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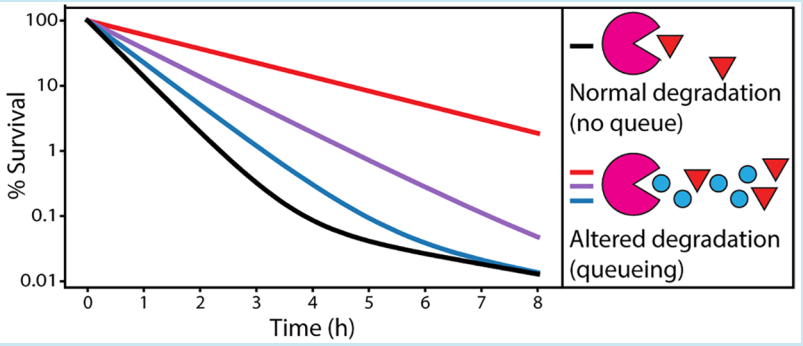
Proteolytic Queues at ClpXP Increase Antibiotic Tolerance

Heather S. Deter,[†,‡] Alawiah H. Abualrahi,[†,‡] Prajakta Jadhav,[†] Elise K. Schweer,[†] Curtis T. Ogle,[§] and Nicholas C. Butzin*[,†]

[†]Department of Biology and Microbiology, South Dakota State University, Brookings, South Dakota 57006, United States

Supporting Information



ABSTRACT: Antibiotic tolerance is a widespread phenomenon that renders antibiotic treatments less effective and facilitates antibiotic resistance. Here we explore the role of proteases in antibiotic tolerance, short-term population survival of antibiotics, using queueing theory (*i.e.*, the study of waiting lines), computational models, and a synthetic biology approach. Proteases are key cellular components that degrade proteins and play an important role in a multidrug tolerant subpopulation of cells, called persisters. We found that queueing at the protease ClpXP increases antibiotic tolerance ∼80 and ∼60 fold in an *E. coli* population treated with ampicillin and ciprofloxacin, respectively. There does not appear to be an effect on antibiotic persistence, which we distinguish from tolerance based on population decay. These results demonstrate that proteolytic queueing is a practical method to probe proteolytic activity in bacterial tolerance and related genes, while limiting the unintended consequences frequently caused by gene knockout and overexpression.

KEYWORDS: *antibiotic tolerance, persistence, antibiotic resistance, queueing, protease, synthetic biology, ClpXP*

The discovery of penicillin in the 1920s led to a new age of human and animal medicine as many antibiotics were quickly identified and developed, but the subsequent explosion of antibiotic treatments and applications has simultaneously driven microbial evolution and the development of widespread resistance.[1,2] A significant contributing factor to the abundance of antibiotic-resistant microorganisms are subpopulations of cells that survive antibiotic treatment without a genetic mutation, antibiotic tolerant and persistent cells.[3,4] Persistence is a physiological state that enables cells to survive antibiotic treatment *via* temporary changes in phenotype, such as slowed growth and biosynthesis, rather than genotype (*e.g.*, antibiotic resistance).[5] Although persistence has been studied for over 70 years, there has been a lack of specificity in the literature between antibiotic tolerance and persistence.[5,6] Recently, a consensus statement that was released after a discussion panel with 121 researchers defined antibiotic persistence as a tolerant subpopulation of cells that result in a distinct phase of population decay.[5] We use population decay to differentiate between tolerance and persistence in this work (Figure 1a).

The widespread nature of persistence suggests that similar mechanisms exist to trigger the persistent state in prokaryotes. These mechanisms include many common systems, *e.g.*, toxin−antitoxin (TA) systems and proteases. Although the precise role of TA systems in persistence is unclear due to the complications of knocking out all TA systems (*E. coli* has >45 known and predicted TA systems[7−9]) and their interrelated role in cellular responses to stress,[10] toxins in TA systems can trigger persistence when at a higher level than their cognate antitoxin.[10−12] Within the cell, the ratio of toxin to antitoxin is regulated during protein production[13−15] and through degradation by proteases.[16,17] Proteases, such as Lon and ClpP, are largely responsible for protein degradation and cell maintenance.[18,19] They provide an essential level of protein regulation throughout the cell, including degradation of RpoS (a transcription factor that responds to stress)[20] and tagged polypeptides (incomplete proteins) synthesized by stalled ribosomes that have been rescued by the trans−translation system.[21] In *E. coli*, *ssrA* (tmRNA) and *smpB* are the primary genes responsible for trans−translation, a cellular mechanism for recovering stalled ribosomes. A tmRNA molecule acts as a tRNA by binding to the A-site of a stalled ribosome. The ribosome then translates the protein-coding region of the

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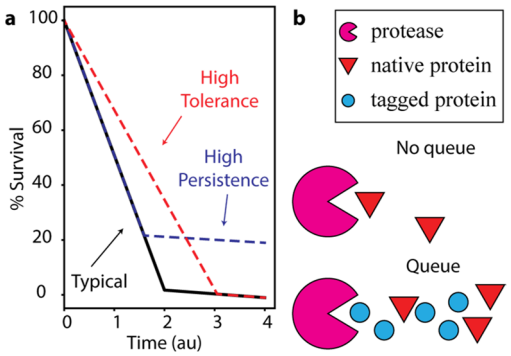

Figure 1. (a) Examples of population decay in typical (black), high persistence (blue), and high tolerance (red) populations. A shift in tolerance can be distinguished from a change in the number of persisters. For example, a high persistence population can initially have the same decay rate as a typical population but have higher survival because of more persisters (dotted blue line). A high tolerance population can have the same persister level as a typical population but have a shift in the initial decay rate (dotted red line). (b) A simple model of proteolytic queueing. When native proteins have low competition for the protease, there is no queue. Induction of synthetic tagged proteins competes with the native proteins for the protease and overloads the protease, which results in a proteolytic queue (bottleneck).

tmRNA, which adds an amino acid tag to target the polypeptide for degradation by ClpXP.[21] While *ssrA* is not essential in *E. coli*, *ssrA* knockouts cause growth defects, increase susceptibility to certain antibiotics,[22] and affect persistence.[10,23−25] Proteases and related chaperones are also consistently identified as persister-related genes in gene knockout experiments[26,27] and transcriptome analysis.[28] Indeed, a drug that targets persisters, acyldepsipeptide (ADEP4), activates the protease ClpP and lowers persister levels.[29] While most published articles focus on methods that reduce persister levels, conditions that increase their levels are integral to understanding the causative mechanisms of action and developing new drugs. As many persister studies incidentally examine antibiotic tolerance,[5,6] it follows that some of the above mechanisms may play a role in antibiotic tolerance.

Synthetic biology takes advantage of these mechanisms to develop new cellular circuits. For example, synthetic oscillators require rapid degradation of proteins, which is accomplished using the *ssrA* degradation tag;[30−32] the ssrA degradation tag is the amino acid sequence AANDENYALAA,[21] which we abbreviate to LAA throughout. Previous work establishes that multiple circuits can be coordinated by overproduction of a common degradation tag to target proteins to a protease.[33,34] When a protease is overloaded, protein species compete for degradation; the enzyme is unable to keep up with the influx of new proteins.[35] This phenomenon can be explained by queueing theory, in which one type of customer competes for processing by servers, which has traditionally been applied to systems such as computer networks and call centers. Limited processing resources in a cell (*e.g.*, proteases) cause biological queues[31,36] (Figure 1b). The queueing effect at the protease ClpXP is essential in allowing for oscillation of the highly used synthetic oscillator (often called Stricker oscillator or dual-feedback oscillator).[30,32] Variations of this oscillator have been used in different strains of *E. coli*,[30,33,34,37] and in *Salmonella* ser. Typhimurium,[38] indicating that queueing at ClpXP is not specific to one strain or species. The coupling of otherwise independent synthetic systems *via* proteolytic queueing demonstrates that queueing affects protein degradation and thus provides a tunable method of studying proteolytic degradation with little effect on cell growth[31,33,34,36] compared to gene knockouts and overexpression of proteases.[18,39,40]

We set out to test the hypothesis that proteolytic queueing at the ClpXP complex affects survival of *E. coli* during antibiotic treatment. Previous studies have used knockout mutants to disrupt activity of specific proteases in *E. coli*, but these studies yielded mixed results.[24,27,41,42] The variability between results of knockout mutations could be due to differences in growth rates and metabolism, which would modulate antibiotic efficacy.[43,44] Proteases are essential to regulating many biological networks and simply removing them likely has downstream effects. For example, ClpXP is known to degrade at least 50 proteins in *E. coli*,[45] and many of them are transcription factors like RpoS, the global regulator of stationary phase.[46,47] Many proteins are regulated at the proteolytic level by ClpXP,[19] including RpoS,[48] and simply removing ClpXP disrupts this regulation and any quantification of persistence or tolerance is indirectly measuring an alteration in the levels of proteins regulated by ClpXP degradation.

Proteolytic queueing is preferred over protease knockouts when probing antibiotic efficacy because while protease knockouts often result in growth defects,[18,39] proteolytic queueing does not noticeably affect cell growth or death[31,33,34,36] (Figure S1). Our results show that during antibiotic treatment, degradation plays a role in cell survival and the effect is tunable using queue formation. Proteolytic queueing at ClpXP increases antibiotic survival and analysis of population decay with and without a queue demonstrates that queueing specifically increases antibiotic tolerance. We hypothesize that the queue is affecting the degradation of one or many regulatory molecules within the cell that cause downstream effects and enhance antibiotic tolerance. These results demonstrate that proteolytic queueing provides a new method to probe proteolytic activity in antibiotic tolerance and persistence.

RESULTS

Proteolytic Queueing Affects Tolerance. Cultures were grown to stationary phase and incubated for 24 h prior to dilution into fresh media containing ampicillin to quantify persistence (see Materials and Methods). A proteolytic queue was induced *via* the production of a ssrA tagged fluorescent protein, CFP-LAA, expressed under an IPTG inducible promoter, $P_{lac/ara-1}$. No apparent change in growth was observed by induction (Figure S1) as reported previously.[33,34] The effects of queue formation on antibiotic survival are shown as the percentage of the population that survived ampicillin treatment (Figure 2). When CFP alone (no degradation tag control) was overexpressed during ampicillin treatment, there was no significant effect on persister levels ($p > 0.2$, Figure 2a). Queue formation (overexpression of CFP-LAA) during ampicillin treatment led to a 25-fold increase in survival after 3 h in a concentration-dependent manner (Figure 2b; $p < 0.0001$, $n \geq 12$).

When a queue was induced for 24 h prior to ampicillin treatment the surviving population at 3 h was over 80-fold higher than the uninduced population, only if induction was maintained during ampicillin treatment. However, if the inducer was removed during ampicillin treatment, the initial

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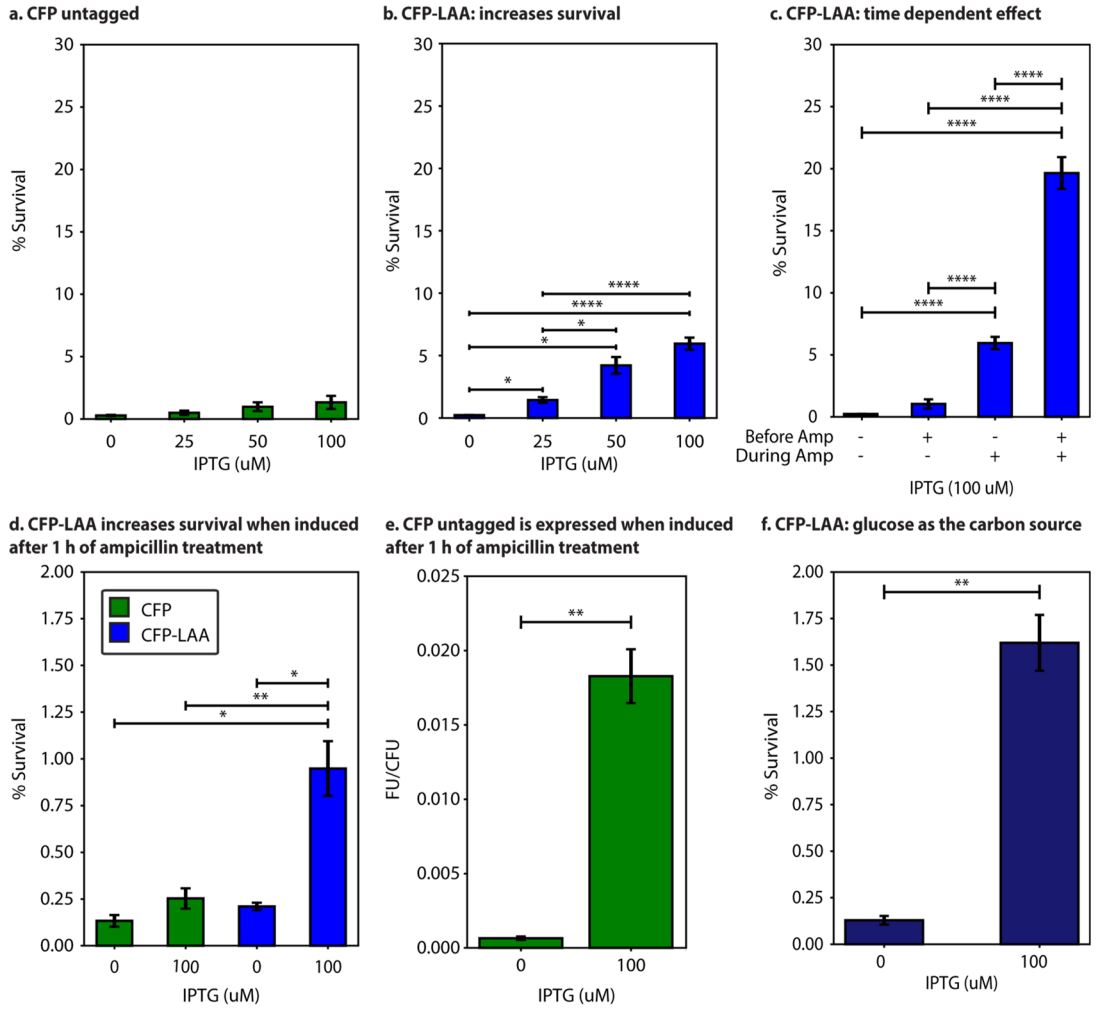

Figure 2. Proteolytic queueing affects survival of cells treated with the antibiotic ampicillin. (a) Induction of untagged CFP during antibiotic treatment has no significant effect on survival ($p > 0.2$). (b) Induction of CFP-LAA during antibiotic treatment causes an increase in survival. (c) CFP-LAA was induced (+) with 100 μM of IPTG or not induced (−). Induction before ampicillin lasted 24 h in stationary phase prior to antibiotic treatment. Queueing affects survival if the queue is maintained during ampicillin treatment. (d,e) Expression of CFP or CFP-LAA was induced with IPTG 1 h into the 3 h antibiotic treatment. Induction of CFP alone (no queue) had no significant effects on survival. Induction of CFP-LAA increased survival (d). Population fluorescence was measured for untagged CFP after antibiotic treatment, demonstrating that CFP is being produced *via* induction (e). (f) Induction of CFP-LAA during antibiotic treatment causes an increase in survival with glucose as a carbon source rather than glycerol, demonstrating that it is not a solely a carbon-specific phenomenon. Cultures were treated with ampicillin (100 μg/mL). Error bars represent SEM $n \geq 3$. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. ****$p < 0.0001$.

24 h of queueing had a minimal effect on survival at 3 h ($p > 0.01$, Figure 2c). These results indicate that survival was affected by queue formation rather than CFP itself, and that the size of the queue (level and length of induction) determines the effect. To confirm that these results are due to induction during antibiotic treatment, we waited 1 h into ampicillin treatment before inducing expression of the fluorescent protein. As we previously observed, induction of untagged CFP had no apparent effect on persister levels (Figure 2d), while quantification of fluorescence after ampicillin treatment confirmed that CFP was produced (Figure 2e). Overexpression of CFP-LAA for 2 h of ampicillin treatment still increased cell survival compared to the uninduced and untagged CFP populations (Figure 2d).

We did further testing to confirm that this effect is not specific to glycerol as a carbon source or ampicillin as the antibiotic. When glucose was the carbon source rather than glycerol, survival still increased due to CFP-LAA induction (Figure 2f), which demonstrates that the effect is not directly related to the carbon source. We then tested the effects of queueing against the antibiotic ciprofloxacin, because ciprofloxacin targets DNA gyrase[49] while ampicillin targets the cell wall.[50] CFP alone caused a slight increase in survival (Figure 3a), however the CFP-LAA tag led to a 60-fold increase in survival (Figure 3b).

Chloramphenicol Inhibits the Synthetic Queue. Neither ampicillin nor ciprofloxacin directly affect production of the fluorescent protein (*i.e.*, target transcription or translation) and thus should not prevent queue formation. On the other hand, an antibiotic that affects protein production should prevent queue formation, and therefore CFP-LAA induction would not affect survival in the presence of such an antibiotic. We found this to be the case when testing the effects of queueing on the survival of cells treated with chloramphenicol. Chloramphenicol is an antibiotic that inhibits protein translation by binding to bacterial ribosomes and inhibiting protein synthesis, thereby inhibiting bacterial growth.[51] Induction of CFP-LAA does not increase survival of

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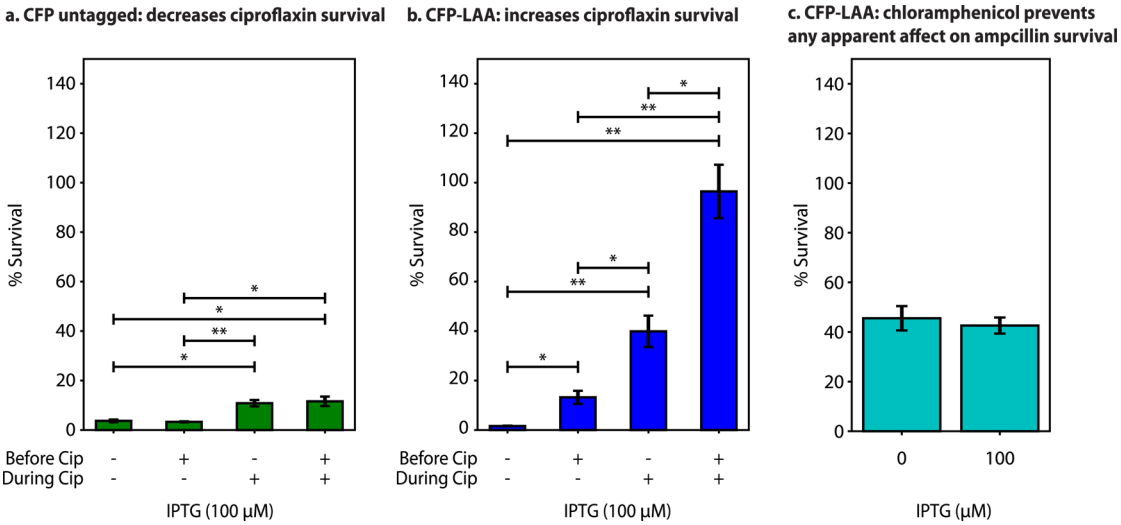

Figure 3. Proteolytic queueing effects in the presence of ciprofloxacin and chloramphenicol. (a) Induction of untagged CFP during ciprofloxacin treatment increases survival less than 4-fold. (b) Induction of CFP-LAA during ciprofloxacin treatment increases survival ~60-fold. (c) Induction of CFP-LAA during ampicillin and chloramphenicol treatment has no apparent effect on survival ($p > 0.7$). *X*-axis labels correspond to Figure 2. Cultures were treated with ciprofloxacin (1 μg/mL) or chloramphenicol (5 μg/mL) respectively. Error bars represent SEM $n \geq 3$. *$p < 0.05$. **$p < 0.01$.

antibiotic treatment when treated with chloramphenicol alone (Figure S2), but chloramphenicol is not bactericidal, so we cotreated cultures with both ampicillin and chloramphenicol. The overall percent survival with chloramphenicol is much higher than with ampicillin alone, which is consistent with the literature.[52] As expected, cotreatment with ampicillin and chloramphenicol had no apparent effect on cell survival, supporting that even when CFP-LAA was induced the queue could not form if translation was blocked (Figure 3c).

Proteolytic Queueing Affects Population Decay. To gain further insight into the relationship between proteolytic queueing, tolerance, and persistence, we measured how a proteolytic queue affects population decay by measuring survival for up to 8 h of ampicillin treatment. Our results show a typical biphasic curve indicative of persister cells in the uninduced population. When the population is induced 24 h prior to and during antibiotic treatment this curve shifts as the rate of population decay slows compared to uninduced cultures. The addition of the inducer exclusively during antibiotic treatment takes a similar effect between two and 3 h into treatment. If the queue is induced 24 h prior to antibiotic treatment, but the queue is not maintained (*i.e.*, the inducer is removed during antibiotic treatment), the effect of the queue dissipates between one to 2 h. There is no apparent difference between induced and uninduced cultures after 8 h, which suggests there is little to no effect on persistence (Figure 4a).

Computational Modeling Supports Queueing-Tolerance. On the basis of the *in vivo* results, we considered a simple computational model of population decay during antibiotic treatment modified from Kussel *et al.*[53] In our model, the persister population (P) has a lower death rate than the susceptible population (N), where the death rates are represented by μ_p and μ_n respectively. We estimated μ_p and μ_n based on the experimentally determined decay rate of the uninduced population before and after 2 h, and set the initial persister population to 0.2% of the total population (Figure 4b). Normal (susceptible) cells enter persistence at rate α, and persister cells return to the normal state at rate β. The rates α and β were set relative to μ_n based on the relationship between these values in Kussel *et al.*[53] Our base model resembles population decay as measured in experimental tests. We use the model to determine whether the increase in overall population survival due to queue formation can be attributed to an increased rate of entering persistence (α) or increased tolerance (*i.e.*, decreased μ_n). Exploration of these parameters using stochastic simulations shows that increasing the rate at which normal cells become persisters (α) shortens the first phase of population decay and increases the number of persisters (Figure 4c). Decreasing the rate of normal cell death (μ_n) lengthens the first phase of population decay but has little to no effect on the number of persisters (Figure 4d).

Overexpression of RpoS Does Not Reproduce Queueing-Tolerance. An increase in tolerance in response to proteolytic queueing at ClpXP is likely due to an increase in the number of one or many proteins. A good candidate is the transcription factor RpoS, a persister related gene[27] that is responsible for regulation of stationary phase, affected by the level of *ssrA*, and regulated by proteolytic degradation.[20] As such, we tested the effects of increasing RpoS levels by gene overexpression using the same vector, promoter, and ribosome binding sites as used to overexpress CFP and CFP-LAA. We found that overexpression of RpoS does not cause a significant increase in tolerance, especially when compared to proteolytic queueing (Figure 5).

DISCUSSION

Proteolytic queueing is an integral component of native systems, and synthetic queues have great potential for studying systems at the proteolytic level. Here we show that queueing provides a tunable method to interfere with protease degradation and affect antibiotic tolerance. Increased antibiotic tolerance in response to queueing was independent of the carbon source (glycerol or glucose) and antibiotic class (β-lactam or fluoroquinolone). When we prevented queue formation using chloramphenicol, adding the inducer did not affect cell survival under the treatment of ampicillin. While CFP production alone slightly increased survival for cipro-

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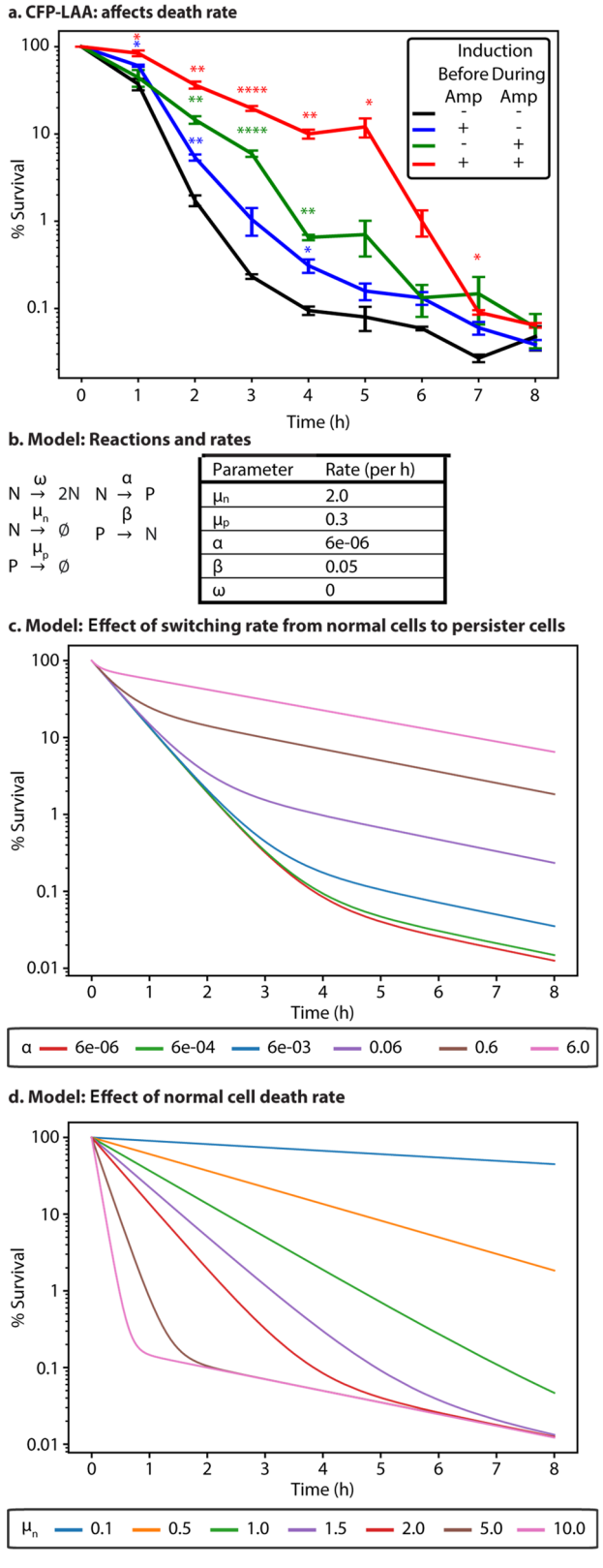

Figure 4. Time of queue formation influences survival. (a) Stationary phase cells were diluted 1/100 into fresh media containing ampicillin (100 μg/mL) and sampled every hour for 8 h ($n \geq 3$). Symbols ($\mp$) correspond to Figure 2c. Error bars represent SEM. Asterisks indicate p-value (compared to no induction (black)) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. There is 100% survival at time zero, because percent survival is determined based on the surviving CFU/mL compared to the CFU/mL at time zero. (b–d) Stochastic model

Figure 4. continued

of population decay with antibiotic treatment. (b) Reactions for the model (left) and baseline rates used for the simulations (right) unless stated otherwise (red lines below). Normal cell division (ω) was set to zero as dividing cells die during ampicillin treatment. (c) Increasing the rate of entering persistence (α) increases cell number during the second phase of population decay. (d) Decreasing the rate of normal cell death (μ_n) causes the first phase of population decay to lengthen. Y-axes are in logarithmic scale for a, c, and d.

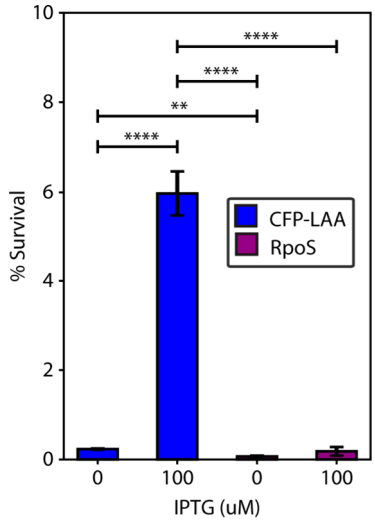

Figure 5. Induction of RpoS during antibiotic treatment has no significant effect on survival after 3 h of ampicillin treatment ($p > 0.4$). The same CFP-LAA data was used in Figure 2f.

floxacin, we suspect that high production of CFP with no apparent method of removal (besides cell division; minimal degradation) causes cell stress and affects survival, especially since high levels of fluorescent proteins can cause oxidative stress,[54,55] which is known to increase persistence.[56−58] However, because CFP-LAA is removed *via* degradation (indicated by lower fluorescence than CFP-untagged), the effects seen *via* overexpression of CFP should be less prominent during CFP-LAA overexpression. The results we describe here would not have been identified in a *clpP* knockout, because *clpP* knockouts break cellular systems and detrimentally affect cellular processes,[59] as evidence by growth defects.[40] Similarly, studies of TA systems and their role in antibiotic survival are confounded by the fact that TA systems make up highly interconnected networks with built in redundancy so that removal of several TA systems does not fully disclude activities by the others[10,60,61] and can even affect growth.[62] As such, changes in tolerance are difficult to differentiate from affects caused by permanent alterations in system dynamics resulting from genetic mutations. Here we demonstrate the utility of proteolytic queueing to study antibiotic survival, while minimizing negative effects of protease knockouts that could obfuscate the phenomenon of interest.

In some cases, the change in survival at 3 h might be interpreted as a change in persistence; however, the shift in decay rates (as described in Figure 1a) clearly demonstrates that queueing increases antibiotic tolerance rather than persistence. Furthermore, the effects caused by adding or removing the inducer during antibiotic treatment suggest that

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the change in antibiotic tolerance is due to an active response to the queue, which must be maintained to affect survival. Although persistence does not appear to be affected by the proteolytic queue at ClpXP, further overloading ClpXP is possible and we simply may not be able to measure an affect at this level. However, persisters are considered metabolically dormant and an active response to the queue could explain why tolerance is affected but not persistence. Alternatively, the synthetic queue may not actually form in persister cells due to slowed translation and transcription. Our model supports that antibiotic tolerance is being affected by queueing rather than persistence, as altering survival of the "normal" population (*i.e.*, tolerance) more closely resembles the effects of proteolytic queueing than altering the rate of switching into persistence. While these results are specific to queueing at ClpXP, tags are available to test the effects of queueing at other proteases (*e.g.*, Lon and ClpAP).[36]

Queueing at ClpXP is likely affecting the proteome of the cell, either directly or indirectly, and pleiotropic effects on protein content and gene regulation could be limiting antibiotic efficacy. We suspect that queue formation increases the intracellular concentration of one or multiple protein species causing a regulatory cascade. When considering proteins both degraded by ClpXP and related to persistence, TA systems are unlikely to be the causative factor, because decreasing degradation should increase antitoxin levels and decrease survival rather than increase survival as we observe. Instead, we consider regulatory proteins as candidates for the causative factor in queueing effects on tolerance. Several regulatory proteins are degraded by ClpXP[45] including RpoS and DksA, proteins that have been implicated in persistence[24,58,63] and may be involved in tolerance.

We have tested the effect of overexpressing RpoS under the same conditions as proteolytic queueing to see if we could replicate the queueing-tolerance phenotype. Our results show that overexpression of RpoS does not significantly affect antibiotic tolerance. These results do not confirm that RpoS alone is responsible for changes in tolerance, especially considering that RpoS levels range broadly under different stress conditions and that over 23% of the *E. coli* genome is regulated by RpoS.[47] However, several other regulatory and stress response proteins are degraded by ClpXP,[45] and increased concentrations of one or several of these proteins due to slowed degradation could be causing the downstream effects that lead to increased tolerance. In a similar vein, computational modeling has shown that altering degradation of MarA (a regulatory protein degraded by Lon that is related to antibiotic tolerance) leads to increased coordination of downstream genes.[64]

The increase in antibiotic tolerance due to queue formation at ClpXP may be specific to overexpression of the LAA-tag, especially when considering that the number of LAA tagged proteins naturally increases during stress. The number of proteins with LAA tags increase during heat shock,[65] and queue formation at the proteases is likely a consequence of the increasing cellular traffic. If the native LAA tag is removed from SsrA while maintaining the ribosome rescue function, the survival of ampicillin treatment decreases in *E. coli*.[24] As the LAA tag could be a measurement of environmental stress, cells may have evolved to increase tolerance in response to increased queueing *via* LAA. Since ribosome rescue and proteolytic queueing are common across species, stress signaling *via* proteolytic queueing could be a general mechanism to regulate survival related genes. Considering that proteolytic queueing is a natural phenomenon and synthetic queues have fewer negative effects compared to protease knockouts, our work demonstrates that proteolytic queueing is a viable alternative method to study proteolytic degradation by specific proteases. In the case of antibiotic tolerance, identifying the key proteins affected by the queue during bacterial tolerance and then understanding how these proteins interact has the potential to determine new drug targets for killing bacterial pathogens.

MATERIALS AND METHODS

Strains and Plasmids. All strains are derived from *E. coli* DH5αZ1, and contain plasmids with the synthetic circuits, p24KmNB82 (CFP-LAA) and p24KmNB83 (untagged CFP) as described in ref 36, and p24KmAA01, which contains RpoS cloned downstream of $P_{lac/ara}$ promoter of p24Km (kanamycin 25 μg/mL) as in ref 36. As such, CFP, CFP-LAA and RpoS are all expressed under identical promoters and ribosome binding sites. DH5αZ1 was derived from *E. coli* K12 (arguably the most studied bacteria strain[66]); it is used by many in synthetic biology, and outside the field,[67−70] this strain has previously been used to study persistence/tolerance or mechanisms related to them (*e.g.*, toxin−antitoxin systems),[71−73] and our previous queueing experiments used these derivatives.[36]

The cultures were grown in modified MMA media,[74] which we will refer to as MMB. MMB media 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 $MgSO_4{\cdot}7H_2O$, 100 μM $CaCl_2$, thiamine (10 μg/mL), 0.5% glycerol, and amino acids (40 μg/mL). Cultures grown on glucose as the carbon source included 0.5% glucose instead of glycerol. 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. Persisters were quantified by comparing colony-forming units per milliliter (CFU/mL) before antibiotic treatment to CFU/mL after antibiotic treatment. The procedure for quantifying persister levels is based on previous research[71,75,76] (Figure S3). Briefly, overnight cultures were diluted 1/100 into fresh media and grown until they reach between OD_{600} 0.2−0.3. A reduced volume of culture (20 mL) was aliquoted into a 125 mL flask, and grown for 16 h to enter stationary phase. Once in stationary phase, cultures were divided into two flasks with 0.2% arabinose; one flask of each replicate was also treated with 100 μM IPTG to induce expression under $P_{lac/ara\text{-}1}$.

Arabinose was added to both induced and uninduced cultures to maintain consistency (Figure S4). All flasks were incubated for 24 h before taking samples for plating and antibiotic treatment; cells were diluted 1/100[71,75] into glass tubes, treated with 10× the MIC of ampicillin (100 μg/mL; Figure S5) or 100× MIC of ciprofloxacin (1 μg/mL) at 37 °C and shaken at 250 rpm for select time periods, 3 h unless otherwise stated. Ampicillin solutions were stored at −80 °C and only thawed once to reduce variability.[22,77] When indicated, samples were treated with chloramphenicol (5 μg/mL); cultures treated with chloramphenicol alone were diluted 1/10. Samples for quantification of CFU/mL were kept on ice and diluted using cold MMB before plating on LB/Km (25

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μg/mL) agar plates. Cultures treated with ciprofloxacin were centrifuged at 16 000*g* for 3 min then washed with cold MMB to dilute ciprofloxacin before taking samples for quantification. LB agar plates were incubated at 37 °C for 40−48 h, then scanned using a flatbed scanner.[78,79] Custom scripts were used to identify and count bacterial colonies[80] then used to calculate CFU/mL and persister frequency. Colonies were tested periodically for resistance, and we found no resistance in >350 colonies tested.

Quantification of CFP. Cells were grown and treated with ampicillin as described in quantification of persistence above. After antibiotic treatment, 300 μL of cell culture was added to individual wells in a 96-Well Optical-Bottom Plate with Polymer Base (ThermoFisher) for fluorescence measurement using FLUOstar Omega microplate reader. The excitation and emission (Ex/Em) used for CFP measurement was 440/480. Readings were measured after 4 min of shaking to decrease variability between wells. Background fluorescence (mean fluorescence of MMB media) was subtracted from the raw reads. Fluorescence values were normalized by CFUs as determined by quantification of persistence, which was carried out simultaneously. Mean and SEM for fluorescence was determined across four biological replicates and three technical replicates.

Computational Modeling. Our model is modified from Kussel *et al.*,[53] where P is the persister population and N is the susceptible population (Figure 4b). Initial species counts P and N were set to 99 800 and 200, respectively, for all simulations, which we based on the percent survival of uninduced cultures. The death rate of N (μ_n) and P (μ_p) and the rate of entering (α) and exiting (β) persistence were set as shown in Figure 4b unless otherwise stated. The rate of susceptible cell division (ω) was set to zero, as normal cells cannot divide without lysis during ampicillin treatment.[81] All simulations were performed using a custom implementation of the Gillespie algorithm[82] in Python leveraging optimizations made possible by the Cython library.[83] Libraries from the SciPy stack[84] were used for analysis.

Statistics. All data is presented as mean ± SD or SEM of at least 3 biological replicates as appropriate.[85] Statistical significance for populations with the same number of replicates (n) was determined using one-way *f*-test to determine variance ($p < 0.001$ was considered to have significant variance) followed by a Student's *t* test (no variance) or a Welch's *t* test (significant variance). Populations with different n values were compared using a Welch's *t* test. All statistical tests were run in Python using libraries from SciPy on groups with at least three biological replicates.

Calculation of Doubling Times. Optical density (OD) was measured at 600 nm in a microplate reader (see Quantification of CFP). Doubling time (t_d) was determined as described in ref 86. Briefly, we calculated the linear regression of the natural logarithm (ln) of the OD over time (t). The equation of the line can thus be derived from a logarithmic growth curve and solved for t_d (eqs 1, 2).

$$\mathrm{OD} = \mathrm{OD}_0 e^{[\ln(2)/t_d]t} \tag{1}$$

$$\ln(\mathrm{OD}) = \frac{\ln(2)}{t_d} t + \ln(\mathrm{OD}_0) \tag{2}$$

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00358.

Figure S1: Induction of untagged CFP and CFP-LAA tag has no apparent effect on growth in MMB+ media; Figure S2: Induction of CFP-LAA does not increase survival of cells treated with chloramphenicol; Figure S3: Persister assay flowchart; Figure S4: The addition of arabinose had no apparent effect on the tolerance/persister level during ampicillin treatment; Figure S5: Determination of minimal inhibitory concentration (MIC) for ampicillin (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: nicholas.butzin@gmail.com.

ORCID

Nicholas C. Butzin: 0000-0003-3807-8666

Present Address

§Independent Researcher, Prosser, Washington 99350, United States.

Author Contributions

‡H.S.D. and A.H.A. contributed equally. H.S.D. wrote the manuscript, developed custom code for colony counting, measured bacterial growth rates, and ran statistical analyses. A.H.A. performed ampicillin and ciprofloxacin persister assays. P.J. performed plate reader fluorescence assays. E.S. performed chloramphenicol persister assay. C.T.O. and H.S.D. adapted the persister model and ran stochastic simulations. N.C.B. initiated and directed the project. All authors contributed to discussing and editing the manuscript.

Notes

The authors declare no competing financial interest.

Code used for model simulations is available on GitHub at https://github.com/ctogle/mini_gillespiem. Code used for colony counting is available on GitHub at https://github.com/hdeter/CountColonies.

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