Regulatory proteins direct a transcriptional network in response to antibiotic stress

Heather S. Deter[1] and Nicholas C. Butzin*[2]

[1] Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, 61801, USA
[2] Department of Biology and Microbiology. South Dakota State University. Brookings, SD. 57006. USA.
*Corresponding author

Abstract:

In a given bacterial population, antibiotic treatment will kill a large portion of the population, while a small, tolerant subpopulation will survive. Tolerant cells disrupt the efficacy of antibiotic treatment and increase the likelihood that a population gains antibiotic resistance. When a population becomes resistant, antibiotic treatment fails, which is a major health concern. Since antibiotic tolerance often leads to resistance, we have taken a systems biology approach to examine how transcriptional networks respond to antibiotic stress so that cells can survive and recover after antibiotic treatment. We have compared gene expression with and without ampicillin in *E. coli*. While many previous studies have identified aspects of the antibiotic stress response at either the protein or transcriptional level, our work leverages existing knowledge of transcriptional regulation to link the dynamics of the two allowing for a more holistic approach. Here, we are the first to develop a whole-cell, transcriptional regulatory network (TRN) of antibiotic tolerant subpopulations and examine how cells respond at the transcriptional level to bactericidal concentrations of an antibiotic. Using TRN analysis, we show how certain sigma and transcription factors are affecting transcriptional regulation under antibiotic stress, and that changes in gene expression specific to ampicillin treatment can be directly linked to cell survival and recovery. The resulting network demonstrates that cells are mounting an active and coordinated response to the antibiotic that spans multiple systems and pathways. The redundancy and interconnectivity of the networks suggest that accounting for whole-cell dynamics may be crucial when modeling and studying antibiotic tolerance in the future.

Introduction

When disaster strikes, a coordinated response is required to mitigate the effects to 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 degradational). The identification of individual regulatory interactions in combination with bioinformatics approaches has resulted in multiple databases detailing regulation throughout the cell, especially in *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 pool 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 as to 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 dormant, 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. What is currently lacking is a more holistic understanding at the network level. 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 there is a transcriptional regulatory network underlying the antibiotic stress response.

Here we take a systems biology, top-down approach to explore antibiotic survival of tolerant populations. We have obtained deep, RNA-seq data on both antibiotic-treated and -untreated populations emerging from stationary phase, and the differences in the transcriptional regulatory networks (TRNs) provide key insights into the molecular mechanisms of antibiotic tolerance. Our results show that cells have an active and coordinated response that is associated with altered activity of specific sigma factors and transcription factors (TF) as a result of lethal antibiotic stress. It has been a long-standing goal to identify a gene or a set of genes unique to antibiotic persistence, but our TRN analysis makes it is clear that tolerance is controlled via a network response consisting of smaller, overlapping networks, some of which are redundant.

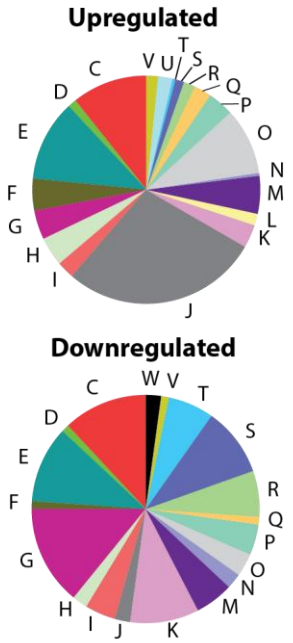
Results and Discussion

We isolated and sequenced RNA from three different populations, namely: *stationary phase cells* and populations incubated for three hours in fresh media without ampicillin (*untreated*) or with lethal concentrations of ampicillin (*treated*). 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 recovering 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 and the same strains of *E. coli*[29]. The sequencing data was analyzed using DESeq (see Methods). Based on this analysis, we consider significant changes in gene expression to be greater than two-fold with an adjusted p-value <0.1. To start, we examined 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), which suggests 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 growth in new media, and thus, we can focus on changes specific to ampicillin by identifying differences in gene expression unique to the treated population. These changes are particularly important because we are using bactericidal concentrations of ampicillin, which necessitates that changes in network dynamics are keeping *E. coli* alive, while preparing for recovery after removal of the antibiotic.

Data analysis using Clusters of Orthologous Groups (COGs)

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 method of grouping genes to identify important trends in the data. When we analyze the COGs within the ampicillin treated TRN, we find a few categories that stand out (Fig. 1). The most upregulated category unique to the treated population is translation, ribosomal structure and biogenesis (68 genes). While the accumulation of translational machinery has previously been shown in persister cells[25], 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). While we will mainly focus on these groups with known functions, seven genes categorized as “function unknown” or “general function prediction only” were also shown to be differentially expressed during ampicillin treatment (Fig. 1). The identification of upregulated genes of unknown function, suggests their function is related to survival.



Code	Functional category	Up	Down
C	Energy production & conversion	26	11
D	Cell cycle control, cell division, chromosome partitioning	3	1
E	Amino acid transport & metabolism	28	10
F	Nucleotide transport & metabolism	11	1
G	Carbohydrate transport & metabolism	10	13
H	Coenzyme transport & metabolism	10	2
I	Lipid transport & metabolism	6	4
J	Translation, ribosomal structure & biogenesis	68	2
K	Transcription	8	9
L	Replication, recombination & repair	4	0
M	Cell wall/membrane/envelope biogenesis	13	5
N	Cell motility	1	2
O	Posttranslational modification, protein turnover, chaperones	23	3
P	Inorganic ion transport & metabolism	9	4
Q	Secondary metabolites biosynthesis, transport & catabolism	6	1
R	General function prediction only	4	6
S	Function unknown	3	9
T	Signal transduction mechanisms	1	6
U	Intracellular trafficking, secretion, & vesicular transport	5	0
V	Defense mechanisms	4	1
W	Extracellular structures	0	2
Total		243	92

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[1]. Upregulated: increased >2-fold gene expression. Downregulated: decreased <2-fold gene expression.

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

To contextualize changes in gene expression, we applied transcriptional regulatory network (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 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 different circumstances. Thus, the activity of some sigma factors can be altered by environmental conditions with little change in their mRNA level[34].

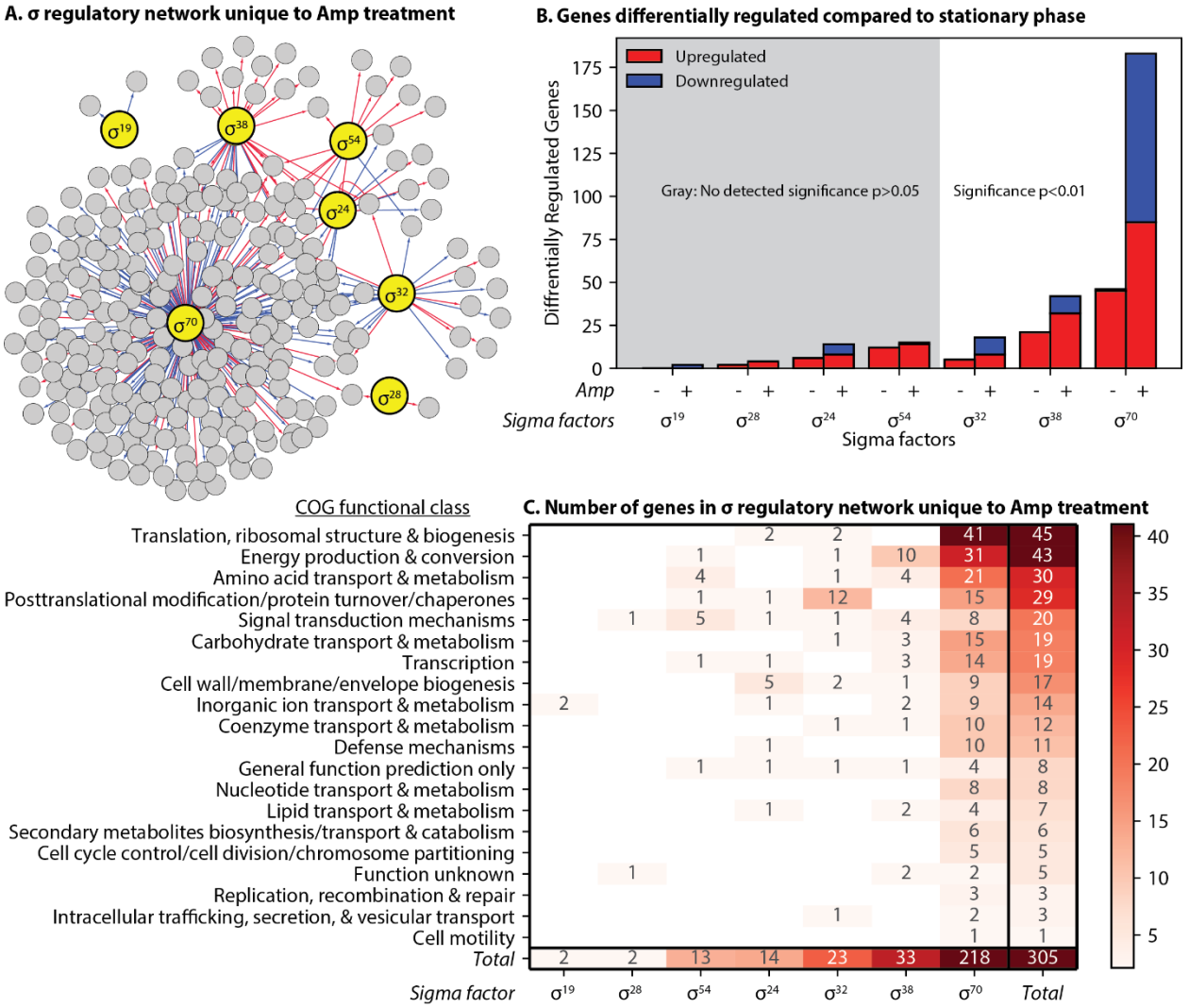


Fig. 2. Sigma factors mapped to changes in expression. Sigma factors act as global regulators that control several redundant and overlapping pathways related to antibiotic stress. Genes with changes in expression over two-fold (adjusted $p < 0.1$) were mapped to sigma factors responsible for their transcription. **A.** Sigma regulatory network unique to the ampicillin treated population compared to stationary phase Red lines: decreased expression. Blue lines: increased expression. Large yellow circles: sigma factors. Small grey circles: genes regulated by the sigma factors. **B.** Counts of differentially regulated genes for the untreated and treated populations compared to stationary phase. The grey background indicates no detected significance ($p>0.05$) in the number of genes that changed expression when comparing untreated (Amp -) and treated (Amp +) groups based on the Fisher's exact test. The white background indicates a significant difference ($p<0.01$). 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. A high number of genes differently expressed are specific to a few COGs, and sigma factors 70, 38, and 32 regulated the majority of these genes.

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]. While sigma factors by their nature upregulate gene expression, we also mapped decreases in gene expression to the sigma factor(s) regulating those genes, because these changes could be due to lower activity of a specific sigma factor or affected by other forms of transcriptional regulation (i.e. TFs). 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 expression of many genes regulated by RpoS (σ^{38}; associated with stationary phase and stress[41]) and RpoH (σ^{32}; associated with heat shock[42]), both of which have previously been associated with antibiotic tolerance and cellular stress response pathways[25-26]. 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, which we will discuss later in the manuscript. Meanwhile, the sigma factor RpoS regulates several genes related to energy production and conversion (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 (can activate or repress transcription) and regulate hundreds of genes (e.g. CRP[43]), while other TFs may simply be responsible for regulating a few genes[44]. As with sigma factors, the activity of many TFs, especially global TFs, is regulated post-translationally (i.e. at the protein level)[43,45-47], 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], and our results support their hypothesis. Furthermore, both *fnr* and *crp* have previously been identified in gene knockout studies as being related to antibiotic tolerance[48-49]. The impact of global TFs on the system is not entirely surprising as they are critical factors for a healthy cell physiology, as is evidenced by the growth defect of a CRP knockout[50].

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

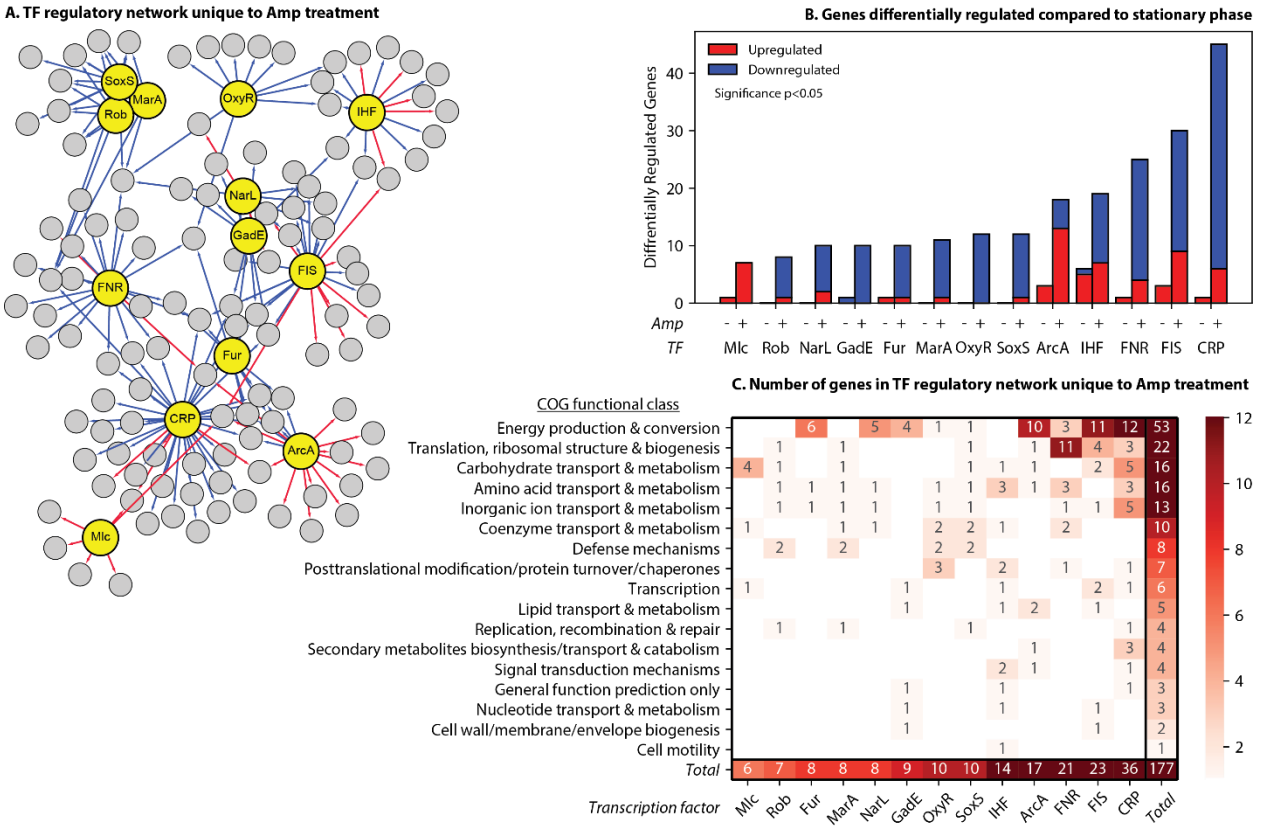


Fig. 3. Transcription factors (TFs) mapped to changes in expression. TFs act as global regulators that control a number of redundant and overlapping pathways related to antibiotic stress. Genes with changes in expression over two-fold (adjusted p < 0.1) were mapped to TFs responsible for their transcription. **A.** TF network unique to the ampicillin treated population compared to stationary phase. 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. 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 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. Amp: ampicillin.

homeostasis and the oxidative stress response. Moreover, enterobactins protect intracellularly against oxidative stress independently of iron availability[53].

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 that are 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].

Identifying the regulation of genes by these TFs shows how the activity of those TFs is changing. These findings highlight a restraint of our study, namely that we are only mapping regulatory and not deregulatory events. Albeit, derepression (removal of repression) is another

form of regulation that affects antibiotic survival. In particular, 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[54]. The same can be said for the identification of 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 and 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.

Another group of local TFs that play a role in the TRN of the antibiotic-treated tolerant subpopulation 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]. The Rob TF is in an inactive state until it is induced by binding to specific substrates[56], 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. Constitutive expression of SoxS has been shown to cause multiple antibiotic resistance in clinical isolates of *E. coli*[57]. 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[58]. MarA is expressed stochastically and is primarily regulated by degradation at the Lon protease[59-60]. 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[61]. It is important to note that the level of *marA* or *soxS* mRNA may not significantly change but the concentration of the proteins may. This underscores the importance of analyzing the MarA and SoxS transcriptional networks rather than focusing on their individual gene mRNA levels.

Altered proteolytic activity can be linked to transcriptional regulation during antibiotic stress

Beyond 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]. Our work suggests that many active transcription factors during antibiotic tolerance are controlled at the degradation level and that disruption of degradation during environmental stress leads to an increase in the activity of these TFs. In fact, the same is true for sigma factors, as RpoS and RpoH are rapidly degraded by ClpXP and FtsH, respectively[64-65]. The findings of REF[25] demonstrate that lower ATP levels in persister cells lead to protein aggregation. This is likely a result of protease activity slowing because many of them require ATP to function[66]. Furthermore, our previous work shows that interfering with protein degradation, specifically at ClpXP, increases antibiotic tolerance[29]. This work suggests that the transcriptional response to the accumulation of proteins such as sigma factors and TF during antibiotic tolerance is to increase expression of genes critical for the recovering from the tolerant state. As mentioned earlier, several genes coordinated under RpoH are related to proteolysis. Critically, *clpB* and *dnaK* both increase in expression; the respective proteins are critical to resuscitation after removal of the antibiotic[25] and the expression of these genes indicates that cells are preparing to enter a recovery state. Additionally, *lon* and *ftsH* increase in expression as well. As Lon degrades MarA and SoxS, and FtsH degrades RpoH and SoxS, increased expression of these proteases may also prepare the cell for rapid recovery from persistence. 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.

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. Our TRN analysis supports a coordinated transcriptional response to an antibiotic in the tolerant subpopulation with the expression of many systems that are redundant, as exemplified by the overlap between the Rob, MarA, and SoxS regulons. As we touched on earlier, there are some constraints to our analysis of TRNs in antibiotic tolerance. We used *E. coli* as our model organism because its regulatory networks are currently the most well defined of known bacteria. Nonetheless, this analysis depends on the current state of knowledge as the regulatory networks and the contexts in which TFs and sigma factors regulate gene expression. Additionally, we tested one antibiotic (ampicillin) under a single growth condition (emergence from stationary phase), and so there may be responses 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. The use of other strains of *E. coli* or different microorganisms (though the networks are poorly defined in most microbes compared to *E. coli*) would significantly improve our understanding of the regulatory networks across strains and species.

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[67-69]. Indeed, in our very recent work, we demonstrated for the first time that a bacteria population lacking any known or predicted TA systems have both antibiotic tolerant and persister subpopulation to different types of antbiotics[68]. Based on ours and others' work, we propose that a systems biology approach would be a fruitful approach in 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) indicates that there are likely systems common to antibiotic tolerance across several species. As such, our analysis is a good step towards developing a comprehensive, systems-level perspective of antibiotic tolerance and recovery from antibiotic stress.

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[70]. To compare results from this work to our previous work[70] we used this same strain, but did not induce the production 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 p24Km and derivatives were grown in MMB+ kanamycin (Km, 25 µg/ml) or on Miller's Lysogeny broth (LB) agar plates + Km (25 µg/ml). All cultures were incubated at 37° C and broth cultures were shaken at 250 rpm.

Quantification of persistence

Persistence 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[71-72]) 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[73]. Rapid filtration allow 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[74-76]. 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[77] 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[77]. 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[78]. 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 generated a network graph made up of directed edges toward any gene with a significant change in expression from all transcription factors that may be an effector of that change (positive/negative). 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[7,79].

COGs

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

Author contributions:

H.S.D. wrote the manuscript, performed experiments and ran data analysis. 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.

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).

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.

References

1. Santos-Zavaleta, A.; Salgado, H.; Gama-Castro, S.; Sanchez-Perez, M.; Gomez-Romero, L.; Ledezma-Tejeida, D.; Garcia-Sotelo, J. S.; Alquicira-Hernandez, K.; Muniz-Rascado, L. J.; Pena-Loredo, P.; Ishida-Gutierrez, C.; Velazquez-Ramirez, D. A.; Del Moral-Chavez, V.; Bonavides-Martinez, C.; Mendez-Cruz, C. F.; Galagan, J.; Collado-Vides, J., RegulonDB v 10.5: tackling challenges to unify classic and high throughput knowledge of gene regulation in E. coli K-12. *Nucleic Acids Res.* **2019,** *47* (D1), D212-D220.
2. Tatusov, R. L.; Galperin, M. Y.; Natale, D. A.; Koonin, E. V., The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* **2000,** *28* (1), 33-6.
3. Karp, P. D.; Weaver, D.; Paley, S.; Fulcher, C.; Kubo, A.; Kothari, A.; Krummenacker, M.; Subhraveti, P.; Weerasinghe, D.; Gama-Castro, S.; Huerta, A. M.; Muniz-Rascado, L.; Bonavides-Martinez, C.; Weiss, V.; Peralta-Gil, M.; Santos-Zavaleta, A.; Schroder, I.; Mackie, A.; Gunsalus, R.; Collado-Vides, J.; Keseler, I. M.; Paulsen, I., The EcoCyc Database. *EcoSal Plus* **2014,** *6* (1), ESP-0009-2013: 1-13.
4. Martinez-Antonio, A.; Janga, S. C.; Thieffry, D., Functional organisation of *Escherichia coli* transcriptional regulatory network. *J. Mol. Biol.* **2008,** *381* (1), 238-47.
5. Jensen, P. A.; Zhu, Z.; van Opijnen, T., Antibiotics Disrupt Coordination between Transcriptional and Phenotypic Stress Responses in Pathogenic Bacteria. *Cell Rep* **2017,** *20* (7), 1705-1716.
6. Kang, Y.; Liow, H. H.; Maier, E. J.; Brent, M. R., NetProphet 2.0: mapping transcription factor networks by exploiting scalable data resources. *Bioinformatics* **2018,** *34* (2), 249-257.
7. Sastry, A. V.; Gao, Y.; Szubin, R.; Hefner, Y.; Xu, S.; Kim, D.; Choudhary, K. S.; Yang, L.; King, Z. A.; Palsson, B. O., The Escherichia coli transcriptome mostly consists of independently regulated modules. *Nat Commun* **2019,** *10* (1), 5536.
8. Tan, J.; Sastry, A. V.; Fremming, K. S.; Bjorn, S. P.; Hoffmeyer, A.; Seo, S.; Voldborg, B. G.; Palsson, B. O., Independent component analysis of E. coli's transcriptome reveals the cellular processes that respond to heterologous gene expression. *Metab. Eng.* **2020**.
9. Liu, R.; Liang, L.; Freed, E. F.; Choudhury, A.; Eckert, C. A.; Gill, R. T., Engineering regulatory networks for complex phenotypes in *E. coli*. *Nat Commun* **2020,** *11* (1), 4050.
10. Levin-Reisman, I.; Ronin, I.; Gefen, O.; Braniss, I.; Shoresh, N.; Balaban, N. Q., Antibiotic tolerance facilitates the evolution of resistance. *Science* **2017,** *355* (6327), 826-830.

11. Van den Bergh, B.; Michiels, J. E.; Wenseleers, T.; Windels, E. M.; Boer, P. V.; Kestemont, D.; De Meester, L.; Verstrepen, K. J.; Verstraeten, N.; Fauvart, M.; Michiels, J., Frequency of antibiotic application drives rapid evolutionary adaptation of *Escherichia coli* persistence. *Nat Microbiol* **2016,** *1*, 16020.
12. Windels, E. M.; Michiels, J. E.; Fauvart, M.; Wenseleers, T.; Van den Bergh, B.; Michiels, J., Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. *ISME J* **2019**.
13. Wu, N.; He, L.; Cui, P.; Wang, W.; Yuan, Y.; Liu, S.; Xu, T.; Zhang, S.; Wu, J.; Zhang, W.; Zhang, Y., Ranking of persister genes in the same *Escherichia coli* genetic background demonstrates varying importance of individual persister genes in tolerance to different antibiotics. *Front Microbiol* **2015,** *6*, 1003.
14. Wang, W.; Chen, J.; Chen, G.; Du, X.; Cui, P.; Wu, J.; Zhao, J.; Wu, N.; Zhang, W.; Li, M.; Zhang, Y., Transposon Mutagenesis Identifies Novel Genes Associated with *Staphylococcus aureus* Persister Formation. *Front Microbiol* **2015,** *6*, 1437.
15. Hansen, S.; Lewis, K.; Vulic, M., Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. *Antimicrob Agents Chemother* **2008,** *52* (8), 2718-26.
16. Wood, T. K.; Knabel, S. J.; Kwan, B. W., Bacterial persister cell formation and dormancy. *Applied and environmental microbiology* **2013,** *79* (23), 7116-21.
17. Orman, M. A.; Brynildsen, M. P., Inhibition of stationary phase respiration impairs persister formation in *E. coli*. *Nat Commun* **2015,** *6*, 7983.
18. Kim, J. S.; Wood, T. K., Persistent Persister Misperceptions. *Front Microbiol* **2016,** *7*, 2134.
19. Prax, M.; Bertram, R., Metabolic aspects of bacterial persisters. *Front Cell Infect Microbiol* **2014,** *4*, 148.
20. Pu, Y.; Zhao, Z.; Li, Y.; Zou, J.; Ma, Q.; Zhao, Y.; Ke, Y.; Zhu, Y.; Chen, H.; Baker, M. A.; Ge, H.; Sun, Y.; Xie, X. S.; Bai, F., Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells. *Mol. Cell* **2016,** *62* (2), 284-94.
21. Wu, S.; Yu, P. L.; Wheeler, D.; Flint, S., Transcriptomic study on persistence and survival of Listeria monocytogenes following lethal treatment with nisin. *J Glob Antimicrob Resist* **2018,** *15*, 25-31.
22. Long, Y.; Fu, W.; Li, S.; Ren, H.; Li, M.; Liu, C.; Zhang, B.; Xia, Y.; Fan, Z.; Xu, C.; Liu, J.; Jin, Y.; Bai, F.; Cheng, Z.; Liu, X.; Jin, S.; Wu, W., Identification of novel genes that promote persister formation by repressing transcription and cell division in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **2019**.
23. Babin, B. M.; Atangcho, L.; van Eldijk, M. B.; Sweredoski, M. J.; Moradian, A.; Hess, S.; Tolker-Nielsen, T.; Newman, D. K.; Tirrell, D. A., Selective Proteomic Analysis of Antibiotic-Tolerant Cellular Subpopulations in *Pseudomonas aeruginosa* Biofilms. *MBio* **2017,** *8* (5).
24. Mali, S.; Mitchell, M.; Havis, S.; Bodunrin, A.; Rangel, J.; Olson, G.; Widger, W. R.; Bark, S. J., A Proteomic Signature of Dormancy in the Actinobacterium *Micrococcus luteus*. *J. Bacteriol.* **2017,** *199* (14).
25. Pu, Y.; Li, Y.; Jin, X.; Tian, T.; Ma, Q.; Zhao, Z.; Lin, S. Y.; Chen, Z.; Li, B.; Yao, G.; Leake, M. C.; Lo, C. J.; Bai, F., ATP-Dependent Dynamic Protein Aggregation Regulates Bacterial Dormancy Depth Critical for Antibiotic Tolerance. *Mol. Cell* **2019,** *73* (1), 143-156 e4.
26. Radzikowski, J. L.; Vedelaar, S.; Siegel, D.; Ortega, A. D.; Schmidt, A.; Heinemann, M., Bacterial persistence is an active sigmaS stress response to metabolic flux limitation. *Mol. Syst. Biol.* **2016,** *12* (9), 882.
27. Duval, V.; Lister, I. M., MarA, SoxS and Rob of Escherichia coli - Global regulators of multidrug resistance, virulence and stress response. *Int J Biotechnol Wellness Ind* **2013,** *2* (3), 101-124.
28. Sharma, P.; Haycocks, J. R. J.; Middlemiss, A. D.; Kettles, R. A.; Sellars, L. E.; Ricci, V.; Piddock, L. J. V.; Grainger, D. C., The multiple antibiotic resistance operon of enteric bacteria controls DNA repair and outer membrane integrity. *Nat Commun* **2017,** *8* (1), 1444.

29. Deter, H. S.; Abualrahi, A. H.; Jadhav, P.; Schweer, E. K.; Ogle, C. T.; Butzin, N. C., Proteolytic Queues at ClpXP Increase Antibiotic Tolerance. *ACS synthetic biology* **2020,** *9* (1), 95-103.
30. Joers, A.; Kaldalu, N.; Tenson, T., The frequency of persisters in *Escherichia coli* reflects the kinetics of awakening from dormancy. *J. Bacteriol.* **2010,** *192* (13), 3379-84.
31. Gefen, O.; Gabay, C.; Mumcuoglu, M.; Engel, G.; Balaban, N. Q., Single-cell protein induction dynamics reveals a period of vulnerability to antibiotics in persister bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **2008,** *105* (16), 6145-9.
32. Rowe, S. E.; Conlon, B. P.; Keren, I.; Lewis, K., Persisters: Methods for Isolation and Identifying Contributing Factors--A Review. *Methods Mol Biol* **2016,** *1333*, 17-28.
33. Galperin, M. Y.; Makarova, K. S.; Wolf, Y. I.; Koonin, E. V., Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Res.* **2015,** *43* (Database issue), D261-9.
34. Paget, M. S., Bacterial Sigma Factors and Anti-Sigma Factors: Structure, Function and Distribution. *Biomolecules* **2015,** *5* (3), 1245-65.
35. Zhao, K.; Liu, M.; Burgess, R. R., The global transcriptional response of Escherichia coli to induced sigma 32 protein involves sigma 32 regulon activation followed by inactivation and degradation of sigma 32 in vivo. *J. Biol. Chem.* **2005,** *280* (18), 17758-68.
36. Wong, G. T.; Bonocora, R. P.; Schep, A. N.; Beeler, S. M.; Lee Fong, A. J.; Shull, L. M.; Batachari, L. E.; Dillon, M.; Evans, C.; Becker, C. J.; Bush, E. C.; Hardin, J.; Wade, J. T.; Stoebel, D. M., Genome-Wide Transcriptional Response to Varying RpoS Levels in Escherichia coli K-12. *J. Bacteriol.* **2017,** *199* (7).
37. Maciag, A.; Peano, C.; Pietrelli, A.; Egli, T.; De Bellis, G.; Landini, P., In vitro transcription profiling of the sigmaS subunit of bacterial RNA polymerase: re-definition of the sigmaS regulon and identification of sigmaS-specific promoter sequence elements. *Nucleic Acids Res.* **2011,** *39* (13), 5338-55.
38. Koo, B. M.; Rhodius, V. A.; Campbell, E. A.; Gross, C. A., Mutational analysis of Escherichia coli sigma28 and its target promoters reveals recognition of a composite -10 region, comprised of an 'extended -10' motif and a core -10 element. *Mol. Microbiol.* **2009,** *72* (4), 830-43.
39. Dartigalongue, C.; Missiakas, D.; Raina, S., Characterization of the Escherichia coli sigma E regulon. *J. Biol. Chem.* **2001,** *276* (24), 20866-75.
40. Kanhere, A.; Bansal, M., A novel method for prokaryotic promoter prediction based on DNA stability. *BMC Bioinformatics* **2005,** *6*, 1.
41. Battesti, A.; Majdalani, N.; Gottesman, S., The RpoS-mediated general stress response in Escherichia coli. *Annu. Rev. Microbiol.* **2011,** *65*, 189-213.
42. Arsène, F.; Tomoyasu, T.; Bukau, B., The heat shock response of Escherichia coli. *Int. J. Food Microbiol.* **2000,** *55* (1-3), 3-9.
43. Shimada, T.; Fujita, N.; Yamamoto, K.; Ishihama, A., Novel roles of cAMP receptor protein (CRP) in regulation of transport and metabolism of carbon sources. *PLoS One* **2011,** *6* (6), e20081.
44. Browning, D. F.; Busby, S. J., Local and global regulation of transcription initiation in bacteria. *Nat. Rev. Microbiol.* **2016,** *14* (10), 638-50.
45. Dilweg, I. W.; Dame, R. T., Post-translational modification of nucleoid-associated proteins: an extra layer of functional modulation in bacteria? *Biochem. Soc. Trans.* **2018,** *46* (5), 1381-1392.
46. Balleza, E.; Lopez-Bojorquez, L. N.; Martinez-Antonio, A.; Resendis-Antonio, O.; Lozada-Chavez, I.; Balderas-Martinez, Y. I.; Encarnacion, S.; Collado-Vides, J., Regulation by transcription factors in bacteria: beyond description. *FEMS Microbiol Rev* **2009,** *33* (1), 133-51.

47. Jeon, Y.; Lee, Y. S.; Han, J. S.; Kim, J. B.; Hwang, D. S., Multimerization of phosphorylated and non-phosphorylated ArcA is necessary for the response regulator function of the Arc two-component signal transduction system. *J. Biol. Chem.* **2001,** *276* (44), 40873-9.
48. Shan, Y.; Lazinski, D.; Rowe, S.; Camilli, A.; Lewis, K., Genetic basis of persister tolerance to aminoglycosides in Escherichia coli. *mBio* **2015,** *6* (2).
49. Mok, W. W.; Orman, M. A.; Brynildsen, M. P., Impacts of global transcriptional regulators on persister metabolism. *Antimicrob Agents Chemother* **2015,** *59* (5), 2713-9.
50. Perrenoud, A.; Sauer, U., Impact of global transcriptional regulation by ArcA, ArcB, Cra, Crp, Cya, Fnr, and Mlc on glucose catabolism in Escherichia coli. *J. Bacteriol.* **2005,** *187* (9), 3171-9.
51. Mehi, O.; Bogos, B.; Csorgo, B.; Pal, F.; Nyerges, A.; Papp, B.; Pal, C., Perturbation of iron homeostasis promotes the evolution of antibiotic resistance. *Mol. Biol. Evol.* **2014,** *31* (10), 2793-804.
52. Reyes, L. H.; Almario, M. P.; Winkler, J.; Orozco, M. M.; Kao, K. C., Visualizing evolution in real time to determine the molecular mechanisms of n-butanol tolerance in Escherichia coli. *Metab. Eng.* **2012,** *14* (5), 579-90.
53. Peralta, D. R.; Adler, C.; Corbalan, N. S.; Paz Garcia, E. C.; Pomares, M. F.; Vincent, P. A., Enterobactin as Part of the Oxidative Stress Response Repertoire. *PLoS One* **2016,** *11* (6), e0157799.
54. Mettert, E. L.; Kiley, P. J., How Is Fe-S Cluster Formation Regulated? *Annu. Rev. Microbiol.* **2015,** *69*, 505-26.
55. Ezraty, B.; Vergnes, A.; Banzhaf, M.; Duverger, Y.; Huguenot, A.; Brochado, A. R.; Su, S. Y.; Espinosa, L.; Loiseau, L.; Py, B.; Typas, A.; Barras, F., Fe-S cluster biosynthesis controls uptake of aminoglycosides in a ROS-less death pathway. *Science* **2013,** *340* (6140), 1583-7.
56. Griffith, K. L.; Fitzpatrick, M. M.; Keen, E. F., 3rd; Wolf, R. E., Jr., Two functions of the C-terminal domain of Escherichia coli Rob: mediating "sequestration-dispersal" as a novel off-on switch for regulating Rob's activity as a transcription activator and preventing degradation of Rob by Lon protease. *J. Mol. Biol.* **2009,** *388* (3), 415-30.
57. Koutsolioutsou, A.; Pena-Llopis, S.; Demple, B., Constitutive soxR mutations contribute to multiple-antibiotic resistance in clinical Escherichia coli isolates. *Antimicrob Agents Chemother* **2005,** *49* (7), 2746-52.
58. Cohen, S. P.; Hachler, H.; Levy, S. B., Genetic and functional analysis of the multiple antibiotic resistance (mar) locus in Escherichia coli. *J. Bacteriol.* **1993,** *175* (5), 1484-92.
59. Rossi, N. A.; Mora, T.; Walczak, A. M.; Dunlop, M. J., Active degradation of MarA controls coordination of its downstream targets. *PLoS Comput Biol* **2018,** *14* (12), e1006634.
60. El Meouche, I.; Siu, Y.; Dunlop, M. J., Stochastic expression of a multiple antibiotic resistance activator confers transient resistance in single cells. *Sci Rep* **2016,** *6*, 19538.
61. Griffith, K. L.; Shah, I. M.; Wolf, R. E., Jr., Proteolytic degradation of Escherichia coli transcription activators SoxS and MarA as the mechanism for reversing the induction of the superoxide (SoxRS) and multiple antibiotic resistance (Mar) regulons. *Molecular microbiology* **2004,** *51* (6), 1801-16.
62. Frees, D.; Gerth, U.; Ingmer, H., Clp chaperones and proteases are central in stress survival, virulence and antibiotic resistance of Staphylococcus aureus. *Int. J. Med. Microbiol.* **2014,** *304* (2), 142-9.
63. Conlon, B. P.; Nakayasu, E. S.; Fleck, L. E.; LaFleur, M. D.; Isabella, V. M.; Coleman, K.; Leonard, S. N.; Smith, R. D.; Adkins, J. N.; Lewis, K., Activated ClpP kills persisters and eradicates a chronic biofilm infection. *Nature* **2013,** *503* (7476), 365-70.
64. Narberhaus, F.; Balsiger, S., Structure-function studies of Escherichia coli RpoH (sigma32) by in vitro linker insertion mutagenesis. *J. Bacteriol.* **2003,** *185* (9), 2731-8.
65. Ranquet, C.; Gottesman, S., Translational regulation of the *Escherichia coli* stress factor RpoS: a role for SsrA and Lon. *J. Bacteriol.* **2007,** *189* (13), 4872-9.

66. Gottesman, S., Proteases and their targets in Escherichia coli. *Annual review of genetics* **1996,** *30*, 465-506.
67. Goormaghtigh, F.; Fraikin, N.; Putrins, M.; Hallaert, T.; Hauryliuk, V.; Garcia-Pino, A.; Sjodin, A.; Kasvandik, S.; Udekwu, K.; Tenson, T.; Kaldalu, N.; Van Melderen, L., Reassessing the Role of Type II Toxin-Antitoxin Systems in Formation of Escherichia coli Type II Persister Cells. *mBio* **2018,** *9* (3).
68. Hossain, T.; Deter, H. S.; Butzin, N. C., Antibiotic tolerance, persistence, and resistance of the evolved minimal cell, Mycoplasma mycoides JCVI-Syn3B. *bioRxiv ((Submitted to iScience - Cell Press)* **2020 (Submitted)**, 2020.06.02.130641.
69. Pontes, M. H.; Groisman, E. A., Slow growth determines nonheritable antibiotic resistance in *Salmonella enterica*. *Sci Signal* **2019,** *12* (592).
70. Butzin, N. C.; Mather, W. H., Crosstalk between Diverse Synthetic Protein Degradation Tags in Escherichia coli. *ACS synthetic biology* **2018,** *7* (1), 54-62.
71. Bordag, N.; Janakiraman, V.; Nachtigall, J.; Gonzalez Maldonado, S.; Bethan, B.; Laine, J. P.; Fux, E., Fast Filtration of Bacterial or Mammalian Suspension Cell Cultures for Optimal Metabolomics Results. *PLoS One* **2016,** *11* (7), e0159389.
72. Mohammad, F.; Green, R.; Buskirk, A. R., A systematically-revised ribosome profiling method for bacteria reveals pauses at single-codon resolution. *Elife* **2019,** *8*.
73. Latif, H.; Szubin, R.; Tan, J.; Brunk, E.; Lechner, A.; Zengler, K.; Palsson, B. O., A streamlined ribosome profiling protocol for the characterization of microorganisms. *BioTechniques* **2015,** *58* (6), 329-32.
74. Kim, J. S.; Chowdhury, N.; Yamasaki, R.; Wood, T. K., Viable But Non-Culturable and Persistence Describe the Same Bacterial Stress State. *Environ. Microbiol.* **2018**.
75. Ayrapetyan, M.; Williams, T. C.; Baxter, R.; Oliver, J. D., Viable but Nonculturable and Persister Cells Coexist Stochastically and Are Induced by Human Serum. *Infect. Immun.* **2015,** *83* (11), 4194-203.
76. Ayrapetyan, M.; Williams, T. C.; Oliver, J. D., Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends Microbiol.* **2015,** *23* (1), 7-13.
77. Kearse, M.; Moir, R.; Wilson, A.; Stones-Havas, S.; Cheung, M.; Sturrock, S.; Buxton, S.; Cooper, A.; Markowitz, S.; Duran, C.; Thierer, T.; Ashton, B.; Meintjes, P.; Drummond, A., Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **2012,** *28* (12), 1647-1649.
78. Love, M. I.; Huber, W.; Anders, S., Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **2014,** *15* (12), 550.
79. Kim, H. Y., Statistical notes for clinical researchers: Chi-squared test and Fisher's exact test. *Restor Dent Endod* **2017,** *42* (2), 152-155.