

A Quantitative Single-Cell Investigation of
Cell Growth and Antibiotic Tolerance in
Mycobacterium smegmatis

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ABSTRACT

Tuberculosis is a disease which has afflicted humans for millennia and currently holds the dubious title of the greatest cause of mortality by any single infectious agent. *Mycobacterium tuberculosis*—the causative agent of tuberculosis—is exquisitely adapted to the infection of humans, with the ability to persist within the hostile intracellular environment of the macrophage. To enable this, *M. tuberculosis* enacts a dormancy programme that permits long-term survival. This programme has been linked to the requirement for six months treatment to cure tuberculosis since it may enhance drug tolerance—the ability for bacteria to survive antibiotics without genetic resistance. In this thesis, I extensively utilise single-cell time-lapse microscopy coupled with microfluidics to observe *Mycobacterium smegmatis* cells over many generations. I describe in detail a robust methodology for long-term time-lapse and subsequent image analysis workflow, including its application to a challenging dataset. I then investigate how single cells maintain their cell size under diverse environmental conditions, and determine that birth length and division length are intimately linked by a fixed average extension when grown in standard medium—a so-called “adder”. This relationship however breaks down when cells are grown in sub-optimal sole carbon sources. At the population level, I investigate antibiotic tolerance in *M. smegmatis*, and demonstrate that the ability to survive is highly enriched by growth phase, but not by nutrient deprivation. Finally, I describe an experimental platform with which the response of *M. smegmatis* to antibiotics can be observed at the single-cell level, and investigate factors that may predict the survival of *M. smegmatis* cells exposed to the anti-tubercular drug rifampicin, finding that single-cell growth rate and the ability to tolerate rifampicin are uncorrelated. Throughout this thesis, I use quantitative measures of single-cell properties that describe mycobacteria, data which may help develop novel strategies to combat the devastating global burden of tuberculosis.

DECLARATION OF ORIGINALITY

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LIST OF ABBREVIATIONS

(p)ppGpp	Guanosine tetraphosphate and pentaphosphate	119
BCG	Bacillus Calmette–Gueérin	19
BL	Biosafety level	21
CFU	Colony-forming unit	132
CTC	5-cyano-2,3-ditolyl tetrazolium chloride	135
CV	Coefficient of variation	63
DMSO	Dimethyl sulfoxide	132
EM-CCD	Electron-multiplying charge-coupled device	24
HdB	Hartmans–de Bont minimal medium	27
IOP	Irreversibly oxidised protein	128
JSON	JavaScript Object Notation	38
MDR-TB	Multidrug-resistant tuberculosis	17
MIC	Minimum inhibitory concentration	113
MPN	Most probable number	124
MRC	Medical Research Council	19
MRSA	Meticillin-resistant <i>Staphylococcus aureus</i>	111
MSE	Mean squared error	76
OADC	Oleic acid–albumin–dextrose–catalase	66
OD ₆₀₀	Optical density at 600 nm	31
PAS	<i>para</i> -aminosalicylic acid	111
PCR	Polymerase chain reaction	135
PDMS	Polydimethylsiloxane	26
PI	Propidium iodide	146
RNAP	RNA polymerase	116

RNS	Reactive nitrogen species	128
ROS	Reactive oxygen species	128
RR-TB	Rifampicin-resistant tuberculosis	17
rRNA	Ribosomal ribonucleic acid	117
sCMOS	Scientific complementary metal–oxide–semiconductor	24
TA	Toxin–antitoxin	116
tRNA	Transfer ribonucleic acid	117
VBNC	Viable but non-culturable	124
WHO	World Health Organisation	17
XDR-TB	Extensively drug-resistant tuberculosis	17

CHAPTER 1 INTRODUCTION

TUBERCULOSIS is a disease caused by the bacterial pathogen *Mycobacterium tuberculosis*, and associated species. It is currently the leading cause of death by any single infectious agent (WHO 2017c). In 2016, 1.7 million people died from tuberculosis, including almost 400,000 HIV-positive individuals; and 10.4 million people were estimated to be infected with TB, a million of which were HIV positive (WHO 2017c).

Tuberculosis is clearly a global health threat, and disproportionately affects low-income countries, with a huge burden of cases attributable to undernourishment, smoking, and diabetes (WHO 2017c). There is also a worrying increase in drug resistance in tuberculosis: 600,000 new cases were reported with resistance to rifampicin (RR-TB)—the most important of the front-line drugs—in 2016, of which 490,000 were additionally resistant to another front-line drug (multidrug-resistant; MDR-TB) which had a shockingly high fatality rate of 46 % compared to the high treatment success associated with drug-sensitive tuberculosis: 85 % including those lost to follow-up (WHO 2017c). Drug-resistant tuberculosis appears to be especially high in former Soviet Union countries, with 38 % of all reported tuberculosis cases in Belarus being caused by MDR-TB, which threatens to spread into low-burden countries (WHO 2017c). Extensively drug-resistant tuberculosis (XDR-TB) cases—resistant to four first-line drugs, and at least one second-line—were reported in 121 countries in 2016 (WHO 2017c). This is concerning since XDR-TB cases are notoriously hard to treat; one reported outbreak in a South African hospital led to the death of 52 out of 53 patients within sixteen days of diagnosis (Gandhi *et al.* 2006).

The standard therapy for drug-sensitive tuberculosis is onerous, but almost always effective if correctly administered, involving 6 months daily treatment with rifampicin, ethambutol, pyrazinamide, and isoniazid for 2 months followed by daily treatment with rifampicin and isoniazid for 4 months (WHO 2017d). MDR-TB treatment usually consists of 20–24 months of daily therapy with drugs from at least 5 different classes, including daily injectables, many of which have severe side-effects (WHO 2016), and this may encourage a higher rate of treatment default—especially when patients begin to feel better—thus potentially leading to increased resistance. Encouragingly, a new short course for MDR-TB is now being recommended by the World Health Organisation (WHO), and has been adopted in 35 countries. It lasts 9–12 months,

has fewer reported side-effects, and a 87–90 % success rate (WHO 2017c).

In addition to the large burden of active disease, there is a vast reservoir of asymptomatic infection—latent tuberculosis—with up to a third of the global population estimated to be infected latently with *M. tuberculosis* (Dye *et al.* 1999; WHO 2017c). This reflects the spectrum of tuberculosis disease, where an estimated 90 % of new infections will be effectively controlled by the immune system, with a 5–10 % lifetime risk of developing active disease (Sutherland *et al.* 1982; Styblo 1985; Glaziou *et al.* 2014), which increases to a 5–10 % annual risk when the immune system is compromised, such as during HIV co-infection, malnutrition, or old age (Sonnenberg *et al.* 2005; Lodi *et al.* 2013). Latency remains a poorly understood phenomenon however, both in its underlying causes and how best to combat it (Young *et al.* 2009; Esmail *et al.* 2014).

Tuberculosis is an ancient disease, believed to have co-existed and co-evolved with humans since our divergence from proto-human ancestors (Gutierrez *et al.* 2005). It effectively and exclusively infects humans (Smith *et al.* 2006). Direct molecular evidence of tuberculosis in humans has been found in a 9000-year-old Neolithic skeleton in the Mediterranean region (Hershkovitz *et al.* 2008), and analysis of genetic information of modern *M. tuberculosis* strains provides compelling evidence that modern pathogenic mycobacteria arose from a common African origin over 40,000 years ago in humans (Brosch *et al.* 2002; Mostowy *et al.* 2002; Gutierrez *et al.* 2005), and spread worldwide with human migration (Wirth *et al.* 2008; Hershberg *et al.* 2008; Comas *et al.* 2013). *M. tuberculosis* DNA has been isolated from a 5000-year-old Egyptian mummy (Nerlich *et al.* 1997), and from a 1000-year-old Peruvian mummy (Salo *et al.* 1994), highlighting the ancient global spread of tuberculosis. Tuberculosis is described in ancient texts such as the biblical books of Deuteronomy and Leviticus; Hippocrates described the clinical presentation of tuberculosis in detail in Ancient Greece—and noted its high mortality rate—with scattered references until the Renaissance in Italy began to make its way north to France. René Laennec—the inventor of the stethoscope—recounted the pathogenesis of tuberculosis in detail in the early 19th century, and founded the discipline of pulmonary medicine (Daniel 2004). Tuberculosis has been attributed as the cause of mortality in up to a quarter of all deaths in Europe in the early 19th century (Dubos and Dubos 1952)—including Laennec himself—with mortality rates due to tuberculosis reaching 800–1000 per 100,000 each year in cities such as London (Krause 1928).

The discovery of the infectious agent of tuberculosis—*M. tuberculosis*—by Robert Koch (1882), was simultaneously presented with the announcement of the postulates of infection he had developed. Koch had isolated and concentrated culture filtrate, named tuberculin, from *M. tuberculosis* cultures, which he became convinced could provide a cure and marketed it as such (Koch 1890). Tuberculin was not a cure for tuberculosis, but it was found to be useful for diagnostic purposes, a purpose for which it is still used today in its form as the Mantoux skin test. Koch's

aggressive and secretive approach to marketing tuberculin, and its subsequent discreditation, directly led to the development of more evidence-led procedures for the discovery of novel drugs. His contemporary and colleague, Paul Ehrlich, used his experience of his involvement in the development of tuberculin to ensure that he conducted—and published—careful clinical evaluations of his drug against syphilis, arsphenamine (Alt 1909), the first antimicrobial agent.

Death rates due to tuberculosis in Europe began to fall in the mid-19th century (Dubos and Dubos 1952), due to several factors including massive improvements in public hygiene, improved nutrition, and natural selection of more resistant humans (Davies *et al.* 1999). In the 1920s, at the Institut Pasteur de Lille, Albert Calmette and Camille Guérin began to develop a vaccine against tuberculosis by passaging the animal pathogen *M. bovis* hundreds of times to develop an attenuated strain, named Bacille Calmette–Guérin (BCG; Calmette *et al.* 1927a) which was demonstrated to be safe (Calmette *et al.* 1927b) and effective in children—at least for those in the UK (Hart and Sutherland 1977). Mass vaccination with BCG was rolled out massively in the mid-20th century with 14 million people vaccinated over three years—under the auspices of the WHO. BCG efficacy varies wildly, with high effectiveness against childhood tuberculosis—especially disseminated forms of disease (Trunz *et al.* 2006)—but very poor effectiveness against adult pulmonary tuberculosis (Colditz *et al.* 1994; Fine 1995; Pitt *et al.* 2013). The impact of BCG on the prevention of tuberculosis has clearly saved many lives, regardless of its low protective efficacy, but widespread vaccination campaigns still have not prevented tuberculosis from remaining the leading worldwide infectious cause of death. In countries particularly afflicted with a high tuberculosis burden, such as Southern African countries, this might be ascribable to a high level of HIV infection. The risk of contracting active tuberculosis is up to thirty times greater in HIV-positive individuals, and tuberculosis is the primary cause of death in HIV-positive patients (these deaths are internationally classed as HIV deaths). The two infectious diseases together are driving mortality in a devastating co-epidemic (Tiberi *et al.* 2017).

The search for chemotherapeutic solutions for the treatment of tuberculosis resulted in the discovery of *para*-aminosalicylic acid (Lehmann 1946a,b; Ryan 1993)—which improved tuberculosis outcomes but is bacteriostatic rather than bacteriocidal—almost simultaneously with the discovery of streptomycin after an extended search for antimicrobial agents in soil microbes (Schatz *et al.* 1944). Streptomycin was the first truly effective anti-tubercular drug, and was conclusively proven to be effective by the first randomised, controlled trials performed by the UK Medical Research Council (MRC 1950)—now a critical component of any new medical therapeutic agent. This trial noted that streptomycin resistance rapidly emerged in the patients who were treated with streptomycin alone, and when isoniazid—still used in modern tuberculosis therapy—was discovered to be highly effective against *M. tuberculosis*, they performed a landmark trial with each of the three drugs in monotherapy and all combinations were tested, which forms the basis of the current doctrine of multi-class multidrug therapy (MRC 1955).

M. tuberculosis is primarily a pulmonary pathogen, infecting the lungs, and spread by the inhalation of liquid droplets induced by coughing, with a single *M. tuberculosis* cell sufficient to cause disease. Once inhaled, *M. tuberculosis* is detected and internalised by innate immune system cells, primarily patrolling resident alveolar macrophages, as well as neutrophils, monocytes, and dendritic cells (Wallgren 1948; Wolf *et al.* 2007; Kang *et al.* 2011), a niche in which *M. tuberculosis* thrives. *M. tuberculosis* is exquisitely adapted to survival within macrophages—the main function of which is to kill bacteria—and blocks many antimicrobial activities, such as the fusion of the phagosome with the highly acidic and hostile lysosome (Xu *et al.* 1994; Clemens 1996). Bacilli also block the acidification process itself by excluding the vacuolar ATPase from vesicular membranes (Wong *et al.* 2011). In addition to preventing many antimicrobial macrophage functions, *M. tuberculosis* is also apparently able to survive even when exposed to such stresses, demonstrating its multilayer defences against the human attempts to eradicate it (e.g. iron starvation; Kurthkoti *et al.* 2017). After *M. tuberculosis* has established itself within a macrophage, its ESX1 type VII secretion system—a unique feature of the pathogenic mycobacteria, and the major difference between *M. bovis* and the BCG vaccine strain (Pym *et al.* 2002)—exports molecules that induce necrotic cell death in their host cell, as well as antigens that stimulate the immune system, which has the result of increasing the environmental niche into which *M. tuberculosis* can expand (Davis and Ramakrishnan 2009). *M. tuberculosis* appears to also have evolved specific mechanisms to inhibit cell apoptosis (Velmurugan *et al.* 2007; Divangahi *et al.* 2010), either to promote uncontrolled necrotic cell death—which stimulates an immune response—rather than controlled programmed cell death, or to permit sufficient intracellular replication prior to cell escape. The innate immune system is largely unable to restrict *M. tuberculosis* growth during initial infection, and indeed promotes bacterial growth, indicating how *M. tuberculosis* has evolved to intimately subvert its sole environmental niche.

The adaptive immune response to *M. tuberculosis* takes an estimated 42 days from infection to become appreciable (Wallgren 1948; Poulsen 1950), a lengthy period compared to most infectious diseases. The primary adaptive response includes antigen-specific CD4⁺ T-cells in the lung lymph nodes, which are usually able to coordinate a restrictive response against tuberculosis, at which point most individuals will become asymptomatic (Poulsen 1950). This response does not clear bacteria, but maintains the population in an uneasy equilibrium with the host immune system. This is effected by the formation of a large—usually necrotic—lesion called a granuloma, in which the immune system prevents the escape of bacteria contained within (Rubin 2009).

The ability for mycobacteria to survive within the hostile environment of the human granuloma is the result of millennia of adaptation to stressful conditions, including the ability to initiate various dormancy programmes, in which bacteria are able to arrest their growth, and thus avoid the detection of the immune system, and evade cidal—whether immunological or pharmacological in origin—molecules which often depend on active growth to effect their killing

activity. This ability may underlie the ability of *M. tuberculosis* to survive latently in the presence of a fully functional immune system as well as the extensive period of chemotherapeutic intervention required to cure tuberculosis. Surprisingly little is known about how mycobacteria grow however, basic research which may be critical in the design of novel strategies against tuberculosis.

In this thesis, I will investigate single-cell attributes of the mycobacterial model species *M. smegmatis*. *M. smegmatis* was originally identified in a syphilitic patient (Lustgarten 1885), but does not generally infect humans, although a handful of soft-tissue infections have been reported (Newton *et al.* 1993). It is generally considered to be an environmental saprophytic soil-dwelling species. *M. smegmatis* is extensively used as a proxy for investigating the basic properties of mycobacteria, which are largely conserved between species. Its non-pathogenic nature allows its use in biosafety level (BL) 1 laboratories, whereas *M. tuberculosis* research requires the cumbersome use of BL3 facilities. In addition, its doubling time is ~ 3 h under optimal conditions, compared to ~ 24 h for *M. tuberculosis*, which greatly increases the number and speed at which experiments can be performed. Mycobacteria are generally classed as neither Gram-negative or Gram-positive species, but as acid-fast, based on their ability to retain stains after acid treatment. They are more similar to Gram-positives, with a single true membrane and a thick peptidoglycan layer, but they also possess a thick and complex cell wall, which has some of the properties of an outer membrane (Kieser and Rubin 2014). This cell wall is mostly impermeable and highly rigid, and the inherent resistance of mycobacteria to many stresses—including osmotic stress, dessication, and antibiotics—has been attributed to this wall (Jarlier and Nikaido 1994). *M. smegmatis* shares these properties with the pathogenic mycobacteria, and as such is a useful model for many of the basic properties of mycobacteria (Reyrat and Kahn 2001; Tyagi and Sharma 2002). The *M. smegmatis* strain used throughout this thesis is *M. smegmatis* mc²155, a derivative of *M. smegmatis* mc²8 which has been engineered to be genetically tractable (Snapper *et al.* 1990). *M. smegmatis* mc²155 is the most well-characterised mycobacteria in the literature and is considered to be wild-type by others and in this thesis.

In Chapter 2, I discuss the optimisation of a microfluidics-based single-cell time-lapse microscopy platform, with associated analysis workflow and scripts created for the purpose, which are used throughout the subsequent chapters. This thesis relies heavily on the commercial ONIX CellASIC[®] microfluidic platform, and I detail the optimisation of the system, and subsequent image processing, cell detection, and lineage assignment required to process the large amounts of data produced. Microfluidics have been revolutionary in the field of live-cell time-lapse microscopy, allowing long-term observation of bacteria under constant conditions. Mycobacteria are especially difficult to maintain for long periods given their propensity to grow in the Z-plane making long-term cell tracking difficult. Specially constructed microfluidic systems such as the ONIX platform can trap bacteria in three dimensions without causing undue stress, both preventing growth into the Z-plane, and retaining bacteria in a single posi-

tion over time. I demonstrate the use of this platform, as well as the software developed for image analysis, to a challenging dataset: the dynamics of the putative chromosome segregation machinery in *M. smegmatis*. These data were acquired by Iria Uhía, a post-doctoral researcher, and the results have recently been published (Uhía *et al.* 2018). The analysis of the dataset demonstrates the tracking of fluorescent foci in live bacteria and provides quantitative insight into the control and consequences of chromosome segregation in mycobacteria.

In Chapter 3, I determine experimentally the phenomenological model which underlies the decision to divide in *M. smegmatis*, by distinguishing between the three theoretical models of cell-size control in bacteria: “sizer”, “timer”, and “adder”. I find conclusively that *M. smegmatis* single cells follow the adder principle: that they add a fixed length per cell cycle irrespective of length at birth, under standard conditions. I also demonstrate that changing the carbon source provided to *M. smegmatis* can affect this principle, with cells deviating from adder in a “sizer-like” manner, the first time such deviations have been observed in ostensibly fast-growing bacteria. This chapter expands on published work that reports these results (Priestman *et al.* 2017).

In Chapter 4, I attempt to determine factors that might influence the ability to survive antibiotic exposure, specifically the response to rifampicin, the major first-line anti-tubercular drug. Drug tolerance has become a topic of great interest, due to its presumed role in recurrent infections and in dormancy during *M. tuberculosis* infection which may underlie the vast reservoir of latent tuberculosis. My work involves the use of batch culture experiments as well as extensive use of the microfluidics platform and analysis pipeline devised in previous chapters. I demonstrate that tolerance to rifampicin is enhanced in stationary phase, but not by nutrient starvation. Similarly to other authors, I also demonstrate that growth rate at the single-cell level prior to antibiotic treatment does not predict survival, though a reduced interdivision time in cells which ultimately survive hints at some effect.

Throughout this thesis, I make quantitative observations of single mycobacterial cells, with insight into the basic properties of cell growth (Chapter 3), and into how these might affect the ability to survive ostensibly cidal levels of antibiotics (Chapter 4). These considerations are important for the future development of strategies to combat tuberculosis. Novel strategies are urgently required to increase the rate at which tuberculosis cases are declining, especially given the WHO End TB goal of reducing deaths due to tuberculosis to 95 % of their current levels by 2035 (WHO 2015b). Understanding how drugs that are currently available act on mycobacteria is of crucial importance in accelerating cure rates and reducing the terrible burden of tuberculosis.

CHAPTER 2 SINGLE-CELL MICROSCOPY

I make the proportion of one of these small Water-creatures to a Cheese-mite, to be like that of a Bee to a Horse: For, the circumference of one of these little Animals in water, is not so big as the thickness of a hair in a Cheese-mite.

Antonie van Leeuwenhoek (1677)

2.1 Introduction

THE first report of the direct observation of bacteria was by Antonie van Leeuwenhoek in the 17th century, who developed a novel method of producing the high-powered lenses required to observe such small objects (van Leeuwenhoek 1677). For the next two centuries, microscopy was almost exclusively limited to static, fixed samples, with observation by means of specific stains, such as the Gram stain for peptidoglycan (Gram 1884). The development of such stains was crucial in the advancement of the field of microbiology; most cells are transparent with few discernible features under basic light microscopy, thus staining permits investigation and identification of bacteria. Indeed, *Mycobacterium tuberculosis* was only identified by the use of alkaline methylene blue by Robert Koch (1882). The stain that was further developed by Paul Ehrlich (1882)—who also invented pivotal histological methods such as heat-fixing (Ehrlich 1877)—and by Franz Ziehl and Friedrich Neelsen who created the Ziehl–Neelsen stain (Ziehl 1883; Neelsen 1883) that is still used today to identify mycobacteria in clinical samples over 130 years later.

Early approaches to time-lapse microscopy involved projecting the image from a microscope onto paper and manually drawing images at regular intervals, a technique that is clearly subject to observer bias. This technique was pioneered by early embryologists, such as Charles Whitman, who traced the cell lineages and fates deriving from single cells in organisms such as fresh-water leeches (Whitman 1878). The first time-lapse apparatus using cameras was developed from macroscopic photography principles, and was assembled in the late 19th century by Étienne-Jules Marey and Lucien Bull. It captured a sequence of the opening of a flower, involving an intensely convoluted system of pulleys, weights, and moving water to trigger

a camera automatically. This was followed by the development of what they termed micro-cinematography, where a camera was attached to a microscope (as described by Talbot 1913). The first publication of the use of photographic time-lapse microscopy was by Auguste Pizon in the early 20th century, who observed live aquatic organisms (Pizon 1905). This was followed by other innovators such as Julius Ries—who observed cell division and advocated against the prevailing orthodox view of chromatin as a static structure (Ries 1909)—and Jean Comandon, who developed side illumination of samples to image the microscopic ciliate *Paramecium* (Comandon 1909).

As evidenced by Comandon's requirement for side illumination, early studies were severely limited by the nature of the cells they were observing. The transparency of living cells makes internal structures almost impossible to observe under standard conditions. This did not prevent the tracking of large cells, for example the tracking of a complete chicken embryo cell lineage from a blastocyst (Wetzel 1929), but smaller and more transparent cells were very difficult to track. The development of phase-contrast microscopy by Frits Zernike (1942)—a technique which allows the visualisation of changes in the phase of light as differences in amplitude—was a catalysing advancement in microscopy, a development for which he received the Nobel Prize for Physics in 1953. Such changes in phase are common in biological samples due to the differences in refractive index and thickness within the sample. The method therefore permits the observation of fine structure within an ostensibly uniformly transparent object—crucially without the use of lethal staining or fixative techniques. This ability to observe sub-structures in unstained samples was instrumental in the development of live-cell imaging, and when coupled with time-lapse photography became an extremely powerful technique for the observation of dynamic processes. While phase-contrast microscopy is unable to resolve bacterial sub-structures due to the physical limitations of light microscopy, advancements in imaging of larger cells such as yeast and mammalian cells were crucial in driving the development of techniques permitting the observation of live, single bacterial cells.

A critical bottleneck in time-lapse microscopy was the need to develop physical film, meaning images could not be observed and adjusted in real time. This was bypassed by the development of video recorders (described in detail by Inoué and Spring 1997) allowing rapid review of results, and quickly followed by the development of real-time displays. The subsequent invention of digital methods was also a significant milestone, with extremely rapid and sensitive cameras now in use. Indeed, the modern electron-multiplying charge-coupled device (EM-CCD) and scientific complementary metal-oxide-semiconductor (sCMOS) cameras that are used in this thesis are sensitive enough to be able to detect single photons of light and fast enough to capture up to 100 full frames a second (C9100-02 and Flash 4 cameras; Hamamatsu).

Early time-lapses were complicated by the same issues that still regularly affect modern time-lapse microscopy: optimal exposure and maintenance of focus. Exposure times have essentially

been solved with the development of extremely accurate shutter control mechanisms, but maintaining focus still remains a serious issue. The solutions used are almost unchanged in the modern day: sample movement is minimised by attempting to isolate the microscope stage from external vibration, initially with the use of heavy wooden tables and now with the use of well-calibrated anti-vibration tables; focal drift is minimised by attempting to maintain the temperature of all components of the microscope and camera through fine temperature control of the room and the modern use of environmental chambers. The development of fine motor control of the microscopic stage has also helped to address the focus issue. Software is now able to position the field-of-view in three dimensions precisely, and the use of software-based autofocus and hardware-based absolute focus solutions—which can raise and lower the focal plane precisely and appropriately—has facilitated the long-term imaging of live microorganisms now performed routinely.

The imaging of live bacteria necessitates systems which can constrain the cells. Bacteria can be motile, resulting in the cells that are being tracked physically swimming out of the field-of-view, and are extremely susceptible to fluctuations in their environment such as capillary action and small thermal variations that can cause the movement of the liquid they are grown in. Various solutions to these constraints have been developed, which can be separated into microfluidic systems and agarose pad systems.

Agarose pads were developed in the early 21st century and involve the seeding of bacteria onto a small agarose pad that is attached to a standard microscope slide, and followed by the tracking of the growth of microcolonies which are adhered to the surface (see Figures 2.1A and 2.1B). This approach was originally developed to track *Escherichia coli* single cells (Elowitz *et al.* 2002; Stewart *et al.* 2005; Bernhardt and de Boer 2005), and has been applied to many other species including *Mycobacterium smegmatis* (Joyce *et al.* 2011, 2012). The technique is conceptually simple, and cheap, but is limited by issues such as evaporation and drying of the imaging surface, fluctuations in microenvironment as nutrients are consumed during the course of experiments, and by the lack of constraint in the Z-plane, with bacteria able to grow in all three dimensions. The Z-plane is important because bacteria are not guaranteed to divide in a single plane—although bacteria such as *E. coli* usually do—with *M. smegmatis* cells tending to grow in all dimensions making tracking single cells very difficult after more than a few generations (Joyce *et al.* 2011).

Conversely, microfluidic approaches are often complicated to set up, but allow much more control of the microenvironment. Microfluidics involve the manipulation of liquids in the μl scale and below, which is well suited to the scale of bacteria. Microscopy systems involving microfluidics are able to constantly provide growing bacteria with fresh nutrients, in a manner similar to a micro-scale chemostat. These systems also usually trap single cells within a defined area allowing the long-term tracking of bacteria under constant conditions, without having to

account for movement of cells, whether due to bacterial motility or physical forces. One of the first experiments conducted involved sealing a *Pseudomonas aeruginosa* bacterial suspension between a glass coverslip etched with a micro-channel and a microscope slide in order to investigate the response of single cells to antibiotics (see Figures 2.1C and 2.1D; Kyriacou *et al.* 2002; Xu *et al.* 2003; Steel *et al.* 2004). This technique prevents evaporation of the growth medium but does not constrain movement of bacteria or affect consumption of nutrients over time. This has been addressed by the development of microfluidic systems that can continually provide fresh medium to physically immobilised cells, using transparent devices suitable for microscopic imaging. These are manufactured by applying soft lithography techniques to create microstructures in polydimethylsiloxane (PDMS)—a soft, transparent, and gas-permeable polymer (McDonald *et al.* 2000; Unger *et al.* 2000). This approach was used by Nathalie Balaban to develop a microfluidic device with approximately 1 μm grooves inserted into a PDMS surface which is seeded with single *E. coli* cells and covered with a membrane—thus constraining cells to grow in a single dimension along the groove—whilst medium is continually provided by diffusion (perfusion) through the membrane (see Figures 2.1E and 2.1F; Balaban *et al.* 2004). This setup allows for the rapid switching of medium, including the addition of antibiotics for example, whilst retaining bacteria in the same position. The authors used this powerful technique to expose the existence of persister cells (described in Chapter 4) and some of their characteristics (Balaban *et al.* 2004). This design was extended to study bacterial ageing with the development of the “mother machine”, in which trenches are closed at one end; a single progenitor mother cell remains trapped at the closed end of the groove with daughter cells growing along the length until they are ejected at the open end (Wang *et al.* 2010), allowing very long-term observations of a single cell over time, with 300 consecutive generations of single *Bacillus subtilis* cells reported (see Figures 2.1G and 2.1H; Norman *et al.* 2013).

While the microfluidic systems used are extremely powerful, they are often produced in-house using a complicated and lengthy procedure of fabrication. The design of the devices usually requires knowledge of fluid dynamics in order to prevent undue stress on living cells, and templates for casting PDMS are often expensive. Protocols generally involve the manual punching of holes for tubing access, and a bewildering array of tubing and temperamental syringe or peristaltic pumps to power the devices. Thus these systems are often difficult to manufacture reliably and rapidly, and require some expertise to assemble and operate. Commercial systems which can obviate the need for manufacturing microfluidic devices have been developed, including the platform used in this thesis. The ONIX CellASIC® Microfluidic Platform (Millipore) produces quality-controlled microfluidic plates in a 96-well plate standard format, which it produces by casting PDMS over a master template, cutting through-holes with a CO₂ laser, adhering the PDMS device to a plate with a UV adhesive, followed by bonding to #1.5 coverglass (Lee *et al.* 2011). This provides a good source of reliably manufactured microfluidic devices for use. The plates are operated by air pressure via a manifold that attaches

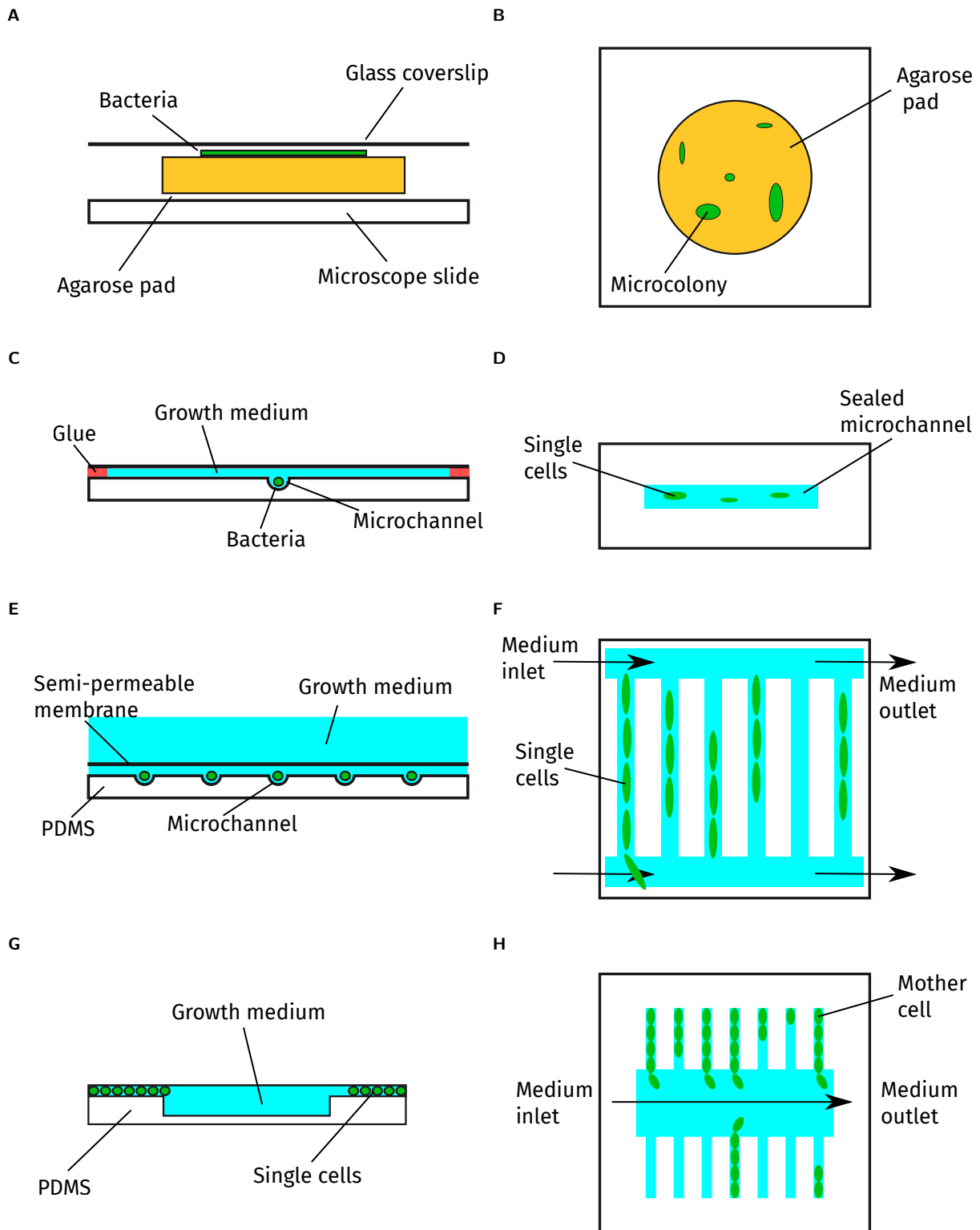


Figure 2.1: Schematic of various single-cell microscopic techniques. Side view (left) and top view (right) of various single-cell microscopic techniques. (A, B) Agarose pad: cells are grown on growth medium-infused agarose in microcolonies without constant medium flow or Z-axis constraint (e.g. Elowitz *et al.* 2002). (C, D) Etched microchannel: cells are grown within a sealed microchannel without medium replenishment (e.g. Kyriacou *et al.* 2002). (E, F) Multiple microchannels: microchannels are created in PDMS and medium constantly replenished by perfusion through a semi-permeable membrane (e.g. Balaban *et al.* 2004). Cells grow out into the medium channels and are washed away into the medium outlet. (G, H) Mother machine: sealed microchannels are created in PDMS and seeded with single cells. Subsequent generations from a single mother are extruded into the medium outlet permitting the observation of a single cell over many generations (e.g. Wang *et al.* 2010).

to each plate, which provides more consistent medium flow compared to syringe or peristaltic pumps. The system is also designed to avoid excessive shear stress on cells within its growth chamber, which can become a problem in other microfluidic devices. The format of the device, and its specific advantages, are described in more detail below (Figure 2.2).

With the ability to observe bacteria over very long-term periods, the volume of data generated by microscopy is significant, and has only increased as cameras and computers have improved. The analysis of microscopy data requires software solutions and automated methods. Many approaches have been developed, ranging from manual measurement of cells using tools such as ImageJ (Abràmoff *et al.* 2004) to fully integrated software packages. I describe some of these packages in Section 2.4.2, including CellProfiler (Carpenter *et al.* 2006), MicrobeTracker (Sliusarenko *et al.* 2011), SchnitzCells (Young *et al.* 2012), and BactImAs (Mekterović *et al.* 2014). Most studies on mycobacterial time-lapses have used the manual ImageJ method, albeit using the ObjectJ plugin (Vischer and Nastase n.d.) which can simplify this process (e.g. Aldridge *et al.* 2012; Wakamoto *et al.* 2013; Santi *et al.* 2013). However, a solution that can increase the throughput of analysis is greatly needed, especially in the mycobacterial field.

In this chapter, I will describe the optimisation of phase-contrast widefield time-lapse microscopy specifically for the observation of *M. smegmatis* single cells, with the application of existing solutions and the creation of novel software to extract data from time-lapse experiments. I will also describe the application of this software, and development of novel software to track chromosome segregation in *M. smegmatis* cells with fluorescently-labelled ParA and ParB—proteins believed to be crucial to this process (Section 2.5). Cells were imaged using the ONIX platform over several generations by Iria Uhía, a post-doctoral researcher, resulting in a challenging dataset to analyse. These data, and my subsequent analysis, have recently been published (Uhía *et al.* 2018).

2.2 Materials

2.2.1 Tyloxapol stock solution

Tyloxapol stock is prepared by weighing out tyloxapol solution (Sigma) and adding an appropriate volume of ddH₂O in a conical flask to produce a 20 % (v/v) stock solution. This is determined by the assumption that pure tyloxapol has a density of 1.1 g ml⁻¹. The solution is stirred for approximately 15 min, sterile filtered, and stored at room temperature.

2.2.2 Hartmans–de Bont minimal medium

Hartmans–de Bont minimal medium (HdB) is a defined medium described by Hartmans and De Bont (1992). It is prepared as described by Smeulders *et al.* (1999), with some modifications.

EDTA	1 g l ⁻¹
MgCl ₂ · 6 H ₂ O	10 g l ⁻¹
CaCl ₂ · 2 H ₂ O	100 mg l ⁻¹
Na ₂ MoO ₄ · 2 H ₂ O	20 mg l ⁻¹
CoCl ₂ · 6 H ₂ O	40 mg l ⁻¹
MnCl ₂ · 2 H ₂ O	100 mg l ⁻¹
ZnSO ₄ · 7 H ₂ O	200 mg l ⁻¹
FeSO ₄ · 7 H ₂ O	500 mg l ⁻¹
CuSO ₄ · 5 H ₂ O	20 mg l ⁻¹

Table 2.1: Chemical composition of 100× trace mineral stock solution. EDTA: ethylenediaminetetraacetic acid

100× trace minerals are prepared by dissolving the chemicals listed in Table 2.1 in ddH₂O, followed by sterile filtering. The solution is wrapped in foil to protect it from light, and stored at room temperature.

100× phosphates are prepared by dissolving K₂HPO₄ (890 mM) and NaH₂PO₄ (708 mM) in ddH₂O, autoclaving at 121 °C for 20 min, and stored at room temperature.

HdB is prepared as follows (in this order): 10 ml of 100× trace minerals are added to approximately 900 ml ddH₂O. 2 g (NH₄)₂SO₄ is dissolved to a final concentration of 15 mM, and 2 ml glycerol is added to a final concentration 0.2 % v/v (27.4 mM). The solution is made up to 940 ml with ddH₂O, and autoclaved at 121 °C for 20 min. It is then permitted to cool to room temperature. 10 ml 100× phosphates are added, and finally 50 ml 20 % tyloxapol stock solution is added to a final concentration of 0.05 % v/v, under sterile conditions. The medium is stored at room temperature.

2.3 Implementation and optimisation of an experimental single-cell platform

The nature of the single-cell growth of mycobacteria raises several problems for conventional single-cell techniques. Mycobacteria tend to grow in all planes including the Z-plane (Joyce *et al.* 2011), thus methods such as agar pads, commonly used for *E. coli* single-cell studies, are often limited. For this reason, a microfluidic approach was used. The CellASIC® ONIX Microfluidic Platform (Millipore) was chosen as a potential solution to directly observe single mycobacterial cells. The platform involves plates containing four independent growth chambers

with variable height traps (0.7, 0.9, 1.1, 1.3, 2.3 and 4.5 μm) which permit the trapping of bacteria in a single plane of focus; for *M. smegmatis* this is the smallest trap with a height of 0.7 μm . Each growth chamber is connected to 5 inlet wells for medium, and a waste well. Growth medium is constantly flowed through the growth chamber using air pressure, this permits the maintenance of constant environmental conditions. These plates are covered with a vacuum-sealed manifold that provides air pressure to the growth medium inlet wells, controlled by the computer (Figure 2.2). The plate is placed on a conventional inverted widefield microscope (phase-contrast), and microscope software allows the definition of fixed XY-positions which can be imaged sequentially in a long-term time-lapse.

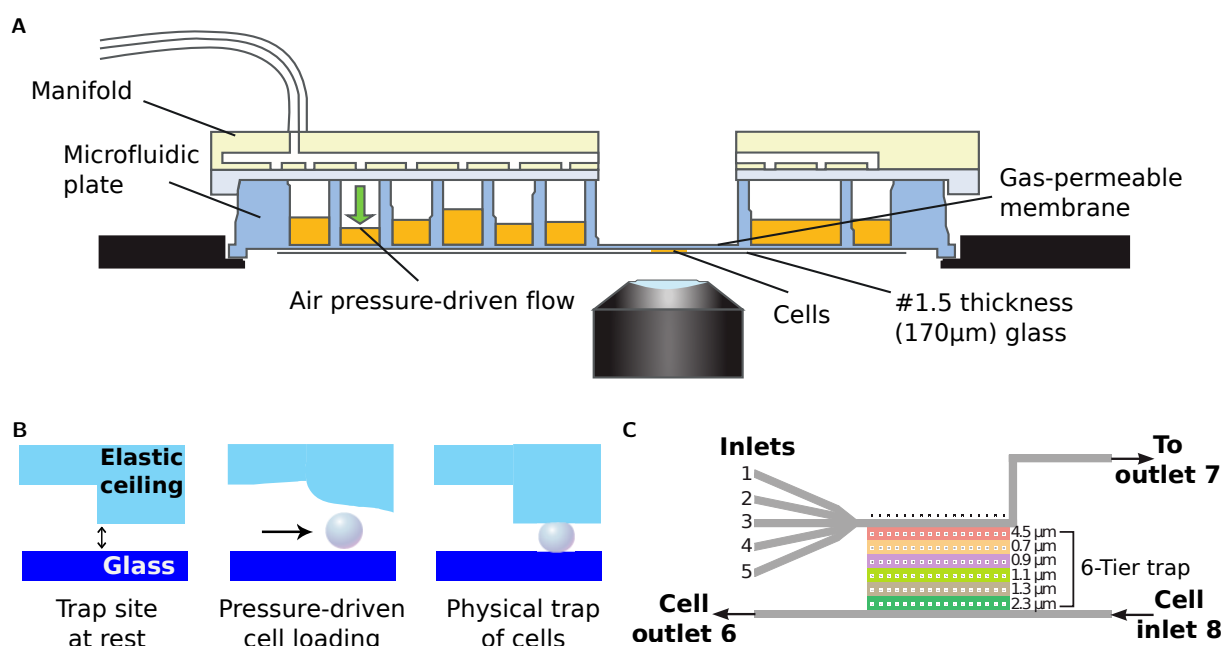


Figure 2.2: CellASIC® ONIX microfluidic platform schematic. Reproduced from Millipore technical datasheets for B04A-04 plates. (A) Depiction of side view of microfluidic plate attached to the manifold. Air is supplied to 5 inlet wells (left) which drives medium flow through the growth chamber (centre) and into the waste well (right). Cells are imaged through glass by the microscope objective lens (bottom). (B) Schematic of cell trapping mechanisms. The elastic ceiling (light blue) of the growth chamber is raised by high pressure and cells are loaded (2nd panel). The release of pressure in the chamber allows the ceiling to rest on cells physically trapping them (3rd panel). (C) Depiction of top view of a single growth chamber (one of four) with inlet well channels (1–5) on the left and outlet well channel (7) on the right. Cells are injected from the cell inlet (8) on the right, and excess and un-trapped cells are ejected through the cell outlet (6) on the left. Each growth chamber consists of 6 traps (centre) with differing trap heights, from top to bottom: 4.5, 0.7, 0.9, 1.1, 1.3, and 2.3 μm .

2.3.1 Troubleshooting

Initial experiments were plagued by the issue of maintaining focus without human intervention over long periods of time. It was clear that the plane of focus was not constant between frames (which were generally taken every 15 min). To address this issue, the microscope software autofocus function was used prior to image capture; this function takes multiple images (using the phase-contrast channel) at different Z-positions and determines the position that has

maximum contrast—which is generally indicative of sharply focused edges. This approach is broadly effective, but generally the autofocus function chooses a Z-position that is a few μm out of focus, thus a strategy incorporating Z-stacks was tested. Z-stacks involve the capture of multiple images at a single XY-position and time-point at multiple Z-positions. At each position, the software autofocus selects the best guess of the focused position, and then positions $5\text{ }\mu\text{m}$ either side of this selected Z-position are captured at $1\text{ }\mu\text{m}$ intervals (10 Z-slices) to attempt to ensure that at least one focused image is always captured for each time-point.

This protocol solves the majority of the focus issues, though not during the first 4 h of most experiments (Figure 2.3A) during which time the rate of change in focal position is often too great for the autofocus to effectively compensate. To solve this, the microscope environmental chamber is pre-heated at least four hours prior to the start of experiments, and the focus is manually adjusted for the subsequent first four hours of every time-lapse. This permits the acquisition of in-focus frames for the full duration of time-lapse experiments (Figure 2.3B).

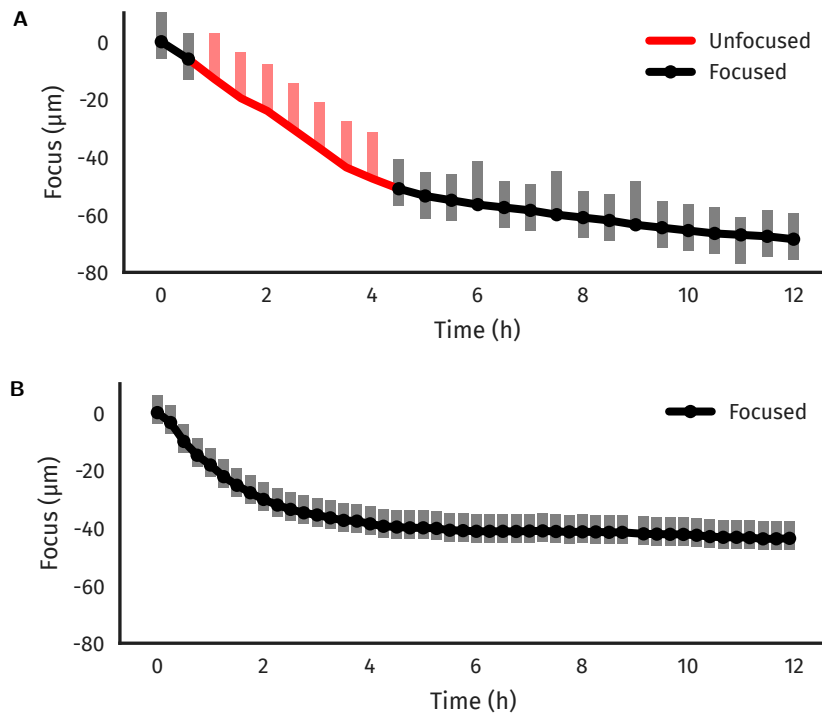


Figure 2.3: Z-drift over time during long-term time-lapse. Z-position relative to the initial starting position is plotted against time. The vertical bars depict the range of each Z-stack for each captured frame with the centre depicting the Z-position selected by the software autofocus. The black line with circles shows which slice in each Z-stack is in focus, the red line shows frames where no slice in the stack is in focus. (A) An early experiment which did not maintain focus. The stack size used in this experiment was $16\text{ }\mu\text{m}$. (B) An experiment in which focus was retained throughout. The stack size used in this experiment was $10\text{ }\mu\text{m}$.

Experiments were also marred by the frequent failure of the plates which can be separated into manufacturing issues and operational issues. Plates were often unable to trap bacteria, with bacteria being washed away after several hours of time-lapse. This was often unpredictable, but was deemed by the manufacturer to be due to the surface of the growth chamber drying

out and delaminating. Quality control in the production line was improved over time and these issues became less frequent. The most common operational issue is the imaging window becoming occluded by condensation after many hours at 37 °C. This was mostly solved by the addition of a small silica tablet into the imaging chamber which reduces the moisture level within the imaging window. Even with this measure, experiments were effectively limited by the accumulation of condensation to 30 hours. This length of time-lapse was however generally more than sufficient for the experiments performed.

2.3.2 Protocol

All experiments were performed in variants of HdB (see Materials section) using *M. smegmatis* mc²155, generally referred to as wild-type. *M. smegmatis* mc²155 is a derivative of a canonical clone of *M. smegmatis* (Snapper *et al.* 1990), which is extremely widely used as the standard wild-type strain for the majority of mycobacterial studies due to its genetic tractability, well-characterised behaviour, and pervasive use in the literature (Reyrat and Kahn 2001; Tyagi and Sharma 2002). *M. smegmatis* cells were grown to stationary phase from glycerol stocks in 5 ml HdB for 48 h in a shaking incubator at 37 °C. Cells were sub-cultured 1:200 in new medium and grown to an optical density at 600 nm (OD₆₀₀) of approximately 0.4 overnight. Cells were diluted to OD₆₀₀ 0.05 and loaded into the microfluidic plate (CellASIC® B04A-03 Microfluidic Bacterial Plates; Millipore). Cells were provided with fresh HdB constantly using a perfusion pressure of 1 psi (6.89 kPa) which corresponds to a flow rate of approximately 5 µl h⁻¹. For each growth chamber (4 independent chambers), 4 XY-positions were chosen and continually imaged at 15 min intervals using phase-contrast and fluorescence channels (when appropriate) with either a 63× oil immersion objective (Zeiss Plan-Apochromat 63× 1.40 Oil Ph3 M27; Zeiss) or a 100× oil objective (Zeiss Plan-Apochromat 100× 1.40 Oil Ph3; Zeiss). Experiments were conducted on a range of microscopes with different cameras and light sources at the Facility for Imaging by Light Microscopy at Imperial College London. All the microscopes have the same objective lens types, fully motorised stages, and environmental chambers. Early experiments were conducted on a Zeiss AxioVert 200M inverted widefield microscope, with a Hamamatsu EM-CCD C9100-02 camera, illuminated with a Xenon lamp, and controlled with SimplePCI or HCImage acquisition software (Hamamatsu). When this microscope was unavailable, I used a different Zeiss AxioVert 200M inverted widefield microscope, with a Hamamatsu Flash 4.0 camera, a pE-4000 CoolLED LED illumination system, and controlled with Volocity software (Perkin Elmer). Later experiments were conducted on a Zeiss Axio Observer inverted widefield microscope, with a Hamamatsu Flash 4.0 camera, a Lumencore SpectraX LED light source, controlled with Zen Blue pro software (Zeiss). The microscopes were all contained within environmental chambers which maintain the temperature at 37 °C. Each position at every time-point was focused using the software autofocus and 10 Z-slices were captured at 1 µm intervals either side of the autofocus-selected position. Each software choice had similar autofocus

functionality, though SimplePCI and Volocity were especially poor. This process resulted in 160 images per time-point (16 positions with 10 Z-slice stacks per position). Experiments were generally conducted for 16–30 h resulting in 10,240–19,200 images per experiment per channel. Files were stored in software proprietary multidimensional formats (SimplePCI and HCSImage create .cxd files, Zen creates .czi files, and Volocity creates .aisf files) and subsequently converted to OME-TIFF format using Fiji (Schindelin *et al.* 2012).

2.4 Image analysis

2.4.1 Initial processing

Each experiment produces a 4-dimensional image file, potentially a 5-dimensional image if fluorescent channels are used. These dimensions are usually summarised as XYZ(C)T; where X and Y refer to the left-to-right and top-to-bottom position within the field-of-view, Z refers to the focal plane within the image stack, C to the fluorescent/brightfield channel, and T to the frame number (i.e. time). In addition to this high dimensionality, each experiment usually captures 16 different fields-of-view. The first step for processing these files is to separate the fields-of-view, a task that is performed either using the software that controls the microscope if it is possible (e.g. Zen) or by using an open source image analysis software application: Fiji, a derivative of ImageJ (Schindelin *et al.* 2012). Once fields-of-view have been separated, images are opened (if not already open) with Fiji. Then, in-focus slices (Z) from each frame (T) are selected using a custom-made Fiji macro (written by Stephen Rothery; part of the Facility for Imaging by Light Microscopy) which extracts each focused slice and concatenates them into a single 3/4-dimensional image: XY(C)T. If fluorescent channels are used, the file is then split according to the channel, resulting in a 3-dimensional image per channel. For further processing, the file must be separated by time (T) with a 2-dimensional image per frame, each of which is saved in a TIFF uncompressed format by Fiji. Each channel is stored in a separate directory and named numerically according to the frame number (T). These steps are summarised in Figure 2.4.

2.4.2 Cell segmentation

Once initial processing is completed, cells in each field-of-view at every time-point must be detected and delineated. To perform this task, several software packages were tested, including CellProfiler (Carpenter *et al.* 2006), SchnitzCells (Young *et al.* 2012), BactImAs (Mekterović *et al.* 2014), and MicrobeTracker (Sliusarenko *et al.* 2011). My requirements for these programs were to detect single cells (segmentation), assemble them into cell lineage trees (cell tracking), and to determine various basic statistics such as cell length.

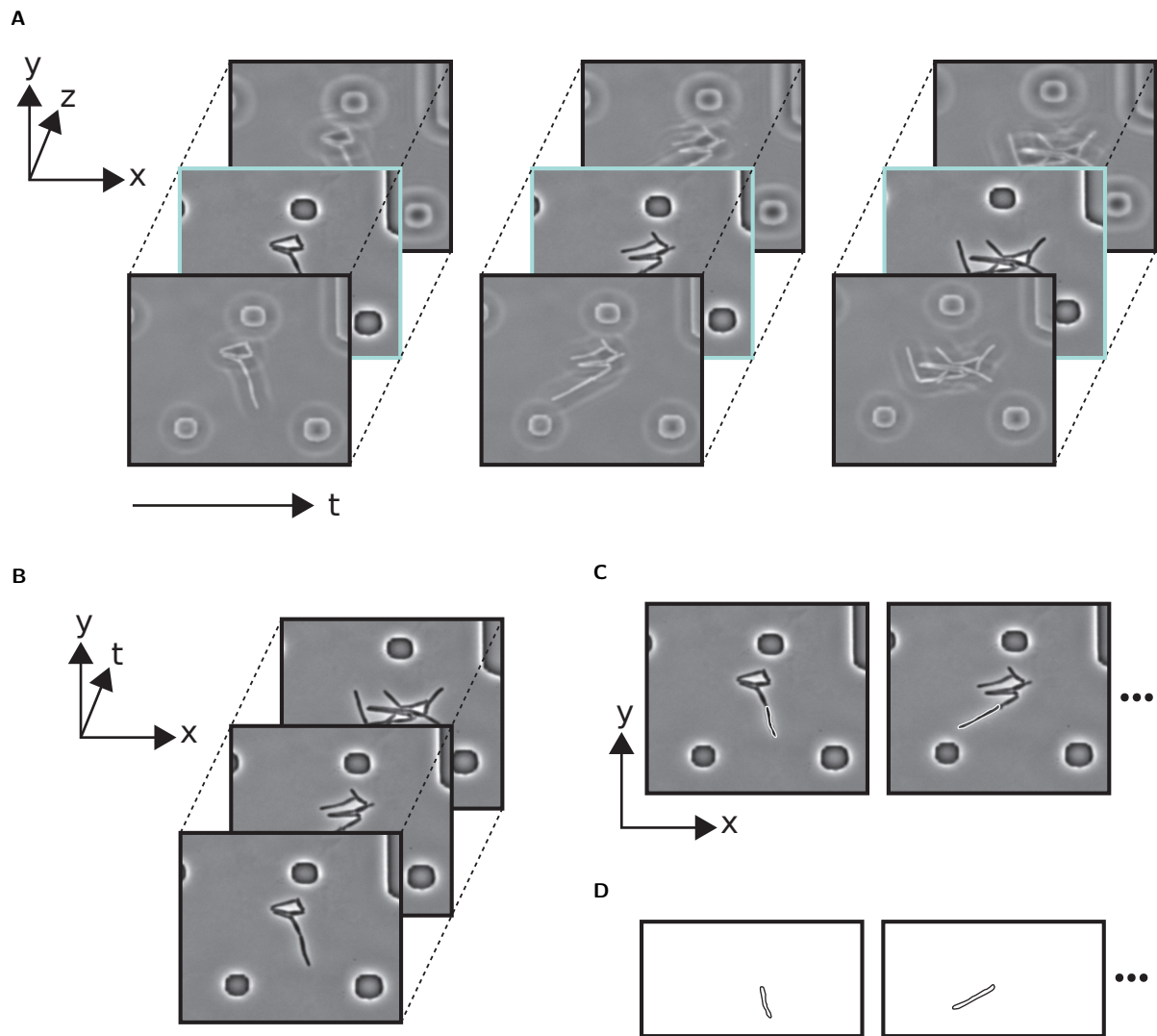


Figure 2.4: Initial image processing steps. Schematic of the process of reducing dimensions from raw microscopy data. (A) 4-dimensional microscopy files are loaded using Fiji with each time-point (t) having 3 dimensions, x and y for the position within the field, and z for the slice within the z -stack. Slices that are in focus are highlighted in cyan. (B) Focused frames are selected using a custom Fiji macro resulting in a 3-dimensional stack. (C) Stacks are saved as individual 2-dimensional TIFF files. A cell is outlined in white in each of the two frames displayed. (D) Each focused frame is analysed by MicrobeTracker, with cells outlined and depicted in black.

CellProfiler (Carpenter *et al.* 2006)

CellProfiler is a stand-alone application designed to automate image processing pipelines without requiring prior experience of programming. It allows users to graphically assemble a series of operations to be performed on a file, verify its effect on a test image, then apply the same set of operations to the whole dataset. However, CellProfiler is primarily designed for classifying much larger cells than bacteria, and has difficulty reliably detecting *M. smegmatis* cells in phase-contrast images, where approaches such as thresholding and watershed algorithms are less effective. When settings are tweaked to permit detection of bacteria in one frame, these settings rarely translate to the whole dataset due to differences in illumination and other factors. Moreover, CellProfiler cell detection algorithms expect somewhat regular cellular morphology of small objects, and attempts to fit the hard-coded models to *M. smegmatis* cells often failed to detect the curvature or irregularity common to *M. smegmatis* cells. These issues alone made CellProfiler unsuitable for analysing *M. smegmatis* time-lapse data.

SchnitzCells (Young *et al.* 2012)

SchnitzCells is a MATLAB analysis package designed for detecting fluorescent protein expression in bacteria in time-lapse experiments, an application which necessitates the outlining of single cells. The package first segments cells automatically according to user-defined parameters, followed by manual correction, assembly of cell lineage trees (cell tracking), and manual correction of lineages. SchnitzCells is optimised for the detection of bacteria from fluorescence channels, where bacteria are constitutively expressing a cytoplasmic fluorescent protein, and was less effective on phase-contrast channels when tested with my data. In addition, mycobacterial cell divisions were poorly detected, and manual correction of division events resulted in poorly outlined daughter cells, especially at the newly created pole. The cell tracking feature of SchnitzCells is based on distance minimisation of the centroid point of all cell pairs between frames (described in detail by Gor *et al.* 2005), and was generally effective for my data. Information for each cell stored by SchnitzCells includes a cell length parameter, but this simply measures the major axis of each segmented object. The major axis is an appropriate estimator of cell length for regularly shaped cells such as *E. coli*, but not for *M. smegmatis* cells which are often curved and irregularly shaped. Given these issues, alternative software solutions were investigated.

BactImAs (Mekterović *et al.* 2014)

BactImAs is a Java-based stand-alone software (as well as an Icy plugin) designed specifically for the detection of irregularly shaped bacteria, with mycobacteria as a stated target. The package is extensively based around ImageJ (Abràmoff *et al.* 2004), with frequent use of manual

user interaction. For example, the cells in the first frame of the time-lapse must be manually delineated, every division event must be defined by the user, and subsequent daughter cells must again be delineated. While BactImAs was generally effective at detecting *M. smegmatis* cells—as it was designed with this purpose in mind—it uses a relatively basic outlining procedure without sub-pixel resolution, and requires a large amount of manual intervention.

MicrobeTracker (Sliusarenko *et al.* 2011)

MicrobeTracker is a MATLAB package originally designed to track *Caulobacter crescentus* cells, and this provides a good starting point for identifying *M. smegmatis* cells since they share some similarities—such as curvature—that make software packages designed for other bacterial species and organisms less suitable. MicrobeTracker is able to draw boundaries around phase-contrast images of mycobacteria with relatively high accuracy, and with a good level of automation coupled with manual flexibility, and was used throughout this thesis. The program splits the procedure into three general stages which are summarised below, and described in detail by Sliusarenko *et al.* (2011).

1. Crude segmentation

This stage separates the first frame into cells or cell clusters using pixel-based morphological operations such as thresholding, then separates single cells using operations such as edge-detection algorithms.

2. Fine segmentation

This stage improves the rough outlines produced by the first stage by employing a procedure known as active contours or morphological snakes. This models the outlines as a contour which responds to physical-like image forces pulling the contour towards regions of high image contrast and pushing it away from low contrast using energy minimisation. This results in a path that can accurately follow the “contours” of the image, thus defining the edges of cells in phase-contrast images. The contours are controlled by a series of parameters that define what characteristics the final shape is permitted to hold, such as smoothness, and minimum and maximum area. This approach to segmentation is very useful especially for the delineation of mycobacteria, which are often irregularly shaped. This stage is relatively accurate, but usually requires significant manual correction to ensure accurate cell boundaries, though MicrobeTracker provides a useful and effective method of correcting cells (detailed below).

3. Cell tracking

This stage involves detecting cells throughout the time-lapse. MicrobeTracker uses the contours from the previous frame as an initial estimate of cell boundaries in the new

frame. This permits the use of the relationship between frames to speed up cell detection. Using these guesses, it applies the active contour model to refine the guess to accurately outline the cell in its new frame. This has the added theoretical benefit of ensuring the continuity of a single cell between frames, allowing the tracking of its history over the course of the time-lapse, including subsequent divisions to create lineages.

In my experience, MicrobeTracker is able to detect cells well, but performs poorly at detecting cell divisions, which requires intervention to mark division events. This is probably due to the manner in which mycobacteria usually divide, which involves a forceful separation of daughter cells to form a characteristic “V” shape, often referred to as “V-snapping” (Dahl 2004; Thanky *et al.* 2007; see Figure 2.5). This mode of division usually involves a significant movement of cells away from the position of their mother cell which MicrobeTracker is unable to account for. Thus, during analysis of time-lapses, cell outlines must generally be added manually for daughter cells, which prevents the automated internal tracking of genealogies within MicrobeTracker. This characteristic of mycobacterial division may have been one of the reasons that the makers of BactImAs decided to require manual assignment of divisions. However, I found that the MicrobeTracker mode of manual assignment is less intrusive than that of BactImAs—since only the approximate centre line of cells requires manual definition—which is followed by automatic refinement by MicrobeTracker similarly to step 2 of the automated procedure. This is faster, more accurate, and more objective than the fully manual approach required by BactImAs.

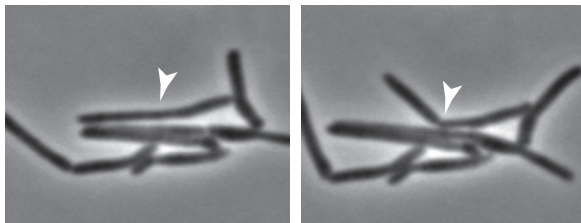


Figure 2.5: Mycobacterial cell division: V-snapping. Mycobacteria divide in a characteristic manner involving a movement away from the axis of the mother cell during division to form a V-shape. *M. smegmatis* cells shown here were imaged during a time-lapse in an ONIX CellASIC® microfluidic plate, and perfused constantly with HdB minimal medium. The mother cell is shown in the left-hand panel, and its two daughter cells in the right-hand panel.

Each cell tracked by MicrobeTracker is defined by a “cell mesh”. This mesh is characterised by a central line placed equidistantly between the cell edges between both poles, with perpendicular ribs protruding from this central line to cell edges, distributed equally along the central line (Figure 2.6). The central line length is used to define cell length since it accurately reflects any irregular curvature in the cell, and is used as a coordinate system for defining other parameters, such as division site placement, and fluorescent foci positioning.

2.4.3 Cell tracking

Given the difficulties that MicrobeTracker faces with tracking cell division in *M. smegmatis* time-lapse experiments, I wrote a series of custom Python scripts which handle this aspect of

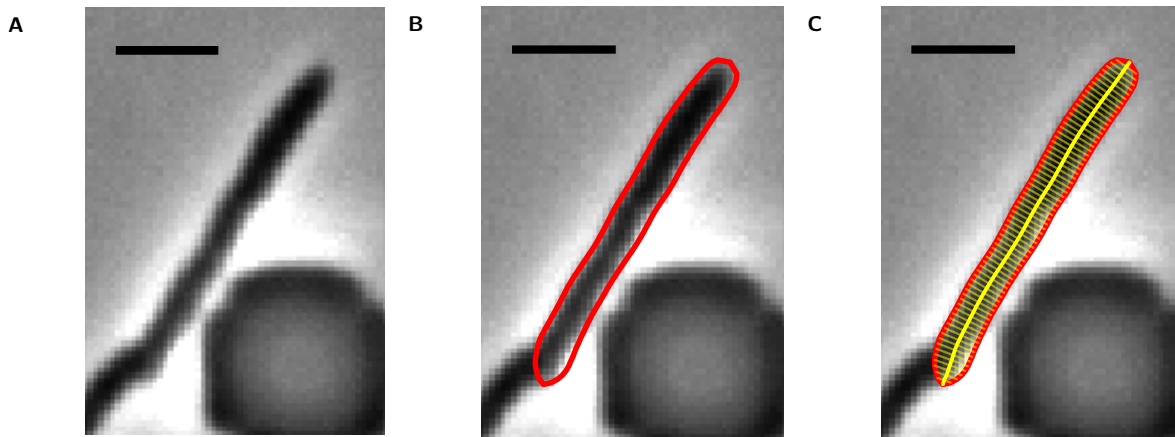


Figure 2.6: Cell mesh defined during image analysis by MicrobeTracker. Bacteria are detected by MicrobeTracker which defines a cell boundary around each cell. This boundary is characterised by a line along the centre of the cell with ribs extending to the cell edges: the cell mesh. (A) Un-annotated cell. (B) The outline of the region defined by MicrobeTracker is shown as a red boundary. (C) The central line (yellow) has ribs placed equidistantly along its length extending to the cell edges perpendicular to the central line (light yellow). The black scale bar denotes 2 μm .

single-cell image analysis. These interdependent scripts are assembled into a package which I have named `lineage`. The names of the major scripts are listed in Table 2.2.

The vast majority of the functionality is encoded in the script `track.py` (`lineage_lib.track`). This file contains classes and functions that are used extensively by all the other scripts, including the most important part of the `lineage` software package: the assignment of cells into lineages.

Script	Description
<code>lineage_lib.track</code>	Defines the majority of the package functionality, all scripts below depend on <code>track</code> .
<code>lineage_lib.poles</code>	Contains logic that allows poles to be defined automatically with a graphical user interface when human input is required.
<code>lineage-bin</code>	Calls <code>lineage_lib.track</code> functions to assign lineages.
<code>lineage_plot</code>	Plots single cell lineages and collated statistics.
<code>lineage_stats</code>	Generates figures of experiment statistics.
<code>lineage_tree</code>	Collates time-lapse data and allows its interrogation interactively.
<code>noisy_linear_map</code>	Calculates and generates figures used in Chapter 3.
<code>lineage_noise</code>	Determines noise within single-cell statistics by bootstrapping, used in Chapter 3.

Table 2.2: List of the major scripts that form the `lineage` package.

`track.py` contains the important classes `Lineage` and `SingleCellLineage` described below.

The Lineage class

This class contains the most important features of `lineage`; when called it loads the MicrobeTracker files that define the time-lapse data: cell meshes and frame-to-frame alignment. Cell meshes are as described above, and the alignment details how frames are aligned relative to each other—necessary given the fluctuations in the absolute positioning of the field-of-view between frames. The file containing cell meshes stores these data as a large multi-dimensional matrix of which the first dimension is image frames, the second dimension details each cell within each frame, and the other dimensions describe features of each cell. The Lineage class loads alignment data and cell mesh information, and assigns a unique identifier to every cell in every frame, which are saved to a file (`uuids.json`) and re-used by all subsequent runs of any of the `lineage` scripts. The `uuids.json` file uses cell numbering defined by MicrobeTracker in each frame, and links each to a unique identifier, storing this information in a JavaScript Object Notation (JSON)-encoded file.

The Lineage class contains the function `guess_lineage`, which attempts to determine two factors: the relationship between the same cell between frames, and the relationship between mother and daughter cells between frames. These relationships are determined by the relative positioning of each cell centre between frames (after offsetting by the MicrobeTracker-defined alignments).

Starting from the first cell in the first frame, the central point of the cell is determined (the halfway point on a straight line drawn between the cell poles) and compared to all the cell centres in the subsequent period. The cell in the next frame with the shortest distance from the cell in question is tentatively assigned as the same cell. The length (central axis) of both cells is then compared, and if the length of the potential cell is within 25 % of the cell under investigation, the two cells are determined to be the same cell at two time-points. If no subsequent cell satisfies these requirements, the function attempts to search for daughter cells.

The daughter search procedure (`search_daughters` function) takes a slightly alternative approach. It compares pole positions as well as central points, based on the nature of V-snapping, where the new poles of the daughters created by division are generally close to the original central point of the mother cell, whereas the old poles inherited from the mother are usually shifted away from their original positions. The daughter search routine first determines the positions of the two poles of the mother cell, then for each potential daughter cell in the subsequent frame, determines the distances between one of the mother cell poles and both of the potential daughter poles. It assumes that the pair with the shortest distance is the same inherited pole (i.e. an “old pole”), and the other pole must be a “new pole”. To test this relationship, it then measures the distance between the assumed new pole and the mother central point, and the distance between the assumed old pole and both the mother poles, and

summarises this by summing all three values. It performs the same aggregation in the opposite direction (old pole to mother centre, new pole to mother poles) and selects the lowest of the two aggregate values as a ‘score’ for the potential daughter. A score is assigned to every cell in the frame, and the two cells with the lowest score are assigned as the daughter cells of the cell in question.

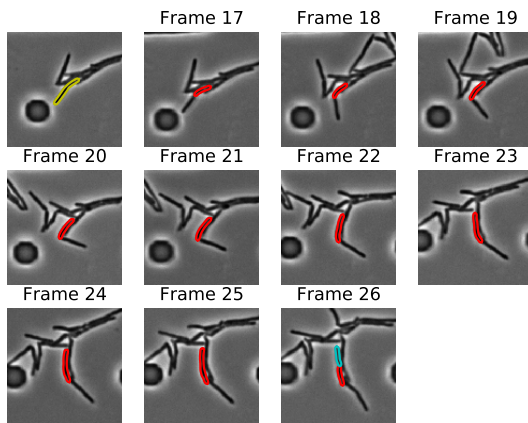
Cells are tracked by the `guess_lineage` function from the first frame to the last frame of the time-lapse, following the first daughter cell (the order of daughters is determined by scoring but is not meaningful) at each division event, and backtracking to the second daughter cell in each branch if it is unable to continue, until all branches of the single-cell lineage through all frames have been guessed. This procedure is fully automatic, but can result in the incorrect assignment of relationships, thus manual verification and correction is required. This functionality is provided by the `interactive_track` function of the `Lineage` class.

The `interactive_track` function provides a graphical method for connecting cells between frames, denoting division events, and terminating any branches in which cells can no longer be tracked. The procedure acts conceptually in a similar manner to `guess_lineage`, with cells tracked through all their branches from the first frame to the last in a frame-by-frame basis. The series of frames that show each cell from birth to division are displayed according to the guessed lineages, which can be accepted or re-assigned using keyboard controls (Figure 2.7A). In the event that the guess is wrong, the interface moves into the re-assignment mode, in which the interface is split into three windows which display the previous, current, and next frames side-by-side (Figures 2.7B and 2.7C). The cell in question is outlined in the previous frame and the current frame, and all cells in the next frame are shaded in, with the guess highlighted. The user can confirm the guess, or deselect the highlighted cell(s) and click on the correct cell(s). If a single cell is selected, the interface progresses to the next frame; if two cells are selected, a division event is assigned, and the interface switches to track the first daughter cell branch; and if no cells are selected, the lineage is terminated and the program will backtrack to the nearest untracked branch (Figure 2.7). This procedure will repeat for all cells until all branches are terminated, and information is saved to the file `lineages.json`.

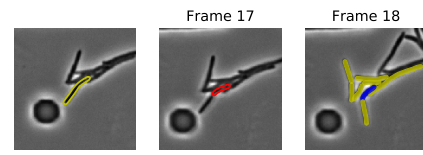
This file encodes the information as a dictionary of key-value pairs, with keys being cell identifiers, and values being arrays of cell identifiers, and stores this information in a JSON-encoded format. If a “growth event” occurs between frames (i.e. no division) the array will contain a single cell identifier; if a division event occurs it will contain two identifiers; and if a termination event occurs it will contain no identifiers. By traversing the structure from cells known to be in the first frame, the entire lineages of all cells can be reconstructed from this file (coupled with the identifier file `uuids.json`). This structure is stored in a `Frames` class object. This object is iterable and acts as a list of all frames within the time-lapse, each frame (`Frame` object) contains a list of all cells (`Cell` object) within that frame. Each `Cell` object contains

A

Lineage preview. Press ENTER to accept or CTRL to re-assign.



B



C

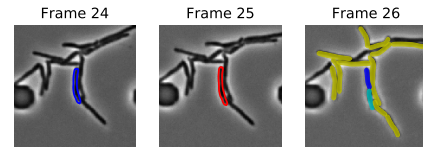


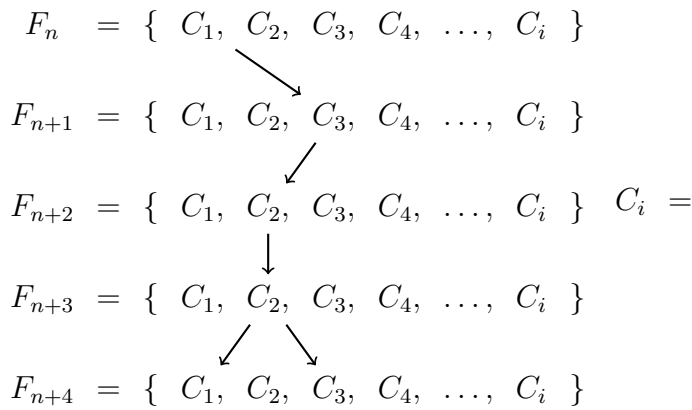
Figure 2.7: Graphical interface for interactive assignment of cell lineages. (A) Guessed lineages are displayed in a preview window from birth until division for each cell, with the parent cell outlined in yellow in the first frame, and subsequent “growth events” outlined in red. Division is denoted by two outlined cells (red and cyan). Guessed assignments can be accepted by pressing enter, or edited by pressing ctrl. (B,C) The $n - 2$ frame is shown in the left panel, and is outlined in yellow if it is the parent of the current cell track, the previous frame ($n - 1$) is shown in the centre panel, outlined in red, and the frame to be edited (n) is shown in the right panel. Cells can be selected or deselected by clicking on them with the mouse cursor, with all potential candidate cells highlighted in yellow. Division events (C) are denoted by highlighting two cells in blue and cyan. Pressing enter increments the frame of each panel by one, unless no cell has been selected or two cells have been selected, in which case the next cell track preview window is shown. The process repeats until all cell tracks have been assigned to the last frame of the experiment.

various attributes including: its “parent” in the previous frame (a cell identifier), its “child” in the next frame (a cell identifier), its length, its mesh data, and the time the frame was captured.

The Lineage class links cells directly between frames by identifying how a cell in frame F_n corresponds to a cell in frame F_{n+1} . This can be considered by the analysis workflow, where frame F_n contains i cells: $F_n = \{C_1, \dots, C_i\}$. The ordering of cells in each frame cannot be guaranteed to be consistent between frames, therefore it is possible but not necessarily true that $C_i|_{F_n} = C_i|_{F_{n+1}}$. For example, Figure 2.8A depicts a structure representing a hypothetical lineage in which the same cell in different frames is not numbered consistently.

To denote this relationship, where each C_i is a Cell object, a parent–child terminology is used, where each cell has a “parent” in the previous frame, and a “child” in the subsequent frame, regardless of whether division actually occurs (Figure 2.8). In the case of division, a Cell object will simply have two children designated (as occurs at the end of the hypothetical lineage above). While this parent–child terminology is useful for constructing paths through each frame, it forces every lineage to be traversed through every frame to obtain useful statistics (such as growth rate) which can be cumbersome.

In addition to this requirement for iteration through many frames for every cell lineage, cells are not guaranteed to be oriented in the same direction between frames, i.e. the first coordinate

A: Frames class**B: Cell class**

id	=	id
frame	=	n
length	=	L
mesh	=	$\begin{Bmatrix} x_1, \dots, x_k \\ y_1, \dots, y_k \end{Bmatrix}$
time	=	t
parent	=	id
child	=	id

Figure 2.8: Representation of data structures encoded by lineage. (A) Idealised and simplified representation of a Frames object, as constructed by the lineage script. Each of n frames (F_n) in an experiment contains an array of i detected cells $C_1, \dots, C_i$. Cells are not guaranteed to be ordered identically between frames, due to cell division and manual re-assignments during the segmentation workflow with MicrobeTracker. A hypothetical cell “lineage” is represented with arrows, connecting the same cell as it grows in the first 4 frames, followed by division in the last hypothetical frame. (B) Each cell C_i contained within the Frames object is an instance of the Cell class, which contains various attributes, a subset of which are shown here. The mesh attribute is itself an array of coordinates delineating the boundary of the cell. The (x_1, y_1) first coordinate pair can be either pole of the cell depending on how the cell was defined by MicrobeTracker, and is not guaranteed to be consistent between frames. The parent and child attributes link the cell to the previous frame and to the next frame respectively, where parent always contains a single id, and child can contain one id (denoting a growth event) or two ids (denoting a division event).

specified by the mesh attribute of each Cell object could be either pole, and is not necessarily consistent between frames. This occurs when cells are added manually during the segmentation process using MicrobeTracker, depending on which pole is first clicked when defining the central spine of the cell.

The SingleCellLineage class

This class was designed to address some of the limitations with the above structures, and includes an automated pole-tracking routine to allow the assignment of pole ages. SingleCellLineage arranges cells into “true” lineages, with “growth” events abstracted behind instances of the class. Each instance contains a list of all the Cell objects—as defined above—as an attribute, but also has a parent and a children attribute—which refer to true parent cells and pairs of daughter cells as is expected intuitively. Instances are also able to contain attributes such as interdivision time, growth and elongation rate, birth and division length, and pole age (described below) which must be calculated in a convoluted manner when using the base Lineage class. In essence, this class places an additional layer over the base Cell objects, allowing more complex attributes to be assigned, and true lineages to be constructed.

Poles are tracked firstly by ensuring the correct orientation of cells, by determining distances

between poles (as in the `search_daughter` function), and by human intervention. Consistent orientation between frames is not guaranteed by the MicrobeTracker workflow, especially when errors are corrected manually, thus `SingleCellLineage` attempts to ensure this. It performs this by measuring the distance between pole positions between frames: if inverting the cell mesh results in a shorter overall distance, then the cell mesh is overwritten with the consistent orientation. If this distance is greater than a threshold value (15 μm), then a manual intervention is requested in which the user must click the same pole in the same cell presented side-by-side in two windows. Pole inheritance is determined by measuring distances between mother and daughter poles, where the pole with the shortest distance to one of the mother poles is assumed to be the old pole in daughter cells, and the other assumed to be the new pole. If the distances are too similar, or too great, then user interaction is required in a similar manner to above. Pole age is calculated based on the inheritance data, with new poles assigned an age of 0, and old poles incrementing their age by 1 at every division. Cells present in the first frame of any time-lapse have an unknown age, and are flagged as such, though their values are still incremented allowing the new/old pole distinction to be determined in a greater number of cells than strict specific age can be.

2.4.4 Single-cell parameters

The `lineage` main script records and calculates several single-cell statistics including: birth length, division length, cell length (at each frame), interdivision time, elongation rate, growth rate, division asymmetry, and pole age.

Lengths

Lengths are assigned to cells in every frame by measuring the length of the central axis defined by MicrobeTracker. Birth and division length are related to the length of a cell in its first frame (termed initial length) and its last frame (final length), but these values are necessarily an overestimate and an underestimate of birth and division length respectively, due to the capture interval of the time-lapse (usually 15 min). Addressing this difference depends on the manner in which single cells grow and is discussed in detail in Chapter 3 (Section 3.2.5). In short, if the growth model is known, a more accurate estimate of birth and division length can be interpolated if the growth rate and interdivision time are known (which they are).

Interdivision time

This value is estimated by the difference in the capture timestamp of the first and last frames of a cell, but is—similarly to birth and division length—affected by the capture frequency of the time-lapse. As above, a more accurate estimate of interdivision time can be obtained by

interpolation if the growth model of the cell is known. This is discussed in detail in Chapter 3 (Section 3.2.5) where the growth model is indeed determined.

Elongation and growth rate

These statistics are two measures of how the length of a single cell changes over time, dependent on the growth model. Determination of the model followed is made in Chapter 3 (Section 3.1.4). Estimates of these rates were determined from the time-series of cell length $L_1, \dots, L_n$ at discrete frames at times $t_1, \dots, t_n$.

Elongation rate describes the linear change in cell length over time, and is calculated by ordinary least squares linear regression of cell length against time:

$$L(t) = \lambda t + L_1 \quad (2.1)$$

Where L is cell length at time t , t is the time since the first time-point, λ is the elongation rate, and L_1 is length in the first frame (initial length). The units of λ are $\mu\text{m h}^{-1}$.

Growth rate describes growth under an exponential model, where incremental change in length is size-dependent. It can be conceptualised as how many times the cell size doubles per unit time, and is determined by ordinary least squares linear regression of the logarithm of cell length against time since the first frame (Equation 2.2), which allows cell length at any time-point to be described as in Equation 2.3.

$$\ln(L(t)) = \gamma t + \ln(L_1) \quad (2.2)$$

$$L(t) = L_1 e^{\gamma t} \quad (2.3)$$

Where L is cell length at time t , t is the time since the first frame, γ is the growth rate, and L_1 is cell length at the first frame (initial length). The units of γ are h^{-1} .

Division asymmetry

Division asymmetry is estimated by simply determining the ratio of the initial length of daughter cells to the sum of the initial lengths of both daughter cells. Each division event results in two daughter cells C_1 and C_2 with birth lengths L_{C_1} and L_{C_2} . The asymmetry of C_1 is calculated trivially as $L_{C_1}/(L_{C_1} + L_{C_2})$. C_1 can be selected arbitrarily as the smaller of the two daughter cells, or according to the age of its pole, to determine new-pole daughter asymmetry for example.

This ratio can also be interpolated by comparing birth lengths instead of initial lengths, as discussed in Chapter 3.

Pole age

This parameter is discussed above, and assigns a generation number to each cell pole, according to how many times it has been inherited from its mother. New poles are assigned an age of 0, and old poles an age of 1 or above. This permits the discrimination of new-pole inheritor cells (maximum pole age of 1) and old-pole inheritors (maximum pole age above 1) which is used extensively in Chapter 3, and is crucial in the analysis of chromosome segregation below. This procedure is described in detail in the `SingleCellLineage` class above.

2.5 Single-cell analysis of chromosome segregation

To demonstrate the applicability of this software and analysis pipeline, I analysed a challenging dataset: the dynamics of chromosome segregation in *M. smegmatis*. This data was acquired using a similar acquisition workflow as described above by Iria Uhía (Department of Medicine, Imperial College London), using the ONIX CellASIC® microfluidic platform.

Chromosome segregation in *M. smegmatis* is believed to be performed by the ParABS system, originally described as a low-copy plasmid segregation system (Gerdes *et al.* 2002) but now known to be involved in chromosome partitioning (Jakimowicz *et al.* 2007; Casart *et al.* 2008; Maloney *et al.* 2009; Nisa *et al.* 2010; Chaudhuri and Dean 2011; Ginda *et al.* 2013; Trojanowski *et al.* 2015; Santi and McKinney 2015). ParA is an ATPase that binds non-specifically to DNA, and interacts directly with ParB. ParB is a DNA-binding protein that binds specifically to *parS* sites clustered near the origin of replication (Jakimowicz *et al.* 2002, 2007; Livny *et al.* 2007; Casart *et al.* 2008). The ParABS system has been extensively studied in *C. crescentus* (Ptacin and Shapiro 2010) in which centromere-like structures are formed by ParB binding to *parS* sites (Graham *et al.* 2014). ParA dimerises when in its ATP-bound form, and binds non-specifically to DNA, coating the chromosome. ParB-*parS* complexes trigger ATP hydrolysis in ParA-ATP, resulting in dissociation of ParA from the chromosome. This induces the movement of ParB-*parS* towards regions of higher ParA, and the segregation of the chromosome (Graham *et al.* 2014). ParA is thought to form a concentration gradient along the chromosome, biasing movement of ParB to effect chromosome segregation, but the mechanism underlying this is unclear (Ptacin *et al.* 2010; Lim *et al.* 2014; Hu *et al.* 2015; Surovtsev *et al.* 2016). When bound to one of the newly-replicated chromosomes, ParB must move towards one of the cell poles, whilst the other chromosome—also ParB-bound—must remain closer to the other pole. In *C. crescentus* and the actinobacteria *Corynebacterium glutamicum*, it is known that *ori* is positioned closer to the pole that was inherited from the mother cell (the old pole) initially, thus some mechanism

must anchor one chromosome close to the old pole, whilst ParB moves the other chromosome towards the new pole (Donovan *et al.* 2010). However, in mycobacteria, *ori* is localised close the mid-cell at birth, moving towards quarter-cell positions upon replication (Trojanowski *et al.* 2015; Santi and McKinney 2015).

To attempt to observe real-time dynamics of the interplay between ParA and ParB in *M. smegmatis*, Iria Uhía constructed strains carrying a plasmid encoding the fluorescent fusions ParA-mCherry and ParB-EGFP in a $\Delta parAB$ background ($\Delta parAB$ [pMEND-AB]). Cells were grown in the ONIX microfluidic device generally as described above, with Z-stacks of phase-contrast, mCherry, and EGFP fluorescence channels captured at 15 min intervals for up to 9 h. 124 individual cells simultaneously expressing ParA and ParB at close to native levels were tracked using the lineage scripts, allowing multiple cell divisions to be observed and lineages to be tracked. This dataset was challenging, with low fluorescence and non-optimal illumination, but provided a useful dataset to develop and extend my time-lapse analysis software. This work was recently published (Uhía *et al.* 2018), in which the data acquired by Iria Uhía coupled with the analysis described below is detailed.

ParA-mCherry is observed to form a diffuse patch along the cell body, with an apparent migration towards the new cell pole as the cell progresses towards division (Figure 2.9A).

ParB-EGFP is observed to form fluorescent foci, usually a single focus at birth which is situated close to mid-cell, which splits into two partway through the cell cycle, which segregate towards opposing sub-polar positions prior to division (Figure 2.9B).

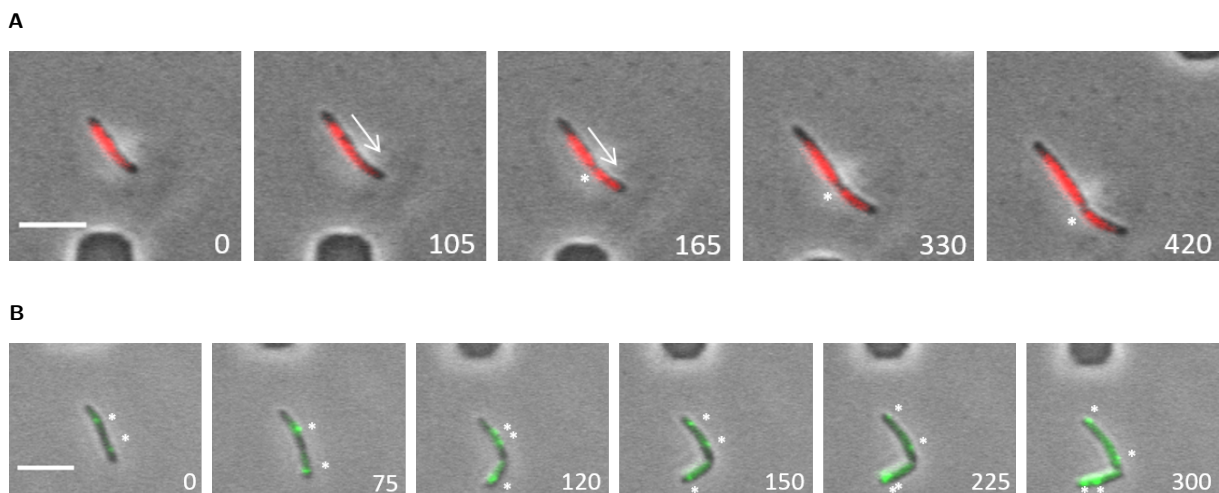


Figure 2.9: Time-lapse of ParA-mCherry and ParB-EGFP during a single cell cycle. Selection of images from 9 h time-lapses with the dynamics of ParA-mCherry and ParB-EGFP shown separately. Scale bars denote 5 μm , and numbers in the bottom right of each frame denote minutes since birth. (A) ParA-mCherry forms a diffuse patch along the cell body which migrates during the cell cycle (white arrow) and intensifies prior to division (septum formation is denoted by a white star). (B) ParB-EGFP forms distinct foci (denoted with white stars) which duplicate and segregate to opposing ends of the cell body during the cell cycle.

The image analysis workflow described above was followed, with focused frames selected with Fiji, and cells segmented using MicrobeTracker. Lineages were constructed using the lineage set of scripts, with associated single-cell parameters. To allow tracking of protein dynamics I developed a suite of additional scripts specifically for the analysis of ParA-mCherry and ParB-EGFP fluorescence profiles, which rely on the lineage scripts, which I named `spot_analysis`. After cells are detected with MicrobeTracker and lineages assigned using `lineage`, the `spot_analysis` script determines fluorescent profiles along the major axis of each cell, and defines both the ParA-mCherry maximum and ParB-EGFP foci within each cell along this axis.

2.5.1 Spot detection

Each fluorescent channel image is smoothed using a Gaussian filter (with a standard deviation of 2 pixels) to reduce noise in the image. Using the mesh defined by MicrobeTracker, the mean fluorescence of all pixels within each segment along each cell is calculated from both the smoothed and raw fluorescence data. This reduces the 3-dimensional fluorescence data (image x and y coordinates, and fluorescence intensity) to a 2-dimensional representation—the mean fluorescence of equally-spaced segments along the cell body. This therefore describes potentially curved cells with a 1-dimensional line from the new pole to the old pole, forming an alternative coordinate system (distance from the new pole) with associated mean fluorescence along this line. The orientation of cells according to inherited pole age is crucial for tracking of fluorescence, and relies on assigned pole ages from the `lineage` script. A crude background subtraction is then performed by defining the background as the simple mean of the entire raw fluorescence field-of-view, based on the assumption that cells are distributed sparsely within this space. Since ParA-mCherry does not form foci (Figure 2.9A) its maximum value along the cell body is used to define its “position”. ParB-EGFP forms one or more foci, a number which can increase during the cell cycle (Figure 2.9B). A peak finding algorithm is used to detect these foci, provided by the `peakutils` python package (Negri and Vestri 2017), which detects points at which the first order differential of the fluorescence profile becomes zero (i.e. an inflection point) with a 5 pixel sliding window and a minimum height of one standard deviation above the mean. To improve this guess, random Gaussian noise—with a standard deviation of half that of the underlying fluorescence—is added to the fluorescence profile, followed by Gaussian smoothing, and the peak detection algorithm repeated. This is performed 20 times, and peaks which are detected more than 10 times in the repeated simulations are retained with greater confidence. Any detected peaks within 2 pixels of either pole are excluded.

Since lineages are defined by the `lineage` scripts, dynamics of both ParA-mCherry and ParB-EGFP between birth and division can then be observed and quantified. ParA-mCherry is defined as a single maximum intensity value along the cell coordinate system. A trace of its position

over time can therefore be obtained trivially, though the concentration profile along the whole cell is also a useful measure, given hypotheses that this forms a gradient which directs the movement of ParB (Graham *et al.* 2014). ParB-EGFP data consists of multiple spots which can duplicate as the cell cycle progresses, and drawing conclusions from the movements of these spots requires that they be tracked. To track these, a partially automated procedure to “connect” spots between frames was developed. This procedure takes every spot in each frame and connects it to the closest spot in each subsequent frame in a similar manner to the lineage assignment procedure above, though since spot positions are effectively 1-dimensional, the procedure is slightly simplified. Each spot is projected onto the cell in the next frame as if there is no growth or movement and the spot is anchored to mid-cell (defined as the midpoint of the cell). Every spot in this next frame is compared to each “expected” position, and the closest focus to each spot is connected as a single “trace”. Any spots in the next frame which are unconnected after this procedure are considered as new spots, and treated in the same manner for subsequent iterations. Since only one connection can be made between spots between frames, traces may terminate prematurely (assuming that ParB foci do not disappear *in vivo*), which is exacerbated by the occasional frame in which ParB peaks are not detected. In addition, ParB splitting events are not considered at all, a process which must occur during DNA replication. This procedure is therefore verified and curated manually with two scripts: `spot_process` and `spot_connect`.

The `spot_process` script provides a graphical interface by which spots can be added, removed and inspected. Each cell is shown frame-by-frame from birth to division, with the fluorescence image shown with the cell outline and detected spots highlighted, the cell fluorescence profile, and an overview of the detected spots from birth to division. Using this interface, automatic spot detection can be overridden for each cell (see Figure 2.10 and legend for further description). As can be seen in the example, automated traces of ParB foci are inaccurate, with two orphaned nodes and the clear duplication event unannotated.

The `spot_connect` script provides a graphical interface by which spots can be connected in time. For the example cell shown in Figure 2.10, the initial guess is depicted in Figure 2.11A, essentially the same panel as the bottom-right of the `spot_process` interface. Spots can be disconnected and connected to one another by clicking on the spot that should be connected and then clicking on the spot which it should be connected to (or outside the frame to disconnect it). The correction of this process for the example cell is shown in Figure 2.11B. This procedure requires human input which is very difficult to automate. Triggering a duplication event is also performed using mouse controls, except the spot that is thought to be duplicated is right-clicked, followed by left-clicking on the spot that it should be connected to. This is represented in Figure 2.11C to produce two corrected ParB traces. Each trace is stored as an array of time, position, and intensity values with duplicated spots appearing in both traces (as in Figure 2.11C).

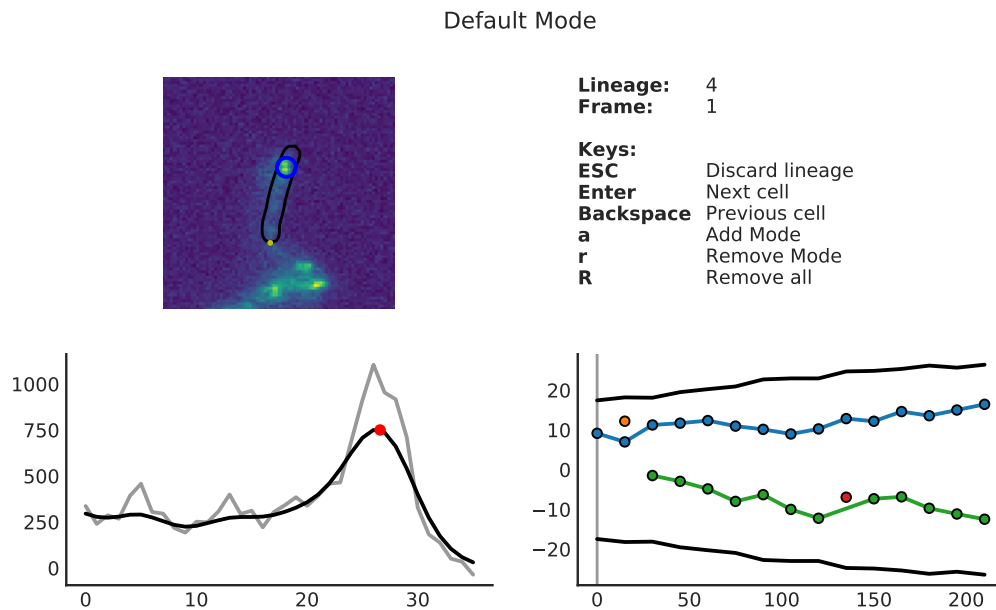


Figure 2.10: Graphical interface to modify fluorescent spot detection. The `spot_process` interface provides 4 panels: top-left, fluorescence image with cell outlined in black, the new-pole marked with a yellow spot, and detected foci with large blue open circles; top-right, cell (lineage) and frame number and interface key commands; bottom-left, fluorescence profile along the cell axis from new to old pole with smoothed fluorescence in black and raw fluorescence in grey, and foci marked with red circles; bottom-right, overview of the cell from birth to division, with the cell boundaries marked in black, the current frame marked with a grey vertical line, and fluorescent spot trajectories marked with black outlined coloured circles. Modes can be switched using the keyboard. The escape key accepts all the spot assignments for the cell and moves to the next, the enter key moves to the next frame (or to the next cell if the last frame), and the backspace key moves to the previous frame. Pressing a switches the interface to “add mode”, in which spot positions can be added by clicking directly on the fluorescence profile panel (bottom-left). Pressing r switches to “remove mode”, in which spots can be removed by clicking on them in the bottom-left fluorescence profile panel. Pressing shift-r removes all spots in the current frame.

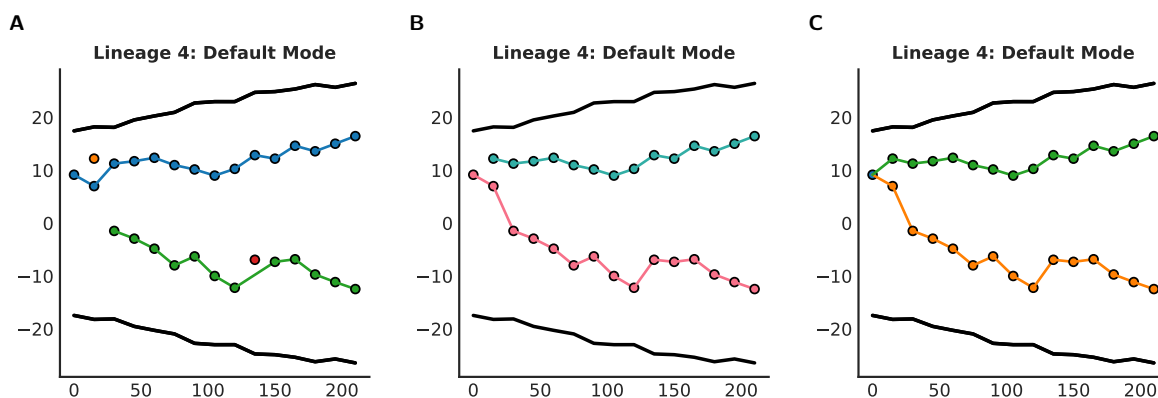


Figure 2.11: Graphical interface to connect fluorescent foci into coherent traces. The `spot_connect` interface shows the position of detected fluorescent foci over time with the cell boundaries denoted with a black line (where distances are relative to mid-cell with the new pole at the bottom of the figure). Existing traces are shown by coloured lines (the colour is chosen randomly). The initial guess for spot traces for an example cell is shown in panel A. Spots can be connected by left-clicking on spot pairs, which automatically updates the view, as in panel B. Duplication events can be indicated by right-clicking on the first spot (e.g. in frame 1 in this example), then left-clicking on the unconnected spot (e.g. the top trace in this example). The final modified traces are then shown for verification in panel C. Changes can be accepted with the enter key, and rejected with the escape key.

2.5.2 ParA dynamics

ParA appears to accumulate over the cell cycle, initially concentrated near mid-cell, where the chromosome is expected to be located at birth. As the cell cycle progresses, ParA-mCherry intensifies, and concentrates in a single region of the cell, which often appears to be inherited by a single daughter cell. Given that cell lineages have been tracked, this inheritance pattern of ParA-mCherry can be quantified. The total fluorescence of ParA-mCherry appears to be directly proportional to cell area at birth, implying that daughter cells receive an equal concentration of ParA-mCherry (Pearson's $r = 0.73$; Figure 2.12A) in a manner that is not related to size. Proportional inheritance of total fluorescence is expected if ParA is partitioned randomly with cellular contents. However, the maximum intensity of ParA-mCherry inherited is not correlated with cell area at birth ($r = 0.03$; Figure 2.12B). This implies that while cells which are born large inherit more total ParA-mCherry, they are not more likely to inherit the most intensely concentrated region of ParA-mCherry.

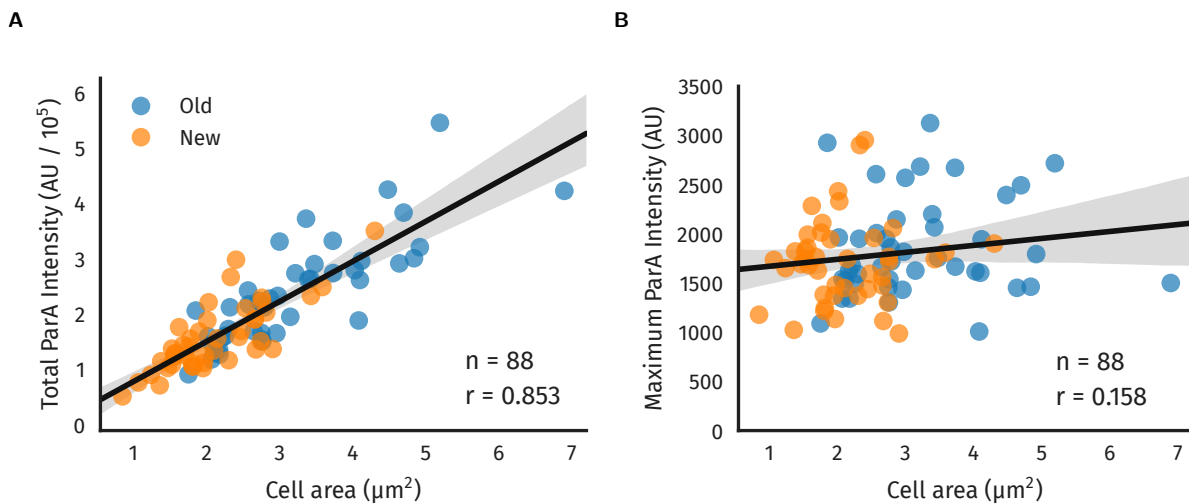


Figure 2.12: Inheritance of ParA-mCherry at birth: total and maximum intensity. Total (A) and maximum (B) intensity of ParA-mCherry is determined at birth, and plotted against cell area (filled circles). Cells which inherit the old pole from their mother are shown in blue, and cells which inherit the new pole are shown in orange. An ordinary least squares linear regression line is fitted to these data, and depicted with a solid black line, with 95 % confidence intervals displayed as a shaded region. The Pearson correlation coefficient (r), and the number of cells analysed (n) is shown in the bottom right.

When this analysis is repeated on sibling pairs however, differences emerge. The sibling which inherits a higher total ParA is significantly more likely to be larger than its sibling (Figure 2.13A), as expected by the population relationship (Figure 2.12A), but the cell which inherits a higher maximum intensity of ParA is also more likely to be larger than its sibling (Figure 2.13B). An example of a cell that inherits the ParA maximum, and the manner in which it is analysed is shown in Figure 2.13C. To determine whether this inheritance might be due to a specific mechanism, I compared the inheritance of the peak to the probability that a randomly distributed point is inherited: the ratio of daughter cell size to parent cell size. The sum of

these ratios over all cell divisions (44 divisions) indicates that the expected number of peaks inherited as predicted purely by cell size in 26.38, and the true number observed is 27. This indicates that the inheritance of the ParA maximum is consistent with a size-based non-specific mechanism that may be a consequence of asymmetric division.

Interestingly, cells which inherit the higher total ParA-mCherry or the maximum intensity region are both more likely to grow faster (Figures 2.13D and 2.13E). However, when larger sisters are compared to their sibling irrespective of ParA inheritance, they do not have a statistically significant difference in growth rate (Figure 2.13F). This may indicate that inheritance of the ParA maximum could confer a growth advantage independently of cell size.

2.5.3 ParB dynamics

Once the fluorescence profile for ParA, and foci positions for ParB have been determined by the above procedures, their simultaneous movements over the cell cycle can be visualised and quantified. The visualisation that can integrate cell length, cell cycle time, and both ParA and ParB can be considered as a form of kymograph in which each frame between birth and division is positioned vertically along the cell axis from new pole to old pole with a heatmap representing ParA-mCherry intensity. ParB-EGFP foci are then overlaid as a standard line plot against time. A cell which is born with a single ParB-EGFP focus is depicted in Figure 2.14. This focus splits into two in the second frame (15 min after birth) which begin to separate as the cell cycle progresses. One focus stays close to the new pole (bottom of the plot), and remains associated with a high concentration of ParA-mCherry, which accumulates near the new pole as the cell cycle progresses. The other ParB-EGFP focus migrates towards the old pole, away from the region of highest ParA-mCherry concentration.

This activity can be quantified in all cells using the underlying data, by determining the specific velocity relative to each pole. This is calculated by fitting a straight line to each ParB focus position over time using ordinary least squares regression. Foci can be measured relative to distance to the nearest pole, in which case three forms of movement can be observed: movement away from the pole, movement towards the pole, and no relative movement. These are classified by a threshold of $0.15 \mu\text{m h}^{-1}$. 52 % of 217 ParB-EGFP spots in 124 cells have no relative movement compared to their nearest pole, indicating that they may be anchored in some manner, moving away from mid-cell at the same rate as cell growth. 16 % of spots move towards the nearest pole at a rate faster than growth, and 31 % move away from it, implying that their movement is not passive. These proportions are similar whether spots are originally observed closer to the old pole or to the new pole, thus the activity observed in Figure 2.14 does not appear to be a conserved feature. When analysing these results relative to the peak of ParA-mCherry fluorescence, movement can again be classified in three forms: away from, towards, and no relative movement (“with”). Equal numbers of ParB-EGFP foci move towards or

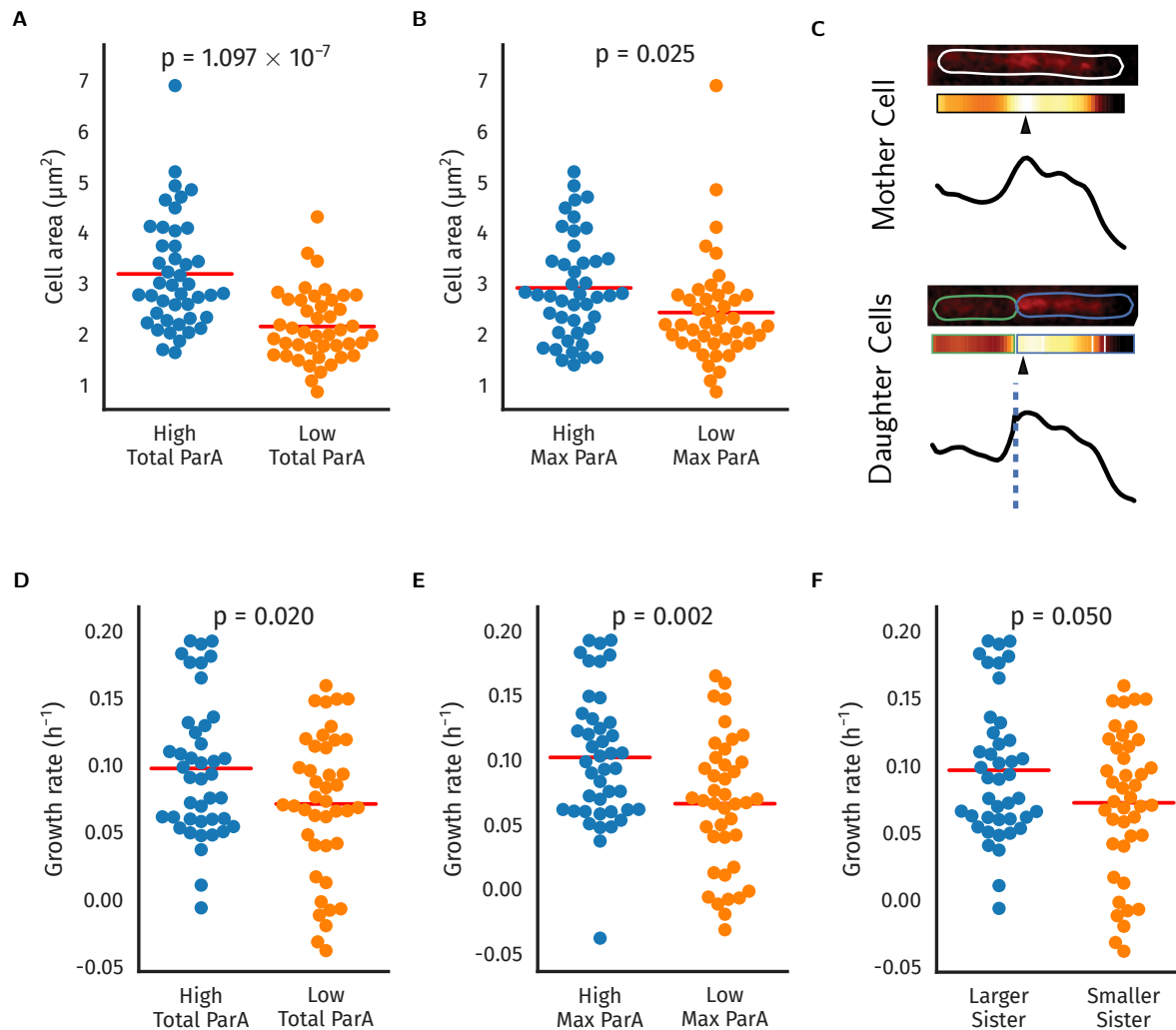


Figure 2.13: Inheritance of total and maximum intensity ParA-mCherry in sibling cells. Swarm plots representing the effect of ParA-mCherry inheritance (total or maximum) on cell area and exponential growth rate. p -values comparing each subset as determined by Wilcoxon's signed-rank test are shown above each plot. Mean values for each data subset are depicted as a red horizontal line. (A) Cell area of cells which inherit a higher total ParA-mCherry are compared to siblings which inherit a lower total ParA-mCherry. (B) Cell area of cells which inherit the highest intensity region of ParA-mCherry compared to their sibling. (C) Example of the distribution of ParA-mCherry in a mother cell and its two daughter cells, in which the larger daughter inherits the highest intensity region of ParA-mCherry. (D) Exponential growth rate of cells which inherit higher total ParA-mCherry compared to siblings which inherit a lower total. (E) Exponential growth rate of cells which inherit the highest intensity region of ParA-mCherry compared to their sibling. (F) Exponential growth rate of cells which are born larger than their sibling, regardless of ParA-mCherry inheritance.

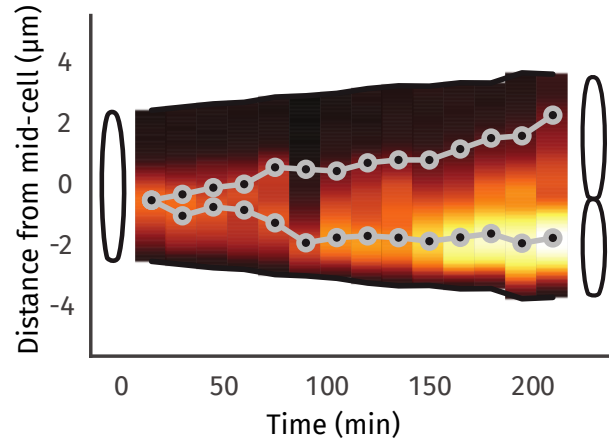


Figure 2.14: Kymograph of ParA-mCherry and ParB-EGFP for a single cell. A single cell from birth to division is shown with ParA-mCherry intensity displayed as a heatmap along the cell axis from new pole to old pole. ParB-EGFP foci are overlaid as traces over the ParA-mCherry intensity plot. Cells are centred around mid-cell with poles marked by a black line. Cell length at birth, and cell length of the two daughter cells are indicated by cartoons on the left and right of the plot.

with the ParA-mCherry peak as they move away from it, in line with chromosome segregation where one chromosome is thought to associate with the region of highest ParA concentration.

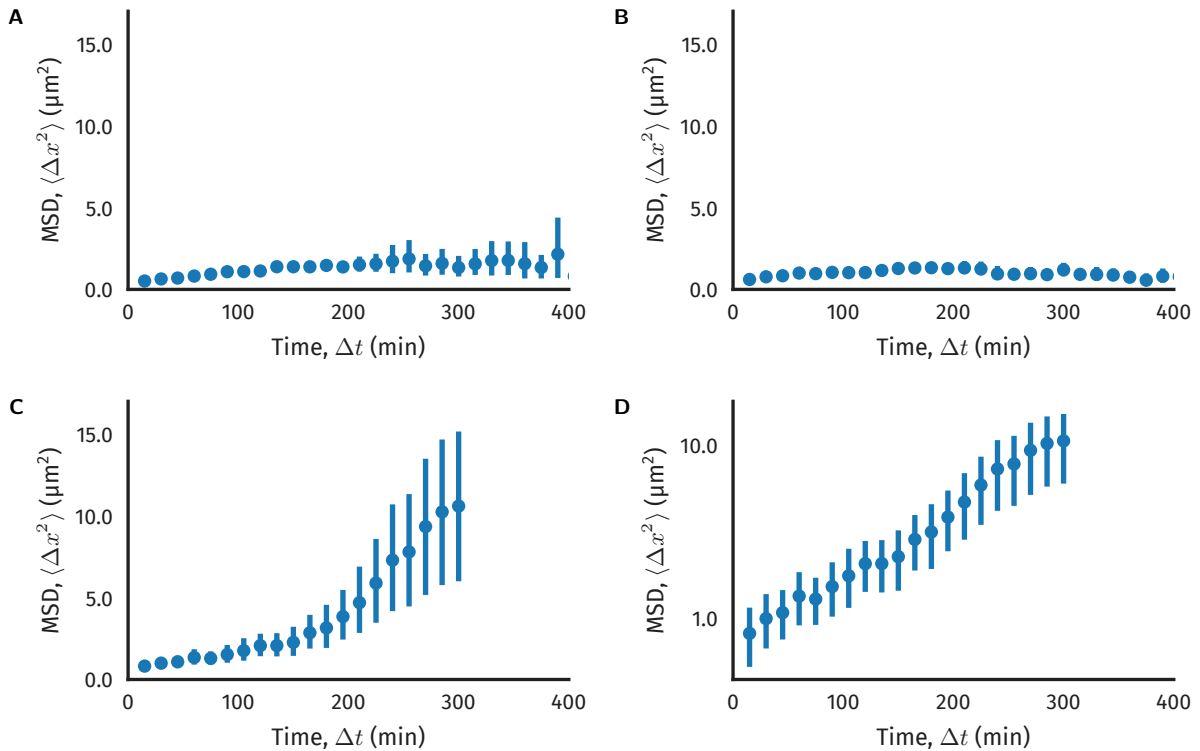


Figure 2.15: Diffusion profiles of ParB focus movement over time according to distance from the ParA peak concentration. Mean squared displacement ($\langle \Delta x^2 \rangle$) of ParB-EGFP foci for all time steps (Δt) are plotted for foci originating $<1.5 \mu\text{m}$ from the ParA-mCherry peak of fluorescence intensity (A), $1.5\text{--}3 \mu\text{m}$ from the peak (B), and $3\text{--}4.5 \mu\text{m}$ from the peak (C and D). For each Δt , the mean squared displacement is plotted, with errors indicating the 95% confidence interval of all spots (determined by bootstrapping). Panels A–C are plotted with a linear y -axis, while panel D is plotted with a log scale.

To further investigate this potential non-passive movement, the diffusion constant (D) of ParB-EGFP movement was determined by fitting the equation for 1-dimensional mean squared

displacement (MSD) $\langle \Delta x^2 \rangle = 2D(\Delta t)^\beta$ for all Δt time windows between the appearance of a spot and spot duplication or cell division. The exponent β of the equation indicates the form of diffusion, where a purely linear relationship ($\beta = 1$) implies passive diffusion or Brownian motion, a β of less than 1 implies anomalous diffusion or sub-diffusion, and a β of greater than 1 implies active movement (Golding and Cox 2006). Fitting the equation to all spots over all time windows provides poor results regardless of the value of the exponent, thus spots were binned based on their distance from the ParA-mCherry peak of fluorescence at the first time-point. The MSD for foci that originate close to the peak of ParA-mCherry ($<1.5 \mu\text{m}$) is low, with $\beta = 0.394$ indicating sub-diffusion that implies molecular crowding or a molecular interaction (Figure 2.15A). This remains similar for foci less than $3 \mu\text{m}$ from the ParA-mCherry peak (Figure 2.15B), but greatly changes for foci more than $3 \mu\text{m}$ away, with a poor fit of the 1-dimensional MSD equation (Figure 2.15C). There appears to be an exponential relationship between MSD and time, indicating rapid, active movement of foci that originate far from the ParA-mCherry intensity peak (Figure 2.15D).

2.6 Discussion

In this chapter I describe the optimisation of a commercial microfluidic platform to allow long-term time-lapse microscopy of *M. smegmatis* cells continuously cultured in growth medium. I discuss the image analysis pipeline, which combines existing software and software that I wrote to allow analysis of single *M. smegmatis* cells. Finally, I demonstrate how this software was applied to an experimental dataset, and how the data structures it produces can be extended to the analysis of a specific experimental question. The analysis of this question, along with the data acquired by Iria Uhía, is described in a recently published manuscript (Uhía *et al.* 2018).

Microfluidics have become a vital tool in bacteriology, since they permit the constant observation of single cells under constant conditions. Microfluidics greatly improve upon previous time-lapse experimental strategies, such as agar pads, since they provide a constant supply of nutrients to cells to maintain their environment. However, most microfluidic systems require complicated in-house fabrication capabilities, and the use of temperamental platforms. The CellASIC® ONIX microfluidic platform provides a reproducible, quality-controlled source of microfluidic plates that permit repeated experiments under similar experimental conditions. Maintaining focus in long-term experiments has been a difficult problem in time-lapse microscopy since the advent of the technique, and this is no different with regards to microfluidic systems. Environmental chambers ostensibly maintain their temperature at a desired setting, however since the chamber is large, gradients between the chamber edges and the heating element can form, leading to fluctuations in temperature. These changes result in the expansion and contraction of elements of the microscope, and thus the movement of the sample. Since the difference between a perfectly focused image and an unusable out-of-focus image can be as low as $0.5 \mu\text{m}$, these

fluctuations are a significant problem. To address this, aside from the conventional use of an anti-vibration table, I preheat the environmental chamber to 37 °C for at least 4 h prior to each experiment, and preheat the microfluidic plate and the growth medium. For each time-point, software autofocus is used to maximise image contrast, then a Z-stack with 10 1 µm slices is used to ensure that at least one slice is in focus for all time-points. Once images have been captured, a Fiji (Schindelin *et al.* 2012) macro is used to allow selection of the slice that is in focus to reduce the dimensionality of the data (Figures 2.4A and 2.4B).

To segment cell objects from time-lapse data, I tested a number of software approaches designed for the detection of single cells, and decided to use MicrobeTracker which is able to detect cell outlines using an active contour model (Sliusarenko *et al.* 2011), and provides a convenient method of correcting errors and designating new cells (especially after division). MicrobeTracker does not reliably detect mycobacterial cell division events, which must be annotated manually in the majority of cases, though the software package does provide several useful tools to perform this easily. To assign lineages, I developed my own suite of Python scripts which I named `lineage`, which in essence replicates many of the functions of the `SchnitzCells` package (Gor *et al.* 2005). This script largely automates the task of tracking single cells through multiple frames and over multiple divisions, and collates these data into a form of tree structure. From these data, fundamental parameters describing single cells can be obtained, including length at birth, length at division, interdivision time, and linear and exponential models of elongation. More specific parameters such as pole age can be defined by ensuring the consistent orientation of cells between frames, and tracking the inheritance of poles from mother to daughter.

The workflow developed essentially uses the segmentation power of MicrobeTracker (Sliusarenko *et al.* 2011), the manual division annotation process used by `BactImAs` (Mekterović *et al.* 2014), and the lineage construction procedure encoded by `SchnitzCells` (Gor *et al.* 2005), to provide a unified system tailored specifically for mycobacteria grown in the ONIX microfluidic system.

These processes are mostly automated, with a visual verification procedure via an interactive graphical interface to correct cell tracking and daughter assignment after division (Figure 2.7). To determine pole inheritance, cell orientation must be maintained, and this is performed automatically by measuring the distance between poles, with additional graphical intervention when automatic orientation cannot be performed. When cells divide, there is usually a significant movement of daughter cells away from their mother cell, as they undergo “V-snapping” (Figure 2.5). This can complicate pole tracking between divisions, and when movement is too significant to allow automatic assignment, another graphic interface is used to provide manual confirmation. These interfaces and automatic procedures form the basis of the `lineage` scripts, and act together to provide a lineage structure that completely describes single cells in long-term time-lapse experiments.

The coupling of the MicrobeTracker mesh (Figure 2.6) with the lineage structure obtained by the lineage scripts provides a powerful tool for investigating the single-cell characteristics of mycobacteria. To demonstrate and test the utility of the scripts, I applied them to a challenging dataset acquired by Iria Uhía, a post-doctoral researcher in my group. She constructed a plasmid containing fluorescently-tagged variants of ParA and ParB—proteins thought to be crucial in chromosome segregation in mycobacteria—and imaged a *M. smegmatis* $\Delta parAB$ deletion strain expressing these tagged proteins, using the microfluidic platform and a similar protocol reported above. ParB binds specifically to *parS* sequences found near to the origin of replication in mycobacteria, whereas ParA is a non-specific DNA-binding ATPase which can bind anywhere on the chromosome when in its ATP-bound form (Jakimowicz *et al.* 2002, 2007; Livny *et al.* 2007; Casart *et al.* 2008). When ParB interacts with ParA–ATP, it stimulates ATP hydrolysis, and disruption of the ParA–DNA interaction. This results in a gradient of ParA–ATP along the chromosome, which—by an unknown mechanism—biases the movement of ParB–*parS* in a manner that results in chromosome segregation (Ptacin *et al.* 2010; Lim *et al.* 2014; Hu *et al.* 2015; Surovtsev *et al.* 2016). The dynamics of ParA and ParB in live cells—at the time of the project—was well known in *C. crescentus* (Ptacin and Shapiro 2010) but not in mycobacteria.

To allow investigation of ParA-mCherry and ParB-EGFP in single cells, I developed scripts which extend the lineage scripts, and make use of the mesh structure determined by MicrobeTracker. Since ParA binds non-specifically, it does not form clear fluorescent foci: the overall gradient and local concentration of the protein are more pertinent, and a more simple computational problem to address (Figure 2.9A). ParB binds only to *parS* sites, thus forms distinct foci that mark the origin of replication in single cells (Figure 2.9B). To detect ParB foci and define peak ParA concentration, I developed a script which reduces the fluorescent signal from the whole cell to a one-dimensional intensity profile representing the mean fluorescence at segments along the cell body (from new pole to old pole). ParB foci positions relative to the new pole are determined in a partially automated manner by determining inflection points in the fluorescence profile, then requiring manual correction and verification with a graphical interface (Figure 2.10). Focus traces within a single cell, including duplication events, are also assigned in a semi-automated manner, and corrected with a graphical interface (Figure 2.11), which allows individual “ParB traces” to be defined for every cell.

ParA position is defined trivially as its highest intensity value along the cell axis dimension, but this is not necessarily the most useful measure. Instead a more holistic view of ParA is used, which generates interesting relationships. The inheritance of ParA at division appears to be non-equal from naive inspection of the raw data (e.g. Figure 2.9A). To quantify this, I determined the total intensity value inherited by daughter cells according to cell area at birth, and observed that there is a direct linear relationship (Figure 2.12A). This relationship is expected if ParA-mCherry is partitioned non-specifically, since it is in proportion to cell area. However, the highest intensity value of ParA-mCherry—assumed to indicate the region

with highest local concentration—is not inherited in proportion with area at birth, with no correlation observed (Figure 2.12B). This implies that cells which are born larger are not more likely to inherit the most intense region of ParA-mCherry. While these relationships are true when quantified without considering cell lineages, differences appear when sibling cells are directly compared. The cell which inherits more total ParA is more likely to be larger than its sibling (Figure 2.13A), as expected from the population measure, but the cell which inherits the higher maximum intensity region is also more likely to be larger (Figure 2.13B), which is not indicated by plotting area against maximum intensity for all cells. This implies that size may play a role in the specific inheritance of the most concentrated region of ParA intensity, in a manner that is not seen when cell division is ignored, or in a manner that is blind to the specific intensity value. This implication can be tested by comparing the probability that a randomly distributed point is inherited according to septum positioning: the ratio of daughter cell size to its parent cell size. The expected number of inheritance events predicted by size ratio is not different from the true number observed to be inherited by the larger daughter at division, implying that asymmetric division alone may drive the observed inheritance pattern. Cells which inherit either a higher amount ParA-mCherry or a higher maximum intensity are significantly more likely to grow faster than their sibling (Figures 2.13D and 2.13E), a difference that is not observed when siblings are compared purely based on size (Figure 2.13F). This has the direct implication that the inheritance of a greater amount or intensity of ParA could trigger faster growth in daughter cells independently of cell size. Investigation of any possible growth advantage conferred by inheritance of ParA would require significant additional experiments, and crucially would require the use of a strain carrying tagged ParA in its native genetic environment (especially with its native promoter). This does however demonstrate the importance of quantifying relationships that appear from basic inspection of raw data.

The movement of ParB over the cell cycle was of great interest, given that ParB has been proposed as the major component that effects chromosome segregation, forming centromere-like structures close to the origin that permit the separation of the nascent chromosomes to each daughter cell. ParB-EGFP generally forms a distinct focus that marks the origin of replication (e.g. Figure 2.9B) which duplicates as DNA replication occurs, and these spots proceed to move apart concurrently with segregation, in line with its role. The analysis of ParB-EGFP in live cells results in a record of the distance of every spot relative to the pole formed at birth (the new pole). Thus changes in this distance can reflect cell growth as the overall distance between both poles increases. This requires reformulating this distance relative to mid-cell, defined simply as the mid-point between both poles, and visualising this in a manner that combines ParA distribution, cell length, and spot positioning. This is implemented with the use of a kymograph of ParA intensity at each time-point relative to mid-cell, with plots of ParB foci positions overlaid (e.g. Figure 2.14). Movement of these traces can be considered relative to mid-cell, their closest pole, or relative to the peak of ParA intensity. The magnitude of this

movement is determined by linear regression of distance from the relative marker against time, to obtain the velocity of spot movement. If movement is defined as a velocity above a threshold value of $0.15 \mu\text{m h}^{-1}$, we can see that just over half of all ParB spots do not move relative to their nearest pole, suggesting that some form of anchoring could be taking place, as has been suggested to occur in *C. crescentus* via the TipN protein (Ptacin and Shapiro 2010; Ptacin *et al.* 2010), or via a similar interaction to that which has been proposed in mycobacteria with Wag31 anchoring ParA to the old pole (Ginda *et al.* 2013; Meniche *et al.* 2014). The anchoring of ParB to a single pole (e.g. the new pole) could explain how segregation occurs mechanistically. Since ParB is thought to be biased in its movement towards ParA, this raises the question of how only one focus moves towards ParA whilst the other remains fixed or moves in the opposite direction to allow segregation to occur. Approximately one third of spots move away from their nearest pole at a rate faster than growth, and the remaining sixth move towards it, which is broadly consistent with chromosome segregation. When analysed relative to the peak of ParA-mCherry fluorescence, equal numbers of spots move towards the peak or remain close to it, or move away from the peak. This is also consistent with the model in which one ParB-*parS* centromere-like structure is closely associated with ParA whilst the other is segregated away from it.

While these numbers provide a general overview of ParB dynamics relative to the polar regions and to ParA, a more quantitative approach was sought by determining the diffusion characteristics of ParB position relative to the ParA peak. This determined that spots which are first observed close to the ParA peak do not move significantly over time, with sub-diffusive motion indicative of a highly crowded local environment, or a molecular interaction that retains ParB (Figures 2.15A and 2.15B). When spots are first observed more than $3 \mu\text{m}$ away from the most concentrated region of ParA-mCherry however, a non-passive diffusion profile is observed, where ParB moves at a greater rate that is expected by Brownian motion (Figure 2.15C). This relationship appears to be exponential (Figure 2.15D), which may imply an active process is governing the motion of ParB. This might be envisioned as a process by which ParB foci which are close to ParA are “captured” by ParA, or some other polar determinant, restricting their movement. If this interaction is saturating, when the *parS* region of the chromosome is duplicated during DNA replication, this focus can then move further away from the ParA peak concentration by diffusion, until it is captured by some other interaction, perhaps with the opposite pole, which then drives rapid segregation. However, this mechanism is highly speculative, and much further work is required.

This dataset provides a good example of the utility of the software that I have developed to analyse single-cell time-lapse images, and demonstrates how the software can be extended to provide additional quantitative insight. The software is used throughout the rest of this thesis: to demonstrate that mycobacteria grow exponentially at the single-cell level, and the phenomenological manner in which they control their cell size in Chapter 3; and to investigate

how single cells respond to antibiotics, focusing especially on the characteristics of surviving cells that are phenotypically tolerant in Chapter 4. The ONIX microfluidic platform provides an easily adaptable experimental system in which growth medium can be maintained or switched at will, with the optimisation reported here providing a reproducible and robust methodology with which to perform such experiments.

CHAPTER 3 CELL-SIZE CONTROL

Tuberculosis

Why o why o why do you

Decide to divide?

Maya Caspari, Emily Mercer (2018)

3.1 Introduction

ALL living organisms must control their cell size in order to ensure that daughter cells receive sufficient material to maintain the concentration of crucial proteins, such as those involved in protein synthesis or central metabolism, and to provide sufficient space for the incorporation of nascent DNA (Shahrezaei and Marguerat 2015). It is well known that bacteria are able to maintain their average size within steady state populations, where average size is thought to be a function of the population doubling time (Schaechter *et al.* 1958; Chien *et al.* 2012). Cell-size homeostasis is determined at the single-cell level by the decision to divide, since this sets both the initial and final length of cells. This decision must be balanced with DNA replication, and be resistant to fluctuating sources of noise, such as stochastic gene expression, heterogeneity in growth, and variation in microenvironment. Strategies to decide when to divide, with some molecular-level insight, are now being investigated in detail, primarily in model bacterial species such as *Escherichia coli* and *Caulobacter crescentus* (Campos *et al.* 2014; Taheri-Araghi *et al.* 2015). Information regarding mycobacteria—at the time of this project—was lacking, thus a definitive model of the cell-size control strategy *M. smegmatis* mc²155 follow was sought.

In this chapter, I discuss published results (Priestman *et al.* 2017) that describe the relationship between birth length and division length, and other single-cell parameter relationships, which provides a principle by which *M. smegmatis* cells can maintain their cell size under standard laboratory conditions. I also determine that this relationship can be modified by the usage of alternative sole carbon sources, strongly emphasizing the necessity of considering the growth medium in *in vitro* models of pathogenic organisms. I investigate the role of pole age in determining growth rate and cell size, and how this might underlie observations that pole age

affects survival to antibiotics by other authors (e.g. Aldridge *et al.* 2012; Richardson *et al.* 2016).

3.1.1 Cell-size control models: sizer, timer, and adder

Three general principles that describe cell-size control have been proposed, usually termed “sizer”, “timer”, and “adder”, which can be described by a single phenomenological model which simply relates birth length to division length with a straight line: known as a noisy linear map (Jun and Taheri-Araghi 2015; Tanouchi *et al.* 2015):

$$L_d = aL_b + b + \eta, \quad (3.1)$$

where L_d is length at division, L_b is length at birth, η is a noise term centred around zero, and a and b are the slope and y -intercept. The a and b terms can be estimated by ordinary least squares linear regression of L_d against L_b , and their values inform the strategy that cells follow:

Sizer: $a = 0$ $L_d = b + \eta$. Cells divide at a fixed average length b , with no correlation between division length and birth length.

Adder: $a = 1$ $L_d = L_b + b + \eta$ Cells divide after incorporating a fixed average length b between birth and division, with complete dependence of division length on birth length.

Timer: $a = 2$ $L_d = 2L_b + b + \eta$ Cells divide after a fixed time interval, which requires a precise doubling in cell size between birth and division, with the implication that b is 0, and the assumption that cells halve their size at division.

Both sizer and adder models provide a principle by which cell size can be maintained (see Figures 3.1 and 3.2). Sizer resets cell size at every division according to the threshold implied by the model, and any noise introduced by the η term is removed at every division, i.e. cell-size history is “forgotten” at birth (see Figure 3.2; Taheri-Araghi *et al.* 2015). Adder also provides cell-size homeostasis, since if a cell is born larger than average, its progeny will be smaller according to the adder principle—assuming division results in an approximate halving of cell size on average—and a cell that is born smaller than average will produce progeny that are larger than itself at birth. This results in average length at birth being adjusted constantly towards the population mean value over multiple generations (Taheri-Araghi *et al.* 2015), where the mean birth length is b (see Figure 3.1). In this manner, cell size history plays a significant role in the cell size distribution over time.

The timer principle—fixed interdivision time—while conceptually simple and easily linked to molecular mechanisms such as DNA replication, does not provide cell-size homeostasis (Amir

2014; Iyer-Biswas *et al.* 2014), since any stochastic change in length at birth cannot be reliably reset, with smaller cells becoming progressively shorter and larger cells becoming progressively larger. Thus, a timer in this context can only result in cell-size homeostasis when no noise is present (see Figure 3.3), a biological impossibility. This can be seen purely from the model, when $L_d = 2L_b + b + \eta$, both b and η —the noise term—must be 0, otherwise L_d will never reach steady state (assuming average $\langle L_b \rangle$ is half average $\langle L_d \rangle$ in the population). However, a pure timer only specifies that cell cycle time is fixed, and $a = 2$ is derived from the assumption that growth rate is constant and birth size-independent, a cell could theoretically have a fixed interdivision time with $a < 2$, therefore the true test of a timer is probably not $a = 2$, but independence of interdivision time from birth size.

The distribution of the noise term is generally assumed to be Gaussian: $\eta \sim \mathcal{N}(0, \sigma)$, and this has been used in most modelling studies which explicitly model noise with the noisy linear map, which have resulted in predictions that match experimental data well (e.g. Amir 2014; Bertaux *et al.* 2018).

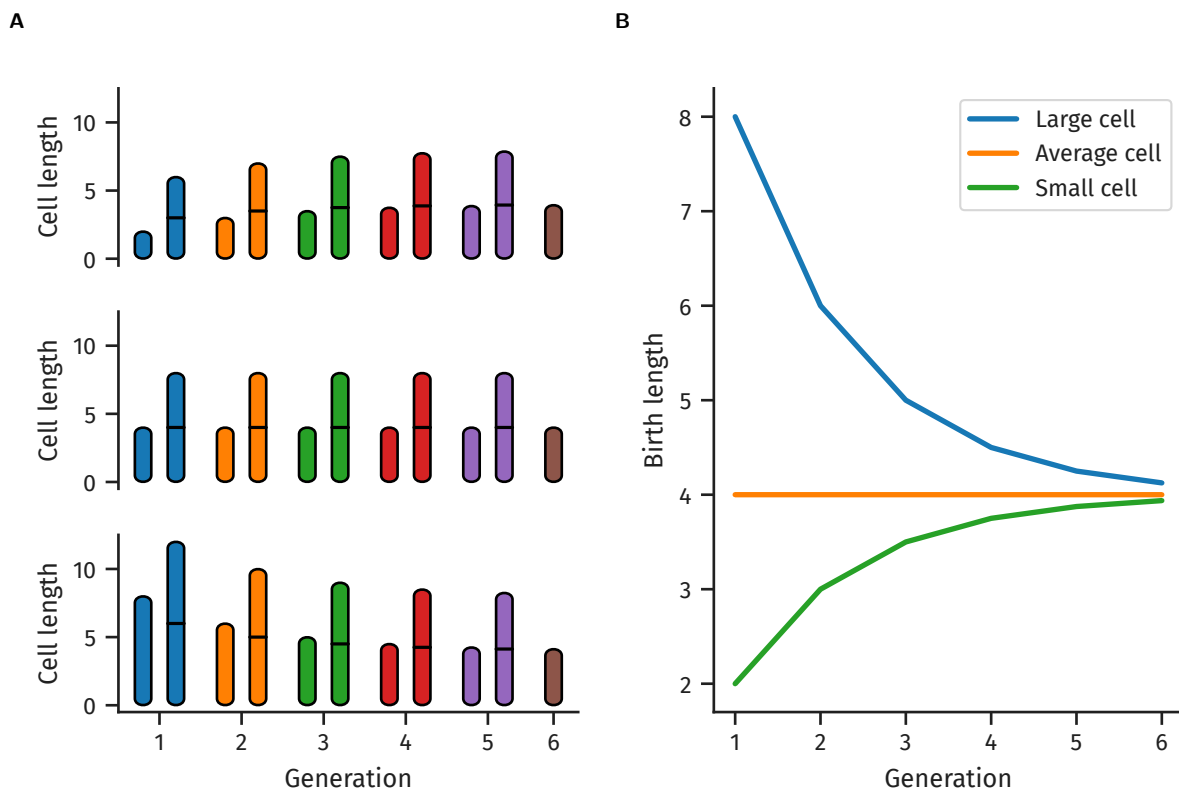


Figure 3.1: Cell-size homeostasis in an idealised adder. Simulated cell length data in which there is no noise with cells behaving as perfect adders at each generation. Added length each generation is set to 4 μm . Small cells are set to a birth length of 2 μm , large cells to 8 μm , and average cells to 4 μm . (A) Cells that are born small (top), average (middle), or large (bottom) grow by a fixed length each generation, with division occurring at exactly mid-cell. Cells are depicted at their birth length (left) and division length (right) for subsequent generations, with mid-cell marked with a black line. (B) Birth length over multiple generations is depicted for the simulated data displayed in panel A.

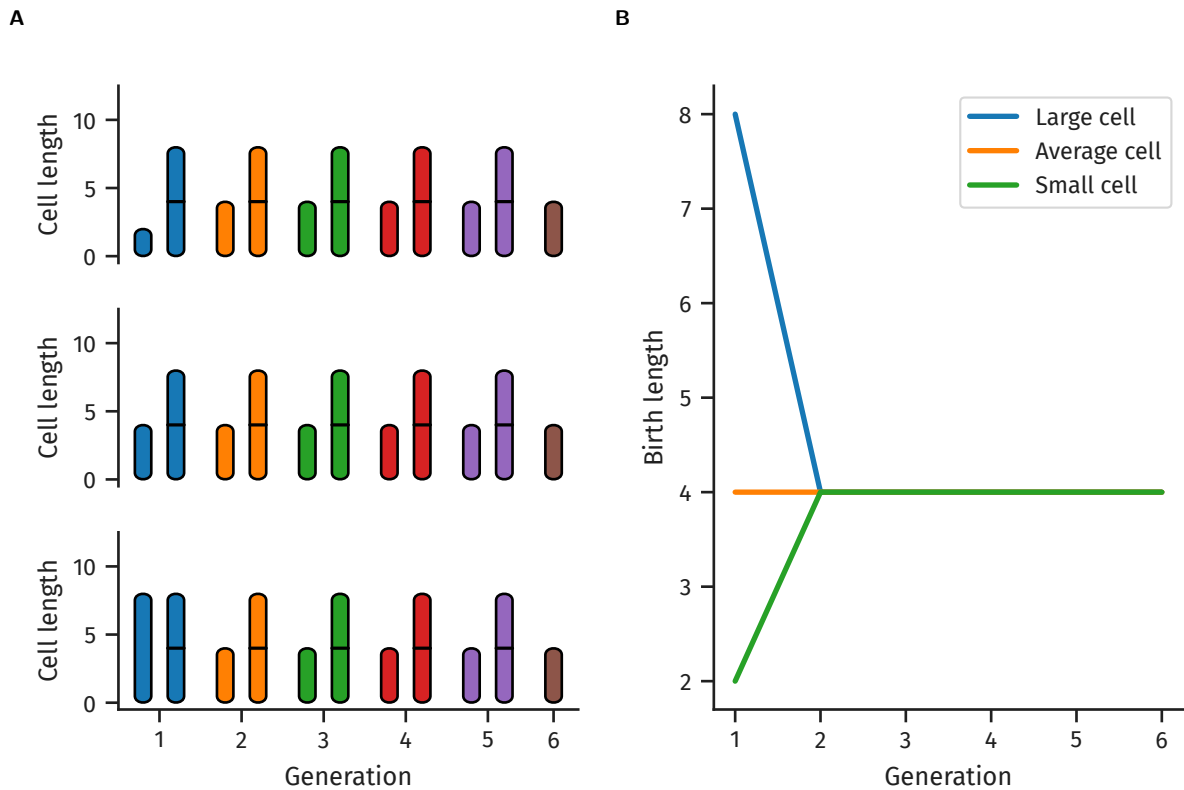


Figure 3.2: Cell-size homeostasis in an idealised sizer. Simulated cell length data for cells behaving as perfect sizers. Division length is set to 8 μm . See Figure 3.1.

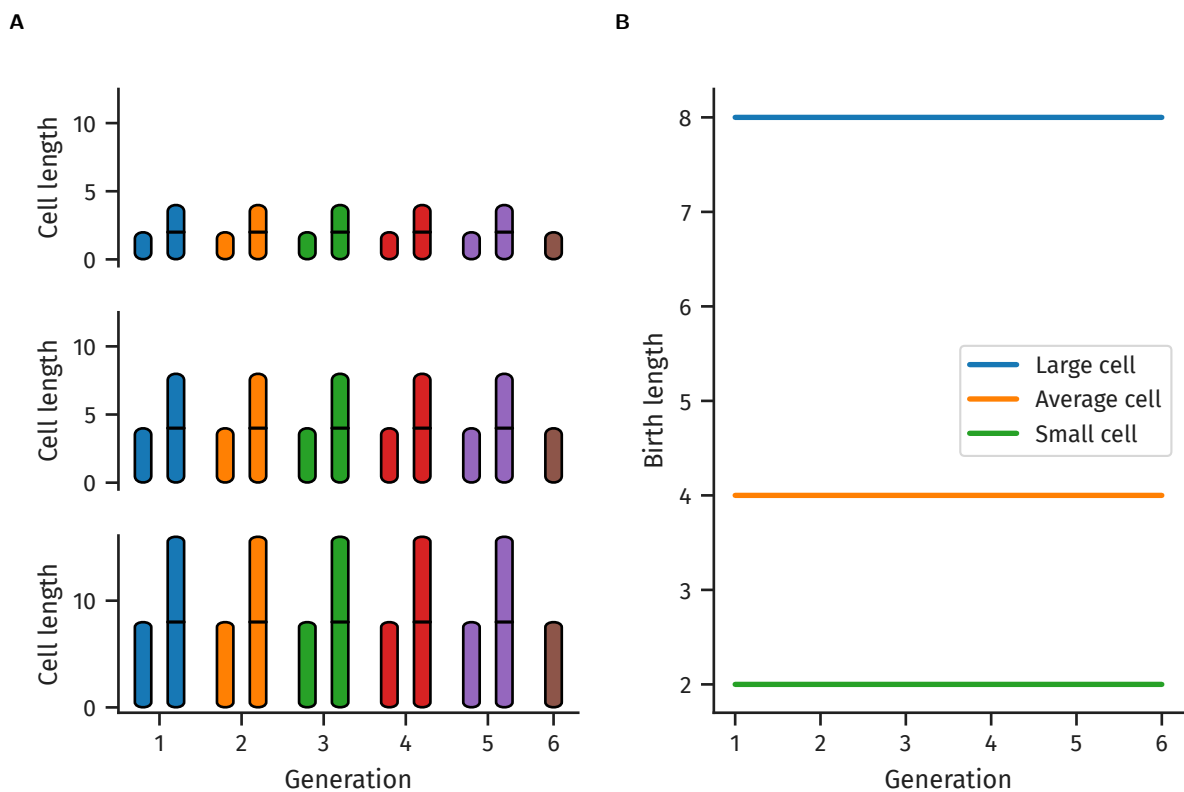


Figure 3.3: Cell-size homeostasis in an idealised timer. Simulated cell length data for cells behaving as perfect timers. Division length is set to double birth length. See Figure 3.1.

3.1.2 Evidence of the adder principle—and its underlying mechanism—in non-mycobacterial species

The concept of cell-size control has been recognised for some time, with suggestions of the sizer and timer models as plausible principles for size homeostasis. Early observations that mean population cell size varies with the population doubling rate indicated that size is indeed controlled (Schaechter *et al.* 1958), and the relationship with doubling rate hinted at a time-based model. Observations that the coefficient of variation (CV) of division length is less than CV of birth length led to the prediction that size control acts at division, and is related to cell size (Koch and Schaechter 1962). However, correlation between birth length and division length was observed in early studies which rules out a pure sizer model (Koppes *et al.* 1980). The paucity of modelling approaches limited the field of cell-size control to a binary choice between sizer and timer, and conflicting evidence produced for both cases. Thus observations that birth length is correlated with division length led to suggestions that cells follow a timer model (e.g. Aldridge *et al.* 2012), and observed negative correlation of interdivision time and birth length led to suggestions that cells are sizers (e.g. Robert *et al.* 2014). The realisation that adder is a plausible model of cell-size control allows these studies to be retrospectively realised as conforming to the adder principle, and the contradictions resolved (Taheri-Araghi *et al.* 2015).

Analysis of many bacterial species has revealed strong evidence that bacteria may universally follow the adder principle, including in gram-negative species such as *E. coli* (Amir 2014; Campos *et al.* 2014; Taheri-Araghi *et al.* 2015), *Pseudomonas aeruginosa* (Deforet *et al.* 2015), and asymmetric *C. crescentus* (Campos *et al.* 2014), in gram-positive species such as *Bacillus subtilis* (Taheri-Araghi *et al.* 2015), and in coccoid species such as the cyanobacterial species *Synechocystis* sp. with its complex circadian rhythm (Yu *et al.* 2017). Adder has also been demonstrated in archaea (Eun *et al.* 2018); in unicellular eukaryotes, such as the fission yeast *Schizosaccharomyces pombe* (Sveiczzer *et al.* 1996) and the budding yeast *Saccharomyces cerevisiae* (Di Talia *et al.* 2007); and even in mammalian single cells (Cadart *et al.* 2017).

Mechanisms underlying the adder principle are unclear, but they require a method by which added length can be kept constant regardless of birth length. Some have suggested a protein-based mechanism, whereby some protein is allocated equally to daughter cells, regardless of their size, and accumulation occurs up to a threshold which triggers division, acting as a form of molecular ruler that reports cell-size increment (Taheri-Araghi *et al.* 2015; Harris and Theriot 2016; Sauls *et al.* 2016). Some authors have suggested that a protein involved in DNA replication initiation could be a likely candidate (e.g. Wallden *et al.* 2016). At the population level it has been proposed that maintenance of mean cell size is controlled by DNA replication (Cooper and Helmstetter 1968; Cooper 1969; Helmstetter 1969), and has been investigated at the single-cell level in *E. coli* (Wallden *et al.* 2016; Si *et al.* 2017), with the hypothesis that DNA replication is initiated at a fixed ratio of origins of replication (*ori*) to cell size, followed by a

fixed time interval for replication to complete (Campos *et al.* 2014; Robert 2015). This in effect is a mixture of sizer and timer models, with an initial threshold length defined by the ratio of *ori* to cell size (i.e. sizer), followed by a fixed time (i.e. timer). Other “mixer” models include the “adder-per-origin” model, in which there is a constant added length between one initiation event and the next (post division in the next cell cycle), proportional to the number of origins of replication, followed by a constant time to division, in which DNA replication occurs (Cooper and Helmstetter 1968; Amir 2014; Ho and Amir 2015; Amir 2017), in effect an adder/timer mixer model. The “parallel adder”, recently formulated, proposes that two adder principles are acting on cell length that act concurrently, with one acting from DNA initiation until the next DNA initiation, and a second from DNA initiation until division (Logsdon *et al.* 2017). This differs from the adder-per-origin model in that instead of fixed time between initiation and division, there is a fixed length added. Mixer models are indistinguishable from an adder with a noisy linear map, since these cells indeed behave as adders in the purest sense—length added between birth and division is constant. The basis of the sizer phase (fixed ratio of *ori* to cell size) is derived from observations that average initiation mass is constant in *E. coli*, regardless of the population doubling rate (Cooper and Helmstetter 1968; Donachie 1968), which has been somewhat confirmed at the single-cell level (Wallden *et al.* 2016; Si *et al.* 2017), though a contradictory report has recently been published (Huls *et al.* 2018).

C. crescentus has been proposed to divide utilising such mixer models, with stalked cells growing exponentially for a fixed time, in a size-independent manner, followed by a pure adder phase between septum formation—concurrent with termination of DNA replication—and cytokinesis (Banerjee *et al.* 2017). A mathematical basis and framework underlying this mixer model has been devised, and many predictions of a pure adder from birth to division hold for the mixer model, for example variation in size is most sensitive to partitioning noise (i.e. asymmetry in division; Modi *et al.* 2017). This emphasizes the phenomenological nature of the noisy linear map, it does not represent a mechanism by which size is controlled, it describes the relationship between division length and birth length only.

More recent evidence of the role of DNA replication in cell-size control has suggested that size homeostasis may be unrelated to the ratio of origins to cell size in *E. coli* (Huls *et al.* 2018). Newborn *E. coli* cells indeed appear to have equal amounts of DNA at birth, a prerequisite of the mixer model, but only in slow-growing cells; fast-growing cells—undergoing multifork replication—do not maintain this constant amount, and the authors propose that this makes it unlikely that the constant length increment in the adder principle is derived from synthesis of equal amounts of DNA (Huls *et al.* 2018). Moreover, they also conclude that large and small newborn cells initiate DNA replication at different times, precluding the mixer model as described above, but still maintain a fixed period between initiation of DNA replication and cell division, deriving adder by an alternative mechanism (Huls *et al.* 2018). Indeed, considering the proposal naively, one of the principles underlying the adder principle is that larger cells will

undergo cell division earlier than smaller cells, with the same added length (under exponential single-cell growth), thus the existence of a fixed time to replicate DNA conferring adder would seem to contradict this assumption, unless the ratio of *ori* to cell size at initiation is not fixed, or the post-replication phase does not behave as an adder. This issue is hinted at by Wallden *et al.*, who predict deviation from adder at very slow growth rates in *E. coli*, where chromosome replication occurs synchronously with birth and division—i.e DNA replication is initiated and completed within the same cell—a case that is not usual for *E. coli* at fast growth rates (Wallden *et al.* 2016). This is interesting from the perspective of mycobacteria, in which overlapping replication cycles are thought to occur much less frequently than in *E. coli* (Santi *et al.* 2013).

The observation of the adder principle in such a diverse range of bacteria, archaea, and eukaryotic cells may suggest that there is no universal mechanism underlying constant added length, but that it may arise as a consequence of the relationships between DNA, growth rate, and size that govern all cells (Schmoller 2017).

3.1.3 Cell-size control in mycobacteria

Mycobacteria have key differences in how they grow and divide compared to *E. coli* and other model species, especially in the accuracy of septum placement; tightly controlled close to mid-cell in *E. coli* (Trueba 1982; Yu and Margolin 1999) by several molecular systems, such as nucleoid occlusion (Wu and Errington 2012), and the MinCDE system (Raskin and de Boer 1999). In comparison, mycobacterial division site selection is much less tightly controlled (Thanky *et al.* 2007; Farnia *et al.* 2010; Singh *et al.* 2010a; Joyce *et al.* 2012; Aldridge *et al.* 2012; Singh *et al.* 2013), which introduces significant heterogeneity in cell length at every cell cycle. *E. coli* cells have birth size distributions with a CV of 12 % (Campos *et al.* 2014; Iyer-Biswas *et al.* 2014) compared to 19–22 % in *M. smegmatis* (this work; Priestman *et al.* 2017; Logsdon *et al.* 2017) and *M. bovis* BCG (Logsdon *et al.* 2017). In addition, mycobacteria incorporate new cell wall material exclusively at their poles, and preferentially at a single pole—the pole inherited from the previous generation (Thanky *et al.* 2007; Hett and Rubin 2008; Joyce *et al.* 2012; Aldridge *et al.* 2012; Santi *et al.* 2013; Meniche *et al.* 2014; Manina *et al.* 2015; Botella *et al.* 2017; Rego *et al.* 2017)—which has been proposed to introduce additional heterogeneity in growth rate due to differential age of the inherited pole by some authors (Aldridge *et al.* 2012). This differs significantly from *E. coli* which incorporate new cell wall material along their lateral wall, and for which the age of the inherited pole has no intrinsic effect on growth rate (Margolin 2009; Typas *et al.* 2012; Wang *et al.* 2012a). This concept of inheritance of an active pole creates a simple method of distinguishing mycobacterial sibling pairs, according to the age of the poles inherited by their mother. Each cell creates a new pole at division, at the septum, and inherits the other pole from its mother (the “old pole”). In mycobacteria, this old pole is generally considered to be site of cell wall incorporation, for at least the majority of the subsequent cell

cycle. At division, daughter cells can either inherit this active old pole, or the inactive new pole, thus can clearly be distinguished at birth as “new-pole inheritors” or “old-pole inheritors”, terminology which I use throughout this thesis.

Asymmetry does not rule out the adder model *per se*; *C. crescentus* cells conform rigidly to the adder principle whilst producing stalked and swarmer cells at every division, which have markedly different sizes, life cycles, and growth rates (Campos *et al.* 2014; Iyer-Biswas *et al.* 2014). However, mycobacterial asymmetry is not fixed in a similar manner to *C. crescentus*, resulting in daughter cells which have ostensibly identical lifestyles. It is more variable compared to *E. coli* (Aldridge *et al.* 2012), which tightly control septum placement at mid-cell, thus the impact of this additional significant source of noise on adherence to adder was unknown at the time of the initiation of this project, especially given the high importance of partitioning noise in cell-size control theory (Modi *et al.* 2017).

Investigation of the cell-size control principle in *M. smegmatis* has shown little evidence for the sizer model, with sizer ruled out using time-lapse microscopy, and the suggestion that cell cycle times are correlated, hinting at a timer model (Aldridge *et al.* 2012), though this was published before the adder principle had been described. Similarly, Santi *et al.* (2013) describe the relationship between added length and birth length in *M. smegmatis* and conclude that they are neither sizers nor timers, though they observed weak correlation between the two ($r = 0.20$), which also hints at “timer-like” behaviour ($a > 1$)¹. While these studies suggest a timer, or at least an imperfect timer model for *M. smegmatis* (since a perfect timer would be largely incompatible for cell-size homeostasis; see Figure 3.3), the timer model was explicitly rejected in the latter study, since interdivision time is correlated with birth size (Santi *et al.* 2013), and indeed Aldridge *et al.* describe an adder model, with the correlation between added length and birth length being 0.00, though they do not name it as such (Aldridge *et al.* 2012). Differences in correlation between added length and birth length observed in the two studies could be explained by the different conditions used in each experiment. Santi *et al.* used a strain of *M. smegmatis* mc²155 carrying Wag31-GFP integrated into the chromosome—a cell division protein that marks the cell poles and the septum (Nguyen *et al.* 2007; Kang *et al.* 2008; Jani *et al.* 2010)—and used 7H9 rich medium supplemented with FM4-64, a membrane dye (Santi *et al.* 2013). The 7H9 they used was also supplemented with OADC (a mycobacterial growth supplement containing oleic acid, albumin, dextrose, and catalase), glycerol, glucose, and Tween-80 detergent, all of which are utilisable as carbon sources by mycobacteria (Dubos and Davis 1946; Dubos 1947; Mizuno and Tsukamura 1978). Their experiment therefore exposed a strain carrying an additional copy of a cell division protein to two wavelengths of excitation light, in a complex medium, and a membrane-intercalating dye (Santi *et al.* 2013), which may

¹Note that linear regression of added length (ΔL) against birth length (L_b) is the same procedure as division length (L_d) against birth length: since $\Delta L = L_d - L_b$, it follows that $\Delta L = (a - 1)L_b + b + \eta$ using the noisy linear map, with a simple subtraction of the slope to convert the two (see Equation 3.2 below).

have affected native cell-size control. Aldridge *et al.* (2012) used a strain of *M. smegmatis* mc²155 carrying an empty integrated kanamycin cassette (for unknown reasons), and used 7H9 complex medium supplemented with glycerol, OADC, Tween-80, and the antibiotic kanamycin. Cell size measurements were generally taken whilst cells were exposed to excitatory wavelengths of light in addition to brightfield. These differences in strain, culture conditions, and any stress induced by imaging conditions may have affected cell size distributions, though it is likely that the latter conditions were less onerous, especially given the role of Wag31 in cell division. Irrespective of the potential effects of differing methodology, differences in cell-size control—specifically the relationship between division/added length and birth length as defined by the noisy linear map—were reported in two independent studies at the single-cell level.

The implications of cell-size control model may have significant impact on bacterial survival strategies, and thus on the development of new therapeutic interventions. Differences in growth rate between sibling cells are observed in the pathogenic *M. tuberculosis*, with cells inheriting the non-growing pole (the new pole formed when the mother cell was born) having a fractionally faster exponential growth rate compared to those that inherit the growing, old pole (Manina *et al.* 2015). Differences between siblings have been observed to have a large effect on survival to antibiotics, with *M. smegmatis* old-pole inheritors more able to survive rifampicin (Aldridge *et al.* 2012; Richardson *et al.* 2016), and new-pole inheritors more able to survive cell wall-targeting antibiotics including meropenem, D-cycloserine, and isoniazid (Aldridge *et al.* 2012). The introduction of variability by asymmetric division on growth rate and cell size has recently been observed to contribute to antibiotic tolerance and virulence in clinical strains of *M. tuberculosis*, where tolerance appears to be related to the variability of the cell size distribution within a strain, highlighting the potential importance of cell-size control in the pathogenic mycobacteria (Vijay *et al.* 2017). Other authors have suggested that pole age and growth rate have no impact on survival to isoniazid in either *M. smegmatis* (Wakamoto *et al.* 2013) or *M. tuberculosis* (Manina *et al.* 2015). Given these discrepancies, it is important that the effects of pole age on factors such as cell size and growth rate are determined precisely, as well as the cell-size control principle which mycobacteria follow.

3.1.4 Measuring growth at the single-cell level

The above differences may also reflect fundamental differences in how growth rate is determined at the single-cell level. Growth has been defined in many ways in the literature (Mitchison 2005; Cooper 2006), including as the inverse of interdivision time (divisions or doubling per unit time), as the linear fit of cell length against time (e.g. Aldridge *et al.* 2012, regarding mycobacteria), and as the exponential fit of cell length against time (e.g. Wakamoto *et al.* 2013; Santi *et al.* 2013; Manina *et al.* 2015, regarding mycobacteria). Doublings per unit time is primarily used as a measure of growth of populations rather than as a single-cell measure of growth, whereas linear

and exponential fits of cell length over time are—necessarily—single-cell measures. Throughout this thesis, I refer to the linear fit as “elongation rate” and the exponential fit as “growth rate”. *E. coli* cells are known to grow exponentially at the single-cell level (Godin *et al.* 2010), which is consistent with lateral insertion of nascent cell wall material (Margolin 2009; Typas *et al.* 2012; Wang *et al.* 2012a), since the surface area available for insertion increases in a size-dependent manner; and other diverse species such as *B. subtilis* (Daniel and Errington 2003) and mammalian cells (Tzur *et al.* 2009; Mir *et al.* 2011) are also known to grown exponentially. However, since mycobacteria are known to elongate from their tips, an exponential model of single-cell growth is less clear (Daniel and Errington 2003). Santi *et al.* (2013) investigated the size dependence of what they termed “instantaneous elongation velocity”, which reflects the increase in length between two adjacent time-points divided by the time difference, and determined this value is size-dependent—i.e. larger cells add more length between time-points than smaller cells—which is strongly indicative of exponential growth at the single-cell level. Exponential growth can be conceptualised as the proportion of cell size that is incorporated per unit time. The distinction between single-cell exponential and linear growth has important implications in cell-size control, and remains an unresolved question in the field of mycobacterial single-cell studies. For example, a time-based division mechanism can result in adder if cells grow linearly at the single-cell level, since constant linear growth of a small cell compared to a large cell would result in the same added length, whereas exponential single-cell growth would result in larger cells adding more length than smaller siblings and rapid divergence of cell size. In addition, exponential growth rate in the adder model implies several other key relationships, such as the prediction that cells that are born larger will divide earlier—i.e. negative correlation of birth length and interdivision time—whereas linear elongation predicts no correlation between the two.

3.1.5 Role of growth medium in cell-size control

In addition to discrepancies between single-cell growth measures, as mentioned above, the growth medium itself has varied between studies, and has hinted at differences in the cell-size control principle (Aldridge *et al.* 2012; Santi *et al.* 2013). Different growth media can modify the growth rate of the population, and this has been proposed to determine the average cell size of the population, as was shown 6 decades ago in *Salmonella typhimurium* (Schaechter *et al.* 1958), long before the advent of detailed microfluidic-based single-cell studies. The authors used a chemostat-like device that maintains populations at constant log-phase growth in various different media with growth rates (as defined by number of doublings per hour; measured by optical density) ranging from 2.8 doublings/h in brain and heart infusion to 0.62 doublings/h in a minimal medium supplemented with lysine (Schaechter *et al.* 1958). They observed that average cell size—as determined by phase-contrast microscopy, and measurement of cellular mass—scales exponentially with the doubling rate, in a precisely predictable manner, i.e. population

doubling rate and doubling rate alone determines mean cell size (Schaechter *et al.* 1958), an observation that is termed the “Schaechter-Maaløe-Kjeldgaard growth law” by some authors (Iyer-Biswas *et al.* 2014; Taheri-Araghi *et al.* 2015; Vadia and Levin 2015; Kennard *et al.* 2016). Therefore, the different media used in the two mycobacterial studies—specifically the addition of glucose by Santi *et al.* (2013)—might affect the cell size distribution of the population, and explain the observed minor correlation between birth and division length that is inconsistent with an adder model. However, close examination of this published data does not appear to show any gross change in population doubling rate with interdivision times of 3.00 ± 0.75 h (Santi *et al.* 2013) and an estimated—by visual approximation—mean of 2.8 h (Aldridge *et al.* 2012), which translate to population doubling rates of approximately 0.33 doublings/h and 0.36 doublings/h respectively. However these values are not quoted in the latter study, thus it may or may not be a contributing factor in disparities in apparent cell-size control. Pathogenic mycobacteria are widely thought to survive and grow intracellularly under carbon-limited conditions, with cholesterol as the primary carbon source (Pandey and Sasseti 2008; Salamon *et al.* 2014; Vromman 2014), thus it is important to determine how culture medium affects cell size and its control in mycobacteria.

3.1.6 Summary of the chapter

This chapter investigates cell-size control in wild-type *M. smegmatis* mc²155 single cells using microfluidics to maintain constant conditions and time-lapse microscopy (described in Chapter 2) to determine the relationship between birth length and division length primarily, and the relationship of other single-cell parameters. Using this methodology, *M. smegmatis* cells are shown to definitively behave as adders, with constant length added between birth and division. In addition, the manner in which single cells grow is determined by model selection between linear and exponential models. An exponential model of single-cell growth is found to fit individual cells much more accurately than a linear model when many cells are analysed, in line with hints from other authors. Given discrepancies in the relationships between birth length and division length reported by other authors, culture medium composition is also modified to determine the role of carbon source in cell-size control, as well as the investigate the role of modulation of growth rate. I replace glycerol provided as a sole carbon source in minimal medium with pyruvate or acetate and determine that cells deviate from adder towards sizer-like behaviour in a pole age-dependent manner with only minor differences in growth rate, a surprising result. The analysis and experiments presented in this chapter have been published (Priestman *et al.* 2017), and are expanded in detail below. At the time of publication, it was the first report that focused on cell-size control in *M. smegmatis*, and it was the first definitive report of a bacterial species that can deviate from the adder principle without a drastic change in doubling time. Shortly after, an additional publication focusing on cell-size control in *M. smegmatis* was published (Logsdon *et al.* 2017) which corroborates many of the

conclusions presented here. A comparison of the two methodologies and results is presented in the Discussion section.

3.2 Methods

3.2.1 Variants of HdB

All cells were grown in HdB (see Chapter 2 Materials section), which contains 0.2 % glycerol as a sole carbon source, or variants of HdB. For alternative carbon source variants, glycerol is replaced with 0.2 % w/v potassium acetate (Sigma) or 0.2 % w/v sodium pyruvate (Sigma) for all experiments.

3.2.2 Microscopy and analysis

For the purposes of determining cell-size control models in *M. smegmatis*, time-lapses were performed generally as described in Chapter 2. Wild-type *M. smegmatis* mc²155 cells were inoculated from frozen glycerol stocks into HdB, and grown twice overnight to stationary phase. Cells were sub-cultured at a 1:200 ratio into fresh medium (HdB or variants) and grown to mid-exponential phase (an OD₆₀₀ of 0.4–0.6). Cells were diluted to an OD₆₀₀ of 0.05–0.1 just prior to starting each experiment. The microscope was pre-warmed to 37 °C for at least 4 h (often overnight) prior to the start of experiments. B04A-03 microfluidic plates (Millipore) were prepared with 350 µl HdB, or variants thereof, in the first medium inlet well, and 100 µl medium and cells added to the cell inlet wells. Microfluidic plates were pre-warmed in a static incubator prior to addition of cells. Plates were placed on the microscope, cells loaded, and medium flow initiated at 1 psi for the entire duration of the experiment. Regions with cells were observed with the microscope, and XYZ-positions recorded for the motorised stage using the microscope software (SimplePCI, HImage, Volocity, or Zen). Images were acquired with brightfield illumination only (100–200 ms; 5–7 V) with a 63× phase-contrast objective (Zeiss Plan-Apochromat 63× 1.40 Oil Ph3 M27). Images were captured with a Hamamatsu EM-CCD C9100-02 camera, or a Hamamatsu Flash 4.0 sCMOS camera, using either a Zeiss AxioVert 200M or a Zeiss Axio Observer inverted microscope. In-focus images were acquired as described in Chapter 2 with software autofocus, 10 1 µm-spaced Z-slices, and a 15 min acquisition frequency. 16 positions were generally tracked, with 4 fields-of-view per growth chamber of the microfluidic plate.

For the analysis, Fiji was used to determined in-focus frames, and MicrobeTracker used to detect cell boundaries in each frame, with division defined as the point at which visible septum constriction or clear cytokinesis (e.g. V-snapping) can be observed. The custom package lineage was used to register movement and growth of individual cells between frames and

to construct cell lineages (see Chapter 2). A custom script was written for the purposes of analysing cell-size control, which records birth length, division length, interdivision time, elongation rate, growth rate, pole age, and division asymmetry for every division observed during the experiment. This information was stored as a Python pandas object (McKinney 2010) for further manipulation (generally performed by the same script).

3.2.3 Noisy linear map

As described in the Introduction, the noisy linear map (Equation 3.1) relates birth length to division length with a simple linear model (Taheri-Araghi *et al.* 2015):

$$L_d = aL_b + b + \eta, \quad (3.1 \text{ revisited})$$

where L_d is length at division, L_b is length at birth, a and b describe the linear relationship, and η is a noise term describing biological variation. a , b , and η can be estimated by ordinary least squares linear regression of L_d against L_b , where a is the slope of the regression line, b is the y -intercept, and η describes the residuals from the model. This is performed using Python, either with the use of the NumPy package (Oliphant 2007) with the `numpy.polyfit` function with a degree of 1, or the Statsmodels package (Seabold and Perktold 2010) via the OLS linear model class to the same effect.

Other authors express the relationship between birth and added length (ΔL) rather than division length, in which case the equation is trivially adapted from Equation 3.1:

$$\begin{aligned} \Delta L &= L_d - L_b, \\ L_d &= \Delta L + L_b = aL_b + b + \eta, \\ \Delta L &= (a - 1)L_b + b + \eta. \end{aligned} \quad (3.2)$$

Thus a slope of 1 of L_d against L_b —a perfect adder—gives a slope of 0 for ΔL against L_b . This formulation is convenient for statistical testing of the linear model, since an F -test can be used to compare if the slope is significantly different from 0, and this is equivalent to testing whether a is significantly different from 1 according to Equation 3.2.

3.2.4 Discrimination of single-cell elongation models

Two major models of single-cell growth have been postulated that apply to mycobacteria: linear and exponential. These models can be described by the relationship between cell length and cell cycle time, and are described in Chapter 2 (Elongation and growth rate).

If cell length is considered as a time series of n time-points between the first frame after birth

and the last frame before division with cell lengths $L_1, \dots, L_n$ at discrete time-points $t_1, \dots, t_n$, then linear elongation rate is described by the function (Equation 2.1):

$$L(t) = \lambda t + L_1, \quad (2.1 \text{ revisited})$$

where $L(t)$ describes cell length L at time t , t describes time since the first frame, L_1 is cell length at $t = t_1$, and λ is the linear elongation rate, in units of $\mu\text{m h}^{-1}$.

Exponential growth rate is described by the equation (Equation 2.3):

$$L(t) = L_1 e^{\gamma t}, \quad (2.3 \text{ revisited})$$

where γ describes the exponential growth rate in units of h^{-1} .

λ can be estimated by linear regression of $L(t)$ against t , and γ can be estimated by linear regression of the logarithm of cell length $\ln(L(t))$ against t , where the slope is the rate, and the y -intercept is L_1 or $\ln(L_1)$ respectively.

For model selection of the most appropriate fit, cell growth is described by the equation:

$$\frac{d}{dt}L = \gamma f(L) + \sigma \epsilon_t, \quad (3.3)$$

where $\epsilon_t \sim \mathcal{N}(0, 1)$ is normally-distributed white noise with unit variance, and γ is the growth rate (i.e. γ from Equation 2.3) or elongation rate (i.e. λ from Equation 2.1). To represent both models, $f(L) = 1$ for the linear and $f(L) = L$ for the exponential growth model. Each $L(t)$ path is discretised in time steps $\delta t_i = t_{i+1} - t_i$, i.e. the difference in time between time-points, giving a path-likelihood for either model:

$$\mathcal{L}(\hat{\gamma}, \hat{\sigma}^2 | \{L_1, \dots, L_n\}) = \prod_{i=1}^{n-1} \frac{e^{-\left(\frac{(L_{i+1} - L_i - \delta t_i \hat{\gamma} f(L_i))^2}{2 \delta t_i \hat{\sigma}^2}\right)}}{\sqrt{2\pi \hat{\sigma}^2 \delta t_i}}. \quad (3.4)$$

The maximum likelihood estimates are

$$\hat{\gamma} = \frac{\sum_{i=1}^{n-1} \delta t_i (L_{i+1} - L_i) f(L_i)}{\sum_{i=1}^{n-1} \delta t_i f^2(L_i)}, \quad (3.5)$$

and

$$\hat{\sigma}^2 = \frac{1}{n-1} \sum_{i=1}^{n-1} \delta t_i \left(\frac{L_{i+1} - L_i}{\delta t_i} - \hat{\gamma} f(L_i) \right)^2. \quad (3.6)$$

According to Akaike's information criterion (Akaike 1973), the model with highest likelihood is then selected.

3.2.5 Interpolation of birth length and division length

Time-lapse data is limited by the frequency at which images can be captured—4 frames per hour, or every 15 min in these experiments—which results in overestimation of birth length, and underestimation of division length and interdivision time. These parameters can be adjusted to provide more accurate estimates of their true values by interpolation, as long as the single-cell elongation model is known.

If cell length in the first frame observed is termed initial length L_{init} at initial time t_{init} , and cell length in the last frame is termed final length L_{final} at final time t_{final} , the true estimates of birth length L_b , division length L_d , and interdivision time τ_d can be calculated based on knowledge of how these cells grow in the missing period between division and birth. This also requires that the elongation or growth rate of both the mother and the daughters are known. If this is the case, the length L_m of the mother cell can be extended into the future based on its elongation or growth rate, and the lengths of the two daughter cells L_{c_1} and L_{c_2} can be projected into the past based on their individual elongation or growth rates. The time-point at which the sum of the L_{c_1} and L_{c_2} is equal to L_m can then be considered to be the point of division, where t is equal to t_{final} of the mother, and t_{init} of both daughters, and the length of the mother cell at this point to be its division length L_d , and the lengths of the two daughters to be their birth lengths L_b . Interdivision time can only be interpolated for cells in which both birth length and division length have been interpolated, in which case it is simply $\tau_d = t_{\text{final}} - t_{\text{init}}$ for that cell.

Therefore if cell length is considered as the function $L(t)$ according to Equation 2.1 or Equation 2.3, with $L_m(t)$ describing the length of the mother cell, and $L_{c_1}(t)$ and $L_{c_2}(t)$ describing the daughter cells: the precise time of cell division (t_{final} of the mother cell and t_{init} for the daughter cells) can be estimated by minimising the value of:

$$|L_m(t) - L_{c_1}(t) - L_{c_2}(t)|, \quad (3.7)$$

and subsequent estimates of L_b of the daughter cells and L_d of the mother cell can be determined at $t = t_{\text{final}}$ and L_b of each daughter cell at $t = t_{\text{init}}$ according to the elongation or growth rate of each cell.

3.2.6 Population and lineage-weighted statistics

General population statistics are obtained by averaging all cells observed within an experiment. Lineage-weighted statistics—which reduce the bias introduced by the over-representation of the descendants of fast-dividing cells early during a time-lapse—are determined by reconstructing random paths through the data, which is represented as a tree with each cell as a node, and edges representing division events. If x_{ij} represents the data of the i^{th} cell of N_j cells in the

j^{th} lineage of N_L lineages, where each lineage is a single path through the whole tree, the lineage-weighted average of the data function f is:

$$\bar{f} = \sum_{j=1}^{N_L} \sum_{i=1}^{N_j} w_j f(x_{ij}). \quad (3.8)$$

The weights w_j for each lineage are defined by:

$$w_j = \frac{2^{-D_j}}{N_T N_j}. \quad (3.9)$$

Where D_j is the total number of divisions in lineage j , which is determined by the probability of choosing a random path through N_T trees: $(2^{-D_j}) / N_T$ (Nozoe *et al.* 2017). Lineage-weighted statistics are then bootstrapped by re-sampling data x_{ij} with probability given by the lineage weights w_j . In effect, this scales population statistics by the number of divisions that occur within each lineage individually to reduce the effect of over-representation of fast-dividing cells. This procedure was devised and performed by Philipp Thomas (Department of Mathematics) on the data that I acquired.

3.2.7 Statistics

Unless otherwise stated, all statistics are expressed as a 95 % confidence interval, or $\pm$ their 95 % confidence interval if this is symmetric. This interval is usually determined by bootstrapping, which makes no assumptions about the underlying distribution of the data, usually with 10,000 resampling iterations. This is performed either using custom functions, or using the Seaborn package (Waskom *et al.* 2017) via `seaborn.algorithms.bootstrap` and `seaborn.utils.ci`. Hypothesis tests are stated with their associated p -values, and are usually performed using the SciPy stats package (Jones *et al.* 2001). Estimating the uncertainty of linear regression coefficients is based on Student's t -distribution, which assumes that residuals are distributed accordingly.

3.3 Results

3.3.1 Data acquisition

For this chapter, I performed a series of independent experiments that grew wild-type *M. smegmatis* mc²155 in HdB, or variants, with brightfield illumination only, conditions which provide the minimum possible perturbation of bacteria by imaging stress. Cells were grown for up to 24 hours with images acquired every 15 min, and analysed using the workflow described

in Chapter 2 and above. For cells grown in glycerol, 544 cell division events were observed over seven independent experiments; for cells grown in pyruvate, 367 cell division events were observed over seven independent experiments; and for cells grown in acetate, 383 cell division events were observed over six independent experiments. For the remaining part of this chapter, I refer exclusively to these data for all subsequent analysis.

3.3.2 Determination of the single-cell growth model—exponential or linear?

There has been some discussion in the field about the model by which bacteria grow at the single-cell level, with different measures used, such as linear elongation rate (e.g. Aldridge *et al.* 2012) or exponential growth rate (e.g. Wakamoto *et al.* 2013). I specifically use the terminology “elongation rate” to refer to a linear model, in which length is added incrementally and at a constant rate, with units of $\mu\text{m h}^{-1}$. The term “growth rate” is reserved for the exponential model, in which length is added at an accelerating rate, and is expressed in units of h^{-1} (see Chapter 2, Elongation and growth rate).

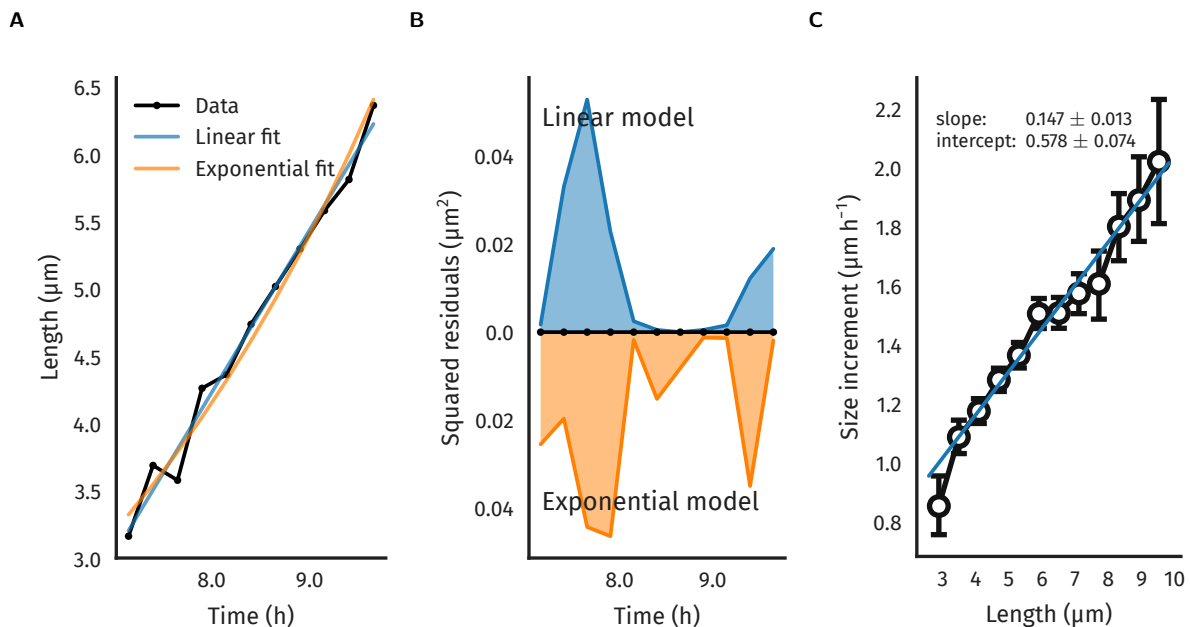


Figure 3.4: Comparison of single-cell growth models. A linear elongation model and an exponential growth model are fitted to the cell length of a single cell. The linear model is determined by ordinary least squares regression of length $L(t)$ against time t . The exponential model is determined by ordinary least squares regression of $\ln(L(t))$ against t . (A) Cell length is plotted against time (black) with the linear fitted model plotted in blue and the exponential model in orange. (B) Squared residuals for each model at each time-point are plotted, with the linear model above the origin line, and the exponential model below it. (C) Average increase in cell length over time (size increment) scales linearly with cell length when cells are grown in glycerol, suggesting exponential growth. The blue line indicates the ordinary least squares linear regression fitted to 11,088 individual increments, the fit coefficients are indicated in the top left of the figure. The black line with white unfilled circles indicates the mean of data binned by length, with 95 % confidence intervals indicated with error bars (determined by bootstrapping). The bins (and fitted data) were included if they had more than 100 observations, with 265, 810, 1403, 1849, 1920, 1632, 1303, 847, 519, 266, 172, and 102 observations in each bin (in order of increasing length).

It is generally difficult to distinguish between linear and exponential models over a single *M. smegmatis* cell cycle, especially considering measurement error from the analysis routine. An example random selected cell trace is shown in Figure 3.4A, in which both linear and exponential models are fitted to lengths between birth and division. For this particular cell, the elongation rate as determined by linear regression of length over time (Equation 2.1) is $1.208 \mu\text{m h}^{-1}$ [1.098–1.318] (95 % confidence interval), and the growth rate as determined by linear regression of the logarithm of length over time (Equation 2.3) is 0.262 h^{-1} [0.231–0.294]. Both models appear to fit the data equally well (visually), and when plotting the residuals of the regression fit, there is very little obvious difference the two (Figure 3.4B). The mean squared error (MSE) of the residuals is $0.016 \mu\text{m}^2$ for the linear model, and $0.022 \mu\text{m}^2$ for the exponential model for this particular cell which implies that both models effectively fit the data equally well. If both models are fitted to all cells within the glycerol dataset (in which there are more than 5 length observations) the mean MSE of 1319 cells is $0.038 \mu\text{m}^2$ [0.035–0.042] (95 % confidence interval determined by bootstrapping) for the linear model, and $0.035 \mu\text{m}^2$ [0.031–0.038] for the exponential model, which is not statistically significantly different ($p = 0.146$; Welch's *t*-test).

When cell length increment between frames ($\Delta L/\Delta t$) is plotted as a function of cell length (L), with over 11,000 individual observations, cells appear to elongate in a length-dependent manner—i.e. larger cells increase their length by more than smaller cells in the next time step—and this dependency appears to be linear ($r = 0.205$, $p = 1.04 \times 10^{-105}$) with an ordinary least squares regression line slope coefficient of $0.147 \pm 0.013 \text{ h}^{-1}$ (Figure 3.4C), which implies exponential growth.

To attempt to determine definitively which model more appropriately fits my time-lapse data, a model selection procedure was performed in collaboration with Philipp Thomas (Department of Mathematics, Imperial College London). This method is detailed in the Methods section (Discrimination of single-cell elongation models). Using this model selection method on all cells demonstrates that over 90 % of cells preferentially fit the exponential model over the linear model. This does not suggest that some bacteria are growing by alternative models, rather that all cells are fitted better by an exponential model with 90 % confidence.

Given this information, initial length, final length, and time between the first and last frame can be interpolated (see Methods section: Interpolation of birth length and division length) using an exponential model of cell growth (Equation 2.3) to estimate birth length L_b , division length L_d , and interdivision time τ_d with greater confidence. Unless otherwise stated, all statistics used below use interpolated values.

3.3.3 Cell-size control with glycerol as a sole carbon source

For cells grown in unmodified HdB, with glycerol as a sole carbon source, I observed 544 cell division events, for which I recorded birth length (L_b), division length (L_d), interdivision length (τ_d), exponential growth rate (γ), and age of the oldest inherited pole. The relationships between these parameters, for all pairwise combinations, according to the age of the inherited pole are depicted in Figure 3.15 for reference purposes.

Ordinary least squares linear regression of division length against birth length according to the noisy linear map (Equation 3.1) results in $a = 0.996 \pm 0.102$ and $b = 3.525 \pm 0.453$ (Figure 3.5A) for cells grown in glycerol. This indicates almost complete dependence of division length on birth length, implying that cells on average add a fixed length each cell cycle ($\sim 3.5 \mu\text{m}$) and conform to the adder model of cell-size control ($a = 1$; $p = 0.938$, ANOVA F -test²). Other authors (e.g. Aldridge *et al.* 2012) have suggested that *M. smegmatis* cells follow a timer model, with fixed interdivision time, which predicts that interdivision time has no dependency on birth length, however, interdivision time is negatively correlated with birth length (Pearson's $r = -0.354$; Figure 3.5B) indicating that cells that are born larger spend less time before dividing again, which is inconsistent with a timer model. A timer model also predicts that cells will double their size on average each cell cycle, or a slope of $a = 2$, which is not observed in these data (Figure 3.5A).

Given these relationships, it can be concluded that *M. smegmatis* mc²155 cells closely follow an adder model of cell division in its purest sense—cells divide after increasing by a fixed length—when grown with glycerol as a sole carbon source.

Other consequences of the adder model predicted and observed in *E. coli* are not necessarily applicable to mycobacteria however. Added length is loosely correlated with growth rate ($r = 0.285$; Figure 3.6A), which is not observed in other bacterial species (Campos *et al.* 2014; Osella *et al.* 2014; Taheri-Araghi *et al.* 2015). This observation might suggest that added length is not constant, and indeed growth rate is negatively correlated with birth length, which implies that cells that are born larger grow at a slower rate than cells that are born small ($r = -0.451$; Figure 3.6B). However, this clearly does not affect the mean added length with respect to birth length as shown by $a = 0.996 \pm 0.102$ (Figure 3.5A). This can probably be explained by the small range of growth rate variation (Figure 3.6B), so while there is significant correlation between birth length and growth rate, the effective impact on growth rate is minor for even relatively large differences in birth length. In addition, the correlation between growth rate and added length is low (Figure 3.6A), so changes in growth rate do not dramatically affect added length.

²This test compares the slope of added length against birth length (Equation 3.2) to 0; equivalent to testing whether the slope of division length against birth length (Equation 3.1) is different from 1.

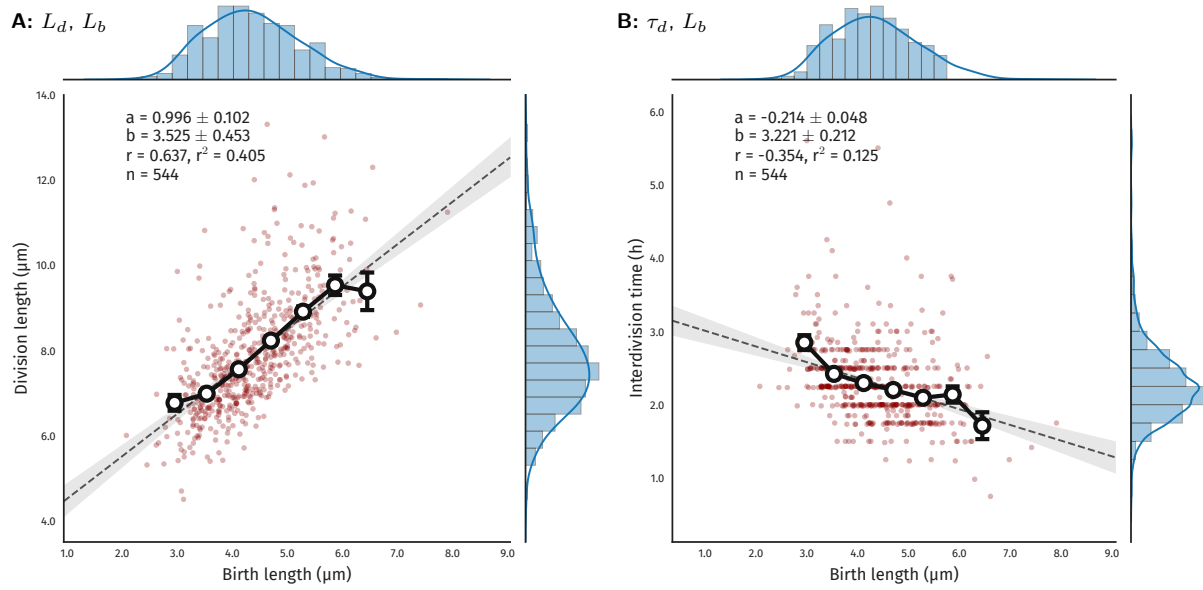


Figure 3.5: Relationship between birth length and other statistics for single cells grown in glycerol. (A) Relationship between birth length (L_b) and division length (L_d) for *M. smegmatis* mc²155 cells grown with glycerol as a sole carbon source. Red circles denote single cell division event measurements; a linear regression line fitted to these data is shown as a dotted line, with 95 % confidence expressed as a light shaded area (determined by bootstrapping); white filled circles represent the mean division length binned by birth length with error bars denoting the 95 % confidence interval of each bin (as determined by bootstrapping); histograms show the distribution of birth length (top) and division length (right), with kernel density estimates as solid blue lines; the key indicates the fitted parameters—‘slope’, the slope of the regression line; ‘intercept’, the y -intercept of the regression line; r , the Pearson correlation coefficient of the data, and its squared value r^2 ; n , the number of cell divisions observed and plotted. All statistics are interpolated where possible. (B) Relationship between birth length (L_b) and interdivision time (τ_d), with key as for panel A.

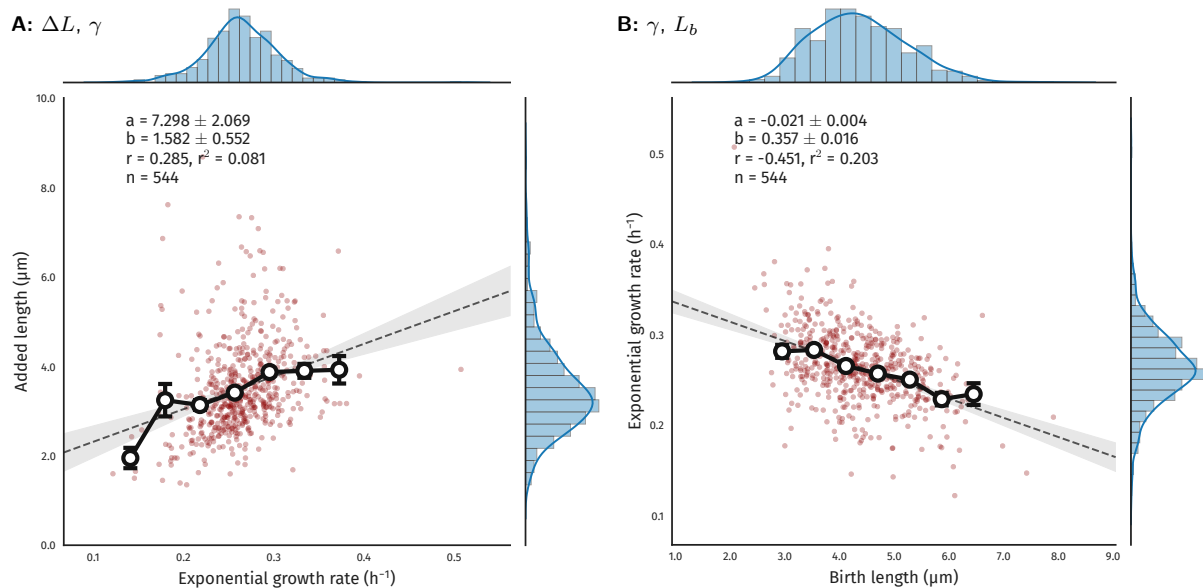


Figure 3.6: Unusual relationships between statistics for single cells grown in glycerol. (A) Relationship between exponential growth rate (γ) and added length (ΔL) for *M. smegmatis* mc²155 cells grown with glycerol as a sole carbon source. (B) Relationship between birth length and exponential growth rate. Key as for Figure 3.5.

Mycobacteria are well known to divide asymmetrically, dependent on pole age. Every cell division creates a new pole at the septum, which grows more slowly than the existing pole (Aldridge *et al.* 2012; Joyce *et al.* 2012; Meniche *et al.* 2014; Botella *et al.* 2017; Rego *et al.* 2017). Cells which inherit this new pole from their mother are characterised as new-pole inheritors or “new-pole daughters”, and cells which inherit the existing pole are characterised as old-pole inheritors, or “old-pole daughters”.

There are significant differences in single cell statistics for new- and old-pole daughters, including birth length, division length, added length, and exponential growth rate (detailed in Table 3.1). In short, old-pole inheritors are statistically significantly larger at birth and at division, and add more length between birth and division compared to new-pole inheritors. In keeping with the relationship between birth length and growth rate (Figure 3.6B), old-pole daughters also grow significantly slower than new-pole daughters. However, there is no statistically significant difference in interdivision time between new- and old-pole daughters.

Statistic	New-pole		Old-pole		p-value*
Birth length (μm)	4.024	[3.931–4.117]	4.706	[4.613–4.800]	2.47×10^{-22}
Division length (μm)	7.399	[7.260–7.544]	8.343	[8.187–8.502]	1.58×10^{-17}
Added length (μm)	3.375	[3.264–3.493]	3.637	[3.514–3.767]	0.003
Interdivision time (h)	2.305	[2.244–2.370]	2.268	[2.209–2.323]	0.393
Growth rate (h^{-1})	0.271	[0.266–0.276]	0.257	[0.253–0.261]	2.69×10^{-5}
Elongation rate ($\mu\text{m h}^{-1}$)	1.488	[1.456–1.521]	1.621	[1.588–1.654]	2.73×10^{-8}
Asymmetry	0.477	[0.470–0.485]	0.555	[0.547–0.564]	1.15×10^{-35}

Table 3.1: Single-cell statistics for cells grown in glycerol according to pole inheritance. Mean and 95 % confidence intervals in brackets (determined by bootstrapping) for cells grown with glycerol as a sole carbon source, split into cells that inherit pole that was created in the previous generation from their mother (“new-pole”) and those that inherit the pole that their mother inherited (“old-pole”). **p*-value as determined by Welch’s *t*-test.

Even given these marked differences in basic parameters, new- and old-pole daughters conform rigidly to the adder model. When, as above, the dependence of division length on birth length is determined by linear regression, $a = 0.948 \pm 0.162$ and $b = 3.882 \pm 0.774 \mu\text{m}$ for daughters that inherit the old pole ($n = 274$; Figure 3.7A), and $a = 0.894 \pm 0.149$ and $b = 3.801 \pm 0.612 \mu\text{m}$ for new-pole daughters ($n = 270$; Figure 3.7B). These values are not significantly different from $a = 1$ (a perfect adder) with $p = 0.529$ and $p = 0.164$ for old- and new-pole daughters respectively (ANOVA *F*-test), and additionally are not significantly different from each other, with $p = 0.631$ (ANCOVA). Therefore, cells inheriting the old, presumably growing, pole follow the same cell-size control mechanism compared to daughter cells which inherit the new pole, even though they grow slower, are born and divide larger, and add more length per cell cycle.

The data that these relationships are determined from allows the construction of cell lineages up to eleven generations before overgrowth or experimental time constraints prevent further

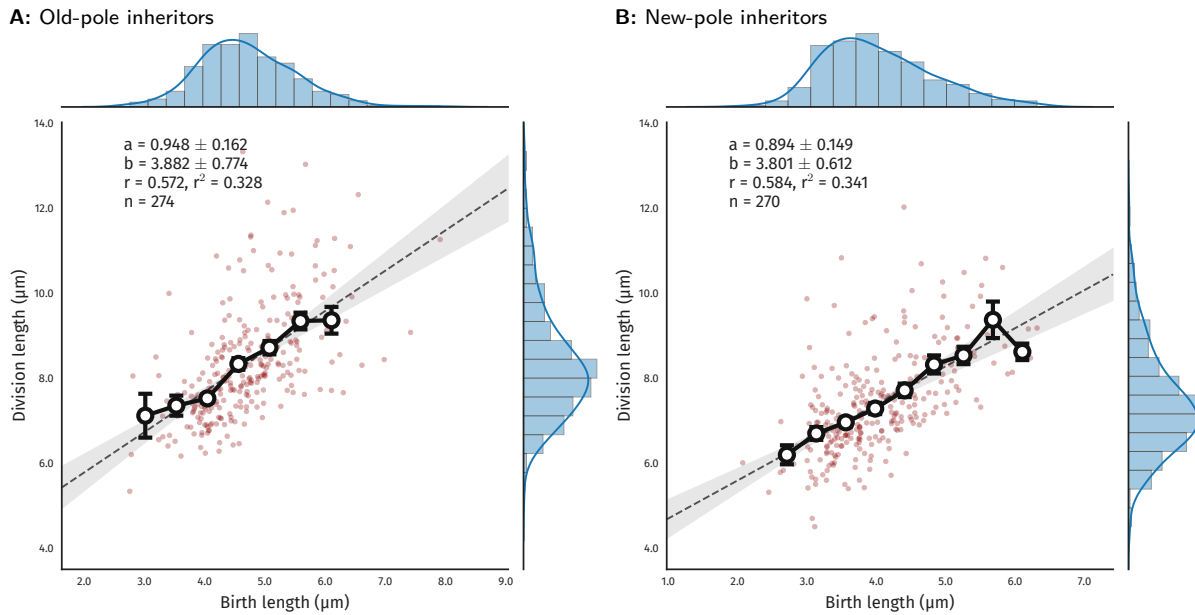


Figure 3.7: Cell-size control according to pole inheritance for cells grown in glycerol. (A) Relationship between birth length and division length for *M. smegmatis* mc²155 cells which inherit the old pole from their mother, grown with glycerol as sole carbon source. (B) Relationship between birth length and division length for cells which inherit the new pole from their mother. Key as for Figure 3.5.

tracking. This allows the impact of pole inheritance to be extended beyond the old/new pole dimension to allow analysis of the impact of pole age itself. This dataset is restricted in size, since age can only be defined when a pole is observed to be created by cell division and tracked over multiple generations. However, there are 170 cells with definitively known old pole ages: 99 with an age of two generations, 39 with an age of three, 21 with an age of four, 8 with an age of five, and 1 with ages of six, seven, and eight generations. For statistical purposes, the 11 cells with a pole age of five or more are excluded from any analysis.

As pole age increases, birth and division length increase (Figures 3.8A and 3.8B), growth rate reduces (Figure 3.8C)—albeit marginally—whilst added length and interdivision time appear to remain constant (Figures 3.8D and 3.8E). As when new- and old-pole inheritors are considered as groups, all pole age subsets conform to the adder principle, although cells with four-generation-old poles may verge towards greater than $a = 1$. Fitted values for a increase from 1.022 ± 0.281 for two-generation-old poles, 1.229 ± 0.562 for three-generation-old poles, to 1.446 ± 1.195 for four-generation-old poles, with associated error also increasing (Figure 3.8F). No significant deviation from adder can be observed for any pole age, and ANOVA-derived p -values for deviation from adder are 0.877, 0.415, and 0.070 respectively, as indicated by confidence intervals which overlap with $a = 1$ (Figure 3.8F).

Given these data, I can conclude—with confidence—that *M. smegmatis* mc²155 cells grown with glycerol as a sole carbon source on average add a constant length between birth and division, regardless of their initial birth length—they behave as adders in the purest sense.

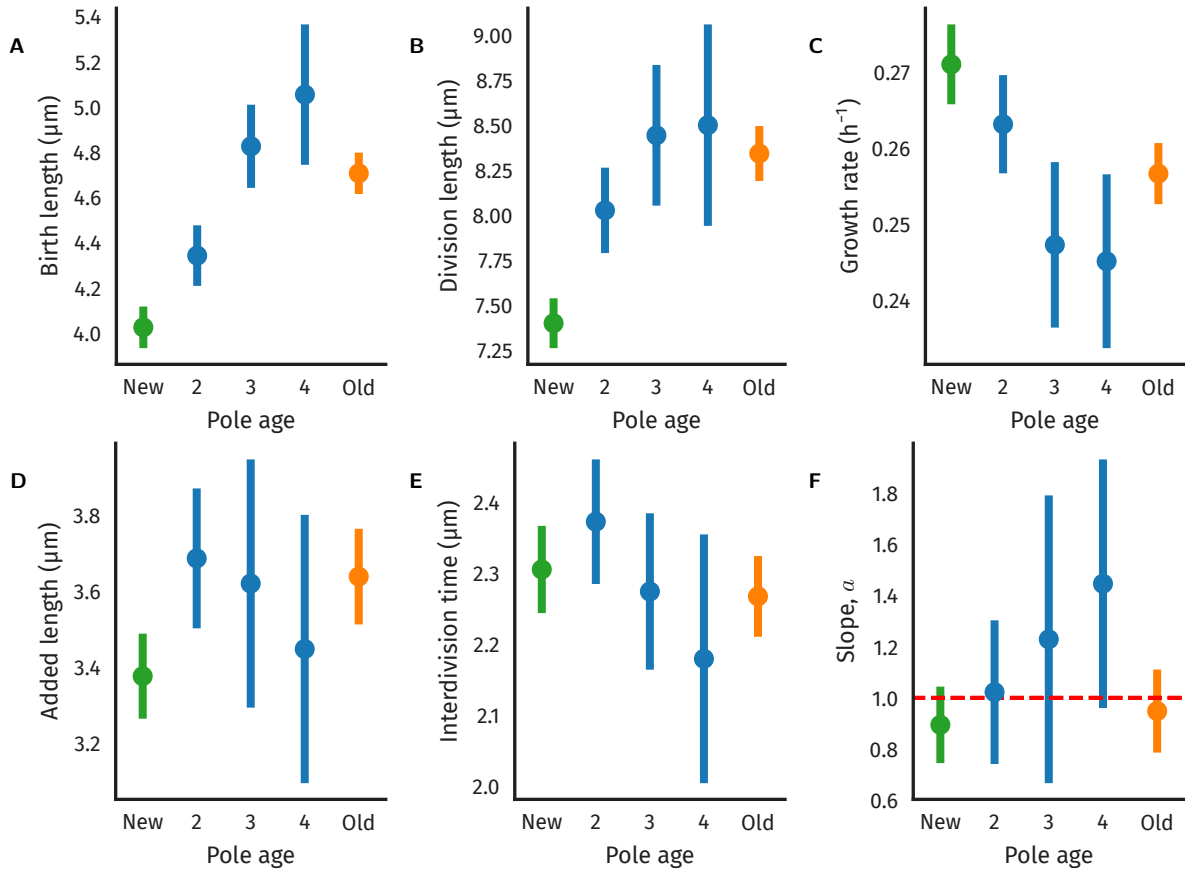


Figure 3.8: Effect of pole age on single cell statistics for cells grown in glycerol. Mean and 95 % confidence intervals (determined by bootstrapping) are plotted for a variety of single cell statistics according to the known age of the old pole inherited (blue). New-pole daughters (green) and all old-pole daughters (orange) are also plotted for each statistic. (A) Birth length, (B) division length, (C) growth rate, (D) added length, (E) interdivision time, and (F) slope (a) of the ordinary least squares linear regression line fitted to division length against birth length. The red dotted line in this panel indicates $a = 1$, or a perfect adder.

3.3.4 Noise in the noisy linear map

The noisy linear map (Equation 3.1) includes the noise term η which is not often discussed. It is a noise term that is centred around zero, therefore should not affect regression of birth and division length used to determine the value of a . However, the shape of the term, while often assumed to be normally distributed: $\eta \sim \mathcal{N}(0, \sigma^2)$ (e.g. Amir 2014; Bertaux *et al.* 2018), is not known in mycobacteria. The distribution can be estimated by analysing the residuals of ordinary least squares linear regression of division length against birth length (Figure 3.5A). The residuals have a mean value of zero, as they are derived from a least squares approximation, with a standard deviation of $1.03 \mu\text{m}$, however, a plot of a normal distribution with $\mu = 0$ and $\sigma = 1.03$: $\eta = \mathcal{N}(0, 1.03^2)$, is a poor fit for the underlying distribution of the residuals (Figure 3.9A). Using a Shapiro–Wilk test of normality, the null hypothesis that the residuals are normally distributed is strongly rejected with $p = 1.28 \times 10^{-14}$. Additionally, a quantile–quantile plot comparing the residuals to a normal distribution shows significant deviation from normal at all quantile positions (Figure 3.9B). This implies that η is not normally distributed for cells grown in glycerol, at least when estimated by ordinary least squares. This is due to

a consistent right-skew in the residuals with a significant skewness of 1.17 ($p = 6.01 \times 10^{-20}$; tested according to D’Agostino *et al.* 1990). The implications of this noise distribution are unclear.

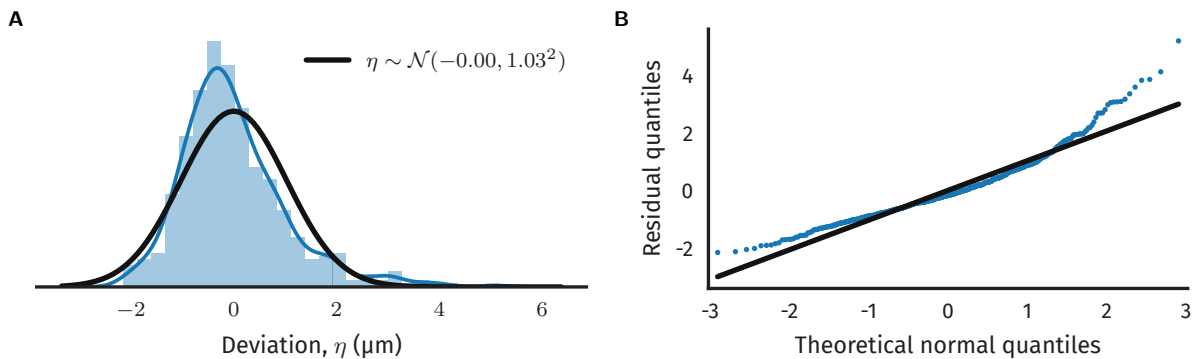


Figure 3.9: Analysis of the noisy linear map noise term. The ordinary least squares linear regression of division length against birth length provides coefficients that reflect a and b in the noisy linear map (Equation 3.1), and the residuals of the fit approximate the shape of the noise term η . (A) Distribution of residuals of the linear fit of division length against birth length depicted as a histogram shaded in blue with a kernel density estimate as a solid blue line. A fitted normal distribution with the same standard deviation is overlaid in black. (B) Quantile–quantile plot of residual data quantiles against theoretical normal distribution quantiles (blue circles) with a perfect fit denoted with a black line.

3.3.5 Cell-size control with alternative sole carbon sources

The natural environment that mycobacteria inhabit is unlikely to be similar to laboratory media which optimise growth rate; *M. smegmatis* is a soil bacterium which probably has limited access to the glycerol provided by HdB, and *M. tuberculosis* is thought to catabolise cholesterol as its primary carbon source when living intracellularly within the phagosome (Salamon *et al.* 2014; Vromman 2014). Therefore I investigated how *M. smegmatis* cells behaved when provided with sub-optimal carbon sources—pyruvate and acetate—as their sole carbon source, replacing glycerol. Pyruvate and acetate (via acetyl-CoA) happen to be metabolic products of cholesterol metabolism in mycobacteria (Griffin *et al.* 2012), but it is unlikely that *M. smegmatis* has evolved to utilise cholesterol to the extent that *M. tuberculosis* has. Rather, the research question was whether changing carbon source would have any effect at all, and whether this would need to be considered in future experiments.

Cells were grown in acetate or pyruvate in the microfluidic device in an identical manner to cells grown in glycerol, simply replacing 0.2 % glycerol in HdB with 0.2 % w/v potassium acetate or sodium pyruvate (see Methods section: Variants of HdB). 383 cell division events were observed for cells grown in acetate, and 367 cell division events were observed for cells grown in pyruvate. For reference purposes, all pairwise combinations of the data acquired during these experiments, according to the age of the pole inherited are depicted in Figure 3.16 for cells grown in acetate, and in Figure 3.17 for cells grown in pyruvate. Cells have reduced cell sizes at birth and increased interdivision time compared to cells grown in glycerol, indicating the paucity of

the medium is having an effect (detailed in Table 3.2). When dependency of division length on birth length is determined for cells grown in these alternative carbon sources, significant differences in cell-size control emerge compared to cells grown in glycerol, a surprising result given the apparent robustness of the adder principle in glycerol.

Statistic	Glycerol		Acetate		Pyruvate	
Birth length (μm)	4.310	[4.253–4.383]	3.898	[3.838–3.967]	3.523	[3.474–3.573]
Division length (μm)	8.012	[7.872–8.138]	7.457	[7.383–7.563]	6.816	[6.730–6.899]
Added length (μm)	3.701	[3.620–3.805]	3.559	[3.481–3.629]	3.294	[3.209–3.377]
Growth rate (h^{-1})	0.263	[0.260–0.266]	0.265	[0.261–0.267]	0.235	[0.232–0.239]
Interdivision time (h)	2.262	[2.208–2.307]	2.485	[2.430–2.539]	2.868	[2.803–2.934]

Table 3.2: Single-cell statistics for cells grown in different sole carbon sources. Mean and 95 % confidence intervals in brackets (determined by bootstrapping) for cells grown with glycerol, acetate, or pyruvate as a sole carbon source.

For cells grown in acetate, the relationship between division length and birth length as determined by ordinary least squares linear regression results in $a = 0.964 \pm 0.140$ (Figure 3.10A), consistent with the adder principle ($p = 0.616$; F -test). When cells are divided according to the age of their inherited pole, old-pole inheritors have a slope of 1.181 ± 0.212 , consistent with an adder ($p = 0.095$; F -test; Figure 3.10C), but new-pole inheritors have a slope of 0.682 ± 0.187 , which is not ($p = 0.001$; F -test; Figure 3.10D). This indicates that new-pole inheritors grown with acetate as a sole carbon source are deviating from the adder principle, and have acquired “sizer-like” properties.

However, close observation of the raw data shows that the binned values do not conform to the linear model very well (Figure 3.10A) with cells that are born large potentially skewing the dataset. These large cells are old-pole inheritors, as indicated by the poor fit of binned data to the linear model for old-pole inheritors (Figure 3.10C) but not for new-pole inheritors, which are modelled relatively well (Figure 3.10D). This raises the possibility that cells that are born very large are skewing the linear fit for the population and old-pole inheritors, with a potential overestimation of the value of a . When data is restricted to exclude cells which are born at sizes larger than $5 \mu\text{m}$ (16 cells), the regression coefficients are altered—providing a much more effective linear fit to the binned data (Figure 3.10B)—resulting in $a = 0.730 \pm 0.154$ for all cells, which is inconsistent with an adder model. Restricted old-pole inheritors also have a reduced $a = 0.883 \pm 0.233$, which is still consistent with an adder, but verging on sizer-like behaviour. Restricted new-pole inheritors, while fitted well without excluding large cells, also have a reduced $a = 0.576 \pm 0.212$, remaining sizer-like for this sub-population. This raises the possibility that all cells grown in acetate may be acting in a sizer-like manner, though the effect of these very large potential outliers may be reflecting a biological phenomenon.

For cells grown in pyruvate, the slope of the ordinary least squares regression line of division

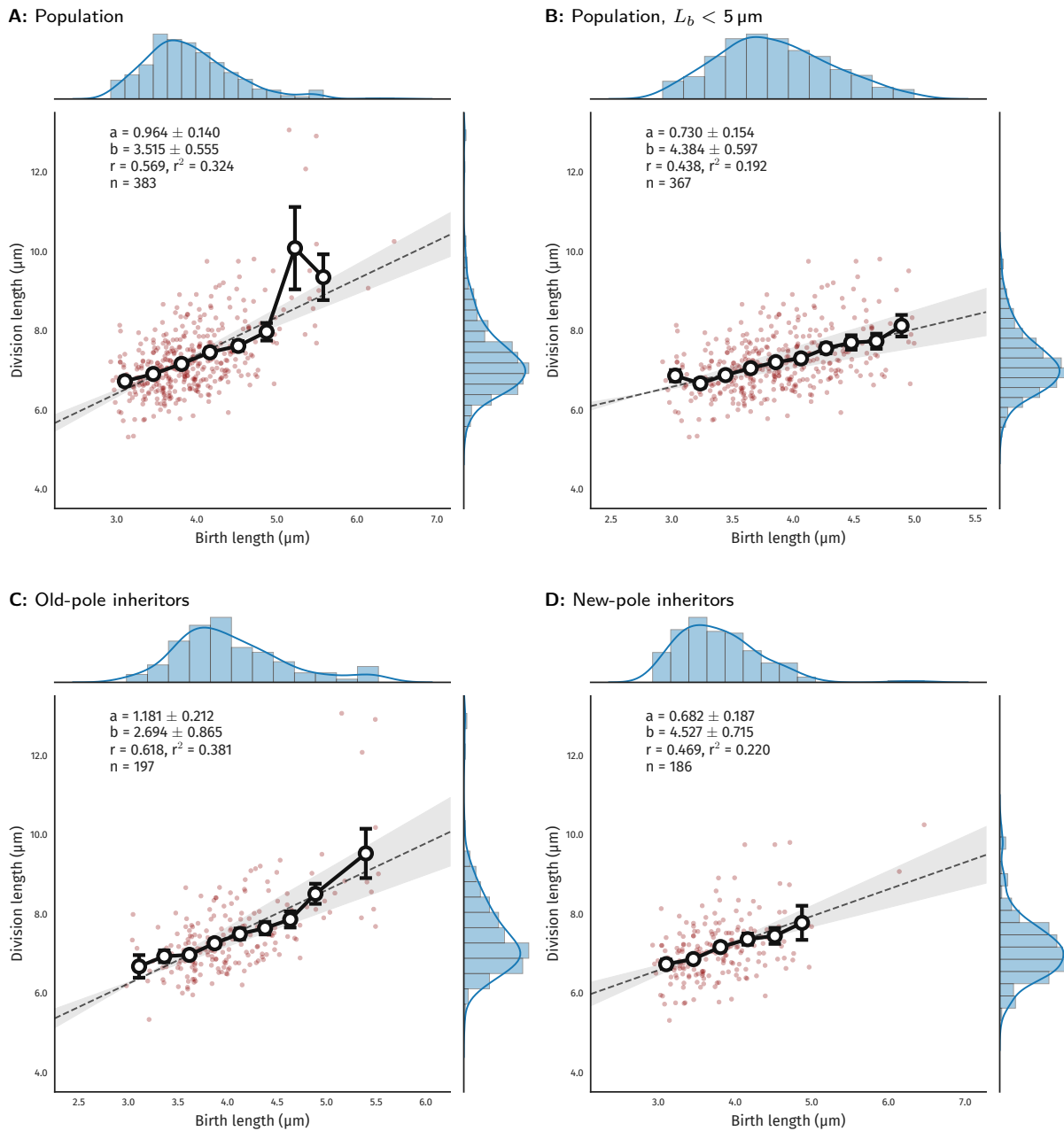


Figure 3.10: Relationship between birth length and division length for single cells grown in acetate. (A) Relationship between birth length and division length for *M. smegmatis* mc²155 cells grown with acetate as a sole carbon source. (B) Relationship between birth length and division length for cells with birth lengths less than 5 μm . (C) Relationship between birth length and division length for cells which inherit the old pole from their mother. (D) Relationship between birth length and division length for cells which inherit the new pole from their mother. Key as for Figure 3.5.

length against birth length is 0.832 ± 0.137 , which is inconsistent with the adder principle. This difference is maintained when old- and new-pole inheritors are analysed separately with $a = 0.767 \pm 0.192$ ($n = 197$; Figure 3.11B) and $a = 0.815 \pm 0.221$ ($n = 170$; Figure 3.11C) respectively. This demonstrates that all cells grown in pyruvate are behaving in a “sizer-like” manner, with $p = 0.016$ (F -test) regardless of which pole is inherited. This may relate to the more significant effect on growth rate observed in this medium (Table 3.2), where there is very little difference in single-cell exponential growth rate between cells grown in glycerol (0.263 h^{-1} [0.260–0.266]) compared to those grown in acetate (0.265 h^{-1} [0.261–0.267]; $p = 0.705$, Welch’s t -test), whereas cells grown in pyruvate are significantly slower (0.235 h^{-1} [0.232–0.239]; $p = 8.26 \times 10^{-29}$, Welch’s t -test).

A: Population

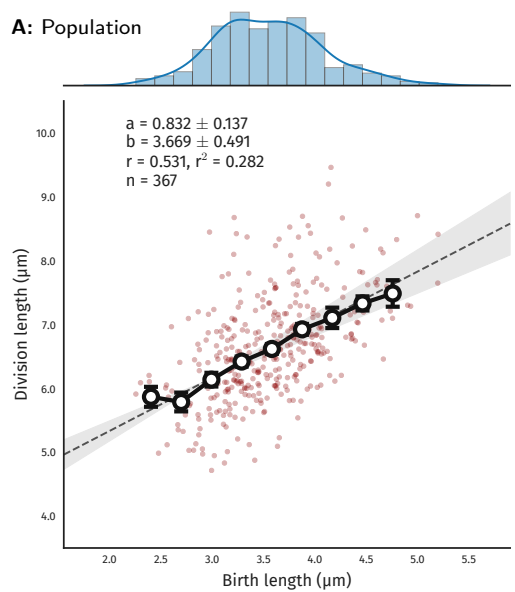
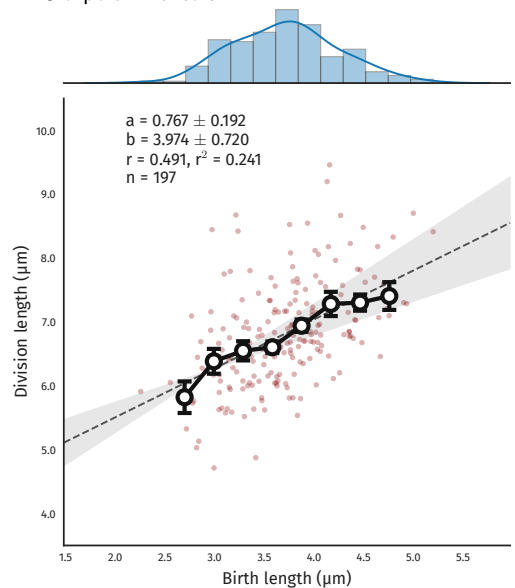
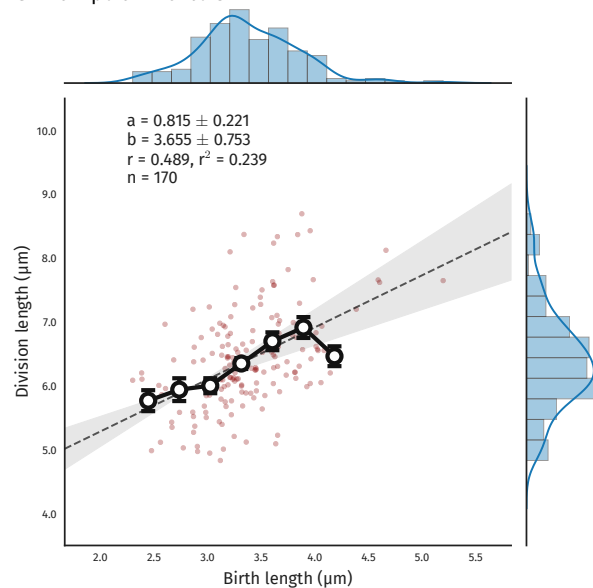


Figure 3.11: Relationship between birth length and division length for single cells grown in pyruvate. (A) Relationship between birth length and division length for *M. smegmatis* mc²155 cells grown with pyruvate as a sole carbon source. (B) Relationship between birth length and division length for cells which inherit the old pole from their mother. (C) Relationship between birth length and division length for cells which inherit the new pole from their mother. Key as for Figure 3.5.

B: Old-pole inheritors



C: New-pole inheritors



3.3.6 Impact of cell-size control on variability

Given that cells grown in the sub-optimal carbon sources appear to deviate from theadder principle compared to cells grown in glycerol, I investigated the implications of the difference. Other authors have suggested that birth length and interdivision time scale in direct proportion to their mean—i.e. the distributions collapse when normalised to their mean value (Taheri-Araghi *et al.* 2015; Kennard *et al.* 2016). This prediction does not hold for *M. smegmatis* mc²155 cells grown with acetate or pyruvate, where interdivision time and growth rate change.

Birth length distributions for cells grown in each carbon source vary significantly (Table 3.2) with cells grown in pyruvate and acetate having much narrower distributions compared to cells grown in glycerol (Figure 3.12A). This is a predicted consequence of reduced growth rate; however, when values are normalised by the mean value, distributions do not collapse (Figure 3.12B). This is indicated by the coefficient of variation (CV) of birth length for each carbon source, with 19.53 % [18.52–20.55] (95 % confidence interval), 14.23 % [12.73–15.30], and 14.84 % [13.89–15.79] for cells grown in glycerol, acetate, and pyruvate respectively, indicating that cells grown in glycerol are inherently more variable in birth length (Figure 3.13A).

Conversely, interdivision times—although coupled with inherent technical noise due to the sampling frequency of the time-lapse (15 min) though they are interpolated where possible—appear to collapse when scaled by their means (Figures 3.12C and 3.12D). Normalised interdivision time distributions have the same height and width in all three carbon sources (Figure 3.12D) as demonstrated by similar CVs, with 22.57 % [19.93–25.39], 20.01 % [17.29–21.76], and 22.94 % [20.54–25.20] for glycerol, acetate, and pyruvate respectively (Figure 3.13C).

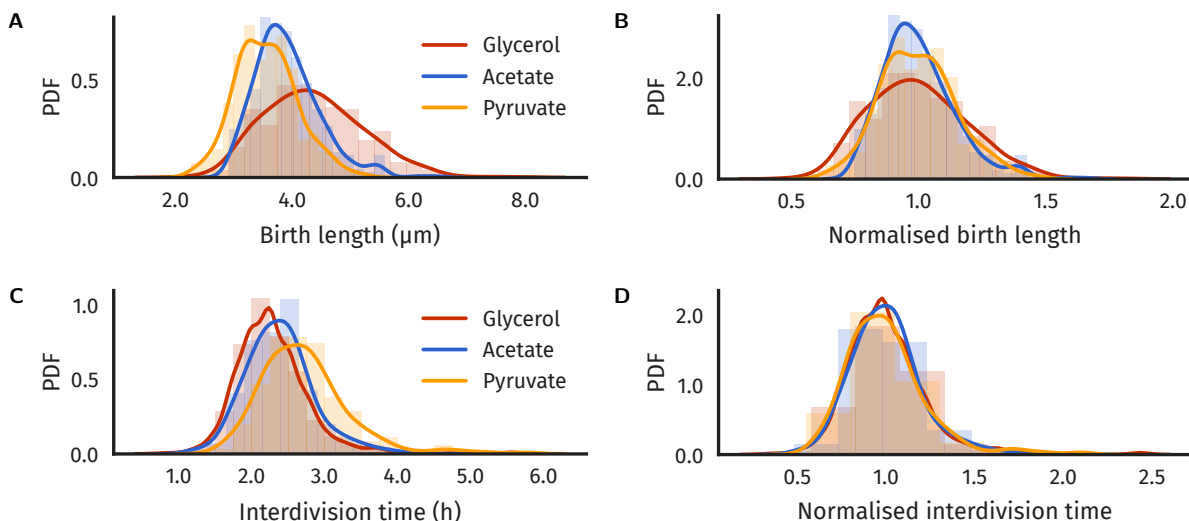


Figure 3.12: Scaling and collapse of birth length and interdivision time distributions for different carbon sources. Plots depict histograms and kernel density estimates for single *M. smegmatis* mc²155 cells grown in glycerol (red), acetate (blue), or pyruvate (yellow) as a sole carbon source. (A) Distribution of birth length for single cells grown in different carbon sources. (B) Distribution of birth length scaled by mean birth length for each carbon source. (C) Distribution of interdivision time. (D) Distribution of interdivision time scaled by mean interdivision time.

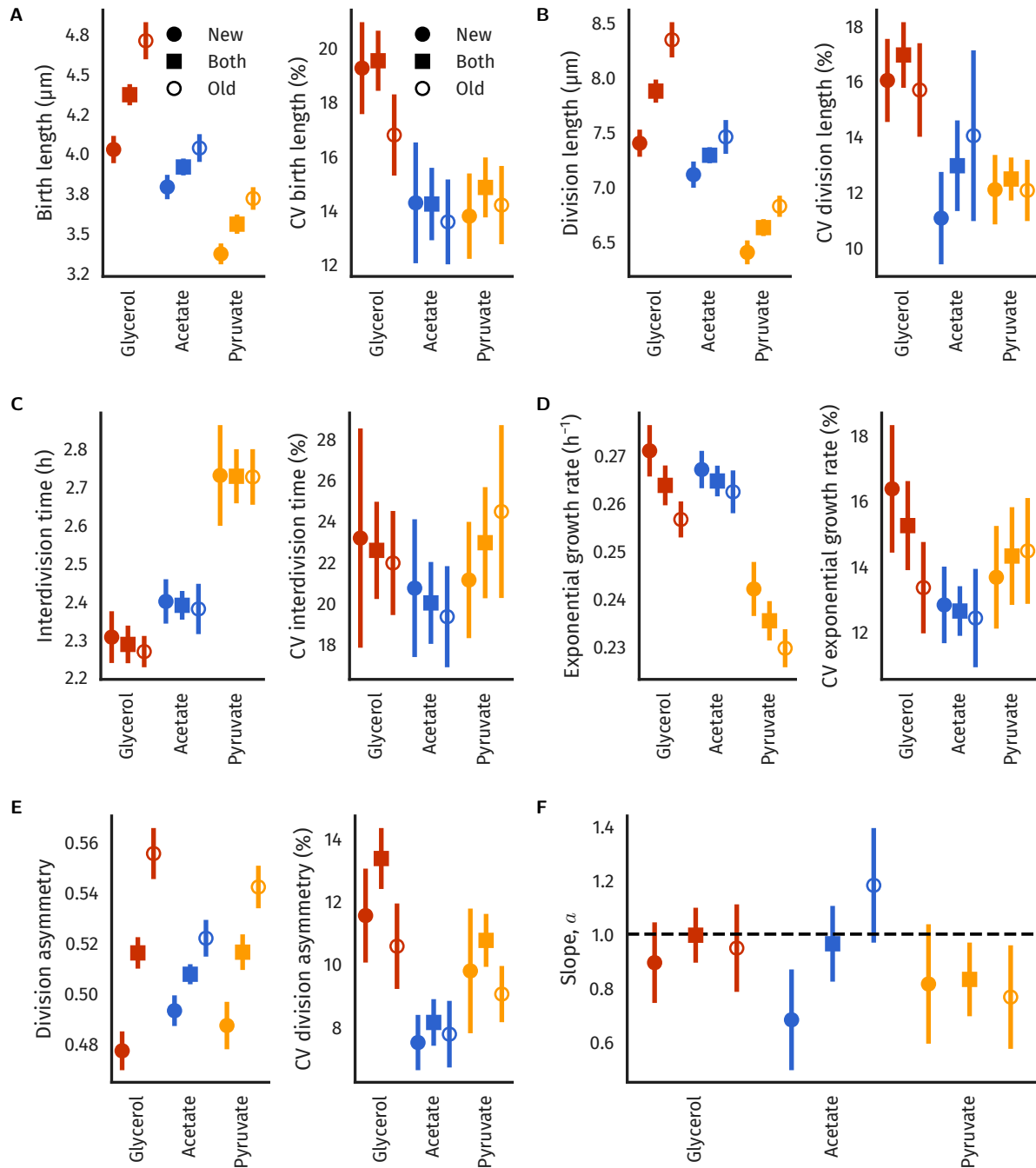


Figure 3.13: Variability in single-cell statistics for cells grown in different carbon sources. Representation of single-cell statistics separated by the age of the pole inherited: new-pole inheritors (closed circles), old-pole inheritors (open circles), and combined datasets (closed squares) with error bars depicting 95 % confidence intervals (determined by bootstrapping) for cells grown in glycerol (red), acetate (blue), or pyruvate (yellow). (A–E) The left side of each panel depicts the absolute values, and the right side depicts the coefficient of variation (CV; standard deviation divided by the mean). (F) The slope of division length against birth length as determined by ordinary least squares linear regression.

A key prediction of cell-size control, especially between the sizer and adder models is that the sizer principle strongly predicts that noise should be reduced, particularly in division length, since size is “reset” at every cell division, correcting any stochastic variation acquired in the previous cell cycle. The adder model predicts that variations are inherited for several generations before cells return to the population mean value. Since new-pole daughters grown in acetate and both old- and new-pole daughters in pyruvate have slopes significantly less than 1 in the cell-size control linear model ($a < 1$), this directly predicts that distributions for division length should be reduced compared to cells grown in glycerol for sub-populations that are acting in the “sizer-like” manner. This is indeed observed with a division length CV of new-pole daughter cells grown in acetate of 11.06 % [9.39–12.51] compared to a division length CV of cells grown in glycerol of 16.94 % [15.76–17.95], and CVs for cells grown in pyruvate of 12.47 % [11.69–13.39], 12.08 % [10.83–13.67], and 12.06 % [10.95–13.35] for the population, new-, and old-pole daughters respectively (Figure 3.13B). Indeed, division length CVs appear to directly follow differences in cell-size control slope (Figure 3.13F) with new-pole daughters grown in acetate—which behave in a sizer-like manner—having a lower CV than old-pole daughters (14.03 % [10.95–17.64]), which ostensibly behave as adders, whereas cells grown in pyruvate appear to have lower CVs compared to cells grown in glycerol regardless of pole age reflecting their general sizer-like nature (Figures 3.13B and 3.13F).

Interestingly, variability in birth length does not appear to be correlated with slope, with all cells grown in acetate and pyruvate having lower birth length CVs compared to cells grown in glycerol with 19.53 % [18.11–20.40], 14.23 % [13.13–15.48], and 14.84 % [13.75–15.86] for glycerol, acetate, and pyruvate respectively (irrespective of inherited pole age; Figure 3.13A). This may reflect differences in division asymmetry observed between these carbon sources, with size differences between new- and old-pole daughters being much greater for cells grown in glycerol compared to cells grown in acetate or pyruvate (Figures 3.13A and 3.13E). Division asymmetry is also expected to be increased in old-pole daughters, given that birth length increases with pole age for cells grown in glycerol (Figure 3.8A), and to a lesser extent in cells grown in the alternative sources. This difference is however not apparent in CVs for either asymmetry or birth length for cells grown in glycerol, with old-pole daughters having lower variation in birth length (16.78 % [15.29–18.24]) compared to new-pole daughters (19.25 % [18.08–20.42]; Figures 3.13A and 3.13E).

Few differences in variability are observed in interdivision time between carbon sources (Figure 3.13C), reflecting the scaling of interdivision time by mean value (Figures 3.12C and 3.12D). Differences in variability in growth rate are also relatively low between carbon sources and pole age, although old-pole inheritors grown with glycerol may have a hint of a lower CV, as well as cells grown in acetate or pyruvate, though the implications of this are unclear (Figure 3.13D).

In summary, cells which do not conform to the adder principle, with $a < 1$, display reduced

variability in their length at division, in qualitative agreement with the extent of their deviation from adder.

3.3.7 Population and lineage-weighted statistics

		Population	Lineage
Glycerol	Birth length (μm)	4.310 [4.253–4.383]	4.194 [4.159–4.224]
	Division length (μm)	8.012 [7.872–8.138]	7.984 [7.946–8.030]
	Added length (μm)	3.701 [3.620–3.805]	3.790 [3.741–3.830]
	Interdivision time (h)	2.262 [2.208–2.307]	2.275 [2.252–2.298]
	Growth rate (h^{-1})	0.263 [0.260–0.266]	0.270 [0.269–0.272]
Acetate	Birth length (μm)	3.898 [3.838–3.967]	3.896 [3.870–3.926]
	Division length (μm)	7.457 [7.383–7.563]	7.476 [7.423–7.515]
	Added length (μm)	3.559 [3.481–3.629]	3.580 [3.541–3.606]
	Interdivision time (h)	2.485 [2.430–2.539]	2.756 [2.728–2.780]
	Growth rate (h^{-1})	0.265 [0.261–0.267]	0.242 [0.241–0.244]
Pyruvate	Birth length (μm)	3.523 [3.474–3.573]	3.466 [3.441–3.494]
	Division length (μm)	6.816 [6.730–6.899]	6.798 [6.749–6.840]
	Added length (μm)	3.294 [3.209–3.377]	3.332 [3.283–3.365]
	Interdivision time (h)	2.868 [2.803–2.934]	3.097 [3.054–3.132]
	Growth rate (h^{-1})	0.235 [0.232–0.239]	0.223 [0.222–0.225]

Table 3.3: Population and lineage-weighted statistics for single-cell data. Estimated mean values and bootstrapped 95 % confidence intervals for single-cell parameters in each of three carbon sources are represented. Population statistics represent the average of all observed cells, and lineage statistics represent the values adjusted for bias introduced by the age structure of the lineage trees (see Methods section: Population and lineage-weighted statistics).

Statistics of tree-structured data, such as single-cell lineages, can become biased due to the over-representation of cells that divide more rapidly than others: a single cell that has a stochastically shorter interdivision time at the start of an experiment will result in more descendants observed at the end of an experiment, even if all its progeny divide at the mean division rate (Powell 1956; Hashimoto *et al.* 2016; Thomas 2017). The effect of such age structure on the data can be reduced by using lineage-weighting in which statistics are adjusted to represent the average of random paths through the lineage tree (see Methods section: Population and lineage-weighted statistics). Random paths through the data are termed “forward lineages”, as opposed to the average of all the data, as used above, which is termed the “population”. This analysis was performed by Philipp Thomas (Department of Mathematics) on the same data used to draw the above conclusions. Interdivision time is the key parameter in the biasing of population statistics, and indeed interdivision time is lower in the population compared to forward lineages for cells grown in acetate (2.485 h [2.430–2.539] and 2.756 h [2.728–2.780] respectively) and for cells grown in pyruvate (2.868 h [2.803–2.934] and 3.097 h [3.054–3.132]; Table 3.3). These differences imply that cells that divide faster contribute more to the population mean than cells

that divide more slowly. This difference implies that other cell statistics should differ between lineage-weighted and population measures, such as growth rate, which is indeed observed, cells grown in acetate and pyruvate appear to grow faster when analysed as a population compared to lineage-weighted values, with mean growth rates of 0.265 h^{-1} [0.261–0.267] and 0.242 h^{-1} [0.241–0.244] for populations and lineages grown in acetate respectively, and 0.235 h^{-1} [0.232–0.239] and 0.223 h^{-1} [0.222–0.225] for populations and lineages grown in pyruvate (Table 3.3). Although growth rate estimates from the population appear to be biased towards faster growth and greater interdivision times, this appears to have little impact on birth and division length, with significantly overlapping confidence intervals for both these statistics (Table 3.3). This implies that any population structural bias in these datasets does not affect the determination of the relationship between birth and division length, and adherence or non-adherence to theadder principle.

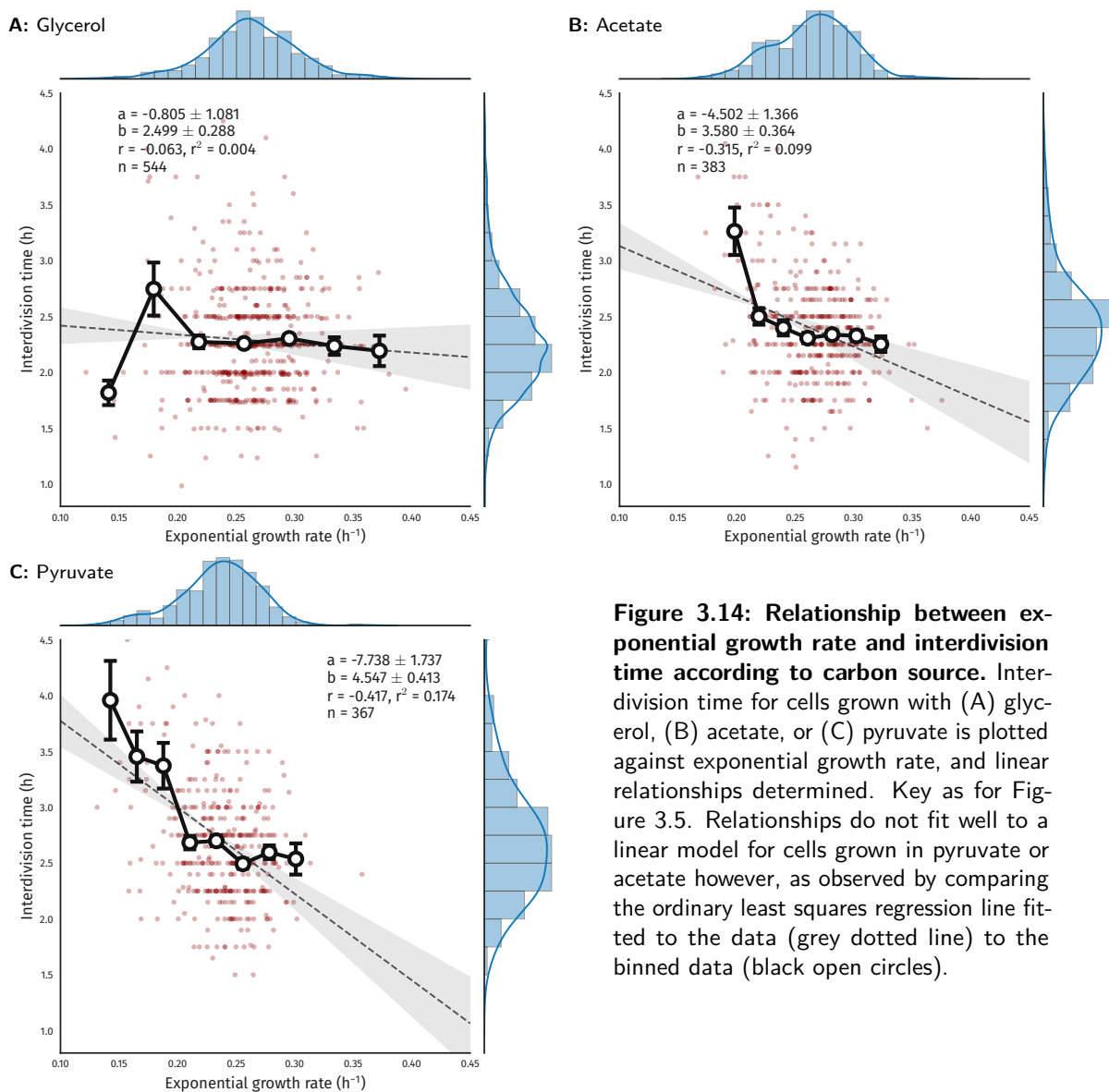


Figure 3.14: Relationship between exponential growth rate and interdivision time according to carbon source. Interdivision time for cells grown with (A) glycerol, (B) acetate, or (C) pyruvate is plotted against exponential growth rate, and linear relationships determined. Key as for Figure 3.5. Relationships do not fit well to a linear model for cells grown in pyruvate or acetate however, as observed by comparing the ordinary least squares regression line fitted to the data (grey dotted line) to the binned data (black open circles).

While cells grown in acetate and pyruvate display these features, differences between population and lineage-weighted mean interdivision times appear not to differ for cells grown in glycerol, with 2.262 h [2.208–2.307] and 2.275 h [2.252–2.298] for population and forward lineages respectively (Table 3.3), suggesting that population measurements for cells grown in glycerol are not biased by cells that divide faster. Moreover, while growth rate is skewed in favour of the population statistic for cells grown in alternative carbon sources, it is skewed in the opposite way for cells grown in glycerol, with 0.263 h^{-1} [0.260–0.266] and 0.270 h^{-1} [0.269–0.272] for populations and lineages respectively (Table 3.3). This is surprising, especially since there is no apparent biological bias in the dataset. This may reflect a flaw in the weighting strategy, or may be a statistical anomaly, especially since the magnitude of the difference is very small with 0.007 h^{-1} difference in means. Also differing from data obtained from cells grown in acetate or pyruvate, there are statistically significant differences in birth length—but not division length—for population and lineage-weighted measures. Mean birth length is $4.310 \mu\text{m}$ [4.253–4.383] for the population and $4.194 \mu\text{m}$ [4.159–4.224] for lineages (Table 3.3). However, as for growth rate differences, the magnitude of the difference is small, with $0.116 \mu\text{m}$ between the two measures (approximately 1–1.5 pixels), thus should not affect conclusions drawn from population measures.

The lack of bias for glycerol data may relate to the lack of observable correlation between growth rate and interdivision time for cells grown in glycerol, with Pearson's $r = -0.063$ (Figure 3.14A), which is not observed for cells grown in acetate ($r = -0.315$; Figure 3.14B) or pyruvate ($r = -0.417$; Figure 3.14C).

It appears that the relationship is non-linear for acetate and pyruvate however, since the ordinary least squares regression lines fit the binned data poorly (Figures 3.14B and 3.14C). Regardless of the poor linear fit, a correlation between growth rate and interdivision time is expected to be necessary for fast growers to dominate populations, and the lack of this correlation may explain why population measures are not biased for cells grown in glycerol.

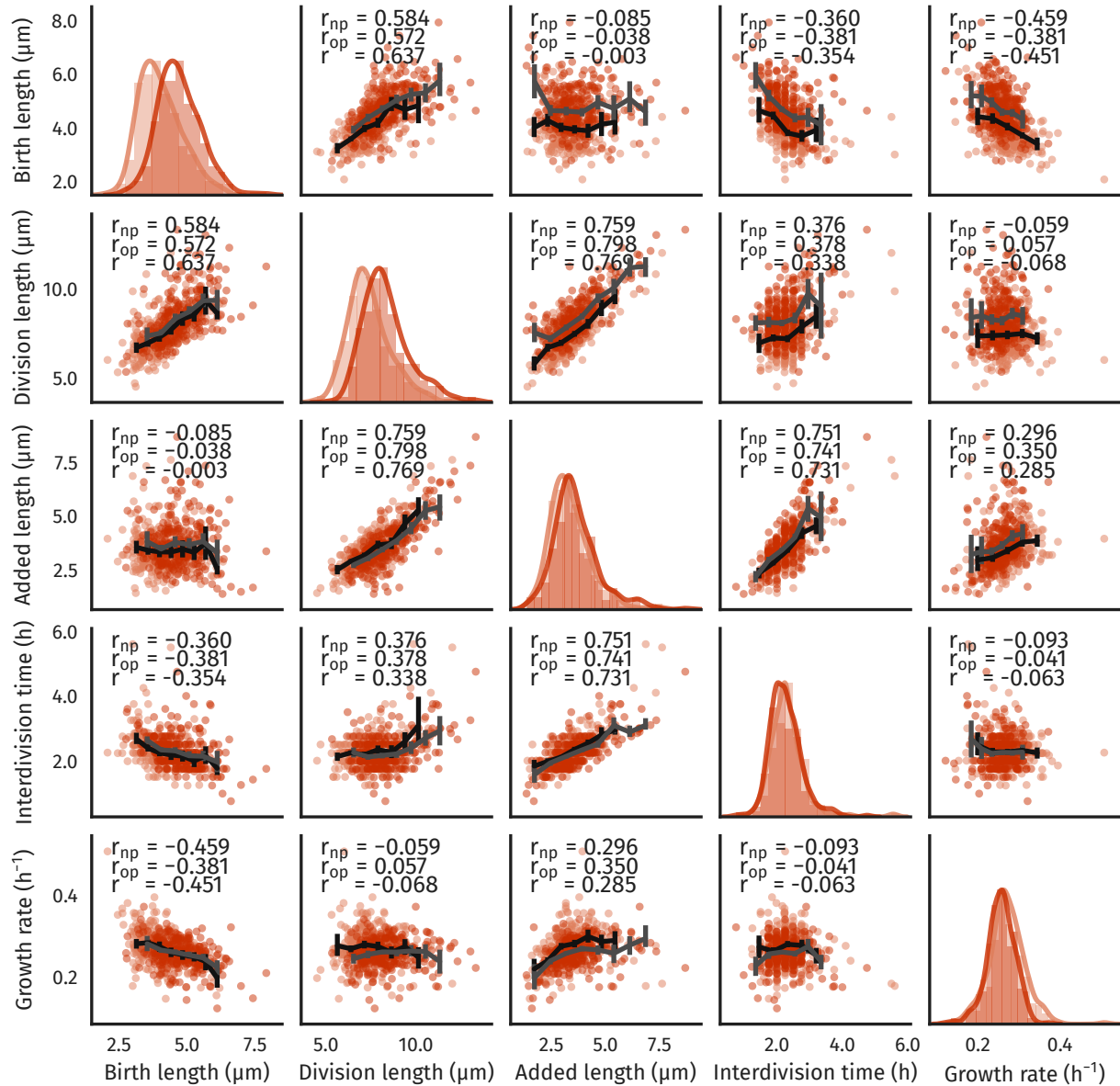


Figure 3.15: All pairwise interactions between single-cell parameters for new- and old-pole inheritors grown in glycerol. Birth length, division length, added length, interdivision length, and growth rate are plotted pairwise on a grid. New-pole inheritors are plotted in a lighter shade than old-pole inheritors. Diagonals represent histograms of the x -axis data, with kernel density estimates plotted as a solid line. For non-diagonals, the underlying data is plotted as circles, with the lines representing y -axis data binned by x (for bins with more than 5 data-points), with error bars denoting the 95 % confidence interval of each bin, with new-pole inheritors in grey, and old-pole inheritors in black. The Pearson correlation coefficient r is annotated in the top left of each comparison.

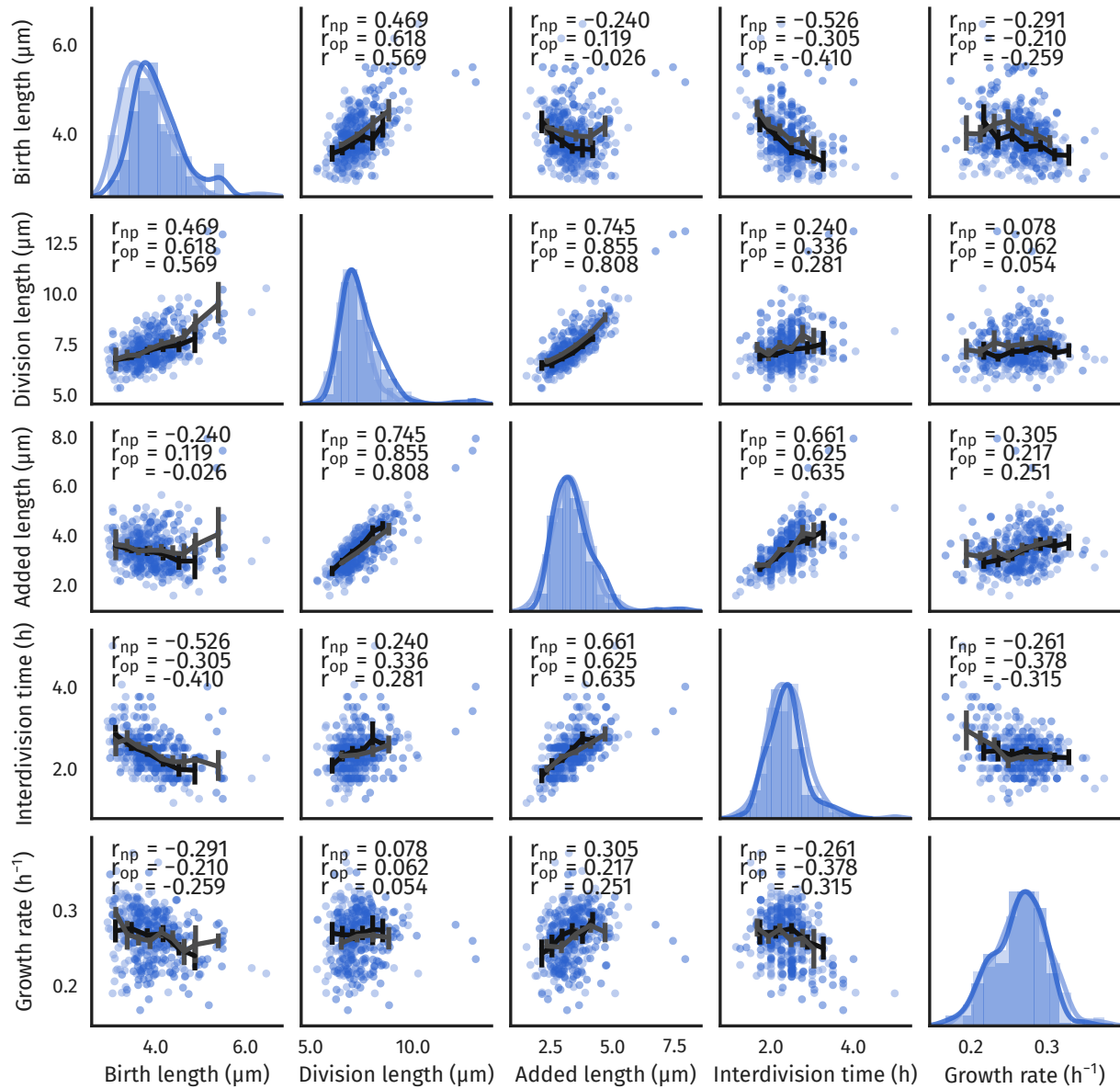


Figure 3.16: All pairwise interactions between single-cell parameters for cells grown in acetate. Key as for Figure 3.15.

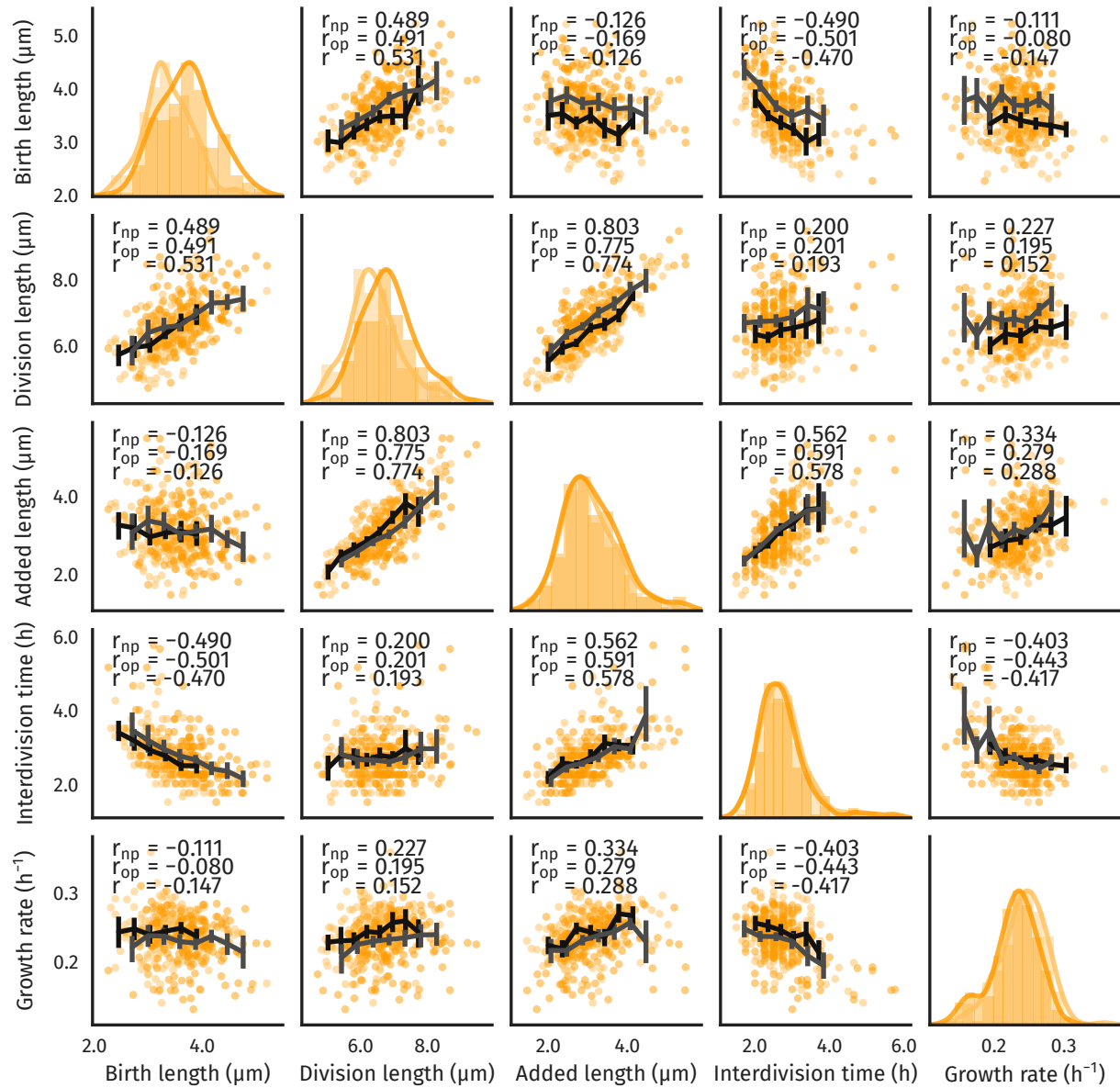


Figure 3.17: All pairwise interactions between single-cell parameters for cells grown in pyruvate. Key as for Figure 3.15.

3.4 Discussion

3.4.1 Summary of major findings

Using careful analysis of basic time-lapse microscopy data, *M. smegmatis* mc²155 cells can be shown to divide by the adder principle: they add a fixed length between birth and division, with a slope determined by linear regression between division length and birth length of $a = 0.996 \pm 0.102$ and a Pearson correlation coefficient of $r = 0.637$ (Figure 3.5A). This indicates that *M. smegmatis* behaves as a perfect adder (with respect to the noisy linear map) with remarkable correspondence to various model predictions. The data also show that cell cycle time is not constant as predicted by a timer, with interdivision time correlated with birth length ($r = -0.354$; Figure 3.5B). The adder model has been extended to make several predictions that depend on how single cells elongate, and observation of how cell length increases over time. Model selection on these data provides strong evidence that *M. smegmatis* single cells grow exponentially at the single cell level (Figure 3.4). This implies that predictions such as the division of larger cells earlier than smaller cells (negative correlation of interdivision time and birth length) apply, and indeed they do (Figure 3.5A).

Previous investigations of cell-size control in mycobacteria have used laboratory media, and the two prior studies have used 7H9 rich medium supplemented with glycerol (Aldridge *et al.* 2012) or both glycerol and glucose (Santi *et al.* 2013), and both enriched with oleic acid–albumin–dextrose–catalase (OADC) growth supplement and the detergent Tween-80 which include additional carbon sources. These two studies—which included comparing added length to birth length—found differing results which may indicate a role for culture medium in how birth and division length are related. For this reason, as well as in an attempt to adjust the population doubling rate, I used a minimal medium containing acetate or pyruvate as a sole carbon source, and compared data to standard minimal medium containing glycerol as a sole carbon source. In this manner, I control for the complex components of 7H9 and OADC, simply changing the carbon source and the carbon source alone. When determining the relationship between birth length and division length, *M. smegmatis* mc²155 cells deviate from adder in a pole age-dependent manner when grown with acetate (Figure 3.10A)—though this may be skewed by large cells—and in a pole age-independent manner when grown with pyruvate (Figure 3.11A). Acetate-grown cells which inherit the new pole (new-pole inheritors) from their mother behave in what I have termed a “sizer-like” manner, in which the slope of the linear regression of division length on birth length is $a = 0.682 \pm 0.187$ which is significantly different from 1 ($p = 0.001$; F -test; Figure 3.10D), whereas old-pole inheritors remain consistent with the adder principle (Figure 3.10C). Cells grown in pyruvate behave in this sizer-like manner regardless of inherited pole age, with $a = 0.832 \pm 0.137$ for all cells (Figure 3.11A). This work was published in Priestman *et al.* 2017, and at the time was the first definitive result demonstrating bacteria

that could deviate from the adder principle without requiring a drastic reduction in growth rate. Shortly after this, another report describing cell-size control in *M. smegmatis* was published (Logsdon *et al.* 2017), and I will discuss my results in context with this.

3.4.2 Single-cell exponential growth

Many organisms have been conclusively determined to grow exponentially at the single-cell level, including *E. coli* (e.g. Godin *et al.* 2010), which is consistent with the incorporation of cell wall material along the cell body, which provides an exponentially increasing surface area in which nascent material can be introduced (Margolin 2009; Typas *et al.* 2012; Wang *et al.* 2012a). Mycobacteria however elongate exclusively from their polar tips (Thanky *et al.* 2007; Hett and Rubin 2008), and preferentially from a single pole (Joyce *et al.* 2012; Aldridge *et al.* 2012; Santi *et al.* 2013; Meniche *et al.* 2014; Manina *et al.* 2015; Botella *et al.* 2017; Rego *et al.* 2017). Since cell width is constant during the cell cycle, the surface area available for incorporation of cell wall during growth does not increase, thus exponential single-cell growth has been less certain in mycobacteria (Daniel and Errington 2003), with authors using both linear and exponential growth measures (e.g. Aldridge *et al.* 2012; Wakamoto *et al.* 2013, respectively). The difficulty in distinguishing between the models is partially due to the slow doubling time of mycobacteria compared to other bacterial species. This necessitates long-term time-lapses, which limits the maximum possible frequency of imaging during one cell cycle, since phototoxic effects become significant at these time scales. This time constraint results in single-cell length traces in which both linear and exponential fits to a single cell are hard to distinguish. In addition, measurement noise—which is a consistent occurrence in single-cell microscopic studies of bacteria—can disguise any underlying model, especially since the differences between the two models are minor even in theory.

The linear model, described in Chapter 2 (Equation 2.1), is $L(t) = \lambda t + L_1$ where $L(t)$ is a function describing length L at time t , t is time from birth (the first frame), L_1 is initial length, and λ is the linear elongation rate. The exponential model, described in Chapter 2 (Equation 2.3), is $L(t) = L_1 e^{\gamma t}$, where γ is the exponential growth rate. In my data, a randomly chosen cell trace has a mean squared error (MSE) of the residuals of the linear fit of $0.016 \mu\text{m}^2$, and $0.022 \mu\text{m}^2$ for the exponential fit (Figures 3.4A and 3.4B), highlighting the similarities between the models. When all cells grown in glycerol are considered ($n = 1319$), MSE for the linear model is $0.038 \mu\text{m}^2$ [0.035–0.042] and $0.035 \mu\text{m}^2$ [0.031–0.038] for the exponential model (95 % confidence interval), with significant overlap of the confidence intervals indicating that the models cannot be distinguished on a cell-by-cell basis. Santi *et al.* addressed this issue by comparing size increment (change in length) in 30-minute time intervals from birth to division for 50 individual cells and found that this increment increases as the cell cycle progresses, which hints that growth is exponential at the single-cell level in mycobacteria (Santi *et al.* 2013).

The approach taken here attempts to confirm this result under my conditions (HdB minimal medium). Plotting mean size increment per unit time ($\Delta L/\Delta t$) between sequential frames against length in the first of the two frames for 1319 cells shows that size increment is indeed correlated with length, i.e. single-cell growth is size-dependent (Figure 3.4C), indicative of exponential growth and in agreement with Santi *et al.* 2013. Furthermore, to demonstrate exponential growth, model selection was performed to compare linear and exponential models, and found that the exponential model fits single-cell data in 90 % of cases, also strongly indicating that *M. smegmatis* mc²155 cells grow exponentially at the single-cell level. Logsdon *et al.* also attempt to determine the single-cell growth model in *M. smegmatis*, and conclude similarly that single traces are insufficient to discriminate between models (Logsdon *et al.* 2017). They compared single cells to a key prediction of single-cell exponential growth, that interdivision time τ_d is proportional to the natural logarithm of the ratio of division size to birth length³: $\tau_d \propto \ln(L_d/L_b)$ and to the linear model, in which interdivision time is proportional to added length⁴: $\tau_d \propto (L_d - L_b)$ (Logsdon *et al.* 2017). They observe that the exponential prediction is a better fit for 98.5 % of their dataset ($n = 391$), and conclude that exponential growth is more likely, in close agreement with my data (Logsdon *et al.* 2017). Therefore, a consensus appears to have been built that *M. smegmatis* mc²155 single cells elongate exponentially, in conformance with other bacteria, using multiple approaches (Santi *et al.* 2013; Priestman *et al.* 2017; Logsdon *et al.* 2017). A true confirmation of this would require more complex methods however, such as precise measurement of cell mass (Godin *et al.* 2010; Cermak *et al.* 2016). I also observe better exponential fits using model selection for cells grown in the alternative carbon sources pyruvate and acetate (data not shown), indicating that predictions of adder cell-size control given exponential growth also hold for these datasets.

This result suggests that single-cell elongation is not rate-limited by the surface area available to incorporate nascent cell wall material, as has been suggested (Daniel and Errington 2003). Exponential single-cell growth can also be considered in terms of cellular mass, of which the majority is cytoplasmic; cytoplasm is able to produce more cytoplasm through protein synthesis by ribosomes, and the production of more ribosomes by such proteins; therefore, the clear expectation is that cytoplasmic mass increase must be exponential (Cooper 2006), and thus the capacity to add new cell wall material may therefore depend on cytoplasmic mass rather than existing surface area in which to incorporate it.

3.4.3 Emergence of adder cell-size control

Early reports of cell-size control in mycobacteria measured birth length L_b against added length ΔL and determined the correlation between the two. This resulted in two reports of $r = 0.03$

³This is derived from the exponential growth function: $L(t) = L_1 e^{\gamma t}$ (Equation 2.3) at $t = \tau_d$, where $L(t)|_{t=\tau_d} = L_d$ and $L_1 = L_b$ to give $\tau_d = \ln(L_d/L_b)/\gamma$ (Soifer *et al.* 2016).

⁴derived from the linear growth function (Equation 2.1).

(Aldridge *et al.* 2012), and $r = 0.20$ (Santi *et al.* 2013). The noisy linear map used in this chapter describes the relationship between L_d and L_b , but can be trivially converted to compare ΔL to L_b (Equation 3.2), thus a finding of no linear correlation is indicative of an adder ($a = 1$), and a positive correlation of timer-like behaviour (Aldridge *et al.* 2012; Santi *et al.* 2013). However, as discussed above, these studies used different formulations of 7H9 rich medium, including several simultaneous carbon sources—glycerol, glucose, oleic acid, dextrose, and Tween-80. Thus, I set out to determine the cell-size control principle in *M. smegmatis* mc²155 cells in a defined minimal medium, using glycerol as a sole carbon source.

For *M. smegmatis* cells grown in glycerol, the linear regression of division length and birth length (interpolated as per the Methods section: Interpolation of birth length and division length, with the assumption of exponential single-cell elongation) is $a = 0.996 \pm 0.102$ with a Pearson correlation coefficient of $r = 0.637$ (Figure 3.5A). This is highly indicative of a basic adder principle, in which added length between birth and division is constant. This is in close correspondence with the observations of no correlation between birth length and added length (Aldridge *et al.* 2012), and more recent results that report a slope of 0.05 between the two, which is equivalent to a slope of 1.05 according to the noisy linear map (Logsdon *et al.* 2017). The latter report also shows a Pearson correlation of $r = 0.65 \pm 0.06$ (Logsdon *et al.* 2017), again very similar to my observed values. This is not however in agreement with other authors who observe a correlation of $r = 0.20$ (Santi *et al.* 2013). This might be explained by their use of a strain carrying an additional integrated copy of Wag31 tagged with GFP (Santi *et al.* 2013). Wag31 is a cell division protein intimately involved in the polar incorporation of peptidoglycan in mycobacteria, and the preferential incorporation at the old-pole (Nguyen *et al.* 2007; Kang *et al.* 2008; Jani *et al.* 2010; Santi *et al.* 2013; Meniche *et al.* 2014). The merodiploid strain used by Santi *et al.* (2013) has been shown to result in some marked effects on cell division and growth, including symmetric elongation from both poles (Botella *et al.* 2017), thus may have affected the relationship between birth and division length (Logsdon and Aldridge 2018). My approach has the advantage of using wild-type *M. smegmatis* mc²155 cells, and demonstrates a reliable adder principle. While my results are in agreement with others, they still use complex media and genetic mutants (Aldridge *et al.* 2012; Logsdon *et al.* 2017).

In addition to demonstrating that added length is independent of birth length, I also show that *M. smegmatis* cells are not timers. The noisy linear map (Equation 3.1) does not consider interdivision time, which is constant in the timer model, but the timer model can be tested by determining the dependence of interdivision time on birth length, which should be uncorrelated. This is not observed in my data, with a Pearson correlation coefficient of $r = -0.354$ (Figure 3.5B), indicating that *M. smegmatis* mc²155 single cells grown in glycerol are not timers. This is in agreement with data from other authors, who report that *M. smegmatis* cells are neither sizers or timers (Santi *et al.* 2013), and that *M. smegmatis* cells are not sizers (Aldridge *et al.* 2012). The latter study reported that size control was time-based, but they published several

years before the adder model was widely disseminated, and came to the conclusion based on lack of conformity to the sizer model rather than evidence of timer itself (Aldridge *et al.* 2012). Subsequent analysis with the adder in mind shows that they also observe adder behaviour with $r = 0.03$ as discussed above. The only manner in which interdivision time can be uncorrelated to birth length, with single-cell exponential elongation, is if division length is a multiple of birth length⁵, and this multiple must be 2 if division is symmetric, hence $a = 2$ for a timer in the noisy linear map, which is definitively not true based on the relationship between birth length and division length that I observe (Figure 3.5A).

There are several predictions of the adder principle which are not necessarily reflected in mycobacteria. The core principle of the adder model is that added length is constant (Taheri-Araghi *et al.* 2015). However, the above data only determines that added length ($L_d - L_b$) is independent of birth length (L_b) (Figure 3.5A). When added length is compared to growth rate, there is dependency observed, with $r = 0.285$ (Figure 3.6A) which implies that added length is not constant. This relationship has not been reported by others, but appears to hold for *E. coli* single cells according to the dataset of Taheri-Araghi *et al.* 2015 (personal communication). Additionally, growth rate itself is negatively correlated with birth length with $r = -0.451$ (Figure 3.6B), which other authors have also noted in *M. smegmatis* and in *M. bovis* BCG (Logsdon *et al.* 2017). These factors might indicate at first approximation that added length cannot be independent from birth length, since larger daughter cells have a lower growth rate, and cells with a lower growth rate have a lower added length. However, there is clearly no dependence between the two in my data (Figure 3.5A). This may reflect the simplicity of the noisy linear map as a description of the complex underlying biological interplay between growth, division, and cell-size homeostasis. The other factor is that the correlations are not strong, especially between added length and growth rate, with less than 30 % of the variation explained by the dependence. In addition, since the impact of birth length on growth rate is minor in magnitude (Figure 3.6B)—coupled with the low correlation of growth rate with added length—the overall impact on the relationship between birth length and division length is negligible.

The noisy linear map equation includes the noise term η , which has generally been assumed to be normally distributed (e.g. Amir 2014; Bertaux *et al.* 2018). This term can be estimated by examining the residuals of the linear fit of division length to birth length, since the mean of η is zero (according to least-squares approach). My data reveals that η is apparently not normally distributed for cells grown in glycerol ($p = 1.28 \times 10^{-14}$, Shapiro–Wilk test; Figure 3.9A), with a significant right-skew (1.17 ; $p = 6.01 \times 10^{-20}$). This indicates that positive errors are more likely than negative errors, or that division length is more commonly over-estimated than under-estimated. This is reflected by visual examination of the raw data where larger deviations

⁵As stated above, based on the exponential growth function (Equation 2.3) evaluated at $t = \tau_d$, $\tau_d = \ln(L_d/L_b)/\gamma$; therefore for τ_d not to depend on L_b , L_d must be a multiple of L_b .

can be observed above the linear fit compared to below (Figure 3.5A). This makes sense as an experimentally-derived error, since division is assigned manually by the analysis pipeline (see Chapter 2), and there are some cells in which division might have occurred but not be immediately obvious (e.g. septum formation without clear pinching) that might lead to over-estimation of division length. Alternatively, given the correlation between added length and growth rate (Figure 3.6A), this non-normal error might be biological in nature, and could reflect an inability for larger cells to regulate their cell size. Indeed, the residuals appear to be positively correlated with division length (Pearson's $r = 0.77$) and with growth rate ($r = 0.28$; data not shown).

3.4.4 Role of pole age in cell-size control

Given that mycobacteria are believed to grow preferentially from a single pole, specifically the pole inherited from their mother (Thanky *et al.* 2007; Hett and Rubin 2008; Joyce *et al.* 2012; Aldridge *et al.* 2012; Santi *et al.* 2013; Meniche *et al.* 2014; Botella *et al.* 2017; Rego *et al.* 2017), this immediately provides a mechanism by which heterogeneity between daughters is established. At each division, a new pole is created at the septum, resulting in each daughter cell receiving a pre-existing pole and a new pole. Since only one of these poles is thought to be actively growing (the pole inherited from the previous generation), one daughter will receive this active pole and one will receive an inactive pole. The active pole is known to be the pre-existing pole inherited from its mother, thus inheritance of this “old pole” is expected to confer a difference compared to the daughter that inherits the inactive “new pole”. Other authors have noted asymmetry between daughter cells in size at birth, with old-pole inheritors consistently larger than new-pole inheritors (Aldridge *et al.* 2012; Santi *et al.* 2013), and I observe similar differences, with mean cell size at birth increasing as a function of age of the inherited pole (Figure 3.8A and Table 3.1). In addition, other single-cell parameters change in response to pole age, including division length which progressively increases (Figure 3.8B and Table 3.1), and growth rate which progressively decreases (Figure 3.8C and Table 3.1). Reduction in growth rate is somewhat surprising, since old-pole inheritors ostensibly inherit the pole that was actively growing in the previous generation, thus might be expected to grow faster than new-pole inheritors. However, growth from both poles may be initiated between septum formation and cell division, i.e. prior to birth (Santi *et al.* 2013)—in a manner somewhat analogous to the principle of “new end take off” in *Schizosaccharomyces pombe*, which switch from unipolar to bipolar growth during their cell cycle (Martin and Chang 2005)—which may negate this age factor on the specific growth at each pole. Additionally, since old-pole daughters are larger than their new-pole sibling, the principles of exponential growth might predict that larger cells should grow faster than small cells. However, the prediction is based on absolute added length per unit time rather than relative increase in length, i.e. linear elongation rate (added length over time) should be higher in old-pole inheritors, and that is indeed what is

observed (Table 3.1). Other authors have also reported that old-pole inheritors have higher linear elongation rates (Aldridge *et al.* 2012; Santi *et al.* 2013), consistent with my results. Active growth at a single pole does not necessarily imply a faster exponential growth rate in the cell that inherits it, though it might be expected intuitively. No significant differences in growth rate of *M. smegmatis* new- and old-pole inheritors have been observed in two independent studies (Wakamoto *et al.* 2013; Santi *et al.* 2013), which is not consistent with my data, but interestingly, *M. tuberculosis* new-pole inheritors do have a higher exponential growth rate (Manina *et al.* 2015), as I observe in *M. smegmatis*. While growth rate is higher in new-pole inheritors, this does not appear to translate to a change in interdivision time, with no clear difference according to pole age (Figure 3.8E). This is in agreement with the observation that there is no correlation between growth rate and interdivision time (Figure 3.14A), a surprising result that is discussed below.

Given these differences, especially in birth length and division length, differences in cell-size control between new- and old-pole inheritors might be expected. However, both new- and old-pole inheritors have a linear relationship between L_b and L_d which is consistent with $a = 1$, with $a = 0.894 \pm 0.149$ (Figure 3.7B) and $a = 0.948 \pm 0.162$ (Figure 3.7A) respectively. When old-pole inheritors are separated by known pole age, the slope does not significantly deviate from $a = 1$ regardless of pole age (Figure 3.8F). Therefore, it can be concluded that pole age does not affect adherence to the adder principle: the relationship between birth length and division length is not significantly different from an idealised adder, irrespective of the age of the inherited pole.

Other authors have observed that distributions of birth length and interdivision time scale with their mean values when single cells follow the adder principle, implying that these distributions normalised by their mean are identical (Taheri-Araghi *et al.* 2015; Kennard *et al.* 2016). This implies that CV ($\sigma_b/\langle L_b \rangle$) of birth length L_b should not be affected by mean birth length $\langle L_b \rangle$. I can test this hypothesis on my glycerol dataset, since new- and old-pole inheritors both behave as adders, but have significantly different birth lengths ($4.02 \mu\text{m}$ [3.94–4.11] and $4.71 \mu\text{m}$ [4.60–4.78] respectively; $p = 2.47 \times 10^{-22}$; Table 3.1). However, their CVs are not equal, with 19.3 % [17.5–21.2] and 16.8 % [15.1–17.8] respectively (Figure 3.13A). This implies that—contrary to expectations of adder control in *E. coli* (Taheri-Araghi *et al.* 2015; Kennard *et al.* 2016)—birth length distributions do not collapse.

3.4.5 Deviation from adder in alternative carbon sources

The observation that diverse single-cell parameters can vary significantly between cells—such as birth length, division length, and growth rate—under constant conditions, yet cells apparently follow the adder principle may suggest that the principle might be universally conserved among bacteria (Taheri-Araghi *et al.* 2015). This is rooted in the long-known observation that mean

cell size scales with the population doubling rate, even in diverse media (Schaechter *et al.* 1958). *E. coli* cells have been predicted and observed to deviate from the adder principle at very slow growth rates, due to the initiation and completion of DNA replication within one cell division cycle (Wallden *et al.* 2016). Deviations from adder have also been hinted at in *M. smegmatis* (Santi *et al.* 2013) and in *M. tuberculosis* (Manina *et al.* 2015) as discussed above, and these studies utilise a different medium composition compared to other authors who report conformance to adder (Aldridge *et al.* 2012; Logsdon *et al.* 2017). Santi *et al.* use 7H9 rich medium supplemented with glycerol, glucose, Tween-80, and OADC—containing oleic acid and dextrose—which can all be used as carbon sources. Since culture medium might affect cell size, I determined how changing the carbon source in a more controlled manner might affect this, by using HdB minimal medium, replacing glycerol as the sole carbon source with pyruvate or acetate (see Methods section: Variants of HdB). When grown in these media, *M. smegmatis* mc²155 cells have higher mean interdivision rates, with 2.262 h, 2.485 h, and 2.868 h for glycerol, acetate, and pyruvate respectively (Table 3.2 and Fig. 3.13C), which is predicted to determine the cell size distribution of the population, at least in *E. coli* (Schaechter *et al.* 1958). This indeed appears to be the case, with mean birth and division length scaling inversely with interdivision time in the three media (Table 3.2 and Figs. 3.13A and 3.13B). Interestingly, although interdivision time is altered, growth rate does not reflect these changes, with cells grown in acetate having no significant difference in mean growth rate compared to cells grown in glycerol— 0.263 h^{-1} and 0.265 h^{-1} respectively—while cells grown in pyruvate have significantly reduced mean growth rate of 0.235 h^{-1} (Table 3.2 and Fig. 3.13D).

When the relationship between birth length and division length is determined for acetate, $a = 0.964 \pm 0.140$, fully consistent with the adder principle (Figures 3.10A and 3.13F) as evidenced by the overlap of the confidence interval with $a = 1$. However, when cells are separated according to the age of their inherited pole, interesting deviations emerge. Old-pole inheritors have a slope of $a = 1.181 \pm 0.212$ (Figure 3.10C), also consistent with adder, though the overlap is small ($p = 0.095$; F -test), but new-pole inheritors significantly deviate from adder, with $a = 0.682 \pm 0.187$ ($p = 0.001$; Figure 3.10D). These values are reflected, as predicted by the noisy linear map, by the correlation of added length with birth length with Pearson's correlation coefficients of $r = 0.119$ and $r = -0.240$ for old- and new-pole inheritors respectively. I term this new-pole behaviour as “sizer-like”, in that added length is negatively correlated with birth length, but not completely negatively correlated as in a pure sizer.

This discrepancy between new-pole inheritors and both poles together may be partially explained by the effect of cells that are born very large, which appear to deviate from the population cell-size control programme as shown by deviation of the linear fit from the binned data (Figure 3.10A). With cells born at above $5 \mu\text{m}$ excluded ($n = 16$), the linear fit is in close accordance with the binned data, and gives a much lower estimate of $a = 0.730 \pm 0.154$ (Figure 3.10B). This indicates that, if large cells are excluded, cells grown in acetate appear to act in a sizer-like

manner, regardless of the age of the inherited pole. However, these large cells may reflect an underlying biological process by which cell-size control is subverted: it might be expected that these large cells have more DNA than smaller cells, and are perhaps undergoing multifork replication. As posited by Wallden *et al.*, adder cell-size control might be a consequence of overlapping replication cycles (discussed below), thus these larger cells may be acting under a different principle to cells born small with acetate as a sole carbon source.

For cells grown with pyruvate as a sole carbon source, $a = 0.832 \pm 0.137$, inconsistent with the adder principle as a population ($p = 0.016$; Figure 3.11A), though this reduction is only observed in old-pole inheritors ($a = 0.767 \pm 0.192$; $p = 0.017$; Figure 3.11B) and not new-pole inheritors ($a = 0.815 \pm 0.221$; $p = 0.101$; Figure 3.11C). However, given that the population as a whole appears to act in this sizer-like manner, this may be a consequence of the lower n -values of old-pole inheritors observed dividing ($n = 197$ and 170 for old- and new-pole inheritors respectively).

The differences from adder for cells grown in acetate and pyruvate are surprising, given how robust the adder principle appears to be for cells grown in glycerol. At first glance, the differences in interdivision time and growth rate might underlie these differences, given that cells spend a longer time between birth and division when grown in acetate or pyruvate, especially when coupled with no change in growth rate for cells grown in acetate (Figures 3.13C and 3.13D and Table 3.2). An increase in interdivision time with no concurrent decrease in growth rate directly implies that the mean ratio between division length and birth length (specifically the logarithm of this ratio) must increase for cells grown in acetate compared to cells grown in glycerol, given that cells grow exponentially at the single-cell level. As mentioned above, this is derived directly from the cell length function (Equation 2.3) at $t = \tau_d$ (interdivision time):

$$L(t)|_{t=\tau_d} = L_d = L_b e^{\gamma \tau_d}, \quad (3.10)$$

$$\gamma \tau_d = \ln \left(\frac{L_d}{L_b} \right) \quad (3.11)$$

This is indeed observed, with a log-ratio of 0.595 [0.582 – 0.608] (95 % confidence interval) for glycerol-grown cells compared to 0.624 [0.612 – 0.636] for acetate-grown cells ($p = 0.001$; Welch's t -test). This log-ratio increase is also observed for cells grown in pyruvate, with 0.626 [0.612 – 0.640], which may imply that the relative scaling of growth rate and interdivision time do not compensate for one another, thus requiring the ratio to change.

The increase in interdivision time for cells grown in acetate or pyruvate may explain their sizer-like behaviour, given the prediction and observation of sizer-like behaviour in *E. coli* at very slow growth rates (Wallden *et al.* 2016). The prediction in *E. coli* is based on models in which cell size is controlled by DNA initiation, with initiation triggered at a fixed cell length relative to the number of origins (Cooper and Helmstetter 1968; Cooper 1969; Helmstetter

1969), followed by a fixed time to complete DNA replication. At fast growth rates, this results in adder cell-size control, due to initiation occurring in the mother cell. However, at slow growth rates, initiation in *E. coli* occurs in the same birth–division cycle resulting in higher dependence of division length on birth length, due to the constant size at initiation rule—i.e. sizer-like behaviour (Wallden *et al.* 2016). In *M. smegmatis*, multifork replication is believed to be limited (Santi *et al.* 2013; Trojanowski *et al.* 2015; Logsdon *et al.* 2017), thus only a small change in interdivision time—as observed in acetate and pyruvate—may be necessary to result in synchrony between the division cycle and the DNA replication cycle, and thus sizer-like behaviour according to the Wallden *et al.* model. As discussed above, potential outliers that are born large when cells are grown in acetate appear to skew the cell-size control model towards adder (i.e. a is reduced when they are excluded; Figure 3.10B), and since larger cells might be expected to have more DNA than smaller cells, this may provide some evidence for this in *M. smegmatis*. Knowledge of DNA replication in these cells would be required to confirm this model however.

Also potentially explaining differences between acetate and pyruvate is the observation that asymmetry between new- and old-pole inheritors is reduced for cells grown in acetate compared to glycerol (Figure 3.13E) with smaller differences in birth length (Figure 3.13A) and in division length (Figure 3.13B). Asymmetry is also reduced for cells grown in pyruvate, but not to such a great extent as for cells grown in acetate. This might underlie the observation that both old- and new-pole inheritors appear to act in a sizer-like manner when cells are grown in pyruvate, compared to only old-pole inheritors in acetate. Elucidating this potential relationship would require decoupling asymmetry from other single-cell parameters, especially interdivision time and growth rate. Loss of asymmetry has recently been reported in a $\Delta lamA$ strain, a protein that appears to regulate the targeting of new cell wall material preferentially to the old pole (Rego *et al.* 2017), and analysis of cell-size control in this strain could be illuminating.

Interestingly, cells grown in glycerol have no significant correlation between interdivision time and growth rate with $r = -0.063$ (Figure 3.14A). This lack of correlation is not observed for cells grown in acetate with $r = -0.315$ (Figure 3.14B), or for cells grown in pyruvate with $r = -0.417$ (Figure 3.14C). In comparison, Santi *et al.* observe strong negative correlation between interdivision time and growth rate ($r = -0.84$), although they observe positive correlation between birth length and division length as discussed above (Santi *et al.* 2013). Indeed, it is very surprising that there is no correlation for glycerol-grown cells, since it implies that cells that grow faster *do not* divide faster, which might be expected to be a universal feature of bacterial growth. The impact of this difference is difficult to unpick, but given Equation 3.11: $\gamma\tau_d = \ln(L_d/L_b)$, this might imply that growth rate and interdivision time can scale freely to maintain the adder principle for cells in glycerol. For cells grown in pyruvate and acetate—sub-optimal carbon sources—where it might be possible that DNA replication and division occur synchronously, this lack of freedom constrains cells to sizer-like behaviour, as predicted by

Wallden *et al.* 2016.

Other authors have investigated the role of increased interdivision time on cell-size control in *M. smegmatis*, with the use of carbon-limited media. Logsdon *et al.* increased the mean interdivision time of *M. smegmatis* cells from 3.1 h in rich 7H9 medium to 4.9 h by modifying the composition of 7H9 to omit oleic acid, reduce albumin from 10 % to 1 %, and omit glycerol completely (Logsdon *et al.* 2017). The authors track DNA replication with the use of a SSB-GFP reporter, and note that the E period of the bacterial cell cycle—when extra rounds of chromosome replication occur—is highly rare for cells grown in carbon-limited medium (Logsdon *et al.* 2017). Critically, they determine that the slope of added length against birth length is -0.4 , i.e. $a = 0.6$ according to the noisy linear map, highlighting how lack of an E period caused by a high interdivision time could lead to sizer-like activity (Logsdon *et al.* 2017). They also show a similar slope for BCG grown in rich medium, which has a very slow doubling time (~ 20 h) similar to pathogenic *M. tuberculosis*, implying that the slow-growing mycobacteria may well also act in this sizer-like manner.

3.4.6 Implications of deviation

The key difference between the cell-size control models concerns how biological noise in size is controlled. The timer model cannot provide cell-size homeostasis, as discussed above (see Figure 3.3), since there is no mechanism by which deviations from mean size at birth or division can be corrected. The adder model corrects errors by allowing cell size to converge to the mean over successive generations, since cells that are born smaller result in larger daughters, and cells that are born larger result in smaller daughters (see Figure 3.1). The sizer model provides immediate correction at every division, since division occurs at a critical size (see Figure 3.2; Taheri-Araghi *et al.* 2015). Therefore, the key difference between adder and sizer, with regards to cell-size homeostasis, is that size at birth and division should be less variable in sizers. This prediction should hold for cells that act in a sizer-like manner ($a < 1$).

I observe that division length variability is 16.9 % [15.9–18.0] (CV; standard deviation divided by mean) for cells grown in glycerol, which behave as adders, compared to 12.9 % [11.2–15.3] and 12.5 % [11.5–13.3] for cells grown in acetate and pyruvate respectively (Figure 3.13B). This appears to reflect the sizer-like nature of cells grown in these alternative carbon sources: with both new- and old-pole inheritors grown in glycerol behaving as adders, with similar high CVs of 16.0 % [14.5–17.2] and 15.7 % [14.0–17.3] respectively (Figure 3.13B). For cells grown in pyruvate, which act in a sizer-like manner as a population, both new- and old-pole inheritors have similar low CVs of 12.1 % [10.8–12.9] and 12.1 % [10.7–13.4] respectively (Figure 3.13B). Cells grown in acetate reflect this prediction very well, since new-pole inheritors behave in a sizer-like manner with a low division length CV of 11.1 % [9.5–12.5], whereas old-pole inheritors behave as adders, and potentially in a timer-like manner, with a corresponding higher CV

of 14.0 % [11.6–16.8] (Figure 3.13B). Indeed, within each carbon source, division length CV appears to directly reflect corresponding values of a (Figure 3.13F).

These differences in CV are however not entirely reflected in birth length CVs, with 19.5 % [18.5–20.5], 14.2 % [13.0–15.3], and 14.8 % [13.6–15.6] for cells grown in glycerol, acetate, and pyruvate respectively (Figure 3.13A). While CVs are similar for both new- and old-pole inheritors for pyruvate, as expected (13.8 % [11.8–15.3] and 14.2 % [12.8–15.2] respectively; Figure 3.13A), they are also similar for all cells grown in acetate regardless of pole age (14.3 % [12.3–16.1] and 13.6 % [12.2–14.8] for sizer-like new-pole inheritors and adder-conforming old-pole inheritors respectively; Figure 3.13A). The clear explanation underlying this difference is that asymmetry varies between carbon sources (Figure 3.13E), and cell-size control principles act on division length, not on birth length. Thus the specific difference between birth lengths of new- and old-pole inheritors grown in acetate is much lower than for cells grown in pyruvate or glycerol (Figure 3.13A). This can be calculated by determining the ratio of each daughter to the sum of its length and its sibling's length for new- and old-pole daughters. In glycerol, this asymmetry reflects the large difference in length with ratios of 0.477 [0.472–0.483] in new-pole inheritors compared to 0.493 [0.488–0.500] for new-pole inheritors in acetate, and 0.487 [0.480–0.496] in pyruvate (Figure 3.13E). Therefore, division is close to symmetric for cells grown in acetate, meaning that birth length variability is expected to be inherently lower than that of the more asymmetric divisions of cells grown in glycerol or pyruvate, regardless of cell-size control strategy. It is notable that theoretical approaches have suggested that the most significant source of variability in cell-size is partitioning noise (Modi *et al.* 2017), which may be reflected in my data.

Logsdon *et al.* have also investigated variability in single-cells and observe a very similar birth length CV of 19 % for cells grown in 7H9 rich medium (Logsdon *et al.* 2017), compared to my observed CV of 19.5 % for cells grown in glycerol (Figure 3.13A), and an identical division length CV of 17 % (Logsdon *et al.* 2017), compared to my observation of 16.9 %, implying that many of their conclusions may well be applicable to my results. They however observe an increase in birth length CV to 22 % for cells grown in carbon-limited medium, potentially inconsistent with their observations of $a < 1$, though the asymmetry of division in this medium is not reported (Logsdon *et al.* 2017). However, while they do not report CV of division length—the more important parameter in the context of cell-size control—they provide figures for these values from which CV can be estimated. CV of division length for cells grown in their carbon-limited medium appears to be ~15 %, indeed a reduction compared to their rich medium conditions (Logsdon *et al.* 2017), and conforming to my observations of sizer-like cells grown in acetate and pyruvate (Figure 3.13B).

3.4.7 Role of population age structure

The age structure of populations, especially at the single-cell level, is predicted to be biased by a form of founder effect, whereby cells which happen to divide faster in the initial stages of growth will have more descendants than cells which divide slower initially, even if mean interdivision times are the same between the descendants (Powell 1956; Wakamoto *et al.* 2012; Hashimoto *et al.* 2016; Thomas 2017). This over-representation introduces a bias into single-cell studies, where lineages derived from cells with a stochastically low interdivision time will dominate single-cell statistics. This bias can be accounted for by constructing “lineage-weighted” statistics, essentially by scaling parameters by the number of cells contained in each lineage (Methods section: Population and lineage-weighted statistics). Lineage-weighted values can be compared to population values to determine whether this potential bias affects the statistics from which the above conclusions are drawn. For cells grown in glycerol, the statistics for population and lineage-weighted measures are very similar, indicating that fast dividers are not biasing the population measures for this carbon source (Table 3.3). This may reflect the lack of correlation between growth rate and interdivision time for cells grown in glycerol (Figure 3.14A), where increases in growth rate do not translate to more frequent division. However, for cells grown in acetate or pyruvate, interdivision time is lower in the population compared to lineage-weighted values, indicating that cells which divide faster are contributing more to the population average (Table 3.3). This appears to have little impact on the other statistics, except for growth rate, where fast dividers appear to grow faster and bias the population measures in this manner (Table 3.3). This probably also reflects the correlation that is observed between growth rate and interdivision time for these carbon sources (Figures 3.14B and 3.14C), where cells which grow faster by chance also divide more frequently, thus introducing bias into the population measure. However, any differences are generally very minor in magnitude, and the bias—if any—is very weak, allowing conclusions drawn from the population measures to be made with high confidence.

3.4.8 Context of the work

There has been mounting evidence that bacteria universally follow the adder principle, and at the time of publication of the majority of the work discussed above, this was simultaneously the first definitive report that mycobacteria follow the adder principle—although prior evidence was strong through the adder lens (Aldridge *et al.* 2012; Santi *et al.* 2013)—and the first report that they can deviate from it (Priestman *et al.* 2017). This deviation is significant since it occurs with only a minor change in environment, whereas the only other sizer-like behaviour reported (post adder model) has been due to very severe growth reduction in *E. coli* (Wallden *et al.* 2016). Very shortly after publication, another investigation of cell-size control in *M. smegmatis* was reported, in which adder cell-size control in single *M. smegmatis* cells is clearly demonstrated—

they add a fixed length between birth and division—when grown in rich laboratory media, with very similar results to mine (Logsdon *et al.* 2017). They also track DNA replication, in order to determine the relationship with cell size as per the Cooper–Helmstetter model (Cooper and Helmstetter 1968; Cooper 1969; Helmstetter 1969). They argue that *M. smegmatis* cells are not behaving as adders between birth and division, but between initiation and initiation, simultaneously with behaving as adders between initiation and division, which they term a “parallel adder” (Logsdon *et al.* 2017). However, this model completely conforms to a birth-to-division adder as I observe, and their results are essentially identical to mine. Logsdon *et al.* also test cell-size control in a carbon-limited medium and in *M. bovis* BCG—conditions under which interdivision time is much greater than wild-type *M. smegmatis* in permissive media—and demonstrate that the birth-to-division adder model breaks down, providing confirmation of my results in pyruvate and acetate (Priestman *et al.* 2017; Logsdon *et al.* 2017). Their model however holds for these cells, and they argue—convincingly—that the birth-to-division adder is in effect a coincidental property of the parallel adder that they propose (Logsdon *et al.* 2017). This work, with my results, highlights the need to take carbon source and growth medium into account when assessing basic bacterial growth parameters. This may well have important implications for studies on *M. tuberculosis*, especially since the intracellular environment in which it generally survives is thought to be highly carbon-limited.

Cell-size control is a highly important part of bacterial survival, since it determines the inheritance of critical cellular components, and may well have an impact on survival to antibiotics and survival in the face of immune attack. Much remains undiscovered in this context, including definitive mechanistic understanding, and given apparent rigid control across the bacterial kingdom, may well provide significant new targets and strategies for the killing of pathogenic species, including *M. tuberculosis*.

CHAPTER 4 ANTIBIOTIC TOLERANCE AT THE SINGLE-CELL LEVEL

When I woke up just after dawn on September 28, 1928, I certainly didn't plan to revolutionize all medicine by discovering the world's first antibiotic, or bacteria killer. But I suppose that was exactly what I did.

Alexander Fleming

4.1 Introduction

SINCE their discovery, antibiotics have revolutionised modern medicine thanks to their ability to treat bacterial infections effectively and rapidly. Child mortality has dramatically fallen, surgeries that would have led to deadly infections are now comparatively safe, and many diseases that once were major causes of death have been relegated to interesting case studies.

The first antibacterial compounds were discovered in the early 20th century by Paul Ehrlich. He speculated that since the dyes he was using could selectively stain bacteria, compounds could be created that would be able to selectively kill bacteria. He discovered arsphenamine (also known as salvarsan), an arsenic-based compound, in 1909 in a systematic screen of hundreds of dyes—arsphenamine was initially termed compound 606 for this reason—and found that it could be used to treat syphilis (Alt 1909), a massive improvement over the therapies mostly centred around repeated large doses of pure mercury at the time (Abraham 1948). Arsphenamine was produced and used in massive amounts, but was also an important stepping stone in the development of how such drugs are tested. Paul Ehrlich was a contemporary and colleague of Robert Koch who had developed an extract of tuberculosis that he termed 'tuberculin' which he had become convinced could be used to cure tuberculosis, and aggressively marketed it as such (Koch 1890). However, while Koch claimed that he had tested tuberculin successfully on guinea pigs, he was unable to prove the same, and the lack of a systematic method of testing drugs led to an enthusiastic welcoming of tuberculin by doctors and the general public despite this lack of objective evidence (Gradmann 2006). Tuberculin was first demonstrated to be ineffective

against tuberculosis by Rudolf Virchow in 1891 (Virchow 1891a,b) and Koch was thoroughly discredited. Given this experience, Ehrlich was keen to avoid such a failure, and conducted and published careful clinical evaluations of arsphenamine (Alt 1909), and though some doubt was cast over its effectiveness and toxicity a few years later (Yakimoff and Kohl-Yakimoff 1911; McDonagh 1912), arsphenamine was seen as a miracle cure for syphilis. This move towards clinical rigour was further driven by the outbreak of World War I: the sudden loss of arsphenamine availability in Britain, given that it was manufactured in Germany, created an immediate local demand which led to the commercial synthesis of the drug in the UK (by Burroughs Wellcome, stimulating the start of a drug-production industry in Britain) and its assessment by the Medical Research Committee—an early incarnation of the modern Medical Research Council (MRC)—which stressed the importance of testing actual clinical efficacy over chemical purity (Williams 2005). This groundwork was invaluable when it came to the mass-production of insulin in the 1920s, when the MRC oversaw a systematic set of clinical trials, though they were still not formally regulated in the modern sense (Cox-Maksimov 1997).

Penicillin, the β -lactam antibiotic, is produced by the fungus *Penicillium chrysogenum* (previously *Penicillium notatum*) which Alexander Fleming found growing on a *Staphylococcus* culture plate and inhibiting its growth in 1928 (Fleming 1929). The demonstration of the utility of penicillin in clinical settings was not appreciated or tested until later, with Cecil Paine treating the first human patients successfully in 1930, though this work was not published (Wainwright and Swan 1986). Penicillin was clearly much more effective than arsphenamine, and much less toxic, but could not be produced in large enough amounts to treat humans (preparations by Fleming are thought to be only 1 % penicillin). Howard Florey and Ernst Chain were instrumental in producing penicillin at the high purity required for routine therapy, and in proving their efficaciousness in animal models (Chain *et al.* 1940; Abraham *et al.* 1941), though not in the vast quantities required. This work was taken over by the Northern Regional Research Laboratory in the United States—at the instruction of the War Production Board who perceived its usefulness in treating wounded soldiers in World War II—whose research into mass production techniques allowed several million doses to be produced prior to the Allied invasion of Normandy in 1944 (Parascandola 1980), in contrast to the 10 doses that Howard Florey and collaborators had been able to produce.

Penicillin ushered in the “antibiotic era”, with novel classes of antibiotics isolated and manufactured regularly for the next 50 years. The discovery of streptomycin in 1944 by means of a lengthy search for antimicrobial agents in soil (Schatz *et al.* 1944) was especially important, since it was the first drug that was shown to be effective in the treatment of tuberculosis, a disease that is unaffected by arsphenamine, penicillin, or sulfonamide—the three antibiotics available at the time—and was the cause of approximately 20,000 annual deaths in England and Wales (55 deaths per 100,000) at the time, making it a serious public health problem in the eyes of the authorities (PHE 2013). Streptomycin was discovered almost simultaneously with another

antibiotic effective against tuberculosis: *para*-aminosalicylic acid (PAS), discovered based on the observation that *Mycobacterium tuberculosis* rapidly metabolises salicylic acid (Lehmann 1946a,b). This was followed by the discovery of the first highly effective anti-tuberculosis drug—isoniazid—in 1951 (Robitzek *et al.* 1952; Singh and Mitchison 1955). These three drugs were tested in randomised controlled clinical trials devised by the MRC—now considered to be a gold standard in demonstrating drug efficacy—as monotherapy, and in all combinations (MRC 1950, 1955). This led to the rapid adoption of these antibiotics into tuberculosis therapy in the UK and worldwide, and led to an astonishingly rapid reduction in TB mortality, dropping from 50 to 7 deaths per 100,000 between 1945 and 1956 in UK (PHE 2013). The MRC observed rapid loss of efficacy of these drugs when used in monotherapy (MRC 1950, 1955), demonstrating the worrying tendency of bacteria to develop resistance to antibiotics, often rapidly after their introduction into clinical settings.

4.1.1 Drug resistance

Antibiotic resistance is a critical modern problem, recognised by the World Health Organisation (WHO) as “one of the biggest threats to global health, food security, and development” (WHO 2017a), with an estimated 10 million excess deaths predicted by 2050, and 100 trillion US dollars in lost economic output (WHO 2015a; O’Neill 2016; WHO 2017b). Resistance has emerged to all classes of antibiotics rapidly after their introduction (Palumbi *et al.* 2001; Clatworthy *et al.* 2007, Figure 4.1). There are at least 50,000 deaths caused by antibiotic-resistant infections annually in Europe and the USA alone (O’Neill 2014), and an estimated 700,000 worldwide (O’Neill 2016). Some species of bacteria appear to be especially prone to acquisition of drug resistance: methicillin-resistant *Staphylococcus aureus* (MRSA) infections have been extensively reported in the media, and in the early 2000s 40–60 % of *S. aureus* infections were thought to be caused by MRSA in the UK and the US (Weinstein 2001; EARSS 2002), and worryingly approximately 50 % in Portugal and Romania in 2016 (ECDC 2016). Bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella* spp. are also however acquiring resistance rapidly and becoming a serious health risk worldwide. The rise of drug resistance in tuberculosis is of considerable concern; in 2016, the WHO estimated that there were 600,000 new cases of tuberculosis resistant to at least rifampicin, including 490,000 cases of multidrug-resistant tuberculosis (MDR-TB)—resistant to at least two first-line anti-tuberculosis drugs—of which there were an estimated 240,000 deaths globally (WHO 2017c). In addition, extensively drug-resistant tuberculosis (XDR-TB)—resistant to all four first-line drugs—cases were reported in 121 countries in 2016 (WHO 2017c). Worryingly, only 54 % of MDR-TB cases were reported as being treated successfully, compared to 85 % of sensitive TB cases (WHO 2017c), though this number includes a high proportion of patients that are lost to follow-up as well as rare deaths due to treatment failure (WHO 2017c). MDR-TB therapy is arduous, which may partially explain the high rate of patient loss, treatment in most countries takes up to 20

months of daily antibiotics from at least 5 different classes simultaneously (WHO 2011)—many of which have severe side-effects and are administered by injection—which may encourage higher rates of treatment default (Franke *et al.* 2008; Ahmad and Mokaddas 2014). The WHO started to recommend a shorter course in 2016 which lasts 9–12 months (WHO 2016) and this may help to increase patient retention. This has only been adopted in 35 countries to date, though they have reported an encouragingly high proportion of treatment success (87–90 %; WHO 2017c).

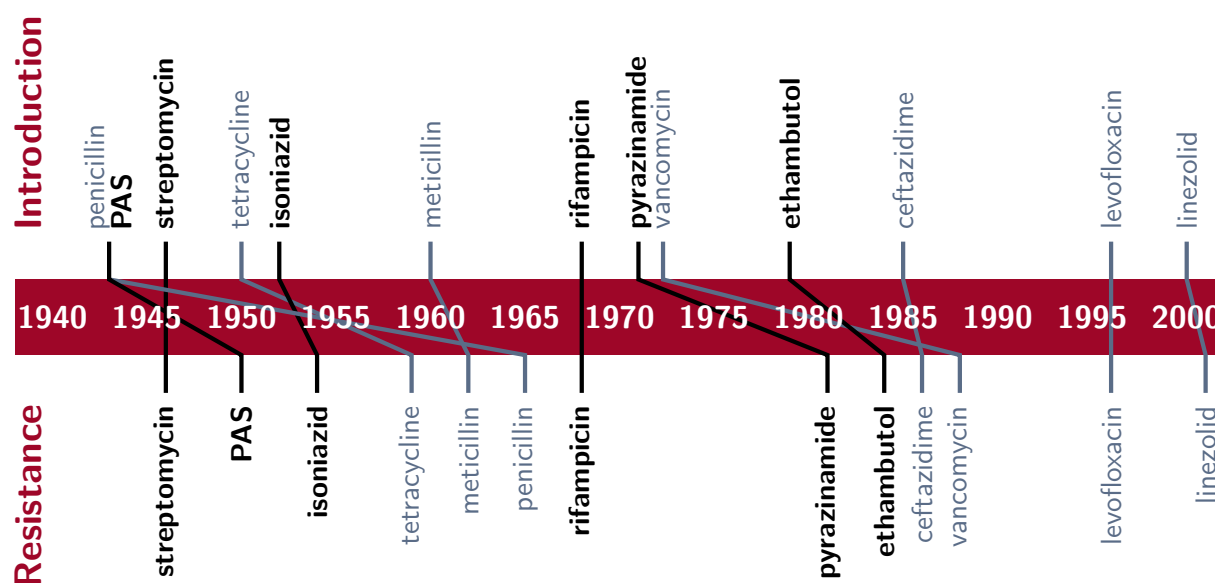


Figure 4.1: Timeline of the introduction and emergence of resistance to antibiotics. A selected subset of antibiotics are depicted comparing the approximate date of their introduction into the clinic (top) to the approximate first emergence of reports of resistance to that antibiotic (bottom). Drugs that are relevant in the treatment of tuberculosis are highlighted in bold. PAS: *para*-aminosalicylic acid.

Drug resistance has been investigated for almost as long as antibiotics have been widely available (Bryskier 2005), and we now understand many of the mechanisms by which bacteria can acquire resistance and how this resistance is effected. Most antibiotics are derived from soil fungi and bacteria—especially actinomycetes (Kieser *et al.* 2000)—which are thought to produce them to avoid competition for resources with other bacteria (Waksman and Woodruff 1940), and the majority of resistance mechanisms are thought to have arisen from environmental sources rather than in response to human use (Martínez 2008; Davies and Davies 2010). This evolutionary arms race has been occurring since microorganisms first began producing antibiotics, and the soil not only provides a huge potential reservoir of novel antibiotics, but also associated resistance to them (D’Costa *et al.* 2006; Davies and Davies 2010). Bacteria develop resistance by two pathways: *de novo* mutation (Martínez and Baquero 2000) and by horizontal gene transfer (Davies 1994; Boto and Martínez 2011). Mutations that lead to resistance fall into a small set of categories: those that modify the target of the antibiotic, those that up-regulate transporters that can export the antibiotic (e.g. efflux pumps) or down-regulate those that import the antibiotic, or those that can modify the antibiotic itself directly (Martínez 2014). Horizontal

gene transfer allows the movement of non-native genes between bacteria, whether pathogenic, commensal, or environmental. Clinically-relevant antibiotic resistance is thought to originate primarily from environmental bacteria via horizontal transfer to pathogens (Davies and Davies 2010). Environmental bacteria are thought to possess antibiotic resistance mechanisms both as a selective advantage against competitors producing antibiotics, and also—perhaps more importantly—to prevent self-inflicted damage in the antibiotic-producing bacteria themselves (Benveniste and Davies 1973). However, the provenance of the majority of the resistance genes found in human pathogens is unknown, aside from two which are derived from non-antibiotic-producing bacteria—quinolone resistance from *Shewanella algae* (Poirel *et al.* 2005) and β -lactamase activity from *Kluyvera* (Cantón *et al.* 2012)—which might suggest otherwise. Indeed, antibiotic resistance may be a side-effect of other processes in many environmental bacteria (Piepersberg *et al.* 1988), such as the detoxifying effect of multidrug efflux pumps on heavy metals or other toxic compounds that bacteria may encounter (Nies 2003; Poole 2005). Indeed, historical *E. coli* strains that were stored before the advent of the antibiotic era have been shown to be resistant to modern drugs (Smith 1967; Houndt and Ochman 2000), suggesting that the evolution of drug resistance occurred well before their use in humans. Supporting this view, analysis of the sequence divergence of β -lactamases suggests that they may have first arisen over 2 billion years ago, and moved to plasmids up to 150 million years ago (Feng *et al.* 1997; Hall and Barlow 2004). Interestingly, horizontal gene transfer appears to be completely absent in *M. tuberculosis* (Achtman 2008; Eldholm and Balloux 2016), aside from evidence of ancient acquisition of virulence factors that promoted its pathogenicity (Reva *et al.* 2015), which implies that drug resistance mutations in *M. tuberculosis* have occurred exclusively *de novo* and have been spread by clonal expansion.

4.1.2 Drug tolerance and the phenomenon of persistence

Drug resistance is generally defined as the heritable genotypic ability for bacteria to grow unimpeded in the presence of high concentrations of an antibiotic (Scholar and Pratt 2000) with an associated increase in the minimum inhibitory concentration (MIC). However, bacteria are also able to survive antibiotics by other mechanisms including drug tolerance and persistence.

Drug tolerance can be defined as the ability (whether heritable or not) for microorganisms to survive a temporary exposure to high concentrations of antibiotics without an increase in MIC (Handwerger and Tomasz 1985; Kester and Fortune 2014). Drug tolerance can be acquired by genetic changes that allow bacteria to adapt to their environment in a manner that renders them insensitive to antibiotics, for example nutrient deprivation signalling pathways that can lead to drug tolerance. The ability to arrest growth and reduce metabolism under stressful conditions is a key aspect of drug tolerance, and can be induced by exposure to antibiotics themselves (Handwerger and Tomasz 1985). A major defining feature of drug tolerance is that

longer exposure to antibiotics results in bacterial killing and this may explain in part why some clinical infections take so long to treat, as well as highlighting the importance of course completion during treatment.

Persistence is the ability of a sub-population of a genetically homogeneous bacterial population to transiently acquire drug tolerance, usually in a growth-arrested state, and in a non-heritable manner (Lewis 2007). The phenomenon of persistence was reported shortly after the introduction of penicillin into routine therapy by the doctor Joseph Bigger in 1944, who observed that he could not completely sterilise genetically homogeneous *Staphylococcus* spp. cultures using penicillin (Bigger 1944). Bigger made a series of conclusions:

1. Only a small subset of the population is able to survive—which he termed “persisters”.
2. Persisters are not resistant mutants.
3. Persisters can both pre-exist and be induced by penicillin.
4. Persistence is not hereditary—descendants of persisters are equally likely to be killed.
5. Persisters are likely to be in a transient, dormant, non-dividing phase of growth.

These conclusions were not confirmed for sixty years, when these characteristics were observed at the single-cell level (Balaban *et al.* 2004; Keren *et al.* 2004a; Wiuff *et al.* 2005). Persistence appears to be universal property of bacteria, and persister cells have been observed in every species tested to date (Lewis 2001; Lewis 2010; Cohen *et al.* 2013).

Bigger demonstrated that persister cells occur at about a frequency of one cell in a million in his *Staphylococcus* cultures (Bigger 1944), and this is true in *E. coli*, but dependent on the growth phase of the population: 10^{-6} in early exponential, 10^{-4} in mid-exponential, and a maximum of 10^{-2} during stationary phase (Lewis 2007). Moreover, pre-existence of persister cells has been confirmed subsequently by repeated sub-culture of early exponential cultures, resulting in the complete lack of persister cells, demonstrating that most persister cells are pre-formed during stationary phase (Keren *et al.* 2004a; Wiuff *et al.* 2005). This frequency still implies a non-deterministic mechanism of formation, since only one in a hundred cells appear to be persistent in stationary phase, though clearly environmental cues must be involved in the enrichment during this phase. Persisters result in highly characteristic biphasic killing curves when populations are exposed to antibiotics, with a rapid initial linear decrease when the majority of the population is killed, followed by a slow rate of cell death as the persister sub-population is revealed (Lewis 2010; Kint *et al.* 2012). The slope of the first phase (death phase) is dependent on the antibiotic concentration, whereas the second phase (persister phase) is independent, since the rate of switching between persister and susceptible states is thought to be random (Balaban *et al.* 2004; Lewis 2010; Cohen *et al.* 2013, see Figure 4.2).

The phenomenon of persistence was largely neglected following its discovery due to the focus on antibiotic resistance, rapidly reported after their introduction (Figure 4.1), which

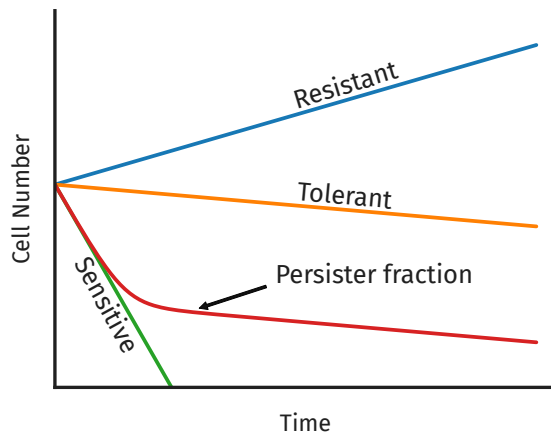


Figure 4.2: A biphasic killing curve reveals persister cells. Idealised plot of cell number (log-scale) against time for different responses to antibiotics. Data was generated computationally. Drug-sensitive cells (green) rapidly die when exposed to antibiotics. Drug-resistant cells (blue) continue to grow normally. Drug-tolerant cells (orange) arrest growth and die slowly over time. Populations harbouring a persister sub-population (red) behave as sensitive cells initially with a rapid death phase, followed by a slow rate of cell death indicative of drug tolerance.

was combatted by the frequent discovery of novel antimicrobial agents during the 1940–1960 golden era (Walsh 2003). The significance of persistence was not revisited until the 1980s, when Harris Moyed became interested in non-heritable variation and searched for *E. coli* mutants that were more able to survive antibiotics, *without* increasing the MIC of the population—a key feature of persistence—and discovered a gain-of-function mutation in the *hip* operon (*hipA7*) which enhanced the persister fraction by a thousand-fold (Moyed and Bertrand 1983; Moyed and Broderick 1986; Black *et al.* 1991, 1994). There are many non-genetic conditions that trigger an increase in persistence, such as high cell density via quorum sensing (Leung and Lévesque 2012), the SOS response (Bernier *et al.* 2013), and internalisation in macrophages in pathogens (Helaine *et al.* 2014). Interestingly, while transposon and gene knockout screens investigating persistence have been conducted, they have not discovered genes that abrogate persister formation, and only found strains that increase persistence (Hu and Coates 2005; Spoering *et al.* 2006; Hansen *et al.* 2008). This may support the hypothesis that persistence may be an intrinsic feature of bacterial growth in general, and that drug tolerance is an emergent property.

Mechanistically, several specific means to acquire transient drug tolerance have been suggested. Various efflux pump genes have been reported to become up-regulated in response to drug exposure, including in response to clinical drugs, such as *iniA* in response to isoniazid and ethambutol (Colangeli *et al.* 2005), and *rv1258c* in response to rifampicin and isoniazid (Siddiqi *et al.* 2004; Jiang *et al.* 2008); both of which appear to enhance tolerance to their inducer. Indeed, *M. tuberculosis*—which appears to be especially proficient at tolerating antibiotics—has at least 26 chromosomally-encoded efflux pumps, the majority of which are multidrug exporters: considerably more than the 8 known in the more “resistant” MRSA (Poole 2004, 2005; De Rossi *et al.* 2006). Many of these efflux pumps have been shown to be up-regulated by macrophage internalisation in the fish pathogen *M. marinum*, where their over-expression leads to tolerance to drugs in zebrafish *in vivo* models of infection (Adams *et al.* 2011). Notably, this induction occurs in actively growing mycobacteria, suggesting that growth arrest may not be a universal feature of drug tolerance, though this may be specific to *M. marinum* (Adams *et al.* 2011).

A more recent underlying mechanism that confers rifampicin tolerance has been observed in *M. smegmatis*, whereby the error rate in protein synthesis appears to be deliberately increased to generate a pleiotropic effect, crucially in the expression of RNA polymerase (RNAP)—the target of rifampicin. Canonical genetic resistance to rifampicin is generally caused by a handful of specific mutations within an 81-bp region of the β -subunit of RNAP (*rpoB*), known as the rifampicin resistance-determining region (Ramaswamy and Musser 1998). These mutations are so common (95–97 % of resistant isolates) that tests for rifampicin resistance in clinical settings can be performed by simply sequencing this region of *rpoB*, a test which has a very low false-negative rate (van Klingeren *et al.* 2007; Lawn and Nicol 2011). Javid *et al.* demonstrate that mistranslation of the *rpoB* from wild-type mRNA can produce proteins which exhibit the same protein sequence as these resistance mutations would produce (Javid *et al.* 2014). This in effect provides a sub-population of RNAP molecules within the larger pool of RNAP which happen to be resistant to the effects of rifampicin (Javid *et al.* 2014). This resistance is non-heritable—aside from by protein inheritance at division—thus forms a true mechanism of transient phenotypic drug resistance, highly similar to—and potentially indistinguishable from—the transient drug tolerance observed in persistence.

There remains a lack of consensus in the field on whether persistence is a specific strategy evolved to combat antibiotics or whether it is a side-effect of other pathways (Balaban *et al.* 2013; Levin *et al.* 2014). Many authors have suggested that growth arrest as a mechanism to avoid cell death in a small proportion of a population is an example of ecological bet-hedging (Kussell and Leibler 2005; Veening *et al.* 2008; Lennon and Jones 2011). Bet-hedging is a general mechanism by which a population will diversify phenotypes under identical environmental conditions to those which might be better adapted to a potential future environment, at the expense of optimal fitness under the current conditions (Seger 1987; Philippi and Seger 1989; Simons 2011; Starrfelt and Kokko 2012). However, there is mounting evidence that suggests the vast majority—if not all—persister cells form deterministically (Kim and Wood 2016), and pre-formed cells (as described above) are cells that arrested growth during stationary phase with longer lag times (Fridman *et al.* 2014), and are derived from the inoculum in *in vitro* experiments and not formed during exponential growth at all (Keren *et al.* 2004b; Orman and Brynildsen 2013). This conflicts with early reports that persister cells form stochastically during exponential phase (Balaban *et al.* 2004). In addition, experiments demonstrating that transient induction of efflux pumps confers drug tolerance in *M. marinum* also show that this induction confers *enhanced* growth within macrophages in the absence of antibiotics (Adams *et al.* 2011), suggesting that phenotypic drug tolerance may occur as a side-effect of increased efflux.

As mentioned above, the *hip* operon was noted to greatly affect persistence in *E. coli* over 30 years ago (Moyed and Bertrand 1983), which encodes the *hipBA* toxin–antitoxin (TA) system. TA systems involve a stable toxin (HipA in this case) that inhibits a crucial biological process, and a labile antitoxin (HipB in this case) that prevents its action, which are simultaneously

expressed (Yamaguchi *et al.* 2011; Page and Peti 2016). These molecular systems have been repeatedly identified in screens for persister cells (e.g. Keren *et al.* 2004a,b; Shah *et al.* 2006), and they appear to cause growth arrest when induced, which may provide another mechanistic underpinning for non-replicating persistence.

Toxin–antitoxin systems

TA systems were first identified as a plasmid stabilisation system, where the loss of the plasmid would lead to the degradation of the inherently unstable antitoxin, the release of the stable toxin, and the post-segregational death of any cell that had lost the plasmid (Gerdes *et al.* 1986; Thisted *et al.* 1994; Jensen and Gerdes 1995). Chromosomal TA systems were subsequently discovered, and a similar mechanism of stabilisation of chromosomal regions proposed and a role in programmed cell death (Aizenman *et al.* 1996), though most chromosomal systems appear to trigger reversible growth arrest rather than death (Tsilibaris *et al.* 2007; Fu *et al.* 2009; Ramisetty *et al.* 2016b). TA systems may be the effectors of persistence, whereby triggering them causes growth arrest, and consequent tolerance to antimicrobials, most of which require active growth to kill bacteria. They can be used to explain both the consistent persister fraction found in homogeneous populations and the deterministic increase under certain conditions such as stationary phase. TA systems are generally controlled by the expression of the antitoxin in excess of the toxin, and strong autorepression of the system. Stochasticity in gene expression—observed in most biological systems (McAdams and Arkin 1999; Elowitz *et al.* 2002; Losick and Desplan 2008)—can lead to stochastic spikes in toxin expression, and mathematical modelling predicts that such spikes can result in a transient increase in toxin, an inhibition of growth, and a persistent phenotype (Rotem *et al.* 2010; Gelens *et al.* 2013; Feng *et al.* 2014). They can also be triggered deterministically by the repression or degradation of antitoxin components leading to release of toxins.

Most bacteria encode multiple TA systems, *E. coli* K-12 has at least thirty-nine (Yamaguchi *et al.* 2011; Tan *et al.* 2011; Wang *et al.* 2012b; Guo *et al.* 2014; Page and Peti 2016), *Salmonella enterica* serovar Typhimurium at least eighteen (Lobato-Márquez *et al.* 2015), and *M. tuberculosis* at least eighty-eight (Ramage *et al.* 2009; Gupta 2009; Han *et al.* 2010). Transcriptomic analysis of bacteria that survive antibiotic exposure shows a general up-regulation of TA systems in *E. coli* pointing to their role in persistence (Keren *et al.* 2004b; Shah *et al.* 2006).

The TA systems can be divided into at least five classes based on their mechanism of interaction between toxin and antitoxin. Type II TA systems are the best characterised and most numerous, with both toxin and antitoxin components being small proteins, with the antitoxin disabling the toxin by direct binding. Most of the type II systems act by blocking translation, mostly by cleavage of RNA, whether mRNA, transfer RNA (tRNA), or ribosomal RNA (rRNA) (Christensen *et al.* 2001; Winther and Gerdes 2011; Schifano *et al.* 2013; Zhang *et al.* 2003b). Type II antitoxins

are unstable due to intrinsic disorder (Oberer *et al.* 2007; Li *et al.* 2008), and are degraded by proteases, notably Lon protease, which is involved in the stringent response to amino acid deprivation (Van Melderren *et al.* 1996; Smith and Rawlings 1998). The *hipA7* allele identified by Moyed and Bertrand (1983) involves the *hipBA* type II TA system, with a mutation in *hipA* that prevents the binding of the HipB antitoxin to HipA leading to greatly increased toxin activity (Korch *et al.* 2003). HipA does not cleave RNA, rather it inhibits glutamyl-tRNA synthetase preventing the charging of tRNA^{Glu} (Winther and Gerdes 2011).

Role and activation of the TA systems

The extensive proliferation of TA systems in bacteria raises the question of why so many modules are required, when only a single TA system is ostensibly required to cause growth arrest. In *Salmonella*, internalisation by macrophages induces the up-regulation of at least fourteen TA systems, and deletion of single systems reduces the proportion of growth-arrested bacteria by 10–30 % (Helaine *et al.* 2014) indicating that these systems may be additive rather than redundant during infection by *Salmonella*, or that antibiotic tolerance is a poor indicator of the ability to survive in macrophages. In *E. coli* however, deletion of five TA systems had no effect on levels of persistence (Tsilibaris *et al.* 2007), but *E. coli* has almost ten times this number of TA systems. While early reports suggested that the deletion of 10 TA systems ($\Delta 10TA$) in *E. coli* led to a decrease in persister levels (Maisonneuve *et al.* 2011, 2013), these results were challenged strongly (Kim and Wood 2016; Ramisetty *et al.* 2016a; Van Melderren and Wood 2017). The $\Delta 10TA$ strain has been re-evaluated and is now known to have no defect in persistence (Shan *et al.* 2017; Harms *et al.* 2017). This has led to the retraction of both of the papers describing this mutant, ostensibly due to evidence of polar off-target effects and a phage contamination (Maisonneuve *et al.* 2018a,b). These developments point to the possibility that these systems are not redundant, and have both distinct triggers and distinct roles, including those outside persistence. The role of the TA systems themselves has also been subsequently questioned, with observations that induction of the TA systems in *E. coli* under several stresses does not lead to a concurrent increase in antibiotic tolerance (Shan *et al.* 2017), and the observation that deletion of multiple TA systems in *S. aureus* has no effect on persistence (Conlon *et al.* 2016).

However, even given the conflicting evidence, there still appears to be strong evidence that the TA systems do play a role in persistence, though this may be through distinct pathways and specific mechanisms according to the type of stress applied rather than via a single master pathway (Lewis 2010; Dörr *et al.* 2010; Norton and Mulvey 2012; Wood *et al.* 2013; Helaine *et al.* 2014; Verstraeten *et al.* 2015; Harms *et al.* 2016; Fisher *et al.* 2017). The mechanism underlying the *hipBA* TA system also provides an interesting model. HipA phosphorylates glutamyl-tRNA synthetase, preventing the charging of tRNA^{Glu} (Germain *et al.* 2013). The

presence of uncharged tRNAs bound to ribosomes leads to the activation of the stringent response to amino acid starvation, with the activation of RelA, which produces the alarmone guanosine tetraphosphate and pentaphosphate ((p)ppGpp; Gentry and Cashel 1996). (p)ppGpp leads to the production of polyphosphate which binds to inactive ribosomes and targets them for degradation (Akiyama *et al.* 1992, 1993; Kuroda *et al.* 2001). This response leads to a dramatic down-regulation and redirection of protein synthesis (Braeken *et al.* 2006; Kanjee *et al.* 2012; Mechold *et al.* 2013; Hauryliuk *et al.* 2015). The retracted papers linked the stringent response directly to activation of the TA systems, but there is still other evidence that (p)ppGpp and the stringent response play a role in TA system activation, though potentially less directly (Viducic *et al.* 2006; Fung *et al.* 2010; Nguyen *et al.* 2011; Verstraeten *et al.* 2015; Gaca *et al.* 2015; Corrigan *et al.* 2016). Indeed, basic evidence that (p)ppGpp levels correlate with growth rate suggests a role in drug tolerance (Ryals *et al.* 1982).

The role of the TA systems in mycobacteria

As mentioned above, *M. tuberculosis* encodes a vast array of TA systems on its chromosome, with at least 88 identified and confirmed as active (Ramage *et al.* 2009). This raises the question of why *M. tuberculosis* requires such an extensive repertoire of such systems, if as was suggested, they may perform similar functions in growth arrest during stress. This implies that the systems are non-redundant in *M. tuberculosis*, and may provide highly specific functions and respond to a large array of signals, though evidence for this hypothesis is not forthcoming. Evidence for their role in host survival and drug tolerance is growing however.

TA systems are known to be up-regulated in *M. tuberculosis* during cellular stress, such as HigBA and VapBC-15 during hypoxia, and VapBC-11 and VapBC-3 during macrophage infection (Ramage *et al.* 2009). Deletion of three *mazF* loci in *M. tuberculosis* has also been shown to result in decreased tolerance to levofloxacin and rifampicin (Silva-Herzog *et al.* 2015; Tiwari *et al.* 2015). The stringent response has been implicated in the long-term survival of *M. tuberculosis in vitro* (Primm *et al.* 2000), and *in vivo* mouse infections (Dahl *et al.* 2003). Starvation of *M. tuberculosis*—as for *E. coli*—induces the stringent response and the formation of (p)ppGpp (Primm *et al.* 2000). In *E. coli*, RelA synthesises (p)ppGpp, and SpoT both synthesises and hydrolyses it, with the deletion of either gene resulting in cells that cannot survive in minimal medium and poorly in stationary phase (Cashel *et al.* 1996). *M. tuberculosis* has a single RelA/SpoT homologue—Rel_{Mtb}—which both produces and degrades (p)ppGpp, and in Δrel_{Mtb} deletion strains, survival under long-term nutrient deprivation, prolonged aerobic culture, and hypoxic shock is severely reduced (Primm *et al.* 2000). However, this growth defect does not cause any reduction in survival within THP-1 human macrophage-like cells (Primm *et al.* 2000), but does in an *in vivo* mouse model of infection (Dahl *et al.* 2003). These reports indicate that the stringent response may have a significant role in *M. tuberculosis* survival during infection. Proteomic

analysis of nutrient-deprived *M. tuberculosis* cells shows that thirteen toxins are up-regulated, including two with more than 100-fold increase in abundance suggesting that there is indeed a link (Albrethsen *et al.* 2013).

Expansion of the TA systems appears to be exclusively limited to the pathogenic species of *Mycobacterium* (Ramage *et al.* 2009), which may imply a connection between pathogenicity and the TA systems in mycobacteria. The model species *M. smegmatis* encodes only three known TA systems: *vapBC*, *mazEF*, and *phd/doc*, thus investigations of their role may have little impact on understanding in *M. tuberculosis*. Notwithstanding this difference, *M. smegmatis* is far more tractable to genetic manipulation and phenotyping, and the knockout of three loci is much less likely to cause polar effects compared to the Herculean task of TA knockouts in *M. tuberculosis*. Single, double, or triple knockouts of any of the three systems appears to have no phenotypic effect in *M. smegmatis*, including their response to antibiotics (Frampton *et al.* 2012), although exposure times were extremely brief in their assay. The only phenotypes apparent in a triple knockout of all three TA systems were a survival defects in complex medium after 15 d, in response to oxidative (H₂O₂) stress, and to heat shock (Frampton *et al.* 2012). The authors suggest that growth in a complex medium may enhance oxidative stress on cells, which they are less able to combat due to the lack of the TA systems, though the relevance of this observation to either antibiotic tolerance or host survival in *M. tuberculosis* is unclear.

Persistence in clinical disease

The role of persistence in disease is not fully established, but the phenomenon of persistence could be a large component in recurrent infections. Many infections recur after treatment, and evidence suggests that these repeated episodes are caused by clonal descendants of the original agent in many cases, such as in *Chlamydia trachomatis* infection (Mpiga and Ravaoarinoro 2006), *E. coli* in urinary tract infections (Mysorekar and Hultgren 2006), *Borrelia burgdorferi* in Lyme disease (Berndtson 2013), and during fungal infection with *Cryptococcus neoformans* (Spitzer *et al.* 1993). Selection of drug-tolerant persister cells by treatment could explain how reservoirs of bacteria could remain viable until reactivation, and underlie the recurrence of symptoms. Evidence of the clinical significance of persistence has been found in several species. In *P. aeruginosa* infections in cystic fibrosis patients, high persistence mutants appear to be selected for, with late isolates having up to a 100-fold increase in levels of persistence compared to early isolates in the same patient (Mulcahy *et al.* 2010). Whole genome sequencing of one such pair reveals that mutations conferring resistance, such as those that up-regulate the expression of the MexXY-OprM multidrug efflux pump, are not responsible for the increase in persistence, since complete removal does not affect persistence levels (Mulcahy *et al.* 2010). Thus in a clinical setting, *P. aeruginosa* cells are acquiring mutations that increase their persister fraction, permitting survival in the face of treatment, and contributing to the recalcitrance of

this infection in cystic fibrosis patients (Mulcahy *et al.* 2010). This is supported by evidence that biofilms—a common problem during *P. aeruginosa* infection—harbour high levels of drug-tolerant bacteria (Brooun *et al.* 2000; Spoering and Lewis 2001). Cells isolated from patients with recurrent *Candida albicans* infections have also been observed to possess mutations that enhance persistence (Lafleur *et al.* 2010). Uropathogenic *E. coli* infections, notorious for their recalcitrance to treatment and recurrence, have been noted to acquire mutations in *hip*, the first observed mechanism underlying persistence *in vitro* (Schumacher *et al.* 2015).

The requirement for extensive therapy (at least 6 months) to treat tuberculosis successfully has been proposed to be related to persistence (McDermott 1958), and the presence of viable bacilli after treatment has been demonstrated in a mouse model (McCune and Tompsett 1956; McCune *et al.* 1956, 1966a,b), with subsequent support from clinical data (Fox *et al.* 1999; Jindani *et al.* 2003). Live *M. tuberculosis* have been found in lesions and potential persister-like cells in adipose tissue (Neyrolles *et al.* 2006; Agarwal *et al.* 2014), as well as sputum (Jindani *et al.* 2003; Garton *et al.* 2008; Walter *et al.* 2015; Honeyborne *et al.* 2016) during treatment. Sputum studies have identified kill curves on initiation of anti-tubercular therapy that closely resemble characteristic persister kill curves (Figure 4.2), with 94 % of bacteria dying within the first four days, while complete sterilisation takes six months, suggesting that *M. tuberculosis* treatment length may be significantly lengthened by the presence of persisters (Walter *et al.* 2015; Honeyborne *et al.* 2016). The key anti-tuberculosis drug pyrazinamide specifically targets non-growing cells by membrane depolarisation (Zhang *et al.* 2003a) and inhibition of *trans*-translation (Shi *et al.* 2011). *Trans*-translation is the mechanism by which stalled ribosomes are rescued and is a crucial process in adapting to stress by the removal of toxic protein products (Li *et al.* 2013). It has been shown *in vitro* to be an important factor in persistence (Withey and Friedman 2003; Wilson and Nierhaus 2007; Li *et al.* 2013; Amato and Brynildsen 2015), thus implying that pyrazinamide may be targeting persisters specifically. It is notable that pyrazinamide greatly reduces treatment time when included in tuberculosis treatment regimens, and treatment outcomes are always inferior when it is excluded (Tasneen *et al.* 2008; Drusano *et al.* 2010; Tasneen *et al.* 2011), which may indirectly implicate the role of non-growing, potentially persistent bacteria during tuberculosis treatment.

In addition to complicating treatment, persistence may also underlie virulence itself. Similarly to resistance mechanisms, drug tolerance may have evolved as a side-effect of stress response mechanisms that lead to growth arrest (Hayes and Low 2009; Baek *et al.* 2011) and these mechanisms are probably selected for in pathogens to allow bacteria to survive immune attack by their host. Common strategies by immune cells such as macrophages and neutrophils that combat intracellular bacterial infections include the restriction of nutrients and essential metal ions such as iron and manganese (Jabado *et al.* 2000; Appelberg 2006; Flannagan *et al.* 2015; Sprenger *et al.* 2018), the over-supply of other metals such as copper or zinc to normally toxic levels (Botella *et al.* 2011, 2012), and the acidification of the bacteria-containing phagosome

(Vieira *et al.* 2002; Russell *et al.* 2009; Sun-Wada *et al.* 2009), many of which are conditions that are known to enhance survival and drug tolerance in *in vitro* and *in vivo* models (Bernier *et al.* 2013; Helaine *et al.* 2014; Kurthkoti *et al.* 2017). This hypothesis is supported by the observation that uropathogenic *E. coli* adaptations that enhance drug tolerance also enhance survival in the face of immune attack (Putrinš *et al.* 2015). This concept also highlights the differences between *in vitro* and *in vivo* models of persistence, where exposure to antibiotics only plays one part in infection, experiments that increase tolerance *in vitro* often do not transfer to *in vivo* systems—such as overexpression of the toxin *relE* in *M. tuberculosis* that enhances persistence in culture, but not survival in mice (Singh *et al.* 2010b)—and vice versa—such as high persistence *M. tuberculosis* mutants isolated from isoniazid-treated mice that do not display increased tolerance in culture (Dhar and McKinney 2010).

There is also increasing evidence that the acquisition of mutations that enhance drug tolerance may pre-dispose bacteria to acquire drug resistance, blurring the lines between phenotypic and genotypic resistance (Levin and Rozen 2006; Smith and Romesberg 2007; Hansen *et al.* 2008; Cohen *et al.* 2013; Wakamoto *et al.* 2013; Levin-Reisman *et al.* 2017). This is proposed to be due to the fact that persisters are exposed to antibiotics for longer, increasing the statistical probability of acquiring resistance mutations, as well as the increased mutation rate itself due to stress (Cohen *et al.* 2013; Levin-Reisman *et al.* 2017).

4.1.3 Latency in tuberculosis

Related to persistence in other bacteria, *M. tuberculosis* has long been characterised by its ability to become dormant. This introduces an additional concept to bacterial growth, already complicated by overlapping and poorly defined terminology. Dormancy is a phenotypic state of reversible quiescence that may underlie the significant issue of latent tuberculosis, the asymptomatic infection that may affect an estimated third of the global population (Dye *et al.* 1999). In addition to this huge reservoir of infection—while the relationship between latency and drug resistance is largely unknown—the high current incidence of resistant cases (490,000 MDR-TB cases in 2016; WHO 2017c) implies that there is also a huge reservoir of drug-resistant latent tuberculosis, which has very concerning implications for progress towards complete eradication, especially given the usage of pre-emptive prophylactic isoniazid monotherapy for latent tuberculosis in close contacts of active patients (Jagger *et al.* 2018).

Only 5–10 % of new *M. tuberculosis* infections are thought to result in active disease, with the remaining cases resulting in latent tuberculosis (Sutherland *et al.* 1982; Styblo 1985; Esmail *et al.* 2012; Zumla *et al.* 2013; Glaziou *et al.* 2014), though this value may be higher dependent on age, geographical location, or immunological status as has been suggested in other studies (Shanaube *et al.* 2009; Sloot *et al.* 2014). This is either due to effective immune control of bacteria, or a specific adaptation of *M. tuberculosis* to host-living whereby growth is severely limited,

or indeed both. The absolute distinction between latent and active infection may however conceal the complexity that underlies tuberculosis. Bacteria are generally contained within a large lesion of immune cells known as a granuloma, where they are sealed away from the body by the immune system. Uncontrolled bacterial replication within the granuloma leads to the breakdown of the structure, and release of bacteria into the airways, whereupon they can induce the formation of new granulomas, or be ejected from their host by coughing to effect transmission. This provides the basis of the *M. tuberculosis* life cycle, and underlies latent infection (Ehlers and Schaible 2012). Latency can be considered as the state in which the integrity of each granuloma is maintained in a restrictive state, thereby preventing active symptoms, and transmission. However, recent evidence points to a more dynamic picture of latency, whereby each granuloma may behave independently, forming and re-forming constantly (Egen *et al.* 2008; Kling *et al.* 2014; Coleman *et al.* 2014; Lenaerts *et al.* 2015; Martin *et al.* 2017). Latency in this context can be considered as a constant battle between immune containment and frequent bacterial release, where active disease occurs when the immune system can no longer keep up. Progression from latency to active disease may lie along a continuous spectrum of balance between immune control and bacterial replication, which is exacerbated by conditions that suppress the immune system, such as HIV co-infection (Young *et al.* 2009).

It is well known that *M. tuberculosis* induces a dormancy programme when subjected to a number of conditions that are relevant to human infection, especially hypoxia which is thought to be a major factor within the granuloma that cells generally reside within (Tsai *et al.* 2006). This programme involves the up-regulation of a large number of genes involved in stress responses, and the down-regulation of genes involved in growth, especially genes involved in central metabolism (Muttucumaru *et al.* 2004). The DosRS two-component system has been frequently referred to in the context of dormancy, where the DosR response regulator induces a set of around fifty genes (known as the *dos* regulon; Park *et al.* 2003; Voskuil *et al.* 2004). This regulon includes genes involved in metabolism such as the glyoxylate shunt—which permits the utilisation of alternative carbon sources when glycolysis is limited (Lorenz and Fink 2002)—fatty acid metabolism, and nitrogen assimilation pathways (Wayne and Lin 1982; Voskuil *et al.* 2003).

Though these genes might be expected to be highly related to persistence, *in vitro* persister cells do not appear to activate the *dos* regulon, as determined by transcriptomics (Keren *et al.* 2011) and proteomics (Albrethsen *et al.* 2013). Additionally, deletion of *dosR* only causes a minor decrease in viability in murine models (Rustad *et al.* 2008). However, the induction of the *dos* regulon may be a transient effect, with almost half of the genes induced returning to normal levels after 24 hours of hypoxia (Rustad *et al.* 2008). This may imply that *dos* is important in initial adaptation to hostile conditions during early infection, but is not involved in long-term survival, and indeed artificial induction of the *dos* regulon does not cause growth arrest *in vitro* (Minch *et al.* 2012). Investigation of long-term microaerophilic cultures demonstrates

the induction of a different set of genes, sometimes known as the enduring hypoxic response, defined as genes that are induced after the DosR-induced genes return to pre-hypoxic levels, and includes 230 genes that are significantly up-regulated after up to seven days of hypoxia (Rustad *et al.* 2008). These genes may play a significant role in the longevity of *M. tuberculosis* within its intracellular niche.

While dormancy may or may not be directly related to persistence, features are clearly in common. Both transcription and translation are down-regulated during dormancy (Bunker *et al.* 2015), and growth is delayed (Flores Valdez and Schoolnik 2010)—features which are thought to be important in persistence. Additionally, under microaerophilic conditions—a trigger of dormancy—*M. tuberculosis* appears to be insensitive to isoniazid (Park *et al.* 2003; Bartek *et al.* 2009) suggesting some overlap between the phenomena of dormancy and drug-tolerant persistence. Other factors other than hypoxia also appear to trigger dormancy, such as acid stress (Byrne *et al.* 2007; Cho *et al.* 2007; Deb *et al.* 2009), in common with persister cells *in vitro*. *In vivo*, other shared triggers are observed, such as induction after macrophage internalisation (Schnappinger *et al.* 2003), in mice (Karakousis 2004), and in guinea pigs (Sharma *et al.* 2006). As with persistence, dormancy also appears to be a reversible and somewhat heterogeneous phenotype, and may underlie the spectrum of disease from latent to active tuberculosis, with bacteria switching between non-growing dormancy and replicating phases within granulomas (Esmail *et al.* 2012; Esmail *et al.* 2014).

Related to dormancy, and often equated with it, is the phenomenon of the viable but non-culturable (VBNC) state. Cells that have been cultivated for extensive periods in stationary phase appear to lose the ability to grow on solid medium (Kaprelyants *et al.* 1993). This state is presumed to be highly similar to the dormant state, and it is unclear what differences there are between the two. VBNC bacteria are most commonly identified by comparing colony-forming units (CFUs) on solid medium to estimates of bacterial numbers in liquid medium by serial dilution: by use of a most probable number (MPN) assay (de Man 1975). MPN estimates are often increased by the addition of spent culture filtrate to the liquid growth medium, implying the presence of a secreted factor that promotes growth (Shleeve *et al.* 2002; Shleeve *et al.* 2004). Early evidence for VBNC mycobacteria has been seen in the Cornell mouse model of infection, in which viable numbers (by CFU counting) of *M. tuberculosis* in mice treated with antibiotics falls to zero over time (Wayne 1994), but appreciable amounts of DNA are still recovered indicative of high numbers of bacteria within organ systems which are ostensibly sterile (de Wit *et al.* 1995), though this may also suggest high numbers of dead cells which are not cleared for unknown reasons. It is notable that the Wayne microaerophilic model does not induce a VBNC state, whilst inducing apparently dormant cells, suggesting that there may be a difference between the two states. The VBNC state in mycobacteria is significantly enriched after extensive stationary phase—four months in the case of *M. tuberculosis* (Shleeve *et al.* 2002)—and by growth in nitrogen- or carbon-limiting conditions in *M. smegmatis* (Shleeve *et al.*

2004) which implies an important role of metabolism in this growth-arrested state. VBNC cells may significantly affect experimental systems that rely on regrowth as a measure of viability, especially in the context of those that investigate the response of bacteria to antibiotics.

4.1.4 Role of growth rate in antibiotic susceptibility

There is a significant diversity in terms which describe states associated with drug tolerance, but a common theme appears to be the role of growth rate: non-replicating persistence, dormancy, and the VBNC state all imply growth arrest. This association with increased drug tolerance is consistent with the long-held view that most antibiotics are more effective on actively growing bacteria; indeed, many cidal agents appear to require active growth, such as the canonical β -lactams (Tomasz 1979). Drug tolerance has been linked extensively with the population growth rate, most notably with the association between growth phase and survival, with survival increasing from 10^{-6} in exponential phase to 10^{-2} in stationary phase in *E. coli* (Keren *et al.* 2004a,b; Lewis 2007). In addition, non-dividing cells are enriched in *E. coli* persisters (Shah *et al.* 2006) and in *Salmonella* persisters recovered from macrophages (Helaine *et al.* 2010, 2014). At the single-cell level, *E. coli* persisters can be identified prior to exposure to antibiotics by their reduction in growth rate (Balaban *et al.* 2004), again demonstrating a strong link between growth arrest and ultimate survival.

Growth arrest and quiescence is strongly associated with a dramatic metabolic shutdown, in which transcription and translation are greatly down-regulated. This has been observed in the eukaryote *Saccharomyces cerevisiae*, with a 3–5-fold decrease in transcription (Choder 1991) and a 20-fold decrease in translation (Fuge *et al.* 1994) during quiescence. This is also observed in very slow-growing *E. coli*, with a dramatic decrease in both transcription and translation initiation events (Pederson 1985). In mycobacteria, this effect is also observed, but coupled with a significant 15-fold increase in mRNA stability (Rustad *et al.* 2013), thought to enable the maintenance of essential proteins during dormancy (Rao and Li 2009), which is also seen in *S. aureus* (Anderson *et al.* 2006) and *S. cerevisiae* (Jona *et al.* 2000) upon sustained quiescence. This shutdown of protein synthesis is not an uncontrolled global shutdown, as indicated by continued expression of essential proteins, but is finely choreographed upon starvation. In *E. coli*, immediately after the onset of starvation, protein synthesis actually increases 5-fold due to the large induction of proteases (Shaikh *et al.* 2010). These proteases act to degrade a large proportion of the proteome to provide the raw materials for stress-related proteins to be expressed (Reeve *et al.* 1984), such as those controlled by σ^S (Talukder *et al.* 1996). Indeed, protein expression appears to be maintained during dormancy, as indicated by the ability for RNA polymerase and DNA gyrase inhibitors to kill non-replicating mycobacteria (Betts *et al.* 2002; Sala *et al.* 2010). In addition, maintenance of the proton-motive force is essential for long-term survival under dormancy (Rao *et al.* 2008), indicative of the maintenance of turnover

of essential proteins. This is sometimes referred to as a “non-growing but metabolically active” state, which may or may not refer to the same phenomenon as the dormant or VBNC states. These factors imply that simple growth arrest is not sufficient to confer multidrug tolerance, since drug targets which are ostensibly indicative of active growth must be maintained to retain viability.

In addition to the additional layers of complexity underlying growth arrest in dormancy, the universality of growth arrest in drug tolerance itself has been called into question in several studies. As mentioned above, *M. marinum* can acquire drug tolerance by the transient over-expression of multidrug efflux pumps, which are specifically enriched in actively growing mycobacteria (Adams *et al.* 2011). At the single-cell level, isoniazid-tolerant cells have also been shown to maintain growth during drug exposure, with survival linked to expression of KatG, the bacterially-encoded enzyme that converts isoniazid into its active form (Wakamoto *et al.* 2013). The suggestion that persister cells arrest their growth prior to addition of the drug has also not been observed in mycobacteria (as discussed in more detail below; Aldridge *et al.* 2012; Wakamoto *et al.* 2013; Manina *et al.* 2015). These studies strongly question the role of growth arrest in phenotypic drug tolerance, at least in mycobacteria.

4.1.5 Antibiotics at the single-cell level

Single-cell studies are crucial in the investigation of antibiotic tolerance, since they provide a means to identify transient phenotypes, and to distinguish sub-populations whose averaged behaviour may conceal any underlying complexities. Early work catalysing renewed interest in persisters was performed using a custom microfluidic device which allows the monitoring of *E. coli* single cells by trapping them in grooves under a membrane through which medium is perfused (see Figures 2.1E and 2.1F; Balaban *et al.* 2004). This permits the exchange of the medium whilst permitting the continued observation of the same single-cell lineages, and was used to great effect to demonstrate that *E. coli* that are able to survive ampicillin exposure pre-exist with reduced growth rate (Balaban *et al.* 2004). Fluorescence-activated cell sorting (FACS) of cells expressing a fixed amount of fluorescent protein that dilutes at each cell division has also been used to determine that *S. enterica* serovar *Typhimurium* cells internalised within macrophages arrest their growth (Helaine *et al.* 2010), and that these cells are drug-tolerant (Helaine *et al.* 2014).

With regards to mycobacteria, microfluidic approaches have been used extensively to investigate the response to antibiotics at the single-cell level. Aldridge *et al.* used a microfluidic device that provides a growth chamber for cells which constrains them in the Z-dimension—similar to the ONIX system that I use—in which they demonstrate that asymmetry in cell division leads to cells which are differentially susceptible to different classes of antibiotics (Aldridge *et al.* 2012). They also show that the pre-existing, growth-arrested cells reported by Balaban *et al.* (2004),

are not observed in *M. smegmatis* cells in response to any of the four antibiotics which they tested (Aldridge *et al.* 2012). Asymmetry here refers to the inheritance of differentially aged cell poles (as described in Chapters 2 and 3), whereby cells which inherit the pole formed by their mother at birth (the new-pole inheritors) appear to have enhanced survival to the cell wall targeting antibiotics meropenem, cycloserine, and isoniazid compared to cells which inherit the pole formed at least two generations prior (the old-pole inheritors) which are apparently more tolerant to rifampicin (Aldridge *et al.* 2012).

Following this study, Wakamoto *et al.* also used similar custom microfluidics to determine the single-cell response of *M. smegmatis* to isoniazid. They also did not observe any cells that arrested their growth rate prior to exposure to drugs, demonstrating that the pre-exposure growth rate (exponential model) is identical between survivors and non-survivors (Wakamoto *et al.* 2013). Moreover, they demonstrate that surviving cells continue to grow in the presence of isoniazid, albeit at a greatly reduced rate (Wakamoto *et al.* 2013), consistent with suggestions that drug-tolerant *M. marinum* cells maintain growth (Adams *et al.* 2011), and that dormant cells retain biosynthetic capability (Betts *et al.* 2002; Sala *et al.* 2010). Similarly to the efflux pump mechanism of drug tolerance in *M. marinum*, isoniazid tolerance is correlated with transient and stochastic levels of expression of a bacterial protein: KatG (Wakamoto *et al.* 2013). KatG is a catalase-peroxidase that converts isoniazid from its pro-drug form into its active cidal form (Vilchèze and Jacobs 2007; Suarez *et al.* 2009), thus increased expression of KatG leads to increased conversion of isoniazid, and decreased survival. Cells which avoid stochastic expression of KatG are therefore more able to survive (Wakamoto *et al.* 2013).

Two investigations of single-cell antibiotic responses in mycobacteria—including pathogenic *M. tuberculosis*—were published in quick succession by the same group in 2015. They both used the same custom microfluidic device as Wakamoto *et al.* (2013) which permits very long-term observation of single cells, a necessity given the interdivision time of *M. tuberculosis* (~24 h), with up to 24 day continuous time-lapses reported.

Manina *et al.* investigated rRNA expression in *M. tuberculosis* single cells (Manina *et al.* 2015). Using an unstable GFP integrated into the chromosome at the *rrn* rRNA locus, they demonstrate large heterogeneity in ribosome activity between single cells, with growth rate positively correlated to GFP fluorescence—as predicted by ribosome scaling (Klumpp and Hwa 2014)—and negatively correlated to CV (i.e. cells that grow faster have more ribosomes and less variation). Interestingly, inheritance of GFP fluorescence (ribosomes) was equal between daughter cells in terms of total fluorescence, even given the smaller size of new-pole inheritors—asymmetry which is also observed by many other authors in *M. smegmatis* (such as Aldridge *et al.* 2012; Santi *et al.* 2013), as well as in my own data (Figure 3.8A and Table 3.1; Priestman *et al.* 2017)—suggesting that ribosome concentration is higher in new-pole inheritors (Manina *et al.* 2015). Given known correlations between growth rate and ribosome numbers (Klumpp and Hwa

2014), *M. tuberculosis* new-pole inheritors also have higher growth rates as expected (Manina *et al.* 2015), in agreement with my *M. smegmatis* data demonstrating higher growth rate in new-pole inheritors (Figure 3.8C and Table 3.1; Priestman *et al.* 2017). The authors tested the response of *M. tuberculosis* single cells to isoniazid, with survival measured by Sytox blue staining (which stains permeabilised cells). They observed no correlation between growth rate prior to isoniazid treatment and survival, similarly to Wakamoto *et al.* (2013), and did not observe any difference among survivors between non-growing cells and cells which could recover growth after removal of isoniazid (Manina *et al.* 2015). They did however note that new-pole inheritors lysed more frequently and more rapidly after the introduction of isoniazid compared to old-pole inheritors, in contrast to reports by Aldridge *et al.* (2012), implying a strong role for pole age in tolerance to isoniazid. They interestingly *only* observed new-pole inheritors to survive isoniazid exposure (Manina *et al.* 2015) even though their lysis rate was increased, although their numbers are too small to draw strong conclusions.

Closely following this report, Vaubourgeix *et al.* (2015) published a further investigation of the role of asymmetry in drug tolerance in *M. tuberculosis*. They investigated how the inheritance of proteins irreparably damaged by oxidation could affect antibiotic susceptibility, using the same custom microfluidics and time-lapse microscopy as Wakamoto *et al.* (2013) and Manina *et al.* (2015). In response to reactive oxygen species (ROS) and reactive nitrogen species (RNS), proteins accrue oxidative damage, usually in the form of carbonylation (Dukan and Nystrom 1998). Excessive oxidation can lead to the exposure of hydrophobic regions, misfolding, and toxic consequences (Hartl *et al.* 2011). Misfolded proteins are usually detected by hydrophobic-binding chaperones which target them for proteolysis, however some proteins can become so extensively carbonylated that they can no longer enter the proteolytic channel present on most general proteases (Grune *et al.* 2004), in which case they are considered to be irreversibly oxidised proteins (IOPs), which are usually toxic, and require constant sequestration by chaperones to prevent undue adverse effects. Vaubourgeix *et al.* demonstrate that IOPs accumulate under a variety of stresses relevant to immunological attack including nutrient deprivation, hydrogen peroxide (which generates ROS), and acidified sodium nitrite (which generates RNS). They additionally show that IOPs accumulate when *M. smegmatis* cells are exposed to sub-inhibitory concentrations of kanamycin—which targets ribosomal protein synthesis to increase the translation error rate—leading to aggregated patches of IOPs which are closely associated with the chaperone ClpP (Vaubourgeix *et al.* 2015). These patches are distributed unevenly along the cell, with the vast majority of IOPs near a single pole. This leads directly to asymmetric inheritance of the majority of the IOPs by a single daughter cell, and this has a significant detrimental effect on these daughters as shown by reduced linear elongation rate, and reduced survival when challenged again with a lethal concentration of kanamycin (Vaubourgeix *et al.* 2015). The same effect is observed when both *M. smegmatis* or *M. tuberculosis* single cells are exposed to clinically-relevant levels of isoniazid, indicating that accumulation of IOPs is not limited to

translation-targeting antibiotics—although the active form of isoniazid is likely a free radical that may cause oxidative damage itself. This report again stresses the importance of asymmetry in mycobacteria with respect to antibiotic tolerance, though the authors do not specify whether pole age is an important factor.

Further increasing the breadth of microfluidic investigations of mycobacterial drug tolerance, Richardson *et al.* determined the response of single *M. smegmatis* cells to rifampicin and attempted to determine factors that might predict survival (Richardson *et al.* 2016). They developed another novel custom microfluidic device permitting the long-term observation of mycobacteria while allowing precise control of their environment and growth medium. They observe that *M. smegmatis* cells which ultimately die after rifampicin exposure are likely to be at extremes of the cell cycle: small and early in the cell cycle, or large and late in the cell cycle, at least at the point of drug administration (Richardson *et al.* 2016). Tolerant cells conversely, while at generally normal length upon treatment, were much more likely to be born at a larger than average size, and significantly more likely to have inherited the old pole from their mother. This again highlights interesting and repeated observations of the importance of pole age in mycobacteria (Aldridge *et al.* 2012; Manina *et al.* 2015). In concordance with the other single-cell studies, they observed no significant difference in growth rate between survivors and non-survivors to rifampicin treatment (Richardson *et al.* 2016).

Most recently, the role of asymmetry in drug tolerance has been investigated more directly, by using an assay of metabolic activity in single cells which reflects the inheritance of cytoplasmic contents at birth. Cells that are more able to convert a fluorescent precursor (calcein) into its fluorescent form are less likely to divide after rifampicin treatment, indicating that survival is negatively correlated with the ability to metabolise calcein (Rego *et al.* 2017). The authors performed a screen of a transposon library to find insertions which would alter the fluorescence distribution of calcein. They identified an interesting gene—annotated as *mmpS3*—which greatly reduces variability in fluorescence between cells, which they renamed *lamA* (loss of asymmetry mutant A). In a clean $\Delta lamA$ background, susceptibility to rifampicin is greatly increased, which the authors hypothesise is due to the loss of tolerant sub-populations. Mycobacteria are largely thought to incorporate nascent cell wall material preferentially at the old pole (Thanky *et al.* 2007; Hett and Rubin 2008; Joyce *et al.* 2012; Aldridge *et al.* 2012; Santi *et al.* 2013; Meniche *et al.* 2014; Botella *et al.* 2017) which provides the basis for cell growth differences between daughter cells. This preferential unipolar growth is also thought to lead to the size difference between daughters, with the septum correctly placed at mid-cell and the period between septum formation and cytokinesis leading to greater elongation at the old pole and subsequent asymmetric division (Aldridge *et al.* 2012; Meniche *et al.* 2014; Botella *et al.* 2017). Therefore, the prevention of preferential unipolar incorporation might lead to symmetric division in $\Delta lamA$, and indeed the $\Delta lamA$ mutant appears to incorporate cell wall equally at both new and old poles, without affecting the overall growth rate of the cell (Rego *et al.*

2017). This symmetry is apparently caused by the derepression of cell wall synthesis at the new pole, usually performed by LamA (Rego *et al.* 2017). The authors hypothesise that asymmetry provides a useful mechanism to rapidly generate heterogeneity in the population, with sub-populations more able to survive certain stresses, such as antibiotic insult. In accordance with this hypothesis, symmetric division in $\Delta lamA$ appears to greatly reduce tolerance to rifampicin, vancomycin, and several β -lactams in both *M. smegmatis* and in *M. tuberculosis* (Rego *et al.* 2017).

4.1.6 Summary of this chapter

In this chapter I use *M. smegmatis* mc²155 to investigate some features that permit mycobacteria to survive exposure to the front-line antibiotic rifampicin—potentially the most important of the four standard regimen drugs—firstly at the batch culture population level, then at the single-cell level, extensively using techniques described and developed in Chapter 2.

The original basis of the project was to determine how growth rate affects survival, to attempt to resolve the discrepancy between the growth arrest that is observed to precede ampicillin exposure in *E. coli* (Balaban *et al.* 2004) and the lack of correlation reported in the context of isoniazid tolerance in *M. smegmatis* (Wakamoto *et al.* 2013; Manina *et al.* 2015). Specifically, given that isoniazid requires conversion to its active form by mycobacterial KatG (Vilchèze and Jacobs 2007; Suarez *et al.* 2009), any growth rate differences might be masked by the expression level of KatG. This work was started prior to the publication of evidence that this difference is also not observed prior to rifampicin exposure (Richardson *et al.* 2016), but this provides a useful comparison with my data.

Initially, I determined the role of population growth phase on survival. This has been described in *E. coli*, where 10^{-6} cells survive in exponential phase compared to 10^{-4} in late exponential phase, and 10^{-2} in stationary phase (Lewis 2007). Dormancy models in *M. tuberculosis* have demonstrated increased survival to rifampicin using gradual oxygen deprivation which may overlap somewhat with stationary phase (Wayne 1994), and others have investigated long-term nutrient starvation which greatly increases survival to rifampicin (Betts *et al.* 2002). Studies demonstrating increased dormancy and VBNC state in cells that are starved or maintained at long-term stationary phase also very much imply that survival increases to rifampicin under these conditions. However, a general comparison of survival after rifampicin treatment at different growth phases has not been described, and I use a batch culture method to investigate this. I also compare how the survival fraction changes with different initial conditions, such as in low carbon and nitrogen media, and in the alternative sole carbon sources used in Chapter 3: acetate and pyruvate.

Since survival—at least prior to the retraction of the two Maisonneuve *et al.* papers—has been

extensively linked to expression and activation of the TA systems, I also tested how survival at different growth phases in knockout *M. smegmatis* strains might differ. I received the same strains used by Frampton *et al.* (2012), and tested their response to rifampicin. In collaboration with a Master's student—Nicola Harris—I also designed several plasmids expressing different toxin and antitoxin combinations to permit overexpression of individual components in knockout backgrounds. These results were largely negative and are not discussed in depth here.

I then move on to single-cell time-lapse microscopy experiments, using an altered protocol in which rifampicin can be perfused into the ONIX growth chambers at will. Life and death at the single-cell level is—perhaps surprisingly—hard to determine, and I develop, test, and refine several single-cell measures of viability that can be coupled with time-lapse microscopy. Using the most accurate measure in my estimation, I then analyse the single-cell factors that are over-represented in cells that are able to survive rifampicin, making extensive use of the software I developed and described in Chapter 2.

4.2 Methods

4.2.1 OADC enrichment

Middlebrook 7H10 oleic acid–albumin–dextrose–catalase growth supplement (OADC; USBiological) is prepared by slowly adding 31.616 g OADC powder to 350 ml ddH₂O pre-warmed to 55 °C and stirring well on a hotplate. As much powder is permitted to dissolve as possible by heating and stirring continuously for approximately 15 min. The suspension is made up to 400 ml with ddH₂O, transferred to 50 ml Falcon tubes, and spun at 3000 *g* for 30 min at 4 °C. Supernatants are sterile filtered, and stored at 4 °C.

4.2.2 7H9 liquid medium

Difco™ Middlebrook 7H9 broth (BD) is prepared according to the manufacturer instructions, with the use of 0.05 % (v/v) tyloxapol—a non-hydrolysable detergent—instead of Tween-80. The preparation of tyloxapol stock solution is described in the Chapter 2 Methods section (Tyloxapol stock solution). 4.7 g 7H9 powder is added to 850 ml Milli-Q water (ddH₂O) containing 2 ml glycerol (to a final concentration of 0.2 % v/v) and mixed well, then made up to 875 ml with ddH₂O. The solution is autoclaved at 121 °C for 20 min, and permitted to cool. 100 ml OADC enrichment is added to the cooled 7H9 solution, followed by 25 ml of 20 % v/v tyloxapol stock solution, under sterile conditions. 7H9 medium is stored at 4 °C.

4.2.3 7H11-agar solid medium

Difco™ Mycobacteria 7H11 Agar (7H11-agar; BD) is prepared according to the manufacturer instructions, as follows: 21 g of 7H11-agar powder is dissolved in 900 ml ddH₂O containing 5 ml glycerol (to a final concentration of 0.5 % v/v), mixed well, autoclaved at 121 °C for 20 min, and permitted to cool in a 55 °C incubator for 1 h. The preparation is further cooled by stirring at 4 °C for 5 min before adding 100 ml OADC enrichment, and any other supplementation required (such as antibiotics) under sterile conditions. Approximately 25 ml is poured into 90 mm Petri dishes and permitted to set in a laminar flow hood for 10 min, then dried in the same hood for another 10 min, before storage at 4 °C.

4.2.4 Hartmans–de Bont minimal medium

Hartmans–de Bont minimal medium (HdB) is prepared as described in the Chapter 2 Materials section (Hartmans–de Bont minimal medium).

Variants of HdB were made by modifying components. For carbon-limited HdB (cHdB), the glycerol concentration is reduced to 0.08 % v/v (0.8 ml per litre). For nitrogen-limited HdB (nHdB), (NH₄)₂SO₄ is reduced to 1 mM, to 0.15 mM for nitrogen-deprived HdB (xHdB), and removed completely for nitrogen starvation experiments (ΔN HdB). For alternative carbon sources, as in Chapter 3 (Methods section: Variants of HdB), glycerol is replaced with 0.2 % w/v potassium acetate (Sigma) or 0.2 % w/v sodium pyruvate (Sigma).

4.2.5 Rifampicin stock

Rifampicin stock solution is prepared by dissolving rifampicin powder (Sigma) in dimethyl sulfoxide (DMSO; Sigma) to a final concentration of 32 mg ml⁻¹, approximately 1000×MIC for wild-type *M. smegmatis* (Alexander *et al.* 2003, see also below). Solutions are aliquoted and kept frozen at –80 °C, and discarded after use (to prevent any freeze–thaw cycles).

4.2.6 Theophylline stock

5× theophylline stock solution is prepared by dissolving powder (Sigma) to a final concentration of 10 mM in the growth medium of each particular experiment. Solutions are sterile filtered, and stored at 4 °C for up to a month.

4.2.7 Batch culture killing curves

To determine the kinetics of killing by antibiotics in batch culture, a simple approach assaying colony-forming units (CFUs) over time is used. *M. smegmatis* mc²155 (referred to generally

as wild-type) or other strains are inoculated from frozen glycerol stock using a sterile loop into 3–5 ml 7H9 or HdB medium. Cultures are incubated in a shaking incubator at 37 °C overnight twice (40–48 h) to stationary phase. Cultures are sub-cultured 1:200 in fresh medium (appropriate for the experiment, e.g. a different carbon source), and grown overnight (~16 h) to mid- to late exponential phase. OD₆₀₀ is measured for the culture, and 50 ml Falcon tubes are inoculated to OD₆₀₀ 0.05 in at least 20 ml of the appropriate medium in duplicate, with one designated as a control tube (i.e. no antibiotic) and the other as a treatment tube. Tubes are incubated at 37 °C in a shaking incubator.

At approximately 3 h intervals, 1 ml culture is taken from both tubes and serially diluted using a 96-well plate. The plate is prepared with 180 µl HdB or PBS in the first six rows of the plate, and enough columns for the target dilution. 20 µl of culture is added to alternate rows of the plate from each tube, allowing three technical replicates of the serial dilution. If OD₆₀₀ measurements are desired, they are measured using the remaining sample. Dilutions are performed using a multi-channel pipette taking 20 µl from each column and mixing it into the subsequent column. Fresh pipette tips are used for each dilution. To determine CFU counts, 20 µl is taken from the final dilution (or multiple dilutions in initial experiments) using the multi-channel pipette with three tips with empty spaces between each, and spotted onto a 7H11-agar plate. The spacing allows appropriate spreading of spots on the plate without resulting in mixing of spots, whilst maximising the space used on each plate allowing up to twelve spots per plate. For initial experiments, where the appropriate dilution was unknown, a broad range of dilutions was spotted, and subsequent CFU counts used for future runs to limit the requirement for large numbers of plates.

Plates are incubated for at least two nights in a 37 °C static incubator, until visible colonies can be observed. Colonies are counted using a colony counter and the CFUs/ml in the undiluted sample are calculated from the knowledge of the volume spotted and the dilution.

According to the experiment being performed, rifampicin is added to a final concentration of 5×MIC (160 µg ml⁻¹) to the treatment tube, and an equivalent volume of DMSO added to the control tube. Tubes are then wrapped completely in aluminium foil to reduce light-induced degradation. For all experiments, rifampicin treatment is continued for 48 h, at which point both control and treatment tubes are spun at 1690 *g* at room temperature for 10 min and the pellet resuspended in an identical volume of fresh medium. Cells are washed in this manner twice more before re-incubating the tubes and continuing the experiment.

Depending on the experiment, time-points are selected in advance to permit the broadest range of coverage without requiring too many overnight time-points to be taken. For example, if rifampicin is to be added after 16 h, the experiment is started at around 5 pm and left overnight, allowing drug to be added the subsequent morning and multiple time-points to be taken during

that day to record the initial action of the drug. Conversely, if rifampicin is to be added after 6 h, the experiment is started as early as possible in the morning to allow rifampicin to be added with sufficient time for multiple time-points on that same day.

4.2.8 Determination of rifampicin MIC by broth dilution

A 96-well clear plate (Greiner) is filled with 100 μl HdB in 10×8 wells. Solutions of 512 $\mu\text{l ml}^{-1}$ and 768 $\mu\text{l ml}^{-1}$ rifampicin are prepared by diluting rifampicin stock (32 mg ml^{-1}) in HdB. 100 μl of the 512 $\mu\text{l ml}^{-1}$ solution is added to the first well of the first 4 rows, and 100 μl of the 768 $\mu\text{l ml}^{-1}$ solution is added to the first well of the last 4 rows. Using a multichannel pipette, 100 μl of each column is taken and added to the subsequent column, resulting in a two-fold dilution series. The 100 μl excess volume from the ninth column is discarded, resulting in a 0 $\mu\text{g ml}^{-1}$ control column (tenth). The concentration layout of a typical plate is depicted in Table 4.1. 5 μl of undiluted *M. smegmatis* mc²155 stationary phase culture is added to all wells in rows 2–3 and 6–8 (leaving rows 1 and 5 as sterile controls), the plate is sealed and incubated at 37 °C in a shaking incubator for 4 d. Wells are then well-mixed by pipetting without introducing air bubbles, and the optical density of each well is read at 620 nm using a plate reader. The lowest concentration of rifampicin in a well with no detectable growth is taken to be the MIC.

	1	2	3	4	5	6	7	8	9	10
1–4	256	128	64	32	16	8	4	2	1	0
5–8	384	192	96	48	24	12	6	3	1.5	0

Table 4.1: Concentration of rifampicin in a broth dilution microtitre plate. Values depict concentration of rifampicin in $\mu\text{g ml}^{-1}$ in each column and row.

4.2.9 Determination of fluorescent protein half-life

Fluorescent protein half-life is determined by a plate-based time-course experiment in which fluorescence in a strain induced for fluorescent protein expression is placed in PBS (in the absence of inducer) and fluorescence measured over time using a plate reader. This is compared to an uninduced control, and PBS alone. Cultures are grown to mid-exponential phase, and induced for protein expression overnight (or not for controls), culture tubes are kept wrapped in aluminium foil throughout. Cultures are washed twice in PBS supplemented with 0.05 % tyloxapol (PBS-tyloxapol) by centrifugation at 1690 g for 10 min, then diluted to an OD_{600} of 0.5 in PBS-tyloxapol and incubated at 37 °C in a shaking incubator. Approximately every hour, 100 μl samples of induced and uninduced cultures, and a 100 μl PBS-tyloxapol control are placed into a black, flat-bottomed 96-well plate (Greiner), and fluorescence measured using a plate reader with an exposure time of 1 s, a 460 nm excitation filter, and a 520 nm emission filter. Fluorescence over time is fitted to an exponential decay curve, $N(t) = N_0 e^{-\lambda t}$, where $N(t)$ is

fluorescence at time t , N_0 is fluorescence at $t = 0$, and λ is the exponential decay constant. The half-life (τ) of the fluorescence signal is determined as $\tau = \ln 2 / \lambda$.

4.2.10 Viability staining of bacteria with CTC

M. smegmatis mc²155 cultures are grown in 7H9 for two days to stationary phase at 37 °C in a shaking incubator, then rifampicin added to 5×MIC (160 µg ml⁻¹), or an equivalent volume of DMSO for 48 h at 37 °C in a shaking incubator. 100 µl samples are removed and incubated with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to a final concentration of 40 µM (dissolved in ddH₂O) for 30 min at 37 °C. 20 µl of each sample is spread onto a glass microscope slide, and air-dried. 20 µl of Mowiol® 4-88 mounting medium (Sigma) is added, and a #1 thickness glass coverslip placed onto the slide. Slides are left to set overnight in the dark before imaging.

4.2.11 Cloning

pST5552

pST5552 is a mycobacterial shuttle vector containing both *E. coli* and mycobacterial origins of replication, with a kanamycin resistance cassette, EGFP with an upstream truncated constitutive Phsp60 promoter, and a theophylline riboswitch leader containing the ribosome-binding site (Seeliger *et al.* 2012). The riboswitch functions by preventing the binding of the transcript to the ribosome unless it has bound theophylline, it is both very tightly controlled, dose-dependent, and strongly inducible.

pRIEST5

pRIEST5 is a variant of pST5552 with a degradation tag fused to the C-terminus of EGFP. I initially cloned EGFP from pST5552 by polymerase chain reaction (PCR) using primers EGFP-F, which anneals to pST5552 at the EcoRI restriction site within EGFP, and EGFP-R4, which replaces the stop codon from EGFP with the ASV variant of the *ssrA* degradation tag (AANDENYAASV), and incorporates a SalI restriction site downstream of this tag (see Primers section below). This PCR product was cloned back into pST5552 using the restriction enzymes EcoRI and SalI to produce the pRIEST5 plasmid. Successful replacement of EGFP with EGFP-ASV in the pST5552 background was confirmed by Sanger sequencing with primers pST F and pST5552-rev3 (see Primers section below). *M. smegmatis* mc²155 was transformed by electroporation with pRIEST5.

Primers

Name	Sequence (5'–3')
EGFP-F	TGCAGAATTCGTGAGCAAG
EGFP-R4	AGCAGTCGACTTAAACTGATGCAGCGTAGTTTTTCGTCGTTTGCTGCCTTGTACAGCTCGTCCATG
pST F	CTTCTTGCACTCGGCATAGG
pST5552-rev3	AGCTGATCACCGCGGCCATG

4.3 Results

4.3.1 Determination of the MIC of rifampicin

The minimum inhibitory concentration (MIC) of rifampicin for *M. smegmatis* mc²155 used throughout this thesis was 32 µg ml⁻¹, as determined by Alexander *et al.* (2003). However, I wished to verify this under my conditions, and conducted a broth dilution assay in a 96-well plate (see Methods section: Determination of rifampicin MIC by broth dilution). I observed that the minimum concentration of rifampicin that prevented growth was 24–32 µg ml⁻¹ in 3 independent experiments, i.e. not significantly different from the published value.

4.3.2 Role of growth phase in antibiotic tolerance

It is known that many bacterial species have different susceptibilities to antibiotics under different growth phases, with survival generally increasing as the population growth rate falls. To determine whether this relationship holds for *M. smegmatis* cells in response to rifampicin, I used a batch cultures grown for different lengths of time before the addition of rifampicin for 48 h. After this period of exposure, cells were washed with fresh medium, and permitted to grow again. CFUs/ml were determined at approximately 3 hour intervals by serial dilution and spotting onto plates (see Methods section: Batch culture killing curves).

Growth phases were determined empirically by preliminary growth curves (Figure 4.3). Exponential phase was defined to occur after 6 hours when population growth is at maximal levels, stationary phase after 48 hours when growth begins to plateau, and an intermediate point—with intermediate growth—selected at 16 hours post inoculation termed “late exponential”.

Using these time-points, *M. smegmatis* cells were exposed to rifampicin at 5×MIC for 48 h and CFU counts determined before and after treatment to determine overall survival. Survival to this high dosage of rifampicin was dramatically affected by growth phase, with a 2-log increase in populations exposed to drug during stationary phase compared to those exposed during exponential phase (Figure 4.4D), with 0.08 ± 0.03 % (± the standard error of the mean

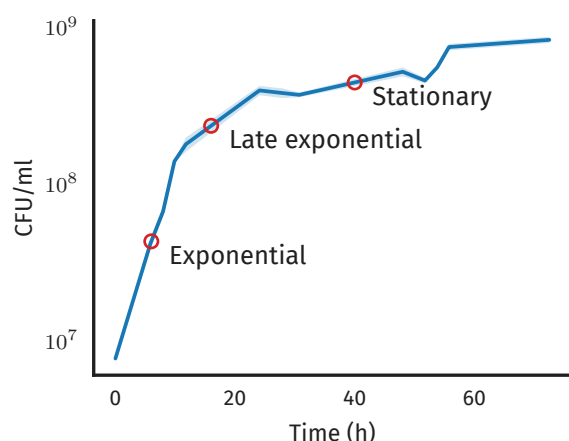


Figure 4.3: Representative growth curve of *M. smegmatis* cells grown in HdB minimal medium in batch culture. Bacteria were grown in Hartmans–de Bont minimal medium for up to 80 hours with colony-forming units measured approximately every three hours by serial dilution and plating on 7H11 agar. Time points for subsequent experiments at different growth phases were determined from this dataset, with “exponential” defined at 6 h after inoculation, “late” defined at 16 h, and “stationary” defined at 40 h, as shown by the red unfilled circles, and associated labels.

of multiple experiments, $n = 4$) of cells surviving at exponential (Figure 4.4A), 1.13 % ($n = 1$) at late exponential (Figure 4.4B), and 21.85 ± 6.17 % ($n = 7$) at stationary phase (Figure 4.4C).

I observed apparent increases in CFU/ml counts during the rifampicin exposure period that might indicate the acquisition of drug resistance (e.g. in Figure 4.4A), so I tested the MIC of cells pre- and post-exposure to rifampicin by two methods. I plated 100 μ l of culture on 7H11-agar plates supplemented with 32 μ g ml⁻¹ rifampicin (1 $\times$ MIC) prior to exposure and observed no visible colonies after several days incubation at 37 °C, and repeated this procedure with cells from the end of the experiment, and again observed no visible colonies on 7H11-agar plates supplemented with rifampicin. In addition, I repeated broth dilution assays at these same time-points, and observed no change in MIC in bacteria exposed to rifampicin in this manner.

4.3.3 Effect of changing nutrient conditions on survival to rifampicin—batch culture

Since nutrient deprivation has been considered to be a major factor in the genesis of persister cells, I investigated how restricting nutrients might affect population survival. Initially, I used nitrogen-modulation as a model for nutrient deprivation, by reducing the concentration of ammonium sulphate ((NH₄)₂SO₄) provided in the culture medium. Initial experiments utilised batch culture to determine killing kinetics, but this raises various issues that make accurate nutrient deprivation difficult since bacteria constantly consume nutrients as they grow. Thus, to attempt to investigate nutrient deprivation in a simple manner in batch culture, I adjusted the nitrogen levels of the starting culture medium, from 10 mM in the standard HdB medium, to 1 mM, and 0.15 mM, which I have termed nHdB and xHdB respectively (see Methods section).

The expectation of nitrogen limitation in this manner was that cells would be placed into a more antibiotic-tolerant state as a population, since pathways involved in persistence might be triggered by the nutrient deprivation signal. However, I did not observe this in my experiments. Survival was greatly reduced when cells were grown in nutrient-limited (nHdB) or nutrient-deprived (xHdB) media, even when cells were grown for 40 h before the addition of rifampicin

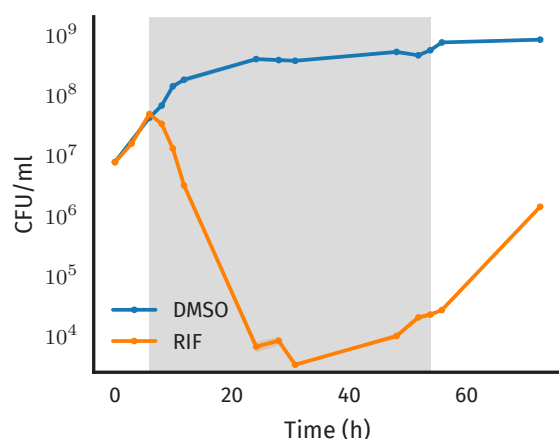
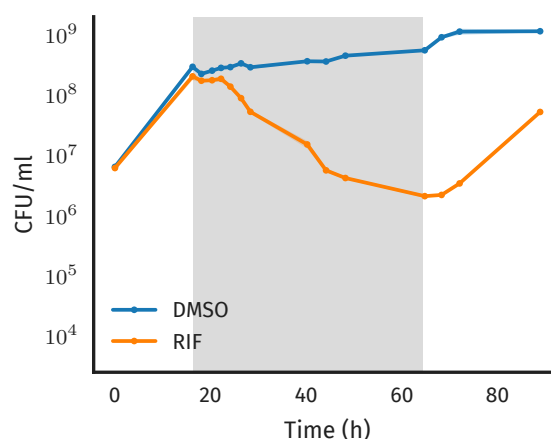
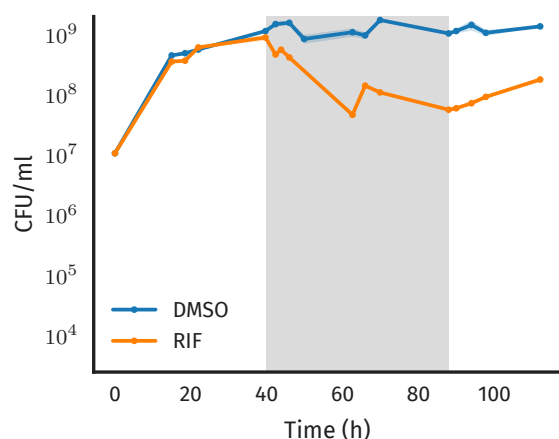
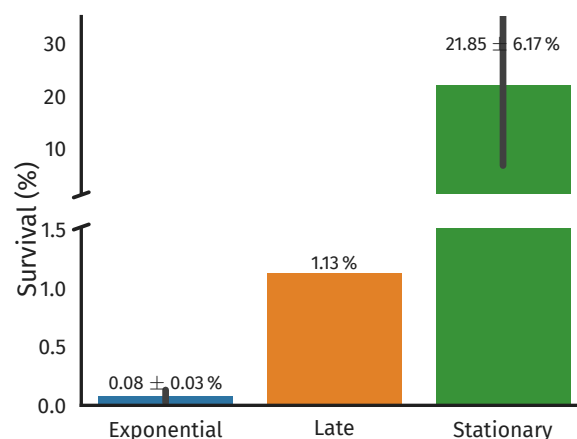
A: Exponential phase, 6 h**B:** Late exponential phase, 16 h**C:** Stationary phase, 40 h**D**

Figure 4.4: Impact of growth phase on antibiotic survival. *M. smegmatis* mc²155 was grown in Hartmans-de Bont minimal medium from an optical density of 0.05 at 600 nm for 6 h (A, exponential), 16 h (B, late exponential), or 40 h (C, stationary) before the addition of 5×MIC rifampicin (160 µg ml⁻¹) or an equivalent volume of DMSO for 48 h (grey box). Samples were taken approximately every three hours, serially diluted, and plated on 7H11-agar for counting of colony-forming units. CFUs/ml against time are plotted for DMSO control (blue line), and rifampicin cultures (orange) for each condition. (D) Percentage survival is determined by dividing CFUs/ml at the time-point that rifampicin was added by the lowest CFUs/ml count through the treatment period. Survival percentages were averaged from multiple independent experiments with error bars depicting the standard deviation, and number labels representing the mean survival ± the standard error of the mean. $n = 4$ for exponential, $n = 1$ for late exponential, and $n = 7$ for stationary. Panels A–C depict representative experiments.

(Figure 4.5D). Compared to 21.85 ± 6.17 % survival at stationary phase for cells grown in HdB (Figure 4.4), only 1.14 % of cells grown in nHdB (Figure 4.5A), and only 0.11 ± 0.10 % of cells grown in xHdB survived (Figure 4.5B). Cultures appear to reach very similar cell densities after 40 h in nitrogen-limited conditions (nHdB) compared to nitrogen-replete conditions (HdB), with approximately 10^9 CFUs/ml; thus a delayed stationary phase does not appear to explain the reduction in survival in this medium (Figure 4.5A). However, under nitrogen-deprived conditions (xHdB), cell density at 40 h is reduced to approximately 10^8 CFUs/ml. This may suggest that bacteria have not truly entered into stationary phase when they are deprived of nitrogen (Figure 4.5B)—although growth appears to have plateaued—and could potentially underlie some part of the dramatic change in survival compared to nitrogen-replete conditions.

The response to nitrogen limitation and deprivation is contrasted by the effect of limiting carbon in batch culture, in which reduction of glycerol from 0.2 % to 0.08 % does not cause such a drastic reduction, with survival in the same order of magnitude (4.09 ± 0.09 %; Figures 4.5C and 4.5D) as for standard HdB (21.85 ± 6.17 %; Figures 4.4C and 4.4D), at least when cells are exposed after 40 h of growth, hinting that there may be specific effects that nitrogen limitation induces that enhance killing with rifampicin.

I also tested the response to rifampicin exposure for cells grown with different sole carbon sources, including glycerol (standard HdB), pyruvate, and acetate, as in Chapter 3. When cells are grown in these sub-optimal carbon sources, survival is greatly increased when rifampicin is added ~ 6 h into the experiment—as for the exponential phase experiments above (Figure 4.4A)—with 0.04 ± 0.01 % survival in glycerol (Figure 4.6A), 47.42 ± 6.84 % survival in acetate (Figure 4.6B), and 33.64 ± 6.62 % survival in pyruvate (Figures 4.6C and 4.6D).

4.3.4 Role of the toxin–antitoxin systems in survival of *M. smegmatis*

Given the evidence that the toxin–antitoxin systems are involved in persistence and antibiotic tolerance, deletion mutants in each of the three systems as well as a triple deletion strain—originally created by, and kindly provided by Frampton *et al.* (2012)—were treated with $5 \times \text{MIC}$ rifampicin for 48 hours, and percentage survival determined by CFU counts before and after exposure, as previously. These strains were $\Delta mazEF$, $\Delta phd/doc$, $\Delta vapBC$, and $\Delta mazEF \Delta phd/doc \Delta vapBC$ ($\Delta triple$). Each strain, as well as mc^2155 wild-type, was treated 40 hours (stationary phase) post-inoculation, and percentage survival was determined after 48 hours (Figure 4.7). The stationary phase time-point was selected given the expectation that deletion of the TA systems would decrease survival. Survival was determined from kill-curves as 7.20 ± 4.22 % ($\pm$ the standard error of the mean) in $\Delta phd/doc$ (Figure 4.7A), 11.62 ± 7.95 % in $\Delta mazEF$ (Figure 4.7B), 41.75 ± 3.78 % in $\Delta vapBC$ (Figure 4.7C), and 31.61 ± 7.39 % in $\Delta triple$ (Figure 4.7D), as shown in Figure 4.7E. While this appears to show significantly reduced survival in $\Delta phd/doc$ and $\Delta mazEF$ compared to wild-type mc^2155 , these differences were not recapitulated in the $\Delta triple$

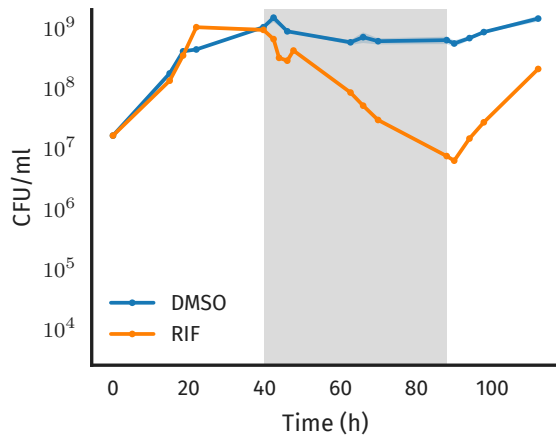
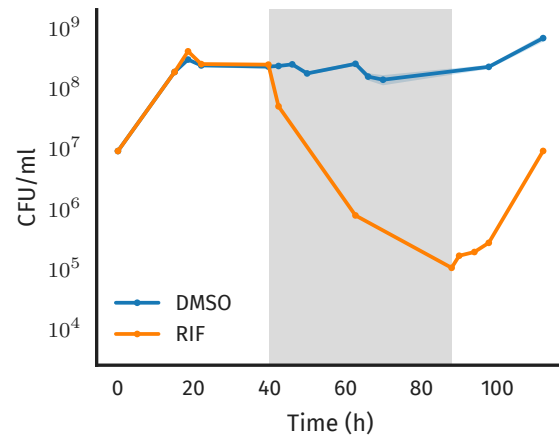
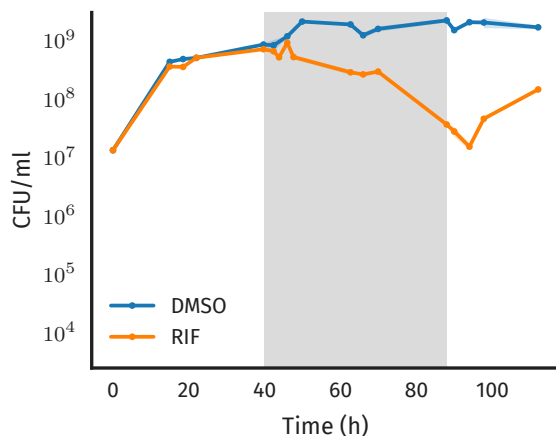
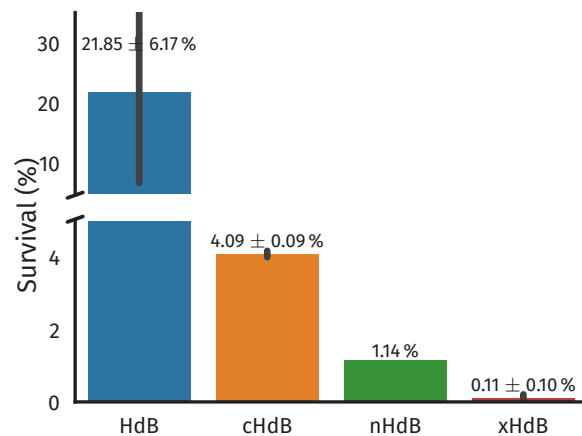
A: Nitrogen-limited nHdB**B:** Nitrogen-deprived xHdB**C:** Carbon-limited cHdB**D**

Figure 4.5: Impact of medium composition on survival at stationary phase. *M. smegmatis* mc²155 was grown to stationary phase similarly to Figure 4.4C in Hartmans-de Bont minimal medium variants containing different amounts of nitrogen or carbon. (A) Nitrogen-limited nHdB, 1 mM (NH₄)₂SO₄. (B) Nitrogen-deprived xHdB, 0.15 mM (NH₄)₂SO₄. (C) Carbon-limited cHdB, 0.08 % glycerol. CFUs/ml against time are plotted for DMSO control (blue line), and rifampicin cultures (orange) for each condition. (D) Percentage survival is determined by dividing CFUs/ml at the time-point that rifampicin was added by the lowest CFUs/ml count though the treatment period. Survival percentages were averaged from multiple independent experiments with error bars depicting the standard deviation, and number labels representing the mean survival ± the standard error of the mean. *n* = 1 for nHdB, *n* = 2 for xHdB, and *n* = 2 for cHdB. Survival for mc²155 at stationary phase is shown for comparison, with *n* = 7 (Figure 4.4). Panels A–C depict representative experiments.

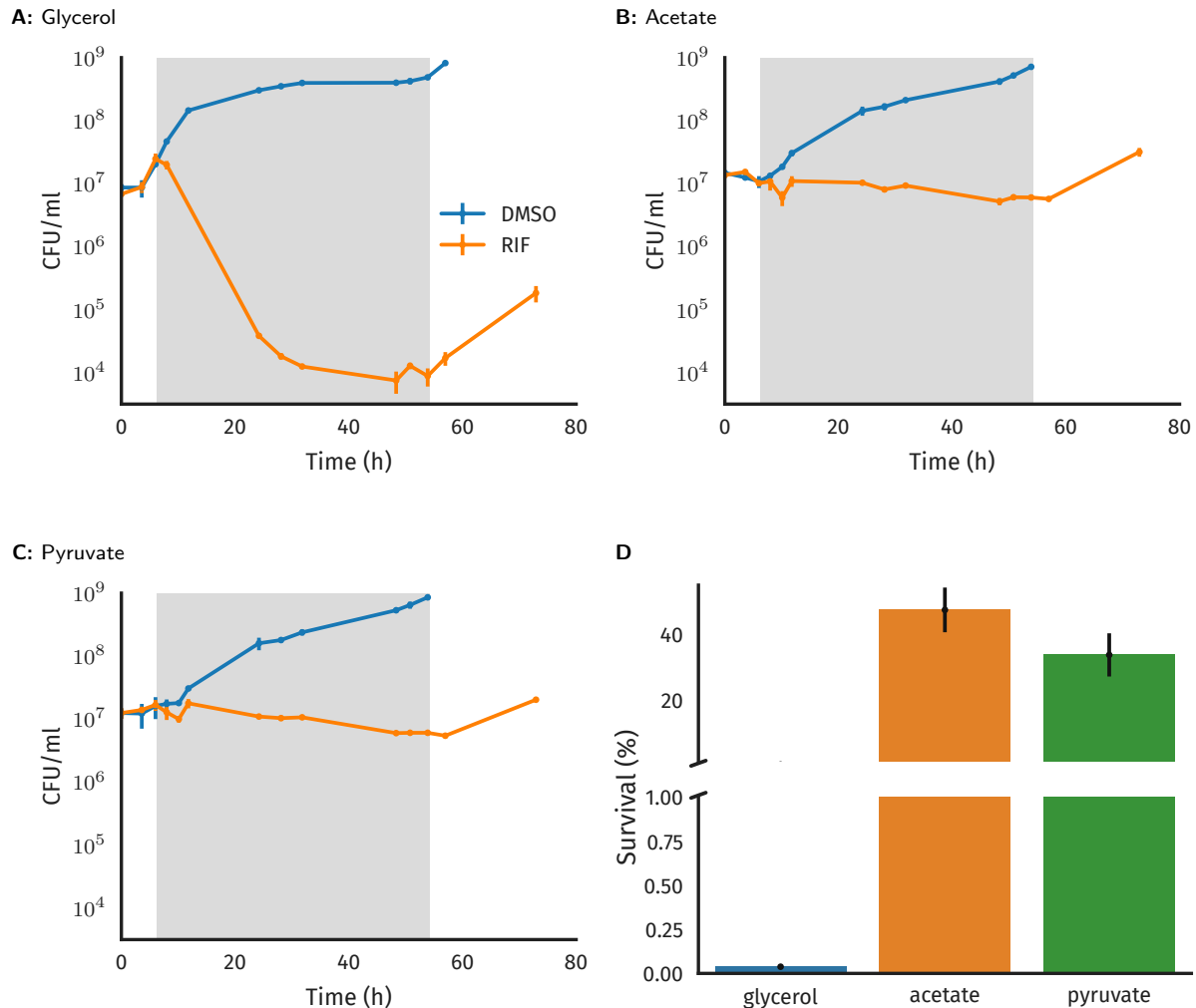


Figure 4.6: Impact of alternative sole carbon sources on survival at exponential phase. *M. smegmatis* mc²155 cells were grown to exponential phase similarly to Figure 4.4A in Hartmans-de Bont minimal medium variants containing either glycerol (A), acetate (B), or pyruvate (C). CFUs/ml against time are plotted for DMSO control (blue line), and cultures exposed to rifampicin (orange line) for each medium (panels A–C). Error bars denote the 95 % confidence interval of CFU counts of three independent serial dilutions from the same biological replicate. The grey shaded region denotes the time period in which rifampicin was introduced to the culture tubes. (D) Percentage survival as determined by dividing CFUs/ml at the time-point that rifampicin is added by the lowest CFUs/ml count throughout the treatment period. Error bars denote the 95 % confidence interval of the combined error in the CFU counts at both start and maximum positions. $n = 4$ for glycerol, $n = 2$ for acetate, and $n = 2$ for pyruvate.

strain (which was tested more times), indicating that these results may be anomalies of the inaccurate technique used, as shown by very large errors between experiments.

Survival at exponential phase was also assessed, with 0.283 % of cells surviving in $\Delta phd/doc$, 0.041 % in $\Delta mazEF$, 0.430 % in $\Delta vapBC$, and 0.060 % in $\Delta triple$ (Table 4.2). While this experiment was only performed once, these values are all within the same order of magnitude as for wild-type, and apparent increases in survival observed in $\Delta phd/doc$ and $\Delta vapBC$ were again not recapitulated in the $\Delta triple$ strain.

Strain	Exponential	<i>n</i>	Stationary	<i>n</i>
mc ² 155	0.08 ± 0.03 %	4	21.85 ± 6.17 %	7
$\Delta phd/doc$	0.283 %	1	7.20 ± 4.22 %	2
$\Delta mazEF$	0.041 %	1	11.62 ± 7.95 %	2
$\Delta vapBC$	0.430 %	1	41.75 ± 3.78 %	2
$\Delta triple$	0.060 %	1	31.61 ± 7.39 %	6

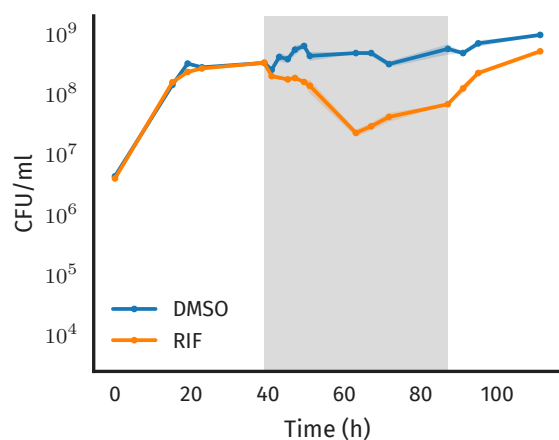
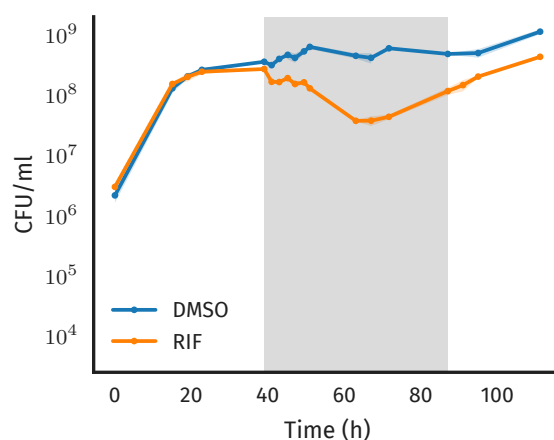
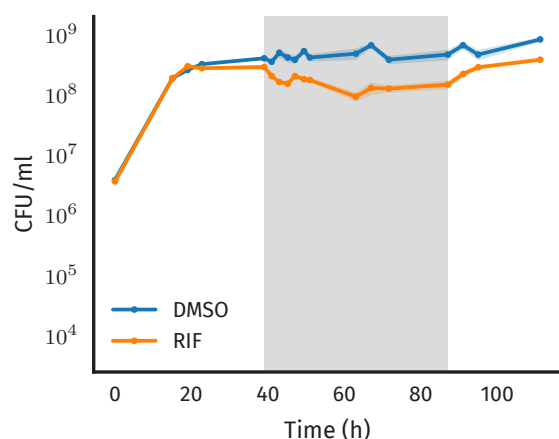
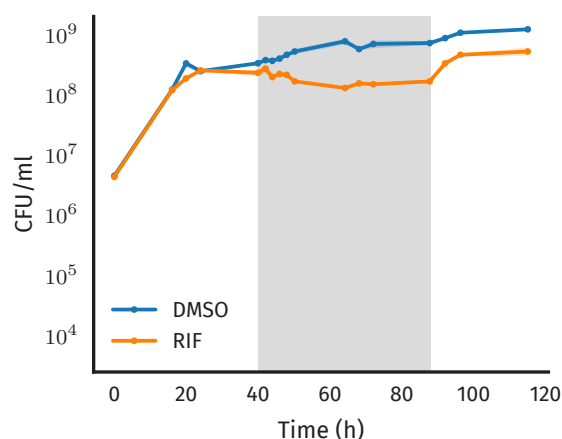
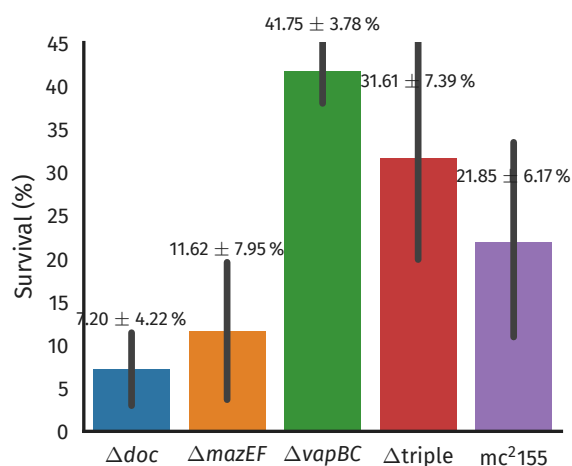
Table 4.2: Survival of toxin–antitoxin mutants at exponential and stationary phase. Cells were exposed to 5×MIC rifampicin (160 µg ml^{−1}) for 48 h and survival determined after 48 h by counting colony-forming units. Cells were grown for 6 h prior to treatment for exponential, and 40 h for stationary. See also Figures 4.4 and 4.7. Where *n*-values are greater than 1, percentage survival is expressed as the mean ± the standard error of the mean.

Given these results, it appears that the TA systems may not be involved in drug tolerance to rifampicin in *M. smegmatis*, at least under these *in vitro* conditions.

In addition to these preliminary results, in collaboration with Nicola Harris, various plasmids were constructed to attempt to dissect the role of the TA systems. This included inducible over-expression constructs of toxins, antitoxins, and both simultaneously in each of their corresponding deletion strain backgrounds. However, overexpression of either toxin, or antitoxin, had no effect on survival to rifampicin in wild-type or $\Delta triple$ backgrounds, indicating that these molecules may indeed not play a role in rifampicin tolerance under these conditions (unpublished data), I therefore decided not to pursue the toxin–antitoxin systems in *M. smegmatis* further.

4.3.5 Assaying life and death at the single-cell level

The batch culture experiments were severely limited by a lack of reproducibility—for example, survival of wild-type *M. smegmatis* at stationary phase in HdB ranges from 5.6 % to 37.9 % in individual experiments under ostensibly the same conditions. This may be due to biological factors such as the age of the inoculum, or variable lag time and initial growth rates which may affect cell numbers and states when rifampicin is added; or due to technical factors such as the accuracy of CFU counting and sampling of the cultures. In addition to these sources of variability, the chemical composition of the medium varies during the experiment due to consumption of nutrients during batch culture, thus confounding nutrient limitation experiments.

A: $\Delta phd/doc$ **B:** $\Delta mazEF$ **C:** $\Delta vapBC$ **D:** $\Delta triple$ **E****Figure 4.7: Impact of toxin–antitoxin system deletions on survival at stationary phase.**

M. smegmatis toxin–antitoxin mutants were grown in Hartmans–de Bont minimal medium to stationary phase as in Figure 4.4C. (A) $\Delta phd/doc$. (B) $\Delta mazEF$. (C) $\Delta vapBC$. (D) $\Delta triple$. CFUs/ml against time are plotted for DMSO control (blue line), and rifampicin cultures (orange) for each condition. (E) Percentage survival as determined by dividing CFUs/ml at the time-point that rifampicin was added by the lowest CFUs/ml count though the treatment period. Survival percentages were averaged from multiple independent experiments with error bars depicting the standard deviation, and number labels representing the mean survival $\pm$ the standard error of the mean. $n = 2$ for $\Delta phd/doc$, $n = 2$ for $\Delta mazEF$, $n = 2$ for $\Delta vapBC$, and $n = 6$ for $\Delta triple$. Survival for mc^2155 at stationary phase is shown for comparison, with $n = 7$ (Figure 4.4). Panels A–D depict representative experiments.

To address these issues, I moved to a single-cell approach using the ONIX microfluidic platform, as described in Chapter 2. The platform permits the rapid switching of media without causing bacteria to flow away, thus allows the long-term observation of single-cell responses to antibiotics during time-lapse experiments. Experiments were conducted as described in Chapter 2, except rifampicin was perfused with the growth medium after a period of pre-exposure growth for ~ 7 h. A period of pre- and post-exposure growth was required to allow statistics such as growth rate to be determined, thus an experimental design with 7 h pre-exposure to observe growth and to allow bacteria to adapt to the conditions, 7 h of rifampicin treatment, and a 7–10 h post-exposure observation was followed. The period of exposure would ideally be 48 h to emulate the batch culture experiments, but the plates were not stable enough to support such long time-lapses.

Initial experiments simply involved determining cell survival by growth in the post-exposure period, which is an accurate measure of viability, but does not necessarily distinguish all viable cells: i.e. cells that regrow can be considered to be alive, but cells that do not regrow cannot be assumed to be dead, especially in the somewhat brief 7 h window in which cells are observed post-exposure. Indeed, preliminary experiments showed that very few cells could be observed to regrow when treated with rifampicin in the microfluidic device. Cells present prior to the addition of rifampicin can be tracked as lineages and their elongation and growth rates determined after removal of the drug. In one experiment, no lineages of 71 lineages present upon treatment can be observed to regrow after exposure to $5\times\text{MIC}$ rifampicin. I also tested how cells reacted to $1\times\text{MIC}$ rifampicin, and only 7 of 47 pre-treatment lineages regrow (14.9 % survival). An example of a cell that regrows after rifampicin is removed is shown in Figure 4.8.

To investigate the drastic decrease in survival observed for cells cultivated in limited nitrogen (Figure 4.5), I also cultivated *M. smegmatis* mc²155 cells starved of nitrogen and observed their response to rifampicin by time-lapse microscopy. Cells were grown from glycerol stocks in standard HdB containing 15 mM $(\text{NH}_4)_2\text{SO}_4$ for approximately 48 h before sub-culturing in HdB containing no nitrogen (ΔN HdB) at 17 h and 5 h prior to the start of imaging. Cells were subsequently placed into a microfluidic plate and cultured for an additional 7 h in ΔN HdB. After 7 h growth, the medium was switched to ΔN HdB containing $5\times\text{MIC}$ rifampicin. This treatment was maintained for 7 h before switching medium to fresh standard HdB to observe any regrowth as above. This was designed to determine the difference in response to rifampicin after 24 h and 12 h starvation.

Initial cell densities were significantly different between the populations, even though cells were diluted to the same optical density (OD_{600} 0.05) prior to cultivation in the plate, with 142 cells observed in the initial frame for the 24 h starvation experiment and 36 cells observed for the 12 h starvation experiment. This may reflect differences in size between the two populations, where cells become smaller with starvation, thus contribute less to optical density. Indeed, simply

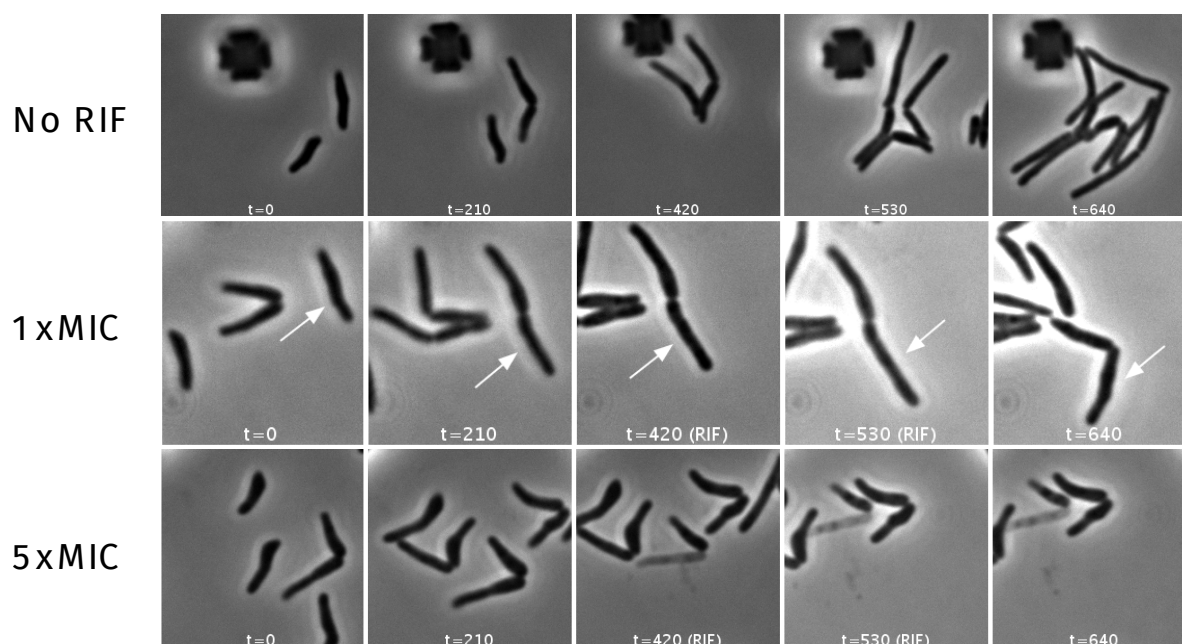


Figure 4.8: Time-lapse microscopy of cells exposed to rifampicin and monitored for regrowth. *M. smegmatis* mc²155 cells were grown at 37 °C in a B04A-03 microfluidic plate (Millipore) with Hartmans-de Bont minimal medium (HdB) for 7 h, the medium was switched to HdB containing either no rifampicin, 1×MIC rifampicin (32 µg ml⁻¹), or 5×MIC rifampicin for 7 h, before switching back to fresh medium for another 7 h. Images were acquired at 15 min intervals on a Zeiss AxioVert 200M inverted microscope, with a 100× objective (Zeiss Plan-Apochromat 100× 1.40 Oil Ph3) with phase-contrast acquired with a Hamamatsu EM-CCD C9100-02 camera. In-focus images were obtained by the use of software autofocus (HClmage), and 1 µm-spaced Z-stacks. The figure depicts three growth chambers from the experiment, exposed to no rifampicin (top), 1×MIC (middle), or 5×MIC (bottom), cropped to highlight single cells. Frames every 210 min are shown to represent the whole time-lapse, with rifampicin being added in the third frame depicted. A cell that regrows is highlighted with a white arrow in the 1×MIC experiment, and a cell that lyses can be observed from the $t = 420$ frame in the 5×MIC experiment.

determining the length of cells in the first frame reveals that cells which have been starved for a longer period are shorter, with 3.33 µm [3.21–3.47] (95 % confidence interval) for the 24 h experiment compared to 3.62 µm [3.33–3.92] for the 12 h experiment, though this difference is not statistically significant. Since these cells are at random points within their cell cycle, their lengths might be expected to be inherently variable. When birth length for cells which are observed to divide is determined, the cells in the 24 h experiment are significantly smaller than those in the 12 h experiment, with 4.01 µm [3.91–4.10] and 4.401 µm [4.26–4.56] respectively.

Division frequency in the initial 7 h incubation period was also markedly different between the two experiments, with only 29 division events observed in the 24 h experiment (20.4 %), compared to 41 division events observed in the 12 h experiment (114 %). This implies a significant lag phase associated with long-term starvation, or greatly arrested growth, though this was not reflected by mean interdivision rate, with 1.87 h [1.56–2.21] and 1.98 h [1.78–2.17] respectively, a non-significant difference. However, this number only reflects the interdivision time for cells that divide at least once during the growth period; the true division rate is probably reflected more accurately by the number of divisions observed. Reflecting this difference in division rate,

cells starved for the longer period also have significantly reduced single-cell growth rates with 0.188 h^{-1} [0.177–0.199] compared to 0.239 h^{-1} [0.223–0.254] for the 24 h and 12 h experiments respectively.

Compared to unstarved control cells, starved cells have a noticeably increased rate of apparent lysis, with rapid loss of contrast between frames observed (e.g. frame 3 of Figure 4.9). For cells that could be tracked, of 70 cells present at the point of addition of rifampicin, only 2 cells are observed to lyse after 12 h starvation (2.86 %), compared to 42 lysis events observed among the 273 cells present at the start of treatment after 24 h starvation (15.4 %). This might help to explain the difference in response to rifampicin in batch culture when cells are cultivated under nitrogen-limiting conditions (Figure 4.5).

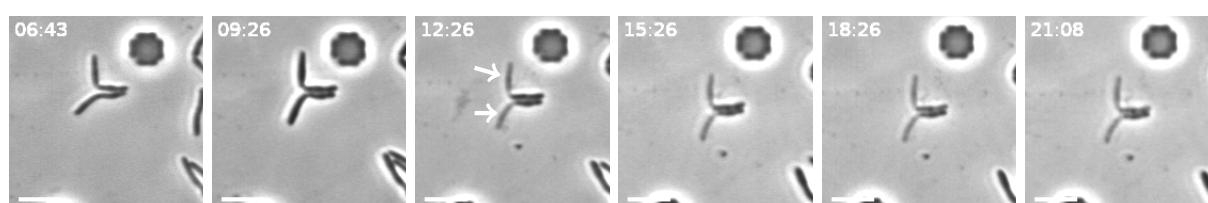


Figure 4.9: Time-lapse microscopy of cells exposed to rifampicin in the absence of a nitrogen source. Cells were pre-cultured in Hartmans-de Bont minimal medium (HdB) completely lacking $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source (ΔN) for 17 h, then loaded into a B04A-03 microfluidic plate. Cells were perfused with ΔN for 7 h. The medium was switched to ΔN containing $5\times\text{MIC}$ rifampicin for 7 h (frames 2–4), then switched to nitrogen-replete HdB (15 mM $(\text{NH}_4)_2\text{SO}_4$) for the remainder of the experiment (frames 5–6). Images were acquired at 15 min intervals on a Zeiss AxioVert 200M inverted microscope, with a $63\times$ objective (Zeiss Plan-Apochromat $63\times$ 1.40 Oil Ph3) with phase-contrast captured with a Hamamatsu EM-CCD C9100-02 camera. The time since the start of the time-lapse is shown in the top left corner of each frame, and the scale bar in the bottom left denotes $5 \mu\text{m}$. Cells which appear to lyse are highlighted with white arrows in frame 3.

Post-exposure regrowth under standard conditions—while a definitive indicator of viability—is not consistent with expected survival, with much fewer cells observed to regrow compared to batch culture expectations. This may indicate that the 7–10 h post-exposure window may not be sufficient to allow all survivors to regrow, which might explain why no cells are observed regrowing in the $5\times\text{MIC}$ preliminary experiment. In addition to this, lysis—while a definitive indicator of *lack* of viability—is clearly not a common occurrence under standard conditions, rather being indicative of a starvation condition induced by lack of nitrogen. Therefore, I investigated methods to determine viability at the single-cell level in the absence of regrowth or lysis. These methods can be divided into those that identify dead cells, and those that identify live cells.

Staining dead cells: propidium iodide

Propidium iodide (PI) is a chemical that binds strongly to DNA and fluoresces when bound. It is unable to cross the cell membrane, thus can only stain cells when this is compromised, which is a strong indicator that cells are dead. PI was added at $10 \mu\text{g ml}^{-1}$ throughout the time-lapses, and both fluorescent and phase-contrast channels were acquired during each experiment. Cells

were observed to become fluorescent as rifampicin was added and throughout the exposure period (Figure 4.10).

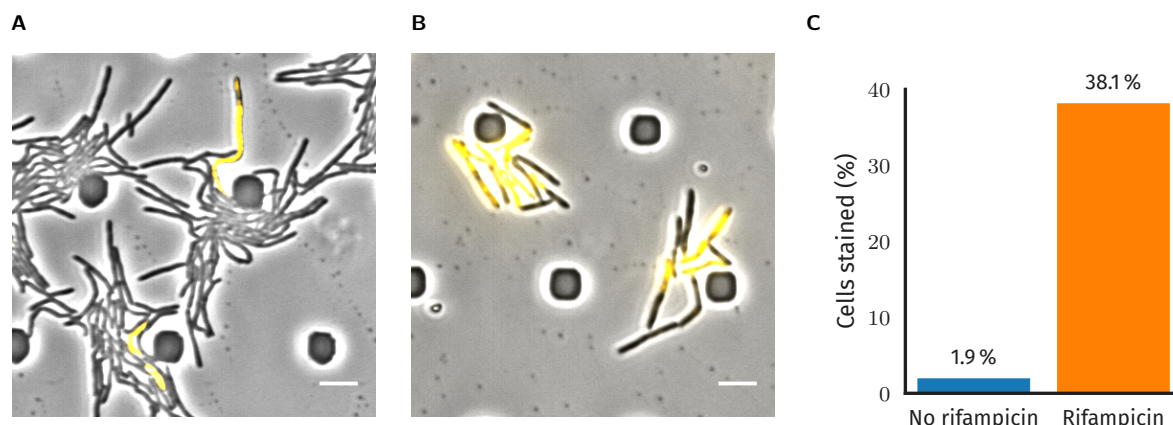


Figure 4.10: Propidium iodide staining of dead bacilli. *M. smegmatis* mc²155 cells were grown in a microfluidic chamber with Hartmans–de Bont minimal medium supplemented with propidium iodide (HdB/PI), at 37 °C in a B04A-03 microfluidic plate (Millipore). Images were acquired at 15 min intervals on a Zeiss AxioVert 200M inverted microscope, with a 63× objective (Zeiss Plan-Apochromat 63× 1.40 Oil Ph3 M27) with phase-contrast (200 ms exposure) and fluorescence (600 ms exposure, TxRed-4040C filter (Semrock): excitation 562/35 nm, emission 624/40 nm, and 593 nm dichroic beam splitter) channels acquired with a Hamamatsu EM-CCD C9100-02 camera. In-focus images were obtained by the use of software autofocus (HCIImage) and 1 μm-spaced Z-stacks. (A) Cells were cultured with constant flow of HdB/PI for 22 h. (B) Cells were cultured with constant flow of HdB/PI for 7 h, followed by HdB/PI with 160 μg ml⁻¹ rifampicin (5×MIC) for 7 h, and returned to HdB/PI for a further 8 h. Panels A and B depict merged fluorescence (yellow) and phase-contrast (grey) channels at the 14 h time-point (when rifampicin is removed). Stained cells are presumed to be dead cells and appear prominently and brightly in yellow. (C) Cells from all fields at the 14 h time-point were counted manually, and the proportion of cells stained is shown in this panel. 6 of 308 cells were stained in the control chamber, and 43 of 113 cells were stained in the rifampicin chamber.

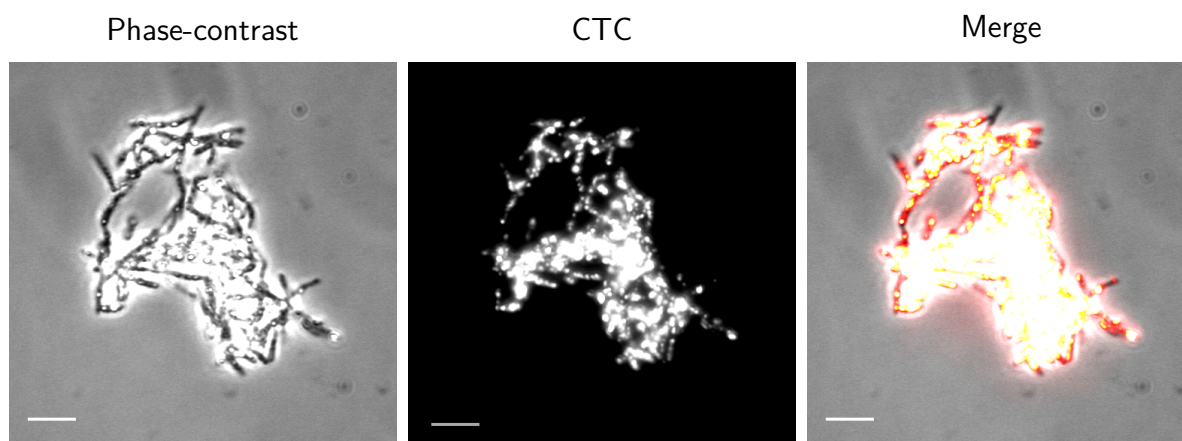
To determine survival in a manner that might be compared with batch culture CFU measures of survival, I counted all cells in all fields of a time-lapse, and determined what percentage were stained with PI. In one experiment, 6 of 308 total cells (1.9 %) were stained after 14 h of growth in HdB, and 43 of 113 cells (38.1 %) were stained when cells were grown for 7 h in HdB and treated with 5×MIC rifampicin for 7 h (Figure 4.10). Approximately 8.35 % [6.79–10.09] (95 % confidence interval) of cells survive after approximately 6 h exposure in the exponential phase batch culture model¹, equivalent to a death proportion of 89.91–93.21 %. Given this rate of death in the batch culture model, a similar rate of staining by PI might be expected, but instead less than half this number appear stained (Figure 4.10C). This indicates that either the batch culture experiments overestimate the number of dead cells, or staining with PI underestimates this number. Overestimation might indicate the presence of non-culturable cells that would reduce the apparent number of surviving cells in batch culture, since cells are exclusively determined as viable by plating on 7H11-agar. Underestimation by PI staining could indicate that cells take a long time to take up the dye after death, even after rifampicin is removed, or

¹Determined by dividing the CFUs/ml at the closest point to 7 h exposure by the CFUs/ml at the point of addition of rifampicin, e.g. in Figure 4.4A

that membrane integrity in *M. smegmatis* is more robust after death compared to other bacterial species. Indeed, the mycobacterial cell envelope is highly complex and contains an external envelope—sometimes referred to as the “mycomembrane”—that is very hydrophobic and may exclude the dye even after cell death.

Staining live cells: 5-cyano-2,3-ditolyl tetrazolium chloride

A: No rifampicin



B: 5×MIC rifampicin

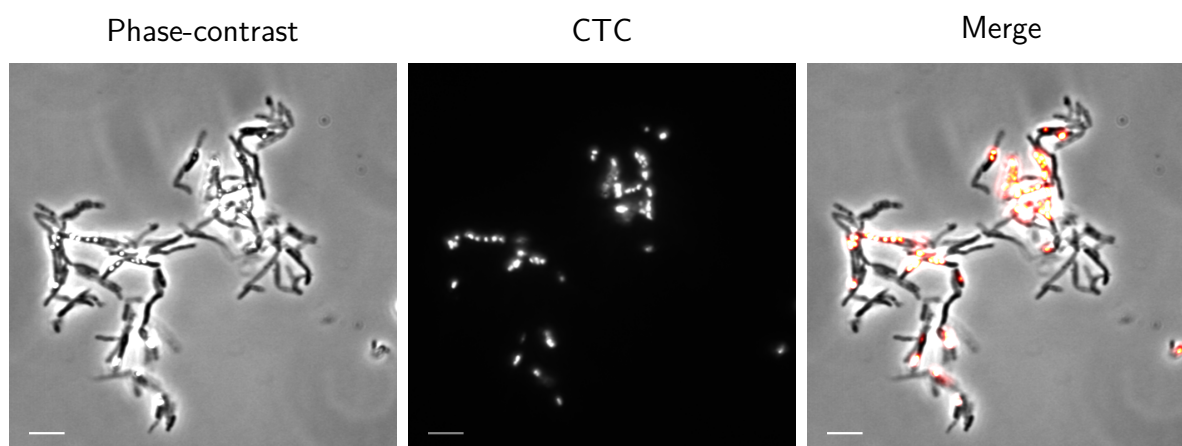


Figure 4.11: Staining of *M. smegmatis* viable cells with 5-cyano-2,3-ditolyl tetrazolium chloride. Two *M. smegmatis* mc²155 cultures were grown in 7H9 for two days to stationary phase, then rifampicin added to 5×MIC (160 µg ml⁻¹), or an equivalent volume of DMSO for 48 h at 37 °C. Samples were incubated with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) for 30 min in the dark at a concentration of 40 µM at 37 °C. Cells were dried onto a microscope slide, and mounted with Mowiol and a cover-slip. Images were acquired with a Zeiss AxioVert 200M inverted microscope with a 100× objective (Zeiss Plan-Apochromat 100× 1.40 Oil Ph3) with phase-contrast (100 ms exposure) and fluorescence (1 s exposure; FITC Quad filter cube: excitation 485/20 nm, emission 520/21 nm) channels acquired with a Hamamatsu EM-CCD C9100-02 camera. For no rifampicin control (A) and cells incubated with 5×MIC rifampicin (B) phase, fluorescence, and merged channels (with fluorescence in red) are shown from left to right. The scale bar denotes 5 µm.

Since the effects of PI staining were not a conclusive measure of cell viability, or rather that PI might not be a reliable indicator of *lack* of viability, I turned to more direct measures of

cell viability. 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is a compound that is converted from its non-fluorescent tetrazolium salt form into an insoluble—and thus retained by the cell—fluorescent formazan by the redox activity of the electron transport chain. Thus CTC can provide a direct measure of metabolic activity, and viability, of cells. To determine the utility of CTC as a viability measure in single cells, I stained untreated and treated *M. smegmatis* mc²155 cells with CTC and mounted them on microscopic slides, and imaged these with a fluorescent microscope (see Methods section: Viability staining of bacteria with CTC).

CTC appears to stain untreated cells more readily than treated cells, indicating that it might function correctly, at least in a qualitative sense (Figures 4.11A and 4.11B). However, there are severe apparent changes in morphology in stained bacteria noticeable on the phase-contrast channel, with large occlusions and bright puncta visible (Figure 4.11). This reflects one of the underlying problems with the use of CTC—it is toxic, thus can only be used as an endpoint measure of death. Ideally I wished to be able to observe death dynamically, allowing access to details such as time-of-death, which an endpoint measure is not able to capture. Additionally, growth rates post-exposure were of interest, and factors such as lag time have been strongly implicated in the generation of persister cells. These requirements were not compatible with a toxic endpoint dye, I therefore tested genetic approaches.

Dynamic viability: unstable EGFP

To obtain such a dynamic and non-lethal measure, I considered inducible expression of plasmid-borne fluorescent proteins such as EGFP. Theoretically, an appropriate system would show viable cells as highly fluorescent, with dead cells losing this as they died. However, EGFP is highly stable within bacterial cells, with a half-life of up to seven days in mycobacteria (Tombolini *et al.* 1997; Blokpoel *et al.* 2003), thus dead cells that retain some structural integrity upon death will retain EGFP fluorescence. Fluorescent protein stability can be modified by the addition of an 11-amino acid C-terminal degradation tag (AANDENYALAA) that targets proteins for *ssrA*-mediated proteolysis (Muto *et al.* 1998). The final three amino acids (native LAA) can modify the rate at which proteolysis occurs (Keiler and Sauer 1996). Such variants have been tested in *M. smegmatis* by Blokpoel *et al.* who tested the half-life of GFPmut2 fused to the degradation tag with LAA, ASV, and AAV terminal tripeptides, and observed half-lives of 165, 110, and 70 min respectively, compared to an effectively infinite stability observed in untagged GFPmut2 (Blokpoel *et al.* 2003). The authors used an acetamide-inducible system, which is a solvent, and given that the ONIX microfluidic plates are somewhat incompatible with solvents, I decided to clone the C-terminal tags into a different expression vector derived from pST5552 (Seeliger *et al.* 2012), which encodes EGFP controlled by a theophylline-inducible riboswitch. Additionally, EGFP has a higher quantum yield and a faster maturation time compared to GFPmut2 (McRae *et al.* 2005; Iizuka *et al.* 2011), and the riboswitch is much less leaky than the

acetamide-inducible system (Schnappinger and Ehrt 2014).

I created a *M. smegmatis* mc²155 strain carrying the plasmid pRIEST5, encoding the ASV unstable variant of EGFP (EGFP-ASV) under the control of a theophylline-inducible promoter (see Methods section: Cloning for construction of this plasmid). The half-life of EGFP-ASV fluorescence was determined by a plate reader time-course experiment (see Methods section: Determination of fluorescent protein half-life) as 41.9 ± 7.0 min (standard error of the mean of three independent experiments) as shown in Figure 4.12.

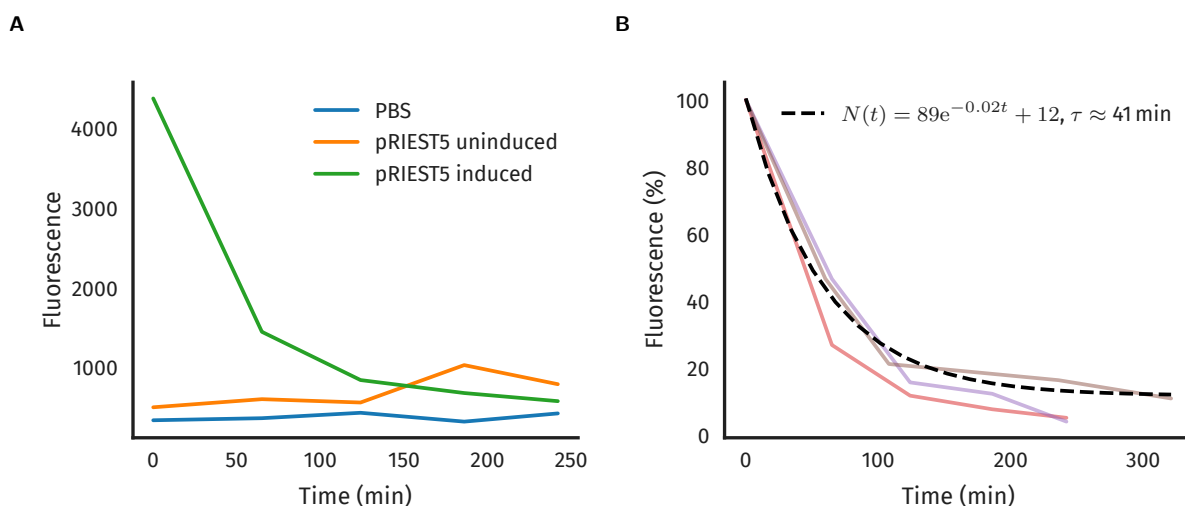


Figure 4.12: Determination of EGFP-ASV half-life. *M. smegmatis* mc²155 cells harbouring the pRIEST5 plasmid were grown in Hartmans-de Bont minimal medium supplemented with $50 \mu\text{g ml}^{-1}$ kanamycin, in the presence or absence of 2 mM theophylline (induced and uninduced respectively), transferred into PBS and EGFP fluorescence measured over time. (A) Representative experiment showing fluorescence (arbitrary units) over time for a PBS control, uninduced pRIEST5, and induced pRIEST5. (B) Three independent repeats of the same experiment are depicted (induced sample only), with mean uninduced fluorescence subtracted as background, and as a percentage of fluorescence at $t = 0$ (purple faded lines). The black dotted line shows an exponential decay curve ($N(t) = N_0 e^{-\lambda t}$) fitted to the mean of all three experiments, with its formula and calculated half-life (defined as $\tau = \ln 2 / \lambda$) shown in the panel legend.

I envisioned two potential mechanisms by which unstable EGFP-ASV fluorescence could present: ideally cells that died would lose the ability to produce new mRNA, would stop expressing EGFP-ASV, and the remaining pool of EGFP-ASV would be degraded by proteases remaining within the cell; alternatively the degradation machinery could be disabled resulting in EGFP-ASV present within the cell being retained for long periods of time, in which event I would modify experiments to stop inducing expression, allowing viable cells to degrade EGFP-ASV and lose fluorescence over time. However, conceptually it would be impossible to distinguish between the two models if EGFP-ASV was induced prior to introduction of rifampicin and the inducer retained, since cells which remained fluorescent could be both interpreted as having no viable proteases thus dead, or retaining the ability to express protein and thus alive. Therefore, the second approach was taken, cells were induced for fluorescence in the 7 h pre-treatment period, then the inducer was removed when $1 \times \text{MIC}$ rifampicin was added for the 7 h treatment

period (note $1 \times \text{MIC}$ was used to ensure that survivors could be observed). When rifampicin was washed out with fresh medium, inducer was again added for a 2 h period then removed again (Figure 4.13 for an example microcolony).

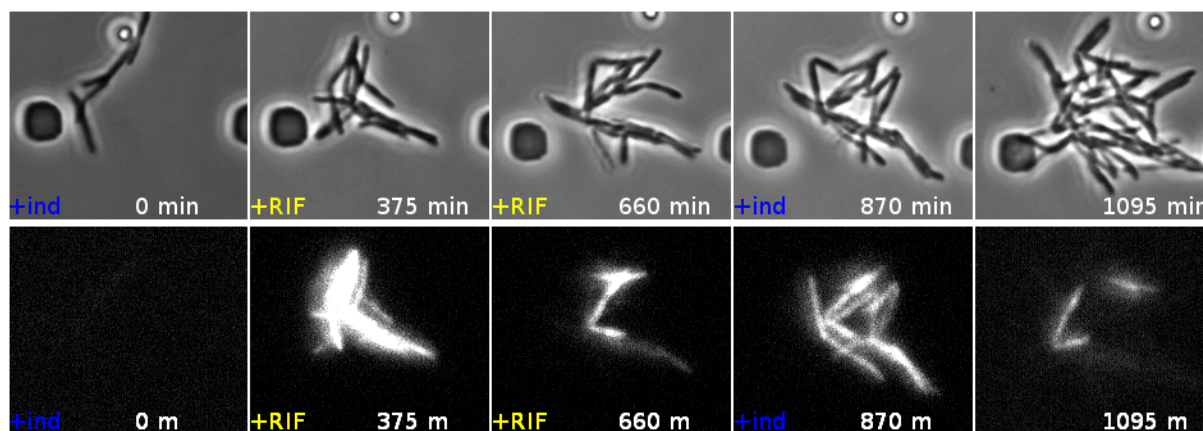


Figure 4.13: Example of a microcolony carrying unstable EGFP-ASV exposed to rifampicin. *M. smegmatis* cells carrying the pRIEST5 plasmid, with an inducible unstable EGFP (EGFP-ASV) cassette, were grown at 37 °C in a B04A-03 microfluidic plate (Millipore). Hartmans-de Bont minimal medium was supplemented with inducer (2 mM theophylline) in the pre-treatment 7 h window (first frame), then medium with $1 \times \text{MIC}$ rifampicin and no inducer added for 7 h (second and third frames). Rifampicin was washed out and inducer re-introduced for 2 h (fourth frame), followed by growth in fresh medium without inducer (fifth frame). These periods are annotated on the figure, with phase-contrast images on the top row, and fluorescence on the bottom row. Images were acquired at 15 min intervals on a Zeiss AxioVert 200M inverted microscope, with a $63\times$ objective (Zeiss Plan-Apochromat 63×1.40 Oil Ph3 M27) with phase-contrast (200 ms exposure, 6.4 V lamp power) and fluorescence (1 s exposure, GFP-3035B filter (Semrock): excitation 472/30 nm, emission 520/35 nm, and 495 nm dichroic beam splitter) channels acquired with a Hamamatsu EM-CCD C9100-02 camera. In-focus images were obtained by the use of software autofocus (HCIImage) and $1\ \mu\text{m}$ -spaced Z-stacks.

In the example time-lapse, cells can be observed increasing their fluorescent signal upon induction of EGFP-ASV as they grow and divide (Figure 4.13, frames 1–2) with relatively uniform expression, though the experiment is hampered by the overlapping of cells. When rifampicin is added, and theophylline withdrawn, the EGFP signal is lost progressively over the 7 h treatment window, except for in three cells which did not lose fluorescence (frame 3). When theophylline is re-introduced (frame 4), fluorescence increases in almost all the cells that lost fluorescence in the microcolony, and then lost again when theophylline is again withdrawn (frame 5). This form of experiment is difficult to interpret: the cells that retain fluorescence can be assumed to have lost the ability to degrade EGFP-ASV, and thus to die early in the treatment period, but the number of cells which display this behaviour is low. Indeed, bacteria might die at any point within the rifampicin exposure period (as seen in the PI experiments), thus the level of EGFP-ASV remaining within the cell at the point of death will be highly variable. This means that cells without fluorescence at the end of the treatment window cannot be assumed to be alive. This is demonstrated by the inability to induce fluorescence in the post-treatment induction step in several cells. This trial experiment convinced me that unstable EGFP-ASV expression probably over-complicates the apparently simple question of whether a cell is alive or not. It is also flawed as a dynamic measure of death, since fluorescence levels at time-of-death

would be highly variable.

Metabolic activity: inducible EGFP

This experiment did however lead to the final measure of viability, simple induction of a fluorescent protein in the post-treatment period. This is a clear indication of metabolic activity, and therefore viability. It also has the advantage of not requiring the exposure of bacteria to high-powered excitation light until the final post-treatment period. The disadvantage of using induction in this manner is that dynamic observation of death is not possible—similarly to the CTC measure—this is an endpoint-only method. It is also potentially affected by the regrowth issue, though to a lesser extent, cells may not recover the ability to synthesise protein for some time after the drug pressure is removed, thus cells that do not fluoresce might ultimately do so at some time-point beyond the observation period of the experiment. However, I believe that this is less likely, since metabolic activity must be observed much earlier than growth which requires a large diverse set of proteins to initiate.

Cells were transformed by electroporation with pST5552 plasmid (Seeliger *et al.* 2012, described in Methods section: pST5552), bearing theophylline-inducible EGFP. Cells were grown in the microfluidic device to test the functionality of inducible EGFP as an endpoint measure of viability. In short, cells were grown in normal medium supplemented with kanamycin (to maintain the plasmid) for approximately 8 h and then exposed to rifampicin at $5\times\text{MIC}$ for 7 h, whilst acquiring phase-contrast images only. Fresh medium supplemented with both kanamycin and theophylline to a final concentration of 2 mM was then added to induce EGFP fluorescence in any metabolically active survivors. Phase-contrast images were captured throughout the experiment, and concurrently with the addition of inducer (2 mM theophylline), cells were additionally imaged for EGFP fluorescence. Cells which had high fluorescence, had divided, or were observed to grow were denoted as survivors (Figure 4.14 for an example microcolony with two survivors). There was usually an initial high background of fluorescence (e.g. frame 5 of Figure 4.14), which was quickly bleached to leave highly fluorescent cells which could be deemed to have survived.

The analysis of repeated experiments shows that 25 of 171 lineages present at the point of introduction of rifampicin fluoresce when induced after exposure to $5\times$ rifampicin, indicating a survival proportion of 14.6 %, which is greater than the range predicted by the batch culture exponential phase model (6.79–10.09 %). If 8.35 % (mean survival as measured by CFUs at 6 h) of lineages are expected to survive, 15 lineages might be expected to be observed in the microfluidic experiments. Conducting a binomial test with these numbers suggests that the difference is significant ($p = 0.0047$). This measure of survival—ignoring such factors—is both conceptually simple and technically easy to perform, whilst minimising interference with normal cell growth due to exposure to the high energy light required for fluorescence imaging,

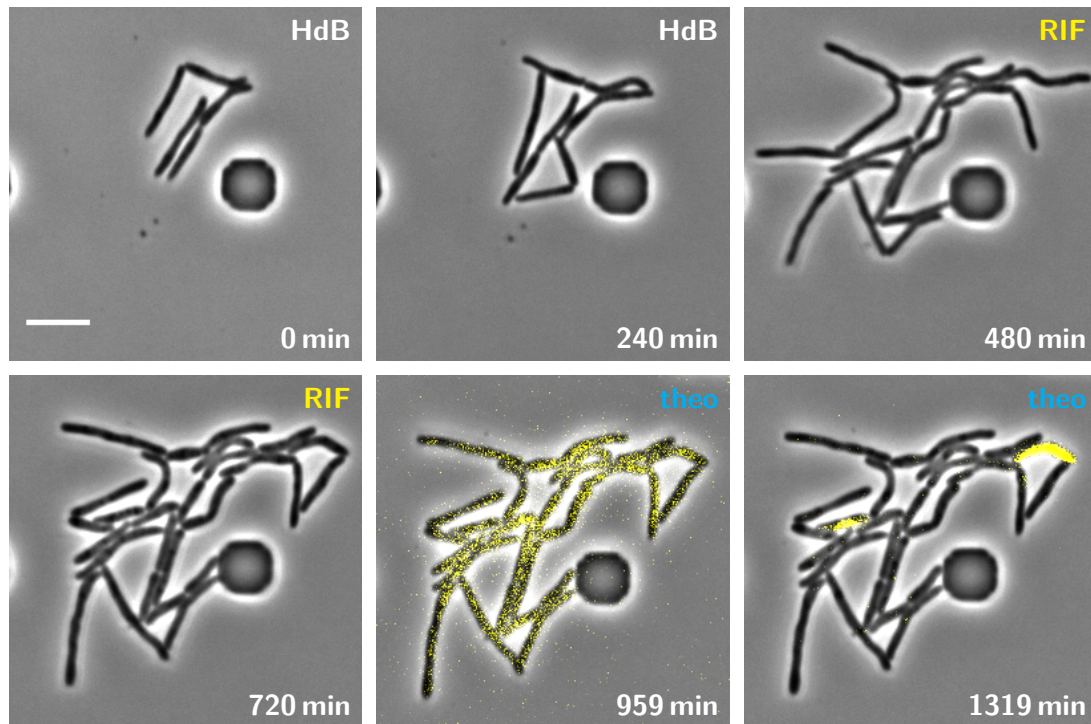


Figure 4.14: Time-lapse series of the determination of viability by induction of EGFP. *M. smegmatis* cells carrying the pST5552 plasmid, with an inducible EGFP cassette, were grown at 37 °C in a B04A-03 microfluidic plate (Millipore). Cells were grown in Hartmans–de Bont minimal medium (HdB) for 8.5 h (frames 1–2), then 5×MIC rifampicin (RIF) was added for 7 h (frames 3–4), before the medium was switched to HdB supplemented with inducer (2 mM theophylline) for a final 6.5 h (frames 5–6). Medium composition is annotated in the top right corner of each frame, and timestamps are annotated in the bottom right corner of each frame. The scale bar in frame 1 denotes 5 μ m. Images were captured at 15 min intervals on a Zeiss Axio Observer inverted widefield microscope, with a 63× objective, with phase-contrast (100 ms exposure, 7.6 V lamp power), acquired with a Hamamatsu Flash 4.0 camera. In-focus images were obtained with the use of software autofocus (Zen) and 1 μ m-spaced Z-stacks. Frames 5–6 were additionally imaged with fluorescence (1 s exposure) with a GFP 38HE filter (Zeiss): excitation 470/40 nm, emission 525/50 nm, dichroic 495 nm, and displayed as merged images, with GFP fluorescence in yellow and phase-contrast in grey.

and several experiments were performed using this method.

4.3.6 Single-cell factors that affect survival

We can use the measures of treatment failure or success above to investigate single cell factors that may affect survival to rifampicin. The time-lapse experiments can be divided into three major phases: growth, treatment, and post-treatment; and into two minor phases: pre-treatment and initial treatment. In these lineages, a surviving cell in the post-treatment period is determined and tracked backwards through all divisions to first frame of the experiment, a structure that can be termed a “back lineage”. From this back lineage, statistics from the different experimental phases can be determined. The phases are defined as follows:

- Growth: the period after the first observed cell division until treatment².

²The first cell is omitted due to adaptation to the microfluidic environment, and since factors such as birth length,

- Pre-treatment: the 2 h period prior to introduction of rifampicin.
- Treatment: the 7 h period in which cells are exposed to 5×MIC rifampicin.
- Initial treatment: the 1 h period after introduction of rifampicin.
- Post-treatment: defined as the period after which rifampicin is removed.

Each phase has various statistics attached to it for each lineage, including linear elongation rate, exponential growth rate, added length, and length at the start of the phase. These statistics are limited to the portion of each cell lifetime that occurs within that phase. Many lineages will undergo cell division during one of the phases, in which case the statistics are averaged for each cell. This analysis of back lineages is depicted in Figure 4.15 for three selected surviving cells, and all statistics are shown in Table 4.3. Note that the original aim of these experiments was to determine whether survivors had unusual characteristics compared to the population, and as such surviving lineages are overrepresented in the data. The following analysis generally compares branches of the same lineage which do not survive to branches which do survive. There are however around four divisions during the growth phase prior to treatment, which may allow for sufficient diversification between lineage branches.

When *M. smegmatis* cells carrying pST5552 are grown in glycerol, with a ~8 h growth phase, a 7 h treatment phase (5×MIC), and a ~8 h post-treatment phase with induction of EGFP: 25 of 171 cells observed to enter the treatment phase have appreciable levels of fluorescence in the post-treatment phase (e.g. Figure 4.14). It is notable that almost no cells observably regrow during the post-treatment period (see Figures 4.16C and 4.16D with values near zero), in line with the experiments measuring survival by regrowth where no cells are observed to regrow after withdrawal of 5×MIC rifampicin (Figure 4.8), although some cells are able to divide and grow post-exposure (see the top lineage in Figure 4.15).

Pre-treatment growth and elongation rate have frequently been analysed by other authors in the context of antibiotic survival (e.g. Aldridge *et al.* 2012; Wakamoto *et al.* 2013; Richardson *et al.* 2016), given the strong link between growth arrest and persistence. *E. coli* cells are known to arrest their growth prior to antibiotic exposure, in a potentially stochastic manner (Balaban *et al.* 2004). However, in these data, there is no evidence that *M. smegmatis* cells that survive grow more slowly prior to exposure than those that do not. There were 100 lineages at the start of the pre-treatment phase—many of which divide during this time, hence the discrepancy with lineages at the start of the treatment phase—of which 12 survive and 88 do not. The survivors have a growth rate of 0.245 h^{-1} [$0.224\text{--}0.263 \text{ h}^{-1}$] (95 % confidence interval of the mean as determined by bootstrapping) compared to 0.263 h^{-1} [$0.251\text{--}0.274 \text{ h}^{-1}$] in non-survivors, and elongation rates of $1.657 \mu\text{m h}^{-1}$ [$1.437\text{--}1.851 \mu\text{m h}^{-1}$] and $1.575 \mu\text{m h}^{-1}$ [$1.492\text{--}1.658 \mu\text{m h}^{-1}$] respectively. These results are not significantly different, with $p = 0.144$ and 0.506 respectively (Welch's *t*-test; Figures 4.16A and 4.16B and Table 4.3). These results

interdivision time, and the age of the inherited pole cannot be determined.

	Survivors		Non-survivors		p*
Number of lineages	25		143		
Growth phase					
Length (μm)	4.748	[4.450–5.285]	3.997	[3.731–4.298]	0.083
Elongation rate (μm h ^{−1})	1.501	[1.243–1.825]	1.535	[1.439–1.638]	0.863
Growth rate (h ^{−1})	0.236	[0.218–0.268]	0.271	[0.260–0.283]	0.144
Length added (μm)	3.643	[2.640–5.585]	3.673	[3.198–4.216]	0.978
Pre-treatment phase					
Length (μm)	5.617	[4.979–6.341]	5.154	[4.879–5.467]	0.261
Elongation rate (μm h ^{−1})	1.657	[1.437–1.851]	1.575	[1.492–1.658]	0.506
Growth rate (h ^{−1})	0.245	[0.224–0.263]	0.263	[0.251–0.274]	0.144
Length added (μm)	2.005	[1.512–2.517]	1.874	[1.688–2.067]	0.651
Initial treatment phase					
Length (μm)	5.494	[4.990–6.027]	5.427	[5.215–5.638]	0.819
Elongation rate (μm h ^{−1})	0.969	[0.843–1.095]	0.893	[0.831–0.955]	0.296
Growth rate (h ^{−1})	0.165	[0.150–0.181]	0.154	[0.145–0.162]	0.244
Length added (μm)	0.924	[0.790–1.054]	0.832	[0.769–0.895]	0.236
Treatment phase					
Length (μm)	5.494	[5.001–6.019]	5.373	[5.171–5.578]	0.679
Elongation rate (μm h ^{−1})	0.350	[0.294–0.407]	0.427	[0.387–0.471]	0.041
Growth rate (h ^{−1})	0.055	[0.048–0.062]	0.071	[0.065–0.078]	0.002
Length added (μm)	1.377	[1.188–1.557]	1.131	[1.035–1.228]	0.028
Post-treatment phase					
Length (μm)	6.338	[5.655–7.039]	6.715	[5.304–8.311]	0.677
Elongation rate (μm h ^{−1})	0.004	[−0.014–0.027]	0.004	[−0.016–0.024]	0.985
Growth rate (h ^{−1})	0.000	[−0.003–0.004]	−0.005	[−0.010–0.000]	0.136
Length added (μm)	−0.039	[−0.114–0.038]	0.047	[−0.040–0.126]	0.162

Table 4.3: Single-cell statistics of lineages exposed to rifampicin and monitored for survival. Numbers indicate the mean and ranges in brackets indicate the 95 % confidence interval, as determined by bootstrapping. Survivors and non-survivors refer to whether these lineages have any descendants which are observed to fluoresce in the post-treatment period upon induction of EGFP. The length parameter is defined as the average length at the *start* of the period (i.e. birth length for the growth phase, but average length at entry for all other phases). **p*-value for comparing survivor and non-survivor means using Welch's *t*-test.

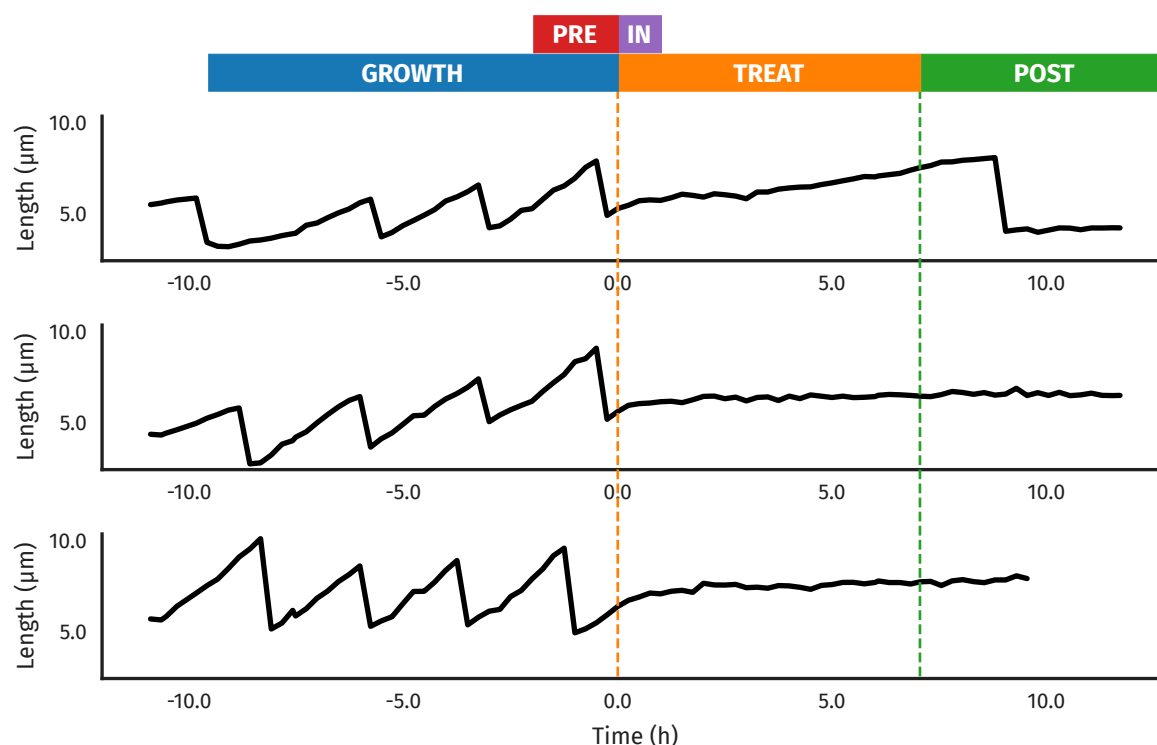


Figure 4.15: Cell length of back lineages that survive exposure to rifampicin. Cells were grown as in Figure 4.14, and survivors determined by fluorescence during the induction period of the experiment. Three lineages that result in survivors are depicted here, one of which (top) is able to continue growing during the treatment phase and subsequently divide. Cell length over time relative to the start of rifampicin treatment is graphed for all members of each lineage. Phases of the experiment are represented above this trace, with “growth” (blue) indicating the time from the first division until rifampicin is added when cells are growing freely; “treat” (orange) indicating the treatment phase from this time-point until rifampicin is removed, and “post” for the remainder of the experiment, at which point inducer is added to the growth medium. The phase boundaries are denoted by coloured dotted lines. Two additional phases are shown above the major phases: “pre” indicates 2 h pre-treatment, and “in” indicates the initial 1 h of treatment. These phases are used for analysis of the bacteria. Underneath the growth trace, the pole inheritance status of each cell is marked, with old-pole-inheritors in light orange, and new-pole-inheritors in light blue.

imply that there is no significant difference in growth rates just prior to addition of rifampicin.

I also investigated other single-cell parameters that might differ between survivors and non-survivors and noted some interesting differences in how cells responded upon extended treatment. In the treatment phase, survivors were significantly more likely to grow more slowly than non-survivors, with growth rates of 0.055 h^{-1} [$0.048\text{--}0.062 \text{ h}^{-1}$] compared to 0.071 h^{-1} [$0.065\text{--}0.078 \text{ h}^{-1}$] ($p = 0.002$; Welch’s t -test; Figure 4.17A), and elongation rates of $0.350 \mu\text{m h}^{-1}$ [$0.294\text{--}0.407 \mu\text{m h}^{-1}$] and $0.427 \mu\text{m h}^{-1}$ [$0.387\text{--}0.471 \mu\text{m h}^{-1}$] respectively ($p = 0.041$; Welch’s t -test; Figure 4.17B), with 25 surviving and 142 non-surviving lineages (Table 4.3). This result indicates that surviving cells may be able to arrest their growth upon exposure to rifampicin more readily than those that do not survive.

Interestingly, surviving lineages also add a statistically significant *greater* length during this pe-

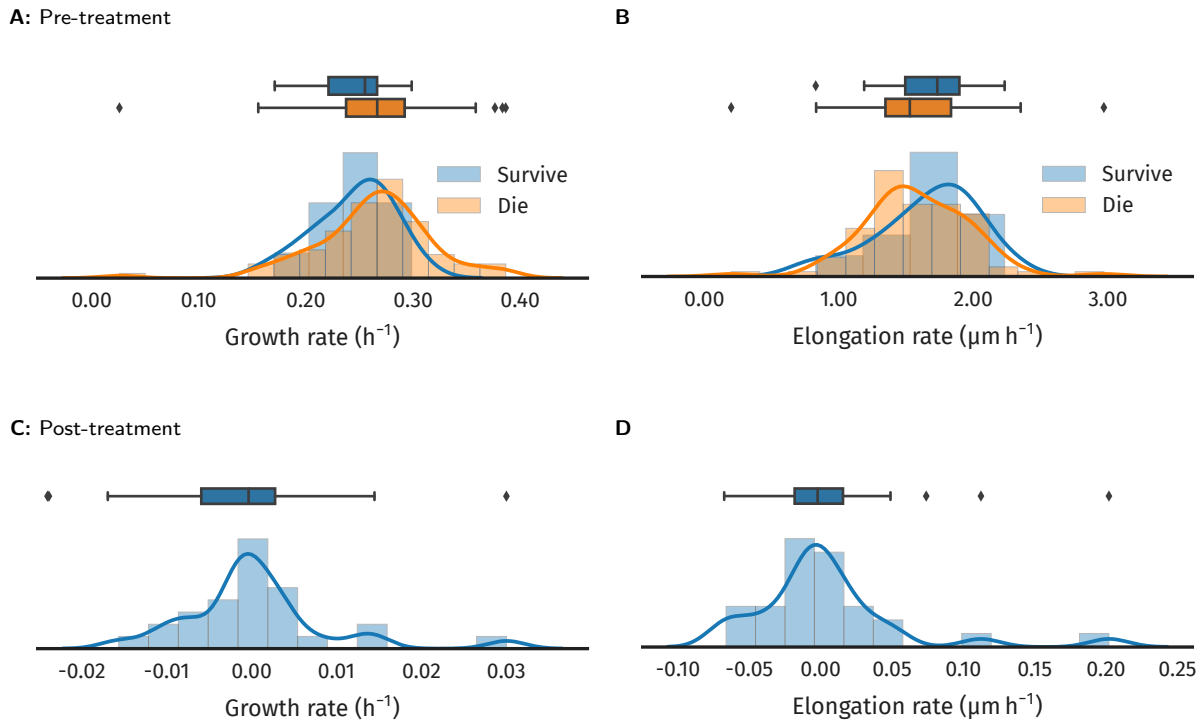


Figure 4.16: Growth of surviving cells treated with rifampicin. Cells were grown as in Figure 4.14 and back lineages constructed based on fluorescence levels in the final few frames. (A) Mean growth rate for lineages in the 2 h prior to addition of rifampicin (pre-treatment phase); and (B) elongation rate for pre-treatment lineages; survivors ($n = 12$) are shown in blue, and non-survivors ($n = 88$) are shown in orange. (C) Mean growth rate for surviving lineages in the period after rifampicin is removed (post-treatment phase); and (D) elongation rate for the post-treatment lineages; $n = 28$. Data are shown as histograms of survivor values, with kernel density estimates as a solid line, and a boxplot above, with the box denoting 25 %, 50 %, and 75 % quartiles, whiskers denoting 1.5 times the interquartile range, and outliers outside this range as diamonds.

riod with $1.377 \mu\text{m}$ [$1.188\text{--}1.557 \mu\text{m}$] added by survivors compared to $1.131 \mu\text{m}$ [$1.035\text{--}1.228 \mu\text{m}$] by non-survivors ($p = 0.028$; Welch's t -test; Figure 4.17C and Table 4.3). Given that survivors appear to grow more slowly, this difference is unexpected. This may reflect differences in division between survivors and non-survivors: only 3 of 25 surviving lineages (12.0 %) include a division event during the treatment phase, whereas 36 of 142 non-surviving lineages (25.4 %) include a division event. This difference is not statistically significant—non-survivors do not divide significantly more or less often than survivors—with $p = 0.201$ (Fisher's exact test; odds ratio 0.402). Regardless, given that *M. smegmatis* cells probably grow exponentially at the single-cell level (Chapter 3, Section 3.3.2), cells will incorporate more length per unit time the closer they get to division. Therefore, since non-survivors include more dividing cells than survivors, mean added length may consequently be greater. The discrepancy between growth and elongation rate, and added length might also be explained by the method by which the different statistics are measured: elongation rate and growth rate are calculated by fitting ordinary linear regression lines to length data, minimising error in frame-to-frame measurement errors, whereas added length is calculated simply as the length in the last frame (or division) subtracted by the length in the first frame (or at birth). Furthermore, although differences in mean

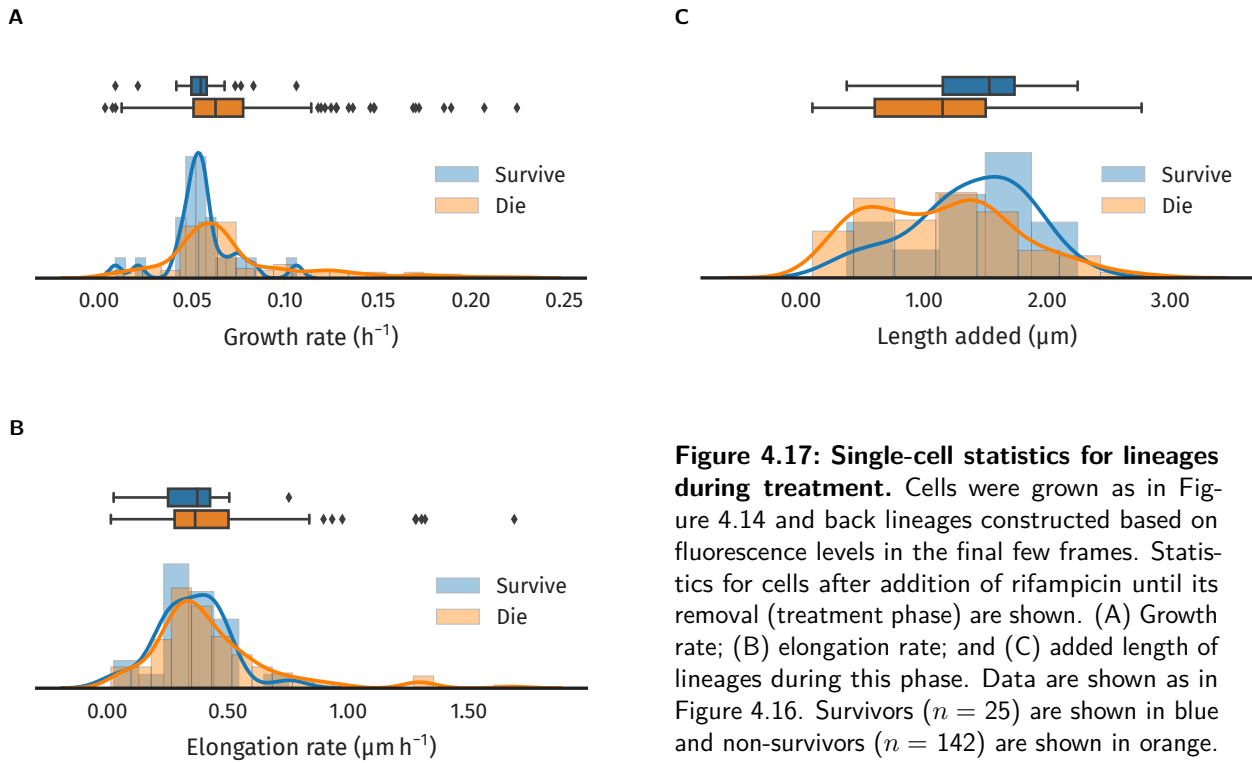


Figure 4.17: Single-cell statistics for lineages during treatment. Cells were grown as in Figure 4.14 and back lineages constructed based on fluorescence levels in the final few frames. Statistics for cells after addition of rifampicin until its removal (treatment phase) are shown. (A) Growth rate; (B) elongation rate; and (C) added length of lineages during this phase. Data are shown as in Figure 4.16. Survivors ($n = 25$) are shown in blue and non-survivors ($n = 142$) are shown in orange.

growth and elongation rate are statistically significant in the treatment phase (as determined by Welch's t -test), there are a large number of outliers in these datasets, especially apparent in the non-survivor lineages (Figures 4.17A and 4.17B), therefore I also checked that median values were different, which should be more robust to outliers. This reveals that survivors still have a significantly different growth rate compared to non-survivors ($p = 0.0376$; Kruskal-Wallis H-test), but do not have a significantly different elongation rate ($p = 0.270$). This still supports the possibility that survivors might be able to arrest their growth upon addition of rifampicin more effectively however.

While there are significant differences in parameters during the treatment phase for survivors compared to non-survivors, there is no significant difference in any of these statistics in the initial treatment phase, which is comprised of the same data as for the treatment phase, truncated to the first hour (Table 4.3).

I was also interested whether survivors had a different history compared to non-survivors in their growth phase, especially with respect to their division characteristics. Key questions were: do statistics such as interdivision time, birth length, division length, pole age inheritance, or asymmetry differ between lineages; and whether surviving cells or non-surviving cells follow different models of cell-size control. To determine these data, lineages were constructed from cells at the start of each experiment, and traced through their divisions until rifampicin was added (the growth phase). The first division was discarded due to lack of information about

birth length and pole age, and the last cell was discarded since its division status could not be determined. From each of these lineages, and depending on whether these lineages ultimately resulted in a cell that survives treatment, statistics of each cell division event were recorded. As a consequence of experimental design, more division events were observed in lineages that would survive (65) compared to those that would not (47), since these lineages were retrieved precisely because they resulted in surviving cells. However, this provides insight into what may differ between sibling lineages, when one branch will ultimately survive, and the other will not. An example of this can be seen in Figure 4.19A, where the surviving lineage is shown in black and its sibling non-surviving lineages in grey. There are no significant differences in division statistics between cells from surviving lineages compared to their non-surviving siblings, including birth length ($4.36\ \mu\text{m}$ [$4.12\text{--}4.60\ \mu\text{m}$] in survivors compared to $4.44\ \mu\text{m}$ [$4.22\text{--}4.67\ \mu\text{m}$] in non-survivors; Figure 4.19B), division length ($8.09\ \mu\text{m}$ [$7.76\text{--}8.41\ \mu\text{m}$] and $7.94\ \mu\text{m}$ [$7.60\text{--}8.30\ \mu\text{m}$] respectively; Figure 4.19C), and added length ($3.72\ \mu\text{m}$ [$3.51\text{--}3.96\ \mu\text{m}$] and $3.50\ \mu\text{m}$ [$3.31\text{--}3.71\ \mu\text{m}$] respectively; Figure 4.19D). There is however a significant difference in interdivision time, with surviving lineages dividing with a mean interdivision time of $2.36\ \text{h}$ [$2.23\text{--}2.50\ \text{h}$] compared to $2.10\ \text{h}$ [$2.01\text{--}2.19\ \text{h}$] among their non-surviving siblings (Figure 4.19E) with $p = 0.0024$ (Welch's t -test). This fits into the hypothesis that growth arrest of cells may aid survival of cells, given that survivors appear to divide less frequently than their non-surviving siblings.

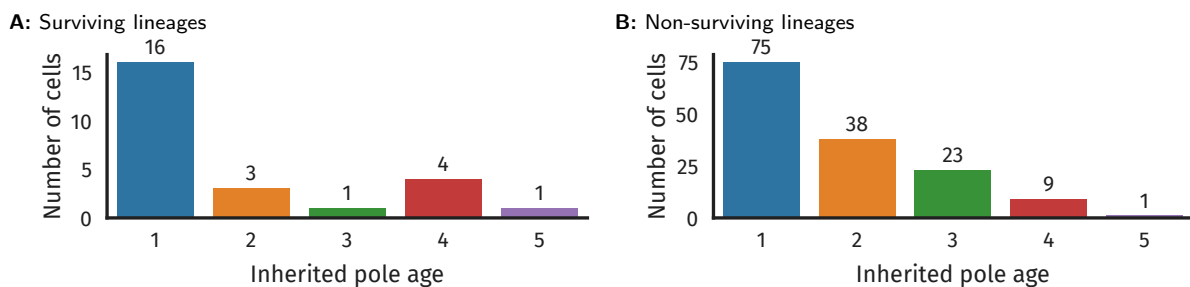


Figure 4.18: Pole age of cells at the point of rifampicin treatment. Poles were tracked during time-lapse using the lineage scripts and pole ages assigned during the growth phase. Each cell present at the point of addition of rifampicin (start of the treatment phase) which had an descendant which survived exposure was classed as a “surviving lineage”, and any cell which had no descendants which survived were classed as a “non-surviving lineage”. Counts of specific pole ages are plotted for survivors (A) and non-survivors (B). A pole age of 1 denotes a new-pole inheritor, and a pole age of above 1 denotes an old-pole inheritor. Colours are arbitrary.

Regarding pole inheritance, pole ages can be tracked throughout the growth period, where poles are known, and the inheritance of the new- or old-pole from the mother cell can be determined. In the growth phase, I observe that survivors inherit the old-pole in 25 divisions, and the new-pole in 40 divisions, whereas non-survivors appear to exhibit no difference in inheritance of old or new pole from their mother, with 23 cells inheriting the new pole compared to 24 cells inheriting the old pole. While this difference is not statistically significant ($p = 0.33$; Fisher's exact test), this could potentially hint at a difference between survivors and non-survivors that should be investigated further, especially given the asymmetry observed that depends on pole age. If pole age is determined at the point of rifampicin addition, of the 25 surviving

lineages, 16 were new-pole inheritors, and 9 were old-pole inheritors (Figure 4.18A). Of 146 non-surviving lineages at the point of treatment, 75 were new-pole inheritors, and 71 were old-pole inheritors (Figure 4.18B). This again shows that there is no significant difference in pole age between survivors and non-survivors in my dataset ($p = 0.20$; Fisher's exact test), though my cell numbers are clearly low indicating a lack of statistical power.

Considering pole age in terms of size asymmetry between daughters, I also observe no statistically significant difference in asymmetry between daughter cells in lineages that result in survivors compared to those that do not. If asymmetry is defined as $L_{b1}/(L_{b1}+L_{b2})$, where L_{b1} is the smaller daughter and L_{b2} is the larger daughter, division events in survivor lineages result in mean asymmetry of 0.446 [0.437–0.454] compared to 0.452 [0.442–0.461] in non-survivor lineages.

Given the deviation of cells from the adder model of cell-size control when grown in pyruvate or acetate (Chapter 3, Figure 3.13F) coupled with the observation that survival is greatly enhanced when cells are grown in these carbon sources in batch culture (Figure 4.6), I also wanted to test whether surviving lineages also diverged from adder. To do this, I performed ordinary least squares linear regression of division length (L_d) against birth length (L_b) to fit the equation for the noisy linear map $L_d = aL_b + b + \eta$ with slope a , y-intercept b , and noise η (Chapter 3, Equation 3.1). The value of a determines the cell-size control model that cells follow, with $a = 1$ defining an adder, and indicating that L_d is completely dependent on L_b . When survivors and non-survivors in the growth phase are fitted to the noisy linear map, $a = 0.97 \pm 0.24$ and $b = 3.86 \pm 1.08 \mu\text{m}$ for survivors, and $a = 1.29 \pm 0.25$ and $b = 2.23 \pm 1.14 \mu\text{m}$ for non-survivors (Figure 4.19F). This difference is not statistically significant as determined by analysis of covariance (ANCOVA), with $p = 0.101$ implying that there is no difference in adherence to adder in survivors compared to non-survivors.

4.4 Discussion

In this chapter, I have investigated the response to *M. smegmatis* to rifampicin in a number of conditions, and demonstrated commonalities with other bacteria, such as dramatic enrichment of the apparent persister fraction in stationary phase (Figure 4.4). Confirming previously published results (Frampton *et al.* 2012), I observe no effect on survival to rifampicin in toxin-antitoxin knockout mutants (Figure 4.7), raising questions as to their role in *M. smegmatis*. I also uncover some interesting effects of nutrient deprivation which were unexpected, such as dramatic reduction in survival when nitrogen is limited (Figure 4.5), surprising given the strong role of nutrient deprivation in *E. coli*, in triggering dormancy in *M. tuberculosis*, and the probable low nitrogen state at entry into stationary phase. This drastic decrease is not observed for cells grown in a low carbon medium (Figure 4.5C), though there is still a decrease, implying

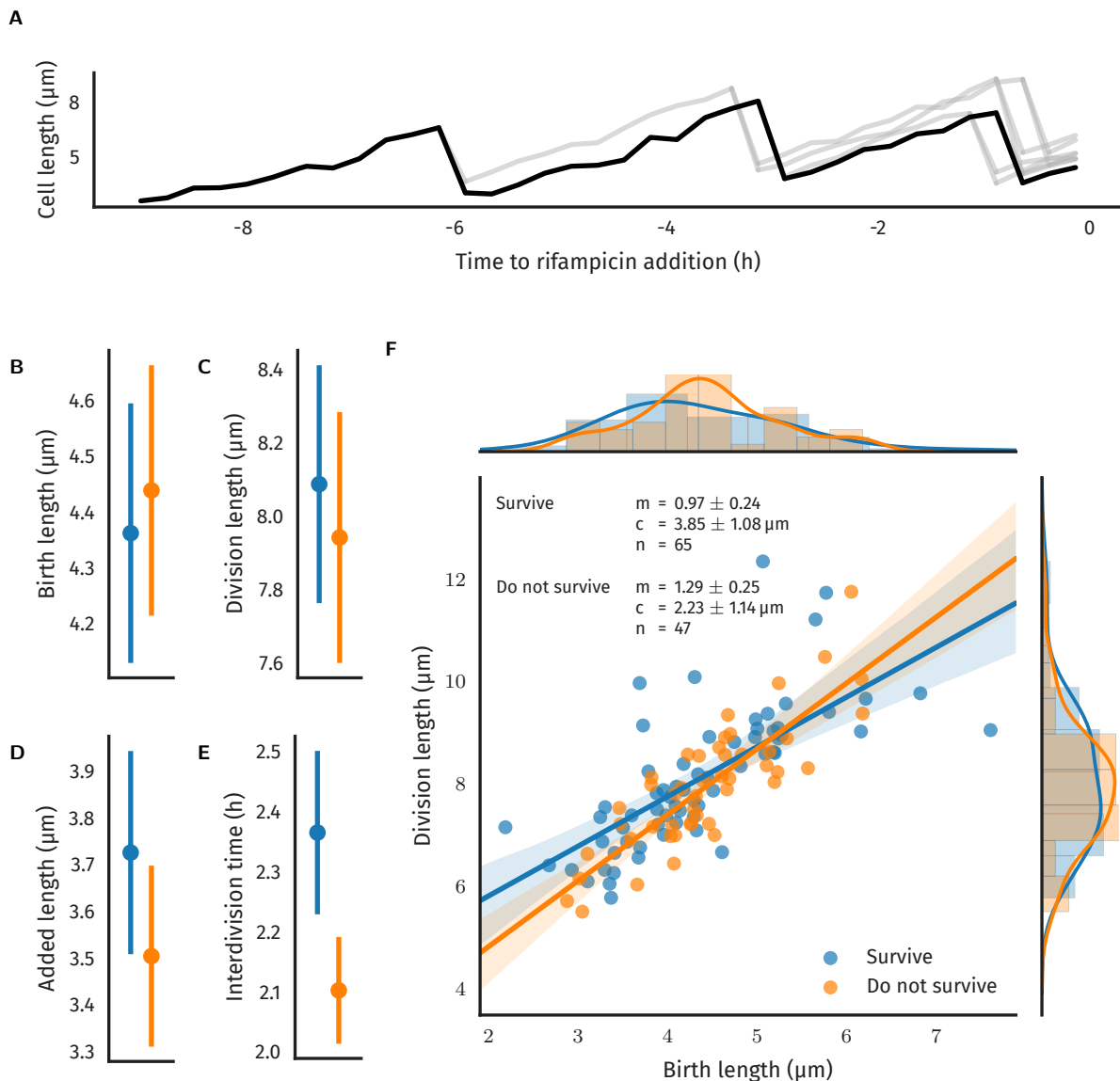


Figure 4.19: Analysis of cell division statistics in the growth phase for survivors compared to non-survivors. Cells grown as in Figure 4.14 were analysed in their growth phase (period from the first division of the lineage to the point at which rifampicin is added). (A) Cell length against time is plotted for a surviving lineage with non-surviving siblings shown in grey. (B–E) Birth length, division length, added length, and interdivision time for survivors (blue) and non-survivors (orange) observed to divide within the growth phase. Data are plotted as the mean value with error bars denoting the 95 % confidence interval as determined by bootstrapping. (F) Scatter plot of birth length against division length for all cells within the growth phase, cells from surviving lineages are depicted in blue, and cells from non-surviving lineages in orange. Linear regression lines as determined by ordinary least squares regression are drawn, with 95 % confidence limits depicted as a shaded region. Histograms indicating the distribution of each variable are shown above (birth length) and on the right (division length) of the scatter plot, with kernel density estimates depicted as solid lines. The slope (m), y-intercept (c), and number of divisions observed (n) are given in the top left corner.

that nutrient state itself may not trigger drug tolerance. When cells are grown in alternative carbon sources, which decreases the population doubling rate, survival is greatly enhanced, pointing to a stronger role for minor changes in population doubling rate (Figure 4.6).

Since batch culture experiments were limited by a severe lack of reproducibility, I developed and refined time-lapse methods to determine the response of *M. smegmatis* to rifampicin at the single-cell level. This uses methods developed in previous chapters to provide a relatively robust and reproducible platform to expose growing mycobacteria to antibiotics at will. However, determining viability at the single-cell level is non-trivial, since regrowth of single cells is non-indicative of survival and lysis of cells in response to rifampicin is rare under standard conditions (Figure 4.8). I therefore test several methods of determining viability in microfluidic experiments, and determine that an endpoint measure of metabolic activity—the induction of a fluorescent protein—is a conceptually simple and robust measure of viability (Figure 4.14). Using this assay, I determine single-cell characteristics of cells that survive rifampicin exposure, and demonstrate, as other authors have, that growth rate prior to drug treatment does not appear to predict survival (Figure 4.16A). Instead, survivors may be able to more rapidly arrest growth upon contact with rifampicin, and interestingly maintain very low level growth throughout rifampicin treatment, in contrast to non-surviving cells which do not arrest their growth immediately (Table 4.3). This results in an apparent paradox of surviving cells increasing their length to a greater degree than non-surviving cells during treatment (Figure 4.17), whilst simultaneously arresting their growth more effectively. Regarding cell-size control as determined in Chapter 3, I also determine that surviving cells grown with standard HdB do not diverge from the adder principle, though there is a hint that they may increase their cell cycle time prior to the addition of rifampicin (Figure 4.19E).

I demonstrate here the importance of single-cell studies in determining antibiotic response profiles, especially important given the prominence of sub-populations in the current literature, and the further usefulness of the image analysis pipeline that I developed in Chapter 2.

4.4.1 Batch culture experiments

Enhanced drug tolerance has long been associated with growth rate, with Joseph Bigger noting that persisters were likely to be in a transient dormant state (Bigger 1944). He noted that approximately 10^{-6} *Staphylococcus* spp. were able to survive penicillin, and a similar fraction is observed in *E. coli* in exponential phase (Bigger 1944; Lewis 2007). This fraction increases from 10^{-6} to 10^{-4} in late exponential and to 10^{-2} in stationary phase (Lewis 2007). The observation that maintenance of *E. coli* cells at exponential phase almost entirely removes the surviving fraction implies that persister cells only form in stationary phase (Keren *et al.* 2004a), and that this drug tolerance may have evolved as a mechanism to enhance survival in stationary phase.

To investigate survival in *M. smegmatis*, I developed a simple killing assay in which CFUs/ml are measured over time, as in a standard growth curve. At time-points corresponding to different growth phases of the population, I added rifampicin at a high dose for 48 h, and determined what proportion of cells were able to survive by comparing CFU counts after this period to counts prior to it. Rifampicin dosage was calculated based on the MIC, determined from the literature (Alexander *et al.* 2003) and by a broth dilution assay as $32\ \mu\text{g ml}^{-1}$. In an attempt to ensure that cells were being killed and not simply ceasing growth, and to decrease the chance of rifampicin resistance arising, I generally used five times this value ($5\times\text{MIC}$) for experiments in this chapter. Growth phases were selected from a growth curve in the absence of antibiotics from an initial optical density at 600 nm of 0.05, corresponding to approximately 10^6 CFUs/ml in standard HdB. Exponential phase was selected as 6 h post-inoculation, and stationary phase as 40 h post-inoculation from this growth curve (Figure 4.3).

Survival was determined from CFU counts as $0.08 \pm 0.03\%$ ($\sim 10^{-3}$) in exponential phase (Figure 4.4A) and $21.85 \pm 6.17\%$ ($\sim 10^{-1}$) in stationary phase (Figure 4.4C). This recapitulates observations in *E. coli* with an approximate 3-log increase in survival between exponential and stationary phases (Lewis 2007). However, the proportion is clearly greatly increased: there are similar numbers of rifampicin survivors observed at exponential phase in *M. smegmatis* compared to survivors at stationary phase in *E. coli* (Figure 4.4D). This data represents—to my knowledge—the first report of the impact of growth phase itself on rifampicin tolerance in mycobacteria. The underlying cause of this increase in survival at stationary phase is largely unknown, but it may be related to stress responses that allow survival during this non-growing phase as well as growth arrest itself. The greatly increased survival fraction in *M. smegmatis* compared to *E. coli* may reflect an inherent mycobacterial property of general drug insensitivity, related to its cell wall structure and impermeability (Kieser and Rubin 2014).

If stationary phase is assumed to be associated with a lack of nutrients that prevents further population growth, this may imply that nutrient deprivation plays some role in increasing the tolerant fraction. Nutrient deprivation has been linked to drug tolerance by other authors, which is highlighted by the apparent role of the stringent response to amino acid starvation in long-term survival of *M. tuberculosis* *in vitro* (Primm *et al.* 2000) and *in vivo* (Dahl *et al.* 2003), and of *E. coli* in minimal and stationary phase (Cashel *et al.* 1996). To investigate this, I performed the same survival assay at stationary phase using variants of HdB with a lower concentration of $(\text{NH}_4)_2\text{SO}_4$ (the sole nitrogen source) or glycerol (the sole carbon source). However, restriction of carbon under these conditions does not enhance survival, it instead causes a reduction in the survival proportion from $21.85 \pm 6.17\%$ to $4.09 \pm 0.09\%$ (Figure 4.5C). This effect is even more stark when nitrogen is limited, with only 1.14% ($\sim 10^{-2}$) of cells surviving in 1 mM $(\text{NH}_4)_2\text{SO}_4$ (Figure 4.5A), and $0.11 \pm 0.10\%$ ($\sim 10^{-3}$) surviving in 0.15 mM $(\text{NH}_4)_2\text{SO}_4$ (Figure 4.5B). To investigate this difference, I also analysed the effect of nitrogen starvation at the single-cell level (discussed below) and observed a significant increase in the rate of cell lysis which might

play some part in explaining this increase in death. This plays into a hypothesis proposed by Frampton *et al.* (2012), in which oxidative stress is increased in complex media, or when cells are deprived of nutrients (Frampton *et al.* 2012). This might lead to increased sensitivity to antibiotics under conditions of increased metabolic stress. It remains difficult however to reconcile the large increase in survival at stationary phase, and its reduction under nutrient deprivation. Since the time between inoculation and plateauing of cell numbers appears to be largely unchanged in these different conditions (Figures 4.5A to 4.5C) compared to standard HdB (Figures 4.4A to 4.4C), batch culture nutrient limitation may not cause growth rate changes at all, at least not when rifampicin is added. These experiments should be repeated with a larger range of initial nutrient concentrations to attempt to investigate this further. It is interesting that others have noted a strong increase in dormancy due to nutrient deprivation or extended stationary phase (Kaprelyants *et al.* 1993; Shleeva *et al.* 2004) which might imply an increase in the survival fraction, yet I do not observe this. This could also be explained by an increase in the VBNC state, which my assay does not capture. To address this, MPN assays in fresh broth and broth supplemented with culture filtrate would need to be performed in parallel with CFU counts.

Given the extensive work conducted on TA systems with respect to their role in drug tolerance, and the huge number of them encoded by *M. tuberculosis* (Ramage *et al.* 2009), I utilised *M. smegmatis* knockout strains constructed by Frampton *et al.* (2012). Since *M. smegmatis* only has three TA systems—MazEF, VapBC, and Phd/Doc—the authors constructed single deletion strains ($\Delta mazEF$, $\Delta vapBC$, and $\Delta phd/doc$), and a triple deletion strain ($\Delta triple$). When Frampton *et al.* tested the response of $\Delta triple$ to rifampicin, they observed no difference in survival compared to wild-type *M. smegmatis*. However, they only exposed cells to rifampicin for 2 h (whilst in exponential phase), less than a single doubling time of *M. smegmatis*, albeit at very high concentration (80 mg ml⁻¹ or 2500×MIC).

I therefore attempted to determine if there was any difference in survival in my assay, with 48 h exposure and 5×MIC rifampicin. At exponential phase—though performed only once—I observed no significant differences in survival between the strains and wild-type *M. smegmatis* with percentages within an order of magnitude of each other (Table 4.2). At stationary phase, there were differences in survival, such as an apparent reduction from 21.85 ± 6.17 in wild-type to 7.20 ± 4.22 in $\Delta phd/doc$ (Figure 4.7A). However, these differences were not observed in the other single deletion strains, and crucially not observed in the $\Delta triple$ strain, implying that this was a spurious observation (Figure 4.7 and Table 4.2). Since a Master's student was undertaking investigation into modulation of the TA systems in these mutant backgrounds, I decided not to pursue this avenue further, especially given my apparent negative results. Given the recent retraction of the *E. coli* studies directly linking the TA systems and persistence (Maisonneuve *et al.* 2018a,b), the role of the TA systems in triggering drug tolerance is less clear, though there is still strong evidence for their involvement.

The batch culture experiments were very variable under ostensibly the same conditions. For example, survival of wild-type *M. smegmatis* at stationary phase in standard HdB ranged from 5.6 % to 37.9 % in independent experiments. The initial cell density was very difficult to keep constant between experiments, since it was estimated by optical density, which is an unreliable measure of CFUs/ml in mycobacteria, as evidenced by variability in my experiments, sometimes differing by almost an order of magnitude between experiments. This probably reflects cell size changes that occur in the inoculating culture as they reach different growth phases. As a consequence of altered initial CFU counts, experiments could not be directly compared in a similar manner to optical density killing curves, hence the use of representative experiments in all cases. Note that survival could not be estimated by optical density, since cells killed by rifampicin still appear to contribute to the turbidity of the culture (data not shown). The state of the initial inoculum has also been noted to greatly affect the number of persistent cells in *E. coli* experiments (e.g. Keren *et al.* 2004a), since the majority of tolerant cells are derived from stationary phase, therefore might well affect the surviving fraction in my experiments.

4.4.2 Single-cell experiments

The ONIX microfluidic device, as described extensively in previous chapters, provides a robust and powerful platform to perform experiments on the effect of antibiotics in single cells. Microfluidics have been used extensively in the mycobacterial field to investigate single-cell responses to antibiotics (Aldridge *et al.* 2012; Wakamoto *et al.* 2013; Manina *et al.* 2015; Vaubourgeix *et al.* 2015; Richardson *et al.* 2016; Rego *et al.* 2017), which are notoriously difficult to monitor using conventional live-cell microscopy.

I initially determined the single-cell response of *M. smegmatis* grown in standard HdB to 7 h exposure to rifampicin, preceded by a ~ 7 h initial growth period, and followed by a ~ 7 –10 h post-growth recovery period. I tested $1\times$ MIC and $5\times$ MIC rifampicin to optimise the platform. I assessed survival by regrowth in the post-growth period, and observed very few cells that regrow. At $1\times$ MIC, only 7 of 47 (14.9 %) lineages present at the start of treatment were observed to regrow, and 0 of 71 lineages observed to regrow at $5\times$ MIC (Figure 4.8). My expected survival proportion, given the batch culture experiments at exponential phase—which the microfluidic system most closely represents—was approximately 8 % (Figure 4.4A). Given that my recovery period is relatively short, I surmised that it was too short to allow sufficient observable regrowth. All current reports of the single-cell antibiotic response to rifampicin have used regrowth or cell division as the primary marker of viability in *M. smegmatis* (Aldridge *et al.* 2012; Richardson *et al.* 2016).

The former study observed that approximately 20 % of cells regrow after withdrawal of rifampicin, with a 8 h post-treatment recovery (Aldridge *et al.* 2012). However, the authors used $1\times$ MIC rifampicin for their experiment, and observe similar survival levels to my experiment at

1×MIC (14.9 %). The latter study used 3×MIC, with a 9.75 h recovery period, and noted 19.2 % of 208 cells present at the start of treatment were tolerant—i.e. they regrew—they however also tested 2×MIC rifampicin and observed as high as 47.9 % survival by regrowth (Richardson *et al.* 2016), a much higher proportion than observed at 1×MIC previously (Aldridge *et al.* 2012). Notwithstanding this discrepancy, they extended the regrowth period to 16 h to determine whether this fraction might increase, and added propidium iodide to stain cells with compromised membranes, and did not observe a significantly higher number of cells recovering or labelled as non-viable compared to their standard design (Richardson *et al.* 2016). Interestingly, their survival fraction did appear to significantly vary between experiments, with 12 % of 92 cells in the propidium iodide experiment, 19.2 % of 208 cells in the 9.75 h recovery experiments, and 29 % of 286 cells in the extended recovery experiment, implying some significant source of variability in their experiments (Richardson *et al.* 2016).

Therefore, while other authors observe significant regrowth after rifampicin removal, I could not recover any cells that regrew at 5×MIC, and I believe this might be due to the higher drug concentration compared to other studies, in which case a longer recovery period might well reveal regrowth of cells post treatment. However, the constraints of the physical operation of the plates prevented significantly extending this period, due to a tendency for steam to occlude the imaging window after overnight experiments (described in Chapter 2).

I was also interested in investigating the response of nitrogen-starved cells to rifampicin at the single-cell level, given the large observed decrease in survival in batch culture (Figures 4.5A and 4.5B). I cultivated cells in HdB containing no nitrogen source (Δ N HdB), for 5 h and 17 h prior to loading, and then for the standard further 7 h in the device—i.e. 12 h and 24 h starvation respectively. I designed the experiment to use Δ N HdB rather than nHdB (1 mM ammonium sulphate) or nHdB (0.15 mM ammonium sulphate) due to the constant perfusion used in the microfluidic device. Since fresh medium is constantly provided to cells within the microfluidic device, nutrient limitation becomes a much harder problem, since it is not completely clear at what level nitrogen becomes “limiting”. Growth rate in this pre-treatment period was significantly reduced compared to cells grown in nitrogen-replete conditions, reflective of the starved condition, with 0.239 h^{-1} [0.223–0.254] and 0.188 h^{-1} [0.177–0.199] observed in the 12 h and 24 h experiments respectively, compared to 0.263 h^{-1} [0.260–0.266] observed for cells grown in HdB (Chapter 3, Table 3.2). The observation that cells starved of nitrogen for 17 h prior to imaging can grow at all was relatively surprising, but *M. smegmatis* is well known to have excellent nitrogen scavenging, storage, and recycling capabilities which may permit this form of long-term maintenance of growth during starvation (Amon *et al.* 2009; Jenkins *et al.* 2012, 2013; Williams *et al.* 2013; Elharar *et al.* 2014). I observed significantly increased lysis in the 24 h starvation condition (15.4 %; Figure 4.9) compared to both 12 h starvation (2.86 %), and unstarved cells (very rare but not measured, an example of a lysed cell in standard HdB can be seen in Figure 4.8). This may underlie the decreased survival fraction observed in batch

culture, since increased lysis implies a weakened cell wall, and increased stress on cells which have been starved for longer, a condition that is likely to be true for the low initial nitrogen (1 mM) compared to the very low nitrogen (0.15 mM) conditions used.

Other authors have used cell lysis as a measure of cell death, coupled with post-treatment regrowth as a measure of viability in the context of isoniazid treatment (Wakamoto *et al.* 2013; Manina *et al.* 2015). However, isoniazid is a cell wall-targeting antibiotic, and as such appears to cause a very high lysis rate. Rifampicin conversely targets RNA polymerase, thus does not directly affect the cell wall, implying that lysis events are a consequence of events significantly downstream of its direct target. Therefore, additional stress placed upon cells grown in nitrogen-limited media, or starved completely of nitrogen must render cells more susceptible to cell wall damage and subsequent lysis. This marked increase in lysis also reflects the inappropriateness of lack of lysis as a robust measure of viability, since a tiny fraction of cells appear to lyse under standard conditions, or even after 12 h nitrogen starvation.

Given that both post-treatment regrowth and lysis during treatment appear to be poor measures of viability or lack of viability respectively, I developed and tested several alternative single-cell measures of survival. As mentioned above, regrowth after rifampicin treatment has been used as the primary measure of viability in the literature, and other authors observe high proportions of cells that are able to resume growth post-treatment—indeed more than might be expected from batch culture experiments (Aldridge *et al.* 2012; Richardson *et al.* 2016), although I use a significantly higher concentration of rifampicin. For experiments with isoniazid, lysis is additionally used as a marker of death (Wakamoto *et al.* 2013; Manina *et al.* 2015). Two studies have used an endpoint marker of membrane permeability to demonstrate non-viable cells, including Sytox blue (Manina *et al.* 2015) and propidium iodide (Richardson *et al.* 2016). These measures assay cell death (lysis or membrane permeability) and cell survival (regrowth) accurately, since cells with damaged membranes can be assumed to be truly dead, and cells which resume growth can be assumed to be alive, but may not reflect cells which are not captured by either assay technique.

Therefore, I first tested the applicability of propidium iodide to assay cell survival. Propidium iodide is a DNA-binding dye which fluoresces brightly when bound to DNA, but it is excluded from membranes with an active potential across them—i.e. viable cells. In the control experiment which was not exposed to rifampicin, 1.9 % of cells were stained with propidium iodide after 14 h growth indicating the basal level of cell death in the ONIX microfluidic device, and 38.1 % of 113 cells were stained after 7 h growth and 7 h exposure to 5×MIC rifampicin (Figure 4.10). This demonstrates that 61.9 % of cells are able to maintain their membrane integrity after 7 h rifampicin treatment, a much higher survival fraction than expected if propidium iodide is an accurate measure of viability. I therefore tested a direct marker of cell viability: 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). CTC is converted by the bacterial electron transport

chain from its non-fluorescent salt form into an insoluble fluorescent formazan form. CTC fluorescence therefore denotes cells which are able to maintain an active redox potential to drive the electron transport chain, and thus can be assumed to be viable. I performed this assay in batch culture at stationary phase with either DMSO or 5×MIC rifampicin added for 48 h, then incubated cells with CTC and observed them mounted on glass slides. The number of viable cells qualitatively matched expectations—fewer cells were stained on the rifampicin slide than the control slide—but severe morphological changes were observed (Figure 4.11). Most noticeably, there were bright puncta visible on the phase-contrast channel which corresponded with regions of high fluorescence. Formazan is insoluble, therefore presumably aggregates within cells to form these regions, but considering the difficulty of distinguishing cells from one another with such change, and my interest in post-exposure growth, I decided not to pursue this measure. In addition, I ideally wished to have a dynamic measure of viability, in which the point of death could be measured, which CTC endpoint staining does not permit.

To attempt to find a real-time readout of viability during rifampicin exposure, I considered the use of inducible fluorescent protein expression as a proxy for metabolic activity—and therefore viability. Ideally, live cells—in the presence of an inducer—would constantly produce fluorescent protein, signalling their viability, and dead cells would be unable to maintain expression resulting in loss of fluorescence. However, fluorescent proteins are highly stable, generally only being removed by dilution caused by cell growth (Tombolini *et al.* 1997; Blokpoel *et al.* 2003), and indeed this is the basis of assays of intracellular growth that identify *Salmonella* persisters induced by macrophage internalisation (Helaine *et al.* 2010, 2014). Therefore, I attempted to construct an inducible EGFP system in which fluorescence would be degraded over time in dead cells. To do this I fused EGFP to a previously described degradation tag that targets cells for proteolysis (Muto *et al.* 1998; Blokpoel *et al.* 2003), coupled to a theophylline-inducible promoter. I confirmed that half-life of unstable EGFP (EGFP-ASV) was 41.9 ± 7.0 min in an ensemble plate-based assay (Figure 4.12). Experiments with 1×MIC rifampicin (chosen to increase the surviving fraction) demonstrated that fluorescence is indeed degraded when theophylline is withdrawn (Figure 4.13), with some cells retaining fluorescence throughout the treatment period. I reasoned that this was due to dead cells losing the ability to degrade EGFP-ASV rather than continued expression of new EGFP-ASV. When rifampicin was removed, and EGFP again induced however, not all the cells which had degraded their fluorescent signal recovered fluorescence. This implies that degradation of EGFP-ASV is not an accurate measure of viability.

This can be explained by considering the kinetics of cell death. Cells presumably die at effectively random times after exposure to rifampicin, therefore will degrade their initial EGFP-ASV levels to different extents. Cells which die near the end of the treatment period will effectively have degraded the vast majority, or all their EGFP-ASV, and will appear to be live cells by the degradation metric. As a measure of time-to-death, EGFP-ASV fluorescence is therefore

probably an unreliable method, since fluorescence levels will be variable throughout the treatment period. Alternatively, while it is clear that some cells—presumably dead—lose the ability to degrade EGFP-ASV, it is not clear that all dead cells lose this ability. Residual proteases could retain their activity for some time after the loss of viability, and thus degrade remaining EGFP-ASV molecules. These considerations also ruled out the use of an alternative assay of dynamic death, in which EGFP-ASV is induced throughout the treatment period, with dead cells losing fluorescence, since the response of dead cells is unclear, cells might lose the ability to degrade or the ability to synthesis new protein at different stages of death.

For these reasons, I decided not to pursue unstable EGFP as a measure of viability. Instead, I decided to use induction of EGFP fluorescence as an endpoint measure (Figure 4.14). This is similar to the CTC experiment, in that it measures metabolic activity (the ability to synthesise new protein), except it does not involve toxic products, or affect cell morphology. In repeated experiments, 25 of 171 cells (14.6 %) present at the point of introduction of rifampicin had appreciable levels of fluorescence post-treatment. This value is greater than the range predicted by batch culture experiments (6.79–10.09 %; Figure 4.4A), but given reports of enrichment of VBNC cells after drug treatment *in vivo* (Wayne 1994), this might be expected to be reflected by an increased survival proportion at the single-cell level. Alternatively, given the differences between the batch culture experiments and the single-cell experiments, this may reflect a true difference. Regardless, EGFP inducibility in the post-treatment period is a simple method—conceptually and practically—that indicates metabolically active and viable cells at close to the levels expected by batch culture experiments.

4.4.3 Single-cell response to antibiotics

Using EGFP signal in the post-treatment phase, I attempted to determine single-cell factors that distinguish surviving lineages. Of particular interest were reports that growth rate prior to treatment has no effect on survival post-treatment in mycobacteria (Aldridge *et al.* 2012; Wakamoto *et al.* 2013; Manina *et al.* 2015; Richardson *et al.* 2016), in contrast to reports in *E. coli* which observe growth arrest prior to drug exposure (e.g. Balaban *et al.* 2004). To address this I calculated the growth rate of cells in the 2 h period prior to addition of rifampicin, and compared lineages in which at least one descendant survived to lineages in which no descendants survived. I termed this period the “pre-treatment phase”. This is very similar to the analysis strategy used by Wakamoto *et al.* (2013), who test the response to *M. smegmatis* to isoniazid. Survivors have no significant difference in growth rate in the pre-treatment phase compared to non-survivors with a *p*-value of 0.144 (Table 4.3 and Fig. 4.16A). This is consistent with others, whether they analyse the response to isoniazid by exponential growth rate (Wakamoto *et al.* 2013; Manina *et al.* 2015) or rifampicin by linear elongation rate (Aldridge *et al.* 2012; Richardson *et al.* 2016). This suggests that mycobacterial drug-tolerant cells either do not pre-exist, or that growth rate

does not correlate with drug tolerance.

Although there is no apparent difference in growth rate in the pre-treatment phase, I also analysed the interdivision time within the growth phase (from the start of the experiment until addition of rifampicin). Lineages that ultimately survive have a statistically significantly greater interdivision time in the growth phase compared to lineages that do not survive with $p = 0.0024$ (Figure 4.19E). Given that I observe no correlation between growth rate and interdivision time (Chapter 3, Figure 3.14A) for cells grown in standard HdB, interdivision time difference might indicate a different form of growth arrest, whereby division is delayed in the surviving fraction of cells, which confers drug tolerance to their descendants in some manner, whilst single-cell elongation is unchanged. Interdivision time is also the single-cell parameter that is most closely related to the population doubling rate, which is the key variable that changes during stationary phase.

There are also clearly observable differences in growth rate during treatment. Lineages that survive have a significantly lower overall growth rate during the treatment phase compared to non-surviving lineages with $p = 0.002$ (Table 4.3 and Fig. 4.17A). This might imply that survivors are more readily able to arrest their growth upon introduction of rifampicin. However, estimates of growth rate are unlikely to be accurate when growth arrest is involved, since ordinary least squares linear fits are probably not appropriate. Therefore, I restricted analysis to the first hour of treatment—the “initial treatment phase”—to attempt to reduce this increased source of error. Growth rate in this initial treatment phase is not significantly different between surviving and non-surviving lineages, implying that ability to arrest growth is not a factor in ultimate survival. This might be expected since the action of rifampicin itself probably causes growth arrest, rather than induction of a potential survival programme.

Another interesting difference is added length during the treatment phase. Surviving cells add a significantly greater length during treatment compared to non-surviving cells with $p = 0.028$ (Table 4.3 and Fig. 4.17C). Since added length is an absolute measure—i.e. not fitted—this may imply the opposite effect to the growth rate implication: cells that survive are able to *maintain* growth during treatment. This might be expected of viable cells, since a good indicator of viability is growth itself. Indeed, I observe some lineages which are able to grow significantly during the treatment window (e.g. Figure 4.15). However, growth arrest generally occurs rapidly after introduction of rifampicin in my experiments by naive observation (e.g. cell numbers over time), and I did not expect this result. A recent paper that investigated the single-cell response of *M. smegmatis* to rifampicin also mentions that some cells are able to maintain growth during treatment, however they use sub-MIC concentrations of rifampicin (Rego *et al.* 2017) which might be expected to result in some variability in responses.

Other authors have noted that rifampicin-susceptible cells appear to be in either the early or

late stages of their cell cycle (i.e. just divided or just about to divide) when rifampicin is added (Richardson *et al.* 2016). I do not track the cell cycle directly, but I can observe cell division events during the treatment phase. Non-surviving lineages appear to be more likely to divide during the treatment phase (25.4 % of 142 cells divide during treatment) compared to surviving lineages (12.0 % of 25), which implies that non-surviving lineages are closer to the end of the cell cycle compared to surviving lineages at the point of treatment. However, this difference is not significant ($p = 0.201$; Fisher's exact test), which may be a consequence of the low numbers of lineages that I observe to survive. Richardson *et al.* (2016) also report that non-surviving lineage cell lengths were more likely to be either small or large when rifampicin is added, reflecting their cell cycle state (early or late). I do not observe any significant difference in cell length at the start of treatment however ($p = 0.819$; Table 4.3).

Pole age has been a significant factor in prior investigations. Aldridge *et al.* (2012) and Richardson *et al.* (2016) report that *M. smegmatis* old-pole inheritors are more likely to survive rifampicin exposure, and Manina *et al.* (2015) report that *M. tuberculosis* old-pole inheritors are less likely to lyse during isoniazid treatment. In my data, when the ages of the inherited poles of cells at the start of the treatment period are determined, there are 9 old-pole inheritors and 16 new-pole inheritors (Figure 4.18A) among surviving lineages, which is not significantly different from the age distribution of non-surviving lineages ($p = 0.20$) which are relatively evenly divided (Figure 4.18B). However, this analysis does not have enough statistical power to allow a conclusion to be drawn concerning the role—or lack of a role—of pole age in tolerance to rifampicin under my conditions.

Given that growth in the alternative carbon sources acetate and pyruvate leads to deviation from adder cell-size control (Chapter 3), and given that the survival fraction at exponential phase in batch culture is greatly increased in these media (Figure 4.6), the phenomenological strategy by which cells divide might have some role in enhancing antibiotic survival, especially given that growth rate does not appear to differ greatly between standard HdB and the two alternative sources at the single-cell level (Table 3.2). I therefore determined the relationship between birth and division length according to the noisy linear map for the growth phase of the antibiotic experiments.

The preliminary hypothesis was that cultivation in sub-optimal carbon sources might bias the population towards sizer-like behaviour, which might confer drug tolerance. While I did not conduct antibiotic experiments using the alternative carbon sources, I determined whether lineages that resulted in survivors differed in their cell-size control during the growth phase compared to non-surviving lineages. I did not observe any significant change in slope with $a = 0.97 \pm 0.24$ for survivors, and $a = 1.29 \pm 0.25$ for non-survivors (Figure 4.19) with $p = 0.101$ (ANCOVA). While this might imply that non-survivors do deviate from adder (the slope does not overlap with $a = 1$), the numbers were relatively small with 65 divisions observed in

surviving lineages and 47 observed in non-surviving lineages.

4.4.4 Context of the work

Antibiotics have been a critical component of modern medicine since shortly after their discovery, and have been credited with the prevention of hundreds of millions of deaths over the past 70 years. Resistance to antibiotics was observed clinically almost simultaneously with their discovery and has remained a huge problem now and in the future, with an estimated 10 million excess deaths predicted annually by 2050 (O'Neill 2016). Tolerance, as defined as the ability to survive antibiotics in a non-heritable manner, was also observed (Bigger 1944) but was not as high priority compared to understanding and combatting drug resistance, especially during the “golden era” of antibiotic drug discovery. However, new classes of antibiotics with broad activity and few side-effects are not being discovered quickly enough to address growing insensitivity. Therefore, it is important that we understand how bacteria respond to currently existing drugs, and how we can enhance killing of transiently tolerant sub-populations, which may underlie many recurrent infections and the relapse of apparently cured individuals. *M. tuberculosis*—currently the leading cause of death by any single infectious agent (WHO 2017c)—appears to be particularly proficient at switching to a drug-tolerant state, requiring at least 6 months of antibiotic therapy to cure.

Therefore, in this chapter, I have described some basic properties of drug tolerance in the mycobacterial model species *M. smegmatis*. I demonstrate—as in *E. coli* (Lewis 2007)—that the fraction of cells able to survive rifampicin greatly increases in stationary phase, but that this does not appear to be related to nutrient availability or in response to nutrient starvation. Indeed, single-cell growth rate prior to introduction of rifampicin, as reported by others, appears to be unrelated to ability to survive. This work has raised a somewhat philosophical question of what cell death actually consists of at the single-cell level. Ability to regrow and membrane permeability, while used by others, do not appear to accurately represent the survival fraction expected, but metabolic activity does. This definition of survival may be related to reports of non-growing but metabolically active cells (Manina *et al.* 2015), which may be similar to the viable but non-culturable state (Kaprelyants *et al.* 1993). My data appears to suggest that there are very few single-cell characteristics that can predict survival prior to administration of rifampicin. I do not observe differences in pole age, growth rate (either exponential or linear), or in cell-size control in survivors compared to non-survivors. I do however observe an apparent delayed cell cycle, with increase interdivision time, which may hint at some form of delay in division permitting survival to rifampicin. This work is preliminary in nature, it requires many more cells to be analysed, since the number of cells which are able to survive high dosage rifampicin ($160\text{ }\mu\text{g ml}^{-1}$) is apparently low. Future experiments should also include the response of *M. smegmatis* to rifampicin in alternative carbon sources, and with different concentrations

of nutrients. I have conducted many experiments which were not analysed fully, thus there is a large dataset available for future work, including nitrogen starvation experiments—do any single-cell parameters predict lysis?—and some killing kinetics information which can be derived from experiments performed with propidium iodide in a similar vein. This chapter also demonstrates the power of microfluidic experiments, with the ability to change the medium rapidly and consistently, whilst observing the same cell for long periods of time. It makes extensive use of the scripts which I developed in Chapter 2 as a basis for all the single-cell analysis performed in this chapter, demonstrating their utility in another application.

CHAPTER 5 THESIS DISCUSSION

THERE is evidence that tuberculosis first emerged as a killer of humans as long as 40,000 years ago, and spread worldwide along with human expansion concurrent with the migration of humans out of Africa (Wirth *et al.* 2008; Hershberg *et al.* 2008; Comas *et al.* 2013). Definitive evidence of *M. tuberculosis* has been found in 9000-year-old Neolithic human bones in Europe (Hershkovitz *et al.* 2008), in a 5000-year-old Egyptian mummy (Nerlich *et al.* 1997), and in a 1000-year-old Peruvian remains (Salo *et al.* 1994), demonstrating that *M. tuberculosis* has been killing humans for millenia. Despite advances in medicine—including the development of germ theory, bacteriology, epidemiology, and public health—tuberculosis remained the leading cause of death by any cause up to at least the 19th century. In the UK, gripped by the fervour of the industrial revolution, tuberculosis caused half of all deaths in Bristol in the last decade of the 18th century, and a quarter of all deaths in London in the early 19th century (Dubos and Dubos 1952; Chalke 1959). This terrible mortality rate probably drove the perverse romanticisation of tuberculosis, highlighting the scale of the disease at the time. Tuberculosis deaths began to fall in the mid to late 19th century, with the introduction of public health measures and improving living conditions, long before the production of the BCG vaccine. However, the burden of disease remained high, fuelling the search for chemotherapeutic solutions, in the era following the discovery of arsphenamine (Alt 1909) and penicillin (Fleming 1929): highly efficacious against many previously fatal diseases. The first drugs effective against tuberculosis were discovered in mid-20th century: streptomycin (Schatz *et al.* 1944), *para*-aminosalicylic acid (Lehmann 1946a), and isoniazid (Robitzek *et al.* 1952), which were tested using a novel approach which has become the foundation of the verification of any new treatment: the randomised controlled trial (MRC 1950, 1955). The modern treatment regime for tuberculosis: rifampicin, isoniazid, ethambutol, and pyrazinamide, is highly effective against drug-sensitive infection, resulting in a cure in the vast majority of cases when treatment is completed (WHO 2017d). However, tuberculosis still remains the greatest cause of death by any single infectious agent, with 1.7 million deaths worldwide in 2016 (WHO 2017c).

The lack of success in reducing tuberculosis deaths could be ascribed to the ability for *M. tuberculosis* to survive within its host, a niche to which it has adapted to since the expansion of modern humans. Indeed, *M. tuberculosis* is very host-specific, and does not routinely infect

non-human animals, nor is it found in the environment, implying that *M. tuberculosis* has evolved to survive in humans and only humans. Unlike the common theme of a zoonotic source for many diseases, with jumps from animal reservoirs into humans, the opposite appears to be true in the pathogenic mycobacteria. *M. bovis* causes natural infection in all warm-blooded vertebrates (Sakamoto 2012), thus it was thought for some time that *M. tuberculosis* derived from *M. bovis* after the domestication of cattle (Stead *et al.* 1995). However, comparative genomics strongly suggest that *M. bovis* is derived from *M. tuberculosis*, implying that tuberculosis in animals—currently a significant veterinarian problem—evolved from a reverse zoonosis event from contact with humans (Brosch *et al.* 2002).

Host specificity has shaped *M. tuberculosis* into a bacterium which is able to subvert many of the sophisticated immunological defence mechanisms in humans, including robust innate immune attacks via macrophages and neutrophils, and a strong adaptive response involving many arms of the complex system. *M. tuberculosis* has evolved to thrive within such an environment, with evidence suggesting that a robust immune response may even be provoked in order to allow *M. tuberculosis* to expand within its preferred niche: the human macrophage (Baena and Porcelli 2009). Indeed, it is notable that tuberculosis most commonly affects humans in the 20–30 year age category (Marais *et al.* 2004), an age group in which the immune response is expected to be powerful and effective.

The ability for *M. tuberculosis* to survive such stresses may directly translate into its ability to survive antibiotic treatment, and may underlie the extended six-month treatment required to cure tuberculosis. This is related to the concept of drug tolerance—the ability for bacteria to survive exposure to an antibiotic without genetic resistance, in a transient manner—first noted shortly after the discovery of penicillin in *Staphylococcus* spp. (Bigger 1944). Drug tolerance occurs in a subset of bacterial populations, in a phenomenon known as bacterial persistence, and has been observed in all species in which it has been investigated (Lewis 2001; Lewis 2010; Cohen *et al.* 2013), implying that it is a universal aspect of bacterial physiology. However, mechanistic understanding of the development of persistence is lacking, notwithstanding a focus on the toxin–antitoxin systems which has subsequently been attacked from multiple angles. Persistence is thought to underlie the clinical presentation of recurrent infections (Mpiga and Ravaoarinoro 2006; Mysorekar and Hultgren 2006; Mulcahy *et al.* 2010; Berndtson 2013; Schumacher *et al.* 2015), and the lengthy treatment required for treatment of tuberculosis (McDermott 1958; Jindani *et al.* 2003; Agarwal *et al.* 2014; Walter *et al.* 2015; Honeyborne *et al.* 2016). There have been strong indications that growth arrest is involved in drug tolerance in non-mycobacterial bacterial species (e.g. Balaban *et al.* 2004), but evidence is not strong in mycobacteria (Aldridge *et al.* 2012; Wakamoto *et al.* 2013; Manina *et al.* 2015). This discrepancy reflects a surprising lack of understanding of the basic features of mycobacterial physiology, including the mode in which they grow, and in their decision to divide.

To address this, I have used long-term time-lapse microscopy coupled with microfluidics to observe *M. smegmatis* single cells as they grow and divide. This has required the optimisation of a protocol suitable for observing mycobacteria, and a robust analysis workflow, which I describe in detail in Chapter 2. This chapter reports the development of an analysis pipeline that I believe is well-suited to mycobacterial time-lapses, which are often problematic. Other authors mostly use ImageJ (Abràmoff *et al.* 2004) or its descendant Fiji (Schindelin *et al.* 2012) with the ObjectJ plugin, which involves significant manual intervention. Here I use MicrobeTracker (Sliusarenko *et al.* 2011) followed by custom Python scripts. This approach is mostly automated, with acceptable levels of manual intervention, and couples the power of the MicrobeTracker mesh concept with the lineage tracking developed in other analysis software packages such as SchnitzCells (Young *et al.* 2012).

I applied this workflow to a dataset acquired by Iria Uhía, a post-doctoral researcher within the group, regarding the chromosome segregation machinery of *M. smegmatis*. I demonstrate the extension of the software to track fluorescent proteins quantitatively and how this was used to draw conclusions based on the dataset. Specifically, we show that ParA may be inherited preferentially by larger daughter cells at division, and that this might confer a growth advantage to these cells. We also show that ParB—which binds to ParA and to *parS* sites near the origin of replication—moves actively to effect chromosome segregation, ruling out passive models of this process. This work was published recently (Uhía *et al.* 2018).

In Chapter 3, I observe cells growing with three carbon sources: standard glycerol, acetate, and pyruvate, using the microfluidic protocol developed in Chapter 2, and analyse cells using the same scripts. Using these data, I demonstrate that *M. smegmatis* cells most likely grow exponentially at the single-cell level, which was an open question at the time of the research, though a clear consensus is now forming in its favour. I then show that length at birth predicts length at division in a linear fashion, with the length added being constant on average for cells grown in glycerol. This demonstrates conformity to the adder principle of cell-size control, as opposed to the timer model—in which cell cycle time is fixed—and the sizer model—in which size at division is fixed (Jun and Taheri-Araghi 2015; Tanouchi *et al.* 2015). I show that this is robust to heterogeneity in the population generated by pole age inheritance, where cells which inherit the pole formed by their mother (new-pole inheritors) differ from cells which inherit the pole that their mother inherited (old-pole inheritors) in cell length, growth rate, and even added length. However, the adder principle breaks down when cells are grown in alternative carbon sources, with cells acquiring “sizer-like” activity. This work was published in July 2017 (Priestman *et al.* 2017), and was rapidly confirmed by another paper published in November of the same year which demonstrated almost identical results (Logsdon *et al.* 2017). At the time of publication, this was the first definitive report of adder behaviour in mycobacteria, and the first report in any bacterial species of deviation from adder cell-size control without severe growth retardation. Deviation from adder control has been proposed theoretically at very slow growth

rates, and shown in *E. coli* (Wallden *et al.* 2016), but growth rates in acetate and pyruvate are very close to growth rates in glycerol. The key questions arising from this work are the role of DNA replication in size control, though this has been partially addressed by others (Logsdon *et al.* 2017), and the effect of other medium perturbations. Growth in cholesterol as a sole carbon source would be interesting, given that *M. tuberculosis* may utilise it as a preferred carbon source *in vivo* (Pandey and Sasseti 2008; Salamon *et al.* 2014; Vromman 2014). However, cholesterol is unlikely to be a major physiological carbon source for *M. smegmatis*, limiting its potential applicability to the pathogenic mycobacteria. Cell-size control in pathogenic mycobacteria has not been widely addressed, presumably due to technical constraints given the requirement for BL3 facilities and their slow growth rate, though some work has been conducted on *M. bovis* BCG (Logsdon *et al.* 2017). BCG has the advantage of maintaining many of the pathogenic properties of *M. tuberculosis* but is classed as a BL2 biological agent, requiring fewer practical considerations. Attenuated forms of *M. tuberculosis*, such as the $\Delta leuD \Delta panCD$ double auxotroph (Sampson *et al.* 2004), are also classed as BL2, and could be used in more practical settings. Testing the response of BCG or *M. tuberculosis* to alternative carbon sources, especially cholesterol, could provide a very interesting and relevant indicator of the state of *M. tuberculosis* during infection, and provide insight into its intracellular physiology which may be of importance in the development of novel therapeutics.

The physiology of mycobacteria is important in understanding their response to antibiotics, and the phenomenon of bacterial persistence which potentially underlies the massive reservoir of tuberculosis infection worldwide. With this in mind, in Chapter 4 I address how *M. smegmatis* responds to the front-line antibiotic rifampicin, potentially the most important drug of the four used in the standard regimen. I demonstrate that, in batch culture, survival to rifampicin is intimately linked to the population growth phase, greatly increasing as cells approach and enter stationary phase. However, this change appears not to be due to nutrient status, since cultivation in carbon- or nitrogen-limited media reduces the surviving fraction, and to a very high degree during nitrogen limitation. To my knowledge, the increase in survival fraction has not been formally reported in mycobacteria, nor the apparent reduction in survival observed when nutrients are limited. Nitrogen limitation appears to greatly increase cell lysis at the single-cell level. Since lysis is uncommon under normal conditions in response to rifampicin, a philosophical question of the nature of death in single cells arises. There is frequent mention of non-culturable cells in the literature (Kaprelyants *et al.* 1993), and a large interest in dormancy in the context of latent tuberculosis infection (Esmail *et al.* 2014). Thus lack of regrowth—which is easily observable at the single-cell level—is not a definitive indicator of non-viability.

I therefore test several means of determining viability at the single-cell level, including membrane integrity with propidium iodide, active oxidative phosphorylation with CTC, and active protein synthesis and degradation machinery with an unstable fluorescent protein variant. None of these measures appear to accurately reflect cell viability, or are too complex to confi-

dently analyse. I instead use a measure of metabolic activity post-treatment: the induction of EGFP expression. Using endpoint metabolic activity, I demonstrate that cell lineages which survive do not have a significantly different growth rate compared to non-surviving lineages, in agreement with others in the field (Aldridge *et al.* 2012; Wakamoto *et al.* 2013; Manina *et al.* 2015; Richardson *et al.* 2016). However, I do observe a reduction in interdivision time, which implies that cells which survive may have a delayed cell cycle that might confer some protection. I also observe differences in growth arrest upon treatment, with rifampicin survivors apparently more able to reduce their growth rate rapidly, but also more able to maintain low-level growth throughout treatment. Unlike other authors (Richardson *et al.* 2016), I do not observe any bias in pole age between survivors and non-survivors, though this may reflect a lack of statistical power rather than a true reflection of single-cell behaviour. Finally, I show that cells which survive rifampicin do not appear to have altered cell-size control compared to cells which do not survive, suggesting that the phenomenological model of cell-size control might not have a role in predicting antibiotic tolerance.

Future work to investigate this should be performed, crucially using alternative carbon sources for which I have preliminary data (not shown). I observe that survival is greatly increased at the population level when cells are grown in pyruvate or acetate, which raises a speculative question: given that growth rate does not predict survival for cells grown in glycerol, could the deviation fromadder cell-size control be related to increased survival for cells grown in pyruvate or acetate? Unpicking the relationship between nutrient starvation and the response to antibiotic response, especially with nitrogen starvation, might also provide an interesting avenue of research: if cells are more drug-tolerant at stationary phase, why are they not more tolerant when they are starved of nitrogen? This may be related to the stress response of mycobacteria, and other authors have suggested that medium-specific effects can increase the oxidative burden of cells (Frampton *et al.* 2012), which might result in a greater impact on cell wall integrity and the increased lysis rate that is observed when cells are starved of nitrogen. Additionally, it could be assumed that the capacity for cells to remodel and repair their cell wall might be compromised when starved of nitrogen. Stationary phase occurs via a gradual transition from exponential phase which might allow cells to prepare more readily and comprehensively than is permitted by relatively sudden starvation.

In summary, I believe that this work demonstrates the utility of quantitative single-cell methodology in understanding basic mycobacterial physiology, and how this might impact important higher-level questions including that of antibiotic tolerance. Such approaches may be vital in addressing the current worldwide tuberculosis epidemic, especially with respect to enhancing the efficacy of current drug treatments.

BIBLIOGRAPHY

- Abraham E, Chain E, Fletcher C, Gardner A, Heatley N, Jennings M, and Florey H (1941). Further observations on penicillin. *The Lancet* 238(6155), pp. 177–89. DOI: 10.1016/S0140-6736(00)72122-2.
- Abraham JJ (1948). Some account of the history of the treatment of syphilis. *The British Journal of Venereal Diseases* 24(4), pp. 153–61.
- Abràmoff MD, Magalhães PJ, and Ram SJ (2004). Image Processing with ImageJ. *Biophotonics International* 11, pp. 36–42.
- Achtman M (2008). Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. *Annual Review of Microbiology* 62(1), pp. 53–70. DOI: 10.1146/annurev.micro.62.081307.162832.
- Adams KN, Takaki K, Connolly LE, Wiedenhof H, Winglee K, Humbert O, Edelstein PH, Cosma CL, and Ramakrishnan L (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 145(1), pp. 39–53. DOI: 10.1016/j.cell.2011.02.022.
- Agarwal P, Khan SR, Verma SC, Beg M, Singh K, Mitra K, Gaikwad AN, Akhtar MS, and Krishnan MY (2014). *Mycobacterium tuberculosis* persistence in various adipose depots of infected mice and the effect of anti-tubercular therapy. *Microbes and Infection* 16(7), pp. 571–80. DOI: 10.1016/j.micinf.2014.04.006.
- Ahmad S and Mokaddas E (2014). Current status and future trends in the diagnosis and treatment of drug-susceptible and multidrug-resistant tuberculosis. *Journal of Infection and Public Health* 7(2), pp. 75–91. DOI: 10.1016/J.JIPH.2013.09.001.
- Aizenman E, Engelberg-Kulka H, and Glaser G (1996). An *Escherichia coli* chromosomal “addiction module” regulated by guanosine 3',5'-bispyrophosphate: a model for programmed bacterial cell death. *Proceedings of the National Academy of Sciences of the United States of America* 93(12), pp. 6059–63.
- Akaike H (1973). “Information theory and an extension of the maximum likelihood principle”. *2nd International Symposium on Information Theory, Tsahkadsor, Armenia, USSR, September 2–8, 1971*. Budapest: Akadémiai Kiadó, pp. 267–81.

- Akiyama M, Crooke E, and Kornberg A (1992). The polyphosphate kinase gene of *Escherichia coli*. Isolation and sequence of the *ppk* gene and membrane location of the protein. *The Journal of Biological Chemistry* 267(31), pp. 22556–61.
- Akiyama M, Crooke E, and Kornberg A (1993). An exopolyphosphatase of *Escherichia coli*. The enzyme and its *ppx* gene in a polyphosphate operon. *The Journal of Biological Chemistry* 268(1), pp. 633–9.
- Albrethsen J, Agner J, Piersma SR, Højrup P, Pham TV, Weldingh K, Jimenez CR, Andersen P, and Rosenkrands I (2013). Proteomic profiling of *Mycobacterium tuberculosis* identifies nutrient-starvation-responsive toxin–antitoxin systems. *Molecular & Cellular Proteomics* 12(5), pp. 1180–91. DOI: 10.1074/mcp.M112.018846.
- Aldridge BB, Fernandez-Suarez M, Heller D, Ambravaneswaran V, Irimia D, Toner M, and Fortune SM (2012). Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science* 335(6064), pp. 100–4. DOI: 10.1126/science.1216166.
- Alexander DC, Jones JRW, and Liu J (2003). A rifampin-hypersensitive mutant reveals differences between strains of *Mycobacterium smegmatis* and presence of a novel transposon, IS1623. *Antimicrobial Agents and Chemotherapy* 47(10), pp. 3208–13. DOI: 10.1128/AAC.47.10.3208–3213.2003.
- Alt K (1909). Behandlungsversuche mit Arsenophenylglyzin bei Paralytikern. *Muenchener Medizinische Wochenschrift* 56, pp. 1457–9.
- Amato SM and Brynildsen MP (2015). Persister heterogeneity arising from a single metabolic stress. *Current Biology* 25(16), pp. 2090–8. DOI: 10.1016/j.cub.2015.06.034.
- Amir A (2014). Cell size regulation in bacteria. *Physical Review Letters* 112(20), p. 208102. DOI: 10.1103/PhysRevLett.112.208102.
- Amir A (2017). Is cell size a spandrel? *eLife* 6, e22186. DOI: 10.7554/eLife.22186.
- Amon J, Titgemeyer F, and Burkovski A (2009). A genomic view on nitrogen metabolism and nitrogen control in mycobacteria. *Journal of Molecular Microbiology and Biotechnology* 17(1), pp. 20–9. DOI: 10.1159/000159195.
- Anderson KL, Roberts C, Disz T, Vonstein V, Hwang K, Overbeek R, Olson PD, Projan SJ, and Dunman PM (2006). Characterization of the *Staphylococcus aureus* heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover. *Journal of Bacteriology* 188(19), pp. 6739–56. DOI: 10.1128/JB.00609–06.
- Appelberg R (2006). Macrophage nutritive antimicrobial mechanisms. *Journal of Leukocyte Biology* 79(6), pp. 1117–28. DOI: 10.1189/jlb.0206079.
- Baek S, Li AH, and Sassetti CM (2011). Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PLOS Biology* 9(5), e1001065. DOI: 10.1371/journal.pbio.1001065.
- Baena A and Porcelli SA (2009). Evasion and subversion of antigen presentation by *Mycobacterium tuberculosis*. *Tissue Antigens* 74(3), pp. 189–204. DOI: 10.1111/j.1399-0039.2009.01301.x.

- Balaban NQ, Gerdes K, Lewis K, and McKinney JD (2013). A problem of persistence: still more questions than answers? *Nature Reviews Microbiology* 11(8), pp. 587–91. DOI: 10.1038/nrmicro3076.
- Balaban NQ, Merrin J, Chait R, Kowalik L, and Leibler S (2004). Bacterial persistence as a phenotypic switch. *Science* 305(5690), pp. 1622–5. DOI: 10.1126/science.1099390.
- Banerjee S, Lo K, Daddysman MK, Selewa A, Kuntz T, Dinner AR, and Scherer NF (2017). Biphasic growth dynamics during *Caulobacter crescentus* division. *bioRxiv*, p. 047589. DOI: 10.1101/047589.
- Bartek I, Rutherford R, Gruppo V, Morton R, Morris R, Klein M, Visconti K, Ryan G, Schoolnik G, Lenaerts A, and Voskuil M (2009). The DosR regulon of *M. tuberculosis* and antibacterial tolerance. *Tuberculosis* 89(4), pp. 310–6. DOI: 10.1016/j.tube.2009.06.001.
- Benveniste R and Davies J (1973). Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 70(8), pp. 2276–80.
- Berndtson K (2013). Review of evidence for immune evasion and persistent infection in Lyme disease. *International Journal of General Medicine* 6, p. 291. DOI: 10.2147/IJGM.S44114.
- Bernhardt TG and de Boer PAJ (2005). SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over chromosomes in *E. coli*. *Molecular Cell* 18(5), pp. 555–64. DOI: 10.1016/j.molcel.2005.04.012.
- Bernier SP, Lebeaux D, DeFrancesco AS, Valomon A, Soubigou G, Coppée JY, Ghigo JM, and Beloin C (2013). Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. *PLOS Genetics* 9(1), e1003144. DOI: 10.1371/journal.pgen.1003144.
- Bertaux F, Marguerat S, and Shahrezaei V (2018). Division rate, cell size and proteome allocation: impact on gene expression noise and implications for the dynamics of genetic circuits. *Royal Society Open Science* 5(3), p. 172234. DOI: 10.1098/rsos.172234.
- Betts JC, Lukey PT, Robb LC, McAdam RA, and Duncan K (2002). Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Molecular Microbiology* 43(3), pp. 717–731. DOI: 10.1046/j.1365-2958.2002.02779.x.
- Bigger JW (1944). Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *The Lancet* 244(6320), pp. 497–500.
- Black DS, Irwin B, and Moyed HS (1994). Autoregulation of *hip*, an operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis. *Journal of Bacteriology* 176(13), pp. 4081–91.
- Black DS, Kelly AJ, Mardis MJ, and Moyed HS (1991). Structure and organization of *hip*, an operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis. *Journal of Bacteriology* 173(18), pp. 5732–9.

- Blokpoel MCJ, O'Toole R, Smeulders MJ, and Williams HD (2003). Development and application of unstable GFP variants to kinetic studies of mycobacterial gene expression. *Journal of Microbiological Methods* 54(2), pp. 203–211. DOI: 10.1016/S0167-7012(03)00044-7.
- Botella H, Peyron P, Levillain F, Poincloux R, Poquet Y, Brandli I, Wang C, Tailleux L, Tilleul S, Charrière GM, Waddell SJ, Foti M, Lugo-Villarino G, Gao Q, Maridonneau-Parini I, Butcher PD, Castagnoli PR, Gicquel B, Chastellier C de, and Neyrolles O (2011). Mycobacterial P₁-type ATPases mediate resistance to zinc poisoning in human macrophages. *Cell Host & Microbe* 10(3), pp. 248–59. DOI: 10.1016/J.CHOM.2011.08.006.
- Botella H, Stadthagen G, Lugo-Villarino G, Chastellier C de, and Neyrolles O (2012). Metallobiology of host–pathogen interactions: an intoxicating new insight. *Trends in Microbiology* 20(3), pp. 106–12. DOI: 10.1016/J.TIM.2012.01.005.
- Botella H, Yang G, Ouerfelli O, Ehrt S, Nathan CF, and Vaubourgeix J (2017). Distinct spatiotemporal dynamics of peptidoglycan synthesis between *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. *mBio* 8(5), e01183–17. DOI: 10.1128/mBio.01183-17.
- Boto L and Martínez JL (2011). Ecological and temporal constraints in the evolution of bacterial genomes. *Genes* 2(4), pp. 804–28. DOI: 10.3390/genes2040804.
- Braeken K, Moris M, Daniels R, Vanderleyden J, and Michiels J (2006). New horizons for (p)ppGpp in bacterial and plant physiology. *Trends in Microbiology* 14(1), pp. 45–54. DOI: 10.1016/j.tim.2005.11.006.
- Brooun A, Liu S, and Lewis K (2000). A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrobial Agents and Chemotherapy* 44(3), pp. 640–6.
- Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, Garnier T, Gutierrez C, Hewinson G, Kremer K, Parsons LM, Pym AS, Samper S, van Soolingen D, and Cole ST (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proceedings of the National Academy of Sciences of the United States of America* 99(6), pp. 3684–9. DOI: 10.1073/pnas.052548299.
- Bryskier A, ed. (2005). *Antimicrobial Agents: Antibacterials and Antifungals*. Washington, D.C.: ASM Press. DOI: 10.1086/503956.
- Bunker RD, Mandal K, Bashiri G, Chaston JJ, Pentelute BL, Lott JS, Kent SBH, and Baker EN (2015). A functional role of Rv1738 in *Mycobacterium tuberculosis* persistence suggested by racemic protein crystallography. *Proceedings of the National Academy of Sciences of the United States of America* 112(14), pp. 4310–5. DOI: 10.1073/pnas.1422387112.
- Byrne ST, Gu P, Zhou J, Denkin SM, Chong C, Sullivan D, Liu JO, and Zhang Y (2007). Pyrrolidine dithiocarbamate and diethyldithiocarbamate are active against growing and nongrowing persister *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 51(12), pp. 4495–7. DOI: 10.1128/AAC.00753-07.
- Cadart C, Monnier S, Grilli J, Attia R, Terriac E, Baum B, Cosentino-Lagomarsino M, and Piel M (2017). Size control in mammalian cells involves modulation of both growth rate and cell cycle duration. *bioRxiv*, p. 152728. DOI: 10.1101/152728.

- Calmette A, Guérin C, Boquet A, and Négre LL (1927a). *La Vaccination préventive contre la tuberculose par le "BCG"*. Paris: Masson et cie.
- Calmette A, Guérin C, and Négre LL (1927b). Sur la vaccination préventive des enfants nouveau-nés contre la tuberculose par le BCG. *Annales De l'Institut Pasteur* 41, pp. 201–32.
- Campos M, Surovtsev IV, Kato S, Paintdakhi A, Beltran B, Ebmeier SE, and Jacobs-Wagner C (2014). A constant size extension drives bacterial cell size homeostasis. *Cell* 159(6), pp. 1433–46. DOI: 10.1016/j.cell.2014.11.022.
- Cantón R, González-Alba JM, and Galán JC (2012). CTX-M enzymes: origin and diffusion. *Frontiers in Microbiology* 3, p. 110. DOI: 10.3389/fmicb.2012.00110.
- Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, and Sabatini DM (2006). CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biology* 7(10), R100. DOI: 10.1186/gb-2006-7-10-r100.
- Casart Y, Gamero E, Rivera-Gutierrez S, González-y-Merchand JA, and Salazar L (2008). *par* genes in *Mycobacterium bovis* and *Mycobacterium smegmatis* are arranged in an operon transcribed from 'SigGC' promoters. *BMC Microbiology* 8, p. 51. DOI: 10.1186/1471-2180-8-51.
- Cashel M, Gentry DR, Hernandez VJ, and Vinella D (1996). "The stringent response". *Escherichia coli and Salmonella: Cellular and Molecular Biology*. Ed. by FC Neidhardt, R Curtis, JL Ingraham, ECC Lin, KB Low, B Magasanik, WS Reznikoff, M Riley, M Schaechter, and HE Umberger. 2nd ed. Washington, D.C.: American Society of Microbiology Press, pp. 1488–96.
- Cermak N, Olcum S, Feijó Delgado F, Wasserman SC, Payer KR, Murakami MA, Knudsen SM, Kimmerling RJ, Stevens MM, Kikuchi Y, Sandikci A, Ogawa M, Agache V, Baléras F, Weinstock DM, and Manalis SR (2016). High-throughput measurement of single-cell growth rates using serial microfluidic mass sensor arrays. *Nature Biotechnology* 34(10), pp. 1052–9. DOI: 10.1038/nbt.3666.
- Chain E, Florey H, Gardner A, Heatley N, Jennings M, Orr-Ewing J, and Sanders A (1940). Penicillin as a chemotherapeutic agent. *The Lancet* 236(6104), pp. 226–8. DOI: 10.5555/URI:PII:S0140673601087281.
- Chalke HD (1959). Some historical aspects of tuberculosis. *Public Health* 74(3), pp. 83–95. DOI: 10.1016/S0033-3506(59)80055-X.
- Chaudhuri BN and Dean R (2011). The evidence of large-scale DNA-induced compaction in the mycobacterial chromosomal ParB. *Journal of Molecular Biology* 413(5), pp. 901–7. DOI: 10.1016/j.jmb.2011.08.002.
- Chien A, Hill NS, and Levin PA (2012). Cell size control in bacteria. *Current Biology* 22(9), R340–R349. DOI: 10.1016/j.cub.2012.02.032.
- Cho SH, Warit S, Wan B, Hwang CH, Pauli GF, and Franzblau SG (2007). Low-oxygen-recovery assay for high-throughput screening of compounds against nonreplicating *Mycobacterium*

- tuberculosis. Antimicrobial Agents and Chemotherapy* 51(4), pp. 1380–5. DOI: 10.1128/AAC.00055-06.
- Choder M (1991). A general topoisomerase I-dependent transcriptional repression in the stationary phase in yeast. *Genes & Development* 5(12A), pp. 2315–26. DOI: 10.1101/GAD.5.12A.2315.
- Christensen SK, Mikkelsen M, Pedersen K, and Gerdes K (2001). RelE, a global inhibitor of translation, is activated during nutritional stress. *Proceedings of the National Academy of Sciences of the United States of America* 98(25), pp. 14328–33. DOI: 10.1073/pnas.251327898.
- Clatworthy AE, Pierson E, and Hung DT (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nature Chemical Biology* 3(9), pp. 541–8. DOI: 10.1038/nchembio.2007.24.
- Clemens DL (1996). Characterization of the *Mycobacterium tuberculosis* phagosome. *Trends in Microbiology* 4(3), pp. 113–8. DOI: 10.1016/0966-842X(96)81528-9.
- Cohen NR, Lobritz MA, and Collins JJ (2013). Microbial persistence and the road to drug resistance. *Cell Host & Microbe* 13(6), pp. 632–42. DOI: 10.1016/j.chom.2013.05.009.
- Colangeli R, Helb D, Sridharan S, Sun J, Varma-Basil M, Hazbón MH, Harbacheuski R, Megjugorac NJ, Jacobs Jr WR, Holzenburg A, Sacchettini JC, and Alland D (2005). The *Mycobacterium tuberculosis iniA* gene is essential for activity of an efflux pump that confers drug tolerance to both isoniazid and ethambutol. *Molecular Microbiology* 55(6), pp. 1829–40. DOI: 10.1111/j.1365-2958.2005.04510.x.
- Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, and Mosteller F (1994). Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. *JAMA* 271(9), p. 698. DOI: 10.1001/jama.1994.03510330076038.
- Coleman MT, Chen RY, Lee M, Lin PL, Dodd LE, Maiello P, Via LE, Kim Y, Marriner G, Dartois V, Scanga C, Janssen C, Wang J, Klein E, Cho SN, Barry III CE, and Flynn JL (2014). PET/CT imaging reveals a therapeutic response to oxazolidinones in macaques and humans with tuberculosis. *Science Translational Medicine* 6(265), 265ra167. DOI: 10.1126/scitranslmed.3009500.
- Comandon J (1909). Cinématographie, à l’ultra-microscope, de microbes vivants et des particules mobiles. *Comptes Rendus Des Séances De l’Académie Des Sciences* 149, pp. 938–41.
- Comas I, Coscolla M, Luo T, Borrell S, Holt KE, Kato-Maeda M, Parkhill J, Malla B, Berg S, Thwaites G, Yeboah-Manu D, Bothamley G, Mei J, Wei L, Bentley S, Harris SR, Niemann S, Diel R, Aseffa A, Gao Q, Young D, and Gagneux S (2013). Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. *Nature Genetics* 45(10), pp. 1176–82. DOI: 10.1038/ng.2744.
- Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, Clair G, Adkins JN, Cheung AL, and Lewis K (2016). Persister formation in *Staphylococcus aureus* is associated with ATP depletion. *Nature Microbiology* 1(5), p. 16051. DOI: 10.1038/nmicrobiol.2016.51.

- Cooper S (1969). Cell division and DNA replication following a shift to a richer medium. *Journal of Molecular Biology* 43(1), pp. 1–11. DOI: 10.1016/0022-2836(69)90074-6.
- Cooper S (2006). Distinguishing between linear and exponential cell growth during the division cycle: single-cell studies, cell-culture studies, and the object of cell-cycle research. *Theoretical Biology and Medical Modelling* 3(1), p. 10. DOI: 10.1186/1742-4682-3-10.
- Cooper S and Helmstetter CE (1968). Chromosome replication and the division cycle of *Escherichia coli* B/r. *Journal of Molecular Biology* 31(3), pp. 519–40. DOI: 10.1016/0022-2836(68)90425-7.
- Corrigan RM, Bellows LE, Wood A, and Gründling A (2016). ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 113(12), E1710–9. DOI: 10.1073/pnas.1522179113.
- Cox-Maksimov D (1997). “The making of the clinical trial in Britain, 1910–1945: expertise, the state and the public”. PhD thesis. University of Cambridge, pp. 138–82.
- D’Agostino RB, Belanger A, and D’Agostino RB (1990). A suggestion for using powerful and informative tests of normality. *The American Statistician* 44(4), p. 316. DOI: 10.2307/2684359.
- D’Costa VM, McGrann KM, Hughes DW, and Wright GD (2006). Sampling the antibiotic resistome. *Science* 311(5759), pp. 374–7. DOI: 10.1126/science.1120800.
- Dahl JL (2004). Electron microscopy analysis of *Mycobacterium tuberculosis* cell division. *FEMS Microbiology Letters* 240(1), pp. 15–20. DOI: 10.1016/j.femsle.2004.09.004.
- Dahl JL, Kraus CN, Boshoff HIM, Doan B, Foley K, Avarbock D, Kaplan G, Mizrahi V, Rubin H, and Barry III CE (2003). The role of Rel_{Mtb}-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. *Proceedings of the National Academy of Sciences of the United States of America* 100(17), pp. 10026–31. DOI: 10.1073/pnas.1631248100.
- Daniel RA and Errington J (2003). Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. *Cell* 113(6), pp. 767–76. DOI: 10.1016/S0092-8674(03)00421-5.
- Daniel TM (2004). René Théophile Hyacinthe Laënnec and the founding of pulmonary medicine. *The International Journal of Tuberculosis and Lung Disease* 8(5), pp. 517–8.
- Davies J (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science* 264(5157), pp. 375–82. DOI: 10.1126/SCIENCE.8153624.
- Davies J and Davies D (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews* 74(3), pp. 417–33. DOI: 10.1128/MMBR.00016-10.
- Davies RP, Tocque K, Bellis MA, Rimmington T, and Davies PD (1999). Historical declines in tuberculosis in England and Wales: improving social conditions or natural selection? *The International Journal of Tuberculosis and Lung Disease* 3(12), pp. 1051–4.

- Davis JM and Ramakrishnan L (2009). The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 136(1), pp. 37–49. DOI: 10.1016/J.CELL.2008.11.014.
- Deb C, Lee CM, Dubey VS, Daniel J, Abomoelak B, Sirakova TD, Pawar S, Rogers L, and Kolattukudy PE (2009). A novel *in vitro* multiple-stress dormancy model for *Mycobacterium tuberculosis* generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLOS ONE* 4(6), e6077. DOI: 10.1371/journal.pone.0006077.
- Deforet M, Ditmarsch D van, and Xavier JB (2015). Cell-size homeostasis and the incremental rule in a bacterial pathogen. *Biophysical Journal* 109(3), pp. 521–8. DOI: 10.1016/j.bpj.2015.07.002.
- Dhar N and McKinney JD (2010). *Mycobacterium tuberculosis* persistence mutants identified by screening in isoniazid-treated mice. *Proceedings of the National Academy of Sciences of the United States of America* 107(27), pp. 12275–80. DOI: 10.1073/pnas.1003219107.
- Di Talia S, Skotheim JM, Bean JM, Siggia ED, and Cross FR (2007). The effects of molecular noise and size control on variability in the budding yeast cell cycle. *Nature* 448(7156), pp. 947–51. DOI: 10.1038/nature06072.
- Divangahi M, Desjardins D, Nunes-Alves C, Remold HG, and Behar SM (2010). Eicosanoid pathways regulate adaptive immunity to *Mycobacterium tuberculosis*. *Nature Immunology* 11(8), pp. 751–8. DOI: 10.1038/ni.1904.
- Donachie WD (1968). Relationship between cell size and time of initiation of DNA replication. *Nature* 219(5158), pp. 1077–9. DOI: 10.1038/2191077a0.
- Donovan C, Schwaiger A, Krämer R, and Bramkamp M (2010). Subcellular localization and characterization of the ParAB system from *Corynebacterium glutamicum*. *Journal of Bacteriology* 192(13), pp. 3441–51. DOI: 10.1128/JB.00214–10.
- Dörr T, Vulić M, and Lewis K (2010). Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. *PLOS Biology* 8(2), e1000317. DOI: 10.1371/journal.pbio.1000317.
- Drusano GL, Sgambati N, Eichas A, Brown DL, Kulawy R, and Louie A (2010). The combination of rifampin plus moxifloxacin is synergistic for suppression of resistance but antagonistic for cell kill of *Mycobacterium tuberculosis* as determined in a hollow-fiber infection model. *mBio* 1(3), e00139–10. DOI: 10.1128/mBio.00139–10.
- Dubos RJ and Davis BD (1946). Factors affecting the growth of tubercle bacilli in liquid media. *The Journal of Experimental Medicine* 83(5), pp. 409–23. DOI: 10.1084/JEM.83.5.409.
- Dubos RJ (1947). The effect of lipids and serum albumin on bacterial growth. *The Journal of Experimental Medicine* 85(1), pp. 9–22. DOI: 10.1084/JEM.85.1.9.
- Dubos RJ and Dubos J (1952). *The White Plague: Tuberculosis, Man and Society*. Boston: Little, Brown and Company.

- Dukan S and Nystrom T (1998). Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. *Genes & Development* 12(21), pp. 3431–41. DOI: 10.1101/gad.12.21.3431.
- Dye C, Scheele S, Dolin P, Pathania V, and Raviglione MC (1999). Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. *JAMA* 282(7), pp. 677–86. DOI: 10.1001/jama.282.7.677.
- EARSS (2002). *Annual Report 2002*. European Antimicrobial Resistance Surveillance System.
- ECDC (2016). *Surveillance of antimicrobial resistance in Europe*. Solna, Sweden: European Antimicrobial Resistance Surveillance Network, European Centre for Disease Prevention and Control (ECDC).
- Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, and Germain RN (2008). Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity* 28(2), pp. 271–84. DOI: 10.1016/j.immuni.2007.12.010.
- Ehlers S and Schaible UE (2012). The granuloma in tuberculosis: dynamics of a host-pathogen collusion. *Frontiers in Immunology* 3, p. 411. DOI: 10.3389/fimmu.2012.00411.
- Ehrlich P (1877). Beiträge zur Kenntniss der Anilinfärbungen und ihrer Verwendung in der mikroskopischen Technik. *Archiv für Mikroskopische Anatomie* 13(1), pp. 263–277. DOI: 10.1007/BF02933937.
- Ehrlich P (1882). Über die Färbung der Tuberkelbazillen. *Deutsche Medizinische Wochenschrift* 1, pp. 269–70.
- Eldholm V and Balloux F (2016). Antimicrobial resistance in *Mycobacterium tuberculosis*: the odd one out. *Trends in Microbiology* 24(8), pp. 637–48. DOI: 10.1016/J.TIM.2016.03.007.
- Elharar Y, Roth Z, Hermelin I, Moon A, Peretz G, Shenkerman Y, Vishkautzan M, Khalaila I, and Gur E (2014). Survival of mycobacteria depends on proteasome-mediated amino acid recycling under nutrient limitation. *The EMBO Journal* 33(16), pp. 1802–14. DOI: 10.15252/embj.201387076.
- Elowitz MB, Levine AJ, Siggia ED, and Swain PS (2002). Stochastic gene expression in a single cell. *Science* 297(5584), pp. 1183–6. DOI: 10.1126/science.1070919.
- Esmail H, Barry III CE, Young DB, and Wilkinson RJ (2014). The ongoing challenge of latent tuberculosis. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 369(1645), p. 20130437. DOI: 10.1098/rstb.2013.0437.
- Esmail H, Barry III CE, and Wilkinson RJ (2012). Understanding latent tuberculosis: the key to improved diagnostic and novel treatment strategies. *Drug Discovery Today* 17(9), pp. 514–521.
- Eun Y, Ho P, Kim M, LaRussa S, Robert L, Renner LD, Schmid A, Garner E, and Amir A (2018). Archaeal cells share common size control with bacteria despite noisier growth and division. *Nature Microbiology* 3(2), pp. 148–54. DOI: 10.1038/s41564-017-0082-6.
- Farnia P, Mohammad RM, Merza MA, Tabarsi P, Zhavnerko GK, Ibrahim TA, Kuan HO, Ghanavei J, Farnia P, Ranjbar R, Poleschuyk NN, Titov LP, Owlia P, Kazampour M, Setareh

- M, Sheikolslami M, Migliori GB, and Velayati AA (2010). Growth and cell-division in extensive (XDR) and extremely drug resistant (XXDR) tuberculosis strains: transmission and atomic force observation. *International Journal of Clinical and Experimental Medicine* 3(4), pp. 308–14.
- Feng D, Cho G, and Doolittle RF (1997). Determining divergence times with a protein clock: update and reevaluation. *Proceedings of the National Academy of Sciences of the United States of America* 94(24), pp. 13028–33. DOI: doi.org/10.1073/pnas.94.24.13028.
- Feng J, Kessler DA, Ben-Jacob E, and Levine H (2014). Growth feedback as a basis for persister bistability. *Proceedings of the National Academy of Sciences of the United States of America* 111(1), pp. 544–9. DOI: 10.1073/pnas.1320396110.
- Fine P (1995). Variation in protection by BCG: implications of and for heterologous immunity. *The Lancet* 346(8986), pp. 1339–45. DOI: 10.1016/S0140-6736(95)92348-9.
- Fisher RA, Gollan B, and Helaine S (2017). Persistent bacterial infections and persister cells. *Nature Reviews Microbiology* 15(8), pp. 453–64. DOI: 10.1038/nrmicro.2017.42.
- Flannagan R, Heit B, and Heinrichs D (2015). Antimicrobial mechanisms of macrophages and the immune evasion strategies of *Staphylococcus aureus*. *Pathogens* 4(4), pp. 826–68. DOI: 10.3390/pathogens4040826.
- Fleming A (1929). On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. *British Journal of Experimental Pathology* 10(3), pp. 226–36.
- Flores Valdez MA and Schoolnik GK (2010). DosR-regulon genes induction in *Mycobacterium bovis* BCG under aerobic conditions. *Tuberculosis* 90(3), pp. 197–200. DOI: 10.1016/j.tube.2010.04.001.
- Fox W, Ellard GA, and Mitchison DA (1999). Studies on the treatment of tuberculosis undertaken by the British Medical Research Council Tuberculosis Units, 1946–1986, with relevant subsequent publications. *The International Journal Tuberculosis and Lung Disease* 3(10), S231–79.
- Frampton R, Aggio RBM, Villas-Bôas SG, Arcus VL, and Cook GM (2012). Toxin–antitoxin systems of *Mycobacterium smegmatis* are essential for cell survival. *The Journal of Biological Chemistry* 287(8), pp. 5340–56. DOI: 10.1074/jbc.M111.286856.
- Franke MF, Appleton SC, Bayona J, Arteaga F, Palacios E, Llaro K, Shin SS, Becerra MC, Murray MB, and Mitnick CD (2008). Risk factors and mortality associated with default from multidrug-resistant tuberculosis treatment. *Clinical Infectious Diseases* 46(12), pp. 1844–51. DOI: 10.1086/588292.
- Fridman O, Goldberg A, Ronin I, Shores N, and Balaban NQ (2014). Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature* 513(7518), pp. 418–21. DOI: 10.1038/nature13469.

- Fu Z, Tamber S, Memmi G, Donegan NP, and Cheung AL (2009). Overexpression of MazF_{sa} in *Staphylococcus aureus* induces bacteriostasis by selectively targeting mRNAs for cleavage. *Journal of Bacteriology* 191(7), pp. 2051–9. DOI: 10.1128/JB.00907–08.
- Fuge EK, Braun EL, and Werner-Washburne M (1994). Protein synthesis in long-term stationary-phase cultures of *Saccharomyces cerevisiae*. *Journal of Bacteriology* 176(18), pp. 5802–13. DOI: 10.1128/JB.176.18.5802–5813.1994.
- Fung DKC, Chan EWC, Chin ML, and Chan RCY (2010). Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. *Antimicrobial Agents and Chemotherapy* 54(3), pp. 1082–93. DOI: 10.1128/AAC.01218–09.
- Gaca AO, Colomer-Winter C, and Lemos JA (2015). Many means to a common end: the intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis. *Journal of Bacteriology* 197(7), pp. 1146–56. DOI: 10.1128/JB.02577–14.
- Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, and Friedland G (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *The Lancet* 368(9547), pp. 1575–80. DOI: 10.1016/S0140–6736(06)69573–1.
- Garton NJ, Waddell SJ, Sherratt AL, Lee SM, Smith RJ, Senner C, Hinds J, Rajakumar K, Adegbola RA, Besra GS, Butcher PD, and Barer MR (2008). Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLOS Medicine* 5(4), e75. DOI: 10.1371/journal.pmed.0050075.
- Gelens L, Hill L, Vandervelde A, Danckaert J, and Loris R (2013). A general model for toxin–antitoxin module dynamics can explain persister cell formation in *E. coli*. *PLOS Computational Biology* 9(8), e1003190. DOI: 10.1371/journal.pcbi.1003190.
- Gentry DR and Cashel M (1996). Mutational analysis of the *Escherichia coli* *spoT* gene identifies distinct but overlapping regions involved in ppGpp synthesis and degradation. *Molecular Microbiology* 19(6), pp. 1373–84. DOI: 10.1111/j.1365–2958.1996.tb02480.x.
- Gerdes K, Bech FW, Jørgensen ST, Løbner-Olesen A, Rasmussen PB, Atlung T, Boe L, Karlstrom O, Molin S, and von Meyenburg K (1986). Mechanism of postsegregational killing by the *hok* gene product of the *parB* system of plasmid R1 and its homology with the *relF* gene product of the *E. coli* *relB* operon. *The EMBO Journal* 5(8), pp. 2023–9.
- Gerdes K, Møller-Jensen J, and Bugge Jensen R (2002). Plasmid and chromosome partitioning: surprises from phylogeny. *Molecular Microbiology* 37(3), pp. 455–66. DOI: 10.1046/j.1365–2958.2000.01975.x.
- Germain E, Castro-Roa D, Zenkin N, and Gerdes K (2013). Molecular mechanism of bacterial persistence by HipA. *Molecular Cell* 52(2), pp. 248–54. DOI: 10.1016/j.molcel.2013.08.045.
- Ginda K, Bezulska M, Ziółkiewicz M, Dziadek J, Zakrzewska-Czerwińska J, and Jakimowicz D (2013). ParA of *Mycobacterium smegmatis* co-ordinates chromosome segregation with the

- cell cycle and interacts with the polar growth determinant DivIVA. *Molecular Microbiology* 87(5), pp. 998–1012. DOI: 10.1111/mmi.12146.
- Glaziou P, Sismanidis C, Floyd K, and Raviglione M (2014). Global epidemiology of tuberculosis. *Cold Spring Harbor Perspectives in Medicine* 5(2), a017798. DOI: 10.1101/cshperspect.a017798.
- Godin M, Feijó Delgado F, Son S, Grover WH, Bryan AK, Tzur A, Jorgensen P, Payer K, Grossman AD, Kirschner MW, and Manalis SR (2010). Using buoyant mass to measure the growth of single cells. *Nature Methods* 7(5), pp. 387–90. DOI: 10.1038/nmeth.1452.
- Golding I and Cox EC (2006). Physical nature of bacterial cytoplasm. *Physical Review Letters* 96(9), p. 098102. DOI: 10.1103/PhysRevLett.96.098102.
- Gor V, Elowitz M, Bacarian T, and Mjolsness E (2005). “Tracking cell signals in fluorescent images”. *Computer Vision Methods for Bioinformatics (CVMB) workshop, 2005 IEEE Computer Society Conference on Computer Vision and Pattern Recognition (CVPR)*. San Diego, California, USA: IEEE, p. 142. DOI: 10.1109/CVPR.2005.544.
- Gradmann C (2006). Robert Koch and the white death: from tuberculosis to tuberculin. *Microbes and Infection* 8(1), pp. 294–301.
- Graham TGW, Wang X, Song D, Etson CM, Oijen AM van, Rudner DZ, and Loparo JJ (2014). ParB spreading requires DNA bridging. *Genes & Development* 28(11), pp. 1228–38. DOI: 10.1101/gad.242206.114.
- Gram HC (1884). Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten. *Fortschritte der Medizin* 2, pp. 185–89.
- Griffin JE, Pandey AK, Gilmore SA, Mizrahi V, McKinney JD, Bertozzi CR, and Sassetti CM (2012). Cholesterol catabolism by *Mycobacterium tuberculosis* requires transcriptional and metabolic adaptations. *Chemistry & biology* 19(2), pp. 218–27. DOI: 10.1016/j.chembiol.2011.12.016.
- Grune T, Jung T, Merker K, and Davies KJ (2004). Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and ‘aggresomes’ during oxidative stress, aging, and disease. *The International Journal of Biochemistry & Cell Biology* 36(12), pp. 2519–30. DOI: 10.1016/J.BIOCEL.2004.04.020.
- Guo Y, Quiroga C, Chen Q, McAnulty MJ, Benedik MJ, Wood TK, and Wang X (2014). RalR (a DNase) and RalA (a small RNA) form a type I toxin–antitoxin system in *Escherichia coli*. *Nucleic Acids Research* 42(10), pp. 6448–62. DOI: 10.1093/nar/gku279.
- Gupta A (2009). Killing activity and rescue function of genome-wide toxin–antitoxin loci of *Mycobacterium tuberculosis*. *FEMS microbiology letters* 290(1), pp. 45–53. DOI: 10.1111/j.1574-6968.2008.01400.x.
- Gutierrez MC, Brisse S, Brosch R, Fabre M, Omais B, Marmiesse M, Supply P, and Vincent V (2005). Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PLOS Pathogens* 1(1), e5. DOI: 10.1371/journal.ppat.0010005.

- Hall BG and Barlow M (2004). Evolution of the serine β -lactamases: past, present and future. *Drug Resistance Updates* 7(2), pp. 111–23. DOI: 10.1016/J.DRUP.2004.02.003.
- Han J, Lee JJ, Anandan T, Zeng M, Sripathi S, Jahng WJ, Lee SH, Suh J, and Kang C (2010). Characterization of a chromosomal toxin–antitoxin, Rv1102c–Rv1103c system in *Mycobacterium tuberculosis*. *Biochemical and Biophysical Research Communications* 400(3), pp. 293–8.
- Handwerger S and Tomasz A (1985). Antibiotic tolerance among clinical isolates of bacteria. *Annual Review of Pharmacology and Toxicology* 25(1), pp. 349–80. DOI: 10.1146/annurev.pa.25.040185.002025.
- Hansen S, Lewis K, and Vulić M (2008). Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* 52(8), pp. 2718–26. DOI: 10.1128/AAC.00144–08.
- Harms A, Fino C, Sørensen MA, Semsey S, and Gerdes K (2017). Prophages and growth dynamics confound experimental results with antibiotic-tolerant persister cells. *mBio* 8(6), e01964–17. DOI: 10.1128/mBio.01964–17.
- Harms A, Maisonneuve E, and Gerdes K (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science* 354(6318), aaf4268. DOI: 10.1126/science.aaf4268.
- Harris LK and Theriot JA (2016). Relative rates of surface and volume synthesis set bacterial cell size. *Cell* 165(6), pp. 1479–92. DOI: 10.1016/j.cell.2016.05.045.
- Hart PD and Sutherland I (1977). BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life. *British Medical Journal* 2(6082), pp. 293–5.
- Hartl FU, Bracher A, and Hayer-Hartl M (2011). Molecular chaperones in protein folding and proteostasis. *Nature* 475(7356), pp. 324–32. DOI: 10.1038/nature10317.
- Hartmans S and De Bont JAM (1992). “The genus *Mycobacterium* – nonmedical”. *The Prokaryotes, a handbook on the biology of bacteria: ecophysiology, isolation, identification, application*. Ed. by A Balows, HG Truper, M Dworkin, W Harder, and KH Schleifer. 2nd edition. New York, N. Y.: Springer-Verlag New York Inc., pp. 1215–37.
- Hashimoto M, Nozoe T, Nakaoka H, Okura R, Akiyoshi S, Kaneko K, Kussell E, and Wakamoto Y (2016). Noise-driven growth rate gain in clonal cellular populations. *Proceedings of the National Academy of Sciences of the United States of America* 113(12), pp. 3251–6. DOI: 10.1073/pnas.1519412113.
- Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, and Gerdes K (2015). Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nature Reviews Microbiology* 13(5), pp. 298–309. DOI: 10.1038/nrmicro3448.
- Hayes CS and Low DA (2009). Signals of growth regulation in bacteria. *Current Opinion in Microbiology* 12(6), pp. 667–73. DOI: 10.1016/J.MIB.2009.09.006.
- Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews Sa, and Holden DW (2014). Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. *Science* 343(6167), pp. 204–8. DOI: 10.1126/science.1244705.

- Helaine S, Thompson Ja, Watson KG, Liu M, Boyle C, and Holden DW (2010). Dynamics of intracellular bacterial replication at the single cell level. *Proceedings of the National Academy of Sciences of the United States of America* 107(8), pp. 3746–51. DOI: 10.1073/pnas.1000041107.
- Helmstetter CE (1969). Sequence of bacterial reproduction. *Annual Review of Microbiology* 23(1), pp. 223–38. DOI: 10.1146/annurev.mi.23.100169.001255.
- Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, Homolka S, Roach JC, Kremer K, Petrov DA, Feldman MW, and Gagneux S (2008). High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. *PLOS Biology* 6(12), e311. DOI: 10.1371/journal.pbio.0060311.
- Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OY, Gernaey AM, Galili E, Eshed V, Greenblatt CL, Lemma E, Bar-Gal GK, and Spigelman M (2008). Detection and molecular characterization of 9,000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. *PLOS ONE* 3(10), e3426. DOI: 10.1371/journal.pone.0003426.
- Hett EC and Rubin EJ (2008). Bacterial growth and cell division: a mycobacterial perspective. *Microbiology and Molecular Biology Reviews* 72(1), pp. 126–56. DOI: 10.1128/MMBR.00028-07.
- Ho P and Amir A (2015). Simultaneous regulation of cell size and chromosome replication in bacteria. *Frontiers in Microbiology* 6, p. 662. DOI: 10.3389/fmicb.2015.00662.
- Honeyborne I, McHugh TD, Kuittinen I, Cichonska A, Evangelopoulos D, Ronacher K, van Helden PD, Gillespie SH, Fernandez-Reyes D, Walzl G, Rousu J, Butcher PD, and Waddell SJ (2016). Profiling persistent tubercle bacilli from patient sputa during therapy predicts early drug efficacy. *BMC Medicine* 14, p. 68. DOI: 10.1186/s12916-016-0609-3.
- Houndt T and Ochman H (2000). Long-term shifts in patterns of antibiotic resistance in enteric bacteria. *Applied and Environmental Microbiology* 66(12), pp. 5406–9. DOI: 10.1128/AEM.66.12.5406-5409.2000.
- Hu L, Vecchiarelli AG, Mizuuchi K, Neuman KC, and Liu J (2015). Directed and persistent movement arises from mechanochemistry of the ParA/ParB system. *Proceedings of the National Academy of Sciences of the United States of America* 112(51), E7055–64. DOI: 10.1073/pnas.1505147112.
- Hu Y and Coates ARM (2005). Transposon mutagenesis identifies genes which control antimicrobial drug tolerance in stationary-phase *Escherichia coli*. *FEMS microbiology letters* 243(1), pp. 117–24. DOI: 10.1016/j.femsle.2004.11.049.
- Huls PG, Vischer NOE, and Woldringh CL (2018). Different amounts of DNA in newborn cells of *Escherichia coli* preclude a role for the chromosome in size control according to the “adder” model. *Frontiers in Microbiology* 9, p. 664. DOI: 10.3389/fmicb.2018.00664.

- Iizuka R, Yamagishi-Shirasaki M, and Funatsu T (2011). Kinetic study of *de novo* chromophore maturation of fluorescent proteins. *Analytical Biochemistry* 414(2), pp. 173–8. DOI: 10.1016/J.AB.2011.03.036.
- Inoué S and Spring KR (1997). *Video microscopy: the fundamentals*. 2nd edition. Plenum Press.
- Iyer-Biswas S, Wright CS, Henry JT, Lo K, Burov S, Lin Y, Crooks GE, Crosson S, Dinner AR, and Scherer NF (2014). Scaling laws governing stochastic growth and division of single bacterial cells. *Proceedings of the National Academy of Sciences of the United States of America* 111(45), pp. 15912–7. DOI: 10.1073/pnas.1403232111.
- Jabado N, Jankowski A, Dougaparsad S, Picard V, Grinstein S, and Gros P (2000). Natural resistance to intracellular infections: natural resistance–associated macrophage protein 1 (NRAMP1) functions as a pH-dependent manganese transporter at the phagosomal membrane. *The Journal of Experimental Medicine* 192(9), pp. 1237–48.
- Jagger A, Reiter-karam S, Hamada Y, and Getahun H (2018). National policies on the management of latent tuberculosis infection: review of 98 countries. *Bulletin of the World Health Organization* 96(3), 173–184F. DOI: 10.2471/BLT.17.199414.
- Jakimowicz D, Brzostek A, Rumijowska-Galewicz A, Żydek P, Dołzbłasz A, Smulczyk-Krawczyszyn A, Zimniak T, Wojtasz Ł, Zawilak-Pawlik A, Kois A, Dziadek J, and Zakrzewska-Czerwińska J (2007). Characterization of the mycobacterial chromosome segregation protein ParB and identification of its target in *Mycobacterium smegmatis*. *Microbiology* 153(12), pp. 4050–60. DOI: 10.1099/mic.0.2007/011619-0.
- Jakimowicz D, Chater K, and Zakrzewska-Czerwińska J (2002). The ParB protein of *Streptomyces coelicolor* A3(2) recognizes a cluster of *parS* sequences within the origin-proximal region of the linear chromosome. *Molecular Microbiology* 45(5), pp. 1365–77. DOI: 10.1046/j.1365-2958.2002.03102.x.
- Jani C, Eoh H, Lee J, Hamasha K, Sahana M, Han J, Nyayapathy S, Lee J, Suh J, Lee S, Rehse SJ, Crick DC, and Kang C (2010). Regulation of polar peptidoglycan biosynthesis by Wag31 phosphorylation in mycobacteria. *BMC Microbiology* 10(1), p. 327. DOI: 10.1186/1471-2180-10-327.
- Jarlier V and Nikaido H (1994). Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS Microbiology Letters* 123(1), pp. 11–8.
- Javid B, Sorrentino F, Toosky M, Zheng W, Pinkham JT, Jain N, Pan M, Deighan P, and Rubin EJ (2014). Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance. *Proceedings of the National Academy of Sciences of the United States of America* 111(3), pp. 1132–7. DOI: 10.1073/pnas.1317580111.
- Jenkins VA, Barton GR, Robertson BD, and Williams KJ (2013). Genome wide analysis of the complete GlnR nitrogen-response regulon in *Mycobacterium smegmatis*. *BMC Genomics* 14(1), p. 301. DOI: 10.1186/1471-2164-14-301.

- Jenkins VA, Robertson BD, and Williams KJ (2012). Aspartate D48 is essential for the GlnR-mediated transcriptional response to nitrogen limitation in *Mycobacterium smegmatis*. *FEMS Microbiology Letters* 330(1), pp. 38–45. DOI: 10.1111/j.1574-6968.2012.02530.x.
- Jensen RB and Gerdes K (1995). Programmed cell death in bacteria: proteic plasmid stabilization systems. *Molecular Microbiology* 17(2), pp. 205–10. DOI: 10.1111/j.1365-2958.1995.mmi_17020205.x.
- Jiang X, Zhang W, Zhang Y, Gao F, Lu C, Zhang X, and Wang H (2008). Assessment of efflux pump gene expression in a clinical isolate *Mycobacterium tuberculosis* by real-time reverse transcription PCR. *Microbial Drug Resistance* 14(1), pp. 7–11. DOI: 10.1089/mdr.2008.0772.
- Jindani A, Doré CJ, and Mitchison DA (2003). Bactericidal and sterilizing activities of antituberculosis drugs during the first 14 days. *American Journal of Respiratory and Critical Care Medicine* 167(10), pp. 1348–54. DOI: 10.1164/rccm.200210-11250C.
- Jona G, Choder M, and Gileadi O (2000). Glucose starvation induces a drastic reduction in the rates of both transcription and degradation of mRNA in yeast. *Biochimica et Biophysica Acta* 1491(1-3), pp. 37–48. DOI: 10.1016/S0167-4781(00)00016-6.
- Jones E, Oliphant T, Peterson P, et al. (2001). *SciPy: Open source scientific tools for Python*. URL: <http://www.scipy.org/>.
- Joyce G, Robertson BD, and Williams KJ (2011). A modified agar pad method for mycobacterial live-cell imaging. *BMC Research Notes* 4, p. 73. DOI: 10.1186/1756-0500-4-73.
- Joyce G, Williams KJ, Robb M, Noens E, Tizzano B, Shahrezaei V, and Robertson BD (2012). Cell division site placement and asymmetric growth in mycobacteria. *PLOS ONE* 7(9), e44582. DOI: 10.1371/journal.pone.0044582.
- Jun S and Taheri-Araghi S (2015). Cell-size maintenance: universal strategy revealed. *Trends in Microbiology* 23(1), pp. 4–6. DOI: 10.1016/j.tim.2014.12.001.
- Kang C, Nyayapathy S, Lee J, Suh J, and Husson RN (2008). Wag31, a homologue of the cell division protein DivIVA, regulates growth, morphology and polar cell wall synthesis in mycobacteria. *Microbiology* 154(3), pp. 725–35. DOI: 10.1099/mic.0.2007/014076-0.
- Kang DD, Lin Y, Moreno JR, Randall TD, and Khader SA (2011). Profiling early lung immune responses in the mouse model of tuberculosis. *PLOS ONE* 6(1), e16161. DOI: 10.1371/journal.pone.0016161.
- Kanjee U, Ogata K, and Houry WA (2012). Direct binding targets of the stringent response alarmone (p)ppGpp. *Molecular Microbiology* 85(6), pp. 1029–43. DOI: 10.1111/j.1365-2958.2012.08177.x.
- Kaprelyants AS, Gottschal JC, and Kell DB (1993). Dormancy in non-sporulating bacteria. *FEMS Microbiology Letters* 104(3–4), pp. 271–286. DOI: 10.1111/j.1574-6968.1993.tb05871.x.

- Karakousis PC (2004). Dormancy phenotype displayed by extracellular *Mycobacterium tuberculosis* within artificial granulomas in mice. *Journal of Experimental Medicine* 200(5), pp. 647–57. DOI: 10.1084/jem.20040646.
- Keiler KC and Sauer RT (1996). Sequence determinants of C-terminal substrate recognition by the Tsp protease. *Journal of Biological Chemistry* 271(5), pp. 2589–93. DOI: 10.1074/jbc.271.5.2589.
- Kennard AS, Osella M, Javer A, Grilli J, Nghe P, Tans SJ, Cicuta P, and Cosentino Lagomarsino M (2016). Individuality and universality in the growth-division laws of single *E. coli* cells. *Physical Review E* 93(1), p. 012408. DOI: 10.1103/PhysRevE.93.012408.
- Keren I, Kaldalu N, Spoering A, Wang Y, and Lewis K (2004a). Persister cells and tolerance to antimicrobials. *FEMS Microbiology Letters* 230(1), pp. 13–8. DOI: 10.1016/S0378-1097(03)00856-5.
- Keren I, Minami S, Rubin E, and Lewis K (2011). Characterization and transcriptome analysis of *Mycobacterium tuberculosis* persisters. *mBio* 2(3), e00100–11. DOI: 10.1128/mBio.00100-11.
- Keren I, Shah D, Spoering A, Kaldalu N, and Lewis K (2004b). Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *Journal of Bacteriology* 186(24), pp. 8172–80. DOI: 10.1128/JB.186.24.8172-8180.2004.
- Kester JC and Fortune SM (2014). Persisters and beyond: mechanisms of phenotypic drug resistance and drug tolerance in bacteria. *Critical Reviews in Biochemistry and Molecular Biology* 49(2), pp. 91–101. DOI: 10.3109/10409238.2013.869543.
- Kieser KJ and Rubin EJ (2014). How sisters grow apart: mycobacterial growth and division. *Nature Reviews Microbiology* 12(8), pp. 550–62. DOI: 10.1038/nrmicro3299.
- Kieser T, Bibb MJ, Buttner MJ, Chater KF, and Hopwood DA (2000). “General introduction to actinomycete biology”. *Practical Streptomyces genetics*. Norwich, UK: John Innes Foundation. Chap. 1.
- Kim J and Wood TK (2016). Persistent persister misperceptions. *Frontiers in Microbiology* 07, p. 2134. DOI: 10.3389/fmicb.2016.02134.
- Kint CI, Verstraeten N, Fauvart M, and Michiels J (2012). New-found fundamentals of bacterial persistence. *Trends in Microbiology* 20(12), pp. 577–85. DOI: 10.1016/j.tim.2012.08.009.
- Kling HM, Nau GJ, Ross TM, Evans TG, Chakraborty K, Empey KM, and Flynn JL (2014). Challenges and future in vaccines, drug development, and immunomodulatory therapy. *Annals of the American Thoracic Society* 11(Supplement 4), S201–10. DOI: 10.1513/AnnalsATS.201401-036PL.
- van Klingeren B, Dessens-Kroon M, van der Laan T, Kremer K, and van Soolingen D (2007). Drug susceptibility testing of *Mycobacterium tuberculosis* complex by use of a high-throughput, reproducible, absolute concentration method. *Journal of Clinical Microbiology* 45(8), pp. 2662–8. DOI: 10.1128/JCM.00244-07.

- Klumpp S and Hwa T (2014). Bacterial growth: global effects on gene expression, growth feedback and proteome partition. *Current Opinion in Biotechnology* 28, pp. 96–102. DOI: 10.1016/j.copbio.2014.01.001.
- Koch AL and Schaechter M (1962). A model for statistics of the cell division process. *Journal of General Microbiology* 29(3), pp. 435–54. DOI: 10.1099/00221287-29-3-435.
- Koch R (1882). Die Ätiologie der Tuberkulose. *Berliner Klinische Wochenschrift* 19, pp. 221–30.
- Koch R (1890). “Über bakteriologische Forschung”. *Verhandlungen des X. Internationalen Medizinischen Kongresses*. August Hirschwald, pp. 650–60.
- Koppes LJ, Meyer M, Oonk HB, Jong MA de, and Nanninga N (1980). Correlation between size and age at different events in the cell division cycle of *Escherichia coli*. *Journal of Bacteriology* 143(3), pp. 1241–52.
- Korch SB, Henderson TA, and Hill TM (2003). Characterization of the *hipA7* allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. *Molecular Microbiology* 50(4), pp. 1199–213. DOI: 10.1046/j.1365-2958.2003.03779.x.
- Krause AK (1928). Tuberculosis and public health. *American Review of Tuberculosis* 18, pp. 271–322.
- Kuroda A, Nomura K, Ohtomo R, Kato J, Ikeda T, Takiguchi N, Ohtake H, and Kornberg A (2001). Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science* 293(5530), pp. 705–8. DOI: 10.1126/science.1061315.
- Kurthkoti K, Amin H, Marakalala MJ, Ghanny S, Subbian S, Sakatos A, Livny J, Fortune SM, Berney M, and Rodriguez GM (2017). The capacity of *Mycobacterium tuberculosis* to survive iron starvation might enable it to persist in iron-deprived microenvironments of human granulomas. *mBio* 8(4), e01092–17. DOI: 10.1128/mBio.01092–17.
- Kussell E and Leibler S (2005). Phenotypic diversity, population growth, and information in fluctuating environments. *Science* 309(5743), pp. 2075–8. DOI: 10.1126/science.1114383.
- Kyriacou SV, Nowak ME, Brownlow WJ, and Xu XN (2002). Single live cell imaging for real-time monitoring of resistance mechanism in *Pseudomonas aeruginosa*. *Journal of Biomedical Optics* 7(4), p. 576. DOI: 10.1117/1.1506707.
- Lafleur MD, Qi Q, and Lewis K (2010). Patients with long-term oral carriage harbor high-persister mutants of *Candida albicans*. *Antimicrobial Agents and Chemotherapy* 54(1), pp. 39–44. DOI: 10.1128/AAC.00860–09.
- Lawn SD and Nicol MP (2011). Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. *Future Microbiology* 6(9), pp. 1067–82. DOI: 10.2217/fmb.11.84.
- Lee P, Gaige T, and Hung P (2011). “Microfluidic systems for live cell imaging”. *Methods in Cell Biology*. Vol. 102. Academic Press. Chap. 4, pp. 77–103. DOI: 10.1016/B978-0-12-374912-3.00004-3.

- van Leeuwenhoek A (1677). Observations, communicated to the publisher by Mr. Antony van Leewenhoek, in a Dutch letter of the 9 Octob. 1676 here English'd: Concerning little Animals by him observed in Rain-Well-Sea- and Snow water; as also in water wherein Pepper had lain infused. *Philosophical Transactions of the Royal Society of London* 12(133-142), pp. 821–31. DOI: 10.1098/rstl.1677.0003.
- Lehmann J (1946a). Determination of pathogenicity of tubercle bacilli by their intermediate metabolism. *The Lancet* 247(6384), pp. 14–5. DOI: 10.1016/S0140-6736(46)91184-1.
- Lehmann J (1946b). Para-aminosalicylic acid in the treatment of tuberculosis. *The Lancet* 247(6384), pp. 15–6. DOI: 10.1016/S0140-6736(46)91185-3.
- Lenaerts A, Barry III CE, and Dartois V (2015). Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunological Reviews* 264(1), pp. 288–307. DOI: 10.1111/imr.12252.
- Lennon JT and Jones SE (2011). Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology* 9(2), pp. 119–30. DOI: 10.1038/nrmicro2504.
- Leung V and Lévesque CM (2012). A stress-inducible quorum-sensing peptide mediates the formation of persister cells with noninherited multidrug tolerance. *Journal of Bacteriology* 194(9), pp. 2265–74. DOI: 10.1128/JB.06707-11.
- Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shores N, and Balaban NQ (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science* 355(6327), pp. 826–30. DOI: 10.1126/science.aaj2191.
- Levin BR and Rozen DE (2006). Non-inherited antibiotic resistance. *Nature Reviews Microbiology* 4(7), pp. 556–62. DOI: 10.1038/nrmicro1445.
- Levin BR, Concepción-Acevedo J, and Udekwu KI (2014). Persistence: a copacetic and parsimonious hypothesis for the existence of non-inherited resistance to antibiotics. *Current opinion in microbiology* 21, pp. 18–21. DOI: 10.1016/j.mib.2014.06.016.
- Lewis K (2001). Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy* 45(4), pp. 999–1007. DOI: 10.1128/AAC.45.4.999-1007.2001.
- Lewis K (2007). Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology* 5(1), pp. 48–56. DOI: 10.1038/nrmicro1557.
- Lewis K (2010). Persister cells. *Annual Review of Microbiology* 64, pp. 357–72. DOI: 10.1146/annurev.micro.112408.134306.
- Li G, Zhang Y, Inouye M, and Ikura M (2008). Structural mechanism of transcriptional autorepression of the *Escherichia coli* RelB/RelE antitoxin/toxin module. *Journal of Molecular Biology* 380(1), pp. 107–19. DOI: 10.1016/J.JMB.2008.04.039.
- Li J, Ji L, Shi W, Xie J, and Zhang Y (2013). Trans-translation mediates tolerance to multiple antibiotics and stresses in *Escherichia coli*. *Journal of Antimicrobial Chemotherapy* 68(11), pp. 2477–81. DOI: 10.1093/jac/dkt231.

- Lim HC, Surovtsev IV, Beltran BG, Huang F, Bewersdorf J, and Jacobs-Wagner C (2014). Evidence for a DNA-relay mechanism in ParABS-mediated chromosome segregation. *eLife* 3, e02758. DOI: 10.7554/eLife.02758.
- Livny J, Yamaichi Y, and Waldor MK (2007). Distribution of centromere-like *parS* sites in bacteria: insights from comparative genomics. *Journal of Bacteriology* 189(23), pp. 8693–703. DOI: 10.1128/JB.01239-07.
- Lobato-Márquez D, Moreno-Córdoba I, Figueroa V, Díaz-Orejas R, and García-del Portillo F (2015). Distinct type I and type II toxin–antitoxin modules control *Salmonella* lifestyle inside eukaryotic cells. *Scientific Reports* 5, p. 9374. DOI: 10.1038/srep09374.
- Lodi S, del Amo J, d’Arminio Monforte A, Abgrall S, Sabin C, Morrison C, Furrer H, Muga R, Porter K, and Girardi E (2013). Risk of tuberculosis following HIV seroconversion in high-income countries. *Thorax* 68(3), pp. 207–13. DOI: 10.1136/thoraxjnl-2012-201740.
- Logsdon MM and Aldridge BB (2018). Stable regulation of cell cycle events in mycobacteria: insights from inherently heterogeneous bacterial populations. *Frontiers in Microbiology* 9, p. 514. DOI: 10.3389/fmicb.2018.00514.
- Logsdon MM, Ho PY, Papavinasasundaram K, Richardson K, Cokol M, Sassetti CM, Amir A, and Aldridge BB (2017). A parallel adder coordinates mycobacterial cell-cycle progression and cell-size homeostasis in the context of asymmetric growth and organization. *Current Biology* 27(21), 3367–3374.e7. DOI: 10.1016/j.cub.2017.09.046.
- Lorenz MC and Fink GR (2002). Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryotic Cell* 1(5), pp. 657–62. DOI: 10.1128/EC.1.5.657-662.2002.
- Losick R and Desplan C (2008). Stochasticity and cell fate. *Science* 320(5872), pp. 65–8. DOI: 10.1126/science.1147888.
- Lustgarten S (1885). The bacillus of syphilis. *The Lancet* 125(3214), pp. 609–10. DOI: 10.1016/S0140-6736(02)17741-5.
- Maisonneuve E, Castro-Camargo M, and Gerdes K (2013). (p)ppGpp controls bacterial persistence by stochastic induction of toxin–antitoxin activity. *Cell* 154(5), pp. 1140–50. DOI: 10.1016/j.cell.2013.07.048. (Retraction published Feb 22, 2018, *Cell*, 172.5, p. 1135).
- Maisonneuve E, Castro-Camargo M, and Gerdes K (2018a). Retraction: (p)ppGpp controls bacterial persistence by stochastic induction of toxin–antitoxin activity. *Cell* 172(5), p. 1135. DOI: 10.1016/J.CELL.2018.02.023.
- Maisonneuve E, Shakespeare LJ, Jørgensen MG, and Gerdes K (2011). Bacterial persistence by RNA endonucleases. *Proceedings of the National Academy of Sciences of the United States of America* 108(32), pp. 13206–11. DOI: 10.1073/pnas.1100186108. (Retraction published March 12, 2018, *Proc Natl Acad Sci*, 115.12, p. E2901).
- Maisonneuve E, Shakespeare LJ, Jørgensen MG, and Gerdes K (2018b). Retraction for Maisonneuve *et al.*, Bacterial persistence by RNA endonucleases. *Proceedings of the National Academy of Sciences of the United States of America* 115(12), E2901. DOI: 10.1073/pnas.1803278115.

- Maloney E, Madiraju M, and Rajagopalan M (2009). Overproduction and localization of *Mycobacterium tuberculosis* ParA and ParB proteins. *Tuberculosis* 89 Suppl 1, S65–9. DOI: 10.1016/S1472-9792(09)70015-0.
- de Man J (1975). The probability of most probable numbers. *European Journal of Applied Microbiology* 1(1), pp. 67–78. DOI: 10.1007/BF01880621.
- Manina G, Dhar N, and McKinney JD (2015). Stress and host immunity amplify *Mycobacterium tuberculosis* phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host & Microbe* 17(1), pp. 32–46. DOI: 10.1016/j.chom.2014.11.016.
- Marais BJ, Gie RP, Schaaf HS, Hesselning AC, Obihara CC, Starke JJ, Enarson DA, Donald PR, and Beyers N (2004). The natural history of childhood intra-thoracic tuberculosis: a critical review of literature from the pre-chemotherapy era. *The International Journal of Tuberculosis and Lung Disease* 8(4), pp. 392–402.
- Margolin W (2009). Sculpting the bacterial cell. *Current Biology* 19(17), R812–22. DOI: 10.1016/j.cub.2009.06.033.
- Martin CJ, Cadena AM, Leung VW, Lin PL, Maiello P, Hicks N, Chase MR, Flynn JL, and Fortune SM (2017). Digitally barcoding *Mycobacterium tuberculosis* reveals *in vivo* infection dynamics in the macaque model of tuberculosis. *mBio* 8(3), e00312–17. DOI: 10.1128/mBio.00312-17.
- Martin SG and Chang F (2005). New end take off: regulating cell polarity during the fission yeast cell cycle. *Cell Cycle* 4(8), pp. 4046–9. DOI: 10.4161/cc.4.8.1853.
- Martínez JL and Baquero F (2000). Mutation frequencies and antibiotic resistance. *Antimicrobial Agents and Chemotherapy* 44(7), pp. 1771–7. DOI: 10.1128/AAC.44.7.1771-1777.2000.
- Martínez JL (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science* 321(5887), pp. 365–7. DOI: 10.1126/science.1159483.
- Martínez JL (2014). General principles of antibiotic resistance in bacteria. *Drug Discovery Today: Technologies* 11, pp. 33–39. DOI: 10.1016/J.DDTEC.2014.02.001.
- McAdams HH and Arkin A (1999). It's a noisy business! Genetic regulation at the nanomolar scale. *Trends in Genetics* 15(2), pp. 65–9. DOI: 10.1016/S0168-9525(98)01659-X.
- McCune RM, Feldmann FM, Lambert HP, and McDermott W (1966a). Microbial persistence: the capacity of tubercle bacilli to survive sterilization in mouse tissues. *The Journal of Experimental Medicine* 123(3), pp. 445–68. DOI: 10.1084/JEM.123.3.445.
- McCune RM, Feldmann FM, and McDermott W (1966b). Microbial persistence: characteristics of the sterile state of tubercle bacilli. *Journal of Experimental Medicine* 123(3), pp. 469–86. DOI: 10.1084/jem.123.3.469.
- McCune RM, McDermott W, and Tompsett R (1956). The fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique: the conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. *The Journal of Experimental Medicine* 104(5), pp. 763–802. DOI: 10.1084/JEM.104.5.763.

- McCune RM and Tompsett R (1956). Fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique: the persistence of drug-susceptible tubercle bacilli in the tissues despite prolonged antimicrobial therapy. *The Journal of Experimental Medicine* 104(5), pp. 737–62. DOI: 10.1084/JEM.104.5.737.
- McDermott W (1958). Microbial persistence. *The Yale Journal of Biology and Medicine* 30(4), pp. 257–91.
- McDonagh JER (1912). Some toxic effects of Salvarsan. *BMJ* 1(2666), p. 272. DOI: 10.1136/bmj.1.2666.272-b.
- McDonald JC, Duffy DC, Anderson JR, Chiu DT, Wu H, Schueller OJA, and Whitesides GM (2000). Fabrication of microfluidic systems in poly(dimethylsiloxane). *Electrophoresis* 21(1), pp. 27–40. DOI: 10.1002/(SICI)1522-2683(20000101)21:1<27::AID-ELPS27>3.0.CO;2-C.
- McKinney W (2010). “Data structures for statistical computing in Python”. *Proceedings of the 9th Python in Science Conference*, pp. 51–6.
- McRae SR, Brown CL, and Bushell GR (2005). Rapid purification of EGFP, EYFP, and ECFP with high yield and purity. *Protein Expression and Purification* 41(1), pp. 121–7. DOI: 10.1016/J.PEP.2004.12.030.
- Mechold U, Potrykus K, Murphy H, Murakami KS, and Cashel M (2013). Differential regulation by ppGpp versus pppGpp in *Escherichia coli*. *Nucleic Acids Research* 41(12), pp. 6175–89. DOI: 10.1093/nar/gkt302.
- Mekterović I, Mekterović D, and Maglica Ž (2014). BactImAS: a platform for processing and analysis of bacterial time-lapse microscopy movies. *BMC Bioinformatics* 15(1), p. 251. DOI: 10.1186/1471-2105-15-251.
- Van Melder L, Thi MHD, Lecchi P, Gottesman S, Couturier M, and Maurizi MR (1996). ATP-dependent degradation of CcdA by Lon protease: effects of secondary structure and heterologous subunit interactions. *Journal of Biological Chemistry* 271(44), pp. 27730–8. DOI: 10.1074/jbc.271.44.27730.
- Van Melder L and Wood TK (2017). Commentary: what is the link between stringent response, endoribonuclease encoding type II toxin–antitoxin systems and persistence? *Frontiers in Microbiology* 8, p. 191. DOI: 10.3389/fmicb.2017.00191.
- Meniche X, Otten R, Siegrist MS, Baer CE, Murphy KC, Bertozzi CR, and Sasseti CM (2014). Subpolar addition of new cell wall is directed by DivIVA in mycobacteria. *Proceedings of the National Academy of Sciences of the United States of America* 111(31), E3243–51. DOI: 10.1073/pnas.1402158111.
- Minch K, Rustad T, and Sherman DR (2012). *Mycobacterium tuberculosis* growth following aerobic expression of the DosR regulon. *PLOS ONE* 7(4), e35935. DOI: 10.1371/journal.pone.0035935.
- Mir M, Wang Z, Shen Z, Bednarz M, Bashir R, Golding I, Prasanth SG, and Popescu G (2011). Optical measurement of cycle-dependent cell growth. *Proceedings of the National Academy*

- of Sciences of the United States of America 108(32), pp. 13124–9. DOI: 10.1073/pnas.1100506108.
- Mitchison JM (2005). Single cell studies of the cell cycle and some models. *Theoretical Biology and Medical Modelling* 2(1), p. 4. DOI: 10.1186/1742-4682-2-4.
- Mizuno S and Tsukamura M (1978). [Utilization of tween 80 as carbon source for growth of slowly growing mycobacteria]. *Kekkaku* 53(11), pp. 537–40. Article in Japanese.
- Modi S, Vargas-Garcia CA, Ghusinga KR, and Singh A (2017). Analysis of noise mechanisms in cell-size control. *Biophysical Journal* 112(11), pp. 2408–18. DOI: 10.1016/j.bpj.2017.04.050.
- Mostowy S, Cousins D, Brinkman J, Aranaz A, and Behr MA (2002). Genomic deletions suggest a phylogeny for the *Mycobacterium tuberculosis* complex. *The Journal of Infectious Diseases* 186(1), pp. 74–80. DOI: 10.1086/341068.
- Moyed HS and Bertrand KP (1983). *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *Journal of Bacteriology* 155(2), pp. 768–75.
- Moyed HS and Broderick SH (1986). Molecular cloning and expression of *hipA*, a gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *Journal of Bacteriology* 166(2), pp. 399–403. DOI: 10.1128/JB.166.2.399-403.1986.
- Mpiga P and Ravaoarino M (2006). *Chlamydia trachomatis* persistence: An update. *Microbiological Research* 161(1), pp. 9–19. DOI: 10.1016/J.MICRES.2005.04.004.
- MRC (1950). Treatment of pulmonary tuberculosis with streptomycin and *para*-aminosalicylic acid; a Medical Research Council investigation. *British Medical Journal* 2(4688), pp. 1073–85.
- MRC (1955). Various combinations of isoniazid with streptomycin or with P.A.S. in the treatment of pulmonary tuberculosis; seventh report to the Medical Research Council by their Tuberculosis Chemotherapy Trials Committee. *British Medical Journal* 1(4911), pp. 435–45.
- Mulcahy LR, Burns JL, Lory S, and Lewis K (2010). Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *Journal of Bacteriology* 192(23), pp. 6191–9. DOI: 10.1128/JB.01651-09.
- Muto A, Ushida C, and Himeno H (1998). A bacterial RNA that functions as both a tRNA and an mRNA. *Trends in Biochemical Sciences* 23(1), pp. 25–9. DOI: 10.1016/S0968-0004(97)01159-6.
- Muttucumaru DGN, Roberts G, Hinds J, Stabler RA, and Parish T (2004). Gene expression profile of *Mycobacterium tuberculosis* in a non-replicating state. *Tuberculosis* 84(3–4), pp. 239–46. DOI: 10.1016/j.tube.2003.12.006.
- Mysorekar IU and Hultgren SJ (2006). Mechanisms of uropathogenic *Escherichia coli* persistence and eradication from the urinary tract. *Proceedings of the National Academy of Sciences of the United States of America* 103(38), pp. 14170–5. DOI: 10.1073/pnas.0602136103.

- Neelsen F (1883). Ein casuistischer Beitrag zur Lehre von der Tuberkulose. *Zentralblatt für die medizinischen Wissenschaften* 28, pp. 497–501.
- Negri LH and Vestri C (2017). *lucashn/peakutils: v1.1.0*. DOI: 10.5281/ZENODO.887917.
- Nerlich AG, Haas CJ, Zink A, Szeimies U, and Hagedorn HG (1997). Molecular evidence for tuberculosis in an ancient Egyptian mummy. *The Lancet* 350(9088), p. 1404. DOI: 10.1016/S0140-6736(05)65185-9.
- Newton Jr JA, Weiss PJ, Bowler WA, and Oldfield EC (1993). Soft-tissue infection due to *Mycobacterium smegmatis*: report of two cases. *Clinical Infectious Diseases* 16(4), pp. 531–3.
- Neyrolles O, Hernández-Pando R, Pietri-Rouxel F, Fornès P, Tailleux L, Payán JAB, Pivert E, Bordat Y, Aguilar D, Prévost M, Petit C, and Gicquel B (2006). Is adipose tissue a place for *Mycobacterium tuberculosis* persistence? *PLOS ONE* 1(1), e43. DOI: 10.1371/journal.pone.0000043.
- Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, McKay G, Siehnel R, Schafhauser J, Wang Y, Britigan BE, and Singh PK (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* 334(6058), pp. 982–6. DOI: 10.1126/science.1211037.
- Nguyen L, Scherr N, Gatfield J, Walburger A, Pieters J, and Thompson CJ (2007). Antigen 84, an effector of pleiomorphism in *Mycobacterium smegmatis*. *Journal of Bacteriology* 189(21), pp. 7896–910. DOI: 10.1128/JB.00726-07.
- Nies DH (2003). Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiology Reviews* 27(2–3), pp. 313–39. DOI: 10.1016/S0168-6445(03)00048-2.
- Nisa S, Blokpoel MCJ, Robertson BD, Tyndall JDA, Lun S, Bishai WR, and O’Toole R (2010). Targeting the chromosome partitioning protein ParA in tuberculosis drug discovery. *The Journal of Antimicrobial Chemotherapy* 65(11), pp. 2347–58. DOI: 10.1093/jac/dkq311.
- Norman TM, Lord ND, Paulsson J, and Losick R (2013). Memory and modularity in cell-fate decision making. *Nature* 503(7477), pp. 481–6. DOI: 10.1038/nature12804.
- Norton JP and Mulvey MA (2012). Toxin–antitoxin systems are important for niche-specific colonization and stress resistance of uropathogenic *Escherichia coli*. *PLOS Pathogens* 8(10), e1002954. DOI: 10.1371/journal.ppat.1002954.
- Nozoe T, Kussell E, and Wakamoto Y (2017). Inferring fitness landscapes and selection on phenotypic states from single-cell genealogical data. *PLOS Genetics* 13(3), e1006653. DOI: 10.1371/journal.pgen.1006653.
- O’Neill J (2014). *Antimicrobial resistance: tackling a crisis for the health and wealth of nations*. London: The Review on Antimicrobial Resistance.
- O’Neill J (2016). *Tackling drug-resistant infections globally: final report and recommendations*. London: The Review on Antimicrobial Resistance. DOI: 10.1016/j.jpha.2015.11.005.
- Oberer M, Zangger K, Gruber K, and Keller W (2007). The solution structure of ParD, the antidote of the ParDE toxin antitoxin module, provides the structural basis for DNA and toxin binding. *Protein Science* 16(8), pp. 1676–88. DOI: 10.1110/ps.062680707.

- Oliphant TE (2007). Python for Scientific Computing. *Computing in Science & Engineering* 9(3), pp. 10–20. DOI: 10.1109/MCSE.2007.58.
- Orman MA and Brynildsen MP (2013). Establishment of a method to rapidly assay bacterial persister metabolism. *Antimicrobial Agents and Chemotherapy* 57(9), pp. 4398–409. DOI: 10.1128/AAC.00372-13.
- Osella M, Nugent E, and Cosentino Lagomarsino M (2014). Concerted control of *Escherichia coli* cell division. *Proceedings of the National Academy of Sciences of the United States of America* 111(9), pp. 3431–5. DOI: 10.1073/pnas.1313715111.
- Page R and Peti W (2016). Toxin–antitoxin systems in bacterial growth arrest and persistence. *Nature Chemical Biology* 12(4), pp. 208–14. DOI: 10.1038/nchembio.2044.
- Palumbi SR, Mooney HA, Lubchenco J, and Melillo JM (2001). Humans as the world’s greatest evolutionary force. *Science* 293(5536), pp. 1786–90. DOI: 10.1126/science.293.5536.1786.
- Pandey AK and Sassetti CM (2008). Mycobacterial persistence requires the utilization of host cholesterol. *Proceedings of the National Academy of Sciences of the United States of America* 105(11), pp. 4376–80. DOI: 10.1073/pnas.0711159105.
- Parascandola J (1980). *The history of antibiotics: a symposium*. American Institute of the History of Pharmacy, p. 136.
- Park H, Guinn KM, Harrell MI, Liao R, Voskuil MI, Tompa M, Schoolnik GK, and Sherman DR (2003). Rv3133c/dosR is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. *Molecular Microbiology* 48(3), pp. 833–43. DOI: 10.1046/j.1365-2958.2003.03474.x.
- Pederson S (1985). “The chain growth rate for protein synthesis varies in *Escherichia coli*”. *Molecular biology of bacterial growth: a symposium held in honor of Ole Maaløe, at the University of Alabama, Tuscaloosa*. Ed. by M Schaechter, F Neidhardt, J Ingraham, and N Kjeldgaard. Boston, MA: Jones and Bartlett, pp. 13–20.
- PHE (2013). *TB mortality and mortality rate, England and Wales, 1913 to 2013*. London: Public Health England.
- Philippi T and Seger J (1989). Hedging one’s evolutionary bets, revisited. *Trends in Ecology & Evolution* 4(2), pp. 41–4. DOI: 10.1016/0169-5347(89)90138-9.
- Piepersberg W, Distler J, Heinzel P, and Perez-Gonzalez J (1988). Antibiotic resistance by modification: many resistance genes could be derived from cellular control genes in actinomycetes – a hypothesis. *Actinomycetologica* 2(2), pp. 83–98. DOI: 10.3209/saj.2_83.
- Pitt JM, Blankley S, McShane H, and O’Garra A (2013). Vaccination against tuberculosis: How can we better BCG? *Microbial Pathogenesis* 58, pp. 2–16. DOI: 10.1016/J.MICPATH.2012.12.002.
- Pizon A (1905). “Une nouvelle application de la chronophotographie: la Biotachygraphie”. *Compte-rendu des séances du sixième Congrès international de zoologie, tenu à Berne du 14 au 19 août 1904*. Vol. 14, pp. 404–9.

- Poirel L, Rodriguez-Martinez J, Mammeri H, Liard A, and Nordmann P (2005). Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrobial Agents and Chemotherapy* 49(8), pp. 3523–5. DOI: 10.1128/AAC.49.8.3523–3525.2005.
- Poole K (2004). Efflux-mediated multiresistance in Gram-negative bacteria. *Clinical Microbiology and Infection* 10(1), pp. 12–26. DOI: 10.1111/j.1469-0691.2004.00763.x.
- Poole K (2005). Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* 56(1), pp. 20–51. DOI: 10.1093/jac/dki171.
- Poulsen A (1950). Some clinical features of tuberculosis. 1. Incubation period. *Acta tuberculosea Scandinavica* 24(3-4), pp. 311–46.
- Powell EO (1956). Growth rate and generation time of bacteria, with special reference to continuous culture. *Journal of General Microbiology* 15(3), pp. 492–511. DOI: 10.1099/00221287-15-3-492.
- Priestman M, Thomas P, Robertson BD, and Shahrezaei V (2017). Mycobacteria modify their cell size control under sub-optimal carbon sources. *Frontiers in Cell and Developmental Biology* 5, p. 64. DOI: 10.3389/fcell.2017.00064.
- Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, and Barry III CE (2000). The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. *Journal of Bacteriology* 182(17), pp. 4889–98. DOI: 10.1128/JB.182.17.4889–4898.2000.
- Ptacin JL, Lee SF, Garner EC, Toro E, Eckart M, Comolli LR, Moerner W, and Shapiro L (2010). A spindle-like apparatus guides bacterial chromosome segregation. *Nature Cell Biology* 12(8), pp. 791–8. DOI: 10.1038/ncb2083.
- Ptacin JL and Shapiro L (2010). Initiating bacterial mitosis: Understanding the mechanism of ParA-mediated chromosome segregation. *Cell Cycle* 9(20), pp. 4033–4. DOI: 10.4161/cc.9.20.13521.
- Putrinš M, Kogermann K, Lukk E, Lippus M, Varik V, and Tenson T (2015). Phenotypic heterogeneity enables uropathogenic *Escherichia coli* to evade killing by antibiotics and serum complement. *Infection and Immunity* 83(3), pp. 1056–67. DOI: 10.1128/IAI.02725-14.
- Pym AS, Brodin P, Brosch R, Huerre M, and Cole ST (2002). Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Molecular Microbiology* 46(3), pp. 709–17. DOI: 10.1046/j.1365-2958.2002.03237.x.
- Ramage HR, Connolly LE, and Cox JS (2009). Comprehensive functional analysis of *Mycobacterium tuberculosis* toxin–antitoxin systems: implications for pathogenesis, stress responses, and evolution. *PLOS Genetics* 5(12), e1000767. DOI: 10.1371/journal.pgen.1000767.
- Ramaswamy S and Musser JM (1998). Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tubercle and Lung Disease* 79(1), pp. 3–29. DOI: 10.1054/tuld.1998.0002.

- Ramisetty BCM, Ghosh D, Roy Chowdhury M, and Santhosh RS (2016a). What is the link between stringent response, endoribonuclease encoding type II toxin–antitoxin systems and persistence? *Frontiers in Microbiology* 7, p. 1882. DOI: 10.3389/fmicb.2016.01882.
- Ramisetty BCM, Raj S, and Ghosh D (2016b). *Escherichia coli* MazEF toxin–antitoxin system does not mediate programmed cell death. *Journal of Basic Microbiology* 56(12), pp. 1398–1402. DOI: 10.1002/jobm.201600247.
- Rao P and Li Q (2009). Protein turnover in mycobacterial proteomics. *Molecules* 14(9), pp. 3237–58. DOI: 10.3390/molecules14093237.
- Rao SPS, Alonso S, Rand L, Dick T, and Pethe K (2008). The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America* 105(33), pp. 11945–50. DOI: 10.1073/pnas.0711697105.
- Raskin DM and de Boer PA (1999). Rapid pole-to-pole oscillation of a protein required for directing division to the middle of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 96(9), pp. 4971–6. DOI: 10.1073/PNAS.96.9.4971.
- Reeve CA, Amy PS, and Matin A (1984). Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. *Journal of Bacteriology* 160(3), pp. 1041–6.
- Rego EH, Audette RE, and Rubin EJ (2017). Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* 546(7656), pp. 153–7. DOI: 10.1038/nature22361.
- Reva O, Korotetskiy I, and Ilin A (2015). Role of the horizontal gene exchange in evolution of pathogenic *Mycobacteria*. *BMC Evolutionary Biology* 15(Suppl 1), S2. DOI: 10.1186/1471-2148-15-S1-S2.
- Reyrat J and Kahn D (2001). *Mycobacterium smegmatis*: an absurd model for tuberculosis? *Trends in Microbiology* 9(10), pp. 472–3. DOI: 10.1016/S0966-842X(01)02168-0.
- Richardson K, Bennion OT, Tan S, Hoang AN, Cokol M, and Aldridge BB (2016). Temporal and intrinsic factors of rifampicin tolerance in mycobacteria. *Proceedings of the National Academy of Sciences of the United States of America* 113(29), pp. 8302–7. DOI: 10.1073/pnas.1600372113.
- Ries J (1909). Kinematographie der Befruchtung und Zellteilung. *Archiv für Mikroskopische Anatomie* 74(1), pp. 1–31. DOI: 10.1007/BF02979930.
- Robert L (2015). Size sensors in bacteria, cell cycle control, and size control. *Frontiers in Microbiology* 6, p. 515. DOI: 10.3389/fmicb.2015.00515.
- Robert L, Hoffmann M, Krell N, Aymerich S, Robert J, and Doumic M (2014). Division in *Escherichia coli* is triggered by a size-sensing rather than a timing mechanism. *BMC Biology* 12(1), p. 17. DOI: 10.1186/1741-7007-12-17.
- Robitzek EH, Selikoff IJ, and Omstein GG (1952). Chemotherapy of human tuberculosis with hydrazine derivatives of isonicotinic acid; preliminary report of representative cases. *Quarterly Bulletin of Sea View Hospital* 13(1), pp. 27–51.

- De Rossi E, Aínsa JA, and Riccardi G (2006). Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS Microbiology Reviews* 30(1), pp. 36–52. DOI: 10.1111/j.1574-6976.2005.00002.x.
- Rotem E, Loinger A, Ronin I, Levin-Reisman I, Gabay C, Shores N, Biham O, and Balaban NQ (2010). Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. *Proceedings of the National Academy of Sciences of the United States of America* 107(28), pp. 12541–6. DOI: 10.1073/pnas.1004333107.
- Rubin EJ (2009). The granuloma in tuberculosis—friend or foe? *New England Journal of Medicine* 360(23), pp. 2471–3. DOI: 10.1056/NEJMcibr0902539.
- Russell DG, Cardona P, Kim M, Allain S, and Altare F (2009). Foamy macrophages and the progression of the human tuberculosis granuloma. *Nature Immunology* 10(9), pp. 943–8. DOI: 10.1038/ni.1781.
- Rustad TR, Minch KJ, Brabant W, Winkler JK, Reiss DJ, Baliga NS, and Sherman DR (2013). Global analysis of mRNA stability in *Mycobacterium tuberculosis*. *Nucleic Acids Research* 41(1), pp. 509–17. DOI: 10.1093/nar/gks1019.
- Rustad TR, Harrell MI, Liao R, and Sherman DR (2008). The enduring hypoxic response of *Mycobacterium tuberculosis*. *PLOS ONE* 3(1), e1502. DOI: 10.1371/journal.pone.0001502.
- Ryals J, Little R, and Bremer H (1982). Control of rRNA and tRNA syntheses in *Escherichia coli* by guanosine tetraphosphate. *Journal of Bacteriology* 151(3), pp. 1261–8.
- Ryan F (1993). *The forgotten plague: how the battle against tuberculosis was won—and lost*. Boston: Little, Brown.
- Sakamoto K (2012). The pathology of *Mycobacterium tuberculosis* infection. *Veterinary Pathology* 49(3), pp. 423–39. DOI: 10.1177/0300985811429313.
- Sala C, Dhar N, Hartkoorn RC, Zhang M, Ha YH, Schneider P, and Cole ST (2010). Simple model for testing drugs against nonreplicating *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 54(10), pp. 4150–8. DOI: 10.1128/AAC.00821-10.
- Salamon H, Bruiners N, Lakehal K, Shi L, Ravi J, Yamaguchi KD, Pine R, and Gennaro ML (2014). Cutting edge: vitamin D regulates lipid metabolism in *Mycobacterium tuberculosis* infection. *The Journal of Immunology* 193(1), pp. 30–4. DOI: 10.4049/jimmunol.1400736.
- Salo WL, Aufderheide AC, Buikstra J, and Holcomb TA (1994). Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. *Proceedings of the National Academy of Sciences of the United States of America* 91(6), pp. 2091–4. DOI: 10.1073/pnas.91.6.2091.
- Sampson SL, Dascher CC, Sambandamurthy VK, Russell RG, Jacobs WR, Bloom BR, and Hondalus MK (2004). Protection elicited by a double leucine and pantothenate auxotroph of *Mycobacterium tuberculosis* in guinea pigs. *Infection and Immunity* 72(5), pp. 3031–7. DOI: 10.1128/IAI.72.5.3031-3037.2004.

- Santi I, Dhar N, Bousbaine D, Wakamoto Y, and McKinney JD (2013). Single-cell dynamics of the chromosome replication and cell division cycles in mycobacteria. *Nature Communications* 4, p. 2470. DOI: 10.1038/ncomms3470.
- Santi I and McKinney JD (2015). Chromosome organization and replisome dynamics in *Mycobacterium smegmatis*. *MBio* 6(1), e01999–14. DOI: 10.1128/mBio.01999-14.
- Sauls JT, Li D, and Jun S (2016). Adder and a coarse-grained approach to cell size homeostasis in bacteria. *Current Opinion in Cell Biology* 38, pp. 38–44. DOI: 10.1016/j.ceb.2016.02.004.
- Schaechter M, Maaløe O, and Kjeldgaard NO (1958). Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. *Journal of General Microbiology* 19(3), pp. 592–606. DOI: 10.1099/00221287-19-3-592.
- Schatz A, Bugle E, and Waksman SA (1944). Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. *Proceedings of the Society for Experimental Biology and Medicine* 55(1), pp. 66–9. DOI: 10.3181/00379727-55-14461.
- Schifano JM, Edifor R, Sharp JD, Ouyang M, Konkimalla A, Husson RN, and Woychik Na (2013). Mycobacterial toxin MazF-mt6 inhibits translation through cleavage of 23S rRNA at the ribosomal A site. *Proceedings of the National Academy of Sciences of the United States of America* 110(21), pp. 8501–6. DOI: 10.1073/pnas.1222031110.
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J, White DJ, Hartenstein V, Eliceiri K, Tomancak P, and Cardona A (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods* 9(7), pp. 676–82. DOI: 10.1038/nmeth.2019.
- Schmoller KM (2017). The phenomenology of cell size control. *Current Opinion in Cell Biology* 49, pp. 53–8. DOI: 10.1016/J.CEB.2017.11.011.
- Schnappinger D and Ehrt S (2014). Regulated expression systems for mycobacteria and their applications. *Microbiology Spectrum* 2(1), MGM2-0018-2013. DOI: 10.1128/microbiolspec.MGM2-0018-2013.
- Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, Dolganov G, Efron B, Butcher PD, Nathan C, and Schoolnik GK (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages. *The Journal of Experimental Medicine* 198(5), pp. 693–704. DOI: 10.1084/jem.20030846.
- Scholar EM and Pratt WB (2000). “Drug resistance”. *The antimicrobial drugs*. 2nd ed. New York: Oxford University Press. Chap. 2, p. 37.
- Schumacher MA, Balani P, Min J, Chinnam NB, Hansen S, Vulić M, Lewis K, and Brennan RG (2015). HipBA-promoter structures reveal the basis of heritable multidrug tolerance. *Nature* 524(7563), pp. 59–64. DOI: 10.1038/nature14662.
- Seabold S and Perktold J (2010). “Statsmodels: Econometric and statistical modeling with python”. *9th Python in Science Conference*.

- Seeliger JC, Topp S, Sogi KM, Previti ML, Gallivan JP, and Bertozzi CR (2012). A riboswitch-based inducible gene expression system for mycobacteria. *PLOS ONE* 7(1), e29266. DOI: 10.1371/journal.pone.0029266.
- Seger J (1987). What is bet-hedging? *Oxford Surveys in Evolutionary Biology* 4, pp. 182–211.
- Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, and Lewis K (2006). Persisters: a distinct physiological state of *E. coli*. *BMC Microbiology* 6, p. 53. DOI: 10.1186/1471-2180-6-53.
- Shahrezaei V and Marguerat S (2015). Connecting growth with gene expression: of noise and numbers. *Current Opinion in Microbiology* 25, pp. 127–35. DOI: 10.1016/j.mib.2015.05.012.
- Shaikh AS, Tang YJ, Mukhopadhyay A, Martín HG, Gin J, Benke PI, and Keasling JD (2010). Study of stationary phase metabolism via isotopomer analysis of amino acids from an isolated protein. *Biotechnology Progress* 26(1), pp. 52–6. DOI: 10.1002/btpr.325.
- Shan Y, Brown Gandt A, Rowe SE, Deisinger JP, Conlon BP, and Lewis K (2017). ATP-dependent persister formation in *Escherichia coli*. *mBio* 8(1), e02267–16. DOI: 10.1128/mBio.02267–16.
- Shanaube K, Sismanidis C, Ayles H, Beyers N, Schaap A, Lawrence K, Barker A, and Godfrey-Faussett P (2009). Annual risk of tuberculous infection using different methods in communities with a high prevalence of TB and HIV in Zambia and South Africa. *PLOS ONE* 4(11), e7749. DOI: 10.1371/journal.pone.0007749.
- Sharma D, Bose A, Shakila H, Das TK, Tyagi JS, and Ramanathan V (2006). Expression of mycobacterial cell division protein, FtsZ, and dormancy proteins, DevR and Acr, within lung granulomas throughout guinea pig infection. *FEMS Immunology & Medical Microbiology* 48(3), pp. 329–36. DOI: 10.1111/j.1574-695X.2006.00160.x.
- Shi W, Zhang X, Jiang X, Yuan H, Lee JS, Barry III CE, Wang H, Zhang W, and Zhang Y (2011). Pyrazinamide inhibits *trans*-translation in *Mycobacterium tuberculosis*. *Science* 333(6049), pp. 1630–2. DOI: 10.1126/science.1208813.
- Shleeve MO, Bagramyan K, Telkov MV, Mukamolova GV, Young M, Kell DB, and Kaprelyants AS (2002). Formation and resuscitation of ‘non-culturable’ cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged stationary phase. *Microbiology* 148(5), pp. 1581–91. URL: <http://mic.sgmjournals.org/content/148/5/1581.long>.
- Shleeve MO, Mukamolova GV, Young M, Williams HD, and Kaprelyants AS (2004). Formation of ‘non-culturable’ cells of *Mycobacterium smegmatis* in stationary phase in response to growth under suboptimal conditions and their Rpf-mediated resuscitation. *Microbiology* 150(Pt 6), pp. 1687–97. DOI: 10.1099/mic.0.26893-0.
- Si F, Li D, Cox SE, Sauls JT, Azizi O, Sou C, Schwartz AB, Erickstad MJ, Jun Y, Li X, and Jun S (2017). Invariance of initiation mass and predictability of cell size in *Escherichia coli*. *Current Biology* 27(9), pp. 1278–87. DOI: 10.1016/j.cub.2017.03.022.

- Siddiqi N, Pathak N, Banerjee S, Ahmed N, Katoch VM, Hasnain SE, and Das R (2004). *Mycobacterium tuberculosis* isolate with a distinct genomic identity overexpresses a Tap-like efflux pump. *Infection* 32(2), pp. 109–11. DOI: 10.1007/s15010-004-3097-x.
- Silva-Herzog E, McDonald EM, Crooks AL, and Detweiler CS (2015). Physiologic stresses reveal a *Salmonella* persister state and TA family toxins modulate tolerance to these stresses. *PLOS ONE* 10(12), e0141343. DOI: 10.1371/journal.pone.0141343.
- Simons AM (2011). Modes of response to environmental change and the elusive empirical evidence for bet hedging. *Proceedings of the Royal Society B: Biological Sciences* 278(1712), pp. 1601–9. DOI: 10.1098/rspb.2011.0176.
- Singh B and Mitchison DA (1955). Bactericidal activity of streptomycin and isoniazid in combination with *p*-aminosalicylic acid against *Mycobacterium tuberculosis*. *Journal of General Microbiology* 12(1), pp. 76–84. DOI: 10.1099/00221287-12-1-76.
- Singh B, Ghosh J, Islam NM, Dasgupta S, and Kirsebom LA (2010a). Growth, cell division and sporulation in mycobacteria. *Antonie van Leeuwenhoek* 98(2), pp. 165–77. DOI: 10.1007/s10482-010-9446-0.
- Singh B, Nitharwal RG, Ramesh M, Pettersson BMF, Kirsebom LA, and Dasgupta S (2013). Asymmetric growth and division in *Mycobacterium* spp.: compensatory mechanisms for non-medial septa. *Molecular Microbiology* 88(1), pp. 64–76. DOI: 10.1111/mmi.12169.
- Singh R, Barry III CE, and Boshoff HIM (2010b). The three RelE homologs of *Mycobacterium tuberculosis* have individual, drug-specific effects on bacterial antibiotic tolerance. *Journal of Bacteriology* 192(5), pp. 1279–91. DOI: 10.1128/JB.01285-09.
- Sliusarenko O, Heinritz J, Emonet T, and Jacobs-Wagner C (2011). High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. *Molecular Microbiology* 80(3), pp. 612–27. DOI: 10.1111/j.1365-2958.2011.07579.x.
- Sloot R, Van Der Loeff MFS, Kouw PM, and Borgdorff MW (2014). Risk of tuberculosis after recent exposure: a 10-year follow-up study of contacts in Amsterdam. *American Journal of Respiratory and Critical Care Medicine* 190(9), pp. 1044–52. DOI: 10.1164/rccm.201406-11590C.
- Smeulders MJ, Keer J, Speight Ra, and Williams HD (1999). Adaptation of *Mycobacterium smegmatis* to stationary phase. *Journal of Bacteriology* 181(1), pp. 270–283.
- Smith ASG and Rawlings DE (1998). Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain, and antidote degradation requires the Lon protease. *Journal of Bacteriology* 180(20), pp. 5458–62.
- Smith DH (1967). R factor infection of *Escherichia coli* lyophilized in 1946. *Journal of Bacteriology* 94(6), pp. 2071–2.
- Smith NH, Kremer K, Inwald J, Dale J, Driscoll JR, Gordon SV, van Soolingen D, Glyn Hewinson R, and Maynard Smith J (2006). Ecotypes of the *Mycobacterium tuberculosis* complex. *Journal of Theoretical Biology* 239(2), pp. 220–5. DOI: 10.1016/J.JTBI.2005.08.036.

- Smith PA and Romesberg FE (2007). Combating bacteria and drug resistance by inhibiting mechanisms of persistence and adaptation. *Nature Chemical Biology* 3(9), pp. 549–56. DOI: 10.1038/nchembio.2007.27.
- Snapper SB, Melton RE, Mustafa S, Kieser T, and Jacobs Jr WR (1990). Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Molecular Microbiology* 4(11), pp. 1911–9. DOI: 10.1111/j.1365-2958.1990.tb02040.x.
- Soifer I, Robert L, and Amir A (2016). Single-cell analysis of growth in budding yeast and bacteria reveals a common size regulation strategy. *Current Biology* 26(3), pp. 356–61. DOI: 10.1016/j.cub.2015.11.067.
- Sonnenberg P, Glynn JR, Fielding K, Murray J, Godfrey-Faussett P, and Shearer S (2005). How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African gold miners. *The Journal of Infectious Diseases* 191(2), pp. 150–8. DOI: 10.1086/426827.
- Spitzer E, Spitzer S, Freundlich L, and Casadevall A (1993). Persistence of initial infection in recurrent *Cryptococcus neoformans* meningitis. *The Lancet* 341(8845), pp. 595–6. DOI: 10.1016/0140-6736(93)90354-J.
- Spoering AL and Lewis K (2001). Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *Journal of Bacteriology* 183(23), pp. 6746–51. DOI: 10.1128/JB.183.23.6746-6751.2001.
- Spoering AL, Vulić M, and Lewis K (2006). GlpD and PlsB participate in persister cell formation in *Escherichia coli*. *Journal of Bacteriology* 188(14), pp. 5136–44. DOI: 10.1128/JB.00369-06.
- Sprenger M, Kasper L, and Hube B (2018). Metabolic adaptation of intracellular bacteria and fungi to macrophages. *International Journal of Medical Microbiology* 308(1), pp. 215–27. DOI: 10.1016/J.IJMM.2017.11.001.
- Starrfelt J and Kokko H (2012). Bet-hedging—a triple trade-off between means, variances and correlations. *Biological Reviews* 87(3), pp. 742–55. DOI: 10.1111/j.1469-185X.2012.00225.x.
- Stead WW, Eisenach KD, Cave MD, Beggs ML, Templeton GL, Thoen C, and Bates JH (1995). When did *Mycobacterium tuberculosis* infection first occur in the new world? An important question with public health implications. *American Journal of Respiratory and Critical Care Medicine* 151(7), pp. 1267–8. DOI: 10.1164/ajrccm.151.4.7697265.
- Steel C, Wan Q, and Xu XN (2004). Single live cell imaging of chromosomes in chloramphenicol-induced filamentous *Pseudomonas aeruginosa*. *Biochemistry* 43(1), pp. 175–82. DOI: 10.1021/BI035341E.
- Stewart EJ, Madden R, Paul G, and Taddei F (2005). Aging and death in an organism that reproduces by morphologically symmetric division. *PLOS Biology* 3(2), e45. DOI: 10.1371/journal.pbio.0030045.

- Styblo K (1985). The relationship between the risk of tuberculous infection and the risk of developing infectious tuberculosis. *Bulletin of the International Union Against Tuberculosis and Lung Disease* 60(3), pp. 117–9.
- Suarez J, Rangelova K, Jarzecki AA, Manzerova J, Krymov V, Zhao X, Yu S, Metlitsky L, Gerfen GJ, and Magliozzo RS (2009). An oxyferrous heme/protein-based radical intermediate is catalytically competent in the catalase reaction of *Mycobacterium tuberculosis* catalase–peroxidase (KatG). *The Journal of Biological Chemistry* 284(11), pp. 7017–29. DOI: 10.1074/jbc.M808106200.
- Sun-Wada G, Tabata H, Kawamura N, Aoyama M, and Wada Y (2009). Direct recruitment of H⁺-ATPase from lysosomes for phagosomal acidification. *Journal of Cell Science* 122(Pt 14), pp. 2504–13. DOI: 10.1242/jcs.050443.
- Surovtsev IV, Lim HC, and Jacobs-Wagner C (2016). The slow mobility of the ParA partitioning protein underlies its steady-state patterning in *Caulobacter*. *Biophysical Journal* 110(12), pp. 2790–9. DOI: 10.1016/j.bpj.2016.05.014.
- Sutherland I, Svandová E, and Radhakrishna S (1982). The development of clinical tuberculosis following infection with tubercle bacilli. 1. A theoretical model for the development of clinical tuberculosis following infection, linking from data on the risk of tuberculous infection and the incidence of clinical tuberculosis in the Netherlands. *Tubercle* 63(4), pp. 255–68.
- Sveiczzer A, Novak B, and Mitchison J (1996). The size control of fission yeast revisited. *Journal of Cell Science* 109(12), pp. 2947–57.
- Taheri-Araghi S, Bradde S, Sauls JT, Hill NS, Levin PA, Paulsson J, Vergassola M, and Jun S (2015). Cell-size control and homeostasis in bacteria. *Current Biology* 25(3), pp. 385–91. DOI: 10.1016/j.cub.2014.12.009.
- Talbot FAA (1913). *Practical cinematography and its applications*. W. Heinemann.
- Talukder AA, Yanai S, Nitta T, Kato A, and Yamada M (1996). RpoS-dependent regulation of genes expressed at late stationary phase in *Escherichia coli*. *FEBS Letters* 386(2-3), pp. 177–80. DOI: 10.1016/0014-5793(96)00426-7.
- Tan Q, Awano N, and Inouye M (2011). YeeV is an *Escherichia coli* toxin that inhibits cell division by targeting the cytoskeleton proteins, FtsZ and MreB. *Molecular Microbiology* 79(1), pp. 109–18. DOI: 10.1111/j.1365-2958.2010.07433.x.
- Tanouchi Y, Pai A, Park H, Huang S, Stamatov R, Buchler NE, and You L (2015). A noisy linear map underlies oscillations in cell size and gene expression in bacteria. *Nature* 523(7560), pp. 357–60. DOI: 10.1038/nature14562.
- Tasneen R, Li S, Peloquin CA, Taylor D, Williams KN, Andries K, Mdluli KE, and Nuermberger EL (2011). Sterilizing activity of novel TMC207- and PA-824-containing regimens in a murine model of tuberculosis. *Antimicrobial Agents and Chemotherapy* 55(12), pp. 5485–92. DOI: 10.1128/AAC.05293-11.

- Tasneen R, Tyagi S, Williams K, Grosset J, and Nuermberger E (2008). Enhanced bactericidal activity of rifampin and/or pyrazinamide when combined with PA-824 in a murine model of tuberculosis. *Antimicrobial Agents and Chemotherapy* 52(10), pp. 3664–8. DOI: 10.1128/AAC.00686–08.
- Thanky NR, Young DB, and Robertson BD (2007). Unusual features of the cell cycle in mycobacteria: polar-restricted growth and the snapping-model of cell division. *Tuberculosis* 87(3), pp. 231–6. DOI: 10.1016/j.tube.2006.10.004.
- Thisted T, Sørensen NS, Wagner EG, and Gerdes K (1994). Mechanism of post-segregational killing: Sok antisense RNA interacts with Hok mRNA via its 5'-end single-stranded leader and competes with the 3'-end of Hok mRNA for binding to the *mok* translational initiation region. *The EMBO Journal* 13(8), pp. 1960–8.
- Thomas P (2017). Single-cell histories in growing populations: relating physiological variability to population growth. *bioRxiv*, p. 100495. DOI: 10.1101/100495.
- Tiberi S, Carvalho ACC, Sulis G, Vaghela D, Rendon A, Mello FCdQ, Rahman A, Matin N, Zumla A, and Pontali E (2017). The cursed duet today: tuberculosis and HIV-coinfection. *La Presse Médicale* 46(2), e23–e39. DOI: 10.1016/J.LPM.2017.01.017.
- Tiwari P, Arora G, Singh M, Kidwai S, Narayan OP, and Singh R (2015). MazF ribonucleases promote *Mycobacterium tuberculosis* drug tolerance and virulence in guinea pigs. *Nature Communications* 6, p. 6059. DOI: 10.1038/ncomms7059.
- Tomasz A (1979). The mechanism of the irreversible antimicrobial effects of penicillins: how the beta-lactam antibiotics kill and lyse bacteria. *Annual Review of Microbiology* 33(1), pp. 113–37. DOI: 10.1146/annurev.mi.33.100179.000553.
- Tombolini R, Unge A, Davey ME, De Bruijn FJ, and Jansson JK (1997). Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. *FEMS Microbiology Ecology* 22(1), pp. 17–28. DOI: 10.1016/S0168-6496(96)00068-2.
- Trojanowski D, Ginda K, Pióro M, Hołowka J, Skut P, Jakimowicz D, and Zakrzewska-Czerwińska J (2015). Choreography of the *Mycobacterium* replication machinery during the cell cycle. *mBio* 6, e02125–14. DOI: 10.1128/mBio.02125–14.
- Trueba FJ (1982). On the precision and accuracy achieved by *Escherichia coli* cells at fission about their middle. *Archives of Microbiology* 131(1), pp. 55–9. DOI: 10.1007/BF00451499.
- Trunz BB, Fine P, and Dye C (2006). Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *The Lancet* 367(9517), pp. 1173–80. DOI: 10.1016/S0140-6736(06)68507-3.
- Tsai MC, Chakravarty S, Zhu G, Xu J, Tanaka K, Koch C, Tufariello J, Flynn J, and Chan J (2006). Characterization of the tuberculous granuloma in murine and human lungs: cellular composition and relative tissue oxygen tension. *Cellular Microbiology* 8(2), pp. 218–32. DOI: 10.1111/j.1462-5822.2005.00612.x.

- Tsilibaris V, Maenhaut-Michel G, Mine N, and Van Melderen L (2007). What is the benefit to *Escherichia coli* of having multiple toxin–antitoxin systems in its genome? *Journal of bacteriology* 189(17), pp. 6101–8. DOI: 10.1128/JB.00527–07.
- Tyagi JS and Sharma D (2002). *Mycobacterium smegmatis* and tuberculosis. *Trends in Microbiology* 10(2), pp. 68–9. DOI: 10.1016/S0966–842X(01)02296–X.
- Typas A, Banzhaf M, Gross CA, and Vollmer W (2012). From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nature Reviews Microbiology* 10(2), pp. 123–36. DOI: 10.1038/nrmicro2677.
- Tzur A, Kafri R, LeBleu VS, Lahav G, and Kirschner MW (2009). Cell growth and size homeostasis in proliferating animal cells. *Science* 325(5937), pp. 167–71. DOI: 10.1126/science.1174294.
- Uhía I, Priestman M, Joyce G, Krishnan N, Shahrezaei V, and Robertson BD (2018). Analysis of ParAB dynamics in mycobacteria shows active movement of ParB and differential inheritance of ParA. *PLOS ONE* 13(6), e0199316. DOI: 10.1371/journal.pone.0199316.
- Unger MA, Chou HP, Thorsen T, Scherer A, and Quake SR (2000). Monolithic microfabricated valves and pumps by multilayer soft lithography. *Science* 288(5463), pp. 113–6. DOI: 10.1126/SCIENCE.288.5463.113.
- Vadia S and Levin PA (2015). Growth rate and cell size: a re-examination of the growth law. *Current Opinion in Microbiology* 24, pp. 96–103. DOI: 10.1016/j.mib.2015.01.011.
- Vaubourgeix J, Lin G, Dhar N, Chenouard N, Jiang X, Botella H, Lupoli T, Mariani O, Yang G, Ouerfelli O, Unser M, Schnappinger D, McKinney JD, and Nathan C (2015). Stressed mycobacteria use the chaperone ClpB to sequester irreversibly oxidized proteins asymmetrically within and between cells. *Cell Host & Microbe* 17(2), pp. 178–90. DOI: 10.1016/J.CHOM.2014.12.008.
- Veening J, Smits WK, and Kuipers OP (2008). Bistability, epigenetics, and bet-hedging in bacteria. *Annual Review of Microbiology* 62(1), pp. 193–210. DOI: 10.1146/annurev.micro.62.081307.163002.
- Velmurugan K, Chen B, Miller JL, Azogue S, Gurses S, Hsu T, Glickman M, Jacobs Jr WR, Porcelli SA, and Briken V (2007). *Mycobacterium tuberculosis nuoG* is a virulence gene that inhibits apoptosis of infected host cells. *PLOS Pathogens* 3(7), e110. DOI: 10.1371/journal.ppat.0030110.
- Verstraeten N, Knapen WJ, Kint CI, Liebens V, Van den Bergh B, Dewachter L, Michiels JE, Fu Q, David CC, Fierro AC, Marchal K, Beirlant J, Versées W, Hofkens J, Jansen M, Fauvart M, and Michiels J (2015). Obg and membrane depolarization are part of a microbial bet-hedging strategy that leads to antibiotic tolerance. *Molecular Cell* 59(1), pp. 9–21. DOI: 10.1016/j.molcel.2015.05.011.
- Viducic D, Ono T, Murakami K, Susilowati H, Kayama S, Hirota K, and Miyake Y (2006). Functional analysis of *spoT*, *relA* and *dksA* genes on quinolone tolerance in *Pseudomonas*

- aeruginosa* under nongrowing condition. *Microbiology and Immunology* 50(4), pp. 349–57. DOI: 10.1111/j.1348-0421.2006.tb03793.x.
- Vieira OV, Botelho RJ, and Grinstein S (2002). Phagosome maturation: aging gracefully. *The Biochemical Journal* 366(Pt 3), pp. 689–704. DOI: 10.1042/BJ20020691.
- Vijay S, Vinh DN, Hai HT, Ha VTN, Dung VTM, Dinh TD, Nhung HN, Tram TTB, Aldridge BB, Hanh NT, Thu DDA, Phu NH, Thwaites GE, and Thuong NTT (2017). Influence of stress and antibiotic resistance on cell-length distribution in *Mycobacterium tuberculosis* clinical isolates. *Frontiers in Microbiology* 8, p. 2296. DOI: 10.3389/fmicb.2017.02296.
- Vilchèze C and Jacobs Jr WR (2007). The mechanism of isoniazid killing: clarity through the scope of genetics. *Annual Review of Microbiology* 61(1), pp. 35–50. DOI: 10.1146/annurev.micro.61.111606.122346.
- Virchow R (1891a). Remarks on the effect of Koch's remedy on the internal organs of tuberculous patients. *British Medical Journal* 1(1568), pp. 127–32.
- Virchow R (1891b). Über die Wirkung des Koch'schen Mittels auf innere Organe Tuberculöser. *Deutsche Medizinische Wochenschrift* 17, pp. 131–4.
- Vischer N and Nastase S (n.d.). *ObjectJ*. URL: <https://sil.s.fnwi.uva.nl/bcb/objectj/>.
- Voskuil MI, Visconti KC, and Schoolnik GK (2004). *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis* 84(3–4), pp. 218–27. DOI: 10.1016/j.tube.2004.02.003.
- Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, and Schoolnik GK (2003). Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *The Journal of Experimental Medicine* 198(5), pp. 705–13. DOI: 10.1084/jem.20030205.
- Vromman F (2014). Exploitation of host lipids by bacteria. *Current Opinion in Microbiology* 17, pp. 38–45. DOI: 10.1016/j.mib.2013.11.003.
- Wainwright M and Swan HT (1986). C.G. Paine and the earliest surviving clinical records of penicillin therapy. *Medical history* 30(1), pp. 42–56.
- Wakamoto Y, Dhar N, Chait R, Schneider K, Signorino-Gelo F, Leibler S, and McKinney JD (2013). Dynamic persistence of antibiotic-stressed mycobacteria. *Science* 339(6115), pp. 91–5. DOI: 10.1126/science.1229858.
- Wakamoto Y, Grosberg AY, and Kussell E (2012). Optimal lineage principle for age-structured populations. *Evolution* 66(1), pp. 115–34. DOI: 10.1111/j.1558-5646.2011.01418.x.
- Waksman SA and Woodruff HB (1940). The soil as a source of microorganisms antagonistic to disease-producing bacteria. *Journal of Bacteriology* 40(4), pp. 581–600.
- Wallden M, Fange D, Lundius EG, Baltekin Ö, and Elf J (2016). The synchronization of replication and division cycles in individual *E. coli* cells. *Cell* 166(3), pp. 729–39. DOI: 10.1016/j.cell.2016.06.052.
- Wallgren A (1948). The time-table of tuberculosis. *Tubercle* 29(11), pp. 245–51.
- Walsh C (2003). *Antibiotics: actions, origins, resistance*. Washington, D.C.: ASM Press.

- Walter ND, Dolganov GM, Garcia BJ, Worodria W, Andama A, Musisi E, Ayakaka I, Van TT, Voskuil MI, De Jong BC, Davidson RM, Fingerlin TE, Kechris K, Palmer C, Nahid P, Daley CL, Geraci M, Huang L, Cattamanchi A, Strong M, Schoolnik GK, and Davis JL (2015). Transcriptional adaptation of drug-tolerant *Mycobacterium tuberculosis* during treatment of human tuberculosis. *Journal of Infectious Diseases* 212(6), pp. 990–8. DOI: 10.1093/infdis/jiv149.
- Wang P, Robert L, Pelletier J, Dang WL, Taddei F, Wright A, and Jun S (2010). Robust growth of *Escherichia coli*. *Current Biology* 20(12), pp. 1099–103. DOI: 10.1016/J.CUB.2010.04.045.
- Wang S, Furchtgott L, Casey Huang K, and Shaevitz JW (2012a). Helical insertion of peptidoglycan produces chiral ordering of the bacterial cell wall. *Proceedings of the National Academy of Sciences of the United States of America* 109(10), E595–E604. DOI: 10.1073/pnas.1117132109.
- Wang X, Lord DM, Cheng H, Osbourne DO, Hong SH, Sanchez-Torres V, Quiroga C, Zheng K, Herrmann T, Peti W, Benedik MJ, Page R, and Wood TK (2012b). A new type V toxin–antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. *Nature Chemical Biology* 8(10), pp. 855–61. DOI: 10.1038/nchembio.1062.
- Waskom M, Botvinnik O, O’Kane D, Hobson P, Lukauskas S, Gemperline DC, Augspurger T, et al. (2017). *mwaskom/seaborn: v0.8.1 (September 2017)*. DOI: 10.5281/zenodo.883859.
- Wayne LG (1994). Dormancy of *Mycobacterium tuberculosis* and latency of disease. *European Journal of Clinical Microbiology & Infectious Diseases* 13(11), pp. 908–14.
- Wayne LG and Lin KY (1982). Glyoxylate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. *Infection and Immunity* 37(3), pp. 1042–9.
- Weinstein RA (2001). Controlling antimicrobial resistance in hospitals: infection control and use of antibiotics. *Emerging Infectious Diseases* 7(2), pp. 188–92. DOI: 10.3201/eid0702.700188.
- Wetzel R (1929). Untersuchungen am Hühnchen. Die Entwicklung des Keims während der ersten beiden Bruttage. *Wilhelm Roux’ Archiv für Entwicklungsmechanik der Organismen* 119(1), pp. 188–321. DOI: 10.1007/BF02111186.
- Whitman CO (1878). Memoirs: The embryology of *Clepsine*. *Journal of Cell Science* s2-18(71), pp. 215–315.
- WHO (2011). *Guidelines for the programmatic management of drug-resistant tuberculosis (2011 update)*. Geneva, Switzerland: World Health Organisation.
- WHO (2015a). *Global action plan on antimicrobial resistance*. Geneva, Switzerland: World Health Organisation.
- WHO (2015b). *WHO End TB Strategy*. Geneva, Switzerland: World Health Organisation.
- WHO (2016). *Treatment guidelines for drug-resistant tuberculosis (2016 update)*. Geneva, Switzerland: World Health Organization.
- WHO (2017a). *Antibiotic resistance fact sheet*. Geneva, Switzerland: World Health Organisation.

- WHO (2017b). *Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics*. Geneva, Switzerland: World Health Organisation.
- WHO (2017c). *Global tuberculosis report 2017*. Geneva, Switzerland: World Health Organisation.
- WHO (2017d). *Guidelines for treatment of drug-susceptible tuberculosis and patient care 2017*. Geneva, Switzerland: World Health Organisation.
- Williams KJ (2005). “British pharmaceutical industry, synthetic drug manufacture and the clinical testing of novel drugs 1895–1939”. PhD thesis. University of Manchester, p. 204.
- Williams KJ, Bryant WA, Jenkins VA, Barton GR, Witney AA, Pinney JW, and Robertson BD (2013). Deciphering the response of *Mycobacterium smegmatis* to nitrogen stress using bipartite active modules. *BMC Genomics* 14(1), p. 436. DOI: 10.1186/1471-2164-14-436.
- Wilson DN and Nierhaus KH (2007). The weird and wonderful world of bacterial ribosome regulation. *Critical Reviews in Biochemistry and Molecular Biology* 42(3), pp. 187–219. DOI: 10.1080/10409230701360843.
- Winther KS and Gerdes K (2011). Enteric virulence associated protein VapC inhibits translation by cleavage of initiator tRNA. *Proceedings of the National Academy of Sciences of the United States of America* 108(18), pp. 7403–7. DOI: 10.1073/pnas.1019587108.
- Wirth T, Hildebrand F, Allix-Béguec C, Wölbeling F, Kubica T, Kremer K, van Soolingen D, Rüsch-Gerdes S, Loch C, Brisse S, Meyer A, Supply P, and Niemann S (2008). Origin, spread and demography of the *Mycobacterium tuberculosis* complex. *PLOS Pathogens* 4(9), e1000160. DOI: 10.1371/journal.ppat.1000160.
- de Wit D, Wootton M, Dhillon J, and Mitchison D (1995). The bacterial DNA content of mouse organs in the Cornell model of dormant tuberculosis. *Tubercle and Lung Disease* 76(6), pp. 555–62. DOI: 10.1016/0962-8479(95)90534-0.
- Withey JH and Friedman DI (2003). A salvage pathway for protein synthesis: tmRNA and trans-translation. *Annual Review of Microbiology* 57(1), pp. 101–23. DOI: 10.1146/annurev.micro.57.030502.090945.
- Wiuuff C, Zappala RM, Regoes RR, Garner KN, Baquero F, and Levin BR (2005). Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. *Antimicrobial Agents and Chemotherapy* 49(4), pp. 1483–94. DOI: 10.1128/AAC.49.4.1483-1494.2005.
- Wolf AJ, Linas B, Trevejo-Nuñez GJ, Kincaid E, Tamura T, Takatsu K, and Ernst JD (2007). *Mycobacterium tuberculosis* infects dendritic cells with high frequency and impairs their function *in vivo*. *Journal of Immunology* 179(4), pp. 2509–19. DOI: 10.4049/JIMMUNOL.179.4.2509.
- Wong D, Bach H, Sun J, Hmama Z, and Av-Gay Y (2011). *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) excludes host vacuolar-H⁺-ATPase to inhibit phagosome acidification. *Proceedings of the National Academy of Sciences of the United States of America* 108(48), pp. 19371–6. DOI: 10.1073/pnas.1109201108.

- Wood TK, Knabel SJ, and Kwan BW (2013). Bacterial persister cell formation and dormancy. *Applied and environmental microbiology* 79(23), pp. 7116–21. DOI: 10.1128/AEM.02636-13.
- Wu LJ and Errington J (2012). Nucleoid occlusion and bacterial cell division. *Nature Reviews Microbiology* 10(1), pp. 8–12. DOI: 10.1038/nrmicro2671.
- Xu S, Cooper A, Sturgill-Koszycki S, Heyningen T van, Chatterjee D, Orme I, Allen P, and Russell DG (1994). Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. *Journal of Immunology* 153(6), pp. 2568–78.
- Xu XN, Brownlow W, Huang S, and Chen J (2003). Single-molecule detection of efflux pump machinery in *Pseudomonas aeruginosa*. *Biochemical and Biophysical Research Communications* 305(1), pp. 79–86. DOI: 10.1016/S0006-291X(03)00692-2.
- Yakimoff W and Kohl-Yakimoff N (1911). Der Einfluss von Mikroben auf die Wirkung des Salvarsan. *Muenchener Medizinische Wochenschrift* 58, pp. 2601–4.
- Yamaguchi Y, Park J, and Inouye M (2011). Toxin–antitoxin systems in bacteria and archaea. *Annual review of genetics* 45, pp. 61–79. DOI: 10.1146/annurev-genet-110410-132412.
- Young DB, Gideon HP, and Wilkinson RJ (2009). Eliminating latent tuberculosis. *Trends in Microbiology* 17(5), pp. 183–8. DOI: 10.1016/j.tim.2009.02.005.
- Young JW, Locke JCW, Altinok A, Rosenfeld N, Bacarian T, Swain PS, Mjolsness E, and Elowitz MB (2012). Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. *Nature Protocols* 7(1), pp. 80–8. DOI: 10.1038/nprot.2011.432.
- Yu FB, Willis L, Chau RMW, Zambon A, Horowitz M, Bhaya D, Huang KC, and Quake SR (2017). Long-term microfluidic tracking of coccoid cyanobacterial cells reveals robust control of division timing. *BMC Biology* 15(1), p. 11. DOI: 10.1186/s12915-016-0344-4.
- Yu XC and Margolin W (1999). FtsZ ring clusters in *min* and partition mutants: role of both the Min system and the nucleoid in regulating FtsZ ring localization. *Molecular Microbiology* 32(2), pp. 315–26. DOI: doi:10.1046/j.1365-2958.1999.01351.x.
- Zernike F (1942). Phase contrast, a new method for the microscopic observation of transparent objects. *Physica* 9(7), pp. 686–698. DOI: 10.1016/S0031-8914(42)80035-X.
- Zhang Y, Wade MM, Scorpio A, Zhang H, and Sun Z (2003a). Mode of action of pyrazinamide: disruption of *Mycobacterium tuberculosis* membrane transport and energetics by pyrazinoic acid. *Journal of Antimicrobial Chemotherapy* 52(5), pp. 790–5. DOI: 10.1093/jac/dkg446.
- Zhang Y, Zhang J, Hoefflich KP, Ikura M, Qing G, and Inouye M (2003b). MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. *Molecular Cell* 12(4), pp. 913–23. DOI: 10.1016/S1097-2765(03)00402-7.
- Ziehl F (1883). Über die Färbung des Tuberkelbacillus. *Deutsche Medizinische Wochenschrift* 9, pp. 247–9.
- Zumla A, Raviglione M, Hafner R, and von Reyn FC (2013). Tuberculosis. *New England Journal of Medicine* 368(8), pp. 745–55. DOI: 10.1056/NEJMra1200894.