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Heterogeneity in efflux pump expression predisposes antibiotic-resistant cells to mutation

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Abstract

Antibiotic resistance is often the result of mutations that block drug activity; however, bacteria also evade antibiotics by transiently expressing genes such as multidrug efflux pumps. A crucial question is whether transient resistance can promote permanent genetic changes. Previous studies have established that antibiotic treatment can select tolerant cells that then mutate to achieve permanent resistance. Whether these mutations result from antibiotic stress or preexist within the population is unclear. To address this question, we focused on the multidrug pump AcrAB-TolC. Using time-lapse microscopy, we found that cells with higher *acrAB* expression have lower expression of the DNA mismatch repair gene *mutS*, lower growth rates, and higher mutation frequencies. Thus, transient antibiotic resistance from elevated *acrAB* expression can promote spontaneous mutations within single cells.

Antibiotic resistance is a major public health problem and is primarily the result of genetic changes that allow microorganisms to overcome the effects of antimicrobial drugs (1). However, genetic changes are not the only way that bacteria can tolerate antibiotics. Transient resistance mechanisms allow cells to temporarily resist drug treatment (2), playing a critical role in recalcitrant and recurrent infections (3). Examples include bacterial persistence, where cells temporarily enter a dormant state to block drug activity (4), and expression of efflux pumps to export antibiotics (5–7). We asked whether transient antibiotic resistance can lead to permanent antibiotic resistance by providing a window of opportunity in which cells can mutate. A recent study showed that bacterial persistence precedes

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Data and materials availability: Data have been archived at Dryad (37).

SUPPLEMENTARY MATERIALS
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resistance. In this state, tolerant cells can subsequently acquire mutations conferring resistance (8). Additionally, antibiotics often induce stress response mechanisms, which can lead to mutations. For instance, the low-fidelity, mutation-prone polymerasesPol II, Pol IV, and Pol V are induced during the SOS response to DNA damage (9). A question remains whether differences in mutation frequency predate antibiotic treatment or whether they are induced by the stress.

We focused on transient resistance arising from heterogeneity in expression of the AcrAB-TolC efflux pump found in many pathogens (6, 7, 10). The pump recognizes and exports β-lactam, tetracycline, and fluoroquinolone antibiotics, among others (11), by using the inner membrane protein AcrB, which works together with the periplasmic linker AcrA and the outer membrane channel TolC (10, 12). *acrA* and *acrB* are commonly arranged together on an operon, whereas *tolC* is expressed elsewhere in the genome.

Recent reports have highlighted the importance of cell-to-cell variability in pump expression (6, 7). For example, in *Escherichia coli*, AcrAB-TolC pumps partition heterogeneously, with pumps accumulating at the old pole, resulting in increased resistance levels in the subset of cells with higher efflux pump expression (7). We asked whether cell-to-cell heterogeneity in pump expression results in differences in the spontaneous mutation rate in addition to its known role in producing single-cell differences in transient antibiotic resistance.

We first calculated the spontaneous mutation frequency in *E. coli* strains with and without efflux pumps. To identify mutations not induced by stress, we performed these measurements in the absence of antibiotics. We plated mid–exponential phase cultures on LB agar with and without rifampicin and calculated the mutation frequency by dividing the number of colony-forming units (CFU) per milliliter on rifampicin plates by the number of CFU per milliliter on LB plates (Fig. 1A) (13).

We first compared wild-type *E. coli* strains with and without overexpression of the *acrAB* operon. Although AcrA and AcrB function together with TolC, overexpression of the *acrAB* operon alone is sufficient to increase resistance (14, 15). We found that the wild-type strain had a significantly lower mutation frequency than the strain overexpressing *acrAB* (Fig. 1B). These mutation frequencies correspond to mutation rates of 1.06×10^{-8} and 2.84×10^{-8} mutations per generation in the wild-type and *acrAB* strains, respectively (table S1), providing resistance without incurring a major fitness cost (16). Deleting *acrB*, which inactivates the entire efflux pump (14), also significantly decreased the mutation frequency relative to that of the wild-type strain (Fig. 1B). Complementing the Δ*acrB* strain with a plasmid containing the *acrAB* operon restored and further increased the mutation frequency over that of the wild type. Using sequencing, we confirmed that the resistance originated from mutations within the *rpoB* gene, which is a known target for rifampicin (17) (table S2). Taken together, these results suggest that elevated expression of the AcrAB efflux pump, which plays a critical role in transient resistance, can also increase the frequency of spontaneous mutations.

We asked whether our findings could be generalized to other bacterial species by focusing on the pathogen *Salmonella enterica* serovar Typhimurium LT2 (10). We introduced a plasmid overexpressing *acrAB* into *S*. Typhimurium and again observed an increase in the spontaneous mutation frequency (Fig. 1C).

We also measured mutation frequencies in *E. coli* Δ*acrB* with and without *acrAB* overexpression by using ciprofloxacin, tetracycline, and chloramphenicol. In each case, we used antibiotic concentrations that exceeded the minimum inhibitory concentration for the strain with *acrAB* overexpression to ensure that we were measuring the mutation frequency and not differences due to drug efflux (15, 18) (fig. S1). Consistent with our results in rifampicin, overexpression of *acrAB* significantly increased the mutation frequency in all three antibiotics relative to those of the strains lacking the pumps (Fig. 1D).

To test whether the differences in mutation frequency are due to pump activity, we generated a catalytically compromised mutant expressing *acrB* with a Phe^{610}→Ala (F610A) mutation (19). In contrast to bacteria with functional AcrB, the Δ*acrB* strain complemented with a plasmid containing *acrAB* F610A had no change in the mutation frequency relative to the Δ*acrB* strain (fig. S2), suggesting that pump activity is critical for the mutation rate differences.

MutS is involved in DNA mismatch repair, which is a crucial step in preventing mutations. MutS deficiency leads to a hypermutable phenotype (20). Recent studies have revealed heterogeneity in the expression of DNA repair enzymes between single cells, highlighting the importance of single-cell–level effects in the emergence of resistance (21–23). To study the link between *acrAB* and *mutS* expression, we constructed a double-color plasmid to report expression simultaneously from the *acrAB* and *mutS* promoters. We fused the *acrAB* promoter to the gene for red fluorescent protein (RFP) (yielding P_{acrAB}-*rfp*)

and the *mutS* promoter to the gene for yellow fluorescent protein (YFP) (yielding P_{mutS}-*yfp*). Using fluorescence microscopy, we observed cell-to-cell variation in both reporters, with cells expressing either P_{acrAB}-*rfp* or P_{mutS}-*yfp* but not both (Fig. 2, A and B). This indicates that cells with higher efflux pump expression, which are more antibiotic resistant (7), also have less mismatch repair, making them more mutation prone (21).

To check whether this effect is due to AcrAB, we introduced the double-color reporter into the Δ*acrB* strain. This decreased the population of cells with high P_{acrAB} and low P_{mutS} expression (Fig. 2, A, C, and D) and potentially indicates positive feedback between AcrAB and its promoter. Complementing the Δ*acrB* strain with a plasmid containing *acrAB* restored cells with higher levels of P_{acrAB} and lower levels of P_{mutS} expression (fig. S3). To rule out spurious plasmid effects as the cause of the inverse relationship, we also tested double-color reporters in which we replaced the P_{acrAB} and P_{mutS} promoters each with a constitutive promoter and no longer observed the reciprocal relationship between the two colors (fig. S4).

Transcriptional fusions between promoters and reporters give an indirect measurement of protein levels; therefore, we next sought to verify our findings by using translational fusions of AcrAB and MutS with fluorescent reporters. We observed a similar relationship between AcrAB-RFP and MutS-YFP, with a subset of cells containing higher levels of AcrAB efflux pumps and lower MutS expression (Fig. 2, E and F).

When we overexpressed *acrAB* in a Δ*mutS* strain, we observed no significant difference in the spontaneous mutation frequency between the strains with and without *acrAB* overexpression (fig. S5). Because MutS is involved in mutation repair, the overall mutation rate is higher in the Δ*mutS* strain than in wild-type cells (24). The similar mutation frequencies observed with and without *acrAB* overexpression may be due to the role of MutS as an effector in the AcrAB-dependent mutation increase, or alternatively, the strong mutator phenotype may simply mask any differences.

The AcrAB pump provides transient antibiotic resistance but can be costly to express. Overexpression alters membrane fluidity, slows growth, and can cause cells to pump out essential metabolites (10, 25). Thus, there is a trade-off between pump expression and fitness (25). It is well known that mutation rates are dependent on growth rates in *E. coli*, and *mutS* expression is repressed in nutritionally stressed cells (26–28). We found that the total number of CFU per milliliter decreased when *acrAB* was overexpressed (fig. S6). Using time-lapse microscopy, we grew wild-type cells containing the double-color transcriptional reporter (P_{acrAB}-*rfp* and P_{mutS}-*yfp*) on agarose pads. Single cells with high P_{acrAB} and low P_{mutS} expression grew more slowly than those with low P_{acrAB} and high P_{mutS} expression (Fig. 3A and movie S1). We quantified P_{acrAB} and P_{mutS} expression and growth rates across many growing microcolonies (n = 3213 cells) and again observed an inverse relationship between P_{acrAB} expression and P_{mutS} expression (Fig. 3B). Overlaying the growth rate onto these data, we found that the slowest-growing cells were those with high P_{acrAB} and low P_{mutS} expression (Fig. 3, B to D). Measurements with the AcrAB-RFP and MutS-YFP translational fusion strain also showed slower growth in this subpopulation of cells (fig. S7 and movie S2).

Growth rate–dependent effects disappeared in a Δ*acrB* background, with cells growing at similar rates across all levels of P_{acrAB} expression (Fig. 3E and movie S3). Quantification across microcolonies confirmed this finding, and growth rates were roughly constant, regardless of P_{acrAB} or P_{mutS} expression (Fig. 3, F to H). Together, these results demonstrate that *acrAB* expression affects single-cell growth rates and that cell-to-cell differences in pump expression result in a subpopulation of cells with high P_{acrAB} expression, low P_{mutS} expression, and a low growth rate.

The AcrAB efflux pump is regulated by the transcription factor MarA (29). Mutations in *marA* and its regulator *marR* frequently arise in clinical isolates and antibiotic resistance studies (30, 31). In addition, MarA expression is heterogeneous and dynamic within isogenic single cells, and its stochastic expression is associated with elevated transient resistance (5, 32). Using fluorescence-activated cell sorting, we found that cells with higher *marA* expression were more mutation prone than those with low *marA* expression and that this effect was due predominantly to the AcrAB pump (fig. S8 and supplementary text).

These results demonstrate a link between transient resistance and heterogeneity in spontaneous mutation frequencies. Our findings indicate that the AcrAB efflux pump, which plays a known role in multidrug resistance, can also affect the initial stages of the evolution of permanent antibiotic resistance. Our results suggest that heterogeneity in AcrAB is correlated with expression of the mismatch repair enzyme MutS in individual cells and that elevated levels of *acrAB* expression decrease the growth rate. In our work, this role for AcrAB was shown in the absence of antibiotic stress, so these differences in mutation frequency are not induced by antibiotic treatment.

Even modest increases in the mutation rate can drive the evolution of resistance under selective pressure. For instance, weak mutator phenotypes have been shown to play a critical role in the evolution of resistance to ciprofloxacin in *E. coli* and *Staphylococcus aureus* (16, 33). Achieving resistance to clinical levels of antibiotics is often a multistep process, requiring several mutational events. As an example, *acrAB*-related genes, including the regulators *acrR*, *marR*, *soxR*, and *marA*, appeared frequently in a microbial evolution and growth arena (MEGA)–plate study in which *E. coli* evolved resistance to trimethoprim (30).

Our findings open the door for further studies of the molecular mechanism by which AcrAB affects mutation frequency. Mutation rates have been shown to depend on the cell growth rate and population density (26, 27). Given the link between pump expression and growth, it is likely that other growth-related phenomena are influenced by single-cell–level differences in pump expression. Efflux pumps may contribute to increases in the mutation rate by influencing growth alone or by exporting compounds involved in cell-to-cell interactions and the methyl cycle (13). Multidrug efflux pumps and DNA repair enzymes are widespread (20, 34, 35). Understanding the initial evolutionary trajectory of resistant strains may suggest strategies for treating infections, such as combination therapies involving antibiotics and efflux pump inhibitors (36).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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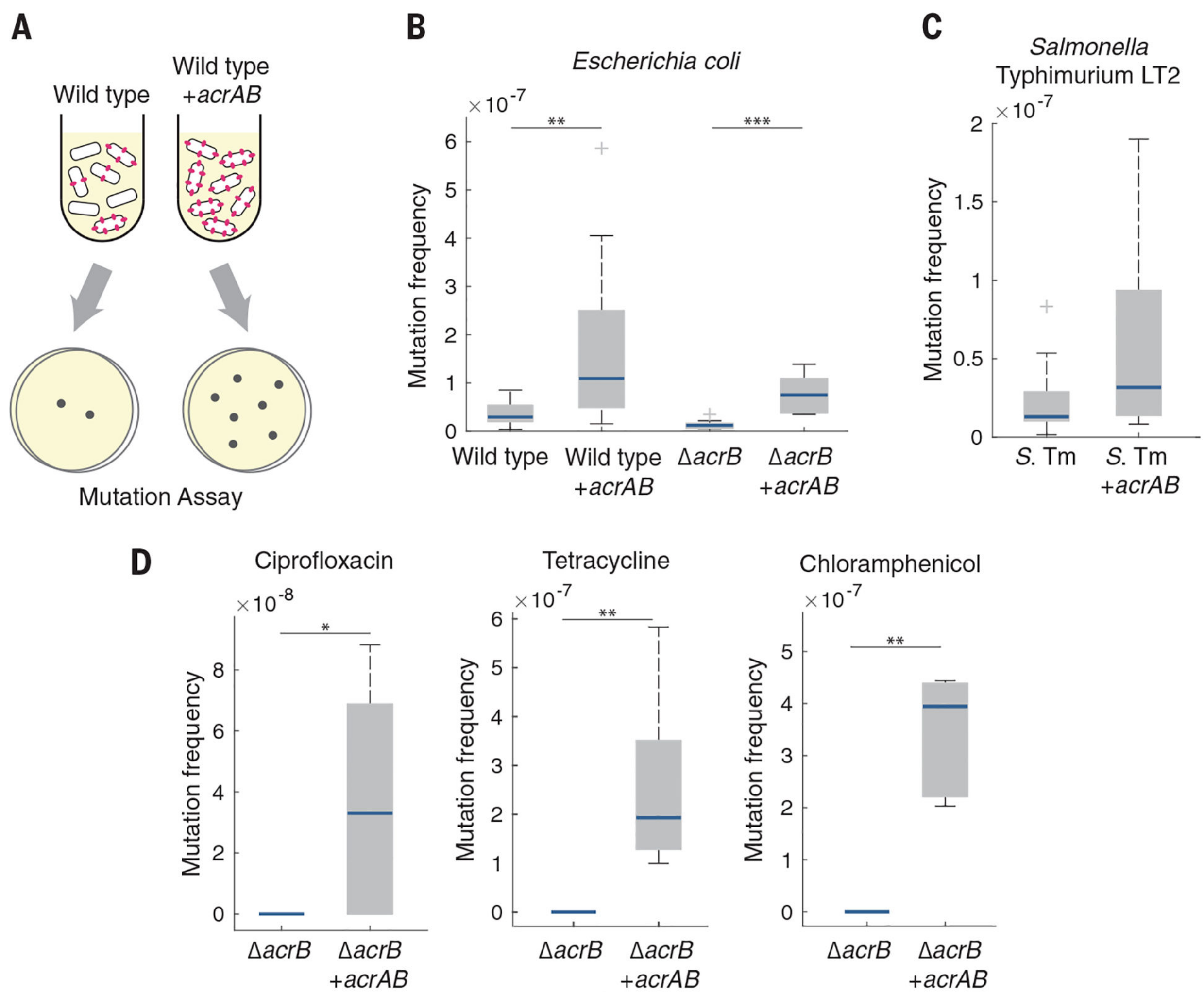

Fig. 1. Overexpression of AcrAB increases the spontaneous mutation frequency.

(**A**) Schematic showing an increase in spontaneous mutations in cells with higher efflux pump expression. (**B**) Rifampicin mutation frequency in *E. coli* wild-type and Δ*acrB* strains with and without *acrAB* overexpression. $n \geq 8$ biological replicates. (**C**) Rifampicin mutation frequency in *S.* Typhimurium (S. Tm) LT2. $n \geq 12$ biological replicates. (**D**) Ciprofloxacin, tetracycline, and chloramphenicol mutation frequencies in *E. coli* Δ*acrB*. $n \geq 5$ biological replicates. The Δ*acrB* strain in (D) did not produce any mutants in the presence of any of the antibiotics; mutants were observed for all antibiotics in the *acrAB* overexpression strain. For (B) to (D), blue bars show the median values, gray boxes indicate the interquartile range, and whiskers show the maximum and minimum values. Box plot raw data are shown in fig. S9A. Strains without *acrAB* overexpression contained an equivalent plasmid expressing *cfp* in place of *acrAB*. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Mann-Whitney rank sum test.

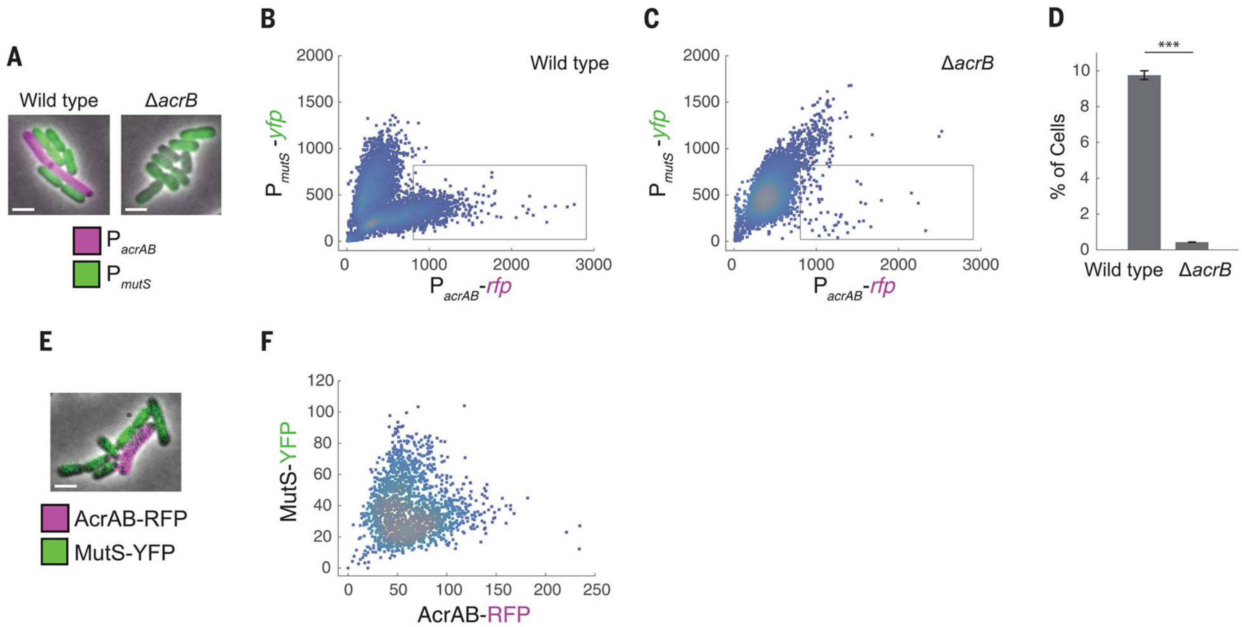

Fig. 2. Inverse relationship between *acrAB* expression and *mutS* expression in single cells.
(**A**) Fluorescence microscopy images of *E. coli* wild-type and Δ*acrB* strains containing the double-color reporter P_{acrAB}-*rfp* + P_{mutS}-*yfp*. Scale bars, 2 μm. (**B** and **C**) RFP fluorescence reflecting *acrAB* promoter activity versus YFP fluorescence reflecting *mutS* promoter activity in (B) wild-type and (C) Δ*acrB* strains. Each dot corresponds to one cell. The gray box indicates the region of interest used for (D). (**D**) Percentages of the cell populations that fall within the region-of-interest box. Error bars, ± SEM. ***$P < 0.001$, two-sample *t* test. (**E**) Wild-type *E. coli* containing translational fusion P_{acrAB}-*acrAB-rfp* + P_{mutS}-*mutS-yfp*. (**F**) AcrAB-RFP versus MutS-YFP. All fluorescence data were obtained after background subtraction to remove autofluorescence. Values in (B), (C), and (F) are expressed in arbitrary units.

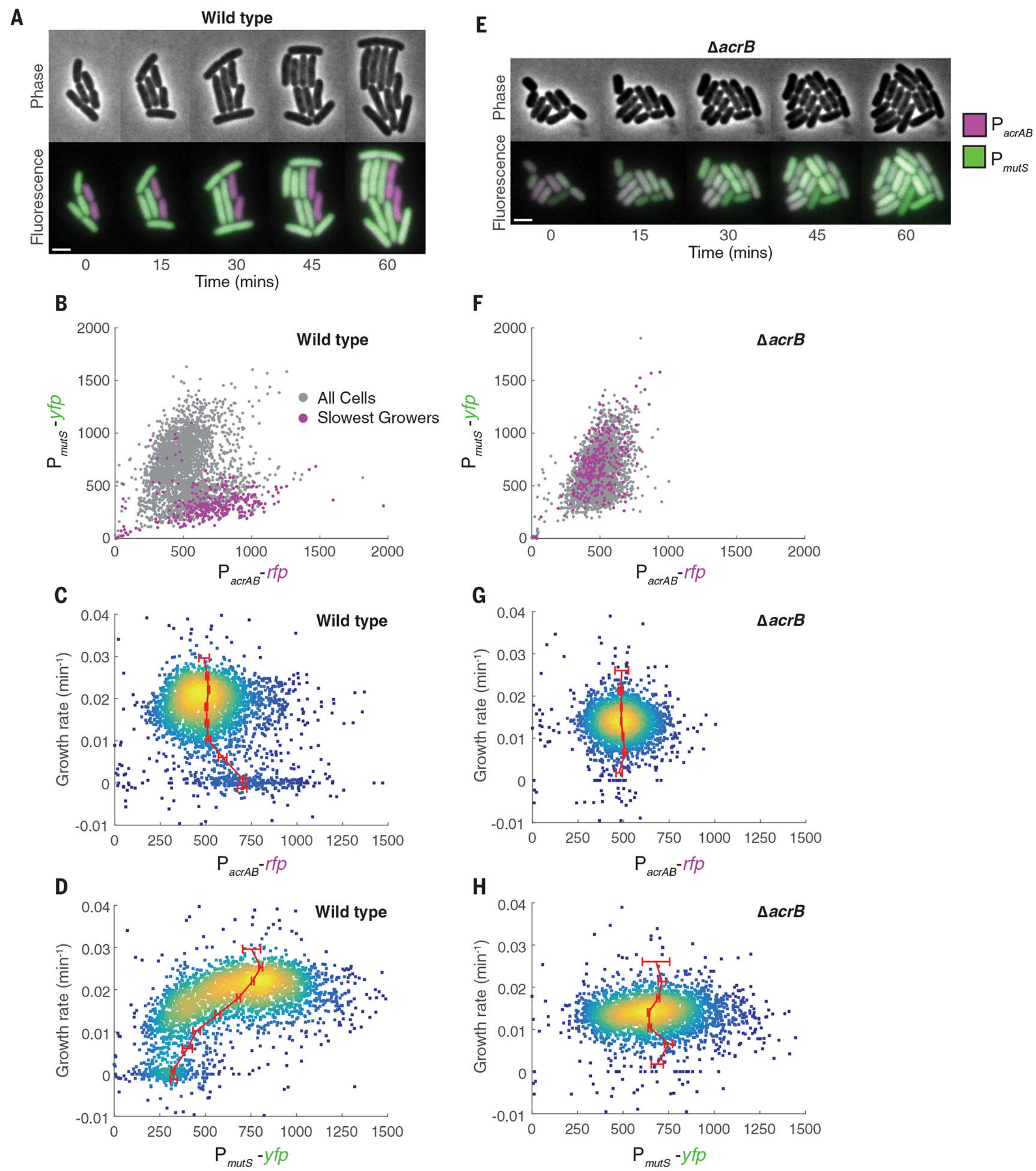

Fig. 3. Reduced growth rate in single cells with high P_{acrAB} and low P_{mutS} expression.

(**A**) Time-lapse microscopy images of wild-type cells expressing P_{acrAB}-*rfp* + P_{mutS}-*yfp*. Scale bar, 2 μm. (**B**) P_{acrAB}-*rfp* expression versus P_{mutS}-*yfp* expression in the wild-type strain. The purple dots correspond to cells whose growth rate falls in the bottom 10% of those measured. (**C**) P_{acrAB}-*rfp* expression and (**D**) P_{mutS}-*yfp* expression versus the growth rate in the wild-type strain. (**E**) Δ*acrB* cells expressing P_{acrAB}-*rfp* and P_{mutS}-*yfp*. (**F**) P_{acrAB}-*rfp* expression versus P_{mutS}-*yfp* expression in Δ*acrB* cells. (**G**) P_{acrAB}-*rfp* expression and (**H**) P_{mutS}-*yfp* expression versus the growth rate in the Δ*acrB* strain. Red lines in (C), (D), (G), and (H) plot the mean fluorescence of cells binned across growth rate in increments of 0.004 min^{-1}, where each bin has a minimum of 15 cells. Error bars, ± SEM. Negative growth rates arise when the automated cell identification process identifies a cell in a subsequent frame as having a smaller number of pixels; however, this is an infrequent event (~2% of cells). Values in (B) to (D) and (F) to (H) are expressed in arbitrary units.