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Single-cell Physiological Response of *Escherichia coli* to Suppressive Antibiotic Combinations

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Abstract

In the natural environment, bacteria are exposed to varying levels of nutrient availability which significantly affect cellular physiology and the efficacy of antibiotics. Changes in nutrient availability and growth rate also impose physiological constraints on the cell's capacity to respond to stress, such as DNA damage, and can have important consequences on treatment outcomes, but this effect is under-appreciated. Bacteria deal with DNA damage by inducing the SOS response which plays an important role in survival under DNA stress and contributes to antibiotic resistance. In clinical practice, antibiotics are increasingly used in combination to improve efficacy against infectious diseases and to limit the emergence of resistance. For some antibiotic combinations, bacterial inhibition decreases when one antibiotic is added to another. This is called a suppressive drug interaction and is common between DNA synthesis inhibitors and translation inhibitors. Despite having a lower efficacy, suppressive drug combinations have potential in treating bacterial infections because they select against antibiotic resistance. However, the relationship between bacterial physiology and susceptibility to suppressive antibiotic combinations is not yet clearly understood.

Previous studies on SOS response induction and suppressive drug combinations rely on bulk-level experiments which lack insight into the underlying population dynamics and the inherent heterogeneity of single-cell growth and gene expression. Single-cell approaches are key in informing a mechanistic understanding of the cellular response to DNA damage and antibiotic combinations. In this thesis, we used a single-cell microfluidic device called the 'Mother Machine' and quantitative time-lapse microscopy to investigate growth-dependence in SOS response induction in *Escherichia coli*. We directly measured the rate of SOS induction in a strain experiencing sublethal, replication-dependent DNA damage and found that it was higher in fast-growth conditions. However, using a population dynamics model, we revealed that the proportion of high-SOS cells in a growing culture is not only a function of the rate of SOS induction but also depends upon competition with low-SOS, fast-dividing cells. Next, we investigated the physiological mechanisms that give rise to the suppressive interaction between ciprofloxacin (a DNA synthesis inhibitor) and tetracycline (a translation inhibitor). Our results show that an improvement in cell survival, rather than increased growth and division, is the dominant mechanism leading to suppression between ciprofloxacin and tetracycline. Furthermore, the suppressive effect was amplified in fast-growth conditions which could have important implications on treatments in fluctuating nutrient environments. Altogether, our

results highlight the utility of single-cell time-lapse methods in revealing population dynamics and single-cell heterogeneity that would otherwise be masked in bulk-level methods. In furthering our understanding of bacterial responses to antibiotics and their combinations, we aim to contribute to the development of more effective treatment strategies.

Lay Summary

Bacteria in the natural environment face varying nutrient levels which influence bacterial responses to antibiotics and DNA damage. Bacterial infections are increasingly difficult to treat, and we require a better understanding of their responses in different environments to combat this. In this thesis, we focus on the DNA damage response and suppressive antibiotic combinations, where the two antibiotics are less effective when they are combined. Unlike previous studies, this research takes a single-cell approach, considering the response of each individual cell in a population rather than the overall population response, which offers a more detailed understanding.

An innovative microfluidic technology called the ‘Mother Machine’, in combination with time-lapse microscopy, was used to track the response of hundreds of individual bacterial cells. The results revealed that the DNA damage response (known as the SOS response) is expressed more frequently in rich nutrient conditions. Surprisingly, the proportion of highly responsive cells in a growing culture depends not only on SOS induction frequency but also on competition with fast-dividing cells with lower response levels. This is because the DNA damage response also slows cell division which introduces variation in division times of individual cells.

This research also explored the suppressive drug interaction between a DNA targeting-antibiotic and a ribosome-targeting antibiotic. The results revealed that suppression is caused by enhanced bacterial survival rather than promoting growth. The suppressive interaction was also found to be amplified in rich nutrient conditions, which may have important implications for antibiotic treatments in fluctuating nutrient environments.

The results presented here underscore the importance of considering bacterial population dynamics and single-cell behaviours in antibiotic research. This research contributes valuable insights for developing more effective treatment strategies against bacterial infections.

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Declaration

I declare that this thesis has been composed by myself and that the work has not been submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications has been included. My contribution and those of the other authors to this work have been explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in Chapter 4 was previously published in *Molecular Systems Biology* as “Growth-dependent heterogeneity in the DNA damage response in *Escherichia coli*” by Sebastian Jaramillo-Riveri, James Broughton (author of this declaration), Alex McVey, Teuta Pilizota, Matt Scott, and Meriem El Karoui (supervisor). This study was conceived by all of the authors. I carried out all mother machine experiments, data analysis, and figure preparation. Contributions from Sebastian Jaramillo-Riveri include: strain construction, design of the mother machine, assistance with data analysis, and derivation of the population dynamics model.

James K. Broughton

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Chapter 1: Introduction

Bacteria in the natural environment experience varying levels of nutrient availability which determines their growth rate. The physiological composition of a cell is known to respond to nutrient availability by optimising the allocation of cellular resources in order to maximise growth. As a result, some antibiotics that target growth-related processes, such as DNA and protein synthesis, are more effective in fast-growing cells. Moreover, changing growth rates imposes constraints on the capacity of bacteria to modify their proteome (Scott *et al*, 2010; Hui *et al*, 2015), potentially influencing the capacity of cells to respond to stress.

The SOS response is an important mechanism in bacteria that facilitates survival under DNA-damaging conditions and can contribute to antibiotic resistance due to increased mutagenesis (Maslowska *et al*, 2019). DNA damage can occur spontaneously during DNA replication or via numerous exogenous sources such as antibiotics. The formation of DNA double-strand breaks is the most toxic form of DNA damage and is repaired via homologous recombination mediated by induction of the SOS response (Maslowska *et al*, 2019). At the single-cell level, expression of the SOS response has been shown to be highly heterogeneous (Jaramillo-Riveri, 2019; Jones & Uphoff, 2021) which could have important consequences on the outcome of individual cells suffering DNA damage. However, the effect of changing growth rate on the variability of SOS response expression in different growth conditions has not yet been quantified. Furthermore, SOS expression inhibits cell division which leads to heterogeneity in cell size and division rates in the population. This introduces further complexity in the population dynamics that can only be deconvolved using single-cell methods. Investigating heterogeneity in SOS expression and its growth dependence is important for understanding bacterial responses to antibiotics in different nutrient environments.

In addition to understanding the bacterial response to antibiotics, novel treatment strategies are also required. Our society is dealing with major negative clinical outcomes associated with antibiotic resistance and persistent bacterial infections (WHO AGISAR, 2019). As the development of new antibiotics has stalled in recent years, we urgently need improved treatment strategies. In clinical practice, antibiotics are increasingly used in combination to increase efficacy against infectious diseases and combat antibiotic resistance (Coates *et al*, 2020). Antibiotic interactions are determined by whether their combined efficacy is equal to (additive), stronger than (synergistic), or weaker than (antagonistic) than expected. For some antibiotic combinations, the combined efficacy is weaker than that of one of the antibiotics

used alone. This is called a suppressive drug interaction and is common between DNA synthesis inhibitors and translation inhibitors (Roemhild *et al*, 2022). Despite having a lower combined efficacy, suppressive drug combinations can slow or reverse the evolution of antibiotic resistance (Singh & Yeh, 2017). Yet, these drug interactions remain relatively understudied. Although suppressive interactions have been described extensively in the literature (Bollenbach *et al*, 2009; Roemhild *et al*, 2022), the relationship between bacterial physiology and susceptibility to suppressive antibiotic combinations is not yet clearly understood.

Stochastic fluctuations in growth rate and gene expression can result in a heterogeneous response to antibiotic treatment among cells within a population. However, most studies of bacteria and their response to antibiotics overlook the physical and temporal heterogeneity within bacterial populations. Traditionally, *in vitro* experiments measure population-averaged behaviour. But, as new microfluidic, imaging, and data analysis technologies have been developed, we are now able to precisely monitor the response of individual cells within a population. For example, a custom microfluidic device called the ‘Mother Machine’ enables long-term tracking of hundreds of individual cells under the microscope (Wang *et al*, 2010). Previous studies on SOS response induction and suppressive drug combinations rely on bulk-level experiments which lack insight into the underlying population dynamics and the inherent heterogeneity of single-cell growth and gene expression. Single-cell approaches are key in informing a mechanistic understanding of the cellular response to DNA damage and antibiotic combinations. Furthermore, single-cell microscopy can reveal the presence of various sub-populations as well as rare and non-heritable, highly resistant phenotypes, such as persister cells, that often emerge in response to antibiotic treatment (Balaban *et al*, 2004).

In this thesis, we use a single-cell approach to investigate the physiological mechanisms that give rise to the suppressive interaction between ciprofloxacin (a DNA synthesis inhibitor) and tetracycline (a translation inhibitor) at sub-lethal concentrations. In addition, we evaluate the growth rate dependence of SOS response expression and, with the help of a mathematical model, compare results with bulk-level observations. A microfluidic device called the ‘Mother Machine’ and fluorescence time-lapse microscopy are used to track single-cell growth and gene expression dynamics in the model organism *Escherichia coli*, which is the causative agent for several human diseases (Johnson & Russo, 2002; Mobley *et al*, 2009; Poolman & Anderson, 2018). In furthering our understanding of bacterial responses to antibiotics and their combinations, we aim to contribute to the development of more effective treatment strategies.

Chapter 2: Literature review

2.1 *Escherichia coli* as a model organism

Escherichia coli is a rod-shaped, Gram-negative bacterium frequently used as a model organism in microbiological research. It is typically found colonising the intestinal tract of humans and animals and can also be found in dilute environmental waters such as lakes and rivers (Savageau, 1983). It is part of the Enterobacteriaceae family, making it closely related to pathogens such as *Klebsiella* and *Salmonella* (Brenner *et al*, 2005). Though usually commensal¹, *E. coli* is the causative agent for several human diseases, such as urinary tract infections, septicaemia and meningitis (Johnson & Russo, 2002; Mobley *et al*, 2009; Poolman & Anderson, 2018). There are also several types of pathogenic strains that produce highly damaging virulence factors², for example enterohemorrhagic *E. coli* produces a shiga-like toxin that destroys small blood vessels leading to abdominal pain, diarrhoea, and is potentially fatal if left untreated (Robinson *et al*, 2006; Blount, 2015). Besides its clinical significance, research on *E. coli* has led to numerous breakthroughs in molecular biology and genetic engineering, and is used extensively today in the biotechnology industry (Blount, 2015).

E. coli K-12 is the most common bacterial strain used in laboratories around the world today. It was first isolated in 1922 from a diphtheria patient and has since become one of the most widely studied organisms (Browning *et al*, 2023). Until the development of cryopreservation, *E. coli* K-12 has been repeatedly sub-cultured in the laboratory and thus has acquired mutations and evolved to adapt to growth in rich nutrient conditions outside of its natural environment. As a result, the sub-strains used today carry various genetic changes. For example, descendant strains of the original *E. coli* K-12 isolate, MG1655 and W3110, contain the following genetic changes: deficient in the F plasmid and bacteriophage λ ; mild pyrimidine starvation due to a frameshift in *rph* resulting in decreased *pyrE* expression; loss of O-antigen synthesis due to a mutation at *rfb-50*; among others (Browning *et al*, 2023). Although the typical laboratory-evolved *E. coli* strains are non-pathogenic and have diverged from their true wild-type counterparts, their use as a model organism in fundamental and applied bacteriology remains indispensable to this day. However, we should always bear in mind the caveats of working with

¹ Commensalism refers to the growth of two organisms in close association, such that one organism benefits without harming the other.

² Virulence factors are molecules that allow a bacterial pathogen to colonise its host and include functions such as adhesion, biofilm formation, motility, toxin activity, and protection against host defences (Pakbin *et al*, 2021).

this organism, in particular genetic differences which should inform our interpretation of results.

2.2 Bacterial physiology: Theory and consequences

2.2.1 Growth laws

Research into cellular physiology over the last 70 years has revealed important correlations, or ‘growth laws’, between the processes of cell division, cell size, and macromolecular composition of the bacterial cell. The broadest of these correlations is the exponential dependence of cell size with growth rate when the latter is modulated by nutrient availability. At the macromolecular level, the abundance of RNA, DNA, protein, and mass per cell scales exponentially with growth rate (Kjeldgaard *et al*, 1958; Schaechter *et al*, 1958; Bremer & Dennis, 2008). These correlations hold for exponentially growing cells and is commonly referred to as ‘balanced’ or ‘steady-state’ growth. Global gene expression is also regulated in a growth-dependent manner (Klumpp *et al*, 2009).

2.2.2 Empirical constraints on the proteome

The growth rate of the cell is fundamentally linked to its ribosome abundance during balanced growth. The ribosome is responsible for protein synthesis and is thus the catalytic unit for growth. This is because translation rates by the ribosome are relatively fixed and therefore the number of ribosomes imposes a strong constraint on the achievable growth rate of a cell. In fact, the protein mass fraction of ribosomes in the cell is directly proportional to the growth rate (Neidhardt & Magasanik, 1960). Concomitantly, protein mass fraction of constitutively expressed genes – those that are un-regulated – was found to be negatively correlated with growth rate (Klumpp *et al*, 2009). Assuming that translation rates are invariant to growth rate (Klumpp *et al*, 2009), the only way the cell can increase its protein production rates is by increasing its ribosome content (Figure 1A). However, this is understood to come at the expense synthesising “other” proteins (i.e., unregulated constitutive proteins) (Figure 1B). This also holds true in the reverse scenario: when the ribosomal fraction decreases (due to poor nutrient availability), the constitutive protein fraction increases. Thus, there is a linear constraint between these two sectors of the proteome (Scott *et al*, 2010) (Figure 1C). In order to maximise growth, the cell manages its translational resources between production of new ribosomes and production of constitutive proteins in a growth rate-dependent manner. This is, of course, a very simple model of the bacterial proteome. The growth-dependent components

of the *E. coli* proteome make up around 50% of the proteome by mass and is comprised of diverse functional sectors each with distinct growth rate dependencies (Hui *et al*, 2015).

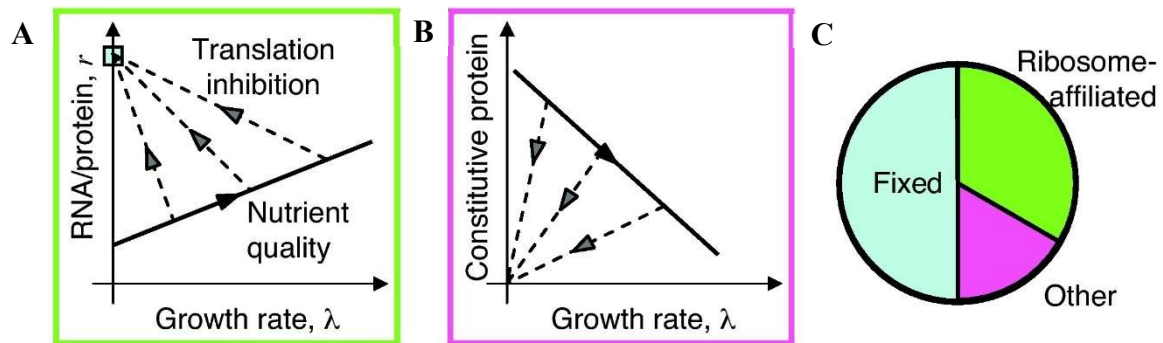


Figure 1. Illustration of bacterial empirical growth laws on proteome partitions. **A.** Relation between growth rate and ribosome abundance within the cell. Ribosomal content (RNA/protein) increases linearly with growth rate when modulated by nutrient quality (solid line). Ribosomal content is inversely correlated with growth rate under translation inhibition by sub-inhibitory levels of antibiotic (dashed line). **B.** Mass fraction of unregulated non-ribosomal proteins (constitutive) is inversely correlated with growth rate under nutrient limitation (solid line) and will be reduced by a larger extent in slow growth conditions under translation inhibition. **C.** Empirical constraints on the proteome. The bacterial proteome is partitioned between a growth-invariant fixed fraction and the growth-dependent fractions which can be coarsely separated between ribosomal and other (constitutive) proteins. Proteome partitions are constrained: Increasing the ribosomal fraction must come at the expense of the constitutive protein fraction. Figure reproduced from Scott *et al.* (2011) with permission from Elsevier (License: 5607230268037).

How do these proteome partitions change when other aspects of cellular physiology are perturbed? In general, ribosome abundance is positively correlated with growth rate when modulated by nutrient quality of the medium. However, when growth rate is modulated by changes in translation efficiency, such as by a translation inhibitor, the contrary is observed: an inverse relation between ribosome abundance and growth rate (Figure 1A, dashed lines). Moreover, the efficacy of the translation inhibitor is also growth-dependent, with certain translation inhibitors being more effective at faster growth rates (see slope of dashed lines in Figure 1A). This can be explained by the higher capacity of slow-growing cells to increase their ribosome levels under translation inhibition. Cells growing near their maximum growth rate are close to their ceiling in terms of ribosome levels, and thus have a smaller capacity to increase ribosomal levels in response to translation inhibition.

These observations have been generalised into empirical growth laws which have been used as a foundation for resource allocation modelling to predict the relation between proteome partitions and growth rate (Klumpp *et al*, 2009; Scott *et al*, 2010; Hui *et al*, 2015), and have

since been used to predict physiological responses to translation inhibitors (Greulich *et al*, 2015). Recently, resource allocation models have been developed that incorporate an increasing number of growth-dependent proteome sectors, such as catabolism, anabolism, stress response sectors, and many more (Hui *et al*, 2015; Mori *et al*, 2021; Bren *et al*, 2022). These finer-grained models enable predictions to be made about how the cells' physiology responds to perturbations including various types of nutrient limitations (carbon, nitrogen, phosphate, and amino acid limitations) and stress (antibiotics, osmolarity, temperature, and oxidative stress).

2.2.3 DNA replication and cell-cycle control

The cell coordinates DNA replication and cell division to reconcile differences in growth rate. During cell division, two events must take place: replication of the chromosome and then chromosome segregation and cell division. These events take approximately 40 minutes and 20 minutes to complete (a total of 60 minutes), respectively, and are invariant with growth rate (Cooper & Helmstetter, 1968). However, we know that *E. coli* can double as fast as every 20 minutes which is three times quicker than it takes to replicate and segregate its chromosome (i.e. the growth rate surpasses the rate of DNA replication). The cell reconciles this difference by initiating an additional round of replication before the previous round has completed (Cooper & Helmstetter, 1968; Trojanowski *et al*, 2018). During slow growth with >60 minute doubling times, a single round of DNA replication occurs per cell-cycle, whereas faster growing cells with <60 minute doubling times undergo overlapping rounds of replication (Figure 2). Overlapping rounds of replication – known as multi-fork replication – allows DNA replication to keep up with faster cell division and growth of the cell. Thus, cells in slow-growth regimes may behave vastly different than in fast-growth regimes. In faster growing cells, increased DNA abundance (chromosomal copies) and the presence of multiple replication forks may have implications on the efficacy and physiological responses to antibiotics, particularly DNA-targeting antibiotics.

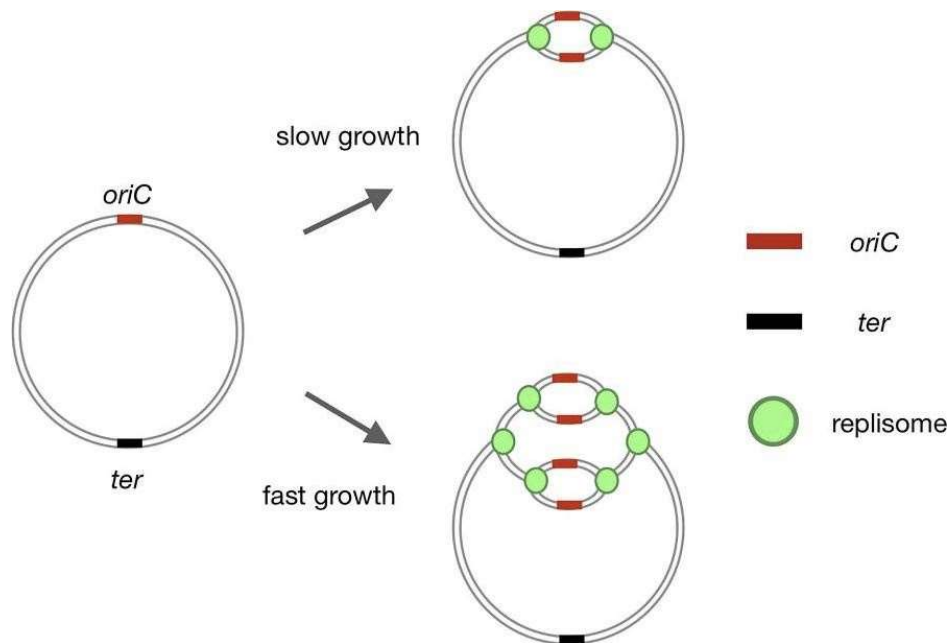


Figure 2. **Replication of the chromosome in slow and fast growth regimes.** Replication is initiated at the *oriC* region, proceeds along both arms of the chromosome and terminates at the *ter* region. In slow growing cells, replication is initiated once per cell-cycle, whereas in fast growing cells, a second replication fork initiates before the previous one has been completed. Figure reproduced from Trojanowski et al. (2018) (use permitted under the Creative Commons Attribution License CC BY 4.0).

2.2.4 Consequences of changing bacterial physiology

Physiological changes imposed by the growth medium can have important consequences on the cell's response to antibiotics, not only for translation inhibitors, but also for DNA-targeting antibiotics. Bacteria cultured at different growth rates are associated with different proteomic allocations (Figure 1) and DNA abundance (Figure 2) and thus may have different translational capacities to induce stress responses and repair their DNA. Additionally, growth-related changes in the target abundance of translation inhibitors (ribosomes) will affect their efficacy (Greulich *et al*, 2015).

2.3 Antibiotics target different components of cell physiology

Antibiotics target essential physiological processes in the cell such as DNA synthesis, RNA transcription, protein translation, or cell wall synthesis. Understanding their individual effects on cell physiology, and how the cell responds to this stress, is vital for developing a mechanistic understanding of antibiotic combinations.

2.3.1 Antibiotics targeting DNA topoisomerase: Fluoroquinolones

Fluoroquinolones are bactericidal³ antibiotics that inhibit the activity of the type II DNA topoisomerases, DNA gyrase and topoisomerase IV (Drlica & Zhao, 1997; Drlica *et al*, 2009; Aldred *et al*, 2014). Type II topoisomerases function by forming protein-bridged double-stranded breaks (DSBs), enabling DNA strand passage through a neighbouring DNA strand, followed by ligation of the DNA ends (closing the previously formed DSB). In doing so, type II topoisomerases relieve supercoiling strain and entanglement during DNA replication and transcription (Drlica *et al*, 2009). Fluoroquinolones act by binding to type II topoisomerases which form a stable complex on the DNA and inhibits the enzyme's ligase activity resulting in the introduction of DNA DSBs on the chromosome. These complexes also act to block the progress of DNA replication forks and transcription (Aldred *et al*, 2014). It is thought that the release of 'naked' DSBs from the fluoroquinolone-topoisomerase-DNA complex could result from collision of replication forks with the complex or some process that releases the enzyme from the DNA such as fluoroquinolone-mediated gyrase subunit dissociation, proteolysis, or denaturation (Drlica *et al*, 2009). The accumulation of DSBs subsequently fragments the chromosome leading to cell death (Malik *et al*, 2006). The introduction of DSBs triggers the SOS response and DNA repair processes which are described in detail later (Section 2.4.2).

Fluoroquinolones such as ciprofloxacin (CIP) can induce significant changes in cell morphology. In particular, cell elongation or filamentation is commonly associated with sub-MIC⁴ CIP treatment (Wojnicz *et al*, 2007; Bos *et al*, 2015; Butler *et al*, 2023). Filamentation is caused by the SOS-mediated inhibition of cell division with continued cell growth. After extended CIP exposure, several phenotypes have been observed such as 'ghost' cells (lysed cells having lost the cytoplasm but with cell wall still intact), cytoplasmic vacuolation, and cytoplasmic shrinkage (Wojnicz *et al*, 2007).

2.3.1.1 ROS-mediated lethality by fluoroquinolones

Besides chromosome fragmentation, a second pathway for fluoroquinolone-mediated killing has been discovered. Fluoroquinolones have been shown to result in the generation of reactive oxygen species (ROS) that cause secondary cellular damage and contribute to cell death (Goswami *et al*, 2006; Dwyer *et al*, 2007, 2014; Kohanski *et al*, 2007; Wang & Zhao, 2009; Belenky *et al*, 2015; Kottur & Nair, 2016; Smirnova *et al*, 2017; Hong *et al*, 2019, 2020; Pribis

³ Bactericidal refers to the activity of an antimicrobial that primarily kills bacteria.

⁴ See Box 1, page 13

et al, 2019; Wong *et al*, 2021). ROS are highly reactive molecules, such as hydroxyl radicals ($\text{OH}\cdot$), hydrogen peroxide (H_2O_2) and superoxide radicals ($\text{O}_2\cdot^-$), that can directly damage various cellular components including DNA, proteins, and lipids (Dwyer *et al*, 2009; Van Acker & Coenye, 2017; Butler *et al*, 2023). In particular, ROS can oxidise the nucleotide pool and peptides, resulting in DNA breaks and misfolded proteins (Foti *et al*, 2012; Butler *et al*, 2023).

ROS are generated as by-products of aerobic respiration and oxidative phosphorylation – processes involved in the generation of cellular energy: ATP (Imlay & Fridovich, 1991). The mechanism linking ROS production to oxidative phosphorylation and central metabolism is summarised in Figure 3. Extensive research has linked cellular respiration and central metabolism (associated with the citric acid cycle) to the lethal activity of bactericidal antibiotics (Kohanski *et al*, 2007; Akhova & Tkachenko, 2014; Dwyer *et al*, 2014; Belenky *et al*, 2015; Lobritz *et al*, 2015; Gutierrez *et al*, 2017; Lopatkin *et al*, 2019). For example, Kohanski *et al*. (2007) found that NADH consumption surged following norfloxacin (a fluoroquinolone similar to CIP) treatment. Dwyer *et al*. (2014) reported a similar surge in oxygen consumption under norfloxacin treatment. Since NADH and oxygen are substrates required to drive oxidative phosphorylation/respiration (Figure 3A), the authors deduced that this surge in substrate consumption resulted in a burst of ROS generation, which has been observed previously (Dwyer *et al*, 2007). Lobritz *et al*. (2015) further proved that cellular respiration accelerates under norfloxacin treatment. Considering other components of cell metabolism, Belenky *et al*. (2015) reported elevated central carbon metabolites and reduced nucleotide and lipid levels in cells treated with norfloxacin and other bactericidal antibiotics. Moreover, the oxidative stress response genes *oxyR* and *soxR* have been shown to be induced in response to fluoroquinolones (Akhova & Tkachenko, 2014). The examples given above are a snapshot of the literature in this complex area of research and are all consistent with the notion that bactericidal antibiotics, including fluoroquinolones, result in the generation of ROS dependent on an elevated respiratory and metabolic state.

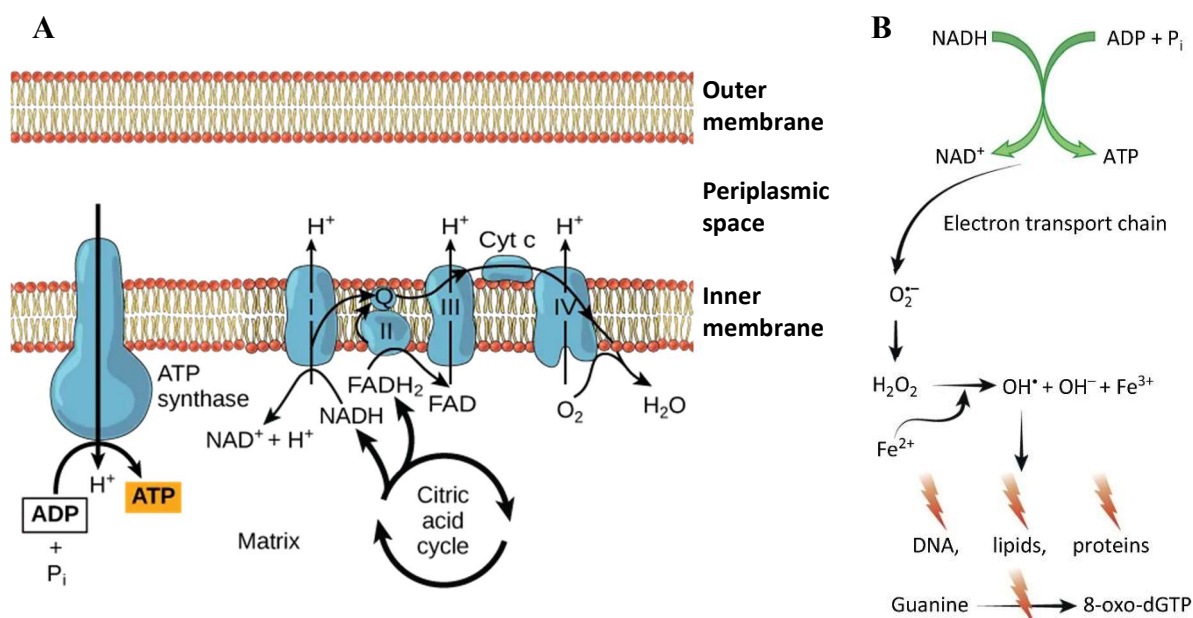


Figure 3. Overview of the role of oxidative phosphorylation and metabolism in the generation of reactive oxygen species. **A.** Oxidative phosphorylation is driven by NADH and FADH₂, which are products of the citric acid cycle (a major pathway in central metabolism), and oxygen. The transfer of electrons through the electron transport chain (ETC), which consists of several membrane-bound enzymes, generates a proton gradient across the inner membrane which drives the synthesis of adenosine triphosphate (ATP) by ATP synthase. Figure modified from Rye *et al.* (2016) (use permitted under the Creative Commons Attribution License CC BY 4.0). **B.** Fluoroquinolones can stimulate hyper-activation of the ETC and oxidation/depletion of NADH (which is dependent upon the citric acid cycle). Acceleration of the ETC generates superoxide radicals (O₂^{•-}) and hydrogen peroxide (H₂O₂) which can destabilise Fe²⁺ from iron-sulphur clusters (Kohanski *et al.*, 2007). The reaction of freed Fe²⁺ with H₂O₂ in the Fenton reaction generates hydroxide radicals (OH[•]) which damages vital physiological components and oxidises the deoxynucleotide pool leading to cell death (Belenky *et al.*, 2015). In particular, oxidation of guanine and subsequent incorporation into DNA can result in double-stranded DNA breaks (Foti *et al.*, 2012). Figure reproduced from Van Acker *et al.* (2017) with permission from Elsevier (License: 5607191062382).

However, ROS-mediated killing has been disputed as a lethal mechanism for bactericidal antibiotics (Keren *et al.*, 2013; Liu & Imlay, 2013; Imlay, 2015). Reasons given include questionable reliability of measuring ROS formation and contradicting results. Recently, Pribis *et al.* (2019) conducted a single-cell study that showed high-ROS formation in only a small minority (<0.3%) of cells up to 4 ng/mL CIP which then jumps to ~20% of cells at 8.5 ng/mL, and then decreases again at higher concentrations. This suggests ROS formation by antibiotics is highly heterogeneous at the single-cell level and is also concentration-dependent, which could explain some of the variable results in this field of research. Given the large body of evidence linking ROS generation and oxidative damage in response to bactericidal antibiotics,

it is hard to argue that the link is not there. Nevertheless, more accurate methods to measure ROS formation in cells are needed to conclusively settle this debate.

2.3.1.2 Clinical significance of fluoroquinolones

Fluoroquinolones are classified by the World Health Organisation (WHO) as a critically important antibiotic used to treat serious bacterial infections and are thus significant for human medicine (WHO AGISAR, 2019). The most commonly prescribed fluoroquinolone is CIP which is widely used to treat a variety of bacterial infections, in particular urinary tract infections (UTIs) and septicemia (Makvana & Krilov, 2015). Resistance to fluoroquinolones is prevalent, occurring in approximately 5-50% of *E. coli* isolates in European countries in 2021 (CAESAR, 2023).

2.3.2 Antibiotics targeting protein synthesis: Tetracycline

Tetracycline (TET) is a bacteriostatic⁵ antibiotic that targets the ribosome to inhibit protein translation (Figure 4). It acts by reversibly binding to the 30S subunit of the ribosome which prevents binding of amino acyl-tRNAs to the ribosome thereby limiting polypeptide chain elongation (Pioletti *et al*, 2001; Poehlsgaard & Douthwaite, 2005). In response, the cell increases ribosome synthesis rates which constrains synthesis of non-ribosomal proteins (see Section 2.2.2) including metabolic enzymes responsible for amino acid supply (Scott *et al*, 2014). With a lower amino acid flux, mass accumulation and therefore growth is slower or stalled depending on the concentration of TET. It has also been proposed that, despite an increase in ribosomal content, the reduction in growth rate is due to the depletion of the pool of free RNA polymerase (RNAP) available for transcription of ribosomal and non-ribosomal genes. However, the expression of ribosomal genes are affected less by this due to the higher affinity of RNAP with ribosomal promoters (Zhang *et al*, 2020). TET and other translation inhibitors have been shown to induce global gene expression changes, including the upregulation of the cold shock response, stringent response, purine biosynthesis, and ribosome associated genes (VanBogelen & Neidhardt, 1990; Bollenbach *et al*, 2009; Mathieu *et al*, 2016; Li *et al*, 2017; Mitosch *et al*, 2017; Møller *et al*, 2020). Tetracyclines have also been linked to potential antioxidant activity, which could limit the oxidative damage to the cell from ROS (Kraus *et al*, 2005; Wang *et al*, 2010b; Kładna *et al*, 2012) – though, antioxidant activity is significantly lower for the parent TET compound compared to its derivatives (Kraus *et al*,

⁵ Bacteriostatic refers to the activity of an antimicrobial that prevents bacterial growth without causing cell death.

2005). This is consistent with the observation that translation inhibitors reduce rates of respiration and metabolic flux (Lobritz *et al*, 2015).

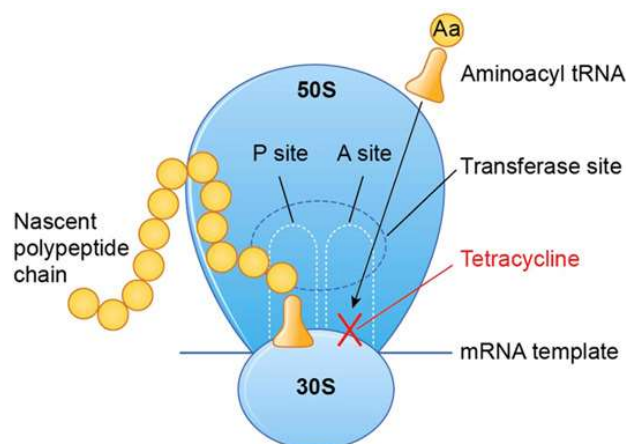


Figure 4. **Illustration of protein synthesis inhibition by tetracycline.** Tetracycline binds to the 30S subunit of the ribosome which allosterically inhibits binding of amino acyl-tRNA to its corresponding mRNA codon at the A-site (acceptor site). Thus, polypeptide chain elongation by the addition of new amino acids (translation) is prevented. Figure reproduced from Graber (2021) (use permitted under the Creative Commons Attribution License CC BY-NC-ND 4.0).

2.3.2.1 Clinical significance of tetracycline

The use of TET in human medicine, classified as highly important by the WHO (WHO AGISAR, 2019), has gradually decreased but is still used in the treatment of various infections, including zoonotic brucellosis, peptic ulcer disease, borreliosis (Lyme disease), chlamydia, and as a malarial prophylactic for mefloquine-resistant *P. falciparum* (van der Hulst *et al*, 1996; Pradines *et al*, 2000; Koehne *et al*, 2021). Resistance to TET is prevalent, occurring in up to 66.9% of *E. coli* isolates in European countries in 2011 (Jones *et al*, 2014b). Resistance to TET is mediated by either energy-dependent efflux pumps, target site mutations, or enzymatic inactivation of the drug (Grossman, 2016).

2.4 Bacterial responses to antibiotics

2.4.1 Growth-dependent susceptibility to antibiotics

Bacterial physiology can strongly affect antibiotic susceptibility, particularly for antibiotics that target growth-related processes. Growth rate-dependent susceptibility of *E. coli* to many antibiotics has been long established, with slow growth commonly associated with reduced antibiotic susceptibility (Tuomanen *et al*, 1986; Millar & Pike, 1992; Greulich *et al*, 2015; Lee *et al*, 2018; Smirnova & Oktyabrsky, 2018; Haugan *et al*, 2019; Zheng *et al*, 2020; Angermayr

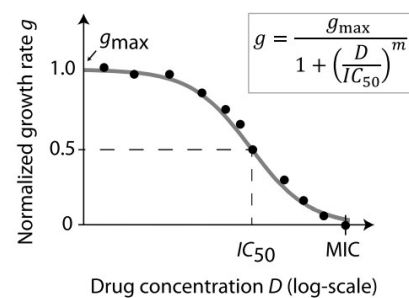
et al, 2022; Smirnova *et al*, 2022, 2023). The reason is intuitive: the activity and cellular concentration of antibiotic targets (e.g., DNA replication proteins, RNA polymerase, ribosomes) correlate positively with growth rate – with fewer targets and slower processing, the action of the antibiotic is limited. For CIP, efficacy may depend on the growth-dependent activity and prevalence of its target DNA gyrase. As for TET, we know that ribosomes are more prevalent in fast growing cells. However, the growth rate dependent efficacy of ribosome-targeting antibiotics also depends on their target binding affinity

(Greulich *et al*, 2015). Specific mechanisms can also influence growth rate-dependence and the shape of the dose-response curve (see Box 1). For example, a growth-dependent negative feedback loop is responsible for the extremely shallow trimethoprim dose-response curve in slow growth conditions (Angermayr *et al*, 2022). For CIP, killing efficacy also depends on metabolic activity (described earlier in Section 2.3.1.1). Therefore, slower growth and metabolic dormancy can render cells more tolerant to CIP (Lobritz *et al*, 2015; Gutierrez *et al*, 2017).

For different translation inhibitors, Greulich *et al*. (2015) observed qualitatively different growth responses, despite the antibiotics sharing the same target (Figure 5). The authors found that growth-dependent susceptibility and shape of the dose-response curve depends on the reversibility of antibiotic membrane transport and target binding (Greulich *et al*, 2015). Susceptibility to irreversibly binding translation inhibitors (streptomycin and kanamycin) are negatively correlated with growth rate and have steep dose-response curves, while susceptibility to reversibly binding translation inhibitors (tetracycline and chloramphenicol) are positively correlated with growth rate and have shallow dose-response curves (Greulich *et al*, 2015). Moreover, it has recently been shown that macrolides (antibiotics that also target the

Box 1: Methods used to quantify antibiotic efficacy

Dose-response curves are commonly used to quantify the concentration-dependent effects of antibiotics on bacterial growth. The dose-response curves are usually described by a Hill function (inset). Other measurements include: minimum inhibitory concentration (MIC), the lowest concentration at which no growth is observed; and IC_{50} , the concentration that reduces growth by 50% compared to a no-drug control. Figure reproduced from Mitosch *et al*. (2014) with permission from Elsevier (License: 5610940042683).



ribosome) have reduced efficacy in fast growth conditions due to higher drug efflux in these conditions (Łapińska *et al*, 2022). Thus, it does not necessarily follow that antibiotics with similar targets will exhibit identical growth rate dependencies.

The threshold-like growth-dependency of ‘irreversible’ translation inhibitors (Figure 5A, B) can be viewed as a competition between antibiotic influx and ribosome production. Incoming antibiotics will bind and inactivate free ribosomes, but if the cell is able to produce ribosomes fast enough, it will neutralise incoming antibiotic and continue growing. As the antibiotic concentration increases, it eventually overwhelms the cell’s ability to neutralise the antibiotic, resulting in a state with no free ribosomes and consequently no growth (Greulich *et al*, 2015). Since fast growing cells have a higher ribosome content (and fast ribosome synthesis), they can tolerate a higher antibiotic concentration than slower growing cells. In contrast, for ‘reversible’ translation inhibitors, there exists an equilibrium between free and antibiotic-bound ribosomes; this equilibrium shifts when the antibiotic concentration increases or the ribosome content changes due to nutrient availability (Greulich *et al*, 2015). This mechanism produces a shallow dose-response curve (Figure 5C, D). In response to the latter, the cell increases ribosome production but the capacity to do so is also growth-dependent: fast growing cells have a lower capacity to increase ribosome production compared to slow growing cells (see slope of line in Figure 1A) and, thus, are more susceptible to ‘reversible’ translation inhibitors (Greulich *et al*, 2015).

The shape of the steeper, threshold-like dose-response curves suggest growth bimodality may occur at concentrations around the steep drop. This can indeed occur as noted by Deris *et al*. (2013) who observed the coexistence of growing and non-growing cells under chloramphenicol treatment due to expression of a chloramphenicol resistance gene. Here it is useful to think of fitness landscapes, where the growth of the culture changes abruptly across this steep decline, or plateau. Collecting single-cell data is vital for studying antibiotics with the potential to induce bi-stability (discussed further in Section 2.6). Due to the stochastic nature of bacterial growth, the response of individual cells can vary significantly at concentrations around this steep decline. Moreover, steeper dose-response curves can accelerate the evolution of antibiotic resistance (Hermsen *et al*, 2012; Deris *et al*, 2013), particularly in spatial gradients of an antibiotic. Antibiotic concentrations can vary significantly within different organs and tissues of the human body (Elliott *et al*, 1995; Onetto & Sharif, 2021). Evaluating growth-dependent antibiotic efficacy is clinically important since the growth environment can vary significantly

within the human body. For example, *E. coli* doubling times can range from 20-30 minutes in the bladder to 3 hours in the intestinal tract (Myhrvold *et al*, 2015; Forsyth *et al*, 2018).

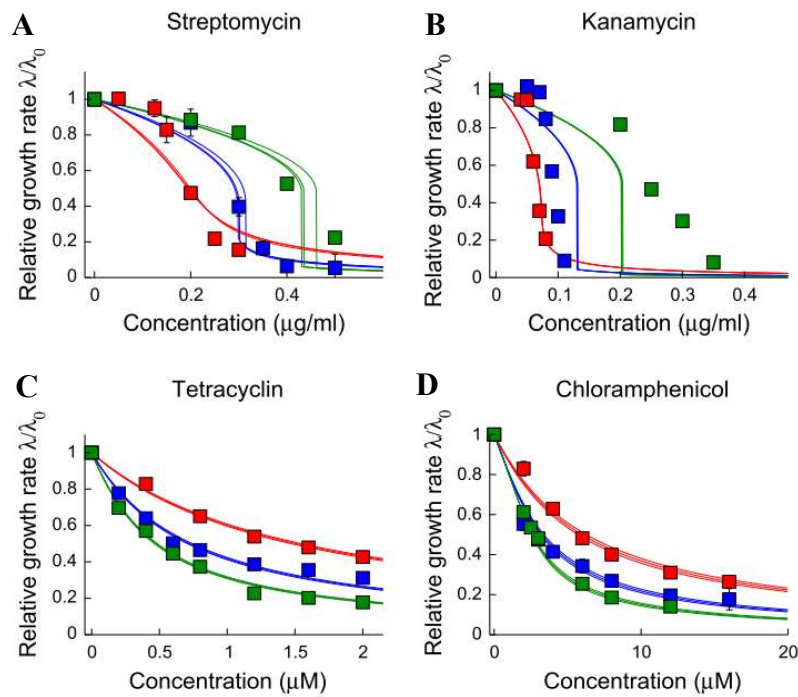


Figure 5. **Growth-dependent susceptibility of ribosome-targeting antibiotics.** The growth-dependency and shape of the curve depends on the reversibility of transport and target binding for the respective drug. **A-B.** Streptomycin and kanamycin are ‘irreversible’ antibiotics which are more effective against slow growing bacteria and have a steep curve. **C-D.** Tetracycline and chloramphenicol are ‘reversible’ antibiotics which are more effective against fast growing bacteria and have a shallow curve. The y-axis represents growth rate relative to a no-drug control. Square symbols are experimental data; lines are the fitted Hill functions. Colours represent different growth media. Red: slow growth (glycerol minimal). Blue: intermediate growth (glycerol + casamino acids). Green: fast growth (glycerol rich-defined medium). Figure reproduced from Greulich *et al.* (2015) (use permitted under the Creative Commons Attribution License CC BY 4.0).

2.4.2 The SOS response

In *E. coli* the SOS response coordinates the cellular response to DNA damage and is specifically triggered by the presence of single-stranded DNA gaps (SSGs) and double-stranded DNA breaks (DSBs). DNA breaks can arise spontaneously due to errors in DNA replication or via exogenous sources including ultraviolet light and DNA-targeting antibiotics such as CIP (Shee *et al*, 2013; Maslowska *et al*, 2019). The SOS operon consists of around 40 genes with diverse functions including DNA repair mechanisms, translesion DNA synthesis, and cell division arrest (Kreuzer, 2013; Baharoglu & Mazel, 2014). Delaying cell division (mediated by the cell division inhibitor SulA (Huisman & D’Ari, 1981)) allows the cell enough

time to repair the damage before segregation of chromosomes into daughter cells (Kreuzer, 2013).

The SOS response is primarily under the regulation of two proteins, RecA and LexA. LexA is a transcriptional repressor of the SOS response. During normal growth conditions, it binds to the promoter region of the SOS operon which blocks transcription of SOS genes by RNA polymerase (pol). Under DNA damaging conditions, the ends of DSBs are recognised and bound by the RecBCD complex which generates single-stranded DNA (ssDNA) overhangs (RecB and RecD: helicase activity; RecC: exonuclease activity). RecA binds to the ssDNA ends to form a nucleoprotein filament which triggers self-cleavage of LexA, leading to expression of the SOS operon (Kreuzer, 2013; Baharoglu & Mazel, 2014). In the case of SSGs, RecA loading is mediated by the RecFOR pathway (Morimatsu & Kowalczykowski, 2003).

DSBs are repaired by homologous recombination (HR), a process in which a homologous sequence from an intact copy of the chromosome is used as a template for repair. The RecA-bound nucleoprotein filament searches for homologous sequences, and once found, initiates strand invasion. Next, error-prone DNA polymerases (Pol II, Pol IV & Pol V) enable repair of the DNA break using the template to synthesise the missing sequence. The crossing of DNA strands results in the formation of a Holliday junction which is resolved by the RuvABC complex (Michel & Leach, 2012; Maslowska *et al*, 2019). The error-prone nature of SOS-mediated DNA replication makes it highly mutagenic which can lead to the development of antibiotic resistance (Dörr *et al*, 2009; Pribis *et al*, 2019). As a result, research on the SOS response has received much attention and is important to consider for studies involving a DNA-targeting antibiotic.

2.4.2.1 Heterogeneity in the SOS response

The expression of SOS genes are not triggered equally; rather, the timing and strength of induction of different genes depends on the amount of DNA damage, duration of the inducing signal, and different LexA-promoter binding affinities (Culyba *et al*, 2018; Maslowska *et al*, 2019). Some genes are induced early in the response, such as *recA* and *uvr* genes (involved in nucleotide excision repair), whereas others are tightly controlled and require a stronger inducing signal, such as the SOS-controlled DNA polymerases and *sulA* (Culyba *et al*, 2018).

Induction of the SOS response has been shown to be highly heterogeneous at the single-cell level (McCool *et al*, 2004; Kamenšek *et al*, 2010; Uphoff *et al*, 2016; Uphoff, 2018; Mitosch *et al*, 2019; Vincent & Uphoff, 2020; Jones & Uphoff, 2021; Jaramillo-Riveri *et al*, 2022).

Sources of heterogeneity in the SOS response include variability in the degradation of LexA, the degree of DNA damage, and intrinsic variation in DNA repair processes. Such heterogeneity could have important consequences on the survival of individual cells.

2.4.3 Other stress responses

2.4.3.1 RpoS-mediated general stress response

The general stress response in *E. coli* is characterised by global changes in metabolism and gene expression that renders cells broadly resistant to a wide range of stressors, including nutrient starvation, oxidative damage, DNA damage, and variations in pH, temperature, and osmolarity (Battesti *et al*, 2011). The general stress response contrasts with specific stress responses in that it is induced by multiple different stresses. It is regulated by the alternative sigma factor RpoS (σ^S) that binds to free RNAP which triggers transcription of RpoS-dependent genes involved in multiple stress responses and slowing metabolic activity and growth (Lange & Hengge-Aronis, 1991). RpoS induction is a complex system regulated at multiple levels: transcriptional, translational, target-binding competition with RpoD (σ^{70}), and protease degradation (Lange & Hengge-Aronis, 1994; Battesti *et al*, 2011). RpoS levels in the cell correlate with growth rate and are very low during exponential phase growth due to dilution and negative regulation. In stationary phase cells and/or under stress conditions, several mechanisms allow the RpoS protein to stabilise, increase in number, and outcompete σ^{70} in binding to RNAP. σ^{70} promotes the transcription of growth-related genes, thus, when RNAP is bound by σ^S , growth is inhibited. The small-molecule alarmone (p)ppGpp, an activator of the stringent response to starvation, is also involved at multiple levels in RpoS induction (Battesti *et al*, 2011). In particular relevance to this study, RpoS has been shown to be involved in improving cell survival under DNA damage (Merrikh *et al*, 2009).

2.4.3.2 Oxidative stress response

Protection against oxidative damage caused by ROS in the cell is primarily mediated by enzymes that either repair ROS-induced damage or neutralise the ROS molecule (Imlay, 2008). Two transcriptional regulators, OxyR and SoxRS, are responsible for sensing oxidative damage and activating the expression of genes involved in mitigating oxidative damage. These include the scavenging activities of superoxide dismutases (SODs), which neutralise superoxides, and catalases/peroxidases which neutralise hydrogen peroxide (Imlay, 2008). Other mechanisms include SoxRS-mediated upregulation of the AcrAB drug-export system.

2.4.3.3 (p)ppGpp-mediated stringent response

The stringent response in bacteria is a signalling pathway associated with starvation and the transition from rapid growth to stationary phase growth. It is mediated by the small alarmone molecule guanosine (tetra) pentaphosphate (p)ppGpp. (p)ppGpp is produced by two proteins, RelA and SpoT, that are activated under amino acid, carbon, nitrogen, iron, phosphate, and fatty acid limitation (Irving & Corrigan, 2018). Under conditions of nutrient deficiency, intracellular (p)ppGpp levels rise and inhibit the expression of genes required for rapid growth, including those involved in transcription, DNA synthesis, ribosome synthesis, and amino acid biosynthesis (Huemer *et al*, 2020). The stringent response has also been linked to activation of toxin-antitoxin modules and persistence (Shan *et al*, 2017; Huemer *et al*, 2020), however, this hypothesis is disputed (Kim & Wood, 2016). There is also a direct link between the stringent response and translation inhibitors. An accumulation of amino acids due to translation inhibition leads to an upregulation of ribosome synthesis due to a decreasing ppGpp pool (Scott & Hwa, 2022). Thus, translation inhibition by TET can mimic a rich nutrient environment with respect to the stringent response.

2.4.4 Tolerance, persistence, and VBNC cells

Sub-populations of dormant bacteria have been shown to exhibit non-heritable resistance to antibiotic treatment in the form of tolerance, persistence, and viable but non-culturable (VBNC) cells. Tolerance refers to the ability of the whole population of cells to survive prolonged antibiotic treatment at high concentrations. This manifests as a longer duration needed to kill an equivalent number of susceptible cells (Figure 6A). Persistence is a phenomenon where a small sub-population of isogenic cells undergoes a temporary phenotypic switch to a non-growing, metabolically dormant state enabling survival under lethal antibiotic exposure (Balaban *et al*, 2004). A defining characteristic of persistence is a biphasic killing curve (Figure 6B) and the ability to resume growth once antibiotic treatment is stopped. Similar to persister cells, VBNC cells can survive prolonged antibiotic treatment and do not grow or divide. However, though they remain viable, VBNC cells do not resume growth following antibiotic treatment (Ayrapetyan *et al*, 2018). VBNC cells can eventually resuscitate but only after prolonged periods of resuscitation-promoting conditions (Li *et al*, 2014). Thus, VBNC cells may represent a deeper state of dormancy than persisters which led to the proposal that the persister and VBNC phenotype are part of a shared dormancy continuum – with persister cells representing an intermediate phenotype between growing cells and VBNC cells

(Ayrapetyan *et al*, 2015). More recently, a phenomenon called ‘perseverance’ has been observed where a sub-population of cells is able to maintain growth for up to 10 generations at lethal antibiotic concentrations (Brandis *et al*, 2023).

Besides a lowering of activity of drug targets, persister and VBNC cells exhibit antibiotic tolerance in other ways. Persister and VBNC cells have been shown to have elevated expression of genes encoding the multi-drug efflux pump AcrAB-TolC and *ompW* (encoding an outer membrane protein) which could reduce intracellular antibiotic levels and improve survival (Asakura *et al*, 2008; Pu *et al*, 2016; Bamford *et al*, 2017). Although persister and VBNC cells are non-growing, they differ from dead cells because they maintain a baseline level of metabolic activity and gene expression (Li *et al*, 2014; Bamford *et al*, 2017). Persister and VBNC cells are clinically important due to their link to chronic bacterial infections, treatment failure (if an antibiotic course is stopped prematurely by a patient), and the development of resistance (Levin-Reisman *et al*, 2017; Barrett *et al*, 2019; Huemer *et al*, 2020).

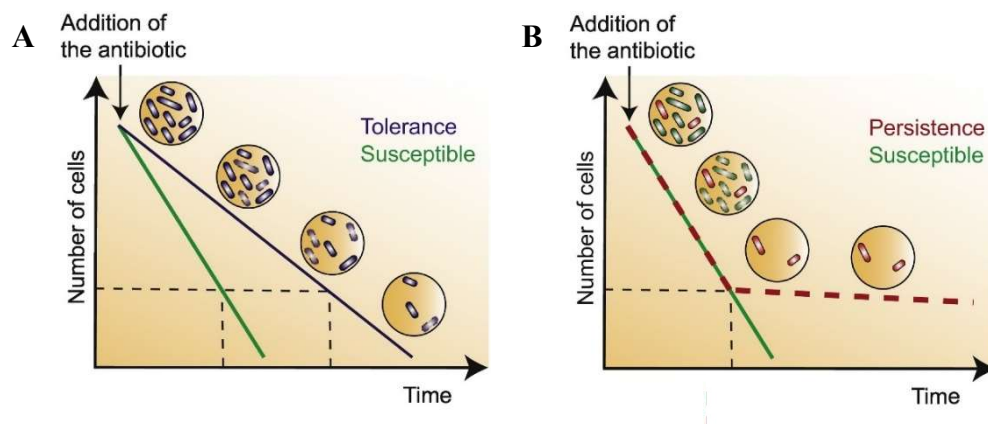


Figure 6. **Illustration of antibiotic tolerance and persistence.** **A.** Antibiotic tolerance reflects reduced susceptibility of the whole population of cells. **B.** A biphasic killing pattern caused by the presence of persister cells. The susceptible population is rapidly killed. A tail in the curve is characteristic for the presence of persister cells in the population. Figure reproduced from Ronneau *et al.* (2019) with permission from Elsevier (License: 5611430234495).

The formation of persister and VBNC cells in the population is said to be linked to inherent, stochastic variability in growth and gene expression dynamics in the population and are generated (depending on the definition) before antibiotic treatment (Ayrapetyan *et al*, 2018). Thus, persistence has been proposed as a bet-hedging strategy employed by bacteria where a population prepares for future stressors by keeping a fraction of cells in a less fit, but more tolerant state (Balaban *et al*, 2004). Persistence is also linked to the growth phase of the culture.

Persister cells are more frequent in stationary phase dense cultures than in exponential phase cultures (Gutierrez *et al*, 2017).

Several mechanisms have been linked to persister and VBNC cell formation, including toxin-antitoxin modules (discussed below), the alarmone (p)ppGpp (stringent response), reduced ATP levels, and intracellular pH (Dörr *et al*, 2010; Gutierrez *et al*, 2017; Ronneau & Helaine, 2019; Pontes & Groisman, 2020; Manuse *et al*, 2021, Goode *et al*, 2021). However, the role of these three mechanisms are disputed as, in some cases, they have been shown to be dispensable for persistence (Shan *et al*, 2017; Goormaghtigh *et al*, 2018; Pontes & Groisman, 2019). Indeed, there is much confusion in this area of research compounded by poorly designed experiments or misinterpretation of data (Kim & Wood, 2016). There has been some debate on the classification of persister-like phenotypes that centres on how they are formed, whether stochastically pre-stress or triggered in response to stress. For example, CIP has been shown to induce persister formation in *E. coli* (Dörr *et al*, 2010). This led to the definition of two types of persistence: triggered persistence (type I persisters), and spontaneous persistence (type II persisters). Nonetheless, what is common among persister-like cells is a very slow growth rate, or lack of growth entirely, and slowed metabolism (Gefen & Balaban, 2009; Pontes & Groisman, 2019; Bakshi *et al*, 2021).

2.4.5 Toxin-Antitoxin modules

Toxin-antitoxin (TA) modules are genetic elements present in the bacterial chromosome or on a plasmid that can cause growth arrest and/or programmed cell death (Page & Peti, 2016). They consist of two genes encoding the toxin and antitoxin. The toxin can damage/interfere with components of the cell such as DNA replication, translation, ATP synthesis, and cell membrane integrity (Page & Peti, 2016). The antitoxin interacts directly or indirectly with the toxin to inactivate it or stop its expression/translation. The antitoxin is usually a short-lived, labile protein that requires continuous production to inactivate the toxin. During periods of stress, the antitoxin is proteolysed by stress-induced proteases (Lon and ClpX-ClpP) which frees the toxin or enables its production (Yamaguchi & Inouye, 2011). There are several categories of TA modules, and we will define two of them here due to their relevance to this thesis. Type I TA modules contain an antitoxin that expresses a noncoding small RNA (sRNA) that base pairs with the toxin mRNA to inhibit its translation. The toxins of type I TA modules are typically hydrophobic peptides that disrupt the cell membrane which can lead to loss of proton motive force, stalled ATP synthesis, and growth arrest. For type II TA modules, the antitoxin is a

protein that binds to the toxin which inhibits its activity, and in some cases the antitoxin or toxin-antitoxin complex can also bind to the TA promoter to repress its transcription (Yamaguchi & Inouye, 2011; Page & Peti, 2016).

2.4.5.1 SOS-regulated toxin-antitoxin modules

Besides activation of DNA repair mechanisms, induction of the SOS response also upregulates several TA modules. TA modules under regulation of the SOS response include *tisB/istR*, *symER* (type I TA modules), *mazEF*, *hokE/sokE*, *dinJ/yafQ*, and *yafNO* (type II TA modules) (Yamaguchi & Inouye, 2011). Induction of the SOS response in exponentially growing *E. coli* has been linked to improved survival under CIP treatment and the formation of persister cells via activation of the TisB toxin (Dörr *et al*, 2009, 2010). The TisB toxin forms pores in the inner membrane thereby disrupting the proton motive force and ATP synthesis, which leads to growth arrest due to inhibition of transcription, translation, and DNA replication (Unoson & Wagner, 2008). Reduced cell growth leads to improved tolerance to antibiotics due to lower activity of the antibiotic target. But, when TisB is overexpressed, such as from a high-copy plasmid, significant cell death occurred after 60 minutes, with dead cells typically retaining their shape while some lysis was observed (Unoson & Wagner, 2008). This is likely a result of the complete loss of membrane integrity due to an overwhelming number of TisB proteins. Su *et al.* (2022) further proved the involvement of TisB in cell death under trimethoprim treatment by showing that survival of a $\Delta tisB$ mutant was 20-fold higher than the wildtype. Thus, there is a strong link between SOS-induced TA toxins and cell death, but also antibiotic tolerance, which depends on the expression level of *tisB*. Several other TAs mediated by the SOS response affect the cell in different ways, including ribonucleolytic activity and inhibition of translation (Prysak *et al*, 2009; Zhang *et al*, 2009; Yamaguchi & Inouye, 2011).

2.5 Antibiotic combinations

The development of novel antimicrobials has slowed significantly in an era of rapidly rising antibiotic resistance. Clinicians need alternative strategies to treat drug-resistant bacteria. Monotherapy is traditionally preferred by clinicians but treatment with a single antibiotic presents the risk of resistance development and treatment failure. Antibiotic combination therapy has enormous potential, and is currently used successfully in some treatments, for example the trimethoprim-sulfamethoxazole pair for treating UTIs, yet many drug combinations remain under-studied. Improving understanding of how antibiotics interact with

each other has the potential to improve treatment efficacy, extend the usefulness of existing antibiotics, and enable discovery of novel treatment combinations (Tyers & Wright, 2019).

2.5.1 Classification of antibiotic interactions

The interaction between two drugs when combined are typically classified as additive, synergistic, or antagonistic (Figure 7). This classification is based on whether the combined efficacy of two drugs is equal to, stronger than, or weaker than expected based on their individual effects, respectively (Loewe, 1928, 1953; Bliss, 1939; Yeh *et al*, 2006). Suppression is a type of hyper-antagonism where the growth inhibition is not only weaker than expected, but also weaker than that of one or both antibiotics alone (Bollenbach, 2015). There are two models commonly used to define an antibiotic interaction: Bliss independence (Bliss, 1939) and Loewe additivity (Loewe, 1928, 1953). These models and their interpretation are summarised in Figure 7. Determining antibiotic interactions involves measuring either growth rate inhibition (Figure 7A), survival fractions (Figure 7B), or MICs (Figure 7C) determined for the antibiotics alone and in combination and comparing to the model definitions.

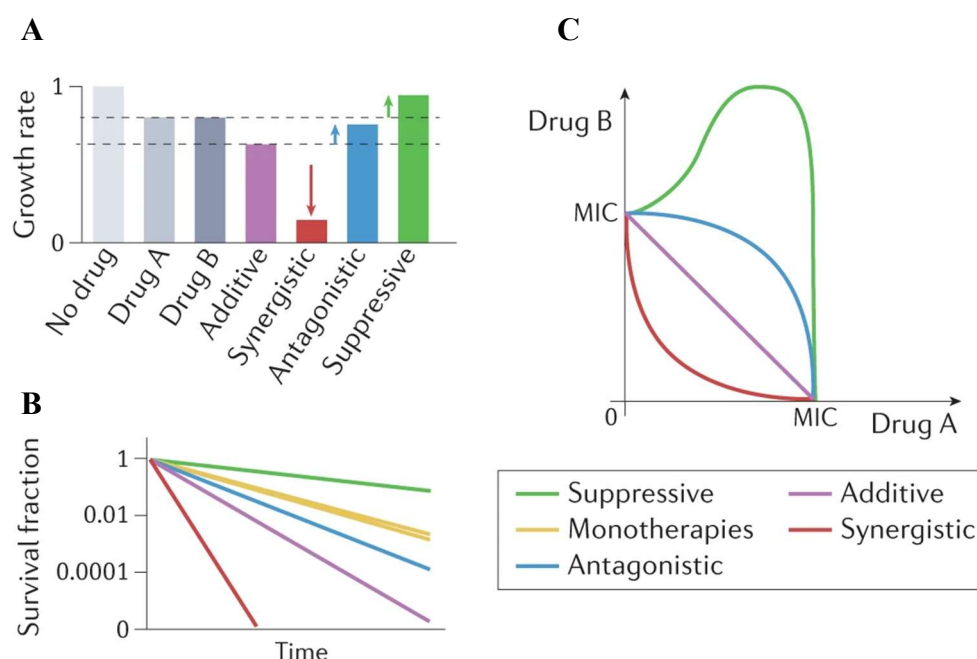


Figure 7. **Classification methods of antibiotic interactions.** **A.** Illustration of the Bliss independence model for determining antibiotic interactions based on growth rate measurements under two drugs applied individually and in combination. The Bliss definition assumes that antibiotics act independently and their individual effects can be multiplied to calculate the expected growth rate (denoted as an ‘additive’ interaction in the figure) under the drug combination. If the growth rate under drug combination is lower than the additive expectation, it is classified as synergistic; if it is higher, it is antagonistic; and if it is higher than at least one of the drugs applied individually, it is a suppressive interaction. **B.** The Bliss

definition can also apply to time-kill assays such as survival fractions. C. Illustration depicting the Loewe definition for determining drug interactions. An isobologram (isoboles represent lines of constant growth rate or the MIC) is generated based on a 2D dose-response surface. The Loewe definition assumes straight isoboles represent an additive interaction; concave isoboles are synergistic; convex isoboles are antagonistic; and a bulging convex shape is suppressive. Figures were reproduced from Roemhild et al. (2022) with permission from Springer Nature (License: 5607130676689).

Much work has been done evaluating the interactions of many pairwise drug combinations for *E. coli* (Yeh *et al*, 2006; Yeh & Kishony, 2007; Bollenbach *et al*, 2009; Mitosch & Bollenbach, 2014; Chevereau & Bollenbach, 2015; Zhou *et al*, 2015; Nguyen *et al*, 2016; Brochado *et al*, 2018; Yilancioglu, 2019; Kavčič *et al*, 2020, 2021; Roemhild *et al*, 2022). A major finding is the robustness of drug interactions among antibiotics sharing a target. In other words, we can confidently predict a drug interaction based on what cellular function is targeted. This has led to the development of drug interaction networks (Figure 8) which greatly simplifies complex networks of many pairwise drug combinations (Yeh *et al*, 2006). For example, combinations of DNA synthesis inhibitors and translation inhibitors are suppressive, while aminoglycosides and cell wall inhibitors are synergistic. Although certain genes can affect the sensitivity to drug combinations, the emergent interaction, whether synergistic or antagonistic, was found to be robust to most genetic perturbations (Chevereau & Bollenbach, 2015). However, there are exceptions, as seen for some drug-resistant mutants (Wood *et al*, 2014) and mutants of genes affecting the target of the drugs (Bollenbach *et al*, 2009; Chevereau & Bollenbach, 2015). This suggests a level of commonality in the response to antibiotics with similar modes of action from which generalised principles can be established. Identifying generalised principles in this way facilitates our understanding of drug interactions and enables predictions to be made (Wood *et al*, 2014; Tekin *et al*, 2016, 2018).

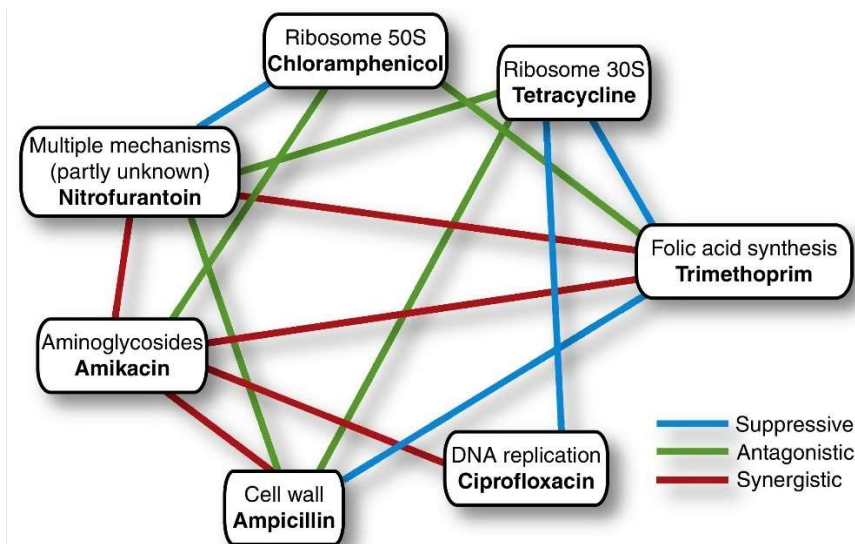


Figure 8. **Antibiotic interaction network typical for *E. coli*.** Lines indicate the drug interactions (shown in different colours) between different classes of antibiotics grouped by their mode of action. Figure reproduced from Bollenbach (2015) (use permitted under the Creative Commons Attribution License CC BY 4.0).

2.5.2 Why study suppressive interactions?

Combining multiple drugs with different modes of action can improve treatment efficacy. For example, synergism can increase the efficacy of the treatment while allowing lower dosages to minimise toxicity to the patient. Moreover, they may be less prone to treatment failure due to resistance development, since one of the drugs may still be effective if resistance develops for the second drug. Synergistic drug interactions have understandably received the most attention for their therapeutic advantages, however, they have been shown to select for and accelerate the development of antibiotic resistance (Hegreness *et al*, 2008; Pena-Miller *et al*, 2013). Though synergistic drug combinations may be more effective in treating infections in the short-term, they would be rendered completely useless if resistance to both drugs develops. This could defeat the purpose of finding improved treatments if they are only effective in the short-term and contribute to antibiotic resistance development.

In the case of antagonistic and suppressive drug interactions, treatment efficacy is reduced, and higher, potentially toxic, therapeutic doses are required (or longer treatment periods). Such interactions should generally be avoided for the patients' interests. Therefore, research on these drug interactions is important so clinicians know which drug combinations result in antagonistic or suppressive interactions. Although considered undesirable for treating infections, antagonistic and suppressive drug combinations have been shown to select against and slow the evolution of antibiotic resistance (Chait *et al*, 2007; Michel *et al*, 2008; Torella *et*

al, 2010; Baym *et al*, 2016; Singh & Yeh, 2017). Suppression allows susceptible cells to outcompete those that have developed resistance to one of the two drugs, whereas synergy selects for resistant mutants (Chait *et al*, 2007; Baym *et al*, 2016; Singh & Yeh, 2017). This is because resistant mutants often incur a fitness cost – a result of expressing the gene conferring resistance – thus favouring the growth of susceptible strains at sub-MIC concentrations. Moreover, rates of adaptation (development of resistance mutations) were found to be lower for suppressive drug pairs than synergistic pairs (Hegreness *et al*, 2008). These observations highlight the importance of considering population dynamics in drug interaction studies, particularly where growth heterogeneity among sub-populations is expected.

A more holistic approach is needed for treating infections and controlling antibiotic resistance. Suppressive drug interactions have comparatively received less attention than synergistic interactions, but given their potential in fighting resistance development, a deeper understanding is certainly warranted.

2.5.3 Mechanisms of antibiotic interactions

The specific mechanism underlying antibiotic combinations is a major topic of study, and some interactions are still poorly understood. For some drug combinations, the interaction operates indirectly through their effects on cellular physiology. For example, synergy between trimethoprim and sulfamethoxazole results from sequential inhibition at different steps in the biosynthesis pathway for folate – an essential cofactor in the synthesis of thymidine, purines, methionine and glycine (Kwon *et al*, 2008). And the suppressive interaction that occurs between DNA synthesis inhibitors and translation inhibitors (described in more detail later) is proposed to be a result of correcting an imbalance in DNA and protein synthesis (Bollenbach *et al*, 2009). However, the complex interplay between bacterial physiology and susceptibility to drug combinations requires further study. Although it is well-known that many individual antibiotics exhibit growth-dependency, less is known how this translates to combinations of antibiotics. Given that the internal macromolecular composition of the cell changes with growth rate, the efficacy of drug combinations that target molecules whose abundance is growth-dependent will likely be affected. The majority of research on pairwise drug combinations have been performed in a single, rich growth medium (typically LB medium), but not much is known how physiological drug interactions are influenced by changing nutrient conditions, particularly at the single-cell level.

2.5.3.1 Antibiotic combinations and gene expression

Gene expression responses to antibiotic monotherapies has been well characterised (Davies *et al*, 2006), but expression profiles under antibiotic combination treatment is less well studied, yet some progress has been made in the past decade (Bollenbach & Kishony, 2011; Chevereau & Bollenbach, 2015; Qi *et al*, 2021). Antibiotics with different modes of action will elicit a unique gene expression response in the cell. But what does the gene expression landscape look like when antibiotics are combined? Can we predict gene expression changes for a drug combination based on the drugs' known individual effects? In many cases, combining antibiotics can result in regulatory conflicts: a gene is upregulated under one antibiotic, but downregulated under the other – so how does the cell respond? Bollenbach *et al.* (2011) addressed this question and found that gene regulatory conflicts are solved in the cell by either prioritising or averaging the response to the individual antibiotics and that this applied to 25% of promoters across the entire genome. The remaining genes (75%) responded to the growth inhibition caused by the antibiotics – this makes it difficult to determine whether gene expression changes are due to changes in growth rate or a specific response to the antibiotic. The response, whether prioritised or averaged, depends on the specific drug combination. For example, the trimethoprim-spiramycin pair (antagonistic interaction) prioritised responses to the drug with the strongest effect on growth, while the tetracycline-spiramycin pair (additive interaction) showed an averaged response to the effects of the individual drugs (Bollenbach & Kishony, 2011). Although translation inhibitors are known to upregulate ribosomal gene expression, this response is dose-delayed (i.e. occurs at higher concentrations) when combined with a DNA synthesis inhibitor (Bollenbach *et al*, 2009).

The response to antibiotic combinations is therefore a complex phenotype that depends on the antibiotic modes of action and how the cell reconciles gene expression responses to the individual antibiotics. In addition, antibiotic interactions in some cases can differ between bacterial species and strains (Brochado *et al*, 2018). Although much progress has been made, a deeper understanding of the link between cellular physiology and drug interactions is still needed.

2.5.3.2 Growth rate dependence of suppressive antibiotic interactions

Since antibiotic efficacy is known to be generally correlated with growth rate, does this hold for antibiotic combinations? Growth rate dependence is important to consider for treating infections since nutrient availability can vary at different sites of infection within the host

(Smith & Holt, 1996). We discussed earlier how the cells' physiological composition changes with nutrient availability which implies that physiological interactions for suppressive drug combinations could change across different nutrient environments. Evidence linking growth rate to susceptibility to antibiotic combinations is severely lacking, but there are some indications that the CIP+TET interaction could be sensitive to changes in nutrient quality. In a supplementary figure, Bollenbach et al. (2009) observed enhanced suppression in M9-based medium supplemented with 0.4% glucose compared to growth in LB medium (Figure 9). LB medium supports a faster growth rate than M9+glucose medium. This suggests that CIP+TET suppression may be enhanced at slower growth rates. However, LB is a complex medium that could affect the cells' physiological response to antibiotics in unknown ways. Therefore, further experiments should be conducted to evaluate growth dependence of suppression in controlled environments.

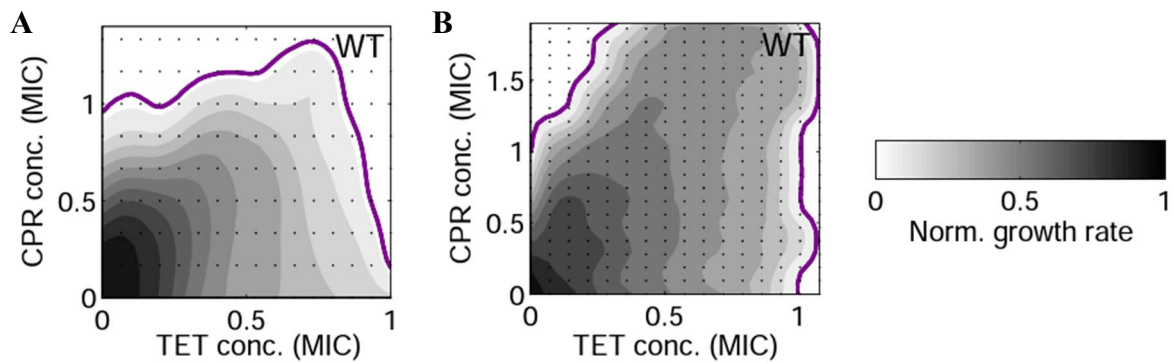


Figure 9. **Suppression is medium-dependent.** Growth rates (grey) and MIC (purple line) for a two-dimensional concentration grid of ciprofloxacin (CPR/CIP) and tetracycline (TET) in LB medium (A) and M9+glucose medium (B). The suppressive effect is stronger in M9+glucose medium. *E. coli* K-12 strain MG1655 was used. Concentrations on the axes are relative to the minimum inhibitory concentration (MIC). Growth rates are normalised to growth without antibiotic. Black dots represent concentrations where growth rates were measured. Figure reproduced and modified from Bollenbach et al. (2009) with permission from Elsevier (License: 5613641241597).

Although suppression can be observed at the MIC (e.g. see purple line in Figure 9), we can also observe suppression on bulk growth rates at sub-MIC levels. The study of sub-MIC antibiotic responses are relevant for the following reasons. Firstly, antibiotics administered orally or intravenously result in a concentration spike followed by a gradual decay in the body's organs, tissues, and bloodstream (Kuriyama *et al*, 2014). In addition, antibiotic levels can vary significantly between different organs and tissues in the body (Elliott *et al*, 1995; Onetto & Sharif, 2023). This can result in antibiotic concentrations that vary temporally and spatially in the human body during which bacteria can be exposed to sub-MIC levels of antibiotic.

Secondly, bacteria are frequently exposed to sub-MIC antibiotic concentrations in the environment, including rivers, sewage, and soils (Polianciuc *et al*, 2020). Lastly, antibiotic resistance can develop at below-MIC levels (Brandis *et al*, 2023).

2.5.3.3 Suppression and cellular physiology

A suppressive drug interaction is often observed between DNA synthesis inhibitors (fluoroquinolones and folate inhibitors) and translation inhibitors. These antibiotics target different physiological processes that are essential for growth. A potential mechanism governing this interaction suggests that these physiological processes become unbalanced under DNA synthesis inhibition, and that this balance is partially restored by the action of the translation inhibitor (Bollenbach *et al*, 2009). As discussed above, in order to maximise growth, the cell optimises its allocation of limited resources for synthesising cellular components (DNA, ribosomes, etc.). Transcription of ribosomal operons, which determines the levels of ribosomes in the cell, are tightly controlled to maintain maximal growth in varying nutrient conditions. When the nutrient environment is poor, intracellular levels of cellular resources (nucleotides, amino acids) decrease and ppGpp levels increase, causing downregulation of ribosomal transcription and lowering growth rates (Dennis *et al*, 2004; Gralla, 2005). Thus, ribosome levels are maintained at their optimal level for the given nutrient environment.

But *E. coli* may not be able to optimally regulate ribosomal synthesis under DNA stress (Bollenbach *et al*, 2009). When exposed to a DNA synthesis inhibitor, such as CIP, the rate of DNA replication slows or stalls completely. This leads to an excessively high, and wasteful, global protein synthesis rate (relative to the rate of DNA replication) due to a non-optimal regulation of ribosome synthesis, and is indicated by a highly elevated protein-to-DNA ratio (Bollenbach *et al*, 2009). An overproduction of ribosomes will lead to excess protein synthesis which will deplete cellular resources at the expense of other cellular processes leading to sub-optimal growth (Nomura, 1999; Gralla, 2005). This imbalance can be extrinsically corrected when protein production is partially impaired by adding a translation inhibitor, such as TET, leading to faster growth (Bollenbach *et al*, 2009). In support of this hypothesis, the authors found that genetically limiting ribosome production reproduced the suppressive effect with CIP. Moreover, a $\Delta relA \Delta spoT$ mutant (which is unable to upregulate ppGpp), enhanced suppression under the drug combination. A quantitative model (Figure 10) linking growth to the distribution of cellular resources between protein and DNA synthesis successfully captured the suppressive effect (Bollenbach *et al*, 2009). Essentially, the addition of the translation

inhibitor acts as a ‘physiological antidote’⁶ where one drug alleviates the effect of the other. This effect was found to be robust for many drug pairings between these two classes of antibiotics (Yeh *et al*, 2006), emphasising the role of cellular physiology in this interaction.

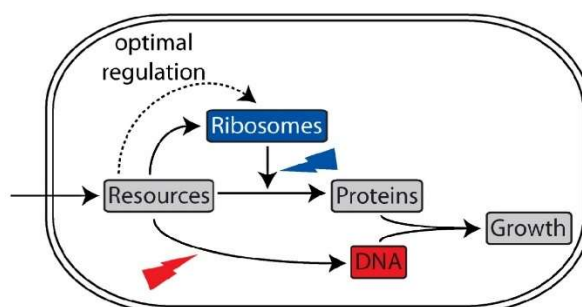


Figure 10. **Illustration of the resource allocation model proposed by Bollenbach et al. (2009).** Cellular resources enter the cells at a fixed rate and are distributed between ribosome, protein, and DNA synthesis. Optimal growth is achieved when the levels of protein synthesis and DNA synthesis are balanced. Ribosome synthesis determines the rate of protein synthesis and depends on the intracellular levels of resources. Antibiotics either inhibit protein synthesis (translation inhibitors) or DNA synthesis. When DNA synthesis is inhibited (red), excess ribosomal activity (dashed line) disrupts the balance between protein and DNA levels, leading to sub-optimal growth. A translation inhibitor (blue) partially restores this balance, resulting in improved growth. Figure reproduced from Bollenbach et al. (2009) with permission from Elsevier (License: 5613641241597).

Although this is a compelling hypothesis explaining the mechanism of the suppressive interaction, it was concluded based on population-averaged data. In fact, almost all antibiotic combination research in the literature has been conducted using bulk experiments. For the suppressive interaction between DNA synthesis inhibitors and translation inhibitors, an improvement in overall growth rate of the population is observed. But it is not fully understood what leads to this improved growth. Bollenbach et al. (2009) provides a reasonable explanation for this based on the correction of excess protein synthesis (described above). However, this hypothesis assumes that the growth rate of individual cells (elongation and division) are improved without considering if cell death plays a role. Growth rates measured at the population-level represent the ‘net’ growth of the population of cells. Net growth is a function of the growth (mass accumulation), division and death rate of cells. So, do individual cells improve growth rates, or is the suppressive interaction due to a lower rate of cell death? Perhaps

⁶ Concept originally proposed by Thomas Fraser in 1872 to describe antagonism of a physiological nature between two poisons, physostigma and atropia.

it is a combination of the two. Untangling these factors is impossible using bulk experiments, but they can be measured separately using single-cell methods.

2.6 The single-cell approach

2.6.1 Sources of single-cell heterogeneity

Growth heterogeneity can lead to varied single-cell responses to antibiotic treatment including drug tolerance. Therefore, it is important to understand the sources of single-cell heterogeneity. Sources of single-cell heterogeneity are diverse and are a result of the inherently stochastic nature of various microbial and biochemical processes. These include: intrinsic stochasticity in gene expression and biochemical reactions, fluctuations in metabolic networks, asymmetric cell division, uneven partitioning of molecular components at cell division, and extrinsic environmental heterogeneity (Elowitz *et al*, 2002; Huh & Paulsson, 2011; Kiviet *et al*, 2014; Thomas *et al*, 2018; Julou *et al*, 2020; Schreiber & Ackermann, 2020; Bi *et al*, 2023). Fluctuations in gene expression has also been linked to gene dosage fluctuations during the DNA replication cycle (Jones *et al*, 2014a; Peterson *et al*, 2015; Wang *et al*, 2019). Gene expression fluctuations can be caused by the presence of additional gene copies from sister chromosomes which are transiently present in the cell when the chromosome is undergoing replication before it has separated. Variations in cell growth can also be propagated via feedback mechanisms. Fluctuations in the expression of genes whose products are important for growth, such as catabolic enzymes, can lead to fluctuations in growth rates which in turn can affect their own expression and that of other genes (Kiviet *et al*, 2014). The dynamics of mRNAs coding for nutrient transporters and enzymes were identified as a major source of growth rate fluctuations (Thomas *et al*, 2018).

Fluctuations in growth rate and gene expression also depend on the nutrient environment and have been shown to decrease with mean growth rate (Kiviet *et al*, 2014; Kennard *et al*, 2016; Thomas *et al*, 2018; Urchueguía *et al*, 2019). This is suggested to be due to saturating levels of growth, consistent with Monod growth laws, where there is little variation in growth due to the abundance of nutrients and ribosomes (Thomas *et al*, 2018). In slower growth conditions, the low abundance of ribosomes and metabolic components contributes to growth fluctuations. And under antibiotic treatment, growth heterogeneity has been predicted to be highly complex (Thomas *et al*, 2018). Growth rate fluctuations, though transient, may have important implications on antibiotic efficacy, particularly where susceptibility is growth rate dependent,

and are expected to contribute to heterogeneity in single-cell responses to the antibiotics and combinations thereof.

Not all cells in a population perceive a uniform antibiotic concentration. Drug levels can vary depending on the spatial heterogeneity of the environment bacteria grow in, for example different organs in the body or in biofilms, which can result in drug-induced growth heterogeneity. Even when cells are exposed to a uniform antibiotic concentration, individual cells can experience heterogeneity in the intracellular concentrations of antibiotics. Łapińska *et al.* (2022) showed, in the mother machine, variability in the uptake of different fluorescently tagged antibiotics. These differences could be due to stochasticity in the uptake and efflux of the antibiotics in individual cells.

2.6.2 Advantages of single-cell methods

The study of the efficacy of antibiotics has traditionally focussed on population-averaged metrics. However, these methods give a limited insight into cell-to-cell variation metrics, while ignoring both spatial and temporal heterogeneity inherent in bacterial populations. Recent advances in microfluidics and high-resolution microscopy have allowed scientists to precisely quantify the magnitude of cellular heterogeneity, investigate their sources, and determine their consequences on population dynamics and response mechanisms. Single-cell research has revealed, in isogenic populations, substantial cell-to-cell heterogeneity in gene expression, cell-cycle dynamics, and growth rates (Elowitz *et al.*, 2002; Potvin-Trottier *et al.*, 2018; Şimşek & Kim, 2018; Thomas *et al.*, 2018; Sampaio *et al.*, 2022).

Single-cell distributions are more informative than population averages (Figure 11A), as the shape and modality of probability distributions provides more insight into the underlying response of individual cells and how they propagate at the population-level (Potvin-Trottier *et al.*, 2018). Heterogeneity measured at the single-cell level can manifest as Gaussian distributions with varying widths or as multi-modal distributions indicating the presence of sub-populations. Thus, single-cell methods can reveal the underlying population structure. These differences would be masked by population-averaging methods.

Moreover, the ability to generate time-series data can provide even more information about the dynamics of a system such as different timescales of gene expression (Figure 11B). Single-cell variability has important implications for population fitness and the outcome of treating infections; for example, a small number of persister cells can result in re-establishment of an

infection after antibiotic treatment (Dhar & McKinney, 2007). Single-cell fluctuations in stress responses and growth rates can significantly affect antibiotic susceptibility and the fate of individual cells (Patange *et al*, 2018; Sampaio *et al*, 2022). This highlights the necessity of tracking individual cells if we are to fully understand how bacteria respond to antibiotic exposure.

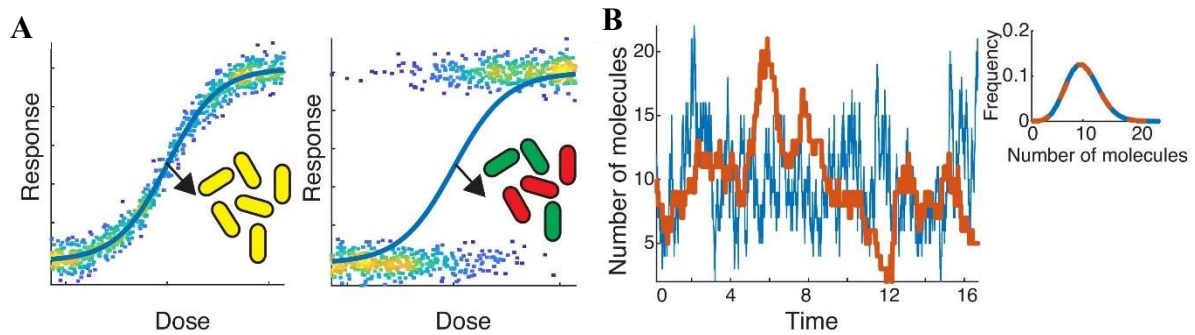


Figure 11. Single-cell and time-lapse experiments are more informative than population-averaged experiments. **A.** Two dose response scenarios with different single-cell behaviour (dots) but identical average behaviours (blue line). Left: Single-cell behaviour is captured by the population average. Right: Single-cell responses are bimodal and vary significantly around the middle of the average dose-response curve. **B.** Stochastic processes (e.g. gene expression) can have very different fluctuation timescales, but can have identical probability distributions (upper right). Figure reproduced from Potvin-Trottier *et al.* (2018) with permission from Elsevier (License: 5613671025600).

2.6.3 The mother machine

The mother machine is a microfluidic device that enables long term tracking of single bacterial cells for hundreds of generations under controlled physiological conditions (Wang *et al*, 2010a; Yang *et al*, 2016). It consists of an array of dead-end microchannels which are approximately the size of a bacterial cell (Figure 12A). The microchannels are connected to a main feeding trench that supplies fresh growth medium and flushes away metabolic waste and surplus cells. Thus, exponential growth of cells can be theoretically maintained indefinitely (at least until the influent medium runs out or biofilms develop). Another advantage of the mother machine is the ability to rapidly change culture conditions. When loading the device with a bacterial culture, cells are trapped in the microchannels and are allowed to grow and divide to produce progeny. Progeny are pushed out into the main trench and flushed away, however a cell always remains at the bottom of the microchannel. This is called the mother cell (which gives the device its name) and we call its progeny daughter cells (Figure 12B, C). We can track the mother cell lineage using quantitative microscopy and measure various parameters such as cell length, growth rate and fluorescent markers of gene expression.

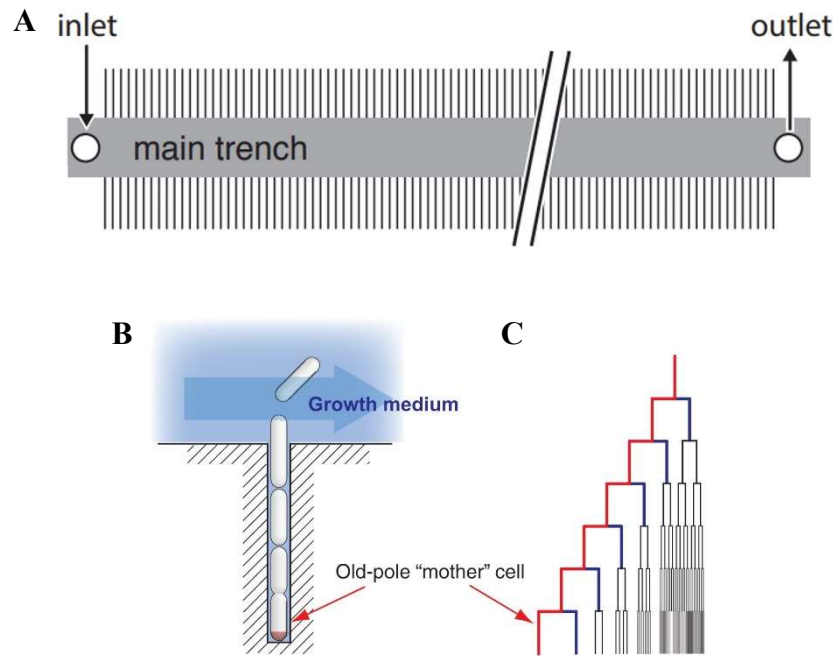


Figure 12. **The ‘mother machine’ design and concept.** **A.** Schematic of the mother machine design. A main trench is flanked by an array of microchannels (cell traps). Fresh medium flows through the main trench and excess cells are washed away. Figure modified from Sauls et al. (2019) (use permitted under the Creative Commons Attribution License CC BY-NC-ND 4.0). **B.** The old pole of the ‘mother’ cell is trapped at the bottom of the microchannel. **C.** Lineage tree representing the old pole mother cell (red) and her progeny, or ‘daughter’ cells. Natural selection is eliminated since only one cell is kept at each generation regardless of its fitness. Figure reproduced from Wang et al. (2010) with permission from Elsevier (License: 5613610495092).

The mother machine was first developed by Wang et al. (2010) who demonstrated robust long-term growth dynamics of mother cells for hundreds of generations. Since then, the mother machine has revolutionised the study of bacteria in several fields of study. For example, single-cell data from the mother machine confirmed the ‘adder’ model and refuted the ‘sizer’ and ‘timer’ models as the mechanism of cell size control in *E. coli* (Taheri-Araghi et al, 2015; Panlilio et al, 2021). The mother machine also allows us to quantify population heterogeneity and determine its effect on the response to antibiotics. For example, Mitosch et al. (2017) demonstrated that noisy response to antibiotic stress correlates with cell survival times. In addition, it has enabled detection of rare phenotypes such as persister and VBNC cells (Bamford et al, 2017; Bakshi et al, 2021; Kaplan et al, 2021), antibiotic susceptibility (Ocampo et al, 2014; Rossi et al, 2019; Łapińska et al, 2022; Brandis et al, 2023; Zhang et al, 2023), and cell-to-cell heterogeneity in stress responses (Patange et al, 2018; Jones & Uphoff, 2021; Vincent & Uphoff, 2021; Sampaio et al, 2022; Choudhary et al, 2023). Recent advances in image analysis techniques incorporating machine learning has significantly improved the

throughput and precision of cell segmentation and lineage tracking (Ollion *et al*, 2019; Ollion & Ollion, 2020; O'Connor *et al*, 2022). The mother machine design has also been used commercially in the medical sector to rapidly (< 30 minutes) determine antibiotic susceptibility at the single-cell level (Baltekin *et al*, 2017).

2.6.3.1 Mother machine considerations

There are some caveats to consider when using the mother machine design. Since cells are physically constrained and separated from each other, growth conditions are obviously different from crowded growth in liquid cultures. For example, physical contact with surfaces can cause a decrease in respiration (Geng *et al*, 2014). Care should also be taken when designing feature dimensions of the device. Yang *et al*. (2018) showed that the dimension of the microchannels can affect growth and division of cells. For example, microchannels that are too tight (narrow channel widths) or too long can impede elongation and division of individual cells (Yang *et al*, 2018).

There is a concern that the limited space surrounding cells in the mother machine microchannels may hinder the diffusion of compounds of interest. Although the diffusion of antibiotic to the mother cells has been shown to be not significantly limited by neighbouring cells (Cama *et al*, 2020), this may not apply to other compounds. Survival under oxidative stress (by hydrogen peroxide) has been shown to improve for cells shielded by their neighbours in the mother machine (Choudhary *et al*, 2023). In this case, expression of scavenging enzymes created a hydrogen peroxide concentration gradient across the length of the microchannels; thus, mother cells were more protected from oxidative damage compared to cells that were closer to the microchannel openings. However, this did depend on the relative width of the channels and cells which determines the reaction-diffusion dynamics of stressor compounds. The mother machine can also impose unique constraints on the biology of the system. For example, Bergmiller *et al*. (2017) found that biased partitioning of cell wall constituents at division led to the accumulation of the AcrAB-TolC multi-drug efflux pump at the old-pole of mother cells. Thus, mother cells exhibited higher drug efflux and were more tolerant (faster growth and division) than their progeny to several translation inhibitors, including TET (Bergmiller *et al*, 2017). Therefore, care should be taken to consider the caveats of using the mother machine and the biology of the system.

The population dynamics of the system is different too. The mother machine maintains a fixed number of mother cells in the microchannels, while a liquid bulk culture is subject to

competitive dynamics among an expanding population of cells. Fast growing cells within a growing population have a competitive advantage and will be over-represented in the population. Their impact on population growth will therefore not be represented by averaging single-cell distributions from the mother machine (Thomas, 2018). Though, methods have been developed to relate single cell distributions to the population level (Thomas, 2017, 2018).

2.7 Motivation and scope of this thesis

Antibiotics are increasingly used in combination to improve efficacy against bacterial infections. The suppressive interaction between DNA synthesis inhibitors and translation inhibitors is a result of the antibiotic's effects on different physiological components of the cell. However, the relationship between bacterial physiology and the suppressive drug interaction is not fully known. Though previous reports have characterised this drug interaction, the behaviour of single cells has not been evaluated. It is currently unknown how the underlying population dynamics contributes to suppression observed at the population level, nor if suppression is growth rate dependent. The SOS response plays an important role in the survival of cells experiencing DNA damage. Previous reports have shown that a higher proportion of cells have elevated SOS expression levels in slow growing cultures, but the underlying single-cell behaviour is not understood. Single-cell methods can reveal important insights into the population dynamics and the physiological response of individual cells that are masked in bulk-level measurements. Gaining a deeper understanding of the bacterial response to DNA damage and antibiotic combinations is important for the design of more effective treatment strategies.

In Chapter 4, we investigated induction of the SOS response at different growth rates and at the single-cell level in order identify mechanisms giving rise to bulk-level observations gathered in previous work. In Chapter 5 we used the mother machine to investigate the suppressive antibiotic combination between a DNA synthesis inhibitor and a translation inhibitor at the single-cell level. We measured single-cell elongation rates, division rates, survival rates, SOS response expression, and constitutive gene expression under the antibiotic combination in different growth conditions. Since the results chapters cover different, but related, topics, we chose to discuss each chapter separately followed by a conclusion to tie the results together.

Chapter 3: Materials and Methods

3.1 Culture conditions and antibiotics

For pre-culturing (cell revival from frozen glycerol stocks, -80°C) and strain construction, cells were grown overnight (16-18 hours) in LB medium or LB agar supplemented with the corresponding selection markers. All cultures were grown in 50 mL falcon tubes agitated at 37°C (300 rpm) with no more than 5 mL of liquid volume, unless otherwise stated. Concentrations employed for selection markers were as follows: ampicillin 100 $\mu\text{g/mL}$, kanamycin 50 $\mu\text{g/mL}$, and gentamycin 10 $\mu\text{g/mL}$.

For all microscopy and batch experiments, cell cultures were grown in M9-based medium. The composition of the M9 salts was as follows: 49 mM Na_2HPO_4 , 22 mM KH_2PO_4 , 8.6 mM NaCl , 19 mM NH_4Cl , 2 mM MgSO_4 , and 0.1 mM CaCl_2 (adjusted to pH 7). This was supplemented with either 0.5% v/v glycerol (Sigma-Aldrich, G9012), 0.5% w/v glucose (D(+)-glucose anhydrous, Scientific Laboratory Supplies, CHE1806), or 0.5% w/v glucose and a mix of amino acids (1 $\times$ MEM Non-Essential Amino Acids and 1 $\times$ MEM Amino Acids, Gibco™). These media are labelled as ‘gly’, ‘glu’, and ‘gluaa’, respectively, throughout this study. Another medium used (labelled as ‘glyaan’) consisted of M9 supplemented with 0.5% w/v glycerol, amino acids, and nucleotides (10X ACGU solution, Teknova). The pH of the 10X ACGU solution was lowered slightly (using concentrated NaOH) to re-solubilise precipitated nucleotides and then filter sterilised (0.22 μm) before aliquoting and storing at -20°C . Aliquots were only ever thawed once before use.

Antibiotics used in this study are listed as follows: ciprofloxacin hydrochloride (APExBIO, C5539), tetracycline hydrochloride (Fisher BioReagents, BP912-100), and trimethoprim (Sigma-Aldrich, SBR00024). Antibiotic stocks were made up in sterile distilled water and filter-sterilised (0.22 μm). Stock concentrations were 5 mg/mL for ciprofloxacin (pH-adjusted to 4.62) and 10 mg/mL for tetracycline. Trimethoprim was ordered as a ready-made solution in DMSO at 25 mg/mL. Antibiotic stocks were aliquoted into small volumes (< 200 μL) and frozen at -20°C . For each experiment, new antibiotic stocks were thawed on the same day and never re-used. Working stocks were diluted using sterile distilled water or an M9-based medium. Fresh tetracycline stocks were made up annually using a new powder batch due to its low long-term stability. When used, antibiotics were always shielded from light.

3.1.1 Culture preparation

Steady-state exponential cell cultures were prepared as follows: Cells were taken from frozen stocks at -80°C and grown for 10–16 hours in LB medium. They were then grown overnight (after a 1:1000 dilution) in their respective M9-based medium, plus appropriate antibiotics in case of a selection marker. These overnight cultures were diluted 1:200 in fresh medium (without antibiotic) and grown until $\text{OD}_{600} = 0.1$ (approximately three division times). From there, they were diluted again in fresh medium (with dilution in the order of 10^{-5} – 10^{-6}) with or without antibiotic so that experiments could be performed the next day. These last dilution factors were calculated to allow for at least 12 division times (without reaching an OD_{600} higher than 0.15) before any measurement to ensure balanced growth. During the day, samples were diluted when necessary to prevent reaching OD_{600} higher than 0.15. All culturing was carried out in 50 mL falcon tubes agitated at 37°C (300 rpm), with no more than 5 mL volume.

3.2 Strain construction

Escherichia coli K-12 MG1655 was used as wild-type (WT) strain in this study. A genetically-modified ‘two palindrome strain’ (2-pal), which has two palindromic sequences inserted on opposite sides of the chromosome, was used to artificially increase SOS induction in a replication-dependent manner. In this study, strains were constructed by inserting gene expression reporters (SOS expression: *PsulA-mGFP*; constitutive expression: *PtetOI-mKate2*), which were cloned into pOSIP plasmids, into the chromosome by clone-integration (CLI) (St-Pierre *et al*, 2013). Insertion of interrupted palindromes *ascB::pal246* and *lacZ::pal246* was done using P1 transduction (Thomason *et al*, 2007). For the *motA* gene deletion strain, a single gene deletion knockout (from the Keio deletion collection (Baba *et al*, 2006)) was transferred to our strain using P1 transduction. Antibiotic resistance selection markers were removed from pOSIP integrants by transformation of the pE-FLP plasmid (St-Pierre *et al*, 2013). After construction, inserts were checked by PCR amplification of the modified chromosomal region. Strains SJR203, SJR206, SJR302, and plasmids were constructed by Jaramillo-Riveri (2019). The strains, plasmids, and primers used are listed in Appendix A.

3.3 Checkerboard assay: Dose-response surface

The inoculum was prepared as follows: An LB overnight culture was prepared as before. Cells were diluted (1:2000) into 5 mL of M9-based medium and incubated overnight at 37°C with

agitation (300 rpm). In the morning, another 1:200 dilution was performed and the culture was incubated until $OD_{600} = 0.2$. In flat-bottomed 96-well plates (Corning Costar), a concentration series for each antibiotic was set up in all columns (drug A) or rows (drug B) as follows: Antibiotics were made up at double the highest concentration desired in 5 mL M9-based medium. A gradient dilution was set up on the x-axis by adding 100 μ L of drug A to all wells in the first column and a serial dilution was performed for the remaining columns of wells up to column 10, with no drug added to column 11 (Figure 13). Thus, each well was made up to a volume of 100 μ L with double the desired drug concentration. A second drug gradient was similarly set up across the y-axis (rows) for drug B, again at double the desired concentration, with no drug added to row H. The desired concentration was achieved in drug combination wells when the two antibiotic solutions are added (100 μ L drug A + 100 μ L drug B = 1:2 dilution). For wells administered with a single drug (column 11 and row H), 100 μ L sterile medium was added to make up the final concentration (1:2 dilution). All wells were then mixed using a pipette. No drugs were added to column 12 which contained the positive and negative controls. At one edge of the plate (column H), four wells were filled with 200 μ L growth medium and inoculum (positive growth controls) and the other four with just 200 μ L growth medium (negative controls for contamination). All wells of the plate (except the negative controls) were inoculated at a 1:1000 dilution using the prepared culture described above. All solutions were pre-warmed at 37°C.

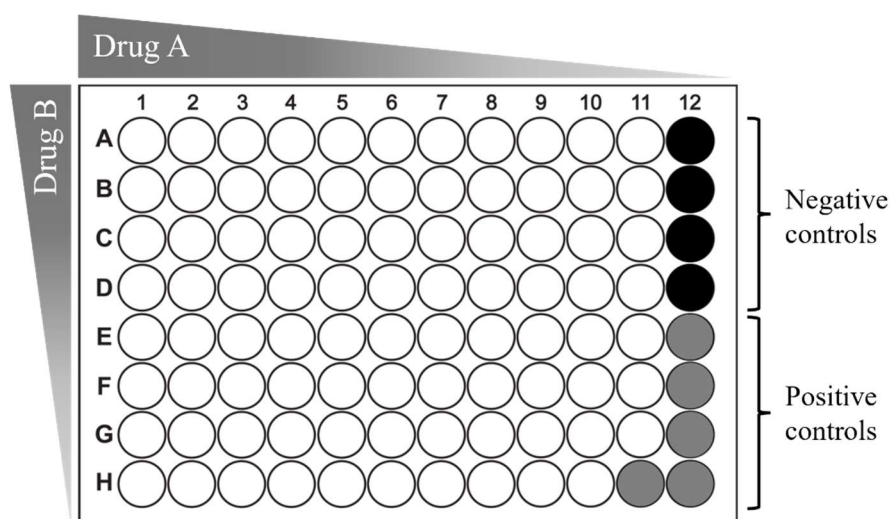


Figure 13. **Depiction of checkerboard assay in a 96-well plate used to evaluate interactions between antibiotic combinations.** A 2D concentration gradient is set up for drug A and drug B along the X and Y axis. Single drug concentration gradients are set up in Row H and Column 11 for drug A and B, respectively. The positions of the negative (black wells) and positive controls (grey wells) are indicated.

A lid was taped onto the 96-well plate to minimise evaporation and debris formation (the tape prevents movement of the lid during shaking). The 96-well plate was then inserted into the FLUOstar Omega microplate reader (BMG LABTECH) and the optical density (OD) at 600 nm (OD_{600}) was measured for each well every 7 min for 28-50 hours depending on the growth medium. The platereader maintained a temperature at 37°C and shaking at 700 rpm using the double-orbital shaking mode. All ODs were blank corrected (subtracted by initial OD of sterile medium) and then exported to an Excel spreadsheet and imported to MATLAB for further analysis.

Growth curves from the platereader were noisy and sometimes contained extreme values considered to be measurement errors. Therefore, growth curves were smoothed with a 5-window moving median and outliers corrected using the *filloutliers* function in MATLAB. Outliers that were missed were manually corrected. Growth rates in each well were then measured via linear regression (*regress* function) to log-transformed OD_{600} from the exponential phase of the growth curve (between $0.025 < OD_{600} < 0.25$). Fits with an R^2 value below 0.8 were discarded. Growth rates were then normalised by the control growth rate (averaged from five wells). To reduce noise, a cubic smoothing spline was used for interpolation to the measured growth rates using the *csaps* MATLAB function. The dose response surface (linearly interpolated isoboles) was then plotted using the *contour* function in MATLAB.

3.4 Bulk growth rates

Population (bulk) growth rates were measured using OD_{600} readings over time taken manually using a spectrophotometer (Jenway 7200) or automatically using OGI-BIO bioreactors.

Batch growth rates from colony-forming units (CFUs) were determined as follows: Steady-state exponential cultures were prepared as described previously (Section 3.1.1). Measurements were taken every 15–30 min depending on the growth condition. For each measurement, the optical density (OD_{600}) was read and then serial dilutions (in steps of 1:10) were performed in a 96-well plate up to a dilution of 10^{-6} . Sterile, pre-warmed PBS was used as diluent. A total of 100 μ L of cells were transferred to pre-warmed LB agar plates and the culture was spread using approximately 10–15 sterile glass beads. The plates were allowed to dry before removing the beads and inverting. Plates were incubated at 37°C overnight. Plate counts were performed in duplicate for each serial dilution where possible. Colonies were counted and CFU/mL calculated for plates containing 30–300 CFUs. CFU-derived growth

rates were estimated via linear regression to the log-transformed CFU/mL growth curves during the exponential growth phase.

3.5 Fluorescence microscopy

All images were captured using a Nikon Ti-E inverted microscope equipped with EMCCD Camera (iXion Ultra 897, Andor), a SpectraX Line engine (Lumencor) and a 100× Nikon TIRF objective (NA 1.49, oil immersion). Nikon Perfect-Focus system was used for continuous maintenance of focus. The filter set for imaging *mGFP* consisted of ET480/40× (excitation), T510LPXR (dichroic), and ET535/50m (emission); whereas for *mKate2* the set ET572/35× (excitation), T590LPXR (dichroic), and ET632/60m (emission) was used. Filters used were purchased from Chroma. *mGFP* fluorescence was measured using 80 ms exposure, whereas *mKate* fluorescence was imaged for 100 ms, both at minimal gain and maximum lamp intensity. Brightfield images were also acquired at each frame using a 20 ms exposure. The microscope and temperature chamber was turned on at least 2 hours before imaging. Microscope was controlled from MATLAB via MicroManager (Edelstein *et al*, 2014) using a custom-made user interface developed by Sebastian Jaramillo-Riveri (Jaramillo-Riveri, 2019). Code accessible at <https://gitlab.com/MEKlab/MicroscopeControl>. Images were saved in *mat* or *tiff* format as one file per fluorescence channel per frame.

3.6 Mother machine experiments

3.6.1 Microfluidics design and fabrication

We used a mother machine design similar to Wang *et al.* (2010), consisting of an array of closed-end microchannels connected to a large flow channel (Figure 14). The device was designed using OpenSCAD and the photomask was manufactured by Compugraphics International Ltd. The master moulds (wafers) were manufactured at the Scottish Microelectronics Centre, Edinburgh, or outsourced to Micro Resist Technology GmbH⁷, using standard photolithography techniques and SU-8 photoresists on a 4" silicon wafer. This was done in two steps: the first layer for the microchannels and the second layer for the flow channel. The length of the microchannels was ~25 µm and the height of the flow channel was 25 µm. The size of *E. coli* cells change depending on the medium used – this scales with growth rate. To accommodate for this, our design consisted of microchannels with a range of widths

⁷ Manufacturing of silicon wafers was outsourced to save time since it was a highly technical and time-consuming process.

(from 0.9 to 1.9 μm) and several master moulds were fabricated corresponding to different heights (0.9–1.35 μm). Appropriate dimensions were tested and selected for each growth condition (Table 1).

Table 1. **Mother machine feature dimensions used for each growth medium.** For each result chapter, wafer dimensions differed due to manufacturer-specific differences.

Growth medium	Microchannel Height (μm)	Microchannel Width (μm)	Microchannel Length (μm)	Flow channel height (μm)
<i>Chapter 4 experiments*</i>				
Gluaa	1.10-1.35	1.3	26	22
Glu	0.91	1.1-1.3	23-28	23
Gly	0.91	1.1-1.2	23-27	23
<i>Chapter 5 experiments**</i>				
Gluaa	1.10	1.2	26	25
Glu	1.10	1.1	26	25
Gly	0.95	1.1	24	25

* Wafers manufactured at the Scottish Microelectronics Centre

** Wafers manufactured by Micro Resist Technology GmbH

Polydimethylsiloxane (PDMS) chips were made using the Silicone Elastomer Kit 184 (Sylgard, Dow Corning) with a 1:10 ratio of curing agent to base. First, the surface of the master wafer was treated with silane to facilitate removal of PDMS from the surface (wafers manufactured by Micro Resist Technology GmbH were already surface treated on arrival). The wafer was then taped to the bottom of a large petri dish to secure it in place. Mixed PDMS (1:10 ratio of curing agent to base) was poured onto the master mould to achieve a thickness of approximately 5 mm. The freshly poured PDMS was degassed for ~1 hour in a vacuum bell jar to remove bubbles followed by curing at 65°C for at least 4 hours or left overnight⁸. After cooling to room temperature, chips were then carefully cut out using a scalpel and feeding channels created using a 0.75 mm (ID) biopsy punch (World Precision Instruments Limited). Chips were cleaned by sonicating in isopropanol for 30 min (two 15 minute rounds, transferred to fresh isopropanol for second round) and then left to air dry overnight at 65°C in a closed petri dish (features facing up). Chips were then inspected for remaining dirt; if found, the chips were rinsed with 70% ethanol and left to air dry. Coverslips (Duran, 24x60 mm, #1.5) were cleaned by sonicating in 1 M KOH for 30 min, followed by rinsing three times in Milli-Q water, and then sonication for a further 30 min in Milli-Q water. Coverslips were left to dry overnight at 65°C.

⁸ Curing PDMS for long durations results in a harder, more brittle material which is prone to tearing when punching the inlet holes and can increase the chance of leaks. I found that shorter curing times (4 hours) avoided this.

Before bonding, chips and coverslips were surface-activated in an oxygen plasma cleaner (supplied with ambient air) operated at high intensity under vacuum for 60 s. Bonded chips were then left at 65°C for at least 5 min (to improve bonding) followed by storage in parafilm-sealed petri dishes at room temperature.

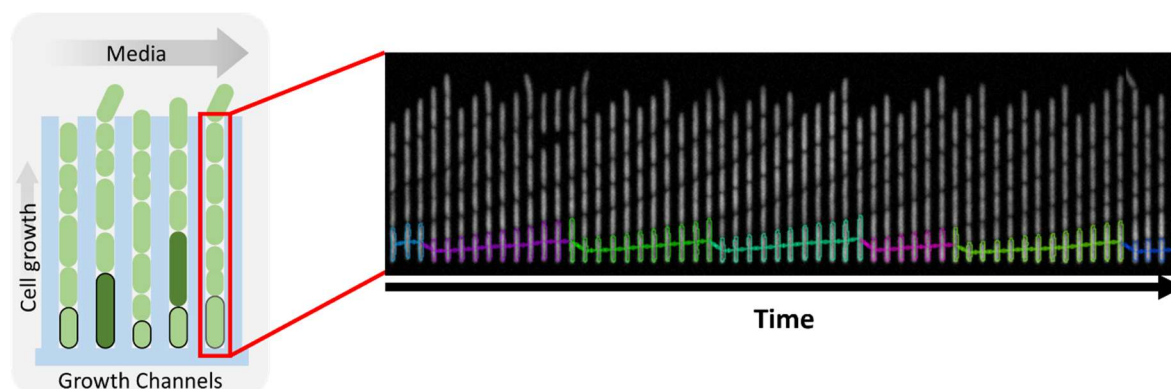


Figure 14. **Illustration of the mother machine.** Left: Cells are trapped in microchannels with dimensions mimicking the size of one cell. Cells at the bottom of the microchannels are the ‘mother cells’ which produce daughter cells that are flushed away by constant flow of medium. Flow of medium supplies fresh nutrients and flushes away waste to maintain exponential bacterial growth. Right: A kymograph showing a sequence of frames from a single microchannel. The mother cell lineage is tracked over time.

3.6.2 Mother machine culture preparation and loading the microfluidic device

For Chapter 4 experiments, exponential cultures were prepared as described previously (Section 3.1.1). Culture preparation for Chapter 5 experiments were amended, for convenience, as follows: As before, cells were resuscitated during overnight growth in LB medium. The next morning (day of experiment), cells were sub-cultured (1:1000 dilution factor) into 2X 10 mL M9-based medium in 50 mL falcon tubes and incubated at 37°C with shaking (300 rpm). When the culture reached an OD_{600} of 0.2, cells were ready for harvesting. For the slow growth medium (gly), an extra step was added: After 1:1000 dilution from the LB overnight into M9-based medium (late afternoon), the culture was incubated overnight. The next morning, cells were diluted again (1:200) and allowed to grow until $OD_{600} = 0.2$. This amendment was deemed acceptable since balanced growth should be achieved during several hours of growth in the mother machine before the experiment begins.

Once the culture was ready for harvesting, Tween-20 Surfact-Amps detergent solution (Thermo Scientific, 85113) was added to prevent cell clumping (0.01% final concentration) before being concentrated 100-fold by centrifugation (4000 rpm for 5 min). Before sample

loading, the chip was passivated with Tween-20 (0.01% final concentration) for at least 1 hour. The concentrated cell culture was briefly vortexed and then injected into the inlets of the microfluidic chip (Figure 15A) using a 1 mL syringe with a 21-gauge blunt needle (OctoInkjet), while taking care not to damage channel inlets. The channel inlets were sealed temporarily using adhesive tape and left at 37°C for ~30 min to allow cells to diffuse into the microchannels. To further assist cell loading, the cells were then spun into the microchannels by centrifugation at 4000 rpm (3220 g) for 5 min using a custom-built mount. Centrifugation was essential for loading of non-motile cells (strain JKB43). The chip was then mounted on the microscope using a custom-built mount and the loading efficiency was evaluated.

Before and after each experiment, the inlet and outlet tubing (0.44 mm ID) were sterilised with 1% bleach, followed by 10% ethanol, rinsed with Milli-Q water, and then primed with fresh medium. Inlet and outlet tubing was then connected to the inlet and outlet channels via 21-gauge blunt needles (Figure 15B) with the medium reservoir on one end and effluent reservoir on the other end. Tween-20 (0.01% final concentration) was also added to the medium reservoir to prevent cell adhesion and biofilm formation within the tubing and device. Medium flow was resumed to flush cells out the main trench using a peristaltic pump (Ismatec IPC ISM932D) at 1.5-2 mL/h and then lowered to 1 mL/h for the duration of the experiment (Figure 15Cii). Cells in the channels were allowed to recover/acclimate for at least 2 hours at 37°C before imaging started.

For antibiotic exposure experiments (Chapter 5), antibiotics were made up to the desired concentration in M9-based medium in separate 50 mL falcon tubes and was done on the morning of the experiment. These were kept at 37°C for the duration of the experiment and wrapped in foil to protect from light. Tubing was connected to the falcon tubes via a custom-built manifold (Figure 15Ci). Tubing was then connected to three-way valves (two inlets, one outlet) which were used to switch between no-drug and antibiotic influent falcon tubes. Valves were controlled automatically via MATLAB communication with a data acquisition device (DAQ, Measurement Computing, USB-1408FS). Valve-outlet tubing was then connected to a peristaltic pump which ‘pushed’ (positive-pressure flow is advantageous as it avoids feature collapse due to negative-pressure driven flow) medium through the microfluidic chip into the effluent flask (Figure 15Ciii).

Experiments for Chapter 4 were run for 10–42 hours depending on the nutrient conditions. Antibiotic treatment experiments (Chapter 5) were standardised as follows: When imaging

started, all flow channels received fresh medium without antibiotic for 6 hours, followed by 12 hours of antibiotic exposure, and then switched back to medium without antibiotic for at least 10 hours, unless otherwise stated. The pre-antibiotic period ensures all cells have acclimated to the system and are undergoing balanced exponential growth. The post-antibiotic period (or recovery period) allows the resuscitation of persister cells, if present.

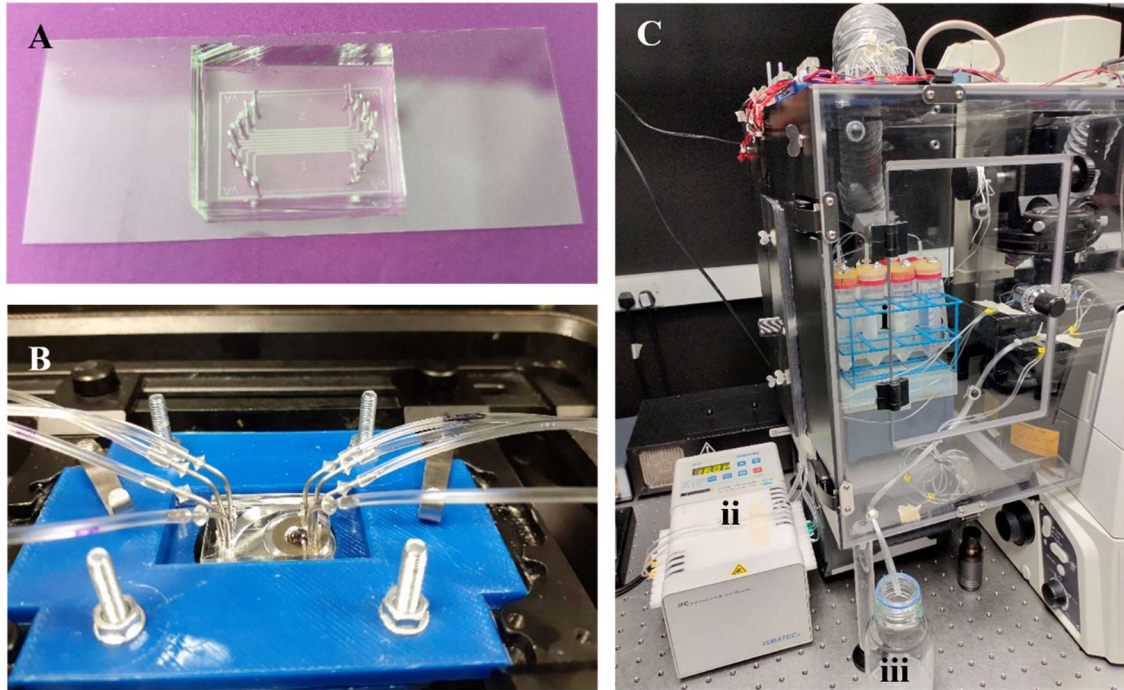


Figure 15. Experimental set-up of mother machine experiments. **A.** Microfluidic chip bound to a glass coverslip, ready for cell loading. **B.** Mother machine mounted on the microscope with inlet and outlet tubing connected via blunt needles. The custom-built mount protects the glass coverslip from cracking during manipulation of the device. **C.** Experimental set-up showing the influent falcon tubes (i), peristaltic pump (ii), and effluent flask (iii).

3.6.3 Image analysis

For mother machine experiments, images were acquired at 5 min intervals for gluaa & glyaa media, 5-10 min for glu medium, and 12 min for gly medium. For Chapter 5 experiments, the imaging interval changed to 5 min for gluaa & glu media, and 10 min for gly medium.

Cell segmentation and lineage tracking were performed using BACMMAN run in Fiji (Ollion *et al*, 2019). The BACMMAN configuration was adapted to segment and track cells based on fluorescence images. Images (*tiff* format) were imported in BACMMAN and pre-processed prior to segmentation. This included image rotation to ensure channels were vertically oriented and the channel opening was at the bottom, and cropping of images to include only the area consisting of microchannels. Background subtraction was performed for both fluorescent

channels using ImageJ's subtract background algorithm (rolling ball method, radius=8). Joint segmentation and tracking of cells were performed using a DistNet model trained on our datasets. DistNet is based on a deep neural network (Ollion & Ollion, 2020). Segmentation was performed on the *mKate* fluorescence channel. Segmentation parameters were optimised for each dataset. Curation of segmentation and tracking was carried out using BACMMAN's interactive graphical interface. Although automated segmentation and tracking was mostly accurate, occasionally errors were produced. Thus, almost every lineage was manually checked, with 2-pal and CIP exposure datasets requiring the most curation mainly due to excessive elongation of cells expressing the SOS response. At this point obvious irregularities were removed including mother cells that did not grow for the duration of the experiment and those that were already excessively elongated at the beginning of the experiment. Positions with channel deformities and where loss of focus occurred were also discarded.

Single-cell fluorescent intensities were calculated by dividing the sum of pixel intensity values by the cell area. Cell length was determined as the maximal distance between two points of the cell contour (FeretMax). Elongation rates were calculated per cell-cycle (generation) by fitting an exponential function, $S_t = S_0 e^{\mu t}$, on the estimated length of cells, where μ is the elongation rate, S_0 is the length at birth, S_t is the length at time t . A minimum of 3 frames per generation was imposed as a fitting constraint. Division rates were calculated as the inverse of the interdivision time. The latter was determined as the time interval between two divisions. Cell fluorescence and morphology measurements were then exported from BACMMAN into excel files for further analysis using custom MATLAB or Python scripts. Kymograph images were constructed by BACMMAN.

3.6.4 Data analysis for Chapter 4

Data analysis and plotting for Chapter 4 was performed using custom MATLAB scripts. The raw image data is available at: <https://www.ebi.ac.uk/biostudies/bioimages/studies/S-BSST833>. The analysis code is available at <https://gitlab.com/MEKlab/growth-sos-msb-2021>. First, the 'mother cell' lineage was isolated. Divisions for the mother cell lineage were recorded with tracking continuing for the cell at the closed end of the channel and discarding the sister cell. All *mGFP* and *mKate* fluorescence values were re-scaled by the mean fluorescence value of the WT strain in each growth medium. We noticed, for some datasets, the cells had yet to adapt to the imaging conditions indicated by a steep initial slope in the *mKate* signal, after which it stabilised (after approximately 2 hours). Therefore, trajectories were truncated based

on stability of the *mKate* signal. *mKate* stability was identified based on a derivative of the median of the *mKate* signal. Stability decision parameters were manually tuned based on visual inspection. At most, 4 hours were truncated from the beginning of datasets. The first and last cell-cycles in a lineage were discarded if they consisted of less than three data points. All non-growing cell-cycles (defined by a minimum growth rate of 0.03 h^{-1}) were removed from the beginning and end of each lineage. This filter also acted to remove cell-cycles for excessively elongated (high-SOS) cells. Thus, all growth and division rates are representative of sub-population n_1 (the low-SOS population) in the fixed-population dynamics model (See Appendix B2 and Section 4.4 for a description of the model). For alpha (α) estimation (the switching rate from low-SOS to high-SOS state), GFP trajectories were smoothed with a 5-point moving average. Data analysis for Chapter 4 was performed with significant help from Sebastian Jaramillo-Riveri.

3.6.4.1 Estimation of high-SOS switching rate

SOS expression levels of single-cell lineages were tracked over time to allow direct estimation of the high-SOS switching rate α . The population dynamics model, which originally represented a growing population, was modified to represent a fixed population size (see Appendix B2). This was necessary since a fixed number of cell lineages were tracked in the mother machine. Estimates for α were only done for 2-pal datasets. Due to low high-SOS induction probability for WT cells and the limited sample size, confident estimates for α could not be obtained for this strain. Alpha was estimated for each medium and experimental replicate and is explained in more detail in Figure 25.

3.6.5 Data analysis for Chapter 5

Data analysis and plotting for Chapter 5 was conducted in Python using Jupyter notebooks. The code is available at: <https://github.com/j-broughton/Data-analysis-backup>. After imaging started, the first 4 hours were cut to avoid *mKate* stability issues described above. For antibiotic treatment experiments, we removed lineages that were initially filamented or non-growing in the first 2 hours. Some cells that filamented extended beyond the length of the microchannels (classified as hyper-filamented cells). We only tracked a cell that filamented up until it reached the maximum microchannel length since cell length measurements beyond that are invalid.

3.6.5.1 Classification of cell fate

First, lineages that excessively filamented (length $>20\ \mu\text{m}$) were separated and classified as ‘hyper-filamented’. These cells extended beyond the length of the microchannels (cell traps). The remaining lineages were classified as ‘died’ if their last recorded elongation rate is below a threshold value, or if they did not divide for 4 hours or more. The elongation rate threshold was set at 20% of the median elongation rate of the no-drug control condition for each medium. In some rare cases, cells lysed – losing their physical integrity – and could no longer be tracked (Figure 40G), therefore escaping the filters set above. These were manually classified as ‘died’. The majority of cells that ceased growth maintained physical integrity and did not resume growth after removal of antibiotic treatment. Each lineage is made up of a succession of cell-cycles before cell death. Thus, we can also determine at which point cell death occurs for each lineage. Within each lineage, we also labelled each cell-cycle to differentiate between periods when the lineage was ‘alive’ and ‘dead’. Classification was performed over the duration of the experiment (24 hours), which included a 10 hour recovery period where fresh no-drug medium was flowed through the device to allow cells to recover from antibiotic exposure – if they can do so. This ensures that cells initially classified as ‘dead’ were not persister cells.

3.6.5.2 Quantification of cell survival and death

Survival analysis was carried out using the *lifelines* package in Python (Davidson-Pilon, 2019). First, we organised our data into two columns, with the first column populated with lineage lifespans (in hours), and the second column denoting whether a death event occurred or not (Boolean, true or false). Lifespans are determined as the time of death or time of censorship, where censorship refers to the end of the experiment. Not all cells died during the experiment – these are known as right-censored individuals and are important to include in our statistic to avoid overestimating the rate of death. The lifespans for each mother cell lineage were determined as the beginning of the cell-cycle where death was estimated to occur (as described above). With our data, we could not determine the precise time of death besides an obvious lytic event. Thus, there is a degree of uncertainty in the lifespan values (particularly in slower growth conditions where cell-cycles are longer) which could be improved upon.

The survival function $S(t)$ was estimated using the Kaplan-Meier (K-M) estimator (Kaplan & Meier, 1958), defined as:

$$\hat{S}(t) = \prod_{t_i < t} \frac{n_i - d_i}{n_i}$$

where d_i are the number of death events at time t_i (where t_i is a time where at least one death event i occurs) and n_i is the number of subjects at risk of death just prior to time t_i . The K-M estimate represents an accumulation of probabilities equivalent to $S(t) = P(\text{surviving past time } t)$. The cumulative hazard $H(t)$

$$H(t) = \int_0^t h(t') dt'$$

was estimated using the Nelson-Aalen (N-A) estimator (Nelson, 1972; Aalen, 1978), defined as:

$$\hat{H}(t) = \sum_{t_i \leq t} \frac{d_i}{n_i}$$

where, h is the instantaneous hazard rate (death rate). Both the K-M and N-A estimators are non-parametric statistics.

3.7 Agarose-pad microscopy and image analysis

Agarose-pad experiments were carried out for the trimethoprim-tetracycline combination. Steady-state exponential cultures were prepared as described previously (Section 3.1.1), except antibiotics were added to the final overnight dilution. Growth rates under each antibiotic were estimated and the dilution ratio adjusted accordingly. As a result, the culture was adapted to growth under the respective antibiotic or drug combination for at least 12 generations of exponential growth. Once the culture reached an OD₆₀₀ of 0.05-0.1 the next day, a 2-5 μ L sample was mounted on 1% agarose pads (Gene-frame 65 μ L) made with the corresponding medium containing the respective antibiotic or antibiotic combination. Antibiotics and nucleotides (for the glycan medium, 10X ACGU, 0.22 μ m filter-sterilised) were added to cooled agarose-medium just before setting in the gene-frame. The samples were added to a small rectangle (approximately 5-by-8 mm) cut from the agarose-pad, and once dried, a coverslip was used to seal the agarose-pad in the gene-frame. Time-lapse images were acquired at intervals approximately one-tenth of the population doubling time for each medium (5 min

for glyaan & 10 min for gly), and for 120 iterations. Around 30-70 stage positions were imaged per condition.

Segmentation of cells and quantification was done using BACMMAN run in Fiji (Ollion *et al*, 2019). The first set of images from the time-lapse experiments were taken for quantification. Cell segmentation was done automatically using fluorescence signal from the constitutive reporter *PtetO1-mKate2*. Segmentation was checked manually to correct for errors. Quantification data (fluorescence intensities and morphology) were exported from BACMMAN and plotted using Python.

Chapter 4: Growth-dependent heterogeneity in the SOS response

4.1 Introduction

The SOS response is an important mechanism in *E. coli* that facilitates survival under DNA-damaging conditions. Bacteria experience various levels of nutrient availability in the environment which affects their growth rate. Changing growth rates (modulated by the growth medium) impose constraints on the capacity of bacteria to modify their proteome (Scott *et al*, 2010; Hui *et al*, 2015), potentially influencing the capacity of cells to induce the SOS response. The connection between growth rate and expression of the SOS response is an understudied topic. Single-cell variability in SOS gene expression could have important consequences on the outcome of individual cells suffering DNA damage. The SOS response has been shown to be highly heterogeneous at the single-cell level (Jones & Uphoff, 2021), but the effect of changing growth rate on the variability of the SOS response in different growth conditions has not yet been quantified.

Recent work by Jaramillo-Rivera *et al.* (2022) revealed the influence of growth rate on the SOS response (Figure 16). In these experiments, batch cultures were grown in three different nutrient conditions, supplemented with either glucose and amino acids (gluaa, doubling time = 37 min), glucose (glu, doubling time = 58 min), or glycerol (gly, doubling time = 100 min). Here, bulk growth rates were measured in steady-state, exponential phase cultures. Samples were taken from the cultures, mounted on agarose-pads, and single-cell SOS expression was quantified (Figure 16A). The distributions suggest the existence of two populations, with a main peak and a secondary peak with high levels of SOS expression in the tail of the distribution (Figure 16A, inset). It is important to note that the distributions represent SOS expression levels at a snapshot in time. The high-SOS fraction of cells were then quantified as those above SOS levels of 5 a.u. (Figure 16B). There was a larger proportion of high-SOS cells in slower growth conditions (Figure 16A, inset).

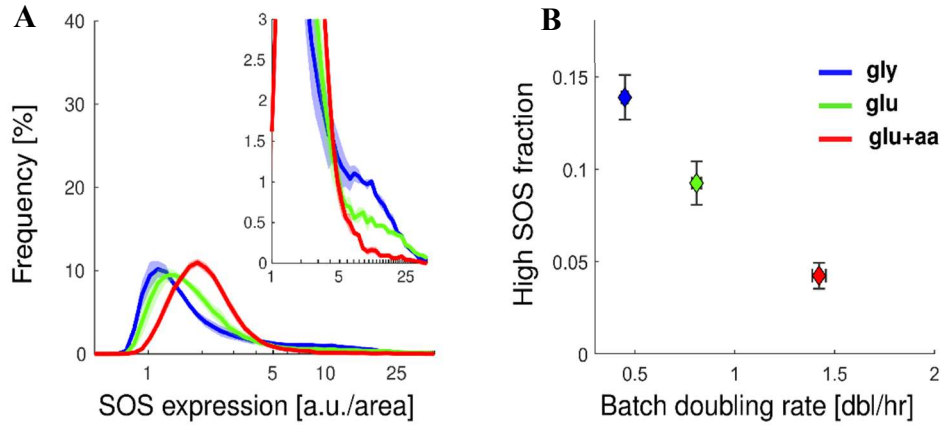


Figure 16. Cells with elevated SOS expression are more frequent in slow growth conditions. **A.** Steady-state distributions of normalised SOS expression (from SOS reporter *PsulA-mGFP*) quantified from single-cells in different growth conditions. SOS expression was normalised to the mean value of the WT strain in each growth condition. Solid line is the average and shaded area the standard error from three experimental replicates. Inset: A magnification of the tail of the distribution. SOS response was induced via replication-dependent DNA damage in the 2-pal strain. Different colours represent the growth condition. **B.** Batch population fractions of high SOS cells in different growth conditions. The high-SOS fraction was determined using a GFP threshold of 5 arbitrary units. Points represent the average and bars the standard error from three biological repeats. Figures reproduced from Jaramillo-Riveri & Broughton et al. (2022) (use permitted under the Creative Commons Attribution License CC BY 4.0).

The results initially suggested that slow-growing cells induce the SOS response to higher levels at a higher frequency, resulting in a larger fraction of high-SOS cells in the population. We can consider that cells ‘switch’ from a low to high-SOS state at a defined and measurable rate (probability per unit of time). However, these observations were made at a snapshot in time after growth in batch culture and thus gave no indication of the rate of switching from a low-SOS to high-SOS state, nor if this rate changes across growth conditions. Moreover, expression of the SOS response is associated with delays in cell division which could have implications on the population dynamics of low-SOS and high-SOS sub-populations. The objective of this chapter was to measure the rate of switching from a low-SOS to high-SOS state. We used time-lapse fluorescence microscopy and a custom microfluidic device, called the ‘mother machine’, to track the growth and SOS expression dynamics of single cells under conditions of chronic sub-lethal levels of DNA damage.

This chapter includes contributions from Sebastian Jaramillo-Riveri: strain construction, design of the mother machine, assistance with data analysis, and derivation of the population dynamics model. Results from this chapter, in combination with Sebastian Jaramillo-Riveri’s

agarose-pad data, were published in Jaramillo-Riveri et al. (2022) of which I am co-first author. A few figures from that publication are reproduced here.

4.2 The SOS response is highly heterogeneous

We tracked the SOS expression of hundreds of single-cell lineages in the mother machine (see Section 2.6.2 and 3.6.1 for a detailed description of the mother machine) for at least 10 hours. SOS gene expression was quantified using a fluorescent reporter coupled to a SOS promoter (*PsulA-mGFP*). Constitutive gene expression was also quantified (*PtetO1-mKate2*) and primarily used for segmentation of cells (Figure 17). We also normalised both gene expression measurements to the mean of the WT in each growth medium. This was done for convenience when setting the GFP thresholds in Section 4.5. To identify growth-dependent influences on SOS expression, cells were cultured in three different growth media corresponding to fast (gluaa), intermediate (glu), or slow growth rates (gly) with batch doubling times of 37, 58, and 100 minutes, respectively. We evaluated spontaneous SOS induction using a wild-type (WT) *E. coli* strain and chronic SOS induction using a mutant strain (referred to as 2-pal). SOS induction in the 2-pal strain occurs in a replication-dependent manner due to the presence of two palindromic sequences inserted into the chromosome. The palindromes lead to site-specific cleavage upon replication (Eykelboom *et al*, 2008), and thus mimics natural replication-dependent DNA breaks (DSBs) but at higher levels that results in moderate SOS induction (Jaramillo-Riveri, 2019).

We observed high single-cell heterogeneity in the SOS response (Figure 17 and Figure 18). SOS expression was either moderate and transient with continued cell division (Figure 17A), or long lasting with higher levels of expression and delayed or arrested cell division, resulting in elongated cells (Figure 17B). Most cells did not recover division from the high-SOS state. These observations are consistent with previous studies that have quantified the SOS response at the single-cell level (McCool *et al*, 2004; Kamenšek *et al*, 2010; Erental *et al*, 2014; Uphoff, 2018; Mitosch *et al*, 2019; Jones & Uphoff, 2021).

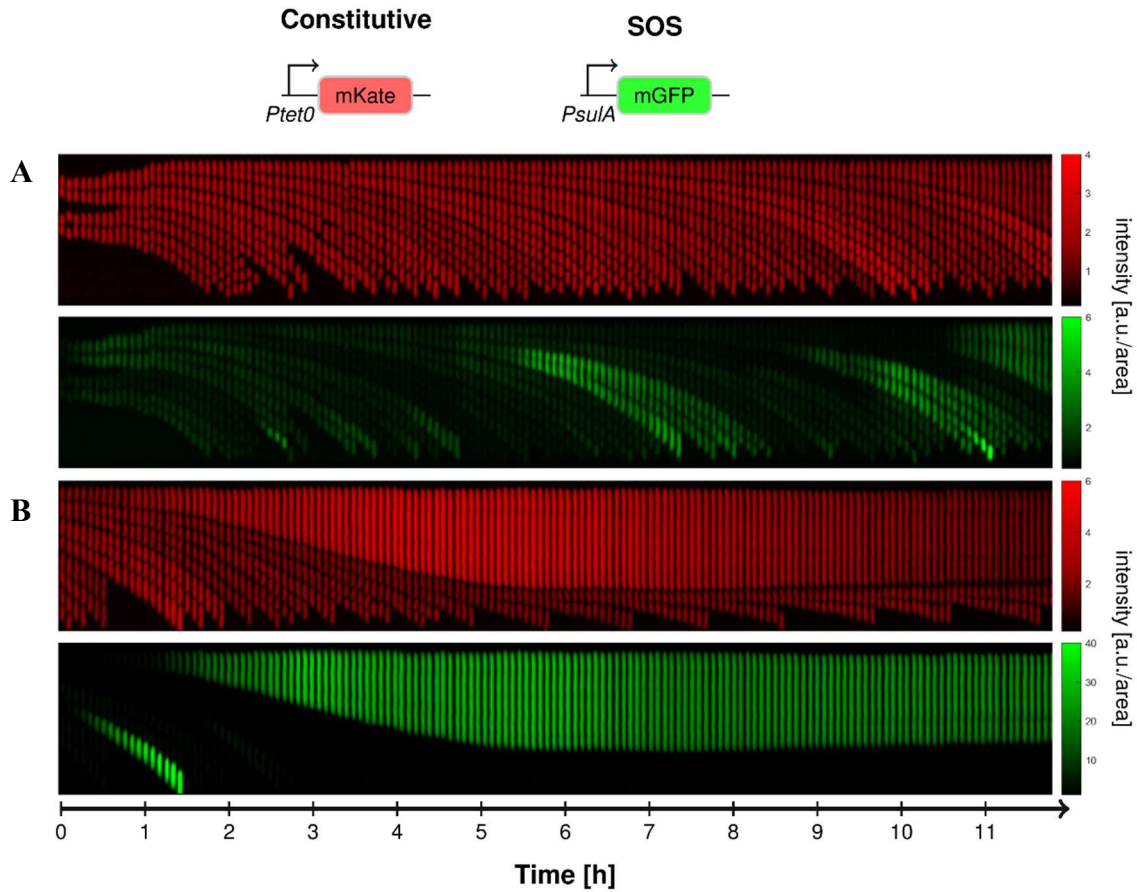


Figure 17. **Representative kymographs depicting single-cell dynamics in the mother machine for the 2-pal strain in fast growth conditions.** **A.** Mother cell lineage exhibiting low levels of SOS induction. **B.** Mother cell lineage exhibiting a high level of SOS induction and inhibition of cell division. For A and B: Top panel represents normalised constitutive expression from *PtetO1-mKate2* (red), and bottom panel represents normalised SOS expression from *PsuIA-mGFP* (green). SOS and constitutive expression was normalised to the mean value of the WT strain. Vertical scale bars represent normalised fluorescent intensity for each fluorescent reporter. Figure reproduced from Jaramillo-Riveri & Broughton et al. (2022) (use permitted under the Creative Commons Attribution License CC BY 4.0).

For the WT strain, we observed a few cells that induced the SOS response above baseline levels (Figure 18A-C). SOS induction was more frequent in *gluaa* (Figure 18C) than in *glu* and *gly* media (Figure 18B, C) likely due to faster DNA replication rates in the fast-growth condition, and hence a higher rate of spontaneous replication errors. SOS induction in the WT strain was transient with most trajectories returning to baseline levels and generally did not reach high SOS levels above 5 a.u., reflecting low levels of DNA damage that is dealt with relatively quickly. Spontaneous DNA damage is estimated to occur only at a rate of 2% per cell division (Shee *et al*, 2013). SOS levels returned to baseline quicker in *gluaa* than in *gly*. This could be a result of a higher rate of dilution of the GFP protein in cells with faster growth rates.

For the 2-pal strain, we observed a higher frequency of cells inducing the SOS response above baseline levels (Figure 18D-F), many of which reached high SOS expression levels above 5 a.u (Figure 18D-F, black dashed line). Cells with high SOS expression levels generally did not return to baseline, with only a few cells exhibiting transient SOS induction to intermediate levels. Stronger and more frequent induction of SOS response for the 2-pal strain (compared to WT) is expected due to replication-dependent acquisition of DSBs. The likelihood of accumulating irreconcilable DNA damage is therefore higher in the 2-pal strain. This can lead to extended periods of very high SOS levels as seen in Figure 17B and Figure 18D-F.

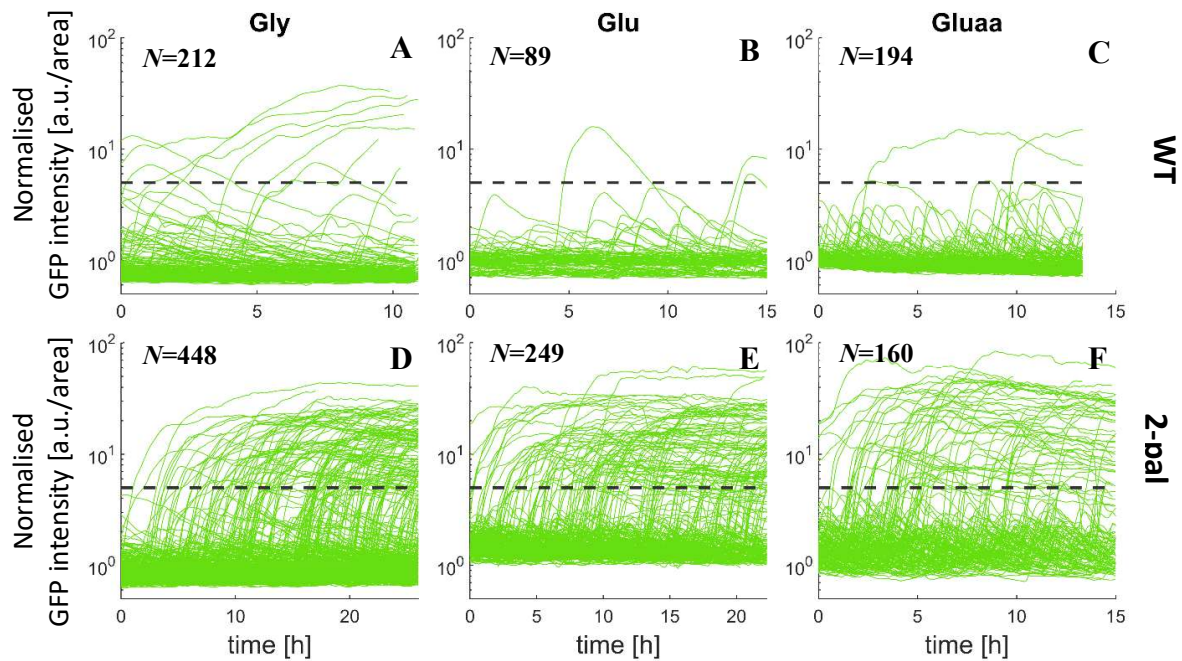


Figure 18. Single-cell SOS expression trajectories for the WT (A-C) and 2-pal (D-F) strain in three growth conditions. One of at least three experimental repeats are shown. Black dashed line indicates a GFP intensity of 5 arbitrary units (a.u.), above which cells are considered to be high-SOS. GFP intensity was normalised to the mean value of the wild-type (WT) datasets in each medium. GFP trajectories were smoothed using a moving average ($n=5$) to reduce noise. The number of mother cell lineages are indicated in each panel.

We then quantified GFP intensity distributions taken over the whole duration of the experiment (Figure 19). Average SOS expression was higher in the 2-pal strain than the WT, and the distributions were wider reflecting higher levels of heterogeneity. For the WT strain, a relatively low proportion of cells reached SOS levels above 5 a.u. as indicated in the tail of the distribution (Figure 19A, inset). The 2-pal strain had a higher frequency of cells with SOS levels above 5 a.u. (Figure 19B, inset). For both spontaneous DNA damage and replication-dependent DNA damage, SOS expression was significantly higher in fast-growth conditions (gluaa & glu) than in slow-growth conditions (gly) (Mann-Whitney U-test, $p<0.005$), with

gluaa and glu having similar levels (Figure 19A, B). The average values for the SOS distributions are listed as follows: 0.90, 1.02, 1.00 a.u. for the WT, and 1.59, 1.84, 1.81 a.u. for the 2-pal strain in gly, glu, and gluaa medium, respectively. Higher SOS levels in faster growth conditions could be due to a higher number of replication forks, and therefore more frequent replication-dependent DSBs.

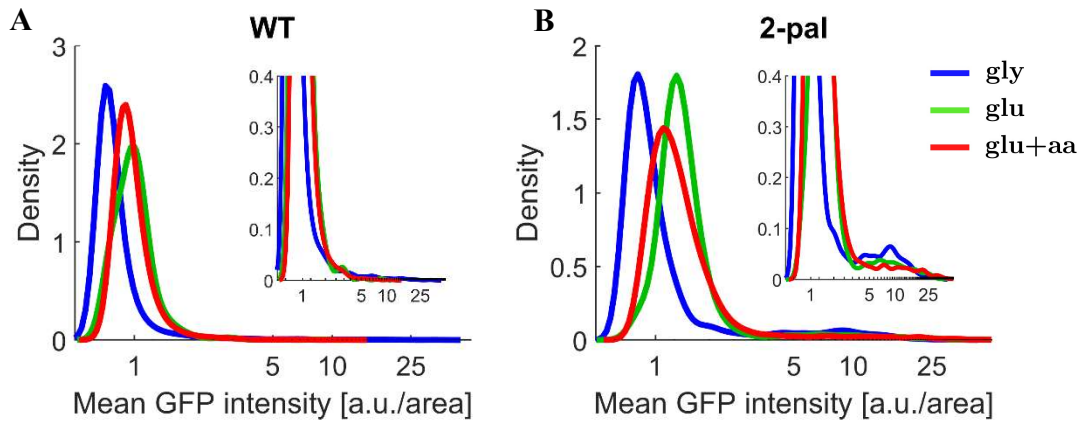


Figure 19. Distributions of cell-cycle averaged SOS expression for the WT (A) and 2-pal (B) strain grown in the mother machine in three different nutrient conditions. Solid lines represent the probability density estimate of data pooled over the duration of the experiment from three experimental replicates. The distributions represent all cell-cycle averaged GFP values at all time points during the experiment. GFP intensity was normalised to the mean value of the wild-type (WT) datasets in each medium. Inset: A magnification of the second peak at high SOS expression. Different colours represent the growth condition. Experimental replicates are plotted individually in Appendix B1 and show good day-to-day reproducibility. All distributions are significantly different from any other distribution (Mann-Whitney U-test, $p < 0.005$).

4.3 High-SOS cells arrest growth and division

Single-cell elongation rates and division times were calculated for each mother cell lineage. As expected, the average elongation rates increased in richer growth media (Figure 20A, C), while the division times decreased (Figure 20B, D; Table 2). Elongation and division rates for the 2-pal strain were significantly slower than the WT strain (Mann-Whitney U-test, $p < 0.005$). This difference reflects the higher frequency of DNA damage and associated physiological burden for the 2-pal strain. Higher SOS induction levels observed for the 2-pal strain are also expected to result in longer division times due to induction of the SOS-dependent cell division inhibitor SulA (Huisman *et al*, 1984).

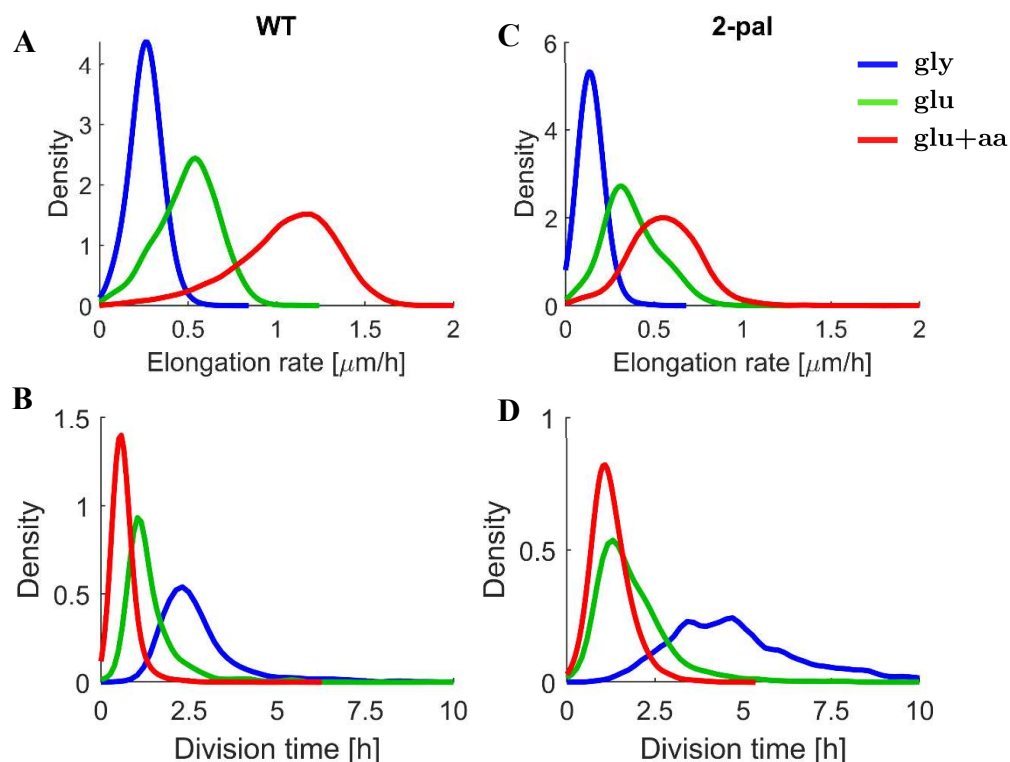


Figure 20. **Distributions of single-cell elongation rates (A, C) and division times (B, D) for the WT (A, B) and 2-pal (C, D) strain in three growth conditions.** Solid lines represent the probability density estimate of data pooled over the duration of the experiment from three experimental replicates. Different colours represent the growth condition. Experimental replicates are plotted individually in Appendix B1 and show good day-to-day reproducibility. All distributions are significantly different from any other distribution (Mann-Whitney U-test, $p < 0.005$).

Table 2. **Averaged single-cell growth parameters for the WT and 2-pal strain in three growth conditions.** Shown is the mean and standard deviation from three experimental replicates.

Strain	Medium	Number of mother lineages	Division times [min]	Elongation rates [h^{-1}]
WT	gly	504	182.8 ± 34.3	0.25 ± 0.03
WT	glu	89	83.1 ± 14.9	0.52 ± 0.07
WT	gluaa	417	38.6 ± 1.39	1.07 ± 0.03
2-pal	gly	603	324 ± 27.5	0.15 ± 0.01
2-pal	glu	899	118 ± 25.0	0.38 ± 0.10
2-pal	gluaa	609	74.2 ± 9.57	0.59 ± 0.06

For the 2-pal strain, the majority of lineages that reached high SOS levels (> 5 a.u.) arrested division and continued elongating (Figure 21D-F). However, for some cells elongation only continued to a point after which elongation stopped as well. Cells reached higher lengths quicker in faster growth conditions which reflects the different growth rates supported by each

medium (Figure 21D-F). For the WT strain, only a few cells filamented reflecting lower levels of SOS induction (Figure 21A-C). Very few cells expressed high levels of the SOS response in the WT, and most of those that did quickly returned to baseline levels, particularly in fast growth conditions (Figure 18A-C). Therefore, it is likely that sustained elevated SOS expression is required for filamentation to occur. A few high-SOS, elongated cells resumed division by budding smaller cells from the end of the filament, which has been reported previously (Raghunathan *et al*, 2020).

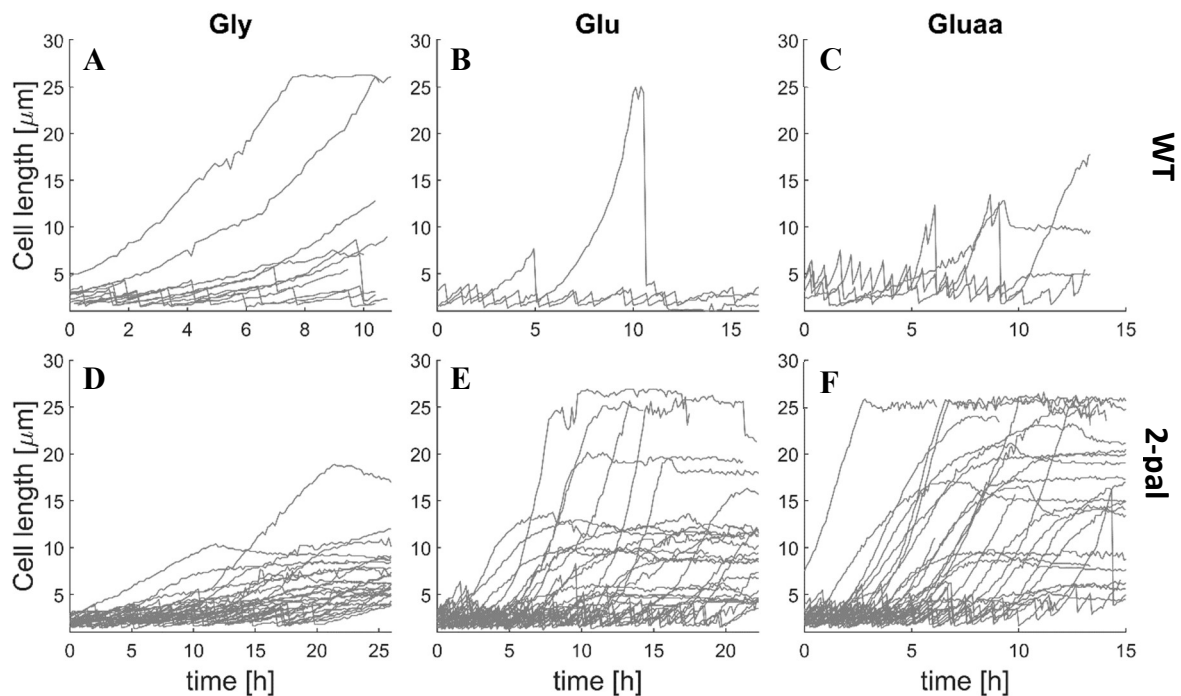


Figure 21. Cell length of high-SOS lineages for the wild-type (A-C) and 2-pal (D-F) strain in three growth conditions. High-SOS lineages were determined if GFP intensity reached a threshold value of 5 arbitrary units. One of at least three experimental repeats are shown. Thirty random lineages were selected for 2-pal data due to a large number of high-SOS lineages.

To investigate the correlation between SOS expression levels and growth arrest, maximum SOS expression was plotted against the maximum time the cell was observed (i.e. between birth and division for each cell-cycle or between birth and the end of the experiment for cells that did not divide – for example Figure 17B and Figure 21D-F) (Figure 22), and elongation rate (Figure 23). We observed two populations of cells in the 2-pal strain. The main population (to the left of the blacked dotted line) displayed low levels of SOS induction with normal division/cell-cycle duration (Figure 22D-F) and elongation rates (Figure 23D-F). The second population displayed high levels of SOS induction (> 5 a.u.) with extended cell-cycle duration (5-20 hours), corresponding to very slow or no division, and slowed elongation rates. As observed before, there was a larger fraction of cells in the second population for the 2-pal strain

compared to the WT. Slowed elongation rates for high-SOS cells is likely a result of excessive levels of DNA damage that the cell is unable to repair, eventually leading to growth arrest and cell death.

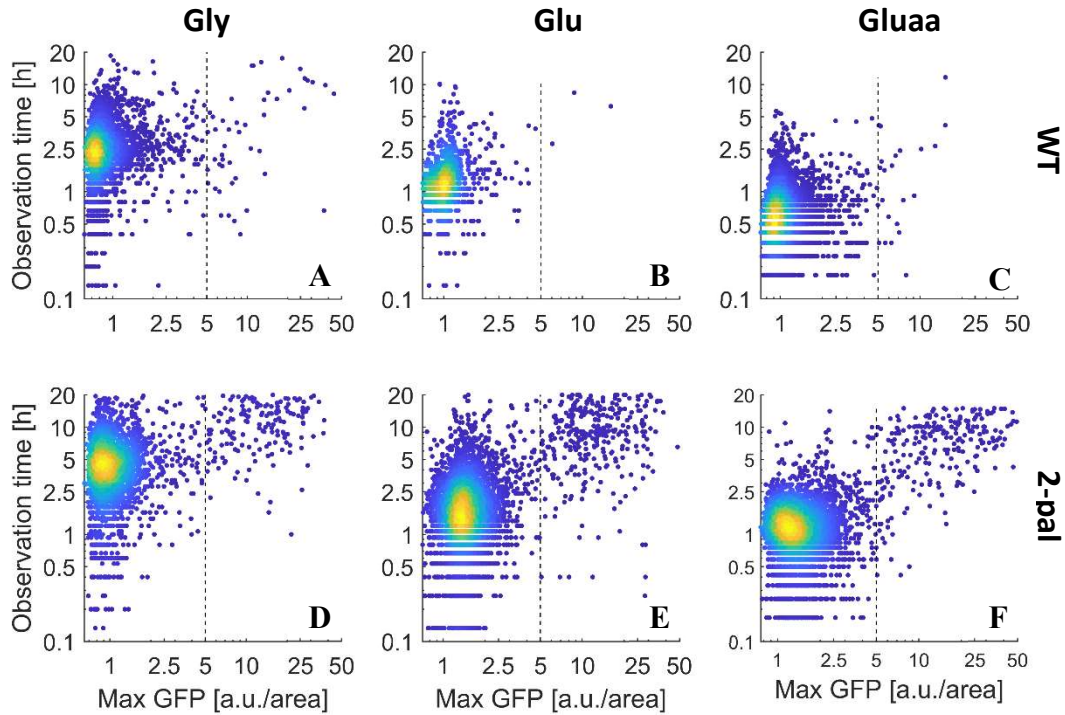


Figure 22. High levels of SOS expression correlates with reduction in cell division. Data is shown for the WT (A-C) and 2-pal (D-F) strain in three growth conditions. Observation time is plotted against the maximum GFP intensity registered for each cell-cycle observation. Observation time is the maximum observed cell-cycle duration before a division or until the end of tracking for each mother cell if it did not divide by the end of the experiment. In this way, data for filaments (which do not end with a division) are captured. Plots consist of data pooled from three experimental replicates. Vertical dashed line represents high-SOS cutoff (at 5 a.u.) that discriminates between low and high-SOS populations. Colours represent the 2D density of data points. Panels A-F consists of data from 4340, 1016, 7393, 2572, 5978, and 9880 cell-cycles, respectively. Figure reproduced with modification from Jaramillo-Riveri & Broughton et al. (2022) (use permitted under the Creative Commons Attribution License CC BY 4.0).

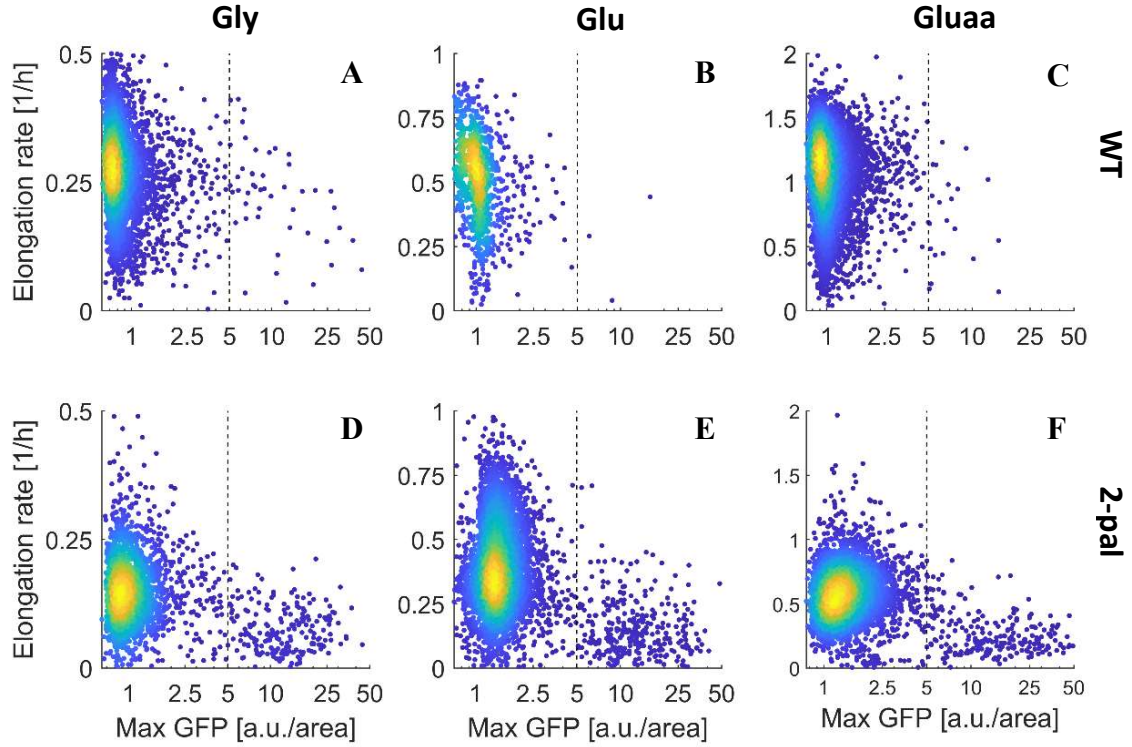


Figure 23. **High levels of SOS expression correlates with reduction in elongation rate.** Data is shown for the WT (A-C) and 2-pal (D-F) strain in three growth conditions. Elongation rate is plotted against the maximum GFP intensity registered for each cell-cycle or cell observation. Here, a proxy for elongation rate was calculated as $\log(l_m - l_0)/t_m$, where l_m and l_0 are the maximum and minimum cell lengths, and t_m is the observation time. Elongation rate was calculated manually here to include cells that did not divide by the end of the experiment (e.g. filaments). Plots consist of data from three experimental replicates. Vertical dashed line represents high-SOS cutoff (at 5 a.u.) that discriminates between low and high-SOS populations. Colours represent the 2D density of data points. Figure reproduced with modification from Jaramillo-Riveri & Broughton et al. (2022) (use permitted under the Creative Commons Attribution License CC BY 4.0).

This data shows, for the 2-pal strain, the existence of two sub-populations that differ with respect to SOS induction and division times. The gene *sulA*, which we quantify directly as a proxy for SOS expression, is part of the SOS operon and is responsible for inhibiting cell division (Huisman *et al*, 1984). Thus, it is expected that higher levels of *sulA* expression correlate with division arrest.

4.4 Modelling low and high-SOS sub-populations

Given the disparity in division rates between low and high-SOS cells, low-SOS cells could outcompete high-SOS cells in an expanding population, i.e. batch culture (Figure 24A). Moreover, the relative abundances between the two populations would change depending on the division rate supported by the growth medium. For example, in fast growth conditions low-

SOS cells would outcompete high-SOS cells to a larger extent than in slow growth conditions (Figure 24A).

The population dynamics involved makes estimation of the rate of high-SOS induction difficult in a growing population. An advantage of using the mother machine is that we can track a fixed population of mother cells. This means measuring changes in the relative fractions of low and high-SOS cells in the mother machine is not confounded by the population dynamics of an expanding population.

We were interested in the rate at which cells induce SOS to high levels. To facilitate this, a two-population mathematical model, previously used to describe population dynamics of persister cells (Balaban *et al*, 2004; Patra & Klumpp, 2013), was adapted to this problem. The model is summarised in Figure 24B and the model derivation is described in Appendix B2. Cells can exist in a low-SOS state (n_1) with division rate λ_1 and switch at rate α to a high-SOS state (n_2) with division rate λ_2 . High-SOS cells switch back to low-SOS state at rate β . We take advantage of the fact that high-SOS cells have significantly slower division rates (Figure 22D-F); and switching back to a low-SOS state was relatively rare (data not shown). Therefore, we assumed λ_2 and β to be negligible. Thus, we can directly estimate α by measuring the fraction of high-SOS cells (f_2) over time in the mother machine.

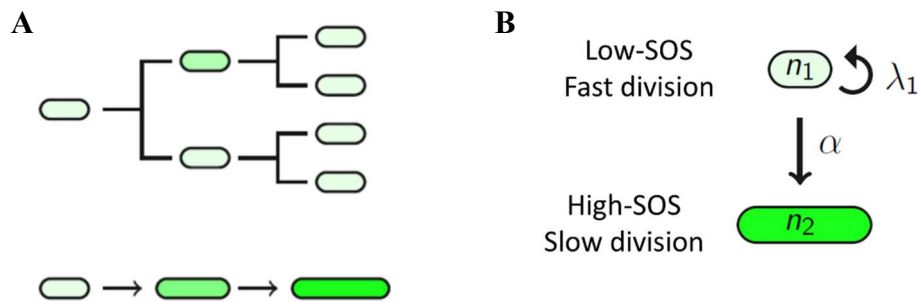


Figure 24. Illustration summarising the fixed population switching model. **A.** In a growing population, low-SOS cells will continue growth and divide, while high-SOS cells arrest division. Over time, the relative fractions will change as the low-SOS population outcompetes the high-SOS population. **B.** Illustration of the mathematical model describing the rate of switching between the low and high-SOS state. For a fixed population of cells, competitive dynamics between the two populations can be ignored. Cells with low levels of SOS induction (n_1) divide at rate λ_1 and switch at rate α to a high-SOS state (n_2). Rates of switching back to n_1 and division rate of n_2 were assumed to be negligible. Derivation of model is described in Appendix B2.

4.5 Estimating switching rate to high-SOS state

Given the low frequency of high-SOS cells observed for the WT strain (Figure 18A-C, Figure 19A), the switching rate α was only estimated for the 2-pal strain. Nevertheless, this data allows us to understand growth-dependence in the SOS response under DNA damage-inducing conditions. Cells were classified as either low-SOS or high-SOS based on their real-time GFP intensity as either below or above a threshold value (Figure 25B). Threshold values were evaluated within a range of 5-20 a.u. where the high-SOS sub-population is clearly observed in all growth conditions (Figure 19B, inset). We used a range of thresholds since these were arbitrarily set. An example of α estimation using a GFP threshold of 5 a.u. is described in Figure 25. The fraction of high-SOS lineages increased approximately linearly over time (Figure 25C). Alpha was estimated (Figure 25D) by linear regression to $-\ln(1-f_2(t))$ (supplementary equation 7), where $f_2(t)$ is the high-SOS fraction of cells as a function of time.

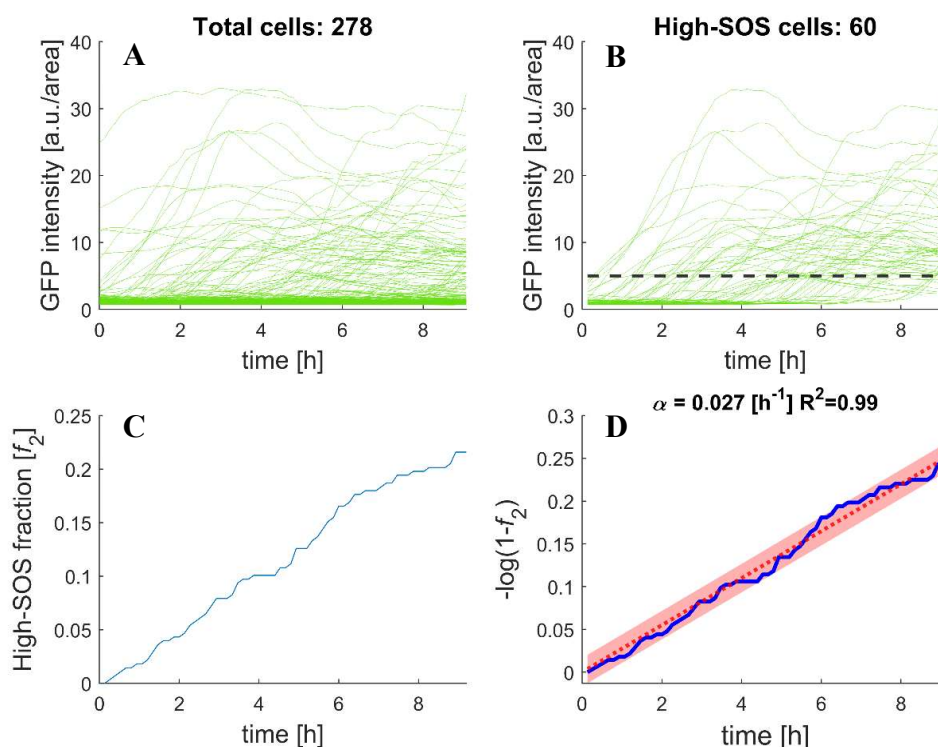


Figure 25. Estimating switching rate to high-SOS state from single-cell SOS trajectories. **A.** SOS expression of single-cell lineages over time. GFP trajectories were smoothed using a moving average ($n=5$) to reduce noise. **B.** High-SOS trajectories were filtered using a GFP threshold, here 5 arbitrary units (a.u.). The black dashed line indicates the threshold (5 a.u.) discriminating between high and low-SOS cells. Lineages with initial GFP intensity values above the threshold were excluded. **C.** Real-time fraction of cells above threshold (high-SOS cells, f_2). Trajectories were counted at their first instance of crossing the GFP threshold (i.e. a cell that crossed the threshold a second time was not counted again) and those that subsequently

dropped below the threshold did not detract from the count. **D.** The switching rate α was estimated by linear regression to $-\ln(1-f_2(t))$ (derivation in Appendix B2). The red dashed line indicates the predicted values. 95% confidence intervals are shaded in red. Since some cells were lost from the microchannels over time, fitting was performed on data where at least 80% of lineages remained. Shown is data from one experimental replicate for the 2-pal strain in the glu medium.

4.6 Switching rate to high-SOS state is growth-dependent

We then estimated α for a range of GFP thresholds (Figure 26). Our results indicate that α is higher for cells in fast-growth conditions when growth is modulated by nutrient quality, and this trend holds across all GFP threshold values. However, lower GFP thresholds resulted in larger differences between the growth conditions. As the GFP threshold increased, α decreased since fewer trajectories pass the threshold. Results were reproducible across three experimental replicates in the three growth conditions (Supplementary Figure 6), and the experimental data fitted the model well over all GFP thresholds tested (Supplementary Figure 7).

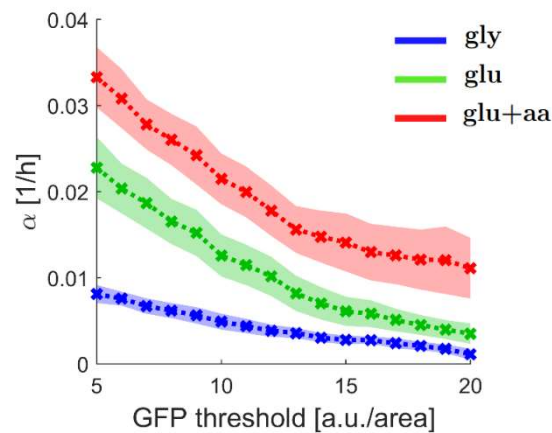


Figure 26. **Switching rate α estimated at multiple GFP thresholds for the 2-pal strain in different growth media.** Points represent the average and the shaded area the standard error from three experimental replicates. Plots for each experimental replicate are shown in Supplementary Figure 6.

Across several GFP thresholds (5, 10 and 15), α increased linearly ($R^2 > 0.98$) at the lower GFP thresholds (5 a.u. & 10 a.u.) with average single-cell division rates (Figure 27). A higher rate of switching to the high-SOS state may reflect a larger degree of DNA damage acquired by faster-growing cells. Since the 2-pal strain experiences replication-dependent DNA breaks, faster growing cells with higher DNA replication rates (higher number of replication forks) likely acquire DNA damage more frequently, thus increasing the probability of acquiring (and accumulating) irreconcilable DNA damage – leading to excessive SOS induction.

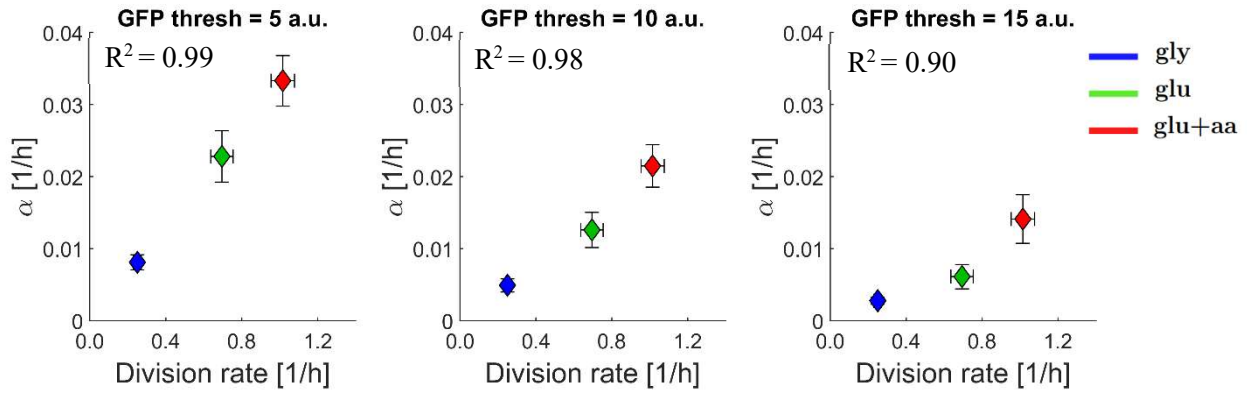


Figure 27. Switching rate to high-SOS state is positively correlated with division rate over a range of GFP thresholds. Switching rate α (diamonds) is shown at selected GFP thresholds (columns) plotted against corresponding average single-cell division rates in three growth media. Division rates shown represent low-SOS cell-cycles only (i.e. cell-cycles with GFP levels below the threshold). Error bars represent standard error for three experimental replicates. Colours represent different growth media as indicated.

4.7 Population dynamics explains elevated SOS expression in slow growing cells for batch experiments

These findings suggest that the larger fraction of high-SOS cells observed in slow growth conditions for steady-state exponential cultures (see Figure 16) is not explained by a higher rate of SOS induction. Given the significant disparity in division rates between low and high-SOS populations, competitive dynamics must play a role in their relative proportions in an expanding population. Here it is important to re-emphasise the difference in growth environments for the mother machine and batch experiments. In the mother machine, a fixed population of cells are maintained throughout the experiment. In a batch culture, the population size expands exponentially. Thus, in the latter, competitive population dynamics will play a role in the relative proportions between sub-populations with very different division rates, particularly where growth and division are de-coupled – as is the case for high SOS expression (Figure 22).

The switching rate and division rates estimated in the mother machine were then used to predict the high-SOS fraction in a steady-state exponential culture. This prediction could then be compared to the experimentally measured steady-state fraction in Figure 16. First, the fixed population model (Figure 24B) was amended to describe a growing population of cells (Figure 24A; see Appendix B3 for model derivation). The predicted high-SOS population fraction can be approximated by $f_2 \approx a/\lambda_1$ (Figure 28A; supplementary equation 15), where λ_1 is the predicted population division rate of low-SOS cells estimated from mother machine division

time distributions using methods described previously (Thomas, 2017; Jaramillo-Riveri *et al*, 2022).

The predicted steady-state high-SOS population (f_2) was found to be higher in slower growth conditions, despite having a lower switching rate (α) (Figure 28B), and was in good quantitative agreement with the observed proportion of high-SOS cells in the fast growth condition (Figure 28C). However, the predicted f_2 for the glu and gly medium was under-estimated, though the overall trend was reproduced. In summary, we show that the higher proportion of cells with elevated SOS levels in batch cultures is a result of the competitive dynamics between low-SOS (fast dividing) and high-SOS (slow dividing) populations.

For transparency, this work (linking the mother machine data I generated to the observed steady-state population fractions) was largely carried out by Sebastian Jaramillo-Riveri and is discussed in more detail in the associated publication (Jaramillo-Riveri *et al*, 2022).

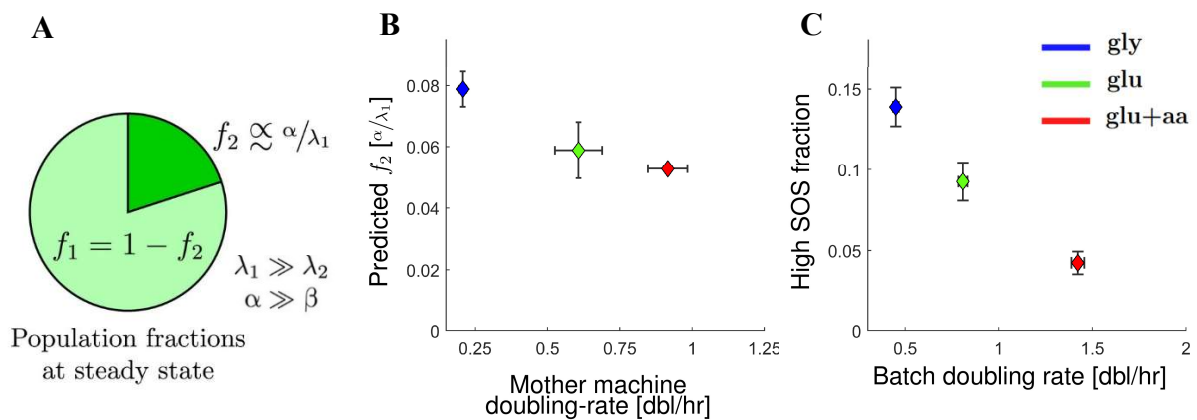


Figure 28. Single-cell measurements predict steady-state high-SOS fraction using a population dynamics model. **A.** The population model predicts the steady-state population of high-SOS cells to be inversely proportional to the division rate λ_1 of the low-SOS population. This assumes that the rate of switching to a high-SOS state α is significantly higher than the reverse ($\alpha \gg \beta$) and the high-SOS cells divide very slowly ($\lambda_1 \gg \lambda_2$). **B.** Predicted steady-state population fractions of high-SOS cells in different growth conditions using parameters estimated from the mother machine. On the x-axis is the population doubling rates converted from the predicted population division rates of low-SOS cells (λ_1) using mother machine data (doubling rate = $\lambda_1 \times \ln 2$ (Monod, 1949)). **C.** Batch population fractions of high-SOS cells for the 2-pal strain in different growth conditions. Figure duplicated from Figure 16B for context. For panels B and C, points represent the average and error bars the standard error from three experimental replicates. A GFP threshold of 5 a.u. was used. Figures reproduced from Jaramillo-Riveri & Broughton et al. (2022) (use permitted under the Creative Commons Attribution License CC BY 4.0).

4.8 Comparison of doubling rates estimated from single-cell mother machine experiments and batch growth experiments

We compared population doubling rates estimated from single-cell distributions to that measured at the bulk level using absorbance-based measurements (OD_{600}) to verify that the conditions are comparable (Table 3, Figure 29A). For the WT strain, population doubling rates estimated from the mother machine was similar to batch doubling rates for the gluaa medium, but lower for the glu and gly medium by approximately 20% (Figure 29A). For the 2-pal strain, doubling rates estimated from the mother machine were significantly lower in all growth media, especially for gluaa which was 35% lower. These differences could be a result of slight physical restriction of cells in the mother machine. Suboptimal microchannel dimensions – including channel width, height, and length – can affect single-cell morphology and slow growth in the mother machine (Yang *et al*, 2018). According to Yang *et al*. (2018), channel lengths of 25 μm (approximate dimensions used in this study; see Table 1) can slow the single-cell growth rates by up to ~14% compared to bulk measurements, despite what others have reported (Wang *et al*, 2010a; Bakshi *et al*, 2021). This is due to increased frictional forces of daughter cells in the microchannels which increase with longer channel lengths.

In addition, batch growth rates could be over-estimated due to the impact of filamentation on OD_{600} measurements (Stevenson *et al*, 2016). Growth rates measured from OD-based growth curves rely on the correlation between absorbance and cell concentration. The presence of elongated/filamented cells leads to higher absorbance values compared to smaller cells, resulting in over-estimated growth rates (Stevenson *et al*, 2016). This could explain the significant disparity for the 2-pal strain in the gluaa medium within which filamentation is more prevalent due to faster growth rates supported by the medium. To investigate this further, we directly quantified cell numbers to measure the batch growth rate by using colony forming units (CFU) instead of OD. We found that CFU-based growth rates were lower than OD-based growth rates and closer to those obtained from the mother machine (Table 3, Figure 29B). Therefore, OD-based measurements over-estimate population growth rates, particularly when cells experience DNA damage in fast-growth conditions. This could also explain the quantitative differences observed in Figure 28B and Figure 28C.

Table 3. **Comparing doubling rates estimated from batch growth curves (OD₆₀₀ and CFUs) and single-cell data from the mother machine for the WT and 2-pal strain under different growth conditions.** All values are averaged from n experimental replicates, and the standard error of the mean is indicated.

Strain	Growth medium	Doubling rates [1/h]					
		OD ₆₀₀		CFUs		Mother machine	
WT	Glu _{aa}	1.61 ± 0.05	$n=7$	1.46 ± 0.0006	$n=2$	1.76 ± 0.04	$n=3$
WT	Glu	1.04 ± 0.04	$n=9$	0.95 ± 0.0004	$n=2$	0.85 ± 0.08	$n=2$
WT	Gly	0.60 ± 0.01	$n=6$	0.58 ± 0.0002	$n=2$	0.37 ± 0.04	$n=3$
2-pal	Glu _{aa}	1.42 ± 0.03	$n=4$	1.08 ± 0.025	$n=2$	0.92 ± 0.07	$n=3$
2-pal	Glu	0.81 ± 0.02	$n=3$	0.72 ± 0.071	$n=2$	0.61 ± 0.08	$n=3$
2-pal	Gly	0.45 ± 0.02	$n=3$	0.33 ± 0.063	$n=2$	0.21 ± 0.01	$n=3$

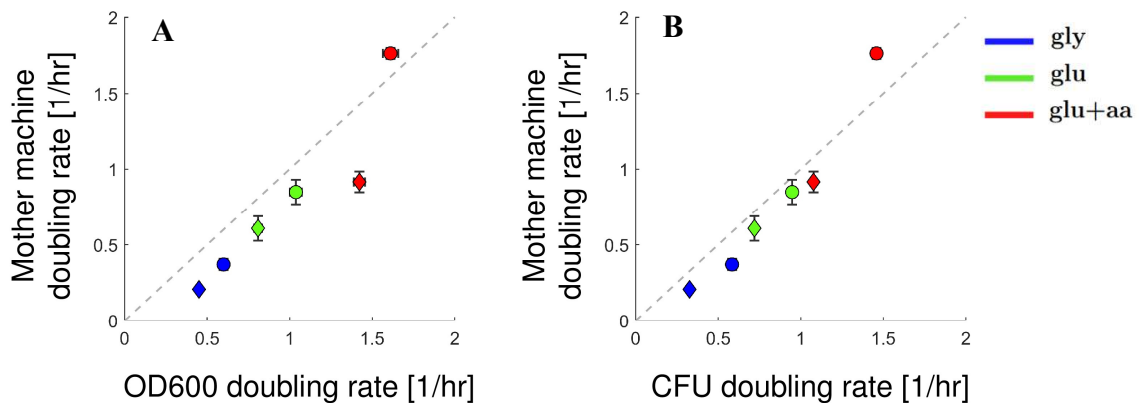


Figure 29. **Doubling rates are over-estimated in batch absorbance-based measurements.** **A.** Comparison between population doubling rates estimated from the mother machine and from batch OD₆₀₀ growth curves in different growth conditions. **B.** Comparison between population doubling rates estimated from the mother machine and from batch CFU/mL (colony forming units) growth curves in different growth conditions. Mother machine doubling rates represent the predicted population doubling rate estimated from the underlying single-cell division time distributions. Circles represent the WT strains and diamonds represent the 2-pal strain. Points represent the average and error bars the standard error from at least two experimental replicates. Figures reproduced from Jaramillo-Riveri & Broughton et al. (2022) (use permitted under the Creative Commons Attribution License CC BY 4.0).

4.9 Discussion

Based on previously acquired SOS distributions from balanced, steady-state batch cultures (Figure 16), we expected a higher rate of SOS induction in slow-growth conditions. However, we found that the opposite was in fact true. By tracking the SOS induction dynamics of individual cells in the mother machine, we accurately measured the rate of high-SOS induction (α) and found that, for the 2-pal mutant, α correlates with an increasing growth rate when this was modulated by nutrient quality (Figure 26, Figure 27). If α is higher in fast-growth conditions, then why does this not reflect in the observed high-SOS population fraction in steady-state batch cultures?

At the single-cell level, SOS induction is highly heterogeneous and a fraction of the population strongly induces the SOS response. Strong SOS induction produces filamentous cells due to continued mass accumulation (elongation) and delayed division from the induction of SulA (a SOS-controlled division inhibitor). Therefore, single-cell growth is de-coupled from division in SOS-inducing cells. As a result, heterogeneity in SOS expression leads to heterogeneity in single-cell division rates. Using a population dynamics model, we showed that the frequency of high-SOS cells is not only a result of the rate of SOS induction, but also depends on the competition between populations of low-SOS (high division rates) and high-SOS (slow division rates) cells with different division rates. In a steady-state batch culture, high-SOS cells are outcompeted (diluted) to a larger extent in faster-growth conditions due to a higher division rate of low-SOS cells supported by the medium.

This work demonstrates the importance of gathering single-cell, time-lapse data for informing interpretations of bulk-level observations. We were able to independently measure the rate of SOS induction and the division rate of different sub-populations which was key in understanding the population dynamics. Identifying the presence and quantifying the relative contribution of sub-populations to overall population behaviour could be vital for understanding antibiotic combinations (investigated in the next chapter), especially when growth and division dynamics are de-coupled as is the case for DNA-targeting antibiotics. Similar studies that quantify the fractions of sub-populations with varying fitness, such as persister cells (Balaban *et al*, 2004; Patra & Klumpp, 2013) or antibiotic-resistant mutants (Deris *et al*, 2013), should take into account how the population dynamics landscape may change in different nutrient environments.

The SOS response is vital for cell survival under DNA damage, and has been shown to increase tolerance to antibiotics (Dörr *et al*, 2009, 2010; Theodore *et al*, 2013). Although single-cell heterogeneity in SOS expression has been observed by others (Jones & Uphoff, 2021), we showed that it is also growth rate-dependent. Heterogeneity in SOS induction could influence the survival of individual cells exposed to DNA-targeting antibiotics, as has been shown for the adaptive response (a response to DNA alkylation damage), acid stress response, and the RpoS-mediated general stress response (Mitosch *et al*, 2017; Patange *et al*, 2018; Rossi *et al*, 2019; Vincent & Uphoff, 2021; Sampaio *et al*, 2022). In the next chapter, we investigated the suppressive interaction between DNA synthesis inhibitors and translation inhibitors. Therefore, investigating induction of the SOS response and heterogeneity therein should prove informative for evaluating the mechanisms underlying suppressive drug interactions for DNA synthesis inhibitors.

We observed a growth-medium dependent effect on the average SOS levels for the low-SOS population of cells for both spontaneous DNA damage and replication-dependent damage – which was positively correlated with growth rate (Figure 19). This is probably due to the higher number of replication forks present in faster growing cells (Cooper & Helmstetter, 1968) which will therefore acquire DSBs from the palindromes more frequently than slow growing cells. These results are representative of a specific mechanism of DNA damage which was chronic, replication-dependent, and always at the same locations. But, do these results hold for other types of DNA damage? For example, DNA damage by the gyrase-poisoning antibiotic CIP is randomly acquired both temporally and physically. This is discussed further in the next chapter.

Additionally, we showed that absorbance-based growth rate measurements could be overestimated in conditions where filamentation is prevalent, such as strong induction of the SOS response. De-coupling growth from division will lead to an increase in cell mass and size, but not an increase in cell number which skews the correlation between absorbance and population size (Stevenson *et al*, 2016). Therefore, care should be taken when interpreting absorbance-based growth rates in conditions where growth is de-coupled from division as is often the case in stressful conditions. It is common to rely on absorbance-based growth rate measurements for cultures exposed to DNA-targeting antibiotics – and other classes of antibiotics can also induce the SOS response and inhibit division (Miller *et al*, 2004). Our results suggest that these measurements should be complemented with growth rates estimated using methods that quantify the cell number directly, such as CFU-based measurements.

The objective of this chapter was to investigate growth-dependency of high levels of SOS induction at the single-cell level to explain what gives rise to population-level behaviours. We found that single-cell switching rates to a high-SOS state is growth rate-dependent. These findings furthered our understanding of how individual cells respond to DNA damage at different growth rates. We demonstrated the utility of single-cell and time-lapse measurements in revealing heterogeneity within a population that would otherwise be overlooked using bulk-level methods.

Chapter 5: Single-cell characterisation of suppression between ciprofloxacin and tetracycline

5.1 Introduction

We urgently need improved treatment strategies to tackle antimicrobial resistance and persistent bacterial infections. Antibiotic combination therapy provides a cost-effective solution that can restore efficacy of old drugs and reduce the evolution of resistance. Antibiotic interactions can be classified in several ways based upon whether their combined efficacy is stronger or weaker than their individual effects. One such classification is called a suppressive interaction, where the combined efficacy of two antibiotics is weaker than that of at least one of the antibiotics used alone. Generally, such combinations should be avoided in clinics, as the weaker efficacy would prolong infection clearance. However, though treatment efficacy is reduced, suppressive antibiotic combinations have some interesting potential applications in treating infections, such as preventing the emergence of antibiotic resistance (Chait *et al*, 2007; Michel *et al*, 2008; Torella *et al*, 2010; Baym *et al*, 2016; Singh & Yeh, 2017) which is a significant threat facing humanity (Antimicrobial Resistance Collaborators, 2022).

The suppressive interaction between ciprofloxacin (CIP) and tetracycline (TET) has been described extensively in literature (Bollenbach *et al*, 2009; Roemhild *et al*, 2022). However, studies so far have been limited to population-level (bulk) methods which overlook the influence of population heterogeneity and important sub-populations. In the previous chapter, we showed that sub-populations can develop under stress and can affect the population average in different ways. Additionally, we showed that acquiring single-cell timelapse data is vital for understanding the population dynamics.

The suppressive interaction observed between CIP and TET manifests as higher than expected bulk growth rates – measured by optical density (OD) – when the drugs are combined compared to the drugs individually. A change in bulk growth rates could indicate increased growth and division of cells, a change in cell viability due to the antibiotic (lower rate of cell death), or a combination thereof. Furthermore, the accuracy of OD-based growth rates – which assumes correlation between cell number and light scatter – for cultures experiencing significant SOS expression could be obfuscated by changes in cell size. In particular, filamentation can lead to overestimation of bulk growth rates as discussed in Chapter 4. Therefore, we decided to address this question at the single-cell level. Using a single-cell microfluidic device (mother machine)

we can accurately measure the growth, division, and death of cells in a population while avoiding biases inherent in bulk-level methods. We can then identify the dominant mechanism leading to improved population growth rates for the suppressive drug combination.

We are also interested in the effect of changing nutrient conditions on the cell's response to the drug combination. Changing growth environments are known to alter a cell's physiological composition which can influence how a cell responds to an antibiotic. CIP and TET target different components of the cell's physiological machinery – CIP inhibits DNA synthesis and TET inhibits protein translation. Susceptibility to CIP and TET is known to be growth-dependent (more susceptible at faster growth rates) as well as carbon source-dependent (Greulich *et al*, 2015; Smirnova & Oktyabrsky, 2018; Smirnova *et al*, 2022). However, growth-rate dependence has not yet been investigated for the drug combination.

Much remains unknown about how suppressive interactions emerge. Explanations for why suppression occurs between DNA and translation inhibitors are based on population-level results that lack insight into the population dynamics (Bollenbach *et al*, 2009). We believe that further characterising this drug interaction at the single-cell level could improve our understanding of suppressive drug interactions. In this chapter, we will quantify single-cell growth and division rates for the CIP+TET drug combination, and the drugs individually, to elucidate how a suppressive drug interaction manifests at the single-cell level. We will also measure SOS gene expression and constitutive gene expression to investigate how the DNA damage stress response and constitutive protein levels are affected, respectively. In addition, we will investigate growth rate-dependency throughout this chapter. As in the previous chapter, cells will be tracked in the mother machine and their response to the antibiotics and the combination will be quantified over time in three growth conditions.

5.2 The suppressive interaction is reproduced in bulk experiments

First, we decided to reproduce bulk-level experiments for the suppressive antibiotic interaction between CIP and TET. In doing so, we were able to establish a concentration range for each antibiotic where a suppressive effect is observed for our particular strain and experimental conditions. A checkerboard assay was performed where a concentration gradient grid was set up for CIP and TET in 96-well plates (Figure 30A-C). Although MIC checkerboard assays are typically carried out with a 2-fold step in antibiotic concentration, we chose to sample a finer step in concentration to better evaluate suppression. Growth rates were measured by OD₆₀₀ measurements in each well in fast (gluaa) and slow (gly) growth conditions (Figure 30B and

Figure 30C, respectively). Doubling rates for growth in the microplate wells were approximately 7% slower than bulk doubling rates measured using the OGI-BIO bioreactors.

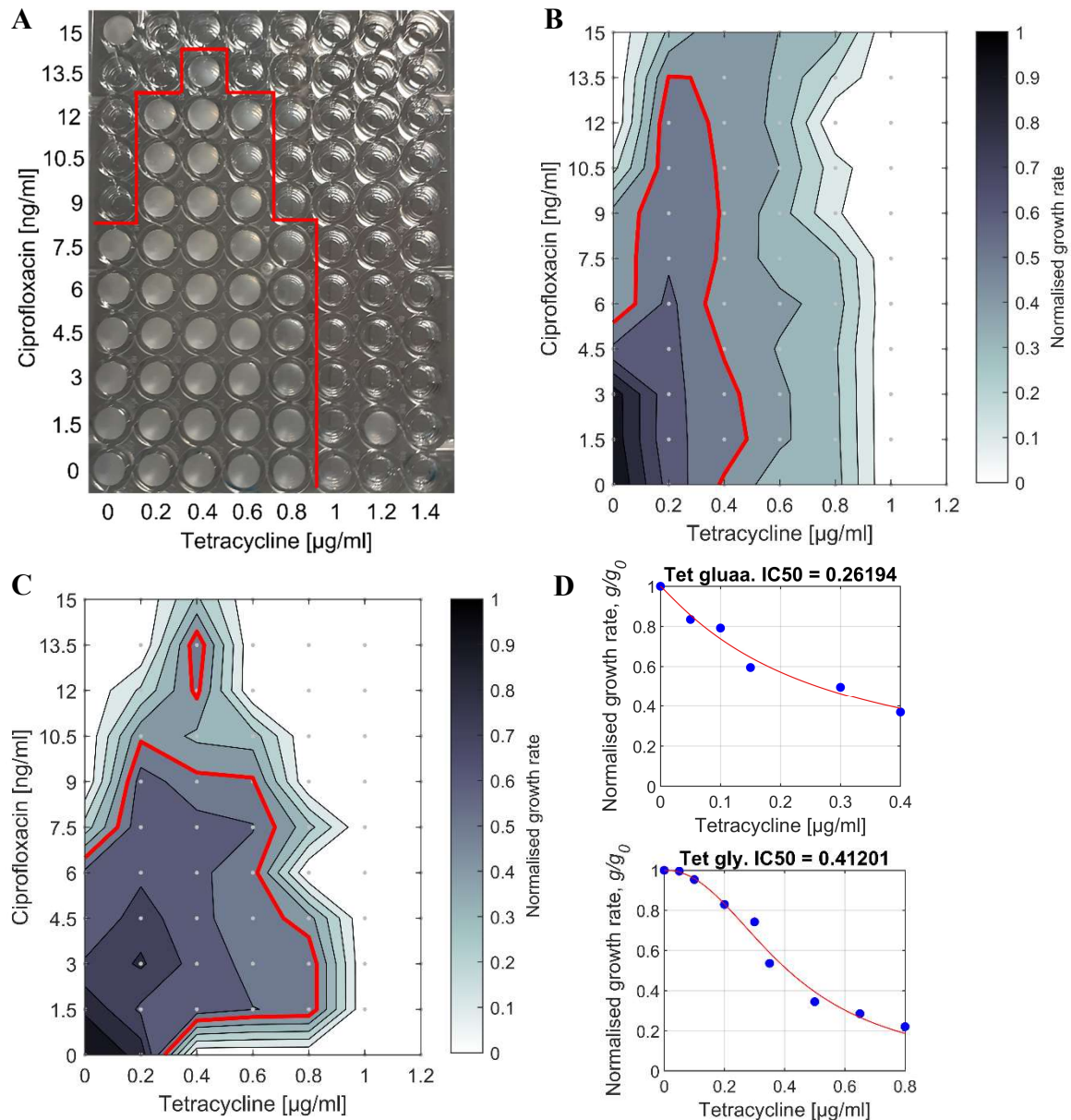


Figure 30. Dose-response relationships between ciprofloxacin and tetracycline. Checkerboard assay reproduced a suppressive interaction between ciprofloxacin and tetracycline (TET) in fast and slow growth conditions for strain SJR206. **A.** Picture of the 96-well plate indicating growth (turbidity) in wells after 28 hours in the gluaa condition. Red line indicates wells with significant growth. Significant growth observed in wells outside this line are likely resistant mutants and were excluded from growth rate analysis. **B-C.** Dose-response surface showing growth rates (blue colour) over a two-dimensional grid of antibiotic concentrations (grey dots) in the gluaa (B) and gly (C) growth condition. Colour represents normalised growth rate where black represents no growth inhibition and white represents no growth. Gradient level lines were derived via interpolation. Red line follows the IC₅₀ isobole

on the dose-response surface. **D.** Single drug dose-response curves for TET in the gluaa and gly growth condition. A Hill function was fit (red line) to the normalised growth rates g/g_0 (blue dots) to estimate the half-maximal inhibitory concentration IC_{50} , indicated in each plot title. Dose-response curves for other treatments and conditions are shown in Appendix C1.

Visual inspection of turbidity in the wells shows no growth above 9 ng/mL CIP and 1.2 μ g/mL TET in the fast growth condition (Figure 30A), which is near, but slightly lower, than the expected minimum inhibitory concentration (MIC) for these antibiotics (expected MIC for CIP=12 ng/mL and TET=1.5 μ g/mL ((Bollenbach *et al*, 2009; Pribis *et al*, 2019). When TET is added (between 0.2-0.6 μ g/mL) to CIP concentrations above 10.5 ng/mL, survival and growth of the culture improved (Figure 30A). This is reflected in the growth rate measurements from each well in Figure 30B, giving a ‘bulge’ – clearly indicated by the IC_{50}^9 isobole¹⁰ (Figure 30B, red line) or the change in MIC – which satisfies the Loewe¹¹ definition of a suppressive drug interaction (Loewe, 1928, 1953). The suppressive effect is directional – it extends parallel to the y-axis with an increasing CIP concentration – which is characteristic for a suppressive interaction between most DNA synthesis inhibitors and translation inhibitors. This means that the suppressive effect is non-reciprocal: one antibiotic (TET) alleviates the effects the other (CIP), but not *vice versa*. Non-reciprocal suppression has been reported previously (Bollenbach *et al*, 2009; Lázár *et al*, 2022). Our plots are similar to those reported (Bollenbach *et al*, 2009; Chevereau & Bollenbach, 2015; Russ & Kishony, 2018) where the suppressive effect is seen in a similar concentration range. The strongest suppressive effect was observed at 0.2 μ g/mL for TET and between 4.5-13.5 ng/mL for CIP.

For the slow growth condition (gly), a suppressive effect was also observed but it appeared weaker than in gluaa (Figure 30C), which suggests positive growth rate-dependence for this antibiotic combination. This contradicts an observation found in the supplementary of Bollenbach *et al*. (2009), where a medium supporting a faster growth rate (LB vs M9-glucose) resulted in weaker suppression. However, as discussed previously, it is difficult to conclude growth-dependence given that LB is a complex medium and the carbon sources differ.

In this experiment, TET appears to be more effective in the slow growth condition which can be seen by comparing the IC_{50} isoboles’ intersection with the axes (Figure 30B, C). Yet, we

⁹ Drug concentration that is required to inhibit the growth rate of a culture by 50%.

¹⁰ A line of constant growth rate.

¹¹ A drug interaction model based on the shape of isoboles on a two-drug dose-response surface. Straight isoboles between the axes indicates no drug interaction. Concave isoboles indicate synergy; convex isoboles indicate antagonism; concave isoboles that bulge in a certain direction indicate suppression.

would expect TET to be more effective in fast growth; i.e. a lower IC_{50} in fast growth (Greulich *et al*, 2015). We suspect that growth rates measured in 96-well plates are underestimated due to sub-optimal mixing and oxygenation in the wells. The single drug dose-response was then measured more precisely (in well-mixed, large volume flasks) and the IC_{50} then estimated (Figure 30D, Supplementary Figure 8). Here we found the expected correlation between IC_{50} and growth rate for TET (Figure 30D). The IC_{50} for CIP ranged between 4.5-5.4 ng/mL and for TET between 0.19-0.41 μ g/mL. These values were in line with those reported in literature (Supplementary Table 5). The dose-response surface from the 96-well plate data should therefore be regarded as an approximation.

From these preliminary experiments we have reproduced a suppressive effect on cell growth for the CIP and TET drug combination according to the Loewe model. Based on this data, we decided to start mother machine experiments with a CIP concentration of 5 ng/mL and TET concentration of 0.2 μ g/mL.

5.3 Optimisation of mother machine experiments

In this chapter, growth in the mother machine was evaluated in more detail and the data analysis pipeline was improved. At this stage, some abnormalities associated with single-cell growth rates and loss of cells from mother machine microchannels were addressed.

5.3.1 Microchannel width

Valuable work by Yang *et al.* (2018) documented the effect of physical constraints associated with the mother machine design (channel width and length) on cell growth. In particular, a too narrow channel width can significantly slow cell growth and should be avoided. We quantified single-cell elongation rates of mother cells in different channel widths (Figure 31) to identify the optimal channel width for our experiments in different growth media. We found that a difference of 0.1 μ m in channel width can significantly impact the measured elongation rates and division times in both fast and slow growing cells ($p < 0.005$, two-sample Kolmogorov-Smirnov test) (Figure 31). For the glyaan¹² medium, elongation rates decreased by 23% from a mean of 1.23 μ m/h (in channel width=1.1 μ m, $N_{\text{cell-cycles}}$ =6921) to 0.95 μ m/h (width=1.0 μ m, $N_{\text{cell-cycles}}$ =81). For the gly medium, elongation rates decreased by 26% from a mean of 0.42 μ m/h (width=1.0 μ m, $N_{\text{cell-cycles}}$ =201) to 0.31 μ m/h (width=0.9 μ m, $N_{\text{cell-cycles}}$ =14). The

¹² In some early experiments we used ‘glyaan’ medium which was subsequently replaced by the ‘gluaa’ medium. Glyaan medium (M9+glycerol+amino-acids+nucleotides) supports a similar growth rate to gluaa medium – 37.5 and 37.8 minutes, respectively – and so results should be roughly comparable.

proportional decrease in elongation rates are similar to those reported by Yang et al. (2018). Therefore, care was taken in future experiments to select microchannel widths that did not significantly affect cell growth for each growth medium.

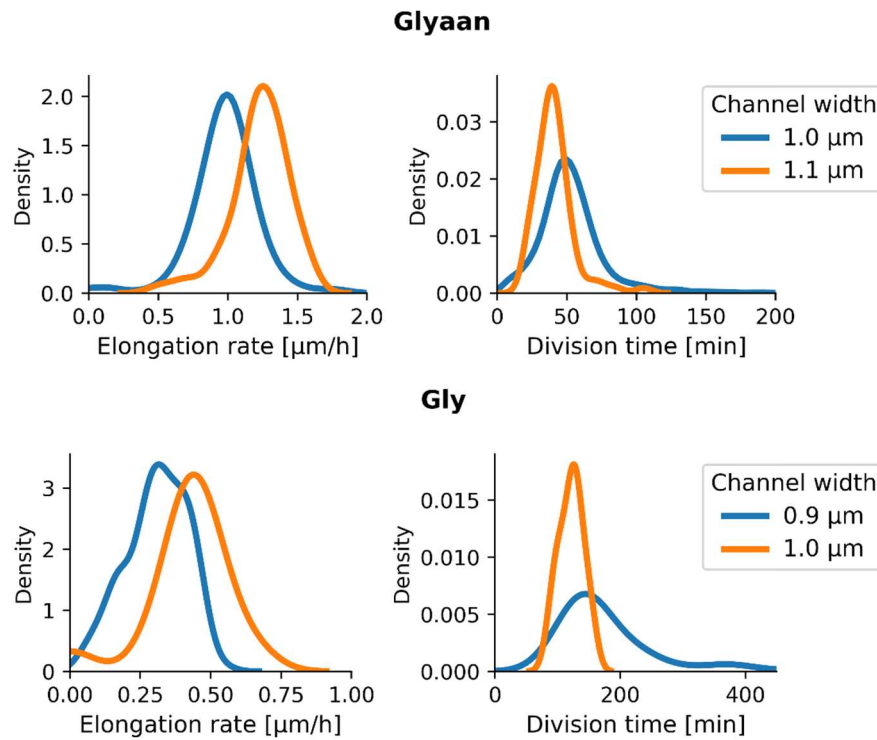


Figure 31. **Effect of microchannel width on cell growth in the mother machine.** Shown are kernel density distributions of elongation rates and division times of cells in different microchannel widths (indicated in the key) grown in glyaan (fast-growth, top row) and gly medium (slow-growth, bottom row). All distributions comparing growth between the two channel widths are significantly different ($p < 0.005$, two-sample Kolmogorov-Smirnov test).

5.3.2 Bacterial motility

In the previous chapter, many cells were lost due to cells swimming out of the microchannels. To address this, we removed the flagellum-associated *motA* gene from our strain which significantly reduced cell motility and improved cell retention in the mother machine. Deletion of flagellum genes, either *motA* or *fliC*, is standard practice for mother machine experiments in literature. Bulk doubling times in LB were almost identical for the parent strain (SJR206) and Δ *motA* mutant strain (JKB43) (22.2 and 22.8 minutes, respectively). We also checked single-cell elongation rates in the mother machine and found no difference between SJR206 and JKB43 in three growth media (Supplementary Table 4). This suggests there was minimal metabolic burden associated with the expression of *motA*, and results from the two strains are comparable.

A technical note: With the *motA* gene intact and its associated increase in cell motility, loading of cells into the mother machine microchannels was relatively easy – cells ‘swam’ into them (but also out of them). With the Δ *motA* mutant, cells had to be forced into the microchannels by centrifugation of the microfluidic chip which introduced the risk of breaking the cover slip and potential stress on the cells. Stress on the cells was mitigated by using low centrifugal forces and allowing cells to recover for at least one hour before imaging.

5.4 Characterising single-cell growth in the mother machine

Cells were cultivated in the mother machine in three growth media (gluaa, glu, and gly). For antibiotic treatment experiments, cells were grown without antibiotic for at least 6 hours to allow adaptation to growth in the mother machine, imaging conditions, and to ensure that all cells were growing exponentially for a few generations. During data analysis, the first 4 hours were discarded to avoid fluctuations due to adaptation. Next, antibiotics were introduced for 12 hours, after which drug-free medium was introduced for at least 10 hours to allow recovery from antibiotic treatment. For each experiment, four treatments were run simultaneously: A no-drug control, CIP 3 ng/mL, TET 0.2 ug/mL, and the drug combination (CIP+TET) at the same concentrations. These concentrations were chosen because they resulted in a suppressive effect while minimising excessive filamentation in the mother machine. At least two experimental replicates were performed, except for CIP in gluaa, the control in gly, and TET in gly, where three replicates were performed.

5.4.1 Growth dynamics in the mother machine under control conditions are stable long term

Cells cultivated under no-drug control conditions in the three growth conditions exhibited stable, long-term dynamics in their average behaviour for elongation rates, SOS expression, and constitutive expression indicating that a steady-state was reached (Figure 32). We observed frequent pulses of SOS expression under no-drug control conditions, with the majority exhibiting short, intermediate level pulses and a few longer-lasting, high-SOS induction events (Figure 32D-F). Such ‘pulsatile’ expression dynamics for spontaneous induction of the SOS response has been previously observed by other groups (Jones & Uphoff, 2021; Sampaio *et al*, 2022), and has been linked to variability in the degradation of LexA (Jones & Uphoff, 2021). Though the cells were unperturbed, spontaneous induction of the SOS response in *E. coli* is known to occur, with an estimated 2% of cells acquiring a spontaneous DNA DSB per cell division (Shee *et al*, 2013). We found that approximately 2.5% of cells induced the SOS

response to high levels (>1000 a.u.). Therefore, SOS expression dynamics in the mother machine are behaving as expected.

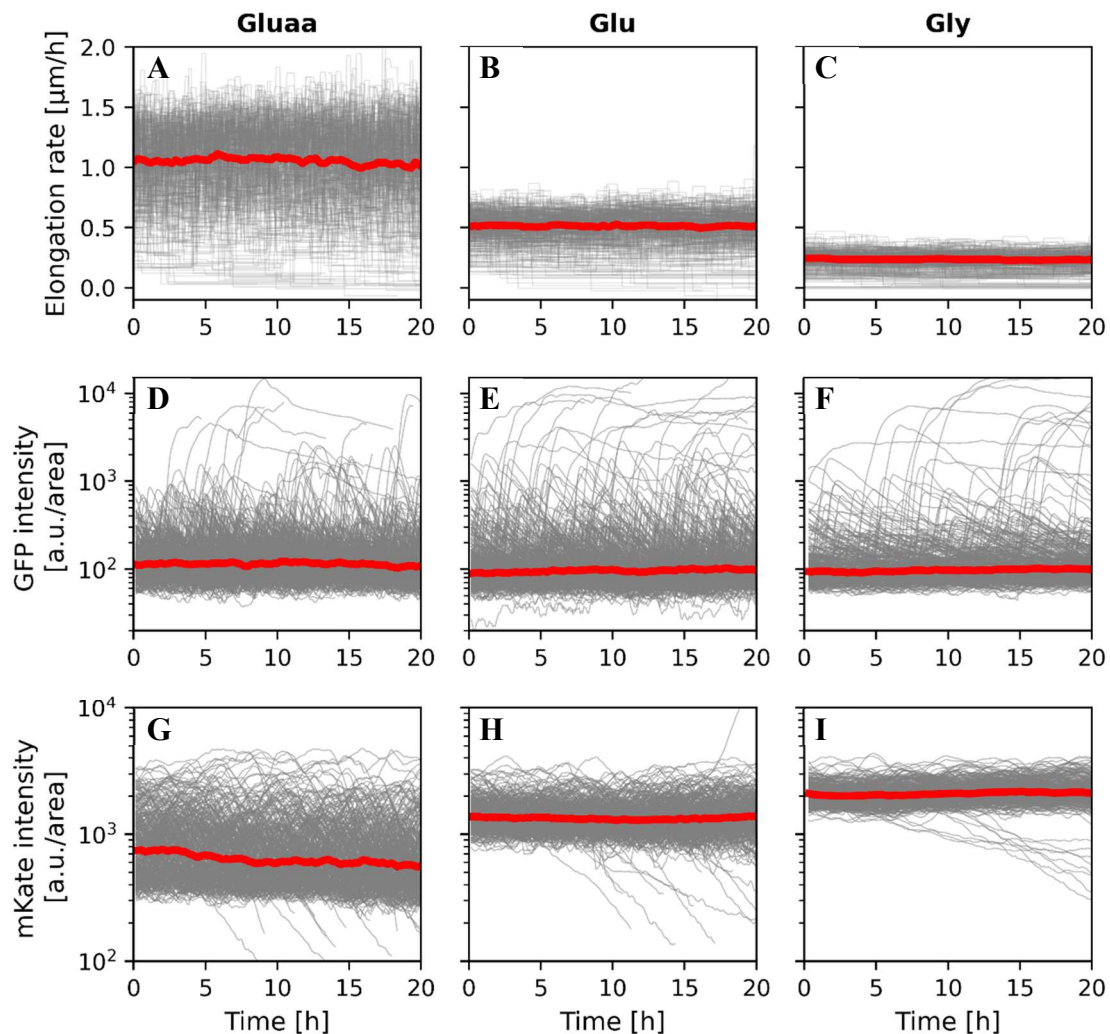


Figure 32. **Single-cell trajectories of elongation rates (A-C), SOS expression from *PsuA-mGFP* (D-F) and constitutive gene expression from *PtetO1-mKate2* (G-I) for different growth media under no-drug control conditions.** The median is indicated by the red lines. Grey lines represent single cell trajectories. Shown is data from one experimental replicate for strain JKB43. *GFP* and *mKate* intensity trajectories have been smoothed with a moving average over a 5-frame window. Gluaa: $N=334$ lineages; Glu: $N=342$; Gly: $N=304$.

The timeseries data was then pooled for the duration of the experiment and presented as a distribution allowing comparison between the growth conditions (Figure 33). Distributions for each experimental replicate are shown in Appendix C2 and show good day-to-day reproducibility. Note that in this chapter fluorescence intensity values were not normalised as done in Chapter 4. Growth-dependent responses of elongation rates, constitutive gene expression, birth lengths, and division lengths were as expected. Birth and division lengths are higher on average in the fast growth condition while the glu and gly distributions are almost

identical (Figure 33E, F). Average birth lengths: $gluaa=2.89\pm0.23\ \mu\text{m}$, $glu=1.85\pm0.11\ \mu\text{m}$, $gly=1.72\pm0.04\ \mu\text{m}$ (Supplementary Table 6). Elongation rates and division times were distinct in the different growth media (Figure 33A, B). Median elongation rates and mean division times: $gluaa=1.13\pm0.01\ \mu\text{m/h}$ & $40.5\pm0.6\ \text{min}$, $glu=0.53\pm0.01\ \mu\text{m/h}$ & $82.5\pm2.0\ \text{min}$, $gly=0.26\pm0.02\ \mu\text{m/h}$ & $187.2\pm19.3\ \text{min}$ (Supplementary Table 6). This data confirmed that the different growth media produced a range of growth rates that will enable growth-dependency investigations for antibiotic combinations.

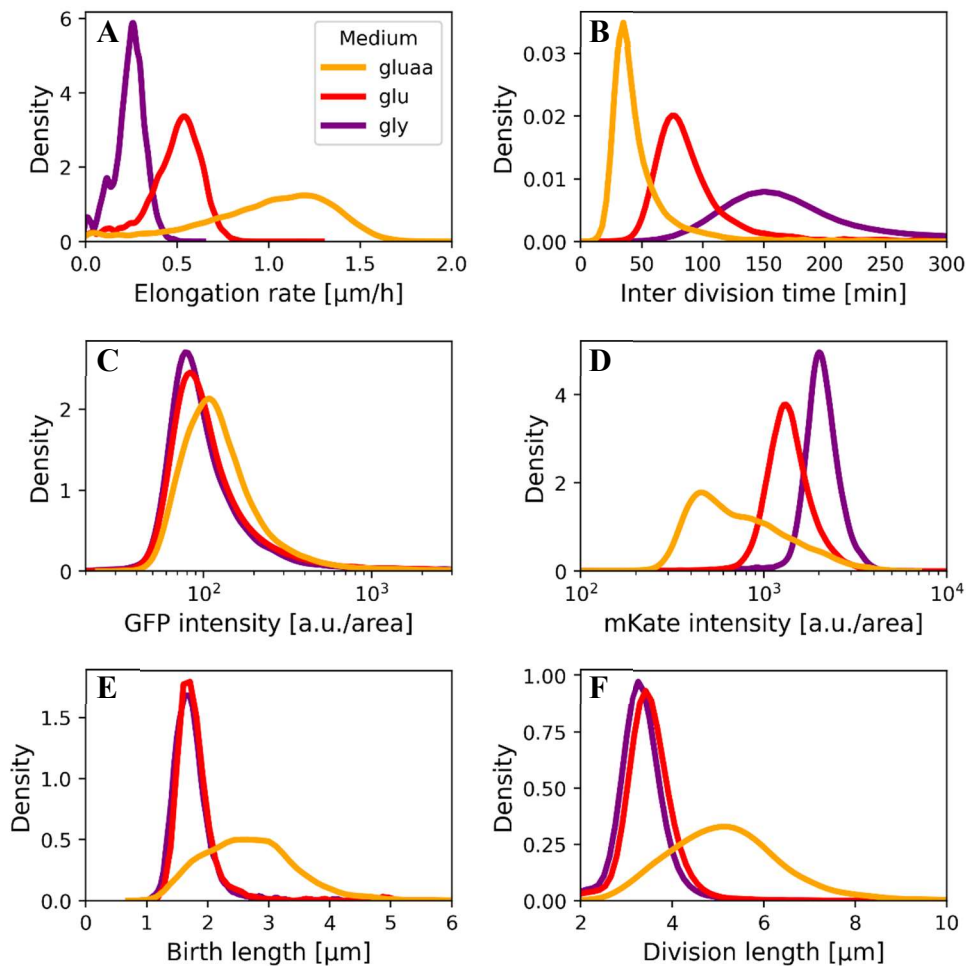


Figure 33. Distributions of single-cell measurements for different growth media under no-drug control conditions. Distributions are kernel density estimates of the underlying histogram pooled from at least two experimental replicates over the entire experiment for strain JKB43. **A.** Elongation rates (averaged for each cell-cycle). **B.** Division times. **C.** SOS expression from the *Psula-mGFP* fluorescent reporter. **D.** Constitutive gene expression from *PtetO1-mKate2* fluorescent reporter. **E-F.** Birth and division length for each cell-cycle, respectively. Different colours represent the growth medium.

SOS gene expression was identical across growth conditions besides a small increase in the fast growth medium (Figure 33C) and is consistent with spontaneous SOS induction observed in Chapter 4 (Figure 19A). Under ‘normal’ unstressed conditions, LexA represses transcription

of SOS genes, so we would not expect average SOS expression to change much with growth rate. Constitutive gene expression is negatively correlated with elongation rates (Figure 33D) which is consistent with universal growth laws for an un-regulated gene (Klumpp & Hwa, 2014). Constitutive expression exhibited higher heterogeneity in fast growth compared to slower growth media (gluaa: $CV \approx 0.6$; glu & gly: $CV \approx 0.2$; Supplementary Table 6).

5.4.2 Optimisation of ciprofloxacin concentration for growth in the mother machine

Initially a CIP concentration of 5 ng/mL, in combination with 0.2 μ g/mL TET, was chosen to evaluate suppression in the mother machine in the fast growth medium (Figure 34). However, we observed excessive filamentation under 5 ng/mL CIP treatment (Figure 34G), leading to all the cells escaping from the microchannels within 5 hours of treatment. This correlated with very high SOS expression (Figure 34E). Since mother cell lineages could not be tracked for long, elongation and division rates could not be estimated for the duration of the experiment (Figure 34A, C). For the CIP-TET combination, cells initially filamented within the first 5 hours but then were able to continue growth and division, and could still be tracked in the mother machine long-term (Figure 34H). However, a few cells did appear to stop growing or die (Figure 34B black arrow, Figure 34H flat trajectories). SOS gene expression initially increased and then levelled off after approximately 5 hours, remaining elevated for the duration of the experiment (Figure 34F). SOS expression did not increase to levels observed under CIP treatment alone (Figure 34E).

From this experiment, we observed improved cell growth and survival under the antibiotic combination which is indicative of a suppressive effect. Although we lost tracking of all cells exposed to 5 ng/mL CIP and could thus not confirm their death, the ability of cells to resume division under CIP+TET represents a significant improvement in their fate. However, this was not a good condition for quantification of growth parameters under CIP treatment.

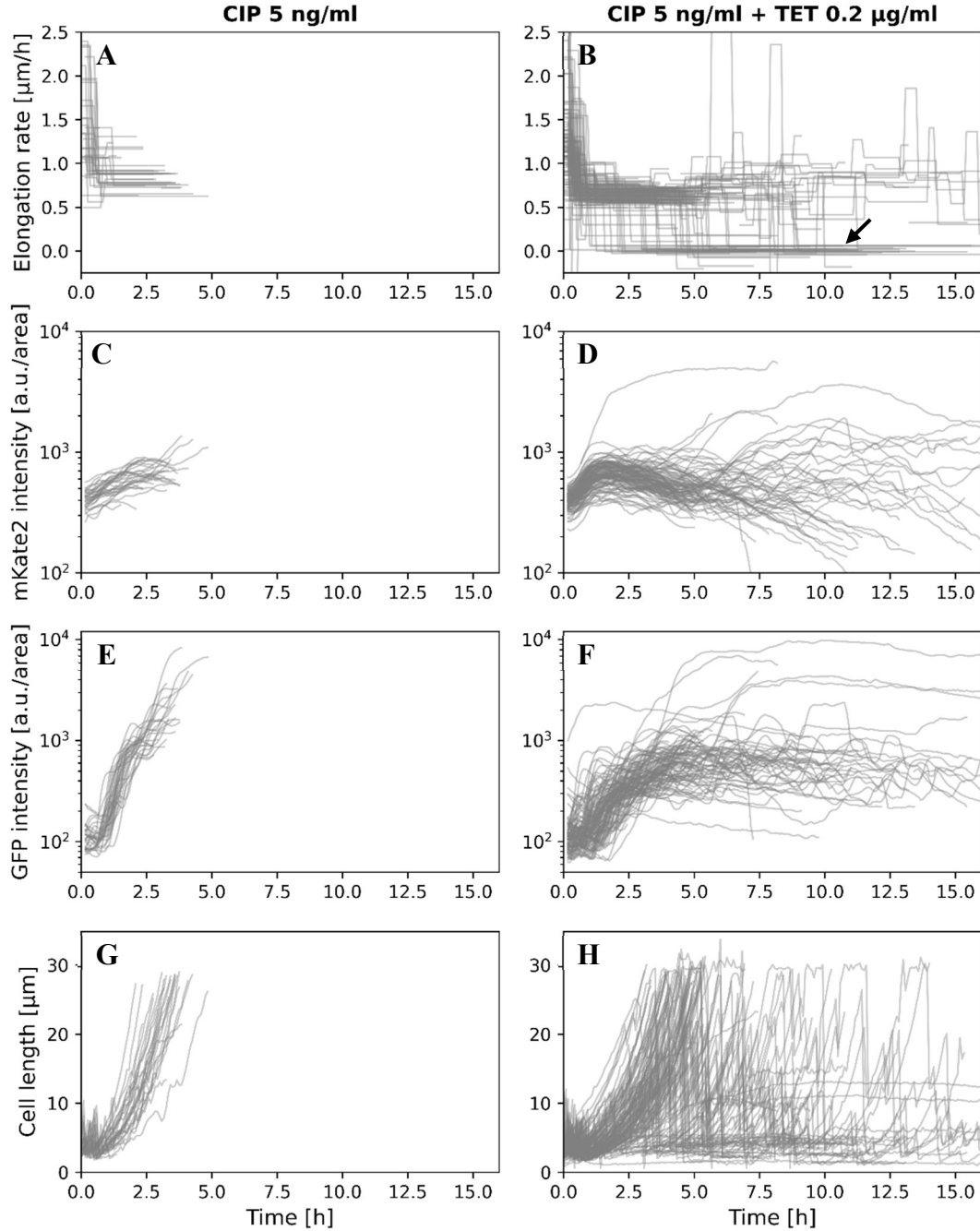


Figure 34. Single-cell trajectories of elongation rates (A-B), constitutive expression from *PtetO1-mKate2* (C-D), SOS expression from *Psula-mGFP* (E-F), and cell length (G-H) for growth in glycan medium under ciprofloxacin and ciprofloxacin-tetracycline treatment. 5 ng/mL ciprofloxacin (CIP) and 0.2 µg/mL tetracycline (TET) was used. Shown is data from one experimental replicate for strain SJR206. *GFP* and *mKate* intensity trajectories have been smoothed with a moving average over a 5-frame window. Antibiotic treatment began at hour 0. CIP: $N=32$ lineages; CIP+TET: $N=121$.

Ideally, we wanted to track the growth of cells for a long duration under CIP treatment. We decided to reduce the CIP concentration to 3 ng/mL to avoid excessive filamentation of cells. However, CIP at 3 ng/mL did not appear to result in a suppressive effect at any TET

concentration in the checkerboard assay (Figure 30B), so we decided to reassess whether suppression is observed in bulk at this concentration using more reliable OD measurements from higher volume, well-mixed flasks (Figure 35). We found that an antagonistic effect for the drug combination was observed at a low CIP concentration of 3 ng/mL, with a significantly higher doubling rate than the Bliss additive¹³ expectation ($p<0.05$, t -test) and a marginally higher, but not significantly, doubling rate than under TET treatment. Therefore, CIP at 3 ng/mL remains a viable concentration for producing at least an antagonistic interaction with TET.

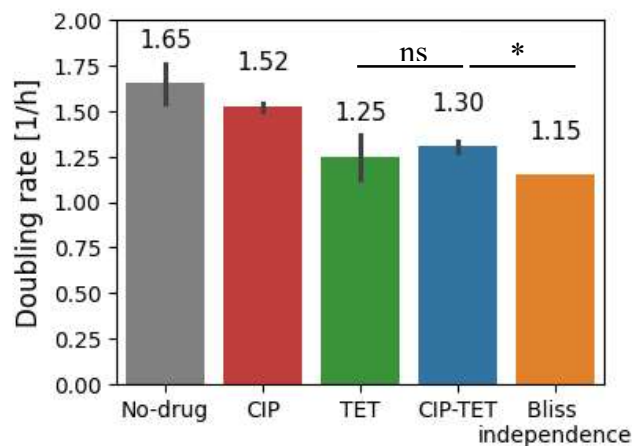


Figure 35. Bulk doubling rates under individual ciprofloxacin and tetracycline treatment and combination treatment in the gluaa growth medium. Shown is the mean value – also indicated above each column – and standard deviation from at least three experimental replicates. The Bliss independence prediction for the doubling rate under the antibiotic combination is calculated according to the Bliss definition of independent drug interactions which assumes that individual drug growth rates can be multiplied: $g_{AB} = g_A \times g_B$, where g_X is the normalised doubling rate in the presence of antibiotic X. Finally, g_{AB} was multiplied by the doubling rate of the no-drug control to get the Bliss prediction. Since CIP+TET doubling rate is higher than the additive expectation value and higher than at least one of the antibiotics, this indicates a suppressive drug interaction. Doubling rates were measured using OD₆₀₀ measurements from OGI-BIO bioreactors for strain JKB43. *: $p<0.05$, ns = not significant (t -test).

¹³ Additivity according to the Bliss independence model refers to the expected effect of a neutral drug combination and is calculated as the summation of their individual effects or product of their individual growth rates (Bliss, 1939).

5.4.3 Single-cell elongation and division dynamics under antibiotic combination treatment

We measured the elongation rates of mother cell lineages over the duration of the experiment (Figure 36). Elongation rate trajectories under CIP treatment remained unchanged for the main population while some cells stopped growth, which can be seen as elongation rates near 0 $\mu\text{m/h}$ (Figure 36A-C, black arrow). Although the population median (red line) decreased during CIP treatment, it does not represent the response very well due to diverging sub-populations. In contrast, for TET and CIP+TET treatment the population uniformly reduced elongation rates by approximately 25%, and then recovered to pre-antibiotic levels once antibiotic was removed (Figure 36D-I). However, for CIP+TET some lineages appeared to stop growing as well (Figure 36G-I). Median elongation rates under TET and CIP+TET changed faster in response to the antibiotics in faster growth media (gluaa & glu), while the response in gly medium was slow, as was the recovery after antibiotic treatment stopped.

The different single-cell responses for CIP and TET (cells dying under CIP and cells slowing growth under TET) are expected for bactericidal and bacteriostatic antibiotics, respectively. Brandis et al. (2023) reported similar results for *E. coli* treated with CIP and TET individually in the mother machine. In particular, the authors also found that 4 ng/mL CIP induced two sub-populations of growing and non-growing lineages.

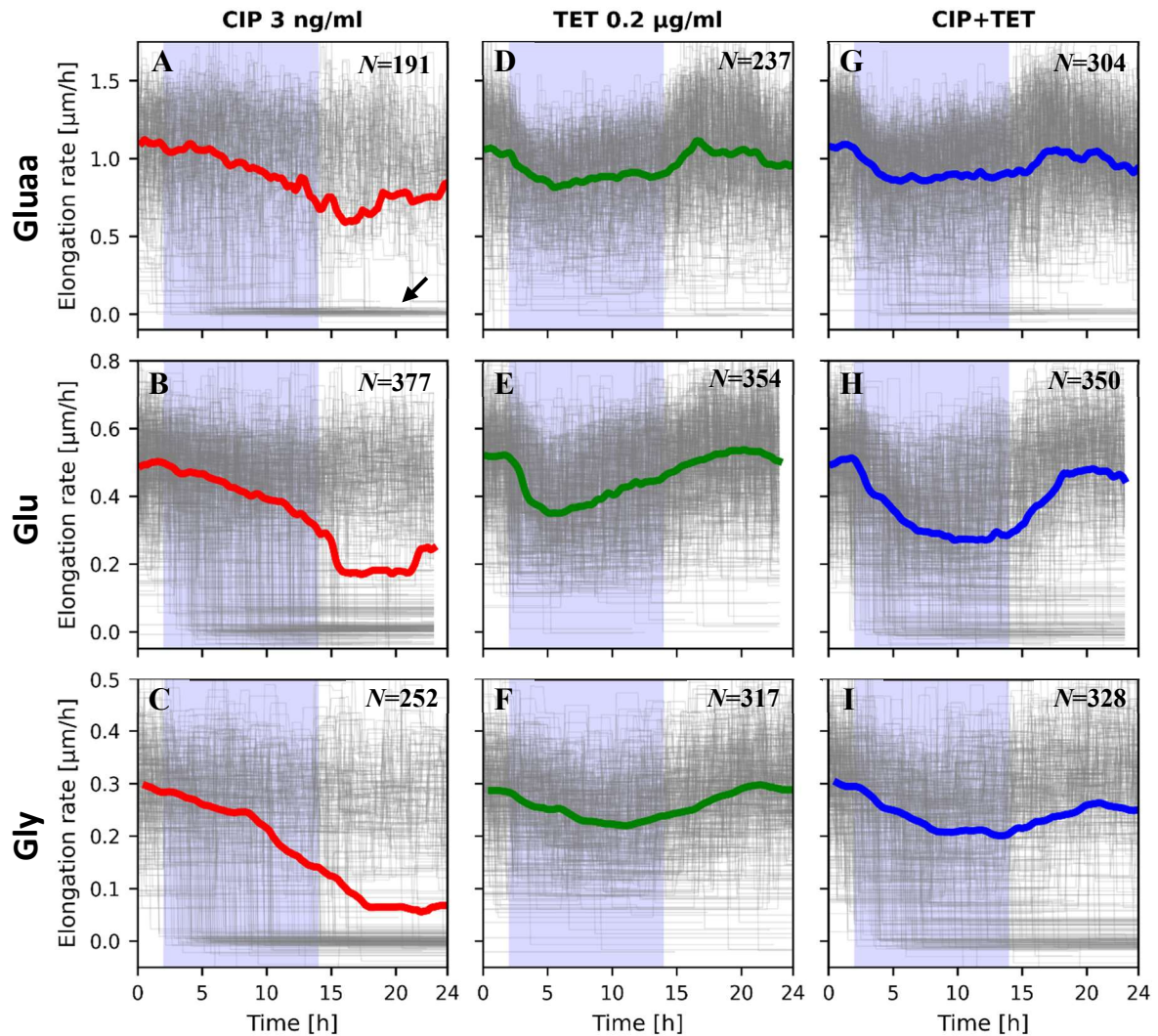


Figure 36. **Single-cell trajectories of elongation rates for different growth media under 3 ng/mL ciprofloxacin (A-C), 0.2 µg/mL tetracycline (D-F) and drug combination (G-I) treatment.** Elongation rates are measured for each cell-cycle. Cells that have stopped growth (example tracks indicated by the black arrow) are represented by long horizontal lines near 0 µm/h. Solid colour lines represent the median and grey lines represent individual lineage trajectories. Shown is data from one experimental replicate for strain JKB43. Antibiotics are introduced between hours 2-14, indicated by the blue shaded area. The number of lineages for each dataset are indicated in each frame.

Another way to represent the data is shown in Figure 37 where distributions are drawn from 2 hour periods. Under CIP treatment, the distribution of elongation rates becomes bimodal from hour 4, with the second peak increasing over time. This reflects an increasing proportion of lineages that stopped growing. The proportion of cells that have stopped growth appear to have increased much slower for CIP+TET treatment (Figure 37D), suggesting that TET may be suppressing cell death caused by CIP. This was investigated in more detail in Section 5.5 below.

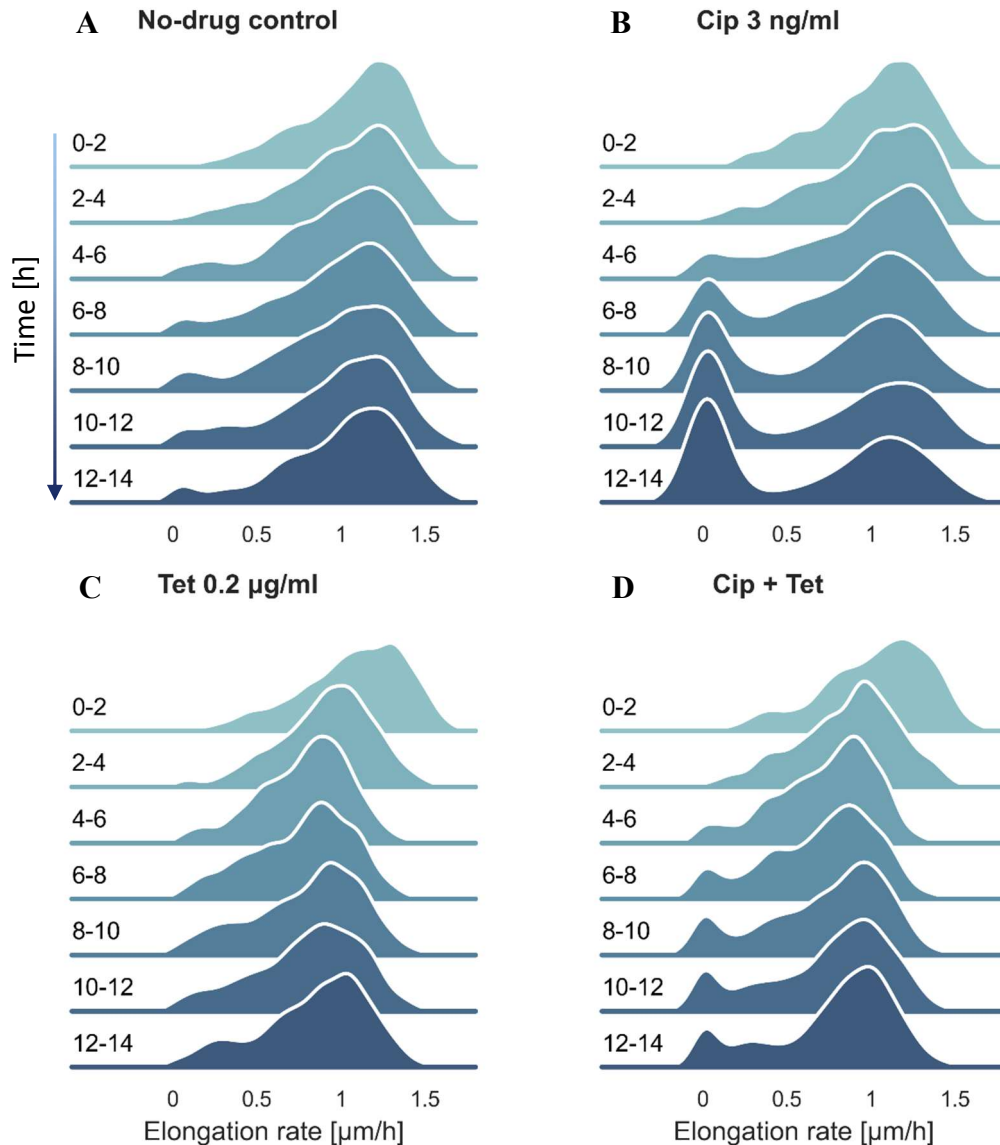


Figure 37. Distributions of single-cell elongation rates drawn from sequential two-hour periods under different antibiotic treatments in the *gluaa* growth medium. **A.** No-drug control. **B.** Under CIP 3 ng/ml treatment. **C.** Under TET 0.2 $\mu\text{g/ml}$ treatment. **D.** Under the drug combination. Distributions are kernel density estimates of the underlying histogram for one experimental replicate for strain JKB43. Time periods, in hours, are indicated to the left of each distribution. The first 2 hours are without antibiotic.

To evaluate the antibiotic effect in different growth conditions, we compared elongation and division rates pooled from the final 2 hours of antibiotic treatment (Figure 38). As observed previously, elongation rate and division rate distributions were bimodal for CIP and CIP+TET treatment in all growth conditions, but the proportion of cells that stopped elongation (secondary peak around 0 $\mu\text{m/h}$) decreased more in faster growing cells under CIP+TET treatment (Figure 38A-C). A similar trend was observed for the division rates (Figure 38D-F).

This data suggests that the suppressive effect on the proportion of non-growing cells (secondary peak) is stronger in fast-growth conditions indicating growth rate dependency.

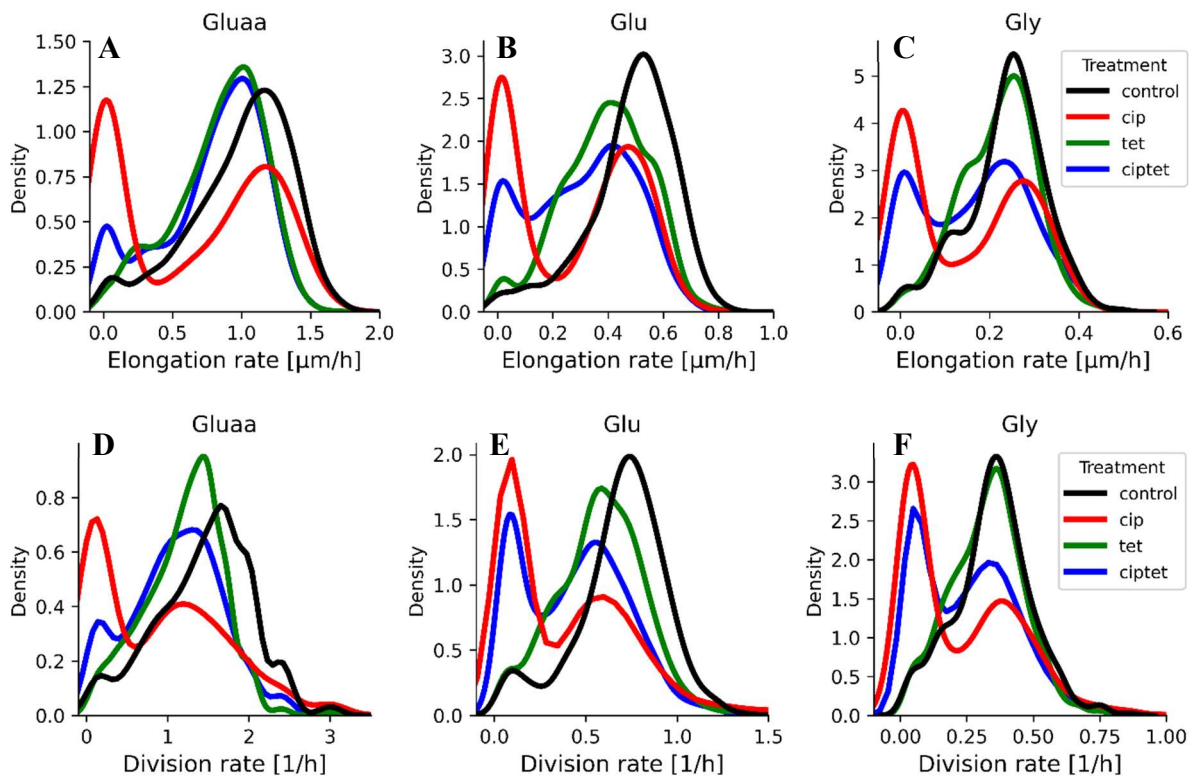


Figure 38. Distributions of single-cell elongation rates (A-C) and division rates (D-F) for different growth media and treatment conditions. Distributions are kernel density estimates of the underlying histogram pooled from the final 2 hours of antibiotic treatment from at least two experimental replicates for strain JKB43. Colours represent the different treatment conditions. Division rates are the inverse of the division times. Distributions for each experimental replicate are shown in Appendix C2 and show good day-to-day reproducibility.

Averages taken from bimodal distributions would be skewed by the non-growing subpopulation. Therefore, we separated the ‘growing’ fraction of cells (described in Section 5.5.2) and compared the medians (Figure 39). When taking the fraction of cells that continue to grow throughout CIP treatment, median elongation rates did not decrease significantly (t -test, $p > 0.05$) (Figure 39A-C), except for a slight decrease of 9% in the glu medium, but the difference was not statistically significant (Figure 39B). Previous reports have also observed unchanged single-cell elongation rates for *E. coli* under CIP treatment in concentrations ranging from 2-16 ng/mL (Bos *et al*, 2015; Ojkic *et al*, 2020; Brandis *et al*, 2023). It is worth noting that CIP concentrations at MIC or above can reduce single-cell elongation rates (Baltekin *et al*, 2017; Brandis *et al*, 2023). Median elongation rates decreased by similar levels under TET (approx. 16%, 18%, 8% in gluaa, glu and gly, respectively) and CIP+TET (approx. 14%, 24%, 13%).

In contrast, median division rates under CIP treatment were lower in faster growing cells (down by 16% in gluaa and 16% in glu), but not in the slow growth condition (Figure 39D-E). This indicates a de-coupling between elongation and division rates under CIP treatment, particularly in faster growth conditions, and has been documented previously (Chapter 4) in conditions with significant SOS induction. In brief, we expect division rates to decrease under CIP due to induction of the SOS-controlled gene *sulA* which inhibits cell division. Median division rates for the growing fraction of cells decreased under TET (approx. 16%, 19%, 5% in gluaa, glu and gly, respectively) and CIP+TET (approx. 20%, 27%, 11%) (Figure 39D-F). Slowed single-cell elongation and division under TET treatment has been observed in the mother machine previously (Ocampo *et al*, 2014; Brandis *et al*, 2023). From this data, we found that the antibiotics were generally more effective (in reducing division rates) in faster growing cells, which is expected for both CIP and TET as observed in bulk conditions (Greulich *et al*, 2015; Gutierrez *et al*, 2017; Smirnova & Oktyabrsky, 2018; Haugan *et al*, 2019).

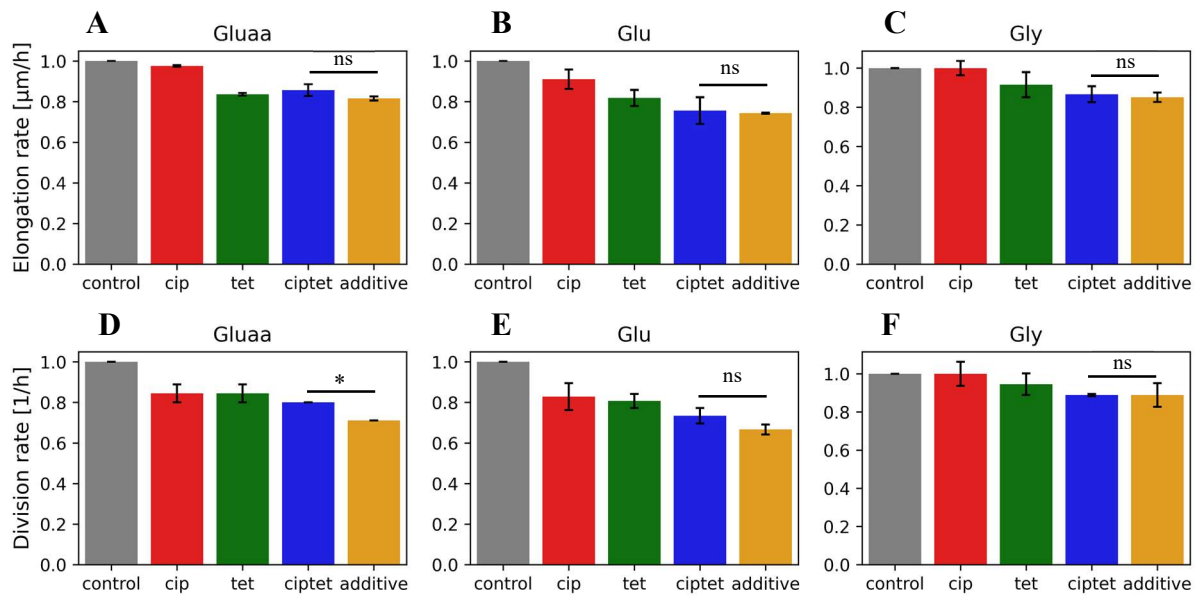


Figure 39. Median single-cell elongation rates (A-C) and division rates (D-F) for the growing fraction of cells in different growth media and treatment conditions. Medians were calculated from data pooled from the final 2 hours of antibiotic treatment and normalised to the no-drug control. Error bars are the standard error of the median of at least two experimental replicates. The additive expectation under the antibiotic combination was calculated as previously described. The Bliss definition of independent drug interactions which assumes that individual drug effects can be multiplied: $g_{AB} = g_A \times g_B$, where g_X is the normalised elongation/division rate in the presence of antibiotic X. Finally, g_{AB} was multiplied by the median elongation/division rate of the no-drug control to get the additive prediction. Statistical analysis: independent *t*-test. *: $p < 0.05$, ns: not significant.

Median elongation rates and division rates (of growing cells) under the drug combination were not significantly higher than that of either of the monotherapies (t -test, $p > 0.05$) (Figure 39A-F). Therefore, we did not observe a suppressive effect for the elongation and division rates of single cells. We also calculated the expected additive effect for the drug combination as done before in Figure 35 – thus, we can classify the interaction according to the formal definition set out by Bliss (1939). Median elongation rates for the drug combination were unchanged compared to the additive expectation (Figure 39A-C), indicating no specific effect on this parameter (additive interaction). But single-cell division rates were higher than the additive expectation in the gluaa and glu medium (Figure 39D, E), indicating an antagonistic effect on this parameter (recall that suppression requires that growth be higher than at least one of the antibiotics as well). Though, antagonism on the division rates is not sufficient to explain suppression observed for this drug combination, especially in the gly medium where the effect is additive (Figure 39F). However, our results so far suggest that the proportion of non-growing or dead cells decreased under the drug combination (compared to CIP treated cells) and that this was growth rate dependent.

In conclusion, we did not observe a suppressive effect on median single-cell elongation rates and division rates in all three growth media. Suppression observed in bulk experiments could therefore be a result of improved cell survival rather than improved single-cell growth/division. To investigate this further, we next quantified cell fate.

5.5 Quantifying single-cell survival and death

Since a large proportion of cells stopped growth and division under CIP treatment, we wanted to quantify cell survival and death to compare the different treatment and growth conditions. First, we considered different phenotypic responses to the antibiotics before implementing a fate classification protocol.

5.5.1 Phenotypic heterogeneity in cell fate

We observed a wide range of phenotypic responses to CIP treatment. Some cells were unaffected (Figure 40A) while others filamented to varying degrees before stopping growth (Figure 40B, C). Some cells that ceased growth did not elongate and usually descended from a mother cell that was filamenting (Figure 40D-F). The majority of non-growing cells remain physically intact for a long duration with a gradual decrease in constitutive fluorescence intensity and did not recover (growth and division) within 10 hours when antibiotic treatment

stopped. A few cells abruptly lost all fluorescence (lysis), but these events were rare (Figure 40G).

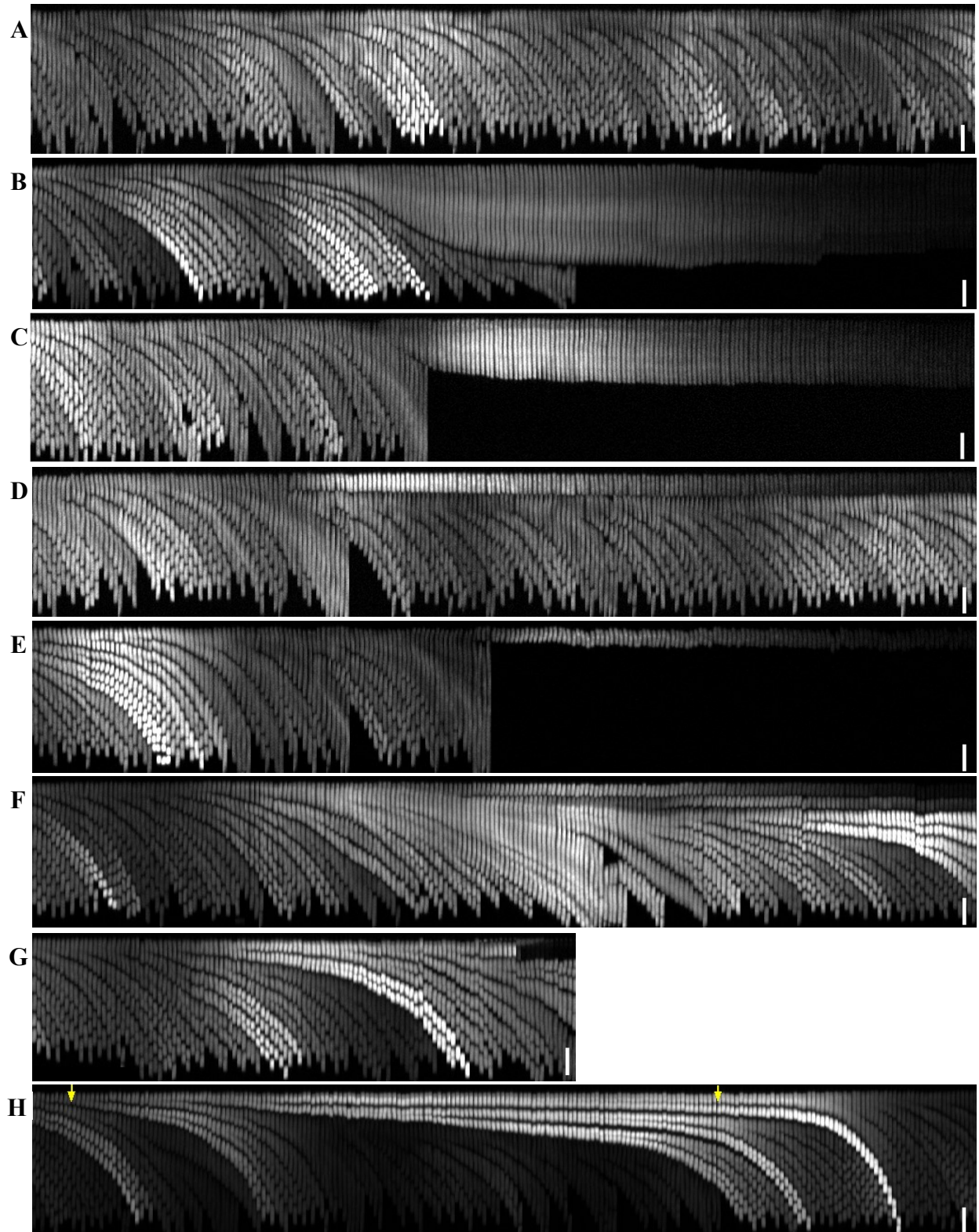


Figure 40. **Kymographs showing phenotypic heterogeneity in cell behaviour under ciprofloxacin treatment at 3 ng/mL in the gluaa growth medium for strain JKB43.** A. A mother cell that is unaffected by antibiotic treatment. B-C. Mother cells that filamented

and eventually stopped growth. **D-F.** Mother cells of normal size that eventually stopped growth. **G.** Cell lysis. **H.** A mother cell significantly slows growth and division during antibiotic exposure and then resumes normal growth and division. Kymographs were constructed as a montage of frames of 5-minute intervals from a single microchannel. The mother cell is the top cell in each kymograph. Shown is fluorescent intensity from the *PtetO1-mKate2* constitutive reporter. The minimum and maximum intensity values have been adjusted in each kymograph to enhance visualisation. In panel H, yellow arrows indicate where ciprofloxacin treatment began and ended. Scale bar represents 5 μm .

In extremely rare cases, cell growth and division slowed markedly during CIP treatment and then resumed when treatment stopped (Figure 40H). The fact that this behaviour aligned well with the antibiotic treatment period is a compelling case that this is a specific response to the antibiotic. However, we found that such events also occurred in untreated cells and were random (data not shown). Therefore, it is unlikely that the behaviour observed in Figure 40H is a specific response to antibiotic treatment, but rather a stochastic occurrence of metabolic dormancy¹⁴ which may confer tolerance to the antibiotics (Lennon & Jones, 2011).

We also investigated the membrane integrity (the lack of which is a hallmark of unrecoverable cell death) of these non-growing cells using a membrane viability dye called SYTOX Green (Appendix C5). We found that, under CIP treatment, 35% of non-growing cells lost membrane integrity, usually after a delay since elongation stopped, while 65% maintained membrane integrity (membrane-viable cells do not take up the dye) (Supplementary Figure 17). However, it is likely that most of the non-growing “viable” cells observed in this experiment will eventually lose membrane integrity (see discussion in Appendix C5), especially since this proportion is much higher than observed previously (Bamford *et al*, 2017; Zhang *et al*, 2023). These non-growing yet viable (specifically membrane-viable) cells are consistent with the VBNC¹⁵ cell phenotype. Yang *et al.* (2019) similarly observed differences in starvation-induced single-cell death trajectories, where some cells stopped growth abruptly with sudden loss of membrane integrity, while others stopped growth but maintained membrane integrity, eventually dying after 10-15 hours. Under CIP treatment (at MIC), Zhang *et al.* (2023) and Bamford *et al.* (2017) also observed different killing phenotypes: cell lysis, cells with disrupted membranes, and cells with intact membranes that did not resume growth even after 21 hours of recovery without antibiotic. The authors classified the latter as VBNC cells due to their

¹⁴ Dormancy refers to a reversible, non-growing state of low metabolic activity that allows cells to survive environmental hazards (Lennon & Jones, 2011).

¹⁵ Viable but non-culturable (VBNC) cells do not divide in the presence of antibiotics or after their removal, but may be revived under specific conditions.

continued dormancy after antibiotic was removed. Hence, some of the non-growing cells observed in our experiments could be VBNC cells. VBNC cells are clinically important; although they generally do not resume division after antibiotic treatment, they can be revived under specific environmental conditions (Li *et al*, 2014). Cell death as a fate is therefore a phenotypically nuanced phenomenon that can manifest or be characterised in different ways. When quantifying cell death, the various phenotypes should be considered.

5.5.2 Classification of cell fate

We then sought to classify mother cell lineages by whether they continue growth, stop growth, or filamented. We classified lineage fate as either ‘survived’, ‘died’, or ‘hyper-filamented’ based on the various phenotypes observed in the kymographs (see Materials and Methods Section 3.6.5.1 for a detailed explanation). Briefly, we relied on an observed cessation in elongation and division, with a few manual designations. We were unable to assess whether hyper-filamented cells survived or died since they were frequently swept out of the microchannels. Thus, they were given a separate category.

An example of mother cell lineages classified by fate is shown in Figure 41. There was good separation between ‘survived’ and ‘dead’ lineages based on the elongation rates (Figure 41A, B). Most cells died during antibiotic treatment or a few hours after, probably because there was still residual antibiotic or residual DNA damage in the cells. For surviving cells, SOS expression did not generally reach high levels (>1000 a.u.) and recovered quickly after drug treatment stopped (Figure 41D). Many lineages that died exhibited strong, persistent induction of the SOS response to very high levels and did not return to baseline levels (Figure 41E). However, this did not appear to be predictive of cell death as many lineages that died did not reach high SOS levels.

Most of the cells classified as ‘dead’ exhibited a gradual decrease in *mKate* intensity (a proxy for constitutive protein concentration) after elongation stopped (Figure 41B, H). Elongation of the cell (a proxy for mass accumulation) effectively dilutes the constituents of the cell. In the case of a constitutive protein, the cell needs to produce the protein at the same rate of dilution to maintain a constant concentration in the cell. Given that elongation (dilution) has ceased, a decreasing constitutive protein concentration cannot be due to dilution, rather, it indicates that expression/protein translation has completely stopped and they are metabolically inactive. For all cells with an exponentially decreasing *mKate* intensity, the slope is roughly the same (Figure

41H) and is probably due to bleaching/biodegradation of the fluorescent protein. Therefore, these non-growing, metabolically inactive cells are likely dead, or in the process of dying.

A few cells classified as dead showed an increase in constitutive expression levels (Figure 41H). This may be due to continued constitutive gene expression when elongation has stopped (therefore lack of dilution) leading to an increase in concentration of fluorescent protein. For most of these cells, *mKate* intensity eventually decreased anyway, indicating that continued production was residual. This may also explain the very high SOS levels observed for many of the dead cells (Figure 41E) and indicates that the SOS promoter is still very active when cells stop elongation.

We identified only five persister cells in our experiments which consisted of between 0.1-1% of all cells. These were non-growing lineages that resumed growth after 30 hours of observation (see Appendix C4). These cells were non-growing at the beginning of the experiment and therefore were not induced by antibiotic exposure; rather, they were likely present in the inoculum and were formed stochastically. These cells also exhibited a decline in constitutive expression but then stabilised around 1000 a.u., after which constitutive levels increased again when division resumed (Supplementary Figure 16). Although a small sample size, this suggests that persister cells maintain a baseline metabolic activity. Studies show that persister cells maintain a residual ATP level, PMF, and protein synthesis (Shan *et al*, 2017; Yin *et al*, 2019; Bergkessel & Delavaine, 2021; Mancini & Pilizota, 2023), which is consistent with our observations.

We cannot exclude the possibility that some cells classified here as ‘dead’, but seem to maintain physical integrity, are persister cells with very long lag times (which has been shown previously (Kaplan *et al*, 2021)). Yet most non-growing cells observed here stop constitutive gene expression entirely and do not maintain any residual expression (Figure 41H), as observed for persister cells (Appendix C4). Thus, this phenotype may reflect an intermediate stage on route to cell death, or a type of “slow death” which has been observed previously for nutrient starved cells (Yang *et al*, 2019).

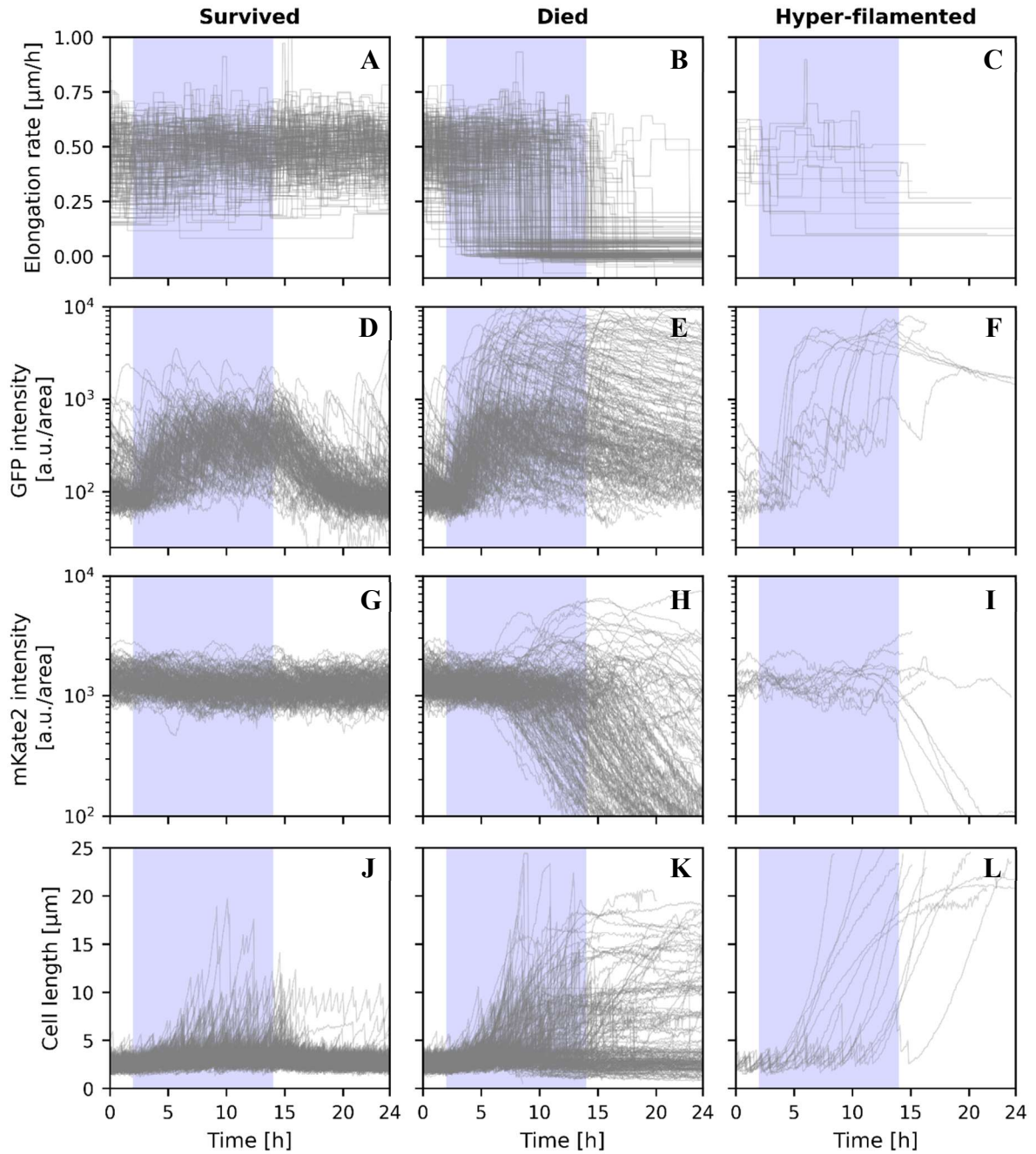


Figure 41. **Single-cell trajectories classified by lineage fate under CIP treatment at 3 ng/mL.** Shown are elongation rates (A-C), SOS expression from *PsuLA-mGFP* (D-F), constitutive expression from *PtetOI-mKate2* (G-I), and cell length (J-L). Lineages were classified by whether they survived, died, or hyper-filamented over the duration of the experiment which included a 10 hour recovery period. Blue shaded area indicates when antibiotic was introduced (between hours 2-14). Shown is data from one experimental replicate for strain JKB43 in the *glu* growth medium. *mGFP* and *mKate* intensity trajectories have been smoothed with a moving average over a 5-frame window.

5.5.3 Quantifying survival probabilities and death rates

We then quantified the survival probability as a function of time and the cumulative hazard function using the Kaplan-Meier (K-M) estimator (Kaplan & Meier, 1958) and Nelson-Aalen (N-A) estimator (Nelson, 1972; Aalen, 1978), respectively (see Materials and Methods Section 3.6.5.2 for a description). These estimators are commonly used in survival analysis studies to estimate the probabilities of an event occurring based on underlying data, for example, deaths of individuals in a cohort of cancer patients. Survival times for each lineage were calculated (Figure 42A) and used as the input for the two estimators. Since some deaths occurred before antibiotic treatment, the first 2 hours were cut from the data passed to the two estimators to avoid bias in the results.

The K-M survival functions indicate significantly reduced survival after 12 hours under CIP treatment in all growth conditions (Figure 42B). However, combining TET with CIP significantly improved the survival probability of cells. Under no-drug and TET treatment, survival probability remained relatively constant and is >0.94 at the end of antibiotic treatment in all conditions. After CIP exposure is stopped (at 12 hours), the slope of the survival curve returned to a rate similar to the no-drug control (Figure 42B). At the end of drug treatment (after 12 hours), survival probability under CIP treatment was 0.49, 0.47, and 0.50 (in gluaa, glu, gly, respectively), and under CIP+TET treatment survival probability was 0.82, 0.72, and 0.69. Thus, we found that the improvement in cell survival under CIP+TET (compared to CIP treatment alone) was growth rate-dependent, with the highest improvement in faster growth conditions (gluaa). Surprisingly, the suppressive effect on cell death was therefore stronger in fast-growth conditions.

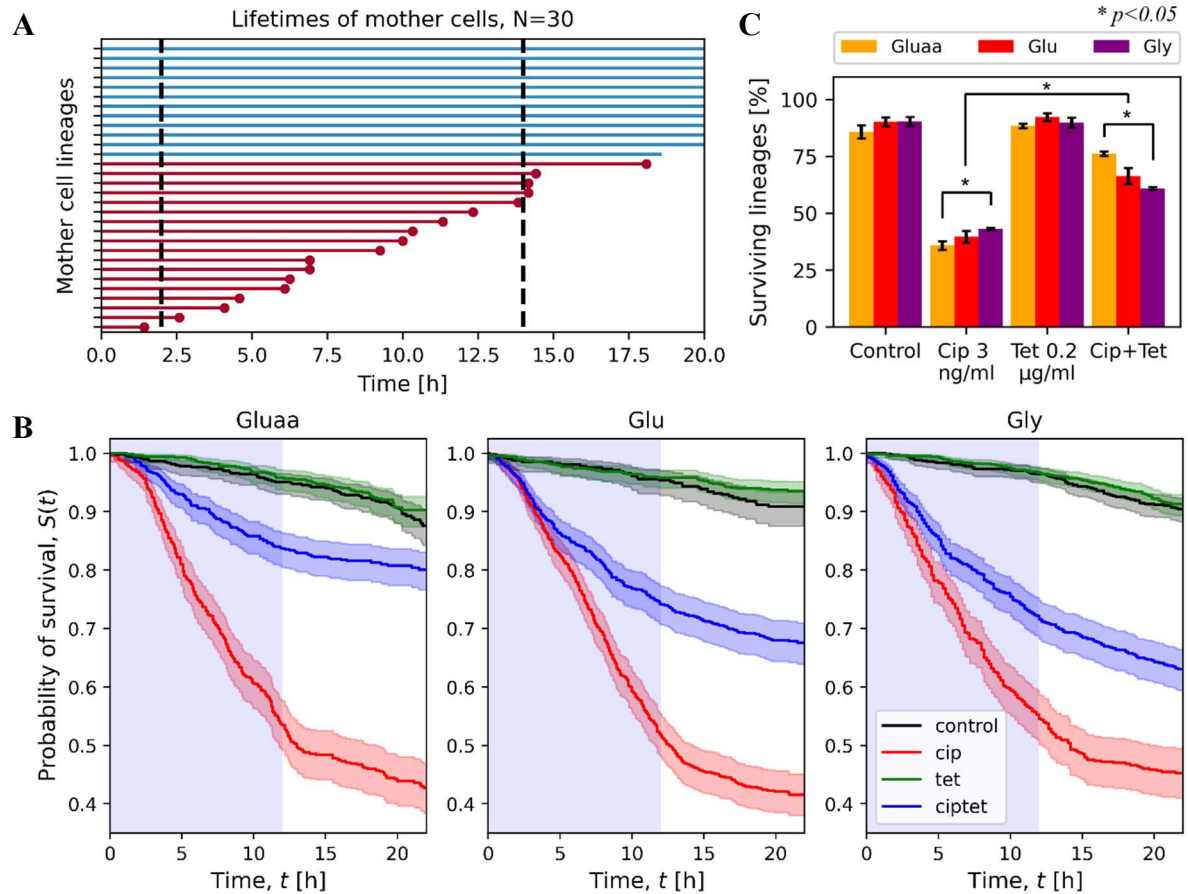


Figure 42. Quantification of single-cell survival dynamics. **A.** Mother cell lifetimes under CIP treatment in the gluaa growth condition. Shown are 30 randomly selected lineages from the population. Blue lines represent lineages that survived and red lines represent lineages that died with their death time indicated by circle points. Antibiotic is introduced between hours 2-14 (black dashed lines). Lineages that died before antibiotic exposure were removed. **B.** Survival functions representing mother cell survival probability under different antibiotic treatments in different growth conditions. Survival probabilities $S(t)$ were estimated with the Kaplan-Meier estimator using mother cell lifetime data pooled from at least two experimental replicates and excludes cells that have hyper-filamented. Each dataset consisted of between 420-1005 mother cell lineages. $S(t)$ is the probability that a cell will survive beyond a certain point in time t . Solid line represents the S at time t and shaded areas the 95% confidence interval. Antibiotics are introduced between hours 0-12 (light blue shaded area). **C.** Percent surviving lineages after 12 hours of drug treatment and 10 hours of recovery for different antibiotic treatments and growth conditions. Different colours represent the growth medium. Bars are the mean and error bars that standard deviation of at least two experimental replicates. The fractions of lineages that survived, died, and hyper-filamented are shown in Supplementary Figure 14. Survival differed significantly between gluaa and gly for CIP ($p=0.027$, independent two-sample t -test) and CIP+TET ($p=0.006$). For all growth conditions, survival for CIP+TET was significantly higher than under CIP-treatment. All data represented is for strain JKB43. $N=592/410/862$ (gluaa/glu/gly) lineages for control, $N=535/792/532$ for CIP, $N=550/696/988$ for TET, and $N=614/691/733$ for CIP+TET.

The log-rank test was used to test for significant differences in the survival functions between each pairwise treatment and growth condition (Figure 42B). The null hypothesis of the log-rank test assumes no difference in the survival distributions between two populations. The survival functions for CIP and CIP+TET differ significantly from each other and the no-drug control in all growth conditions (log-rank test, $p < 0.005$). The no-drug control and TET treatment did not differ significantly from each other ($p > 0.05$). Between the different growth media, the survival functions under CIP, TET, and no-drug treatment did not differ significantly ($p > 0.05$). However, under CIP+TET treatment the survival function for gluaa is significantly higher than both glu and gly, but not between glu and gly (For CIP+TET: gluaa-glu: $p < 0.005$, gluaa-gly: $p < 0.005$, glu-gly: $p = 0.30$).

In Figure 42C, we compare the fraction of surviving lineages at the end of the experiment (including 10 hours of recovery) to better visualise growth-dependent effects. Here, growth-dependency emerges for CIP treatment with significantly higher single-cell survival in slower growth conditions (Figure 42C). This result is consistent with other studies that have reported higher efficacy of CIP against fast-growing *E. coli* (Gutierrez *et al*, 2017; Smirnova & Oktyabrsky, 2018; Biselli *et al*, 2020; Zheng *et al*, 2020; Smirnova *et al*, 2022). For CIP+TET treatment, the inverse was observed: survival was significantly higher in faster growth conditions. Interestingly, survival increased by a larger margin in the fast growth medium than in slow growth: survival increased from 39% under CIP-treatment to 79% under CIP+TET treatment in the gluaa medium (a 40% increase). Therefore, the suppressive effect on cell death is growth rate-dependent and confirms, at least qualitatively, bulk-level observations (Figure 30B, C).

It was unexpected that survival for CIP+TET increased with faster growth rates. Generally, susceptibility to single-administered antibiotics increases with faster growth rates (Tuomanen *et al*, 1986; Millar & Pike, 1992; Greulich *et al*, 2015; Lee *et al*, 2018; Smirnova & Oktyabrsky, 2018; Haugan *et al*, 2019; Angermayr *et al*, 2022; Smirnova *et al*, 2022, 2023), however, growth-dependence established for single antibiotics may not apply when combined with other antibiotics.

We then estimated the cumulative hazard function using the N-A estimator for the population of mother cells over the duration of the experiment (Figure 43A). The cumulative hazard function is defined as the probability that a cell will die at some time, given its survival up to that time. The slope of this curve is effectively the death rate (or hazard rate). Since the slope

of the cumulative hazard function changes over time, the instantaneous death rate is plotted to visualise this (Figure 43B).

For TET and the no-drug control, death is minimal over the duration of the experiment (Figure 43A, B). The lack of cell death under TET treatment is expected due to the bacteriostatic nature of the antibiotic. The survival or death of cells under CIP and CIP+TET was not monotonic. At the beginning of CIP and CIP+TET treatment, the cumulative hazard increased exponentially for the first 3-4 hours (Figure 43A, red and blue lines) which is reflected in the linear increase in death rates during this period (Figure 43B). After this period, the rise in cumulative hazard is roughly linear under CIP with the death rates stabilising between 0.04-0.08 h⁻¹, appearing to reach a steady-state for all growth conditions. After antibiotic treatment stops, death rates decrease rapidly for gluaa and glu, and there is approximately a 2 hour delay before death rate decreases in gly. The fact that the initial death rate for gly starts at a higher value is considered to be an artefact due to the difference in sampling times between gly and gluaa/glu (10 min vs. 5 min).

Under CIP+TET, death rates peak after 3-4 hours – with the lowest peak in fast growing cells – and then begins to gradually slow down in all growth conditions. The observed decrease in death rate during CIP+TET treatment (Figure 43B, blue lines) suggests cells may be adapting to the conditions. A contributing factor could be an accumulation of the multi-drug efflux pump AcrAB-TolC at the old-pole of mother cells over time – which has been demonstrated to be the cause of improved tolerance to TET in aging mother cells (Bergmiller *et al*, 2017). This could also lead to improved tolerance to CIP since overexpression of the AcrAB-TolC efflux pump is known to reduce intracellular antibiotic accumulation (Łapińska *et al*, 2022) and contributes to fluoroquinolone persistence (Pu *et al*, 2016) and resistance (Atac *et al*, 2018; Byrd *et al*, 2021).

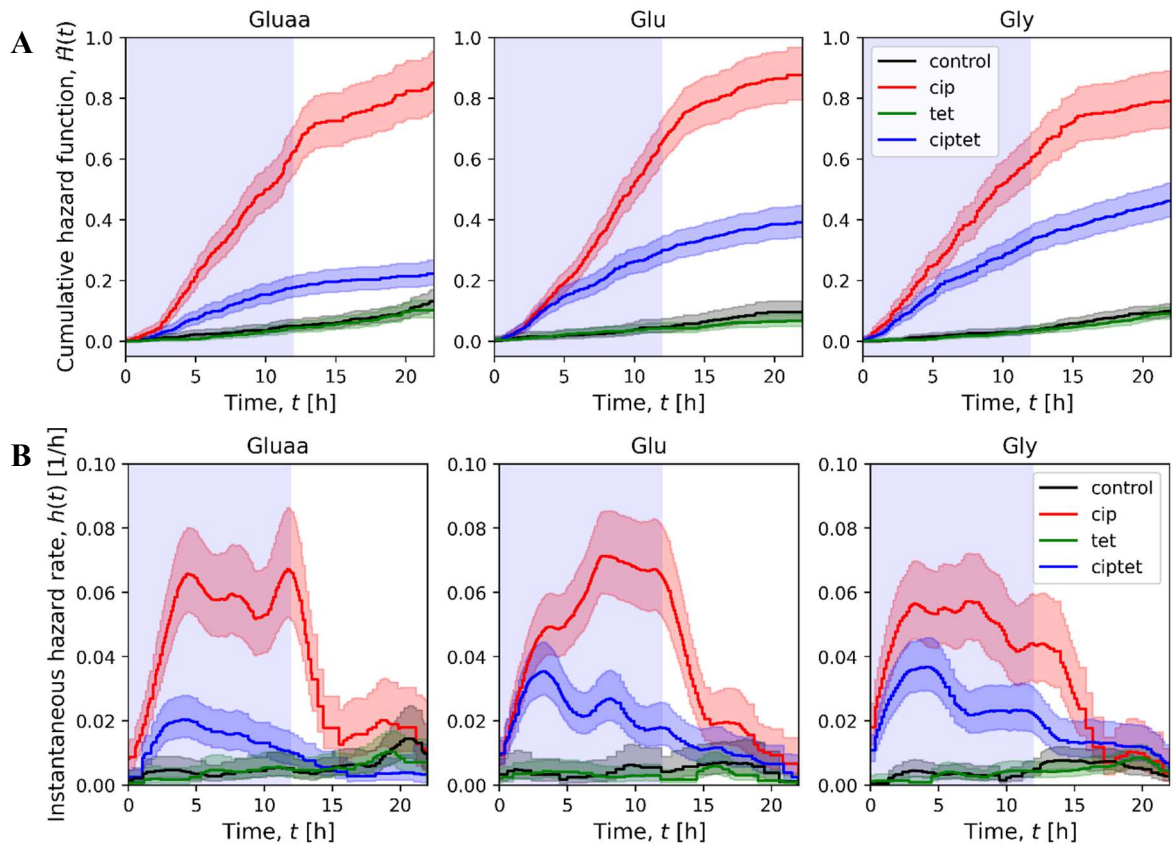


Figure 43. Quantification of single-cell death and death rate dynamics. **A.** Cumulative death function of mother cell lineages under different antibiotic treatments in different growth conditions. **B.** Estimated instantaneous death rates of mother cell lineages under different antibiotic treatments in different growth conditions. The cumulative hazard function $\hat{H}(t)$ and hazard rate $h(t)$ was estimated with the Nelson-Aalen estimator using mother cell lifetime data pooled from at least two experimental replicates and excludes cells that have hyper-filamented. $\hat{H}(t)$ is the probability that a cell will die at some time t , given its survival up to that time. Solid lines represent the average $\hat{H}$ or h at time t and shaded areas the 95% confidence interval. Antibiotics are introduced between hours 0-12, indicated by the light blue shaded area. All data represented is for strain JKB43. $N=592/410/862$ (gluaa/glu/gly) lineages for control, $N=535/792/532$ for CIP, $N=550/696/988$ for TET, and $N=614/691/733$ for CIP+TET.

We previously found that for mother cell lineages that stopped growth and division, often the sister cell was successful in producing progeny (see Figure 40D, F, G). Typically, we only tracked the mother cell dynamics, discounting the dynamics of the neighbouring sister cell and its lineage. Such is the limitation of the mother machine where daughter cell lineages are eventually lost from the microchannels. In the survival analysis (Figure 42) we measured the survival fraction of mother cells as a function of time. Thus, we could be overestimating cell death, since, when we count a dead lineage, its sister cell may survive and could continue propagation of a microbial colony or an infection.

Although the population of cells are genetically identical, we found that death occurred at different times despite being exposed to the same treatment – thus, death appears to be stochastic. But how does stochasticity in cell death arise? Stochastic death of bacteria has been previously documented for antibiotic treated (Coates *et al*, 2018) and nutrient starved *E. coli* (Yang *et al*, 2016, 2019, 2023). In the mother machine, Yang *et al*. (2023) showed that stochastic accumulation of damage over time leads to variation in time of death and that variation in damage decreased with cell age. Also contributing to variation could be heterogeneity in drug uptake which has been demonstrated previously for CIP and TET (Łapińska *et al*, 2022).

In this study, we relied on an observed cessation in elongation and division, with a few manual designations, for our survival analysis. For our purposes, this method was sufficient in differentiating between growing and non-growing sub-populations. Yet, our survival analysis can be improved by using membrane viability dyes which will enable higher precision in determining when a cell has died (according to the membrane disruption definition), as done previously by others (Rossi *et al*, 2019; Yang *et al*, 2019, 2023). Our data lacks some precision because we determine cell death at the beginning of the cell-cycle during which growth or division ceased, yet a cell may have ‘died’ some time after division – for example see residual cell elongation following division in Figure 40C, or residual constitutive gene expression in Figure 40D and Figure 41H. A membrane viability dye could tell us exactly when a cell has lost viability and should be used, along with other death-defining characteristics, to construct survival curves in future experiments. But, it is also important to consider that cell death may occur without or before loss of membrane viability. Additionally, partial membrane depolarisation caused by induction of the TisB toxin could lead to false positives when using membrane viability dyes.

5.5.4 Extended antibiotic treatment

We evaluated survival over a longer treatment duration (up to 48 hours) to see how survival dynamics may change over time (Figure 44; see Supplementary Figure 13 for elongation rates, SOS and constitutive expression dynamics). At the end of the experiment, only 2.6% of cells remained for CIP, 78% for TET, and 43% for CIP+TET (Figure 44A). Cumulative hazard (risk of death) increased exponentially under CIP treatment (Figure 44B), which is reflected in (roughly) linearly increasing hazard rates in Figure 44C. As observed previously, cumulative hazard increased at a much higher rate under CIP, followed by CIP+TET and then TET with

the lowest cumulative hazard. Near the end of the experiment when there are very few cells still alive, the 95% confidence intervals for the hazard rates widen, which reflects the loss of statistical power (Figure 44C). In a similar experiment, except this time for carbon starved cells, Yang et al. (2016) and Yang et al. (2023) observed an exponential increase in the cumulative hazard with increasing age for carbon-starved *E. coli*. We can consider that a population of cells has a baseline rate of death and antibiotics add to this baseline to varying degrees.

The hazard rate for extended CIP+TET treatment (Figure 44C) is similar to what was observed in Figure 43B. The hazard rate initially increased before stabilising long-term. It is likely that the fluctuations in hazard rates observed in Figure 43B is a result of biological noise that is more apparent on the shorter timescale. The hazard rate for extended CIP treatment also differs from those observed in Figure 43B; it continues to increase throughout the treatment period. Again, this difference could be due to stochasticity in the survival times of individual cells.

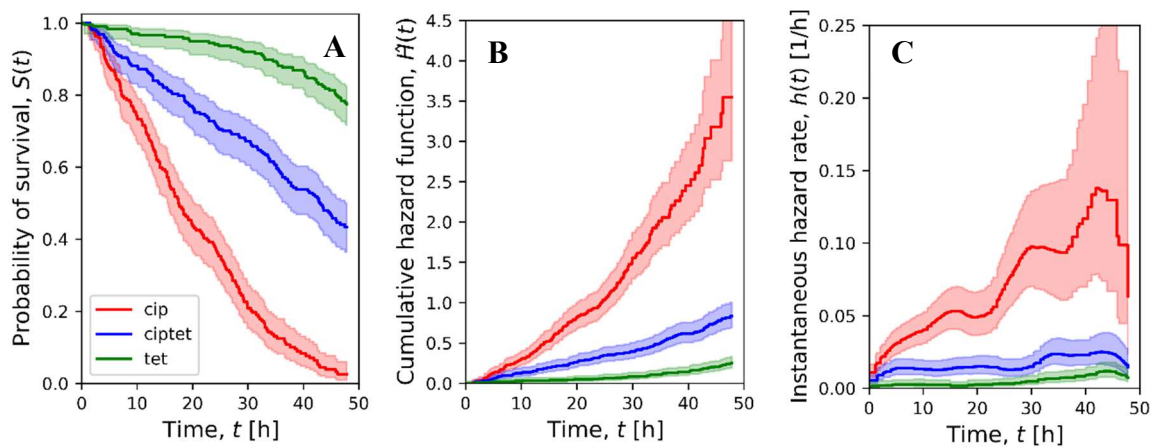


Figure 44. Quantification of single-cell survival and death dynamics under extended antibiotic treatment. Kaplan-Meier survival functions (A), Nelson-Aalen cumulative death function (B), and instantaneous death rates (C) of mother cell lineages under different antibiotic treatments in the gluaa growth medium. Survival probabilities $S(t)$, cumulative hazard function $\hat{H}(t)$ and hazard rate $h(t)$ were estimated as described previously using mother cell lifetime data from a single experiment and excludes cells that have hyper-filamented. Solid line represents the estimated S , $\hat{H}$, or h at time t and shaded areas the 95% confidence interval. Concentrations used were 3 ng/mL for ciprofloxacin (CIP) and 0.2 μ g/mL for tetracycline (TET). $N=247$ for CIP, $N=252$ for TET, and $N=253$ lineages for CIP+TET. Antibiotics are introduced for the entire 48 hour experiment. All data represented is for strain JKB43.

5.6 The SOS response is elevated under DNA damage

The SOS response is vital for the survival of cells experiencing DNA damage. Since cell death was found to be a major factor in the suppressive interaction between CIP and TET, we evaluated SOS gene expression to understand how cells respond to DNA damage in the different treatment conditions.

Overall, SOS expression was highly heterogeneous and fluctuated significantly in all conditions. Median SOS expression under CIP and CIP+TET treatment initially increased and then levelled off, appearing to reach a steady-state after approximately 3-5 hours of antibiotic treatment (Figure 45A-C & Figure 45G-I, respectively). This represents the main population well; however, some cells deviated from the population average and induced the SOS response to very high levels (above 1000 a.u.), and generally did not return to baseline levels. As observed in Figure 41E, all cells with persistent high SOS levels eventually died. Since the level of SOS induction is correlated with the amount of DNA damage (Maslowska *et al*, 2019), these cells have likely accumulated an irreconcilable amount of DNA damage leading to cell death. Median SOS levels increased to a higher level in slower growth conditions but also reached steady-state slower (Figure 45A-C & Figure 45G-I). Also, high-SOS cells appeared to be more frequent in slower growing cells. After the antibiotic was removed, SOS expression returned to pre-exposure levels in all conditions, though the rate of recovery occurred slower in slow growing cells.

Under TET treatment, SOS expression levels were, on average, unaffected (Figure 45D-F) which is expected for an antibiotic that does not damage DNA. However, as observed in the no-drug control (Figure 32D-F), a few cells did show significantly elevated SOS response, but these are likely due to spontaneous DNA damage.

It is interesting to note that the period where steady-state in the SOS response is achieved (Figure 45A-C & Figure 45G-I) corresponds to the levelling off in death rates observed in Figure 43B. This indicates some correlation between SOS response and instantaneous death rates.

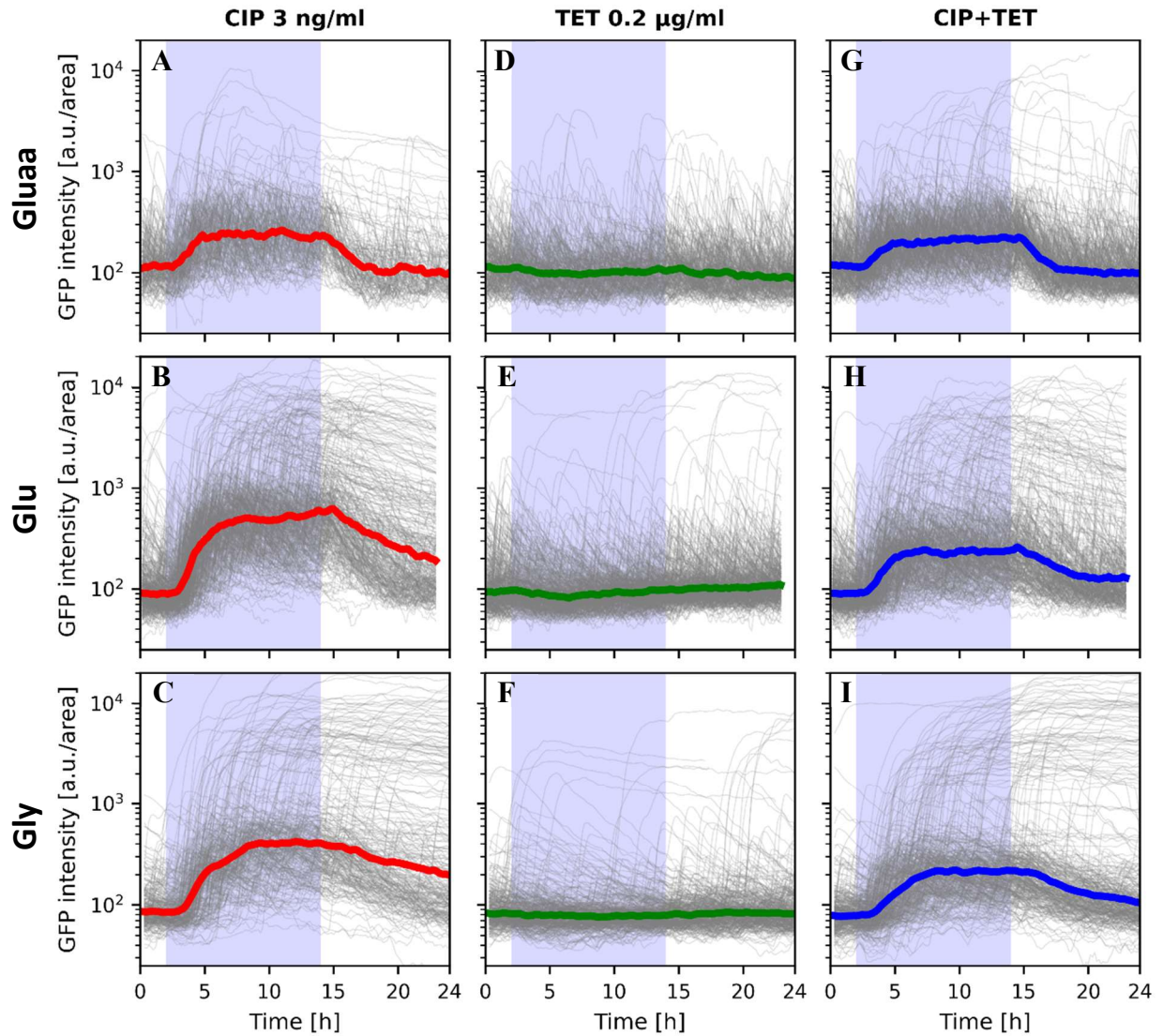


Figure 45. Single-cell trajectories of SOS expression from *PsuA-mGFP* fluorescent reporter for different growth media under 3 ng/mL ciprofloxacin (A-C), 0.2 µg/mL tetracycline (D-F) and drug combination (G-I) treatment. Solid lines represent the median and grey lines represent individual lineage trajectories. Shown is data from one experimental replicate for strain JKB43. Antibiotics are introduced between hours 2-14, indicated by the blue shaded area. GFP and intensity trajectories have been smoothed with a moving average over a 5-frame window.

5.6.1 Example trajectories show high variability in SOS response induction

Taking a few example trajectories under CIP treatment revealed high heterogeneity in the SOS response between individual cells (Figure 46D, H, L). For fast growing cells, SOS expression was noisy, with multiple sharp induction events followed by decreases in SOS expression towards baseline levels (Figure 46D). For slower growing cells, SOS expression dynamics appeared to be less frequent and were slower to recover to normal levels (Figure 46H, I), which

could reflect a lower dilution rate of the fluorescent protein once SOS induction is stopped. In these examples, the first SOS induction event, assumed to be correlated with an accumulation of DNA damage by CIP, occurred at different times, with the first event occurring after 8 hours of exposure for one of the trajectories (Figure 46H). SOS induction for individual cells can vary substantially from the population average shown in Figure 45. Not only do cells differ in the strength of SOS induction (e.g. low and high-SOS cells), there is also significant temporal variability. Variability in the single-cell response times (delay between stress and response induction event) of the SOS response and several other stress responses has been reported previously (Mitosch *et al*, 2017, 2019; Jones & Uphoff, 2021; Vincent & Uphoff, 2021).

This data shows that cells may not perceive DNA damage by CIP uniformly and sometimes induce the SOS response late into CIP exposure. Łapińska *et al.* (2022) showed high cell-cell heterogeneity in the accumulation of CIP, which is in part due to variability in single-cell elongation rates before antibiotic treatment. The authors also reported a relatively long period before significant uptake began (compared to other drugs) – after roughly 40 minutes. However, we note that the authors used fluorescent antibiotic derivatives to measure antibiotic uptake into the cell. These can have significantly higher molecular weights (MW) than the parent antibiotic, for example, ciprofloxacin-HCl MW=367.82 g/mol, fluorescent derivative MW=663 g/mol. It follows that uptake and drug activity will be affected – and the MIC increased 256-fold (Łapińska *et al*, 2022). Nonetheless, our results are consistent with the authors' observations which could explain some of the cell-cell heterogeneity in our data. Heterogeneity in SOS induction could also result from variability in the degradation of LexA protein which is the transcriptional repressor of the SOS operon (Jones & Uphoff, 2021).

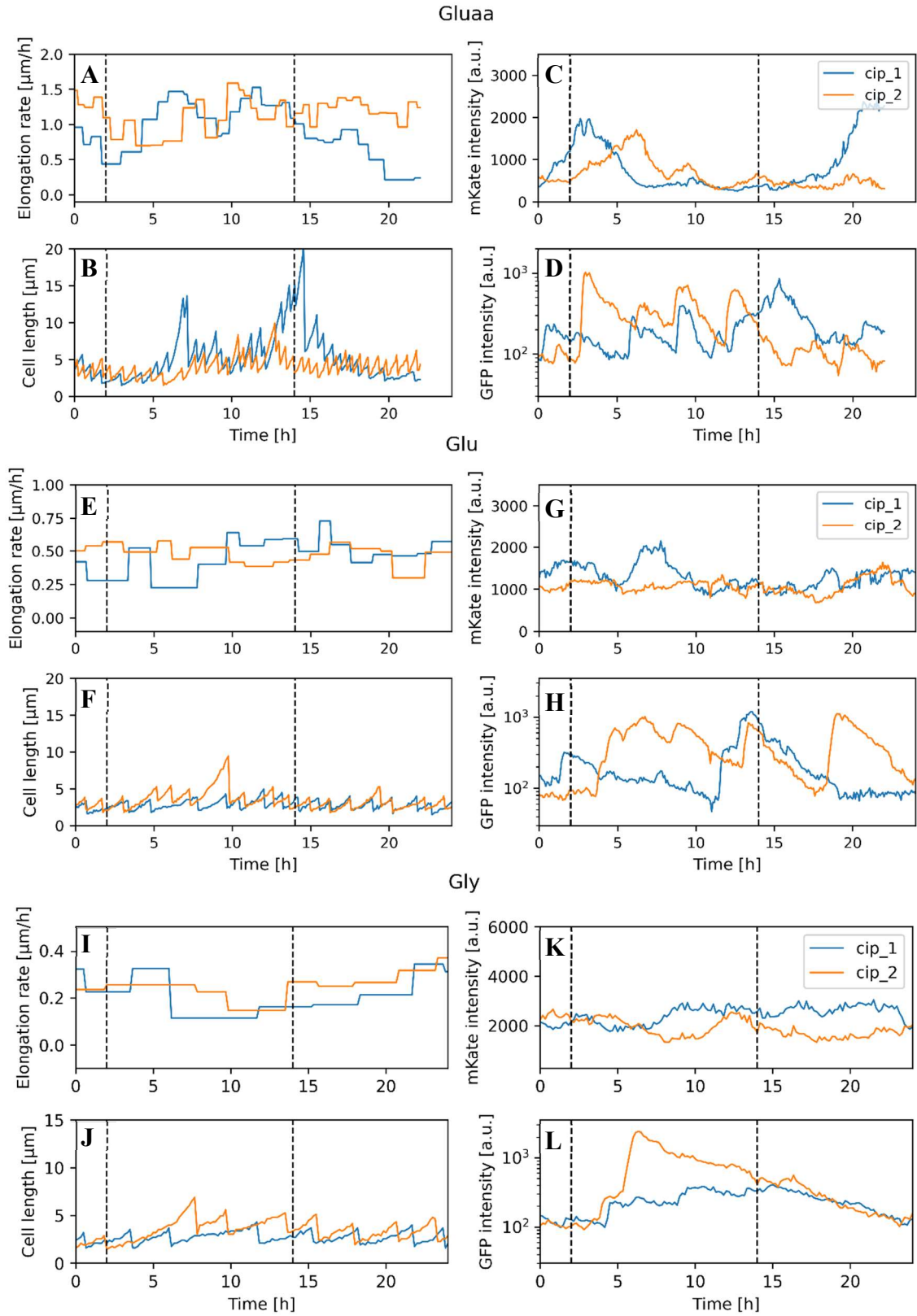


Figure 46. **Example trajectories of single-cell dynamics under 3 ng/mL ciprofloxacin exposure in the gluaa (A-D), glu (E-H), and gly (I-L) growth medium.** Colours represent different single-cell trajectories. Ciprofloxacin (CIP) is introduced between hours 2-14 (black dashed lines).

5.6.2 The SOS response is suppressed when tetracycline is added to ciprofloxacin-treated cells in slow-growth conditions

SOS expression was then quantified over the final 2 hours of antibiotic treatment when average SOS levels were at steady-state (Figure 47). Median SOS expression levels were elevated for CIP in all growth conditions but were more than 2-fold higher in slower growing cells (glu and gly media) (Figure 47A-D). Similar results have been reported previously for *E. coli* exposed to

3 ng/mL CIP on agarose-pads (Jaramillo-Riveri, 2019). This could reflect a higher degree of DNA damage accumulated in slower growing cells (resulting in a stronger induction). A possible explanation for lower SOS expression in the fast growth medium is improved DNA repair (via homologous recombination) due to a higher number of chromosome copies per cell during multi-fork replication. Alternatively, and more likely, it could reflect a growth-dependent impact on SOS expression levels which became unregulated when induced. When the SOS response is induced, it is no longer under LexA repression, and will therefore behave similar to a constitutively expressed (unregulated) gene when growth rate is modulated by nutrient quality. According to established growth laws, the mass fraction (or concentration) of an unregulated non-ribosomal protein will be inversely correlated with growth rate (Scott *et al*, 2010), which may explain the negative correlation between induced SOS levels and growth rate (Figure 47A-D).

For CIP+TET treatment, median SOS expression levels were lower than under CIP treatment in glu and gly growth media, but were almost identical for gluaa (Figure 47D). Inhibition of the SOS response in *E. coli* by translation inhibitors, including TET, has been observed previously in a concentration-dependent manner (Berger *et al*, 2019). Also, median SOS levels were almost identical under CIP+TET in the three growth media, however, they differed with respect to higher levels of SOS induction (Figure 47A-C, discussed below). Although TET treatment alone did not affect the SOS response (as expected since DNA is not damaged), adding TET to CIP-treated cells suppressed the SOS response, but only in slower growth media. Again, this observation can be explained by universal growth laws which tells us that the mass fraction of an unregulated protein will be reduced under translation inhibition and to a larger degree in slower growth media (Scott *et al*, 2010). So, it is expected that the reduction in induced SOS levels by TET will be relatively higher in slow-growing cells. To summarise, we found that levels of SOS gene expression were reduced when a translation inhibitor is added

to cells undergoing DNA damage by CIP in a growth-dependent manner consistent with universal growth laws.

The distributions of SOS expression were bimodal for CIP and CIP+TET except in fast growing cells (Figure 47A-C), reflecting higher variability in SOS expression in slower growing cells (Figure 47E). The second peak corresponds with high-SOS lineages observed in Figure 45. Variability of SOS expression levels were growth rate dependent: CV was consistently higher in slower growing cells and did not change significantly when TET was added to CIP (Figure 47E). We then quantified the high-SOS fraction (>1000 a.u.) of cells and found they were more abundant in slow growth conditions and were reduced for the drug combination compared to CIP treatment (Figure 47F). In contrast, we observed the opposite for the 2-pal strain in the previous chapter – we discuss this further in the discussion.

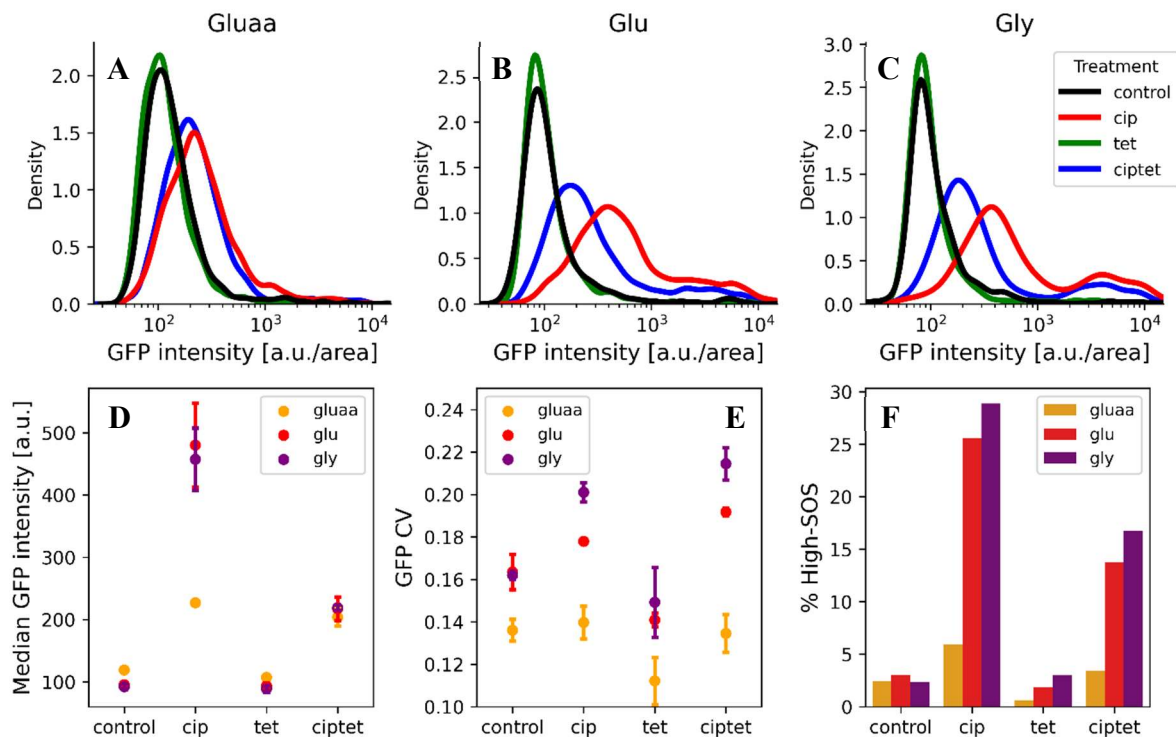


Figure 47. SOS gene expression is suppressed under the antibiotic combination and is growth-dependent. A-C. Distributions of single-cell SOS expression from *PsuA-mGFP* for different growth media and treatment conditions. Distributions are kernel density estimates of the underlying histogram pooled from the final 2 hours of antibiotic treatment from at least two experimental replicates for strain JKB43. Colours represent the different treatment conditions. Distributions for each experimental replicate are shown in Appendix C2 and show good day-to-day reproducibility. D. Median GFP intensity from data shown in panels A-C. Error bars are the standard error of the mean of experimental replicates. E. Coefficient of variation (CV) of data shown in panels A-C. Error bars are the standard error of the mean of experimental replicates. F. Percent of high-SOS cells determined as a GFP intensity above 1000 a.u. For panels D-F, colours represent different growth conditions.

5.6.3 High-SOS cells tend to filament or die

We also classified each cell-cycle within mother cell lineages (Figure 48A) allowing differentiation between SOS gene expression in cells that were either ‘alive’, ‘dead’ or ‘hyper-filamented’ at a certain point in time (Figure 48B-D). Thus, we can see how cell state contributes to the variation observed in the SOS expression distributions (see Figure 47A-C). Cells that had filamented excessively (‘hyperfila’) had the highest SOS levels while growing cells maintained relatively low-SOS levels (Figure 48B-D). Dead cells had median SOS levels somewhere in between with the highest variation. Relating back to Figure 47A-C, the secondary peak representing high-SOS levels (>1000 a.u.) are therefore primarily made up of cells that were either dead (non-growing) or hyper-filamented. We can infer from this data that cell death is associated with an extreme SOS response in many but not all cells, particularly in slower growth conditions.

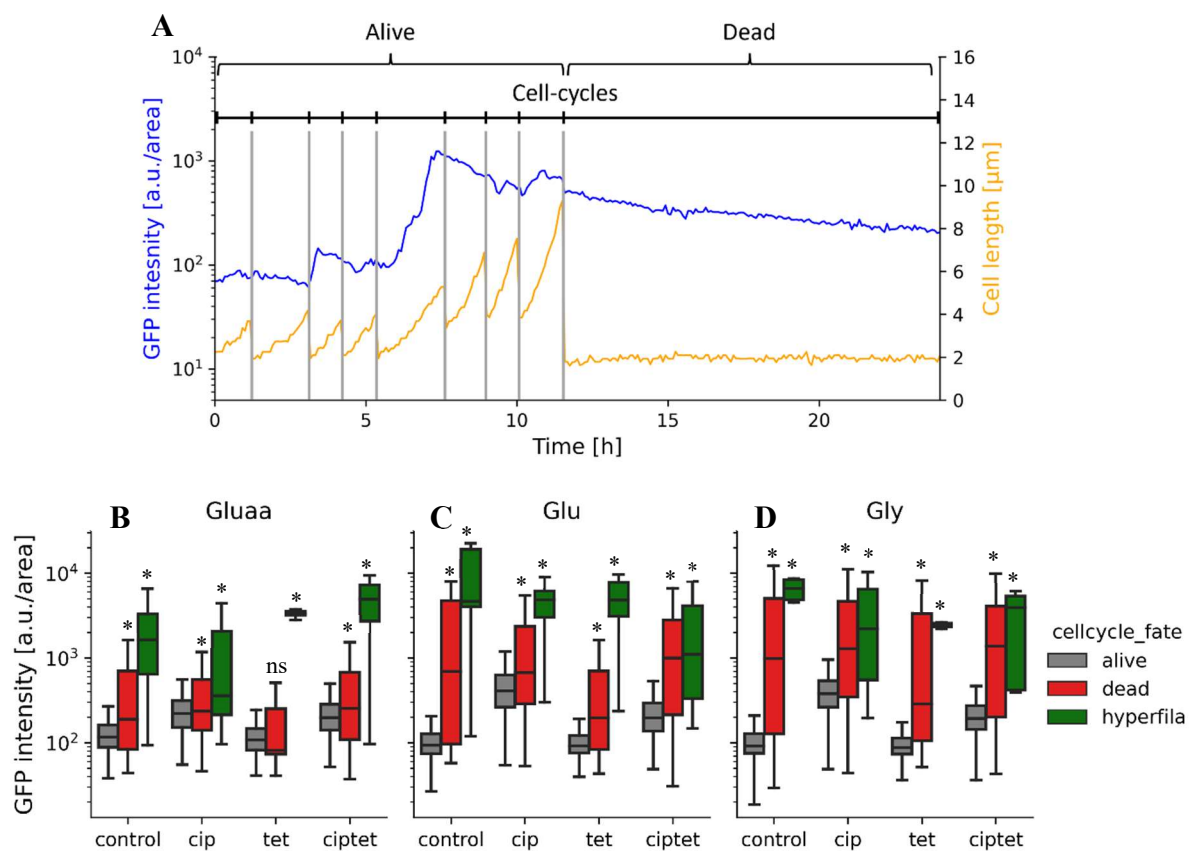


Figure 48. Dead and hyper-filamented cells have high SOS levels. **A.** Illustration of a mother cell trajectory separated by its constituent cell-cycles (grey vertical lines). Classification of ‘alive’ and ‘dead’ cell-cycles are indicated. **B-D.** Box-and-whisker plots showing SOS gene expression from *Psula-mGFP* separated by cell-cycle fate in different growth and treatment conditions. Different colours represent cell-cycles that have been classified as either alive (grey), dead (red), or hyper-filamented (green). GFP intensity was averaged over the duration

of each cell-cycle. Data is same as shown in Figure 47A-C. Statistically significant differences from the ‘alive’ data is indicated for the ‘dead’ and ‘hyperfila’ data in each condition. * $p < 0.005$, ns = not significant (Mann-Whitney U-test).

5.7 Antibiotics exhibit a minimal effect on constitutive gene expression

The effect on overall constitutive gene expression under all antibiotic treatments was minimal in the three growth conditions (Figure 49). A second sub-population of cells deviated from the main population, displaying a terminal decline in *mKate* intensity at a similar rate (likely photobleaching); these were more frequent in CIP and CIP+TET (Figure 49A-C, G-I, indicated by black arrow). These lineages correspond to those that stopped elongation (Figure 41H) and suggests protein synthesis has stopped and the cells are likely dead or dying.

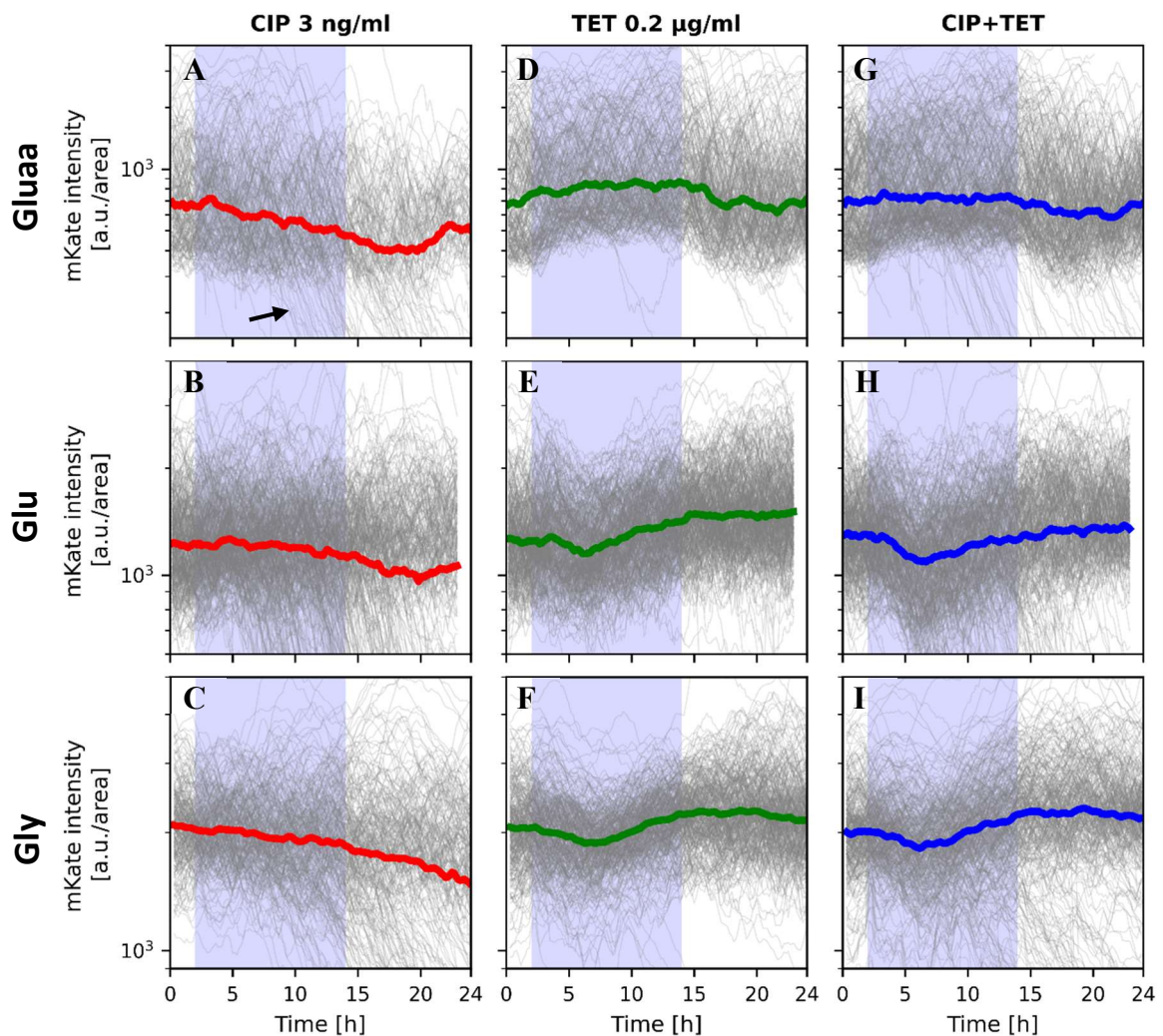


Figure 49. Single-cell trajectories of constitutive expression from *PtetO1-mKate2* fluorescent reporter for different growth media under 3 ng/mL ciprofloxacin (A-C), 0.2 µg/mL tetracycline (D-F) and drug combination (G-I) treatment. Solid lines represent the median and grey lines represent individual lineage trajectories. Black arrow indicates

trajectories that have deviated from the main population. Shown is data from one experimental replicate for strain JKB43. Antibiotics are introduced between hours 2-14, indicated by the blue shaded area. *mKate* intensity trajectories have been smoothed with a moving average over a 5-frame window.

Distributions taken from the final 2 hours of antibiotic treatment show that constitutive levels increased slightly under TET and CIP+TET treatment in fast growing cells (Figure 50A). However, we expect constitutive gene expression for a steady-state culture to decrease when exposed to a translation inhibitor (Scott *et al*, 2010). The effects of TET at different concentrations were investigated further in Appendix C3, but to summarise, we think that the TET concentrations used here were too low to observe the expected decrease in constitutive expression levels. The quantitative difference in response to TET between SOS expression (Figure 47) and constitutive expression was probably due to differences in promoter binding affinity and the fact that the *PtetO1* promoter is not in its native position on the chromosome.

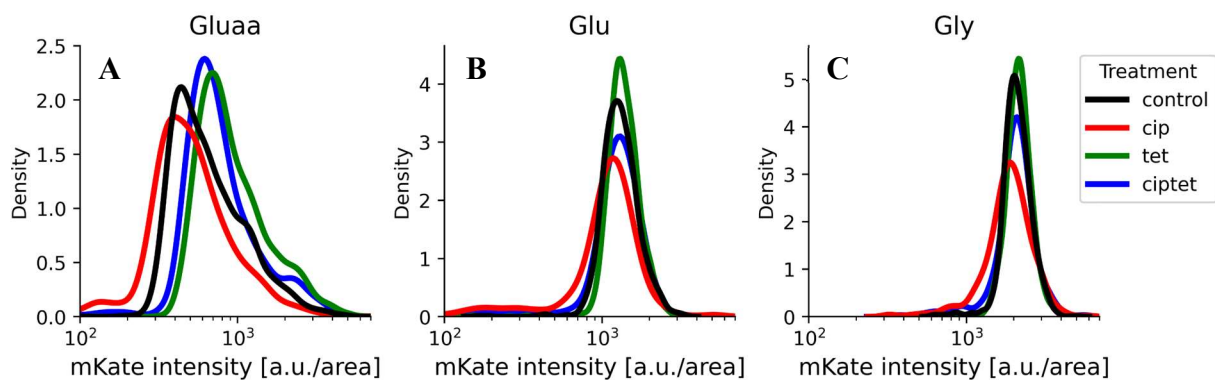


Figure 50. Distributions of single-cell constitutive expression from *PtetO1-mKate2* for different growth media and treatment conditions. Distributions are kernel density estimates of the underlying histogram pooled from the final 2 hours of antibiotic treatment from at least two experimental replicates for strain JKB43. Distributions for each experimental replicate are shown in Appendix C2 and show good day-to-day reproducibility. Colours represent the different treatment conditions.

In addition, translation inhibitors are expected to have a larger effect on constitutive levels in slower growing cells (Scott *et al*, 2010; Klumpp & Hwa, 2014). In *glu* and *gly* media, median *mKate* intensity (for the timelapse plots) for TET and CIP+TET initially decreased slightly before recovering to normal levels (Figure 49D-F), but the effect was subtle, and not reflected in the distributions in Figure 50. Perhaps this represents the cells adapting to TET exposure. So, we observed the expected decrease in constitutive levels in slow growth conditions, but our results show that cells can, on average, return to normal constitutive levels during TET treatment.

5.8 Cell length and filamentation under antibiotic combination

Filamentation was frequent under CIP (Figure 51A-C) and CIP+TET (Figure 51G-I) treatment and rare under TET treatment (Figure 51D-F). Filamentation was also correlated with growth rate, occurring more frequently in fast-growing cells. In glu and gly media, filamenting cells elongated slower and the majority reach intermediate cell lengths, whereas fast-growing cells in gluaa media elongated rapidly and often extended beyond the maximum length of the mother machine microchannels. After antibiotic treatment, filamentation decreased especially for the CIP-treated cells in gluaa (Figure 51A).

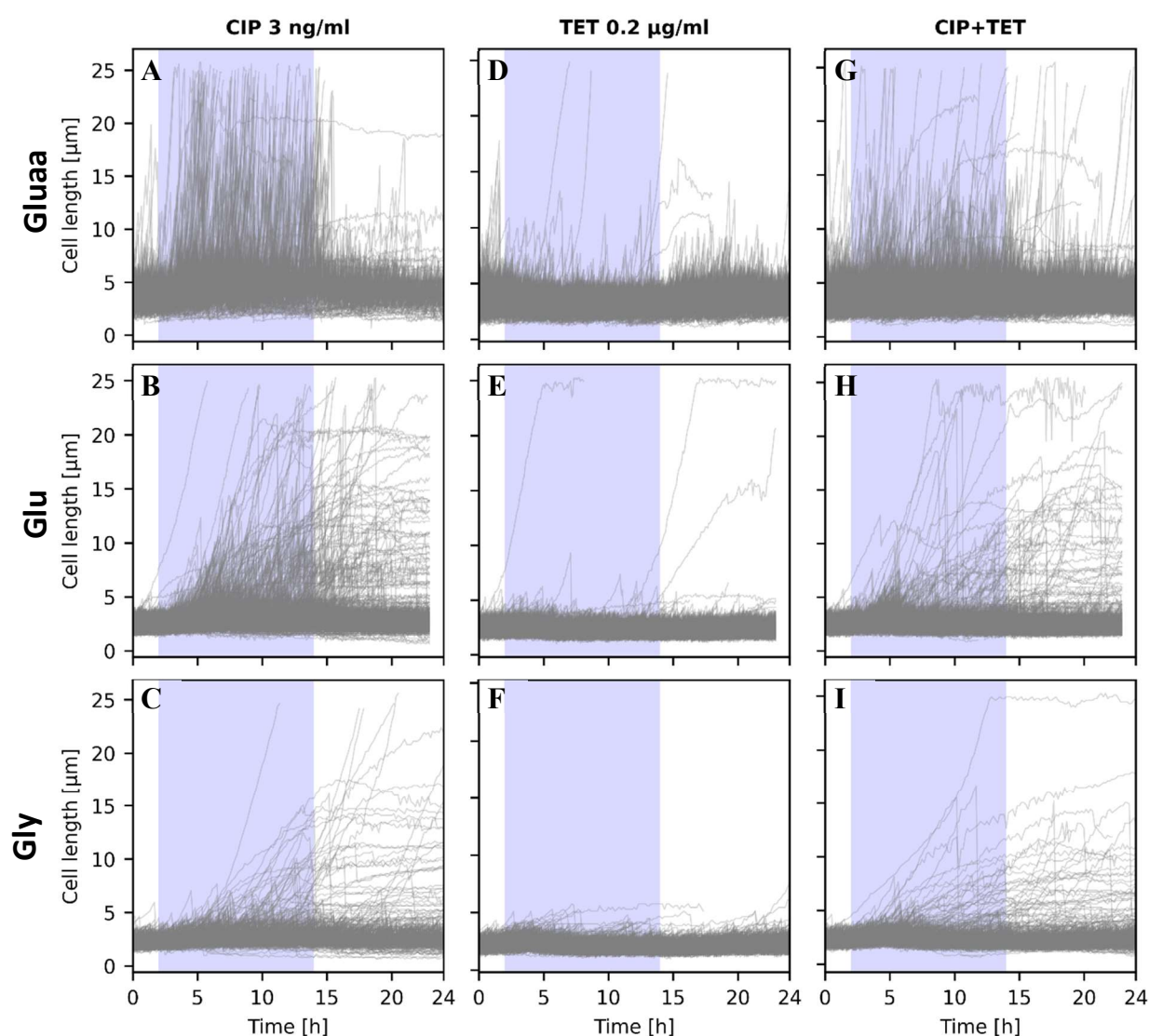


Figure 51. **Single-cell trajectories of cell length for different growth media under 3 ng/mL ciprofloxacin (A-C), 0.2 μg/mL tetracycline (D-F) and drug combination (G-I) treatment.** Grey lines represent individual lineage trajectories. Shown is data from one experimental

replicate for strain JKB43. Antibiotics are introduced between hours 2-14, indicated by the blue shaded area.

In all conditions, the birth and division length of cells under CIP treatment were elevated compared to the no-drug control (Figure 52). Adding TET with CIP, in all growth conditions, restores birth and division lengths to levels similar to the no-drug control (Figure 52, blue distributions), indicating that cell division dynamics returned to normal under the drug combination despite having elevated SOS levels (see Figure 47A-C). Under TET treatment, we observed lower birth and division lengths which is consistent with reduced elongation and division rates observed previously.

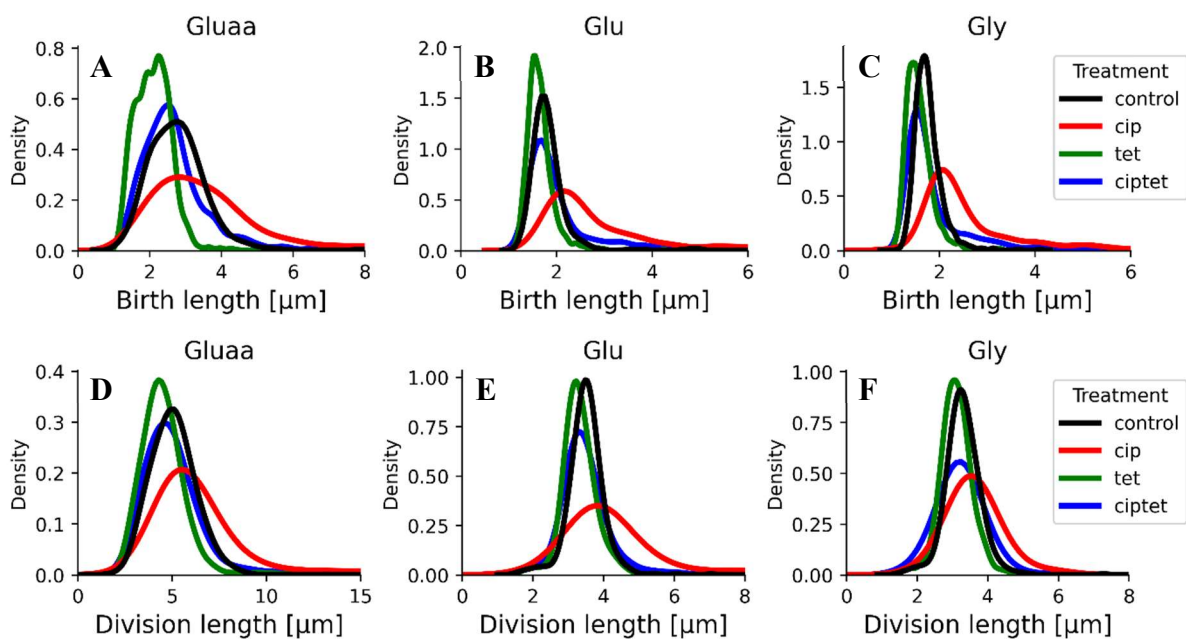


Figure 52. Distributions of single-cell birth (A-C) and division lengths (D-F) for different growth media and treatment conditions. Distributions are kernel density estimates of the underlying histogram pooled from the final 2 hours of antibiotic treatment from at least two experimental replicates for strain JKB43. Distributions for each experimental replicate are shown in Appendix C2 and show good day-to-day reproducibility. Colours represent the different treatment conditions.

5.8.1 Filamentation is suppressed for the antibiotic combination

Lineages that hyper-filamented (length $>20 \mu\text{m}$) made up a relatively small proportion of all lineages (no more than 15.9% for CIP in gluaa) and were more frequent in fast growing cells and under CIP treatment (Figure 53). For the drug combination, hyper-filamentation was significantly reduced compared to CIP treatment in all growth conditions ($p < 0.05$, t -test).

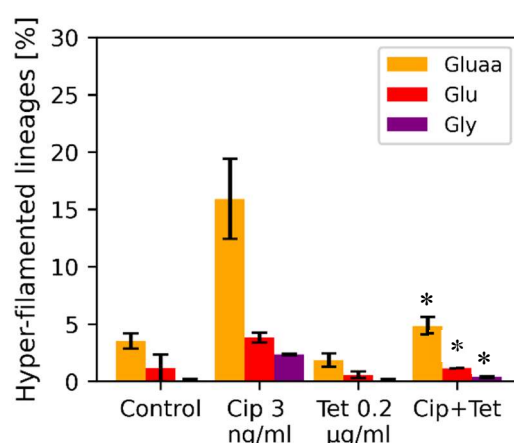


Figure 53. Percent hyper-filamented lineages after 12 hours of drug treatment and 10 hours of recovery for different growth conditions and antibiotic treatments. Hyper-filamented lineages are those that reached cell lengths above 20 μm (which is near the maximum microchannel length in the mother machine) in their final cell-cycle (i.e., lineage fate) and did not resume cell division for the duration of the experiment. Different colours represent the growth medium. Bars represent the mean and error bars the standard deviation of at least two experimental replicates. Data shown is for strain JKB43. Hyper-filamentation for CIP+TET was significantly lower than CIP in all growth media. *: $p < 0.05$, independent samples t -test.

We then calculated the cumulative fraction of lineages that had filamented as a function of time to evaluate whether the frequency of filamentation differs between treatments (Figure 54). Here, ‘filamented’ refers to cells that extend beyond double the average cell length at division for the no-drug control condition for each growth medium (filament threshold: gluaa = 10.55 μm ; glu = 7.26 μm ; gly = 7.06 μm). Since elongation rates differ markedly for each growth medium, the time it takes for cells to reach the filamentation threshold will vary. Thus, without normalising the filamentation threshold to the growth medium, the filamented fraction could be overestimated in the faster growth medium.

A growth-dependent response was observed in the fraction of filamented cells, with the highest frequency observed in faster growth conditions for all treatments (Figure 54). Between treatments, CIP resulted in the most filamentation, followed by CIP+TET, no-drug control, and then TET. Under CIP we expected cell filamentation due to DNA damage induced SOS expression. TET treatment reduced filamentation because it slows growth of the cell by inhibiting translation. Lower frequency of filamentation in CIP+TET was likely due to the action of TET inhibiting protein synthesis, which slows cell elongation (Figure 38), and lowering SOS expression (Figure 47). However, we are unsure whether the effect of TET could have other downstream effects that influence SOS expression and filamentation. Inhibition of

filamentation by translation inhibitors in quinolone-treated *E. coli* has been observed previously (Piddock *et al*, 1990; Ocampo *et al*, 2014).

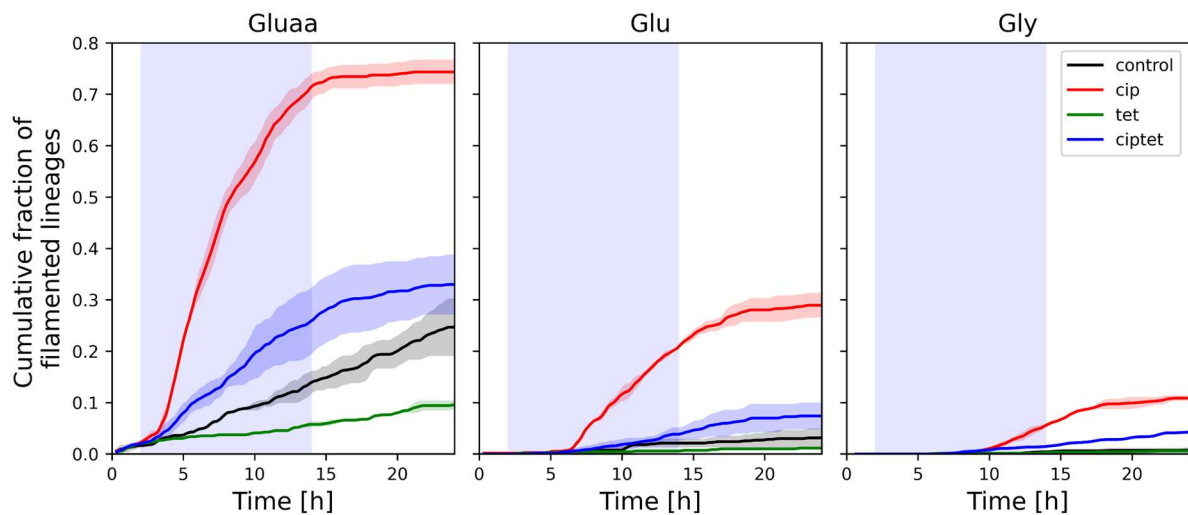


Figure 54. Cumulative filamentation fractions as a function of time for different treatment conditions under different growth conditions. The cumulative fraction is defined as the sum of the number of lineages that have filamented up until a certain point in time divided by the total number of lineages. A filamented cell was determined by a sliding threshold on the cell length, where the threshold was set at double the mean division length of the no-drug control condition for each growth medium. Line represents mean of at least two experimental replicates and shaded area represents standard deviation. Shaded blue area is the antibiotic treatment period. All data represented is for strain JKB43.

After antibiotic treatment ceased, filamentation slowed down (Figure 54). The respective treatments affected the filamentation fraction only after a slight delay, with the delay increasing with growth rate (approximately 3 hours in gluaa, 6 hours in glu, and 9 hours in gly). Although, the filamentation rate between mother and daughter cells has been shown to diverge (higher in mother cells) with increasing replicative age in the mother machine (Wang *et al*, 2010a). But Wang *et al.* (2010) found that filamentation rates only significantly diverged after 50 generations which amounts to approximately 33 hours for our strain in the gluaa medium. Therefore, the filamentation fraction observed in this study is likely representative of the whole population of cells.

5.9 Discussion

5.9.1 Improved cell survival is responsible for suppression between DNA synthesis and translation inhibitors

For many drug combinations, the specific mechanism underlying the interaction is poorly understood. There have been some notable papers in the last few years that have investigated why suppression occurs between DNA synthesis inhibitors and translation inhibitors (Bollenbach *et al*, 2009; Chevereau & Bollenbach, 2015; Angermayr *et al*, 2022). Bollenbach *et al.* (2009) suggested that treatment with a DNA synthesis inhibitor leads to an imbalance in the cellular DNA and protein content as a result of significantly slowed DNA synthesis relative to protein synthesis. This protein overproduction (due to excessive ribosome production) imposes a metabolic burden on the cell, thus reducing growth rates. Generally, rates of DNA synthesis and protein synthesis should be balanced to achieve optimal growth. However, the authors proved that this protein-DNA imbalance was a result of insufficient downregulation of ribosomal promoters since ribosomal genes are not directly regulated by DNA stress (Bollenbach *et al*, 2009). They then showed that the translation inhibitor corrects for this protein-DNA imbalance (by reducing overall protein synthesis), leading to “improved survival and growth”. This hypothesis is grounded in cell physiology and largely argues that cell volume growth (mass accumulation) improves under the drug combination. Yet, the experimental data acquired by Bollenbach *et al.* (2009), as well as most other works that studied antibiotic combinations (Chevereau & Bollenbach, 2015; Qi *et al*, 2021; Angermayr *et al*, 2022), are based upon population-averaged bulk growth rates which cannot discriminate between cell mass accumulation, birth and death, nor can it give an understanding of the underlying population dynamics. Though, their results do show an increase in the MIC under the drug combination – implying improved survival – the authors neglected to include cell death in their model (summarised in Figure 10). The overall (net) growth rate of a population is a function of the difference in the rate of cell growth (birth and elongation) and the rate of cell death. Improved bulk growth rates observed for the drug combination could therefore be due to an increase in single-cell growth and division, reduced cell death, or a combination thereof. So, which is it?

Using single-cell methods, we separately quantified single-cell growth (elongation rate and division rate) and death under single-drug treatment and the drug combination – the latter has not been investigated until now. Our experimental results showed that, at CIP concentrations

well below MIC, cell death occurs at a frequency much higher than without antibiotic (Figure 43), with no significant reduction in elongation rates for the growing fraction of cells (Figure 38A-C, Figure 39A-C). Similar observations have been reported previously for CIP concentrations up to 8 ng/mL (Brandis *et al*, 2023). Not only did we quantify cell survival, but we also accurately measured the dynamic change in the rate of cell death with high reproducibility (Figure 44). We observed a significantly lower rate of death for the drug combination compared to CIP-treated cells. Although division rates were slightly reduced (Figure 39D-F), this data proves that cell death is a dominant outcome for cells exposed to sub-lethal levels of CIP. As for TET, we observed significantly lower single-cell elongation and division rates with no change in death rates, which is expected for a bacteriostatic translation inhibitor. Quantifying the rate of death at the single-cell level as a function of time, to our knowledge, has not been done before for antibiotic and antibiotic combination therapy.

Since elongation rate is a proxy measurement for the rate of mass accumulation in the cell, we showed that mass accumulation (protein production) is largely unaffected by CIP. In fact, this observation is consistent with the Bollenbach *et al.* (2009) observation that ribosomal expression is not sufficiently downregulated under DNA stress. Under the drug combination, single-cell survival significantly increased with no increase in elongation rates compared to TET-treated cells (Figure 42, Figure 43, Figure 44). However, we did observe a slight increase in division rates (only in fast growth conditions) which suggests an antagonistic effect; yet this is not sufficient to produce suppression observed in bulk experiments. Interestingly, single-cell elongation rates, division rates, and constitutive gene expression for the drug combination behaved almost identically to that under TET treatment alone. This is in agreement with reports that show that the growth-limiting antibiotic dominates the cellular response for antagonistic drug pairs (Bollenbach & Kishony, 2011). Therefore, our results show that increased cell survival, rather than increased single-cell growth, is responsible (or the dominant mechanism) for the suppressive effect observed between CIP and TET. Consistent with our results, Coates *et al.* (2018) found that the extinction (death) probability of microbial colonies (using CFU plate counts) reduced significantly when sub-MIC TET is added to CIP. Also, at above MIC levels, Ocampo *et al.* (2014) showed that TET prevents cell death and filamentation from nalidixic acid, another gyrase inhibitor, using CFU plating as well as mother machine experiments (observed an ~84% increase in single-cell survival). To be clear, we are not refuting the model proposed by Bollenbach *et al.* (2009); in fact, their results are convincing and are therefore an important part of the story. But their model does not take into account cell

death and single-cell heterogeneity which may have important consequences on the net growth of a population. Also, absorbance-based growth rate measurements employed by Bollenbach *et al.* (2009) and others could be confounded by cell size changes induced under DNA stress (Stevenson *et al.*, 2016), leading to over-estimations of bulk growth rates as we showed in Chapter 4.

5.9.2 Suppression of cell death is growth-dependent

We found that single-cell survival under CIP+TET was significantly higher in fast-growth conditions (Figure 42B, C). This result is puzzling because susceptibility to CIP and TET individually were found to correlate positively with growth rate (when modulated by nutrient quality) in both bulk and single-cell experiments; and this is the expectation based on the literature (Greulich *et al.*, 2015; Haugan *et al.*, 2019; Angermayr *et al.*, 2022). Improved survival under the CIP+TET combination could, in theory, be due to a lower growth rate imposed by the action of TET. However, we found that survival under the drug combination correlated with faster growth rates which contradicts this notion. Furthermore, when growth (elongation rate) was reduced by 75% via nutrient limitation (from gluaa to gly medium), only a minor increase in survival, about 17%, was observed under CIP treatment. But, when growth was reduced by 15% via translation inhibition, survival increased by up to 53%. Thus, it is unlikely that the reduced efficacy of CIP under translation inhibition can be explained by general physiological responses to slow growth, though it may play a role. However, it should be noted that the physiological composition of the cell under translation inhibition differs markedly from that under nutrient limitation (Scott *et al.*, 2010), so this is not unexpected.

Growth-dependency of suppression may be explained by differences in how the antibiotic is perceived by cells in the different growth media. We exposed cells to 0.2 $\mu\text{g/mL}$ TET in all growth conditions. But the antibiotic is more effective in fast-growing cells (see growth-dependence in the IC_{50} , Figure 30D). This can be seen by the relatively larger effect on single-cell elongation and division rates in faster growing cells (Figure 38C, F). In fact, elongation rates were unchanged in gly media under TET. Therefore, the higher efficacy of TET in fast growth could explain amplified suppression in these conditions because it may be closer to the optimal concentration for suppression in this medium. This is not unexpected since the strength of suppression does change over different TET concentrations around an optimal value (Figure 30B, C).

Fast growing cells likely acquire DSBs more frequently due to the higher number of replication forks and gyrase targets. It is possible that reduced growth from translation inhibition by TET leads to a smaller number of replication forks per cell-cycle (and fewer targets), thereby reducing the efficacy of CIP. This could explain the larger increase in survival for the drug combination observed in the fast-growth medium compared to the slow-growth medium (Figure 42C). According to this model, the reduction in growth imposed by TET would have limited benefit in the slow-growth medium since the average number of replication forks per cell-cycle in this medium are close to zero (Cooper & Helmstetter, 1968).

5.9.3 Cell death is dominant for the non-growing phenotype

Defining if and when a cell is dead is not trivial: It can occur rapidly, expressed as an immediate lysis or bursting of the cell, or resemble a “terminal illness” which eventually leads to the collapse of physical and genetic integrity (Baquero & Levin, 2021). We did not observe significant cell lysis under the antibiotic treatments at the concentrations tested, but we did observe a large proportion of cells that: stopped elongation and division; did not resume growth after antibiotic treatment stopped; and appeared to maintain physical integrity for the remainder of the experiment. We want to be sure that the cells we classified as dead are, in fact, dead. Although we quantified cell survival based upon a lack of cell elongation and division, there is more nuance to this phenotype. Non-growing cells can of course be persister cells or VBNC cells, both of which are clinically significant because they can seed a new infection following antibiotic treatment (and are therefore important to consider). We will discuss the occurrence of both of these below.

Persister cells can be identified as dormant, non-growing cells that survive antibiotic treatment and can resume growth sometime after treatment stops (Balaban *et al*, 2019). The recovery period in our experiments was set at 10 hours. However, lag times for persister cell re-growth can take as long as 30 hours in rare cases (Kaplan *et al*, 2021). We then evaluated whether an extended no-drug period (up to 36 hours) would improve the chances of cell recovery but did not observe a noticeable improvement in recovery of non-growing cells. However, we found a few persister cells (five in total, 0.2-1% of cells) in some of the glycerol datasets (Appendix C4, Supplementary Figure 16). Consistent with our results, Zhang *et al*. (2023), using the mother machine, similarly reported that persistence was extremely rare in stationary phase cells under CIP treatment at MIC (1% of cells) and ampicillin treatment at 25X MIC (0.3% of cells). In our study, these cells were initially not growing from the beginning of the experiment, in

two cases survived antibiotic treatment (CIP and CIP+TET), and then resumed growth after approximately 30 hours, thus satisfying the classical definition for spontaneous persister cells (Balaban *et al*, 2019). Interestingly, these cells all maintained a baseline level of constitutive gene expression (around 1000 a.u. in gly medium) which suggests they still exhibited a low level of protein synthesis and metabolic activity, consistent with previous reports (Bamford *et al*, 2017; Shan *et al*, 2017; Yin *et al*, 2019; Bergkessel & Delavaine, 2021; Mancini & Pilizota, 2023). In contrast, for the vast majority of cells that stopped growth and division in our experiments, constitutive gene expression stopped completely and did not recover (Figure 40B-F, Figure 41H). Therefore, it is likely that the cells observed to stop growth under antibiotic treatment in this study are not persister cells capable of resuming growth.

We also investigated whether these non-growing cells could be VBNC cells by checking their membrane viability. Viability dyes have been used previously to determine cell death and membrane viability in the mother machine (Bamford *et al*, 2017; Rossi *et al*, 2019; Yang *et al*, 2019, 2023; Zhang *et al*, 2023). We exposed cells to 3 ng/mL CIP for 12 hours followed by a 16 hour period without antibiotic. We identified, using a membrane viability dye, that 35% of cells continued growing, 22.5% stopped growth and were non-viable, and 42.5% stopped growth but were membrane-viable (see Appendix C5, Supplementary Figure 17). This suggests that VBNC cells (the latter group) may constitute a significant proportion of cells that stopped growth under CIP treatment, which is significantly higher than observed previously in related studies (Bamford *et al*, 2017; Zhang *et al*, 2023). However, our conditions differ from the cited studies in important ways which could account for these differences. We used a different dye: SYTOX Green instead of propidium iodide which was used in the cited studies. The difference in sensitivity between propidium iodide and SYTOX Green dyes could differ and should be compared in future experiments. We also used much lower CIP concentration: sub-MIC instead of above MIC. The majority of cells (94%) exposed to above MIC CIP concentrations lysed in the mother machine as reported by Bamford *et al*. (2017), whereas cell lysis was rare in our experiments. But, the population fraction of VBNC cells have been shown to be concentration-dependent and antibiotic-dependent (Zhang *et al*, 2023). For example, ampicillin is a cell wall-targeting antibiotic which may disrupt membrane integrity more so than other antibiotics. Furthermore, in this study we used exponential phase cells instead of stationary phase cells for the inoculum, as well as a different growth medium (M9-based instead of LB medium). We also observed, as discussed previously, a consistent decline in constitutive fluorescence levels for the vast majority of non-growing cells which indicates that constitutive gene expression has

stopped completely. In contrast, VBNC cells identified by Bamford et al. (2017) appeared to maintain some level of metabolic activity and gene expression, similar to persister cells.

And lastly, we want to note that membrane depolarisation (dye uptake) typically occurred only after a significant delay since elongation stopped (sometimes up to 18 hours delay; Supplementary Figure 17E-H). Therefore, it is possible that most of the cells would lose viability if monitored for a longer duration, particularly those that stopped growth near the end of the experiment. This is not unsurprising since it is conceivable that cell death could occur without an immediate loss of membrane viability, particularly for antibiotics that do not directly target the cell wall or membrane, and for treatment at sub-MIC levels.

Here we show, as others have previously, that growth arrest is a nuanced phenomenon and can include various clinically important phenotypes (cell death, persisters, VBNC cells). Care should be taken to accurately classify cells that appear phenotypically similar. Quantifying the different fates of antibiotic-treated cells with this level of specificity can enable the optimisation of drug treatments that maximise cell death and minimise tolerant phenotypes.

5.9.4 The SOS response is not responsible for the suppression of cell death

Induction of the SOS response is vital for the survival of cells under DNA stress. We tracked the expression of the SOS response in single cells exposed to the antibiotics and found that SOS induction was growth rate dependent. This may be due to a reduced capacity to induce stress response genes in fast-growing cells because the reallocation of translational resources to sectors other than ribosome synthesis are constrained in fast-growth conditions (Scott *et al*, 2010; Hui *et al*, 2015). Translation inhibition by TET reduced SOS levels in CIP-treated cells in the intermediate and slow-growth medium, but not in the fast-growth medium (Figure 47A-C). In fact, SOS distributions were almost identical for CIP and CIP+TET treatment in fast growth, yet survival was 40% higher in the latter. Therefore, the SOS response, though crucial for cell survival under DNA damage, is not responsible for the suppression of cell death for this antibiotic combination. Some other mechanism is involved in improving cell survival that depends on the growth medium. Further experiments should confirm this using a strain devoid of SOS response induction. Moreover, SOS expression under CIP and CIP+TET in the fast-growth condition almost completely lacked the high-SOS sub-population seen in the intermediate and slow-growth conditions (Figure 47F). This suggests that higher levels of SOS induction may not necessarily mean better survival under DNA stress. In fact, cells with very

high levels of SOS are predominantly those that have stopped growth (dead) or have filamented (Figure 48B-D).

5.9.5 How could tetracycline improve cell survival?

We next asked how translation inhibition could improve cell survival under DNA stress. Besides the physiological mechanism proposed by Bollenbach *et al.* (2009) (described in Section 5.9.1), we sought to identify other mechanisms which could relate directly to cell survival. These could include attenuation of ROS-mediated killing, upregulation of stress responses, and SOS-regulated toxin-antitoxins.

The formation of ROS, which is dependent upon aerobic respiration, and subsequent oxidative damage is a purported secondary effect of fluoroquinolone treatment that contributes to cell death (Goswami *et al.*, 2006; Dwyer *et al.*, 2007, 2014; Kohanski *et al.*, 2007; Wang & Zhao, 2009; Belenky *et al.*, 2015; Kottur & Nair, 2016; Smirnova *et al.*, 2017; Hong *et al.*, 2019, 2020; Pribis *et al.*, 2019; Wong *et al.*, 2021). However, ROS-mediated killing by bactericidal antibiotics has been disputed (Keren *et al.*, 2013; Imlay, 2015). Nonetheless, if we accept the ROS-mediated killing hypothesis, which an increasingly larger body of evidence supports, this opens up another possibility for suppressed killing for the CIP+TET combination.

Cellular respiration, metabolic activity/flux, and ROS levels have been shown to be elevated under fluoroquinolone treatment (Kohanski *et al.*, 2007; Akhova & Tkachenko, 2014; Dwyer *et al.*, 2014; Belenky *et al.*, 2015; Lobritz *et al.*, 2015; Gutierrez *et al.*, 2017; Lopatkin *et al.*, 2019). On the other hand, translation inhibitors have been shown to decrease cellular respiration (Lobritz *et al.*, 2015); downregulate important metabolic pathways including the citric acid cycle, pyruvate metabolism, glycolysis, and gluconeogenesis (Lin *et al.*, 2014); and reduce the accumulation of ROS in fluoroquinolone-treated *E. coli* (Wang *et al.*, 2010b). Lobritz *et al.* (2015) reported significantly improved survival of norfloxacin (a fluoroquinolone similar to CIP) treated cells when combined with translation inhibitors (TET and chloramphenicol) possibly due to suppression of respiration (measured via oxygen uptake rate). Moreover, in *Staphylococcus aureus*, chloramphenicol treatment resulted in the accumulation of ATP, NADH, central carbon metabolites, amino acids, nucleotides, and lipids (Lobritz *et al.*, 2015), which is consistent with reduced respiratory and metabolic rates. To further confirm the role of respiration in cell killing, genetically-enhanced respiration resulted in improved killing, whereas genetically-reduced respiration attenuated killing (Lobritz *et al.*, 2015). Butler *et al.* (2023) found that CIP treatment can increase the levels of misfolded proteins in the cell.

Misfolded proteins (and their loss of function) can result from damage by ROS which are associated with fluoroquinolone treatment (Imlay, 2008). The authors found that cells with fewer misfolded proteins had higher survival times. Since protein synthesis rates are likely correlated with the level of misfolded proteins, and fluoroquinolones are known to result in excess protein synthesis (Bollenbach *et al*, 2009), reducing protein synthesis and protein misfolding via translation inhibitors could improve survival under CIP exposure. Translation inhibition by TET could also limit the synthesis of electron transport proteins, thereby reducing ROS formation through respiration. Given the above, it is possible that reduced metabolic activity induced by TET plays a role in preventing ROS-mediated cell death.

However, the ROS-mediated killing hypothesis does run into a problem. With an increasing growth rate, cells begin to transition to the more proteome-efficient fermentation pathway for ATP synthesis (Basan *et al*, 2015). Fast-growing cells carry out a mixture of respiration and fermentation with an increasing proportion of the proteome devoted to the latter with faster growth rates. Whereas slow-growing cells exclusively carry out respiration (Basan *et al*, 2015). Therefore, the capacity to produce ROS through respiration could be limited in fast-growth conditions which is not consistent with the higher efficacy of CIP observed in fast-growing cells in our experiments (Figure 42C). This adds further uncertainty to the ROS-mediated killing hypothesis which is still controversial in this field of research (Keren *et al*, 2013; Imlay, 2015). Further experiments and the development of more precise techniques to measure ROS and oxidative damage in bacteria are needed.

Besides the role that translation inhibitors play in reducing respiration and subsequent ROS generation, they may attenuate killing by upregulating stress responses. For example, TET has been shown to trigger a rapid induction of the oxidative stress response (2-fold upregulation in oxidative stress promoters) in *E. coli* (Mitosch *et al*, 2017). This may provide a level of protection against ROS-mediated damage induced by CIP.

Lastly, the expression of SOS-controlled TA modules may also contribute to cell death, particularly in cells with high levels of SOS induction. It is possible that translation inhibitors limit synthesis of the toxin, leading to improved cell survival. On the other hand, SOS-controlled TA modules, such as *tisAB*, have been shown to increase tolerance to fluoroquinolones (Dörr *et al*, 2010; Ronneau & Helaine, 2019; Su *et al*, 2022). One theory states that the TisB toxin forms pores in the inner membrane resulting in a decrease of proton motive force and ATP synthesis, thereby inducing dormancy and antibiotic tolerance (Unoson

& Wagner, 2008; Dörr *et al*, 2010). Nonetheless, overexpression of the TisB toxin (but above what would be possible for a single gene copy) has been shown to be highly toxic and can result in a 100-fold decrease in viable cell counts (Unoson & Wagner, 2008). Further experiments should be conducted to confirm the role, if any, of TA modules in the suppression between CIP and TET. For example, one could compare survival with a mutant devoid of several SOS-controlled TA modules.

5.9.6 Growth-dependence of SOS induction depends on the source of DNA damage

In this chapter, we found that the average SOS expression of the main population was higher in slow-growth conditions under DNA damage induced by CIP (Figure 47). However, we observed the opposite trend in cells undergoing chronic, replication-dependent DNA damage (2-pal strain) and spontaneous DNA damage (WT strain) – the average of the main population was higher in fast-growth conditions (see Chapter 4, Figure 19A). This may reflect the difference in how DNA damage occurs in the 2-pal strain and under CIP treatment. DNA damage for the 2-pal strain is replication-dependent, and thus will occur (and accumulate) at a higher frequency in fast-growing cells due to faster DNA replication rates. Whereas for CIP treatment, DNA damage is randomly acquired and can also occur anywhere on the chromosome (DSBs for 2-pal occur at fixed locations). But at the same time, CIP likely has a higher target abundance in fast-growth conditions due to a higher number of replication forks per cell-cycle and therefore, at least intuitively, a higher number of gyrase molecules. A reduced capacity to induce stress response genes in fast-growth conditions may be responsible for lower average SOS expression and the lack of SOS induction to high levels under CIP treatment. This is because reallocation of translational resources to sectors other than ribosome synthesis are constrained in fast-growth conditions (Scott *et al*, 2010; Hui *et al*, 2015).

Furthermore, we hypothesise that improved DNA repair efficiency in the fast-growth condition may be responsible for lower levels of high-SOS induction events. Fast-growing cells with a doubling time below ~60 minutes carry out multi-fork replication and thus will have a higher number of potential alleles as a substrate for HR-mediated DNA repair. This is consistent with our data where we found a significant reduction in high-SOS levels in the *gluaa* growth medium that supported a division rate below 60 minutes (average *gluaa* division rates = 40 min), whereas the high-SOS fractions for growth media supporting division rates above the 60 minute threshold (*glu* and *gly*) were much higher (Figure 47F).

Therefore, we suspect that the replication-dependent nature of DNA damage is far more toxic in fast-growing cells compared to slow-growing cells, leading to higher average SOS levels in the 2-pal strain. Whereas, under CIP treatment, physiological constraints and improved DNA repair may be dominant in producing growth-dependence.

Chapter 6: Conclusions

Single-cell microfluidics combined with time-lapse quantitative microscopy is a powerful technique that gives us the capability to measure different aspects of bacterial growth that are not possible using bulk-level methods. In this thesis, we used this technique to investigate the underlying population dynamics of SOS response induction and growth under a suppressive antibiotic combination. In doing so, we discovered some important insights that were not previously reported.

First, we found SOS induction to be highly heterogeneous at the single-cell level, with a fraction of the population exhibiting high levels of expression. The rate of high-SOS induction for a strain experiencing sub-lethal, replication-dependent DNA damage was positively correlated with growth rate. However, strong induction of the SOS response induced a disparity in division rates among sub-populations with different SOS levels. This has consequences on the population dynamics of a growing culture: Although high-SOS cells are produced more frequently in fast growth, they are outcompeted by low-SOS, fast-dividing cells. High-SOS cells are outcompeted to a larger extent in fast-growth media compared to slow-growth media resulting in a smaller steady-state fraction in bulk cultures. This is consistent with previous bulk-level observations.

Next, we investigated the physiological mechanisms that give rise to the suppressive interaction between CIP (a DNA synthesis inhibitor) and TET (a translation inhibitor). Single-cell methods allowed us to separately quantify the effects of antibiotics and their combinations on elongation rates (mass accumulation), division rates, and death rates. Our results show that an improvement in cell survival, rather than increased biomass growth, is the dominant mechanism leading to suppression between CIP and TET. Single-cell methods proved vital in revealing this fact that was otherwise masked by population-averaged methods and could have implications for how data reported in the literature so far is interpreted. Furthermore, the suppressive effect on survival was amplified in fast-growth conditions which could have important implications on treatments in fluctuating nutrient environments. So far, no studies have thoroughly considered the effect of growth medium on this suppressive interaction.

Interestingly, in the fast growth medium, survival improved under the antibiotic combination, but it did so without any change in SOS expression compared to CIP-treated cells. This could mean that some other mechanism is also responsible for improving cell survival that depends

on growth medium. We discussed several mechanisms that could be involved in the suppression of cell death but further experiments should be conducted to confirm these hypotheses. More definitive studies investigating antibiotic-induced cell death and survival, and its physiological causes, would go a long way in improving understanding of antibiotic combinations and increasing their efficacy in the clinic.

Several coarse-grained models have been developed to predict cellular growth rates and physiological responses under various nutrient and stress conditions. Though these models in many cases successfully approximate bacterial responses, many fail to fully appreciate the influence of heterogeneity and population dynamics at the single-cell level. This is also the case when interpreting data from bulk-level methods. One should consider the context of the system and perturbation in question and whether the perturbation (e.g. antibiotic treatment) induces heterogeneity in the system. Single-cell techniques can give us more insight into the behaviours of individual cells to better estimate emergent population-level effects closer to first principles. Directly measured death rates under antibiotic treatment can be used to optimise the duration of antibiotic treatment, which can have positive clinical outcomes for patients treated with antibiotics that are toxic for the human body.

We started work on another suppressive drug combination: trimethoprim (TMP) and TET (see Appendix C7). TMP is a folate synthesis inhibitor which limits the synthesis of nucleotides and amino acids (Hitchings & Smith, 1980). Like CIP, TMP results in DNA damage and induction of the SOS response, but also has numerous downstream metabolic effects (Rao & Kuzminov, 2019). In studying this combination, we were interested in how suppression emerges for an antibiotic with a slightly different effect than CIP: TMP limits the *synthesis* of nucleotides and is bacteriostatic in its effect. In summary, we found, using agarose-pads, a highly heterogeneous effect on cells exposed to TMP, but only in fast-growth conditions: a significant increase in cell thickness, cell lysis, filamentation, protoplast formation, and frequent occurrence of cytoplasmic voids (Supplementary Figure 20C and Supplementary Figure 22). Adding TET to TMP-treated cells completely removed cell size abnormalities (Supplementary Figure 20D and Supplementary Figure 21D) and reduced SOS gene expression to levels below that of the no-drug control (Supplementary Figure 21A), suggesting a complete reversal in DNA damage induced by TMP. This work shows how suppression between TMP and TET manifests at the single-cell level, which has not been reported previously.

We urgently need improved treatment strategies to tackle antimicrobial resistance. Antibiotic combination therapy provides a cost-effective solution that can restore efficacy of old drugs and reduce the evolution of resistance. Altogether, our results highlight the utility of single-cell time-lapse methods in revealing population dynamics and single-cell heterogeneity that would otherwise be masked in bulk-level methods. In furthering our understanding of bacterial responses to antibiotics and their combinations, we aim to contribute to the development of more effective treatment strategies.

Appendix A: List of strains, plasmids, and primers

Supplementary Table 1. **List of strains.**

Strain	Background	Genotype	Source
eSJR203	MG1655	rph-1 λ^- F- P21: <i>Ptet01-mKate2</i>	Jaramillo-Riveri (2019)
eSJR206	MG1655	rph-1 λ^- F- <i>HK022:PsulA-mGFP</i> P21: <i>Ptet01-mKate2</i>	Jaramillo-Riveri (2019)
eSJR302 (‘2-pal’)	MG1655	eSJR206 <i>lacI^q</i> <i>lacZ::pal246 cynX::Gm^R</i> <i>asbB::pal246 ascF::Kn^R</i>	Jaramillo-Riveri (2019)
JW1879-2	BW25113	rph-1 λ^- F- <i>hsdR514</i> $\Delta(\textit{araD-araB})567$ $\Delta(\textit{rhaD-rhaB})568$ $\Delta\textit{lacZ4787}(\textit{::rrnB-3})$ $\Delta\textit{motA743}::\textit{Kn}^R$	Gift from Rosalind Allen (CGSC 9565) (Baba <i>et al</i> , 2006)
JKB22	MG1655	eSJR206 $\Delta\textit{motA}::\textit{Kn}^R$	eSJR206 P1 using JW1879-2
JKB43	MG1655	JKB22 $\Delta\textit{motA}$	JKB22 pE-FLP using pSJR017
JKB17 (BHC97)	MG1655	rph-1 λ^- F-	Genomic Stock Centre (CGSC7740)
JKB33	MG1655	JKB17 $\Delta\textit{motA}::\textit{Kn}^R$	JKB17 P1 using JW1879-2
JKB51	MG1655	JKB17 $\Delta\textit{motA}$	JKB33 pE-FLP using pSJR017
JKB55	MG1655	JKB51 <i>HK022:PsulA-mKate2::Kn^R</i>	JKB51 CLI using pSJR080
JKB63	MG1655	JKB55 <i>HK022:PsulA-mKate2</i>	JKB55 pE-FLP using pSJR017
JKB18	MG1655	eSJR203 $\Delta\textit{motA}::\textit{Kn}^R$	eSJR203 P1 using JW1879-2
JKB39	MG1655	eSJR203 $\Delta\textit{motA}$	JKB18 pE-FLP using pSJR017

Supplementary Table 2. **List of plasmids.**

Plasmid	Purpose	Source
pSJR017	Clone-integration marker excision	pE-FLP (St-Pierre <i>et al</i> , 2013)
pSJR021	Clone-integration at HK022 site	pOSIP-HK (St-Pierre <i>et al</i> , 2013)
pSJR080	<i>PsulA-mKate2</i> insertion by CLI	pSJR021 backbone (Jaramillo-Riveri, 2019)

Supplementary Table 3. **List of primers.**

Primer	5'-3' Sequence	Purpose
oSJR58	GGAATCAATGCCTGAGTG	HK022 insertion verification
oSJR59	ACTTAACGGCTGACATGG	HK022 insertion verification
oSJR60	ACGAGTATCGAGATGGCA	HK022 insertion verification
oSJR61	GGCATCAACAGCACATTC	HK022 insertion verification
oJB01	CTTTTGGCTTTGCGTCGTTTG	<i>motA</i> deletion verification FW
oJB02	TTCCCAGAATCCTGCCGATA	<i>motA</i> deletion verification RV

We compared mean elongation rates measured in the mother machine between strains SJR206 and JKB43 to evaluate whether removing the *motA* gene alters the physiology of the cell. For instance, the presence of the *motA* gene could add to the metabolic burden of a cell and reduce its growth rate. We found that single-cell elongation rates were not significantly different between the two strains (Supplementary Table 4), and therefore, results from these strains should be comparable.

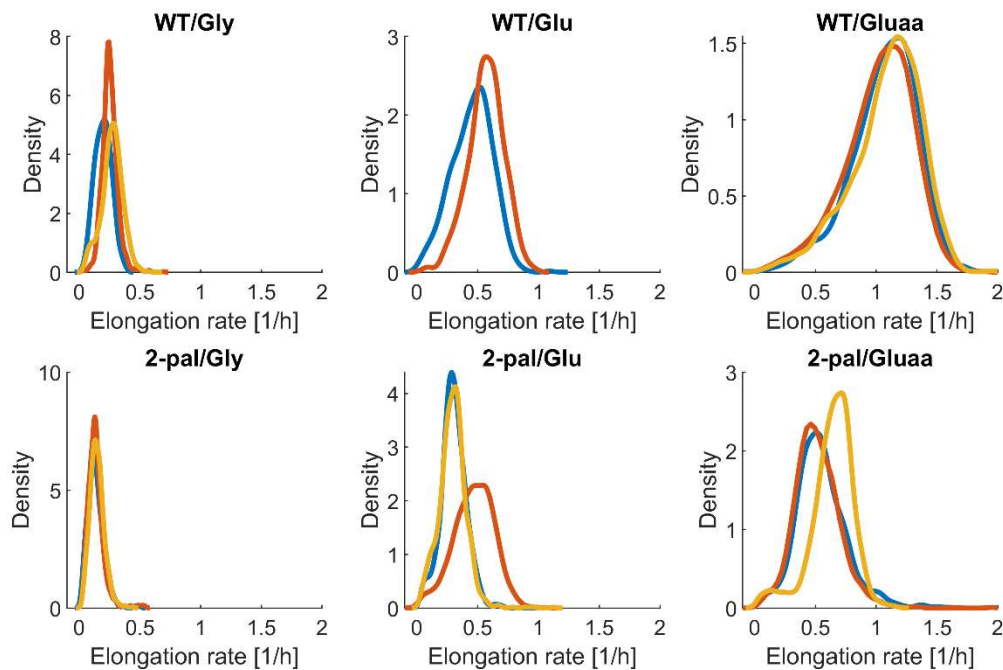
Supplementary Table 4. **Mean single-cell elongation rates for strains with and without the *motA* gene in three different growth media measure in the mother machine.**

Strain	Elongation rates [$\mu\text{m/h}$]		
	Glu _{aa}	Glu	Gly
SJR206	1.09	0.53	0.27
JKB43	1.09	0.52	0.29

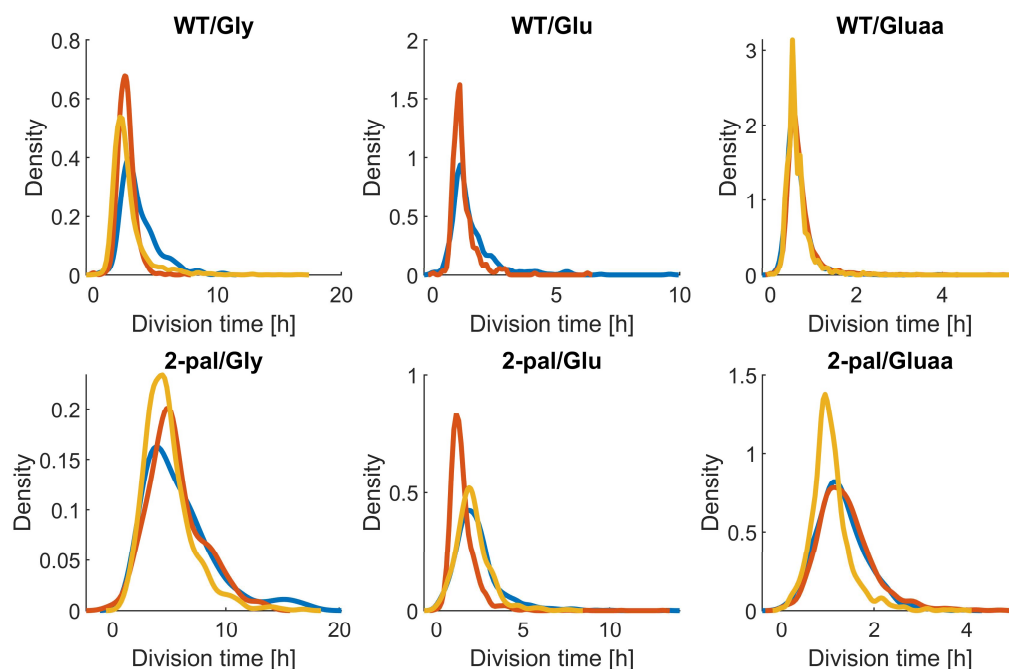
Appendix B: Supplementary for Chapter 4

Appendix B1: Supplementary figures for mother machine experiments

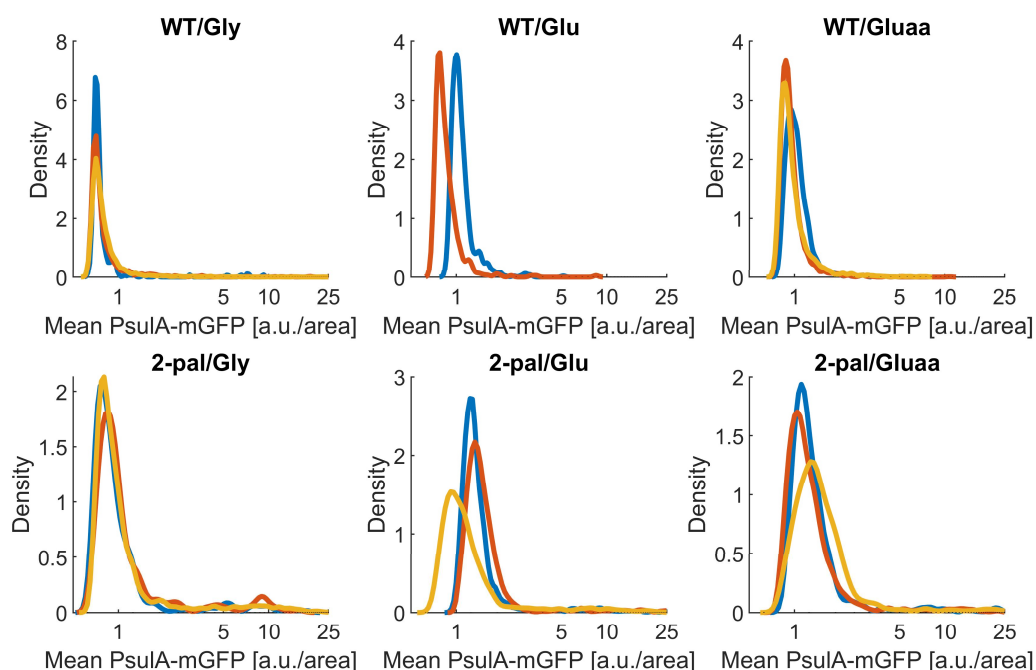
Growth rates and division times were reproducible among experimental replicates except for a single repeat in the 2-pal/glu and 2-pal/glu^{aa} condition. It is suspected that this difference may be due to inter-day variability in the experimental set-up as well as intrinsic variability in growth rates.



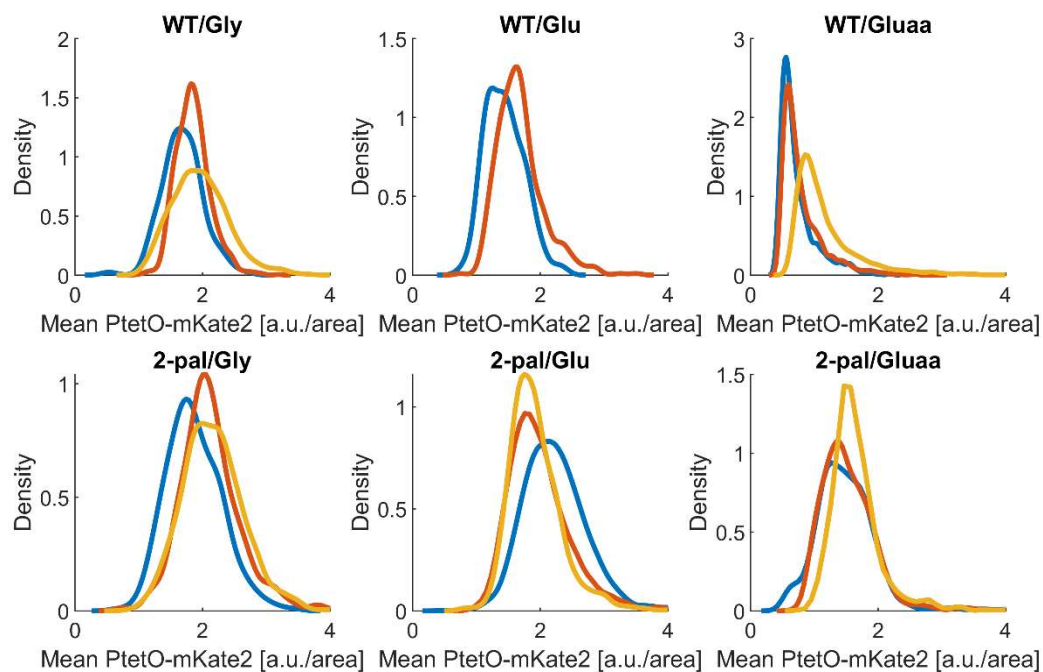
Supplementary Figure 1. **Replicate distributions of single-cell elongation rates for the WT and 2-pal strain in three growth conditions.** Solid lines represent the probability density estimate of data pooled over the duration of the experiment. Different colours represent the experimental replicates.



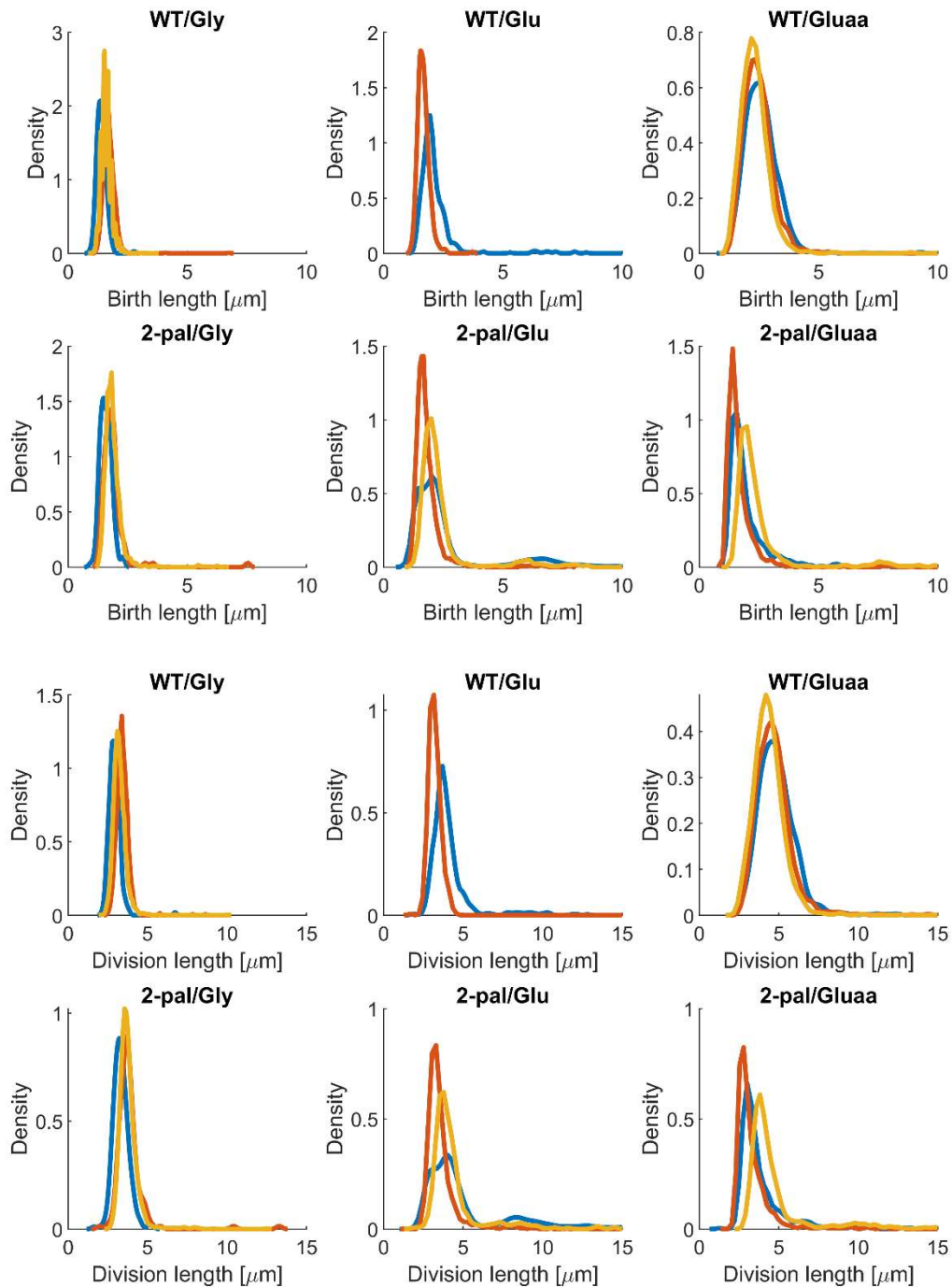
Supplementary Figure 2. **Replicate distributions of single-cell division times for the WT and 2-pal strain in three growth conditions.** Solid lines represent the probability density estimate of data pooled over the duration of the experiment. Different colours represent the experimental replicates.



Supplementary Figure 3. **Replicate distributions of cell-cycle averaged SOS expression for the WT and 2-pal strain in three growth conditions.** Solid lines represent the probability density estimate of data pooled over the duration of the experiment. Different colours represent the experimental replicates.



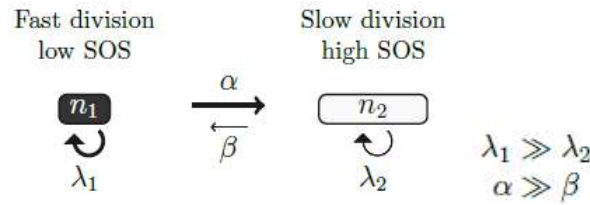
Supplementary Figure 4. **Replicate distributions of cell-cycle averaged constitutive expression for the WT and 2-pal strain in three growth conditions.** Solid lines represent the probability density estimate of data pooled over the duration of the experiment. Different colours represent the experimental replicates.



Supplementary Figure 5. **Replicate distributions of single-cell birth lengths and division lengths for the WT and 2-pal strain in three growth conditions.** Solid lines represent the probability density estimate of data pooled over the duration of the experiment. Different colours represent the experimental replicates.

Summary statistics for Chapter 4 mother machine data can be found at: <https://www.ebi.ac.uk/biostudies/BioImages/studies/S-BSST833>

Appendix B2: Derivation of fixed population model used to estimate switching rate from low-SOS to high-SOS state



Call n_1 and n_2 the number of ‘mother’ cells differentiated by their GFP intensity (SOS) either below or above a specified threshold value. The change in each population can be characterised as:

$$\frac{dn_1}{dt} = -\alpha n_1 + \beta n_2 \quad (1)$$

$$\frac{dn_2}{dt} = \alpha n_1 - \beta n_2 \quad (2)$$

where β and α are the respective conversion rates. Since population size is fixed:

$$\frac{dn_2}{dt} = \alpha(N - n_2) - \beta n_2 \quad (3)$$

where $N = n_1 + n_2$ is the total number of mother cells. This equation can be solved to obtain the number of cells n_2 over time:

$$n_2(t) = N \frac{\alpha}{\alpha + \beta} + \left(n_2(0) - N \frac{\alpha}{\alpha + \beta} \right) e^{-(\alpha + \beta)t} \quad (4)$$

where $n_2(0)$ is the initial number of n_2 cells. In terms of population fractions:

$$f_2(t) = \frac{n_2(t)}{N} = \frac{\alpha}{\alpha + \beta} + \left(f_2(0) - \frac{\alpha}{\alpha + \beta} \right) e^{-(\alpha + \beta)t} \quad (5)$$

where f_2 is the fraction of high-SOS cells. If we assume that the reversion rate β is significantly smaller than α ($\alpha \gg \beta$), the population fraction can be simplified as:

$$f_2(t) = 1 + (f_2(0) - 1)e^{-\alpha t} \quad (6)$$

Thus, assuming $f_2(0)=0$, α can be estimated using the following linear relation:

$$-\ln(1 - f_2(t)) = \alpha t \quad (7)$$

where the values of $f_2(t)$ can be measured using the mother machine experiments.

Model derived by Sebastian Jaramillo-Riveri

The method of estimation of α presented in this thesis represents a preliminary method that was subsequently improved upon using a maximum likelihood estimation. The improved estimation method was published in Jaramillo-Riveri et al. (2022).

Appendix B3: Derivation of expanding population model used to estimate the steady-state high-SOS fraction

Let's consider n_1 and n_2 the number of cells in each state, and λ_1 and λ_2 the respective division rates. We call n_1 the number of “healthy” low-SOS cells, and n_2 the number of slow-dividing cells with high-SOS induction. Let us assume first-order kinetics for conversion between the two populations, and call α the conversion rate constant from population one to population two, and β the rate constant for the reverse reaction. Thus, the population dynamics is given by the following equations:

$$\frac{dn_1}{dt} = (\lambda_1 - \alpha)n_1 + \beta n_2 \quad (8)$$

$$\frac{dn_2}{dt} = (\lambda_2 - \beta)n_2 + \alpha n_1 \quad (9)$$

We are interested in the population fractions and call them $f_1 = \frac{n_1}{n_1+n_2} \in [0,1]$, and $f_2 = \frac{n_2}{n_1+n_2} \in [0,1]$. Converting the dynamics into fractions we get:

$$\frac{df_1}{dt} = -\alpha f_1 + \beta f_2 + f_1 f_2 (\lambda_1 - \lambda_2) \quad (10)$$

$$\frac{df_2}{dt} = \alpha f_1 - \beta f_2 - f_1 f_2 (\lambda_1 - \lambda_2) \quad (11)$$

Notice that the total population growth is given by:

$$\frac{d(n_1 + n_2)}{dt} = (\lambda_1 f_1 + \lambda_2 f_2)(n_1 + n_2) \quad (12)$$

At steady-state, the population fractions are time-invariant. The solution for f_1 and f_2 at steady-state is given by:

$$f_1 = \frac{1}{2} \left(1 + \frac{\alpha + \beta \pm \sqrt{\delta}}{\lambda_2 - \lambda_1} \right) \quad (13)$$

$$f_2 = \frac{1}{2} \left(1 - \frac{\alpha + \beta \pm \sqrt{\delta}}{\lambda_2 - \lambda_1} \right) \quad (14)$$

where $\delta = (\beta + \alpha + \lambda_2 - \lambda_1)^2 - 4\beta(\lambda_2 - \lambda_1)$. The steady-state can be approximated when $\alpha \gg \beta$ (and therefore β is negligible as we assumed previously) and $\lambda_1 \gg \lambda_2$. Under these assumptions we have:

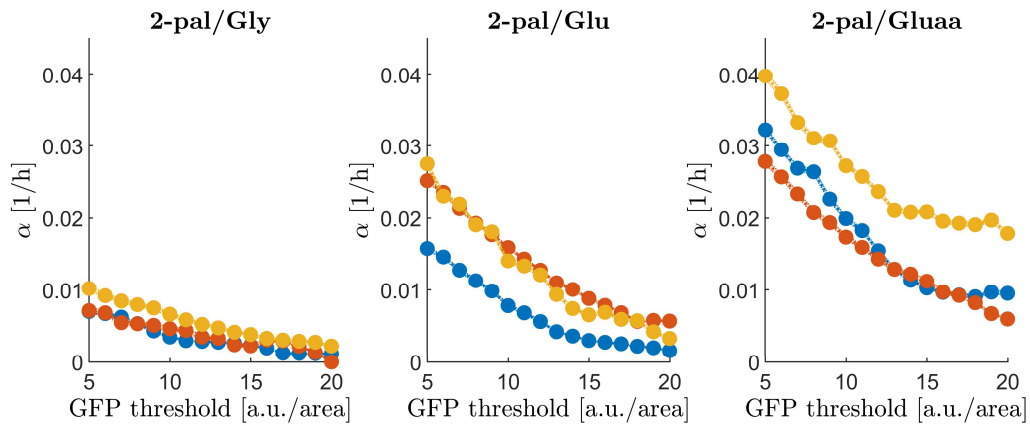
$$f_2 \approx \alpha / \lambda_1 \quad (15)$$

$$f_1 \approx 1 - \alpha / \lambda_1 \quad (16)$$

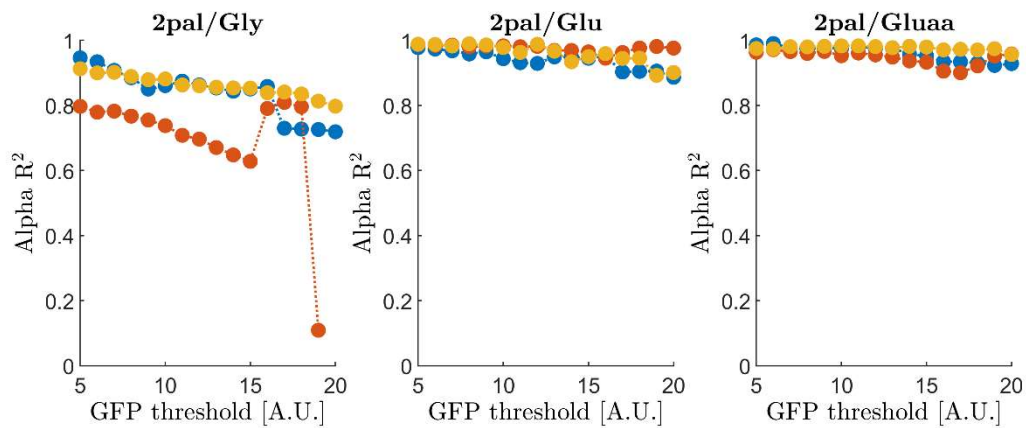
Therefore, using this model, we can predict f_2 (the fraction of high-SOS cells) based on the parameters measured in the mother machine and compare this prediction to the experimentally measured fraction of high-SOS cells.

Model derived by Sebastian Jaramillo-Riveri

Appendix B4: Supplementary figures for estimation of high-SOS switching rate (α)



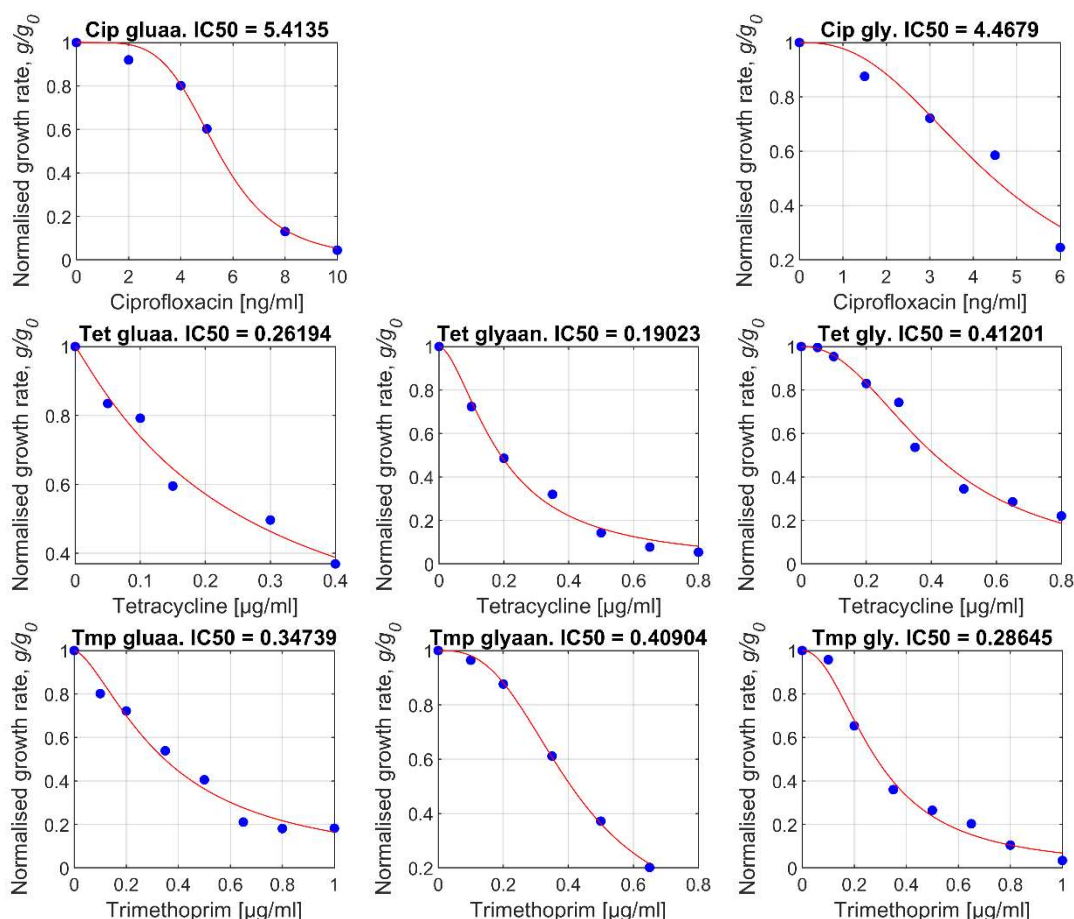
Supplementary Figure 6. **Switching rate α estimated for multiple GFP thresholds for the 2-pal strain in different growth media.** Points represent the estimated value and colours represent different experimental replicates.



Supplementary Figure 7. **R-squared value for fitting α estimated for multiple GFP thresholds for the 2-pal strain in different growth media.** Colours represent different experimental replicates.

Appendix C: Supplementary for Chapter 5

Appendix C1: Supplementary figures for bulk experiments



Supplementary Figure 8. Single drug dose-response curves showing normalised growth rate g/g_0 over a drug concentration gradient in gluaa (left column), glyaan (middle column), and gly (right column) growth medium for *E. coli* strain SJR206. Growth rates under ciprofloxacin (CIP, top row), tetracycline (TET, middle row), and trimethoprim (bottom row) treatment were measured via optical density readings. IC₅₀s (indicated in the title of each panel) were estimated by non-linear least squares regression of the Hill function (fit shown by the red line), defined as $g/g_0 = 1/(1 + (x/IC_{50})^n)$ to the data using *lsqcurvefit* function in MATLAB. x is the antibiotic concentration and n is the Hill coefficient which determines the steepness of the curve. The IC₅₀ is defined as the concentration that reduces the growth rate by 50%. Growth rates are normalised to the no-drug control in each growth medium. Circle points represent the data and red lines represents the Hill function fit.

Supplementary Table 5. **List of antibiotics used and comparison of measured IC₅₀ to other studies with similar medium composition.**

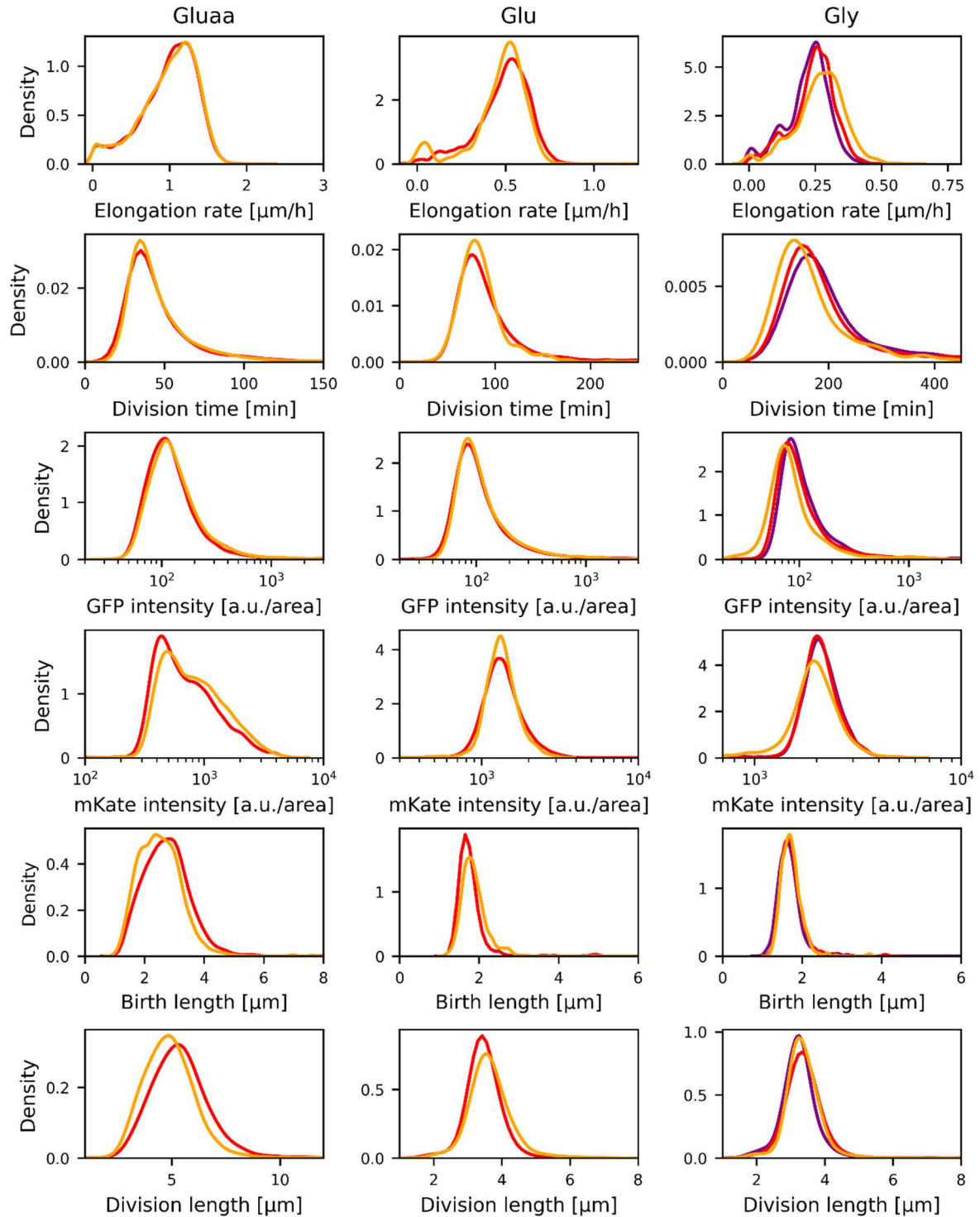
Abbreviation	Antibiotic	Mechanism of action	IC ₅₀ (µg/mL)	Literature IC ₅₀ (µg/mL)
CIP	Ciprofloxacin	DNA Gyrase	0.0054 (gluaa)	0.0055 (Chevereau & Bollenbach, 2015) ¹
TMP	Trimethoprim	Folic acid biosynthesis	0.35/0.41 (gluaa/glyaan)	0.16 (Chevereau & Bollenbach, 2015) ¹
TET	Tetracycline	30S protein synthesis	0.26/0.19 (gluaa/glyaan)	0.32 (Kavčič <i>et al</i> , 2020) ² 0.48 (Greulich <i>et al</i> , 2015) ³

¹ LB medium. 37°C. *E. coli* K-12 (BW25113)

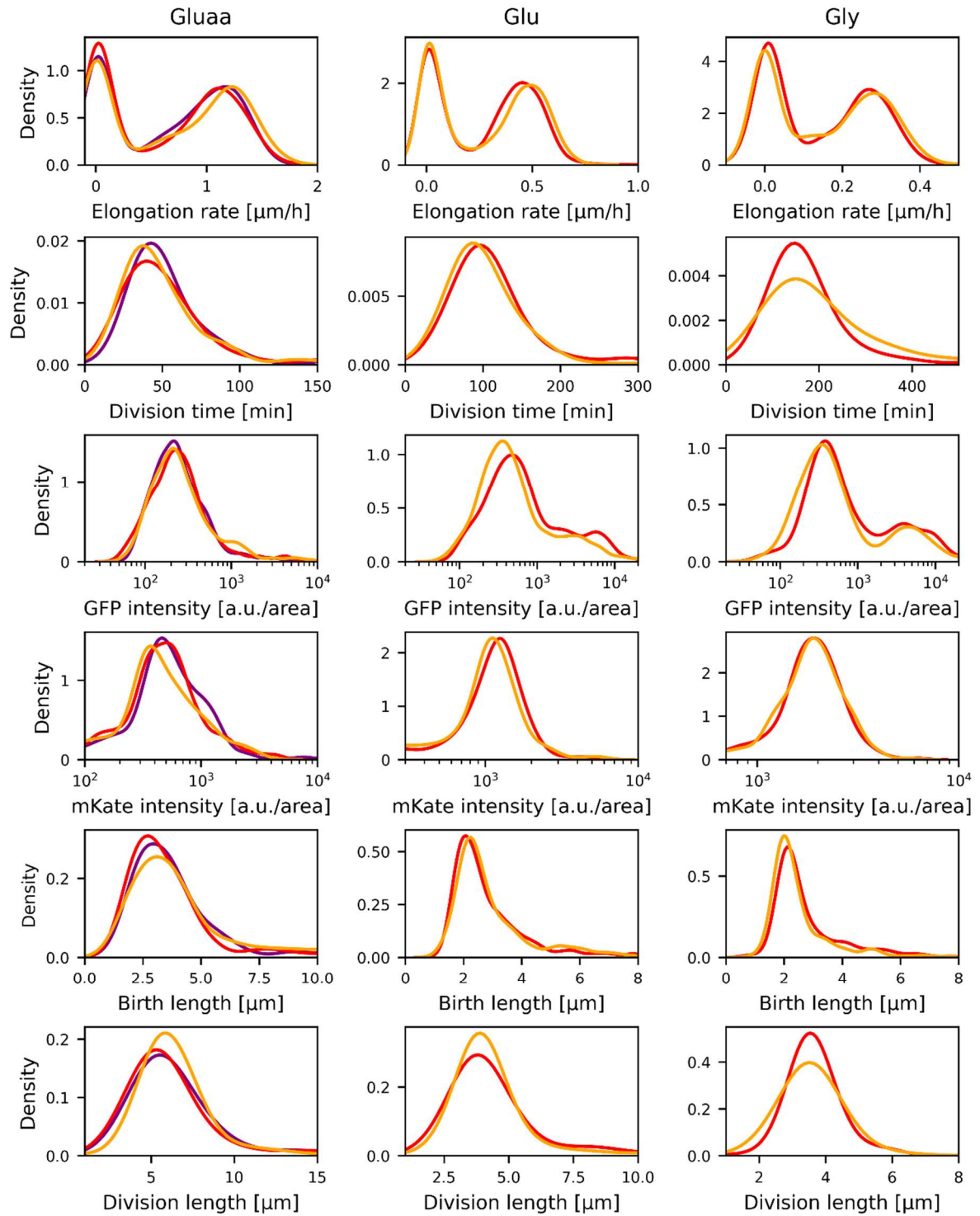
² LB medium. 37°C. *E. coli* K-12 (MG1655)

³ MOPS minimal medium (Neidhardt's), 0.2% glucose, 0.2% casamino acids. 37°C. *E. coli* K-12 (MG1655)

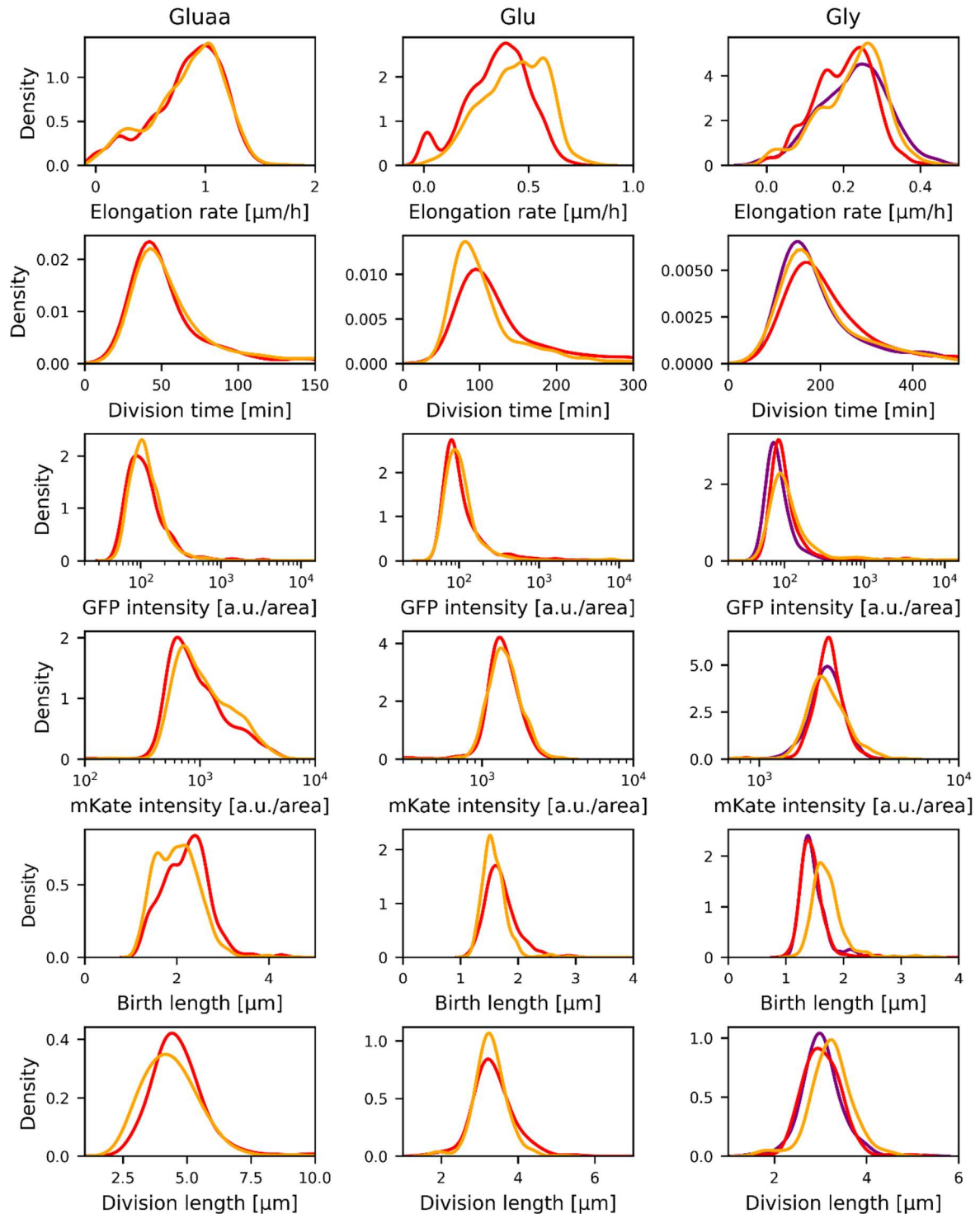
Appendix C2: Supplementary figures for single-cell experiments



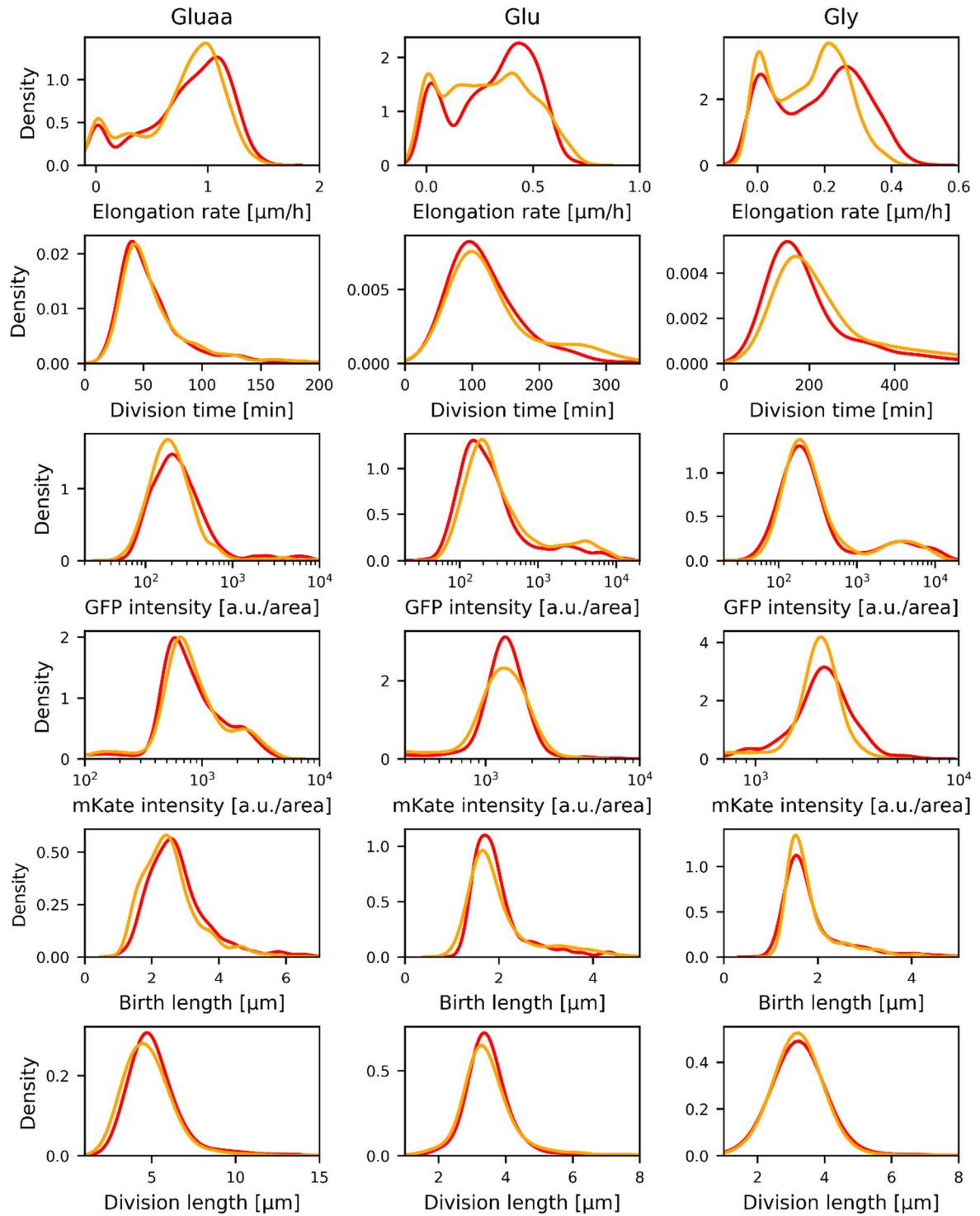
Supplementary Figure 9. **Distributions of single-cell measurements comparing experimental replicates for different growth media under no-drug control condition.** Distributions are kernel density estimates of the underlying histogram pooled from the first 20 hours of antibiotic treatment for strain JKB43. Colours represent the different experimental replicates done on different days.



Supplementary Figure 10. **Distributions of single-cell measurements comparing experimental replicates for different growth media under 3 ng/mL ciprofloxacin treatment.** Distributions are kernel density estimates of the underlying histogram pooled from the final 2 hours of antibiotic treatment for strain JKB43. Colours represent the different experimental replicates done on different days.



Supplementary Figure 11. **Distributions of single-cell measurements comparing experimental replicates for different growth media under 0.2 $\mu\text{g/mL}$ tetracycline treatment.** Distributions are kernel density estimates of the underlying histogram pooled from the final 2 hours of antibiotic treatment for strain JKB43. Colours represent the different experimental replicates done on different days.



Supplementary Figure 12. **Distributions of single-cell measurements comparing experimental replicates for different growth media under 3 ng/mL ciprofloxacin and 0.2 $\mu\text{g}/\text{mL}$ tetracycline treatment.** Distributions are kernel density estimates of the underlying histogram pooled from the final 2 hours of antibiotic treatment for strain JKB43. Colours represent the different experimental replicates done on different days.

Supplementary Table 6. **Single-cell statistics under no-drug control conditions for experimental replicates in different growth media.** Data pooled for the duration of the experiment for strain JKB43.

Growth medium & replicate	Number lineages	Number cell-cycles	Elongation rate [μm/h]			Division time [min]				
			Mean	Median	CV	Mean	Median	CV		
Gluaa_1	283	8919	1.09	1.13	0.27	40.54	35	0.47		
Gluaa_2	334	11581	1.09	1.12	0.27	39.74	35	0.53		
Glu_1	78	1108	0.51	0.52	0.25	81.12	80	0.32		
Glu_2	342	5871	0.53	0.54	0.23	83.97	75	0.39		
Gly_1	188	1978	0.28	0.28	0.29	166.23	150	0.47		
Gly_2	376	3517	0.25	0.26	0.28	191.02	170	0.50		
Gly_3	304	2647	0.24	0.25	0.29	204.31	180	0.57		
	SOS intensity			Constitutive intensity			Birth		Division	
	[a.u./area]			[a.u./area]			length [μm]		length [μm]	
	Mean	Median	CV	Mean	Median	CV	Mean	CV	Mean	CV
Gluaa_1	152.61	115.61	1.18	791.09	626.33	0.60	2.73	0.29	5.00	0.14
Gluaa_2	140.51	107.72	1.20	683.03	531.85	0.63	3.05	0.33	5.55	0.13
Glu_1	151.25	98.35	1.48	1350.84	1312.40	0.21	1.92	0.28	3.73	0.07
Glu_2	150.19	95.59	2.55	1362.13	1306.90	0.25	1.77	0.23	3.53	0.09
Gly_1	119.20	79.39	2.56	1942.07	1928.56	0.24	1.75	0.16	3.38	0.06
Gly_2	149.37	88.68	2.46	2070.40	2028.95	0.17	1.74	0.17	3.43	0.08
Gly_3	167.08	96.46	2.80	2130.10	2093.16	0.17	1.68	0.19	3.26	0.06

Supplementary Table 7. **Single-cell statistics under 3 ng/mL ciprofloxacin for experimental replicates in different growth media.** Data pooled from the final 2 hours of antibiotic treatment for strain JKB43.

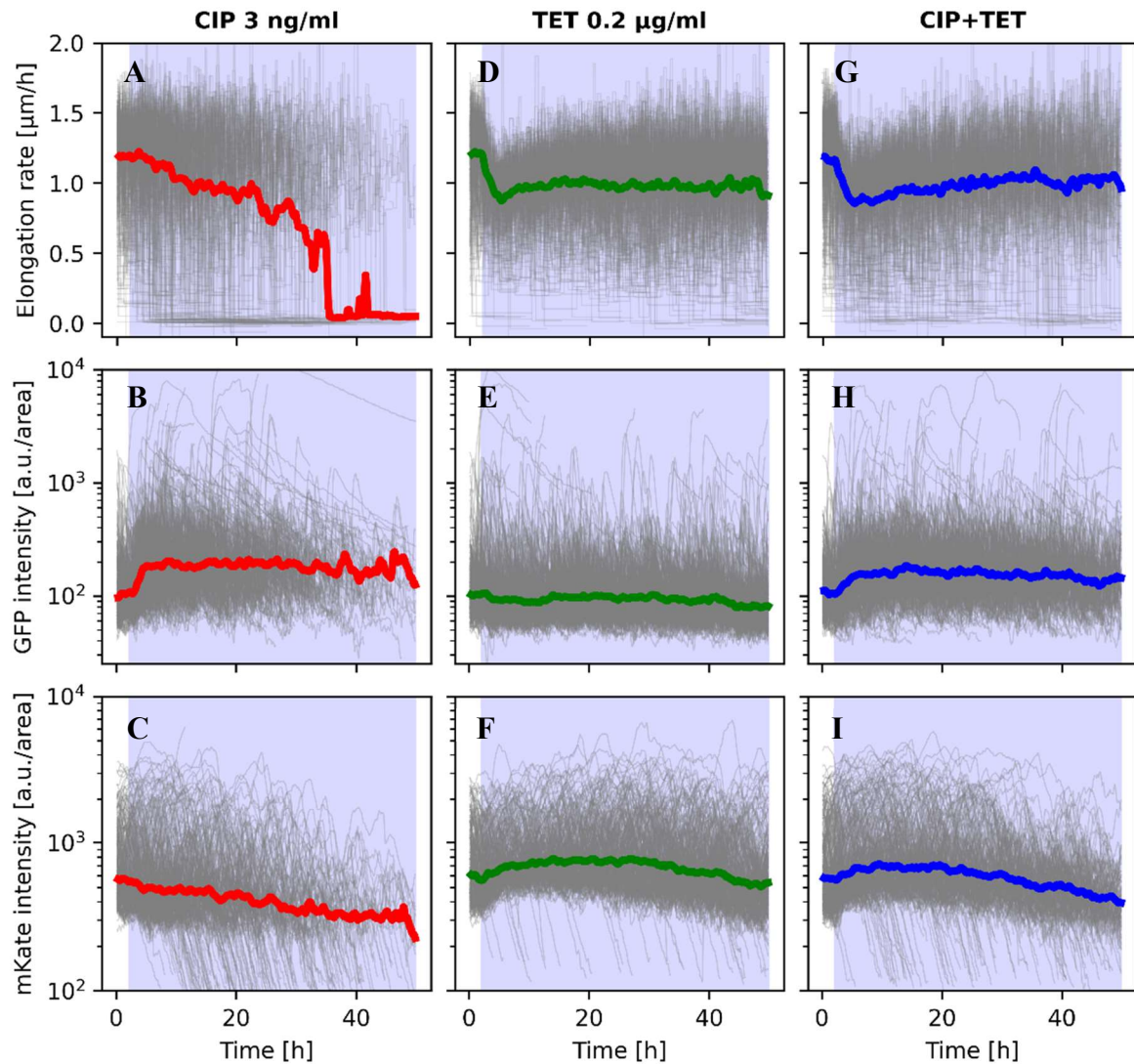
Growth medium & replicate	Number lineages	Number cell-cycles	Elongation rate [μm/h]			Division time [min]				
			Mean	Median	CV	Mean	Median	CV		
Gluaa_1	189	511	0.89	1.04	0.53	45.73	40	0.77		
Gluaa_2	156	423	0.89	1.02	0.51	46.27	40	0.57		
Gluaa_3	182	522	0.95	1.11	0.52	42.57	35	0.76		
Glu_1	379	678	0.36	0.43	0.61	101.39	85	0.86		
Glu_2	357	620	0.34	0.41	0.65	109.60	90	0.80		
Gly_1	251	362	0.18	0.21	0.78	222.51	160	0.98		
Gly_2	307	442	0.17	0.21	0.82	199.46	150	0.81		
	SOS intensity			Constitutive intensity			Birth		Division	
	[a.u./area]			[a.u./area]			length [μm]		length [μm]	
	Mean	Median	CV	Mean	Median	CV	Mean	CV	Mean	CV
Gluaa_1	337.56	235.44	2.24	621.80	508.20	0.81	4.26	0.69	7.18	0.65
Gluaa_2	289.77	226.37	1.01	638.85	495.08	0.79	4.34	0.62	7.78	0.63
Gluaa_3	338.52	219.22	1.76	576.58	420.37	0.83	4.55	0.66	7.80	0.64
Glu_1	893.88	389.61	2.33	1140.08	1109.73	0.51	3.01	0.54	5.19	0.66
Glu_2	1133.67	519.67	1.75	1171.14	1182.86	0.46	2.85	0.52	5.10	0.72
Gly_1	1402.11	386.83	2.28	1958.16	1882.28	0.33	2.47	0.47	3.89	0.58
Gly_2	1598.73	451.36	1.91	1948.16	1898.42	0.34	2.63	0.45	4.14	0.61

Supplementary Table 8. **Single-cell statistics under 0.2 µg/mL tetracycline for experimental replicates in different growth media.** Data pooled from the final 2 hours of antibiotic treatment for strain JKB43.

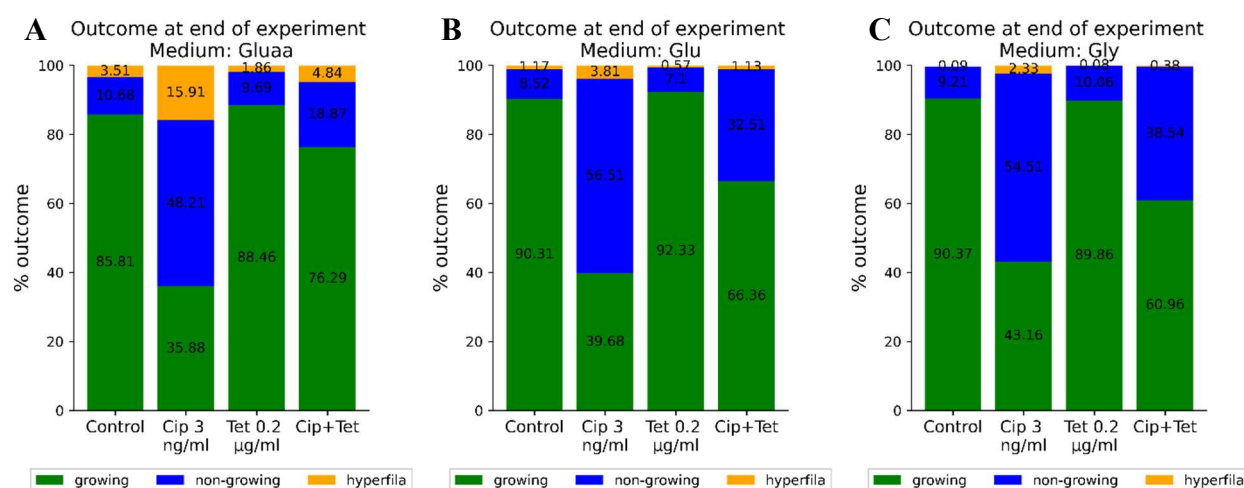
Growth medium & replicate	Number lineages	Number cell-cycles	Elongation rate [μm/h]			Division time [min]				
			Mean	Median	CV	Mean	Median	CV		
Gluaa_1	320	1079	0.91	0.96	0.32	54.43	45	0.73		
Gluaa_2	233	793	0.92	0.96	0.30	50.77	40	0.65		
Glu_1	349	714	0.38	0.40	0.34	118.86	100	0.57		
Glu_2	351	791	0.46	0.48	0.30	101.22	85	0.50		
Gly_1	317	534	0.23	0.23	0.39	202.62	170	0.54		
Gly_2	277	449	0.20	0.21	0.35	229.67	190	0.59		
Gly_3	411	684	0.23	0.25	0.35	205.81	160	0.69		
	SOS intensity			Constitutive intensity			Birth		Division	
	[a.u./area]			[a.u./area]			length [μm]		length [μm]	
	Mean	Median	CV	Mean	Median	CV	Mean	CV	Mean	CV
Gluaa_1	135.06	106.78	0.96	1130.22	862.97	0.64	2.11	0.22	3.73	0.27
Gluaa_2	134.29	100.25	1.25	992.38	765.00	0.66	2.30	0.22	4.03	0.31
Glu_1	162.74	88.29	2.59	1381.54	1329.35	0.22	1.71	0.22	2.97	0.28
Glu_2	157.16	95.57	3.20	1416.62	1362.65	0.24	1.61	0.17	2.84	0.26
Gly_1	123.91	77.37	2.82	2154.14	2142.47	0.19	1.47	0.16	2.43	0.24
Gly_2	146.71	89.56	2.25	2212.07	2204.12	0.16	1.45	0.17	2.38	0.29
Gly_3	280.50	97.44	4.31	2195.34	2101.36	0.23	1.71	0.15	2.83	0.29

Supplementary Table 9. **Single-cell statistics under 3 ng/mL ciprofloxacin and 0.2 µg/mL tetracycline for experimental replicates in different growth media.** Data pooled from the final 2 hours of antibiotic treatment for strain JKB43.

Growth medium & replicate	Number lineages	Number cell-cycles	Elongation rate [μm/h]			Division time [min]				
			Mean	Median	CV	Mean	Median	CV		
Gluaa_1	320	1004	0.87	0.93	0.36	53.33	45	0.59		
Gluaa_2	287	935	0.92	0.98	0.34	49.75	45	0.53		
Glu_1	347	653	0.37	0.40	0.46	121.59	100	0.80		
Glu_2	336	598	0.34	0.36	0.56	134.51	105	0.78		
Gly_1	325	503	0.20	0.23	0.65	212.31	160	0.69		
Gly_2	431	634	0.17	0.19	0.59	237.86	190	0.67		
	SOS intensity			Constitutive intensity			Birth		Division	
	[a.u./area]			[a.u./area]			length [μm]		length [μm]	
	Mean	Median	CV	Mean	Median	CV	Mean	CV	Mean	CV
Gluaa_1	243.99	191.94	1.61	935.18	720.29	0.69	2.63	0.33	4.64	0.44
Gluaa_2	301.30	214.66	1.68	891.93	684.24	0.66	2.90	0.38	5.19	0.47
Glu_1	430.80	194.92	2.45	1326.25	1305.00	0.36	1.91	0.28	3.41	0.48
Glu_2	588.37	229.00	2.04	1280.30	1254.59	0.38	2.01	0.53	3.53	0.66
Gly_1	754.01	205.60	2.40	2194.44	2135.45	0.32	1.80	0.39	2.99	0.58
Gly_2	703.59	219.16	2.19	2027.37	2043.05	0.26	1.78	0.30	2.86	0.55



Supplementary Figure 13. **Single-cell trajectories of elongation rates, SOS expression and constitutive gene expression under extended antibiotic treatment.** 3 ng/mL ciprofloxacin (A-C), 0.2 µg/mL tetracycline (D-F) and drug combination (G-I). Antibiotics were introduced between hours 2-49, indicated by the blue shaded area. Elongation rates are measured over the duration of each cell-cycle. Solid lines represent the median and grey lines represent individual lineage trajectories. Shown is data from one experimental replicate for strain JKB43 in *gluaa* medium. $N=247$ for CIP, $N=252$ for TET, and $N=253$ lineages for CIP+TET.



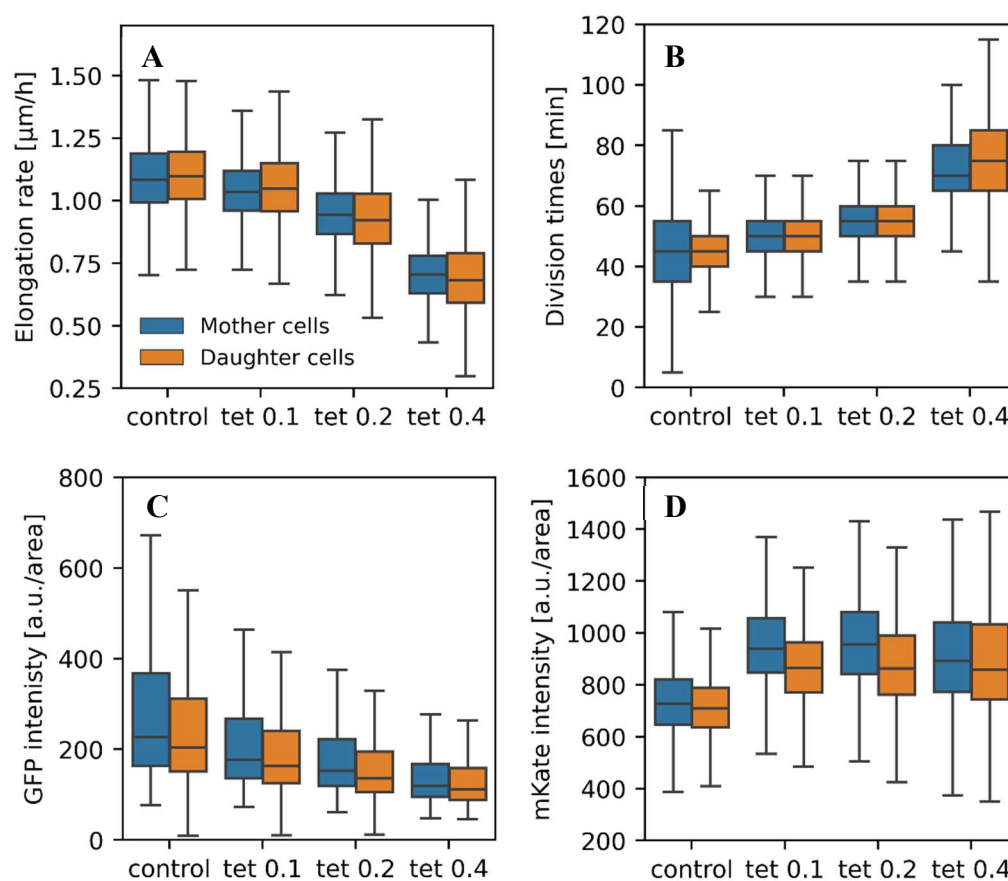
Supplementary Figure 14. **Percent outcome of mother cell lineages after 12 hours of drug treatment and 10 hours of recovery for different antibiotic treatments and growth conditions.** Different colours represent the outcome/fate of mother cell lineages. Values shown are the mean of at least two experimental replicates.

Appendix C3: Single-cell response to tetracycline concentration series and comparison of mother and daughter cell data

The unexpected increase in constitutive levels under TET treatment prompted us to investigate this further. We evaluated the response to TET over a range of concentrations – 0.1, 0.2, and 0.4 µg/mL (Supplementary Figure 15) and found that *mKate* levels were consistently elevated over all TET concentrations tested (Supplementary Figure 15D). Based on the universal growth laws, we expected constitutive gene expression to decrease in response to a translation inhibitor (Scott *et al*, 2010). It is possible that the TET concentrations used here are just too low to observe this expectation. Bollenbach *et al.* (2009) found that *E. coli* only significantly upregulated ribosomal promoter expression in response to TET concentrations that inhibited growth rates by more than 40%, whereas for spiramycin (another translation inhibitor, but targets the 50S ribosomal subunit), upregulation occurred at much lower effective concentrations. Here, 0.4 µg/mL TET resulted in a 36% reduction in elongation rates. This suggests that some variation can be expected when comparing the response between different translation inhibitors. We believe that the TET concentrations used in this study were too low to trigger ribosomal upregulation, and the observed increase in *mKate* intensity could be due to slowed growth/dilution.

Bergmiller *et al.* (2017) found that AcrAB-TolC – a major multidrug efflux pump in *E. coli* – accumulates at the old pole of mother cells due to asymmetric distribution of cell wall constituents between mother and daughter cells at division. This resulted in the mother cells

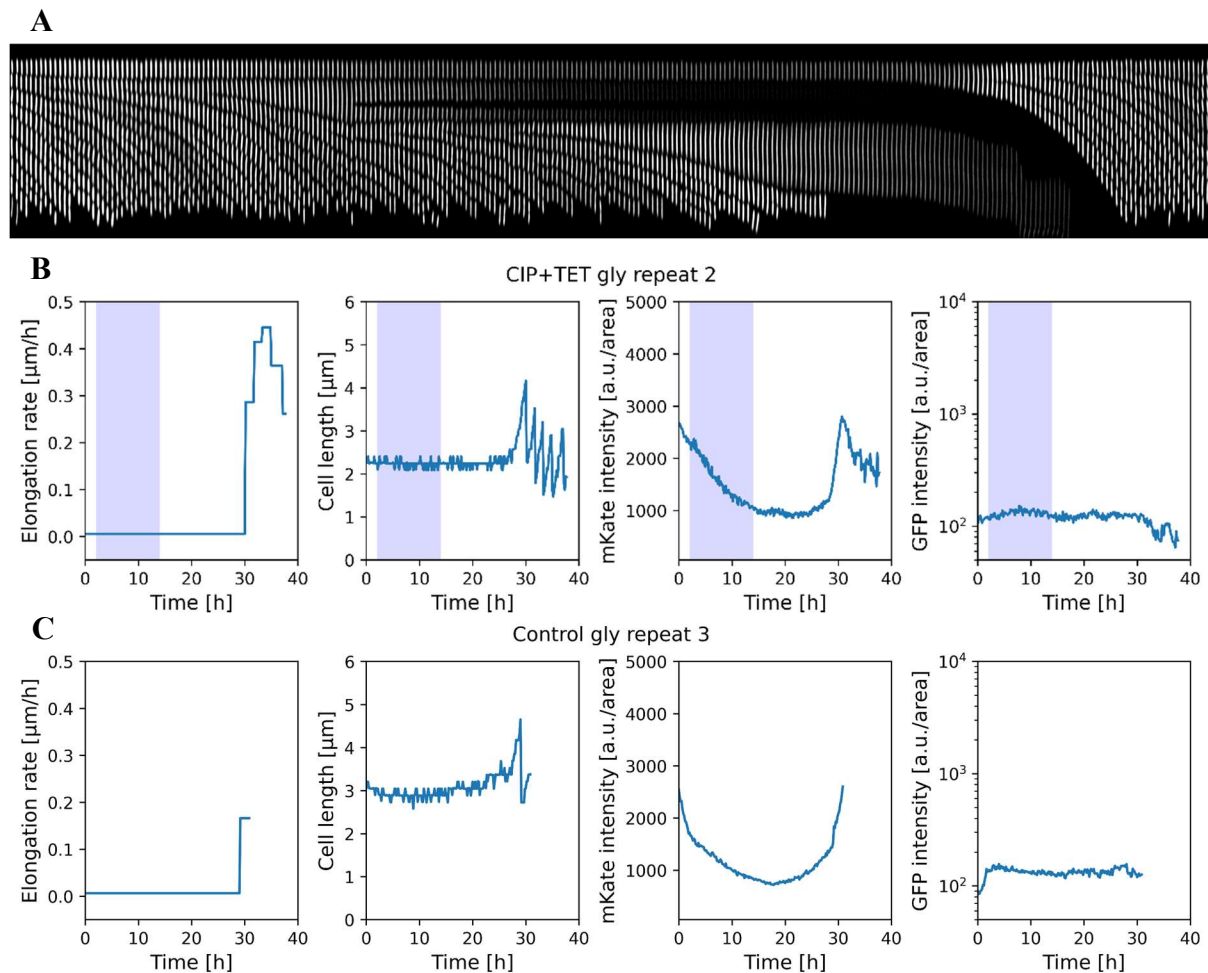
becoming less sensitive to TET with increasing age. Did we observe the same? In the mother machine, daughter cell lineages are also tracked for a short duration until they are lost from the microchannel and allows us to compare mother cell lineage data to daughter cell lineages. We found that elongation rates and division times did not differ significantly between mother and daughter cells at the concentrations tested (Supplementary Figure 15A, B). Our results are consistent with Bergmiller et al. (2017) because they found that elongation rates between mother and daughter cells were only significantly different at TET concentrations above 0.4 $\mu\text{g/mL}$. Moreover, we found that elongation rates maintain a steady-state under prolonged TET treatment (Supplementary Figure 13).



Supplementary Figure 15. **Box plots comparing elongation rates (A), division times (B), SOS expression from *Psula-mGFP* (C), and constitutive expression from *PtetO1-mKate2* (D) for mother cells and daughter cells over a range of different tetracycline concentrations.** Box plots represent the median and inter-quartile range. Whiskers show the remaining distribution excluding outliers. Data shown here are cell-cycle averaged values pooled from the steady-state period. In these experiments strain SJR206 was grown in the glyaan growth medium. Mother cells are indicated in blue and daughter cells in orange. Tetracycline (TET) concentrations are in $\mu\text{g/mL}$ units.

Appendix C4: Persister cells

Almost all cells that stopped growth during antibiotic treatment did not resume growth after the antibiotic was removed, even when the recovery period was extended to 36 hours. However, we did identify a few persister cells: One in the CIP+TET gly condition (0.2% of cells), one in the CIP gly condition (0.2% of cells), and three in the control gly condition (1% of cells) (Supplementary Figure 16). Persister cells were present as non-growing cells at the start of the experiment. Constitutive gene expression decreased and stabilised around 1000 a.u. and increased again when division resumed. This suggests that persister cells maintain a baseline level of metabolic activity which could differentiate them from non-growing cells that eventually die.

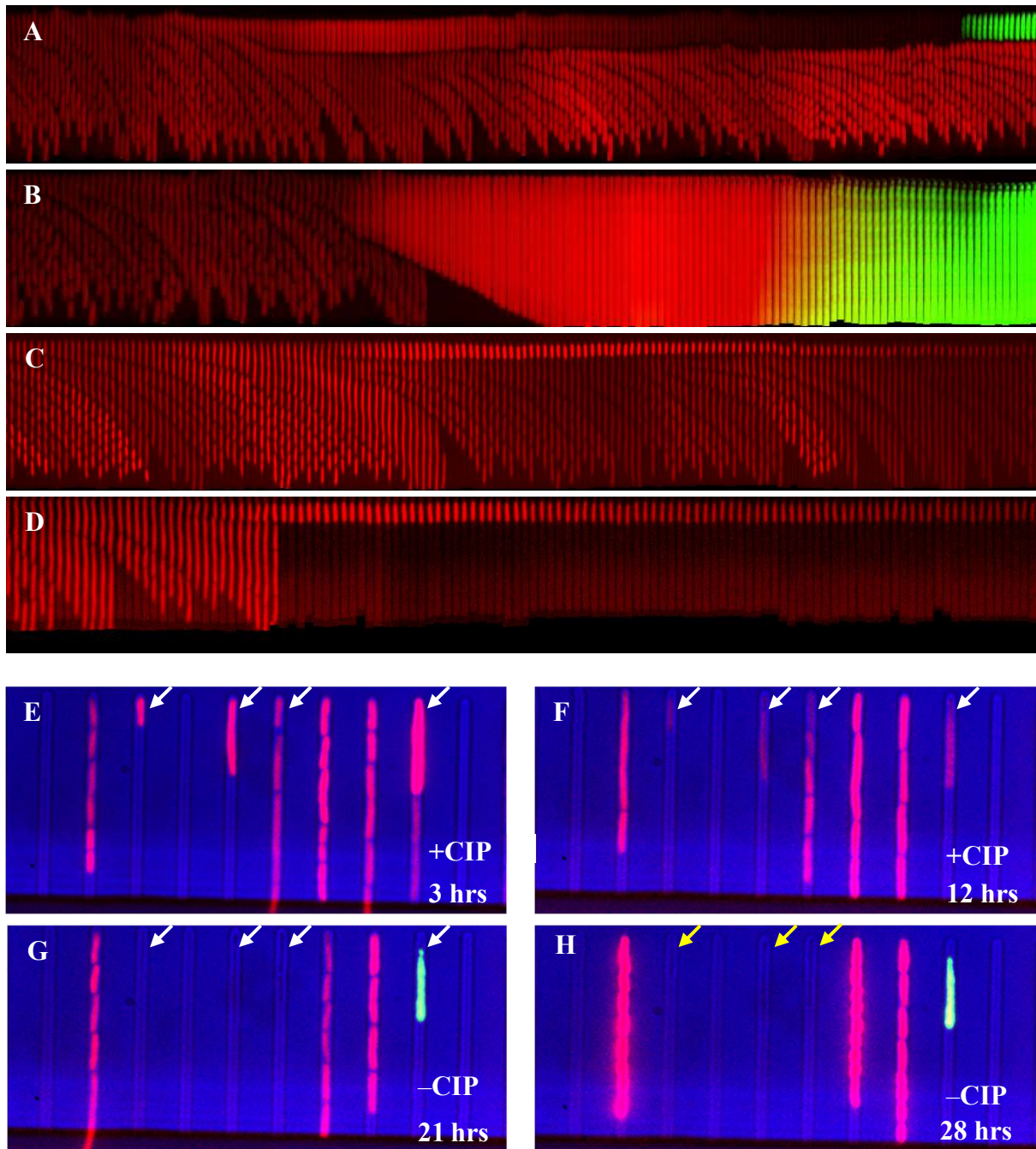


Supplementary Figure 16. **Persister cells observed in the mother machine.** **A.** Kymograph showing persister cell. The cell is not growing from the beginning of the experiment and resume division after approximately 30 hours. **B-C.** Single-cell parameters of two persister cells in the gly growth medium under CIP+TET treatment (B) and for the no-drug control (C). The blue shaded area represents when the antibiotic was introduced. The data in panel B represents the cell in the kymograph in panel A. Only 5 persister cells were identified, all in the gly growth medium.

Appendix C5: Confirming cell death using a membrane viability dye

We found that the vast majority of cells that ceased growth maintained structural integrity (did not lyse). Therefore, we used a membrane viability dye – SYTOX Green Dead Cell Stain (Invitrogen, S34860) – to determine whether these cells were truly ‘dead’ as defined by their membrane integrity. SYTOX Green can only enter cells with compromised membranes (a sign of cell death that is unrecoverable) and is excluded from cells with intact cell membranes. Once inside the cell, the dye will intercalate with DNA and resulting in a 500-1000-fold increase in fluorescence emission than its un-bound form (Roth *et al*, 1997; Thakur *et al*, 2015). Thus, fluorescence indicates a loss of membrane integrity or viability. Though SYTOX Green is not as popular as propidium iodide (PI) (an alternative membrane viability dye), it has been shown to provide better discrimination between live and dead cells than PI (Roth *et al*, 1997).

After treatment with 3 ng/mL CIP for 12 hours in gluaa medium, we observed two phenotypes of non-growing cells: some remained ‘viable’ with their membrane intact (VBNC-like), others became non-viable with disrupted membranes (Supplementary Figure 17). Of a total of 160 mother cells, 56 (35%) continued growing, 68 (42.5%) stopped growth but were viable, and 36 (22.5%) stopped growth and were non-viable. However, we noticed there was often a significant delay between the moment a cell stopped growth and when it lost membrane viability (Supplementary Figure 17A), sometimes as long as 18 hours (Supplementary Figure 17E-H). It is possible that many of the non-growing viable cells counted here would eventually lose viability, particularly those that stopped growth near the end of the experiment.



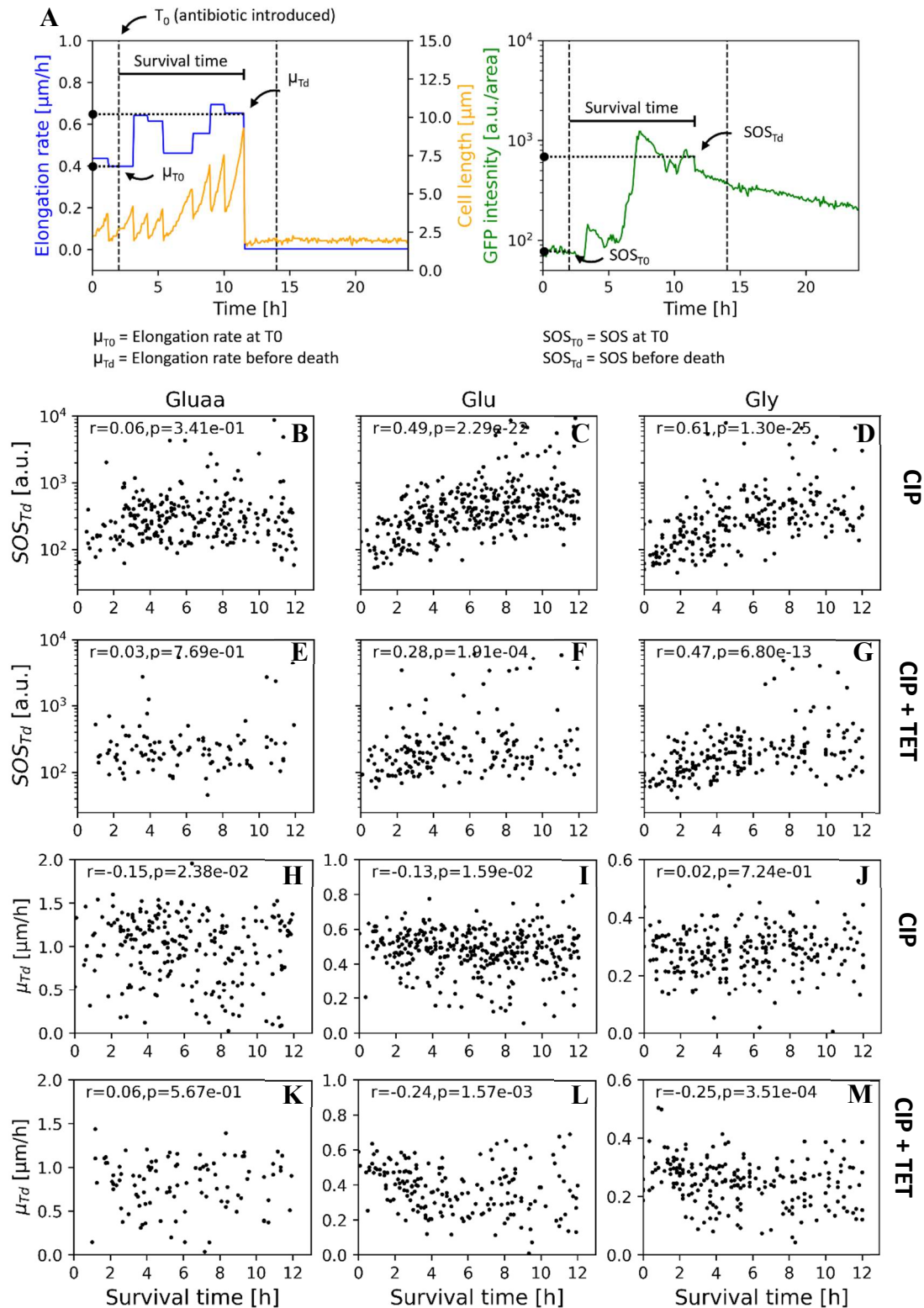
Supplementary Figure 17. **Determining membrane viability of non-growing cells exposed to 3 ng/mL ciprofloxacin in the mother machine.** A-D. Representative kymographs of cells that stopped growth. Red intensity represents constitutive gene expression from the *PtetO1-mKate2* reporter and green intensity represents SYTOX Green dye imaged in the GFP channel. Red and green channels have been merged. Some cells that stop growth eventually lose membrane integrity and take up the dye to fluoresce bright green (panel A) and included a few filamentous cells (B). Some cells that stopped growth did not take up the dye for the remainder of the experiment (C, D). E-H. A series of micrographs of a single XY position in the mother machine over time. After 3 hours of exposure to CIP four cells have stopped growth (panel E, white arrows), all of which eventually lost all constitutive fluorescence (panel F-H) and did not recover growth once antibiotic is removed (panel G, H). After 21 hours (panel G), one of the cells have a disrupted membrane and fluoresces green, while the other three cells, which are

still present in the microchannels as seen in the brightfield channel (yellow arrows), do not take up the dye. Here, brightfield, red and green channels have been merged. Ciprofloxacin (CIP) at 3 ng/mL was introduced for the first 12 hours and then removed for the remainder of the experiment (16 hours recovery period, 28 hours in total). SYTOX Green was added to the influent medium at a concentration of 15 μ M. For all images, GFP intensity (green) was normalised to a minimum of 2000 a.u. and maximum of 10000 a.u. Blue intensity represents brightfield images which enable visualisation of the microchannel and cells that lost fluorescence in both channels. Strain JKB39 was used to make the green channel available for SYTOX Green quantification.

Appendix C6: Initial SOS expression and elongation rate does not correlate with single-cell survival

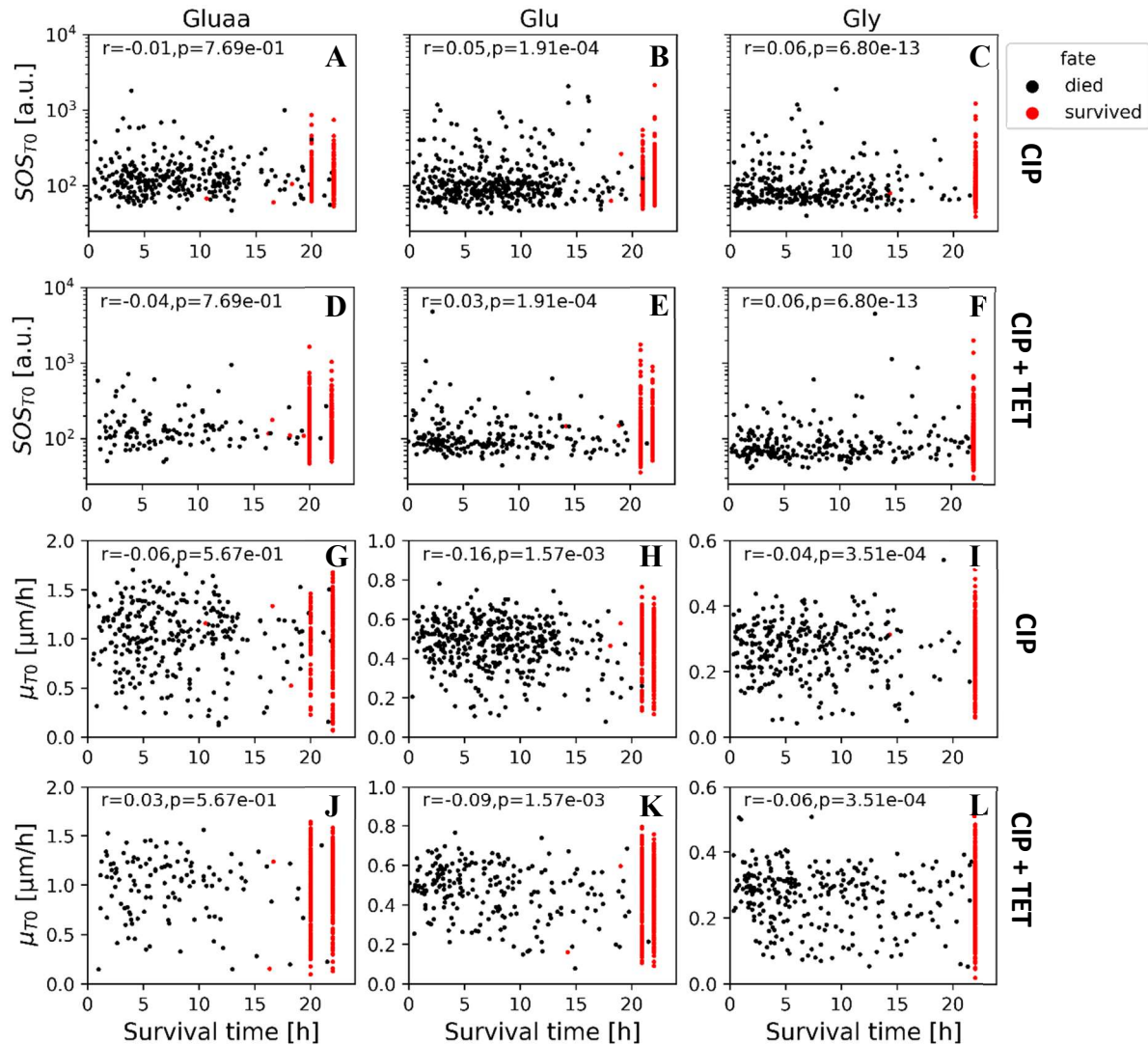
We investigated whether there was a correlation between the survival time and SOS expression or elongation rates measured before cell death or taken at the beginning of antibiotic exposure (Supplementary Figure 18 and Supplementary Figure 19, respectively). We found a moderate correlation between survival time under CIP and CIP+TET and SOS expression just before cell death but not in fast growing cells (Supplementary Figure 18B-G). This data suggests that higher SOS induction may improve the survival probability of single-cells under CIP treatment in a growth-dependent manner. Elevated SOS expression could be responsible for lowering the death rate observed in Figure 43B. The death rate initially rises rapidly during the delay between antibiotic exposure and SOS induction; once the SOS response reaches a high enough level in the population, the death rate is reduced. However, this could be a false correlation since SOS expression levels are elevated anyway after around 5 hours of CIP exposure. No correlation was found between survival and SOS expression at the beginning of antibiotic exposure (Supplementary Figure 19A-F).

We found no significant correlation between survival and elongation rates just before cell death (Supplementary Figure 18H-M). The small correlation observed for CIP+TET (Supplementary Figure 18L, M) is likely due to growth rate reduction from TET.



Supplementary Figure 18. **Correlation of survival time with SOS expression and elongation rate just before cell death.** **A.** Representative trajectory of a lineage that died during antibiotic exposure. Vertical black dashed lines represent period of antibiotic exposure. Elongation rate and SOS expression level at the beginning of antibiotic treatment (T_0) and before death (T_d) was measured as shown. **B-G.** Correlation of survival times with SOS expression before cell death under ciprofloxacin (CIP) (B-D) and ciprofloxacin+tetracycline (CIP+TET) (E-G)

treatment. **H-M.** Correlation of survival times with elongation rates before cell death under CIP (H-J) and CIP+TET (K-M) treatment. Survival times of lineages that died within the antibiotic treatment period (hours 0-12) were plotted against the SOS expression (from *PsulA-mGFP*) or elongation rate at the time of birth of the cell that died. For panels B-G, the Spearman's correlation coefficient (r) was calculated, and the p-values are indicated. For panels H-M, the Pearson's correlation coefficient (r) was calculated. $N=249$, $N=352$, and $N=242$, for *gluaa*, *glu*, and *gly*, respectively.



Supplementary Figure 19. Correlation of survival time with initial SOS expression and elongation rate. **A-F.** Correlation of survival times with SOS expression at the beginning of drug exposure (T_0) under ciprofloxacin (CIP) (A-C) and ciprofloxacin+tetracycline (CIP+TET) (D-F) treatment. **G-L.** Correlation of survival times with elongation rates at the beginning of drug exposure (T_0) under CIP (G-I) and CIP+TET (J-L) treatment. Survival times of lineages that died (black dots) or survived (red dots) within the were plotted against the SOS expression (from *PsulA-mGFP*) or elongation rate at the beginning of antibiotic treatment. For panels A-F, the Spearman's correlation coefficient (r) was calculated, and the p-values are indicated. For panels G-L, the Pearson's correlation coefficient (r) was calculated. $N=249$, $N=352$, and $N=242$, for *gluaa*, *glu*, and *gly*, respectively.

Appendix C7: Preliminary experiments for the trimethoprim-tetracycline suppressive combination

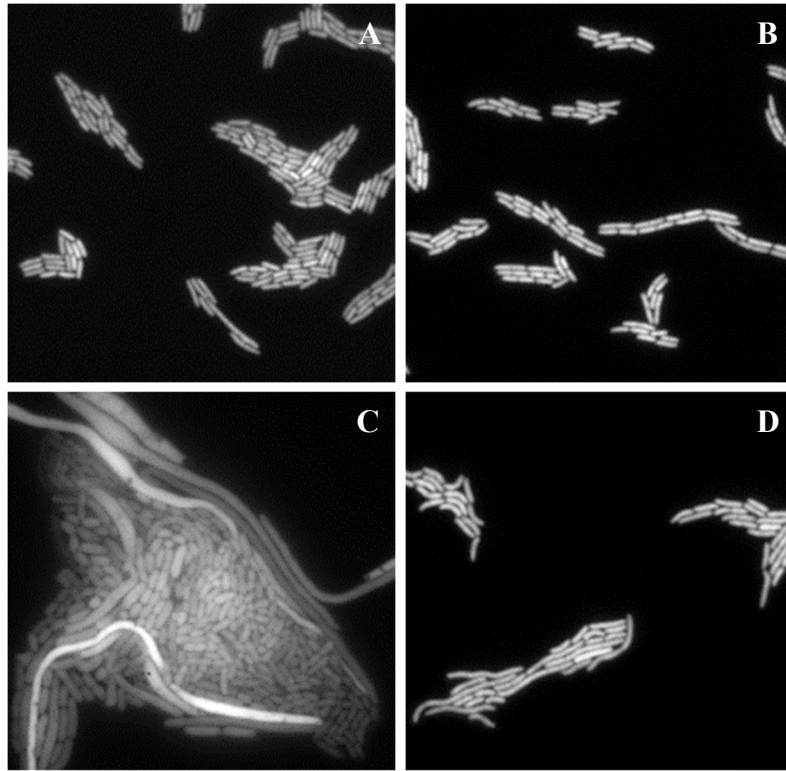
Drug interactions are typically classified between groups of antibiotics that share a common mode of action, for example the suppression between DNA synthesis inhibitors and translation inhibitors. We were interested in how suppression among antibiotics with a slightly different mode of action behaves differently. We started work on another suppressive drug combination: trimethoprim and tetracycline (TMP+TET). TMP is a folate synthesis inhibitor, acting by inhibiting the activity of dihydrofolate reductase (DHFR) (Hitchings & Smith, 1980). Folate is an essential cofactor for the synthesis of thymidine, purines and amino acids (glycine, methionine). Hence, TMP can have wide-ranging downstream effects on the cell, some of which have not yet been fully characterised. TMP acts differently than CIP in that it limits the *synthesis* of nucleotides and is bacteriostatic in its effect. However, TMP can be bactericidal at high concentrations due to a phenomenon known as “thymineless death” – a severe lack of the nucleotide thymine (Ahmad *et al*, 1998; Sangurdekar *et al*, 2010). During DNA replication, thymine starvation results in DNA with single-strand gaps and consequent induction of the SOS response (Rao & Kuzminov, 2019).

We exposed exponential cultures to TMP and TET in either glycan (fast) or gly (slow) medium for several generations before mounting on agarose-pads. Under 0.2 µg/mL TMP (a low concentration resulting in ~15-25% growth rate reduction (Supplementary Figure 8)) in fast growth conditions, cell size was highly heterogeneous consisting of relatively large cells of varying size, elongated cells, and very long filaments (Supplementary Figure 20C). Surprisingly, almost all cells were significantly thicker (in width) than those in other treatments (Supplementary Figure 21D). This outcome is not often reported in *E. coli* under TMP treatment, but was observed over 40 years ago and characterised again recently (Meacock *et al*, 1978; Rao & Kuzminov, 2021). Given the dramatic effect of TMP on cell size and shape, growth rates estimated from optical density readings are potentially misleading (as discussed above) and emphasises the advantage of collecting single-cell data.

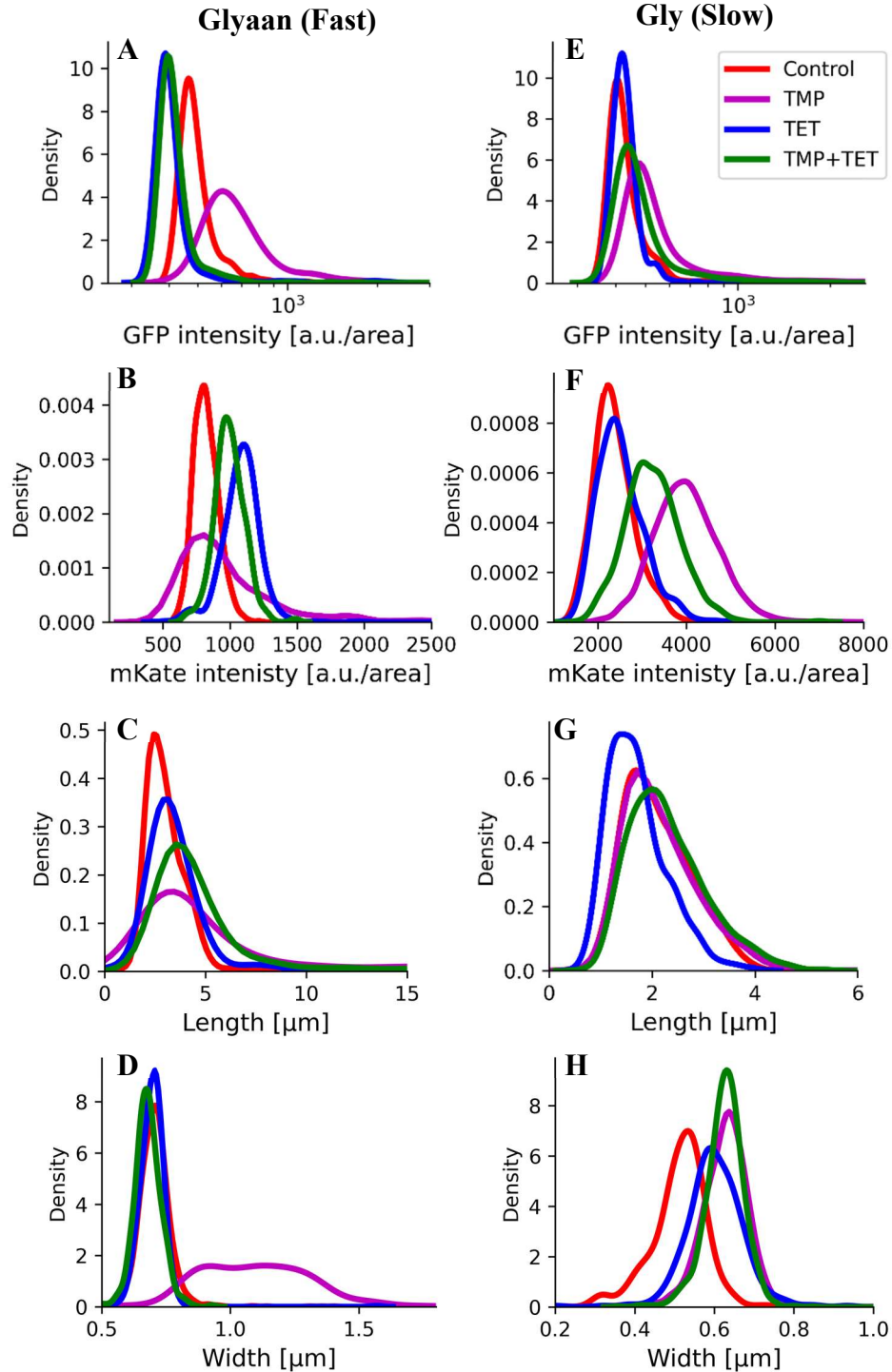
We also observed some interesting phenotypes of TMP-treated cells, including occasional cell lysis, the appearance and disappearance of cytoplasmic voids of various sizes, protoplast formation (cells shedding their cell wall) (Supplementary Figure 22). Similar outcomes have been reported previously and a metabolic link has been established between cell wall components (e.g. exopolysaccharides) and thymidine levels which potentially explains effects on the cell wall (Cushnie *et al*, 2016; Rao & Kuzminov, 2021). Wong *et al*. (2021) also

observed voids in the cytoplasm (which they characterised as “cytoplasmic condensation”), as a result of membrane damage, for *E. coli* treated with CIP at above-MIC levels. They determined that it was correlated with an accumulation of oxidative damage and ROS, which suggest that TMP may also cause ROS-mediated oxidative damage due to the similar phenotypes observed. Swelling of cells from the middle may be caused by interruption of peptidoglycan synthesis. Continued accumulation of cross-link defects in peptidoglycan synthesis (which occurs mostly mid-cell) could create a pore through which the cytoplasm escapes (Cushnie *et al*, 2016). TMP could cause this by preventing the synthesis of amino acids required for peptidoglycan synthesis. Moreover, the formation of protoplasts, or “L-forms”, have clinical importance since they are resistant to β -lactam antibiotics, and have been found in the urine of patients with chronic urinary tract infections (Petrovic Fabijan *et al*, 2022). We did not observe significant changes in cell morphology in TMP treatment in slow growth conditions (Supplementary Figure 21G, H), suggesting that fast growth is required for the effects on cell morphology to occur.

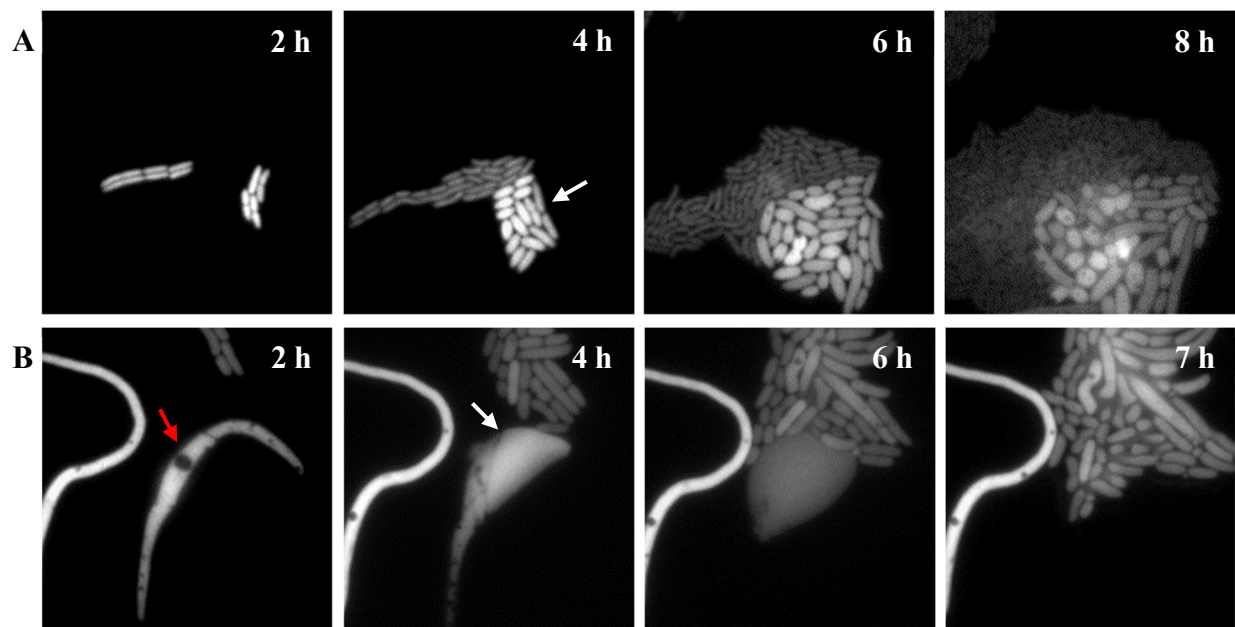
Adding 0.2 $\mu\text{g/mL}$ TET to TMP-treated cells completely removed cell size abnormalities (cell size almost identical to the control; Supplementary Figure 20D and Supplementary Figure 21D) and reduced SOS gene expression to levels below that of the no-drug control (Supplementary Figure 21A). Continued protein synthesis is required for thymineless death to occur in thymine-limited cells (Sangurdekar *et al*, 2010), and is also likely required for the effects on cell morphology. Therefore, it is likely that translation inhibition by TET improves growth and survival by slowing the depletion of thymidine, thereby preventing incorporation of single-strand gaps during DNA replication and deficiencies in cell wall components. We also evaluated growth in the mother machine, however, the increased cell width of TMP-treated cells made this difficult, and we decided to focus on the CIP+TET combination instead.



Supplementary Figure 20. **Agarose-pad snapshots of *E. coli* under different antibiotic treatments in the glyaan growth medium.** **A.** No-drug control. **B.** 0.2 $\mu\text{g/mL}$ tetracycline. **C.** 0.2 $\mu\text{g/mL}$ tetracycline. **D.** 0.2 $\mu\text{g/mL}$ trimethoprim and 0.2 $\mu\text{g/mL}$ tetracycline. Cells were sampled from exponential cultures (strain SJR206) exposed to the respective antibiotic overnight and loaded onto agarose-pads supplemented with glyaan medium and the respective antibiotic or antibiotic combination. Images represent cells following 2 hours of growth on the agarose-pad.



Supplementary Figure 21. **Snapshot distributions of single-cell measurements under different treatment conditions in fast (A-D) and slow (E-H) growth conditions.** Snapshots on agarose-pads were sampled from exponential cultures exposed to the respective antibiotic overnight. Distributions are kernel density estimates of the underlying histogram. Different colours represent the treatment condition. **A/E.** SOS expression from the *PsuA-mGFP* fluorescent reporter. **B/F.** Constitutive expression from *PtetO1-mKate2* fluorescent reporter. **C/G.** Cell length. **D/H.** Cell width. All data represented is for strain SJR206. $N=1721/589$ (glyaan/gly) cells for control, $N=367/4865$ for TMP, $N=537/384$ for TET, and $N=352/1118$ for TMP+TET.



Supplementary Figure 22. **Agarose-pad snapshot images showing unique phenotypes observed for *E. coli* exposed to trimethoprim.** **A.** A colony of cells starts growth with normal size after which it deviates (white arrow) to produce cells that are ovoid in shape. These cells could represent an “L-form” phenotype. Shown is growth on agarose-pads supplemented with 0.2 μg/mL trimethoprim and 0.2 μg/mL tetracycline. **B.** An elongated cell exhibiting cytoplasmic voids (red arrow). Several smaller voids are located throughout the cell. The cell then sheds its cell wall. The cell wall boundary is indicated by the white arrow. Eventually the cell is only encapsulated by the plasma membrane and then lyses presumably due to a lack of structural integrity. Shown is growth on agarose-pads supplemented with 0.2 μg/mL trimethoprim. All images represent growth of strain SJR206 on agarose-pads made up with glycan medium and the stated antibiotic or antibiotic combination.

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