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LETTERS

Bacterial charity work leads to population-wide resistance

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Bacteria show remarkable adaptability in the face of antibiotic therapeutics. Resistance alleles in drug target-specific sites and general stress responses have been identified in individual end-point isolates[1–7]. Less is known, however, about the population dynamics during the development of antibiotic-resistant strains. Here we follow a continuous culture of *Escherichia coli* facing increasing levels of antibiotic and show that the vast majority of isolates are less resistant than the population as a whole. We find that the few highly resistant mutants improve the survival of the population's less resistant constituents, in part by producing indole, a signalling molecule generated by actively growing, unstressed cells[8]. We show, through transcriptional profiling, that indole serves to turn on drug efflux pumps and oxidative-stress protective mechanisms. The indole production comes at a fitness cost to the highly resistant isolates, and whole-genome sequencing reveals that this bacterial altruism is made possible by drug-resistance mutations unrelated to indole production. This work establishes a population-based resistance mechanism constituting a form of kin selection[9] whereby a small number of resistant mutants can, at some cost to themselves, provide protection to other, more vulnerable, cells, enhancing the survival capacity of the overall population in stressful environments.

Antibiotic-resistant bacterial strains continually arise and their increasing prevalence poses significant clinical and societal challenges[7,10]. Functional analyses of resistant mutants and the study of the endogenous processes responsible for resistance by mutation have yielded valuable insights[1–7,11,12]. However, population dynamics and communal interactions that underlie the development of resistance through mutations are often overlooked. To study these neglected aspects, we tracked a bacterial population as it developed antibiotic resistance in a bioreactor.

Starting with an isogenic strain of wild-type *E. coli*, we continuously challenged the population with progressively increasing concentrations of norfloxacin. To provide evolutionary pressure while maintaining a sizeable population, the concentration of antibiotic was chosen such that no more than 60% of growth was inhibited; we defined this concentration as the minimum inhibitory concentration (MIC). Every 24 h, the daily population MIC was determined and the norfloxacin concentration adjusted as tolerated. From each daily sample, 12 individual isolates were randomly chosen and each of their MICs was determined.

We found that the group's MIC was not usually predictive of its constituents' MICs (Fig. 1a). The vast majority of individual isolates were actually less resistant isolates (LRIs), that is, isolates whose MICs were lower than the concentrations of norfloxacin in which they were found and, therefore, also lower than the group MIC. Intriguingly, we also isolated a mutant with an MIC higher than the bioreactor concentration, a highly resistant isolate (HRI). We suspected that our rare detection of HRIs was due to their low abundance in the population throughout most of the experiment. Indeed, when we plated daily populations under norfloxacin selection, we frequently detected low-abundance HRIs that emerged before increases in the group MIC (Fig. 1b). We were, however, surprised by the large number of

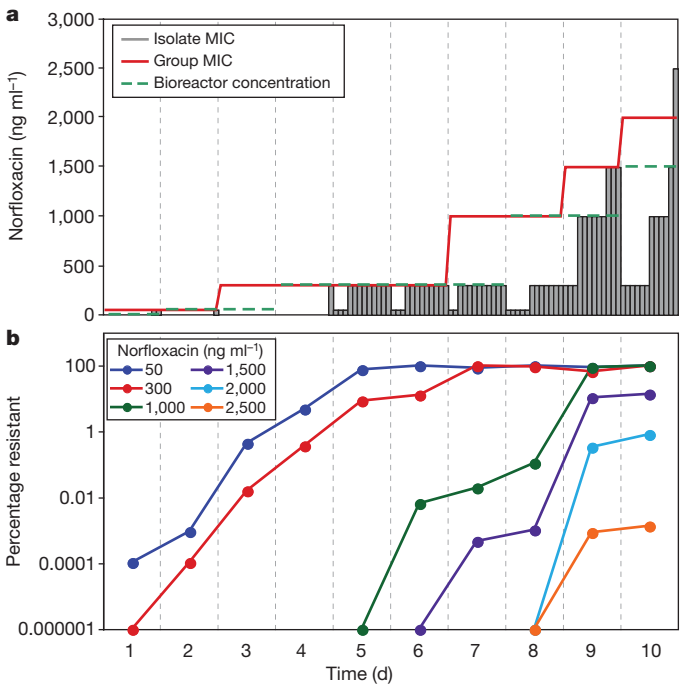

Figure 1 | Tracking a population of *E. coli* developing antibiotic resistance. **a**, A clonal wild-type *E. coli* MG1655 population was continuously cultured in a bioreactor for ten days with increasing concentrations of the quinolone norfloxacin. MIC is defined as the drug concentration inhibiting no more than 60% of unstressed cell growth. The initial bioreactor concentration was set as the MIC of wild-type cells. Every 24 h thereafter, the population MIC (red lines) was measured. Following increases in group MIC, the bioreactor concentration (dashed green lines) was adjusted accordingly at the next sampling interval. Twelve individual isolates were selected from plating daily populations on non-selective plates and their MICs (grey bars) were determined. MICs shown are representative of biological duplicates. **b**, Daily population analysis profiles, representing the fraction of the population resistant to each drug level, were taken throughout the ten days of continuous culture. Daily populations were serially diluted and spotted on plates with a range of norfloxacin concentrations. The percentage resistance (circles coloured according to norfloxacin concentration) was calculated as the number of colonies at specific norfloxacin concentrations relative to the total number of cells (plated on non-restrictive plates). Results shown are representative of biological duplicates.

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LRIs in the population. We speculated that the few HRIs were generating a benefit for the numerous LRIs, thus allowing weaker isolates to endure more antibiotic stress than they could in isolation. Media conditioning by HRIs seemed a plausible mode of protection for LRIs. To test this hypothesis and to examine the conditioning that may be taking place, we focused our studies on the most resistant HRI that we detected: c10,12 (colony number 12 of those isolated on day ten). Supernatant from c10,12 following growth in the presence of norfloxacin was analysed by protein gel electrophoresis. We detected a dominant protein band, along with several weaker protein bands, in the media. We next subjected the observed protein bands to mass spectrometry for identification (Supplementary Table 1). The dominant protein band was identified as TnaA (Fig. 2a). We verified the identification of TnaA by creating the corresponding genetic mutant, c10,12Δ*tnaA*, and analysing its supernatant for protein following growth under antibiotic stress. The dominant band was completely absent from the resulting gel. We also found this to be the case for c10,6, the least resistant isolate found on day 10. We next verified, by Sanger sequencing, that the promoter and coding regions of the *tna* operon of c10,12 had not undergone gain-of-function mutations. *tnaA* codes for the enzyme tryptophanase, whose major enzymatic reaction is the breakdown of tryptophan into ammonia, pyruvate and indole[8].

Importantly, indole is a signalling molecule implicated in stress tolerance in *E. coli*[13–15]. We proposed that indole produced by the HRIs was protecting its less resistant neighbours. To test this, we first quantified the extracellular indole produced by c10,12 (HRI), c10,6 (LRI) and wild type using high-pressure liquid chromatography. Under no antibiotic stress, these isolates were capable of producing up to approximately 300 μM of indole (Fig. 2b). Under antibiotic stress, however, only c10,12 was capable of maintaining its indole production (Fig. 2b). We detected no indole from our tryptophanase mutant, c10,12Δ*tnaA*.

We next sought to determine the protective effect of extracellular indole on an LRI under antibiotic stress. We determined the minimum bactericidal concentration (MBC) of norfloxacin for c10,6, the least resistant isolate found on day ten, with and without addition of indole to the media. Added indole provided a stark survival benefit under drug stress (Fig. 2c). The MBC for c10,6 without indole is 800 ng ml^{-1}, indicating that the day-nine bioreactor concentration (1,000 ng ml^{-1}) would have been sufficient to kill the isolate. Addition of indole boosts the MBC for c10,6 to 1,400 ng ml^{-1}, indicating that the effect of indole can account for much of the isolate's ability to survive in the day-ten bioreactor concentration (1,500 ng ml^{-1}). It is possible that other protein products identified in the supernatant also contribute to the protective effect afforded by HRIs; this warrants further study. Taken together, our results suggested a plausible mechanism for altruistic communal interaction: an HRI conditions the media, in part through the production of indole, to benefit weaker and more vulnerable isolates.

To examine this putative protective communal interaction, we compared growth under antibiotic stress of three isolates—an LRI (c10,6), an HRI (c10,12) and an indole-deficient mutant (c10,12Δ*tnaA*)—in isolation and in co-culture. On the basis of the results reported above, we reasoned that a population dominated by c10,6 would be protected by a minority of c10,12 and that this protective effect would be absent with an indole-deficient HRI, c10,12Δ*tnaA*. Of note, c10,12Δ*tnaA* outgrew c10,12 in isolation. Similarly, in a competitive fitness assay[16] where the proportion of the two isolates was initially balanced, c10,12Δ*tnaA* outcompeted c10,12, leading to a relative fraction of 2.6:1 (c10,12Δ*tnaA*:c10,12), demonstrating that indole production by HRIs under antibiotic stress carries a fitness cost. For the co-culture experiments, we chose a 1:100 (1 in 100) dilution of c10,12 (1%) to c10,6 (99%) to replicate the low abundance of HRIs. As shown in Fig. 2d, a mixture of c10,6 and c10,12 grew better than either isolate individually, indicating that the indole produced by c10,12 enhanced the survival capacity of c10,6 under antibiotic stress, boosting total growth beyond that of c10,12 alone. In contrast, the growth of a mixture of c10,6 and c10,12Δ*tnaA* achieved a level only slightly higher than c10,6 alone; this slight increase was due entirely to growth of c10,12Δ*tnaA* (Supplementary Information).

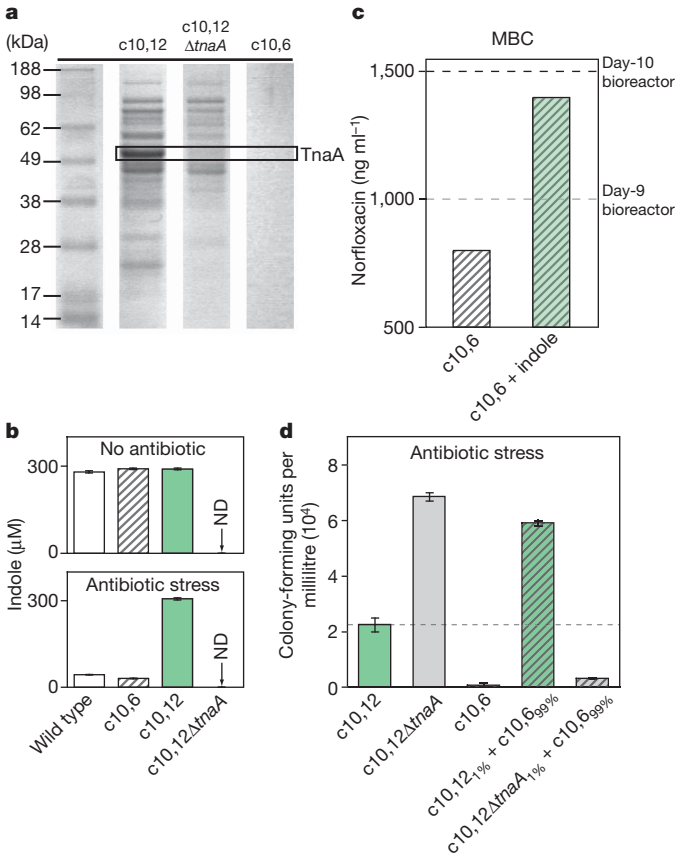

Figure 2 | Indole production by isolates and the protective effect of extracellular indole. **a**, Proteins were detected in the supernatant of c10,12 when grown clonally under the bioreactor concentration of norfloxacin (1,500 ng ml^{-1}). These protein bands were subjected to mass spectrometry for protein identification. The top hit for the dominant protein band matched over 75% of residues for *tnaA*, which encodes the enzyme tryptophanase. The major enzymatic activity of tryptophanase yields indole. This dominant band was absent from the supernatant of c10,12Δ*tnaA*. No proteins were found in the supernatant of c10,6. **b**, Quantification by high-pressure liquid chromatography of extracellular indole production by isolates with varying norfloxacin resistance: wild type (white bars); c10,6 (striped bars); c10,12 (green bars); and c10,12Δ*tnaA* (ND, not detected). With the exception of c10,12Δ*tnaA*, all isolates produce approximately 300 μM of indole in the absence of antibiotic stress. Under norfloxacin stress (1,500 ng ml^{-1}), c10,12 continued to produce up to 300 μM of indole whereas wild type and c10,6 produced $<$50 μM of indole. No indole was detected for c10,12Δ*tnaA*. Data shown, mean ± s.e.m. ($n \geq 3$). **c**, MBC, the minimum concentration of norfloxacin that kills 99.9% of the cells in a culture, is shown for c10,6 with and without the addition of 300 μM of indole. The bioreactor concentrations for day nine (1,000 ng ml^{-1}) and for day ten (1,500 ng ml^{-1}) are also shown. **d**, Total growth of mutants under norfloxacin stress (1,500 ng ml^{-1}) in isolation or in co-culture: c10,6 (striped bars); c10,12 (green bars); and c10,12Δ*tnaA* (grey bars). Each condition starts with the same total number of cells, and co-cultures are mixed in an HRI-to-LRI ratio of 1:100 (1 in 100). Results shown are representative of biological replicates and are expressed as mean ± s.e.m.

We next used genome-wide transcription profiling to explore the physiological role of indole. We measured total transcripts from an indole-deficient mutant with wild-type resistance, MG1655Δ*tnaA*, following exposure to the initial bioreactor concentration of norfloxacin (50 ng ml^{-1}) supplemented with an intermediate concentration of indole (200 μM). We generated an indole transcriptional signature by filtering for differentially expressed genes. Our analysis (Supplementary

Information and Supplementary Table 2) showed that indole upregulated multi-drug efflux pumps such as *mdtE* ($P = 0.026$) and increased production of succinate through upregulation of *astD*[14,15] ($P = 0.025$). Our analysis also revealed downregulation of *oxyS* ($P = 0.004$), which is a small-RNA sensor for intracellular oxidative stress[17]. We also observed a decrease in iron–sulphur-cluster repair and production through downregulation in *iscU* ($P = 0.022$), which has previously been shown to be central to a common mechanism of bactericidal antibiotic-mediated cell death[18–20]. Nitric oxide response systems were also activated through the downregulation of *nsrR* ($P = 0.041$) and the corresponding upregulation in *hmp* ($P = 0.008$), which is a central nitric oxide detoxification gene[21]. These findings indicate that indole induces two modes of antibiotic detoxification: physical export and activation of oxidative-stress protective mechanisms.

Finally, we used whole-genome sequencing to explore the mutations in the HRIs and, more broadly, the mutations present in evolving populations. We selected three HRIs from days eight to ten along with one LRI from day ten; we also sequenced our initial wild type for reference. We identified five single nucleotide polymorphisms (SNPs) not present in our initial wild type (Fig. 3a). As expected, each resistant isolate carried a mutation in a subunit of a known norfloxacin target, the DNA gyrase encoded by *gyrB*[22]. These mutants also carried a SNP in *yciW*, which is a widely conserved putative oxidoreductase. These two mutations were common to all evolved mutants, suggesting that the evolved isolates descended from the same ancestor. We also identified mutations in *marR*, a master regulator of drug efflux pumps, and its paralogue, *soxR*, whose regulon protects the cell from superoxide damage[18,23]. Lastly, we identified a mutation in the promoter for *mdtK*, an efflux pump with high norfloxacin specificity[24]. It is important to note that these mutations are unrelated to indole production, and instead probably serve to confer the level of drug resistance needed to maintain indole production under stress.

To track the emergence and fixation of each mutation in our evolution experiment, we genotyped, using mass spectrometry, our daily populations to estimate the allelic frequency of each SNP. To maximize detection of SNPs in rare abundance, we revived our daily populations in parallel: by non-restrictive growth and by norfloxacin selection with the respective daily bioreactor concentrations. This drug selection precedes the production of indole by the HRIs and therefore enriches for the low-abundance, highly resistant population. As shown in Fig. 3b, mutations in *yciW* and *gyrB* appeared simultaneously in the highly resistant population by the second day. The mutants carrying these two SNPs probably represented the earliest HRI that, through indole production, catalysed the increase in group MIC. The truncated *marR* allele, found in the sequenced day-eight HRI, was so rare as to be undetectable by allelotyping, hinting at a diverse population of low-abundance HRIs. On day nine, mutations appeared in *soxR* and *mdtK*. Although the *soxR* mutation was more abundant than the *mdtK* mutation in the total population, their abundances were roughly equivalent under drug selection, indicating that mutants harbouring each were part of the HRI population. By day ten, however, despite the unchanged total abundance of each SNP, *mdtK* mutants began to dominate the highly resistant subpopulation and *soxR* mutants were relegated to the less resistant subpopulation. This ebb and flow of resistant mutants suggests an environment supportive of a phenotypically diverse population.

Our results establish a population-based antibiotic-resistance mechanism (Fig. 4) based on indole as a cell-signalling molecule. A population of *E. coli* in the absence of stress thrives and naturally exudes the metabolic product indole (Fig. 4a). Under severe antibiotic stress, however, dead and dying cells no longer produce indole in significant quantities (Fig. 4b). An antibiotic-resistant mutant, once it emerges, endures a fitness cost to produce indole, which protects the more vulnerable cells by inducing various antibiotic-tolerance mechanisms (Fig. 4c).

Recognizing that indole would be protective under a variety of antibiotics, we speculated that this population-based resistance mechanism would also arise in response to other antibiotic treatments. To test this hypothesis, we repeated our bioreactor experiment, this time following a continuous culture of *E. coli* facing increasing concentrations of the aminoglycoside gentamicin. As was the case in our

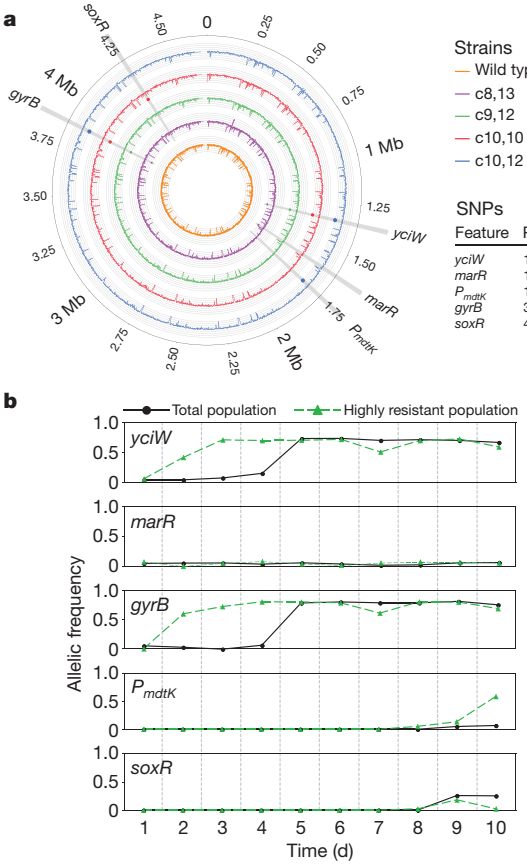

Figure 3 | Whole-genome sequencing of various mutants. **a**, Five total genomes were sequenced using the Solexa GA2: wild type, three HRIs from days eight to ten, and an LRI from day ten. Sequencing coverage for each isolate is plotted, according to colour, on concentric tracks with the wild-type genome (orange) in the centre. Intervals within each track represent ×25 coverage per 1,000 bases (Mb) of the genome. Each SNP, represented by circles coloured according to isolate, is marked at the appropriate genomic position on the genome(s) in which it was found. P_{mdtK} denotes the promoter of the gene *mdtK*. **b**, Allelic frequency of each SNP over the course of the ten-day evolution experiment was estimated, using Sequenom's iPLEX platform, in the total population (black circles) and in an enriched, highly resistant population by norfloxacin selection (green triangles).

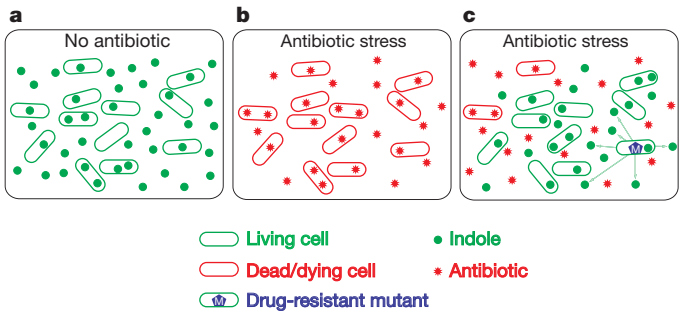

Figure 4 | A population-based antibiotic-resistance mechanism. A bacterial population is shown. **a**, In the absence of antibiotic stress, wild-type cells naturally produce indole. **b**, Under antibiotic stress, wild-type cells stop producing indole and eventually die. **c**, When a drug-resistant mutant emerges, it is able to produce indole even under antibiotic stress. This indole allows the more vulnerable cells in the population to survive the antibiotic stress, by inducing various antibiotic-tolerance mechanisms, thereby boosting the survival capacity of the population.

norfloxacin experiments, we found that individual isolates tended to be less resistant than the population as a whole and that a few highly resistant mutants appeared and preceded a change in the group MIC (Supplementary Fig. 1a, b). On further examination of isolates from day seven, we verified that an HRI was capable of producing high concentrations of indole (>300 μM) under antibiotic stress, but that an LRI was not (Supplementary Fig. 1c). Co-culturing of these two isolates resulted in more robust growth than either of the isolates individually, indicating increased survival for the more vulnerable isolate (Supplementary Fig. 1d). These findings demonstrate that the population-based resistance mechanism illustrated in Fig. 4 is not drug specific.

This work shows that, under antibiotic stress, a few spontaneous drug-resistant mutants will endure a fitness cost to produce and share among the population the metabolite indole, thus shielding the less resistant isolates from antibiotic insult. This altruism allows weaker constituents to survive and concurrently explore the space of beneficial mutations, a phenomenon similar in character to kin selection[9]. These few drug-resistant mutants, by enhancing the survival capacity of the overall population in stressful environments, may also help to preserve the potential for the population to return to its genetic origins should the stress prove transient. Efforts to monitor and combat antibiotic resistance are complicated by these bet-hedging survival strategies and other forms of bacterial cooperation. Deeper exploration into the repertoire of strategic intracellular communication used by bacteria may prove critical for the rational design of effective clinical interventions to face a growing threat of resistant bacterial infections.

METHODS SUMMARY

We performed all experiments with *E. coli* MG1655 (ATCC 700926)-derived strains. *tnaA* deletion mutants were created by P1 transduction and derived from an *E. coli* single-gene knockout library[25]. In the bioreactor, *E. coli* were continuously cultured with a dilution rate of 0.48 h^{-1}. We determined MICs and MBCs by standard methods. Proteins were separated by denaturing polyacrylamide gel electrophoresis (SDS–PAGE) and identified by mass spectrometry[26]. We quantified extracellular indole by means of high-pressure liquid chromatography[27]. The competition assay was performed in continuous culture and relative fractions were derived from Malthusian parameters[16]. Transcriptional profiling was performed as previously described[18] and normalized with FARMS[28]. Whole-genome sequencing was performed using custom adapters on a Solexa GA2[29]. We estimated allelic frequency by matrix-assisted laser desorption/ionization–time of flight mass spectrometry using Sequenom's iPLEX chemistry[30].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions All authors designed the study. H.H.L. and M.N.M. performed and analysed the experiments with input from C.R.C. and J.J.C. All authors prepared and commented on the manuscript.

Author Information The microarray data have been deposited in the NCBI Gene Expression Omnibus under GEO Series accession number GSE22833. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.J.C. (jcollins@bu.edu).

METHODS

Bacterial strains, media, and chemicals. All experiments were performed with *E. coli* MG1655 (ATCC 700926)-derived strains. *tnaA* deletion mutants were created by P1 transduction and derived from an *E. coli* single-gene knockout library[25]. Bacterial cultures were grown at 37 °C in lysogeny broth obtained from Fisher Scientific. Norfloxacin and gentamicin were purchased from Sigma. Kanamycin was purchased from Fisher Scientific. Indole was purchased from Acros Organics.

Bioreactor experiments. An overnight *E. coli* culture, grown from a single colony, was diluted 1:10,000 in the bioreactor and grown, with a dilution rate of 0.48 h^{-1}, to OD_{600} 0.6. The medium was then supplemented with norfloxacin and protected from heat and light. Every 24 h, MICs were determined for the group and individual isolates, respectively. Daily populations, or overnight cultures of individual isolates, were diluted 1:10,000 in 100 μl of lysogeny broth with a range of antibiotic concentrations in 96-well plates. Following overnight growth, OD_{600} was measured using a SPECTRAFluor Plus (Tecan) spectrophotometer and the MIC was determined as the concentration that inhibited no more than 60% of growth.

Protein identification and indole quantification. Supernatant from mutants grown overnight in lysogeny broth with norfloxacin was purified with a 0.2-μm nylon filter (Costar). The protein concentration of each sample was adjusted following quantification of total protein with a BCA protein assay (Pierce) and concentrated by acetone precipitation when needed. Proteins in each sample were separated by denaturing SDS–PAGE with MES buffer on 10% Bis-Tris gels (Invitrogen) and stained with GelCode Blue Stain Reagent (Pierce). Whole gels were submitted to the Boston University Proteomics Core Facility for protein identification by mass spectrometry[26]. Extracellular indole was quantified by high-pressure liquid chromatography as previously described[27]. Filtered supernatant (50 μl) was injected into a Waters Xterra MS C18 column on an HP Agilent 1090 Series II. A standard curve, created by measuring signals of known indole concentrations in lysogeny broth, was used.

MBC determination. A 1:1,000 dilution of an overnight culture of c10,6 was grown in lysogeny broth and supplemented with 300 μM indole or the equivalent concentration of methanol, the solvent for indole. OD_{600} was measured as described above following overnight incubation in norfloxacin at a range of concentrations. Replicate cultures with $OD_{600} \leq 0.1$ were subcultured on non-restrictive lysogeny broth/agar plates. The MBC was determined as the minimum norfloxacin concentration that killed at least 99.9% of an initial c10,6 inoculum.

Competition assay. Overnight cultures of c10,12 and c10,12Δ*tnaA* were combined 1:1 and continuously cultured as described above. The medium was supplemented with norfloxacin (1,500 ng ml^{-1}) and protected from heat and light. Samples were harvested after approximately five volume changes to avoid prolonged exposure to mutagenic effects of norfloxacin, and plated differentially on non-restrictive lysogeny broth/agar plates and lysogeny broth/agar plates supplemented with kanamycin (25 μg ml^{-1}). Relative fractions of each isolate were derived from Malthusian parameters[16] calculated from median cell counts.

Co-culture. Each isolate was grown overnight to OD_{600} >1.5. Mixed cultures consist of an HRI diluted 1:30,000 and a LRI diluted 1:300. In each clonal culture, an isolate was diluted 1:30,000 + 1:300 for a total number of cells equivalent to the mixed cultures. Cultures were incubated overnight in lysogeny broth supplemented with norfloxacin (1,500 ng ml^{-1}). Cell counts were obtained by subculturing on non-restrictive lysogeny broth/agar plates.

Transcriptional profiling. An indole-deficient mutant with wild-type resistance to norfloxacin, MG1655Δ*tnaA*, was seeded and continuously cultured as described above. Total transcripts were prepared and hybridized as previously described[18], from samples collected in serial and representing three distinct conditions. The first sample was collected after a 30-min exposure to norfloxacin (50 ng ml^{-1}). The bioreactor medium was then replaced, by dilution, with non-restrictive lysogeny broth medium. Indole (200 μM) was added, followed by norfloxacin 30 min later. The second sample was collected after 30 min of simultaneous exposure to both indole and norfloxacin. The media was again purified as described. The third sample was collected after a re-exposure to norfloxacin. Resulting CEL files were normalized by FARMS and informative probes selected for analysis[28].

Whole-genome sequencing and allelic frequency estimation. Genomic DNA (gDNA) was extracted from each sample using Qiagen's genomic-tip kit. For whole-genome sequencing, gDNA of each isolate was sonicated (Covaris) to 200 bases pairs. Illumina single-end sequencing libraries for each isolate were prepared, using custom adapters[29] and NEBNext DNA Sample Master Mix Set 1 (New England Biolabs). Libraries were submitted to the Boston University Sequencing Core. For allelic frequency estimation, 100 base pairs surrounding each identified mutation was PCR-amplified from the total population gDNA and the enriched, highly resistant population gDNA, respectively. Amplicons were submitted to Partners Healthcare Center for Personalized Medicine for matrix-assisted laser desorption/ionization–time of flight mass spectrometry using Sequenom's iPLEX chemistry[30].