

# Large mutational target size for rapid emergence of bacterial persistence

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**Phenotypic heterogeneity displayed by a clonal bacterial population permits a small fraction of cells to survive prolonged exposure to antibiotics. Although first described over 60 y ago, the molecular mechanisms underlying this behavior, termed persistence, remain largely unknown. To systematically explore the genetic basis of persistence, we selected a library of transposon-mutagenized *Escherichia coli* cells for survival to multiple rounds of lethal ampicillin exposure. Application of microarray-based genetic footprinting revealed a large number of loci that drastically elevate persistence frequency through null mutations and domain disruptions. In one case, the C-terminal disruption of methionyl-tRNA synthetase (MetG) results in a 10,000-fold higher persistence frequency than wild type. We discovered a mechanism by which null mutations in *transketolase A (tktA)* and *glycerol-3-phosphate (G3P) dehydrogenase (glpD)* increase persistence through metabolic flux alterations that increase intracellular levels of the growth-inhibitory metabolite methylglyoxal. Systematic double-mutant analyses revealed the genetic network context in which such persistent mutants function. Our findings reveal a large mutational target size for increasing persistence frequency, which has fundamental implications for the emergence of antibiotic tolerance in the clinical setting.**

genetic interactions | evolution | adaptation | systems biology

In a classic study conducted in 1944, Joseph W. Bigger discovered that a subpopulation of cells within a genetically homogeneous culture of *Staphylococcus aureus* was consistently able to survive prolonged exposure to bactericidal concentrations of the then-novel antibiotic penicillin (1). The behavior of these cells, called persisters, was not due to a mutation because the initial survivors, once regrown, displayed the same level of sensitivity to penicillin. Instead, persistence has been attributed to noninherited antibiotic resistance (2) and was shown to be a feature of a subpopulation of bacteria exhibiting phenotypic heterogeneity (3). It is thought that this heterogeneity has evolved to ensure the longevity of a population threatened with a potentially catastrophic event such as lethal antibiotic exposure (4). Recent years have witnessed renewed interest in persistence due to its potential role in chronic and recalcitrant infections. Understanding the biology of persistence is thus central to achieving effective antibiotic treatment.

Many years after Bigger's description, a substantial contribution to our understanding of persistence was made by the identification of high persistence (*hip*) mutations in an *Escherichia coli* operon (5). One of these, *hipA7*, increases persistence frequency by 10,000-fold. Using these mutants and microfluidic devices, Balaban and colleagues provided a rich phenotypic description by showing that the persistent subpopulation exists in a slow-growing state in the presence of the  $\beta$ -lactam drug, ampicillin; because ampicillin is a bactericidal agent that targets cell wall biosynthesis, nondividing persister cells are immune to the lethal effects of this drug (6). Although the dormant state of persisters is accompanied by a cessation of most cellular processes, translation continues at a reduced rate (7). In a recent study, Allison and colleagues take advantage of this observation and show that metabolic stimulation of persisters makes them

vulnerable to killing by aminoglycosides (8), which are bactericidal antibiotics that target the ribosome. The enhanced killing of persisters by the addition of metabolites was found to be specific to aminoglycosides, as metabolic stimulation failed to reduce the number of persisters when exposed to quinolones or  $\beta$ -lactam antibiotics (8), which target DNA replication and cell division, respectively.

Despite efforts to identify the genetic determinants contributing to persister cell formation, we still have a limited understanding of the molecular basis of this phenotype. In this study, we applied a highly sensitive experimental strategy for large-scale characterization of the genetic basis of persistence. After a series of selections using a high-density library of transposon-insertional mutants, we combined competitive selection and microarray-based genetic footprinting to measure the contribution of every genetic locus to persistence. Our results implicate a large number of genes that increase persistence by varying degrees and through diverse mechanisms. We used the same framework to carry out global epistasis analyses, which revealed the genetic network context in which these loci function to affect persistence.

## Results and Discussion

### Selection Strategy to Identify Genetic Determinants for Persistence.

We designed and implemented a unique selection strategy to identify the genetic loci contributing to persistence. Because of its widespread occurrence, we hypothesized that persistence is an evolutionary adaptation with a discrete set of genetic determinants. We sought to identify these determinants by exposing a library of high-density transposon-insertional mutants of *E. coli* to several rounds of selection in the presence of lethal antibiotic exposure.

Because persisters are commonly observed in biofilms or associated with a solid surface (9), we expected to observe a more robust persistence phenotype if we used a solid growth medium as a substrate for adhesion. To enrich for mutants exhibiting an increasing propensity for persistence, a culture of transposon-insertional mutants was grown to stationary phase, plated on LB agar containing ampicillin, and incubated at 37 °C for 24 h. The plates were then sprayed with penicillinase to inactivate the ampicillin followed by an additional incubation to permit the growth of surviving colonies. This procedure constituted one round of persister cell enrichment, and we reasoned that multiple cycles of selection

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than persistence. Using two complementary methods of determining the minimal inhibitory concentration (MIC) of these strains to ampicillin, we showed that the persistence phenotype exhibited by the strains listed in Table S1 was not a result of genetically acquired antibiotic resistance (Fig. S3).

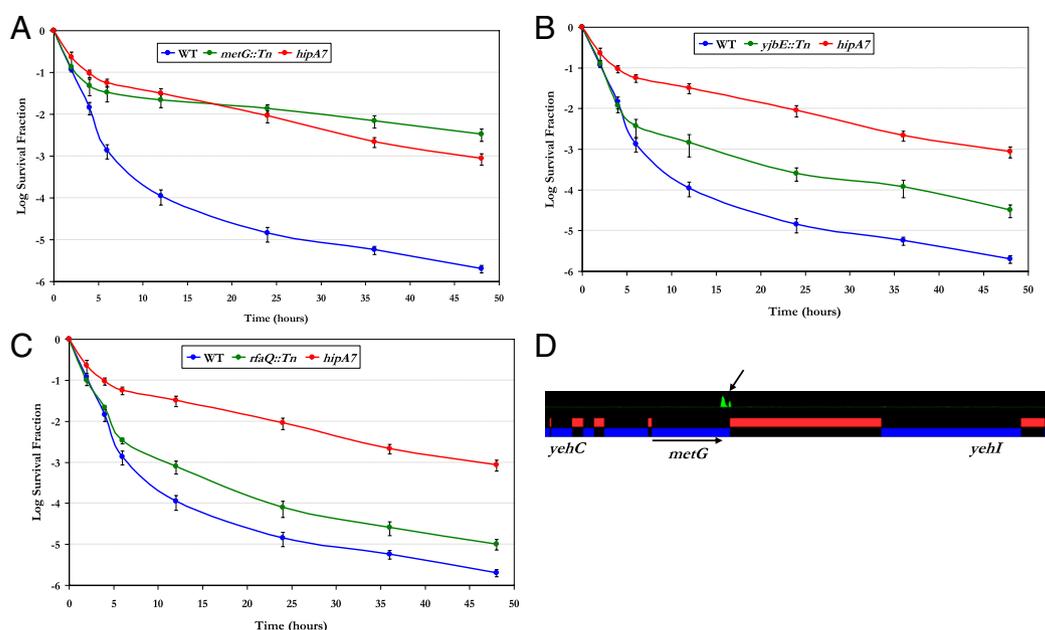
**Quantitative Determination of Persistence Frequency.** Kill curves provide a quantitative measure of persistence frequency by representing the survival fraction of cells as a function of the length of exposure to antibiotics (6). To generate a kill curve, a culture in stationary phase is diluted and plated on LB agar containing ampicillin. At designated time points, penicillinase is sprayed onto the surface of the agar to inactivate the ampicillin. Colonies that form after a subsequent incubation period are counted, and the survival fraction at each time point is calculated relative to the initial number of cells. For each kill curve, the *hipA7* mutant and the wild type are used as strains that represent high- and low-persistence frequency, respectively. To make the comparisons meaningful, the *hipA7* allele was transferred to the same genetic background as the wild type. To transfer this allele, we took advantage of a selectable marker closely linked to *hipA7* and a cold sensitivity phenotype conferred by the allele (13) to transduce *hipA7* from TH1269 (14) to our wild-type *E. coli* MG1655 (Fig. S4). We created quantitative kill curves for a subset of the mutants identified in this study (discussed below).

**Insertions in *metG* Create a Hyper-Persistence Phenotype.** We isolated several mutants with insertions in the 3' region of *metG* (Fig. 1D). The persistence frequency of one of these mutants was evaluated by generating a kill curve, and remarkably, this *metG* mutant showed a level of survival that is very similar to the *hipA7* mutant (Fig. 2A). To ensure that the observed phenotype was due to the insertion in *metG* and not a secondary mutation, we used P1 transduction to move the insertion back into the wild-type parent strain. This strain showed a level of persistence indistinguishable from the original isolate (Fig. S5). The sequencing results revealed that the transposon insertion occurred at the 3' end of *metG*,

presumably disrupting the C-terminal domain and preserving the essential function of the gene product. This observation was confirmed through a high-resolution mapping of transposon-insertion sites using a high-density tiling array (Fig. 2D).

The *metG* gene encodes methionyl-tRNA synthetase (MetRS), an enzyme essential for viability that functions to covalently link methionine amino acids to their cognate tRNA molecules. MetRS is a large protein with multiple domains, and the distal C-terminal domain is involved in dimerization (15). C-terminal truncations of MetRS, leading to the monomeric form of the enzyme, have been reported to have minimal effect on the activity of the enzyme (16). It has been found that monomeric MetRS has reduced binding affinity to the methionine tRNA (tRNA<sup>Met</sup>) (17). The reduced affinity to tRNA<sup>Met</sup> exhibited by the *metG* mutants may be the key factor in this mutant's persistence phenotype. Inefficiencies in translation may lead to a heterogeneous population in which a minority of cells in the population experiences a temporary cessation of growth. The similarities in the kill kinetics of this mutant to the *hipA7* mutant (Fig. 2A) suggest that both mutants exhibit the persistence phenotype by the common mechanism of stochastic switching from the growing to the nongrowing state (6).

**Increased Persistence Frequency Through Activation of the Rcs-Signaling Pathway.** Some of the mutants we identified had insertions in genes encoding cellular structures. For example, many of the constituents of the *rfa* operon, which are associated with LPS synthesis, exhibited high z scores. Mutations in genes comprising the *rfa* operon have been found to activate the Rcs-signaling pathway (18), and activation of this pathway has been linked to increased tolerance to  $\beta$ -lactam antibiotics (19). Because activation of the Rcs pathway by LPS mutations has been found to induce colanic acid capsular polysaccharide synthesis (18), we hypothesized that the increased polysaccharide synthesis displayed by LPS mutants serves as a barrier that reduces antibiotic permeability. We confirmed this hypothesis by disrupting the Rcs pathway in our LPS mutants and observing a persistence frequency comparable to the wild type.



**Fig. 2.** Phenotypic characterization of insertional mutants. (A–C) Kill curves generated for mutants with insertions in *metG* (A), *yjbE* (B), and *rfaQ* (C) are shown in green. For comparison, kill curves were also generated for the wild type (blue) and the *hipA7* strain (red). Error bars represent SDs of three independent measurements. (D) A high-resolution tiling array covering the entire *E. coli* genome with overlapping 25-mer oligos at four base pair resolution was used to display the abundance of insertions in a population of transposon mutants that have been enriched for persistence. This profile of the *metG* locus shows a strong transposon-insertion signal limited to the 3' end of *metG*. Peaks in green represent insertion sites.

Another operon with multiple insertions contributing to persistence was the *yjb* operon. The genes in this operon have no assigned function; however, the *yjbEFGH* locus has been found to be part of the Rcs regulon (20). This locus is also implicated in exopolysaccharide production (21). As with the *rfa* mutants, introducing mutations that block signaling through the Rcs pathway abolished the observed elevation in persistence of *yjb* insertional mutants. Therefore, the persistence phenotype may be more related to the generation of a drug permeability barrier rather than an intracellular state that is impervious to antibiotic effect.

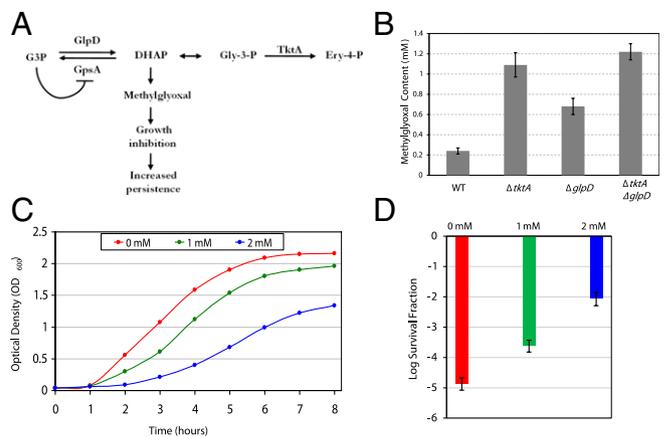
Most of the genes that comprise the *yjb* and *rfa* operons showed high *z* scores in our selection (Fig. 1C), and we isolated a disproportionately large number of mutants with insertions in *yjbE* and *rfaQ* (Fig. 1D). Quantitative kill curves were generated using *yjbE::Tn* (Fig. 2B) and *rfaQ::Tn* (Fig. 2C), which revealed an intermediate level of persistence, below the frequency for *hipA7* yet 10-fold higher than the wild type.

**Increased Persistence Through the Accumulation of the Intracellular Metabolite Methylglyoxal.** Loss of function mutations in two genes associated with metabolism, G3P dehydrogenase (*glpD*) and transketolase A (*tktA*), gave rise to increased persistence frequency. Glycerol-3-phosphate dehydrogenase is encoded by *glpD*, and *tktA* codes for transketolase in *E. coli*. The gene products of *glpD* and *tktA* function at the juncture between respiration and glycolysis. During aerobic respiration, glycerol-3-phosphate (G3P) is converted to dihydroxyacetone phosphate (DHAP) by *GlpD*. The reverse reaction—conversion of G3P from DHAP—is catalyzed by G3P synthase (*GpsA*). In a *glpD* mutant, the accumulation of G3P inhibits *GpsA* by a negative feedback mechanism, which leads to the accumulation of DHAP (22) (Fig. 3A). Likewise, mutations in *TktA* prevent the conversion of glyceraldehyde-3-phosphate (Gly-3-P) to erythrose-4-phosphate (Ery-4-P), which also leads to an accumulation of DHAP (Fig. 3A). Elevated levels of DHAP have been shown to produce methylglyoxal (23), which causes growth arrest (24) and could be responsible for the high-persistence frequency observed by *glpD* and *tktA* mutants.

To determine whether the *glpD* and *tktA* mutants are producing elevated levels of methylglyoxal, we measured the intracellular levels of this metabolite in these mutants. Both the *glpD* and *tktA* mutants showed statistically significant elevated levels of methylglyoxal (Fig. 3B). We created a *glpD-tktA* double mutant and found even higher levels of methylglyoxal in the double mutant than each of the individual mutants (Fig. 3B). This double mutant displayed persistence frequency of 18.2-fold higher than the wild type (Table S1), which is slightly higher than each of the individual mutants.

Next we sought to determine whether exogenous addition of methylglyoxal causally influences persistence rate. We created dose–response curves to monitor sensitivity to various concentration of methylglyoxal, and found an inverse relationship between metabolite concentration and growth rate (Fig. 3C). Next, we measured persistence frequency of the wild type when exposed to these concentrations, and observed that exposure to methylglyoxal increases persistence in a dose-dependent manner (Fig. 3D). These findings confirm our hypothesis that *glpD* and *tktA* loss of function lead to elevated persistence through increased intracellular concentration of methylglyoxal.

**Uncharacterized Genes Contributing to Persistence.** Genes with unknown function that were found to be associated with persistence are *visC*, *yacC*, and *yiiS* (Table S1). *VisC* is a predicted oxidoreductase with an FAD/NAD(P)-binding domain; however, deletion of *visC* has been found to have no effect on aerobic respiration (25). The gene products of *yacC* and *yiiS* have no assigned function, although, *yiiS* has been identified as a member



**Fig. 3.** Role of methylglyoxal in the persistence phenotype of *glpD* and *tktA* mutants. (A) A model illustrating the metabolic consequences of *glpD* and *tktA* disruptions. G3P is converted to DHAP by *GlpD* during aerobic respiration, and the reverse reaction is catalyzed by *GpsA*. DHAP is a precursor to Gly-3-P, which is converted to Ery-4-P by *TktA*. Mutations in *glpD* cause elevated levels of G3P, which inhibits the function of *GpsA* and leads to increased amounts of DHAP. DHAP also accumulates as a consequence of *TktA* malfunction and leads to production of methylglyoxal, which causes growth cessation and increased persistence. (B) Methylglyoxal concentration was determined by ES/LC/MS. Error bars represent SDs of three independent measurements. (C) Dose–response curves were used to monitor sensitivity to methylglyoxal. LB media containing the indicated methylglyoxal concentrations was inoculated with an overnight culture of the wild type. Growth was monitored using  $OD_{600}$  spectrophotometric readings. (D) Survival fractions of the wild type exposed to various concentrations of methylglyoxal were determined. Aliquots were taken three hours postinoculation from the dose–response curves shown in C and plated on LB agar with ampicillin. Plates were sprayed with penicillinase after 24-h incubation at 37 °C and incubated again for colony formation. Error bars represent SDs of three independent measurements.

of the sigma E regulon (26). Further experimentation is required to characterize the role of these genes in persistence.

**Insertions in *hipB* Increase Persistence Frequency.** We identified an insertion in *hipB* (Table S1), whose product is known to suppress persistence by its physical interaction with *HipA* (27). Expression of *hipA* causes an increased rate of persistence through growth cessation (28), and the two substitution mutations in *HipA7*—G22S and D291A—confer a high-persistence phenotype in *hipA7* strains through a weakened *HipA*–*HipB* interaction (29). These observations suggest that the inability of *HipB* to sequester *HipA* is likely the mechanism by which the *hipB* insertional mutant is able to exhibit a 10,000-fold increase in persistence frequency.

**Characterization of Persistence Through Genetic Interaction Mapping.** In this study, we have shown that single-gene disruptions can produce substantial increases in persistence frequency. However, as with other complex phenotypes, persistence is a manifestation of multigene networks. The microarray-based genetic footprinting approach used in the study is well suited for exploring genetic interaction networks. We used this experimental framework to identify genes that interact with *hipA7* and *metG::Tn*, each of which confers a persistence frequency 10,000-fold higher than the wild type. The first step in this process involved creating double-mutant libraries in each of these genetic backgrounds (10). However, because *metG::Tn* already contained a transposon with the same selectable marker used to create libraries, we created a mutant by site-directed recombination to replace 21 nucleotides at the 3' end of *metG* with a selectable marker (30) that was removed using Flippase recombination enzyme (31), resulting in a gene product missing seven amino acids at the C-



recolonization in a wide variety of chronic infections that may be initially cleared by a seemingly successful round of antibiotics. In addition, the large number of persister bacteria constitutes a potentially important reservoir for the emergence of high-level antibiotic tolerance through the acquisition of mobile genetic elements. A better molecular understanding of persistence may lead to the development of drugs that reduce persistence rate to levels low enough to allow successful eradication of the entire population by the application of existing antibiotics. Historically, the genetic basis of persistence has been difficult to study. However, the identification of dozens of loci that substantially alter persistence frequency should aid in future experimental discovery and analysis of persistence pathways.

## Materials and Methods

**Bacterial Strains and Microbiological and Molecular Techniques.** All strains in this study are isogenic derivatives of *E. coli* MG1655 and are listed in Table S2. Bacteriophage and plasmids are listed in Table S3, and oligonucleotide sequences can be found in Table S4. Transposon mutant libraries were constructed using hyperactive Tn5 EZ::TN transposase (1.0 U/μL; Epicentre Technologies) according to the manufacturer's guidelines. Chromosomal markers were transferred by transduction with P1vir. Ampicillin (Sigma) was used in LB agar plates at a concentration of 100 μg/mL. Penicillinase (Sigma) was diluted to 2,500 U/mL in penicillinase buffer consisting of 50 mM of Tris-Cl (pH 7.5), 50mM NaCl, 1 mM EDTA (pH 7.5), 1 mM DTT, and 20% (vol/vol) glycerol, and stored at -80 °C. Before use, penicillinase was diluted to 50 U/mL in LB and filter sterilized. Genetic footprinting and subsequent hybridization to DNA spotted arrays were performed as described previously (10).

**Enrichment for Increased Persistence.** Erlenmeyer flasks containing 50 mL of LB were inoculated with 500 μL of 10<sup>9</sup> transposon-insertional mutants and cultivated at 37 °C with aeration to early stationary phase; 100 μL of each culture was plated onto each of 50 LB plates with 100 μg/mL of ampicillin and incubated at 37 °C for 24 h. Plates were then sprayed with a fine mist of penicillinase solution to destroy the ampicillin in the media. After another incubation period, colonies were eluted from the agar surface, pooled, and 500 μL of the eluent was used to inoculate 50 mL of fresh LB, which was incubated at 37 °C to stationary phase. This procedure constitutes one cycle of enrichment, which was repeated for three rounds.

**Generating Kill Curves.** Kill curves were performed as described previously (6). Briefly, a clonal population was cultivated for 6 h at 37 °C with aeration, diluted, and plated. At specified time points, plates were sprayed with penicillinase and incubated again to permit colony formation. Average survival fractions and SDs were calculated from triplicate sets of plates at each time point.

**Quantification of Methylglyoxal.** Methylglyoxal concentrations were determined by electrospray ionization-liquid chromatography-mass spectrometry as previously described (38) with some modifications. Log-phase cultures were grown in LB at 37 °C, washed twice in deionized H<sub>2</sub>O at 4 °C, followed by sonication at 3 × 5 s 10-W bursts; 5 M hydrochloric acid was added at 0.1 volume and the mixture was centrifuged at 16,000 × *g* for 10 min to remove cell debris and precipitated proteins. The supernatant samples were derivatized at 4 °C for 4 h with 500 nmol of *O*-phenylenediamine (Sigma Chemical), loaded with 2.5 nmol 2,3-hexanedione (Sigma Chemical) as an internal standard, and quantified by ES/IC/MS.

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