Antibiotic tolerance is associated with a broad and complex transcriptional response in *E. coli*

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Summary:

Antibiotic treatment kills a large portion of a population, while a small, tolerant subpopulation survives. Tolerant bacteria disrupt the efficacy of antibiotics and increase the likelihood that a population gains antibiotic resistance, a growing concern. Using a systems biology approach to, we examine how transcriptional networks respond to antibiotic stress to survive and recover from antibiotic treatment. We are the first to apply transcriptional regulatory network (TRN) analysis to antibiotic tolerance in *E. coli*, by comparing gene expression with and without lethal concentrations of ampicillin and leveraging existing knowledge of transcriptional regulation. TRN analysis shows that changes in gene expression specific to ampicillin treatment are likely caused by specific sigma and transcription factors typically regulated by proteolysis. These results demonstrate that altered activity of specific regulatory proteins cause an active and coordinated transcriptional response that leverages multiple gene systems to survive and recover from ampicillin treatment.

Introduction

When disaster strikes, a coordinated response is required to mitigate the effects and survive in the face of adversity. Cells have developed an array of regulatory networks to coordinate cellular activity and respond to their environment. These regulatory events occur simultaneously and at multiple levels, including transcriptional, translational, and post-translational regulation (including degradation). The identification of individual regulatory interactions in combination with bioinformatics approaches has resulted in multiple databases detailing regulation throughout the cell, the most detailed databases are currently for *E. coli* (e.g. RegulonDB[1], EcoCyc[3]). The depth and availability of information regarding regulatory dynamics provide a new framework for analyzing sequencing data in the context of the whole cell. As such, these types of databases have been applied to analyze network topology[4] as well as experimental datasets[5-9]. Here we focus on RNA-sequencing data in the context of cell growth and antibiotic stress. The development and widespread use of antibiotics to treat infections relies on the ability to disrupt microbial systems and cause targeted cell death. Bacterial survival of antibiotics is therefore dependent on cellular networks adjusting to circumvent the physiological impact of the antibiotic.

Antibiotic tolerance, and more specifically antibiotic persisters, are a perfect example of how alterations in metabolic activity (i.e. gene expression and protein activity) lead to changes in cell phenotypes, which can increase the chance of survival. Persisters are a subpopulation of cells that tolerate antibiotics for long periods of time despite being genetically identical to susceptible cells. This surviving population provides a group of cells present after antibiotic treatment that can either mutate or acquire resistance genes from their environment[10-12]. Persistence has been studied extensively, but the heterogeneity of the persister population combined with the variance between antibiotic survival of diverse species and growth conditions has led to a number of different, often conflicting, models regarding the causative factors underlying persistence. Often persistence studies take the approach of using gene knockouts and overexpression to study specific elements of the persistence state[13-15]. Many studies suggest that persisters are metabolically repressed, particularly with respect to translation, while others propose that active metabolism plays a vital role in persistence[16-20]. A few studies have leveraged RNA-sequencing (RNA-seq)[20-22] and proteomic data[23-25], and they have identified several individual genes and systems related to antibiotic tolerance. Sigma factors and transcription factors, regulatory proteins

that affect gene expression, are two classes of protein in which many individual genes have been related to antibiotic stress[15,25-28]. The significance of these genes to antibiotic survival suggests that a broader regulatory network underlies the antibiotic stress response, and while understanding the role of individual genes in persistence is important, what is currently lacking is a more holistic understanding at the network level. While it has been a long-standing goal to identify a gene or a set of genes unique to antibiotic tolerance and persistence, our TRN analysis makes it clear that the transcriptional response to antibiotic treatment is controlled via a whole-cell, network response consisting of smaller, overlapping networks, some of which are redundant.

We have taken a systems biology, top-down approach to explore antibiotic survival of tolerant populations, and obtained deep, RNA-seq data on both antibiotic-treated and -untreated populations emerging from stationary phase. Our results show that cells have an active and coordinated response to lethal antibiotic stress that is associated with altered activity of specific sigma factors and transcription factors (TFs). Existing literature allows us to connect the activity of these regulatory proteins and changes in gene expression to the pathways involved in antibiotic tolerance and recovery. Our findings show that many of the regulatory proteins activated during antibiotic stress are regulated by protein degradation. Protein aggregation was recently demonstrated in persister cells[25], and our previous work shows that interfering with degradation at the protease ClpXP increases antibiotic tolerance in *E. coli*[29]. To confirm that the transcriptional changes are due to altered proteolysis, we then sequenced populations with slowed protein degradation using our previously established system[29]. The analysis of the TRNs in these populations provides evidence that slowed proteolytic processing of native proteins activates a stress response that promotes recovery once the stress is removed.

Results and Discussion

We will initially discuss RNA isolated from three different populations: *a stationary phase population*, which was diluted into fresh media and incubated for three hours without ampicillin (*untreated population*, Amp-), or with lethal concentrations of ampicillin (*treated population,* Amp+). We used rapid filtration to harvest cells followed by flash freezing in liquid nitrogen. Two advantages of this method are it allows for concentration of cells while minimizing time for RNA degradation, and we recover any whole cells present after antibiotic treatment regardless of viability (this includes cells that are considered “viable but not culturable” (VBNC), see Methods). This work builds off our previously reported results with antibiotic tolerance, and uses a similar setup with the same strains of *E. coli*[29]. The sequencing data was analyzed using DESeq and based on this analysis, we consider significant changes in gene expression to be greater than two-fold with an adjusted p-value <0.1. We began by examining changes in gene expression between the stationary phase population and the untreated population. The comparison of these two populations identifies how the cells adjust to their new environment, as diluting the cells in fresh media is a critical aspect of selection for ampicillin tolerance from stationary phase[30-32]. The majority of the genes with significantly different expression in the untreated population decreased from stationary phase (rather than increased), suggesting that cells are expending fewer resources in the new media. This phenomenon is likely due to the introduction of fresh nutrients and dilution of spent media resulting in a less stressful environment. Data for the untreated population provides information on the changes specific to the new environment; thus, we can focus on changes specific to antibiotic stress by identifying differences in gene expression unique to the treated population. These differences provide key insights into the molecular mechanisms of antibiotic tolerance and the role of transcriptional regulatory networks (TRNs) in antibiotic survival and recovery.

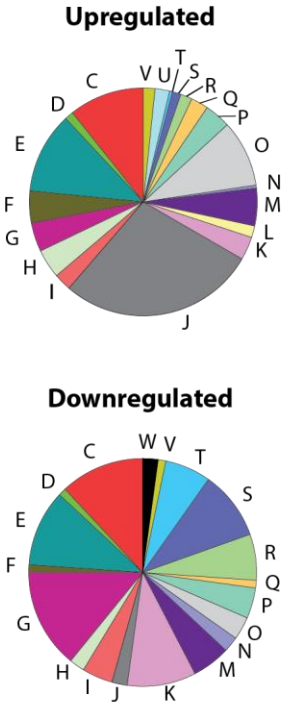
Clusters of Orthologous Groups (COGs) during ampicillin treatment

Using a top-down approach, we analyzed changes in gene expression based on the general functional classification, as defined by Clusters of Orthologous Groups (COGs)[2,33]. Although the

COGs classification system is limited to known functions, more qualitative than quantitative, and often used to study evolutionary relationships; it provides a practical method of grouping genes to identify notable trends in the data. When we analyze the COGs for gene expression unique to the ampicillin treated population, we find a few categories stand out (Fig. 1). The most upregulated category is translation, ribosomal structure and biogenesis (68 genes). While the accumulation of translational machinery has previously been shown in persister cells[25], it is important to note that we removed rRNA during the RNA-seq library preparation process and most of the genes in this category encode ribosomal proteins. Thus, we will not go into depth for this particular group to avoid potential biases in our datasets. The remaining groups with more than 20 upregulated genes are amino acid transport and metabolism (28 genes); energy production and conversion (26 genes); and posttranslational modification, protein turnover, chaperones (23 genes). We explore these groups further in the context of the TRN. While we focus on groups with known functions, seven genes categorized as "function unknown" or "general function prediction only" were also shown to be up regulated during ampicillin treatment, which suggests their function is related to survival or recovery (Fig. 1).

Sigma factor (σ) regulatory network shows increased activity of σ^{70}, σ^{38}, and σ^{32}

To contextualize changes in gene expression, we applied TRN analysis. To do so, we used all the available information from EcoCyc[3] on the direct regulation of all annotated genes. As such, this analysis is limited to regulatory interactions as defined at the time of analysis (File S1). To



Code	Functional category	Up	Down	Genome
C	Energy production & conversion	26	11	239
D	Cell cycle control, cell division, chromosome partitioning	3	1	30
E	Amino acid transport & metabolism	28	10	329
F	Nucleotide transport & metabolism	11	1	93
G	Carbohydrate transport & metabolism	10	13	353
H	Coenzyme transport & metabolism	10	2	148
I	Lipid transport & metabolism	6	4	108
J	Translation, ribosomal structure & biogenesis	68	2	205
K	Transcription	8	9	195
L	Replication, recombination & repair	4	0	109
M	Cell wall/membrane/envelope biogenesis	13	5	187
N	Cell motility	1	2	82
O	Posttranslational modification, protein turnover, chaperones	23	3	128
P	Inorganic ion transport & metabolism	9	4	199
Q	Secondary metabolites biosynthesis, transport & catabolism	6	1	67
R	General function prediction only	4	6	225
S	Function unknown	3	9	182
T	Signal transduction mechanisms	1	6	137
U	Intracellular trafficking, secretion, & vesicular transport	5	0	38
V	Defense mechanisms	4	1	79
W	Extracellular structures	0	2	31

Fig. 1. Cluster of Orthologous Genes (COGs). Changes in gene expression specific to ampicillin treatment are enriched in a few COG categories, but are rare or nonexistent in others. Left: The number of genes (counts) that are differently expressed (adjusted $p<0.1$) in the ampicillin treated population organized according to COGs (Top left: upregulated; Bottom left: downregulated). Only genes that were unique to the treated population (compared to stationary phase) were included; genes that show differential regulation in both the treated and untreated population (compared to stationary phase) were removed. Genes in multiple functional categories were accounted for more than once. Right: Functional categories and codes from REF[2]. Up: increased >2-fold gene expression. Down: decreased <2-fold gene expression. Genome: number of genes with that functional category based on the annotated genome.

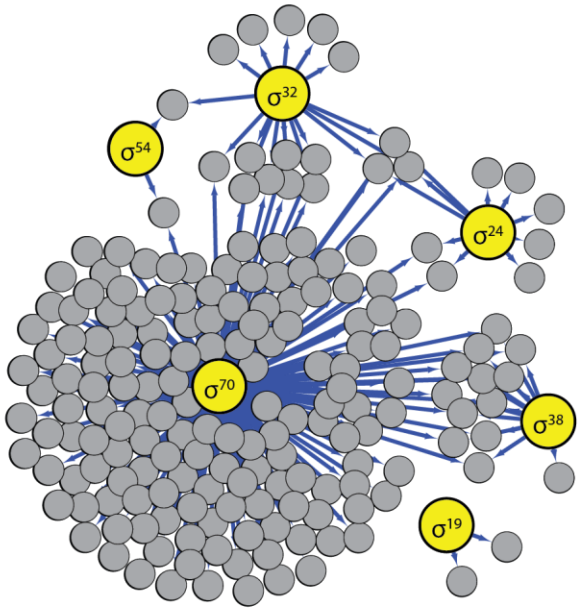
get a global picture of gene expression, we compared overall transcriptional activity by mapping changes in expression to specific sigma factors (master regulators) in the context of COGs (Fig. 2). We are the first (to our knowledge) to use COGs to support a TRN analysis based on experimental data. Sigma factors are essential components of transcriptional regulation, as these proteins interact with RNA polymerase to initiate transcription, and their activity changes depending on cellular conditions. The presence of anti-sigma factors or alterations in protein degradation often regulate sigma factors at the post-translational level, and different sigma factors are active under specific circumstances. Thus, the activity of some sigma factors can be altered by environmental conditions with little change in their mRNA level[34].

Extensive studies on sigma factor activity, both *in vivo* and *in silico*, have identified or predicted the sigma factor(s) responsible for transcription at specific promoters throughout the genome[35-40]. When comparing both the untreated and treated populations to stationary phase, we found that the total number (count) of changes in gene expression is higher in the treated population than in the untreated population as compared to the stationary phase population (Fig. 2B). Many of the genes that increase in expression in response to ampicillin are regulated by both RpoD (σ^{70}) as well as another sigma factor, and thus some genes are accounted for twice (Fig. 2A). As expected, there was an increase in the expression of many genes regulated by sigma factors related to stationary phase and heat shock, which have previously been associated with antibiotic tolerance and cellular stress response pathways[25-26]. Namely, RpoH (σ^{32}; associated with heat shock[41]), RpoS (σ^{38}; associated with stationary phase and stress[42]), and RpoE (σ^{24}; also associated with heat shock[43]) had more active regulons in the treated compared to the untreated population. Our analysis shows that RpoH transcribes many genes related to one of the COG groups noted earlier, 'posttranslational modification, protein turnover and chaperones'. Genes specifically regulated by RpoH encode the proteins ClpB, GroL, Lon, DnaK, DnaJ, and FtsH, many of which are related to protein degradation, which we will discuss later in the manuscript. Meanwhile, the sigma factor RpoS regulates several genes related to energy production and conversion, and RpoE regulates a few genes related to cell wall biogenesis (Fig. 2C). While these trends are exciting to find in our data, it is clear that other components of transcriptional regulation, namely TFs, are playing a critical role in controlling gene expression and the antibiotic stress response.

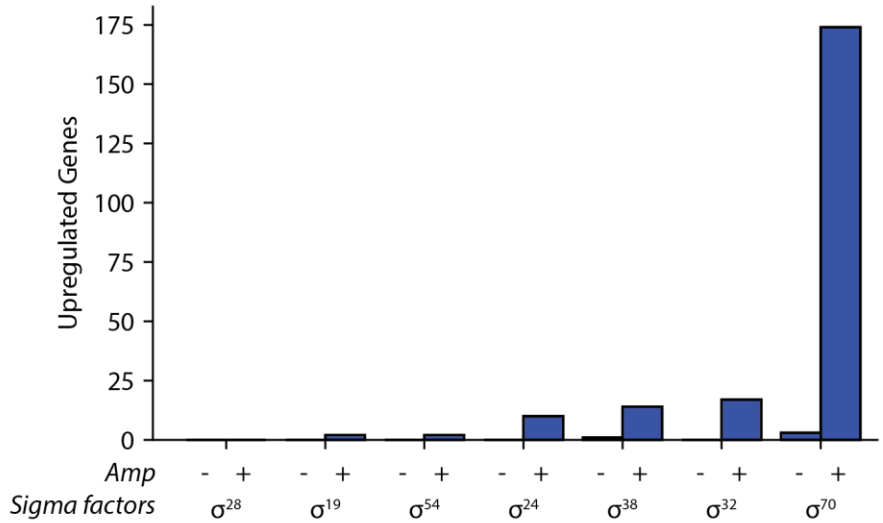
Transcription factor (TF) regulatory network shows increased activity of global regulators

TFs act on a variety of scales; global regulators are typically dual-regulators (the same protein can activate or repress transcription) and regulate hundreds of genes (e.g. CRP[44]), while other TFs may simply be responsible for regulating a few genes[45]. As with sigma factors, the activity of many TFs, especially global TFs, is regulated post-translationally (i.e. at the protein level)[44,46-48], and therefore directly measuring expression of TFs is not typically informative in regards to their activity. To identify the role of TFs that alter gene expression in response to ampicillin, we mapped changes in expression to all possible direct regulatory interactions that could be responsible for that change. Analyzing this network makes it very clear that the regulatory pathways at play in the population's response to ampicillin are highly interconnected and likely redundant (Fig. 3). Several TFs that are more active during ampicillin treatment stand out rather quickly, particularly all seven global regulators CRP, FNR, IHF, FIS, ArcA, NarL and Lrp. Global regulators are responsible for the up and down regulation of genes across a heterogenous array of functional groups, and they are an integral facet of the hierarchical structure of the TRN in *E. coli*[4]. The altered activity of these TFs in the treated population suggests that the cells are actively regulating their metabolism and responding at the whole-cell level. REF[15] found that global regulation is critical to antibiotic survival and the authors suggest that global regulators control several redundant pathways related to antibiotic stress[15], which is also supported by our results. Furthermore, both *fnr* and *crp* have previously been identified in gene knockout studies as being related to antibiotic tolerance[49-50]. The impact of global TFs on the system is not entirely surprising as they are critical factors for a healthy cell physiology.

A. σ regulatory network unique to Amp treatment



B. Genes differentially regulated compared to stationary phase



C. Number of genes in σ regulatory network unique to Amp treatment

COG functional class	σ19	σ54	σ24	σ38	σ32	σ70
Translation, ribosomal structure & biogenesis			2		2	39
Energy production & conversion				7		23
Posttranslational modification/protein turnover/chaperones		1	1		11	12
Amino acid transport & metabolism		1				15
Cell wall/membrane/envelope biogenesis			4		2	7
Coenzyme transport & metabolism				1		9
Nucleotide transport & metabolism						8
Inorganic ion transport & metabolism	2					5
Carbohydrate transport & metabolism				1		5
Defense mechanisms			1			4
Secondary metabolites biosynthesis/transport & catabolism						4
Transcription						4
Intracellular trafficking, secretion, & vesicular transport					1	2
Lipid transport & metabolism			1			2
Cell cycle control/cell division/chromosome partitioning						3
Replication, recombination & repair						3
General function prediction only				1		2
Function unknown						2
Cell motility						1
Total	2	2	9	10	16	150

Sigma factor

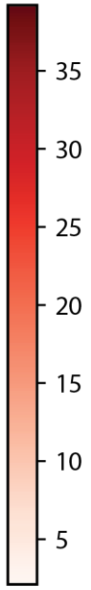


Fig. 2. Stress related sigma factors (σ) are more active in the ampicillin treated population. Sigma factors act as global regulators that control several redundant and overlapping pathways related to antibiotic stress. Genes with increased expression over two-fold (adjusted $p < 0.1$) were mapped to sigma factors responsible for their transcription. The ampicillin treated population had more expression associated with stress related sigma factors, RpoH (σ^{32}), RpoS (σ^{38}), and RpoE (σ^{24}), than the untreated population ($p < 0.001$). **A.** A graphical representation of the sigma regulatory network during antibiotic tolerance inferred from changes in gene expression unique to the ampicillin treated population compared to stationary phase (File S2). Blue lines: increased expression. Large yellow circles: sigma factors. Small grey circles: genes regulated by the sigma factors. **B.** Counts of upregulated genes for the untreated and treated populations compared to stationary phase. Untreated (Amp-): cells incubated for 3 h in fresh media without ampicillin. Treated (Amp+): cells incubated for 3 h in fresh media with ampicillin. Genes with multiple possible sigma factors are accounted for each possible sigma factor. **C.** Changes in gene expression for each sigma factor based on COG functional group; genes uncharacterized by REF[2] were not included.

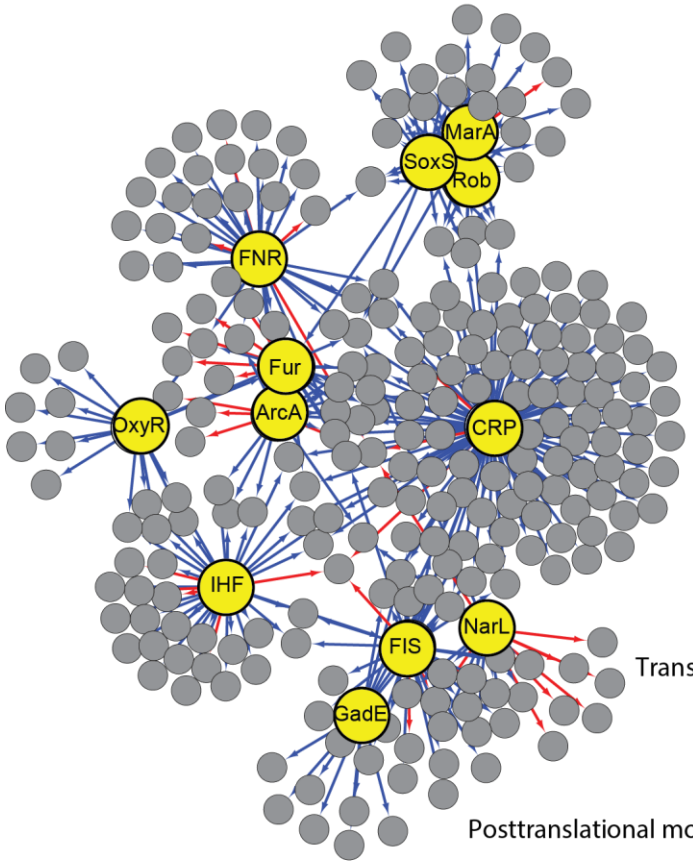
Another global TF of interest is Fur; a Δ*fur* strain has been shown to have decreased levels of tolerance to ciprofloxacin but also have an increased chance of acquiring resistance[51]. Previous work examining expression data of the Δ*fur* strain with and without ciprofloxacin identified *entA, entC* and *sufA* expression as being affected by ciprofloxacin and Fur[51], all of which also show increased expression in our ampicillin treated population. These genes have been identified in other types of tolerance as well, such as n-butanol tolerance[52]. The operon *entCDEBAH* is regulated by both CRP and Fur, encodes enterobactins, and is associated with both iron homeostasis and the oxidative stress response. Moreover, enterobactins protect intracellularly against oxidative stress independently of iron availability[53], supporting that the oxidative stress response is integrated with the antibiotic stress response.

Transcription factor (TF) regulatory network shows increased activity of specific, local regulators

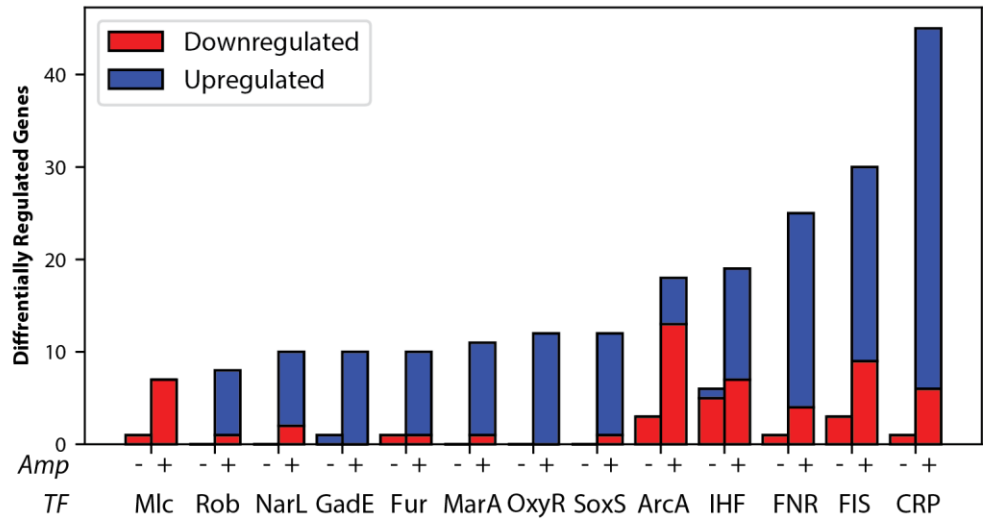
While Fur plays a role at the global level, when we examined pathways related to iron-sulfur (Fe-S) clusters more closely, we identified a group of genes with changes in expression regulated by local regulators OxyR and IscR. Notably, the treated population had increased expression of genes important for the formation of Fe-S clusters: *sufS*, *sufE, sufC*, *iscA, iscR, iscS, iscU* and *iscX*. Fe-S clusters act as protein co-factors and serve a wide variety of functions in the cell; they are suited to a variety of processes including redox sensing, catalysis, or electron transfer. Notably, the clusters are also part of the oxidative stress response[54]. Fe-S clusters have been associated with increased development of antibiotic resistance to ciprofloxacin[51] and uptake of aminoglycosides[55].

Our findings further support that Fe-S clusters are involved in antibiotic tolerance, and this system also highlights a restraint of our study, namely that we are only mapping regulatory and not deregulatory events. Derepression (removal of repression) is another type of transcriptional regulation that affects antibiotic survival. In this instance, when accounting for genes regulated by IscR, the *isc* genes were not included because IscR represses these genes and the genes increased in expression in our network. However, the increased expression is likely due to derepression of IscR, which is supported by the simultaneous increase of the *suf* genes, which are also regulated by IscR[54]. The same can be said with respect to regulation by Fur, which is also associated with iron homeostasis. While Fur was identified as being significantly more active in the treated population, since Fur primarily acts as a repressor, increased expression resulting from derepression by Fur was not accounted for in the TRN analysis. This particular group of TFs demonstrates how changes in regulatory interactions must be identified contextually with respect to when these changes might occur. The context of regulation is particularly significant for dual-regulators (e.g. global regulators), since the same TF can activate and repress different genes depending on the state of the cell.

A. TF regulatory network unique to Amp treatment



B. Genes differentially regulated compared to stationary phase



C. Number of genes in TF regulatory network unique to Amp treatment

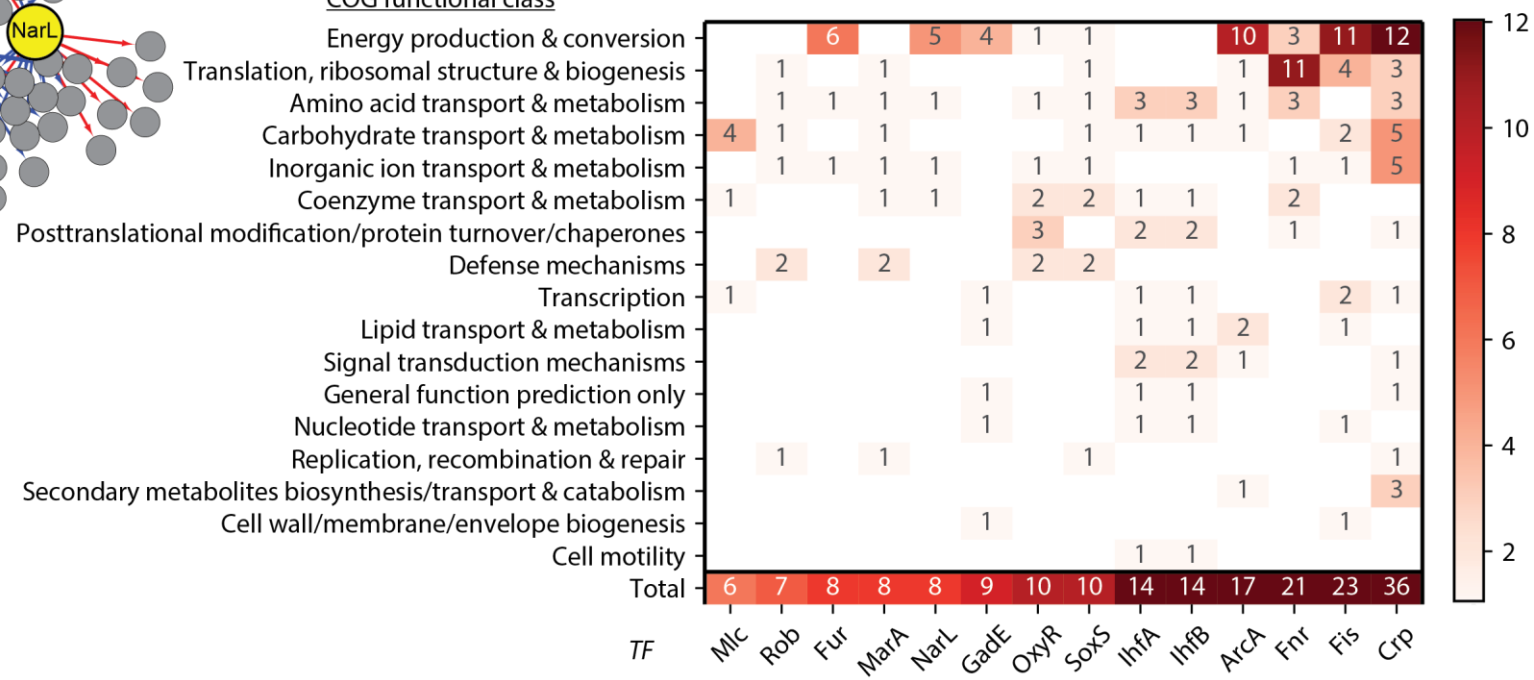


Fig. 3. TFs associated with antibiotic tolerance are more active in the ampicillin treated population. TFs act as transcriptional regulators that control a number of redundant and overlapping pathways related to antibiotic stress. Genes with changes in expression over two-fold (adjusted $p < 0.1$) were mapped to TFs responsible for their transcription. **A.** A graphical representation of the TF regulatory network during antibiotic tolerance inferred from changes in gene expression unique to the ampicillin treated population compared to stationary phase (File S3). Red lines: decreased expression. Blue lines: increased expression. Large yellow circles: select TFs. Small grey circles: genes regulated by select TFs. **B.** Counts of differentially regulated genes for the untreated and treated populations compared to stationary phase. Selected TFs displayed a significant difference ($p<0.05$) in the number of genes that changed expression when comparing untreated (Amp-) and treated (Amp+) groups based on the Fisher's exact test. Amp- (untreated): cells incubated for 3 h in fresh media without ampicillin. Amp+ (treated): cells incubated for 3 h in fresh media with ampicillin. Genes with multiple possible TFs are accounted for each possible TF. **C.** Changes in gene expression for the select TFs based on COG functional group; genes uncharacterized by REF[2] were not included. A high number of genes differently expressed are specific to a few COGs.

Another group of local TFs that play a role in the antibiotic-treated population is the Mar/Rob/SoxS regulon. These three TFs are closely related, with degenerative consensus sequences that result in considerable overlap of regulated genes, and the regulon is highly associated with antibiotic tolerance and multidrug resistance[27-28]. Constitutive expression of SoxS has been shown to cause multiple antibiotic resistance in clinical isolates of *E. coli*[56]. Extensive work has shown that the TF MarA plays a role in antibiotic tolerance, as the operon was named for its role in multiple antibiotic resistance[57]. MarA is expressed stochastically and is primarily regulated by degradation at the Lon protease[58-59]. SoxS is also degraded by Lon as well as the protease FtsH. The rapid degradation of MarA and SoxS enables the cell to rapidly recover once the stress is removed[60]. Meanwhile, the TF Rob is in an inactive state until it is induced by binding to specific substrates[61], which is a perfect example of how analyzing the mRNA level of a TF is not sufficient toward the understanding of a protein's activity. It is important to note that the level of *rob, marA* or *soxS* mRNA may not significantly change, while the concentration and/or regulatory action of the proteins do change. This fact underscores the importance of analyzing transcriptional networks rather than focusing on expression levels of specific TFs.

Altered proteolytic activity affects transcriptional regulation similarly to antibiotic stress

In addition to the regulation of MarA and SoxS through degradation, proteolytic processes play a vital role in antibiotic tolerance, as many previous works have suggested[29,62-63]. These effects on the TRN during ampicillin treatment suggest that the activity of many transcription factors is controlled at the proteolytic level and that disruption of degradation during environmental stress leads to an increase in the activity of these TFs. We have previously demonstrated that interfering with proteolytic activity causes an increase in antibiotic tolerance. To do so, we overexpressed a fluorescent protein tagged with a short amino acid degradation tag that causes it to compete for degradation at the protease complex ClpXP[29]. This competition results in a proteolytic queue, wherein proteins compete to be processed (i.e. degraded); thus the degradation of native proteins effectively slows due to an influx of new proteins with a high affinity for degradation (CFP-LAA; Fig. 4)[64-68].

To identify whether the increase in antibiotic tolerance via proteolytic queueing is due to an amplification of the transcriptional response, we sequenced populations diluted from stationary phase into fresh media containing IPTG (to induce expression of CFP-LAA) and incubated the cells for three hours without ampicillin (q*ueueing population*) or with lethal concentrations of ampicillin (*queueing-tolerant population*). We will thus refer to the previously discussed populations without queueing as *growing* (untreated) and *persistent* (treated), with all changes in gene expression in comparison to stationary phase. If slowed proteolytic activity leads to an increase in antibiotic tolerance, we would expect to see similar behavior of the TRN between the persister and queueing populations, and indeed that is the case. The queueing-tolerant

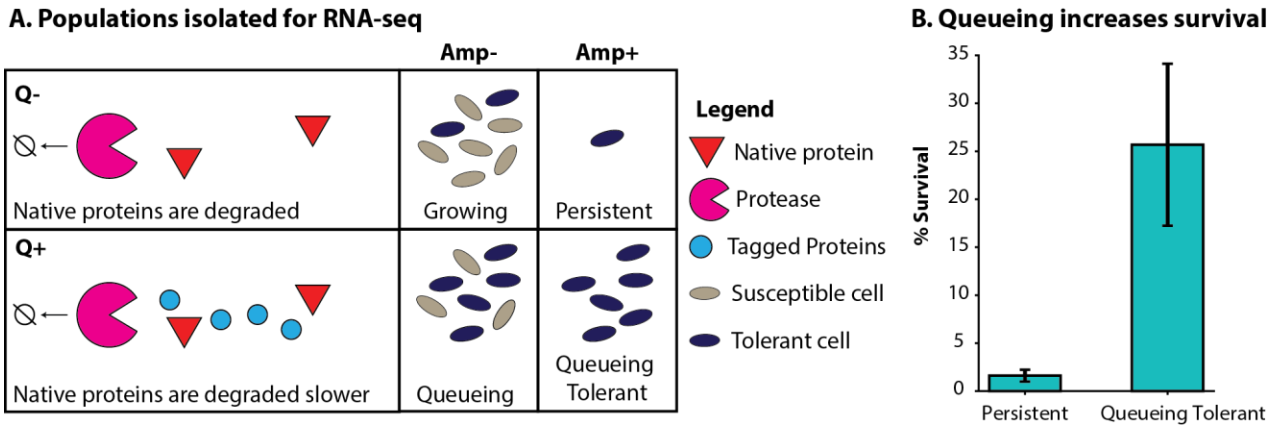


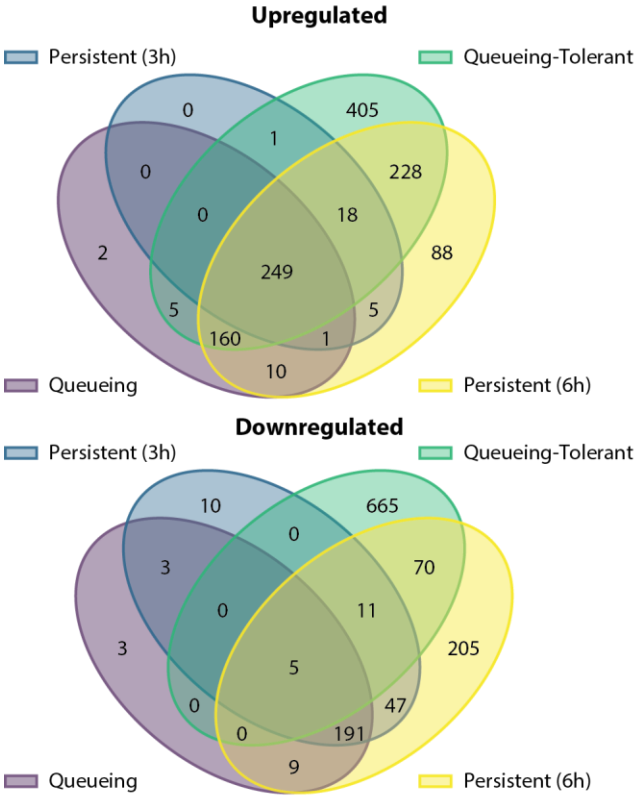
Fig. 4. Proteolytic queueing increases survival of ampicillin. A. We obtained RNA-sequencing (RNA-seq) data on populations with (Q+) or without (Q-) induction of a queue at the protease ClpXP via overexpression of CFP-LAA, and populations with (Amp+) or without (Amp-) ampicillin treatment (100 µg/ml). **B)** Percent survival of cells after ampicillin treatment for three hours without a queue (Persistent) or with a queue (Queueing Tolerant).

population, which is simultaneously responding to the induction of a proteolytic queue and ampicillin treatment, shares most of these similarities as well. However, the transcriptional response seen in the queuing-tolerant population seems to be a magnification of the response seen in either the queueing or persister population alone, with many of the same trends (Fig. 5).

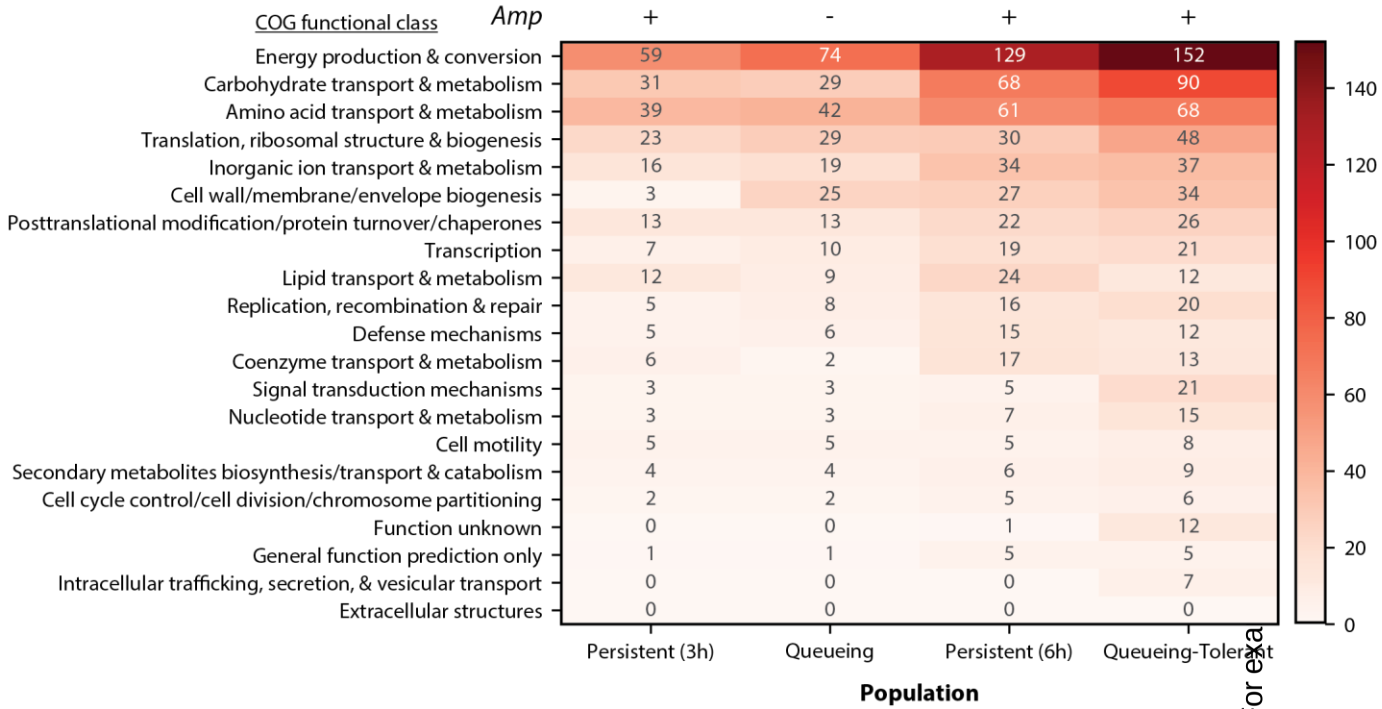
To test whether or not queueing is effectively 'quickening' (i.e. causing certain events to happen at a faster timescale) the response to the antibiotic, we then sequenced the RNA from the treated culture after six hours of treatment (*persistent 6h)*, which demonstrates that the changes in gene expression at three hours hold after six hours with hundreds of additional genes changing expression (Fig. 5A). In general, the trends between gene expression in the persistent, queueing and queueing-tolerant populations are quite similar. Upregulated genes common to the three populations include genes and systems related to tolerance that we discussed earlier, including those related to Fe-S clusters, oxidative stress and heat shock. When analyzing the COGs most highly regulated in queueing-tolerance, it appears that the population is heavily regulating processes related to metabolism and energy (Fig. 5B). It has previously been reported that ATP levels largely affect antibiotic tolerance and persistence[25,69-70]. Particularly relevant to our work, are the findings of REF[25], which demonstrates that lower ATP levels in persister cells lead to protein aggregation.

Mechanistically, protein aggregation likely results from slow protease activity, since many proteases require ATP to function[71]. In comparison, proteolytic queueing via overexpression of CFP-LAA interferes with degradation at ClpXP and causes a build-up of proteins normally degraded by the complex. The queue also affects degradation rates by other proteases[68] and leads to protein aggregation. When looking at sigma factors and TFs that affect the transcriptional response, in this case, a greater number of sigma factors and TFs significantly change activity in queueing-tolerant cells compared to queueing or persistent cells (Fig. 5C-D). We propose that one of the reasons for this is that the magnified response in the queueing-tolerant population expediates the transcriptional response and thus some trends that were present in the persistent population at three hours become more significant after six hours and/or in the queueing-tolerant population, which was only treated for three hours. In the case of sigma factors, we find that RpoS, RpoH and RpoE are more active in every condition compared to the growing (untreated) population (Fig. 5C). Meanwhile, some TFs that were somewhat active in the persistent population at three hours are now significantly engaged in the queueing-tolerant population and

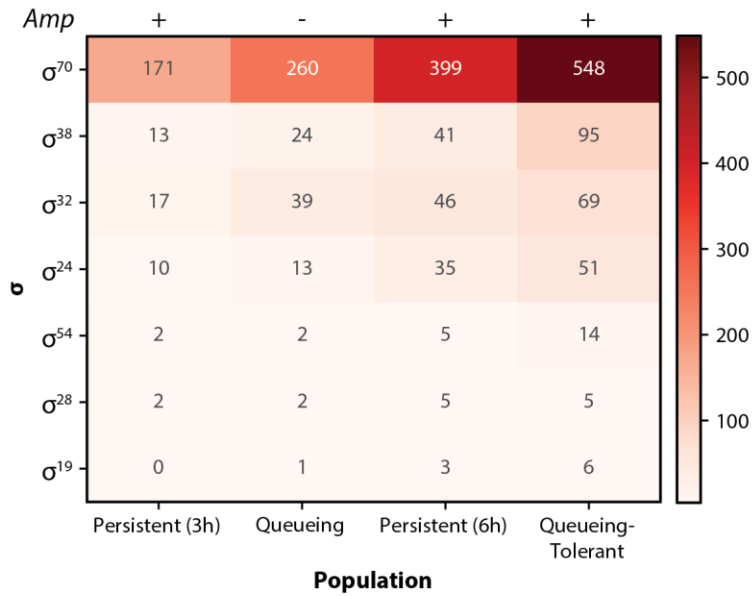
A. Differential gene expression



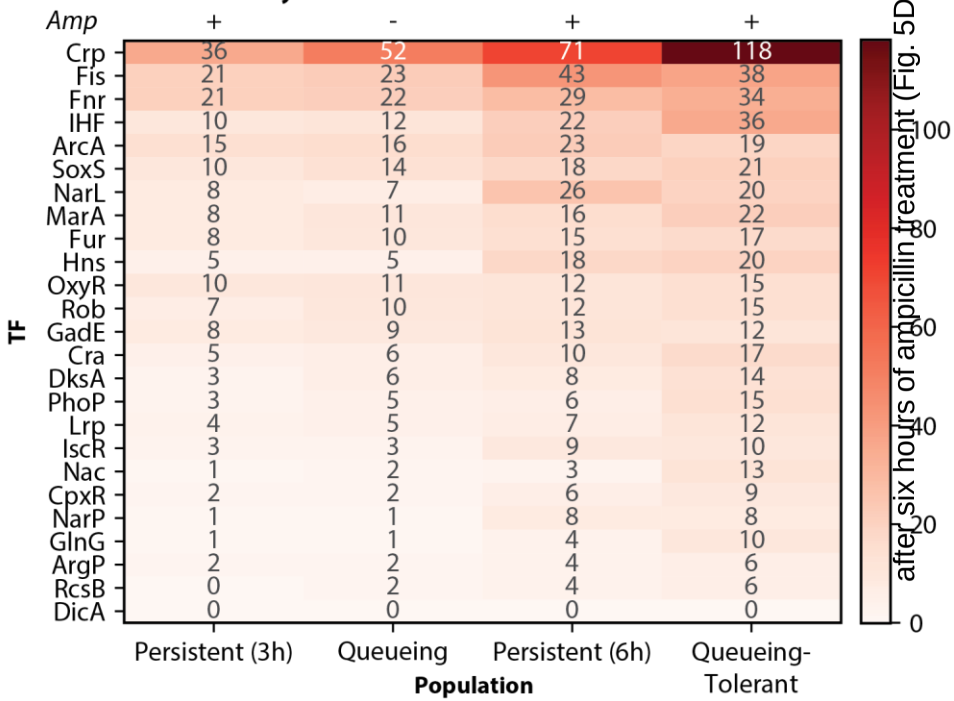
B. Changes in gene expression sorted by COG



C. Trends in σ activity



D. Trends in TF activity



after six hours of ampicillin treatment (Fig. 5D). For exa

Fig. 5. Proteolytic queueing and ampicillin treatment similarly affect gene expression in *E. coli*. Changes in gene expression compared to stationary phase (adjusted p<0.1; with >2-fold change) for each population with/without queueing (Q) and/or ampicillin (Amp). Changes in gene expression present in the growing population were removed. Persistent: Q-, Amp+. Queueing: Q+, Amp-. Queueing-Tolerant: Q+, Amp+. The persistent population was sequenced after three (3h) and six (6h) hours of ampicillin treatment. **A.** Venn diagram comparing each population. Top: Upregulated. Bottom: Downregulated **B.** Changes in gene expression sorted in Clusters of Orthologous (COG) groups for each population. Genes in multiple categories were counted for each category. **C.** Changes in gene expression mapped to sigma factors (σ) for each population. Genes transcribed by multiple sigma factors were counted for each sigma factor. **D.** Changes in gene expression mapped to transcription factors (TFs) for each population. Genes transcribed by multiple TFs were counted for each TF.

persistent population at three hours compared to the growing population, but it is even more active at six hours of ampicillin treatment and in the queueing tolerant population. DksA is particularly interesting because it has previously been shown to play a role in antibiotic tolerance and the stringent stress response[72], and is typically degraded by ClpXP[73-74].

Sigma and transcription factors that control the antibiotic stress response are commonly regulated by proteolytic degradation

As we previously touched on, many of the sigma factors and TFs involved in the antibiotic stress response are regulated by proteolysis. In all the stressed populations (queueing and/or ampicillin), we find that the sigma factors RpoH and RpoS significantly more active compared to the growing population (Fig. 5C). Increased activity of these proteins is likely a result of slowed proteolysis; RpoS and RpoH are rapidly degraded by ClpXP and FtsH, respectively[75-76]. In fact, there is direct evidence showing that RpoS is regulated by ATP levels; degradation of RpoS at the protease complex ClpXP slows when ATP levels are low[77]. Since the induction of CFP-LAA targets queue formation at ClpXP specifically, we have previously attempted to replicate the queueing-tolerance phenomenon by overexpressing RpoS. We found that high expression levels of RpoS alone did not measurably affect antibiotic tolerance[29], but the TRN analysis of the queueing-tolerant population suggests that RpoS is playing a role in cell survival. This highlights the importance of looking at networks (as compared to individual genes) when studying survival mechanisms to antibiotic stress.

It is probable that RpoS must work in concert with RpoH and transcription factors to mediate an effective response, and altered proteolytic activity could act as the coordinating factor underlying this stress response. As mentioned earlier, several genes coordinated under RpoH are related to proteolysis. Particularly noteworthy are *clpB* and *dnaK* which both have increased expression during treatment, and have previously been demonstrated as critical protiensfor resuscitation after removal of the antibiotic[25]. Other genes that would be key to recovering from antibiotic stress are also upregulated in the persistent, queueing and queueing-tolerant populations, such as the proteases *lon* and *ftsH* (both upregulated by RpoH). Increased expression of proteases likely prepares the cell to remove aggregated proteins and recover from the stress. However, both proteases are ATP-dependent and thus their activity is regulated by the availability of ATP (especially important during recovery[25,69-70]).

Future directions to expand our understanding of antibiotic tolerance

Our TRN analysis supports a coordinated transcriptional response to an antibiotic with the expression of many systems that have redundant functions, as exemplified by the overlap between the Rob, MarA, and SoxS regulons. These changes in the TRN indicate that cells are actively preparing for survival and recovery. The mechanism of recovery from persistence is of therapeutic interest because new drugs may be developed in combination with current antibiotics to prevent recovery, and thus leading to bacterial cell death. It is likely that the cells are actively transcribing mRNA during antibiotic treatment, especially when we consider that queueing only affects tolerance if the system is actively transcribed[29]. At the same time, several of our samples are heterogeneous populations (especially when ampicillin is not used for selection) and the data

is an average of that population, which is informative but not comprehensive. It is possible that individual cells have transcriptional profiles different from the average, and as antibiotic treatment continues, different subgroups are killed. Perhaps the surviving population after six hours of ampicillin treatment are a subpopulation present after three hours, but the expression profile is overshadowed by other subpopulations that are killed later. Understanding transcriptomic heterogeneity in the tolerant population would necessitate the use of more advanced techniques such as single-cell transcriptomics, which has only recently been demonstrated in bacteria[78].

Our previous results demonstrate that proteolytic queueing does not improve long-term survival[29], but it is likely that the transcriptional response to proteolytic queueing and antibiotic stress improves short-term survival and recovery while other factors are required to survive for extended periods of time. Many previous works have demonstrated, ribosomes are largely inactive in persister cells[79-82] and thus translation is slow. Therefore, it is possible the transcripts we have measured are slowly building up in the cells and only being translated at low levels if at all. The transcripts could then be translated and readily available to the cell once ribosomes resume activity after removal of the antibiotic. It is also possible that cells are activity translating select proteins, which seems reasonable with the number of genes upregulated during persistence. Regardless, future experiments (such as RNA profiling, mass spectrometry, etc.) are required to understand the cellular response.

As we touched on earlier, there are some constraints to our analysis of TRN in antibiotic tolerance. We tested one antibiotic (ampicillin) under a single growth condition (emergence from stationary phase), and some responses we identified could be specific to these experimental conditions. Our knowledge of the network response to antibiotics could be expanded by using different antibiotics, with varying treatment times and growth conditions. Perhaps, the use of multiple antibiotics will lead to a greater understanding of the networks (or TFs) that are most important for survival. A recent paper highlights the potential in using combinations of antibiotics and staggered treatment of antibiotics where pairing strongly and weakly dependent metabolism antibiotics together are effective in killing persisters[83]. We used *E. coli* as our model organism because its regulatory networks are currently the most well defined of known bacteria, nonetheless our analysis is limited to the current state of knowledge. Future work may be able to incorporate new information regarding the regulatory networks and contexts in which TFs and sigma factors regulate gene expression and may allow this type of analysis to be expanded to other strains of *E. coli* or different microorganisms. Exploring tolerance with a more minimal bacterial genome with less redundancy between networks (e.g. Rob and SoxS, etc.) may allow for clarification of how the TRN affects survival and recovery. Expanding our knowledge of antibiotic tolerance beyond *E. coli* and ampicillin treatment would significantly improve our understanding of the regulatory networks across strains and species. However, as proteolysis is a highly conserved process, it is likely that the coordination of transcriptional regulation is highly conserved during stress.

Conclusions

Our TRN analysis and top-down approach show that studying regulatory networks in antibiotic surviving subpopulations is a powerful method to understand the molecular mechanisms that enable cell survival. Gene knockouts have been useful for identifying aspects of these networks. However, our results support that antibiotic tolerance is a whole-cell response that can occur through multiple pathways. This type of systems biology approach is advantageous when compared to methods focusing on individual genes and specific mechanisms, which are susceptible to the presence of redundant systems and can often lead to contrary results. For example, toxin-antitoxins (TA) systems were once thought by many to be the primary effectors of persistence; however, several studies have suggested that they are not directly related to persistence[84-86]. We instead propose that antibiotic tolerance is a multifaceted response rooted in the general stress response systems of the cell. Indeed, in our very recent work, we demonstrated

that a bacteria population containing primarily essential genes have both antibiotic tolerant and persister subpopulation to different types of antbiotics[85]. Based on ours and others' work, we propose that a systems biology approach would be fruitful for further study of antibiotic tolerance and persistence. The fact that many of the networks we identify and discuss in this work are universal (e.g. proteolysis, oxidative stress response) indicates that there are likely systems common to antibiotic tolerance across several species. As such, TRN analysis of transcriptomic data is a good step towards developing a comprehensive, systems-level perspective of antibiotic tolerance and recovery from antibiotic stress.

Author contributions:

H.S.D. wrote the manuscript, performed the majority of the experiments, and ran data analysis. T.H. performed the 6-hour persister experiments. N.C.B. planned and directed the project. All authors contributed to discussing and editing the manuscript.

Declaration of interests: The authors declare no competing interests.

Acknowledgements

This work is supported by the National Science Foundation award Numbers 1922542 and 1849206, and by a USDA National Institute of Food and Agriculture Hatch project grant number SD00H653-18/project accession no. 1015687. We would also like to thank Drs. Will Mather and Javier Buceta for their advice and expertise.

Materials and Methods

Strains and Plasmids

Strains and plasmids are from REF[29]. Briefly, the strains are derived from *E. coli* DH5αZ1 and contain the plasmid, p24KmNB82 (contains CFP-LAA under the $P_{lac/ara1}$ promoter) as described in REF[68]. To compare results from this work to our previous work[68], we used this same strain, and produced a queueing at ClpXP by induction of CFP-LAA. Cultures were grown in modified MMB media[29], which consists of the following: K_2HPO_4 (10.5 mg/ml), KH_2PO_4 (4.5 mg/ml), $(NH_4)_2SO_4$ (2.0 mg/ml), $C_6H_5Na_3O_7$ (0.5 mg/ml) and NaCl (1.0 mg/ml). Additionally, MMB+ consists of MMB and the following: 2 mM $MgSO_4$ x $7H_2O$, 100 µM $CaCl_2$, thiamine (10 µg/ml), 0.5% glycerol and amino acids (40 µg/ml). Strains containing the plasmid p24KmNB82 were grown in MMB+ kanamycin (Km, 25 µg/ml) or on Miller's Lysogeny broth (LB) agar plates + Km (25 µg/ml). Cultures diluted after stationary phase also contained 0.1% Arabinose for consistency and CFP-LAA was induced by adding 100 µM IPTG (the promoter is $P_{lac/ara1}$). All cultures were incubated at 37° C and broth cultures were shaken at 250 rpm.

Quantification of antibiotic survival

Antibiotic survival was quantified as in REF[29] with the following modifications. Cultures were grown to stationary phase in MMB+ kanamycin, then diluted 1/100 into 200 ml of pre-warmed (37°C; consistency between experiments requires the media to be prewarmed) MMB+ kanamycin+ arabinose (0.2%) and incubated in 250 ml flasks (consistency between experiments requires the use of the same flask size and media volume) for three hours. Cultures with ampicillin were treated at a concentration of 100 µg/ml. Samples were plated before and after ampicillin treatment onto LB+Km agar plates to quantify colony forming units (CFUs) and percent survival.

RNA isolation

Samples were treated with Tween (0.2%) and chloramphenicol (5 µg/ml) then immediately filtered out of solution (using rapid filtration similar to REFs[87-88]) and resuspended in lysis buffer containing 25 mM Tris pH 8.0, 25 mM NH_4Cl, 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); lysis buffer was modified from REF[89]. 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 minutes at 16,000 *x 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.

This isolation method is advantageous because RNA can be harvested from what are considered "viable but not culturable" (VBNC) cells and dying cells (note that all cells during antibiotic treatment are indeed dying cells, and cells only survive because we remove the antibiotic). Recent research provides strong evidence that VBNC cells are persister cells that do not emerge from persistence when quantified using traditional persister plate assays[90-92]. The presence of VBNCs demonstrates the heterogeneity of the population that survive antibiotic treatment. Nevertheless, as our data includes any whole cells present after antibiotic treatment and the systems we have identified as related to antibiotic stress are likely playing a role in a large portion of this population.

DNA-sequencing

DH5αZ1 genomic DNA was harvested and purified using Genomic DNA Purification Kit (ThermoFisher) in accordance with the manufacturer's instructions. The genome was then sequenced using paired-end Illumina sequencing Novogene Ltd. Sequencing libraries were generated from the qualified DNA samples(s) using the NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's protocol. Reads were then assembled using Geneious v. 11.0.3[93] with *E. coli* K12 str. MG1655 (NC_000913.3) as a reference sequence. *E. coli* DH5αZ1 genome is 99.9% identity to MG1655 genome, and due to the more thorough annotation of the MG1655 reference sequence, we used NC_000913.3 as the reference for RNA-seq analysis.

RNA-sequencing data analysis

Fastq files were aligned to NC_000913.3 and the plasmid p24KmNB82 using Geneious v. 11.0.3[93]. Raw read counts were then analyzed using DEseq to compare expression; we used a 2-fold cutoff and adjusted p-values<0.1 to identify significant changes in expression[94]. All conditions tested had three biological replicates.

Transcription factor (TF) network analysis

Information on transcriptional regulation was downloaded using the EcoCyc SmartTables feature[3]. We then used custom Python scripts to generate a network graph made up of directed edges toward any gene with a significant change in expression (>2-fold change in expression, adjusted p<0.01) originating from each transcription factor that may be an effector of the observed change. Visualization of this graph was done using Cytoscape[95]. The total number of genes mapped to a particular TF was compared between populations (i.e. untreated and treated each compared to stationary phase) using a two-sided Fisher's exact test[7,96].

COGs

Data was downloaded from http://www.ncbi.nlm.nih.gov/COG/[2,33].

Data and materials availability: All data that supports the findings of this study are available from the corresponding author upon request (email: nicholas.butzin@gmail.com).

Supplemental information

File S1: Tab-delimited file containing direct regulatory information for each gene according to the Ecocyc[3] database as of February 2020.

File S2: Tab-delimited file containing regulatory interactions used to make network in Fig. 2A

File S3: Tab-delimited file containing regulatory interactions used to make network in Fig. 3A

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