultures of *S. typhimurium* Δ*narV:CmR* (control), Δ*oxyR:CmR*, and Δ*pspBC:CmR* were incubated with indole (0 or 125 μM) for 1 h before treatment with ciprofloxacin (0.5 μg/mL). Error bars represent mean ± SD of 8–13 biological replicates. Stars indicate significance level of two-sided two-sample *t* tests assuming unequal variance (*$P \leq 0.05$; **$P \leq 0.01$). Cohen's effect size (d) using pooled SD was calculated for the difference between indole-treated and untreated cultures compared with the difference observed in the Δ*narV* control; $d > 0.8$ suggests a large effect.

found that indole-induced tolerance was reduced in the Δ*pspBC* mutant and entirely eliminated in the Δ*oxyR* mutant relative to the control (Fig. 2*B*). These results suggest that both the OxyR and phage shock responses are involved in indole-induced antibiotic tolerance in *S. typhimurium*, as was previously observed in *E. coli*. Interestingly, although the mechanism of indole-induced antibiotic tolerance appears to be conserved between species, the role of the phage shock response is attenuated in *S. typhimurium* compared with *E. coli*.

Given the high levels of induction in some aspects of the oxidative stress response (Fig. 2*A*), we hypothesized that the OxyR regulon might play a role in inducing antibiotic tolerance in *S. typhimurium*. To test this hypothesis, we treated *S. typhimurium* cultures with a range of H_2O_2 concentrations 1 h before treating cultures with antibiotic (Fig. 3). We found that low levels of oxidative stress (Fig. S3) could increase antibiotic tolerance of *S. typhimurium* to the same degree induced by indole, suggesting that indole may primarily act through the OxyR regulon to induce tolerance in *S. typhimurium*.

The protective range of H_2O_2 was different for carbenicillin and ciprofloxacin, with better protection against carbenicillin at lower H_2O_2 concentrations and better protection against ciprofloxacin at higher H_2O_2 concentrations. Interestingly, this antibiotic-specific concentration dependence for H_2O_2 treatment (Fig. 3) resembles the concentration dependence observed during indole treatment (Fig. 1*A*). The qualitative similarities in the antibiotic tolerance curves suggest that the OxyR regulon plays a functional role in indole-induced antibiotic tolerance in *S. typhimurium*. These data may additionally suggest that contribution of reactive oxygen species (ROS) to antibiotic lethality differs between carbenicillin and ciprofloxacin, adding further nuance to a growing understanding of antibiotic-induced cell death (25–28).

Having demonstrated that *S. typhimurium* responds to *E. coli*-produced indole by inducing an oxidative stress response, thereby increasing antibiotic tolerance, we sought to determine whether this interspecies signaling occurred in an intestinal environment. We used a *C. elegans* model for *S. typhimurium* infection (18) to explore in vivo the relation between *E. coli*-produced indole and *S. typhimurium* tolerance. This model provides a tractable approach for investigating intestinal mixed-microbial interactions and has been used for determining in vivo antibiotic efficacy against pathogenic bacteria (29). Although clearly less complex than the mammalian intestine, the *S. typhimurium* intestine maintains many critical factors that pertain to microbes, including mixed, semiimmobilized bacterial populations with spatial distributions, innate antimicrobial defenses, and the requirement of pathogen-epithelium adhesion (30).

We used fluorescent microscopy to verify that *E. coli* and *S. typhimurium* established a mixed-microbial community in the *C. elegans* intestine (18). Synchronized cultures of adult *C. elegans* were fed on *E. coli* (mCherry) in tryptophan-free S-medium with or without 125 μM indole then incubated with *S. typhimurium* (GFP) to allow infection; indole was added exogenously to allow precise control of indole concentrations (Fig. 4 *A* and *B*; *SI Materials and Methods*). Worms generally showed observable fluorescence in both channels, indicating the simultaneous presence of *E. coli* and *S. typhimurium* in the intestine. Interestingly, indole-treated cultures (125 μM) showed low-intensity *S. typhimurium* infection with small adhered colonies in nearly all worms sampled (Fig. 4*B*), whereas untreated cultures contained a mix of heavily infected and uninfected worms (Fig. 4*A*). However, by measuring cfu/worm, we found that treatment with 125 μM indole did not affect the average pathogen count per worm (Fig. 4*C*), suggesting that indole altered heterogeneity of the *S. typhimurium* infection in *C. elegans*.

We next sought to determine if indole affected *S. typhimurium* antibiotic tolerance in the *C. elegans* model. Synchronized adult cultures of *C. elegans* in S-medium ± 125 μM indole were fed on *E. coli* (EMG2 Δ*tnaA* Pro; *SI Materials and Methods*). Infection with *S. typhimurium* [chloramphenicol resistance (*CmR*); *SI Materials and Methods*] was then facilitated as described above,

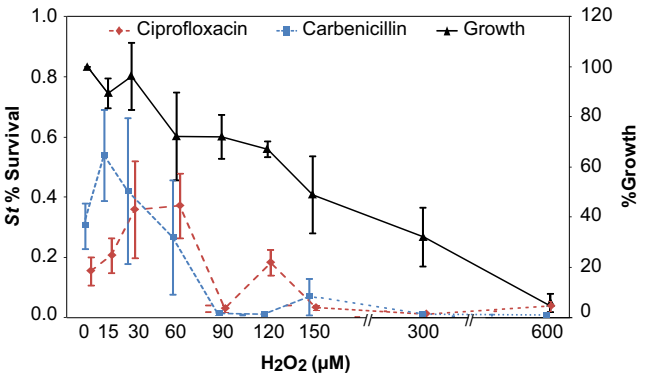

Fig. 3. Hydrogen peroxide induces tolerance in *S. typhimurium*. Exponential-phase cultures of *S. typhimurium* LT2 in M9CG were incubated with H_2O_2 (0–600 μM) for 1 h before treatment with antibiotics. Black line indicates percent growth of LT2 cultures during incubation with hydrogen peroxide, where growth in the absence of hydrogen peroxide was used as reference (100%). Percent survival was determined after 4-h treatment with ciprofloxacin (0.5 μg/mL, dashed red line) or carbenicillin (100 μg/mL, dotted blue line). Error bars represent mean ± SD of at least six biological replicates.

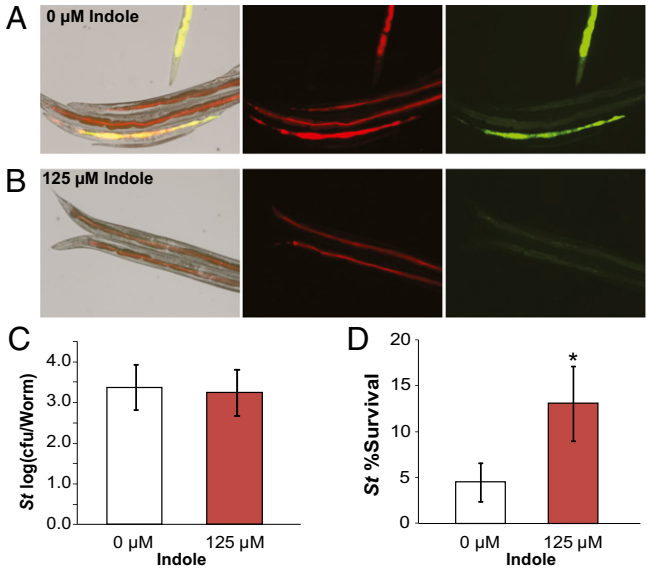

Fig. 4. Indole increases antibiotic tolerance of *S. typhimurium* in the *C. elegans* intestine. All experiments were performed in S-Medium (*SI Materials and Methods*) at 25 °C to ensure sterility and survival of *C. elegans* AU37. Images were selected to represent the variation observable in each experimental condition. (*A* and *B*) Visualization of infection in the *C. elegans* intestine. *C. elegans* fed on *E. coli* EMG2 (mCherry) and infected with *S. typhimurium* (GFP) with (*A*) no indole or (*B*) 125 μM indole added to media were imaged 36 h after initial infection. Differential interference contrast (DIC) images are shown overlaid with red and green fluorescent channels, and fluorescent channels are shown in isolation (*SI Materials and Methods*). (*C*) Average intensity of infection by *S. typhimurium* (*St*). (*D*) Survival of *S. typhimurium* in the *C. elegans* intestine after treatment of worm cultures with ciprofloxacin (2 μg/mL). All error bars indicate mean ± SD of four biological replicates. Stars indicate significance level of one-sided two-sample *t* tests assuming unequal variance, comparing indole-treated and untreated cultures (*$P \leq 0.05$; **$P \leq 0.01$).

and infected cultures were treated with ciprofloxacin to determine antibiotic tolerance. We observed that, although ciprofloxacin was effective in killing *S. typhimurium* in the *C. elegans* intestine, worms incubated with indole carried *S. typhimurium* with higher antibiotic tolerance. This finding suggests that indole increased antibiotic tolerance of *S. typhimurium* in the *C. elegans* host intestine (Fig. 4*D* and Fig. S4).

Having observed that indole could increase *S. typhimurium* tolerance in a host-commensal-pathogen model, we sought to test if indole produced by *E. coli* in this model was sufficient for increasing tolerance. We investigated this by incubating synchronized cultures of adult *C. elegans* in modified S-medium (pH~7) with or without additional tryptophan using *E. coli* wild-type or Δ*tnaA* strains as the *C. elegans* food source (*SI Materials and Methods*). We found that addition of tryptophan to worm media encouraged the formation of small punctate intestinal colonies of *S. typhimurium* when worms were fed on wild-type *E. coli* but not when fed on the Δ*tnaA E. coli* strain (Fig. 5 *A*–*D*). Average pathogen count per worm was unchanged across all experimental conditions (Fig. 5*E*). We found that adding tryptophan to *C. elegans* culture media increased antibiotic tolerance of *S. typhimurium* when wild-type *E. coli* was present, but not in the presence of the Δ*tnaA E. coli* strain (Fig. 5*F*). These results suggest that *S. typhimurium* can intercept *E. coli* indole signaling in the *C. elegans* intestine, allowing the pathogen to increase its tolerance to antibiotics.

Discussion

Here, we report that physiologically relevant concentrations of indole, whether added exogenously or produced by *E. coli* in coculture, enhance the antibiotic tolerance of *S. typhimurium*. Indole-induced tolerance was observed consistently in *S. typhimurium* despite naturally occurring variation in baseline tolerance measurements (31). Physiological responses to indole have been observed in non-indole-producing bacteria (32), suggesting that indole can function as an interspecies signal (2, 33–35). The data presented here implicate indole signaling by commensal bacteria and exploitation of this signal by pathogenic bacteria as a potential factor in establishing antibiotic tolerance of pathogens.

We also showed that indole signaling by *E. coli* enhances *S. typhimurium* tolerance in a *C. elegans* host intestinal model. Despite the relative simplicity of the *C. elegans* model, it has been suggested as a useful model for *S. typhimurium* infection of mammals based on the observation that strains attenuated in virulence in mammals were also attenuated in *C. elegans* (29).

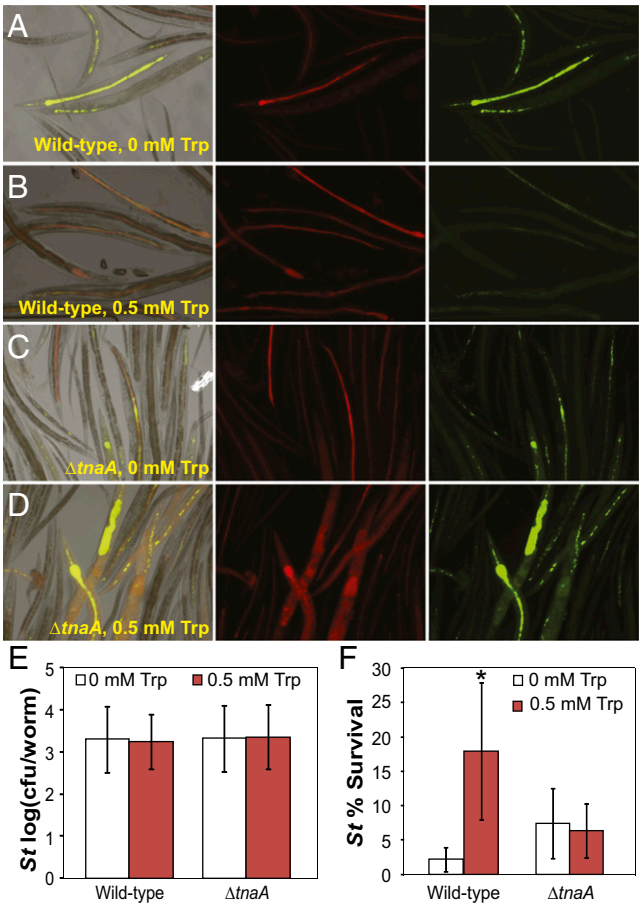

Fig. 5. Indole produced by *E. coli* in a mixed-microbe population induces tolerance of *S. typhimurium* in the *C. elegans* intestine. All experiments were performed in modified S-Medium (*SI Materials and Methods*). Images were selected to represent the variation observable in each experimental condition. (*A*–*D*) Visualization of infection in the *C. elegans* intestine. *C. elegans* fed on (*A* and *B*) *E. coli* EMG2 (mCherry) or (*C* and *D*) Δ*tnaA* (mCherry) and infected with *S. typhimurium* (GFP) with (*A* and *C*) no tryptophan or (*B* and *D*) 0.5 mM tryptophan added to media were imaged 36 h after initial infection. DIC images are shown overlaid with red and green fluorescent channels, and fluorescent channels are shown in isolation. Images were autoscaled using software default settings (*SI Materials and Methods*). (*E*) Average intensity of infection by *S typhimurium* (*St*). (*F*) Survival of *S. typhimurium* in the *C. elegans* intestine after treatment of worm cultures with ciprofloxacin (2 μg/mL). Error bars indicate mean ± SD of four biological replicates. Stars indicate significance level of one-sided two-sample *t* tests assuming unequal variance, comparing tryptophan-treated and untreated cultures (*$P \leq 0.05$; **$P \leq 0.01$).

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The *C. elegans* infection model was used here to create a spatially nonhomogenous mixed-bacterial culture with adhesion of bacteria to an epithelium. Our results with this system indicate that pathogenic *S. typhimurium* are able to "eavesdrop" on commensal bacterial communication by *E. coli* to enhance their antibiotic tolerance within the host intestine (Fig. 6).

We found that indole-induced tolerance in *S. typhimurium* is mediated, in part, by the oxidative stress response and the phage shock response, as was seen previously in *E. coli* (3). Antioxidant capability has previously been linked to antibiotic tolerance in bacteria (36, 37), and in this case, induction of the oxidative stress response appears to be largely responsible for the indole-induced antibiotic-tolerant phenotype. We observed increased transcript levels for *katG*, which acts to protect the cell from damage under conditions of increased oxidative stress (38, 39), but not alkyl hydroperoxide reductase C (*ahpC*), which has been implicated in scavenging of low-level, endogenously produced H_2O_2 (40), suggesting that indole response prepares these bacteria for survival in stressful conditions.

We found some differences in indole-induced changes in transcript abundance between *E. coli* and *S. typhimurium*. For example, while both species show an indole-responsive increase in *pspE* transcript, indole-treated *E. coli* show increased levels of *pspA* transcript, whereas indole-treated *S. typhimurium* do not (3). Although stress response genes are largely conserved and highly homologous between *E. coli* and *S. typhimurium*, there are important functional and regulatory differences between these species (22, 23, 41), and it is therefore expected that indole-based induction of individual genes within the relevant stress response pathways will vary somewhat between species. However, it is notable that the same pathways are observed to respond to indole in both species, suggesting that the mechanism of indole-induced tolerance is conserved. It remains to be tested whether this cross-species protection extends to other bacterial pathogens, including Gram-positives.

Indole signaling may affect stress responses that are important for survival in a host and establishment of a chronic infection. Previous work has shown that survival in macrophages is critical for *Salmonella* virulence (42, 43) and that peroxide catalase activity plays an essential role in survival (39). OxyR-mediated processes may protect *Salmonella* intracellularly by inducing tolerance to the oxidative bursts that immune cells use to kill bacteria. The importance of oxidative stress in establishment and recurrence of *Salmonella* infection is demonstrated by chronic granulomatous disease, a hereditary immunodeficiency in which macrophage cannot generate the oxidative burst; patients with this disease are highly susceptible to bacteremic infection by nontyphoidal *Salmonella* and to recurrences of these infections (44). We found that indole signaling strongly induced expression of OxyR regulon genes in *S. typhimurium*, suggesting that the ability to detect commensal indole signaling may provide the pathogen with a method of altering its physiology to tolerate the stresses of the immune system and antibiotics and thereby establish a persistent infection.

More broadly, our results suggest that persistence of pathogens in the host environment may be induced by the interception of nonnatively produced bacterial-signaling molecules. There is a growing body of work exploring the complex relationship between the host, the innate microbiota, and gastrointestinal pathogens (45–49), and it is increasingly evident that the interplay between the established microbiota and introduced organisms in the intestine may be critical in determining the progress and resolution of infections and the aftermath of disease. Here, we observe a case where a bacterial pathogen has lost the capacity for production of a signal but has retained the ability to respond to signaling produced by commensals in the shared intestinal environment. Further, we show that interception of and response to nonnative signaling produces an antibiotic-tolerant phenotype in a bacterial pathogen, suggesting that interactions between the commensal microbiota and invading pathogens may in some cases improve stress tolerance in pathogens, thereby increasing recalcitrance of bacterial infections.

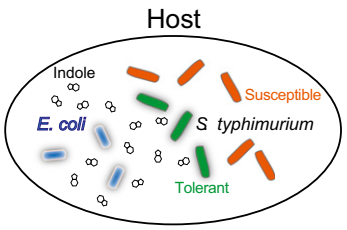

Fig. 6. Bacterial communication, signal interception, and antibiotic tolerance in the host environment. Within the *C. elegans* host intestine, pathogenic *S. typhimurium* encounters indole-producing *E. coli* (7) and is able to intercept indole signaling to enhance its antibiotic tolerance.

Materials and Methods

Bacterial Strains and Strain Construction. All experiments were performed using laboratory strains of *E. coli* and *Salmonella enterica* serovar Typhimurium. Ancestral wild-type *E. coli* K-12 EMG2 +fertility factor plasmid (F+) obtained from Yale *E. coli* Genetic Stock Center (ECGC 4401) was the reference wild-type *E. coli* strain used in all experiments, and *S. typhimurium* LT2 [American Type Culture Collection (ATCC) 700720] was the reference strain of nontyphoidal *Salmonella*. *C. elegans* strains were provided by the *C. elegans* Genetic Stock Center, which is funded by National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). Bacterial strains and primers used in this study are presented in Table S1. Details of strain construction are presented in *SI Materials and Methods*.

Antibiotics and Chemicals. The following concentrations of antibiotics were used in this study: 100 μg/mL carbenicillin, 10–60 μg/mL kanamycin, 0.5–2 μg/mL ciprofloxacin, and 1–5 μg/mL ofloxacin. Strains containing kanamycin-resistance plasmids were grown with 30–60 μg/mL kanamycin for selection, and strains containing spectinomycin-resistance cassettes were grown with 50 μg/mL spectinomycin. For induction of plasmid-borne genes, 25–50 ng/mL anhydrotetracycline (aTc) was added after 2–4 h of growth. Otherwise, antibiotic treatments were ≥10× minimum inhibitory concentration (MIC) to ensure killing of sensitive cells.

Growth and Tolerance Assays. All experiments were performed in accordance with standard protocols unless otherwise stated. Briefly, bacterial cultures were grown in light-insulated shakers at 37 °C with shaking at 300 rpm (14 mL-Falcon tubes, Fisher Scientific) or 900 rpm (96-well, clear, flat-bottom culture plates, Fisher Scientific; with Breathe-Easy adhesive gas-permeable membrane, USA Scientific). Cultures were grown in tryptophan-free rich media (M9 + 0.2% casamino acids + 0.4% glucose, M9CG, pH >7.2) or in complete rich media (Luria-Bertani, LB).

Antibiotic tolerance was assessed by incubating cultures for at least 4 h with antibiotic to allow full killing of sensitive cells. Serial dilution and plating were used to determine cfu/mL before and after treatment (*SI Materials and Methods*).

For coculture experiments, *E. coli* K-12 EMG2 was grown to stationary phase in M9CG + 0–2 mM tryptophan to allow indole production. The Δ*tnaA* knockout was used to prevent indole production. Cultures of *E. coli* Δ*tnaA* pZA21-*tnaA* or pZA21-GFP were grown in M9CG + 2 mM tryptophan, with or without 50 ng/mL anhydrotetracycline (aTc) for induction of plasmid-borne genes. Twenty percent of culture volume was replaced with exponential-phase culture of *S. typhimurium* in M9CG, and cultures were incubated 1 h before treatment with ciprofloxacin.

Indole Quantification. Indole quantification was performed via HPLC. Details of sample preparation and chromatography are presented in *SI Materials and Methods*.

Quantitative PCR. RNA was collected from indole-treated and untreated cultures of *S. typhimurium* LT2. Cultures were inoculated 1:500 from overnight LB cultures into 1 mL of M9CG in 14-mL Falcon tubes and incubated

3.5 h before treatment with indole. After 30-min incubation with indole (0, 50, or 125 μM), cultures were stabilized with RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer's protocol. Details of RNA extraction, cDNA synthesis, and qPCR are presented in *SI Materials and Methods*.

Induction of OxyR. Cultures were inoculated 1:200 (*E. coli*) or 1:500 (*S. typhimurium*) from overnight LB cultures into M9CG (1 mL in 14-mL Falcon tubes or 150 μL in 96-well plates) and allowed to grow to exponential (3.5 h) or stationary phase (24 h) at 37 °C. Cultures were incubated 1 h with hydrogen peroxide (0–600 μM) before treatment with antibiotics. Serial dilution plating was performed before and after 1 h of hydrogen peroxide incubation and after 4 h of ofloxacin treatment to determine survival at each stage.

***C. elegans* Intestinal Model for *S. typhimurium* Infection.** The temperature-sensitive germ line proliferation (*glp*) mitogen-activated protein kinase kinase (*sek*) mutant strain *AU37* [*glp*-4 (*bn2*) I; *sek*-1 (*km4*) X] of *C. elegans* (Caenorhabditis Genetics Center) was used as a model organism for *Salmonella* pathogenesis. *E. coli* OP50 was used as a food source in maintenance cultures. *E. coli* EMG2 and Δ*tnaA* Pro were used as food sources during experiments, and *S. typhimurium* nitrate reductase 2 mutant (Δ*narV*::CmR) was used as a pathogen. For fluorescent microscopy, worms were fed on *E. coli* EMG2 pZS4-mCherry or *E. coli* Δ*tnaA* pZS4-mCherry, and *S. typhimurium* pZA21-GFP was used as the infectious agent. Details of *C. elegans* culture and experimental conditions are given in *SI Materials and Methods*.

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