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Effects of antibiotics on individual bacterial cells

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EFFECTS OF ANTIBIOTICS ON INDIVIDUAL BACTERIAL CELLS

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To all of my teachers

Those whom I have met in person

and those whom I have met through books

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Summary

While antibiotics are a mainstay of contemporary medicine, many basic questions about how they inactivate and kill bacteria are still unresolved. The main goal of this thesis was to address some of these questions. Specifically, we aimed at expanding our understanding of two central themes that are difficult to explore in traditional microbiological approaches. The first theme is about how antibiotics affect the basic components of bacterial growth: cell division and death. We exposed bacterial populations to various concentrations of antibiotics and analyzed how the population growth patterns that we observed emerged from the interplay between cell division and cell death. The second theme is non-genetic factors that contribute to differences in the response and survival to antibiotics, either between individual cells or between environments with plenty or limited resources. We used *Escherichia coli* and ribosome-binding antibiotics as our model systems and employed microfluidics and time-lapse microscopy to investigate the effects of antibiotics at the level of individual cells.

In Chapter 2, we addressed the effect of antibiotic pulses on bacterial cells. We focused on a range of antibiotic doses where, in batch experiments, a net positive or negative population growth rate would mask death or division rates, respectively. Our single-cell approach allowed us to quantify the division events occurring for each cell. This revealed that antibiotics around what is considered the minimal inhibitory concentrations (MIC) in batch

experiments or higher sometimes have no discernible effect on the rate of cell division during the pulse. Informed by this finding and in combination with bulk experiments, we explored the role of time, concentration and total dose (the product of time and concentration) on the probability that bacterial cells would survive an antibiotic pulse. One of our main observations was that total dose had the strongest effect on cell survival, but short pulses of high concentrations or long pulses of low concentrations led to slightly higher mortality than equivalent total doses of intermediate duration and concentration.

In Chapter 3, we asked whether we would be able to predict that a given bacterial cell would survive a pulse of antibiotic, based on features of this cell that we could measure previous, during and immediately after antibiotic exposure. We did not find evidence that the interdivision time previous to exposure (as a proxy for growth rate) neither the time that had elapsed since the last division before the pulse (as a proxy for cell cycle position at the onset of the stress) predicted cell fate. We found, however, that potential persisters (cells not dividing at all in the time monitored previous to exposure) showed higher chances of survival in conditions with high mortality. We also found that cell division patterns immediately after the pulse can predict survival.

In Chapter 4 we investigated how two different ribosome-targeting antibiotics affect bacterial growth in a limited-nutrient environment, particularly disentangling the role of division on the growth dynamics observed at the population level. We further focused on the effect of gentamicin as we found that cells exposed to low concentrations of this drug entered stationary phase earlier. The results of our experiments suggest that bacteria exposed to antibiotics might be depleting resources faster during

exponential phase. It is likely that this phenomenon is specifically related to amino acid consumption.

In summary, this work highlights the relevance of improving our understanding of how antibiotics affect individual bacterial cells. The questions, results and discussions presented in this thesis suggest that single-cell approaches are key to understand bacterial growth dynamics under stress and to fill important gaps related to non-genetic factors that affect survival and response to antibiotic treatment. Such understanding can potentially help in the development of new antibiotics, and help using existing antibiotics more effectively.

Zusammenfassung

Antibiotika spielen eine zentrale Rolle in der zeitgenössischen Medizin. Nichtsdestotrotz verstehen wir aber nicht im Detail, wie Bakterien durch Antibiotika inaktiviert und abgetötet werden. Das zentrale Ziel dieser Dissertation war es, an diesen Wissenslücken zu arbeiten. Konkret haben wir uns auf zwei Aspekte fokussiert, die mit traditionellen Methoden aus der Mikrobiologie schwierig anzugehen sind. Der erste Aspekt ist, wie Antibiotika die grundlegenden Komponenten des bakteriellen Wachstums – Zellteilung und Zelltod – beeinflussen. Wir haben Bakterien verschiedenen Konzentrationen von Antibiotika ausgesetzt und haben untersucht, wie das beobachtete Populationswachstum zu Stande kommt durch ein Wechselspiel von Zellteilung und – tod. Der zweite Aspekt ist, dass nicht-genetische Faktoren einen erheblichen Effekt haben können darauf, wie Bakterien Antibiotika-Exposition überleben, und Unterschiede im Überleben zwischen individuellen Zellen innerhalb von klonalen Population oder zwischen verschiedenen Wachstumsbedingungen verursachen können. Wir haben das Bakterium Escherichia coli als Modellsystem verwendet, und mit Antibiotika gearbeitet, die an das bakterielle Ribosom binden. Um die Effekte von Antibiotika auf Einzelzell-Ebene zu untersuchen, haben wir Experimente in Mikrofluidik-Kammer und mit Zeitraffer-Mikroskopen durchgeführt.

In Kapitel 2 haben wir untersucht, wie diskrete Pulse von Antibiotika auf Bakterien wirken. Wir haben mit Konzentrationen gearbeitet, unter der eine

leichte Zu- oder Abnahme der Populationsgrösse in konventionellen Experimenten Zelltod respektive Zellteilung maskieren. Unsere Einzelzell-Analyse hat uns erlaubt, die Zellteilungsaktivität von individuellen Zellen zu messen. Diese Messungen haben gezeigt, dass Antibiotika-Konzentrationen in der Nähe der in Batchkulturen gemessenen MIC (Minimal Inhibitory Concentration) manchmal keinen messbaren Effekt auf die Zellteilungsrate während dem Antibiotikapuls haben. Wir haben auf diesen Ergebnissen aufgebaut und analysiert, wie die Wahrscheinlichkeit von einzelnen Zellen, einen Antibiotika-Puls zu überleben, von dessen Dauer, der Konzentration und totalen Dosis (dem Produkt aus Dauer und Konzentration) abhängt. Die totale Dosis hatte den stärksten Einfluss auf die Überlebensrate. Interessanterweise war aber nicht nur die totale Dosis entscheidend – kurze starke Pulse und lange schwache Pulse haben zu einem leicht höheren Absterben geführt als Kombinationen von intermediärer Konzentration und Zeitdauer.

In Kapitel 3 haben wir untersucht, ob das Überleben von einzelnen Bakterienzellen unter Antibiotika-Exposition voraussagen können, basierend auf Zelleigenschaften vor, während und unmittelbar nach der Exposition. Wir haben keine Evidenz dafür gefunden, dass die Teilungsrate vor der Exposition oder die Zellzyklusposition während der Exposition eine Voraussage über das Überleben einer Zelle erlauben würden. Wir haben aber gesehen, dass einzelne Zellen sich gar nicht teilten vor der Antibiotika-Exposition, und manche dieser Zellen überlebt haben. Diese Zellen entsprechen potentiell früher beschriebenen ‘Persister’-Bakterien. Unsere Experimente haben auch gezeigt, dass das Zellteilungsmuster unmittelbar nach der Exposition korreliert mit dem längerfristigen Überleben einer Zelle.

In Kapitel 4 haben wir untersucht, wie zwei Ribosomen-bindende Antibiotika das bakterielle Wachstum in nährstoffarmen Bedingungen beeinflussen. Wir haben dabei analysiert, wie das auf Populationsebene beobachtete Wachstum zustande kommt aus einer sich über die Zeit verändernden Zellteilungsrate. Wir haben gefunden, dass das Antibiotikum Gentamycin dazu führt, dass wachsende Bakterienkulturen früher in die Stationärphase eintreten. Unsere Resultate legen nahe, dass Antibiotika-Exposition dazu führt, dass Bakterien Nährstoffe schneller aufbrauchen. Es ist wahrscheinlich, dass dieses Phänomen spezifisch mit dem Verbrauch von Aminosäuren zusammenhängt.

Zusammenfassend zeigt unsere Arbeit, dass es wichtig ist, den Effekt von Antibiotika auf einzelne Zellen zu untersuchen und verstehen. Einzelzell-messungen können aufzeigen, wie die Raten von Zellteilung und -Tod von Antibiotika beeinflusst werden, und welche Rolle nicht-genetische Faktoren dabei spielen. Solche Einsichten können potentiell bei der Entwicklung neuer Antibiotika eine Rolle spielen, und auch helfen, bestehende Antibiotika besser zu nutzen.

Chapter 1

Introduction

Before the advent of antibiotics, doctors' main duty was to predict the course of an illness. Learning "the art of prediction" was the major reason to attend medical school. This, at least, is the view of Thomas Lewis, physician and writer, who saw during his lifetime the discovery, development and rise of antibiotics. In his autobiography, he argued that before the era of antimicrobials, the possibility of an accurate prognosis was "all there was to science in medicine"[1]. Antibiotics thus might have transformed medicine in what he calls "the youngest science".

By revolutionizing medicine, antibiotics had a profound impact on human health. Better living conditions, such as nutrition and housing, improved sanitation and cleaner water, had already reduced the mortality caused by infectious diseases. The discovery of antibiotics and its application accelerated the decline of deaths by this cause. Until the first half of the twentieth century, bacterial infections were the leading cause of human death. In the United States, for example, tuberculosis, pneumonia and diarrhea were responsible for almost 30% of all deaths in 1900[2]. At the beginning of the second half of the century, however, pneumonia dropped to the sixth place and tuberculosis to the 15th [3]. Today, chronic diseases have replaced them to a great degree as the leading causes of death[2], more notoriously in developed countries. Except for lower respiratory infections in 5th and 6th place, there are no other infectious diseases in the top 10

causes of death in upper-middle-income and high-income economics[4]. An increase in hygiene and other environmental factors played a crucial role in this change, but the development of antibiotics was relevant for the effective treatment of bacterial infections.

While antibiotics revolutionized how infections were treated, their efficacy was soon threatened by the evolution of bacteria that were genetically resistant against treatment. The WHO has, in the last decade, continuously warned about the major threat that antibiotic resistance poses to public health and food security[5]. In January 2018, WHO's Global Antimicrobial Surveillance System (GLASS) reported a detailed account of common pathogenic strains that are evolving resistance towards existing drugs. Among the species with higher occurrence of resistance are *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumonia* and *Salmonella* spp (*Mycobacterium tuberculosis* is not included in these data)[6].

While antibiotic resistance understandably receives a lot of attention in the scientific community and the general public, is not the sole problem we are facing with respect to controlling bacterial pathogens. In the last decades, there has been increasing evidence that in clonal populations of genetically nonresistant bacteria, some individuals can survive antibiotic exposure, for instance, by a transient phenotypic state that confer tolerance[7–10]. Other studies point to how the presence of other microorganisms in the vicinity can influence a bacterium's fate upon exposure to antibiotics, through diffusible metabolites, the production of volatile compounds or the expression of antibiotic-degrading enzymes[11–14]. Physical factors matter, too. Cells embedded in a biofilm show increased levels of tolerance towards antimicrobials, potentially due to the polymer matrix structure, which limits antibiotic penetration, and to the physiological state of such cells (e.g. decreased metabolic activity)[15–17]. Moreover, tolerance to antibiotics

conferred by these factors (e.g. growth rate, other cells and space) can also be the first step towards the emergence of resistance[18,19].

In order to better understand how antibiotics can affect different individuals in a clonal population differently, we need to analyze the effects of antibiotics on individual bacterial cells. This approach will allow, in the first place, unraveling basic details about the growth dynamics of bacteria exposed to antibiotics during treatment or in the environment. Additionally, it will increase our knowledge about the heterogeneity of the bacterial response to drugs. Understanding this heterogeneity is critical for controlling bacterial infections, as it will inform us about which bacterial cells survive treatments, how many cells survive, and why they survive.

Growth dynamics: the interplay of birth and death

Microbiology has traditionally relied on population-level experiments. The study of the effect of antibiotics is not the exception. For decades, scientists have assessed the impact of drugs on bacteria by looking at population growth, for example, using turbidity as a proxy of the number of cells in a fixed volume of growth medium. Another established approach is plate counting, a method to estimate the number of culturable cells in a volume. Plate counting combines batch experiments with the enumeration of the number of cells in a volume that are able to divide and give raise to a colony. Evaluating growth by turbidity or by colony forming units (CFUs) on a plate has been fundamental to understand certain aspects of the effect of antibiotics, for example, the minimum inhibitory concentration (MIC), which is the lowest concentration required to prevent visible growth, or to monitor the percentage of cells that are able to grow again after treatment.

However, analyzing the growth dynamics in bulk experiments has a number of limitations. The growth observed at this level is the result of the interplay of birth and death events; one of the limitations is thus the impossibility to disentangle the role of these two processes. Specifically, and in the context of antibiotics, we usually do not know how exposure to a drug affects the division rate and the mortality of bacteria – we usually only know effects on the net population growth rate. Analyzing these two processes separately might not be relevant in scenarios where one of the processes has a rate close to zero, but in an intermediate range between the two extremes of no mortality and no survival, we would certainly benefit from understanding how a given population level growth rate results from the interplay between cell division and mortality. Given the scarcity of research tackling this question, we usually do not even know whether a given experiment falls into this intermediate range where both division and death play a substantial role.

Understanding effects of antibiotics on the division rate and on mortality separately is important for two reasons. The first reason is that this will allow us to better understand how exactly antibiotics impact bacteria, in detail. While the cellular targets of antibiotics are known in most or all cases, the downstream effects that arise after a drug binds to its cellular target are usually much less clear[20–22]. In order to understand these effects, it is important to know how cell division and mortality are both affected by a given drug. The second reason why disentangling division and mortality is important is that these two processes determine the scope for evolution in bacterial populations. Assuming that most mutations occur during DNA replication, the rate of replication and division determines the input of mutations into a bacterial population. To illustrate this point, let us consider a bacterial population whose size does not change over time. A constant population size could either result from a situation where no cells divide and

no cells die; in this case, the input of mutations into the population would be zero. However, and alternatively, a constant population size could also be based on division and mortality occurring at high but equal rates; in that case, the rate of new mutations would be high.

In scenarios where population size decreases, for example, after exposure to a certain stressor, batch experiments are usually interpreted under the implicit assumption that division is not taking place. In a study of *Mycobacterium smegmatis* exposed to the drug isoniazid, Wakamoto *et al* reported that, even at the killing period known as the persistence phase, where only a tiny fraction of cells withstand the drug, the constancy of the population size resulted from a balance of cell division and death[23]. It is critical to be aware of the occurrence of division events in such conditions, for instance, to estimate the probability of the emergence of resistant mutants.

On the other hand, unraveling the role of birth and death is also relevant in scenarios where population size increases. Growth at low, specifically subMIC concentrations, corresponds to the other extreme of high division rate and low death rate. When monitoring the density of bacteria over time in such environments, the positive growth indicates that the division rate is higher than the death rate, but the relative rate of the two processes is generally unknown. Since there is evidence that such conditions can select and enrich for resistant mutants, understanding cell division and death under these conditions is important[24].

Non-genetic factors allowing survival

The idea that bacterial cells are able to survive antibiotic exposure, even without the genetic factors that confer resistance, is not so recent. In the

1940s, Bigger *et al* already had reported the observation that around one cell in a million of a *Staphylococcus pyogenes* population would survive penicillin treatment high enough to kill all the other cells. Their hypothesis was that these cells were not killed because they were in a temporal non-dividing stage and penicillin only kills bacteria in the process of dividing. In their paper, they also stated that descendants of what they called "persisters" were mostly sensitive to the antibiotic[25].

In the last years, aided by new technologies, scientists have been able to confirm the existence of such cells and obtain more insights into the basis of their survival. The hypothesis raised by Bigger, that the persistent subpopulation is growth-arrested, was confirmed by direct microscopic observation of individual cells[26]. While this phenomenon was first directly observed in cells exposed to beta-lactam antibiotics —that were known to target actively dividing cells—, there have been other examples of subpopulations with reduced growth rate that survive bactericidal antibiotics[27,28]. Aminoglycosides, for instance, target the ribosome. Tolerance towards this antibiotic has been associated with ribosome hibernation —ribosomes in an inactive form— in cells at the stationary phase[29].

Reduced growth, triggered by either stochastic mechanisms or by the environment, is not the only factor that has been associated with tolerance to antibiotics. Cell fate in *Mycobacterium smegmatis* exposed to the drug isoniazid is not correlated with growth rate and rather linked to the growth-rate-independent expression of an enzyme[23]. For antibiotics targeting the ribosome, faster growth rates can either increase or decrease susceptibility to subMIC concentrations of the drug depending on the degree of reversibility of binding between the antibiotic molecule and the ribosome[30]. Growth rate is thus an important factor affecting the bacterial response to

drugs but whether slow or fast growth is associated with survival depends on the exact circumstances.

Focus of this thesis

Understanding non-genetic factors that affect survival upon antibiotic exposure and the effects of variable antibiotic concentrations on bacteria, is thus critical, both for learning more about how antibiotics act on bacteria, and for controlling bacterial populations more effectively. This will help answering questions regarding the evasion of treatment, but also offer a better picture of the potential gateways that lead to antibiotic resistance. Given these knowledge gaps and their relevance, the main focus of this thesis is to increase our understanding of bacterial growth and survival in scenarios where an intermediate fraction of the population survives, mainly by temporal or continuous exposure to antibiotic concentrations lower than those used in clinics.

In **Chapter 2**, we investigated how bacteria respond to pulses of antibiotics of different time lengths and concentrations. Given the heterogeneous environments that bacterial cells experience in body compartments during treatment, it is essential to understand how temporal variation in the concentration of an antibiotic affects survival and cell division. We used population- and single-cell experiments to address the effect of concentration, time and total dose (the product of concentration and time) in such environments.

One of the main observations of chapter 2 was that there is a substantial range of antibiotic concentrations and duration of exposure where survival is intermediate, that is, where some cells survive and others are killed during the so-called log-linear death phase. This raises the question whether

we can *predict* which cells will survive, based on measurable properties of single cells. Thus, in **Chapter 3**, we asked whether there are physiological traits, either observed previous to exposure or as part of the early response, that are associated with higher chances to survive. In order to address this question, we evaluated the role of interdivision times previous to exposure, cell cycle stage at the onset of the stress and early divisional behavior after the antibiotic challenge.

Finally, the goal of **Chapter 4** was to understand the role of division in scenarios with continuous exposure to low antibiotic concentrations, given the relevance this has on increasing the chances for the evolution of resistance. One of our initial observations was that the exponential growth rate of bacteria was not affected by the exposure to low levels of gentamicin, but that the effect of the antibiotic becomes visible at the transition to stationary phase. Using a combination of batch- and microfluidic experiments we further investigated the basis of this growth-phase dependent effect of the antibiotic.

Our experimental model: *Escherichia coli* exposed to ribosome-targeting antibiotics

Escherichia coli was the model organism we used to explore all the questions posed in this thesis. A Gram-negative bacterium with a length of about 1μm and a width of about 0.35 μm, it is the most-studied bacterial model system. It is a distinctive member of a healthy gut microbiome in many animals, but also an important human pathogen. Pathogenic *E. coli* is a major cause of urinary tract infections, bloodstream infections and diarrheal diseases, as well as a frequent cause of foodborne infections worldwide[31,32]. As a member of the Enterobacteriaceae, it is closely related to other important pathogens such as *Salmonella* and *Klebsiella* and it might be possible to

translate findings with *E coli* to these other organisms. Moreover, when exploring new experimental approaches, *E. coli* is —due to our good understanding of its biology and its ease of handling— probably the best organism to start with.

All the antibiotics used in this thesis —tetracycline and the aminoglycosides kanamycin and gentamicin— target the ribosome. The rationale for this choice was partly technical: many other antibiotics cause cell elongation in the range of concentrations and exposure times we wanted to explore. This phenotype interferes with the time-lapse recording of cells inside microfluidic devices, as filamentous cells tend to be removed from these devices through flow. Most importantly, however, is the clinical relevance of these antibiotics. Tetracycline is an inexpensive broad-spectrum antibiotic widely used in clinics and agriculture and often used to treat mycoplasmas, rickettsiae and Lyme disease[33]. Aminoglycosides, such as gentamicin and kanamycin, have been used for decades to treat common and potentially dangerous infections caused by Gram-negative bacteria. Moreover, as in the last decade Gram-negatives have shown increased levels of resistance to other type of antibiotics, aminoglycosides have become in the last decade one of the main choices for therapy[34].

Most of the questions asked in this thesis were tackled by the use of microfluidics and time-lapse microscopy, which allowed monitoring single cells over time and rigorously controlling the media they were exposed to. The use of these experimental models with this technical approach will hopefully contribute to the current demands for understanding the effect of antibiotics on bacteria.

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Chapter 2

How to kill most bacteria with a given dose of antibiotics: the effects of time and concentration of an antibiotic pulse

With contributions from:

Martin Ackermann, ETH Zurich and Eawag
Conception and design of the study together with AMMC;
essential involvement throughout the research process;
critical revision of the chapter

Daniel J. Kiviet, ETH Zurich and Eawag
Development of the experimental setup and software for analysis;
critical input throughout the research process

Abstract

When bacterial cells are exposed to antibiotics, the concentrations they experience are rarely constant over time. In clinics, for instance, treatment is frequently administered in series of periodical doses, and the concentration of antibiotics in a patient thus changes dynamically over time. We do not yet understand well how the effects of an antibiotic on bacteria depend on the dynamics of the antibiotic —whether the effect of a certain total dose of antibiotics depends on the details of how its concentration changes over time. Our goal here was to contribute towards filling this knowledge gap. We exposed our model system *Escherichia coli* to pulses of kanamycin. Using population- and single-cell experiments, we asked how features of an antibiotic pulse, such as time, concentration and total dose (the product of time and concentration) affect cell survival. Our first finding was that the main determinant of survival is the total dose, regardless of the duration and concentration that is used to administer this dose. However, we also observed deviations from this rule: administering a given dose with a very high concentration or over a very long time increased mortality levels compared to combinations of intermediate duration and concentration. Our single-cell approach revealed substantial rates of cell division during periods of antibiotic exposure, sometimes even at equal rates as in the absence of antibiotics. These single-cell experiments additionally allowed to assess how features of the antibiotic pulse affect the growth potential of the surviving fraction of the population, i.e. how many divisions those cells lose during and after the pulse. Finally, by studying another feature of the pulse, that is, comparing a gradual versus a stepwise increase of the drug, we found no evidence that the rate at which an antibiotic's concentration increases over time would be an important determinant of survival. Overall, the study of single cells for understanding bacterial responses to bacterial

pulses provided a more accurate quantification of cell survival and offered a detailed account of the divisional pattern of survivors.

Introduction

Bacteria are frequently exposed to fluctuations in antibiotic concentrations for variable periods of times. This is particularly common in the context of antibiotic treatment. Patients usually receive antibiotic doses at periodic points in time, and this creates fluctuations in the antibiotic concentration. In addition, bacteria might experience additional antibiotic concentration gradients resulting from the heterogeneous distribution of drugs between host compartments[1,2]. It is interesting to ask how fluctuations in the concentration of an antibiotic modulate its effect on bacteria. Will a certain dose of antibiotics be more effective if it is administered as a short intense pulse, or over an extended period of time at a low concentration?

The study of the response to treatment for define periods of time has been common practice for decades, for instance, usually expressed through the concept of time-kill curves, by quantifying colony-forming cells after the exposure to a drug concentration over time. These analyses, however, have limitations. If bacterial cell division continues during antibiotic exposure, then population-level measurements cannot disentangle how antibiotics affect division and death. Specifically, we currently do not know how division events occurring during treatment influence the survival rate quantified at the level of the population. We also do not have an understanding of how cells that survive after the pulse are affected by it. We addressed these questions by exposing *Escherichia coli* to pulses of the aminoglycoside kanamycin over a range of exposure times and concentrations where only a fraction of the population survives. Using time-lapse microfluidics, we monitored the division events in single cells previous, during and after the pulse, which offered a detailed understanding of the reproductive behavior of each cell throughout the experiment.

Scientists and clinicians use a similar concept to what we call here the total dose (the product of time and concentration of a rectangular drug pulse): Area Under the drug concentration-time Curve (AUC). The AUC is, however, specifically assigned to the antibiotic concentration measured in serum during treatment[3–5] and has been studied at the level of bacterial populations in a host or in vitro[4,5]. In this chapter, we rather asked how single bacterial cells are affected if we change the concentration of a drug or the period of time during which it is administered.

We furthered explored how the antibiotic pulse affect those cells who survive. Aminoglycosides, such as kanamycin, inhibit protein synthesis by binding to the ribosome. As they interrupt the protein elongation step where the translocation of the mRNA-tRNA complex takes place, these drugs also create mistranslation, and misfolded proteins affect the membrane[6]. Therefore, it is likely that these related events are one of the main causes of the cell death resulting from exposure to aminoglycosides[6,7]. However, in scenarios where cells survive after exposure and given that kanamycin binds in an irreversible manner, a period of time is needed for resynthesizing the lost ribosomes. This postantibiotic effect (PAE)[8] has been measured in batch[9], but it is not clear how the death of some cells influence this quantification. In this work, we are able to study the reproductive behavior of each of the survivors alone, by monitoring single-cell divisions over time.

Finally, we performed experiments where the concentration increases gradually, to investigate how such temporal gradients would affect bacterial survival. Temporal gradients emerge naturally in many clinical situations, whenever antibiotics are administered as discrete doses; in these cases, dissolution of the drug and the subsequent distribution through the body lead to a gradual (but often fast) increase of the drug's concentration in a

given body compartment[1,10]. This is also the case for aminoglycosides, which are sometimes administered as a single dose per day. We think it is interesting to ask whether a gradual increase in a drug's concentration, compared to a step-wise increase, would allow bacteria to physiologically adapt and thereby tolerate higher drug concentrations,

Results and Discussion

How the features of antibiotic pulse affect survival

Our first aim was to understand the role of time, concentration and total dose on the survival of individual bacterial cells. For this purpose, we treated cohorts of *E. coli* MG1655 cells inside a microfluidic chamber with pulses of 4, 8 or 16 μg/ml of kanamycin for a period of 0.5, 1 or 2 hours (we determined the minimal inhibitory concentration, MIC, to be 4 μg/ml of kanamycin for *E. coli* MG1655). This range of exposures led to a range of different survival probabilities from almost 100% to less than 1%; this allowed us to analyze how survival probability depended on the combination of time and concentration at which a given dose was administered. It is important to discuss briefly how we defined the cohort of cells whose survival we analyzed. We started our experiments with a few hundred cells per treatment, and then exposed these cells to antibiotics. As we will discuss below, some of these cells divided during exposure. How did we then keep track of the cells whose survival we studied? At each cell division, we always followed the cell that carried the old cell pole[11,12]. In our microfluidic chip, these cells were always conveniently located at the end of a dead-end channel and were thus not displaced during cell division. When we thus make statements about the fraction of cells that survived a given dose of antibiotic, this refers to the group of old-pole cells that we followed

during the whole experiment. Note that occasionally old-pole cells might have been displaced and eventually removed from the growth channels; in these cases we followed the cell that was located at the end of the dead-end channel.

We monitored the cells' division events during and after the pulse and used the occurrence of division events after exposure as a proxy for survival (Figure 1a). We considered a bacterial cell as having survived exposure if this cell divided at least once during the period of 4 to 10 hours after the pulse (thick blue line in Figure 1a; for further details see Material and Methods). This criterion avoided to falsely classifying cells as dead if these cells were experiencing a postantibiotic effect (PAE), where cells stop growing for a short period of time after exposure[8]. Previous work has shown that PAE for *E. coli* cells treated with aminoglycosides manifests up to 4.5 hours after exposure for 4xMIC and only up to 9 hours for concentrations as high as 64xMIC[9].

As expected, the results showed that time, concentration and total dose played a role in survival of the cells (ANOVA, $p<0.001$). By comparing the absolute survival of the nine conditions and performing a Tukey posthoc ANOVA test, we were able to group these conditions in four homogeneous subsets (A to D in Figure 1b; treatments that belong to the same subset show no significant differences in mean survival among them). In all cases, cells treated with equivalent total doses (shaded in grey) had survival means that belonged to at least one equivalent subset. This suggested that a given dose of antibiotic had more or less the same effect independently of the combination of concentration and time that was used to administer the dose. However, we also found some tentative evidence that increasing the concentration and decreasing the time reduced survival in some cases. For exposure times below one hour, combinations with higher concentrations

sometimes belonged to survival groups with lower means (Figure 2). This analysis suggested a potential slightly larger role of concentration compared to time, but also showed that the main predictor of mortality was the total dose.

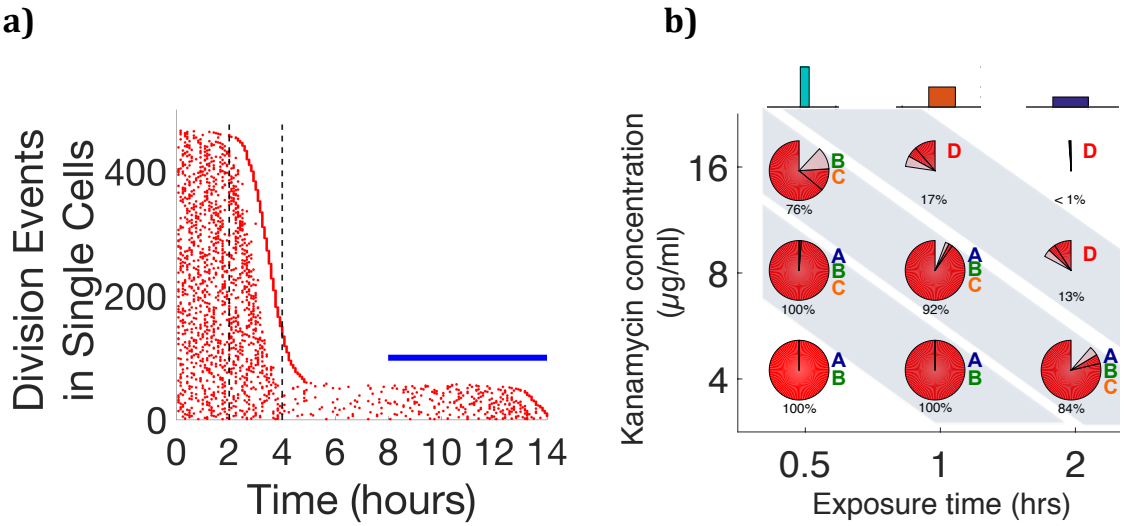

Figure 1. Survival of *E. coli* cells after a pulse of kanamycin, for different combinations of exposure times and antibiotic concentrations. (a) We recorded division events in cohorts of 446 cells from three independent replicates that were exposed to a 2-hour pulse of 8 μg/ml of kanamycin (represented by the dashed vertical lines). The 446 cells are ordered along the y-axis based on the time of their last division. Each red dot corresponds to a division event of a cell; dots that have the same y-coordinate are consecutive divisions of the same cell. The thick blue line indicates the period during which we evaluated whether a cell had survived antibiotic exposure; cells that divided during this period were considered to have survived. **(b)** Percentage of survival normalized to the survival rate in the absence of kanamycin for antibiotic pulses of different durations (x-axis) and concentrations (y-axis). Individual pie charts show mean survival in three replicates in red; the section in pink marks the standard error of the mean. Grey areas group combinations of time and concentration that correspond to the same total dose (concentration x time). The capital letters next to the pie charts refer to the outcome of a Tukey post hoc analysis of variance. Treatments that are marked with the same letter show no significant differences in their mean absolute survival after exposure. The letter A marks the treatments with the highest mean survival and the letter D the group with the lowest mean survival.

Divisions during antibiotic pulses and the implications for CFU analysis

Our next goal was to investigate the occurrence of cell division during antibiotic exposure and the impact of cell divisions on survival estimates. Here, we exposed bacterial cells temporally to concentrations of kanamycin equivalent or higher than the MIC. The MIC is defined as the antibiotic concentrations where visible growth is prevented when measured over a 24-hour period. Yet bacteria exposed to these concentrations for short periods of time (0.5, 1 or 2 hours) divided during exposure in our experiments (Figure 2a). In order to quantify the number of cell divisions, we counted the total number of cell divisions during the antibiotic pulse in our cell cohort, and divided this sum by the number of cells in the cohort to obtain the average number of divisions per cell. We observed no significant differences in the number of divisions in the presence or absence of kanamycin during pulses of half an hour in all the concentrations studied (ANOVA, $p=0.15$). Division rate neither decreased significantly for concentrations up to 8 μg/ml for one hour (ANOVA, $p=0.21$) and two hours (ANOVA, $p=0.19$). Divisions even took place at the highest concentration studied (16 μg/ml, 4 times the MIC); these elevated concentrations of kanamycin only lead to a reduction in the number of divisions by around 50% for 2 hours of exposure (Figure 2a).

These observations raise the question of how the absence of turbidity after overnight exposure to those concentrations might be the result of the interplay between division and death of cells, with potentially a continuous change in the rate of these two processes throughout the 24-hour period of an MIC experiment. Further experiments with cells treated with such doses but longer exposure times (e.g. 24 hours) inside microfluidic chambers will be critical. First, such experiments are required to confirm whether MIC values are equivalent in population-level batch experiments and in single-

cell experiments. Second, such experiments will offer a more accurate picture of how antibiotic concentrations actually affect bacterial growth dynamics over longer periods of time.

The occurrence of divisions during the pulse also affects how we measure bacterial survival when we perform CFUs (colony-forming units) measurements. Conventionally, bacterial survival is estimated by counting CFUs before and after antibiotic exposure. This approach assumes that there are no cell divisions during the period of antibiotic exposure. That divisions *do* take place, at least for the conditions explored here, is expected to lead to an overestimation of survival.

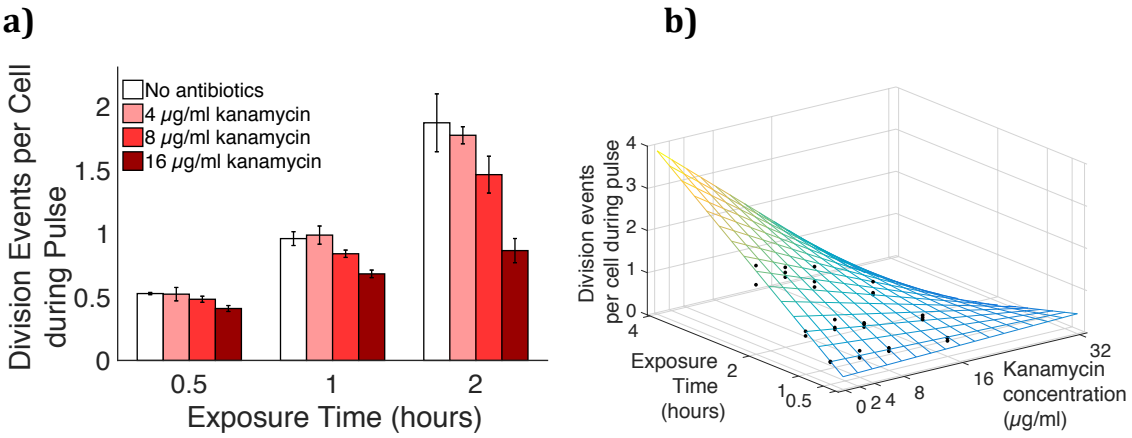

Figure 2. Division events during antibiotic pulses (a) Mean of division events per cell during the antibiotic pulse with different kanamycin concentrations. Error bars indicate standard error of the mean of replicates (2 replicates for the control pulse and 3 replicates for each of the other treatment conditions). **(b)** Multiple linear regression model that includes time and concentration as the explanatory variables to determine the number of divisions taking place during pulses of different durations and intensities (R^2=0.9381). The black dots represent the experimental data shown in panel (a). The surface represents the regression surface that best describes the data.

It is important to note that, notwithstanding this confounding effect of cell divisions on survival estimates, the total number of CFUs after exposure is

also an important and relevant measure: it is a proxy for the total population size after treatment, and thus for the threat posed by a bacterial pathogen for a host. However, here we were interested in the fraction of cells in a cohort that survives antibiotic exposure, and cell division during exposure leads to an overestimation of survival. We think it is interesting to focus on survival in this cohort and not just on the effects on the total population size: we expect that this information is required for better understanding how antibiotics act on single cells. The overestimation of survival is expected to be higher for those conditions where division rate is not significantly affected during exposure. It might be problematic, for instance, when comparing conditions with equivalent total doses but where the division rate during pulse is affected in different ways (e.g. 16 μg/ml of kanamycin in a one-hour pulse versus 8μg/ml of kanamycin in a two-hour pulse, as shown in Figure 2a and our previously described statistic analyses). As we will discuss in the next section, knowing the division rates in different antibiotic regimes allowed us to correct for this effect and derive survival probabilities from population measurements.

Combining population experiments with single-cell data allows extending the range of exposure time and antibiotic concentrations that we can analyze

The above-described measurements of division events in our single-cell data had a useful application: they allowed us to use population measurements for estimating survival probabilities under antibiotic exposure and to correct these population measurements for the overestimation of survival that resulted from cell division during exposure (Figure 2). This also permitted to extend the number of combinations of antibiotic concentrations and exposure times beyond what we had studied with the microfluidic approach. We could thus evaluate how shorter and longer,

milder and more intense pulses affected cell survival. We tested a larger number of combinations (0-32 μg/ml kanamycin administered during 15 minutes to 4 hours) using batch cultures. In these experiments, survival was initially estimated by comparing CFUs before and after the kanamycin treatment (Figure 3a). These results, however, did not take into account the division events occurring during the pulse.

We used the experimental data obtained from the single-cell measurements (Figure 2a) to correct the results obtained in batch for cell division events and thus to obtain more precise survival estimates. We used the division rates that we directly measured in the microfluidic experiments as well as the division rates that we obtained from the multiple linear regression analysis for conditions that were not explored under the microscope (Figure 2b). These rates were used to correct the percentage of surviving cells after the batch cultures were treated with kanamycin (Figure 3b; see Material and Methods for further details).

The survival percentages observed in the nine conditions studied in microfluidics showed no difference to the results obtained in batch after correcting for divisions during pulse (compare Figure 3b to Figure 1b; ANOVA, $p=0.20$). This suggests that the batch data corrected for cell divisions provided a reliable measure of survival. We thus analyzed survival in the whole extended set of combinations of concentration and exposure time (see Figure 3b). We compared the survival of those conditions where we observed non-zero mortality in all replicates after correcting for division events. We again grouped these conditions in homogeneous subsets (Tukey post hoc ANOVA test; $p<0.05$; Figure 3). As in the single-cell experiments, we observed that, in exposures that lasted one hour or less, administering a given dose as a shorter pulse at higher concentrations decreased survival — more dramatically than what we observed in the single-cell experiments—

(Figure 3b). We observed the opposite trend in exposures that lasted for more than one hour: there, administering a given dose as a longer pulse at lower concentrations decreased survival. This suggests that, although total dose was again the main determinant of survival, increasing either the duration or the concentration of a pulse led to more effective killing for a given total dose.

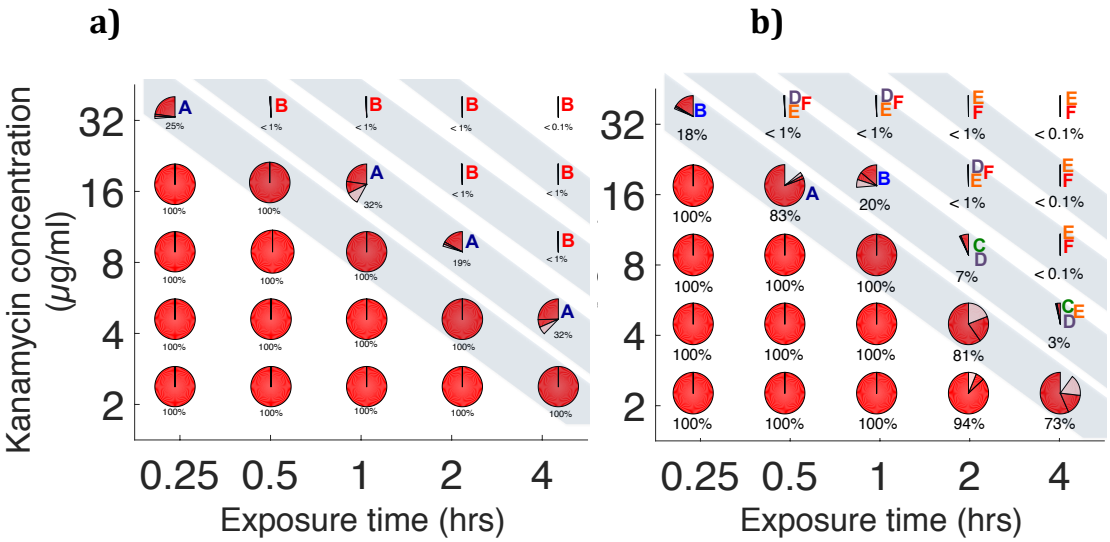

Figure 3. Extension of the range of concentration and pulse duration: experiments performed in batch (a) Percentage of survival after treatment: number of CFUs after antibiotic pulse compared to CFUs before treatment. **(b)** Percentage of survival after treatment: number of CFUs after antibiotic pulse compared to CFUs before treatment corrected by the rate of cell division during the pulse (based on the model in Figure 2b; see text for further detail). In both graphs, the fraction in red represents the mean of the replicates and the sections in pink the standard error of the mean. In cases where the ratio of CFUs before to CFUs after the pulse is above 1, survival is shown as 100%. Grey areas group equivalent total doses. Coloured letters indicate groups without significantly different means in the absolute survival after treatment, where group A has the higher mean and group B and F the lowest in Figure a and b, respectively (Tukey post hoc ANOVA test comparing groups with all replicates showing a ratio of CFUs before to after pulse below 1; $p<0.05$).

As expected, not considering the cells that divide during the pulse results on an overestimation of survival (ANOVA, $p<0.001$). However, the impact that

longer exposures have on survival is only evident when adding those divisions, perhaps because the overestimation of survival is stronger at those scenarios (e.g. 4-hour exposure to 2 and 4 μg/ml of kanamycin). Previous studies have suggested that bacterial mortality caused by aminoglycosides is more dependent on concentration than on time exposure[3], partly due to its prolonged PAE[9,13]. This could be potentially observed in our experiments if the range studied increases, i.e. testing much higher concentrations. However, it is interesting to note that division events in the opposite side (where concentration is lower and time exposure longer) might be masking higher mortality than the one usually observed when only the net population growth is measured.

Understanding survivors: how the pulse affects their growth potential

Up to now, we have focused on exploring the effects of antibiotic pulses on survival within a bacterial population. Next, we extended our analysis to investigate other effects of antibiotics on the exposed populations. Specifically, we investigated the impact of the exposure on bacterial growth potential, i.e. on the reduction of the number of divisions in exposed compared to unexposed populations. For this purpose, we first chose the time period to count divisions; we analyzed periods that extended beyond the time of the antibiotic pulse, as drug exposure usually affect cells even after the antibiotic is removed. This effect is even stronger when using aminoglycosides, as cells need time for resynthesizing ribosomal proteins[9]. It has been established that the postantibiotic effect (PAE), where cells reduce their growth, goes up to 9 hours after the pulse for high concentrations in this type of antibiotics[9]. Given this knowledge and the observation that, in our experiments, surviving cells in all conditions resumed their original division rate within ten hours after exposure (Figure

4), for our analysis we focused on a timespan comprising the pulse plus ten hours after it was removed.

The first interesting finding when observing the impact of the pulse in cell growth was that in some conditions with low total dose, divisions during the observation period were gained rather than lost (green pie charts in Figure 5). This happened, on average, in all pulses of 4μg/ml of kanamycin and in the pulse of 8 μg/ml of kanamycin for half an hour. In the condition with the lowest total dose (4 μg/ml of kanamycin for half an hour) this effect is most pronounced and consistent across all replicates. These gains were not statistically significant compared to the treatments where no kanamycin was added (Tukey post hoc ANOVA test; subsets defined by significantly different means; $p>0.05$). However, it is relevant to observe that, even in scenarios where the cells are exposed to concentrations equivalent to the MIC —as measured in batch—, survivors did not show any reduction in the number of cell divisions during (already seen in Figure 2) and after the pulse (this result might depend on the choice of the period that is used to compare cell divisions; in our case this period was ten hours).

This analysis showed the impact of time, concentration and total dose on those who survived (Figure 5). Higher total doses were once more having a dramatic effect on cells. Conditions with equivalent total doses were mostly grouped in homogeneous subsets (Tukey posthoc ANOVA test; $p<0.05$), suggesting that, at least within this regime, neither concentration nor time had a stronger impact on surviving cells. There was only an observed trend that longer pulses of low concentrations were either excluded from subsets with lower means (4 μg/ml of kanamycin for two hours) or included in subsets with higher means (8 μg/ml of kanamycin for one hour). This suggested a slightly stronger effect of concentration —in contrast with time— on growth potential of survivors.

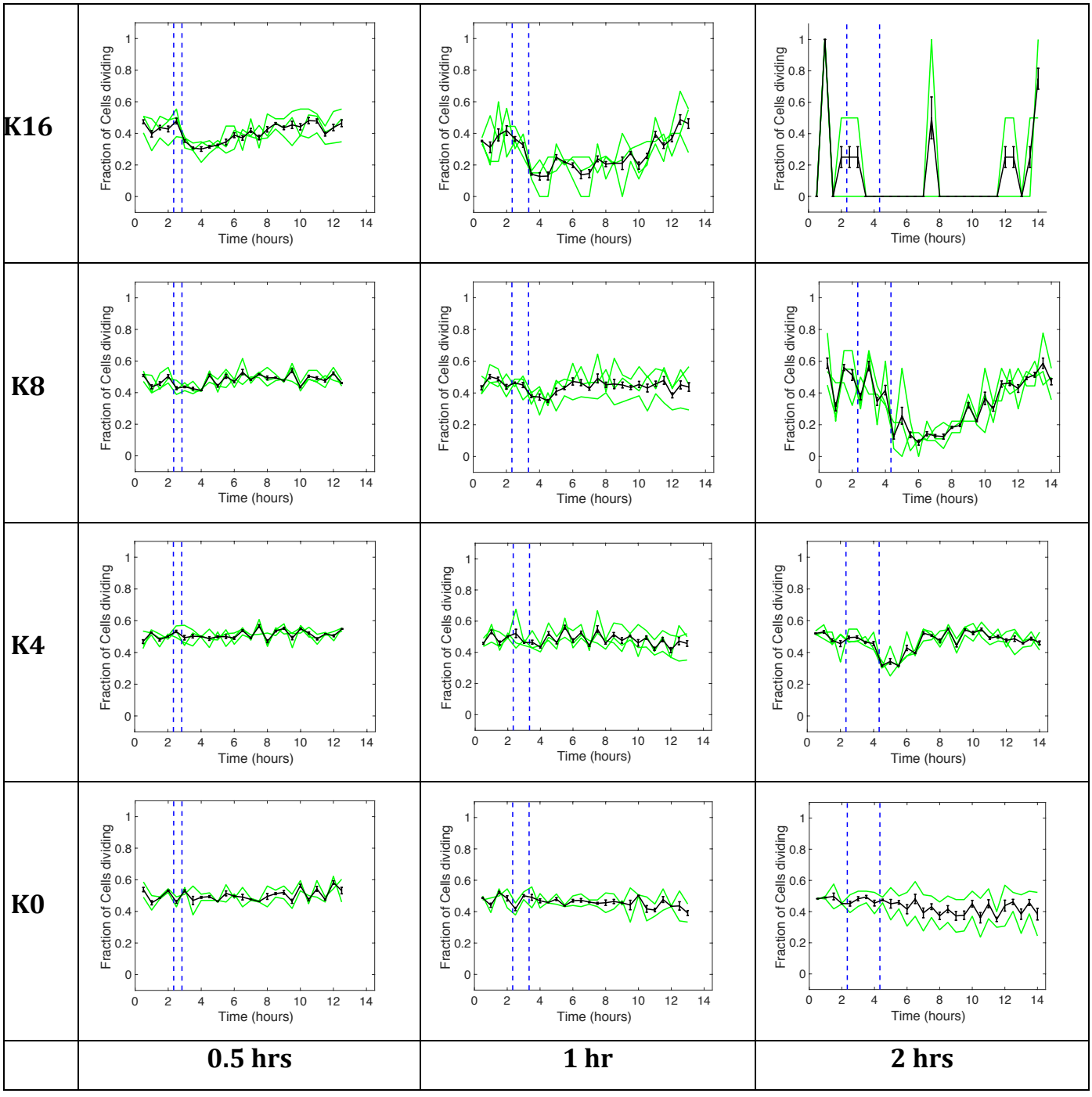

Figure 4. Division behaviour of surviving cells. For each condition studied, the green lines show the fraction of cells dividing every 30 minutes (each dot represents the period comprising the previous half an hour) for each replicate. The black lines show the mean and standard error of these replicates. The pulse is represented with vertical blue dashed lines, shown here with the 20 minutes delay from the time it was applied until it reaches the microfluidic device. It is relevant to note that the pulses that result in

higher mortality (e.g. 2 hours of 16 µg/ml of kanamycin) have a very small sample of surviving cells.

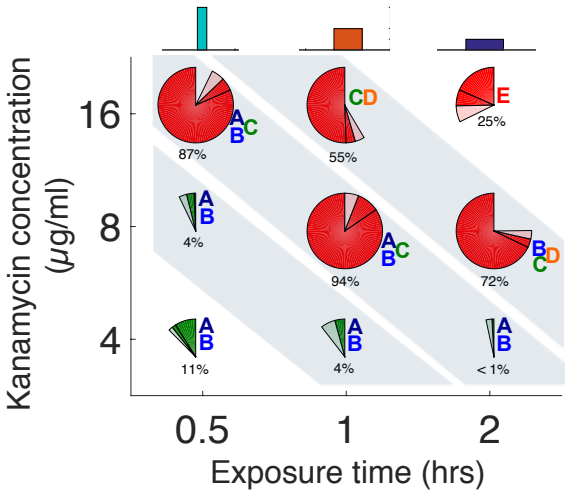

Figure 5. Percentage of divisions in surviving cells during and after the pulse compared to scenarios without antibiotic exposure. Red pies represent conditions in which antibiotic exposure resulted in a reduction of cell divisions; green pies represent conditions with an increase in cell divisions. The sections in pink and light green show the standard error. Grey areas group conditions with equivalent total dose. Identical coloured letters indicate groups without significantly different means in the fraction of divisions gained or lost. Group A has the higher mean and group E the lowest (Tukey post hoc ANOVA test; $p<0.05$).

Step-wise versus gradual increase of antibiotic concentration

Finally, we were interested in exploring whether equivalent total doses applied through a gradual, rather than a stepwise increase in concentration, would influence survival. We were able to create a continuous antibiotic gradient by feeding the chambers with two pumps, one without the drug and the other with the maximum concentration reached (16 µg/ml kanamycin) and setting the rate of the former from 0.5 to 0 ml/hour and the latter from 0 to 0.5 ml during the exposure times tested (1, 2 or 4 hours).

We found that survival neither growth potential of survivors was affected by this modification of the antibiotic pulse (Figure 6). We did this by comparing these results with equivalent doses in step-wise increases of the pulse (Figures 1b and 5) and we found no significant differences between both

types of pulses (all ANOVA; p>0.05, see table S3). These results confirmed the strong role of the total dose we observed throughout the previous experiments.

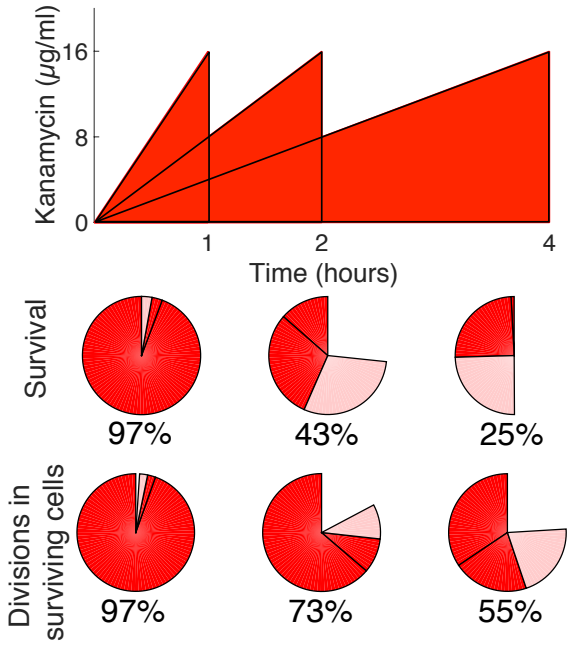

Figure 6. Effect of an antibiotic pulse when the concentration increases gradually. *E. coli* cells were exposed to antibiotic pulses where the drug increased gradually during 1, 2 or 4 hours until reaching 16 μg/ml of kanamycin. The upper row of pie charts shows percentage of survival for each condition while those in the lower row represent the percentage of the number of divisions maintained during and after the pulse. Mean survival of three replicates is shown in red; the section in pink marks the standard error of the mean across the three replicates (150-350 cells were studied for each replicate).

Materials and Methods

The strain used for all the experiments and results exposed in this chapter was *Escherichia coli* MG1655.

Single-cell experiments

Time-lapse microscopy

Cultures of *E. coli* MG1655 strain were grown overnight in M9 media supplemented with 0.4% glucose, to be afterwards inoculated in 1:20 in fresh equivalent media plus 0.01% Tween for two hours, time at which they were at exponential phase. The cells were then introduced into the chamber

of microfluidic devices, where they were fed by M9 media supplemented with 0.4% glucose and 0.01% Tween, using a peristaltic pump. Cells inside the chamber were left overnight with continuous input of fresh media and were then subjected to an antibiotic pulse of 4, 8 or 16 μg/ml during 0.5, 1 or 2 hours. Images were recorded every five minutes 2-3 hours before the pulse, during it and 10-12 hours afterwards. Division rate was subsequently measured manually, with the aid of the software Vanellus[14]. Previous research work evaluating the diffusion of small molecules in microfluidic chambers confirm that there is a separation of timescales between the (fast) diffusion of small molecules and the (slow) uptake of the cell[15], suggesting that the fast diffusion of the antibiotic had likely allowed the cells to experience the pulse during the whole period it lasted.

The protocol for the experiments performed to test the response to gradual increase of antibiotics was mostly equivalent to those performed for the rectangular pulses. The only difference was the pumps used to feed the chambers. We used syringe pumps previous, during and after exposure. For obtaining the gradual increase in antibiotic, we connected two pumps to the input of the chambers; one of the syringes contained media without antibiotics and the other 16 μg/ml of kanamycin. The rate of pumping for the first one went from 0.5 to the 0 ml/hour while the second pumped from 0 to 0.5ml/hour, throughout the 1, 2 or 4 hours of exposure, creating a gradient of the drug.

Division events as a proxy for survival

The period monitored to evaluate if a cell was alive or dead (based on the presence or absence of divisions) comprise the last 6 hours of the 10 studied after the input media with antibiotics was replaced with fresh M9 with glucose (thick blue line in Figure 1a). Survival values are not significantly

affected when division is evaluated in periods expanding to the last 9.5 or reduced to the last 2.5 hours of those 10 hours (ANOVA, p=0.27).

Measuring division events during the antibiotic pulse

We determined the number of division events per cell that took place during the antibiotic pulse (Figure 2a) by averaging the number of divisions of all cells during the treatment. In order to identify the exact moment when the bacteria cells experienced the pulse, we performed tests using the equivalent setup where the input media was food colouring. We found that a change in the media took approximately 20 minutes to reach the cells. Therefore, the division events during the pulse, shown in Figure 2a, were evaluated taking into account this 20-minute delay.

Percentage of divisions in surviving cells compared to those not exposed

In order to obtain the percentages of divisions in surviving cells (Figure 5), we used the six replicates exposed to pulses of media without kanamycin (2 for each exposure time studied). For the nine conditions with kanamycin, we counted the time during the pulse (excluding the first 20 minutes) and the 10 hours after the manual removal of the media. We compared that value with the number of divisions taking place in the 6 replicates of Kan0 for the exact period of time, regardless whether the pulse in the absence of antibiotics was for 0.5, 1 or 2 hours.

Population-level experiments (batch)

Antibiotic pulses for batch cultures

Cultures of *E. coli* MG1655 strain were grown overnight in M9 media supplemented with 0.4% glucose, to be then inoculated in 1:1000 in fresh equivalent media for four hours, time at which they were at exponential phase. The cells were plated and exposed to 0, 2, 4, 8, 16 or 32 μg/ml of kanamycin for 0.25, 0.5, 1, 2 or 4 hours in 96-well plates. The cultures were

plated before and after the pulse in M9 agar plates supplemented with 0.4% glucose. After at least 30 hours, CFUs were counted and survival was determined by the ratio of this measurement before and after treatment.

In order to correct the survival percentage by including divisions during pulse, we modified the CFUs before treatment (n_i) to $\boldsymbol{n_i * 2^d}$, where d is the division events per cell during the antibiotic pulse, obtained from the experiment data if available or from the predictions by the multiple linear regression model (Figure 2b).

Statistical analyses

Single-cell experiments

In order to evaluate whether there were differences in the mean in survival among the nine conditions studied, we performed an analysis of variance (ANOVA) on the arcsine-transformed data ($p<0.001$; same result on data not transformed). The Tukey posthoc test described in Figure 1b also gave the same homogeneous subsets on data not transformed.

When comparing number of divisions in those cells surviving, we performed the Tukey posthoc ANOVA test on (1) the fraction of divisions taking place during and after ten hours after the pulse (values above and below 1; data is not transformed) and (2) comparing absolute number of divisions during pulse and 12 hours since pulse started (noted that these number of hours included the pulse, in order to standardized the number of hours studied in all conditions). Equivalent homogeneous subsets were found in both approaches. In both analyses, the divisions taking place during the first 20 minutes after the manual change of input media are not included, as that is the time period it takes the media to reach the cells.

Batch culture experiments

In order to evaluate whether there were differences in the mean in survival among the conditions studied (those with survival below 100%), we performed an analysis of variance (ANOVA) on the arcsine-transformed data not yet corrected for divisions during pulse ($p<0.001$; same result on data not transformed). The Tukey posthoc test described in Figure 3a gave the same homogeneous subsets on data not transformed. We performed equivalent analyses for the arcsine-transformed data including the division events during treatment ($p<0.001$; same result on data not transformed). The Tukey posthoc test described in Figure 3b was affected when performed on data not yet transformed: all CDEF subsets were grouped in only one homogeneous subset.

Comparing microfluidic versus batch culture experiments

We compared the results obtained for the nine conditions studied in both experiments (4, 8, 16 μg/ml kanamycin for 0.5, 1, 2 hours of exposure). In cases where the ratio of CFUs before to after treatment was above 1 in any of the replicates in batch, this value was modified to 1. The ANOVA was performed on the arcsin transformed data but no significant differences were neither found when analysis was performed on data not transformed ($p = 0.72$). However, when comparing microfluidic experiments versus results in batch without accounting for the divisions during pulse, the ANOVA showed significant differences between both experimental approaches ($p<0.001$ in transformed and untransformed data).

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Supplementary material

	Kan 0		**Kan 4**		**Kan 8**		**Kan 16**	
	Nr Cells	*Survival Fraction*	*Nr Cells*	*Survival Fraction*	*Nr Cells*	*Survival Fraction*	*Nr Cells*	*Survival Fraction*
0.5 h	116 101	1.0000 0.9703	190 247 154	0.9789 0.9919 0.9935	163 140 209	0.9755 0.9571 0.9856	101 198 127	0.6436 0.6061 0.9764
1 h	135 160	0.9778 0.9750	290 128 103	0.9931 0.9844 0.9903	180 80 85	0.8722 0.9375 0.8941	205 86 158	0.2098 0.2326 0.0570
2 h	131 135	0.9695 0.9630	157 73 72	0.8089 0.9041 0.7361	187 169 109	0.1497 0.0533 0.1835	192 134 143	0 0.0149 0.0070

Table S1. Number of cells used for each replicate of the 12 conditions studied in the microfluidic experiments.

	Kan 0	Kan 2	Kan 4	Kan 8	Kan 16	Kan 32
0.25 h	4	4	4	4	4	4
0.5 h	4	4	4	4	4	4
1 h	4	4	4	2	4	4
2 h	4	2	3	4	4	4
4 h	4	4	4	4	4	4

Table S2. Number of replicates for each of the 30 conditions studied in batch culture experiments.

Total doses µg/ml Kanamicin /hour	**Survival (stepwise versus gradual) P-values**	**Growth potential of survivors (stepwise versus gradual) P-values**
32	0.35	0.35
16	0.48	0.15
8	0.24	0.45

Table S3. ANOVA P-values for comparing equivalent total doses in stepwise and gradual increase of antibiotics. Survival: Absolute percentage of survival was used for comparison, p-values shown for data after arcsin transformation; equivalent results ($p>0.05$) were obtained when data was not transformed. **Growth potential of survivors**: p-values shown for data without transformation; equivalent results ($p>0.05$) were obtained when data was arcsin transformed in the cases when this was possible.

Chapter 3

Can we predict cell survival upon an antibiotic pulse?

With contributions from:

Martin Ackermann, ETH Zurich and Eawag
Conception and design of the study together with AMMC;
essential involvement throughout the research process;
critical revision of the chapter

Daniel J. Kiviet, ETH Zurich and Eawag
Development of the experimental setup and software for analysis;
critical input throughout the research process

Abstract

During the last decades, it has become clear that phenotypic differences between genetically identical bacteria can play an important role in determining whether or not individual cells survive exposure to antibiotics. For instance, a tiny fraction of bacterial cells that are in a metabolic inactive state, called persisters, are known to be more tolerant to antibiotics. Little work, however, has been done to understand the factors allowing cell survival in the early phase of killing upon antibiotic exposure, before persister numbers become relevant. In this chapter, we looked for phenotypic predictors of survival at the single-cell level, using information about division events before, during and after *E. coli* cells were exposed to kanamycin pulses, as described in Chapter 2. We found no evidence that interdivision time previous to exposure, as a proxy for growth rate, predicts survival. However, in conditions with low survival (between 5% and 50%), we found that cells that did not divide at all in the hours previous to exposure (potential persisters) showed higher levels of survival. We also asked whether the cell cycle position of a cell at the onset of exposure could influence survival, but found no evidence to support this. Finally, we saw that early divisional behaviour (i.e. division frequency during and immediately after the pulse) can predict survival.

Introduction

Bacterial cells can survive antibiotic treatment either by being genetically resistant or by being in a phenotypic state that confers protection from a drug. As opposite to genetic resistance, which defines the identity of a population, phenotypic traits can sometimes be found in a subpopulation of a clonal group of cells that tolerate only transiently the presence of a drug[1,2]. Persister cells are an example of a subpopulation that can survive antibiotic treatment as a consequence of their phenotypic state[1–4]. Persistence —in one of its definitions— refers to the phenomenon where a minor fraction of cells survives antibiotic exposure a long time after most of the population have been killed in a log-linear fashion[1,4,5]. Their survival is attributed largely to their reduced growth rate[5].

In this chapter, we were interested in exploring potential phenotypic traits associated with survival during the log-linear death phase, before the 'persistent fraction' becomes significant. Are there phenotypic differences that cause some cells to be more sensitive than others during this early phase of death? Little work has been done to understand survival and death in this early phase of the kill curve. But there is evidence that, in scenarios where survival is much higher than the one expected in a population composed only of 'persisters' —in the definition previously described—, individual traits have been associated with higher chances of survival. Cell cycle position in *Caulobacter crescentus*, for example, has a strong impact on survival to sodium chloride[6] and expression of virulent genes in *Salmonella typhimurium* increases survival to antibiotic treatment[7].

Here we studied whether phenotypic traits of individual cells were associated with survival after an antibiotic pulse within the log-linear death phase (Figures 1 and S1). *Escherichia coli* cells were exposed to a range of 4-

16 μg/ml of the aminoglycoside kanamycin for 0.5-2 hours. These experiments are fully described in Chapter 2. In the current chapter we only focus on the five conditions where the survival percentage lies between 5% and 95% (Figures 1a and 1b). We investigated traits that can be obtained from monitoring division events previous, during and shortly after the pulse, such as interdivision times and cell cycle stage at the onset of the exposure.

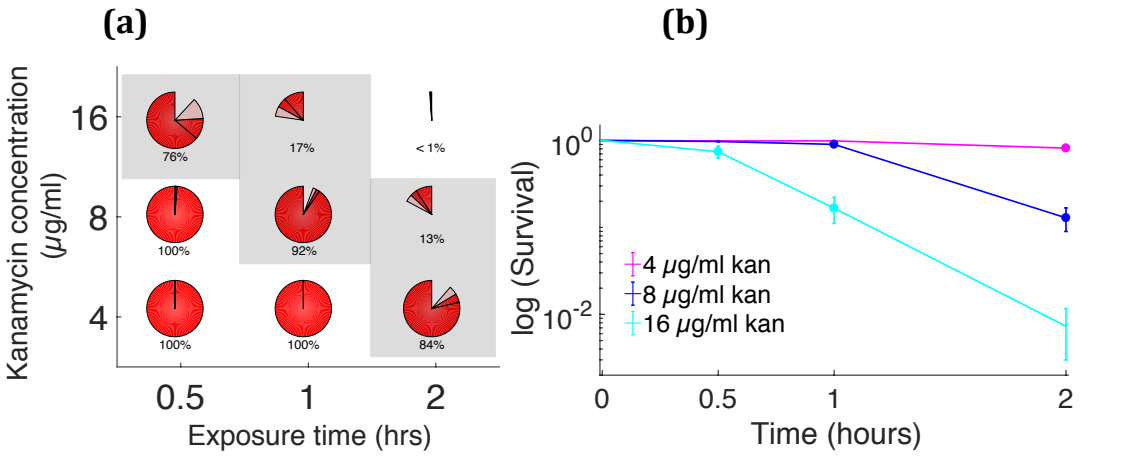


Figure 1. Percentage of survival of *E. coli* cells after a pulse of kanamycin (results taken from Chapter 2). (a) Percentage is normalized to the survival rate in the absence of kanamycin; individual pie charts show mean survival of three replicates in red and the section in pink refers to the standard error of the mean (see Chapter 2 for further details). Conditions shaded in grey are the ones studied in the current chapter. **(b)** Time-kill curves show mean and standard error of survival in the conditions illustrated in Figure 1a; those with a filled circle in the mean value are the ones we studied in this chapter (these are the same conditions as the shaded area in Figure 1a).

Growth rate could be potentially relevant for bacteria exposed to aminoglycosides. These antibiotics target the ribosome, inhibiting protein synthesis and generating protein mistranslation[8,9]. There is, for instance, a correlation between ribosome content and nutrient-dependent growth rate[10], which has been shown to be relevant in response to sublethal concentrations of ribosome-binding antibiotics[11]. Additionally, as cells enter stationary phase, ribosomes enter into an hibernation state that increases

tolerance to aminoglycosides[12]. Associations between the number and the state of ribosomes and growth rate and survival are usually analysed by varying the quality and quantity of nutrients; it is not clear whether phenotypic variation in these traits within one population can also explain variability in survival.

It is also unclear how the cell cycle stage of an individual exposed to antimicrobial treatment impacts its survival. In eukaryotes, where the stages of the cell cycle are well defined, there is evidence of such a role in certain stresses. Yeast cells exposed to copper, for example, have higher chances of survival if they are in G1 phase when they are exposed[13]. Neuroblastoma cells in a tumour also show different response to treatment depending on their cell-cycle position[14]. In bacteria, however, the issue is less clear. The bacterium *Caulobacter crescentus* shows significant differences in survival when exposed to salt stress depending on the cell cycle stage[6]. On the other hand, survival of *E. coli* cells exposed to heat stress has not been found to be influenced by this factor[15]. As far as we know, the role of cell cycle stage in bacteria exposed to antibiotic stress has not been addressed.

Results and Discussion

Interdivision time previous to exposure does not predict survival

In order to evaluate whether growth rate before the antibiotic pulse could influence survival, we monitored the cells two hours previous to exposure (plus the 20-minute delay that it takes for the media to reach the cells, as reported in Chapter 2). We registered the division events in this period and used the length of time between the penultimate and the last division event before the pulse as a proxy for growth rate. We then applied a logistic

regression using the minutes of this interdivision period to test whether it could be used as a predictor for survival. We implemented this procedure for each replicate in all the conditions studied (5 conditions and 3 replicates each, giving a total of 15 experiments) and obtained their corresponding regression models as shown in Figure 2a (the red line represents the only case where the relationship between both variables was significant, i.e. one of the three replicates where cells were exposed to 16µg/ml to kanamycin). We then plotted the beta values of all the experiments against their p-values, where the sign of beta indicates whether there is a positive or negative correlation between the two variables and its p designates the significance of this relationship (Figure 2b). Except for the one replicate mentioned, none of the logistic regression models showed a significant association between the length of the interdivision times and the chance of survival (Figure 2b).

We hypothesized that growth rate could influence the cell fate after the antibiotic exposure due to different reasons. In first place, there are several studies indicating that slow-growing populations are more tolerant to antibiotics. This, however, has been mainly done through manipulating growth rate by modifying the growth condition[4,16,17] (i.e. increased tolerance found at the level of population, not heterogeneously in a same clonal group exposed to the same environment) or, through the study of persisters, which is a very small number not necessarily relevant for the survival percentage we see in our conditions[4,5,18]. Growth rate could also potentially be relevant for this specific type of drug, as kanamycin targets ribosomes. Ribosome content is linearly related to growth rate, when growth rate is manipulated by nutrient quality[10], and this variation is known to affect how bacteria respond to ribosome-targeting antibiotics, at least at subinhibitory concentrations[11]. However, it is unknown whether the linear correlation between ribosomes and growth rate is maintained within a clonal population experiencing the same nutrient quality. It is also unknown how

much variation exists in the ribosome content within a clonal population, although superresolution fluorescence microscopy might be helpful to soon unravel this question[19,20].

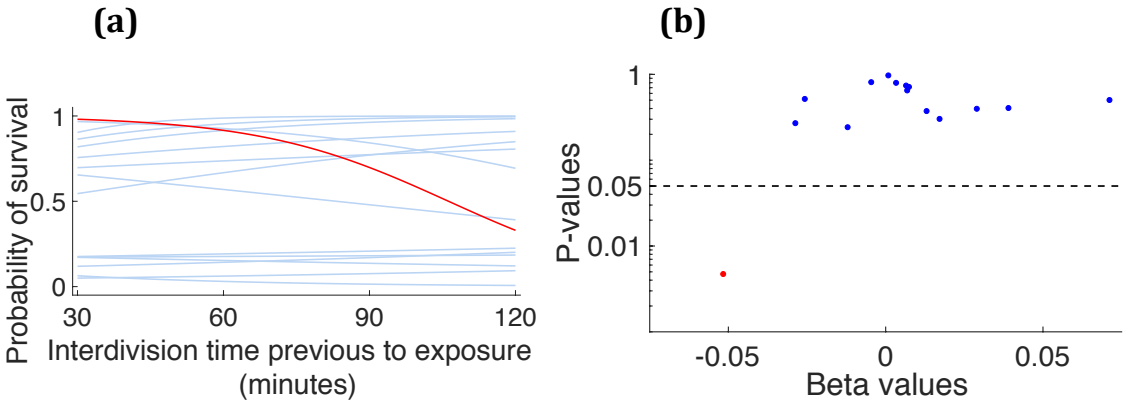


Figure 2. Logistic regression to evaluate the role of interdivision time previous to exposure on survival. (a) Logistic regression model for the 15 experiments performed (5 conditions, 3 replicates each). Except for the one experiment marked with a red line, none of the experiments showed a significant effect of the interdivision time on the probability of surviving exposure to antibiotics. **(b)** The beta values and significance values (p-values) obtained from the regression models in Figure 2a are plotted. The beta value describes how the probability of survival changes with increasing interdivision time; a positive beta means that survival increases with longer interdivision times (as we would expect if cells that grow more slowly are more tolerant); a negative value means that survival decreases with increasing interdivision time. The p-value associated with each beta-value denotes whether the effect is statistically significant. The dashed line separates significant ($p<0.05$) from nonsignificant coefficient estimates.

Our results, however, suggested that interdivision time previous to exposure did not predict survival. Research on single-cells has shown that growth rates and inverse interdivision times are correlated across slow growth conditions[21] —as the one studied in this chapter—, so it is possible to translate these results into the observation that the variation in growth rate among the cells in our experimental setting did not seem to influence survival probabilities upon exposure to kanamycin. One possible

explanation for this finding is that the variation in growth rate and interdivision time in clonal populations under the conditions studied here might be too small to manifest as survival differences. In particular, the variation in growth rate might not be linked with ribosome content or proton motive force, and thus might not translate into variation in phenotypic traits that are linked with tolerance to aminoglycosides.

Potential role of persister-like cells in survival in conditions with high mortality

In the previous analysis, we only included cells that had a defined interdivision time (i.e. two division cells during the monitored period previous to treatment). However, there was a small fraction of cells that did not divide throughout the 140 minutes before the pulse. We do not know the last time these cells divided; they could potentially have been in a growth-arrested state for a long period of time, a characteristic of persister cells. Persister cells, in that definition, are usually present as a very small fraction in a population (10^{-4} to10^{-6} in traditional studies of exposure to beta-lactam antibiotics[5,18]; these numbers are usually defined by surviving cells, not by growth rate previous to treatment).

The potential persister cells in our experiments might come from two different sources: they could have emerged during stationary phase (type I persisters) or have been produced through phenotypic switching during exponential phase: (type II persisters)[5]. There is a feature of our experimental system that increases the probability of having type I persisters compared to a batch experiment that has grown for the same amount of time in exponential phase. This is the fact that cells monitored in our experiments were placed inside the microfluidic chambers after two hours of exponential growth of a population coming from an overnight

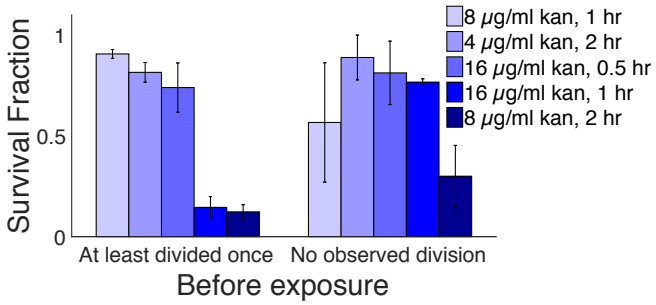
culture, and then fed with continuous media without antibiotics for 20-27 hours before treatment. The majority of cells that entered into exponential phase during the first two hours —before being placed inside the chambers— were already in a balanced growth when they were exposed to kanamycin. However, the minority of cells that had not yet initiated growth and ended up in the bottom of a chamber did not go through the dilution process they would have gone through in a bulk experiment. While we thus do not know whether the persister-like cells observed here were formed during stationary or exponential phase, it is plausible that a substantial fraction derived from stationary phase and were protected from dilution by fast-growing cells during growth in our microfluidic device.

We therefore investigated whether these cells in apparent growth-arrested state showed higher levels of survival. We grouped all the cells studied in two categories: those that at least divided once in the lapse monitored before the pulse and those that did not divide at all (on average 3% of the population studied). When comparing the fraction of divisions in both categories, we found no significant differences in the fraction of survival for conditions where survival was high (above 75% of the population in average; ANOVA, p-values: 0.32, 0.58, 0.73 for 8, 4, 16 μg/ml of kanamycin for 1, 2, 0.5 hours respectively). However, in conditions where survival was low, i.e. only 10-20% of cells survived, the fraction of survivors was higher for the potential persister population, although only significant in one of the two conditions (ANOVA p-values: $p<0.001$ and $p=0.32$ for 16 and 8 μg/ml of kanamycin for 1 and 2 hours, respectively). The different fractions of survival in both categories and the number of potential persisters are shown in Figure 3.

These findings suggested that the growth-arrested state of cells was conferring higher survival in conditions where the total dose was high.

However, the presence of persisters only explained to a very small degree the fate of cells in the log-linear death phase. The survival fraction in the category of cells dividing once (Figure 3a) illustrates this.

(a) **(b)**



Number and (fraction) of cells with no observed division			
	10 (0.06)	1 (0.01)	1(0.01)
	3 (0.02)	2 (0.03)	1 (0.01)
	2 (0.02)	6 (0.03)	16 (0.13)
	5 (0.02)	4 (0.05)	4 (0.03)
	4 (0.02)	5 (0.03)	5 (0.05)

Figure 3. Survival fraction of potential persistent cells (a) Survival fraction of each condition studied for cells dividing at least once during the 2:20 hours monitored previous to exposure and those with no observed division (potential persisters). Mean and standard error of three replicates for each case is shown. When comparing both categories of cells, there is only a significant increase of survival in potential persisters for those exposed to 16 μg/ml of kanamycin during one hour (ANOVA, $p<0.001$). **(b)** Number of cells with no division observed for each of the replicates and the fraction of the population that it represents.

Cell cycle stage at the moment of pulse does not predict survival

We then tested whether the cell cycle position of each cell at the onset of the antibiotic exposure affected the probability to survive. We used the minutes that had elapsed since the last cell division as a proxy for cell cycle stage and categorized each cell based on this variable. By comparing the survival fraction of cells for each category in all the conditions studied, we found no evidence that the cell cycle stage of a cell had an effect on that cell's survival (Figure 4; ANOVA p values for each conditions $p>0.05$; see table S1).

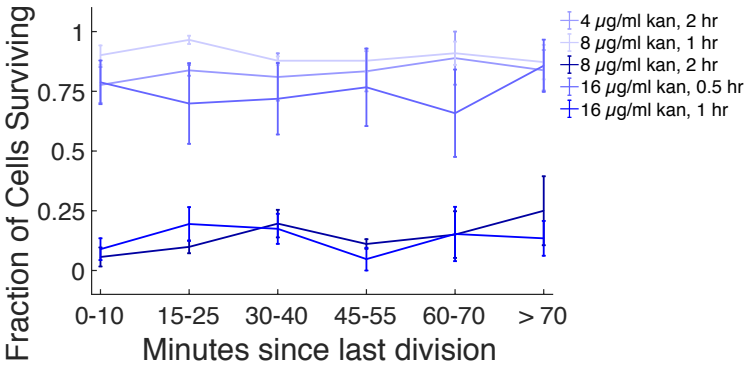


Figure 4. Fraction of cells surviving the antibiotic pulse as a function of the cell cycle stage at the beginning of the pulse. Fraction of cells surviving depending on the number of minutes since the last division at the moment of the stress (a proxy for cell cycle position). When comparing all six categories of cell cycle stage in all five conditions with three replicates each, none of them showed a significant change in survival (ANOVA; all p-values >0.05). The intensity of the blue lines depicts mortality.

Whether the cell-cycle position of a bacterial cell influences its response to stress is a question that needs further exploration. In contrast with growth rate, where there is a more general trend in the observations (slow growth rate correlates with higher survival, with few exceptions), the role of cell cycle stage might be much more variable across species and dependent of the nature of the stress that is studied. Variability across species might be expected, for instance, given the different strategies for chromosome replication, which is the target of some stressors. We would expect a role of this trait on survival to aminoglycoside exposure if drug uptake or ribosomal content would be influenced by the cell cycle stage.

Early reproductive behaviour after the pulse predicts cell survival

Finally, we explored how early it was possible to determine whether a cell would eventually survive or die after the pulse of kanamycin. We considered the number of divisions during and shortly after the pulse (30 to 120 minutes after the end of the pulse) and used them as the explanatory variable for survival at the single-cell level. We performed a logistic

regression on the 15 experiments we performed (3 replicates for 5 conditions) and obtained their beta estimates and their respective p-values, where the sign of beta indicates a positive or negative correlation between number of divisions during the period studied and the fate of the cell (as described in the legend of figure 2). Figure 5a shows this procedure applied to the period of the pulse plus 90 minutes after it was removed. All beta values in this case are higher than zero, i.e. implying a positive correlation between the number of divisions in this interval and the probability of surviving antibiotic exposure; 12 of the 15 (0.8 fraction of the experiments) are significant. We applied the same procedure for the period comprising the pulse plus 30, 60 and 120 minutes. These results were plotted on Figure 5b, where the bars represent the fraction of all the 15 experiments that showed a positive correlation between number of divisions and survival, showing in blue those with significant p-values ($p>0.05$) and in black those not significant.

We found that the early reproductive behaviour of cells, i.e. their division frequency immediately after the pulse, was a good predictor of survival. The positive correlation was visible as early as when the studied period comprised the pulse plus half an hour, even if the p-values were not yet significant. This might reflect the fast bactericidal action of the antibiotic.

The absence of an early predictor given this data, though, encourages further investigation, testing for instance individual variation in gene expression or macromolecular content. Potential candidates for such a role are the individual variation in the expression of the outer-membrane porins[22], where aminoglycosides enter through, and single-cell variation in the number of ribosomes[20].

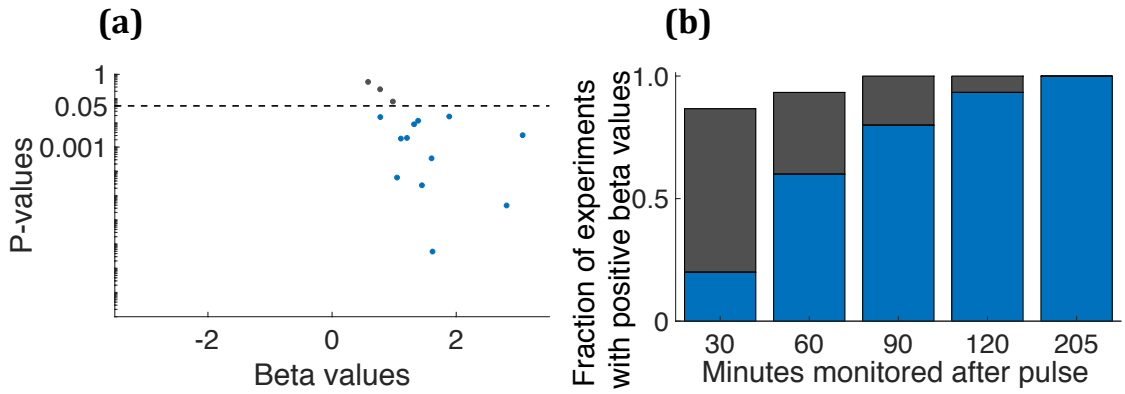


Figure 5. Early reproductive behaviour after the pulse predicts survival. (a) The beta and their associated p-values plotted for the logistic regression performed for each replicate of each condition, where the number of divisions of a cell during and 90 minutes after the pulse were used as predictors of survival. All beta values are positive and 12 of the 15 experiments showed significant p-values (blue), indicating that those cells with higher number of division in this period have higher chances of survival. **(b)** The same analysis than the one performed in (a) was applied for 30, 60 and 120 minutes after the pulse. The blue bars indicate the fraction of experiments with significant positive beta values and the stacked gray represent those positive but not significant beta values. Significance in all positive beta values is reached until studying 205 minutes after the pulse.

Materials and Methods

The strain used for all the experiments and results exposed here was *Escherichia coli* MG1655. All the analysis performed in the current chapter was based on the experimental data described in Chapter 2.

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Supplementary material

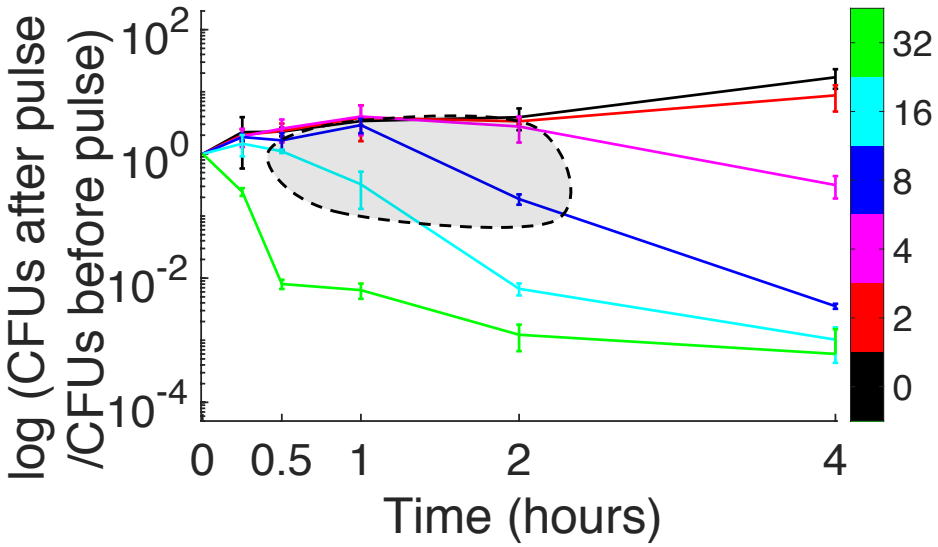

Figure S1. Time-kill curve of *E. coli* cells exposed to kanamycin (data obtained from batch experiments in Chapter 2). The graph plots CFUs after variable concentrations of kanamycin during 0.25-4 hours. The shaded area indicates the regime we explored in this chapter in the context of this traditional data illustration (see figure 1b for the exact conditions analysed with single-cell data).

ANOVA p-values for cell cycle analysis	
4 μg/ml during 2 hours	0.96
8 μg/ml during 1 hour	0.70
8 μg/ml during 2 hours	0.56
16 μg/ml during 0.5 hour	0.94
16 μg/ml during 1 hour	0.72

Table S1. ANOVA p-values for comparing survival fractions depending on cell cycle stage at the beginning of the antibiotic pulse. Six categories (as in Figure 4a) were compared for each condition (three replicates per condition).

Chapter 4

The effects of sublethal concentrations of antibiotics on single-cell growth parameters during feast and famine

With contributions from:

Martin Ackermann, ETH Zurich and Eawag
Conception and design of the study together with AMMC; essential involvement throughout the research process; critical revision of the chapter

Bruce Levin and **Véronique Perrot**, Emory University
Conception and design of the study at early stages

Daniel J. Kiviet, ETH Zurich and Eawag
Development of the experimental setup and software for analysis; revision of the chapter

Abstract

Bacteria are often exposed to antibiotics at concentrations that are lower than those used in clinics. In spite of the ubiquity of these conditions and their role in promoting the evolution of antibiotic resistance, we only have limited understanding of how they affect bacterial physiology. Our goal here was to analyze how low concentrations of ribosome-targeting antibiotics affect bacteria at different stages of a growth cycle. We exposed populations of *Escherichia coli* to gentamicin or tetracycline and analyzed the impact on the growth dynamics. At very low concentrations, gentamicin did not affect the growth rate during exponential phase but reduced the growth yield. In contrast, tetracycline affected the growth rate at the exponential phase but had a lower impact on the yield attained. In order to gain insights into the single-cell basis of these observations, we performed time-lapse microscopy of bacteria that experienced the feast and famine conditions that are typical for batch growth. Our results suggested that the observations at the population level were explained, to a large extent, by how antibiotics affected the division rate of individual cells. It was particularly interesting to confirm that, by being exposed to low concentrations of gentamicin, cells stopped dividing earlier and entered into stationary phase, in accordance to the reduced yield observed at the population level. A subsequent series of experiments suggested that cells exposed to antibiotics could be depleting resources faster during exponential phase. This was in agreement with recent findings that cells exposed to subinhibitory concentrations deplete amino acids faster than unexposed cells. Further preliminary experiments, however, suggested that this phenomenon was not occurring when a single sugar, e.g. glucose, was the main carbon source. Increasing our understanding of how bacterial physiology is affected by low antibiotic concentrations is crucial both for its clinical implications and for what it reveals about how bacteria respond to low levels of stress.

Introduction

For several decades, humans have used antibiotics to fight bacterial infections. Since the early days of the discovery of antibiotics, the treatment doses were chosen to be high enough to eradicate the pathogen without being toxic towards the host. In most clinical settings, this simple logic has been maintained up to now, only being recently questioned, for example, by an increasing number of scientists and medical doctors advocating for individualized antibiotic dosing taking into account other factors[1]. However, given the purpose of antibiotic use, scientific research was for a long time mainly focused on studying how high antibiotic concentrations —the ones employed for medical treatment— affect or kill bacteria.

Bacteria are nonetheless frequently exposed to antibiotic concentrations that are lower than those used in the clinical context. These concentrations are commonly referred to as non-lethal, subinhibitory or subMIC, as they are below the known amounts needed to inhibit growth after 24 hours of exposure (the minimal inhibitory concentration, MIC). Bacteria can encounter temporal and spatial gradients that include low concentrations in common environments, such as in sewage water, lakes, and rivers, but also inside hosts, e.g. patients and livestock undergoing treatment[2]. Many factors contribute to the presence of such gradients for instance the natural production of these molecules by a wide range of organisms, the dosing rationale in clinics and the distribution of antibiotics inside the patient body. Given the ubiquity of such low concentrations, it is relevant to understand how they affect the rates of bacterial cell division and death.

In addition to their occurrence in numerous environments, subinhibitory drug concentrations have been recently pointed out as important factors in bacterial infection and treatment. Importantly, they influence the evolution

of resistance as they select and enrich for potential mutants already present in a population[3]. These concentrations are also known to trigger variability at the genetic and phenotypic level, by affecting horizontal gene transfer and recombination rates, inducing mutagenesis or simply affecting the physiological state of the exposed cells[4,5]. Recent studies also suggest a role of antibiotics at low concentrations acting as signaling molecules[6–8], for instance, affecting various aspects of bacterial behavior, such as virulence[6] and biofilm formation[6,9].

However, most studies exploring bacterial responses to low antibiotic concentrations have been focused on periods of exponential growth. We therefore lack, for instance, knowledge about how the effects of antibiotics at low concentrations interact with nutrient depletion. Moreover, bacterial responses to low concentrations of antibiotics are typically measured at the population level and it is often not clear how effects of antibiotics on single cells scale up to give rise to patterns observed at the population level.

In this work, we explored the growth response to two antibiotics targeting the bacterial ribosome: gentamicin, which binds to the ribosome in an irreversible way, and tetracycline, which binds reversibly. Our results show that, at concentrations equivalent or below half the MIC, the two antibiotics differ in their effect on the different growth phases. By performing single-cell experiments to study this response, we learnt that the division rate of cells was notably influencing the dynamics observed at the population level.

We then investigated bacterial responses to low concentrations of gentamicin in more detail, as we found that the growth rate of exposed cells was not affected during exponential growth. Cells, however, had a lower yield than in the absence of the drug and analysis of division rate in single cells suggested that this was due to an early cessation of growth. When we

tested different hypotheses to understand this phenomenon, we found that cells were entering earlier into stationary phase, likely due to an earlier depletion of nutrients.

As our experiments were performed in LB (Lysogeny broth) media (LB 10%, see methods for further details), it is likely that the early entrance in the presence of gentamicin resulted from the faster amino acid depletion, in accordance to recent findings[10]. Adding a carbon source, glucose, during the transition to stationary phase, increased the yield of exposed cells, without increasing the yield of those not exposed. When we asked whether this faster depletion of nutrients could also be taking place in environments with other types of carbon sources (i.e. sugars, specifically carbon), our preliminary results suggested that this was not the case. In fact, we observed the opposite effect: transition to stationary phase took place later and higher yield was reached in such scenarios.

Together, our data strongly suggest that the type and availability of nutrients play an important role in how cells respond to low concentrations of antibiotics.

Results and Discussion

Bacterial response to low antibiotic concentrations at different growth phases

When we exposed *E. coli* to a range of subinhibitory concentrations of gentamicin and tetracycline, we observed different growth responses for the two antibiotics: gentamicin at low concentrations did not have a significant effect on the growth rate during the exponential phase (ANOVA, p=0.13) but

it reduced the growth yield (ANOVA, $p<0.001$; figures 1a and 1c). The yield continuously decreased with increasing gentamicin concentration, reaching for instance only 40% in populations exposed to half the MIC (0.3 μg/ml); interestingly, even at this concentration of gentamicin, we did not find a significant effect on the growth rate during the exponential phase. In the case of tetracycline exposure, growth rate and yield were both affected at these low concentrations (figures 1b and 1d). The growth rate during exponential phase decreased with increasing tetracycline concentration (ANOVA, $p<0.001$) while the yield increased at extremely low concentrations (0.05 μg/ml tetracycline, ANOVA, $p<0.005$ both after 24 and 40 hours of exposure) and then decreased when the tetracycline concentration increased (but did not exceed the minimal inhibitory concentration; ANOVA, $p<0.001$ both after 24 and 40 hours of exposure). This reduction of yield is, however, less pronounced for tetracycline than for gentamicin. Bacteria exposed to half the MIC of tetracycline (0.2 μg/ml tetracycline) reached 50% of their maximal yield after 24 hours and even 86% after 40 hours.

While both antibiotics target the 30S subunit of the bacterial ribosome and inhibit the step of elongation during protein translation, their different mode of action might partially explain these contrasting observations. Gentamicin binds in an irreversible manner and causes interruption of the amino acid chain elongation. Like other aminoglycosides, it is known to prevent elongation by inhibiting translocation of the mRNA-tRNA complex, causing also protein mistranslation. It is considered a bactericidal antibiotic for *E. coli*, meaning that the antibiotic concentration needed to reduce the viable bacterial density by at least 99.9% under specific conditions in vitro is less than four times the MIC[11]. The mechanisms that induce cell death after gentamicin exposure include ribosomal mistranslation, accumulation of protein aggregates, changes in membrane potential and alterations to the

membrane composition and integrity[12,13]. At the low concentrations used here, however, it is not clear whether the lower yield was a result of cell death or earlier entrance to stationary phase. On the other hand, tetracycline binds to the ribosome in a reversible way, mainly interrupting the delivery of tRNAs, but not creating mistranslation. It is therefore considered to have a bacteriostatic effect, i.e. inhibiting cell division rather than causing cell death. Our first results suggested that this slowdown in division rate was already observed at low concentrations of tetracycline, but the effect on yield was not as pronounced.

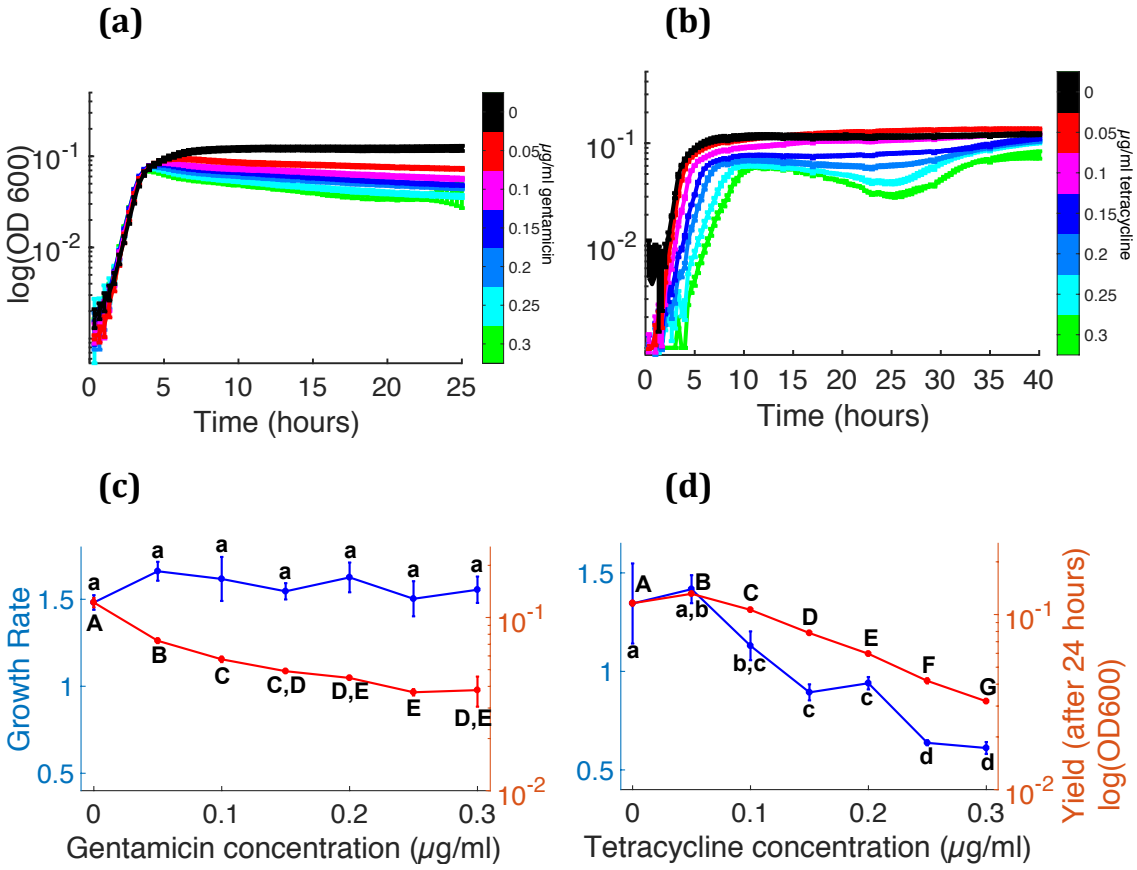

Figure 1. Bacterial growth at subinhibitory concentrations of ribosome-binding antibiotics. **(a,b)** Population growth: mean and standard deviation of the O.D.$_{600}$ of three replicates (*E. coli* MG1655) over 25 hours of exposure to subMIC concentrations of gentamicin (a) and 40 hours of exposure to subMIC concentrations of tetracycline (b) (MIC for gentamicin: 0.6 µg/ml; MIC for tetracycline: 0.4 µg/ml). **(c,d)** Mean and standard deviation of the growth rate (blue) and yield after 24 hours (red) of the three replicates. Identical lowercase letters indicate groups without

significant differences in their growth rate and uppercase letters those without significant differences in their yield (Tukey post hoc ANOVA test; $p<0.05$)

These first experiments performed at the population level did not provide details about the single-cell growth behavior that underlay the population-level observations. In particular, for the experiments with gentamicin, it is unclear whether the lower yield was due to a decline in the division rate of some or all cells or from the death of a fraction of the population.

Disentangling the role of division and death is thus key to get a better understanding of how these antibiotics affect bacterial physiology. This is particularly relevant in the context of the evolution of resistance, where higher rates of division would for instance increase the probability of the emergence of mutants. Additionally, analyzing the growth behavior of individual cells also allows studying the degree of heterogeneity in the response to low concentrations of antibiotics among genetically identical cells.

Division rate is an important factor in the bacterial growth dynamics at low antibiotic concentrations.

To analyze the growth behavior at the single-cell level, we performed single-cell microfluidics monitored through time-lapse microscopy. Our setup consisted on growing cells inside chambers in a microfluidic device. Each chamber fitted around 20 cells and was connected to a wider channel where media flowed at a constant rate. In these experiments, this input channel was connected to a flask with a growing *E. coli* culture. Bacterial cells inside the chambers therefore faced similar conditions to the ones experienced by the culture growing inside the flask. We used time-lapse microscopy to

monitor the division of the cells in the bottom of each chamber by imaging every 5 minutes (figure 2a, see methods).

Using this setup, we carried out equivalent experiments to those in batch and measured the cell division frequency of cells in the absence or presence of 0.05 μg/ml of gentamicin and 0.1 μg/ml of tetracycline. An initial phase of frequent division in both scenarios, followed by a deceleration period up to no division, indicated that cells inside the chambers were indeed experiencing a similar environment to the batch cultures feeding them, moving from exponential to stationary phase (figures 2b and 2c).

The analysis of the frequency of division in these microfluidics experiments suggested that the timing of division of individual cells explained to a large extent the growth dynamics observed at the population level. Cells exposed to sublethal concentrations of gentamicin achieved the same maximal division rate during exponential phase as those not exposed to the antibiotic (ANOVA, $p=0.60$). However, the frequency of division decelerated earlier during batch growth in the presence of gentamicin and exposed cells also stopped dividing earlier (figure 2b). As it will be discussed below, this observation can explain the lower yield observed in the batch experiments. Cells exposed to low levels of tetracycline showed a different division dynamics. During the first hours of growth, i.e. the exponential phase, they divided less frequently than the ones growing in the absence of antibiotics (ANOVA, $p<0.001$). However, during the transition to stationary phase, their frequency of division was similar to the control cells (ANOVA, $p=0.37$) (figure 2c).

Our next goal was to scale up from single cells to populations and ask whether the single-cell growth patterns that we measured can explain the observations at the population level described further above. To do so, we

performed a numerical simulation of the growth of population based on the division dynamics measured at the individual level (see methods for further details). We observed a qualitative agreement between the outcome of this numerical simulation and the patterns observed in batch experiments (figures 2d and 2e). This confirmed both that the cells inside the chambers experienced a similar environment to those grown in batch and that division could partially explain the growth dynamics observed. For gentamicin, the impact of the antibiotic on growth yield was smaller in the batch experiment compared to the simulation based on single-cell data. This could be potentially explained by the fact that the former was measured in terms of optical density (O.D.) while the latter was based on the numbers of cells. As cells are known to be smaller during stationary phase, the difference in terms of O.D. was probably smaller than the difference in the number of cells (figure 2d). The simulation for tetracycline exposure based on the single-cell division dynamics also confirmed the pattern observed at the population level: growth rate during exponential phase was lower in exposed cells and cells, therefore, potentially took longer to reach the same yield as in the absence of the antibiotic (figure 2e).

Death rate was not measured in the single-cell experiments and thus also not included in the numerical simulation (since cells cease to divide upon entering stationary phase, it was not possible to use cessation of cell division as a proxy for cell death). Nevertheless, initial observations of the time-lapse microscopy images suggested that the single-cell mortality rates were low. Further studies should be performed to confirm this, for example, using fluorescent markers that allow distinction between alive and dead cells. From what we observed in both batch and single-cell experiments at the lowest tetracycline concentrations and, given the bacteriostatic nature of this antibiotic, it is likely that death rate barely played a role in the growth dynamics observed (figure 1b). In contrast, in the experiments where cells

were exposed to gentamicin, the O.D. decreased over time during stationary phase (figure 1a), presumably either because of mortality or because of a change in a cellular phenotype that affected the optical density.

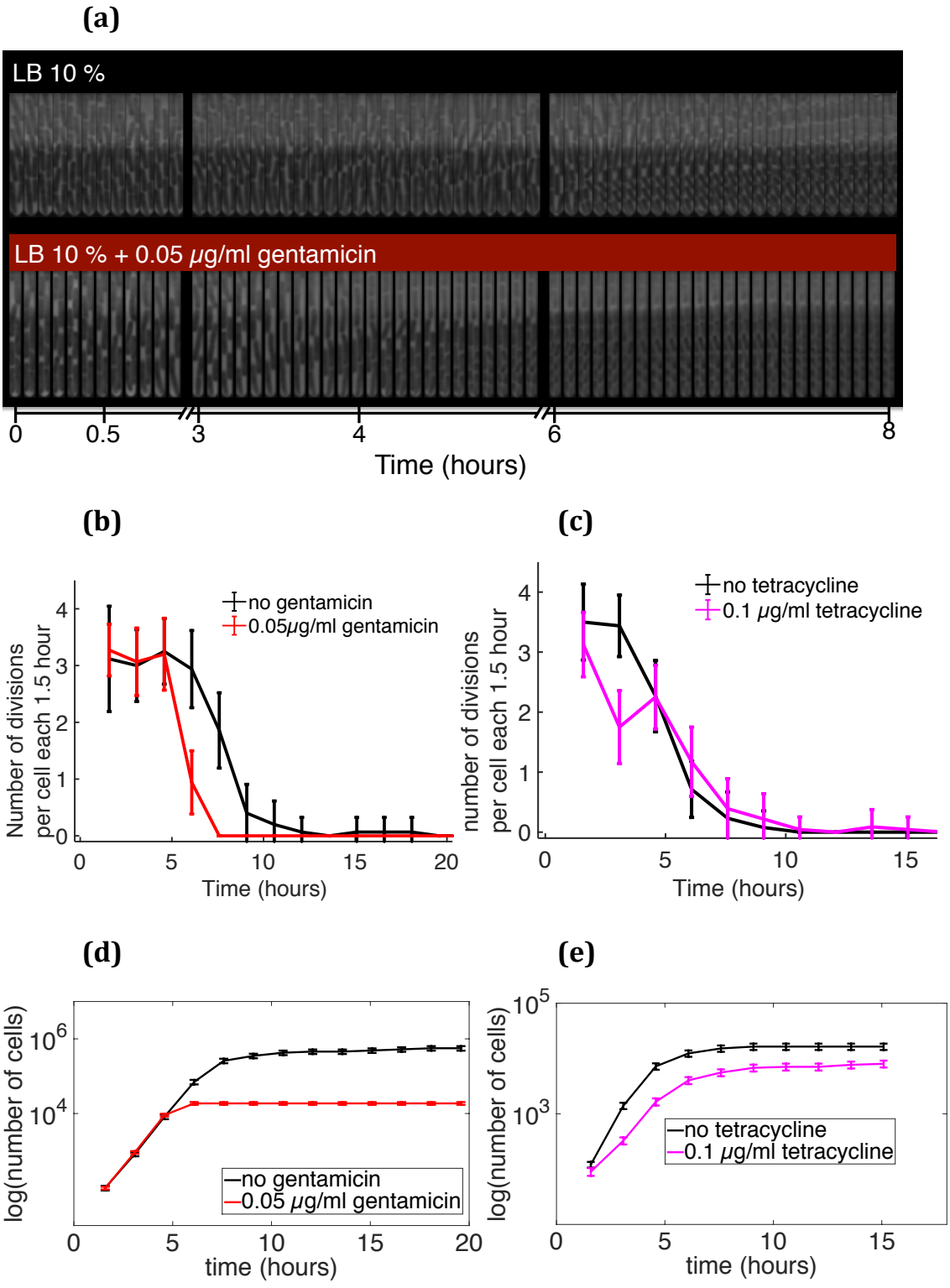

Figure 2. Single-cell growth at subinhibitory concentrations of ribosome-binding antibiotics. **(a)** *E. coli* cells inside the microfluidic channels connected to a batch culture growing in the absence (top row) or presence of gentamicin (bottom row). The temporal montage shows consecutive time frames of the same two channels. During the first hour both cell populations were growing exponentially. However, in the following hours, cells growing in gentamicin slowed down division earlier and entered into stationary phase before the ones without antibiotics. Images were taken every five minutes. **(b,c)** Cell division rate: mean and standard deviation of the frequency of division of ~20 cells of one replicate over 20 hours of exposure to none (black) or 0.05 μg/ml of gentamicin (red) (b) and over 17 hours of exposure to none (black) or 0.1 μg/ml of tetracycline (magenta) (c). **(d,e)** Mean and standard deviation of the change in the population size during batch growth in 10 simulations, based on the frequency of division obtained from the single-cell experiments where cells were exposed to gentamicin (d) and tetracycline (e).

Early transition to stationary phase in low concentrations of gentamicin might be a consequence of faster depletion of nutrients

Next, we were interested in understanding why cells reduced their division rate and entered into stationary phase earlier when exposed to subinhibitory concentrations of gentamicin. One possibility is that in these conditions cells lacked resources for further growth. Alternatively, it is conceivable that the effect of low concentrations of gentamicin increased during stationary phase, i.e. that the inhibitory effect of the antibiotic had a synergistic interaction with either low nutrient concentrations or with metabolites or toxins released during this growth phase. A third possibility is that exposed cells got increasingly damaged over time and were not able to divide further at the onset of stationary phase.

We tested the three different hypotheses and our results suggested that it was the environmental conditions that did not allow further growth. First, yield was not altered when bacteria coming from fresh medium were

introduced at different timepoints (figure 3a: blue series, ANOVA, p=0.80 — not including bacteria added after 8:20 hours, when there was a decline in O.D.). This suggested that the reduction in yield was not a consequence of bacteria accumulating damage during growth in gentamicin. Second, adding gentamicin during the transition to stationary phase did not reduce the growth, but allowed cells to reach a higher yield when it was added at latter points (figure 3a: magenta series; ANOVA, $p<0.01$, posthoc analysis indicating a significant increase when gentamicin was added after 5:40, 7:00 and 8:20 hours, likely because of cells already having grown in the absence of the drug). This suggested that the reduction in yield was not a consequence of the growth-suppressing effect of gentamicin increasing at the onset of stationary phase. Finally, single-cell experiments with continuous flow of 0.05 μg/ml of gentamicin without resource limitation indicated that cells maintained the same growth rate after 12 hours of exposure (figure 3b). This supported the conclusion from above that damage accumulation during prolonged exposure to gentamicin was not responsible for the decrease in yield. Together, these observations strongly suggested that it was the media which was not allowing further growth.

In order to evaluate whether faster nutrient depletion could explain the early entrance into stationary phase, we added different types of nutrients five hours after the experiment started, i.e. during the transition to stationary phase. Among the nutrients tested, we included glucose —as a carbon source— and $MgSO_4$ and $CaCl_2$ —sources of divalent cations, which could be potentially limiting factors in LB[14]. In the three cases, statistical analyses indicated an interaction between the addition of the nutrients on growth yield and the presence versus absence of gentamicin in the medium (two-way ANOVA, glucose $p<0.01$; $MgSO_4$ $p<0.001$; $CaCl_2$ $p<0.005$). This meant that the effect of these nutrient additions on growth yield depended on the presence of the antibiotic.

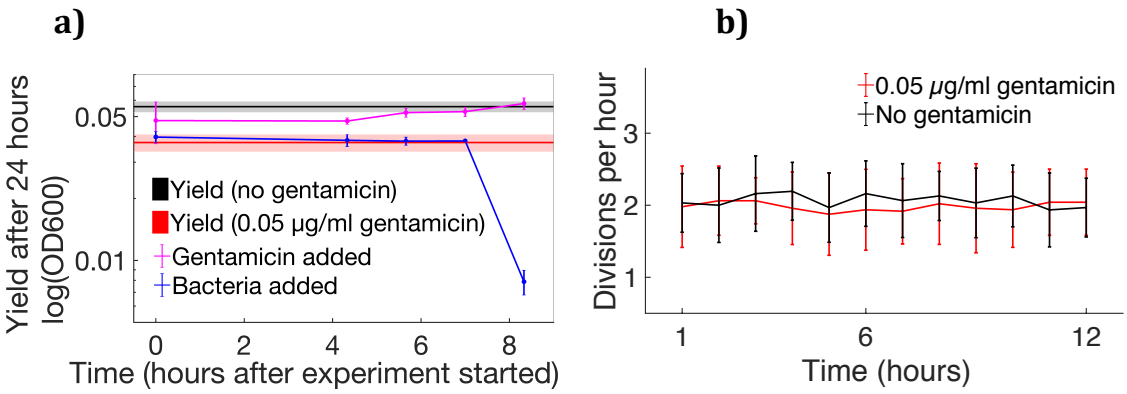

Figure 3. Testing three hypotheses to understand the earlier transition to stationary phase when cells are exposed to low gentamicin concentrations. (a) In blue: changes in the population yield when bacteria grown in the absence of antibiotics were added to a bacterial culture grown in the presence of 0.05 µg/ml of gentamicin at the beginning or during the transition to stationary phase (mean and standard deviation of three replicates); in magenta: changes in population yield when 0.05µg/ml of gentamicin were added to a population of bacteria grown without antibiotics (mean and standard deviation of three replicates). The black line indicates yield in the absence of gentamicin and the red line yield in the presence of gentamicin (line: mean; shaded areas: standard deviation of three replicates). **(b)** Mean and standard deviation of the number of divisions per hour of cells inside a microfluidic chamber exposed to continuous fresh media during 12 hours (LB 10% with or without gentamicin; n=48 and n=31, respectively).

The addition of sources of divalent cations affected the yield in dissimilar ways. In the case of magnesium, the O.D. notably increased regardless of the presence or absence of the antibiotic (Figure 4b), although the effect was significantly different between the two scenarios (ANOVA; $p<0.001$). The increase in yield could be explained by the hypothesis that divalent cations might be the limiting resource for *E.coli* when growing in LB (discussed in Nikaido, H., 2009[14]). The idea is that bacterial cells require a high amount of divalent cations in order to neutralize the negatively charged lipopolysaccharide molecules in the outer membrane, a concentration potentially not available in LB. Our analysis indicated that the increase in

yield was stronger in the absence of the drug. This could be partially explained by the damage that the outer membrane suffers when cells are exposed to aminoglycosides, but it also suggested that another type of resource depletion was responsible for the observed reduction in yield. Interestingly, adding calcium had a different effect (figure 4b): it reduced yield slightly in the absence of gentamicin (ANOVA, $p<0.05$) and had no significant effect in the presence of gentamicin (ANOVA, $p=0.06$). This difference between magnesium and calcium raised doubts about a general effect of cations on growth yield in the presence of gentamicin.

The addition of glucose allowed further growth in cells exposed to gentamicin, while this was not the case for cells growing in media without antibiotics (figure 4a). These results suggested that carbon or energy limitation might prevent cells from dividing further, leading them to enter earlier into stationary cells. Thus, cells exposed to low concentrations of this aminoglycoside might deplete carbon or energy faster than unexposed cells. It is relevant to note that the yield was, however, not fully reached as in the absence of gentamicin. This is likely due to the low glucose concentration added.

The hypothesis of cells consuming higher resources when exposed to low antibiotic concentrations is in line with results obtained by Mathieu *et al* [10]. In this work, Mathieu *et al* discovered that *E. coli* cells increase energy production and translation when exposed to low doses of bactericidal antibiotics, specifically ampicillin, norfloxacin and gentamicin. They reported that cells treated with 50% MIC (which for their strain is 0.5 μg/ml of gentamicin) deplete amino acids faster than in the absence of antibiotics. The lower concentrations of intracellular amino acids induce the stringent response, which leads to early entrance into stationary phase.

Both their[10] and our experiments were performed in LB media. LB contains very low concentrations of fermentable sugars. Amino acids are therefore the major energy source[15]. Preliminary experiments where cells grow on a single sugar (glucose) with no amino acids show that the response to the antibiotic differs from what we observed in LB10% (Figure S1). Our results suggested that yield of cells exposed to gentamicin in minimal media with glucose was at least as high as in the absence of the drug (Figure S1a). Additionally, we found no evidence of an early entrance to stationary phase when glucose was the main source (Figures S1b). Both observations were in accordance to the explanation offered by Mathieu et al[10], where early entrance to stationary phase is linked to the stringent response, i.e. depletion of amino acids.

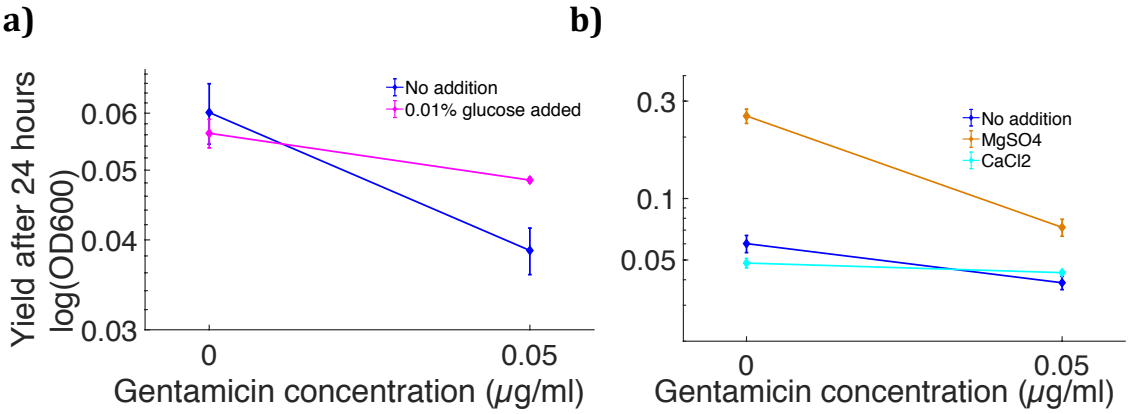

Figure 4. Adding nutrients to the growth media during the transition to stationary phase. (a) Adding 0.01% of glucose 5 hours after the beginning of the experiment had a significantly different effect on yield in the absence or presence of the antibiotic (two-way ANOVA; $p<0.01$). **(b)** The addition of divalent cations sources 5 hours after the start of the experiment had also a significantly different effect on yield in the absence or presence of drug (two-way ANOVA: $MgSO_4$: $p<0.001$; $CaCl_2$: $p<0.005$). In both graphs, diamonds indicate mean of three replicates and error bars refer to the standard deviation.

Materials and Methods

The strain used for all the experiments and results exposed here was *Escherichia coli* MG1655.

LB 10% media is composed by 0.1 fraction of the tryptone and yeast extract that LB (Lysogeny Broth) contains and equivalent NaCl concentration, i.e. 5g NaCl, 1g tryptone, 0.5g yeast extract per liter of water.

We chose LB 10% as cells inside the chambers did not reach stationary phase when they were fed with the batch culture growing in LB. One possible explanation was that the batch culture could have been experiencing a reduction of oxygen before consuming all resources, which may have resulted in a still carbon-rich medium input for the cells inside the microfluidic chambers.

Population-level experiments (batch)

Bacterial response to low antibiotic concentrations

Three independent replicates were grown overnight in LB, then inoculated in 1:1000 in fresh LB 10% + 0.01% Tween20 medium for three hours, time at which they were in exponential phase. Each replicate was then diluted to obtain an O.D. of 0.001 and afterwards inoculated in 96-well plates with 195µl of LB 10% + 0.01% Tween20 with or without antibiotics. The O.D. was measured every 20 minutes during more than 25 hours. In order to test the addition of resources during growth, 5 µl of glucose (for a final concentration of 0.01%), $MgSO_4$ or $CaCl_2$ were added after 5 hours after the experiment started.

Single-cell experiments

Time-lapse microscopy

Cultures of *E. coli* MG1655 strain were grown overnight in LB, to be afterwards inoculated in 1:1000 in LB10% for three hours, time at which they were at exponential phase. The cells were then introduced into the chamber of microfluidic devices, where they were fed by either LB10% or by a growing batch culture in LB10%, both in the absence and presence of antibiotics (0.05 μg/ml gentamicin or 0.1 μg/ml tetracycline) in order to follow single cells throughout the bacterial growth curve. Images were recorded every five minutes during 15-20 hours. Division rate was subsequently measured manually by observing the cells captured.

In silico population growth based on single-cell division dynamics

Matlab simulation

The division frequency of cells was obtained for every 1.5-hour period of time throughout the single-cell microfluidic experiments, allocating each cell value into a vector. Based on this data, 10 simulations in Matlab were performed for population growth in the absence or presence of antibiotics. Each simulation starts with an initial number of ten cells. In order to assign the division frequency to each cell for every 1.5-hour period of time, the division vector was sampled at random, with replacement, and the corresponding cell will divide accordingly during that period. This step is repeated for each period during 15- 20 hours, updating in each of them the number of cells —including the progeny resulting from the division processes—.

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Supplementary Material

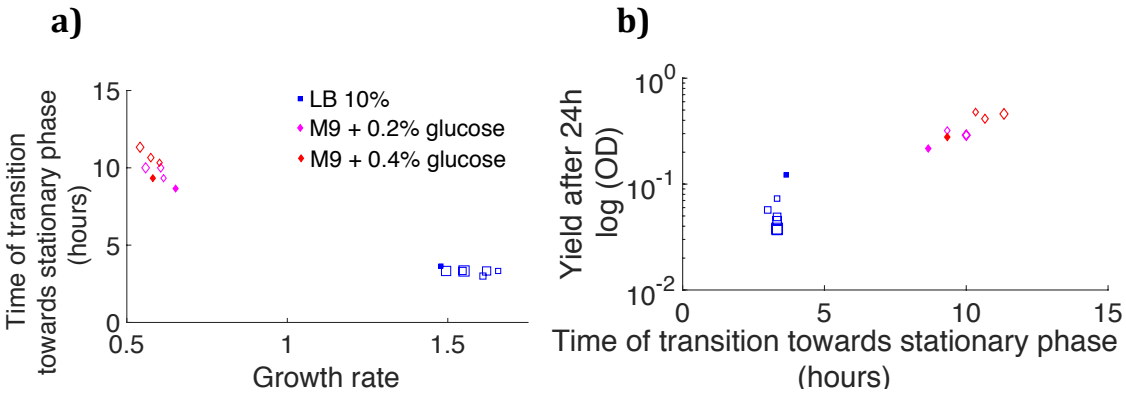

Figure S1. Comparing growth on LB 10% and M9 with glucose (preliminary studies). (a) Growth rate and yield reached after 24 hours for cells growing in LB 10% (blue squares), M9 with glucose 0.2 % (pink rhomboids) and M9 with glucose 0.4 % (red rhomboids). Filled symbols show values in the absence of antibiotics; unfilled symbols show values in the presence of gentamicin (smaller square: 0.05 µg/ml, bigger square: 0.3 µg/ml; smaller rhomboids: 0.01, bigger rhomboids: 0.1). In all cases, mean of three replicates is shown. **(b)** Time of transition to stationary phase (timepoint —hours after the experiment started— where exponential growth rate slows down) versus yield after 24 hours of growth. The same nomenclature as in figure S1a is shown.

Supplementary Methods

Comparing growth on LB 10% and M9 with glucose

The values shown for LB 10% are described in the main Materials and Methods section. For the experiments in M9, three independent replicates were grown overnight in LB, then inoculated in 1:1000 in fresh M9 + 0.2 or 0.4% medium for four hours, time at which they were in exponential phase. Each replicate was then diluted to obtain an O.D. of 0.001 and afterwards inoculated in 96-well plates with 195µl of M9 + 0.2 or 0.4% with or without antibiotics. The O.D. was measured every 20 minutes during more than 30 hours.

Chapter 5

Discussion

The antibiotic crisis we are currently facing prompts us more than ever to better understand how exactly antibiotics act on bacterial cells, and how they affect rates of division and cell death. Extensive research has been done in the last decades to investigate many aspects of this simple question. However, many basic issues remain unresolved, for instance, how the effect of drugs on individual cells give rise to the patterns we observe at the population level, both in the lab and clinics. In this thesis, we took advantage of single-cell experimental approaches to investigate little-explored general aspects of the effect of antibiotics on bacteria. Part of our work focused on disentangling rates of cell division and cell death, specifically by studying division events at the individual cell level. We also explored non-genetic factors affecting survival and response to antibiotics, specifically, phenotypic single cell traits and the role of nutrient depletion during antibiotic exposure.

Regarding **growth dynamics**, the study of division events in single cells exposed to antibiotic pulses strong enough to kill a considerable fraction of cells (**Chapter 2**) and to environments with continuous low antibiotic concentrations with limited resources (**Chapter 4**), provided the following insights:

- Cell division can sometimes continue in the presence of antibiotics: rates of cell division of bacteria exposed to pulses of kanamycin at

concentrations equal to or above the MIC during up to two hours can be as high as in the absence of the drug (**Chapter 2**). That populations do not grow under these conditions might be a consequence of cell death rate.

- Administering a given antibiotic dose at a high concentration or over a long time increased cell mortality levels compared to combinations of intermediate duration and concentration (**Chapter 2**).
- Division rate is an important factor determining bacterial growth dynamics at low antibiotic concentrations (**Chapter 4**).
- Bacterial cells exposed to low gentamicin concentrations in a limited-resource environment enter earlier into stationary phase (**Chapter 4**).

When studying **non-genetic factors** affecting the effect of a drug in these different scenarios (antibiotic pulses and continuous exposure to subMIC antibiotic concentrations with limited resources), our most relevant findings were:

- We found no evidence that interdivision time previous to an antibiotic pulse or the cell cycle stage at the onset of the pulse influences cells survival (**Chapter 3**).
- Potential persister cells (referring to non-dividing cells) in our experiments had higher chances of survival in pulses with high mortality rates (**Chapter 3**).
- Single-cell experiments suggest that the response of bacterial cells to low gentamicin concentration depends on the availability of nutrients. By combining batch experiments with a microfluidic set-up, we found that antibiotic exposure leads to an earlier transition into stationary phase, potentially as a consequence of faster depletion of nutrients during the exponential phase (**Chapter 4**).

It is pertinent to ask whether and how these findings are relevant for clinical application. While our work was focused on studying aspects of the antibiotic effect that are little understood, it cannot directly and immediately be translated into a clinical setting. Nonetheless, we believe that, together with the work of other colleagues, the findings here challenge some ideas and concepts about the use of antibiotics for treating patients.

For instance, even after decades of antibiotic use in modern medicine, scientists and clinicians are still looking for the optimal dosing strategies to treat infections. This is not an easy endeavour, as many elements have to be taken into account —the responses of the patient and pathogen being at the centre of the issue. One of the chapters of this thesis addressed one aspect of this problem: how the effect of a certain total dose of antibiotics on the bacteria depends on the concentration of the antibiotics and the time period during which it is applied. This is not a new question, but by exploring it through the study of single cells, the work here reveals that divisions do take place during antibiotic treatment with concentrations equivalent or higher than the MIC (as measured in batch experiments), questioning traditional quantifications of survival in these scenarios. The occurrence of division events during these pulses, also observed in another work at conditions with even higher death rate[1] should be studied in the context of infection. How relevant, for example, are these division events during longer periods of drug treatment? The effort for finding the optimal antibiotic dosing strategy will probably benefit from the knowledge of division events that an analysis of the net population growth rate usually masks.

This thesis also emphasizes the need to understand variation in survival times between single cells during the log-linear death phase, i.e. before the presence of persisters —understood in the traditional sense of a minority of non-growing cells[2,3]— starts to influence population-level survival

measurement. We explored the question in Chapter 3 and found no evidence that growth rate, a common factor associated with survival, plays a role in such scenario, except for a extremely small fraction of the population, which are likely persister cells. Understanding why some cells die immediately and others survive much longer might prove powerful for treating diseases where this early phase of bacterial death takes long periods of time, such as tuberculosis, and where we want to understand what makes some cells resist the drug for longer than others.

Microfluidics for studying antibiotic effects: relevant for clinics?

Most of the questions that we ask in this thesis can only be answered by monitoring bacteria at the single-cell level, a goal accomplished here by the use of time-lapse microscopy in combination with microfluidics. Studying the effects of antibiotics at the single-cell level offers insights that are difficult to obtain with more conventional population analyses. Two such insights have been mentioned several times throughout this thesis. One is precisely the possibility of visualizing how events (e.g. division) in individual cells impact the response we have for decades observed in batch experiments. The other one is to evaluate whether and how the phenotypic state of a bacterial cell in a clonal population influences its response to antibiotic treatment.

Ignoring the interplay between division and death can affect our understanding of the evolution of resistance under stressful situations for bacteria. Divisions masked by high death rates will lead to underestimating the potential for mutations. This goes hand in hand with a second problem: overlooking death during periods of treatment where population grows can lead to an overestimation of mutation rates under stress. A recent

combination of experimental and theoretical work[4] showed that death events in scenarios where bacteria are exposed to subinhibitory antibiotic concentrations can bias quantification of mutation rates. When cell death was included in the analysis, for instance in scenarios of kanamycin exposure, there was no evidence of stress-induced mutagenesis[4]. Thus, details of growth dynamics during treatment will offer a more accurate view of the evolution of resistance in such environments.

Taking into account heterogeneity in a population or bacterial community is key in our attempt to control infections. Several *in vitro* studies have shown how heterogeneity can play a role in clonal infections[1,5,6]. The next challenge is to study phenotypic heterogeneity in bacterial pathogens under conditions that are closer to the situation inside hosts. Microfluidics can potentially make an important contribution in replicating host conditions experimentally in the laboratory: efforts are currently under way for using microfluidics to mimic biofilms[7,8] and host compartments including blood, urinary tract and simple organs[9,10]. We expect more of these promising developments in the coming years.

Finally, microfluidics have certainly limitations. Such experiments are much more time- and resource- demanding. As a consequence, this approach is mainly a tool in research environments rather than in the clinics. However, we expect that its power in answering relevant questions as the ones discussed throughout this work, will lead to future discoveries about the behaviour and properties of single cells that will be relevant for clinical applications. Another limitation is the current difficulty to observe rare events due to the low number of cells that can be monitored. This challenge is starting to be solved by technical solutions[11,12] and has been addressed previously by the use of mutants (hipA mutants, for example, which show higher levels of persistence[13]).

In summary, the challenges posed by the antibiotic crisis and the development of single-cell and microfluidic technologies, make it necessary and possible to further investigate growth dynamics, phenotypic heterogeneity and the role of individual traits in bacterial infections.

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