The role of population dynamics in driving bacterial persistence



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Declaration

I declare that this thesis was composed by myself and that the work contained is my own except where explicitly stated in the text. This work has not been submitted for any degree or professional qualification except as specified.

Saran Davies, Trinity term 2023

Acknowledgements

I feel as though even the persister cells would be proud of how I have persisted through this DPhil. Though I could not have done this without my incredible support network.

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Publications & Contributions

Publications arising from this thesis include:

- **Davies, S.E.F.**, Choudhary, D., Uphoff, S., Griffin, A.S., and Ghoul, M. "High growth rates promote persistence in *Pseudomonas aeruginosa*". Submitted to *PNAS.*

- Judge, M.*, **Davies, S.E.F.***, Ghoul, M., and Griffin, A.S. "Assessing the role of population dynamics on persister cell formation in *Escherichia coli*". In preparation for submission to *Frontiers in Microbiology*.

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Chapter 2

The following manuscript resulted from this chapter:

- **Davies, S.E.F.**, Choudhary, D., Uphoff, S., Griffin, A.S., and Ghoul, M. "High growth rates promote persistence in *Pseudomonas aeruginosa*". Submitted to *PNAS.*

I completed the majority of the lab work, analyses, and writing. Divya Choudhary assisted with the single cell imaging, and kill curve and growth curve analyses. Prof. Stephan Uphoff provided input on the experimental design for single cell imaging, and feedback on the manuscript. Prof. Ashleigh Griffin and Dr Melanie Ghoul supervised the project, providing input on experimental design, interpretation of results, and comments on the manuscript.

This chapter represents part of an integrated thesis. If this chapter was broken down into traditional sections as part of a non-integrated thesis, the following chapters would be included:

1. "How do changing population dynamics influence the frequency of persister cell formation throughout the growth curve?". Here, I measured persistence every two hours from zero to 36 hours of a growth curve using *Pseudomonas aeruginosa* as my study organism. To accurately measure persistence at each culturing timepoint I conducted a kill curve to observe how cells responded to the antibiotic treatment during the 24-hour treatment period.

2. "Testing the role of population cell division history on persister cell formation". This involved assaying persistence every two hours from zero to 20-hours of culturing in four cultures inoculated with incrementally lower cell densities.

3. "How does cell division history influence population cell-cell heterogeneity?". This required ATP measures, single cell imaging, and further analysis of the growth curve.

Chapter 3

This chapter was completed in collaboration with Madeleine Judge; a fourth year MBiol student I was supervising in the lab. Work from this chapter will be submitted to *Frontiers in Microbiology*:

- Judge, M.*, **Davies, S.E.F.***, Ghoul, M., and Griffin, A.S. "Assessing the role of population dynamics on persister cell formation in *Escherichia coli*". In preparation for submission to *Frontiers in Microbiology*.

Madeleine Judge carried out the majority of the experiments and analysis. I conceived the experimental design, provided training and support within the lab, assisted with the analyses and write-up, and re-drafted the MBiol project dissertation ahead of journal submission. The project was supervised by Prof. Ashleigh Griffin who provided input on the experimental design, interpretation of the results, and comments on the manuscript.

Chapter 4

For chapter 4, I conceived the experimental design, carried out all lab work, and completed all analyses and the write-up. The project was jointly supervised by Prof. Ashleigh Griffin and Dr Melanie Ghoul who both provided input on the experimental design, interpretation of the results, and comments on the manuscript.

Appendix

The appendix contains an outline of future work on RNA sequence data. I designed and carried out the lab work for this project, and I am in the process of analysing the data in collaboration with Simon Kershenbaum, a fellow DPhil student in the lab.

Abstract

Bacterial persister cells can survive a broad range of antibiotics despite being genetically susceptible. This trait is clinically relevant, yet evolutionary explanations for persistence remain mysterious. Significant focus has been given to single cell studies with the aim of identifying genes responsible for persistence. However, other studies have indicated that persistence may not be genetically-driven at all but instead simply a by-product of other cellular processes. Moving away from the single cell approach, more needs to be done to consider persistence at the population level – strikingly, the proportion of persisters is maintained at a remarkably low level across populations and even species, between 0.0001-1%. Uncovering the conditions that promote persistence could tell us more about the regulatory mechanisms behind this trait. In my thesis, I explore the population-wide dynamics behind elevated levels of bacterial persistence in *Pseudomonas aeruginosa*. I first look at how changing bacterial population dynamics (growth rate and cell density) affect persistence by varying the culture inoculum density to detect the effect of growth, while also controlling for age of culture. This reveals inoculum density dependent effects where persistence peaks are not always associated with stationary phase as is commonly assumed in the literature, but perhaps with the number of divisions of individual cells. To expand this work, I replicate the study with, *Escherichia coli,* the most widely studied species in the persister research field. I find that *E. coli* displays different persistence patterns to *P. aeruginosa*, with limited inoculation density-dependent responses. While persistence patterns varied between species, extracellular ATP, which can be an indicator of cell lysis, was consistent with persistence patterns across both species, presenting intriguing avenues to explore in future. Lastly, I look at an unusual phenomenon I came across in my early work where I saw antibiotic-induced growth occurring in *P. aeruginosa* which I term a 'stress divide'. Here, I look at potential links with another antibiotic survival mechanism: heteroresistance, a less understood survival trait whereby a bacterial subpopulation is heterogeneously resistant to the antibiotic treatment. This introduces the concept of looking at how heterogenous phenotypic traits such as persistence and heteroresistance could work synergistically to promote population survival. In summary, by testing a broad range of growth conditions, I have found that persistence is not always greatest in stationary phase, is species-specific, and can work in tandem with traits like heteroresistance to enhance survival. My thesis highlights the significance of examining the impact of population dynamics on persistence, considering the mechanisms driving persistence on a species-by-species basis.

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Chapter 1. Introduction

Thesis aims and outline

In this thesis I explore proximate and ultimate explanations for persister cell formation in bacterial populations. I identify species-specific persister formation patterns and unexpected antibiotic survival dynamics.

In chapter 2, my main data chapter, I look at how population growth dynamics drive differences in persistence patterns within the growth curve. My study organism is the clinically relevant pathogen, *Pseudomonas aeruginosa*. A common assumption within the persistence literature is that persister cells are most likely to be formed from slow-growing stationary phase cells. Despite this association with slow growth, the mechanistic factors driving persistence remain contentious whereby multiple mechanistic pathways have been proposed without the identification of a definitive gene driving persistence. I first test the assumption of stationary phase being a proxy for persistence by measuring changes in persister cell formation over a wide range of growth curve conditions. From finding persistence unexpectedly peaking during exponential growth, I further manipulate growth curve dynamics by altering inoculation density. I find that for *P. aeruginosa*, growth curve phase is not the main determinant for when persistence peaks. Instead, persistence is better predicted by individual cell state heterogeneity driven from the population division history.

In chapter 3, I ask the question: does increased persistence during exponential growth occur in *Escherichia coli*? *E. coli* is the best-studied species in the persistence field and one of the few species where a high persister mutant has been identified. Using similar methodology to chapter

2, I assay persistence in two strains of *E. coli*: the wildtype, MG1655; and the high persister mutant, HipA7. More in line with expectations from the literature, persistence peaks most prominently during stationary phase across all growth conditions. This indicates that the population regulatory networks behind persistence may not be the same for different bacterial species. Despite persistence peaking in stationary phase for both strains, the culturing timepoint of these peaks was strain-specific and correlated with an increase in extracellular ATP, a potential indicator of cell lysis within bacterial populations. Swapping the supernatant between these strains at points of extracellular ATP peaks showed how external signals can influence persistence. This shows how persistence may not solely be driven by intracellular dynamics but could also be driven by external cues.

In chapter 4, I explore how a *P. aeruginosa* lab strain (PAO1) was unexpectedly able to grow through lethal doses of antibiotics (discovered from assays in chapter 2). I term this antibiotic induced growth a 'stress divide'. The stress divide inflates the population size up to several thousand-fold before the population numbers decrease, in a biphasic killing pattern revealing the underlying persister fraction. By inflating the population size, a greater number of persisters can form in response to the antibiotic treatment. To explore potential causes of this stress divide, I assay for heteroresistance, an often overlooked-antibiotic survival phenotype in bacteria. I identified heteroresistance under treatment with two antibiotic classes: beta lactams and aminoglycosides. However, given the low fraction of heteroresistant cells within the population, these were unlikely to be the sole driver of the stress divide. Nonetheless, the coexistence of persistence and heteroresistance within bacterial populations highlights how we may be overlooking how these traits could work synergistically to promote population survival.

Persistence

What is persistence?

Persistence occurs when a subset of cells in a bacterial population adopt a phenotype which enables them to survive antibiotic treatment despite being genetically susceptible (Balaban *et al.*, 2019). The lack of a distinct genetic signature and transient nature of this trait makes the persistence phenotype challenging to study. In addition, the persistence phenotype is rare within bacterial populations, typically comprising less than 1% of a bacterial population (Van den Bergh *et al.*, 2017). I will cover the intricacies of this complex trait in further detail below.

A brief history on antibiotic persistence

The term 'persister' was coined by Joseph Bigger just under 80 years ago (Bigger, 1944) (Fig. 1). Penicillin had only recently been discovered by Alexander Fleming and held great promise for eradicating bacterial infections. In particular, Bigger was interested in the potential use of penicillin for sterilisation. After attempting sterilisation assays using penicillin on *Staphylococcus pyogenes*, Bigger identified that a subset of these cells could survive without acquiring/developing resistance. As the cells were able to 'persist' through the antibiotic treatment, bigger described them as persister cells. Based on a series of experiments, Bigger established key foundational principles about persister cells: (1) Persisters are non-dividing in the presence of the antibiotic; (2) Persisters are not killed by high doses of antibiotics; (3) Persisters are able to resuscitate and re-populate the culture; and (4) Descendants of persisters are no more resistant to antibiotic.

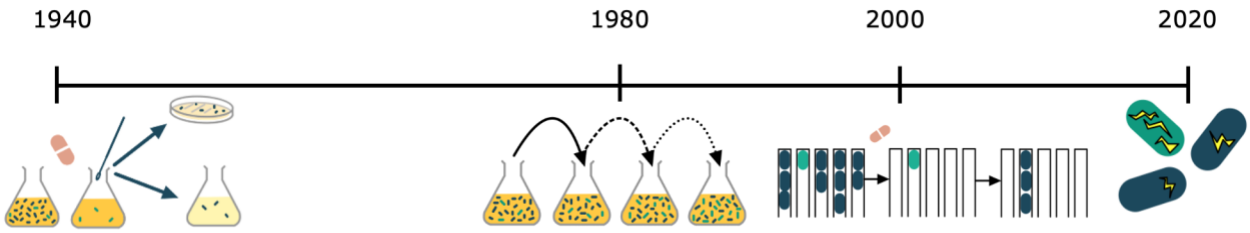


Figure 1. Simplified interpretation of notable studies in the persistence research timeline: from batch culture to single cells. Persisters were first classified by Joseph Bigger in 1944. Owing to the significance of resistance research, few persistence studies were published for the next forty years. In 1983, Moyed & Bertrand used a selection experiment to identify persister-promoting genes (including the *E. coli* high persister mutant HipA7). In 2004, Natalie Balaban's group used cutting-edge microfluidic devices to image and classify persistence at the single-cell level. More recently, studies have been looking at *in vivo* responses within bacteria to establish proximate causes for persistence (Pu *et al*., 2019; Soares *et al*., 2019; Alnahhas & Dunlop, 2023).

Despite Bigger's ground-breaking discoveries in 1944, persistence gained little attention in the context of antibiotic survival mechanisms. In 1940 (within 12 years of the discovery of penicillin), researchers had already identified genetically acquired antibiotic resistance in *E. coli* (Abraham & Chain, 1940), and penicillin-resistant strains of *Staphylococcus aureus* were identified in hospitalised patients by 1942 (Rammelkamp & Maxon, 1942). By the late 1940s, there were over 100 publications on antibiotic resistance each year, and in contrast, there were fewer than 50 publications on persistence in total (Fig. 2; Web of Science, 2023).

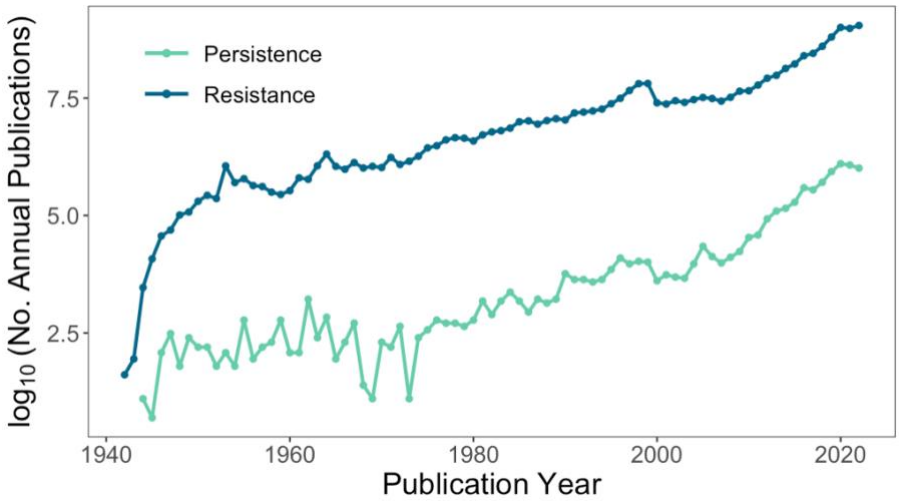


Figure 2. Annual number of publications on resistance *vs* persistence. The data was gathered from a search in Web of Science and has been log transformed for visualisation.

Notable persistence research

It was not until the 1980s that the number of annual persistence publications increased (Fig. 2). In 1983, through a selection experiment where antibiotics were pulse-treated to try and select for a strain with a greater ability to survive this treatment, Moyed and Bertrand identified a high persister mutant in *E. coli* (Moyed & Bertrand, 1983). They named this high persister mutant HipA7 and found it was able to persist up to 10,000-fold greater than the wildtype *E. coli* strain, K-12. Work on persistence steadily increased between 1980-2000. Then, in the early 2000s, a series of notable persistence studies emerged. Most notably, it was Natalie Balaban's work with *E. coli* strains that outlined key assumptions for studying persistence in the 21st century (Balaban *et al.*, 2004). Balaban carried out single cell imaging to gain a better understanding of the persistence phenotype at the cell level. This study enabled Balaban to confirm the persister dormancy phenotype proposed by Bigger (1944), and introduce the concept of different types of persisters: Type I, and Type II. Type I persistence was classified as being most likely to arise during exponential growth, whereas Type II persistence (the dominant type) was more probable in stationary phase. These classifications for persistence have since been updated to 'spontaneous' (Type I) versus 'triggered' (Type II) persistence (Balaban *et al.*, 2019).

Other notable persistence studies were carried out in the early 2000s. Spoering & Lewis (2001) contributed towards the foundational assumptions that persistence was most likely to arise during stationary phase by assessing antibiotic killing dynamics for *P. aeruginosa* in a range of culture conditions including exponential and stationary phase cultures, and biofilms. Keren *et al*. (2004) further supported persistence arising most predominantly in stationary phase, with evidence from assays using multiple bacterial species (*E. coli*, *P. aeruginosa*, and *S. aureus*). Keren *et al*. also

identified that persistence could be eradicated in cultures maintained in exponential growth within a chemostat. Later work by Shah *et al*., (2006) presented possible proximate explanations for the persistence phenotype by isolating dormant cells using fluorescence-activated cell sorting (FACS). Shah *et al*. then looked at RNA expression profiles and identified a potential role for toxin-antitoxin (TA) systems in persistence. Subsequent research has shown that the higher persistence in the HipA7 *E. coli* mutant discovered by Moyed & Bertrand (1983) is associated with a TA system (Germain *et al*., 2013).

TA systems genetically encode a stable toxin (generally a protein) and an unstable antitoxin (either RNA- or protein-based) (Ronneau & Helaine, 2019). Under normal bacterial growth conditions, the antitoxin element of the TA system suppresses the toxin. When the antitoxin is not expressed or repressed, the toxin limits cellular metabolism and contributes towards growth arrest or dormancy (Jurėnas *et al*., 2022). This link to dormancy ignited an interest in testing the role of TA systems in persistence. Maisonneuve *et al*., (2011) created a gene knockout *E. coli* K-12 mutant with the successive deletion of 10 mRNase-encoding TA loci. Successive deletions of the 10 TA systems revealed a reduction in persistence. However, after the discovery of bacteriophage contamination (phi80), the authors had to retract the paper. A subsequent publication from this lab highlighted the variability of persister assay findings depending on the experimental setup (Harms *et al.*, 2017). Later studies have subsequently questioned the role of TA systems in persistence (Goormaghtigh *et al.*, 2018; Pontes & Groisman, 2019; Shi & Zarkan, 2022; Svenningsen *et al.*, 2022). Recent research speculates that TA systems might putatively play a role in persistence (Song *et al*., 2023).

Looking at the role of dormancy in persistence, Orman and Brynildsen (2013) further used FACS and other fluorescence indicators to isolate non-dividing cells and surprisingly found that dormancy prior to antibiotic exposure was not a prerequisite for persistence. The fastest dividing cells could give rise to persister cells, and of the existing dormant cells isolated using FACS, fewer than 1% were persisters. This brought the role of dormancy prior to persistence into question, and indicated that the persister phenotype was multi-faceted with the requirement for a wider range of explanations to characterise this trait. There have been a wide range of mechanisms proposed to lead to persistence, and these mechanisms are outlined in table 1, highlighting the support in favour and against.

Persistence is ubiquitous in bacterial species, and examples of similar behaviour has been observed in eukaryotic cells including fungi (Wuyts *et al.*, 2018), and even cancer cells (Dhanyamraju *et al*., 2022). Nonetheless, the most prominent study organism is *E. coli*. Most studies have been conducted in *E. coli* despite this bacterium representing a narrow range of the vast bacterial diversity. Of the persistence papers published since Joseph Bigger's seminal paper, 32.4% use *E. coli* as the main study organism (Web of Science, 2023). While use of *E. coli* is justified due to the additional context available in this well-characterised bacterium, we may be missing opportunities to obtain a more general understanding from comparisons across species.

In summary, despite the discovery of persistence almost 80 years ago, no single defined mechanistic pathway has been agreed upon. As a result, more authors are suggesting that persistence is not governed by a single mechanism but is instead influenced by a range of conditions (Balaban *et al.*, 2004; Kaldalu *et al.*, 2016; Van den Bergh *et al.*, 2017; Kaplan *et al.*,

2021). Added confusion surrounding persistence mechanisms may in part be due to the wide range of methods and materials used to carry out persister assays: ranging from differences in inoculum densities, chosen growth curve phases, culture media, antibiotics, and bacterial species and strains.

Table 1. Commonly proposed mechanisms behind antibiotic persistence.

Mechanism	Role in persistence	Supported by	Disputed by
Stringent Response	Promotes (p)ppGpp production in response to nutrient stress to downregulate metabolism and gene expression which triggers slow growth/dormancy	Nguyen *et al.*, 2011; Stewart *et al.*, 2015; Svenningsen *et al.*, 2019; Wood & Song, 2020; Song & Wood, 2021 (Thomas Wood)	Wood *et al.*, 2021 (Whitney Wood)
Protein Aggregation	Reduced metabolism/slow growth due to sequestration of cellular components / Indirect triggering of the stringent response	Leszczynska *et al.*, 2013; Pu *et al.*, 2019; Dewachter *et al.*, 2021; Goode *et al.*, 2021	
ATP Depletion	Reduced metabolism leading to a reduction in antibiotic target activity / Indirect triggering of the stringent response	Conlon *et al.*, 2016; Grassi *et al.*, 2017; Dewachter *et al.*, 2019	Pontes & Groisman, 2020; Song & Wood, 2021
Signalling: Quorum Sensing & Indole	Cell-to-cell communication and signalling that can regulate genes involved in the stress response and biofilm formation	Wood, 2016; Fernandez-García *et al.*, 2018; Zarkan *et al.*, 2020	
Intracellular pH Regulation	Reduced metabolism due to lowered intracellular pH	Goode et al., 2021; Van den Bergh et al., 2022	

Population growth curve dynamics

My general approach to understanding persister cell formation is to use the different phases of the growth curve to identify conditions driving variation in persister cell formation. The bacterial growth curve is one of the first principles a student will encounter in microbiology: how unicellular cells transition from low to high densities in batch culture. The sigmoidal curve is characterised

by an initial lag phase, where cells adapt to their environment, before cells divide exponentially until reaching stationary phase where the number of divisions is equal to the number of deaths (Fig. 3). After an extended period of time in stationary phase, the culture can no longer support growth and cells enter death phase where the population size declines.

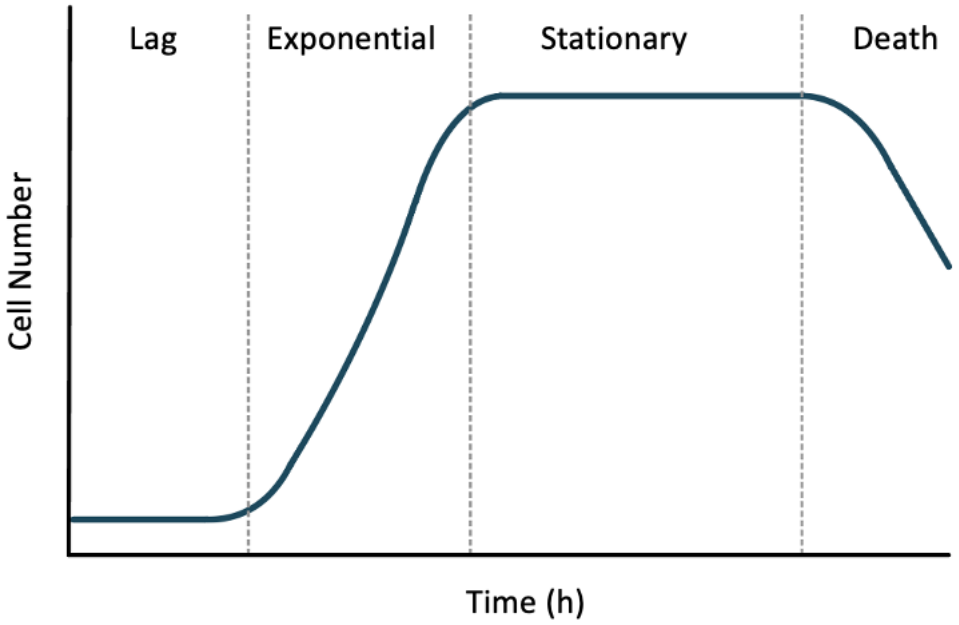


Figure 3. Bacterial growth curve schematic indicating the different phases of growth (dashed lines).

Despite being characterised as a period of 'latency', lag phase is a dynamic and active phase where cells prepare for exponential division (Bertrand, 2019). A study on *Salmonella enterica* by Rolfe *et al.* (2012) found changes in gene expression for over a thousand genes within four minutes of inoculation. This rapid response during 'lag' phase highlights how cells can dynamically transcribe genes within a short period of time, though this ability to coordinate such an activity so quickly remains mysterious (Bertrand, 2019). Once cells have adapted their transcriptome, they exit lag phase by beginning to divide. The root of what initiates this transition is contested, however, a recent study found that exiting lag phase is triggered by a leader cell that initiates division in neighbouring cells (André *et al.*, 2022).

By dividing exponentially, bacterial cells can go from an initial density of around 100 to 4.8×10^{10} within 12 hours, dividing every 20 minutes at their peak (own data). During this phase, cells are considered to be most metabolically active, utilising cellular machinery to replicate DNA, produce proteins, and build cell walls (Bremer & Dennis, 2008). This exponential growth is only sustained in plentiful nutrients, where toxin build-up and competition for space do not act as limiting factors (Dworkin & Harwood, 2022). Due to nutrients generally being low in the natural environment, exponential phase growth is rare within natural bacterial populations, with most cells existing in stationary phase (Hobbie & Hobbie, 2013; Morita, 1988).

Stationary phase is characterised by a general winding down of cellular metabolism and division rates. Ribosomes, responsible for protein synthesis, go into hibernation during stationary phase to conserve energy (Beckert *et al.*, 2018; Gohara & Yap, 2018), and morphologically, cell size decreases (Kolter *et al.*, 1993). A range of stress responses are also thought to be more active during stationary phase in order to endure the environmental limitations (Dworkin & Harwood, 2022; Zhu *et al.*, 2019). For example, the stringent response (which regulates bacterial shutdown) is triggered during stationary phase to aid bacterial survival in unfavourable conditions (Jaishankar & Srivastava, 2017).

Antibiotics

Persister cells enable bacterial populations to survive antibiotic treatments despite being genetically susceptible. Antibiotics are antimicrobial compounds that can be bactericidal (where they kill the bacteria) or bacteriostatic (where they inhibit bacterial growth) (Baquero & Levin, 2021). Over 75% of antibiotics are derived from natural products produced by bacteria and fungi (Hutchings *et al*., 2019; Nothias *et al*., 2016). Since their discovery almost 100 years ago, there are over 100 antibiotics in circulation globally with most belonging to a handful of classes (Coates *et al*., 2011; Dhingra *et al*., 2020). However, the golden age of antibiotic discovery is thought to be over as there has been a dearth in the discovery of novel antibiotic classes in recent years with the last being discovered and brought to market in the 1980s (Silver, 2011). The main classes of antibiotics are highlighted in table 2.

Table 2. Main classes of antibiotics. Table content derived using information from Kohanski *et al.*, 2010 and NCBI's Medical Database: *StatPearls*, 2023. *Asterisks denote antibiotics used for assays within this thesis.

Class	Examples	Mode of Action	Lethality	Main Targets (Gram +ve/-ve)
Beta Lactams:		Inhibit peptidoglycan cross wall linkage by binding to penicillin-binding proteins (PBPs)	Bactericidal	
Penicillins	Penicillin Amoxicillin			+/-
Cephalosporins	Cefalexin			+/-
Carbapenems	Meropenem* Imipenem*			+/-
Monobactams	Aztreonam			-
Fluoroquinolones	Ciprofloxacin* Levofloxacin	Inhibit DNA synthesis by targerting DNA gyrase	Bactericidal	+/-
Aminoglycosides	Tobramycin* Gentamicin	Disrupt protein synthesis by binding to the 30s ribosomal subunit	Bactericidal	-
Tetracyclines	Tetracycline Doxycycline	Inhibit protein synthesis by binding to the 30s ribosomal subunit	Bacteriostatic	+/-
Macrolides	Erythromycin Azyhtromycin	Inhibit protein synthesis by binding to the 50s ribosomal subunit	Bacteriostatic	+
Diaminopyrimidines	Co-trimoxazole	Inhibit tetrahydrofolic acid synthesis	Bacteriostatic	+/-
Glycopeptides	Vancomycin Teicoplanin	Inhibit peptidoglycan cross wall linkage by binding to peptidoglycan units	Bactericidal	+
Lipopeptides	Daptomycin Polymixin B	Disrupt cell membranes by creating pores	Bactericidal	+
Rifamycins	Rifampin Rifapentine	Inhibit RNA synthesis by binding to DNA-dependent RNA polymerase	Bacterostatic	+

Bacterial antibiotic survival dynamics

Competition for resources dominates life in microbial communities (Granato *et al*., 2019). Consequently, many antibiotics derive from compounds produced naturally by microbes as weapons against other microbes, with the most prominent example being penicillin (Fleming, 1941). Given the common occurrence of these interactions within natural environments, bacteria have developed a range of both genetically and phenotypically derived responses to survive.

The standard way to identify the different types of antibiotic survival mechanisms is through using kill curves (Balaban *et al*., 2019). Kill curves reveal how bacteria respond to the antibiotic treatment, where susceptible cells die revealing the composition of resistant/tolerant cells in the population. The E-test method further gives an indication of the minimum inhibitory concentration (MIC) of antibiotics (Brauner *et al*., 2016). To measure the MIC, lawns of bacteria are spread onto agar prior to the addition of an E-test strip where the area of growth inhibition indicates the minimum concentration of antibiotic able to suppress growth of the bacterial population. Below, I outline the different survival pathways and how they compare depending on kill curve dynamics and responses to E-tests (Fig. 4).

Resistance

Resistance is the most studied and well understood antibiotic survival mechanism. Resistance occurs when bacteria either mutate to evade antibiotic treatment or acquire resistance genes from the environment (this includes free DNA and acquisition from neighbouring bacteria) (Reygaert, 2018). Resistance mechanisms can either block the antibiotic from successfully binding to the target, involve the production of enzymes to degrade the antibiotic, or reduce intracellular build-

up of the antibiotic (either through reducing permeability or increasing efflux) (Breidenstein *et al.*, 2011). This means bacteria can grow in the presence of this otherwise bactericidal or bacteriostatic antibiotic. The ability to grow through higher concentrations of antibiotics corresponds with a higher MIC in response to the E-test strip.

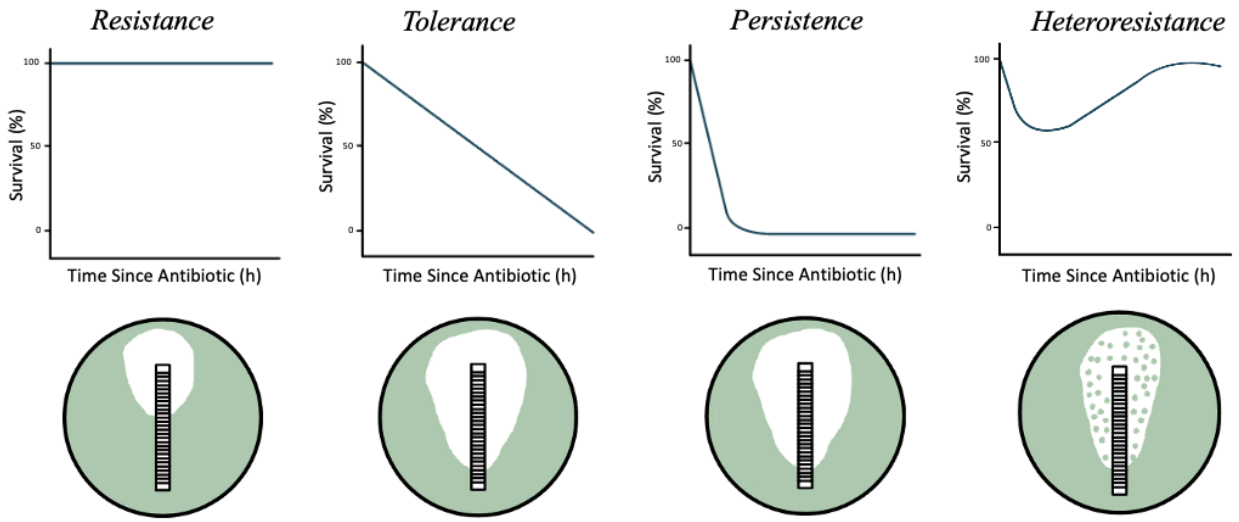


Figure 4. Bacterial antibiotic survival mechanisms. Recording survival post exposure provides an indication of antibiotic susceptibility (top panel). The bottom panel shows E-tests which indicate how the different antibiotic survival mechanisms alter the response to the minimum inhibitory concentration (MIC) of antibiotics. Diagram inspired by (Sherman *et al.*, 2019).

Tolerance

Antibiotic tolerance is a population-wide trait where all cells within the population display a similar ability to tolerate the antibiotic treatment, dying slowly over time (Brauner *et al.*, 2016). Tolerance has been shown to be a slow-growing phenotype which spans across the whole population (Michaux *et al.*, 2022). This leads to a kill curve with a slow decline which ultimately reveals a small proportion of survivors. Due to the eventual sensitivity to the antibiotics, the MIC is expected to be relatively low (Verstraete *et al.*, 2022).

Persistence

Persistence occurs in a small fraction of the population where cells are thought to adopt a phenotype which enables them to evade the antibiotic treatment (Balaban *et al*., 2019). The terms persistence and tolerance are sometimes used interchangeably however, it is important to note that kill curves help distinguish between the increased population-wide ability to withstand antibiotic treatment (tolerance), and the existence of only a subfraction able to survive (persistence) (Shi & Zarkan, 2022). The gold standard measure for persistence is biphasic killing, where susceptible cells die quickly revealing an underlying level of surviving cells that plateaus (Balaban *et al.*, 2019). Bacterial populations with a persistent subfraction will maintain a low MIC despite having a subfraction that is able to survive even at very high antibiotic concentrations as these cells are unable to grow in the presence of the antibiotic (Verstraete *et al.*, 2022).

Heteroresistance

Heteroresistance arises when resistance genes are heterogeneously expressed in a bacterial population conferring antibiotic resistance to some but not all cells (Andersson *et al.*, 2019). In comparison to persistence, the proportion of surviving cells after antibiotic treatment may be higher than the initial cell count as the surviving resistant population regrows (Lin *et al*., 2019). Heteroresistance can further be distinguished from persistence by looking at E-test responses where despite having a low baseline MIC, heteroresistant colonies are able to grow sporadically in the zone of inhibition (Sherman *et al*, 2019). Despite being widely accepted as an antibiotic survival mechanism, there are few examples of heteroresistance within the literature (Andersson *et al*., 2019).

Perseverance

The concept of antibiotic perseverance has recently emerged in a study by Brandis *et al.* (2023). This is proposed to be unique in comparison to resistance, tolerance, persistence, and heteroresistance. Antibiotic perseverance is said to occur when a fraction of the population continues to grow in the presence of the antibiotic despite being genetically susceptible. There is some uncertainty as to the origins of antibiotic perseverance due to only being described in one study thus far (Brandis *et al.*, 2023), and having many similarities with heteroresistance.

Bacterial study organisms

The two main study organisms within this thesis are *P. aeruginosa* and *E. coli.* They are both rod-shaped Gram-negative bacteria of clinical relevance. Both species can be opportunistic pathogens and have been shown to be responsible for causing chronic and recalcitrant infections (El-Mosalamy *et al*., 2012; Hannan *et al*., 2010; Koeva *et al*., 2017; Staudinger *et al*., 2014). As previously mentioned, *E. coli* has been studied broadly within the persistence literature whereas *P. aeruginosa* is studied less. As a result, I chose to work on *P. aeruginosa* due to its lower representation within the literature, and to build on pre-existing expertise on this species within my lab. In terms of antibiotic susceptibility, *E. coli* has been shown to generate a higher proportion of persisters (Keren *et al*., 2004), whereas despite having lower persistence, *P. aeruginosa* has a broad range of intrinsic antibiotic resistance (Breidenstein *et al*., 2011).

The heterogeneity across lab protocols looking at a heterogeneous trait

A recent study by Atolia *et al*., (2020) outlined how variable microbial growth curve dynamics are depending on the initial conditions within the culture. This included: whether or not cultures were

inoculated directly from freezer stocks; the effects of the selected growth media; the knock-on effects of the chosen inoculation density; and, for measuring growth, the position within the 96-well plate. Given the wide range of factors introducing variation, it is not surprising that research into a variable and heterogenous phenotypic trait like persistence has also led to so much variation.

Another aspect that is also often overlooked in persistence research is the effect of different types of antibiotic stressor. Antibiotics have a wide range of mechanisms to arrest growth or bring about cell death, and this should be of significant interest when determining how persisters can evade a broad range of antibiotic mechanisms. If persistence is simply a blanket 'shutdown' mechanism that cells can use to simply ignore their environment, then we would expect limited variation in the percentage of persisters depending on the antibiotic selected. However, if there is significant variation then we would expect that, despite being in the persistence state, cells are interacting with their environment and finding ways of surviving through the antibiotics. Urbaniec *et al.* (2022) recently proposed a novel 'hunker' theory to explain this variation, with specialised cells that have antibiotic-specific persistence mechanisms and do not depend on slow growth/metabolism rates to regulate persistence.

Searching for proximate and ultimate explanations for persister cell formation

Phenotypic heterogeneity within bacterial populations is known to be linked with antibiotic persistence (Gefen & Balaban, 2009; Orman & Brynildsen, 2013; Verstraete *et al.*, 2022), with research even using persister cell formation as a proxy for phenotypic heterogeneity (Smith *et al.*, 2018). The ability of persister cells to survive antibiotic treatment has led to an assumption that persistence is a deterministic survival adaptation, favoured by selection for the same reasons as

genetic resistance (Balaban *et al.*, 2019). However, one of the few studies examining long-term evolution *in situ* suggests that selection to increase persister cell formation is weak or absent (Ghoul et al., 2023).

Whether the driving force behind the generation of phenotypic heterogeneity, and so persistence, is deterministic or stochastic is disputed (Avery, 2006; Ackermann, 2015; Bollen et al., 2023). Five potential avenues for the generation of phenotypic heterogeneity have been put forward by Ackermann (2015): 1) stochastic gene expression; 2) periodic oscillations; 3) cellular aging; 4) cell-cell interactions; and 5) epigenetic modification. Avery (2006) proposed that phenotypic individuality is thought to bolster the fitness of populations, with growing evidence that there is evolutionary selection for diversity-generating mechanisms in microorganisms. Therefore, regardless of whether the underlying processes behind heterogeneity may be deterministic or stochastic, the generation of this variation may provide a selective advantage.

When considering how behaviours evolve, we can use Tinbergen's four questions as a useful framework. Nikolaas Tinbergen was a prominent ethologist of the 20th century who set out four fundamental questions for studying and understanding behavioural characteristics (Tinbergen, 1963). These four questions are what is the (1) 'survival value'; (2) 'ontogeny'; (3) 'evolution'; and (4) 'causation'? – Put simply, (1) what is the trait for; (2) how does it develop during the organism's lifetime; (3) how did it evolve within the species; and (4) what causes it? These questions can be further classified into proximate (2&4) and ultimate (1&3) explanations – the 'how' and 'why' does a characteristic trait arise (Bateson & Laland, 2013).

In terms of understanding persistence as a characteristic, most of the focus within the literature has gone into deriving the mechanistic drivers of persistence ((4) causation – see table 2 above). However, this leaves important aspects of Tinbergen's four questions unanswered. Particularly, by focusing on the wider proximate and ultimate explanations of persistence, we may be able to establish the fundamental principles behind how and why persistence arises. To do this, we can observe persistence through different growth dynamics in bacterial populations ((2) ontogeny), and question how differences in survival patterns change depending on these population dynamics ((1) survival value), so that we may finally begin to question the adaptive nature of persistence to further interrogate if/how it has evolved ((3) evolution).

Empirical studies have explored adaptive explanations for persistence, notably focusing on persistence as a stepping stone to resistance (Vogwill *et al.*, 2016; Levin-Reisman *et al., 2017;* Santi *et al.,* 2021*;* Ghoul *et al.*, 2023), and the adaptive role persisters may play in biofilms (Carvalho *et al*., 2018). However, very few have experimentally tested adaptive explanations for persistence (Stepanyan *et al.*, 2015). A notable mention in terms of exploring adaptive explanations for persistence is a theoretical study by Gardner *et al*. (2007), where the authors suggest a potential cooperative role for persistence. If persister cells act as an "insurance policy", providing a reservoir of cells that can survive catastrophic events, it might be possible to understand the division of a bacterial population into persister and growing cells as a kind of division of labour. A dormant subpopulation of cells may also reduce competition for limiting resources in growing bacterial populations (Fig. 5). In this sense, persistence can be considered as a social trait which is favoured when cells are related and resources are scarce. Along these lines, persistence can act as a bet-hedging strategy whereby the reduced fitness phenotype of a

dormant/slowly dividing cell can provide a selective advantage in certain conditions (such as under antibiotic stress) (Philippi & Seger, 1989; Gardner *et al.*, 2007). However, if the trait itself is costly, it is most advantageous to maintain this phenotype in only a subset of the population (Schröter & Dersch, 2019).

The question still unanswered, therefore, is how is this subpopulation regulated? There may be advantages of maintaining a phenotypically distinct subpopulation, but how do populations ensure that enough cells are actively dividing to compete for resources, and enough cells are in the persistent state to repopulate in case catastrophe strikes? Epigenetic inheritance has been shown to have an effect on bet-hedging in terms of another bistable survival mechanism in bacteria: spore formation in *Bacillus subtilis* (Veening *et al*., 2008). Troy Day proposed a role for epigenetic inheritance to regulate persistence at the population level (Day, 2016). Here, cells accumulate variation and become more heterogenous through the successive inheritance of nongenetic material from cell lineages. This means that persistence may not be genetically regulated *per se*, but instead arises through cells inheriting increasingly 'noisy' cell contents that eventually build up to switch a fraction of cells into the persistence state. Day posits that persistence is the best studied example of evolution by epigenetic inheritance.

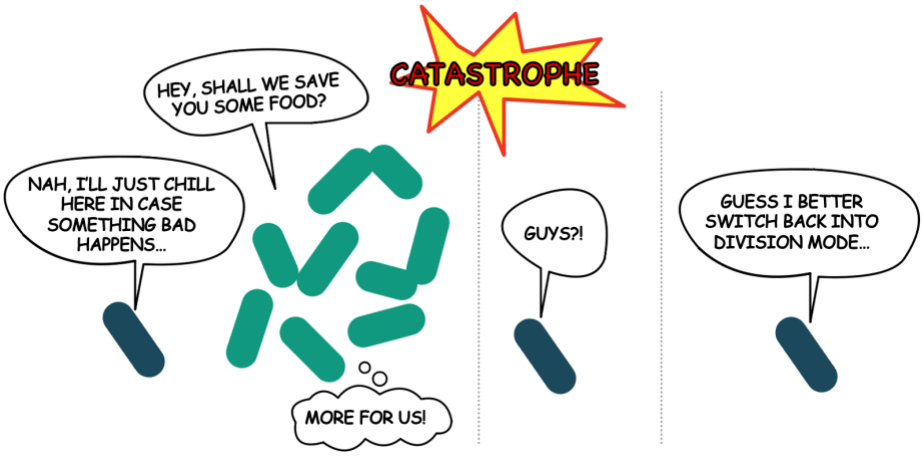


Figure 5. Comic book strip highlighting the division of labour between actively dividing cells which build the population size (turquoise), and persister cells (dark blue) which act as an insurance policy for the population in case catastrophic events arise.

In the context of social evolution, these are the big questions in persistence research that I believe are unresolved within the literature:

1) What environmental conditions select for bacterial persistence?
2) How is persistence regulated at the population level?
3) Is persistence an adaptive trait?
4) What are the evolutionary origins of persistence?
5) Does persistence provide an evolutionary advantage to a bacterial population?

In my thesis, I answer question 1, provide evidence for question 2, and lay the foundations for future studies to address question 3. By addressing questions 1-3, we may begin to posit how persistence evolved and consider the evolutionary advantages behind this trait.

Chapter 2 Motivation

The motivation behind this chapter was to establish how persister levels changed throughout the growth curve in a clinically relevant and lesser-studied bacterial species, *P. aeruginosa*. My goal was to establish how changes in growth rates and cell density influence the underlying persister formation pattern in *P. aeruginosa*. The hypothesis was that persistence formation levels would be higher from stationary phase cells which is in line with expectations from the literature (Keren *et al.*, 2004; Gardner *et al.*, 2007; Gefen *et al.*, 2014; Harms *et al.*, 2017; Khare & Tavazoie, 2020).

I used a small inoculation density to generate a culture with an extended growth curve where cells did not reach stationary phase until ~16 hours of culturing (based on data from previous research within the lab – see (Ghoul et al., 2015; Ghoul, 2014, Chapter 4). This lower inoculation density enabled me to capture several hours of lag, exponential, and stationary phase where I could record data over multiple hours of growth within each phase where cells have different rates of growth and cell densities.

In part, I also completed this initial growth curve persistence assay with the view to designing a competition assay later on during my DPhil. I needed to identify the culturing timepoints and states of population growth where persistence was most likely to increase to compete persisters versus non-persisters. However, initial results indicated that persistence unexpectedly peaked during exponential growth. This led to a series of further experiments where I varied inoculum density to see whether the low initial inoculum density was having an effect on the culturing timepoint of persistence peaks. I had the idea to alter inoculum density after reading Atolia *et al*.'s (2021)

research on the potential knock-on effects from the initial inoculum size on subsequent population growth rate.

To further understand these population dynamics, I also measured ATP and cell size distribution throughout the growth curve to get a sense of whether these parameters were also affected by the inoculum density. My motivation to measure ATP stemmed from reading Mempin *et al.* (2013) where ATP can give an indication of the metabolic effort cells are exerting as well as show changes in ATP levels both intra- and extracellularly. I used cell imaging to specifically identify changes in the patterns of cell size distributions across the growth curve, and see how this variance compared with patterns in persistence depending on inoculum density.

With this chapter, the key questions I wanted to answer included:

1) What environmental conditions select for bacterial persistence?
    a. How do changes in population dynamics influence persistence?
2) How is persistence regulated at the population level?
    a. How does the magnitude of persistence change throughout the growth curve, and what does this tell us about the potential mechanisms that are promoting persistence?

Chapter 2. High growth rates promote persister cell formation in *Pseudomonas aeruginosa*

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Abstract

Subpopulations of bacterial cells, known as persister cells, can survive high doses of antibiotics despite being genetically susceptible. This raises questions about how persistence evolves and what factors may explain intraspecific trait variation? Persister cell formation is associated with arrested growth and thought to predominantly occur during slow growth conditions, such as during stationary phase in bulk culture or within inner regions of biofilms. An evolutionary explanation for this could be that the potential fitness costs of arrested growth are less likely to affect cells that are not growing anyway. To test this, we compared persister fractions at different points of a growth curve in populations of the pathogenic bacterium, *Pseudomonas aeruginosa*. In contrast to expectations, the highest persister cell fraction was measured in samples from a population undergoing exponential growth. To further investigate the effect of growth rate, we compared persister cell levels, ATP concentration, and cell morphology in populations with different growth curve phase durations and growth rates by varying inoculum density. In these assays we identified a role for cell division history in determining when persistence arises within the growth curve. This points towards the state of individual cells being the most accurate predictor of persistence, rather than the population growth phase. Future work should focus on possible advantages of quiescence to cells in growing populations.

Significance

Bacterial cells can exist in a quiescent state known as persistence, which is characterised by arrested growth and high antibiotic tolerance. Persistence is often considered to be a strategy that bacterial cells can adopt in order to survive in the presence of antibiotics, even though the phenotype is only adopted by a small fraction of the population (0.0001-1%). This raises fundamental questions about how persister cell formation evolves and how it might enhance the fitness of individual cells. The physiological switch to persistence has been linked to slow-growing populations, when the potential costs of arrested growth are likely to be at a minimum. This is not the pattern we observed in our experiments; instead we found high persistence levels not only during stationary phase but also prominent levels in actively growing populations. This ability for persistence to peak during different growth curve phases indicates an important role for population cell division history. Persistence also varied depending on antibiotic stressor; a feature that is rarely discussed in persister assays but has potential clinical implications in the case of chronic infections. These results highlight the need for more work to understand the evolutionary drivers of persistence so that new strategies for blocking this route to surviving antimicrobial treatments can be pursued.

Key Words: *Pseudomonas aeruginosa*; antibiotic resistance, tolerance, & persistence; microbiology; bacterial growth; inoculum density

Introduction

Persister cells are phenotypic variants that can survive high doses of antibiotics, despite being genetically identical to antibiotic-susceptible cells (Balaban *et al.*, 2019). They consequently present a serious challenge to treatment of infection across bacterial species (Fisher *et al.*, 2017). In *Pseudomonas aeruginosa*, for example, persister cell formation is thought to contribute to chronic infections in cystic fibrosis patients (Mulcahy *et al.*, 2010; Mojsoska *et al.*, 2019; Rossi *et al.*, 2021). Unlike antibiotic resistant cells, persisters cannot grow in the presence of antibiotics; they are in a quiescent state and can only revive upon removal of the antibiotic stressor (Kaldalu *et al.*, 2016). This phenotype enables a reversible commitment to the new cell state without the associated costs of DNA-driven resistance (Avery, 2006). While this transient state enables persisters to survive antibiotic doses, persistence typically only occurs within 0.001-1% of a bacterial population (Van den Bergh *et al.*, 2017). The phenotypic nature of persistence, and the fact that it is adopted by a consistent but marginal proportion of a bacterial cell population, presents challenges for understanding its evolution.

Research on persister cells has strived to identify genes and cellular mechanisms involved in persistence (Conlon *et al.*, 2016; Pontes & Groisman, 2019; Wood & Song, 2020). However, no definitive regulatory pathway has been identified (Balaban, 2011; Van den Bergh *et al.*, 2017), and the underlying genetics remain mysterious. One dominant finding is that persister cell formation is linked to stress and conditions of slow growth (Pontes & Groisman, 2019). Single cell imaging has enabled researchers to attribute persistence to growth arrest (Balaban *et al.*, 2004; Gefen *et al.*, 2014), and persister formation has been observed deep within biofilms among non-dividing cells (Soares *et al.*, 2019; Spoering & Lewis, 2001). *In vitro*, slowly dividing cells survive

antibiotic treatments better, and studies have generally shown increased antibiotic lethality with increased growth rates (Lee *et al*., 2018) and metabolic activity (Lopatkin *et al*., 2019). In addition, persisters are eradicated in cells maintained in an exponential growth state in chemostats (Keren *et al.*, 2004; Orman & Brynildsen, 2013). Taken together, these results lead to a general expectation of persistence peaking in stationary phase when cell growth rates have decreased (Keren *et al.*, 2004; Gardner *et al.*, 2007; Gefen *et al.*, 2014; Harms *et al.*, 2017; Khare & Tavazoie, 2020). However, the evidence to support this expectation is inconsistent: other studies suggest that fast bacterial growth can reduce antibiotic efficacy (Łapińska *et al.*, 2022), persisters can arise from the fastest dividing cells (Orman & Brynildsen, 2013), and that cell metabolism remains active in persister cells (Amato *et al*., 2014; Pu *et al*., 2019).

More recently, attention has been given to a potential role for population heterogeneity in persistence (Gefen & Balaban, 2009; Ackermann, 2015; Smith *et al.*, 2018; Balaban *et al.*, 2019; Schröter & Dersch, 2019), which has been shown to increase with growth rates (Ackermann, 2015; Goormaghtigh & Melderen, 2019). This is an interesting possibility as it links persister cell formation with a population level feature: heterogeneity rather than the physiological state of a single cell (Gefen & Balaban, 2009; Aspridou & Koutsoumanis, 2015; Day, 2016). This heterogeneity may explain why persister cells make up a small proportion of the population which remains constant even after several rounds of treatment with antibiotics (Balaban *et al.*, 2019). Measuring persistence alongside changes in growth rates has been shown in previous studies which measured persistence across the growth curve for eight hours (Harms *et al.*, 2017), and up to 10 hours of culturing (Keren *et al.*, 2004). Both studies found a consistent pattern of increased persistence during slow growth in stationary phase.

To capture the effect of changes in growth rates on persistence over a broader range of culturing conditions, we use lower inoculum densities and measure persistence for up to 36 hours of culturing time. We use the *P. aeruginosa* PAO1 lab strain and assay persistence (by carrying out kill curves - methodology A), and cell survival (by measuring samples 24-hours after antibiotic treatments - methodology B). We use antibiotic concentrations above MIC to diminish the effects of resistance on cell survival, however, this cannot guarantee that we completely eliminate all resistance effects on survival, such as that of upregulated efflux pumps (Breidenstein *et al*., 2011). Therefore, cell survival after 24-hours under antibiotic treatment could indicate a combination of resistance, tolerance, and persistence which we further confirm with kill curves. Kill curves are the standard for measuring persistence, where biphasic killing is expected: the antibiotic-susceptible fraction of the population die quickly before revealing the underlying persister plateau (Balaban *et al.*, 2019). We use three classes of antibiotics to test for stressor-specific responses: ciprofloxacin (fluoroquinolone); tobramycin (aminoglycoside); meropenem (beta-lactam). These antibiotics have distinct mechanisms of bactericidal action: ciprofloxacin targets DNA gyrase inhibiting DNA replication (LeBel, 1988); tobramycin inhibits the formation of the 70s ribosome thus preventing protein synthesis (Goffic *et al*., 1979); and meropenem targets cross-wall linkage inhibiting cell wall formation (Baldwin *et al.*, 2008). To test the effect of cell doubling rates on cell survival, we compare cell survival across cultures with incrementally lower inoculum densities. Lowering inoculum density has been shown to have a significant effect on increasing lag phase length, maximal growth rates, and delaying the timepoint of entry into stationary phase (Atolia *et al*., 2020). As well as assessing cell survival under changing population growth rates, we also look for an effect more specifically associated with the state of individual cells by collecting data for ATP concentration and cell morphology variation. Intracellular ATP is an

indicator of metabolic rate (Yaginuma *et al*., 2014), and differences in cell-to-cell size provide a measure for population heterogeneity (Bakshi *et al.*, 2021). Our assays enabled us to measure persistence and cell survival over a broad range of growth conditions; from bulk responses in batch culture down to the single-cell level.

Results

High rate of persister cell formation during exponential growth

We sampled a freshly inoculated culture every two hours for 36 hours of growth and characterised variation in persistence by performing a kill curve assay at each time point with three different antibiotic treatments (Fig. 1A-C; Fig. S1-2). We determined growth curve phases by measuring CFU, optical density and growth rate (Fig. 1D; Fig. S3). Persister cell levels peaked prominently during exponential phase for all antibiotic treatments (Fig. 1E). In the subsequent samplings in late exponential phase, persistence decreased before increasing again in stationary phase. The peak in persistence during exponential phase was distinct from the elevated persistence in stationary phase (Fig. 1E). Some antibiotic treatments caused an unexpected increase in cell count in the initial hours of treatment (two-four hours) preceding death (Fig. S4). In some samples treated with meropenem and tobramycin, we also saw an increase in cell count after 18-24 hours of treatment which may have been caused by antibiotic degradation (Medaney *et al.*, 2016). To account for cell count increases in response to the antibiotic treatments, we calculated persister percentages as the proportion of the highest number of cells before numbers dropped, rather than the number of cells pre-antibiotic treatment, and took cell count prior to any potential antibiotic degradation-related regrowth (see methods, supplementary information, and Chapter 4 for further discussion) (Fig. 1B & Fig. S1-2). We noticed that peaks in persistence (methodology A – measuring persistence by

kill curves) and peaks in cell survival (methodology B – measuring cells that survive 24 hours post antibiotic treatment) occurred from cell populations in the same stages of their growth curve (Fig. S5).

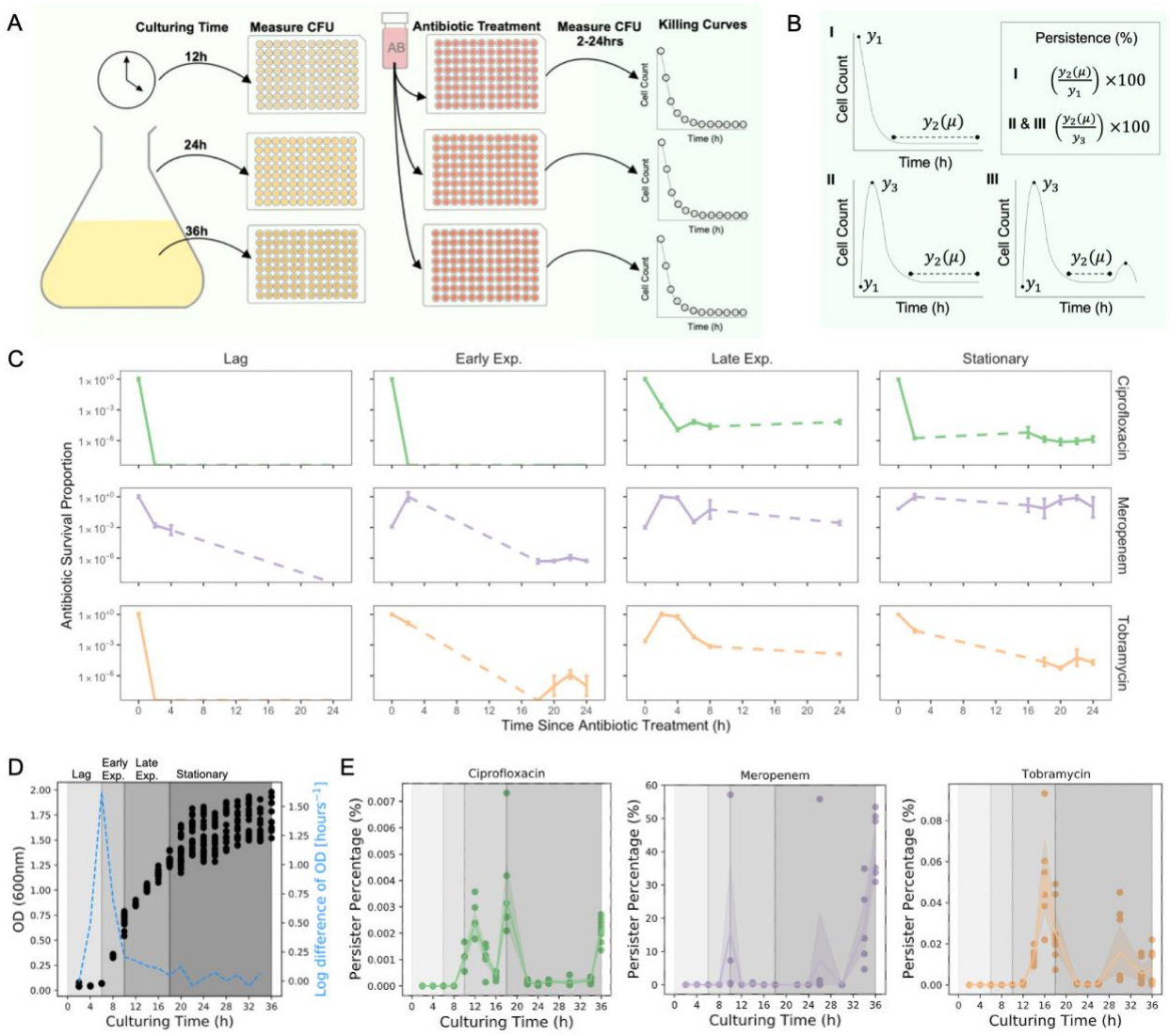


Fig. 1. Persistence peaks in exponential phase. (*A*) Diagram illustrating the kill curve assay procedure to measure persistence. (*B*) Methods used to calculate the persister fraction (minimum cell count value across Y_2 (represented by dashed horizontal line)) from individual kill curves accounting for antibiotic-induced stress divides (II) and regrowth (III) (if present) (Fig. S1-S2). (*C*) Representative kill curves from each phase of the growth curve phases for each antibiotic treatment (Lag – 4h; Early Exponential – 8h; Late Exponential – 14h; Stationary – 26h). The dashed line represents periods between sampling timepoints. The scale has been log transformed and error bars depict standard error of the mean. Individual kill curves for each growth curve timepoint are depicted with error bars in the supplementary information (Fig. S1). (*D*) Growth curve phases (shaded panels) derived from growth rate and optical density: OD_{600} (black dots from individual experimental replicates), and growth rate (blue dashed line). This culture was inoculated with a low inoculum density (10^{-4} dilution of a 0.1 OD_{600}-standardised overnight culture). (*E*) Persister percentage

across growth phases (lag, early exponential, late exponential, and stationary phase – depicted by incrementally darker shaded panels) for three antibiotic treatments: ciprofloxacin (green), meropenem (lilac), and tobramycin (orange). The culture was sampled every two hours from 2 to 36 hours of culturing time. Each datapoint is representative of at least eight technical replicates.

Cell survival is affected by cell division history

We conducted further experiments to identify putative factors responsible for driving the unexpected persister cell peaks in exponential growth phase. We measured cell survival (method B) every two hours for 20 hours of culturing time in four cultures inoculated with diminishing cell densities (10^{-2}-10^{-5}-fold dilutions of a 0.1 OD_{600}-standardised overnight culture) (Fig. 2A). Reducing inoculum density showed how less dense cultures have longer growth curve phases and enter stationary phase at later culturing timepoints (Fig. 2B). By comparing across these growth curves, we tested the hypothesis that the physiological state of individual cells, rather than the population growth phase, was responsible for driving peaks in persister cell levels. We experimentally decoupled cell state from population growth phase by varying inoculum density and measured: (1) the number of doublings following inoculum; (2) ATP concentration per cell; and (3) heterogeneity in cell size. All cultures reached comparable cell densities during stationary phase within our assay regardless of inoculum density (CFU/ml: ~8×10^{12}; Fig. 2B; Fig. S6). Peaks in survival were observed earlier in low density inoculum cultures; and even as early as during lag phase in the lowest inoculum culture (Fig. 2C; Fig. S7-8).

Number of doublings: Cell survival peaks were better predicted by the number of doublings from inoculation rather than growth curve phase transitions. We initially found that as lag phase length increased, cell survival peaked earlier in the growth curve (Kruskal-Wallis: $\chi_2 = 6.63$, df = 1, P = 1.0×10^{-2} (Fig. 2C)). This earlier peak in cell survival was most pronounced in the lowest inoculum density culture which had a long lag phase. Reducing inoculum density also increased the average

number of doublings required to reach stationary phase (Fig. S9). We further looked at the number of doublings prior to peaks in cell survival. The number of divisions prior to cell survival peaks were generally comparable across inoculum dilutions, with the exception of the lowest inoculum density (10^{-5}) under treatment with ciprofloxacin or tobramycin, and the highest inoculum density (10^{-2}) under treatment with meropenem (Fig. 3A).

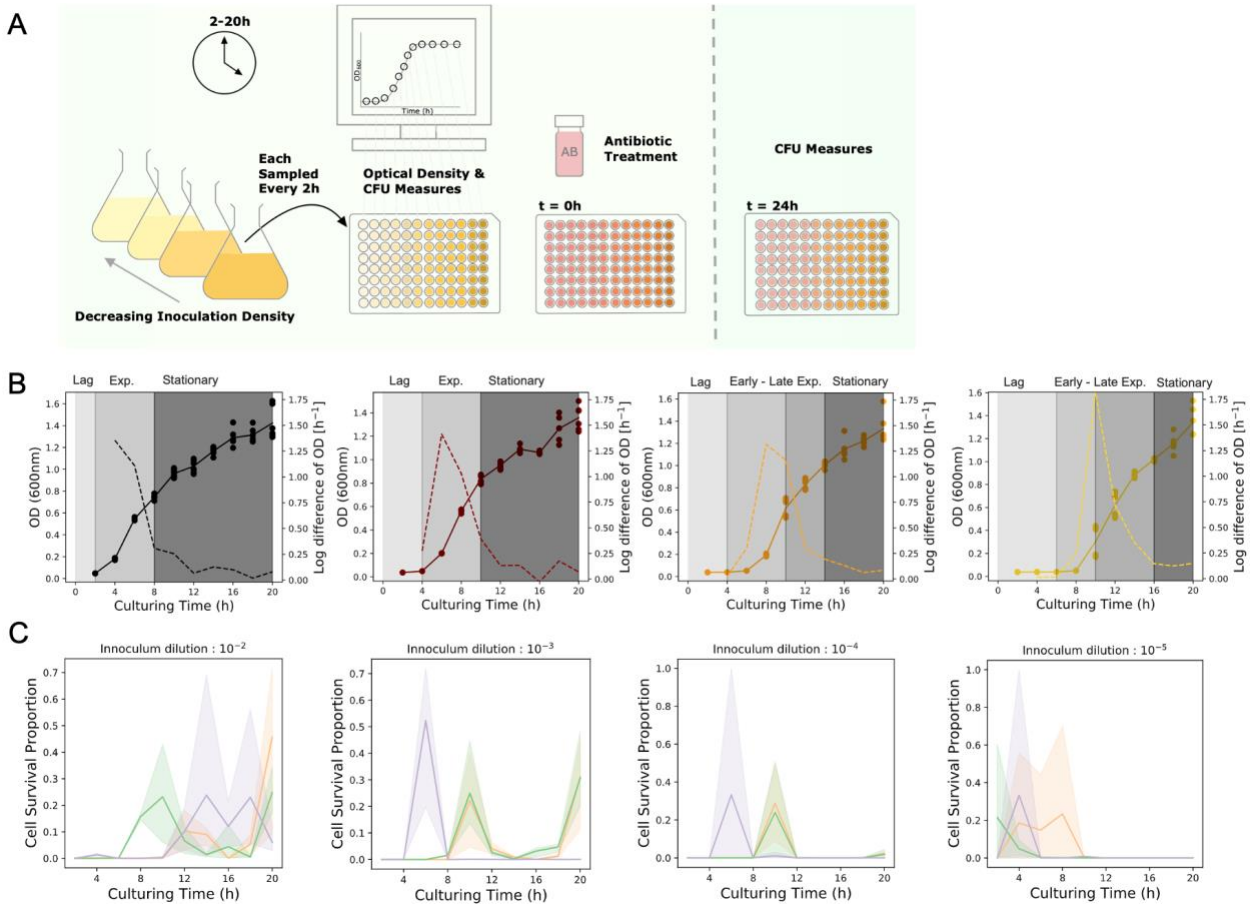


Fig. 2. Cell survival is influenced by population division history. **(***A*) Diagram illustrating the 24-hour antibiotic assay procedure. (*B*) Growth curves (2-20hrs) for cultures inoculated with incrementally lower cell densities, where an overnight culture was standardised to 0.1 OD_{600} before being diluted 10^{-2}-, 10^{-3}-, 10^{-4}-*, 10^{-5}- fold: (black, brown, orange, and yellow, respectively) (*Same inoculum density as the above assay in figure 1). The different phases of growth (shaded regions) are based on growth rate (dashed line) and OD_{600} (solid line with scatter points). (*C*) Survival proportion peaks for three antibiotic treatments: ciprofloxacin (green), meropenem (lilac), and tobramycin (orange). Cell survival proportion has been used for comparison across antibiotic treatments, where survival percentage data has been normalised to represent the proportion of surviving cells relative to the greatest peak in survival across inoculum densities within each antibiotic treatment. Raw cell survival data for each treatment is shown in Table 1 and further in the supplementary information (Fig. S7). Due to a dilution effect in samples treated with ciprofloxacin

in the lowest inoculum dilution culture (10^{-5}), a further assay with a NaCl washing step was carried out to determine the timepoint of the cell survival peak (see supplementary information (Fig. S10)). Each data point is representative of at least three technical replicates (3-6). Shading depicts standard error of the mean.

Intracellular ATP concentration: Higher metabolic rate during lag phase and early exponential phase (as seen in cultures inoculated with low cell densities) coincided with high survival peaks (Fig. 3B). Across the growth curve, the peak concentration of ATP per cell was significantly higher in cultures initiated with low inoculum densities (10^{-4}- and 10^{-5}-fold dilution) (Kruskal-Wallis: $\chi_2 = 21.42$, df = 3, $P = 8.6\times10^{-5}$; Fig. 3B). This is further evidence to support an association between increased cell survival against antibiotics with metabolic effort, as opposed to metabolic shut-down.

Cell size heterogeneity: We assessed heterogeneity in cell size by microscopy snapshots of samples across the growth curves with different inoculum densities. Variation in cell size depended on growth phase (Fig. 3C), with the greatest variance occurring in actively growing cultures initiated from low inoculum densities (Fig. 3D). In low inoculum density culture samples, increased size heterogeneity within the growth curve correlated with high energy availability per cell and increased cell survival. Cell size heterogeneity generally decreased over culturing time which coincided with a decrease in cell survival.

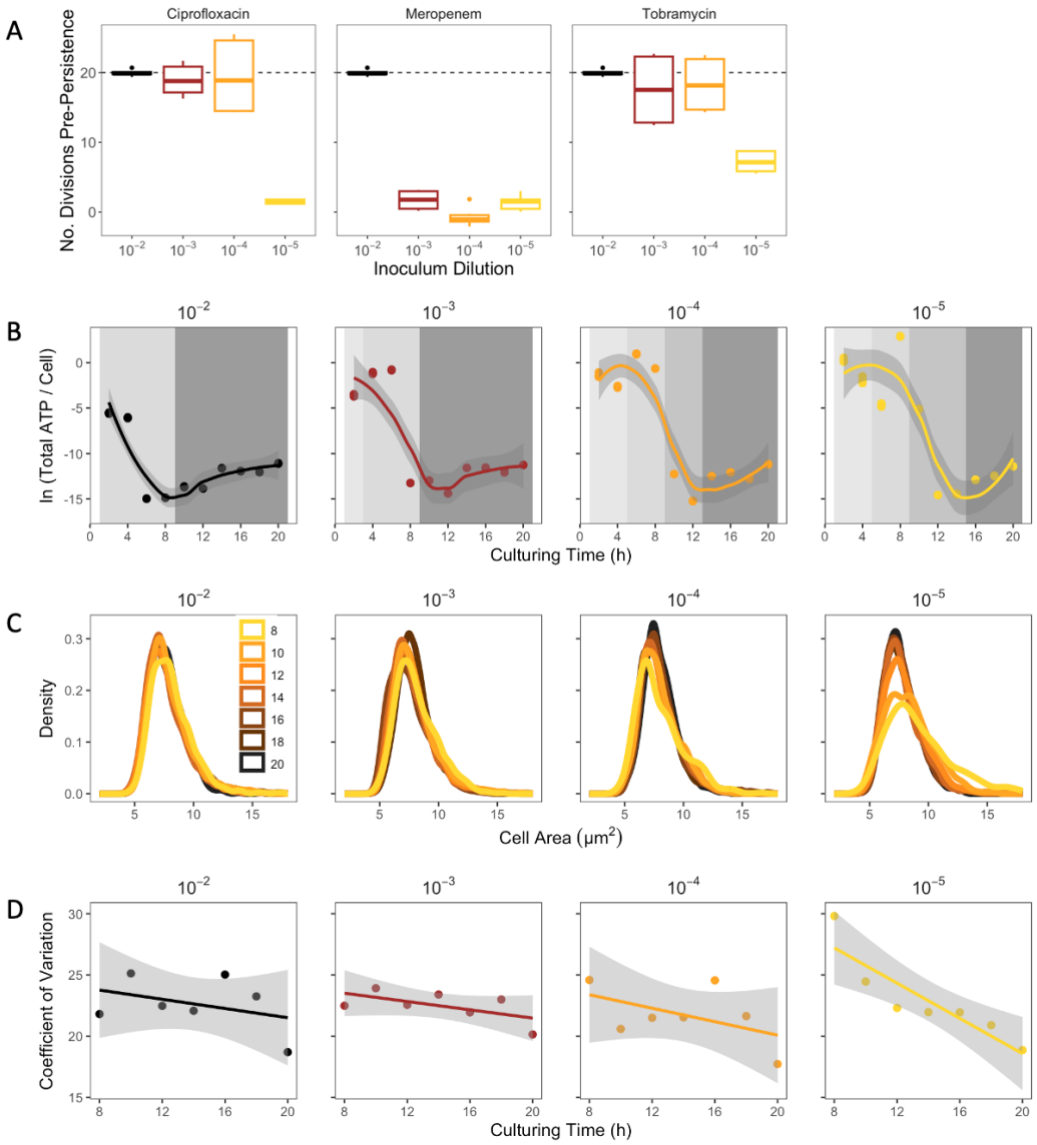


Fig. 3. Growth curve phase transitions and energy availability determine cell-cell heterogeneity. (*A*) Cumulative number of cell divisions prior to persistence peaks forming for cultures inoculated with diminishing cell densities (10^{-2}-10^{-5}-fold dilutions of a 0.1OD_{600} standardised overnight culture). Cell survival peaks occurred at the following culturing times for each antibiotic treatment: ciprofloxacin: 10^{-2} – 10hrs; 10^{-3} – 10hrs; 10^{-4} – 10hrs, 10^{-5} – 2hrs*; meropenem: 10^{-2} – 14hrs; 10^{-3} – 6hrs; 10^{-4} – 6hrs; and 10^{-5} – 4hrs; tobramycin: 10^{-2} – 12hrs; 10^{-3} – 10hrs; 10^{-4} – 10hrs; and 10^{-5} – 6hrs (for 10^{-2} cultures, the number of divisions prior to entry into stationary phase (6hrs) has been used to estimate the number of growth phase divisions prior to cell survival peaks (dashed line)). *The timing of the cell survival peak under ciprofloxacin treatment in the low inoculum density culture (10^{-5}) has been taken from the cell washing assay (See Supp. Info: Fig. S10). (*B*) ATP per cell where total ATP concentration has been divided by total cell number. Data has been log transformed for visualisation. Each datapoint is represented by three technical replicates. (*C*) Cell size (μm²) distribution across the growth curve (8-20 hours) in samples inoculated with incrementally lower cell densities. Each histogram has been generated using measures from 360 cells. (*D*) Coefficient of variation in population cell size (μm²) distribution from 8-20 hours of culturing time for cultures with different inoculum densities (10^{-2}-10^{-5}-fold dilutions). A linear model has been fitted with 95% confidence intervals.

Cell survival in stationary phase

We observed the expected increase in cell survival in stationary phase cultures inoculated with high densities (10^{-2}- and 10^{-3}-fold dilution) with the exception of meropenem (Fig. 2C). The low inoculum density cultures (10^{-4}- and 10^{-5}-fold dilution) did not exhibit the same distinct cell survival peaks during stationary phase (Fig. S8). However, our longer 36-hr growth curve assay, initiated with a low inoculum density (10^{-4} -fold dilution, Fig. 1E), did show increased persistence in late stationary phase across all antibiotic treatments. This suggests that cell survival is not necessarily driven by cell density effects when reaching carrying capacity but instead by the duration of time spent in stationary phase.

Extracellular ATP can be an indicator of population cell lysis rate (Ihssen *et al.*, 2021), or active ATP secretion from cells (Spari & Beldi, 2020). In cultures initiated by high density inoculum, we found that peaks in extracellular ATP coincided with increased cell survival in stationary phase (Fig. S11). These stationary phase peaks in extracellular ATP were only a signature of cultures that had been in stationary phase for an extended period of time (high inoculum density cultures). In contrast, extracellular ATP was significantly lower in stationary phase for cultures initiated with a low inoculum density despite being at comparable cell densities (Kruskal-Wallis: $\chi_2 = 37.25$, df = 3, $P = 4.1\times10^{-8}$). This further highlights the differences in culture conditions driven by population division history.

The magnitude and variation of cell survival depends on antibiotic class and inoculum density

The magnitude of cell survival was not the same across the three antibiotic classes (Kruskal-Wallis: χ_2 = 98.907, df = 2, P = 2.2×10^{-16}) (Fig. 4A). We confirmed that the fold-increase in antibiotic concentration relative to the MIC was unlikely to be responsible for these differences in cell survival (Fig. S12), and that our high antibiotic dose avoided potential prophage induction (Harms *et al*., 2017). Cell survival was highest in cultures treated with meropenem and lowest with ciprofloxacin. These differences in antibiotic-specific response were consistent across all but the highest inoculum density culture (10^{-2}-dilution).

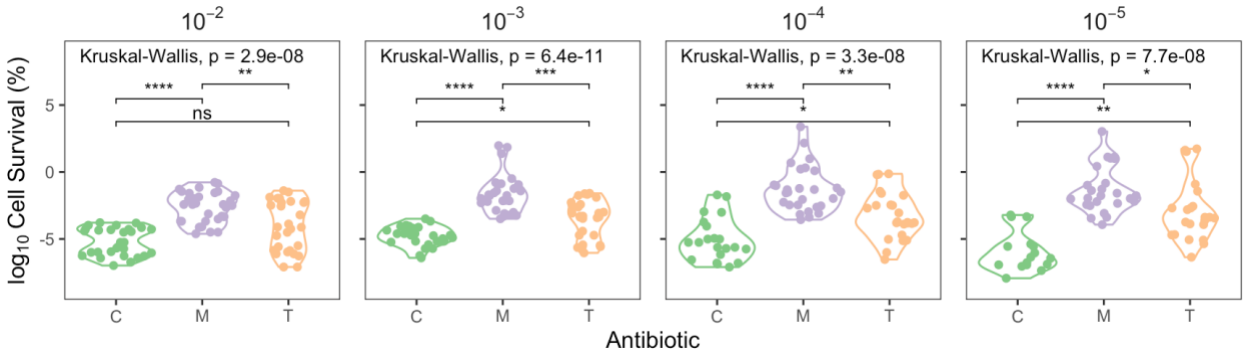


Fig. 4. Antibiotic-specific effects on cell survival. Violin plot showing log transformed persister fractions after treatment with ciprofloxacin (green), meropenem (purple), or tobramycin (orange) integrating all timepoints across the growth curve in cultures inoculated with incrementally lower cell densities (10^{-2}-10^{-5}-fold dilutions). Asterisks denote significance where *: p <= 0.05; **: p <= 0.01; and ***: p <= 0.001.

Variance in cell survival was consistently greatest in low inoculum density treatments (10^{-4}- and 10^{-5}-fold) across all three antibiotic treatments (F = 2.1×10^{-3}, df = 215, P = 2.2×10^{-16}). In both tobramycin and meropenem treatments, the mean and maximum cell survival was higher in low inoculum density treatments (10^{-4} and 10^{-5}-fold dilutions) (Table 1).

Table 1. Summary of cell survival percentages integrating all timepoints across the growth curve depending on antibiotic class and inoculum density. *Value not taken from NaCl washing protocol. **Where 'Survival (%)' is greater than 100%, this indicates growth after addition of the antibiotic (see Supp. Info).

Antibiotic	Inoculum Density Dilution	Mean Survival (%)	Maximum Survival (%)
Ciprofloxacin	10^{-2}	0.0000274	0.000165
	10^{-3}	0.000035	0.000321
	10^{-4}	0.00101	0.0195
	10^{-5}	0.0000431	0.000615*
Meropenem	10^{-2}	0.0178	0.169
	10^{-3}	5.26	94.1
	10^{-4}	71.3	2400**
	10^{-5}	31	1065**
Tobramycin	10^{-2}	0.00381	0.0406
	10^{-3}	0.00337	0.0257
	10^{-4}	0.0595	0.732
	10^{-5}	3.62	53.6

Discussion

In a survey of persister cell levels across different stages of the growth curve, with a range of antibiotic stressors, we found a consistent pattern of persister cell levels peaking during exponential growth. This feature of persister cell formation has received less attention in the context of previous work emphasising the significance of conditions during stationary phase for persister cell formation. The focus on the effect of growth stage is perhaps unhelpful, however, as our results point to persistence being determined primarily by cell state rather than population growth stage. Cell survival measures based on methodology B served as a reliable indicator of where persistence (methodology A – kill curves) was peaking within the growth curve. Cell survival in cultures initiated from low-density inoculum showed the first peak in survival shifting to as early as lag phase. We observed comparability in the number of doublings prior to cell survival peaks across inoculum densities depending on the antibiotic stressor. These antibiotic- and inoculum-dependent effects are further supported by evidence showing that peaks in cell

survival coincided with increased ATP concentration per cell and cell size heterogeneity. Across the three antibiotic classes assayed, the timepoint of cell survival peaks was broadly consistent, however, the magnitude of cell survival varied significantly depending on antibiotic class. Taken together, our results motivate further research into persister cell formation in actively metabolising and growing cells. Not only are different mechanistic explanations required for persister cell formation in different growth phases, different ultimate explanations, about the adaptive value of persister cell formation, are also required.

The presence of persister cells early in the growth curve has received relatively little attention in the literature so far – not because it has not been observed before but because any peaks observed have been relatively small compared to peaks in stationary phase (Goormaghtigh & Melderen, 2019; J.-S. Kim *et al.*, 2018; Orman & Brynildsen, 2013; Spoering & Lewis, 2001; Svenningsen *et al.*, 2022). This focus is potentially problematic given that the patterns observed in this study are not easily explained by the dominant narrative of persister cell formation being associated with stress, such as during stationary phase (Balaban *et al.*, 2019). We also found a peak in persister cell levels in stationary phase and our results are consistent with previous findings; the problem is perhaps one of omission. In surveying experimental methods adopted in other studies, we note that cultures are generally initiated with a relatively high-density inoculum. The importance of experimental protocol in determining the persister cell fraction was previously highlighted by Harms *et al*. (2017), who suggested caution when observing persistence during different phases of the growth curve. Notable persistence studies have used higher inoculum densities in the range of 1×10^4 to 1×10^7 cells/ml (Balaban *et al.*, 2004; Keren *et al.*, 2004; Shah *et al.*, 2006; Gefen *et al.*, 2014), and the lowest inoculum density in Harms *et al*. (2017) was 1×10^5 cells/ml. In contrast, we

undertook a step to standardise our overnight culture (to 0.1 OD_{600}) prior to diluting further into our culture media (up to 100,000-fold, equivalent to 1×10^3 cells/ml) (Table S2). It was in cultures generated using the lowest inoculum densities that we found the most prominent peaks in persistence during exponential growth.

We note that cell counts at high cell densities are above the expected range for a dense stationary phase culture (Fig. S3 & S6). The most likely explanation for this is cell clumping during biofilm formation in stationary phase cells (Beal et al., 2020), potentially exacerbated by conducting high through-put assays robotically, where narrow-bore tips increase cell adhesion. Recognising these potential issues, we also took optical density measures alongside taking CFU counts and found that growth rates derived from either method were consistent (Fig. S3 & S6). Furthermore, our hypotheses were tested by comparing cell counts pre- and post- antibiotic treatment relative to one another within each persister assay.

The importance of cell state for persister formation is consistent with descriptions of spontaneous versus triggered persistence, which is thought to result from response to environmental conditions in stationary phase (Balaban *et al.*, 2019). Our results align with predictions of when cells are expected to switch spontaneously into persistence, with peaks in actively metabolising cells, which are producing ATP over an extended period of time. One divergence from expectations (Balaban 2019), is the observation that this effect shifts to lag phase and is not limited to cells undergoing balanced growth (steady-state exponential growth), though variation in population cell lag-time distributions should also be considered, specifically with tolerance by lag (Fridman *et al.*, 2014). This emphasis on cell-state rather than population growth phase supports two recent theoretical

models into the effects of cell metabolism on persistence. Phenotypic state switching capacity, which could promote persistence, is predicted to be highest when cell energy levels are highest (Kerr *et al.*, 2019). Bruggeman *et al.* (2020) proposed a model predicting that high growth rates lead to preparatory protein expression limitations, which in turn increases the potential for phenotypic diversification. Wood *et al.* (2021) provide empirical support for the limitation of protein translation having the effect of increasing persistence. These mechanisms offer potential explanations for how the switch to persistence operates during exponential growth, and highlights the need for a different mechanistic explanation for persister cell formation in stationary phase when cellular processes may be quite radically different. Several recent reviews provide excellent overviews of the field and the need for caution in applying universal explanations for observations of persistence (Balaban *et al.*, 2019; Verstraete *et al.*, 2022; Alnahhas & Dunlop, 2023).

The major question of whether persistence should be thought of as a cell or group-level adaptation (not mutually exclusive explanations), or an adaptation at all, remains largely unanswered. The switch to a persistent state may be a survival strategy adopted by individual cells. However, this explanation does not easily account for the typically low and remarkably constant proportion of cells in a persistent state across populations and even species (Van den Bergh *et al.*, 2017). Instead, is persistence a population level trait, as a form of bet-hedging through "division of labour", where some cells prioritise growth and others prioritise survival? Or do none of these apply? Is persister cell production adaptive or just a by-product of cellular mechanisms? If persistence is optimized at the population level, the mechanism of how this is achieved is of key interest. How do populations regulate persistence such that only a small fraction of the population enters a phenotypic survival state? We found that early peaks in persistence can be predicted by population

cell division history. This suggests that individual cell state and division history may be an important consideration for predicting when persistence is most likely to arise, rather than growth phase. It is speculated that population cell division history influences epigenetic inheritance patterns which may play a role in persistence (Day, 2016), with regulation based on historical experiences within the culture to support bet-hedging against adverse environmental conditions (Gardner *et al.*, 2007). Persistence arising stochastically in this way agrees with the '*Persistence as Stuff Happens – PaSH*' theory presented by Johnson and Levin (2013), where actively dividing cells may accumulate greater population-wide heterogeneity over successive rounds of division. This may help explain ultimate causes behind persister cell formation where generally only a small fraction of the population undergoes a phenotypic switch.

The response to different antibiotic stressors may be informative when considering proximate and ultimate explanations for persister cell formation. We found significant variation in the magnitude of persistence depending on antibiotic class, despite peaks forming at comparable timepoints within the growth curve. This was true for persistence in both exponential and stationary phase. For meropenem treatment, persistence was consistently higher in stationary phase which is in line with previous findings for beta lactam observations (Khare & Travazoie, 2020). This disparity in magnitude of survival across antibiotic treatments points towards a role for active cell metabolism in persister cell formation. Differences in survival could be due to differences in membrane permeability (Pagès *et al.*, 2008), or drug targeting efficacy (Clarelli *et al.*, 2020). Persisters have also been found to have increased efflux pump activity (Pu *et al.*, 2016), potentially enabling them to reduce the intracellular accumulation of the antibiotic. However, it must be noted that the differences in survival responses across antibiotic treatments from our assay may have been biased

by the chosen antibiotic concentrations (100µl/ml), which despite being added well in excess, varied in multiplication relative to the MIC for each antibiotic. However, we confirmed that antibiotic concentrations in excess of the MIC had limited effects on underlying cell survival. Regardless, the occurrence of persistence peaks at comparable timepoints suggests underlying population heterogeneity exists, however individual cells may interact with the different antibiotics to limit potency to varying degrees. Taking account of this variation is important, especially when comparing findings across different persister assays.

Conclusion

Our results challenge the dominant narrative in the literature that persister cell formation is primarily a response to slow growth conditions. This raises the possibility that we do not yet understand fundamental aspects of why cells switch to a persistent state and makes it difficult to predict cellular responses to different kinds of antibiotic treatment. How persistence varies depending on antibiotic class also has clinical implications. By exploring a wider range of growth conditions in persister cell assays, we may begin to disentangle these confounding relationships and get closer to understanding the mechanisms driving persistence and ultimate causation: is persister cell formation an adaptive strategy or a by-product of a physiological response to internal (or external) state? Is the role of population cell division history in persistence ubiquitous across bacterial species? If it is adaptive, the first step to understanding this trait as a response to selection is to understand how it varies in response to physiological state, heterogeneity, and growth. Further work should question the comparability of previous persister studies, and consider the potential role of population division history in determining when persistence is most likely to arise.

Materials and Methods

Materials

We used wildtype *P. aeruginosa* (PAO1, Copenhagen collection) as the main study organism. To isolate persisters, we used the following antibiotics: ciprofloxacin, tobramycin, and meropenem. Each antibiotic was provided in powdered form from Sigma Aldrich, Cayman Chemical Company, and TOKU-E, respectively. We made liquid stocks of each antibiotic at a concentration of 1.5mg/ml and stored them in a freezer at -20ºC. For optimal growth, we grew all cultures in media generated from powder of Luria Broth (LB Miller – Fisher BioReagents: Tryptone 10g/L, Yeast extract 5g/L, Sodium Chloride 10g/L) and plated cells on pre-buffered LB agar (Fisher BioReagents: Casein Peptone 10g/L, Yeast extract 5g/L, Sodium Chloride 10g/L, Agar 15g/L, TRIS/TRIS HCl 1.5g/L). We used Sodium Chloride solution (0.9%) to carry out serial dilutions. For ATP measures, we used BacTiter-Glo™ according to the manufacturer's instructions.

Culturing conditions

To ensure comparability across growth curves in replicate assays, we standardized the culture inoculum procedure. We initiated all inoculating cultures from the same freezer stocks of PAO1. To generate our overnight culture, we inoculated 6ml of LB media with a crystal of PAO1 freezer stocks and grew for 17-19 hours overnight in glass vials at 37˚C and 220rpm. We then ensured our overnight cultures had reached a sufficient turbidity through measuring optical density (OD_{600}) at 600nm using a spectrophotometer (Spectramax M2) expecting an OD_{600} of 0.45-0.6. To do this, we transferred 100µl of overnight culture into a 1ml cuvette containing 900µl 0.9% NaCl, creating a 10-fold dilution to increase instrument sensitivity and measured OD_{600} after calibrating against a blank. Once sufficient growth had occurred, we standardised the cultures' OD_{600} to 0.1, before

carrying out further dilutions into our experimental cultures. Our four inoculum densities were generated by carrying out serial dilutions of the same standardised culture: 10^{-2}-, 10^{-3}-, 10^{-4}-, and 10^{-5}-fold dilutions. For optimal aeration, we used 250ml flasks (with loose covers) and volumes of 50ml of LB for culture growth. Once inoculated, all experimental flasks were placed in an incubator at 37°C and 220rpm.

Pre-antibiotic cell quantification

To quantify cell density, we measured both OD_{600} and colony forming units (CFU). We measured both OD_{600} and CFU to monitor population growth, and CFU to calculate persistence. For measuring OD_{600}, 170µl samples (x9 replicates) of undiluted experimental culture were transferred into separate wells in a 96-well plate. We used a spectrophotometer (SpectraMax®i3x) to measure OD_{600} for all samples in 96-well plates. Once OD_{600} had been measured, we used aliquots from the same samples to measure CFU. We did this by transferring 20µl from each sample well (again, x9 replicates) in the OD_{600} 96-well plate to another 96-well plate for serial dilution. This left 150µl in each well for the addition of antibiotic, which we will discuss further below. Serial dilution varied depending on the point in the growth curve: samples taken from lag phase had fewer dilutions than exponentially growing cultures (10^{-3} versus 10^{-8}). Once we diluted accordingly, we plated 10µl from each well onto square 15cm. After 18-24 hours in the incubator at 37°C, we counted CFU.

Addition of antibiotic

Once we had sampled the initial cell density, we added antibiotics to each well. To limit potential volume-based bias in 96-well plate conditions, we added all antibiotics at the same volume and

concentration: 10µl of 1.5mg/ml antibiotic solution to create an overall concentration of 100µg/ml. For each timepoint in the growth curve, we had three biological replicates and two technical replicates for each antibiotic. We incubated samples in antibiotic solution for up to 24 hours at 37ºC. After incubation, we measured persister CFU by transferring 20µl from each sample well into 96-well plates for serial dilution. Antibiotic treatment was generally diluted out with serial dilution. To overcome a dilution effect in the early culturing hours (0-10 hrs) with ciprofloxacin treatment in the lowest inoculation density (10^{-5}), we carried out an extra washing step with NaCl (see Supp. Info, S4). To establish the relevant dilution for ample persister CFU counting, we spotted diluted samples onto square LB Petri dishes covering all dilutions. We found that optimum dilutions for samples treated with the following antibiotics were: 10^{-3} for ciprofloxacin, 10^{-4} for tobramycin, and 10^{-6} for meropenem. We counted CFU after plates were incubated for 18-24 hours in the incubator at 37°C.

Ruling out resistance

To limit the potential for resistance to arise, we used antibiotic concentrations several times greater than the minimum inhibitory concentration (MIC): ciprofloxacin – 0.25µg/ml; meropenem – 4µg/ml; tobramycin – 2µg/ml (Table S1). We ascertained MICs following the liquid microdilution method (Andrews, 2001). Since we were administering antibiotics at different points throughout the growth curve, we measured MICs for a range of cell densities. The MIC only increased with cell density for tobramycin-treated samples (from 2 to 4 µg/ml). To ensure the 400 times MIC ciprofloxacin treatment was reliable for persister isolation, we tested a range of concentrations to check for sufficient bactericidal action without the induction of prophages (Fig. S12) (Harms *et al.*, 2017). Our assays involved the use of high-dose antibiotics, therefore, we needed to use

additional tests to ensure our samples had not become resistant to the antibiotic stressors. To test if the antibiotic was suppressing bacterial growth within our cultures after a 24-hour incubation with antibiotics, we spotted 5µl of pure treated sample from each well onto fresh LB agar. To further test for the ability to grow in the presence of antibiotics, we repeated the above steps with LB agar containing 100µg/ml of each relevant antibiotic (Fig. S13).

Persister assay

To produce kill curves and capture cell response after adding the antibiotics, we sampled every two hours for 24 hours to measure the percentage of surviving cells (methodology A). We used a standardised overnight culture inoculation procedure and quantified CFU prior to the addition of antibiotics (following the above methodology). For the kill curve, we treated all samples in 96-well plates using the same volumes of culture and antibiotics (150µl and 10µl, respectively). However, as each well only contained 160µl of antibiotic-culture solution, by taking 20µl samples every two hours, after only 8 sampling points the well volume would be diminished. Therefore, to avoid the issue of diminishing well volumes over time, we used a new well for each timepoint per antibiotic. To account for potential variability using different wells, we used eight technical replicates per time point for each antibiotic treatment. Once treated with antibiotics, we took 20µl from each well for serial dilution every two hours. To precisely capture the most countable CFU for each timepoint, we spotted 5µl from each serially diluted sample onto LB agar to include all dilutions from 10^{-1}-10^{-12}. We extrapolated baseline persistence by selecting the minimum point in survival under antibiotic treatment and normalised relative to the peak initial population size (Fig. S2). For the assays with varied inoculum densities, we treated samples with antibiotics for 24 hours before measuring cell survival (methodology B - after validating that survival peaks after 24 hours

of treatment corresponded with kill curve-derived persistence peaks (Fig. S5)). After allowing the spotted samples to soak into the agar, we placed all plates in the incubator for 18-24 hours at 37˚C.

Cell imaging

We fixed cells for imaging according to methodology used by Jones and Uphoff (2021). We first spun cells down to remove the supernatant before resuspending in 2.5% paraformaldehyde and PBS buffer solution. We incubated cells at room temperature for 30 minutes before a further round of washing in PBS buffer prior to imaging. We transferred 2µl onto 1% agarose M9 pads for imaging. We used a modified combination of MicrobeTracker (Sliusarenko *et al.*, 2011) and SuperSegger (Stylianidou *et al.*, 2016) to segment and measure cells for image analysis.

Calculations

To compare cell survival across the growth curve, we calculated cell survival percentage by dividing post-antibiotic cell number by pre-antibiotic cell number and multiplying by 100, at each time point for each antibiotic. In our analyses, we additionally used growth rate (GR) as a parameter and calculated GR using the following equation:

$$GR = \frac{ln(OD_t) - ln(OD_{t0})}{(t - t_0)}$$

Where OD_t is the OD_{600} measure at time t, and OD_{t0} is the measure at time t_0. We categorised samples into different phases of the growth curve using the points of inflection in growth rate (Fig. S3 & S6).

To estimate the number of cell divisions for each inoculum density, we calculated divisions as follows:

$$N = \frac{ln(\frac{Cell\ No_t}{Cell\ No_{t0}})}{ln(t)}$$

Where N is number of divisions that have occurred, $Cell\ No_t$ is cell count at time t, $Cell\ No_{t0}$ is the cell count at time t_0, and t represents the number of hours between each cell count measure to estimate doubling rate over time.

Statistical tests

We carried out all statistical analysis using R (Version 1.2.1335). To test for differences in cell survival across different classes of antibiotics, we used ANOVA and Kruskal-Wallis tests depending on the normality of the data. We used independent T tests and Mann Whitney U tests to further explore statistical significance in variation during points of interest in the growth curves.

Chapter 2. Supplementary Information

This document contains supporting evidence supporting 'High growth rates promote persister cell formation in *Pseudomonas aeruginosa*'

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Figure S1. Kill curves generated relative to the cell number prior to treatment with antibiotics across ten timepoints within the growth curve.

Figure S2. Mathematical method used to extract persistence measures from kill curves.

Figure S3. Deriving growth phases from optical density and CFU.

Figure S4. Cell survival against antibiotic two hours after treatment.

Figure S5. Cell survival 24 hours after antibiotic addition.

Figure S6. Deriving growth phases from optical density and CFU data for cultures inoculated with incrementally lower cell densities

Figure S7. Raw cell survival data for cultures inoculated with incrementally lower cell densities.

Figure S8. CFU versus cell survival across the growth curve.

Figure S9. Reducing inoculation density delays entry into stationary phase.

Figure S10. Cell survival after carrying out an additional washing step to remove ciprofloxacin treatment for a low inoculation density culture.

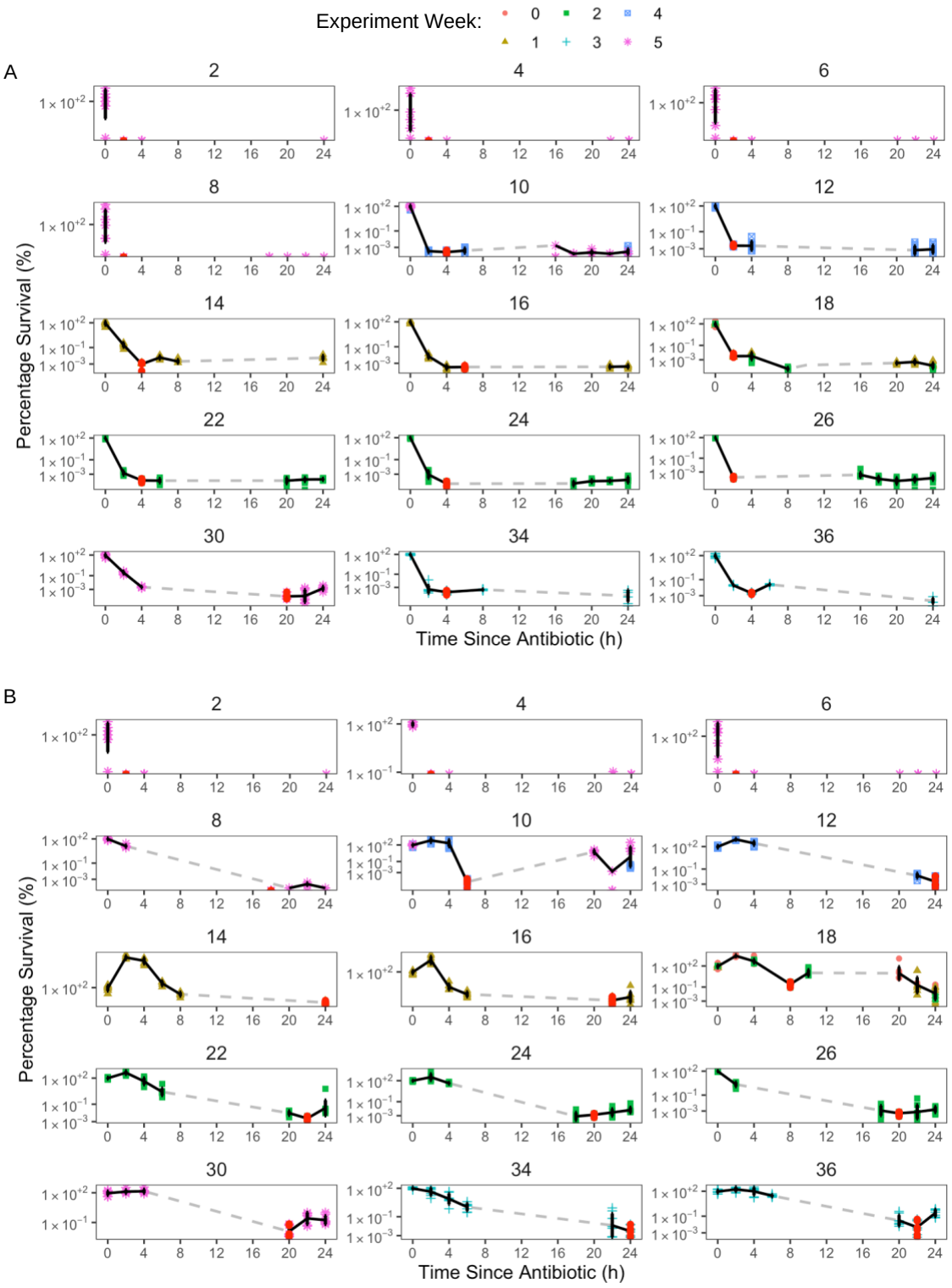
Figure S11. Extracellular ATP peaks in stationary phase.

Figure S12. Cell survival counts after treatment with increasing concentrations of antibiotics.

Figure S13. Antibiotic-treated samples were spotted onto agar to show antibiotic efficacy.

Table S1. Minimum Inhibitory Concentration (MIC) measures for each antibiotic over a range of optical densities.

Table S2. Inoculum density literature comparison for notable persister studies.


A
B
Experiment Week:
0
1
2
3
4
5
2
4
6
8
10
12
14
16
18
22
24
26
30
34
36
Percentage Survival (%)
Time Since Antibiotic (h)

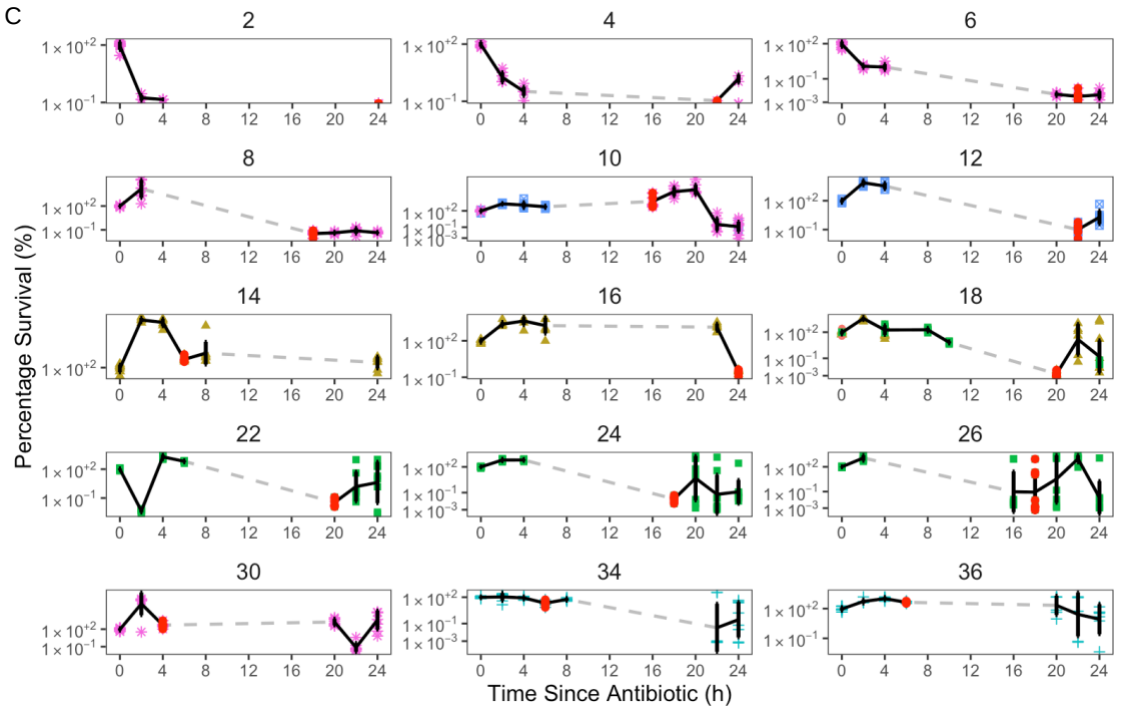


Fig. S1. Kill curves generated relative to the cell number prior to treatment with antibiotics across ten timepoints within the growth curve. Samples were treated with either: (*A*) ciprofloxacin; (*B*) tobramycin; or (*C*) meropenem. Error bars depict standard deviation of the mean. The scale has been log10 transformed for data visualisation. The experiment weeks are color-coded (see legend), with the red point denoting the time since antibiotic treatment timepoint within the kill curve where the persistence value has been extracted for Figure 1C in the main text. The dashed line represents periods between sampling timepoints. Each timepoint (post-AB) is representative of at least eight technical replicates.

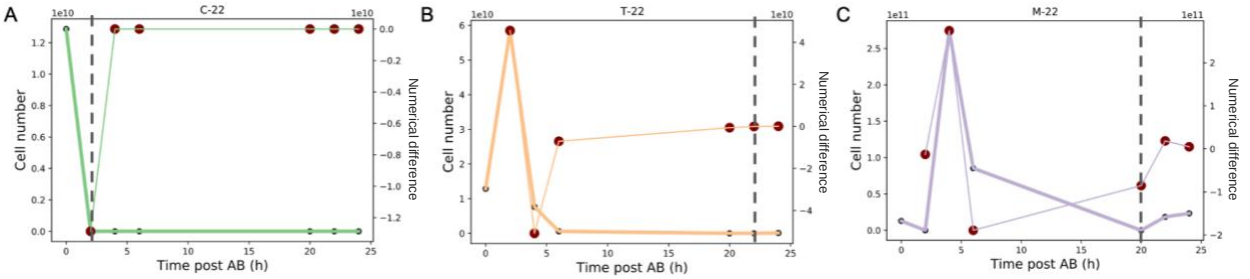


Fig. S2. Illustration of the method used to determine the baseline persister fraction from individual kill curves (with an example of a sample that was cultured for 22hrs prior to antibiotic treatment). Samples were treated with three different antibiotic treatments: *(A)* Ciprofloxacin; *(B)* Tobramycin; and *(C)* Meropenem). The bold line shows the mean cell count after antibiotic treatment and the thin line represents the numerical difference in cell count (which helped illustrate if antibiotic regrowth occurred). For unity across all antibiotic treatments and culturing timepoints, we applied the same method whereby we used an algorithm to select the minimum point of survival prior to any regrowth from potential antibiotic degradation (if present) within the kill curve. The vertical dashed line represents the selected timepoint to determine baseline persistence. We further normalised the baseline persistence values relative to the highest initial cell count (pre-antibiotic treatment at 0h, or 2-4hrs post antibiotic treatment in the case of stress divides).

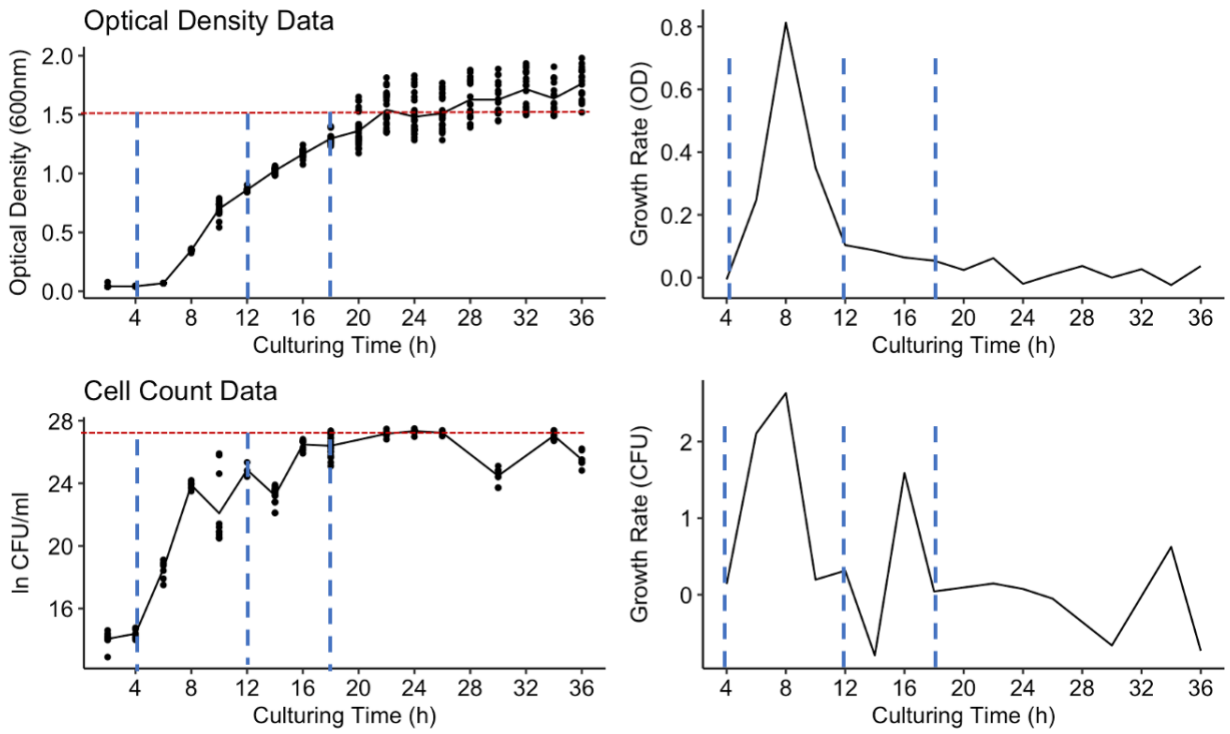


Fig. S3. Growth curve phases derived from Optical Density (OD 600nm) and CFU data. The vertical dashed lines (blue) represent phase transitions where inflection points in growth rates occur (namely from lag, early exponential, late exponential, and stationary phase). Entry into stationary phase has been deduced from where cells have reached saturation (dashed horizontal line – red). Each data point is represented by at least eight technical replicates.

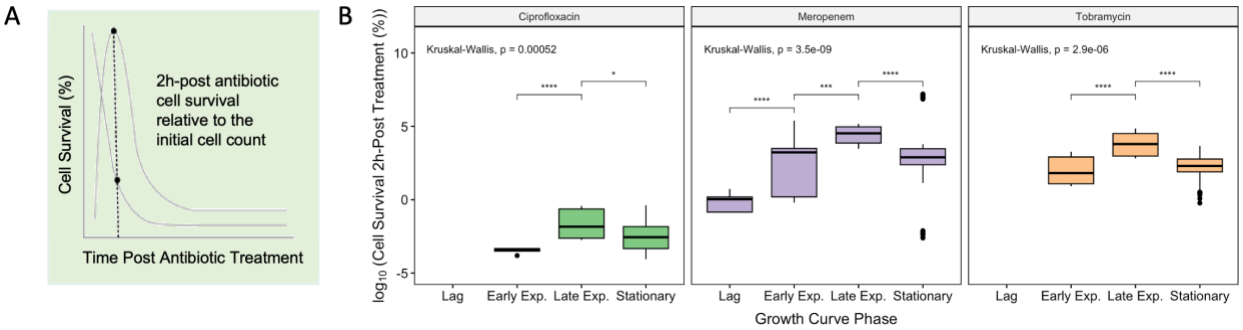


Fig. S4. Cell survival against antibiotic two hours after treatment. (*A*) Schematic diagram representing the timepoint within the kill curve for comparing cell survival differences in the adjacent figure. (*B*) Boxplot comparing cell survival 2-h into antibiotic treatment by growth curve phase. Each data point is representative of at least three technical replicates.

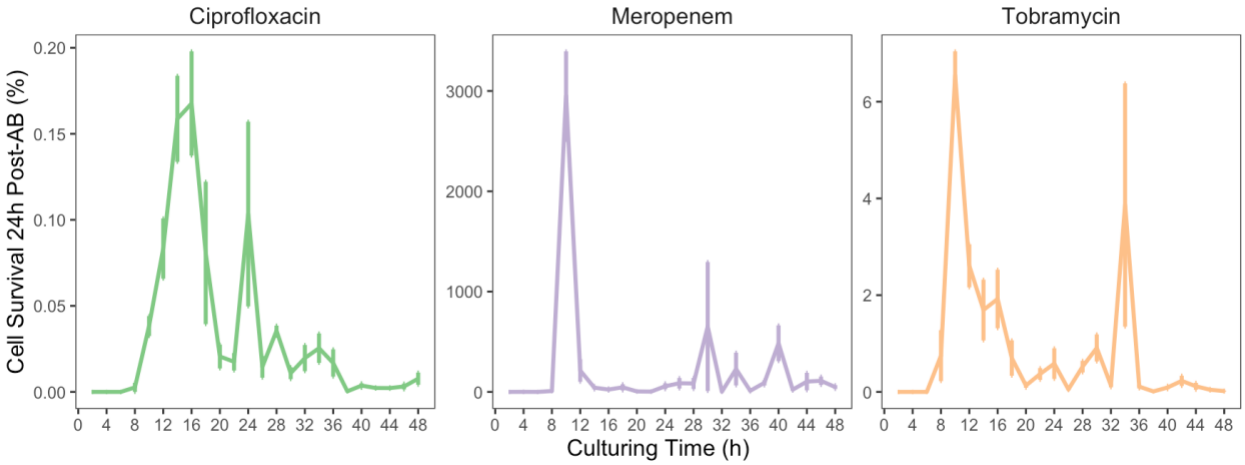


Fig. S5. Antibiotic survival 24 hours after treatment (methodology B). After serial dilution, 20µl of each sample was plated on whole Petri dishes and spread with glass beads and incubated for 24-hours before enumeration. Error bars represent standard error of the mean. Each data point is representative of at least three technical replicates, with hours 2-36 representing at least six replicates (three technical and two biological replicates).

Resistance-Checking Assays

By tracking colony forming units during the kill curves, we found that treatment with tobramycin and meropenem induced a temporary burst of cell division, occurring in the first two to four hours following treatment before the population cell count crashed (mean count increasing up to 28.2-fold (sd = 43.1-fold), and up to 11.3-fold (sd = 18.5-fold) for meropenem and tobramycin, respectively) (Fig. S1). The temporary burst of cell division and initial survival to antibiotic treatment was highest in cultures treated during late exponential phase (Fig. S4). Despite initial survival post-antibiotic treatment also being highest during late exponential phase, we saw no temporary burst of cell division for samples treated with ciprofloxacin.

Due to a significant increase in cell growth in meropenem-treated populations, and higher than expected percentage cell survival in tobramycin treatments, we needed to confirm that the surviving cells counted were persistent and not resistant to the antibiotic concentrations used in our persister assays. We confirmed that the concentration of 100 μg/ml used for each antibiotic were 25, 50, and 400 times higher than the MIC values (4, 2, and 0.25μg/ml) for meropenem, tobramycin, and ciprofloxacin, respectively (Table S1).

To test for antibiotic degradation, we spotted non-diluted antibiotic-treated samples after 24-hours of exposure onto plain LB agar, and no growth was observed in ciprofloxacin and tobramycin treatments, indicating that the antibiotic continues to suppress cell growth. However, meropenem treatment failed to supress growth in samples treated from ten hours of culturing time onwards (until our observations up to 36 hours of culturing time). To ensure that any observed cell growth was not due to resistance emerging within or at 24 hours of antibiotic exposure, we plated aliquots

of the antibiotic-treated populations on agar impregnated with 100 µg/ml of each respective antibiotic, and observed no growth (Fig. S13). This indicates that for the meropenem treatment, higher density populations may degrade the meropenem antibiotic faster allowing for cell growth within our 24-hour antibiotic treatment.

In line with antibiotic degradation, we also identified a regrowth after 20-22 hrs of meropenem treatment despite the high antibiotic concentration (in cultures treated in exponential phase, cell counts were as high as pre-antibiotic treatment counts) (Fig. S1). This regrowth was most pronounced in samples treated during exponential phase. In general, we found that high growth rates prior to antibiotic treatment promoted increased survival against antibiotic treatment.

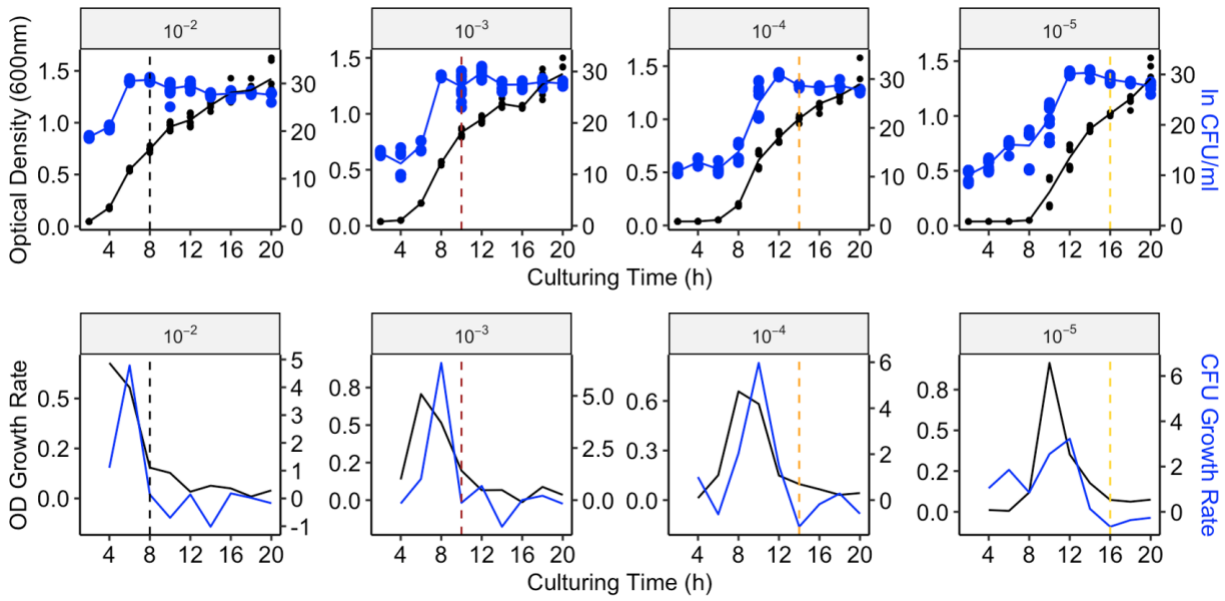


Fig. S6. Growth curve phases derived from Optical Density (OD 600nm) and CFU data in samples inoculated with diminishing cell densities (from 10^{-2}-10^{-5} -fold dilutions of a 0.1 OD_{600} standardised culture). The growth rate was calculated using the log difference (see methods). Growth phase transitions have been derived from points of inflection in growth rate including data from OD and CFU, as well as taking into consideration when cell count has reached saturation and is no longer increasing. The vertical dashed line represents where the culture has entered stationary phase. Each data point is representative of at least three technical replicates.

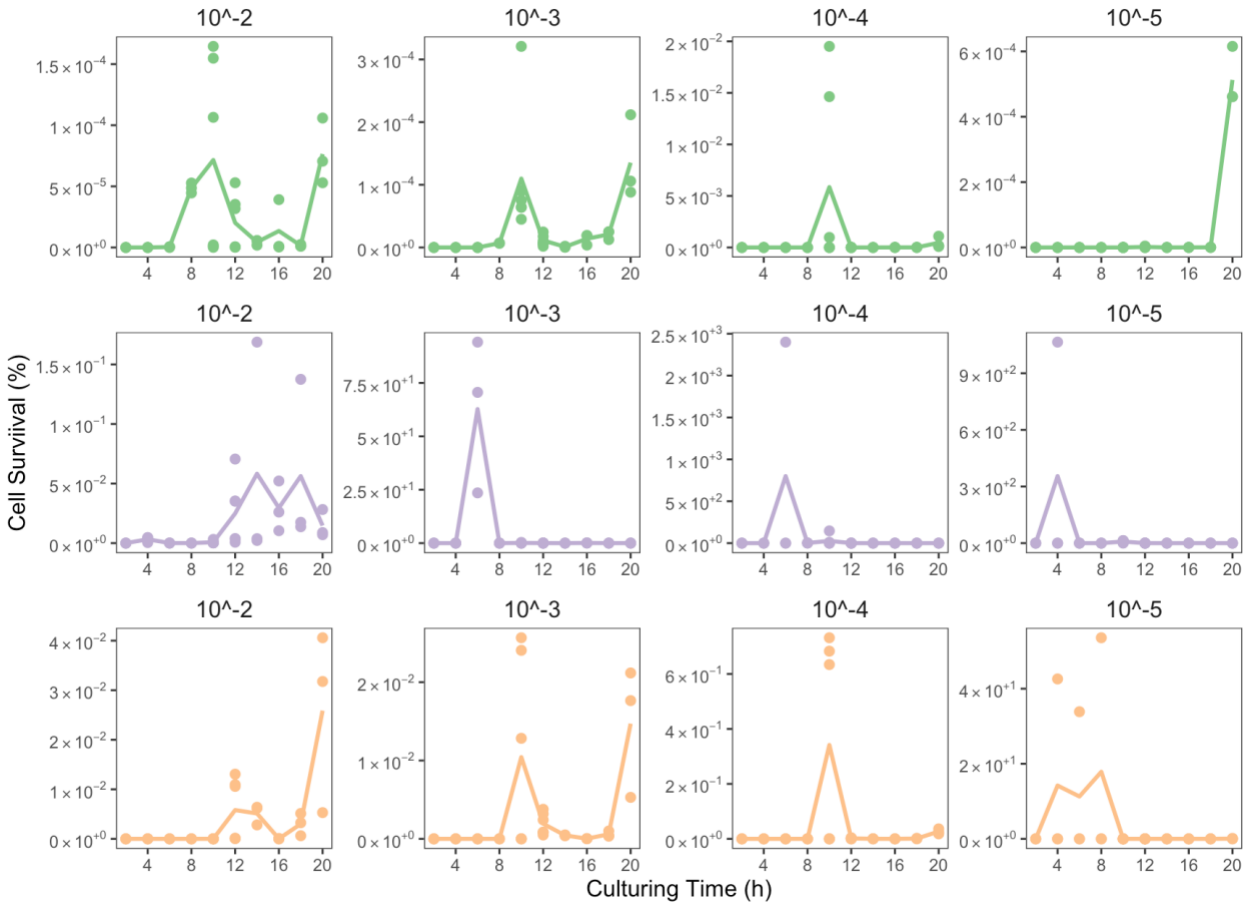


Fig. S7. Raw growth curve cell survival data for cultures inoculated with incrementally lower cell densities (from 10^{-2}-10^{-5}-fold dilutions of a 0.1 OD_{600} standardised culture) (methodology B). The line represents the mean cell survival at each sampling timepoint with individual replicates represented with scatter points. Antibiotic treatments are colour-coded from top to bottom: green – ciprofloxacin; purple – meropenem; and orange – tobramycin. Each datapoint is represented by at least three technical replicates.

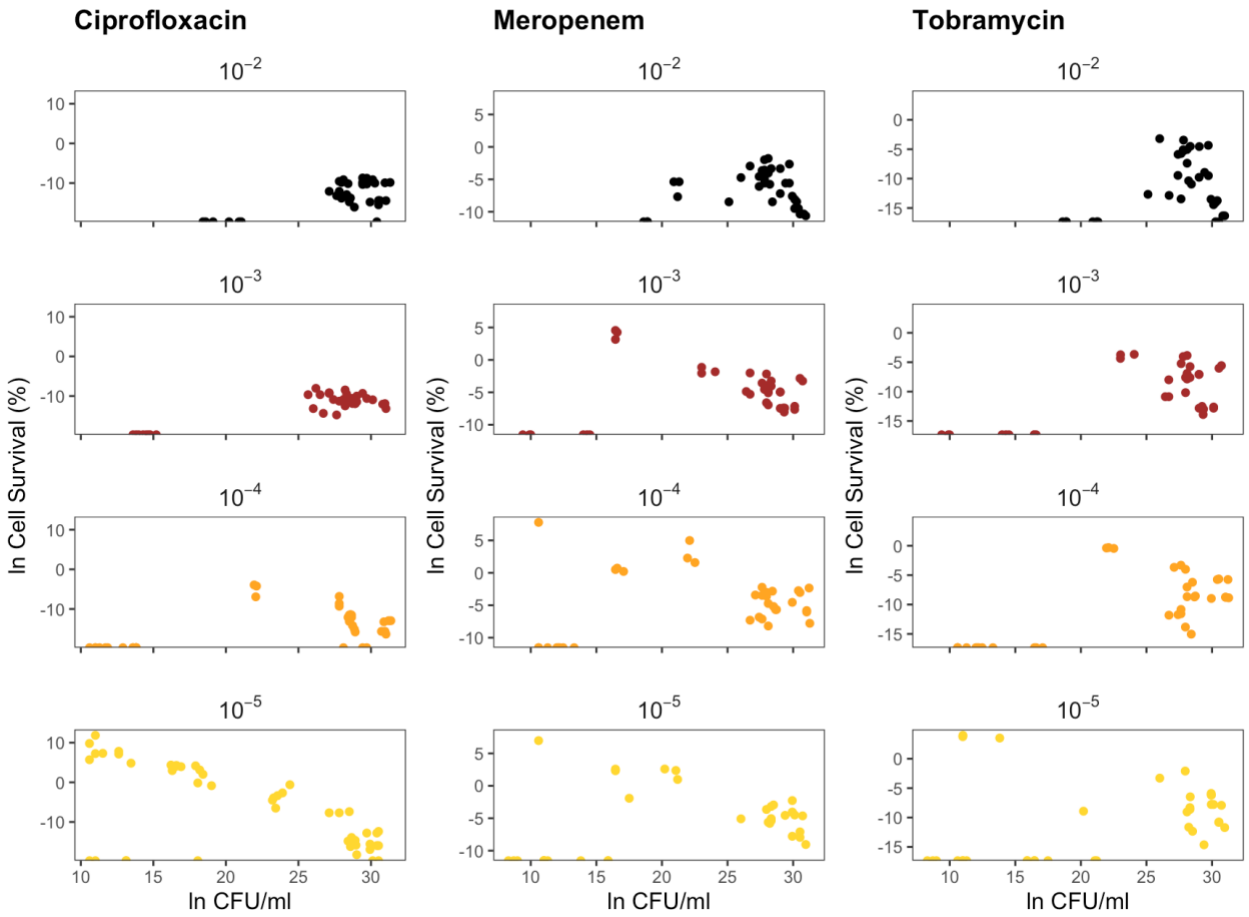


Fig. S8. Cell density versus cell survival across 20 hours of culturing in cultures inoculated with diminishing cell densities (10^{-2}-10^{-5} dilution of a 0.1 OD_{600}-standardised overnight culture) (methodology B). The samples were treated with either ciprofloxacin, meropenem, or tobramycin for 24 hours before being counted. The data has been log transformed for visualisation.

Identifying cell survival peaks in a low-density culture treated with ciprofloxacin

In contrast to treatment with meropenem and tobramycin, between 0-10hrs of culturing we found no survival under ciprofloxacin treatment for the lowest inoculum density culture (10^{-5}) (Fig. 3A & Fig. S3). We hypothesised that this was due to a dilution effect whereby the serial dilution to count persisters after ciprofloxacin treatment did not surpass the minimum inhibitory concentration of the antibiotic. To test this, we added an extra washing step to remove the antibiotic supernatant and resuspend the cells in NaCl prior to serial dilution. This revealed high survival between 2-8hrs of culturing for ciprofloxacin-treated samples, similar to the pattern found with the un-washed tobramycin treatment (Fig. S3-4). When calculating survival percentages for these samples, we found that the cell count had increased up to 57.16-fold (sd = 93.63-fold) relative to the initial cell count. Due to this unexpectedly high fold increase, we found that it was not possible to reliably report cell survival percentages using the NaCl washing step. Nonetheless, we confirmed the presence of surviving cells within these samples.

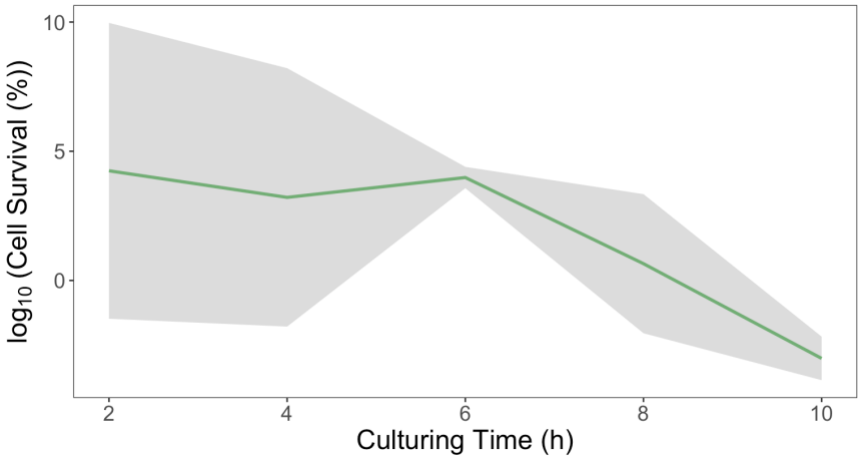


Fig. S9. Cell survival after carrying out an additional washing step to remove ciprofloxacin treatment for a low inoculation density culture (10^{-5}-fold dilution of a 0.1 OD_{600} standardised overnight culture). Samples were treated with antibiotics according to the steps described in Fig. 2A with an additional NaCl (0.9%) washing step prior to carrying out serial dilutions to measure the surviving cell fraction after 24-hours in antibiotic treatment. The data have been log transformed for visualization. The shaded region depicts 95% confidence intervals. Each datapoint is represented by at least 6 technical replicates.

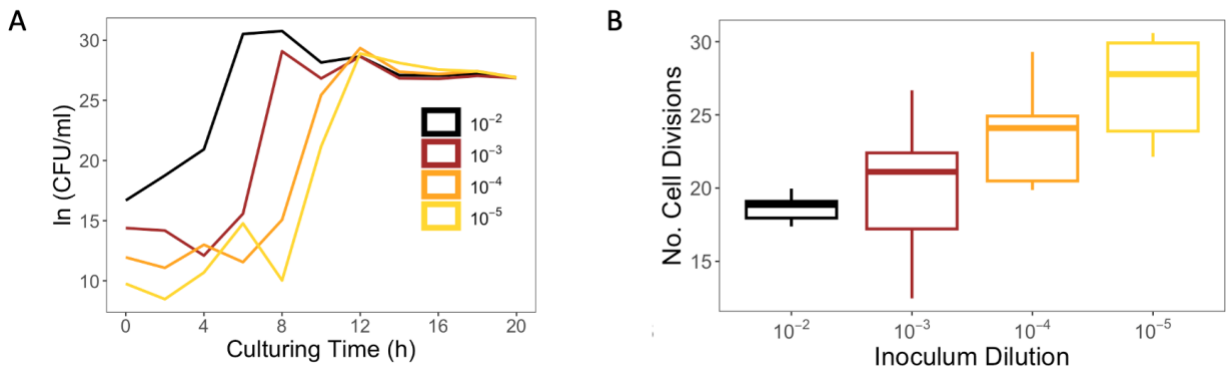


Fig. S10. Reducing inoculation density delays entry into stationary phase. (*A*) Growth curves for cultures inoculated with incrementally lower cell densities. (*B*) Cumulative number of population cell divisions prior to entering stationary phase for cultures inoculated with incrementally lower cell densities (10^{-2}-10^{-5}-fold dilutions of a 0.1OD_{600} standardised overnight culture). Cultures entered stationary phase at the following culturing times: 10^{-2} – 6hrs; 10^{-3} – 10hrs; 10^{-4} – 14hrs, 10^{-5} – 14hrs. Inoculum dilutions are color-coded from high (10^{-2}) to low (10^{-5}). Each data point is representative of at least three technical replicates.

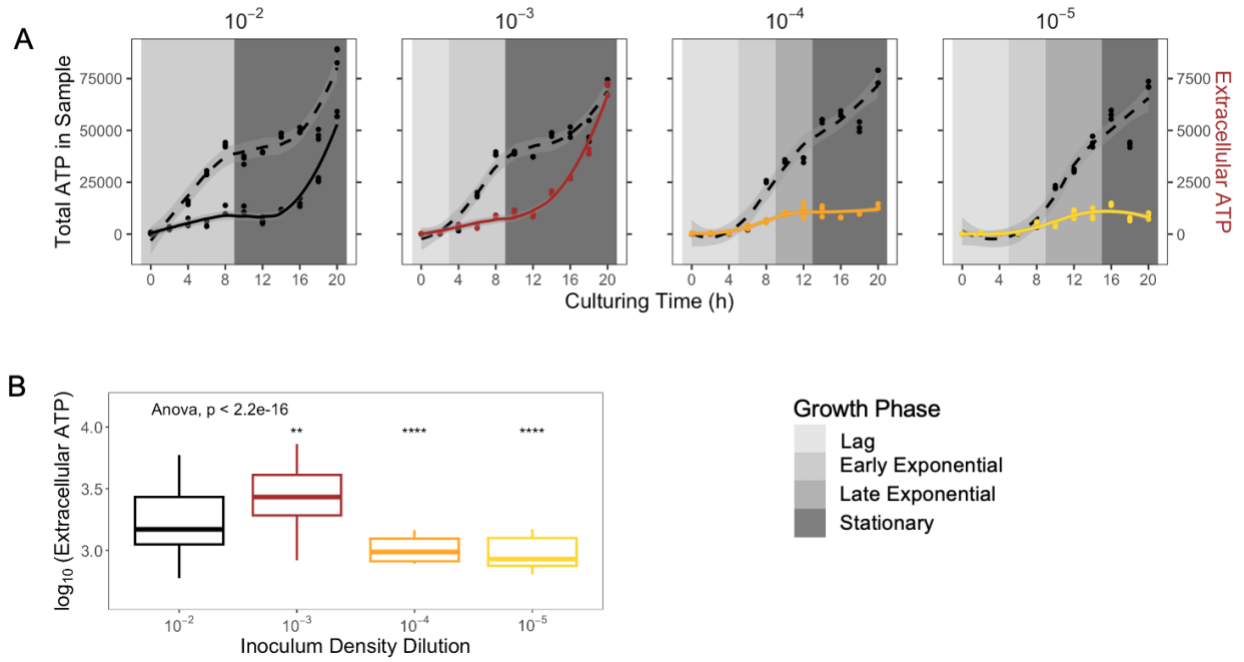


Fig. S11. Extracellular ATP peaks in stationary phase only in high inoculation density cultures. (*A*) Comparison between total ATP (dashed line – left y axis) versus extracellular ATP (solid line – right y axis). Inoculum dilutions are color-coded from high to low. (*B*) Boxplot depicting extracellular ATP measure during peaks in persistence. The widely distributed data points have been log transformed and asterisks denote significance where *: p <= 0.05; **: p <= 0.01; and ***: p <= 0.001. Each data point is representative of at least three technical replicates.

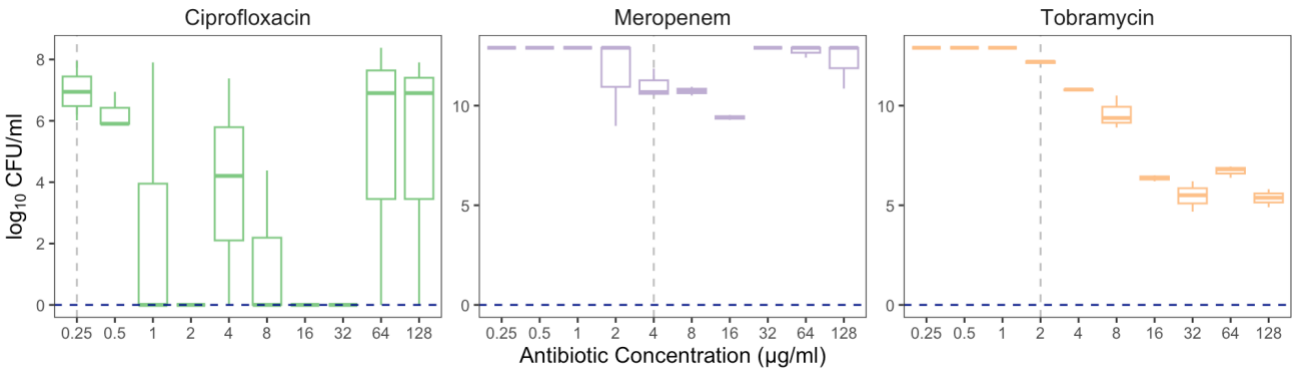


Fig. S12. Cell count after treatment with increasing concentrations of antibiotic (from 0.25-128µg/ml). The data have been log-transformed and a small constant (1) has been added to all values to account for zeros in the dataset (notably ciprofloxacin samples in 1-32µg/ml) (The horizontal dashed line represents the cell detection limit). The vertical dashed line represents the MIC for each antibiotic treatment. Samples were prepared according to the McFarland liquid dose protocol. Each datapoint is representative of three technical replicates.

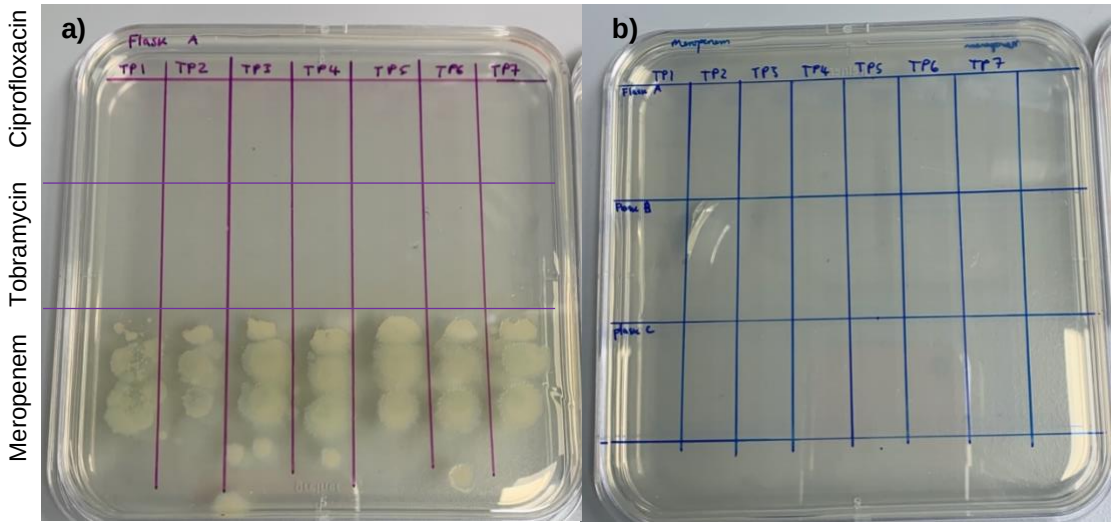


Fig. S13. Antibiotic-treated samples spotted onto agar to show antibiotic efficacy. (*A*) For meropenem, samples were able to grow on LB agar despite being treated with a liquid dose (100 µg/ml) in comparison to no growth under treatment with ciprofloxacin or tobramycin. (*B*) If the LB agar contained 100 µg/ml meropenem, samples were unable to grow.

Table S1. Minimum Inhibitory Concentration (MIC) measures for each antibiotic treatment over a range of cell densities. The MIC was determined using the McFarland liquid dose method and verified using E-Test strips.

Antibiotic	**Minimum Inhibitory Concentration (µg/ml)**			**MIC Multiplication for Assay (100 µg/ml)**
	0.5 McFarland	4 McFarland	5 McFarland	
Ciprofloxacin	0.25	0.25	0.25	400×
Tobramycin	2	4	4	50-25×
Meropenem	4	4	4	25×

Table S2. Inoculum density literature comparison for notable persister studies.

Authors	Inoculum Density	Study Species	Findings
Keren *et al*., 2004	1:1000 dilution of an overnight culture	*E. coli* K12 MG1655, HM22, & HM21 *Staphylococcus aureus* DB *Pseudomonas aeruginosa* PAO1	Persistence increases in mid-exponential phase and is highest in stationary phase
Balaban *et al*., 2004	Inoculation from frozen aliquots of stationary phase cultures (15hrs of growth)	*E. coli* MG1655, HM22, HM21, DS1, KL16, and MGY.	Persisters generated throughout exponential growth but persistence is highest in stationary phase
Shah *et al.*, 2006	1:100 / 1:1,000 dilution of an overnight culture	*E. coli* MG1655	Persisters are a phenotypically distinct subset of cells relative to exponential and stationary phase cells
Harms *et al*., 2017	Up to 1:100,000 dilution of an overnight culture	*E. coli*-K12 MG1655 and a broad range of constructed *E. coli* mutants	Persistence increases throughout exponential growth and cells became tolerant during stationary phase
Pontes & Groisman., 2019	1:200 dilution of overnight culture / frozen aliquots	*Salmonella entericum* serovar Typhurium 14028s and a range of constructed *S. entericum* mutants	Persisters arise from slow growing cells with similar patterns to results found with *E. coli* and *S. aureus*
Svenningsen *et al*., 2019	1:100 dilution of a dense overnight culture	*E. coli*-K12 MG1655 and a broad range of constructed *E. coli* mutants	Persistence is driven by the transcriptional response to increased (p)ppGpp levels which coincidentally increase upon entry into stationary phase
Kim *et al.*, 2022	1:100 dilution of a bacterial culture	*Acinetobacter baumanii* and a broad range of *A. baumanii* strains and mutants	Persistence may be regulated by DNA methyltransferase. Overexpression of DNA methyltransferase leads to a slow growth phenotype.

Chapter 3 Motivation

Owing to findings with *P. aeruginosa*, I wanted to replicate the experimental protocol from Chapter 2 on another bacterial species. I chose *E. coli* as a good baseline for comparison given the breadth of work on persistence that has been completed using this species (Balaban *et al*., 2004; Keren *et al.*, 2004; Shah *et al*., 2006; Kim & Wood, 2010; Harms et al., 2017; Leszczynska *et al.*, 2013; Goormaghtigh & Melderen, 2019; Zarkan *et al*., 2020 ; Svenningsen *et al*., 2022). My motivation with this chapter was to look at inoculum density effects to test theories on the role of cell division history on population heterogeneity.

Alongside wildtype *E. coli*, I further used the *E. coli* high persister mutant (HipA7) to explore how the *HipA* antitoxin system may also influence persistence throughout the growth curve (essentially as growth rates and cell densities change over culturing time). Also, by comparing two strains of the same species, I could further test the effects of changes in cell density and growth rate against the influence of the underlying genetic framework within the cell. My initial assumption was also that HipA7 would reliably produce a high-persister phenotype, again with an interest in laying the foundations for future use of these study systems in competition assays, expanding work similar to that conducted by Balaban *et al*. in 2004 (specifically, the section detailed in the supplementary information).

Despite replicating the methodology used in Chapter 2, I found limited inoculum effects in *E. coli* in comparison to the more obvious effects with *P. aeruginosa*. However, I did find more strain-specific effects – namely differences in the culturing timepoint of persistence peaks as well as different extracellular ATP patterns when comparing the two strains. Further investigation with

ATP measures led to the identification of a potential relationship between these persistence peaks and extracellular ATP. After manipulation of the supernatant, while the direct influence of ATP in persistence was inconclusive, the potential link between external cues and persistence trends presented an interesting potential avenue for future study.

Similarly to Chapter 2, the key questions I wanted to answer included:

1) What environmental conditions select for bacterial persistence?
    a. How do changes in population dynamics influence persistence?
2) How is persistence regulated at the population level?
    a. How does the magnitude of persistence change throughout the growth curve, and what does this tell us about the potential mechanisms that are promoting persistence?
3) Additionally, following a key unanswered question outlined by Verstraete *et al*. (2022): "*Is the effect of environmental conditions for persistence conserved across bacterial species […]*?"
    a. I shine light on this question by replicating methodology for both *P. aeruginosa* (Chapter 2) and *E. coli* (Chapter 3).

Chapter 3. The role of population dynamics on persister cell formation in *Escherichia coli*

M. Judge*, S. E. F. Davies*, M. Ghoul, & A. S. Griffin

***** = co-first author

Abstract

Persister cells represent a subpopulation of bacterial phenotypic variants that can survive antibiotic treatment despite being genetically susceptible. This has led to a widely-held assumption that persister cell formation has evolved as a survival strategy under challenge from antibiotics. Nonetheless, explicit support for this is still lacking and we have yet to understand the basic patterns of variation in persister cell formation. While persister cell formation in *Escherichia coli* has often been reported during slow growth and dormancy, a test designed specifically to assess the importance of growth rate has not yet been developed. This uncertainty leaves the adaptive value of persister cell formation an open question. We assayed persister cell formation in response to multiple antibiotic treatments across a wide range of growth conditions in wildtype *E. coli*. We confirm that, regardless of inoculum density, persister cell formation is most strongly associated with stationary phase, when the fitness costs of arrested growth were likely to be minimal. We further measured persistence in the *E. coli* high persister mutant, HipA7, and found an increase in persistence during stationary phase of comparable magnitude to the wildtype. However, the timing of the peak in persister cell formation in stationary phase was strain-specific and coincided with peaks in extracellular ATP - a potential indicator of cell lysis. We then swapped the supernatant between the strains at these peaks in extracellular ATP and showed a directional influence on persister cell formation, either decreasing or increasing persistence in accordance with the original

observation within the respective strains. Collectively, this research indicates that persister cell formation is both strain- and antibiotic-specific, potentially explaining why broad-spectrum approaches to tackling persister cell formation have been unsuccessful. It contrasts with our previous work on persister peaks in *Pseudomonas aeruginosa*, suggesting that persistence may perform different functions in different species, if it is adaptive at all.

Introduction

Antibiotic persistence confers the ability for a subpopulation of bacterial cells to survive antibiotic treatment despite being genetically susceptible (Balaban *et al.*, 2019). Although the possible advantages of a cell lineage maintaining a subpopulation of cells that can survive a wide range of antibiotics are obvious, evidence that switching to a persistent state is adaptive at the level of a lineage population or even at the single cell level has not yet been provided. We still lack a basic understanding of how the trait varies across strains and under different conditions, which is the necessary first step to hypothesising about what the adaptive advantage might be.

The key distinction between antibiotic resistance and persistence, is that persistence is predominantly a phenotypic trait, affecting a small but relatively predictable proportion of a bacterial population (typically 0.001-1%) (Van den Bergh *et al.*, 2017). This adds an often-underestimated challenge to identifying an adaptive advantage. To date, we lack consensus around the mechanism for persister cell formation, and efforts to identify the underlying metabolic processes involved in persistence are often contested (Song & Wood, 2021). The uncertainty surrounding persistence and the scarcity of persister cells within bacterial populations has prompted inquiries about its evolutionary origins and its influence on individual and population

fitness. The good news is that we do not require a detailed understanding of mechanism, or even genetic basis to understand the adaptive variation of a trait (Darwin, 1859).

One hypothesis is that persister cell formation may be favoured when population growth rate is relatively low as growth arrest, a characteristic of persistence, is likely to be less costly. This hypothesis is supported by studies of *E. coli* in the early 2000s which compared persistence measures in *E. coli* cells either in exponential phase, stationary phase, or within biofilms (Keren *et al.*, 2004; Shah *et al.*, 2006). This comparison clearly pointed towards persistence being most prominent in slow-growing stationary phase or biofilm cultures, and such has been the broad assumption since (Leszczynska *et al.*, 2013; Gefen *et al.*, 2014; Dewachter *et al.*, 2021). However, evidence has also emerged which could question this narrative. Orman and Brynildsen (2013) found that actively dividing *E. coli* cells could produce persister cells at a higher rate than pre-existing dormant cells, and more recently, research on *P. aeruginosa* led to the discovery of prominent peaks in persistence forming during exponential growth and even as early as lag phase (Davies *et al.*, submitted). This points to further questions surrounding how we determine how persister cell formation is affected by growth rate and the associated growth curve phase.

Persister cell formation may not be adaptive at all but may arise simply as a by-product of cellular processes. Even clonal populations of cells differ in state at any point in time due to phenotypic stochasticity (Kussell & Leibler, 2005; Avery, 2006; Lidstrom & Konopka, 2010; Allison *et al.*, 2011). It has been proposed that this variation in cellular physiology within bacterial populations may explain the observed variation in persister cell formation (Avery, 2006; Ryall *et al.*, 2012; Day, 2016; Balaban *et al.*, 2019). Consequently, the mechanisms reportedly underpinning

persistence, such as the stringent response, toxin-antitoxin systems, epigenetics, and quorum sensing, are thought to be heterogeneously expressed (Ryall *et al.*, 2012; Eisenreich *et al.*, 2021). Processes such as stringent response activation are thought to be upregulated when cells are stressed and media is nutrient-limited, resulting in greater persister cell formation in stationary phase (Łapińska *et al.*, 2022). The role of heterogeneity in persister cell formation is in line with the "Persistence as Stuff Happens" theory (PaSH), which states that a multitude of mechanisms are responsible for the stochastic generation of persister cells (Johnson & Levin, 2013). Taken together, this points to an advantage of designing studies which attempt to understand persister cell formation at the population level rather than a cellular level.

Our aim was to characterise variation in persister cell formation in *E. coli* across growth curve phases. To test a broad range of growth conditions, we used four inoculum densities and measured persistence every two hours from zero to 20 hours of the growth curve. To compare the effect of changing growth rates over time, we compared two *E. coli* strains with highly comparable growth dynamics. *E.coli* is a well-studied bacterium within the persistence literature and has a well-characterised high persister mutant, HipA7, which has also been the subject of considerable persister cell formation research (Korch *et al.*, 2003; Balaban *et al.*, 2004; Kaspy *et al.*, 2013). HipA7 has been reported to generate persistence 10-10,000-fold greater than the wildtype, which is attributed to mutations in the toxin-antitoxin system in the *hipA* gene (Moyed & Bertrand, 1983; Korch *et al.*, 2003; Keren *et al.*, 2004; Lewis, 2007). We used three different classes of antibiotics to assess the extent to which persister cell formation is antibiotic-specific: ciprofloxacin (fluoroquinolone), meropenem (beta lactam) and tobramycin (aminoglycoside). Alongside assaying persistence, we measured intracellular and extracellular ATP as indicators of metabolic

effort and ATP release (Mempin *et al.*, 2013). We carried out further manipulations of the supernatant to assess whether changes in persistence were due to intracellular or extracellular cues by repeating assays with washed cells, swapping supernatant between strains at points of interest in the growth curve, and adding exogenous ATP.

Results

Persistence is associated with stationary phase in *E. coli*

We measured the proportion of persister cells in two *E. coli* strains across stages of the growth curve, and inoculated with a range of cell densities (Fig. 1A-B; Fig. S1&2). Persister cell formation was significantly greater in stationary phase than exponential phase in both the WT (MG1655) and "high persister" mutant (HipA7), irrespective of the initial inoculum density or antibiotic type (Fig. 1C-D; GLM: (WT) $t = 9.82$, $P < 0.001$; (HipA7) $t = 10.25$, $P < 0.001$; Fig. S1). The persister fraction significantly correlated with increasing optical density across all treatments in both the WT and the high persister *E. coli* strains across all inoculum densities (Fig. S2, S3 (statistics inset), and Table S1). While population dynamics were highly comparable between the two strains, the timepoint of the persistence peak occurred upon entry into stationary phase in the high persister mutant, whereas the wildtype persistence peak occurred after spending an extended duration of time in stationary phase (Fig. 3D).

Kill curves verified that measurement of the persister fraction 24 hours after antibiotic addition provided an accurate measure of the underlying persister fraction, with no significant difference between the kill curve plateau (6-24 hours) and the 24 hours-post antibiotic measure (Fig. S4;

Wilcoxon tests: Ciprofloxacin W = 26, P = 0.95, Meropenem W = 22, P = 0.61 and Tobramycin W = 36, P = 0.33).

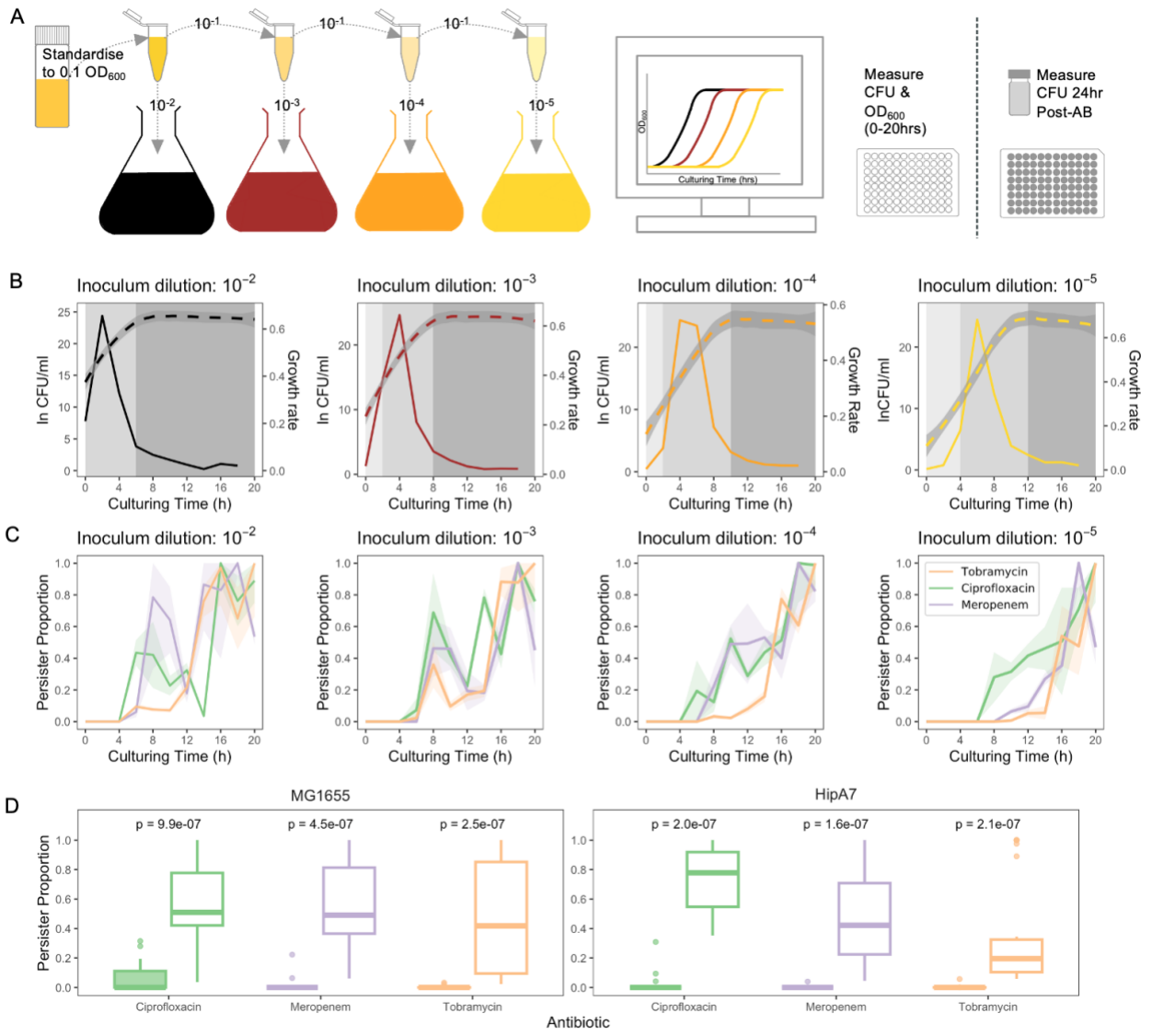


Figure 1. Persistence peaks in stationary phase, with limited inoculum effect. A) Schematic diagram to illustrate the experimental setup for four inoculum densities (10^{-2}-10^{-5}-fold dilutions of a 0.1 OD_{600}-standardised overnight culture). B) Cell count (CFU/ml – dashed line) plotted against growth rate (log difference of CFU/ml – solid line) to compare growth curve phases in cultures inoculated with diminishing inoculum densities: black (10^{-2}); brown (10^{-3}); orange (10^{-4}); yellow (10^{-5}). Shaded panelling represents the different growth curve phases (exponential-stationary for 10^{-2}, and lag-stationary for 10^{-3}-10^{-5}). C) Persistence in WT *E. coli* in cultures inoculated with diminishing cell densities in response to treatment with three antibiotics: ciprofloxacin; meropenem; and tobramycin. Each datapoint is representative of at least six replicates (two biological, and three technical replicates). D) Relative persistence in the WT (left panel – MG1655) and high persister mutant strain (right panel – HipA7) across inoculum densities and grouped by growth phase (exponential versus stationary phase), with colour representing antibiotic type: ciprofloxacin (green), meropenem (purple) and tobramycin (orange). Lefthand boxplots represent

exponential phase (filled) and righthand boxplots represent stationary phase (empty) for each antibiotic treatment. The P values represent Wilcoxon tests between growth phases for each antibiotic. To aid comparability between growth phase, persistence was standardised based on the maximum persistence for each antibiotic and inoculum density. Boxplots for each inoculum density are plotted in the supplementary information (Fig. S1).

Effect of Inoculum Density

Low inoculum density treatments advanced the increase in persistence towards exponential phase (Fig. 1C). Persistence was significantly higher during exponential phase in cultures inoculated with the lowest density of cells when compared to the highest inoculum density cultures (Fig. 2A – statistics reported in figure). Limited persister cell formation was observed in exponential and early stationary phase across inoculum density treatments (Fig. 1C; Fig. S2). Inoculum density did not significantly affect the relationship between optical density and persistence when controlling for antibiotic and strain ($F = 1.89$, $P = 0.13$) (Fig. S3). The growth rates of both the WT and the HipA7 mutant were similar across all inoculum densities (GLM: $t=0.364$, $P=0.716$; Fig. 3A; Fig. S5). This was further supported by comparable measures of total ATP per cell (GLM: $t=0.965$, $P=0.335$; Fig. 3B).

To assess whether persister cell formation in exponential phase was a true signal or represented a carryover from the initial inoculum, we compared persister cell formation between a washed and unwashed overnight culture (Fig 2B). We found that persister cell formation patterns were dependent on culturing time and not influenced by the washing of the culture or the type of antibiotic used (GLM: $t = 3.14$, $P < 0.01$). This confirmed that persister cell formation in late exponential and early stationary phase was a true signal.

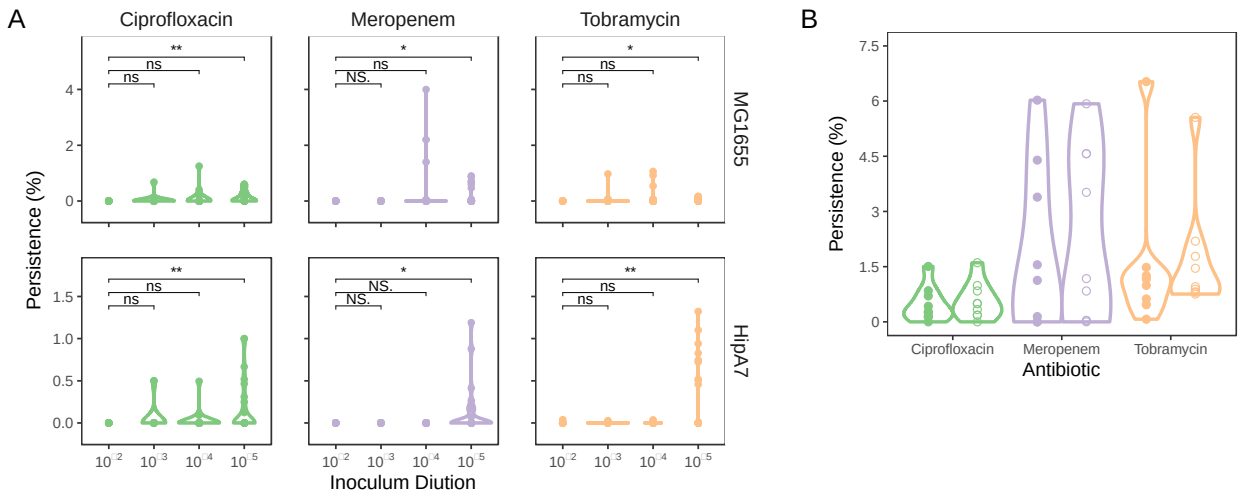


Figure 2. Persistence in exponential phase cultures. A) Percentage of persisters formed during exponential phase growth for each inoculum density culture. Asterisks denote significance where *: p <= 0.05; ** and p <= 0.01. Each datapoint is representative of at least six technical replicates. The data has been log transformed for visualisation. B) Comparison of persister cell formation in washed (filled violin plot and points) versus unwashed (unfilled violin plot and points) for each antibiotic: ciprofloxacin (green), meropenem (purple) and tobramycin (blue) for the wildtype *E. coli* strain (MG1655). Each datapoint is representative of at least six replicates (two biological, and three technical replicates).

Extracellular ATP correlates with persistence peaks

In both strains, extracellular ATP increased over time alongside population cell density, with culturing timepoint-specific peaks for the individual strains (12 hours for the high persister, and 18-20 hours for the wildtype). We found that this peak in extracellular ATP correlated with the prominent peak in persister cell formation for each strain across all inoculum densities (Fig. 3C-D; GLM: t=4.453, P<0.01; Fig. S6).

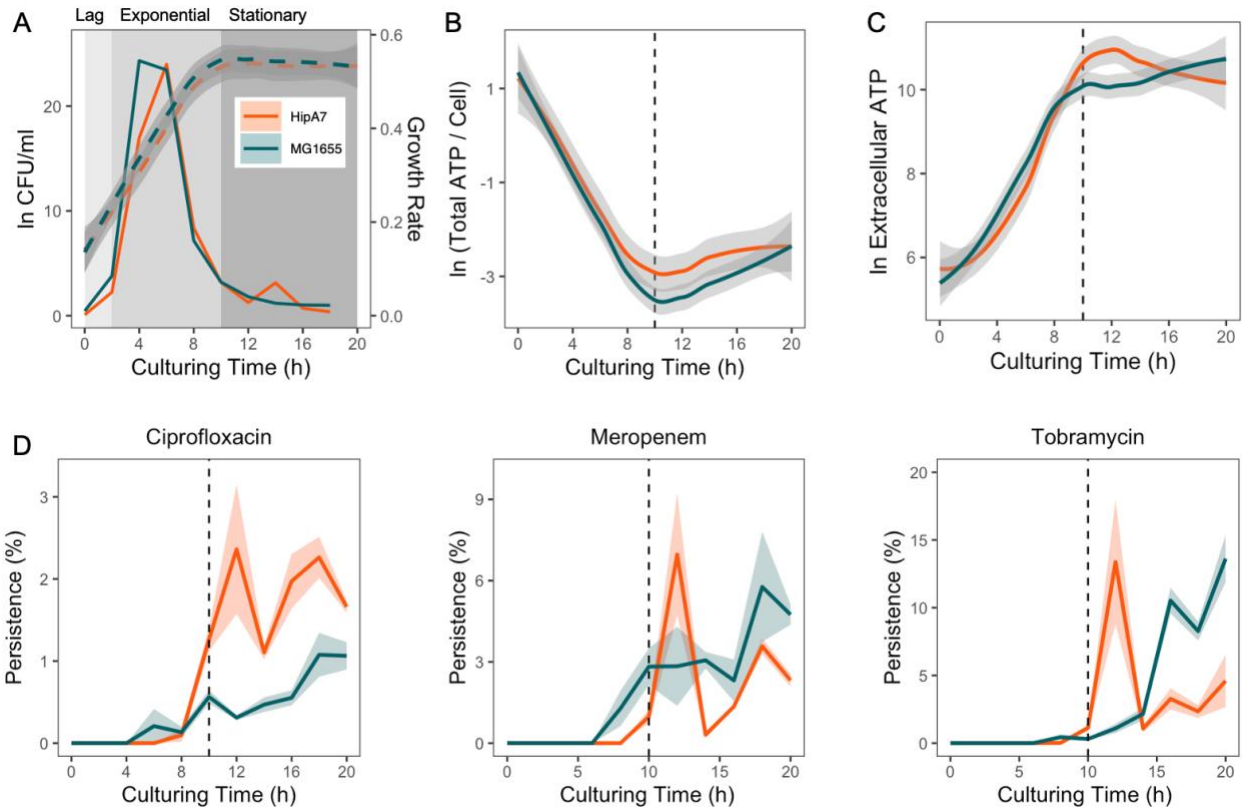


Figure 3. The timing of the peak in persister cell formation in stationary phase is strain-specific. A) Growth parameters in both strains, with the solid lines representing mean growth rate and the dashed lines representing log of initial cell number. Colour represents the *E. coli* strain in all plots, with the wildtype (MG1655) in blue and the high persister mutant (HipA7) in orange. Growth curve phases are denoted with shaded panels. B) & C) Intracellular and extracellular ATP per cell over time in the two strains tested, respectively. The solid line represents a smoothed line fitted to the data using loess, with shaded areas indicating the 95% confidence intervals. The dashed vertical line represents the boundary between exponential and stationary phase. The data has been log transformed for data visualisation. D) Mean persister cell formation over time for two strains when treated with three different antibiotics. The confidence intervals represent the mean persister percentage +/- standard error for each strain. Each point represents at least six replicates (two biological, and three technical replicates). Results representative of one inoculum density culture (10^{-4}) – graphs for the other inoculum density cultures are reported in the supplementary information (Fig. S5-6).

Extracellular supernatant influences persister cell formation

To establish whether persistence patterns were influenced by intracellular or extracellular factors, we swapped the supernatant between the high persister and the wildtype at points of interest when persistence was peaking in the growth curve (Fig. 3D). Based on observations at the 12 hours culturing timepoint (when persistence was peaking in the high persister) (Fig. 3D), swapping the

supernatant had the expected directional effect on persistence in all six experiments (reciprocal swaps in three antibiotic treatments): wildtype supernatant reduced persistence in the high persister, and high persister supernatant increased persistence in the wildtype (Fig. 4). The supernatant swap had less of an expected directional effect at 18 hours of culturing (when persistence was peaking in the wildtype) (Fig. 3D): wildtype supernatant had a weakly positive effect on persistence in the high persister mutant, and high persister supernatant significantly increased persistence in the wildtype (Fig. 4).

Since high persister supernatant had a positive effect on persistence in the wildtype at the 12-hour culturing timepoint when extracellular ATP was peaking, we wanted to test whether we could exaggerate this further by adding exogenous ATP. We established that supernatant manipulation had an instantaneous effect by showing that exogenous ATP is immediately taken up by cells (Fig. S7A). ATP addition had an antibiotic-specific effect on persister cell formation, resulting in a significant increase in persistence in ciprofloxacin and meropenem treatment, but a decrease in persistence in tobramycin treatment (Fig. S7B – Statistics reported in the figure). This antibiotic-specific response was consistent with increased intracellular ATP influencing antibiotic uptake. Hence, our results were not able to replicate the pattern of elevated extracellular ATP resulting in increased persistence as seen in Figure 3D. Instead, our findings indicated that extracellular ATP coincided with, but did not cause, peaks in persister cell formation.

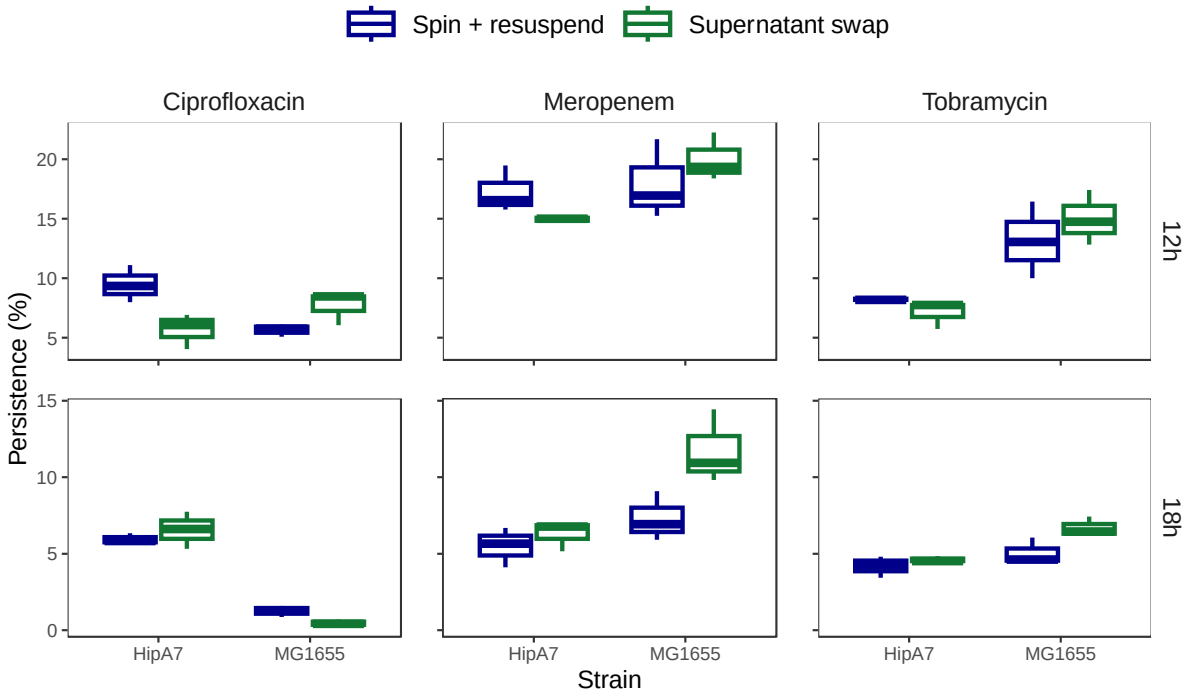


Figure 4. Supernatant swaps between the wildtype and the high persister mutant. Swaps were carried out at either 12- or 18-hrs of culturing. Boxplots show the colour of the treatment where the culture sample was either spun down and resuspended in the same supernatant (blue), or spun down and resuspended in supernatant from the other *E. coli* strain (green). Each datapoint is representative of three technical replicates.

Higher persistence in the high persister mutant is condition-dependent

Given that HipA7 is considered to be a 'high persister' mutant, we compared the magnitude of persistence across stationary phase with the wildtype and found that significantly elevated persistence only occurred in the high persister mutant under ciprofloxacin treatment (Wilcoxon test: Ciprofloxacin: W=574, P=0.028, Meropenem: W=951, P=0.14, Tobramycin: W=810, P=0.93). This was somewhat driven by increased susceptibility to ciprofloxacin in the wildtype strain in comparison to the other antibiotic treatments; persister cell formation was significantly lower when treated with ciprofloxacin than meropenem or tobramycin (Dunn test: Meropenem, Z=-2.72, P <0.01, Tobramycin: Z=-2.60, P <0.01).

Discussion

Analysis of persister cell formation in *E. coli* across a wide range of growth conditions revealed that persister cell formation peaked in stationary phase regardless of the initial inoculum density. Despite comparable growth dynamics in the WT and the high persister mutant, the timing of peaks in persister cell formation differed between strains. The peaks in persistence of both strains correlated with peaks in extracellular ATP, a potential indicator of cell lysis; with extracellular ATP increasing across stationary phase. A reciprocal swap of the supernatant suggests that persistence was not only driven by intracellular conditions but also influenced by extracellular signals. Further manipulation of the supernatant showed that ATP increases alone were unlikely to be the key driver of these cues. By comparing the persister fraction between the WT and the high persister mutant, we also show how the magnitude of persistence in the two strains was broadly comparable, questioning labelling of HipA7 as a "high persister". Taken together, our results confirm conditions in stationary phase as conducive to persister cell formation with evidence that population growth dynamics can influence the exact timing of a peak, and introduce new questions about the role of extracellular signals for further research.

Our results support previous observations indicating persister cell formation occurring predominantly in stationary phase (Balaban *et al.*, 2004; Ryall *et al.*, 2012; Van den Bergh *et al.*, 2017)., with a consistent pattern of persister cell formation seen across treatments. To our knowledge, 20 hours represents the longest growth curve analysis to date, providing further support that persister cell formation peaks in stationary phase, regardless of the initial inoculum density in both *E. coli* strains (Balaban *et al.*, 2004; Keren, *et al.*, 2004; Leszczynska *et al.*, 2013; Harms *et al.*, 2017; Pontes & Groisman, 2019). This contrasts with recent findings on *P.*

aeruginosa, where persistence peaks were predicted by the cell division history, regardless of the initial inoculum density (Davies *et al.*, submitted). It is challenging to pinpoint the precise reason for the species-specific difference in patterns of persister cell formation. For *E. coli* even within the same species, growth parameters (cell count, OD, and growth rate) between the WT and HipA7 were indistinguishable, yet the persister patterns were different. This finding indicates that persistence is not simply about changes in growth rate but is also driven by effects from underlying species and strain-specific differences. Persistence is often thought of as a universal trait across species of bacteria but further comparison across species may reveal that its function varies.

Peaks in persister cell formation consistently coincided with low intracellular ATP levels and high extracellular ATP levels. Previous evidence has suggested that persistence is associated with ATP depletion (Conlon *et al.*, 2016). We tested for a correlation of persistence with extracellular ATP as an assay to further understand the state of cells in different stages of the growth cycle – extracellular ATP, often indicative of cellular stress or damage, can signal shifts in environmental conditions or other challenges faced by cells (Xi & Wu, 2010). Our research revealed that peaks in extracellular ATP levels of each strain correlated with strain-specific peaks in persistence. The source of extracellular ATP is contested, with evidence pointing towards active ATP secretion (Hironaka *et al.*, 2013; Mempin *et al.*, 2013; Spari & Beldi, 2020), and other evidence pointing towards ATP release through lysis (Zhang *et al.*, 2007; Grygorczyk *et al.*, 2021; Ihssen *et al.*, 2021). If lysis is playing a role in promoting persistence, this would be consistent with previous research on the benefits of lysis for population adaptation and survival (Corchero *et al.*, 2001; Noor, 2015; Vos *et al.*, 2019).

Since the magnitude of persistence peaks was broadly comparable between the WT and high persister mutant for two out of three of our antibiotic treatments (tobramycin and meropenem), our findings are consistent with the growing view that the toxin-antitoxin system does not universally drive greater persister cell formation (Van den Bergh *et al.*, 2017; Goormaghtigh *et al.*, 2018; Pontes & Groisman, 2019; Svenningsen *et al.*, 2019; Fraikin *et al.*, 2020; LeRoux & Laub, 2022). Persistence ranging between 1% and 15% in the WT strain was greater than the expected 1% maximum expected (Van den Bergh *et al.*, 2017). Reports of elevated survival at this magnitude are often attributed to tolerance (Balaban *et al.*, 2019; Brauner *et al.*, 2016). However, the presence of a characteristic biphasic killing pattern in kill curves confirms that the cell survival we observed was primarily attributed to persistence rather than tolerance (Balaban *et al.*, 2004; Brauner *et al.*, 2016). One potential explanation for the discrepancy lies in the presentation of persistence data. Persister percentages are often log transformed when reported, which serves as a useful tool for visualisation, but may obscure the raw values. This became evident in previous studies on *E. coli*, where persister formation was found to be around 10%, although this information was not initially apparent as the data was log transformed (Keren, *et al.*, 2004; Harms *et al.*, 2017). The observed variation in persister cell formation of supposedly comparable lab strains highlights the need for caution and careful consideration when comparing results from different studies (Harms *et al.* , 2016).

Conclusion

Analysis of persister cell formation across a wide range of growth rates revealed that persister cell formation consistently increases in stationary phase in the *E. coli* WT and high persister mutant. However, the timing of peak persister cell formation in stationary phase was strain-specific, illustrating the species- and strain-specific nature of persister cell formation. These findings

underscore the limitations of species-wide comparisons of persister cell formation patterns when treated with distinct antibiotic types in different experimental media. This highlights the importance of interpreting persister cell formation patterns in the context of the experimental set-up. This is essential to aid the suitability of meta-analyses of persister cell formation across laboratory studies, a useful tool that will assist in the development of more targeted and effective antibiotic treatments.

Materials and Methods

Strains

Two strains of *E. coli* were used: the wildtype (MG1655) and the high persister mutant (HipA7 - MG1655 *hipA7* zde::tn10). Both strains were shared with us by Yong Zhang.

Lab materials

The three antibiotics used to isolate persistence were ciprofloxacin, meropenem and tobramycin. The antibiotics were provided in powdered form from Sigma Aldrich, APExBio and Cayman Chemical Company, respectively. We made liquid antibiotic stocks at a concentration of 1.5mg/ml and stored them in a freezer at -20°C. We sourced adenosine 5'-triphosphate disodium salt hydrate (A26209-5G) from Sigma Aldrich. For optimal growth prior to antibiotic treatment, we grew samples in pre-mixed capsules of Luria Broth and plated them onto LB agar (both LB Miller – Fisher BioReagents). We used sodium chloride (NaCl) at a 0.9% concentration for serial dilutions. The reagent for the BacTiter-Glo^{TM} Microbial Cell Viability Assay was supplied by Promega UK.

Persister assay

We grew overnight cultures of *E. coli* for 17hrs at 37˚C shaking at 220rpm (reaching an OD_{600} of ~0.6) before standardising to an OD_{600} of 0.1. We diluted this standardised culture into experimental flasks (250ml) containing 100ml of LB media. The four different inoculation densities were: 10^{-2}, 10^{-3}-, 10^{-4}-, and 10^{-5}-fold dilutions of the $0.1OD_{600}$ standardised culture. We then sampled this culture every two hours from 0-20hrs of culturing time (in a shaker at 220rpm and 37˚C). Prior to adding antibiotics, we transferred aliquots (170µl) of each culture to 96-well plates and measured cell density (OD_{600} using a SpectraMax®i3x), cell number (from serial dilution and colony forming unit (CFU) plating), and intracellular and extracellular ATP concentration (using BacTiter-Glo™ following the manufacturer's instructions). We then added 10µl of antibiotic to 150µl of culture in 96-well plates resulting in a final antibiotic concentration of 100µg/ml. All antibiotic treatments were added in excess of the minimum inhibitory concentration (Table S2). We incubated the antibiotic-treated plates for 24hours at 37˚C before serial dilution and CFU plating. For all CFU plating, we spotted 10µl from each serially diluted row in the 96-well plates onto square LB plates. By plating the whole series of serial dilutions, we were able to detect persistence over a broad range of dilutions.

Kill curve assay

To observe how bacterial cultures responded to the antibiotic within the 24h treatment period, we conducted a kill curve assay by measuring the survival at 2h, 4h, 6h, and 24h post antibiotic addition. We carried out these kill curves for both *E. coli* strains at points of interest in the growth curve (8h, 10h, & 12h). We inoculated the cultures following the same method outlined in the persister assay above. Since we carried out multiple measures of the same antibiotic-treated

sample, we used a separate row in the 96-well plate for each post-antibiotic time point measure to avoid volume depletion. We counted surviving cells by serially diluting the samples and spotted 10µl of each dilution onto LB agar plates before placing these plates into a 37˚C incubator for 17h.

Assaying washed cells

To test the effect of potential supernatant carryover from the overnight culture, we compared persister cell formation in cultures inoculated with washed and unwashed cells. To create the washed culture, we spun 1ml of overnight culture in a 1.5ml Eppendorf at 5000rpm for 10 minutes, washed the culture with 1ml NaCl and re-suspended it with 1ml fresh LB. This protocol ensured that cells remain viable and was based on Pembrey *et al*'s findings (Pembrey *et al*., 1999). We standardised to an OD_{600} of 0.1 and diluted 10^{-2}-fold prior to inoculation into experimental media. We selected 10^{-2} as our inoculum density as this treatment was the least diluted and so would contain the greatest carry-over from overnight supernatant. We then compared persister cell formation across exponential and early stationary phase growth in washed and unwashed cultures.

Reciprocal supernatant swap

We conducted the supernatant swap by spinning down and washing cells before either resuspending them in the original supernatant (control) or supernatant from the other strain (reciprocal swap). We quantified cells before and after resuspension to account for the cell number prior to adding antibiotics. We added the same concentration of antibiotics (100µg/ml) outlined in the persister assay above, calculating persistence as a percentage 24-hours after antibiotic treatment.

ATP manipulation assays

We made ATP standard solutions using adenosine 5'-triphosphate disodium salt hydrate and diluted the ATP five-fold using distilled water to achieve the desired concentration. The high-ATP treatment contained 10µM ATP per 1ml (Mempin *et al.*, 2013). For simplicity and comparability across repeats, we considered one inoculum density, 10^{-4}. To assess ATP take up, we measured OD_{600}, intracellular and extracellular ATP, and persistence before and after adding a high-ATP concentration (10µM/ml).

Data processing and statistical tests

We calculated the percentage of persister cells by dividing the number of cells 24 hours after exposure to antibiotics by the initial number of cells before exposure, and multiplied by 100. We performed this calculation for each growth curve time point and antibiotic tested. We used OD_{600} data to calculate growth rate by using the following equation:

$$Growth\ rate = \frac{\ln(OD_{t+1}) - \ln(OD_t)}{(t_{t+1} - t)}$$

Where OD_{t+1} is OD600 at time t_{t+1}, and OD_t is a measure at time t. We identified growth phases by analysing points of inflection in the growth rate across the growth curve.

We conducted all statistical analysis using R (Version 4.2.2). All models used were generalised linear models. As the data was not normally distributed, we used Kruskal-Wallis tests to compare the medians of two or more independent groups. We used Wilcoxon rank-sum tests and Dunn tests to perform pairwise-comparisons of non-normal data. We log transformed data where necessary to control for the effect of extreme values and to assist visualisation.

Chapter 3. Supplementary Information

This document contains supporting evidence supporting '*The role of population dynamics on persister cell formation in Escherichia coli*'

Table of Contents:

Figure S1. Comparison between persistence in exponential versus stationary phase for each culture inoculated with incrementally lower cell densities (10^{-2}-10^{-5}).

Figure S2. Cell count versus persistence shows no inoculum effect in *E. coli* cultures inoculated with diminishing cell densities (10^{-2}-10^{-5} dilutions of an 0.1 OD_{600}-standardised culture).

Figure S3. Increasing optical density has a significantly positive effect on persister cell formation across all treatments (inoculum density, antibiotic, and strain).

Figure S4. Biphasic killing patterns were observed in kill curves for both strains at three time points, using an inoculum dilution of 10^{-2}.

Figure S5. Growth parameters in both *E. coli* strains (MG1655 – wildtype, and HipA7 – high persister mutant).

Figure S6. Persistence correlates with extracellular ATP.

Figure S7. ATP addition reduces optical density and influences persistence in an antibiotic-specific manner in the wildtype.

Table S1. Results from correlation tests between optical density and persistence for both strains under each antibiotic treatment.

Table S2. MIC values from E-test strips for both strains and all antibiotics.

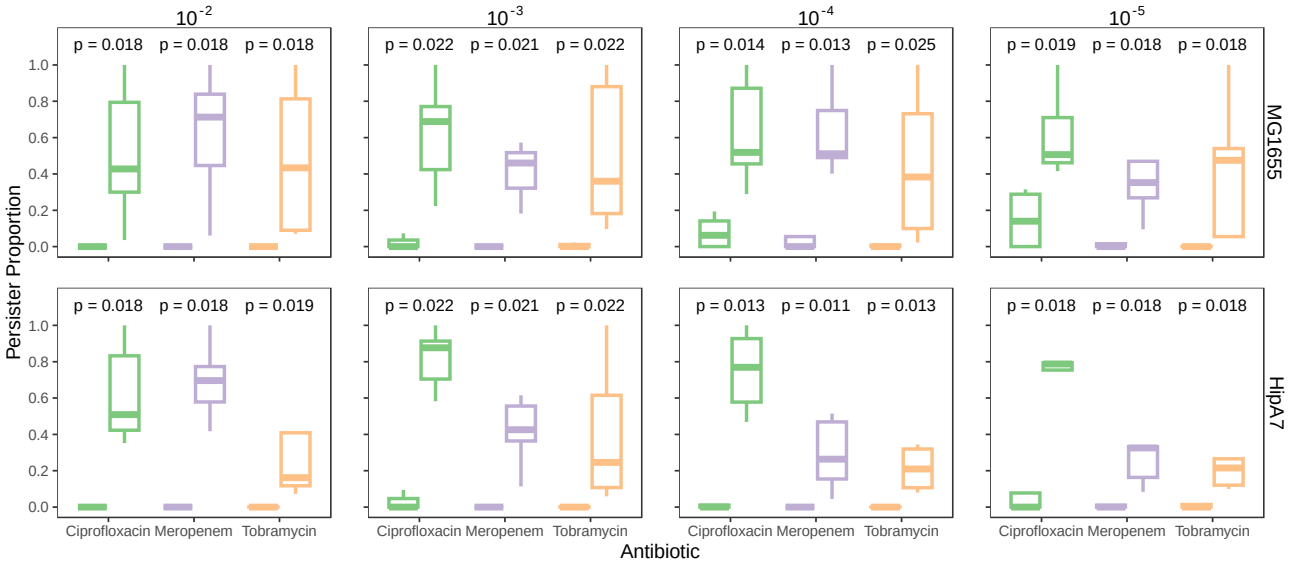


Figure S1. Comparison between persistence in exponential versus stationary phase for each culture inoculated with incrementally lower cell densities (10^{-2}-10^{-5}). Relative persistence in the WT (top panel – MG1655) and high persister mutant strain (bottom panel – HipA7) across inoculum densities and grouped by phase, with colour representing antibiotic type: ciprofloxacin (green), meropenem (purple) and tobramycin (orange). Lefthand boxplots represent exponential phase and righthand boxplots represent stationary phase for each antibiotic treatment. The P values represent Wilcoxon tests between growth phases for each antibiotic. To aid comparability between growth phase, persistence was standardised based on the maximum persistence for each antibiotic and inoculum density.

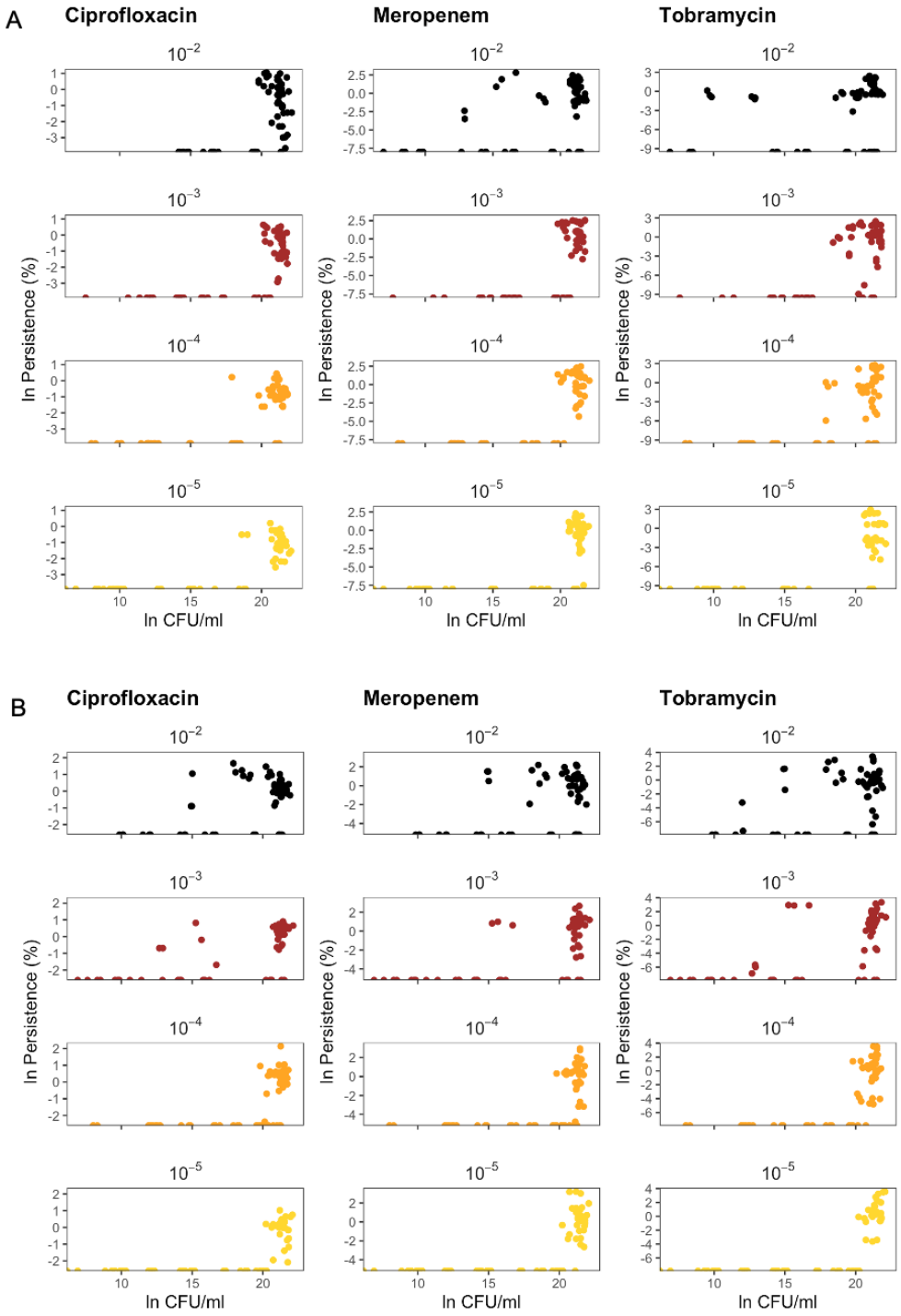


Fig. S2. Cell density versus persistence across 20 hours of culturing in cultures inoculated with diminishing cell densities (10^{-2}-10^{-5} dilution of a 0.1 OD_{600}-standardised overnight culture). A) *E. coli* wt (MG1655) and B) *E. coli* HipA7 (high persister strain). The samples were treated with either ciprofloxacin, meropenem, or tobramycin for 24 hours before being counted. The data has been log transformed for visualisation.

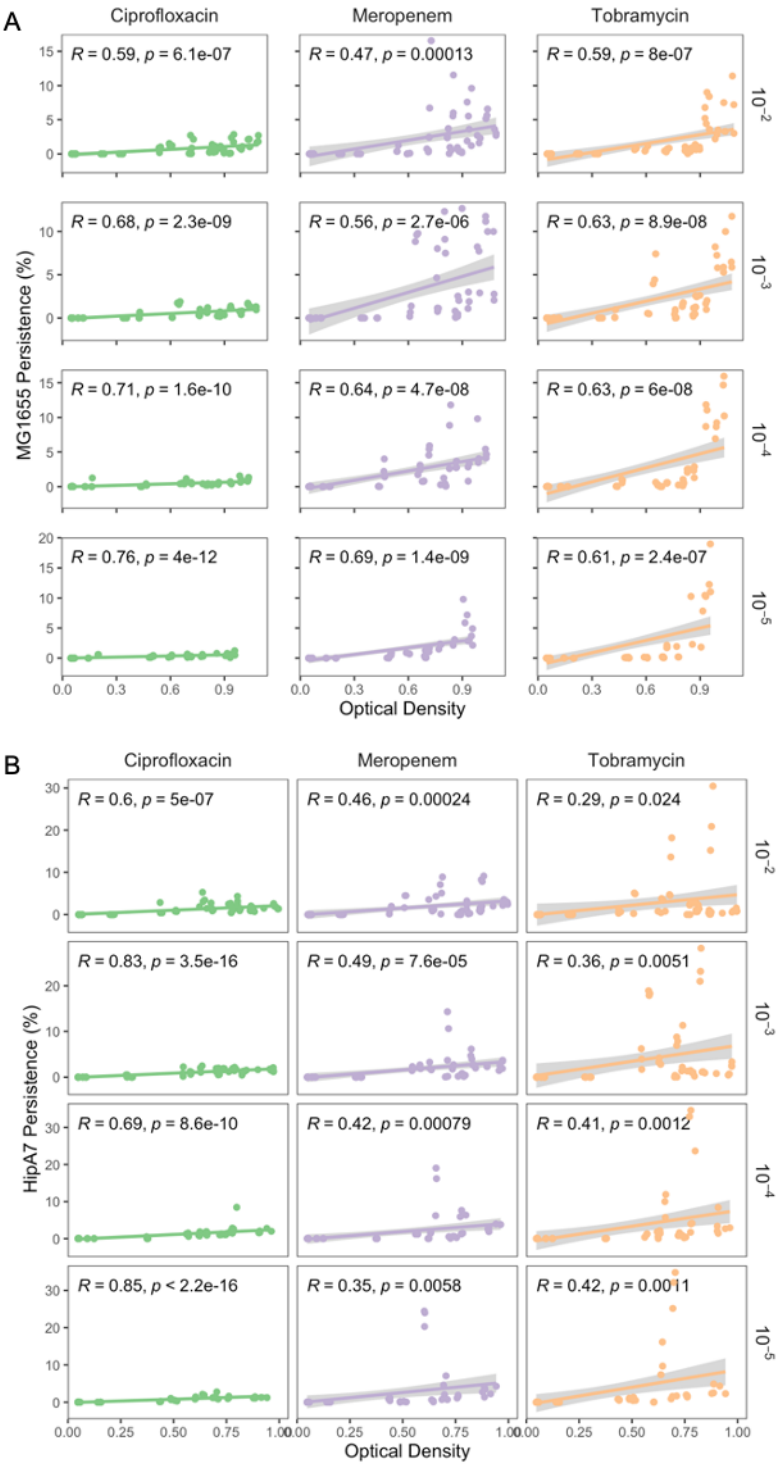


Figure S3. Increasing optical density has a significantly positive effect on persister cell formation across all treatments (inoculum density, antibiotic, and strain). Linear models have been fitted to individual data points and the correlation significance is denoted within each plot. Each datapoint is representative of at least six technical replicates.

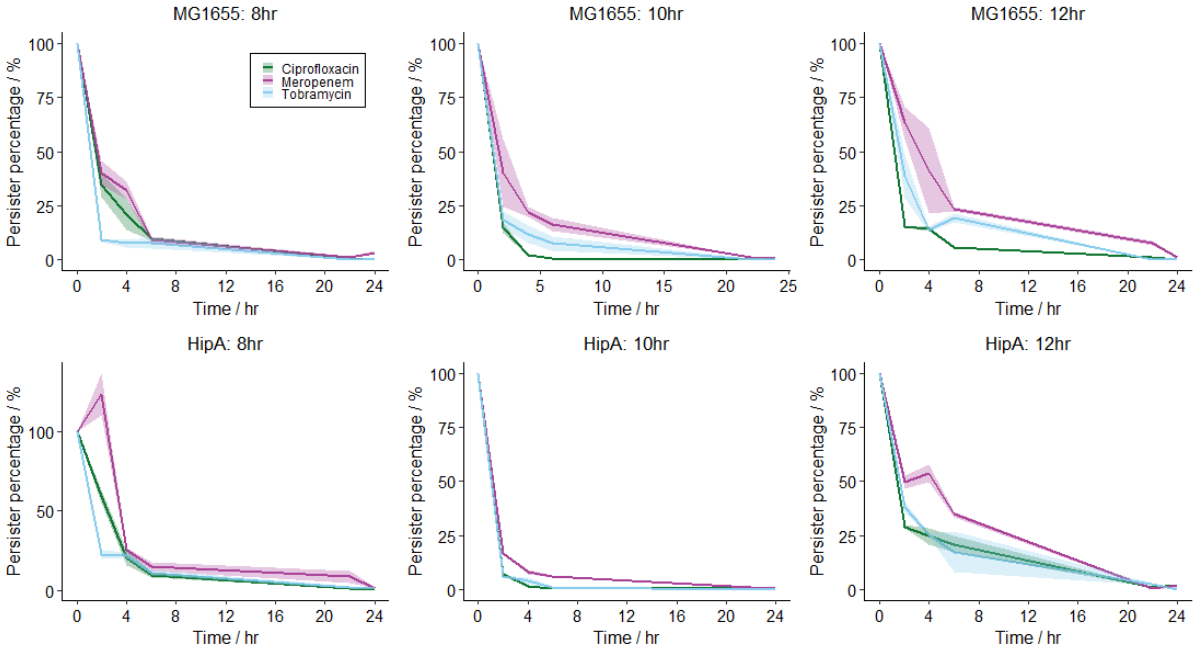


Figure S4. Biphasic killing patterns were observed in kill curves for both strains at three time points, using an inoculum dilution of 10^{-2}. The shaded region represents the mean +/- standard error. Colour denotes antibiotic, with ciprofloxacin in green, meropenem in purple and tobramycin in blue.

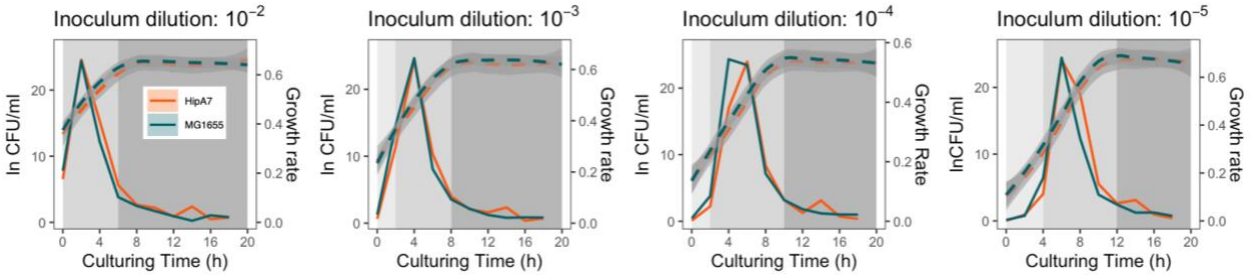


Figure S5. Growth parameters in both strains. The solid lines representing mean growth rate and the dashed lines representing log of the initial cell number. The inoculum dilution denotes the fold dilution applied to a 0.1OD_{600}-standardised overnight culture. Colour represents the *E. coli* strain in all plots, with the wildtype (MG1655) in blue and the high persister mutant (HipA7) in orange. Growth curve phases are denoted with shaded panels that represent the growth curve phases, determined by cell number and optical density data. These phases, consisting of lag, exponential and stationary phase, and are distinguished by progressively darker shading from lag to stationary phase. Each datapoint is representative of at least six technical replicates.

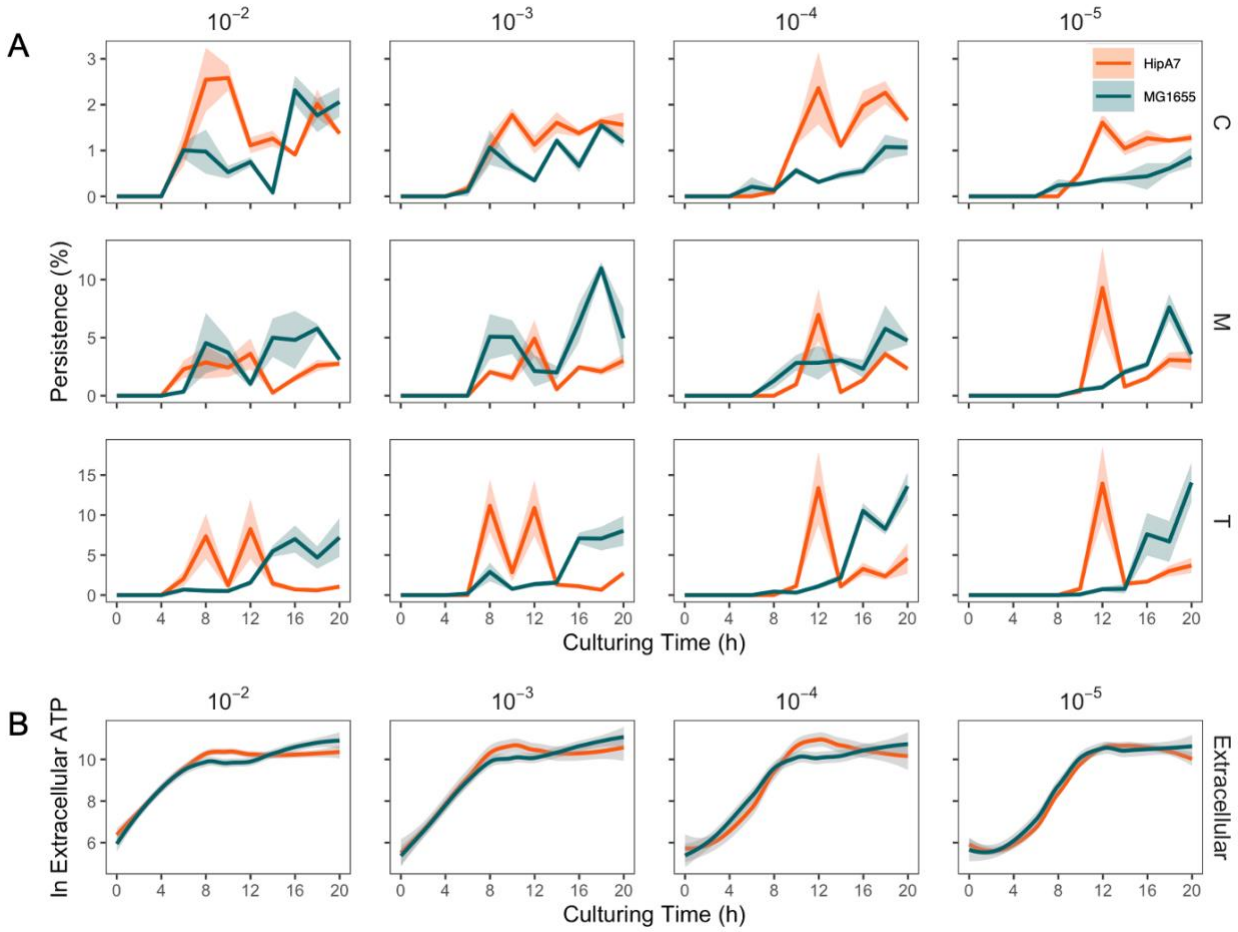


Figure S6. Persistence correlates with extracellular ATP. A) Mean persister cell formation over time for two strains when treated with three different antibiotics for each inoculum density culture. The confidence intervals represent the mean persister percentage +/- standard error for each strain. Each point represents at least two biological replicates and three technical replicates (six replicates in total). B) Extracellular ATP per cell over time in the two strains tested, respectively. The solid line represents a smoothed line fitted to the data using loess, with shaded areas indicating the 95% confidence intervals. Each point represents three technical replicates. The data has been log transformed (natural log) for visualisation.

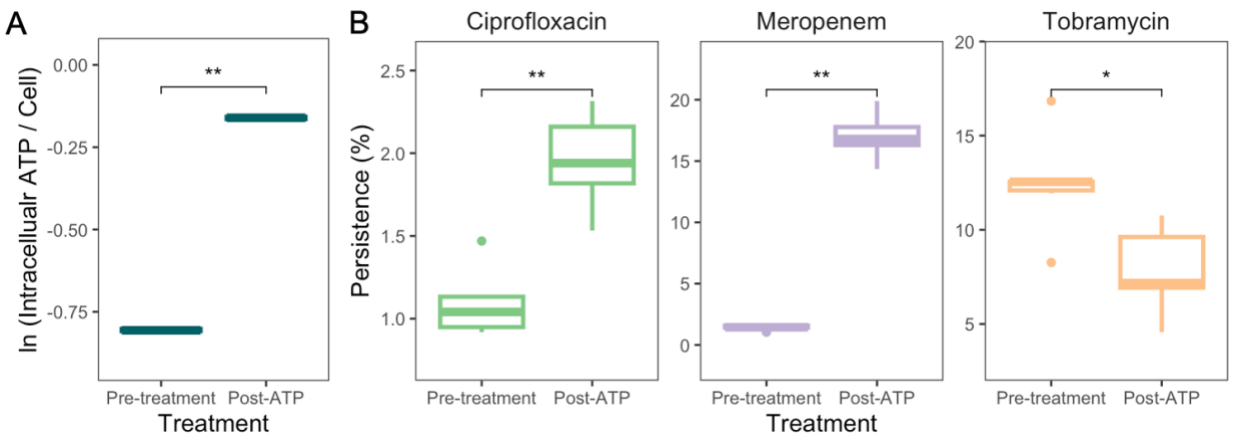


Figure S7. Addition of exogenous ATP causes an antibiotic-specific response in persistence. A) Exogenous ATP addition results in an immediate rise in intracellular ATP. The intracellular ATP measure was taken immediately after ATP addition. B) Persistence before (pre-treatment) and immediately after adding ATP (post-AB). Asterisks denote significance where: * - p <= 0.05; ** - p <= 0.01. Each boxplot is representative of at least six technical replicates.

Table S1. Results from correlation tests between optical density and persistence for both strains under each antibiotic treatment.

Species	Antibiotic	Correlation (Optical Density versus Persistence (%))	P Value
MG1655 (Wildtype)	Ciprofloxacin	0.64	5.1×10^{-29}
	Meropenem	0.57	3.9×10^{-22}
	Tobramycin	0.59	1.1×10^{-23}
HipA7 (High Persister)	Ciprofloxacin	0.71	1.1×10^{-37}
	Meropenem	0.38	9.5×10^{-10}
	Tobramycin	0.36	9.5×10^{-9}

Table S2. MIC values from E-test strips for both strains and all antibiotics.

	MG1655 (µg/ml)	HipA7 (µg/ml)
Ciprofloxacin	0.008	0.016
Meropenem	0.016	0.016
Tobramycin	0.75	0.75

Chapter 4 Motivation

My motivation for Chapter 4 stemmed from trying to disentangle the unexpected antibiotic response profiles seen in Chapter 2. The ability for cultures to continue growing through the early hours of antibiotic treatment presented an intriguing conundrum for further study. This response was antibiotic-specific and enabled bacterial samples to reach high cell densities before the population crashed. This growth after antibiotic addition occurred despite the dose being several times greater than the minimum inhibitory concentration (MIC). I also controlled for resistance mutations by plating cells on antibiotic-impregnated agar of the same concentration and found no growth.

Therefore, this phenotypic survival ability above the MIC led me to investigate a potential role for heteroresistance, whereby a subfraction of a bacterial population can have greater resistance to the antibiotic through heterogeneous expression of resistance genes. I came across heteroresistance through extra reading around antibiotic survival mechanisms (Verstraete *et al*., 2022). An early assumption with this Chapter was that heteroresistance may be responsible for the antibiotic-induced stress divide.

My goal was to a) identify whether there was heteroresistance to my selected antibiotic treatments in *P. aeruginosa*; b) try to disentangle the effects of this heteroresistance in terms of being the driving force behind elevating the population size in response to antibiotics (i.e. through a stress divide); c) explore whether population inflating responses could promote higher persistence by generating a bigger population from which a persister fraction could emerge.

Chapter 4. Growth surge following a lethal antibiotic dose in *Pseudomonas aeruginosa* PAO1

S. E. F. Davies, M. Ghoul, & A. S. Griffin

Abstract

Antibiotic resistant bacterial infections pose a significant threat to human health in the 21st century. Growing evidence suggests that there is more to the evolution of resistance than simple acquisition of a resistant allele through mutation. The pathway to resistance can be facilitated by phenotypic survival mechanisms such as persistence and heteroresistance. Persistence enables a subpopulation of cells to survive despite being genetically susceptible, and heteroresistance enables a subpopulation of cells to have substantially higher resistance to antibiotics relative to the whole population. Here we report an additional strategy – accelerated rate of division in response to antibiotic treatment, "stress divide", that can work synergistically with other strategies to enhance the survival probability of bacterial cells. We observed that this stress divide strategy enables a higher number of cells to persist through the antibiotic treatment - up to several thousand-fold increase in the population size. In addition, we identified heteroresistance within these PAO1 samples in response to the same antibiotic treatments. Taken together, these results provide a potential route to resistance for populations of bacterial cells that would otherwise be eradicated by treatment. More work is needed to understand the potential role of these phenotypic mechanisms working together in clinical settings.

Introduction

Pseudomonas aeruginosa is a clinically relevant pathogen with a known range of antibiotic survival mechanisms (Breidenstein *et al*., 2011). The combination of being a Gram-negative bacterium and having intrinsic resistance to a wide range of antibiotics means *P. aeruginosa* infections can be difficult to eradicate (Høiby *et al*., 2005; Pang *et al*., 2019). In addition, genetically encoded resistance can arise under antibiotic selection creating infectious strains capable of surviving and growing through lethal doses of antibiotics. One such example is carbapenem-resistant *P. aeruginosa* (CRPA) which was recently highlighted by the World Health Organisation as a critical priority pathogen (WHO, 2017). While the acquisition of resistant mutations is key for the evolution of resistance, far less is understood about phenotypic and behavioural strategies *P. aeruginosa* can adopt to increase its chance of survival in the short-term and thereby resistance in the long-term.

While standard assays for resistance provide an indication of antibiotic efficacy, they are not designed to provide detailed information of how the bacterial population responds to the antibiotic. A typical starting point is to measure the minimum inhibitory concentration (MIC) of antibiotic required to suppress bacterial growth. Standard techniques to measure the MIC include the disk diffusion, broth dilution, E-test, and agar dilution methods (Sherman *et al*., 2019). These techniques involve diluting the starting population to a low density prior to treatment, with the goal of assessing susceptibility to the mechanistic action of the antibiotic. As a result, these techniques will fail to detect whether, for example, fast growing cells are more susceptible to antibiotics (Lee *et al*., 2018), or whether cells reduce the bactericidal effect of antibiotics through

biofilm formation (Drenkard, 2003). These dynamics are also important to consider in the context of phenotypic antibiotic survival mechanisms such as persistence and heteroresistance.

Persistence occurs when only a sub-fraction of cells are able to survive antibiotic treatment through entering dormancy (Balaban *et al.*, 2019), whereas heteroresistance occurs when a subfraction of the population heterogeneously express genes that enable them to grow through the antibiotic treatment (this can arise phenotypically or genetically) (Andersson *et al.*, 2019). Both have been shown to act as precursory survival mechanisms prior to genetically encoded resistance arising *in vivo* (persistence: Mulcahy *et al.*, 2010; Dewachter *et al.*, 2019; Windels *et al.*, 2019; heteroresistance: Band & Weiss, 2019). Other instances of non-resistant bacterial populations growing through supposedly lethal doses of antibiotics have also been reported: defined as antibiotic perseverance by Brandis *et al*. (2023), and undefined by Möker *et al*. (2010). Measuring the phenotypic response to antibiotic treatment is a first step towards understanding variation in the evolution of resistance in pathogens such as *P. aeruginosa*, which may be currently unexplained by genotype alone.

We treated populations of *P. aeruginosa* PAO1 cells across a range of different growth conditions by applying antibiotics at different stages of the growth curve in cultures inoculated from different starting densities. We treated cultures antibiotics from three distinct classes with different modes of bactericidal action in order to assess the generality of antibiotic responses depending on the population's underlying growth dynamics prior to treatment: meropenem and imipenem are carbapenems that target cell wall synthesis (Baldwin *et al*., 2008); ciprofloxacin is a fluoroquinolone that inhibits DNA gyrase (LeBel, 1988); and tobramycin is an aminoglycoside

that disrupts protein synthesis (Goffic *et al*., 1979). We used kill curves to look at how cells responded to the antibiotic treatments, we used E-tests to establish the MIC, and we created a population analysis profile (PAP) to measure heteroresistant fractions within the population. An unexpected consequence of our experimental treatment was the observation of a period of accelerated population growth following treatment (a "stress divide") that has not been widely reported before (Brandis *et al*., 2023; Möker *et al*., 2010). We were, therefore, able to assess potential interactions of this phenomenon with other phenotypic survival mechanisms.

Results

Incidence of an antibiotic-induced stress divide

Stress-divides were observed in response to treatment with tobramycin and meropenem. The stress divides were characterised by divisions of up to six divisions/hour, compared to the maximum doubling rate of three divisions/hour in untreated populations (Fig. 1A-B). After an average of four hours after treatment, the cell count decreased and plateaued revealing the underlying persister fraction (Fig. 1B; Fig. S1). Under treatment with ciprofloxacin, regardless of inoculum density, cells died in a biphasic pattern which is typically observed in persistence assays (Fig. 1B).

Consequences for persister cell number

Populations that underwent a stress divide upon addition of the antibiotic had an elevated cell count from which a persister fraction was produced (Table 1). Stress divides facilitated a 10,000-fold and 4,000-fold increase in cell count under meropenem and tobramycin treatment, respectively (Fig. 1C). In order to inflate the population size to this extent, the whole population of bacteria would have to have undergone up to 13 rounds of division. Relative to this stress divide peak in

cell count, the surviving percentage of persisters was 0.27% (sd = 0.16%) for meropenem and 0.014% (sd = 0.0035%) for tobramycin treatment (Fig. 1D). In the meropenem treatment, owing to the stress divide, the surviving number of persister cells 24-hours after treatment was higher than the population cell count prior to antibiotic treatment. This instance of higher mean survival relative to the initial population size 24-hours after meropenem treatment occurred twice within the growth curve at 14- (late exponential) and 34-hrs of culturing (late stationary) (Fig. S2).

Table 1. Cell numbers pre- and post-antibiotic treatment highlighting the presence/absence of an antibiotic-induced stress divide and the consequences for the persisting fraction 24 hours after antibiotic treatment.

Inoculum Dilution	**Antibiotic**	**No. Cells Pre-Antibiotic (cells/ml)**	**No. Cells at Stress Divide Peak (cells/ml)**	**No. Cells 24hrs Post Antibiotic (cells/ml)**	**Persistence Relative to Peak Survival (%)**	**Predicted No. Persister Cells without Stress Divide (cells/ml)**
	Ciprofloxacin		-	4.5×10^{5}	6.5×10^{-3}	-
10^{-4}	Tobramycin	6.9×10^{9}	2.8×10^{12}	3.7×10^{8}	1.4×10^{-2}	9.6×10^{5}
	Meropenem		6.7×10^{12}	1.8×10^{10}	2.7×10^{-1}	1.9×10^{7}

The addition of a beta lactamase inhibitor (cloxacillin) to a culture treated with imipenem, a beta lactam antibiotic similar to meropenem, significantly reduced the initial ability for PAO1 to survive (Fig. S3). This reduction in survival under treatment with cloxacillin and imipenem was also true in high inoculation density cultures. This suggests that regardless of inoculum density and population division history, the survival after treatment with beta lactam antibiotics was enhanced by beta-lactamase production.

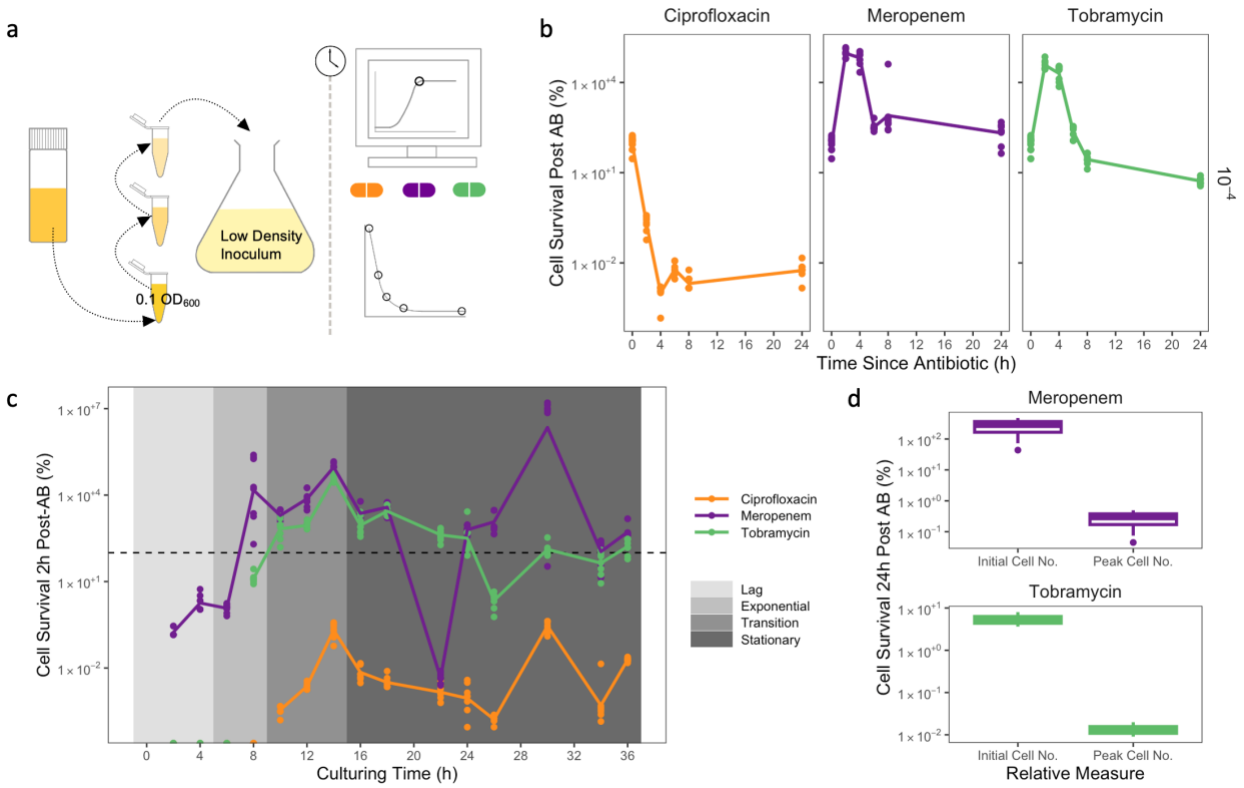


Figure 1. The initial response to antibiotic treatment is influenced by population growth pattern. A) Schematic diagram showing the experimental culture setup from overnight culture to produce a low density (10^{-4}-fold dilution of a $0.1OD_{600}$-standardised overnight culture) inoculum culture prior to treatment with antibiotics. B) Kill curves for cultures treated during stationary phase after initially being inoculated with a low (10^{-4}-fold dilution) inoculum density. The cultures were treated with either: ciprofloxacin (orange); meropenem (purple); or tobramycin (green). Individual replicates (x6) are plotted as points. Note how meropenem and tobramycin treatment in the low-density inoculum cultures (10^{-4}) enables the culture to initially grow through the antibiotic treatment. C) Percentage cell survival two hours after antibiotic treatment for samples treated with either ciprofloxacin (orange), meropenem (purple), or tobramycin (green). The shaded panels denote the different phases of the bacterial growth curve, and the dashed line illustrates the population size prior to antibiotic treatment. D) Persistence is greater in a culture that is able to initially grow through the antibiotic treatment. The boxplots denote the underlying persistence (Cell survival (%)) 24 hours after adding the antibiotic either relative to the initial cell count (pre-antibiotic addition) or the peak cell count (after antibiotic-induced overgrowth). Each data point is representative of at least six technical replicates. All of the axes have been log10-transformed for visualisation.

Identifying heteroresistance in *Pseudomonas aeruginosa* PAO1

We assayed for heteroresistance using E-tests and population analysis profiles for cultures with different inoculum conditions and culturing times (Fig. 2A). We identified heteroresistance to meropenem and tobramycin in all samples, regardless of the population division history. Results

from E-test strips showed clear heteroresistance arising in response to meropenem (Fig. 2B), whereas in tobramycin E-tests, heteroresistance only became apparent in an exponentially growing culture that was inoculated using a low inoculum density (10^{-4}) (highlighted with a magnified image (Fig. S4B)).

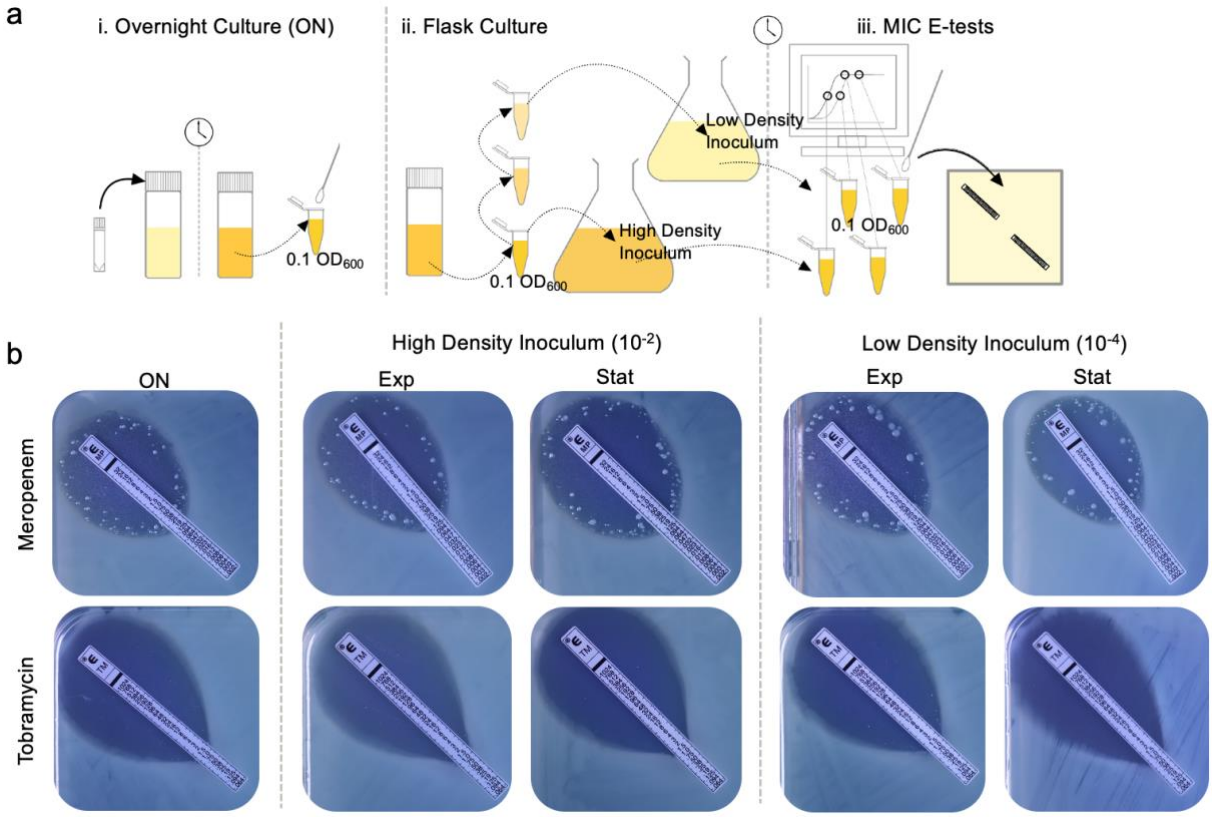


Figure 2. Heteroresistance in the *P. aeruginosa* PAO1 lab strain. A) Schematic diagram illustrating the culturing procedure to measure heteroresistance in i) overnight cultures or ii) flask cultures for carrying out iii) E-test MIC measures. B) E-test strip profiles for bacterial cultures with different cell division histories: overnight (ON) culture (inoculated from freezer stocks); and exponential and stationary phase cultures inoculated with either a high (10^{-2}-fold) or low (10^{-4}-fold) dilution of a $0.1OD_{600}$-standardised overnight culture. All samples were diluted down to $0.1OD_{600}$ prior to being spread on the agar plate. E-test strip results for imipenem can be seen in the supplementary information (Fig. S4A). A magnified image of the low-density inoculum (10^{-4}-fold dilution) exponential phase culture response with mild heteroresistance to the tobramycin E-test strip can be seen in the supplementary information (Fig. S4B). E-test MIC measures were replicated at least three times.

Owing to the limited heteroresistance detection using tobramycin E-test strips, we further conducted a population analysis profile (PAP) (Fig. 3A). Heteroresistance can be confirmed using a PAP when a proportion above 1×10^{-6} of the initial population are able to grow at concentrations

8x the MIC (Andersson *et al.*, 2019). We found that heteroresistance was consistently detectable in overnight cultures for all antibiotic treatments (Fig. 3B). In flask cultures, the proportion of heteroresistant cells was consistently higher in stationary phase in comparison to exponential phase cultures regardless of the inoculum density (Fig. 3C). According to PAP standards, heteroresistance was less detectable in high density inoculum exponential cultures in comparison to the low-density inoculum cultures with extended division histories.

To postulate whether the heteroresistant fraction of cells could contribute towards the antibiotic-induced stress divide, we calculated that in order for ~2×10^{-6} % of cells to inflate the whole population 10,000-fold, these cells would have to divide around 20 times per hour – a division rate of one division every three minutes.

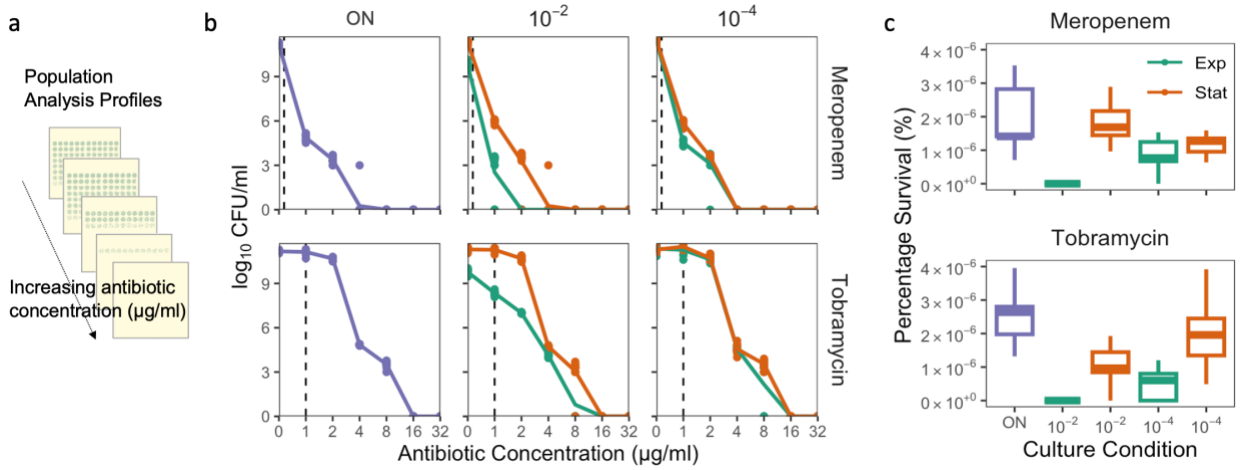


Figure 3. Population analysis profiles to detect heteroresistance. A) Schematic diagram illustrating the procedure to carry out a population analysis profile. B) Population analysis profiles for each culture. The different cultures are colour-coded: overnight (purple); exponential phase (green); and stationary phase (orange). The MIC for each antibiotic is represented with a vertical dashed line. The data has been log-transformed for visualisation. C) The underlying heteroresistant fraction in each culture. All samples are representative of at least 12 replicates (six technical and two biological replicates).

Discussion

We found that populations of PAO1 could initially grow through lethal doses of antibiotics in a process we term a stress divide, before the population crashed. This stress divide facilitated an increase in the number of surviving persister cells, presenting a potentially synergistic relationship between resistance-like survival mechanisms and persistence. The addition of a beta lactamase inhibitor alongside beta lactam antibiotics reduced this initial survival, indicating that the stress divide may somewhat be supported by beta lactamase production under beta lactam treatment. Since PAO1 cannot enzymatically degrade aminoglycosides in the same way, other resistance-like mechanisms were likely being employed. After further exploration of antibiotic survival dynamics, we found that PAO1 was heteroresistant to both meropenem and tobramycin. While mathematically, the ability for the heteroresistant fraction to cause the stress divide seemed implausible, the presence of a heteroresistant subpopulation likely contributed towards increased population survival. Given that both heteroresistance and persistence have been implicated in the development of resistance *in vivo*, future research should focus on the potential selective advantages of these two phenotypic traits working together.

The ability for cells to keep growing through lethal doses of antibiotics is not widely reported in the literature. Hormetic effects, where antibiotic stressors can stimulate growth, are more generally observed at sub-inhibitory concentrations of antibiotics (Iavicoli *et al*., 2021; X. Li *et al*., 2020). However, two notable studies have reported the ability for cells to keep dividing in the presence of antibiotics beyond the minimum inhibitory concentration. These include Möker *et al*.'s (2010) observation of *P. aeruginosa* cultures continuing to grow during carbapenem treatment at concentrations as high as 500µg/ml prior to lysis, and more recently the ability for a subset of

Escherichia coli cells to temporarily sustain division under rifampicin treatment was reported as 'antibiotic perseverance' (Brandis *et al*., 2023). Notably in Möker *et al*.'s (2010) case, the authors did not provide data or further information regarding the initial growth through the antibiotic treatment paying little attention to this phenomenon and its relevance within the wider literature. More work is needed to establish whether this is an adaptive response or a temporary "side-effect" of the antibiotic action.

One possible advantage of a stress divide is the positive effect on the surviving persister fraction. The ability to grow through lethal doses of antibiotics was dependent on the population division history, mainly occurring in cultures with extended division histories as a result of low inoculum densities. By inflating the initial population size, a greater number of persisters were able to form in response to the lethal antibiotic dose. The formation of persister cells during the antibiotic treatment (and not simply depending on the number of persisters at the point of treatment) was recently documented in *Staphylococcus aureus* by Liu *et al*., (2023). Here the authors looked at transcriptomics during the 24-hour antibiotic treatment and showed how persisters formed from metabolically active cells in an antibiotic-specific manner. This ability to modulate persistence during the antibiotic treatment further supports how increasing the population size could enable more cells to become persisters.

It must be noted, the inflated cell counts recorded in response to antibiotic treatment, which we have termed a stress divide, may have been driven in some part by cell adhesion/biofilm formation. While we employed extensive mixing with pipette tips during serial dilutions, it is possible that there may have been carry-over during the serial dilution process. This would go towards

explaining how calculations of up to six divisions per hour in order to elevate the population size are considerably higher than those expected in the literature (Mason, 1935). Nonetheless, it is also possible that unexpected cell responses could result in cells being able to increase the population size through non-conventional division mechanisms such as elongation and filamentation (Blázquez et al., 2006; Frost et al., 2018; Miller et al., 2004). Future directions to verify the primary cause of cell count increases could entail using single cell imaging to record the cellular response upon antibiotic addition.

Interestingly, we could inhibit the initial stress divide under imipenem treatment using the beta lactamase inhibitor, cloxacillin. This suggests that the initial ability to undergo a stress divide under beta lactam treatment is supported by the production of antibiotic degrading enzymes such as AmpC-beta-lactamases (Mirsalehian *et al*., 2014). Chromosomal AmpC is naturally occurring in PAO1 and regulated by AmpR (Kong *et al*., 2005). Coincidentally, AmpR has been shown to regulate multiple genes including mediating resistance to non-beta lactam antibiotics through efflux pump overexpression, OprD under expression (porin restriction), and regulation of virulence (Balasubramanian *et al*., 2012). This means that the underlying genetic features that promote heteroresistance to different antibiotics could be regulated by the same pathway. This would help explain why we see heteroresistance and the stress divide in response to antibiotics from different classes (beta lactams and aminoglycosides).

Observations of heteroresistance in response to beta lactams within PAO1 has been reported before (Ahmed *et al*., 2022; Lu *et al*., 2022), and also in response to quinolones (Li *et al*., 2022). However, to our knowledge, this is the first time heteroresistance in response to aminoglycosides has been

reported in this strain. We show how heteroresistance is not always easily detectable using methods such as E-test strips, as heteroresistance in response to tobramycin was only consistently detected using a population analysis profile (PAP), further supporting the need to use a PAP as the gold standard measure for heteroresistance (Sherman *et al*., 2019). The challenge of detecting heteroresistance likely contributes to its under-reporting in the scientific literature (Andersson *et al*., 2019). This may also apply to the observation of 'antibiotic perseverance' identified by Brandis *et al.* (2023) as it is not clear whether a PAP was used to conclusively rule out heteroresistance. This ambiguity with defining heteroresistance further highlights the need for caution when interpreting antibiotic killing dynamics in bacteria, especially when relatively little attention has been paid to the role of population dynamics in heteroresistant populations (Pereira *et al*., 2021).

Conclusion

Our results show how populations of the PAO1 lab strain of *P. aeruginosa* can undergo a stress divide under lethal antibiotic doses. This stress divide inflated the initial cell number to the advantage of increasing persister cell formation. This points towards a potential role for phenotypic antibiotic survival mechanisms (rapid divisions followed by cell dormancy) to act synergistically to promote survival under antibiotic treatment. Coincidentally, we also found that these populations were heteroresistant to the antibiotic treatments. While we cannot infer a direct causal relationship between heteroresistance and the antibiotic stress divide, the existence of this phenotype in a population able to temporarily grow through antibiotic treatment presents compelling avenues to explore in the context of population survival mechanisms. This is especially relevant given that both persistence and heteroresistance have been shown to act as intermediate steps in the development of antibiotic resistance *in vivo*. This presents questions regarding the

potential adaptive advantage of heterogeneity-driven survival mechanisms within clonal bacterial populations. Further work should consider how population dynamics influence the interaction between these survival phenotypes.

Materials and Methods

Our study organism was the *P. aeruginosa* lab strain, PAO1 (Copenhagen). We cultured and plated all samples in LB Broth (Miller) and LB agar prepared using pre-buffered powders supplied by Fisher Bioreagents. We prepared stock solutions (1.5mg/ml) of each antibiotic from powders supplied by: TOKU-E (meropenem); Cayman (tobramycin); Sigma Aldrich (ciprofloxacin); and Cayman (imipenem); and stored them in the freezer at -20˚C. The beta lactamase inhibitor, cloxacillin, was supplied by Sigma Aldrich.

Culturing conditions

For consistency across biological replicates, we inoculated all samples from freezer stocks into glass vials containing 6ml of LB media and incubated for 17hrs at 220rpm and 37˚C. After overnight incubation, we standardised the samples to an OD_{600} of 0.1 before further diluting into experimental flasks. We diluted this standardised sample either 10^{-2}- or 10^{-4}-fold for the high and low inoculum dilution treatments, respectively. For batch culturing, we used 100ml of LB media in 250ml Erlhenmeyer flasks incubated at 37˚C and 220rpm.

Measuring heteroresistance

We used two techniques to determine the presence of heteroresistance: 1) E-test strips; and 2) a population analysis profile (PAP). We assayed with E-test strips according to the recommended

guidelines: we created a lawn from a sample diluted to an OD_{600} of 0.1 before placing an E-test strip on the surface and incubating for 24 hours at 37˚C. For the PAP, we followed guidelines recommended by Sherman *et al.* (2019).

Kill curve assays

We conducted kill curves by treating cells with an antibiotic concentration of 100µg/ml. We treated all samples with 10µl of 1.5mg/ml antibiotic in 96-well plates (initial volume of 150µl). To assay for the effect of enzymatic degradation of beta lactams, we used liquid doses of the beta lactamase inhibitor, cloxacillin. To ensure cloxacillin was added in excess, we added 15µl of 3mg/ml cloxacillin to create an overall concentration of 300µg/ml. To assess the response to antibiotic treatments, we sampled cells every hour. To avoid sample depletion, we followed the protocol outlined in Davies *et al*. (submitted).

Calculations

To estimate the number of cell divisions for the antibiotic-induced stress divide, we calculated divisions as follows:

$$N = \frac{ln(\frac{Cell\ No_t}{Cell\ No_{t0}})}{ln(t)}$$

Where N is number of divisions that have occurred, $Cell\ No_t$ is cell count at time t, $Cell\ No_{t0}$ is the cell count at time t_0, and t represents the number of hours between each cell count measure to estimate doubling rate over time.

Statistical tests and software

All statistical analyses were carried out in R (version 2023.03.0). Data was checked for normality prior to selection of a parametric, or non-parametric test.

Chapter 4. Supplementary Information

This document contains supporting evidence supporting '*Growth surge following a lethal antibiotic dose in Pseudomonas aeruginosa PAO1*'

Table of Contents:

Figure S1. Representative kill curves relative to peak in cell count after a stress divide.

Figure S2. Percentage cell survival 24 hours after antibiotic treatment.

Figure S3. Addition of a beta lactamase inhibitor can reduce the initial survival to the antibiotic treatment.

Figure S4. E-test strip profiles.

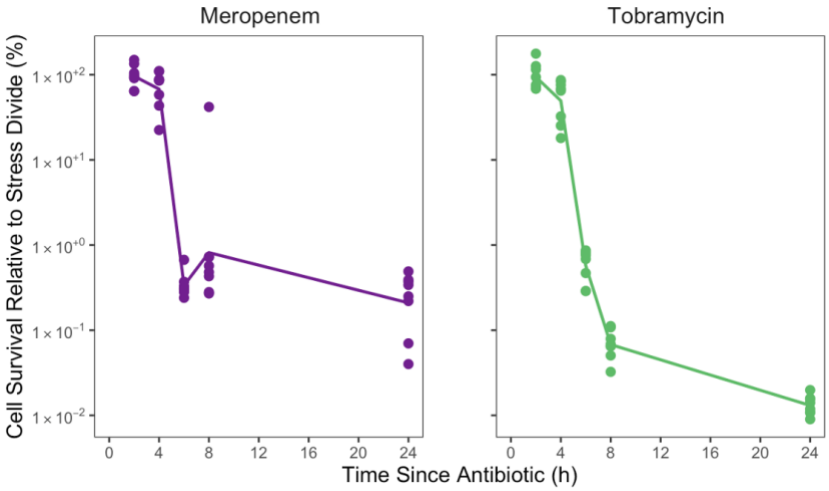


Figure S1. Kill curves relative to the peak in cell count after a stress divide. The cultures were treated with either: meropenem (purple); or tobramycin (green). The percentage of surviving cells has been calculated relative to the mean of the peak in cell survival (stress divide) two hours after antibiotic treatment. By taking this stress divide peak, the antibiotic response represents the more expected biphasic killing pattern seen when assaying for persistence. Each datapoint is representative of six technical replicates.

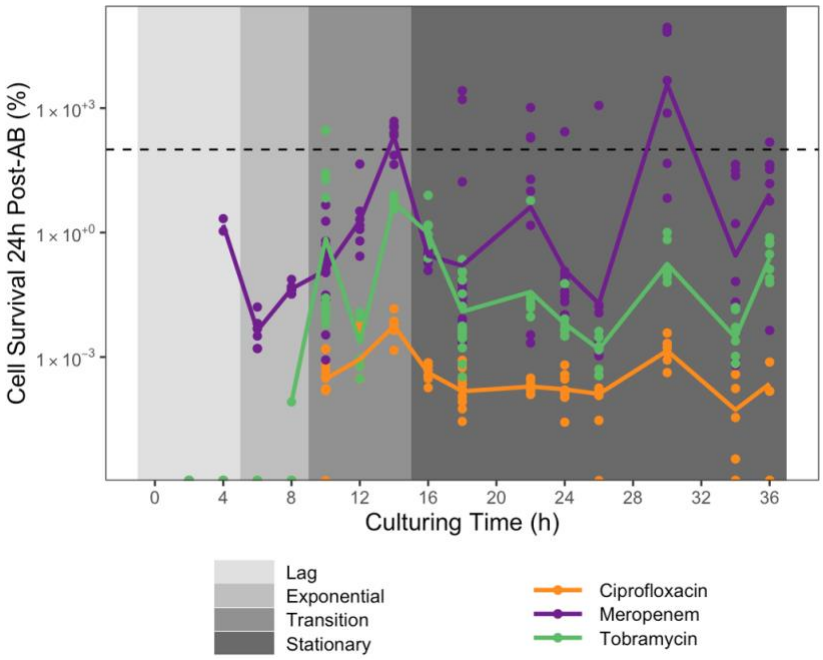


Figure S2. Percentage cell survival two hours after antibiotic treatment. Samples treated with either ciprofloxacin (orange), meropenem (purple), or tobramycin (green). The shaded panels denote the different phases of the bacterial growth curve, and the dashed line illustrates the population size prior to antibiotic treatment (100%). Each data point is representative of at least six technical replicates. The y axis has been log10-transformed for visualisation.

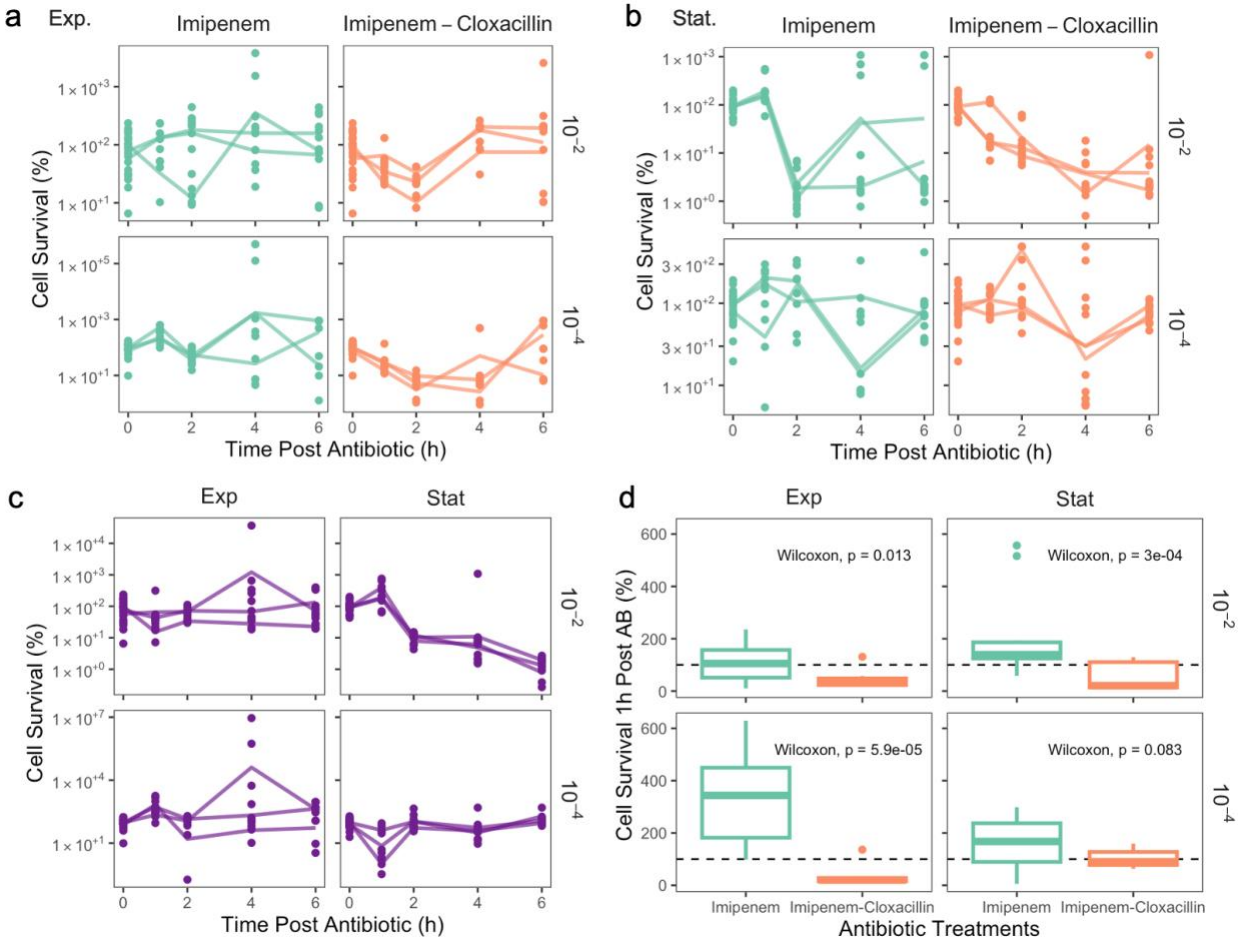


Figure S3. Addition of a beta lactamase inhibitor can reduce the initial survival to the antibiotic treatment. A-C) Antibiotic response curves for a culture treated during exponential or stationary phase after being inoculated with either a high (10^{-2}-fold dilution of a 0.1 OD_{600}-standardised overnight culture) or low (10^{-4}-fold dilution) inoculum density, respectively. The cultures have been treated with imipenem (green), imipenem and cloxacillin (beta lactamase inhibitor) (orange)), or meropenem (purple). The lines represent the mean of the individual biological replicates (with points representing the four technical replicates within each assay (three biological replicates)). The axes have been log10-transformed for visualisation. D) Addition of cloxacillin, a beta lactamase inhibitor, alongside imipenem reduces initial survival (1h post treatment) to the antibiotic. The dashed horizontal line illustrates the initial population size. The antibiotic/antibiotic+inhibitor treatments have been compared using a Wilcoxon test.

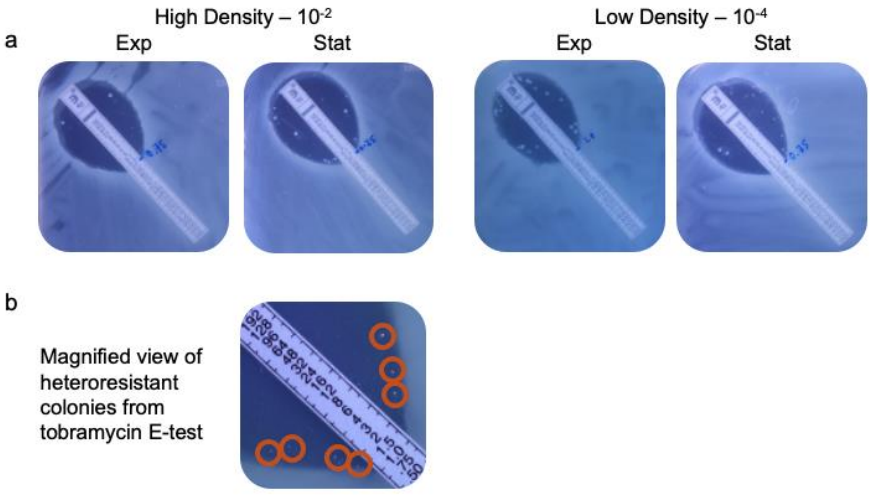


Figure S4. E-test strip profiles. A) Imipenem E-test strips for bacterial cultures with different cell division histories: exponential and stationary phase cultures inoculated with either a high (10^{-2}-fold) or low (10^{-4}-fold) dilution of a 0.1OD_{600}-standardised overnight culture. B) Example of heteroresistant colonies forming in response to the tobramycin E-test strip in the exponential phase low density inoculum culture. All samples were diluted down to 0.1OD_{600} prior to being spread on the agar plate. Each E-test assay was conducted with three technical replicates.

Chapter 5. Discussion

Each of the previous chapters contain a relevant and in-depth discussion. Here, I plan to discuss the key findings and observations from my work, highlighting potential future directions for research on antibiotic persistence.

My aim was to approach antibiotic persistence from an evolutionary perspective, observing how population dynamics influence when this trait is most likely to be expressed. I used the PAO1 lab strain of *P. aeruginosa* as my main study organism. After reviewing the literature, my prediction was that persistence would most likely arise during stationary phase, when bacterial cells are shutting down metabolically, facilitating the transition for a subset of the population to enter into a dormant state. However, initially to my surprise, I found that persistence could peak prominently during exponential growth, when cells are expected to be most metabolically active and dividing to increase the overall population size. In terms of potential fitness costs, this seemed counterintuitive: why would a population of bacteria sacrifice a subset of cells to slow growth/dormancy (i.e. persistence) when the population should be optimising exponential growth?

To further explore why this may be the case, I manipulated growth curve dynamics, decoupling culturing time from growth rate by measuring persistence in cultures inoculated with a range of inoculum densities. This enabled me to identify a potential role for population cell division history in persistence. In this scenario, the growth curve phases were not the main predictive indicator for when persistence was most likely to arise. Instead, persistence increased after the cumulative increase in population-wide heterogeneity through successive rounds of division. In lower inoculum density cultures, which required more rounds of division to reach stationary phase,

persistence peaked as early as lag phase. The detection of persistence during active growth questions the established views within the literature where stationary phase is considered to be a key signature of persister cell formation.

To test whether this phenomenon of persistence arising during active growth was true in other bacterial species, I expanded my persister assay to include two well-characterised strains of *E. coli*: the wildtype (MG1655), and the high persister mutant (HipA7). I conducted this research in collaboration with a Master's student I was supervising in the lab. By replicating the assay methods used previously with *P. aeruginosa*, we were able to see whether we could manipulate cell division history to cause persistence to peak earlier in the growth curve, or whether population cell division history had no effect in *E. coli.* The latter was true, and regardless of inoculum size or antibiotic treatment, persistence consistently peaked during stationary phase growth for both *E. coli* strains. This is consistent with persistence being favoured most strongly when there is limited cost to arrested growth. By characterising the two *E. coli* strains alongside each other, we were able to identify two interesting features: 1) persistence consistently peaked earlier in the growth curve for the high persister mutant; and 2) this earlier peak coincided with an increase in extracellular ATP in this strain. We found that persistence correlated with extracellular ATP in both *E. coli* strains, which can be an indicator of cell lysis. Further manipulation of the supernatant showed how external cues from the strain-specific supernatants can influence persistence. This indicates that persistence may not only be driven by intrinsic factors.

Through manipulating cell division history in *P. aeruginosa* cultures, I also discovered that populations grown from a low inoculum density could unexpectedly grow through the antibiotic

treatment. We termed this antibiotic-induced growth a ‘stress divide’. This stress divide occurred under treatment with antibiotics from two classes: beta lactams and aminoglycosides. This ability to initially survive the antibiotic treatment and inflate the population size had a positive knock-on effect on persistence, sometimes increasing persister cell numbers to the extent where they were even greater than the initial population size. To further investigate potential causes behind why these low inoculum density cultures could grow through lethal doses of antibiotics, I investigated another heterogeneity-driven antibiotic survival mechanism; heteroresistance. My theory here was that heteroresistance was arising in the low-density inoculum cultures due to inoculum density effects. However, I found that regardless of inoculum density, heteroresistance arose against both beta lactams and aminoglycosides. Additionally, I found that the low fraction of heteroresistant cells (~$2x10^{-6}$) was unlikely to be able to facilitate the several thousand-fold cell count increase upon treatment with antibiotics. This meant that I did not find a direct causal link between heteroresistance and the antibiotic-induced stress divide. Nonetheless, heteroresistance in this strain could easily have been missed in the high-density inoculum cultures which largely presented as susceptible populations when only looking at kill curve and E-test results. The extent to which heteroresistance was contributing towards the underlying population survival, and so persistence, is unclear. Either way, this research opened up exciting avenues to consider how heterogenous phenotypic survival mechanisms such as persistence and heteroresistance could work synergistically to increase population survival.

General Remarks

My key findings in this thesis stem from using different inoculum densities to set up bacterial growth curves. By manipulating the growth curve dynamics in this way, I was able to initially

establish the differences between growth rate and cell density-dependent effects on persistence across multiple strains and species. This work has enabled me to find interesting phenomena including persistence during fast growth, persistence patterns alongside extracellular ATP, and even instances of heteroresistance in a lab strain of *P. aeruginosa*. Finding such different effects through simply using a range of inoculum densities highlights the potentially narrowed scope of the literature, where lab-based assays are optimised for working day hours (9am-5pm). In order to take repeated measures of bacterial growth curves up to 48-hours, I had to inoculate cultures as late as midnight, and as early as 5.30am. While more labour-intensive, I believe it opens up new opportunities to explore how monoclonal bacterial populations can generate so much variation depending on their population division history.

Given how population division history can have such a significant effect on persistence, this somewhat agrees with pre-existing theories such as 'persistence as stuff happens' (PaSH) (Johnson & Levin, 2013), and links in with epigenetics playing a role in persistence (Ryall *et al.*, 2012; Day, 2016). The fact that persistence did not consistently increase into stationary phase questions the literature's hard-set views on stationary phase conditions being a key driver behind persistence (Keren *et al.*, 2004; Shah *et al.*, 2006; Leszczynska *et al.*, 2013; Gefen *et al.*, 2014; Dewachter *et al.*, 2021). Additionally, even when I consistently identified persistence during stationary phase in *E. coli*, this was culturing time- and strain-dependent, showing how persistence can be variable even within stationary phase. It is also key to consider that these findings stem from research in optimal conditions with highly nutritious media (LB). My work provides the foundations to replicate these assays under more natural conditions, for example considering media which mimics conditions *in vivo*, and even looking at persistence in the context of mixed communities.

My thesis provides a comprehensive overview of persister cell formation in response to antibiotics from three antibiotic classes. While antibiotics are common-place in clinical settings, in terms of natural communities other bactericidal systems such as phage are abundant (Chevallereau *et al.*, 2022). Phage therapy is increasingly becoming considered as the next line of defence against bacteria, as mainstream Western antibiotics begin to fail (Loh & Leptihn, 2020). There is also emerging evidence that purported persistence-inducing mechanisms such as TA systems actually play a role in phage defence (LeRoux & Laub, 2022; S. Song & Wood, 2020). This means that more of our observations of phenotypic survival mechanisms in bacteria could potentially be further explained by the interaction between bacterial species and phage. Therefore, considering the potential role of phage in persistence will also be key in determining the evolutionary drivers of persistence.

Future directions

Further manipulation of growth curve dynamics, separating the age of culture from growth rate

Since population cell division history had such a significant effect on persistence in *P. aeruginosa*, future experiments could be used to exaggerate the number of divisions to test this relationship. One way to achieve this could be through using turbidostats; where bacteria can be maintained at constant optical densities, and hence growth rates, for extended periods of time. This would help separate the age of culture from inoculum density to test whether successive rounds of division increase or decrease persistence. It must be noted that a previous study showed how persistence can be eradicated by maintaining populations in exponential growth in a chemostat (similar to a turbidostat, but regulates nutrients instead of turbidity) (Wiuff *et al.*, 2005). This ability to eradicate persistence likely arose from creating uniform growth conditions, increasing the

homogeneity of the bacterial population, and thus decreasing heterogeneous traits such as persistence. Therefore, it would be interesting to see if altering the culture inoculum density had any effect on the subsequent persistence patterns after spending an extended duration of time in constant conditions.

An example experimental setup would be to inoculate cultures with different inoculum densities (Fig. 1A), grow them to the same optical density, set this optical density as a constant within the turbidostats, and measure persistence at set intervals over time (0h, 24h, and 48h of culturing within the turbidostat) (Fig. 1B). By comparing the two inoculum densities, it could then be established whether persistence patterns are explained by the number of divisions or the predictability of constant growth rates. It would also be important to conduct killing curves to measure persistence as these would also indicate how the population responds to antibiotics over time: whether persistence remains constant, the population becomes more/less tolerant to the antibiotic, or whether instances of stress divides occur.

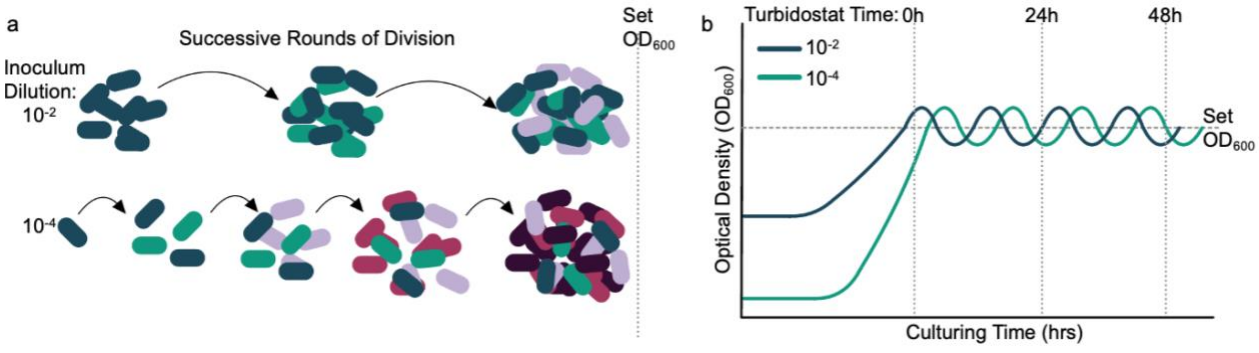


Figure 1. Separating age of culture from growth rate. A) Low density inoculum cultures (10^{-4}) need to go through more rounds of division to reach the same density as high-density inoculum cultures (10^{-2}). A greater number of divisions can create more heterogeneity within the population. B) Turbidostat setup to measure persistence in high versus low density inoculum cultures.

RNAseq analysis

To gain more insight into how the underlying gene expression profiles change within bacterial populations with different cell division histories, I collected RNA sequence data at different points within the bacterial growth curve (see Annex). My key questions were to ascertain: why do we get such different persister patterns in *P. aeruginosa* depending on inoculum density (Fig. 2), and can this be explained by differences in population RNA expression patterns? Transcriptomics studies have been used for many years to interrogate how persistence may be regulated at the population level (Shah *et al.*, 2006; Smith *et al.*, 2018; Deter *et al.*, 2021). The challenge with these population-level approaches arises when you consider how few cells within the bacterial population are actually in the persistence state. To try and address this, recent studies have used single cell RNA sequencing techniques with a specific focus on the mechanistic drivers behind persistence (Alnahhas & Dunlop, 2023). My approach focusses less on specific mechanisms and more on how patterns of heterogeneity change within the population. Analyses of RNA data might reveal gene-specific patterns that change within the population throughout the growth curve. Population-level gene expression may provide valuable information about the circumstances in which persistence is most likely to arise, which can give us further clues about the potential adaptive nature of persistence (Gardner *et al.*, 2007).

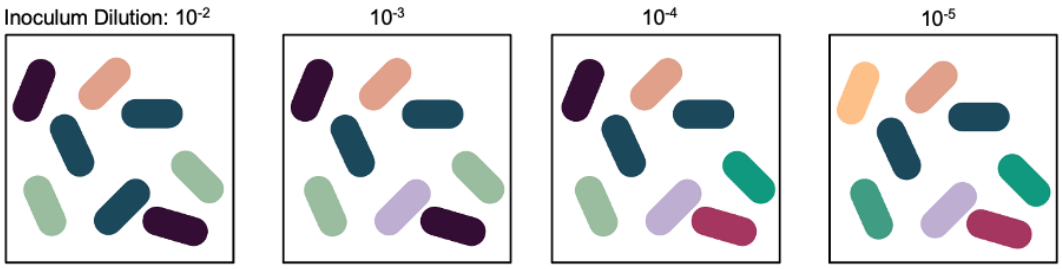


Figure 2. Predicted variation in gene expression profiles in exponential phase for cultures inoculated with incrementally lower cell densities. The colours indicate how cells may have different expression profiles depending on inoculum dilution.

Exploring the source of extracellular ATP

When I first measured ATP as another parameter to study population dynamics, my inspiration came from Mempin *et al.* (2013). This study showed how extracellular ATP could peak during exponential phase growth; something we had observed with persistence. The source of this extracellular ATP is contested, with some studies indicating it is due to cell lysis (Zhang *et al.*, 2007; Grygorczyk *et al.*, 2021; Ihssen *et al.*, 2021), whereas others have shown that ATP can be actively secreted/released from cells (Hironaka *et al.*, 2013; Mempin *et al.*, 2013). Nonetheless, further looking into ATP dynamics alongside persistence produced interesting results – with inoculum density dependent effects in *P. aeruginosa* and higher persistence when cells were metabolically more active (Chapter 2), and instances of pulses in extracellular ATP correlating with increases in persistence in an *E. coli* strain-dependent manner (Chapter 3). Alongside these observations, my interest in ATP as a purinergic signalling molecule was growing, especially after reading studies pointing towards its relevance *in vivo* from both the host and pathogen perspective either increasing the inflammatory response (Coutinho-Silva & Ojcius, 2012), or modulating virulence within the infecting population (Abbasian *et al.*, 2019).

To extend this ATP work further, I would first establish the origin of the changes in extracellular ATP patterns (Fig. 3). My initial questions include: 1) Are cells lysing to release ATP; 2) If not, is this ATP actively secreted? I would then be able to postulate further with questions: 1) What is triggering this ATP release; 2) Are there any potential selective benefits from this ATP release based on when it is occurring; 3) How does this link in, if at all, with persistence? Preliminary data from Professor Craig Maclean's lab showed a positive correlation between extracellular ATP and extracellular DNA which indicates that cell lysis is occurring (data not published). If ATP release

is occurring as a result of lysis, this may have implications on other lysis-related outcomes in bacteria such as the recently proposed concept of 'Sexual selection in bacteria' (Vos *et al.*, 2019). The authors highlight that established cellular systems such as competence, work to deliberately trigger cell lysis in non-competent cells, releasing useful cell contents which could potentially be used as public goods (Steinmoen *et al.*, 2002). By identifying the cause of extracellular ATP increase, this could open up more avenues to explore how persistence links in with the underlying mechanism.

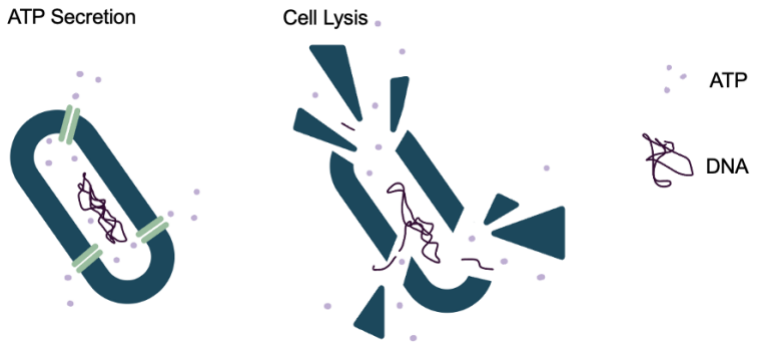


Figure 3. Determining whether increases in extracellular ATP were due to active secretion or cell lysis.

Meta-analysis across strains and species

Despite only assaying two species of bacteria, I found different patterns in persistence. Persistence in the PAO1 lab strain of *P. aeruginosa* was affected by cell division history, whereas cell division history had little to no effect on when persistence arose in two strains of *E. coli* (wildtype – MG1655, and high persister mutant – HipA7). To further explore why this may be the case, I would test more species of bacteria including Gram positive species such as *S. aureus* (Fig. 4). One of the limitations that I believe the literature falls into is carrying universal assumptions about persistence across studies that not only have different experimental setups, but also different species and strains of bacteria. Given the variation I saw across only two species, looking at more species using unified methodology will be essential. By comparing more species, there will also

be more opportunities to explore how underlying genetic features may help explain differences in persistence patterns. Given that we found variation in persistence patterns depending on cell division history and cell density-dependent effects, the expansion into more examples of bacteria could unlock further mysteries behind how persistence arises within bacterial populations.

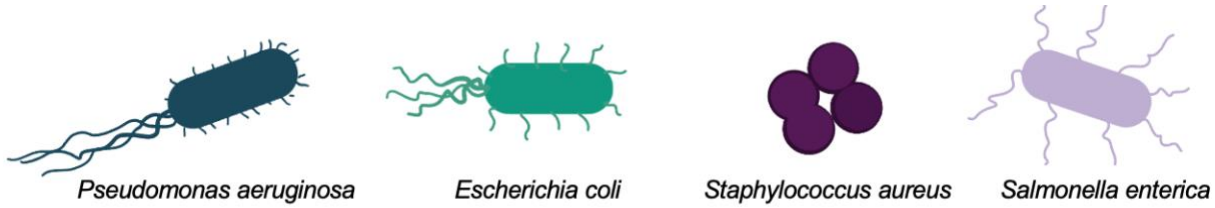


Figure 4. Expanding cross-species persistence comparisons.

Concluding Remark

The fact that bacterial populations can maintain a subpopulation of cells capable of surviving lethal doses of antibiotics is a wonderous phenomenon in its own right. Despite being discovered almost 80 years ago, the secrets of antibiotic persistence continue to challenge researchers, with further mysteries surrounding how and when persistence arises and what can be done, if anything, to stop it. Through simple measurements and manipulations of bacterial growth curves, I have shown how persistence isn't simply a 'stationary phase trait', and the variation brought about through successive rounds of division could play a role in determining when persistence arises. Bacterial populations are capable of bringing about incredible variation to combat a wide range of environmental conditions even though they reproduce clonally. It is therefore no wonder that these bacteria can produce phenotypes that enable them to survive a broad range of conditions. Whether this is regulated and deliberately mechanistically driven, and therefore adaptive, remains to be seen.

Bibliography

Abbasian, B., Shair, A., O'Gorman, D. B., Pena-Diaz, A. M., Brennan, L., Engelbrecht, K., Koenig, D. W., Reid, G., & Burton, J. P. (2019). Potential Role of Extracellular ATP Released by Bacteria in Bladder Infection and Contractility. *mSphere*, *4*(5), 10.1128/msphere.00439-19. https://doi.org/10.1128/msphere.00439-19

Abraham, E. P., & Chain, E. (1940). An Enzyme from Bacteria able to Destroy Penicillin. *Nature*, *146*(3713), Article 3713. https://doi.org/10.1038/146837a0

Ackermann, M. (2015). A functional perspective on phenotypic heterogeneity in microorganisms. *Nature Reviews Microbiology*, *13*(8), 497–508. https://doi.org/10.1038/nrmicro3491

Ahmed, F., Mirani, Z. A., Mirani, P. N., Imdad, M. J., Khan, F. Z., Khan, M. N., Khan, A. B., Li, Y., & Zhao, Y. (2022). *Pseudomonas aeruginosa* Response to Acidic Stress and Imipenem Resistance. *Applied Sciences*, *12*(16), Article 16. https://doi.org/10.3390/app12168357

Alnahhas, R. N., & Dunlop, M. J. (2023). Advances in linking single-cell bacterial stress response to population-level survival. *Current Opinion in Biotechnology*, *79*, 102885. https://doi.org/10.1016/j.copbio.2022.102885

Amato, S. M., Fazen, C. H., Henry, T. C., Mok, W. W. K., Orman, M. A., Sandvik, E. L., Volzing, K. G., & Brynildsen, M. P. (2014). The role of metabolism in bacterial persistence. *Frontiers in Microbiology*, *5*. https://doi.org/10.3389/fmicb.2014.00070

Andersson, D. I., Nicoloff, H., & Hjort, K. (2019). Mechanisms and clinical relevance of bacterial heteroresistance. *Nature Reviews Microbiology*, *17*(8), Article 8. https://doi.org/10.1038/s41579-019-0218-1

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, *48*(suppl_1), 5–16. https://doi.org/10.1093/jac/48.suppl_1.5

Aspridou, Z., & Koutsoumanis, K. P. (2015). Individual cell heterogeneity as variability source in population dynamics of microbial inactivation. *Food Microbiology*, *45*, 216–221. https://doi.org/10.1016/j.fm.2014.04.008

Atolia, E., Cesar, S., Arjes, H. A., Rajendram, M., Shi, H., Knapp, B. D., Khare, S., Aranda-Díaz, A., Lenski, R. E., & Huang, K. C. (2020). Environmental and Physiological Factors Affecting High-Throughput Measurements of Bacterial Growth. *mBio*, *11*(5). https://doi.org/10.1128/mBio.01378-20

Avery, S. V. (2006). Microbial cell individuality and the underlying sources of heterogeneity. *Nature Reviews Microbiology*, *4*(8), Article 8. https://doi.org/10.1038/nrmicro1460

Bakshi, S., Leoncini, E., Baker, C., Cañas-Duarte, S. J., Okumus, B., & Paulsson, J. (2021). Tracking bacterial lineages in complex and dynamic environments with applications for growth control and persistence. *Nature Microbiology*, *6*(6), 783–791. https://doi.org/10.1038/s41564-021-00900-4

Balaban, N. (2011). Persistence: Mechanisms for triggering and enhancing phenotypic variability. *Current Opinion in Genetics & Development*, *21*(6), 768–775. https://doi.org/10.1016/j.gde.2011.10.001

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., Brynildsen, M. P., Bumann, D., Camilli, A., Collins, J. J., Dehio, C., Fortune, S., Ghigo, J.-M., Hardt, W.-D., Harms, A., Heinemann, M., Hung, D. T., Jenal, U., Levin, B. R., … Zinkernagel, A. (2019). Definitions and guidelines for research on antibiotic persistence. *Nature Reviews Microbiology*, *17*(7), 441–448. https://doi.org/10.1038/s41579-019-0196-3

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L., & Leibler, S. (2004). Bacterial Persistence as a Phenotypic Switch. *Science*, *305*(5690), 1622–1625. https://doi.org/10.1126/science.1099390

Balasubramanian, D., Schneper, L., Merighi, M., Smith, R., Narasimhan, G., Lory, S., & Mathee, K. (2012). The Regulatory Repertoire of *Pseudomonas aeruginosa* AmpC ß-Lactamase Regulator AmpR Includes Virulence Genes. *PLOS ONE*, *7*(3), e34067. https://doi.org/10.1371/journal.pone.0034067

Baldwin, C. M., Lyseng-Williamson, K. A., & Keam, S. J. (2008). Meropenem. *Drugs*, *68*(6), 803–838. https://doi.org/10.2165/00003495-200868060-00006

Band, V. I., & Weiss, D. S. (2019). Heteroresistance: A cause of unexplained antibiotic treatment failure? *PLOS Pathogens*, *15*(6), e1007726. https://doi.org/10.1371/journal.ppat.1007726

Baquero, F., & Levin, B. R. (2021). Proximate and ultimate causes of the bactericidal action of antibiotics. *Nature Reviews Microbiology*, *19*(2), Article 2. https://doi.org/10.1038/s41579-020-00443-1

Bateson, P., & Laland, K. N. (2013). Tinbergen's four questions: An appreciation and an update. *Trends in Ecology & Evolution*, *28*(12), 712–718. https://doi.org/10.1016/j.tree.2013.09.013

Beal, J., Farny, N. G., Haddock-Angelli, T., Selvarajah, V., Baldwin, G. S., Buckley-Taylor, R., Gershater, M., Kiga, D., Marken, J., Sanchania, V., Sison, A., & Workman, C. T. (2020). Robust estimation of bacterial cell count from optical density. *Communications Biology*, *3*(1), 1–29. https://doi.org/10.1038/s42003-020-01127-5

Beckert, B., Turk, M., Czech, A., Berninghausen, O., Beckmann, R., Ignatova, Z., Plitzko, J. M., & Wilson, D. N. (2018). Structure of a hibernating 100S ribosome reveals an inactive conformation of the ribosomal protein S1. *Nature Microbiology*, *3*(10), Article 10. https://doi.org/10.1038/s41564-018-0237-0

Bigger, J. W. (1944). Treatment of Staphylococcal Infections with Penicillin by Intermittent Sterilisation. *Lancet*, 497–500.

Blázquez, J., Gómez-Gómez, J.-M., Oliver, A., Juan, C., Kapur, V., & Martín, S. (2006). PBP3 inhibition elicits adaptive responses in Pseudomonas aeruginosa. *Molecular Microbiology*, *62*(1), 84–99. https://doi.org/10.1111/j.1365-2958.2006.05366.x

Bollen, C., Louwagie, E., Verstraeten, N., Michiels, J., & Ruelens, P. (2023). Environmental, mechanistic and evolutionary landscape of antibiotic persistence. *EMBO Reports*, *24*(8), e57309. https://doi.org/10.15252/embr.202357309

Brandis, G., Larsson, J., & Elf, J. (2023). Antibiotic perseverance increases the risk of resistance development. *Proceedings of the National Academy of Sciences*, *120*(2), e2216216120. https://doi.org/10.1073/pnas.2216216120

Brauner, A., Fridman, O., Gefen, O., & Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nature Reviews Microbiology*, *14*(5), Article 5. https://doi.org/10.1038/nrmicro.2016.34

Breidenstein, E. B. M., de la Fuente-Núñez, C., & Hancock, R. E. W. (2011). *Pseudomonas aeruginosa*: All roads lead to resistance. *Trends in Microbiology*, *19*(8), 419–426. https://doi.org/10.1016/j.tim.2011.04.005

Bremer, H., & Dennis, P. P. (2008). Modulation of Chemical Composition and Other Parameters of the Cell at Different Exponential Growth Rates. *EcoSal Plus*, *3*(1). https://doi.org/10.1128/ecosal.5.2.3

Bruggeman, F. J., Planqué, R., Molenaar, D., & Teusink, B. (2020). Searching for principles of microbial physiology. *FEMS Microbiology Reviews*, *44*(6), 821–844. https://doi.org/10.1093/femsre/fuaa034

Carvalho, G., Balestrino, D., Forestier, C., & Mathias, J.-D. (2018). How do environment-dependent switching rates between susceptible and persister cells affect the dynamics of biofilms faced with antibiotics? *Npj Biofilms and Microbiomes*, *4*(1), Article 1. https://doi.org/10.1038/s41522-018-0049-2

Chevallereau, A., Pons, B. J., van Houte, S., & Westra, E. R. (2022). Interactions between bacterial and phage communities in natural environments. *Nature Reviews Microbiology*, *20*(1), Article 1. https://doi.org/10.1038/s41579-021-00602-y

Clarelli, F., Palmer, A., Singh, B., Storflor, M., Lauksund, S., Cohen, T., Abel, S., & Wiesch, P. A. zur. (2020). Drug-target binding quantitatively predicts optimal antibiotic dose levels in quinolones. *PLOS Computational Biology*, *16*(8), e1008106. https://doi.org/10.1371/journal.pcbi.1008106

Coates, A. R., Halls, G., & Hu, Y. (2011). Novel classes of antibiotics or more of the same? *British Journal of Pharmacology*, *163*(1), 184–194. https://doi.org/10.1111/j.1476-5381.2011.01250.x

Conlon, B. P., Rowe, S. E., Gandt, A. B., Nuxoll, A. S., Donegan, N. P., Zalis, E. A., Clair, G., Adkins, J. N., Cheung, A. L., & Lewis, K. (2016). Persister formation in *Staphylococcus aureus* is

associated with ATP depletion. *Nature Microbiology*, *1*(5), 1–7. https://doi.org/10.1038/nmicrobiol.2016.51

Corchero, J. L., Cubarsí, R., Vila, P., Arís, A., & Villaverde, A. (2001). Cell lysis in *Escherichia coli* cultures stimulates growth and biosynthesis of recombinant proteins in surviving cells. *Microbiological Research*, *156*(1), 13–18. https://doi.org/10.1078/0944-5013-00066

Coutinho-Silva, R., & Ojcius, D. M. (2012). Role of extracellular nucleotides in the immune response against intracellular bacteria and protozoan parasites. *Microbes and Infection*, *14*(14), 1271–1277. https://doi.org/10.1016/j.micinf.2012.05.009

Darwin, C., Murray, J., William Clowes and Sons, & Evans, B. &. (1859). *On the origin of species by means of natural selection, or, The preservation of favoured races in the struggle for life* (pp. 1–556). John Murray, Albemarle Street. https://doi.org/10.5962/bhl.title.82303

Davies, S. E. F., Choudhary, D., Uphoff, S., Ghoul, M., & Griffin, A. S. (submitted). *High growth rates promote persister cell formation in Pseudomonas aeruginosa*.

Day, T. (2016). Interpreting phenotypic antibiotic tolerance and persister cells as evolution via epigenetic inheritance. *Molecular Ecology*, *25*(8), 1869–1882. https://doi.org/10.1111/mec.13603

Deter, H. S., Hossain, T., & Butzin, N. C. (2021). Antibiotic tolerance is associated with a broad and complex transcriptional response in *E. coli*. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-85509-7

Dewachter, L., Bollen, C., Wilmaerts, D., Louwagie, E., Herpels, P., Matthay, P., Khodaparast, L., Khodaparast, L., Rousseau, F., Schymkowitz, J., & Michiels, J. (2021). The Dynamic Transition of Persistence toward the Viable but Nonculturable State during Stationary Phase Is Driven by Protein Aggregation. *mBio*, *12*(4), e0070321. https://doi.org/10.1128/mBio.00703-21

Dewachter, L., Fauvart, M., & Michiels, J. (2019). Bacterial Heterogeneity and Antibiotic Survival: Understanding and Combatting Persistence and Heteroresistance. *Molecular Cell*, *76*(2), 255–267. https://doi.org/10.1016/j.molcel.2019.09.028

Dhanyamraju, P. K., Schell, T. D., Amin, S., & Robertson, G. P. (2022). Drug-Tolerant Persister Cells in Cancer Therapy Resistance. *Cancer Research*, *82*(14), 2503–2514. https://doi.org/10.1158/0008-5472.CAN-21-3844

Dhingra, S., Rahman, N. A. A., Peile, E., Rahman, M., Sartelli, M., Hassali, M. A., Islam, T., Islam, S., & Haque, M. (2020). Microbial Resistance Movements: An Overview of Global Public Health Threats Posed by Antimicrobial Resistance, and How Best to Counter. *Frontiers in Public Health*, *8*. https://www.frontiersin.org/articles/10.3389/fpubh.2020.535668

Drenkard, E. (2003). Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes and Infection*, *5*(13), 1213–1219. https://doi.org/10.1016/j.micinf.2003.08.009

Dworkin, J., & Harwood, C. S. (2022). Metabolic Reprogramming and Longevity in Quiescence. *Annual Review of Microbiology*, *76*(1), 91–111. https://doi.org/10.1146/annurev-micro-041320-111014

Eisenreich, W., Rudel, T., Heesemann, J., & Goebel, W. (2021). Persistence of Intracellular Bacterial Pathogens—With a Focus on the Metabolic Perspective. *Frontiers in Cellular and Infection Microbiology*, *10*, 615450. https://doi.org/10.3389/fcimb.2020.615450

El-Mosalamy, H., Salman, T. M., Ashmawey, A. M., & Osama, N. (2012). Role of chronic *E. coli* infection in the process of bladder cancer- an experimental study. *Infectious Agents and Cancer*, *7*(1), 19. https://doi.org/10.1186/1750-9378-7-19

Fernandez-García, L., Blasco, L., Trastoy, R., García-Contreras, R., Wood, T. K., & Tomás, M. (2018). Quorum Sensing Systems and Persistence. In Pallaval Veera Bramhachari (Ed.), *Implication of Quorum Sensing System in Biofilm Formation and Virulence* (pp. 17–27). Springer. https://doi.org/10.1007/978-981-13-2429-1_3

Fisher, R. A., Gollan, B., & Helaine, S. (2017). Persistent bacterial infections and persister cells. *Nature Reviews Microbiology*, *15*(8), 453–464. https://doi.org/10.1038/nrmicro.2017.42

Fleming, A. (1941). Penicillin. *British Medical Journal*, *2*(4210), 386.

Fraikin, N., Goormaghtigh, F., & Van Melderen, L. (2020). Type II Toxin-Antitoxin Systems: Evolution and Revolutions. *Journal of Bacteriology*, *202*(7), 10.1128/jb.00763-19. https://doi.org/10.1128/jb.00763-19

Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., & Balaban, N. Q. (2014). Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature*, *513*(7518), 418–421. https://doi.org/10.1038/nature13469

Frost, I., Smith, W. P. J., Mitri, S., Millan, A. S., Davit, Y., Osborne, J. M., Pitt-Francis, J. M., MacLean, R. C., & Foster, K. R. (2018). Cooperation, competition and antibiotic resistance in bacterial colonies. *The ISME Journal*, *12*(6), 1582–1593. https://doi.org/10.1038/s41396-018-0090-4

Gardner, A., West, S. A., & Griffin, A. S. (2007). Is Bacterial Persistence a Social Trait? *PLOS ONE*, *2*(8), e752. https://doi.org/10.1371/journal.pone.0000752

Gefen, O., & Balaban, N. Q. (2009). The importance of being persistent: Heterogeneity of bacterial populations under antibiotic stress. *FEMS Microbiology Reviews*, *33*(4), 704–717. https://doi.org/10.1111/j.1574-6976.2008.00156.x

Gefen, O., Fridman, O., Ronin, I., & Balaban, N. Q. (2014). Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. *Proceedings of the National Academy of Sciences*, *111*(1), 556–561. https://doi.org/10.1073/pnas.1314114111

Germain, E., Castro-Roa, D., Zenkin, N., & Gerdes, K. (2013). Molecular Mechanism of Bacterial Persistence by HipA. *Molecular Cell*, *52*(2), 248–254. https://doi.org/10.1016/j.molcel.2013.08.045

Ghoul, M., Andersen, S. B., Marvig, R. L., Johansen, H. K., Jelsbak, L., Molin, S., Perron, G., & Griffin, A. S. (2023). Long-term evolution of antibiotic tolerance in *Pseudomonas aeruginosa* lung infections. *Evolution Letters*, *7*(6), 389–400. https://doi.org/10.1093/evlett/qrad034

Ghoul, M., West, S. A., Johansen, H. K., Molin, S., Harrison, O. B., Maiden, M. C. J., Jelsbak, L., Bruce, J. B., & Griffin, A. S. (2015). Bacteriocin-mediated competition in cystic fibrosis lung infections.

Proceedings of the Royal Society B: Biological Sciences, *282*(1814), 20150972. https://doi.org/10.1098/rspb.2015.0972

Ghoul, Melanie. (2014). *Social dynamics in natural populations of Pseudomonas aeruginosa [PhD thesis]*. Oxford University, UK.

Goffic, F. L., Capmau, M.-L., Tangy, F., & Baillarge, M. (1979). Mechanism of Action of Aminoglycoside Antibiotics. *European Journal of Biochemistry*, *102*(1), 73–81. https://doi.org/10.1111/j.1432-1033.1979.tb06264.x

Gohara, D. W., & Yap, M.-N. F. (2018). Survival of the drowsiest: The hibernating 100S ribosome in bacterial stress management. *Current Genetics*, *64*(4), 753–760. https://doi.org/10.1007/s00294-017-0796-2

Goode, O., Smith, A., Łapińska, U., Bamford, R., Kahveci, Z., Glover, G., Attrill, E., Carr, A., Metz, J., & Pagliara, S. (2021). Heterologous Protein Expression Favors the Formation of Protein Aggregates in Persister and Viable but Nonculturable Bacteria. *ACS Infectious Diseases*, *7*(6), 1848–1858. https://doi.org/10.1021/acsinfecdis.1c00154

Goormaghtigh, F., Fraikin, N., Putrinš, M., Hallaert, T., Hauryliuk, V., Garcia-Pino, A., Sjödin, A., Kasvandik, S., Udekwu, K., Tenson, T., Kaldalu, N., & Melderen, L. V. (2018). Reassessing the Role of Type II Toxin-Antitoxin Systems in Formation of *Escherichia coli* Type II Persister Cells. *mBio*, *9*(3). https://doi.org/10.1128/mBio.00640-18

Goormaghtigh, F., & Melderen, L. V. (2019). Single-cell imaging and characterization of *Escherichia coli* persister cells to ofloxacin in exponential cultures. *Science Advances*, *5*(6), eaav9462. https://doi.org/10.1126/sciadv.aav9462

Granato, E. T., Meiller-Legrand, T. A., & Foster, K. R. (2019). The Evolution and Ecology of Bacterial Warfare. *Current Biology*, *29*(11), R521–R537. https://doi.org/10.1016/j.cub.2019.04.024

Grassi, L., Di Luca, M., Maisetta, G., Rinaldi, A. C., Esin, S., Trampuz, A., & Batoni, G. (2017). Generation of Persister Cells of *Pseudomonas aeruginosa* and *Staphylococcus aureus* by

Chemical Treatment and Evaluation of Their Susceptibility to Membrane-Targeting Agents. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01917

Grygorczyk, R., Boudreault, F., Ponomarchuk, O., Tan, J. J., Furuya, K., Goldgewicht, J., Kenfack, F. D., & Yu, F. (2021). Lytic Release of Cellular ATP: Physiological Relevance and Therapeutic Applications. *Life*, *11*(7), 700. https://doi.org/10.3390/life11070700

Hannan, T. J., Mysorekar, I. U., Hung, C. S., Isaacson-Schmid, M. L., & Hultgren, S. J. (2010). Early Severe Inflammatory Responses to Uropathogenic *E. coli* Predispose to Chronic and Recurrent Urinary Tract Infection. *PLOS Pathogens*, *6*(8), e1001042. https://doi.org/10.1371/journal.ppat.1001042

Harms, A., Fino, C., Sørensen, M. A., Semsey, S., & Gerdes, K. (2017). Prophages and Growth Dynamics Confound Experimental Results with Antibiotic-Tolerant Persister Cells. *mBio*, *8*(6). https://doi.org/10.1128/mBio.01964-17

Harms, A., Maisonneuve, E., & Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science (New York, N.Y.)*, *354*(6318), aaf4268. https://doi.org/10.1126/science.aaf4268

Hironaka, I., Iwase, T., Sugimoto, S., Okuda, K., Tajima, A., Yanaga, K., & Mizunoe, Y. (2013). Glucose Triggers ATP Secretion from Bacteria in a Growth-Phase-Dependent Manner. *Applied and Environmental Microbiology*, *79*(7), 2328–2335. https://doi.org/10.1128/AEM.03871-12

Hobbie, J., & Hobbie, E. (2013). Microbes in nature are limited by carbon and energy: The starving-survival lifestyle in soil and consequences for estimating microbial rates. *Frontiers in Microbiology*, *4*. https://www.frontiersin.org/articles/10.3389/fmicb.2013.00324

Høiby, N., Frederiksen, B., & Pressler, T. (2005). Eradication of early *Pseudomonas aeruginosa* infection. *Journal of Cystic Fibrosis*, *4*, 49–54. https://doi.org/10.1016/j.jcf.2005.05.018

Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: Past, present and future. *Current Opinion in Microbiology*, *51*, 72–80. https://doi.org/10.1016/j.mib.2019.10.008

Iavicoli, I., Fontana, L., Agathokleous, E., Santocono, C., Russo, F., Vetrani, I., Fedele, M., & Calabrese, E. J. (2021). Hormetic dose responses induced by antibiotics in bacteria: A phantom menace to be thoroughly evaluated to address the environmental risk and tackle the antibiotic resistance phenomenon. *Science of The Total Environment*, *798*, 149255. https://doi.org/10.1016/j.scitotenv.2021.149255

Ihssen, J., Jovanovic, N., Sirec, T., & Spitz, U. (2021). Real-time monitoring of extracellular ATP in bacterial cultures using thermostable luciferase. *PLoS ONE*, *16*(1), e0244200. https://doi.org/10.1371/journal.pone.0244200

Jaishankar, J., & Srivastava, P. (2017). Molecular Basis of Stationary Phase Survival and Applications. *Frontiers in Microbiology*, *8*, 2000. https://doi.org/10.3389/fmicb.2017.02000

Johnson, P. J. T., & Levin, B. R. (2013). Pharmacodynamics, Population Dynamics, and the Evolution of Persistence in *Staphylococcus aureus*. *PLOS Genetics*, *9*(1), e1003123. https://doi.org/10.1371/journal.pgen.1003123

Jones, E. C., & Uphoff, S. (2021). Single-molecule imaging of LexA degradation in *Escherichia coli* elucidates regulatory mechanisms and heterogeneity of the SOS response. *Nature Microbiology*, *6*(8), Article 8. https://doi.org/10.1038/s41564-021-00930-y

Jurėnas, D., Fraikin, N., Goormaghtigh, F., & Van Melderen, L. (2022). Biology and evolution of bacterial toxin–antitoxin systems. *Nature Reviews Microbiology*, *20*(6), Article 6. https://doi.org/10.1038/s41579-021-00661-1

Kaldalu, N., Hauryliuk, V., & Tenson, T. (2016). Persisters—As elusive as ever. *Applied Microbiology and Biotechnology*, *100*(15), 6545–6553. https://doi.org/10.1007/s00253-016-7648-8

Kaplan, Y., Reich, S., Oster, E., Maoz, S., Levin-Reisman, I., Ronin, I., Gefen, O., Agam, O., & Balaban, N. Q. (2021). Observation of universal ageing dynamics in antibiotic persistence. *Nature*, *600*(7888), Article 7888. https://doi.org/10.1038/s41586-021-04114-w

Kaspy, I., Rotem, E., Weiss, N., Ronin, I., Balaban, N. Q., & Glaser, G. (2013). HipA-mediated antibiotic persistence via phosphorylation of the glutamyl-tRNA-synthetase. *Nature Communications*, *4*(1), Article 1. https://doi.org/10.1038/ncomms4001

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., & Lewis, K. (2004). Persister cells and tolerance to antimicrobials. *FEMS Microbiology Letters*, *230*(1), 13–18. https://doi.org/10.1016/S0378-1097(03)00856-5

Kerr, R., Jabbari, S., & Johnston, I. G. (2019). Intracellular Energy Variability Modulates Cellular Decision-Making Capacity. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-56587-5

Khare, A., & Tavazoie, S. (2020). Extreme Antibiotic Persistence via Heterogeneity-Generating Mutations Targeting Translation. *mSystems*, *5*(1). https://doi.org/10.1128/mSystems.00847-19

Kim, H., Kim, J. H., Cho, H., & Ko, K. S. (2022). Overexpression of a DNA Methyltransferase Increases Persister Cell Formation in Acinetobacter baumannii. *Microbiology Spectrum*, *10*(6), e02655-22. https://doi.org/10.1128/spectrum.02655-22

Kim, J.-S., Yamasaki, R., Song, S., Zhang, W., & Wood, T. K. (2018). Single cell observations show persister cells wake based on ribosome content. *Environmental Microbiology*, *20*(6), 2085–2098. https://doi.org/10.1111/1462-2920.14093

Kim, Y., & Wood, T. K. (2010). Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*. *Biochemical and Biophysical Research Communications*, *391*(1), 209–213. https://doi.org/10.1016/j.bbrc.2009.11.033

Koeva, M., Gutu, A. D., Hebert, W., Wager, J. D., Yonker, L. M., O'Toole, G. A., Ausubel, F. M., Moskowitz, S. M., & Joseph-McCarthy, D. (2017). An Antipersister Strategy for Treatment of Chronic *Pseudomonas aeruginosa* Infections. *Antimicrobial Agents and Chemotherapy*, *61*(12), 10.1128/aac.00987-17. https://doi.org/10.1128/aac.00987-17

Kohanski, M. A., Dwyer, D. J., & Collins, J. J. (2010). How antibiotics kill bacteria: From targets to networks. *Nature Reviews Microbiology*, *8*(6), Article 6. https://doi.org/10.1038/nrmicro2333

Kolter, R., Siegele, D. A., & Tormo, A. (1993). The Stationary Phase of the Bacterial Life Cycle. *Annual Review of Microbiology*, *47*(1), 855–874. https://doi.org/10.1146/annurev.mi.47.100193.004231

Kong, K.-F., Jayawardena, S. R., Indulkar, S. D., del Puerto, A., Koh, C.-L., Høiby, N., & Mathee, K. (2005). *Pseudomonas aeruginosa* AmpR Is a Global Transcriptional Factor That Regulates Expression of AmpC and PoxB β-Lactamases, Proteases, Quorum Sensing, and Other Virulence Factors. *Antimicrobial Agents and Chemotherapy*, *49*(11), 4567–4575. https://doi.org/10.1128/aac.49.11.4567-4575.2005

Korch, S. B., Henderson, T. A., & Hill, T. M. (2003). Characterization of the hipA7 allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. *Molecular Microbiology*, *50*(4), 1199–1213. https://doi.org/10.1046/j.1365-2958.2003.03779.x

Kussell, E., & Leibler, S. (2005). Phenotypic diversity, population growth, and information in fluctuating environments. *Science (New York, N.Y.)*, *309*(5743), 2075–2078. https://doi.org/10.1126/science.1114383

Łapińska, U., Voliotis, M., Lee, K. K., Campey, A., Stone, M. R. L., Tuck, B., Phetsang, W., Zhang, B., Tsaneva-Atanasova, K., Blaskovich, M. A., & Pagliara, S. (2022). Fast bacterial growth reduces antibiotic accumulation and efficacy. *eLife*, *11*, e74062. https://doi.org/10.7554/eLife.74062

LeBel, M. (1988). Ciprofloxacin: Chemistry, Mechanism of Action, Resistance, Antimicrobial Spectrum, Pharmacokinetics, Clinical Trials, and Adverse Reactions. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, *8*(1), 3–30. https://doi.org/10.1002/j.1875-9114.1988.tb04058.x

Lee, A. J., Wang, S., Meredith, H. R., Zhuang, B., Dai, Z., & You, L. (2018). Robust, linear correlations between growth rates and β-lactam–mediated lysis rates. *Proceedings of the National Academy of Sciences*, *115*(16), 4069–4074. https://doi.org/10.1073/pnas.1719504115

LeRoux, M., & Laub, M. T. (2022). Toxin-Antitoxin Systems as Phage Defense Elements. *Annual Review of Microbiology*, *76*(1), 21–43. https://doi.org/10.1146/annurev-micro-020722-013730

Leszczynska, D., Matuszewska, E., Kuczynska-Wisnik, D., Furmanek-Blaszk, B., & Laskowska, E. (2013). The Formation of Persister Cells in Stationary-Phase Cultures of *Escherichia coli* Is Associated with the Aggregation of Endogenous Proteins. *PLOS ONE*, *8*(1), e54737. https://doi.org/10.1371/journal.pone.0054737

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., & Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science*, *355*(6327), 826–830. https://doi.org/10.1126/science.aaj2191

Lewis, K. (2007). Persister cells, dormancy and infectious disease. *Nature Reviews. Microbiology*, *5*(1), 48–56. https://doi.org/10.1038/nrmicro1557

Li, W.-R., Zhang, Z.-Q., Liao, K., Wang, B.-B., Liu, H.-Z., Shi, Q.-S., Huang, X.-B., & Xie, X.-B. (2022). *Pseudomonas aeruginosa* heteroresistance to levofloxacin caused by upregulated expression of essential genes for DNA replication and repair. *Frontiers in Microbiology*, *13*. https://www.frontiersin.org/articles/10.3389/fmicb.2022.1105921

Li, X., Shi, J., Sun, H., & Lin, Z. (2020). Hormetic dose-dependent response about typical antibiotics and their mixtures on plasmid conjugative transfer of *Escherichia coli* and its relationship with toxic effects on growth. *Ecotoxicology and Environmental Safety*, *205*, 111300. https://doi.org/10.1016/j.ecoenv.2020.111300

Lidstrom, M. E., & Konopka, M. C. (2010). The role of physiological heterogeneity in microbial population behavior. *Nature Chemical Biology*, *6*(10), 705–712. https://doi.org/10.1038/nchembio.436

Lin, J., Xu, C., Fang, R., Cao, J., Zhang, X., Zhao, Y., Dong, G., Sun, Y., & Zhou, T. (2019). Resistance and Heteroresistance to Colistin in *Pseudomonas aeruginosa* Isolates from Wenzhou, China. *Antimicrobial Agents and Chemotherapy*, *63*(10), 10.1128/aac.00556-19. https://doi.org/10.1128/aac.00556-19

Liu, S., Huang, Y., Jensen, S., Laman, P., Kramer, G., Zaat, S. A. J., & Brul, S. (2023). *Molecular physiological characterization of the dynamics of persister formation in Staphylococcus aureus* (p. 2023.06.21.545909). bioRxiv. https://doi.org/10.1101/2023.06.21.545909

Loh, B., & Leptihn, S. (2020). A Call For a Multidisciplinary Future of Phage Therapy to Combat Multi-drug Resistant Bacterial Infections. *Infectious Microbes & Diseases*, *2*(1), 1. https://doi.org/10.1097/IM9.0000000000000018

Lopatkin, A. J., Stokes, J. M., Zheng, E. J., Yang, J. H., Takahashi, M. K., You, L., & Collins, J. J. (2019). Bacterial metabolic state more accurately predicts antibiotic lethality than growth rate. *Nature Microbiology*, *4*(12), 2109–2117. https://doi.org/10.1038/s41564-019-0536-0

Lu, Y., Liu, Y., Zhou, C., Liu, Y., Long, Y., Lin, D., Xiong, R., Xiao, Q., Huang, B., & Chen, C. (2022). Quorum sensing regulates heteroresistance in *Pseudomonas aeruginosa*. *Frontiers in Microbiology*, *13*, 1017707. https://doi.org/10.3389/fmicb.2022.1017707

Maisonneuve, E., Shakespeare, L. J., Jørgensen, M. G., & Gerdes, K. (2011). Bacterial persistence by RNA endonucleases. *Proceedings of the National Academy of Sciences*, *108*(32), 13206–13211. https://doi.org/10.1073/pnas.1100186108

Mason, M. M. (1935). A Comparison of the Maximal Growth Rates of Various Bacteria under Optimal Conditions. *Journal of Bacteriology*, *29*(2), 103–110. https://doi.org/10.1128/jb.29.2.103-110.1935

Medaney, F., Dimitriu, T., Ellis, R. J., & Raymond, B. (2016). Live to cheat another day: Bacterial dormancy facilitates the social exploitation of β-lactamases. *The ISME Journal*, *10*(3), Article 3. https://doi.org/10.1038/ismej.2015.154

Mempin, R., Tran, H., Chen, C., Gong, H., Kim Ho, K., & Lu, S. (2013). Release of extracellular ATP by bacteria during growth. *BMC Microbiology*, *13*(1), 301. https://doi.org/10.1186/1471-2180-13-301

Michaux, C., Ronneau, S., Giorgio, R. T., & Helaine, S. (2022). Antibiotic tolerance and persistence have distinct fitness trade-offs. *PLOS Pathogens*, *18*(11), e1010963. https://doi.org/10.1371/journal.ppat.1010963

Miller, C., Thomsen, L. E., Gaggero, C., Mosseri, R., Ingmer, H., & Cohen, S. N. (2004). SOS Response Induction by ß-Lactams and Bacterial Defense Against Antibiotic Lethality. *Science*, *305*(5690), 1629–1631. https://doi.org/10.1126/science.1101630

Mirsalehian, A., Kalantar-Neyestanaki, D., Nourijelyani, K., Asadollahi, K., Taherikalani, M., Emaneini, M., & Jabalameli, F. (2014). Detection of AmpC-β-lactamases producing isolates among carbapenem resistant P. aeruginosa isolated from burn patient. *Iranian Journal of Microbiology*, *6*(5), 306–310.

Mojsoska, B., Cameron, D. R., Bartell, J. A., Haagensen, J. A. J., Sommer, L. M., Lewis, K., Molin, S., & Johansen, H. K. (2019). The high persister phenotype of *Pseudomonas aeruginosa* is associated with increased fitness and persistence in cystic fibrosis airways. *bioRxiv*, 561589. https://doi.org/10.1101/561589

Möker, N., Dean, C. R., & Tao, J. (2010). *Pseudomonas aeruginosa* Increases Formation of Multidrug-Tolerant Persister Cells in Response to Quorum-Sensing Signaling Molecules. *Journal of Bacteriology*, *192*(7), 1946–1955. https://doi.org/10.1128/jb.01231-09

Morita, R. Y. (1988). Bioavailability of energy and its relationship to growth and starvation survival in nature. *Canadian Journal of Microbiology*, *34*(4), 436–441. https://doi.org/10.1139/m88-076

Moyed, H. S., & Bertrand, K. P. (1983). hipA, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *Journal of Bacteriology*, *155*(2), 768–775. https://doi.org/10.1128/jb.155.2.768-775.1983

Mulcahy, L. R., Burns, J. L., Lory, S., & Lewis, K. (2010). Emergence of *Pseudomonas aeruginosa* Strains Producing High Levels of Persister Cells in Patients with Cystic Fibrosis. *Journal of Bacteriology*, *192*(23), 6191–6199. https://doi.org/10.1128/JB.01651-09

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., Britigan, B. E., & Singh, P. K. (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science (New York, N.Y.)*, *334*(6058), 982–986. https://doi.org/10.1126/science.1211037

Noor, R. (2015). Mechanism to control the cell lysis and the cell survival strategy in stationary phase under heat stress. *SpringerPlus*, *4*(1), 599. https://doi.org/10.1186/s40064-015-1415-7

Nothias, L.-F., Knight, R., & Dorrestein, P. C. (2016). Antibiotic discovery is a walk in the park. *Proceedings of the National Academy of Sciences*, *113*(51), 14477–14479. https://doi.org/10.1073/pnas.1618221114

Orman, M. A., & Brynildsen, M. P. (2013). Dormancy Is Not Necessary or Sufficient for Bacterial Persistence. *Antimicrobial Agents and Chemotherapy*, *57*(7), 3230–3239. https://doi.org/10.1128/AAC.00243-13

Pagès, J.-M., James, C. E., & Winterhalter, M. (2008). The porin and the permeating antibiotic: A selective diffusion barrier in Gram-negative bacteria. *Nature Reviews Microbiology*, *6*(12), 893–903. https://doi.org/10.1038/nrmicro1994

Pang, Z., Raudonis, R., Glick, B. R., Lin, T.-J., & Cheng, Z. (2019). Antibiotic resistance in *Pseudomonas aeruginosa*: Mechanisms and alternative therapeutic strategies. *Biotechnology Advances*, *37*(1), 177–192. https://doi.org/10.1016/j.biotechadv.2018.11.013

Pembrey, R. S., Marshall, K. C., & Schneider, R. P. (1999). Cell Surface Analysis Techniques: What Do Cell Preparation Protocols Do to Cell Surface Properties? *Applied and Environmental Microbiology*, *65*(7), 2877–2894.

Pereira, C., Larsson, J., Hjort, K., Elf, J., & Andersson, D. I. (2021). The highly dynamic nature of bacterial heteroresistance impairs its clinical detection. *Communications Biology*, *4*(1), Article 1. https://doi.org/10.1038/s42003-021-02052-x

Philippi, T., & Seger, J. (1989). Hedging one's evolutionary bets, revisited. *Trends in Ecology & Evolution*, *4*(2), 41–44. https://doi.org/10.1016/0169-5347(89)90138-9

Pontes, M. H., & Groisman, E. A. (2019). Slow growth determines nonheritable antibiotic resistance in *Salmonella enterica*. *Science Signaling*, *12*(592). https://doi.org/10.1126/scisignal.aax3938

Pontes, M. H., & Groisman, E. A. (2020). A Physiological Basis for Nonheritable Antibiotic Resistance. *mBio*, *11*(3). https://doi.org/10.1128/mBio.00817-20

Pu, Y., Li, Y., Jin, X., Tian, T., Ma, Q., Zhao, Z., Lin, S., Chen, Z., Li, B., Yao, G., Leake, M. C., Lo, C.-J., & Bai, F. (2019). ATP-Dependent Dynamic Protein Aggregation Regulates Bacterial Dormancy Depth Critical for Antibiotic Tolerance. *Molecular Cell*, *73*(1), 143-156.e4. https://doi.org/10.1016/j.molcel.2018.10.022

Pu, Y., Zhao, Z., Li, Y., Zou, J., Ma, Q., Zhao, Y., Ke, Y., Zhu, Y., Chen, H., Baker, M. A. B., Ge, H., Sun, Y., Xie, X. S., & Bai, F. (2016). Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells. *Molecular Cell*, *62*(2), 284–294. https://doi.org/10.1016/j.molcel.2016.03.035

Rammelkamp, C. H., & Maxon, T. (1942). Resistance of *Staphylococcus aureus* to the Action of Penicillin. *Proceedings of the Society for Experimental Biology and Medicine*, *51*(3), 386–389. https://doi.org/10.3181/00379727-51-13986

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology*, *4*(3), 482–501. https://doi.org/10.3934/microbiol.2018.3.482

Ronneau, S., & Helaine, S. (2019). Clarifying the Link between Toxin–Antitoxin Modules and Bacterial Persistence. *Journal of Molecular Biology*, *431*(18), 3462–3471. https://doi.org/10.1016/j.jmb.2019.03.019

Rossi, E., La Rosa, R., Bartell, J. A., Marvig, R. L., Haagensen, J. A. J., Sommer, L. M., Molin, S., & Johansen, H. K. (2021). *Pseudomonas aeruginosa* adaptation and evolution in patients with cystic fibrosis. *Nature Reviews Microbiology*, *19*(5), Article 5. https://doi.org/10.1038/s41579-020-00477-5

Ryall, B., Eydallin, G., & Ferenci, T. (2012). Culture History and Population Heterogeneity as Determinants of Bacterial Adaptation: The Adaptomics of a Single Environmental Transition.

Microbiology and Molecular Biology Reviews, *76*(3), 597–625. https://doi.org/10.1128/MMBR.05028-11

Santi, I., Manfredi, P., Maffei, E., Egli, A., & Jenal, U. (2021). Evolution of Antibiotic Tolerance Shapes Resistance Development in Chronic *Pseudomonas aeruginosa* Infections. *mBio*, *12*(1), 10.1128/mbio.03482-20. https://doi.org/10.1128/mbio.03482-20

Schröter, L., & Dersch, P. (2019). Phenotypic Diversification of Microbial Pathogens—Cooperating and Preparing for the Future. *Journal of Molecular Biology*, *431*(23), 4645–4655. https://doi.org/10.1016/j.jmb.2019.06.024

Shah, D., Zhang, Z., Khodursky, A. B., Kaldalu, N., Kurg, K., & Lewis, K. (2006). Persisters: A distinct physiological state of *E. coli*. *BMC Microbiology*, *6*(1), 53. https://doi.org/10.1186/1471-2180-6-53

Sherman, E. X., Wozniak, J. E., & Weiss, D. S. (2019). Methods to Evaluate Colistin Heteroresistance in *Acinetobacter baumannii*. *Methods in Molecular Biology (Clifton, N.J.)*, *1946*, 39–50. https://doi.org/10.1007/978-1-4939-9118-1_4

Shi, X., & Zarkan, A. (2022). Bacterial survivors: Evaluating the mechanisms of antibiotic persistence. *Microbiology*, *168*(12). https://doi.org/10.1099/mic.0.001266

Silver, L. L. (2011). Challenges of Antibacterial Discovery. *Clinical Microbiology Reviews*, *24*(1), 71–109. https://doi.org/10.1128/CMR.00030-10

Sliusarenko, O., Heinritz, J., Emonet, T., & Jacobs-Wagner, C. (2011). High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. *Molecular Microbiology*, *80*(3), 612–627. https://doi.org/10.1111/j.1365-2958.2011.07579.x

Smith, A., Kaczmar, A., Bamford, R. A., Smith, C., Frustaci, S., Kovacs-Simon, A., O'Neill, P., Moore, K., Paszkiewicz, K., Titball, R. W., & Pagliara, S. (2018). The Culture Environment Influences Both Gene Regulation and Phenotypic Heterogeneity in *Escherichia coli*. *Frontiers in Microbiology*, *9*. https://www.frontiersin.org/article/10.3389/fmicb.2018.01739

Soares, A., Roussel, V., Pestel-Caron, M., Barreau, M., Caron, F., Bouffartigues, E., Chevalier, S., & Etienne, M. (2019). Understanding Ciprofloxacin Failure in *Pseudomonas aeruginosa* Biofilm: Persister Cells Survive Matrix Disruption. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02603

Song, S., & Wood, T. K. (2020). A Primary Physiological Role of Toxin/Antitoxin Systems Is Phage Inhibition. *Frontiers in Microbiology*, *11*. https://www.frontiersin.org/articles/10.3389/fmicb.2020.01895

Song, S., & Wood, T. K. (2021). Are we really studying persister cells? *Environmental Microbiology Reports*, *13*(1), 3–7. https://doi.org/10.1111/1758-2229.12849

Song, Y., Tang, H., & Bao, R. (2023). Comparative analysis of five type II TA systems identified in *Pseudomonas aeruginosa* reveals their contributions to persistence and intracellular survival. *Frontiers in Cellular and Infection Microbiology*, *13*. https://www.frontiersin.org/articles/10.3389/fcimb.2023.1127786

Spari, D., & Beldi, G. (2020). Extracellular ATP as an Inter-Kingdom Signaling Molecule: Release Mechanisms by Bacteria and Its Implication on the Host. *International Journal of Molecular Sciences*, *21*(15), Article 15. https://doi.org/10.3390/ijms21155590

Spoering, A. L., & Lewis, K. (2001). Biofilms and Planktonic Cells of *Pseudomonas aeruginosa* Have Similar Resistance to Killing by Antimicrobials. *Journal of Bacteriology*, *183*(23), 6746–6751. https://doi.org/10.1128/JB.183.23.6746-6751.2001

StatPearls. (2023). StatPearls Publishing.

Staudinger, B. J., Muller, J. F., Halldórsson, S., Boles, B., Angermeyer, A., Nguyen, D., Rosen, H., Baldursson, Ó., Gottfreðsson, M., Guðmundsson, G. H., & Singh, P. K. (2014). Conditions Associated with the Cystic Fibrosis Defect Promote Chronic *Pseudomonas aeruginosa* Infection. *American Journal of Respiratory and Critical Care Medicine*, *189*(7), 812–824. https://doi.org/10.1164/rccm.201312-2142OC

Steinmoen, H., Knutsen, E., & Håvarstein, L. S. (2002). Induction of natural competence in Streptococcus pneumoniae triggers lysis and DNA release from a subfraction of the cell population. *Proceedings of the National Academy of Sciences*, *99*(11), 7681–7686. https://doi.org/10.1073/pnas.112464599

Stepanyan, K., Wenseleers, T., Duéñez-Guzmán, E. A., Muratori, F., Van den Bergh, B., Verstraeten, N., De Meester, L., Verstrepen, K. J., Fauvart, M., & Michiels, J. (2015). Fitness trade-offs explain low levels of persister cells in the opportunistic pathogen *Pseudomonas aeruginosa*. *Molecular Ecology*, *24*(7), 1572–1583. https://doi.org/10.1111/mec.13127

Stewart, P. S., Franklin, M. J., Williamson, K. S., Folsom, J. P., Boegli, L., & James, G. A. (2015). Contribution of Stress Responses to Antibiotic Tolerance in *Pseudomonas aeruginosa* Biofilms. *Antimicrobial Agents and Chemotherapy*, *59*(7), 3838–3847. https://doi.org/10.1128/AAC.00433-15

Stylianidou, S., Brennan, C., Nissen, S. B., Kuwada, N. J., & Wiggins, P. A. (2016). SuperSegger: Robust image segmentation, analysis and lineage tracking of bacterial cells. *Molecular Microbiology*, *102*(4), 690–700. https://doi.org/10.1111/mmi.13486

Svenningsen, M. S., Svenningsen, S. L., Sørensen, M. A., & Mitarai, N. (2022). Existence of log-phase *Escherichia coli* persisters and lasting memory of a starvation pulse. *Life Science Alliance*, *5*(2). https://doi.org/10.26508/lsa.202101076

Svenningsen, M. S., Veress, A., Harms, A., Mitarai, N., & Semsey, S. (2019). Birth and Resuscitation of (p)ppGpp Induced Antibiotic Tolerant Persister Cells. *Scientific Reports*, *9*(1), 6056. https://doi.org/10.1038/s41598-019-42403-7

Tinbergen, N. (1963). On aims and methods of Ethology. *Zeitschrift Für Tierpsychologie*, *20*(4), 410–433. https://doi.org/10.1111/j.1439-0310.1963.tb01161.x

Urbaniec, J., Xu, Y., Hu, Y., Hingley-Wilson, S., & McFadden, J. (2022). Phenotypic heterogeneity in persisters: A novel 'hunker' theory of persistence. *FEMS Microbiology Reviews*, *46*(1), fuab042. https://doi.org/10.1093/femsre/fuab042

Van den Bergh, B., Fauvart, M., & Michiels, J. (2017). Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. *FEMS Microbiology Reviews*, *41*(3), 219–251. https://doi.org/10.1093/femsre/fux001

Van den Bergh, B., Schramke, H., Michiels, J. E., Kimkes, T. E. P., Radzikowski, J. L., Schimpf, J., Vedelaar, S. R., Burschel, S., Dewachter, L., Lončar, N., Schmidt, A., Meijer, T., Fauvart, M., Friedrich, T., Michiels, J., & Heinemann, M. (2022). Mutations in respiratory complex I promote antibiotic persistence through alterations in intracellular acidity and protein synthesis. *Nature Communications*, *13*(1), Article 1. https://doi.org/10.1038/s41467-022-28141-x

Veening, J.-W., Stewart, E. J., Berngruber, T. W., Taddei, F., Kuipers, O. P., & Hamoen, L. W. (2008). Bet-hedging and epigenetic inheritance in bacterial cell development. *Proceedings of the National Academy of Sciences*, *105*(11), 4393–4398. https://doi.org/10.1073/pnas.0700463105

Verstraete, L., Van den Bergh, B., Verstraeten, N., & Michiels, J. (2022). Ecology and evolution of antibiotic persistence. *Trends in Microbiology*, *30*(5), 466–479. https://doi.org/10.1016/j.tim.2021.10.001

Vogwill, T., Comfort, A. C., Furió, V., & MacLean, R. C. (2016). Persistence and resistance as complementary bacterial adaptations to antibiotics. *Journal of Evolutionary Biology*, *29*(6), 1223–1233. https://doi.org/10.1111/jeb.12864

Vos, M., Buckling, A., & Kuijper, B. (2019). Sexual Selection in Bacteria? *Trends in Microbiology*, *27*(12), 972–981. https://doi.org/10.1016/j.tim.2019.07.009

Web of Science. (2023). *Web of Science search on persistence key words.* [dataset].

WHO. (2017). *WHO publishes list of bacteria for which new antibiotics are urgently needed*. https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

Windels, E. M., Michiels, J. E., Fauvart, M., Wenseleers, T., Van den Bergh, B., & Michiels, J. (2019). Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. *The ISME Journal*, *13*(5), Article 5. https://doi.org/10.1038/s41396-019-0344-9

Wiuff, C., Zappala, R. M., Regoes, R. R., Garner, K. N., Baquero, F., & Levin, B. R. (2005). Phenotypic Tolerance: Antibiotic Enrichment of Noninherited Resistance in Bacterial Populations. *Antimicrobial Agents and Chemotherapy*, *49*(4), 1483–1494. https://doi.org/10.1128/aac.49.4.1483-1494.2005

Wood, T. K. (2016). Combatting bacterial persister cells. *Biotechnology and Bioengineering*, *113*(3), 476–483. https://doi.org/10.1002/bit.25721

Wood, T. K., & Song, S. (2020). Forming and waking dormant cells: The ppGpp ribosome dimerization persister model. *Biofilm*, *2*, 100018. https://doi.org/10.1016/j.bioflm.2019.100018

Wood, W. N., Mohler, K., Rinehart, J., & Ibba, M. (2021). Deacylated tRNA Accumulation Is a Trigger for Bacterial Antibiotic Persistence Independent of the Stringent Response. *mBio*, *12*(3), e01132-21. https://doi.org/10.1128/mBio.01132-21

Wuyts, J., Dijck, P. V., & Holtappels, M. (2018). Fungal persister cells: The basis for recalcitrant infections? *PLOS Pathogens*, *14*(10), e1007301. https://doi.org/10.1371/journal.ppat.1007301

Xi, C., & Wu, J. (2010). dATP/ATP, a Multifunctional Nucleotide, Stimulates Bacterial Cell Lysis, Extracellular DNA Release and Biofilm Development. *PLOS ONE*, *5*(10), e13355. https://doi.org/10.1371/journal.pone.0013355

Yaginuma, H., Kawai, S., Tabata, K. V., Tomiyama, K., Kakizuka, A., Komatsuzaki, T., Noji, H., & Imamura, H. (2014). Diversity in ATP concentrations in a single bacterial cell population revealed by quantitative single-cell imaging. *Scientific Reports*, *4*(1), Article 1. https://doi.org/10.1038/srep06522

Zarkan, A., Matuszewska, M., Trigg, S. B., Zhang, M., Belgami, D., Croft, C., Liu, J., El-Ouisi, S., Greenhalgh, J., Duboff, J. S., Rahman, T., & Summers, D. K. (2020). Inhibition of indole production increases the activity of quinolone antibiotics against *E. coli* persisters. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-020-68693-w

Zhang, Y., Phillips, G. J., & Yeung, E. S. (2007). Real-Time Monitoring of Single Bacterium Lysis and Leakage Events by Chemiluminescence Microscopy. *Analytical Chemistry*, *79*(14), 5373–5381. https://doi.org/10.1021/ac070477u

Zhu, M., Pan, Y., & Dai, X. (2019). (p)ppGpp: The magic governor of bacterial growth economy. *Current Genetics*, *65*(5), 1121–1125. https://doi.org/10.1007/s00294-019-00973-z

Appendix 1

In this section I describe extended RNA sequence data which remains at an early stage of development at the point of thesis submission.

The first batch of RNA sequence data relates to Chapter 2 (Chapter 2: Fig. 2). I collected time series RNA data from all inoculum density treatments (Fig. 1). My aim was to characterise changes in population gene expression profiles across growth curve phases to help explain the variation I observed in persister cell formation.

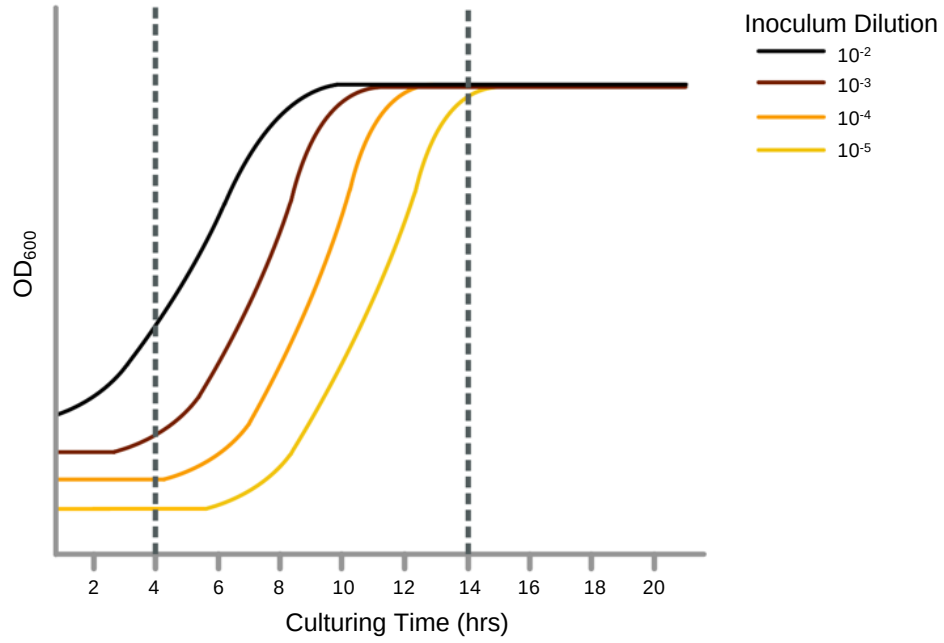


Figure 1. Illustration of the growth curve timepoints where RNA sequence data has been collected from cultures inoculated with incrementally lower cell densities. The data spans lag, exponential, and stationary phase in *P. aeruginosa* PAO1 cultures where inoculum density was shown to have a significant effect on the growth curve timepoints of persistence peaks. RNA was extracted from culture samples every two hours from 4 to 16hrs of culturing time (region represented between dashed vertical lines). The inoculum dilutions are colour-coded (see legend): the inoculum was prepared by standardising an overnight culture to an optical density (OD_{600}) of 0.1 before diluting 10^{-2}-10^{-5}-fold into experimental media. In total there are 24 samples (six for each culture inoculated with a different inoculum dilution).

Specific questions I am exploring with preliminary results:

1) Does the inoculum density determine the population's gene expression profile through the growth curve?

I am interested in how gene expression can be influenced by population division history. We have carried out preliminary exploratory work looking at volcano plots to compare gene expression patterns between cultures inoculated with different starting densities (Fig. 2). Here, we can already see that there's a greater difference in gene expression profiles the more you increase the difference in inoculum density.

2) Can we identify genes of interest which correlate with persistence peaks?

I am interested in identifying genes that are associated with persistence regardless of the culture inoculum density. This also has the benefit of being able to inspect population-level drivers of persistence irrespective of growth curve phase. We clustered all of the genes into 5 categories based on their patterns of gene expression across exponential phase and plotted the mean expression of each gene cluster for each inoculum density (Fig. 3). This allowed us to identify clusters in which genes reach peak expression during the persistence peak. We then visually identified which clusters were peaking at the same culturing timepoints of persistence peaks and compared these genes in a Venn diagram (Fig. 4). The genes which were present in all three inoculum density cultures are reported in Table 1. Some potential genes of interest include *sulA* (involved in the SOS response – inhibiting cell division), *bacA* (bacitracin resistance protein), and *mucC* (involved in biofilm formation). Further work will require a more rigorous protocol to select gene clusters, along with also looking into genes that are downregulated at points of persistence peaks.

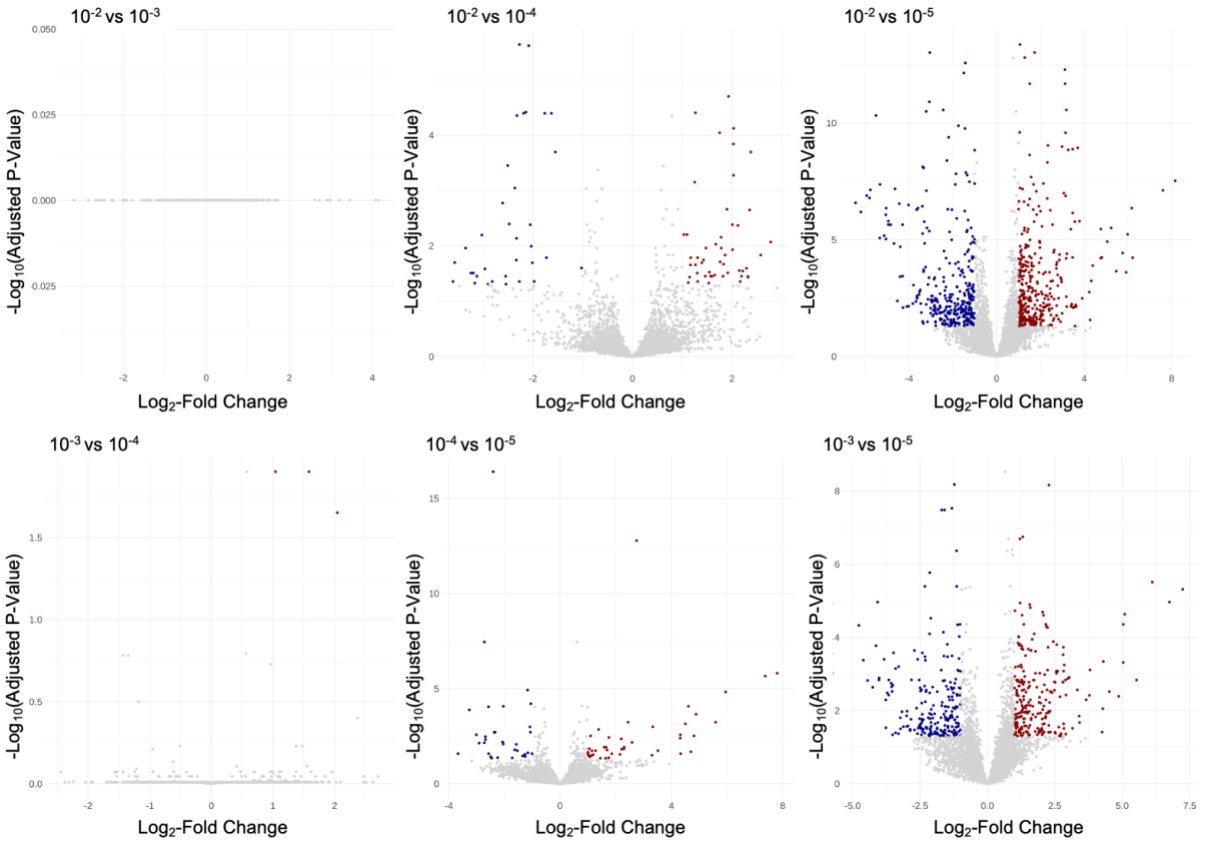


Figure 2. Volcano plots comparing gene expression patterns between cultures inoculated with different starting densities (10^{-2}-10^{-5}-fold dilutions of a 0.1OD_{600}-standardised overnight culture). We selected 8-12hrs of culturing time to inspect potential differences in these cultures.

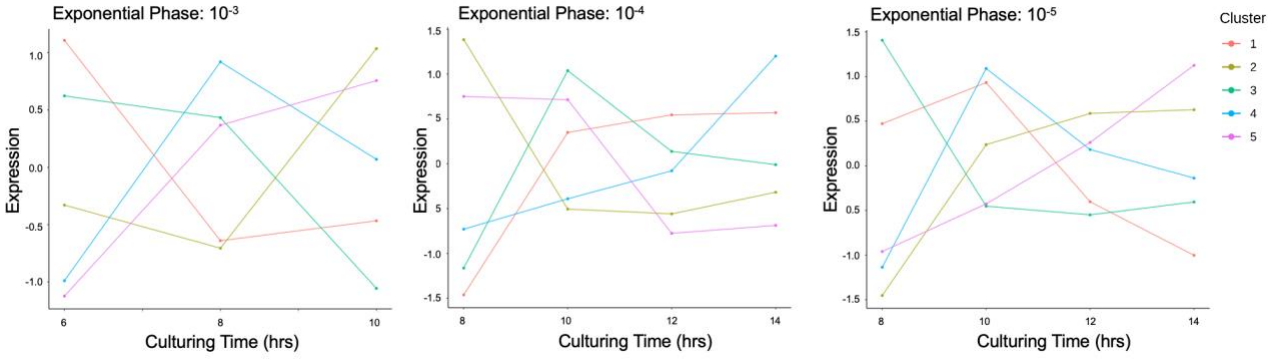


Figure 3. Clustered gene expression over culturing time for exponentially growing cultures inoculated with different starting densities (10^{-3}-10^{-5}-fold dilutions of a 0.1OD_{600}-standardised overnight culture). *10^{-2} omitted due to RNA sampling times not covering exponential phase. **Clusters were defined per inoculum density culture and so clusters 1-5 do not necessarily represent the same underlying genes throughout.

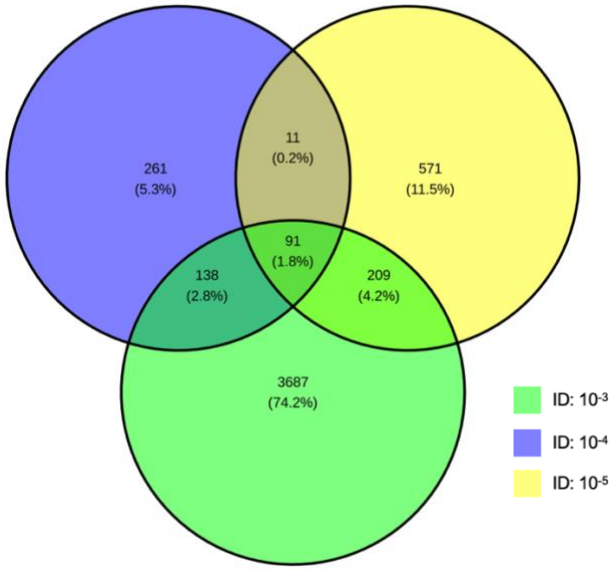


Figure 4. Venn diagram illustrating the overlapping genes that are upregulated at persistence peaks across the three inoculum density cultures (Figure 3: 10^{-3} – gene clusters 2&5; 10^{-4} – gene clusters 3&5; 10^{-5} – gene cluster 3).

Table 1. Overlapping genes expressed at persistence peaks across three inoculum density cultures (outlined in Figure 4).

Gene	Gene Name	Function
PA0005	*lptA*	Lysophosphatidic acid acyltransferase, LptA
PA0285	*pipA*	Cyclic di-GMP phosphodiesterase, PipA
PA0357	*mutM*	Formamidopyrimidine-DNA glycosylase
PA0363	*coaD*	Phosphopantetheine adenylyltransferase
PA0386	_	Probable oxidase
PA0537	_	Conserved hypothetical protein
PA0547	_	Probable transcriptional regulator
PA0551	*epd*	D-erythrose 4-phosphate dehydrogenase
PA0554	_	Hypothetical protein
PA0580	*gcp*	O-sialoglycoprotein endopeptidase
PA0592	*ksgA*	rRNA (adenine-N6, N6)-dimethyltransferase
PA0607	*rpe*	Ribulose-phosphate 3-epimerase
PA0668.2	_	tRNA-Ile
PA0668.3	_	tRNA-Ala
PA0762	*algU*	Sigma factor AlgU
PA0765	*mucC*	Positive regulator for alginate biosynthesis mucc
PA0769	_	Hypothetical protein
PA0770	*rnc*	Ribonuclease III
PA0771	*era*	GTP-binding protein Era

PA0837	*slyD*	Peptidyl-prolyl cis-trans isomerase SlyD
PA0858	_	Conserved hypothetical protein
PA0888	*aotJ*	Arginine/ornithine binding protein AotJ
PA0889	*aotQ*	Arginine/ornithine transport protein AotQ
PA0905.1	_	tRNA-Ser
PA0913	*mgtE*	MgtE
PA1112.1	_	Conserved hypothetical protein
PA1132	_	Hypothetical protein
PA1162	*dapE*	Succinyl-diaminopimelate desuccinylase
PA1170	_	Conserved hypothetical protein
PA1687	*speE*	Spermidine synthase
PA1768	_	Hypothetical protein
PA1775	*cmpX*	Conserved cytoplasmic membrane protein, CmpX protein
PA1776	*sigX*	ECF sigma factor SigX
PA1796	*folD*	5,10-methylene-tetrahydrofolate dehydrogenase / cyclohydrolase
PA1796.1	_	tRNA-Arg
PA1846	*cti*	Cis/trans isomerase
PA1959	*bacA*	Bacitracin resistance protein
PA2023	*galU*	UTP--glucose-1-phosphate uridylyltransferase
PA2291	_	Probable glucose-sensitive porin
PA2541	_	Probable CDP-alcohol phosphatidyltransferase
PA2645	*nuoJ*	NADH dehydrogenase I chain J
PA2744.1	*P13*	P13
PA2819.2	_	tRNA-Gly
PA2819.3	_	tRNA-Glu
PA2983	_	Probable tolq-type transport protein
PA2988	*lolE*	Lipoprotein localization protein LolE
PA3008	*sulA*	Sula (In the SOS Operon - Cell division inhibitor)
PA3112	*accD*	Acetyl-coa carboxylase beta subunit
PA3133.3	_	tRNA-Glu
PA3159	*wbpA*	UDP-N-acetyl-d-glucosamine 6-Dehydrogenase
PA3160	*wzz*	O-antigen chain length regulator
PA3262.1	_	tRNA -Asp
PA3262.2	_	tRNA -Val
PA3466	_	Probable ATP-dependent RNA helicase
PA3480	_	Probable deoxycytidine triphosphate deaminase
PA3579	_	Probable carbohydrate kinase
PA3633	*ygbP*	4-diphosphocytidyl-2-C-methylerythritol synthase
PA3639	*accA*	Acetyl-coenzyme A carboxylase carboxyl transferase (alpha subunit)

PA3685	*yeaZ*	Yeaz (biosynthesis of threonylcarbamoyl adenosine (T6A))
PA3722	_	Hypothetical protein
PA3726	_	Conserved hypothetical protein
PA3817	_	Probable methyltransferase
PA3818	_	Extragenic suppressor protein SuhB
PA3828	*lptF*	Lipopolysaccharide export system permease protein LptF
PA3849	_	Conserved hypothetical protein
PA3949	_	Hypothetical protein
PA3966	_	Hypothetical protein
PA3967	_	Hypothetical protein
PA3979	_	Hypothetical protein
PA4280.4	_	tRNA-Ile
PA4292	_	Probable phosphate transporter
PA4438	_	Conserved hypothetical protein
PA4627	_	Conserved hypothetical protein
PA4646	*upp*	Uracil phosphoribosyltransferase
PA4647	*uraA*	Uracil permease
PA4669.1	_	tRNA-Gln
PA4690.3	_	tRNA-Ala
PA4690.4	_	tRNA-Ile
PA4719	_	Probable transporter
PA4735	_	Hypothetical protein
PA4746	_	Conserved hypothetical protein
PA4746.1	_	tRNA-Met
PA4746.2	_	tRNA-Leu
PA4850	*prmA*	Ribosomal protein L11 methyltransferase
PA4851	_	Hypothetical protein
PA4937.1	_	tRNA-Leu
PA4940	_	Conserved hypothetical protein
PA4956	*rhdA*	Thiosulfate:cyanide sulfurtransferase
PA4961	_	Hypothetical protein
PA4963	_	Hypothetical protein
PA4972	_	Hypothetical protein
PA5045	*ponA*	Penicillin-binding protein 1A
PA5076	_	Putative amino acid ABC transporter substrate-binding protein
PA5174	*fabY*	Beta-acetoacetyl-acyl carrier protein synthase FabY
PA5227.1	*ssrS*	6s RNA
PA5228	_	Conserved hypothetical protein

The second batch of RNA sequence data relates to Chapter 4 (Chapter 4: Fig. 3). I collected RNA pre- and post-treatment with meropenem (Fig. 5). The samples were collected in triplicate from either a high- or low-density inoculum culture in exponential or stationary phase pre- and two hours post treatment with meropenem (a beta lactam antibiotic). I did this with the view to understanding more about the underlying population expression profile that facilitates the ability for the population to undergo a stress divide upon treatment with the antibiotic. This population of *P. aeruginosa* PAO1 was also heteroresistant to beta lactams and aminoglycosides and so RNA sequence data could tell us more about the underlying expression profiles of a bacterial population with a heteroresistant subpopulation.

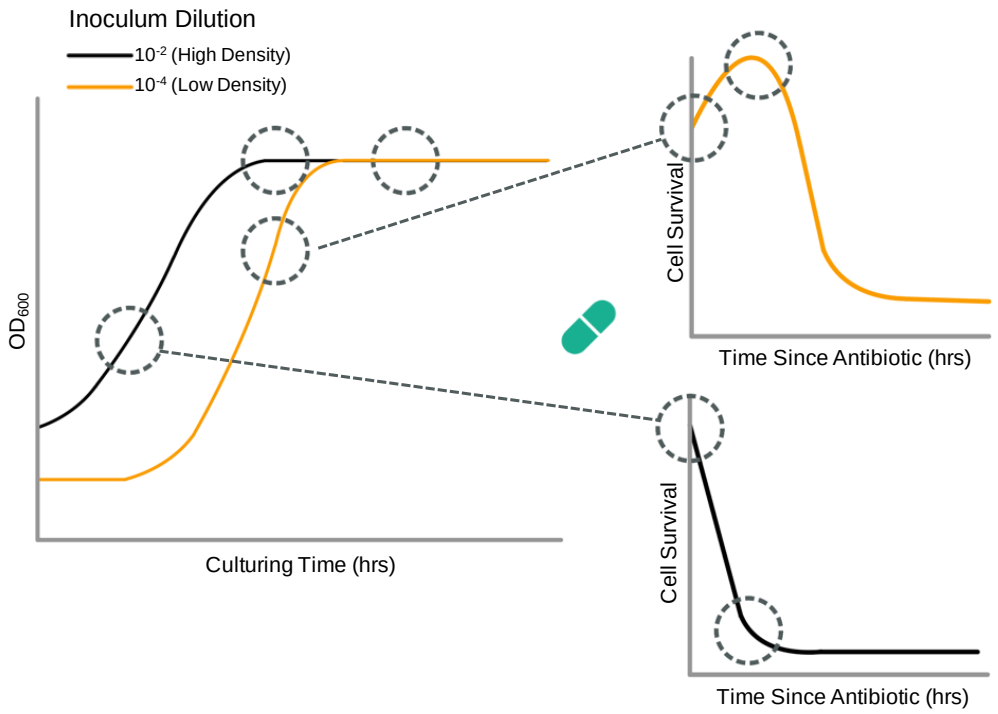


Figure 5. Illustration of the data timepoints where RNA sequence data has been collected from cultures pre- and two hours post-treatment with meropenem (beta lactam antibiotic). Samples have been collected for both exponential and stationary phase timepoints for the respective high and low inoculum density cultures (see legend). The selected timepoints were: 4 (exponential) and 12 hours (stationary) of culturing for the high-density culture; and 12 (exponential) and 18 hours (stationary) of culturing for the low-density culture. After collecting the pre-antibiotic RNA data, samples were treated with meropenem for two hours before further RNA extraction. In total there are 24 RNA transcripts (triplicate samples for each treatment outlined above).

We have yet to generate preliminary results for this portion of the RNA sequence data; however, these are the specific questions I aim to explore:

1) Can we identify which genes are helping the population survive against meropenem?
    a. I aim to compare the gene expression profile before and after treatment with meropenem to determine what genes are affecting survival against meropenem at different points of the growth curve depending on inoculum density.
2) How does the underlying gene expression determine whether a population is able to undergo a stress divide?
    a. Considering the stress divide was inoculum-dependent, I aim to compare the population gene expression profiles prior to treatment with antibiotics across the inoculum density cultures for different stages of the growth curve.

Appendix 2 – Data

Culturing Time	Week	AB	Time Post AB	Serial Dulution	Plated Volume	CFU	CFU/ml
2	5	C	2	10	5	0	0
2	5	C	2	10	5	0	0
2	5	C	2	10	5	0	0
2	5	C	2	10	5	0	0
2	5	C	2	10	5	0	0
2	5	C	2	10	5	0	0
2	5	C	2	10	5	0	0
2	5	C	2	10	5	0	0
2	5	C	4	10	5	0	0
2	5	C	4	10	5	0	0
2	5	C	4	10	5	0	0
2	5	C	4	10	5	0	0
2	5	C	4	10	5	0	0
2	5	C	4	10	5	0	0
2	5	C	4	10	5	0	0
2	5	C	24	10	5	0	0
2	5	C	24	10	5	0	0
2	5	C	24	10	5	0	0
2	5	C	24	10	5	0	0
2	5	C	24	10	5	0	0
2	5	C	24	10	5	0	0
2	5	C	24	10	5	0	0
2	5	C	24	10	5	0	0
4	5	C	2	10	5	0	0
4	5	C	2	10	5	0	0
4	5	C	2	10	5	0	0
4	5	C	2	10	5	0	0
4	5	C	2	10	5	0	0
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4	5	C	2	10	5	0	0
4	5	C	4	10	5	0	0
4	5	C	4	10	5	0	0
4	5	C	4	10	5	0	0

4	5	C	4	10	5	0	0
4	5	C	4	10	5	0	0
4	5	C	4	10	5	0	0
4	5	C	4	10	5	0	0
4	5	C	4	10	5	0	0
4	5	C	22	10	5	0	0
4	5	C	22	10	5	0	0
4	5	C	22	10	5	0	0
4	5	C	22	10	5	0	0
4	5	C	22	10	5	0	0
4	5	C	22	10	5	0	0
4	5	C	22	10	5	0	0
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4	5	C	24	10	5	0	0
4	5	C	24	10	5	0	0
4	5	C	24	10	5	0	0
4	5	C	24	10	5	0	0
4	5	C	24	10	5	0	0
4	5	C	24	10	5	0	0
4	5	C	24	10	5	0	0
4	5	C	24	10	5	0	0
6	5	C	2	10	5	0	0
6	5	C	2	10	5	0	0
6	5	C	2	10	5	0	0
6	5	C	2	10	5	0	0
6	5	C	2	10	5	0	0
6	5	C	2	10	5	0	0
6	5	C	2	10	5	0	0
6	5	C	4	10	5	0	0
6	5	C	4	10	5	0	0
6	5	C	4	10	5	0	0
6	5	C	4	10	5	0	0
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6	5	C	4	10	5	0	0
6	5	C	4	10	5	0	0
6	5	C	20	10	5	0	0
6	5	C	20	10	5	0	0

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14	1	C	2	10000	5	19	38000000
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8	5	C	0	10000000	5	8	1.6E+10
8	5	C	0	10000000	5	13	2.6E+10
8	5	C	0	10000000	5	10	2E+10
8	5	C	0	10000000	5	13	2.6E+10
8	5	C	0	10000000	5	15	3E+10
8	5	C	0	10000000	5	12	2.4E+10
8	5	C	0	10000000	5	16	3.2E+10
10	4	C	0	1000000	20	981	4.905E+10
10	4	C	0	10000000	20	320	1.6E+11
10	4	C	0	10000000	20	353	1.765E+11
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10	5	C	0	1000000	5	4	800000000
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12	4	C	0	10000000	20	202	1.01E+11
12	4	C	0	10000000	20	120	6E+10
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14	1	C	0	10000000	5	9	1.8E+10
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14	1	C	0	10000000	5	9	1.8E+10
14	1	C	0	10000000	5	10	2E+10
14	1	C	0	10000000	5	2	4000000000
14	1	C	0	10000000	5	4	8000000000
14	1	C	0	10000000	5	12	2.4E+10
14	1	C	0	10000000	5	8	1.6E+10
14	1	C	0	10000000	5	8	1.6E+10
14	1	C	0	10000000	5	8	1.6E+10
14	1	C	0	10000000	5	7	1.4E+10
14	1	C	0	10000000	5	6	1.2E+10
14	1	C	0	10000000	5	2	4000000000

16	1	C	0	100000000	5	22	4.4E+11
16	1	C	0	100000000	5	13	2.6E+11
16	1	C	0	100000000	5	9	1.8E+11
16	1	C	0	100000000	5	19	3.8E+11
16	1	C	0	100000000	5	11	2.2E+11
16	1	C	0	100000000	5	19	3.8E+11
16	1	C	0	100000000	5	19	3.8E+11
16	1	C	0	100000000	5	20	4E+11
18	1	C	0	100000000	5	14	2.8E+11
18	1	C	0	100000000	5	10	2E+11
18	1	C	0	100000000	5	19	3.8E+11
18	1	C	0	100000000	5	25	5E+11
18	1	C	0	100000000	5	12	2.4E+11
18	1	C	0	100000000	5	23	4.6E+11
18	1	C	0	100000000	5	31	6.2E+11
18	1	C	0	100000000	5	10	2E+11
18	0	C	0	100000000	5	7	1.75E+11
18	0	C	0	100000000	5	7	1.75E+11
18	0	C	0	100000000	5	4	1E+11
18	0	C	0	100000000	5	13	3.25E+11
18	0	C	0	100000000	5	8	2E+11
18	0	C	0	100000000	5	30	7.5E+11
18	0	C	0	100000000	5	16	4E+11
18	0	C	0	100000000	5	5	1.25E+11
18	2	C	0	100000000	5	35	7E+11
18	2	C	0	100000000	5	34	6.8E+11
18	2	C	0	100000000	5	38	7.6E+11
22	2	C	0	100000000	5	28	5.6E+11
22	2	C	0	100000000	5	43	8.6E+11
22	2	C	0	100000000	5	31	6.2E+11
22	2	C	0	100000000	5	33	6.6E+11
22	2	C	0	100000000	5	36	7.2E+11
22	2	C	0	100000000	5	22	4.4E+11
24	2	C	0	100000000	5	26	5.2E+11
24	2	C	0	100000000	5	39	7.8E+11
24	2	C	0	100000000	5	39	7.8E+11
24	2	C	0	100000000	5	34	6.8E+11
24	2	C	0	100000000	5	40	8E+11
24	2	C	0	100000000	5	37	7.4E+11

24	2	C	0	100000000	5	44	8.8E+11
24	2	C	0	100000000	5	40	8E+11
26	2	C	0	100000000	5	27	5.4E+11
26	2	C	0	100000000	5	30	6E+11
26	2	C	0	100000000	5	33	6.6E+11
26	2	C	0	100000000	5	36	7.2E+11
26	2	C	0	100000000	5	39	7.8E+11
26	2	C	0	100000000	5	38	7.6E+11
26	2	C	0	100000000	5	33	6.6E+11
30	5	C	0	100000000	5	3	6E+10
30	5	C	0	100000000	5	2	4E+10
30	5	C	0	100000000	5	3	6E+10
30	5	C	0	100000000	5	1	2E+10
30	5	C	0	100000000	5	3	6E+10
30	5	C	0	100000000	5	2	4E+10
30	5	C	0	100000000	5	4	8E+10
30	5	C	0	100000000	5	1	2E+10
34	3	C	0	100000000	5	39	7.8E+11
34	3	C	0	100000000	5	34	6.8E+11
34	3	C	0	100000000	5	38	7.6E+11
34	3	C	0	100000000	5	22	4.4E+11
34	3	C	0	100000000	5	29	5.8E+11
34	3	C	0	100000000	5	27	5.4E+11
34	3	C	0	100000000	5	20	4E+11
34	3	C	0	100000000	5	21	4.2E+11
36	3	C	0	100000000	5	6	1.2E+11
36	3	C	0	100000000	5	3	6E+10
36	3	C	0	100000000	5	12	2.4E+11
36	3	C	0	100000000	5	6	1.2E+11
36	3	C	0	100000000	5	5	1E+11
36	3	C	0	100000000	5	5	1E+11
36	3	C	0	100000000	5	11	2.2E+11
36	3	C	0	100000000	5	5	1E+11
2	5	M	2	10	5	1	2000
2	5	M	2	10	5	2	4000
2	5	M	2	10	5	1	2000
2	5	M	2	10	5	0	0
2	5	M	2	10	5	2	4000
2	5	M	2	10	5	1	2000

2	5	M	2	10	5	1	2000
2	5	M	2	10	5	1	2000
2	5	M	4	10	5	0	0
2	5	M	4	10	5	0	0
2	5	M	4	10	5	0	0
2	5	M	4	10	5	0	0
2	5	M	4	10	5	0	0
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2	5	M	4	10	5	1	2000
2	5	M	4	10	5	1	2000
2	5	M	24	10	5	0	0
2	5	M	24	10	5	0	0
2	5	M	24	10	5	0	0
2	5	M	24	10	5	0	0
2	5	M	24	10	5	0	0
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2	5	M	24	10	5	0	0
2	5	M	24	10	5	0	0
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4	5	M	2	100	5	5	100000
4	5	M	2	100	5	2	40000
4	5	M	2	100	5	2	40000
4	5	M	2	100	5	1	20000
4	5	M	2	100	5	1	20000
4	5	M	2	100	5	1	20000
4	5	M	2	100	5	3	60000
4	5	M	4	10	5	4	8000
4	5	M	4	10	5	3	6000
4	5	M	4	10	5	8	16000
4	5	M	4	10	5	3	6000
4	5	M	4	10	5	1	2000
4	5	M	4	10	5	3	6000
4	5	M	4	10	5	3	6000
4	5	M	4	10	5	3	6000
4	5	M	22	10	5	1	2000
4	5	M	22	10	5	0	0
4	5	M	22	10	5	0	0
4	5	M	22	10	5	0	0
4	5	M	22	10	5	0	0

4	5	M	22	10	5	0	0
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4	5	M	22	10	5	0	0
4	5	M	24	100	5	1	20000
4	5	M	24	100	5	2	40000
4	5	M	24	10	5	0	0
4	5	M	24	10	5	0	0
4	5	M	24	10	5	0	0
4	5	M	24	10	5	0	0
4	5	M	24	10	5	0	0
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6	5	M	4	1000	5	8	1600000
6	5	M	4	1000	5	9	1800000
6	5	M	4	1000	5	4	800000
6	5	M	4	1000	5	6	1200000
6	5	M	4	10000	5	2	4000000
6	5	M	20	10	5	2	4000
6	5	M	20	10	5	2	4000
6	5	M	20	10	5	4	8000
6	5	M	20	10	5	4	8000
6	5	M	20	10	5	4	8000
6	5	M	20	10	5	4	8000
6	5	M	20	10	5	3	6000
6	5	M	20	10	5	2	4000
6	5	M	22	10	5	1	2000
6	5	M	22	10	5	2	4000
6	5	M	22	10	5	1	2000
6	5	M	22	10	5	1	2000

6	5	M	22	10	5	2	4000
6	5	M	22	10	5	3	6000
6	5	M	22	10	5	0	0
6	5	M	22	10	5	10	20000
6	5	M	24	10	5	4	8000
6	5	M	24	10	5	2	4000
6	5	M	24	10	5	3	6000
6	5	M	24	10	5	4	8000
6	5	M	24	10	5	10	20000
6	5	M	24	10	5	1	2000
6	5	M	24	10	5	3	6000
6	5	M	24	10	5	1	2000
8	5	M	2	10000000	5	24	4.8E+10
8	5	M	2	100000000	5	22	4.4E+11
8	5	M	2	100000000	5	28	5.6E+11
8	5	M	2	1000000000	5	23	4.6E+12
8	5	M	2	1000000000	5	20	4E+12
8	5	M	2	1E+10	5	22	4.4E+13
8	5	M	2	1E+10	5	25	5E+13
8	5	M	2	1E+10	5	30	6E+13
8	5	M	18	10000	5	4	8000000
8	5	M	18	10000	5	4	8000000
8	5	M	18	10000	5	3	6000000
8	5	M	18	10000	5	1	2000000
8	5	M	18	10000	5	9	18000000
8	5	M	18	10000	5	3	6000000
8	5	M	18	10000	5	6	12000000
8	5	M	18	10000	5	10	20000000
8	5	M	20	10000	5	6	12000000
8	5	M	20	10000	5	4	8000000
8	5	M	20	10000	5	6	12000000
8	5	M	20	10000	5	4	8000000
8	5	M	20	10000	5	5	10000000
8	5	M	20	10000	5	3	6000000
8	5	M	20	10000	5	7	14000000
8	5	M	20	10000	5	8	16000000
8	5	M	22	1000	5	18	3600000
8	5	M	22	10000	5	6	12000000
8	5	M	22	10000	5	11	22000000

8	5	M	22	10000	5	22	44000000
8	5	M	22	10000	5	8	16000000
8	5	M	22	10000	5	7	14000000
8	5	M	22	10000	5	25	50000000
8	5	M	22	10000	5	16	32000000
8	5	M	24	10000	5	4	8000000
8	5	M	24	10000	5	5	10000000
8	5	M	24	10000	5	6	12000000
8	5	M	24	10000	5	5	10000000
8	5	M	24	10000	5	5	10000000
8	5	M	24	10000	5	4	8000000
8	5	M	24	10000	5	6	12000000
8	5	M	24	10000	5	9	18000000
10	4	M	2	1000000000	5	8	1.6E+12
10	4	M	2	1000000000	5	10	2E+12
10	4	M	2	1000000000	5	13	2.6E+12
10	4	M	2	1000000000	5	12	2.4E+12
10	4	M	2	1000000000	5	13	2.6E+12
10	4	M	2	1E+10	5	2	4E+12
10	4	M	2	1E+10	5	2	4E+12
10	4	M	2	1E+10	5	1	2E+12
10	4	M	4	1000000000	5	6	1.2E+12
10	4	M	4	1000000000	5	7	1.4E+12
10	4	M	4	1000000000	5	3	6E+11
10	4	M	4	1000000000	5	4	8E+11
10	4	M	4	1000000000	5	2	4E+11
10	4	M	4	1000000000	5	17	3.4E+12
10	4	M	4	1000000000	5	5	1E+12
10	4	M	4	1E+10	5	11	2.2E+13
10	4	M	6	100000000	5	76	1.52E+12
10	4	M	6	100000000	5	62	1.24E+12
10	4	M	6	100000000	5	25	5E+11
10	4	M	6	100000000	5	37	7.4E+11
10	4	M	6	100000000	5	40	8E+11
10	4	M	6	100000000	5	37	7.4E+11
10	4	M	6	100000000	5	27	5.4E+11
10	4	M	6	100000000	5	16	3.2E+11
10	5	M	16	10000000	5	3	6000000000
10	5	M	16	10000000	5	5	1E+10

10	5	M	16	10000000	5	7	1.4E+10
10	5	M	16	10000000	5	10	2E+10
10	5	M	16	10000000	5	6	1.2E+10
10	5	M	16	100000000	5	14	2.8E+11
10	5	M	16	1000000000	5	11	2.2E+12
10	5	M	16	1000000000	5	11	2.2E+12
10	5	M	18	1000000000	5	13	2.6E+12
10	5	M	18	1000000000	5	14	2.8E+12
10	5	M	18	1000000000	5	4	8E+11
10	5	M	18	1000000000	5	1	2E+11
10	5	M	18	1000000000	5	9	1.8E+12
10	5	M	18	1E+10	5	23	4.6E+13
10	5	M	18	1E+10	5	7	1.4E+13
10	5	M	18	1E+10	5	19	3.8E+13
10	5	M	20	1000000000	5	15	3E+12
10	5	M	20	1000000000	5	15	3E+12
10	5	M	20	1000000000	5	16	3.2E+12
10	5	M	20	1000000000	5	9	1.8E+12
10	5	M	20	1000000000	5	22	4.4E+12
10	5	M	20	1000000000	5	15	3E+12
10	5	M	20	1E+10	5	35	7E+13
10	5	M	20	1.00E+11	5	40	8E+14
10	5	M	22	100	5	8	160000
10	5	M	22	100	5	11	220000
10	5	M	22	1000	5	14	2800000
10	5	M	22	1000	5	15	3000000
10	5	M	22	1000	5	8	1600000
10	5	M	22	1000	5	18	3600000
10	5	M	22	10000	5	22	44000000
10	5	M	22	100000	5	26	520000000
10	4	M	24	100000	5	14	280000000
10	4	M	24	100000	5	7	140000000
10	4	M	24	100000	5	7	140000000
10	4	M	24	100000	5	8	160000000
10	4	M	24	100000	5	10	200000000
10	4	M	24	100000	5	2	40000000
10	4	M	24	1000000	5	4	800000000
10	4	M	24	1000000	5	1	200000000
10	5	M	24	10	5	20	40000

10	5	M	24	10	5	5	10000
10	5	M	24	100	5	14	280000
10	5	M	24	1000	5	29	5800000
10	5	M	24	1000	5	12	2400000
10	5	M	24	1000	5	19	3800000
10	5	M	24	10000	5	11	22000000
10	5	M	24	10000	5	27	54000000
12	4	M	2	1000000000	5	10	2E+12
12	4	M	2	1000000000	5	26	5.2E+12
12	4	M	2	1000000000	5	20	4E+12
12	4	M	2	1000000000	5	13	2.6E+12
12	4	M	2	1E+10	5	6	1.2E+13
12	4	M	2	1E+10	5	6	1.2E+13
12	4	M	2	1E+10	5	3	6E+12
12	4	M	2	1E+10	5	2	4E+12
12	4	M	4	1000000000	5	9	1.8E+12
12	4	M	4	1000000000	5	9	1.8E+12
12	4	M	4	1000000000	5	5	1E+12
12	4	M	4	1000000000	5	5	1E+12
12	4	M	4	1000000000	5	11	2.2E+12
12	4	M	4	1E+10	5	3	6E+12
12	4	M	4	1E+10	5	1	2E+12
12	4	M	4	1E+10	5	4	8E+12
12	4	M	22	10000	5	4	8000000
12	4	M	22	100000	5	6	120000000
12	4	M	22	100000	5	7	140000000
12	4	M	22	100000	5	2	40000000
12	4	M	22	100000	5	2	40000000
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12	4	M	24	100000	5	9	180000000
12	4	M	24	100000	5	21	420000000
12	4	M	24	1000000	5	5	1000000000
12	4	M	24	1000000	5	4	800000000
12	4	M	24	1000000	5	7	1400000000
12	4	M	24	1000000	5	11	2200000000
12	4	M	24	10000000	5	15	3E+10
14	1	M	2	1000000000	5	70	1.4E+13

14	1	M	2	1000000000	5	90	1.8E+13
14	1	M	2	1000000000	5	66	1.32E+13
14	1	M	2	1000000000	5	63	1.26E+13
14	1	M	2	1000000000	5	100	2E+13
14	1	M	2	1000000000	5	61	1.22E+13
14	1	M	2	1000000000	5	43	8.6E+12
14	1	M	2	1000000000	5	43	8.6E+12
14	1	M	4	1000000000	5	57	1.14E+13
14	1	M	4	1000000000	5	74	1.48E+13
14	1	M	4	1000000000	5	73	1.46E+13
14	1	M	4	1000000000	5	29	5.8E+12
14	1	M	4	1000000000	5	59	1.18E+13
14	1	M	4	1000000000	5	58	1.16E+13
14	1	M	4	1000000000	5	15	3E+12
14	1	M	4	1000000000	5	39	7.8E+12
14	1	M	6	10000000	5	21	4.2E+10
14	1	M	6	10000000	5	45	9E+10
14	1	M	6	10000000	5	21	4.2E+10
14	1	M	6	10000000	5	20	4E+10
14	1	M	6	10000000	5	22	4.4E+10
14	1	M	6	10000000	5	25	5E+10
14	1	M	6	10000000	5	19	3.8E+10
14	1	M	6	10000000	5	16	3.2E+10
14	1	M	8	10000000	5	19	3.8E+10
14	1	M	8	1000000000	5	28	5.6E+12
14	1	M	8	10000000	5	18	3.6E+10
14	1	M	8	10000000	5	32	6.4E+10
14	1	M	8	10000000	5	49	9.8E+10
14	1	M	8	10000000	5	38	7.6E+10
14	1	M	8	10000000	5	48	9.6E+10
14	1	M	8	10000000	5	29	5.8E+10
14	1	M	24	10000000	5	26	5.2E+10
14	1	M	24	10000000	5	33	6.6E+10
14	1	M	24	10000000	5	25	5E+10
14	1	M	24	10000000	5	17	3.4E+10
14	1	M	24	10000000	5	15	3E+10
14	1	M	24	10000000	5	23	4.6E+10
14	1	M	24	10000000	5	3	6000000000
14	1	M	24	10000000	5	5	1E+10

16	1	M	2	1000000000	5	34	6.8E+12
16	1	M	2	1000000000	5	35	7E+12
16	1	M	2	1000000000	5	34	6.8E+12
16	1	M	2	1000000000	5	36	7.2E+12
16	1	M	2	1000000000	5	100	2E+13
16	1	M	2	1000000000	5	34	6.8E+12
16	1	M	2	1000000000	5	36	7.2E+12
16	1	M	2	1000000000	5	26	5.2E+12
16	1	M	4	1000000000	5	11	2.2E+12
16	1	M	4	1000000000	5	100	2E+13
16	1	M	4	1000000000	5	51	1.02E+13
16	1	M	4	1000000000	5	100	2E+13
16	1	M	4	1000000000	5	100	2E+13
16	1	M	4	1000000000	5	100	2E+13
16	1	M	4	1000000000	5	100	2E+13
16	1	M	4	1000000000	5	100	2E+13
16	1	M	6	1000000000	5	17	3.4E+12
16	1	M	6	1000000000	5	12	2.4E+12
16	1	M	6	1000000000	5	16	3.2E+12
16	1	M	6	100000000	5	16	3.2E+11
16	1	M	6	1000000000	5	100	2E+13
16	1	M	6	1000000000	5	100	2E+13
16	1	M	6	1000000000	5	100	2E+13
16	1	M	6	1000000000	5	100	2E+13
16	1	M	22	1000000000	5	30	6E+12
16	1	M	22	1000000000	5	41	8.2E+12
16	1	M	22	1000000000	5	56	1.12E+13
16	1	M	22	1000000000	5	24	4.8E+12
16	1	M	22	1000000000	5	21	4.2E+12
16	1	M	22	1000000000	5	8	1.6E+12
16	1	M	22	1000000000	5	20	4E+12
16	1	M	22	1000000000	5	9	1.8E+12
16	1	M	24	1000000	5	5	1000000000
16	1	M	24	1000000	5	5	1000000000
16	1	M	24	1000000	5	3	600000000
16	1	M	24	1000000	5	6	1200000000
16	1	M	24	1000000	5	2	400000000
16	1	M	24	1000000	5	7	1400000000
16	1	M	24	1000000	5	5	1000000000

16	1	M	24	1000000	5	6	1200000000
18	1	M	2	1000000000	5	60	1.2E+13
18	1	M	2	1000000000	5	60	1.2E+13
18	1	M	2	1000000000	5	97	1.94E+13
18	1	M	2	1000000000	5	100	2E+13
18	1	M	2	1000000000	5	75	1.5E+13
18	1	M	2	1000000000	5	100	2E+13
18	1	M	2	1000000000	5	61	1.22E+13
18	1	M	2	1000000000	5	29	5.8E+12
18	1	M	4	100000000	5	6	1.2E+11
18	1	M	4	100000000	5	9	1.8E+11
18	1	M	4	100000000	5	9	1.8E+11
18	1	M	4	100000000	5	3	6E+10
18	1	M	4	100000000	5	100	2E+12
18	1	M	4	100000000	5	100	2E+12
18	1	M	4	100000000	5	6	1.2E+11
18	1	M	4	100000000	5	100	2E+12
18	2	M	4	1000000000	5	18	3.6E+12
18	2	M	4	1000000000	5	3	6E+11
18	2	M	4	1000000000	5	10	2E+12
18	2	M	4	1000000000	5	31	6.2E+12
18	2	M	4	1000000000	5	17	3.4E+12
18	2	M	4	1000000000	5	13	2.6E+12
18	2	M	4	1000000000	5	9	1.8E+12
18	2	M	4	1000000000	5	11	2.2E+12
18	2	M	8	1000000000	5	12	2.4E+12
18	2	M	8	1000000000	5	7	1.4E+12
18	2	M	8	1000000000	5	3	6E+11
18	2	M	8	1000000000	5	4	8E+11
18	2	M	8	1000000000	5	6	1.2E+12
18	2	M	8	1000000000	5	8	1.6E+12
18	2	M	8	1000000000	5	13	2.6E+12
18	2	M	8	1000000000	5	7	1.4E+12
18	2	M	10	10000000	5	15	3E+10
18	2	M	10	10000000	5	24	4.8E+10
18	2	M	10	10000000	5	29	5.8E+10
18	2	M	10	10000000	5	35	7E+10
18	2	M	10	10000000	5	23	4.6E+10
18	2	M	10	10000000	5	32	6.4E+10

18	2	M	10	10000000	5	18	3.6E+10
18	2	M	10	10000000	5	24	4.8E+10
18	1	M	20	10000	5	7	14000000
18	1	M	20	10000	5	2	4000000
18	1	M	20	10000	5	3	6000000
18	1	M	20	10000	5	2	4000000
18	1	M	20	10000	5	3	6000000
18	1	M	20	10000	5	8	16000000
18	1	M	20	1000	5	16	3200000
18	1	M	20	1000	5	17	3400000
18	1	M	22	100000000	5	41	8.2E+11
18	1	M	22	100000000	5	7	1.4E+11
18	1	M	22	100000000	5	24	4.8E+11
18	1	M	22	1000000000	5	19	3.8E+12
18	1	M	22	100000	5	7	140000000
18	1	M	22	1000000	5	4	800000000
18	1	M	24	1000000000	5	29	5.8E+12
18	1	M	24	10000	5	15	30000000
18	1	M	24	10000	5	4	8000000
18	1	M	24	10000	5	14	28000000
18	1	M	24	100000	5	19	380000000
18	1	M	24	1000000000	5	47	9.4E+12
18	1	M	24	10000000	5	30	6E+10
18	2	M	24	100000	5	10	200000000
18	2	M	24	100000	5	7	140000000
18	2	M	24	100000	5	9	180000000
18	2	M	24	100000	5	15	300000000
18	2	M	24	100000	5	20	400000000
18	2	M	24	100000	5	9	180000000
18	2	M	24	100000	5	23	460000000
18	2	M	24	100000	5	7	140000000
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22	2	M	2	10000	5	19	38000000
22	2	M	2	10000	5	14	28000000
22	2	M	2	10000	5	15	30000000
22	2	M	2	10000	5	8	16000000
22	2	M	2	10000	5	14	28000000
22	2	M	2	10000	5	9	18000000
22	2	M	2	10000	5	25	50000000

22	2	M	4	1000000000	5	37	7.4E+12
22	2	M	4	1000000000	5	33	6.6E+12
22	2	M	4	1000000000	5	39	7.8E+12
22	2	M	4	1000000000	5	40	8E+12
22	2	M	4	1000000000	5	100	2E+13
22	2	M	4	1000000000	5	100	2E+13
22	2	M	4	1000000000	5	100	2E+13
22	2	M	4	1000000000	5	100	2E+13
22	2	M	6	1000000000	5	21	4.2E+12
22	2	M	6	1000000000	5	25	5E+12
22	2	M	6	1000000000	5	13	2.6E+12
22	2	M	6	1000000000	5	23	4.6E+12
22	2	M	6	1000000000	5	23	4.6E+12
22	2	M	6	1000000000	5	23	4.6E+12
22	2	M	20	100000	5	14	280000000
22	2	M	20	100000	5	22	440000000
22	2	M	20	100000	5	5	100000000
22	2	M	20	100000	5	39	780000000
22	2	M	20	100000	5	37	740000000
22	2	M	20	100000	5	4	80000000
22	2	M	20	100000	5	5	100000000
22	2	M	20	100000	5	6	120000000
22	2	M	22	100000	5	13	260000000
22	2	M	22	100000	5	38	760000000
22	2	M	22	1000000	5	11	2200000000
22	2	M	22	1000000	5	17	3400000000
22	2	M	22	1000000	5	20	4000000000
22	2	M	22	100000000	5	13	2.6E+11
22	2	M	22	1000000000	5	31	6.2E+12
22	2	M	24	10000	5	11	22000000
22	2	M	24	10000	5	7	14000000
22	2	M	24	1000000	5	48	9600000000
22	2	M	24	10000000	5	32	6.4E+10
22	2	M	24	10000000	5	62	1.24E+11
22	2	M	24	100000000	5	65	1.3E+12
22	2	M	24	100000000	5	61	1.22E+12
22	2	M	24	1000000000	5	33	6.6E+12
24	2	M	2	1000000000	5	16	3.2E+12
24	2	M	2	1000000000	5	14	2.8E+12

24	2	M	2	1000000000	5	35	7E+12
24	2	M	2	1000000000	5	25	5E+12
24	2	M	2	1000000000	5	26	5.2E+12
24	2	M	2	1000000000	5	33	6.6E+12
24	2	M	2	1000000000	5	22	4.4E+12
24	2	M	2	1000000000	5	25	5E+12
24	2	M	4	1000000000	5	29	5.8E+12
24	2	M	4	1000000000	5	25	5E+12
24	2	M	4	1000000000	5	28	5.6E+12
24	2	M	4	1000000000	5	21	4.2E+12
24	2	M	4	1000000000	5	21	4.2E+12
24	2	M	4	1000000000	5	32	6.4E+12
24	2	M	4	1000000000	5	23	4.6E+12
24	2	M	4	1000000000	5	15	3E+12
24	2	M	18	100000	5	18	360000000
24	2	M	18	100000	5	11	220000000
24	2	M	18	100000	5	3	60000000
24	2	M	18	100000	5	11	220000000
24	2	M	18	100000	5	7	140000000
24	2	M	18	100000	5	9	180000000
24	2	M	18	100000	5	5	100000000
24	2	M	18	100000	5	2	40000000
24	2	M	20	10000	5	6	12000000
24	2	M	20	100000	5	6	120000000
24	2	M	20	100000	5	2	40000000
24	2	M	20	10000000	5	35	7E+10
24	2	M	20	100000000	5	22	4.4E+11
24	2	M	20	1000000000	5	52	1.04E+13
24	2	M	20	1000000000	5	36	7.2E+12
24	2	M	20	1000000000	5	100	2E+13
24	2	M	22	10000	5	7	14000000
24	2	M	22	10000	5	79	158000000
24	2	M	22	10000	5	10	20000000
24	2	M	22	10000	5	6	12000000
24	2	M	22	10000	5	4	8000000
24	2	M	22	100000	5	9	180000000
24	2	M	22	100000000	5	14	2.8E+11
24	2	M	22	1000000000	5	51	1.02E+13
24	2	M	24	10000	5	39	78000000

24	2	M	24	100000	5	11	220000000
24	2	M	24	100000	5	14	280000000
24	2	M	24	100000	5	44	880000000
24	2	M	24	100000	5	27	540000000
24	2	M	24	100000	5	36	720000000
24	2	M	24	100000	5	8	160000000
24	2	M	24	1000000000	5	10	2E+12
26	2	M	2	1000000000	5	26	5.2E+12
26	2	M	2	1000000000	5	26	5.2E+12
26	2	M	2	1000000000	5	27	5.4E+12
26	2	M	2	1000000000	5	22	4.4E+12
26	2	M	2	1000000000	5	15	3E+12
26	2	M	2	1000000000	5	100	2E+13
26	2	M	2	1000000000	5	100	2E+13
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26	2	M	16	10000	5	16	32000000
26	2	M	16	10000	5	8	16000000
26	2	M	16	10000	5	20	40000000
26	2	M	16	10000	5	9	18000000
26	2	M	16	10000	5	38	76000000
26	2	M	16	1000000000	5	32	6.4E+12
26	2	M	16	1000000000	5	29	5.8E+12
26	2	M	18	10000	5	4	8000000
26	2	M	18	10000	5	2	4000000
26	2	M	18	100000	5	3	60000000
26	2	M	18	10000	5	5	10000000
26	2	M	18	10000	5	5	10000000
26	2	M	18	100000000	5	7	1.4E+11
26	2	M	18	100000000	5	4	8E+10
26	2	M	18	1000000000	5	29	5.8E+12
26	2	M	20	10000	5	4	8000000
26	2	M	20	10000	5	6	12000000
26	2	M	20	100000	5	4	80000000
26	2	M	20	1000000	5	5	1000000000
26	2	M	20	1000000000	5	69	1.38E+13
26	2	M	20	1000000000	5	43	8.6E+12
26	2	M	20	1000000000	5	54	1.08E+13
26	2	M	20	1000000000	5	34	6.8E+12

26	2	M	22	1000000000	5	30	6E+12
26	2	M	22	1000000000	5	40	8E+12
26	2	M	22	1000000000	5	40	8E+12
26	2	M	22	1000000000	5	51	1.02E+13
26	2	M	22	1000000000	5	44	8.8E+12
26	2	M	22	1000000000	5	3	6E+11
26	2	M	22	1000000000	5	28	5.6E+12
26	2	M	22	1000000000	5	100	2E+13
26	2	M	24	10000	5	39	78000000
26	2	M	24	10000	5	60	120000000
26	2	M	24	10000	5	56	112000000
26	2	M	24	1000	5	46	9200000
26	2	M	24	1000	5	36	7200000
26	2	M	24	1000	5	45	9000000
26	2	M	24	1000	5	81	16200000
26	2	M	24	1000000000	5	39	7.8E+12
30	5	M	2	1.00E+12	5	22	4.4E+15
30	5	M	2	10000000	5	8	1.6E+10
30	5	M	2	1.00E+12	5	20	4E+15
30	5	M	2	1.00E+12	5	32	6.4E+15
30	5	M	2	1.00E+12	5	38	7.6E+15
30	5	M	2	1.00E+12	5	34	6.8E+15
30	5	M	2	1.00E+12	5	24	4.8E+15
30	5	M	2	1.00E+12	5	17	3.4E+15
30	5	M	4	10000000	5	25	5E+10
30	5	M	4	100000000	5	14	2.8E+11
30	5	M	4	100000000	5	8	1.6E+11
30	5	M	4	100000000	5	15	3E+11
30	5	M	4	100000000	5	14	2.8E+11
30	5	M	4	100000000	5	6	1.2E+11
30	5	M	4	100000000	5	16	3.2E+11
30	5	M	4	1000000000	5	7	1.4E+12
30	5	M	20	100000000	5	15	3E+11
30	5	M	20	100000000	5	9	1.8E+11
30	5	M	20	100000000	5	9	1.8E+11
30	5	M	20	100000000	5	23	4.6E+11
30	5	M	20	1000000000	5	12	2.4E+12
30	5	M	20	1000000000	5	6	1.2E+12
30	5	M	20	1000000000	5	15	3E+12

30	5	M	20	1000000000	5	23	4.6E+12
30	5	M	22	10000	5	14	28000000
30	5	M	22	10000	5	11	22000000
30	5	M	22	10000	5	4	8000000
30	5	M	22	10000	5	8	16000000
30	5	M	22	10000	5	7	14000000
30	5	M	22	10000	5	7	14000000
30	5	M	22	100000	5	4	80000000
30	5	M	22	1000000	5	9	1800000000
30	5	M	24	1000000	5	16	3200000000
30	5	M	24	10000000	5	11	2.2E+10
30	5	M	24	100000000	5	18	3.6E+11
30	5	M	24	1000000000	5	11	2.2E+12
30	5	M	24	1E+10	5	21	4.2E+13
30	5	M	24	1E+10	5	18	3.6E+13
30	5	M	24	1E+10	5	18	3.6E+13
30	5	M	24	1E+10	5	16	3.2E+13
34	3	M	2	100000000	5	75	1.5E+12
34	3	M	2	100000000	5	64	1.28E+12
34	3	M	2	100000000	5	50	1E+12
34	3	M	2	100000000	5	51	1.02E+12
34	3	M	2	100000000	5	60	1.2E+12
34	3	M	2	100000000	5	29	5.8E+11
34	3	M	2	100000000	5	11	2.2E+11
34	3	M	2	100000000	5	4	8E+10
34	3	M	4	100000000	5	24	4.8E+11
34	3	M	4	100000000	5	37	7.4E+11
34	3	M	4	100000000	5	13	2.6E+11
34	3	M	4	100000000	5	24	4.8E+11
34	3	M	4	100000000	5	35	7E+11
34	3	M	4	100000000	5	20	4E+11
34	3	M	6	100000000	5	6	1.2E+11
34	3	M	6	100000000	5	2	4E+10
34	3	M	6	100000000	5	6	1.2E+11
34	3	M	6	100000000	5	11	2.2E+11
34	3	M	6	100000000	5	15	3E+11
34	3	M	6	100000000	5	4	8E+10
34	3	M	6	100000000	5	6	1.2E+11
34	3	M	8	100000000	5	10	2E+11

34	3	M	8	100000000	5	17	3.4E+11
34	3	M	8	100000000	5	15	3E+11
34	3	M	8	100000000	5	26	5.2E+11
34	3	M	8	100000000	5	12	2.4E+11
34	3	M	8	100000000	5	15	3E+11
34	3	M	8	100000000	5	25	5E+11
34	3	M	22	1000	5	22	4400000
34	3	M	22	1000	5	30	6000000
34	3	M	22	1000	5	27	5400000
34	3	M	22	1000	5	22	4400000
34	3	M	22	1000	5	20	4000000
34	3	M	22	100000000	5	100	2E+12
34	3	M	22	100000000	5	100	2E+12
34	3	M	24	1000	5	18	3600000
34	3	M	24	1000	5	23	4600000
34	3	M	24	10000	5	60	120000000
34	3	M	24	100000	5	19	380000000
34	3	M	24	1000000	5	47	9400000000
34	3	M	24	10000000	5	67	1.34E+11
34	3	M	24	10000000	5	91	1.82E+11
34	3	M	24	10000000	5	126	2.52E+11
36	3	M	2	100000000	5	27	5.4E+11
36	3	M	2	100000000	5	29	5.8E+11
36	3	M	2	100000000	5	29	5.8E+11
36	3	M	2	100000000	5	28	5.6E+11
36	3	M	2	100000000	5	29	5.8E+11
36	3	M	2	100000000	5	33	6.6E+11
36	3	M	2	100000000	5	100	2E+12
36	3	M	4	100000000	5	37	7.4E+11
36	3	M	4	100000000	5	45	9E+11
36	3	M	4	100000000	5	63	1.26E+12
36	3	M	4	100000000	5	82	1.64E+12
36	3	M	4	100000000	5	86	1.72E+12
36	3	M	4	100000000	5	85	1.7E+12
36	3	M	4	100000000	5	100	2E+12
36	3	M	6	100000000	5	24	4.8E+11
36	3	M	6	100000000	5	22	4.4E+11
36	3	M	6	100000000	5	25	5E+11
36	3	M	6	100000000	5	24	4.8E+11

36	3	M	6	100000000	5	38	7.6E+11
36	3	M	6	100000000	5	35	7E+11
36	3	M	6	100000000	5	36	7.2E+11
36	3	M	20	10000000	5	47	9.4E+10
36	3	M	20	10000000	5	6	1.2E+10
36	3	M	20	10000000	5	28	5.6E+10
36	3	M	20	100000000	5	99	1.98E+12
36	3	M	20	100000000	5	100	2E+12
36	3	M	20	100000000	5	100	2E+12
36	3	M	22	10000	5	27	54000000
36	3	M	22	10000	5	33	66000000
36	3	M	22	10000000	5	36	7.2E+10
36	3	M	22	100000000	5	84	1.68E+12
36	3	M	22	100000000	5	100	2E+12
36	3	M	22	100000000	5	100	2E+12
36	3	M	24	1000	5	29	5800000
36	3	M	24	1000000	5	38	7600000000
36	3	M	24	10000000	5	10	2E+10
36	3	M	24	10000000	5	22	4.4E+10
36	3	M	24	10000000	5	28	5.6E+10
36	3	M	24	10000000	5	29	5.8E+10
36	3	M	24	10000000	5	100	2E+11
2	5	M	0	1000	5	8	1600000
2	5	M	0	1000	5	9	1800000
2	5	M	0	1000	5	2	400000
2	5	M	0	1000	5	6	1200000
2	5	M	0	1000	5	7	1400000
2	5	M	0	1000	5	11	2200000
2	5	M	0	1000	5	6	1200000
2	5	M	0	1000	5	7	1400000
4	5	M	0	1000	5	13	2600000
4	5	M	0	1000	5	9	1800000
4	5	M	0	1000	5	7	1400000
4	5	M	0	1000	5	7	1400000
4	5	M	0	1000	5	8	1600000
4	5	M	0	1000	5	12	2400000
4	5	M	0	1000	5	12	2400000
4	5	M	0	1000	5	6	1200000
6	5	M	0	100000	5	10	200000000

6	5	M	0	100000	5	3	60000000
6	5	M	0	100000	5	8	160000000
6	5	M	0	100000	5	7	140000000
6	5	M	0	100000	5	8	160000000
6	5	M	0	100000	5	2	40000000
6	5	M	0	100000	5	5	100000000
6	5	M	0	100000	5	7	140000000
8	5	M	0	10000000	5	10	2E+10
8	5	M	0	10000000	5	8	1.6E+10
8	5	M	0	10000000	5	13	2.6E+10
8	5	M	0	10000000	5	10	2E+10
8	5	M	0	10000000	5	13	2.6E+10
8	5	M	0	10000000	5	15	3E+10
8	5	M	0	10000000	5	12	2.4E+10
8	5	M	0	10000000	5	16	3.2E+10
10	4	M	0	1000000	20	981	4.905E+10
10	4	M	0	10000000	20	320	1.6E+11
10	4	M	0	10000000	20	353	1.765E+11
10	5	M	0	1000000	5	8	1600000000
10	5	M	0	1000000	5	4	800000000
10	5	M	0	1000000	5	5	1000000000
10	5	M	0	1000000	5	6	1200000000
10	5	M	0	1000000	5	10	2000000000
10	5	M	0	1000000	5	6	1200000000
10	5	M	0	1000000	5	4	800000000
10	5	M	0	1000000	5	4	800000000
12	4	M	0	10000000	20	81	4.05E+10
12	4	M	0	10000000	20	202	1.01E+11
12	4	M	0	10000000	20	120	6E+10
14	1	M	0	10000000	5	4	8000000000
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14	1	M	0	10000000	5	10	2E+10
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14	1	M	0	10000000	5	6	1.2E+10
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16	1	M	0	100000000	5	13	2.6E+11
16	1	M	0	100000000	5	9	1.8E+11
16	1	M	0	100000000	5	19	3.8E+11
16	1	M	0	100000000	5	11	2.2E+11
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22	2	M	0	100000000	5	31	6.2E+11
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10	4	T	2	1000000000	5	3	6E+11
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12	4	T	2	100000000	5	24	4.8E+11
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18	0	T	2	1000000000	5	38	9.5E+12
18	0	T	2	1000000000	5	21	5.25E+12
18	0	T	2	1000000000	5	22	5.5E+12
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18	0	T	2	1000000000	5	38	9.5E+12
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18	2	T	4	1000000000	5	14	2.8E+12
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18	2	T	8	1000000	5	8	1600000000
18	2	T	8	1000000	5	9	1800000000
18	2	T	8	1000000	5	10	2000000000
18	2	T	8	1000000	5	9	1800000000
18	2	T	8	1000000	5	20	4000000000
18	2	T	8	1000000	5	18	3600000000
18	2	T	8	1000000	5	3	600000000
18	2	T	10	10000000	5	16	3.2E+10
18	2	T	10	10000000	5	16	3.2E+10
18	2	T	10	10000000	5	19	3.8E+10
18	2	T	10	10000000	5	29	5.8E+10
18	2	T	10	10000000	5	28	5.6E+10
18	2	T	10	10000000	5	26	5.2E+10
18	2	T	10	100000000	5	15	3E+11
18	2	T	10	100000000	5	24	4.8E+11
18	0	T	20	1000000	5	21	5250000000
18	0	T	20	1000000	5	27	6750000000
18	0	T	20	1000000	5	25	6250000000
18	0	T	20	10000000	5	12	3E+10
18	0	T	20	1000000	5	23	5750000000
18	0	T	20	10000000	5	22	5.5E+10
18	0	T	20	10000000	5	12	3E+10
18	0	T	20	1000000000	5	13	3.25E+12
18	0	T	22	100000	5	22	550000000

18	0	T	22	100000	5	28	700000000
18	0	T	22	100000	5	45	1125000000
18	0	T	22	100000	5	16	400000000
18	1	T	22	10000000	5	39	7.8E+10
18	1	T	22	100000	5	16	320000000
18	1	T	22	10000000	5	40	8E+10
18	1	T	22	100000	5	16	320000000
18	1	T	22	10000	5	55	110000000
18	1	T	22	10000	5	13	26000000
18	1	T	22	10000	5	13	26000000
18	1	T	22	100000	5	30	600000000
18	0	T	24	100000	5	13	325000000
18	0	T	24	100000	5	12	300000000
18	0	T	24	100000	5	22	550000000
18	0	T	24	100000	5	23	575000000
18	0	T	24	100000	5	25	625000000
18	0	T	24	100000	5	8	200000000
18	1	T	24	100000	5	1	20000000
18	1	T	24	100000	5	6	120000000
18	1	T	24	100000	5	3	60000000
18	1	T	24	1000	5	12	2400000
18	1	T	24	1000000	5	2	400000000
18	1	T	24	10000	5	3	6000000
18	1	T	24	100000	5	13	260000000
18	1	T	24	1000	5	6	1200000
18	2	T	24	10000	5	12	24000000
18	2	T	24	10000	5	14	28000000
18	2	T	24	10000	5	10	20000000
18	2	T	24	10000	5	11	22000000
18	2	T	24	10000	5	13	26000000
18	2	T	24	10000	5	16	32000000
18	2	T	24	10000	5	17	34000000
18	2	T	24	10000	5	6	12000000
22	2	T	2	1000000000	5	11	2.2E+12
22	2	T	2	1000000000	5	16	3.2E+12
22	2	T	2	1000000000	5	9	1.8E+12
22	2	T	2	1000000000	5	18	3.6E+12
22	2	T	2	1000000000	5	23	4.6E+12
22	2	T	2	1000000000	5	22	4.4E+12

22	2	T	2	1000000000	5	6	1.2E+12
22	2	T	2	1000000000	5	12	2.4E+12
22	2	T	4	1000000000	5	3	6E+11
22	2	T	4	1000000000	5	1	2E+11
22	2	T	4	1000000000	5	1	2E+11
22	2	T	4	1000000000	5	1	2E+11
22	2	T	4	1000000000	5	5	1E+12
22	2	T	4	1000000000	5	1	2E+11
22	2	T	4	1000000000	5	3	6E+11
22	2	T	4	100000000	5	3	6E+10
22	2	T	6	10000000	5	8	1.6E+10
22	2	T	6	10000000	5	8	1.6E+10
22	2	T	6	10000000	5	6	1.2E+10
22	2	T	6	10000000	5	7	1.4E+10
22	2	T	6	10000000	5	7	1.4E+10
22	2	T	6	10000000	5	2	4000000000
22	2	T	6	10000000	5	10	2E+10
22	2	T	6	100000000	5	7	1.4E+11
22	2	T	20	100000	5	3	60000000
22	2	T	20	100000	5	6	120000000
22	2	T	20	100000	5	5	100000000
22	2	T	20	100000	5	2	40000000
22	2	T	20	100000	5	5	100000000
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22	2	T	22	10000	5	6	12000000
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22	2	T	22	10000	5	9	18000000
22	2	T	22	10000	5	12	24000000
22	2	T	24	100000	5	6	120000000
22	2	T	24	100000	5	6	120000000
22	2	T	24	100000	5	5	100000000
22	2	T	24	100000	5	10	200000000
22	2	T	24	100000	5	8	160000000

22	2	T	24	100000	5	5	100000000
22	2	T	24	100000	5	3	60000000
22	2	T	24	10000000	5	19	3.8E+10
24	2	T	2	1000000000	5	18	3.6E+12
24	2	T	2	1000000000	5	3	6E+11
24	2	T	2	1000000000	5	7	1.4E+12
24	2	T	2	1000000000	5	19	3.8E+12
24	2	T	2	1000000000	5	19	3.8E+12
24	2	T	2	1000000000	5	6	1.2E+12
24	2	T	2	1000000000	5	5	1E+12
24	2	T	2	1000000000	5	100	2E+13
24	2	T	4	100000000	5	16	3.2E+11
24	2	T	4	100000000	5	13	2.6E+11
24	2	T	4	100000000	5	18	3.6E+11
24	2	T	4	100000000	5	26	5.2E+11
24	2	T	4	100000000	5	21	4.2E+11
24	2	T	4	100000000	5	12	2.4E+11
24	2	T	4	100000000	5	14	2.8E+11
24	2	T	4	100000000	5	12	2.4E+11
24	2	T	18	1000	5	8	1600000
24	2	T	18	1000	5	10	2000000
24	2	T	18	1000	5	7	1400000
24	2	T	18	10000	5	5	10000000
24	2	T	18	10000	5	5	10000000
24	2	T	18	10000	5	2	4000000
24	2	T	18	10000	5	8	16000000
24	2	T	18	100000	5	3	60000000
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24	2	T	20	10000	5	5	10000000
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24	2	T	20	10000	5	7	14000000
24	2	T	20	10000	5	4	8000000
24	2	T	20	10000	5	2	4000000
24	2	T	20	10000	5	5	10000000
24	2	T	20	10000	5	6	12000000
24	2	T	22	1000	5	20	4000000
24	2	T	22	1000	5	26	5200000
24	2	T	22	10000	5	10	20000000
24	2	T	22	10000	5	14	28000000

24	2	T	22	10000	5	9	18000000
24	2	T	22	10000	5	15	30000000
24	2	T	22	10000	5	21	42000000
24	2	T	22	100000	5	9	180000000
24	2	T	24	10000	5	22	44000000
24	2	T	24	10000	5	12	24000000
24	2	T	24	10000	5	17	34000000
24	2	T	24	10000	5	17	34000000
24	2	T	24	10000	5	12	24000000
24	2	T	24	10000	5	16	32000000
24	2	T	24	10000	5	31	62000000
24	2	T	24	100000	5	22	440000000
26	2	T	2	10000000	5	9	1.8E+10
26	2	T	2	10000000	5	7	1.4E+10
26	2	T	2	10000000	5	2	4000000000
26	2	T	2	10000000	5	11	2.2E+10
26	2	T	2	10000000	5	11	2.2E+10
26	2	T	2	10000000	5	4	8000000000
26	2	T	2	10000000	5	10	2E+10
26	2	T	2	10000000	5	15	3E+10
26	2	T	18	100	5	31	620000
26	2	T	18	10000	5	5	10000000
26	2	T	18	10000	5	28	56000000
26	2	T	18	10000	5	10	20000000
26	2	T	18	10000	5	9	18000000
26	2	T	18	10000	5	1	2000000
26	2	T	18	10000	5	6	12000000
26	2	T	18	10000	5	2	4000000
26	2	T	20	1000	5	7	1400000
26	2	T	20	1000	5	20	4000000
26	2	T	20	1000	5	20	4000000
26	2	T	20	1000	5	10	2000000
26	2	T	20	1000	5	24	4800000
26	2	T	20	1000	5	20	4000000
26	2	T	20	1000	5	26	5200000
26	2	T	20	1000	5	24	4800000
26	2	T	22	1000	5	7	1400000
26	2	T	22	1000	5	2	400000
26	2	T	22	1000	5	18	3600000

26	2	T	22	1000	5	14	2800000
26	2	T	22	1000	5	20	4000000
26	2	T	22	1000	5	12	2400000
26	2	T	22	10000	5	19	38000000
26	2	T	22	100000	5	13	260000000
26	2	T	24	1000	5	17	3400000
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26	2	T	24	10000	5	6	12000000
26	2	T	24	10000	5	5	10000000
26	2	T	24	10000	5	10	20000000
26	2	T	24	10000	5	6	12000000
26	2	T	24	10000	5	10	20000000
26	2	T	24	10000	5	14	28000000
30	5	T	2	10000000	5	35	7E+10
30	5	T	2	10000000	5	19	3.8E+10
30	5	T	2	10000000	5	28	5.6E+10
30	5	T	2	10000000	5	29	5.8E+10
30	5	T	2	10000000	5	29	5.8E+10
30	5	T	2	10000000	5	30	6E+10
30	5	T	2	10000000	5	23	4.6E+10
30	5	T	2	100000000	5	8	1.6E+11
30	5	T	4	10000000	5	39	7.8E+10
30	5	T	4	10000000	5	32	6.4E+10
30	5	T	4	10000000	5	40	8E+10
30	5	T	4	10000000	5	19	3.8E+10
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30	5	T	4	10000000	5	26	5.2E+10
30	5	T	4	100000000	5	9	1.8E+11
30	5	T	20	1000	5	9	1800000
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30	5	T	20	1000	5	10	2000000
30	5	T	20	1000	5	14	2800000
30	5	T	20	1000	5	9	1800000
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30	5	T	20	10000	5	12	24000000
30	5	T	20	10000	5	17	34000000
30	5	T	22	10000	5	10	20000000
30	5	T	22	10000	5	11	22000000

30	5	T	22	10000	5	13	26000000
30	5	T	22	100000	5	23	460000000
30	5	T	22	100000	5	13	260000000
30	5	T	22	100000	5	11	220000000
30	5	T	22	100000	5	22	440000000
30	5	T	22	100000	5	18	360000000
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30	5	T	24	10000	5	15	30000000
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30	5	T	24	100000	5	24	480000000
30	5	T	24	100000	5	16	320000000
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34	3	T	2	100000000	5	8	1.6E+11
34	3	T	2	100000000	5	22	4.4E+11
34	3	T	2	100000000	5	13	2.6E+11
34	3	T	2	100000000	5	28	5.6E+11
34	3	T	2	100000000	5	22	4.4E+11
34	3	T	2	100000000	5	36	7.2E+11
34	3	T	2	100000000	5	5	1E+11
34	3	T	4	10000000	5	2	4000000000
34	3	T	4	10000000	5	15	3E+10
34	3	T	4	10000000	5	14	2.8E+10
34	3	T	4	10000000	5	20	4E+10
34	3	T	4	10000000	5	26	5.2E+10
34	3	T	4	10000000	5	17	3.4E+10
34	3	T	4	100000000	5	11	2.2E+11
34	3	T	4	100000000	5	13	2.6E+11
34	3	T	6	10000000	5	2	4000000000
34	3	T	6	10000000	5	4	8000000000
34	3	T	6	10000000	5	5	1E+10
34	3	T	6	10000000	5	4	8000000000
34	3	T	6	10000000	5	6	1.2E+10
34	3	T	6	10000000	5	11	2.2E+10
34	3	T	6	1000000	5	9	1800000000
34	3	T	6	1000000	5	8	1600000000
34	3	T	22	1000	5	21	4200000

34	3	T	22	1000	5	20	4000000
34	3	T	22	1000	5	23	4600000
34	3	T	22	10000	5	26	52000000
34	3	T	22	10000	5	17	34000000
34	3	T	22	100000	5	19	380000000
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34	3	T	22	1000000	5	24	4800000000
34	3	T	24	10000	5	7	14000000
34	3	T	24	10000	5	40	80000000
34	3	T	24	10000	5	15	30000000
34	3	T	24	10000	5	11	22000000
34	3	T	24	10000	5	2	4000000
34	3	T	24	10000	5	45	90000000
34	3	T	24	10000	5	2	4000000
34	3	T	24	10000	5	3	6000000
36	3	T	2	100000000	5	4	8E+10
36	3	T	2	100000000	5	14	2.8E+11
36	3	T	2	100000000	5	19	3.8E+11
36	3	T	2	100000000	5	5	1E+11
36	3	T	2	100000000	5	20	4E+11
36	3	T	2	100000000	5	20	4E+11
36	3	T	2	100000000	5	10	2E+11
36	3	T	2	100000000	5	10	2E+11
36	3	T	4	10000000	5	11	2.2E+10
36	3	T	4	10000000	5	14	2.8E+10
36	3	T	4	100000000	5	8	1.6E+11
36	3	T	4	100000000	5	12	2.4E+11
36	3	T	4	100000000	5	14	2.8E+11
36	3	T	4	100000000	5	10	2E+11
36	3	T	4	100000000	5	21	4.2E+11
36	3	T	4	100000000	5	6	1.2E+11
36	3	T	6	10000000	5	12	2.4E+10
36	3	T	6	10000000	5	22	4.4E+10
36	3	T	6	10000000	5	17	3.4E+10
36	3	T	6	10000000	5	14	2.8E+10
36	3	T	6	10000000	5	32	6.4E+10
36	3	T	6	10000000	5	23	4.6E+10
36	3	T	6	10000000	5	19	3.8E+10
36	3	T	6	10000000	5	18	3.6E+10

36	3	T	20	10000	5	7	14000000
36	3	T	20	10000	5	7	14000000
36	3	T	20	10000	5	17	34000000
36	3	T	20	10000	5	10	20000000
36	3	T	20	10000	5	19	38000000
36	3	T	20	10000	5	4	8000000
36	3	T	20	100000	5	16	320000000
36	3	T	20	100000	5	25	500000000
36	3	T	22	1000	5	25	5000000
36	3	T	22	1000	5	27	5400000
36	3	T	22	1000	5	15	3000000
36	3	T	22	1000	5	4	800000
36	3	T	22	1000	5	2	400000
36	3	T	22	10000	5	20	40000000
36	3	T	22	10000	5	18	36000000
36	3	T	22	10000	5	28	56000000
36	3	T	24	10000	5	87	174000000
36	3	T	24	10000	5	52	104000000
36	3	T	24	10000	5	41	82000000
36	3	T	24	100000	5	20	400000000
36	3	T	24	100000	5	36	720000000
36	3	T	24	100000	5	37	740000000
36	3	T	24	1000000	5	5	1000000000
2	5	T	0	1000	5	8	1600000
2	5	T	0	1000	5	9	1800000
2	5	T	0	1000	5	2	400000
2	5	T	0	1000	5	6	1200000
2	5	T	0	1000	5	7	1400000
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4	5	T	0	1000	5	13	2600000
4	5	T	0	1000	5	9	1800000
4	5	T	0	1000	5	7	1400000
4	5	T	0	1000	5	7	1400000
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4	5	T	0	1000	5	12	2400000
4	5	T	0	1000	5	6	1200000

6	5	T	0	100000	5	10	200000000
6	5	T	0	100000	5	3	60000000
6	5	T	0	100000	5	8	160000000
6	5	T	0	100000	5	7	140000000
6	5	T	0	100000	5	8	160000000
6	5	T	0	100000	5	2	40000000
6	5	T	0	100000	5	5	100000000
6	5	T	0	100000	5	7	140000000
8	5	T	0	10000000	5	10	2E+10
8	5	T	0	10000000	5	8	1.6E+10
8	5	T	0	10000000	5	13	2.6E+10
8	5	T	0	10000000	5	10	2E+10
8	5	T	0	10000000	5	13	2.6E+10
8	5	T	0	10000000	5	15	3E+10
8	5	T	0	10000000	5	12	2.4E+10
8	5	T	0	10000000	5	16	3.2E+10
10	4	T	0	1000000	20	981	4.905E+10
10	4	T	0	10000000	20	320	1.6E+11
10	4	T	0	10000000	20	353	1.765E+11
10	5	T	0	1000000	5	8	1600000000
10	5	T	0	1000000	5	4	800000000
10	5	T	0	1000000	5	5	1000000000
10	5	T	0	1000000	5	6	1200000000
10	5	T	0	1000000	5	10	2000000000
10	5	T	0	1000000	5	6	1200000000
10	5	T	0	1000000	5	4	800000000
10	5	T	0	1000000	5	4	800000000
12	4	T	0	10000000	20	81	4.05E+10
12	4	T	0	10000000	20	202	1.01E+11
12	4	T	0	10000000	20	120	6E+10
14	1	T	0	10000000	5	4	8000000000
14	1	T	0	10000000	5	4	8000000000
14	1	T	0	10000000	5	11	2.2E+10
14	1	T	0	10000000	5	9	1.8E+10
14	1	T	0	10000000	5	6	1.2E+10
14	1	T	0	10000000	5	9	1.8E+10
14	1	T	0	10000000	5	10	2E+10
14	1	T	0	10000000	5	2	4000000000
14	1	T	0	10000000	5	4	8000000000

14	1	T	0	10000000	5	12	2.4E+10
14	1	T	0	10000000	5	8	1.6E+10
14	1	T	0	10000000	5	8	1.6E+10
14	1	T	0	10000000	5	8	1.6E+10
14	1	T	0	10000000	5	7	1.4E+10
14	1	T	0	10000000	5	6	1.2E+10
14	1	T	0	10000000	5	2	4000000000
16	1	T	0	100000000	5	22	4.4E+11
16	1	T	0	100000000	5	13	2.6E+11
16	1	T	0	100000000	5	9	1.8E+11
16	1	T	0	100000000	5	19	3.8E+11
16	1	T	0	100000000	5	11	2.2E+11
16	1	T	0	100000000	5	19	3.8E+11
16	1	T	0	100000000	5	19	3.8E+11
16	1	T	0	100000000	5	20	4E+11
18	1	T	0	100000000	5	14	2.8E+11
18	1	T	0	100000000	5	10	2E+11
18	1	T	0	100000000	5	19	3.8E+11
18	1	T	0	100000000	5	25	5E+11
18	1	T	0	100000000	5	12	2.4E+11
18	1	T	0	100000000	5	23	4.6E+11
18	1	T	0	100000000	5	31	6.2E+11
18	1	T	0	100000000	5	10	2E+11
18	0	T	0	100000000	5	7	1.75E+11
18	0	T	0	100000000	5	7	1.75E+11
18	0	T	0	100000000	5	4	1E+11
18	0	T	0	100000000	5	13	3.25E+11
18	0	T	0	100000000	5	8	2E+11
18	0	T	0	100000000	5	30	7.5E+11
18	0	T	0	100000000	5	16	4E+11
18	0	T	0	100000000	5	5	1.25E+11
18	2	T	0	100000000	5	35	7E+11
18	2	T	0	100000000	5	34	6.8E+11
18	2	T	0	100000000	5	38	7.6E+11
22	2	T	0	100000000	5	28	5.6E+11
22	2	T	0	100000000	5	43	8.6E+11
22	2	T	0	100000000	5	31	6.2E+11
22	2	T	0	100000000	5	33	6.6E+11
22	2	T	0	100000000	5	36	7.2E+11

22	2	T	0	100000000	5	22	4.4E+11
24	2	T	0	100000000	5	26	5.2E+11
24	2	T	0	100000000	5	39	7.8E+11
24	2	T	0	100000000	5	39	7.8E+11
24	2	T	0	100000000	5	34	6.8E+11
24	2	T	0	100000000	5	40	8E+11
24	2	T	0	100000000	5	37	7.4E+11
24	2	T	0	100000000	5	44	8.8E+11
24	2	T	0	100000000	5	40	8E+11
26	2	T	0	100000000	5	27	5.4E+11
26	2	T	0	100000000	5	30	6E+11
26	2	T	0	100000000	5	33	6.6E+11
26	2	T	0	100000000	5	36	7.2E+11
26	2	T	0	100000000	5	39	7.8E+11
26	2	T	0	100000000	5	38	7.6E+11
26	2	T	0	100000000	5	33	6.6E+11
30	5	T	0	100000000	5	3	6E+10
30	5	T	0	100000000	5	2	4E+10
30	5	T	0	100000000	5	3	6E+10
30	5	T	0	100000000	5	1	2E+10
30	5	T	0	100000000	5	3	6E+10
30	5	T	0	100000000	5	2	4E+10
30	5	T	0	100000000	5	4	8E+10
30	5	T	0	100000000	5	1	2E+10
34	3	T	0	100000000	5	39	7.8E+11
34	3	T	0	100000000	5	34	6.8E+11
34	3	T	0	100000000	5	38	7.6E+11
34	3	T	0	100000000	5	22	4.4E+11
34	3	T	0	100000000	5	29	5.8E+11
34	3	T	0	100000000	5	27	5.4E+11
34	3	T	0	100000000	5	20	4E+11
34	3	T	0	100000000	5	21	4.2E+11
36	3	T	0	100000000	5	6	1.2E+11
36	3	T	0	100000000	5	3	6E+10
36	3	T	0	100000000	5	12	2.4E+11
36	3	T	0	100000000	5	6	1.2E+11
36	3	T	0	100000000	5	5	1E+11
36	3	T	0	100000000	5	5	1E+11
36	3	T	0	100000000	5	11	2.2E+11

36	3	T	0	100000000	5	5	1E+11