An isogenic *E. coli* population gives rise to multiple persister phenotypes

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Abstract
Bacterial persisters are a multidrug-tolerant subpopulation capable of surviving and resuscitating after lethal antibiotic treatment, leading to relapsing infections and the emergence of antibiotic resistance. We hypothesize that multiple persister subpopulations are within an isogenic *Escherichia coli* population, allowing them to survive lethal antibiotic stress. We conducted transcriptomic analyses at multiple time points with lethal ampicillin (Amp) antibiotic, and as expected, several genes were differentially expressed over time. We identified a subset of genes consistently upregulated by comparing transcription levels at different time points of Amp-treated to untreated. Some genes had previously been associated with persisters, while others were new. Subsequently, network analysis showed the gene response between networks, but could not map hypothetical genes. So, we overexpressed seven hypothetical genes, which resulted in slow growth or no growth, indicating that high production harmed the cell. We then made single gene knockouts, which dramatically reduced persister level by ~4-6 fold at 3 h and ~10-15 fold at 6 h of Amp treatment. However, no significant difference in survival rates was observed at 24 h, indicating the presence of multiple persister subpopulations. Our mathematical model demonstrated a 20-fold decrease in the slow-decaying fraction in the mutant, suggesting the importance of decay kinetics in bacterial survival. These results support the existence of multiple subpopulations of persisters, each characterized by distinct decay rates. These results challenge the idea of complete dormancy, suggest the presence of intricate, multifaceted survival mechanisms, and indicate that the persister population itself is heterogeneous.

Keywords
Antibiotic tolerance, antibiotic persistence, antibiotic resistance, phenotypic heterogeneity, cell-state transitions, phenotypic switching

Significance statement
Bacterial persisters, a subpopulation known for their multidrug tolerance and ability to survive lethal antibiotic treatments, have long posed challenges in understanding their formation and long-term survival. They are a driving force of antibiotic resistance, so it is paramount that we learn more about them as the antibiotic resistance problem continues to grow. Our study challenges the long-held consensus that persisters are completely dormant and are of one single population. Our results clearly show that persisters are not as dormant as once thought, and multiple populations of persisters form during lethal antibiotic treatment despite the cells being genetically identical. We use wet lab and dry lab (mathematical modeling) to demonstrate these new findings.

Introduction
Bacteria inhabit diverse and harsh environments, requiring them to adapt to stress by altering their metabolic states, a phenomenon widely known as persistence[1, 2]. Persistence is a bet-hedging strategy employed by all free-living bacteria to endure stress[2]. Persisters are a small fraction of the bacterial

population that arises from inherent phenotypic heterogeneity (switching or transition) rather than genetic mutations. They show reduced cellular activity and do not divide under stress[3]. When the threat subsides, persister cells can resume their growth, leading to recurrent infections that often require prolonged and repeated antibiotic therapy. Recent studies have demonstrated that persisters also have a high mutation rate[4], which has been linked to the evolution of antibiotic resistance, a pressing public health distress. It is the leading cause of death worldwide, outpacing other pathogens, including diseases such as HIV or malaria[5]. Global death projections are now estimated at 10M/year by 2050[6, 7] unless substantial measures are taken to curb this disturbing trend. Here, we specifically focus on probing the persister survival mechanism under antibiotic stress.

Despite being discovered over eight decades ago, the molecular mechanisms responsible for persistence are still unclear and debated. An excellent approach to determine the essential genes and networks for persistence is to study the transcriptome profile —comprising the entirety of mRNA synthesized by cells at a specific time—of persister cells through RNA sequencing (RNA-Seq). Nevertheless, due to their low abundance and intrinsic transient nature, it is enormously challenging to study their transcriptomic profile. Thus, many previous transcriptomic studies have relied on sub-lethal concentrations or short-term exposure to lethal antibiotic concentrations[8, 9]. The populations under scrutiny often encompass short-term tolerant cells (dividing, slow-growing cells) but not necessarily persisters. It's worth noting that short-term tolerance can mask persistence, and empirical evidence has demonstrated their distinct nature[10]. Our recent work reveals that high persistence levels do not correlate with high short-term tolerance levels. Hence, it becomes imperative to use lethal antibiotic concentrations when investigating the transcriptional regulatory mechanisms of persisters[11].

The genes related to antibiotic survival are heavily disputed because of conflicting results and different interpretations of data. Some argue protein synthesis is slowed in persisters due to slower transcription and translation rates[12], while others provide evidence for ample protein synthesis[13]. Other metabolic processes such as oxidative respiration[14] and antibiotic efflux[15] are also debated[12, 14-17]. The role of adenosine triphosphate (ATP) and the tricarboxylic acid (TCA) cycle is particularly controversial[14, 16-19]. Much of the debate comes from looking at mixed tolerant and persister populations, and only individual genes (not networks or clusters of genes).

In this study, we isolated RNA from persisters treated with high doses of an antibiotic for sufficient time to kill short-term tolerant cells. We used time-course assays followed by mutation studies to test our hypothesis that the persisters transcriptome is not static but changes over time. Our study also highlights that differentially expressed commonly upregulated genes could be a potential regulator for persisters. This study is a follow-up to a previous study where we showed that *E. coli* employs an array of networks to survive antibiotic exposure. These networks collaborate in regulating the cellular response, with no single gene or network solely controlling this intricate response[20]. Here, we conducted a network biology approach to illustrate how gene responses within a network change or adapt at different treatment time points: 3 h, 6 h, and 24 h. Through network centrality analysis, we identified potential key hubs. Our results showed that the gene network responses vastly differ across these three time points. We employed a mutation study with selected hypothetical genes to gain deeper insights and further validate our potential target. We showed that the knockout of a single gene (or the alteration of a single network) can reduce the ability of a population to survive antibiotics at specific time points but not for long-term antibiotic stress. We also used mathematical modeling to elucidate the survival mechanism of persisters in mutant strains when exposed to antibiotic stress, demonstrating the existence of multiple subpopulations of persisters.

Results and Discussion

The overview of the experimental plan is shown in Fig. 1. We harvested RNA from a persister subpopulation treated with high doses of a bactericidal antibiotic (ampicillin, a β-lactam antibiotic that

inhibits transpeptidase enzyme involved in cell-wall synthesis)[21] for sufficient time to kill short-term tolerant cells and then subjected for sequencing to analyze the transcriptome profile of the persisters. We previously showed that the *E. coli* Dh5αZ1 strain displayed a biphasic cell death curve, where the short-term cells died quickly in the first phase, and the remaining cells (persisters) died slowly and steadily, beginning at ~2 h. We used rapid filtration[22, 23] for larger samples, followed by flash freezing. Rapid filtration allows us to isolate and wash cells quickly. Large-scale pelleting can be slow, the transcriptome may alter over time, and RNA can be degraded during harvesting. We have empirically shown that removing dead cells requires several washes when pelleting (but not with rapid filtration)[24]. Evidence supports that most RNA from dead cells is unstable after death[25-27], and RNA is sensitive to intra- and extra-cellular RNases (or ribonucleases) degradation. This is why RT-qPCR is often preferred for detecting food pathogens or bacteria on surfaces over DNA[25, 26, 28-39]. The relative stability of DNA compared to RNA facilitates its detection from dead cells over extended periods. We complemented our approach with mutational studies (gene knockouts and gene overexpression) to validate our transcriptomic findings further.

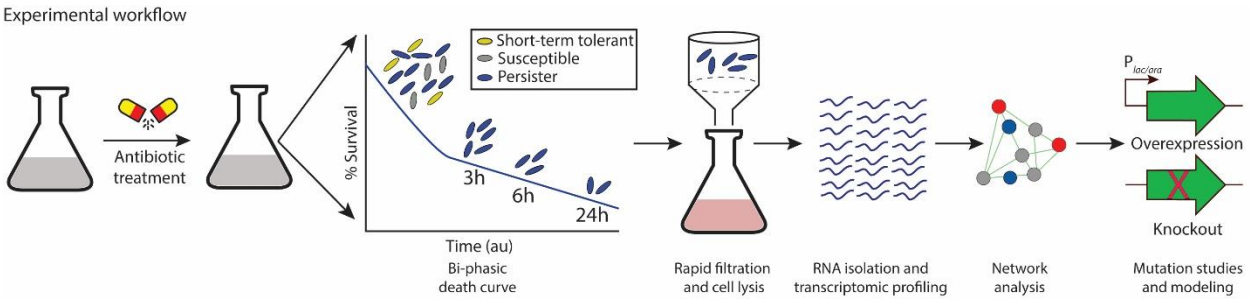


Fig. 1. Schematic diagram of the experimental design. Stationary phase cells were diluted 1:100 times in pre-warmed MMB+ media and treated with Amp (0.1 mg/mL) for 24 h, and samples were isolated for RNA sequencing before antibiotic treatment (0 h) and after antibiotic treatment at 3 h, 6 h, and 24 h. Cells were isolated using rapid filtration to minimize dead cells. Subsequent steps involved RNA extraction, RNA sequencing, and gene network analysis using bioinformatics tools. Overexpression and mutational studies were conducted to test the network analysis and validate the transcriptome-derived findings.

Time-series persister transcriptome analysis showed differential expression of several genes. We compared the transcriptome profiles at different time points to investigate the dynamic changes and/or existence of multiple persister subpopulations in response to lethal antibiotic treatment. We identified substantial differences in gene expression by comparing the expression patterns of a non-treated population at 0 h with persister subpopulations at 3 h, 6 h, and 24 h. Our findings revealed a notable number of differentially expressed genes at each time point: 378 at 3 h, 1478 at 6 h, and 310 at 24 h (Fig. 2 a). Interestingly, these genes showed diverse patterns of upregulation and downregulation. At 3 h, 160 genes were upregulated, and 218 were downregulated. This trend continued at 6 h, where 620 genes were upregulated and 858 were downregulated, and at 24 h, 143 genes were upregulated, and 167 genes downregulated (Fig. 2 a). This observation defies the commonly held hypothesis of a global metabolic slowdown in persister cells. The fact that upregulated genes did not substantially outnumber downregulated genes indicates a complex regulatory mechanism governing gene expression under antibiotic stress. Here, the interactive heatmap showed a clear expression difference of all gene sets at all time points (Fig. 2 b). This observation led us to focus on a particular set of genes, showing a similar expression profile, and we found 27 genes were commonly upregulated at all time points (Fig. 2 c). Among these 27 genes, 8 were identified as hypothetical (Table S1), while 7 were annotated as ribosomal

proteins. There is a variation in ribosome-associated proteins between persister cells and normal cells. This causes the suppression of transcriptional activity in persister cells[40]. However, our particular interest was hypothetical genes. These hypothetical genes have not been previously associated with antibiotic survival. We assume these genes may play a pivotal role in the adaptive response of persister cells, signifying their potential role in antibiotic survival. We wondered if these upregulated genes were solely due to the antibiotic we used, so we tested another type of antibiotic. Of the 27 genes identified with the 6 h Amp treatment, 22 are also commonly upregulated with ciprofloxacin (Cip) treatment at 6 h (Fig S1). This observation demonstrated that the commonly upregulated genes follow consistency with different types of antibiotics; Amp targets cell wall formation[21], while Cip targets DNA synthesis[41].Our goal was to determine how these genes were related to persister survival. We build off what we knew from previous persister and transcriptomic analysis studies[24, 42-48]. Previous studies identified several persister-related genes including *oxyR, dnaK, sucB, relA, rpoS, clpB, mqsR*, and *recA*, which were found to be related to prominent persister formation with multiple antibiotics treatment[42]. We found that these genes showed fluctuating responses at different time points with Amp and Cip treatments. However, our study particularly focused on commonly upregulated genes, including the hypothetical genes and their response to long-term persister survival.

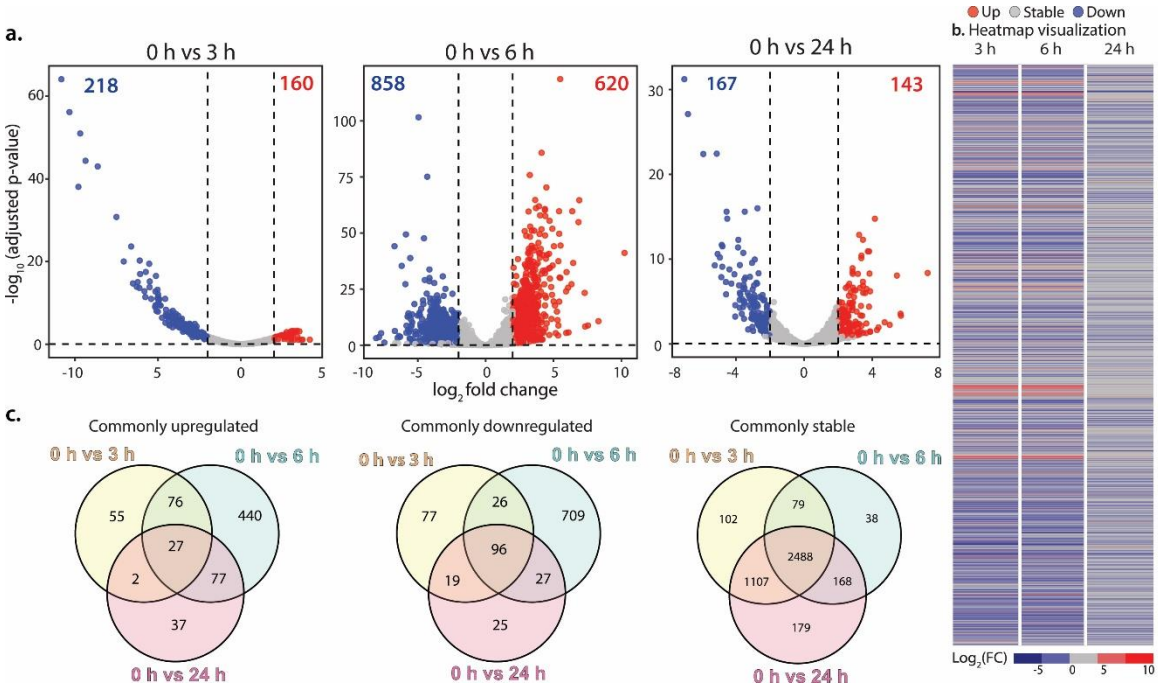


Fig. 2. Comparative transcriptome profile analysis of the population before and after antibiotic treatment shows that the gene expression profile is not the same over time. **(a)** Volcano plots for 0 h (non-persister population) vs 3 h (persister population); 0 h vs 6 h, and 0 h vs 24 h. **(b)** Heatmap visualization for gene expression shows that many genes are differently expressed at different points in Amp treatment. Each row corresponds to a gene, and each column represents treatment times. The intensity of the color at each intersection point indicates the level of gene expression in each time point, with red denoting high expression and blue denoting low expression. The intensity of the colors at each intersection point signifies the gene expression levels in the respective sample. **(c)** Venn diagram for transcriptome comparison of commonly upregulated, downregulated, and stable genes on 0 h vs 3 h, 0 h vs 6 h, and 0 h vs 24 h. We focused on the 27 commonly upregulated genes, hypothesizing that they likely are important for persister long-term survival. Differentially expressed genes are defined to be those with p-adjusted

values <0.1 and fold change higher or lower than ±2 (these same parameter values were employed for all subsequent figures).

Topological variation of differentially expressed genes during antibiotic treatment. Using a cluster-centric top-down approach, we analyzed the changes in gene network response and identified common key network hubs at different time points. Although there are certain limitations of studying protein or gene networks and their pattern of interconnections (refers to network topology) due to minimal resources for predicting network interactions and dynamic changes depending on cell types[49, 50], this type of analysis can be a powerful tool to explore interactions that are challenging to identify by other means. The emergence of systems biology and mathematical modeling, encompassing graph theory, and neural networks, has facilitated the integration of fundamental cellular components (such as genes, RNAs, proteins, etc.) into a unified intricate network[51]. Using this systematic approach, we provided valuable insights into the network responses of differentially expressed genes with lethal doses of antibiotics treatment at different time points (Fig. 3 a). We constructed the gene network and identified major clusters of genes using the molecular complex detection (MCODE) method[52], which detects highly densely connected nodes in a network. We found a significant fluctuation of gene network response at different time points: significant variation of edges (connections) (Fig. 3 b i) and the associated nodes (genes) that form gene clusters (Fig. 3 b ii and Fig. S2 a-c) at different time points. The number of upregulated genes was higher at 6 h compared to 3 h and 24 h treatments. In comparison, the number of downregulated genes was significantly lower at 24 h, indicating a fluctuation of gene network response (Fig. 3 b ii). The stable genes in the clusters may be responsible for maintaining the dormant state, while the downregulated genes may play a role in preserving cellular resources during dormancy. Notably, it is predicted that a network or cluster will undergo minimal to no significant alterations under normal conditions. However, when subjected to external perturbations, there is a high likelihood of experiencing substantial changes in gene expression and network reconfiguration. Based on these observations, we have two competing hypotheses:
Hypothesis 1: Long-time antibiotic exposure may significantly alter network response.
Hypothesis 2: Little or no alterations in the network response. In other words, any variations observed in the network's behavior are due to random chance or noise rather than deliberate or systematic changes in gene expression or network response.

Network analysis shows a dramatic change in gene network response (Fig. 3 b and Fig. S2 a-c). This means that the overall network connectivity of a gene has a greater influence on its expression, and it seems likely that multiple genes or pathways influence how bacteria survive against stress. These results support Hypothesis 1, that multiple persister subpopulation exists, and due to stress, network reconfiguration occurs. This happens due to a 'one-to-many' gene interaction rather than a 'one-to-one' interaction, facilitated by cell-state transitions and network switching. Similar to cancer cell metastasis[53, 54], the dynamic network switching of genes may allow reversible phenotypic plasticity (cell-state transition), causing resistance to drug and survival benefits to the cells. Next, we focused on identifying the subset of genes with the strongest control in the entire network and the potential roles of the key genes (Table S1) in the network at different time points.

Network centrality analysis highlights potential key hubs. Our goal was to identify key gene hubs that are important to surviving antibiotics. We analyzed each gene's network centrality (such as degree, betweenness, and closeness) to demonstrate the gene attributes in a network. These classic network centrality[55] initially developed for characterizing social networks that have been transposed to biological networks to determine the hierarchy of nodes or edges in an intricate network. Thus, it provides information on a gene's importance in a central network based on connectivity, or information flow throughout the network. A high centrality score indicates that a node has a larger-than-average number of connections in a network. We compared the centrality score of 27 genes we identified that are commonly

upregulated throughout a time course of antibiotic treatment to the entire gene sets of the network at different time points. One of the key aspects of our analysis was the comparison of the centrality scores of differentially expressed genes with the average centrality score of all genes within the network. At 3 h, a substantial 23% of expressed genes showed high betweenness centrality scores (Fig. 3 c), while at 6 h and 24 h, the proportion decreased slightly to 21% (Fig. S3 i-ii).

Similarly, when considering closeness centrality, ~33% of genes displayed high scores at 3 h (Fig. 3 c). This percentage slightly diminished to ~32% at 6 h and ~31% at 24 h (Fig. S3 i-ii). These results indicate that a substantial portion of genes maintains a central position regarding their ability to interact with other genes within the network rapidly. A robust node (genes) plays a significant role as this node is minimally affected by disruption of other nodes in a network[56], and this might be the central gene for bacterial persisters. What's particularly intriguing is the overlap between genes showing high betweenness and closeness centrality at all time points. Most of the commonly upregulated genes (excluding the hypothetical genes) showed high betweenness and closeness centrality, suggesting their importance to persister survival. This observation suggests that these genes might play critical regulatory roles early in the process, thereby contributing to the long-term survival of persister cells.

It was unsurprising that 8 genes of the 27 commonly upregulated genes showed lower centrality scores than all genes' average scores because they are hypothetical genes. Hypothetical genes often represent novel or less-studied elements within the network; thus, we often do not know what controls their expression or how their protein productions interact with the expression of other genes. The involvement of these genes should not be disregarded, as they might serve as vital regulatory players in processes yet to be fully understood. But it does highlight the importance of studying how hypothetical genes are related to persisters. In addition, we recognize that the multi-pronged network approach and the resulting data have limitations and can potentially identify false positives that can compromise the prediction of essential nodes in a network. So, experimental validation of this data is indispensable. We considered this aspect by investigating 7 hypothetical genes, recognizing their contribution to the persister survival through wet-lab mutational studies and overexpression experiments (Fig. 4 a-b).

Identifying gene clusters with potential biological functions. We investigated the roles of various genes at different time points by integrating biological interpretation and functional gene grouping by ClueGO, a Cytoscape plug-in[57, 58] (Table S2 and Fig. S4). We focused on known oxidative, cellular, and antibiotic response genes. We found gene (both up and downregulated) clusters with significant fluctuation at different time points (Table S2), suggesting a potential shift in the cellular dynamics or adaptation mechanisms. At 6 h, the highest number of upregulated genes known to be related to oxidative, cellular, and antibiotic response genes were identified. Surprisingly, no known oxidative, cellular, and antibiotic response genes were upregulated at 24 h, This indicates that other genes are more important at 24 h, or the oxidative, cellular, and antibiotic response proteins produced at 6 h were sufficient (intact and functional) to remain by 24 h, thus no increased expression is required. Our observations strongly suggest that the response of genes to antibiotic treatment is dynamic, and the changes in the network response are possibly linked to the long-term survival of persister cells.

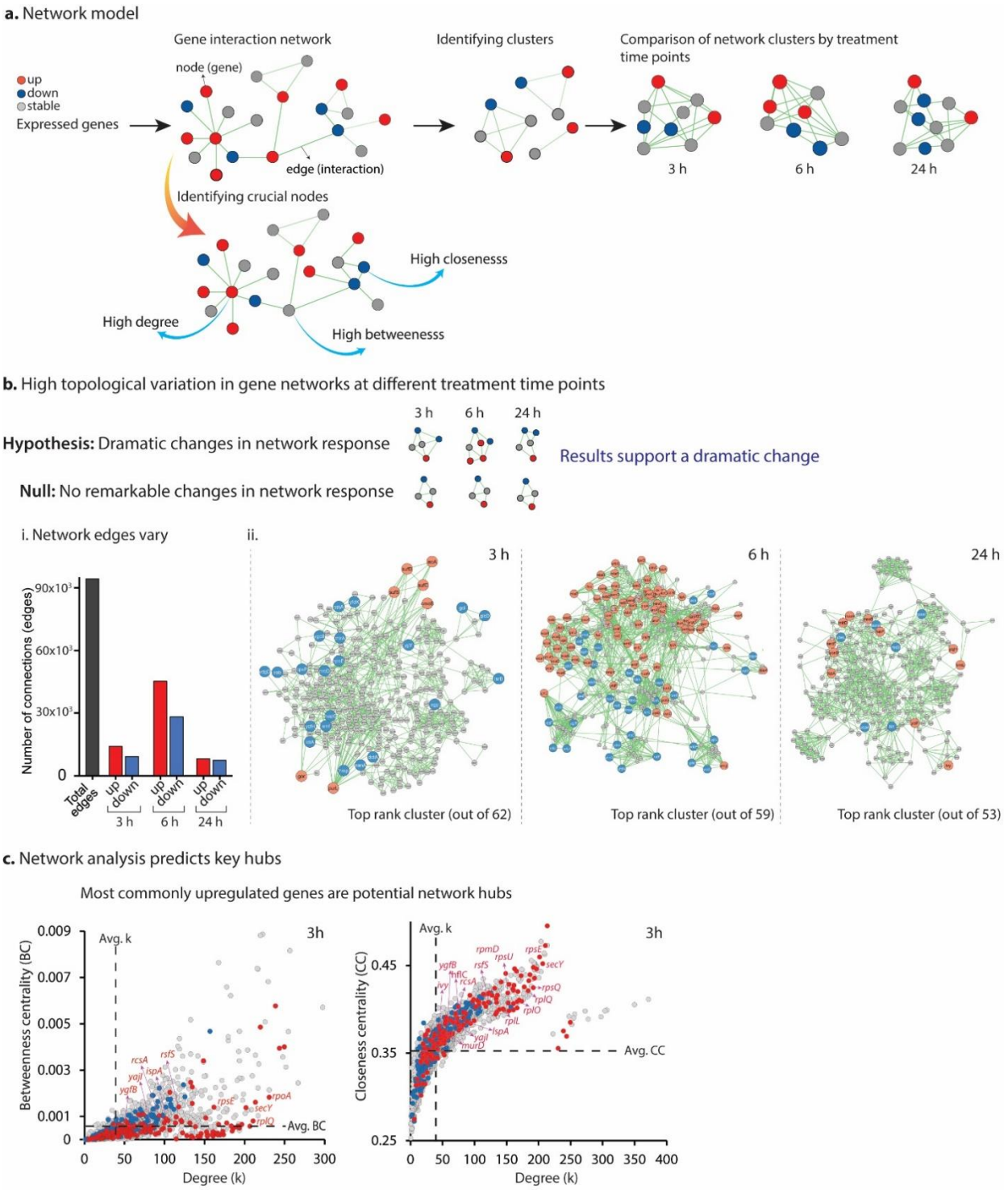


Fig. 3. a. This figure illustrates the construction of a gene interaction network model using upregulated, downregulated, and stable genes. Each gene is represented as a node, and the interactions between genes are depicted as edges. The network was analyzed using MCODE to identify gene clusters at different time points. The clusters were then compared to showcase variations in the gene network responses. Key nodes within the network were identified through centrality analyses involving node degree, betweenness, and closeness. **b.** Network analysis shows dynamic topological variation in gene networks at different treatment time points. **i.** Significant fluctuation in edges: comparing the number of edges within the

network highlights fluctuations relative to the total number of edges. At 6 h treatment time-point, the number of edges is higher than 3 h and 24 h, indicating dynamic network changes. **ii.** Top-ranking clusters are shown at three distinct time points: 3 h (total clusters: 62), 6 h (total clusters: 59), and 24 h (total clusters: 53). Here, we highlight the top rank cluster for each time point, and the remaining clusters for each time-point shown in Fig. S2 a-c. At 3 h, the top rank cluster constituted 321 nodes and 1,115 edges, and the expression of genes vastly fluctuated (i.e., stable: 293; up: 7; down: 21). At 6 h, 278 nodes and 1,882 edges were detected with variable gene expression (i.e., stable: 246; up:19; down:13); and 24 h the top rank cluster was associated with 242 nodes and 914 edges with highly fluctuated gene expression (i.e., 229, up: 6, down: 7). The results indicate that the total number of nodes and edges of the entire networks varied at 3 h, 6 h, and 24 h. **c.** Network analysis predicts key hubs. Identification of potential network hubs at 3 h: Degree (k) vs. Betweenness Centrality (BC) and Degree (k) vs. Closeness Centrality (CC) analyses identify commonly upregulated genes as potential network hubs. The remaining 6 h and 24 h centrality analysis is described in Fig. S3 i-ii. Degree centrality refers to the distribution of nodes' degrees (number of connections) in a network. It provides insights into the network structure and can reveal important connectivity, centrality, and overall network behavior characteristics. Betweenness centrality (also known as a bottleneck) quantifies how often a node appears on the shortest paths between pairs of other nodes in the network[59], whereas closeness centrality quantifies how quickly a node can interact with other nodes in the network[60].

Overexpression of hypothetical genes decreases growth rate. We cloned all the selected hypothetical genes under IPTG/Ara promoter (Fig. 4 b i). When we overexpressed the genes, most of them had significant growth defects. This shows that overexpression disrupts normal cellular processes. Notably, the overexpression of two genes, *yjbE* and *yajI*, led to the cells entering a nondividing state. This state lasted several hours before cells began growing, likely because of a mutation. To explore this, growing cells were subcultured without an inducer and then tested to see if they went into a non-dividing state again. Induced and uninduced cultures had no growth defects, suggesting a mutation (data not shown). Given the well-established connections between the growth rate and both short-term and long-term survival under antibiotic stress[61-64], we chose not to employ overexpression studies to investigate these genes' involvement in persistence. Instead, we employed gene editing to create knockout mutants, enabling us further to explore the role of these hypothetical genes in persistence.

Knockout of hypothetical genes reduces persister survival under antibiotic treatment initially (3 h and 6 h), but not in the long run (24 h or longer). We focused on hypothetical genes not previously shown to play a role in persistence. We first mutated hypothetical gene *yjbE* because of its dynamic expression profile, and it showed the highest expression levels at 6 h (>10-fold) and 24 h (>7-fold) of all the hypotheticals. The knockout mutant was constructed CRISPR/cas9-based genome editing tool[65] (we modified the plasmid and the protocol for this knockout to get the mutants in our strain of *E. coli*, see Methods) to test the effect(s) of the given genes at different time points (Fig. 4 b ii-iii). The *yjbE* knockout mutants did not show any growth defects under normal conditions, which contrasts with the growth defects observed upon gene overexpression. We wanted to know if the other hypothetical genes would act similarly, so we knocked out all of them but *ycfJ* and *ygfB* because getting those knockouts were challenging. All seven knockout mutants did not show any growth defects under normal conditions, which contrasts with the growth defects observed upon gene overexpression. This outcome was unsurprising, given that we grew the cells in defined media with ample nutrition and a well-controlled environment. If these genes are associated with persistence or environmental stress survival, it would be unlikely to observe growth defects under optimal environmental conditions. However, under lethal antibiotic stress, based on our transcriptional analysis, we would expect a dramatic decrease in persister levels in the mutants.

As expected, there was a dramatic decrease in persister levels in the mutants following Amp treatment compared to the wild type: 4-5-fold reduction at 3 h, and 10-14-fold reduction at 6 h (Fig. 4 b ii-iii).

Remarkably, the persister levels in the mutants did not significantly differ from the wild type at 24 h. This result supports the existence of multiple persister subpopulations in an isogenic *E. coli* population, and different persister subpopulations have unique survival mechanisms, each associated with varying decay rates. This led us to propose two plausible hypotheses (Fig. 5a):
(1) Within a given population, multiple phenotypes of persisters result in different subpopulations, and each phenotype dynamically stems from non-persisters. These distinct phenotypes emerge during the logarithmic growth phase or the rapid death phase (of the biphasic death curve), given that susceptible cells perish by the end of the rapid death phase.
(2) One persister phenotype forms dynamically from non-persister populations and subsequently adapts sequentially or non-sequentially to multiple other phenotypes. This could happen during the fast or slow death phase.

Of these two hypotheses, we favor the second one, which involves an adaptive response, as it aligns with our transcriptomic findings. This intriguing possibility suggests that the persister state is highly dynamic and adaptable, and cells have multiple strategies to survive antibiotic stress. Intuitively, both persister and non-persister cells undergo cell-state transitions, giving rise to various persister phenotypes that facilitate bet-hedging and resistance to antibiotic onslaught. However, with the current data, it remains unclear whether the occurrence of various persister phenotypes is a result of random or reversible phenotypic switching, and if these transitions occur before or after antibiotic treatment.

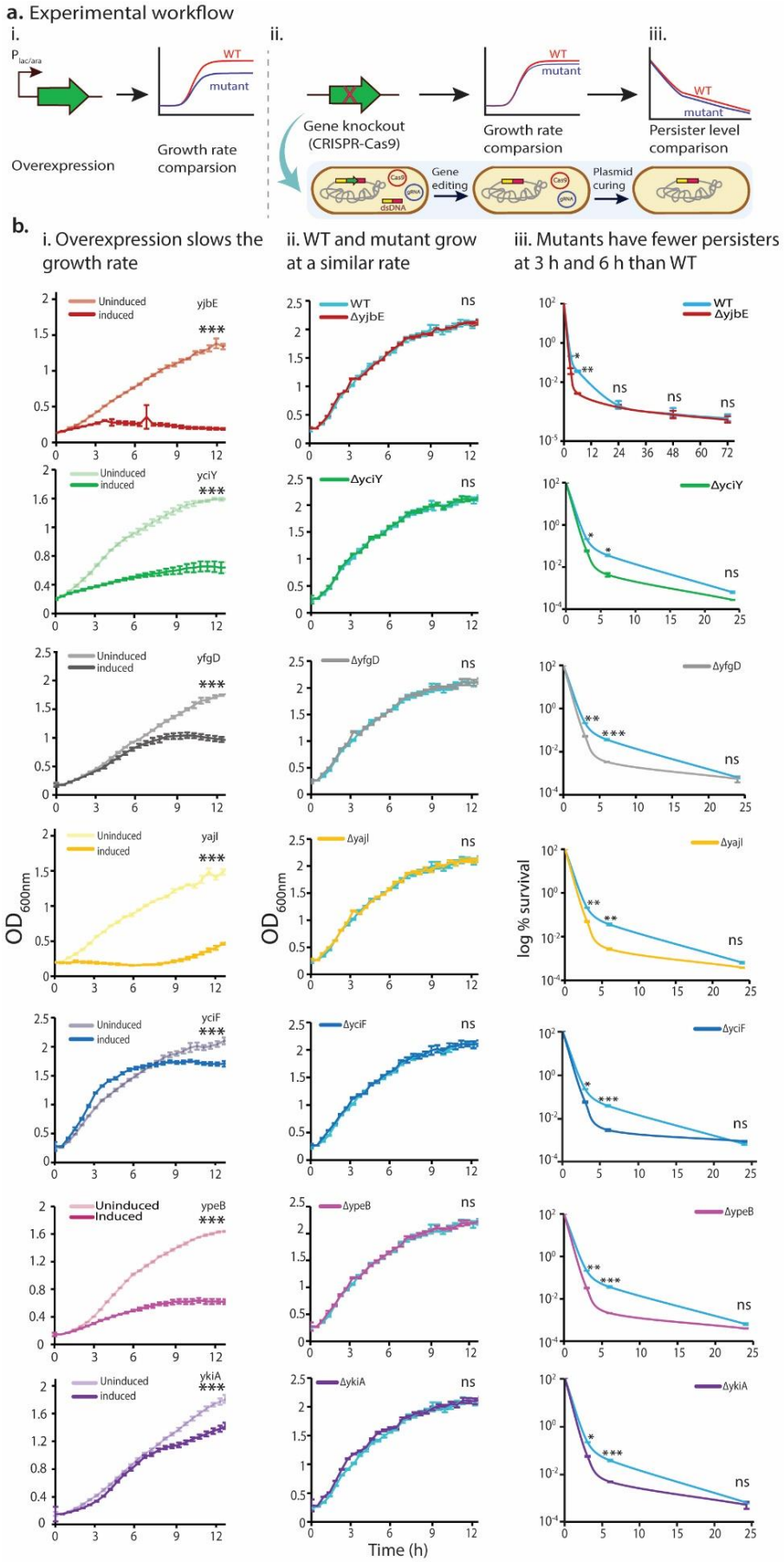
Mutants and wild-type strains showed similar growth rates (Fig. 4b ii), suggesting that any difference in their death rates would be unrelated to their ability to grow and divide. We observed significant variations in persister levels at different time points between wild-type and mutant strains (Fig. 4b iii), illustrating the presence of multiple subpopulations of persisters. When a gene is removed, consequently disrupting certain survival mechanisms, it eliminates persister phenotypes reliant on those mechanisms, leading to decreased early time point persistence levels (at 3 h and 6 h). This phenomenon accounts for the observed reduction in early persistence among mutant strains. Furthermore, it provides compelling evidence that the selected hypothetical genes regulate the persister cell's long-term survival and antibiotic stress response. However, this decrease does not seem to have a significant long-term effect on the cells. In the long run (24 h to 72 h), the persister phenotypes with the slowest decay rates survive regardless of gene disruptions.

Generally, the identification and characterization of persister cells have been based on biphasic death curves[66]. However, the binary nature of this curve may not accurately represent the diverse array of decay rates among persister cells. A recent study demonstrated the advantages of employing multi-phase killing curves. Depending on the intricate interplay of decay rates within persister cell subpopulations, these curves can manifest in various phasic (triphasic, tetraphasic, etc.) patterns. This approach provides more accurate information as it accounts for bacterial survival times that cannot be properly predicted through a singular focus on a biphasic death curve[64]. Our results suggest that the long-term behavior of persisters resembles those of multi-phase killing curves, featuring multiple decay rates (Fig. 4b iii and Fig. 5b). These findings indicate that persister cells are not a homogeneous entity, but consist of multiple distinct subpopulations, each with its unique survival characteristics and decay rates. These challenge the "one-size-fits-all" approach as it has become increasingly evident that persister cells are heterogeneous. They do not conform to a uniform response to antibiotics, which a simple biphasic curve might imply. Instead, the presence of multiple decay rates within persister populations highlights the need for a more subtle and comprehensive approach to understand the intricate mechanism of these cells.

We developed a simple mathematical model (described in the Methods) to explore further the variations in decay fraction rate between *E. coli* wild type and mutant strains in response to lethal antibiotic treatment. We plotted the bacterial survival fractions over time, showing that different persister subpopulations had distinct decay rates. Notably, the slow-decay fraction of the mutant strain exhibited a

significant 20-fold decrease, emphasizing the importance of decay kinetics in bacterial survival. This result again supports our hypothesis that multiple subpopulations of persisters exist, each with distinct decay rates (Fig. 5b).

It is well established that persisters form due to phenotypic heterogeneity in the general *E. coli* population. We identified here that the persister population itself has its own heterogeneity and can switch between different cell-states. This heterogeneity (diversity) in the persister population allows subpopulations of persisters to survive antibiotic stress, but they die at different times, which is evident by the different decay rates.


a. Experimental workflow
i.
ii.
iii.
Overexpression
Growth rate comparsion
Gene knockout (CRISPR-Cas9)
Growth rate comparsion
Persister level comparison
Gene editing
Plasmid curing
b.
i. Overexpression slows the growth rate
ii. WT and mutant grow at a similar rate
iii. Mutants have fewer persisters at 3 h and 6 h than WT
Uninduced
induced
yjbE
yciY
yfgD
yajI
yciF
ypeB
ykiA
WT
ΔyjbE
ΔyciY
ΔyfgD
ΔyajI
ΔyciF
ΔypeB
ΔykiA
ns
OD600nm
log % survival
Time (h)

389

Fig. 4. a. Experimental workflow. **i.** Hypothetical genes were under an inducible promoter ($P_{lac/ara-1}$) and in *E. coli* DH5αZ1 and grown with or without arabinose; uninduced: 0% induced: 0.2% arabinose. **ii.** Hypothetical genes were knocked out using the CRISPR/CAS-9 system, and the growth rate of each mutant was compared with the wild-type strain. **iii.** The persister level of the mutant was compared to the wild type at 3 time points: 3 h, 6 h, and 24 h (normalized by time 0 for each strain). **b.** Results: **i.** Overexpression of the hypothetical genes slows growth or causes cells to enter a non-growth state. **ii.** Wild-type and knockout mutant growth rates are nearly identical in the same media. **iii.** Persister level is significantly reduced in mutants at 3 h and 6 h compared to wild type. However, no significant decrease was observed at 24 h. Persister level comparison was shown up to 72 h for *yjbE* gene, and 24 h for all other mutants. These results show that the 7 hypothetical genes help cells survive in the first 6 h but are not helpful by 24 h. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$.

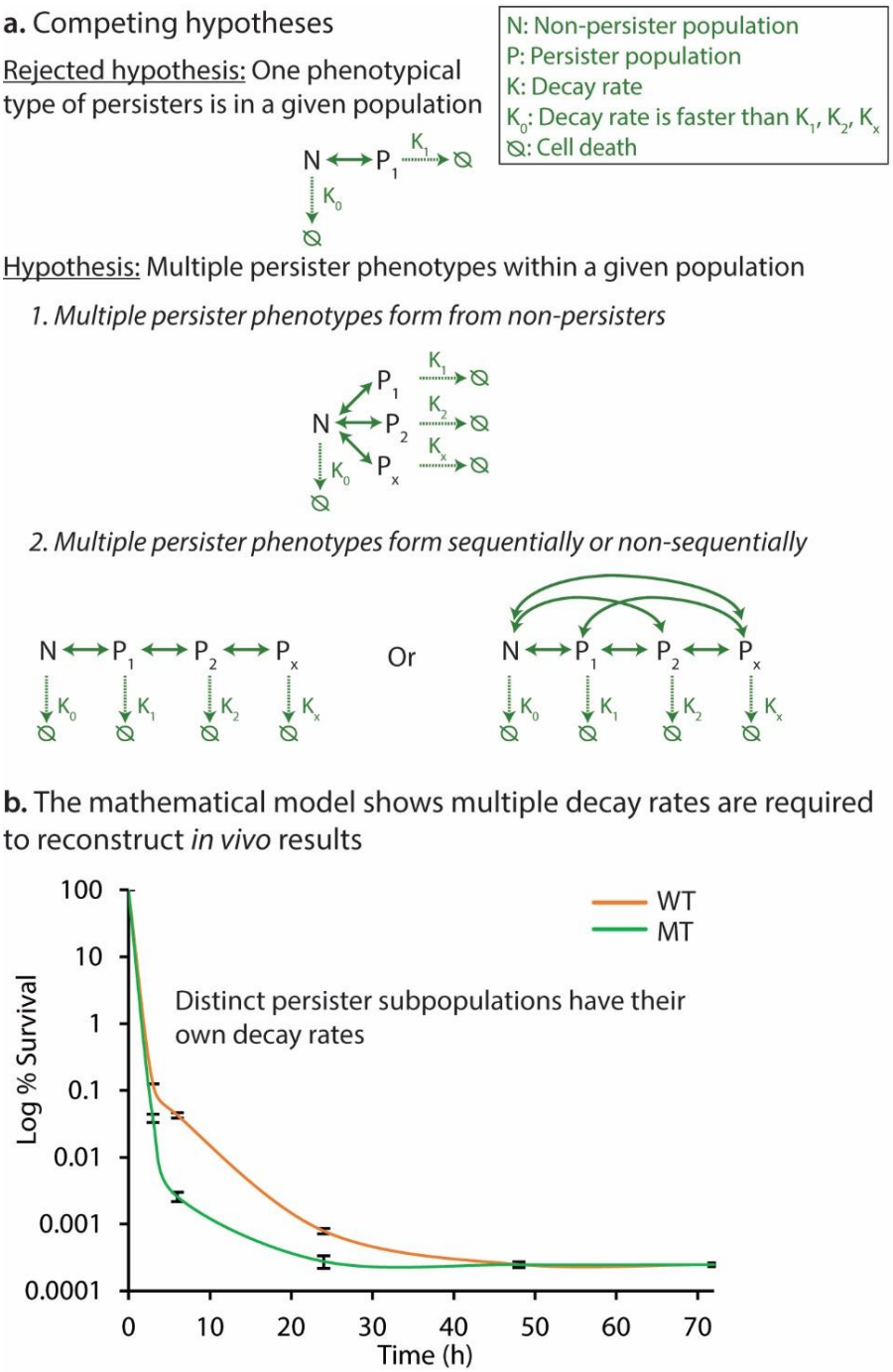


Fig. 5. a. Model figures demonstrating competing hypotheses for multiple persister subpopulations and decay rates a. Null hypothesis: Only one phenotypical type of persisters exists within a population. The persister population (P) is formed from the non-persister population (N). Non-persisters and persisters alternate in the process of persister formation and revival. Alternative hypothesis: (1) Multiple persister phenotypes (P_1, P_2, P_X) exist, and all phenotypes form from non-persisters (N). (2) A single phenotype of persisters forms from non-persister cells, and eventually, that phenotype sequentially or non-sequentially adapts to other phenotypes. Persister formation and revival involve a dynamic interchange between non-persisters and persister subpopulations and simultaneous alternation within the persister subpopulations. In any scenario, non-persisters (susceptible cells) have the highest decay rate

(K_0) compared to the decay rate of any persister subpopulation. **b.** The plot shows the dynamics of the bacterial surviving fraction in response to lethal antibiotic exposure as predicted by the model (see Methods). The model was constructed considering three different subpopulations: a fast-decaying (non-persister population (N) having susceptible and tolerant cells), a slow-decaying (persister subpopulation 1 (P_1)), and a very slow or non-decaying population (persister subpopulation 2 (P_2)). The decay rate of the wild type and the *yjbE* mutant was calculated individually and concurrently, showing a 20-fold decrease in the slow-decaying fraction (P_1) in the mutant, while the non-decaying fraction (P_2) remains the same in the mutant. It indicates that multiple and distinct persister subpopulation maintain their unique decay rates. The plot axes represent percent survival in log scale (Y-axis), time (h) (X-axis).

Outlook

Persisters are often described as metabolically dormant and controlled by one or a few sets of genes[15, 16, 67]. However, although persisters are dormant with respect to growth, they render an active response against antibiotics[16, 20]. Here, we argued that persisters have an altered cellular activity rather than being in a dormant state. We mutated 7 hypothetical genes and observed their effect on persister survival, which validated the biological relevance of our transcriptomic analysis. Our result also shows that not a single gene is responsible for persister formation; it's a total network response, and persister can adjust their transcriptome to survive lethal antibiotic stress. Our 7 mutational experiments and mathematical modeling results clearly demonstrate that more than one subpopulation of persisters can develop from an isogenic *E. coli* population. Another line of evidence supporting this claim is obvious; persister cells continue to die at a slow decay rate (Fig. 1, see death curve). If persisters were of one identical population, the population should die relatively at the same rate. But we always see a slow decay rate because different subpopulations die quicker than others.

What is the selective advantage of having multiple subpopulations of persisters? We propose that, logically, this is a good strategy for survival. Antibiotic-resistant bacteria have molecular mechanisms available to detect a specific threat, respond, and allow for continued growth. However, non-resistant bacteria do not contain the genes to detect a particular threat, so individual cells react to the danger in their own manner. Most cells die, but those that can persist will survive longer. Some persister cells (a subpopulation) have responded in a manner that will allow them to survive longer than other persister subpopulations. This is what our results suggest. It is also possible that one subpopulation of persisters is converting to another subpopulation of persisters, but currently, we have no evidence for this. A simple analogy to this scenario is to imagine boats at sea encountering a devastating, fast-moving storm. An aircraft courier may be large enough and stable enough to weather the storm; they are resistant to it. Small boats would not be resistant to the storm but can be persistent by different manners. For example, boats may speed in different directions, and those who move away from the storm will survive longer than those who stay put or head into the storm. Those who move completely out of the storm have a higher chance of surviving than those who only run in front of it because it may eventually catch them. Others may radio for help, and so on. Bacterial persisters sample different methods to survive, and this heterogeneity in response is what makes them so difficult to kill.

We have previously hypothesized that the presence of a single persister gene is unlikely, but disruption of essential cellular networks leads to persister formation. The results shown here support this. We also hypothesize a "ground state" where cells have entered a non-growing, reduced activity state, but are not quite persisters. Once in the ground state (a transition stage), cells can shift into persistence (a more dormant state) or return to the dividing phase. Cells can reach the ground state by several different means, but the ground state occurs when cells encounter internal or external stress. In this manner, the condition that the cell entered the ground state determines the type of persister that will form. We make this hypothesis based on the fact that persister-related genes are constantly identified, and if one mechanism is reasoned for persister formation by one research group, another's group disclaimed that mechanism[42-46, 68](suggesting not one gene is responsible for persister formation). The minimal genome contains mainly

essential genes (less than 500 genes) and still form persisters (other microbes contain genes that have been to be persister-related, but the minimal genome lacks them)[69], slowed growth either externally[64] or internally[2, 70] appears to increase persister levels (showing that both internal and external stress alters persister levels). Our results clearly show that more than one subpopulation of persisters develops because of different stress response genes. These genes affect the type of persister and how long they will survive antibiotics.

In addition, persisters are present at substantially higher levels in stationary phase compared to log phase[71]. Although different phases can be identified in batch cultures (in this order): lag, log, stationary, death, and long-term stationary (LTS phase), it has been proposed that many bacteria exist in the LTS phase longer than any other phase in natural environments[72, 73]. *E. coli* cultures have been retained in LTS stage (without additional nutrients) for more than 5 years while maintaining viability[72]. Although persisters were identified first, persisters and LTS cells share similar phenotypes. They are unlikely to be in the same state because LTS have been shown to mutate to survive, while persisters can survive without mutation. It is possible that LTS could be cells in the ground state. Here, we specifically use the term "ground state" because the term describes a low-activity state. Though we do not have enough evidence to declare the "ground state" hypothesis correct, we have ample evidence to put it forward to the scientific community.

Methods

Microbial strains and media. *Escherichia coli* DH5αZ1 containing the p24KmNB82 plasmid was used in this study. DH5αZ1 is a derivative of *E .coli* K12 strain, and it has been used in our previous persistence studies[24, 74]. The cultures were grown in MMB+ as described in our previous work[11, 24]. We used 0.5% glycerol as the carbon source and each of the 20 canonical amino acids (0.04 mg/mL) for fast-growing cultures. Cultures were grown in MMB+/kanamycin (Km, 0.025 mg/mL) or on Miller's lysogeny broth (LB) agar plates/Km (0.025 mg/mL). All cultures were incubated at 37 °C and shaken at 300 rpm.

Persister assay. The assay was done as previously described[11, 24]. Briefly, overnight cultures were diluted 1/100 into fresh media and grown until they reached between OD_{600} ~0.1- 0.2. A reduced volume of culture (10 mL) was aliquoted into three 100 mL flasks, and grown for ~2-3 h until the culture reached to mid-log phase having ~1E+8 CFU/mL (~OD 0.5). After that, the culture was treated with a bactericidal concentration of Amp (0.1 mg/mL) for 3 h at 250 rpm, 37°C. Persister percentage was calculated by comparing CFUs per milliliter (CFU/mL) before antibiotic treatment to CFU/mL after antibiotic treatment. Plates were incubated at 37ºC for 48 h, then scanned using a flatbed scanner. Custom scripts were used to identify and count bacterial colonies[75, 76]. For the time-kill curve, antibiotic treatment was extended to 24 h, and the number of persisters was recorded at 3 h, 6 h, and 24 h.

RNA sample preparation and sequencing. We used our previously developed methodology to harvest RNA from persisters[20]. Cultures are first treated with lethal Amp (0.1 mg/ml) concentrations, and their transcriptomes are isolated and compared to an untreated population. In brief, **s**amples were treated with Tween (0.2%) and chloramphenicol (0.005 mg/ml) then immediately filtered out of solution (using rapid filtration) and resuspended in lysis buffer containing 25 mM Tris pH 8.0, 25 mM NH4Cl, 10 mM MgOAc, 0.8% Triton X-100, 0.1 U/µL RNase-free DNase I, 1 U/µL Superase-In, ~ 60 U/µl ReadyLyse Lysozyme, 1.55 mM Chloramphenicol, and 17 µM 5′-guanylyl imidodiphosphate (GMPPNP); Rapid filtration allows for the harvesting of any whole-cell; this minimizes RNA degradation because samples are immediately frozen in liquid nitrogen have suspension in lysis buffer. The solution was then frozen in liquid nitrogen and stored at − 80 °C. Frozen pellets were thawed on ice and centrifuged for 20 min at 16,000×*g* at 4 °C with 0.3% Sodium Deoxychorate to pellet particulate matter. The supernatant was then removed, and then RNA was column purified (Qiagen miRNeasy cat. No. 217004) before removing

rRNA (Invitrogen RiboMinus cat no. K155004) and prepping the cDNA libraries (NEBNext Ultra II cat no. E7103S). Libraries were multiplexed, then pooled and sequenced by Illumina HiSeq paired-end sequencing by GENEWIZ. The data is analyzed using a combination of Geneious Prime NGS software[77], custom Python scripts modified from our previous transcriptome analyses[78], and the DESeq2 R package[79]. We consider significant changes in gene expression to be greater than two-fold with an adjusted p-value <0.1. InteractiVenn[80] was used to generate Venn-diagram.

Network construction and data analyses. We constructed the network and retrieved nodes degree, betweenness and closeness centrality scores using Cytoscape (v3.10.0) StringApp (v2.0.1)[57, 81, 82]. Molecular Complex Detection (MCODE)_v2.0.3 method[52] was used to identify the molecular clusters of the entire network. MCODE used the graph theoretic clustering algorithm to identify densely connected regions in a large protein-protein interaction network (PPIn) representing molecular complexes[52]. We also investigated the functional roles of oxidative, cellular, and antibiotic stress response genes through ClueGO (v2.5.10), a Cytoscape plug-in[57, 58]. We used default parameter values during network construction via String App (i.e., Network type: full String network, Confidence cutoff: 0.4) and selective parameters values during MCODE analysis like, Find Clusters: in Whole Network; Network Scoring - a) Include Loops: Turn off, b) Degree Cutoff: 10; Cluster Finding- a) Haircut: Turn on, b) Fluff: Turn off, c) Node Score Cutoff: 0.2, d) K-Core: 2, e) Max. Depth: 100. During ClueGO analysis, default parameter values were used such as Analysis Mode: Functional Analysis, Visual Style: Groups, Ontologies: Biological and cellular process.

Overexpression of hypothetical genes. Since our control strain contains the plasmid p24KmNB82 (CFP-LAA), we cloned the seven selected hypothetical genes under the same promoter ($P_{lac/ara-1}$) and ribosome binding sites as in REF[83]. Our control strain Dh5αZ1 produces two proteins (genes are on the genome), AraC and LacI, which are used to control the expression of genes through the $P_{lac/ara-1}$ promoter. AraC is an activator in the presence of arabinose, while LacI is a repressor of this promoter. Isopropyl β-D-1-thiogalactopyranoside (IPTG) acts as a repressor of LacI, thus allowing for activating the $P_{lac/ara-1}$ promoter. We used both arabinose (0.2%) and IPTG (0.100 mM) to express the hypothetical genes.

Knockout of hypothetical genes. To knockout the hypothetical genes, we used the clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease 9 (Cas9)-based genome editing tool pEcCas/pEcgRNA system explained in ref[65], but had to modify the protocol and plasmid to get the system to work efficiently with our *E. coli* strain. The pEcgRNA plasmid was originally designed for spectinomycin selection; however, the *E. coli* strain we used in our work contains a streptomycin resistant gene. To facilitate effective selection, the pEcgRNA plasmid was modified by introducing an ampicillin-resistant gene at the BglII/XbaI restriction sites. In brief, 0.040 mL of pre-induced (with 1% arabinose) competent cells containing pEcCas plasmid was added with pEcgRNA plasmid (200 ng - 800 ng), and donor dsDNA (200 ng - 800 ng). Then, the mixture was electroporated in a precooled 2 mm Gene Pulser cuvette (Bio-Rad, Hurcules, USA) at 1.8kV, 25uF, 200 ohms. After electroporation, freshly prepared 1mL of SOC media (containing 1.5% arabinose) was added, and the culture was shaken for 1 h at 250 rpm, 37°C. Then, the culture was plated on LB/Amp (0.1 mg/mL)/Km (0.05 mg/mL) plates and incubated at 37 °C for 24 h. Each knockout was confirmed by colony PCR and sequencing. After the knockout, the edited clone was inoculated into 10 ml of LB with Km (0.05 mg/mL), and 100 mM rhamnose and grew for 12 hours at 37 °C to cure pEcgRNA plasmid. Then, the culture was again transferred to 10 ml of LB with no antibiotics and grew for overnight at 37 °C to cure pEcCas plasmid. Then, the culture was plated on LB agar with 5% sucrose plate and incubated overnight at 37 °C. Finally, ~48 colonies from sucrose plates were streaked on LB, LB/Amp (0.1 mg/mL), and LB/Kan (0.05 mg/mL) plates and incubated at 37 °C

overnight. Colonies that only grew on LB plates were selected as our knockout strain. Finally, we transformed the p24KmNB82 plasmid[24] in each knockout strain to keep it similar to our control strain.

Determination of growth curve. Overnight cultures were diluted into OD 0.1 (measured in SpectronicTM 200E), and 0.03 mL of diluted cultures were inoculated into individual wells containing 0.27 mL of SP16 media in a 96-Well Optical-Bottom Plate with Polymer Base (Thermo Fisher) to measure OD at 600 nm using FLUOstar Omega microplate reader.

Mathematical model. To model the dynamics of the bacterial surviving fraction in response to lethal antibiotic exposure, we consider three different subpopulations: a fast-decaying (non-persister population having susceptible and tolerant cells), a slow-decaying (persister subpopulation 1), and a very slow or non-decaying population (persister subpopulation 2). In persister subpopulation 2, we considered the time scale of decay to be zero in the experimental timespan since the death rate is so low after 24 h based on our experimental result. But it is notable to mention that in real scenarios, the decay rate cannot be zero because no cells can live forever, and at some point, cells will die. However, let the fraction of cells in these populations be f_1, f_2 and f_3, respectively, with corresponding decay rates of γ_1, γ_2, and 0. The fraction of surviving cells $f(t)$ at time t after antibiotic exposure is then given by:

$$f(t) = f_3 + f_2 e^{-\gamma_2 t} + +f_1 e^{-\gamma_1 t}$$

with $f_1 = 1 - f_2 - f_3$. The parameters $f_3, f_2, \gamma_2, \gamma_1$ are estimated by performing a least square fit of $f(t)$ with data.

We initially fitted this equation to data from WT and the yjbE mutant individually and realized the biggest difference was in f_2, the fraction of cells in the slow-decaying state. Next, we fitted the data from both WT and mutant simultaneously, keeping f_3, γ_2, γ_1 fixed between the two genotypes while allowing f_2 to vary between them. Our fitting yields $f_3 \approx 2.5 \times 10^{-6}, \gamma_1 \approx 2.6\, hr^{-1}, \gamma_2 \approx 0.24\, hr^{-1}$ with $f_2 \approx 2 \times 10^{-3}$ (for WT) and $f_2 \approx 10^{-4}$ (for mutant). The model fits are shown in Fig. 5b, where the change in a single parameter, a 20-fold decrease in the slow-decaying fraction in the mutant, is sufficient to capture the decay kinetics of bacterial survival.

Statistical analysis. All data is presented in the manuscript as mean ± SEM of at least three independent biological replicates. Statistical significance was assessed using an f-test to determine variance ($p < 0.05$ was considered to have significant variance), followed by a two-tailed t-test with unequal variances (if F statistic > F critical value) or equal variances (if F statistic < F critical value).

Competing interests

No conflict.

Authors' contributions

K.R., T.H., R.A., performed the experiments, and ran data analysis. A.S. constructed mathematical modeling. N.C.B. and T.H. formalized the research concept and designed the experiments as well as planned and directed the project. All authors contributed to discussing and editing the manuscript.

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Data availability

All the data that supports the findings of this study are included in this published article (and its supplementary information files). Also, any additional data is available from the corresponding author upon request (email: nicholas.butzin@gmail.com).

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