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Signaling-Mediated Bacterial Persister Formation

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Abstract

Here we show that bacterial communication through indole signaling induces persistence, a phenomenon in which a subset of an isogenic bacterial population tolerates antibiotic treatment. We monitor indole-induced persister formation using microfluidics, and identify the role of oxidative stress and phage-shock pathways in this phenomenon. We propose a model in which indole signaling "inoculates" a bacterial sub-population against antibiotics by activating stress responses, leading to persister formation.

Bacterial persisters are dormant cells[1] within an isogenic bacterial population that tolerate antibiotic treatment[2] and have been implicated in chronic and recurrent infections[3–5]. Persister formation occurs heterogeneously within an antibiotic-susceptible population, predominantly at the transition to stationary phase[6,7]. Though numerous genes have been associated with persistence[8–10], a complete understanding of persister formation remains elusive.

There is increasing evidence that bacterial communication via chemical signaling plays a role in establishing population heterogeneity[11]. The bacterial stationary phase signaling molecule indole[12,13] is produced[14,15] (Supplementary Fig. 1) under conditions known to increase persistence. Indole is actively transported by Mtr[16] but may enter the cell by other means[17] (see Supplementary Results). Indole signaling affects membrane stress and oxidative stress responses[18,19] and has been shown to increase antibiotic resistance (MIC) via multi-drug transport[14,19,20]. Given the above, we hypothesized that indole signaling may trigger the formation of bacterial persisters.

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Author Contributions

All authors designed experiments, discussed results, and contributed to the manuscript. N.M.V. performed all experiments. N.M.V., K.R.A., and A.S.K. analyzed data. A.S.K. developed the microfluidics platform and performed the microfluidic experiments.

Competing Financial Interests

The authors declare no competing financial interests.

To test this hypothesis, we incubated exponential phase cultures of wild-type *E. coli* in M9CG medium with physiological levels of indole (500 μM) for one hour, then treated with high concentrations of bactericidal antibiotics (see Supplementary Methods). As expected, wild-type *E. coli* showed different levels of persistence to different antibiotics[7]. Further, we found that incubation with indole increased persister levels to each of the three antibiotics tested by at least an order of magnitude (Supplementary Fig. 2), indicating that the protective effects of indole are not specific to a single antibiotic mode of action and suggesting that indole induces the transition to a persistent state (Supplementary Results, Supplementary Fig. 3 and 4).

To further explore the role of indole in persister formation, we tested the indole-induced persistence of a genetic knockout strain (Δ*tnaA*) unable to catabolize tryptophan to indole. Stationary phase conditions were used to maximize persister levels and indole concentration in the growth medium. As expected, we found no significant difference between survival of the wild-type and Δ*tnaA* strains in tryptophan-free M9CG medium (Fig. 1a, Supplementary Table 1), since wild-type *E. coli* produce very little indole when grown in this medium (Supplementary Fig. 5). By contrast, in rich Luria-Bertani (LB) medium, high levels of extracellular indole were present in wild-type but not Δ*tnaA* cultures (Supplementary Fig. 6), and the Δ*tnaA* mutation decreased persister formation by nearly an order of magnitude (Fig. 1a, Supplementary Table 1). In the Δ*tnaA* strain, incubation with indole increased persister formation by an order of magnitude in both M9CG and LB (Fig. 1a; Supplementary Table 1), and complementation with a plasmid bearing the wild-type *tnaA* gene reversed the low-persistence phenotype observed in rich medium (Supplementary Fig. 7). These results indicated that the effect of the Δ*tnaA* mutation on persister levels was a result of the lack of indole in Δ*tnaA* cultures. Consistent with earlier work[14], we found that the Δ*tnaA* mutant showed a greater deficit in persister formation relative to wild-type at low temperature (Supplementary Fig. 8). The Δ*tnaA* mutation did not completely eliminate persister formation, suggesting that mechanisms in addition to indole signaling are also involved in persister formation. Interestingly, the increased persistence in LB relative to M9CG was abolished in the Δ*tnaA* strain, suggesting that indole signaling in LB may account for the observed difference (Fig. 1a, Supplementary Results).

Having demonstrated that indole signaling induces persister formation, we next sought to determine whether indole uptake plays a role in this process. We assayed persister levels in stationary phase cultures of a mutant strain (Δ*mtr*) with impaired indole import[16]. We verified the role of Mtr in indole import using HPLC (Supplementary Fig. 5) and auxotrophy experiments (Supplementary Results, Supplementary Fig. 9). In M9CG and in LB, we found that the Δ*mtr* strain demonstrated approximately an order of magnitude greater survival than wild-type, even without the addition of indole, and addition of indole did not further induce persistence (Fig. 1a). Overnight incubation of wild-type cultures with 15 μM indole, to mimic indole concentrations in Δ*mtr* cultures, increased wild-type persistence to the levels observed in Δ*mtr* (Supplementary Results, Supplementary Fig. 10). Heterologous expression of *mtr* in the knockout strain restored wild-type persister levels (Supplementary Fig. 7), and eliminating indole production in the Δ*mtr* mutant abolished the high-persistence phenotype

in this strain (Supplementary Fig. 11). These results suggest that indole-induced persistence is, in part, a response to indole levels in the periplasm or extracellular space.

We next sought to determine if the cells with the strongest indole response were persistent to antibiotic treatment. Using fluorescently activated cell sorting (FACS), we confirmed indole response in the fluorescent reporter plasmid P*tnaC* (Supplementary Methods, Supplementary Fig. 13a–c). The Δ*mtr* strain had higher induction than wild-type, suggesting that increasing extracellular indole increases indole response. We sorted wild-type *E. coli* P*tnaC* to obtain sub-populations exhibiting "low" (bottom 10%) and "high" (top 10%) fluorescence and found that the sub-population with "high" fluorescing cells was more persistent to ofloxacin than the "low" fluorescing sub-population (Supplementary Fig. 13d). Similar results were obtained with Δ*tnaA* P*tnaC* + 500 μM indole (Supplementary Fig. 13e).

We sought to directly observe the generation of indole-induced persisters using a microfluidic chemostat (Supplementary Fig. 14, Supplementary Movies 1–3). Low levels of fluorescence were observed during growth of wild-type cells in indole-free media (Fig. 1b, I). During one hour of incubation with 500 μM indole, a heterogeneous increase in fluorescence was evident (Fig. 1b, II). Treatment with high concentrations of ampicillin caused massive lysis (Fig. 1b, III–V). Lysis reached a plateau after one hour of ampicillin treatment (Supplementary Fig. 15), leaving a small number of viable cells (Fig. 1b, V). (Consistent with previous results[1], persister frequency differed between microfluidic and batch cultures.) We found that cells that survived antibiotic treatment had higher indole-responsive fluorescence than cells that did not survive (Fig. 1c), suggesting that cells that sensed indole to a greater degree were more likely to become persisters. These results demonstrate that indole response within a population is heterogeneous and, further, that indole signaling plays an important role in the formation of individual persister cells.

We next sought to investigate the biological effects of indole signaling by examining the genome-wide transcriptional response to indole. RNA from wild-type cultures (exponential and stationary phase) incubated with and without indole was harvested for microarrays as described in Supplementary Methods. Microarray analysis indicated that incubation with indole significantly ($p \leq 0.05$) increased expression of genes in oxidative stress (OxyR) and phage shock (Psp) pathways in stationary phase (Supplementary Fig. 16) and exponential phase cultures (Supplementary Fig. 17). We did not observe statistically significant increases in expression of drug exporter systems (Supplementary Results, Supplementary Table 2), consistent with the hypothesis that the increase in survival after incubation with indole is due to an increase in persister formation rather than antibiotic resistance. qPCR was used to validate microarray results for selected targets (Supplementary Fig. 18). A detailed analysis of microarray data and a comparison to previous indole studies are presented in the Supplementary Results.

Given that both the oxidative stress and phage shock pathways play a protective role during bacterial stasis[21,22], we next used genetic knockouts to determine whether these pathways are involved in indole-induced persistence. The Δ*flu*Δ*oxyR* and Δ*pspBC* mutants were constructed to allow inactivation of the OxyR and phage shock responses, respectively (see Supplementary Methods). We found that indole-induced persistence was substantially

reduced in both the Δ*flu*Δ*oxyR* and Δ*pspBC* mutant strains relative to the parent strains (Fig. 2a, Supplementary Fig. 19). Further, we found that simultaneous inactivation of both pathways (Δ*flu pspBC*Δ*oxyR*) completely abolished indole-induced persistence (Fig. 2a, Supplementary Fig. 19). These results suggest that both the OxyR and phage shock responses are involved in indole-induced persistence.

As non-toxic levels of indole induce persister formation, we sought to determine whether a known antimicrobial agent and OxyR inducer (hydrogen peroxide, H_2O_2)[23] could also induce persistence. Treatment with moderate levels of this agent has been shown to increase tolerance as part of the bacterial adaptive response[24]. We found that pre-incubation of stationary phase cultures with 300–600 μM H_2O_2 increased persister levels by an order of magnitude (Fig. 2b). Using qPCR, we verified that treatment with H_2O_2 (300 μM) induced the OxyR regulon, and we found that it also induced the phage shock response (Supplementary Fig. 18). Interestingly, bactericidal concentrations of H_2O_2 (3 mM) did not have a protective effect (Fig. 2b). These results indicate that activation of the OxyR and phage shock responses in the absence of cytotoxic stress may be sufficient to induce persister formation, suggesting that activation of these responses by non-lethal stimuli “inoculates” a population against future stress.

On the basis of our findings, we propose the following mechanism for indole-induced persister formation (Fig. 2c). The bacterial signaling molecule indole is sensed in a heterogeneous manner by a population of cells, causing induction of oxidative stress (OxyR) and phage shock (Psp) pathways via a periplasmic or membrane component, thereby inducing the creation of a persistent sub-population. Indole is not toxic at physiological levels, but triggers protective responses, acting to “inoculate” a sub-population (persisters) against possible future stress.

Here we have shown that bacterial communication through indole signaling induces persister formation in *E. coli*. This process involves the activation of oxidative stress and phage shock pathways, and allows bacteria to protect a sub-population against antibiotic treatment. These findings add to an understanding of persister formation as a bacterial “bet-hedging” strategy in uncertain environments[25]. Indole, produced under nutrient-limited conditions, allows *E. coli* to alter the frequency of persister formation, thereby providing a mechanism by which a bacterial population can adjust its bet-hedging strategy based on environmental cues. Our findings demonstrate that persister formation is influenced by communication within a population of cells, and it is not simply the result of an isolated, random switching event in individual cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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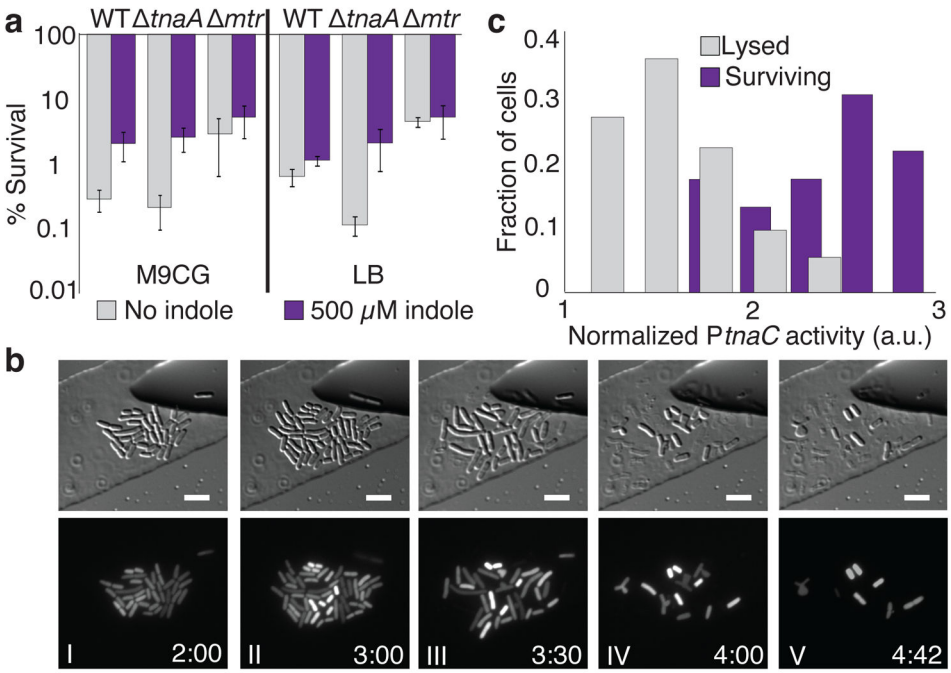

Figure 1. Indole induces persistence in *E. coli*

All experiments were performed at 37°C. **(a)** Percent survival of stationary-phase wild-type, Δ*tnaA*, and Δ*mtr E. coli* in M9CG and in rich media (LB), pre-incubated with and without indole and treated with ofloxacin. Error bars represent mean ± s.d. of at least three biological replicates. (**b**) Direct observation of indole-induced persistence. Representative time-lapse of optical (DIC) and fluorescence (GFP) images of wild-type *E. coli* P*tnaC* grown in the microfluidic chamber in selective media (M9CG + kanamycin) for two hours (I), then treated with 500 μM indole (1 hour, II) before lysis with ampicillin (30 minutes, III; 1 hour, IV; 1 hour ampicillin lysis + 42 minutes in selective media, V). Time (in hours:minutes) depicted within each image corresponds to total time elapsed since the beginning of the experiment. Raw fluorescent images were identically exposed and contrast-scaled. Scale bar is 5 μM. (**c**) Histograms of normalized P*tnaC* activity (see Supplementary Methods), for lysed versus surviving cells in microfluidics experiments. Data were obtained from three biological replicates.

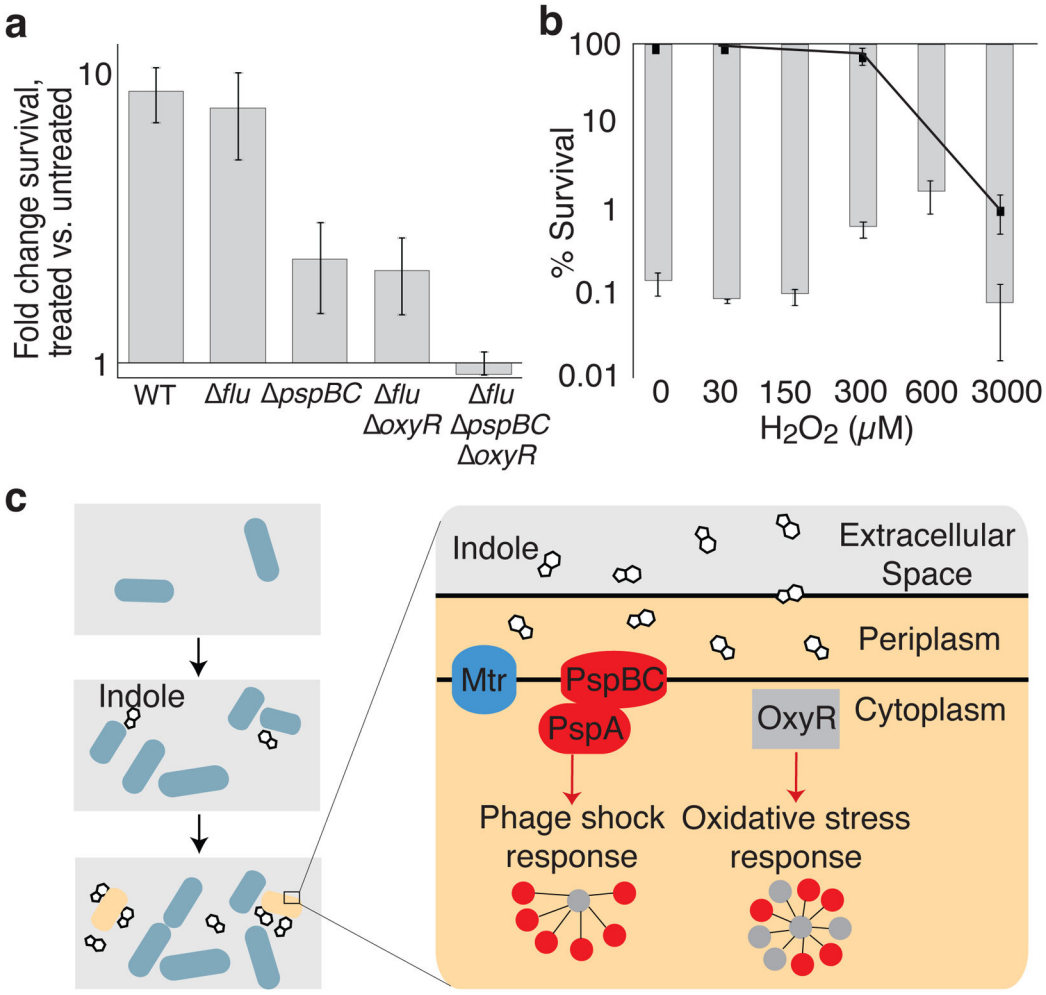

Figure 2. Indole induces persistence through the phage shock and OxyR pathways

All experiments were performed in M9CG. Error bars represent mean ± s.d. of at least three biological replicates. (**a**) Fold change survival in indole-treated versus untreated stationary phase cultures of wild-type *E. coli*, Δ*flu*Δ*oxyR*, and Δ*pspBC* after treatment with ofloxacin. (**b**) Pre-treatment of stationary phase cultures of wild-type *E. coli* with hydrogen peroxide leads to increased survival after subsequent ofloxacin treatment. Black line indicates percent survival of cultures after incubation for 1 hour with hydrogen peroxide. Grey bars indicate percent survival of the same cultures after subsequent ofloxacin treatment, relative to survival after incubation with H_2O_2. **(c)** Proposed mechanism for indole-induced persister formation. Up-regulated gene products and pathways are shown in red and down-regulated gene products and pathways are shown in blue (see Supplementary Fig. 16 and 17).