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Signaling-mediated bacterial persister formation

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

To test this hypothesis, we incubated exponential-phase cultures of wild-type *Escherichia coli* in M9 minimal medium supplemented with casamino acids and glucose (M9CG) with physiological concentrations of indole (500 μM) for 1 h and then treated them with high concentrations of bactericidal antibiotics (**Supplementary Methods**). As we expected, wild-type *E. coli* showed different degrees of persistence to different antibiotics[7]. Further, we found that incubation with indole increased persistence 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 Note 1** and **Supplementary Figs. 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. We found no difference between survival of the wild-type and Δ*tnaA* strains in tryptophan-free M9CG (**Fig. 1a** and **Supplementary Table 1**), which we expected because wild-type *E. coli* produce very little indole when grown in this medium (**Supplementary Fig. 5**). In contrast, in rich LB medium, high concentrations 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** and **Supplementary Table 1**). In the Δ*tnaA* strain, incubation with indole increased persister formation by an order of magnitude in both M9CG and LB medium (**Fig. 1a** and **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], our results showed that the Δ*tnaA* mutant had a greater deficit in persister formation relative to the 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. Remarkably, the increased persistence of cultures grown in LB medium relative to those grown in M9CG was abolished in the Δ*tnaA* strain, suggesting that indole signaling in LB medium may account for the observed difference (**Fig. 1a** and **Supplementary Note 1**).

Having demonstrated that indole signaling induces persister formation, we next sought to determine whether indole uptake has 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 Note 1** and **Supplementary Fig. 9**). In M9CG and LB media, we found that the Δ*mtr* strain showed approximately an order of magnitude greater survival than the wild type, even without the addition of indole, and that 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 the degree of wild-type persistence to that observed with the Δ*mtr* strain (**Supplementary Note 1** and **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 concentrations in the periplasm or extracellular space.

We next sought to determine whether the cells with the strongest indole response were persistent to antibiotic treatment. Using fluorescence-activated cell sorting, we confirmed indole response in the fluorescent reporter plasmid P*tnaC* (**Supplementary Methods** and **Supplementary Fig. 13a–c**). The Δ*mtr* strain had higher induction than the wild type, suggesting that increasing extracellular indole increases indole response. We sorted wild-type *E. coli* P*tnaC* to obtain subpopulations showing 'low' (bottom 10%) and 'high' (top 10%) fluorescence and found that the subpopulation with high-fluorescing cells was more persistent to ofloxacin than the low-fluorescing subpopulation

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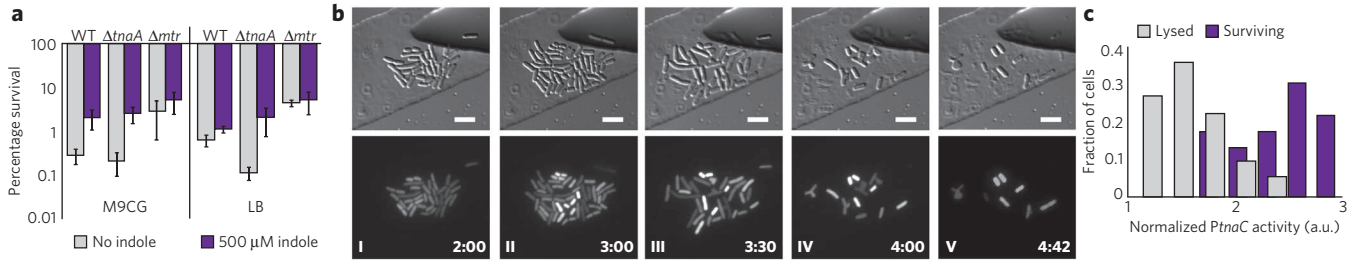

Figure 1 | Indole induces persistence in *E. coli*. (**a**) Percentage survival of stationary-phase wild-type (WT), Δ*tnaA* and Δ*mtr E. coli* in M9CG and in rich medium (LB), preincubated with or without indole and treated with ofloxacin. Error bars represent mean ± s.d. of at least three biological replicates. (**b**) Direct observation of indole-induced persistence. Representative time-lapse of optical (differential interference contrast) and fluorescence (green fluorescent protein) images of wild-type *E. coli* P*tnaC* grown in the microfluidic chamber in selective medium (M9CG plus kanamycin) for 2 h (I), then treated with 500 μM indole (1 h, II) before lysis with ampicillin (30 min, III; 1 h, IV; 1 h ampicillin lysis plus 42 min in selective media, V). Time (in h:min) 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 bars, 5 μm. (**c**) Histograms of normalized P*tnaC* activity (**Supplementary Methods**), for lysed versus surviving cells in microfluidics experiments. Data were obtained from three biological replicates. a.u., arbitrary units. All experiments were performed at 37 °C.

(**Supplementary Fig. 13d**). Similar results were obtained with Δ*tnaA* P*tnaC* plus 500 μM indole (**Supplementary Fig. 13e**).

We sought to directly observe the generation of indole-induced persisters using a microfluidic chemostat (**Supplementary Fig. 14** and **Supplementary Movies 1–3**). Low fluorescence was observed during growth of wild-type cells in indole-free medium (**Fig. 1b**, I). During 1 h 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–IV). Lysis reached a plateau after 1 h of ampicillin treatment (**Supplementary Fig. 15**), leaving a small number of viable cells (**Fig. 1b**, V). Consistent with previous results[1], observed 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 has a substantial 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 or 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 OxyR and phage-shock pathways in stationary-phase (**Supplementary Fig. 16**) and exponential-phase (**Supplementary Fig. 17**) cultures. We did not observe statistically significant ($P \approx 0.28$) increases in expression of drug exporter systems (**Supplementary Note 1** and **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. Quantitative PCR 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 **Supplementary Note 1**.

Given that both the OxyR and phage-shock pathways have a protective role during bacterial stasis[21,22], we next used genetic knockouts to determine whether these pathways are involved in

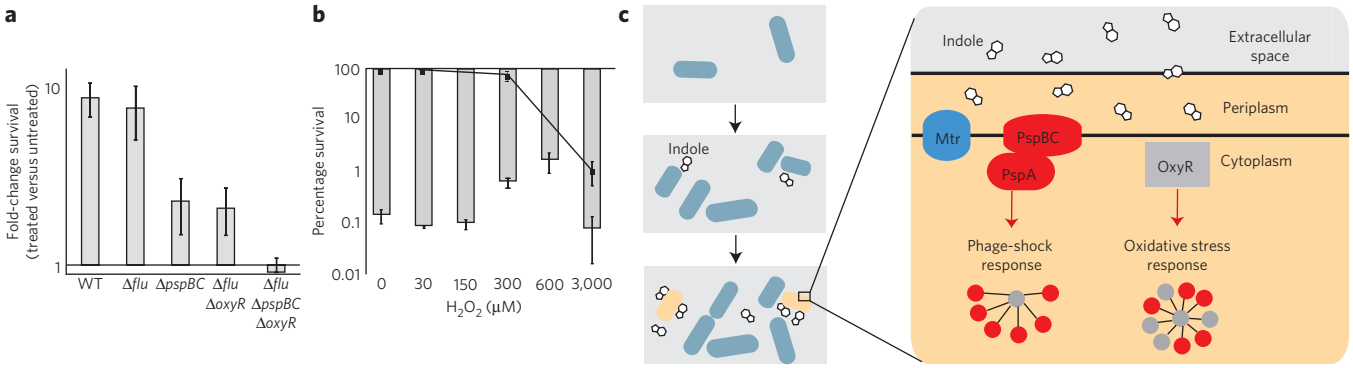

Figure 2 | Indole induces persistence through the phage-shock and OxyR pathways. All experiments were performed in M9CG. Error bars represent mean ± s.d. of at least three biological replicates. (**a**) Fold-change survival in indole-treated versus untreated stationary-phase cultures of wild-type (WT) *E. coli* and Δ*flu* Δ*oxyR* and Δ*pspBC* mutant strains after treatment with ofloxacin. (**b**) Pretreatment of stationary-phase cultures of wild-type *E. coli* with H_2O_2 leads to increased survival after subsequent ofloxacin treatment. Black line indicates percentage survival of cultures after incubation for 1 h with H_2O_2. Gray bars indicate percentage 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. The bacterial signaling molecule indole is produced under nutrient-limited conditions and sensed in a heterogeneous manner by a population of cells, causing induction of OxyR and Psp pathways. Activation of these pathways, via a periplasmic or membrane component, induces high tolerance to antibiotics, thereby creating a persistent subpopulation (orange cells). Upregulated gene products and pathways are shown in red, and downregulated gene products and pathways are shown in blue (**Supplementary Figs. 16** and **17**).

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

As nontoxic concentrations of indole induce persister formation, we sought to determine whether a known antimicrobial agent and OxyR inducer (H_2O_2)[23] could also induce persistence. Treatment with moderate concentrations of this agent has been shown to increase tolerance as part of the bacterial adaptive response[24]. We found that preincubation of stationary-phase cultures with 300–600 μM H_2O_2 increased persister levels by an order of magnitude (**Fig. 2b**). Using quantitative PCR, 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**). Notably, 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 nonlethal 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 OxyR and phage-shock pathways via a periplasmic or membrane component, thereby inducing the creation of a persistent subpopulation. Indole is not toxic at physiological concentrations, but it triggers protective responses, acting to inoculate a subpopulation (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 OxyR and phage-shock pathways and allows bacteria to protect a subpopulation 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 on the basis of environmental cues. Our findings demonstrate that persister formation is influenced by communication within a population of cells and is not simply the result of an isolated, random switching event in individual cells.

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Author contributions

N.M.V., K.R.A., A.S.K. and J.J.C. 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.

Additional information

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