Single-cell imaging of the *Mycobacterium tuberculosis* cell cycle reveals linear and heterogenous growth

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Abstract

Difficulties in antibiotic treatment of *Mycobacterium tuberculosis* (Mtb) are partly thought to be due to heterogeneity in growth. Although the ability of bacterial pathogens to regulate growth is crucial to control homeostasis, virulence, and drug responses, single-cell growth and cell cycle behaviours of Mtb are poorly characterised. Here, we use time-lapse, single-cell imaging of Mtb coupled with mathematical modelling to observe asymmetric growth and heterogeneity in cell size, interdivision time, and elongation speed. Contrary to *Mycobacterium smegmatis*, we find that Mtb initiates cell growth not only from the old pole, but also from new poles or both poles. Whereas most organisms grow exponentially at the single-cell level, Mtb exhibits a linear growth mode. Our data demonstrate that growth behaviour of Mtb diverges from that of model bacteria, provide detail into how Mtb grows and creates heterogeneity, and suggest that growth regulation may also diverge from that in other bacteria.

Introduction

Tuberculosis remains challenging to treat, requiring lengthy multidrug treatment due to heterogeneity in host response and the behaviors of individual *Mycobacterium tuberculosis* (Mtb) bacilli[1–6]. Drug-tolerant subpopulations of Mtb exhibit different characteristics in growth, metabolic state, and gene regulation[7–13]. Therefore, the ability of Mtb to develop heterogeneity in many cellular processes, including growth behaviors, is thought to be a major obstacle in developing shortened therapies[3,14–16]. Phenotypic switching to a slow growth state to create persistence has been observed in other bacteria, including *Escherichia coli* (*E. coli*)[17] and Salmonella[18,19]. Despite the critical role of growth variation in the ability of bacteria to tolerate drug treatment, the basic growth behaviors and heterogeneity in growth characteristics of Mtb are unknown, making it challenging to study how growth variation is created and maintained in this pathogen.

Single-cell level growth studies of mycobacteria have focused on the non-pathogenic species *Mycobacterium smegmatis* (*M. smegmatis*), which is faster growing and larger than Mtb[20–26]. Mycobacteria elongate from their poles, and *M. smegmatis* grow and divide asymmetrically, creating variation in growth behaviors between sister cells (Supplementary Figure 1, first row). *M. smegmatis* elongate more from their old poles, and the sister inheriting the old pole (accelerator cell) is longer at birth and grows faster than its sister (alternator cell). Our understanding of growth behaviors in *M. smegmatis* is based on data from live-cell and fixed-cell imaging, and practical challenges have made it difficult to perform analogous live-cell imaging experiments in Mtb due to its slow-growing nature, small size, and the requirement for handling Mtb in a biosafety level-3 conditions. As a result, the collective understanding of growth behaviors in Mtb is predominantly based on those detailed studies of *M. smegmatis* and limited verification by fixed-cell imaging in Mtb[20,22,24,26–29]. Although there is evidence that Mtb can grow and divide asymmetrically[30,31], it may be that the growth behaviors of Mtb are not very similar to those of *M. smegmatis,* given the difference in time scales and dimensions. For example, the doubling time of *M. smegmatis* ranges from 3-5 hours[25,32–34] whereas Mtb doubles every ~18h in rich medium which increases in host-mimicking conditions[3,35–37]. The fundamental differences in size, growth rate, and ribosome density of *M. smegmatis* and Mtb suggest that understanding the growth behaviors of Mtb requires a direct study of Mtb using time-lapse imaging rather than a transfer of learning from *M. smegmatis*[31,38].

Although cells double in number in each generation with their population growing exponentially, it is unclear *a priori* how a single cell grows. Analysis of single-cell data in most bacteria, including *M. smegmatis* and even archaea, suggest exponential growth, attributed to ribosome self-production and protein synthesis (Supplementary Figure 1, second row)[39–46]. *E. coli* exhibits super-exponential growth during later cell cycle stages[47–49], while *Bacillus subtilis* (*B. subtilis)* follows a biphasic growth pattern with linear growth until a critical size is reached, followed by exponential growth for a fixed time[50]. *C. glutamicum*, a tip grower like mycobacteria, exhibits asymptotic linear growth, in which the rate-limiting step for growth is the synthesis of the polar cell wall[51]. Complex growth rate trends are observed in eukaryotic organisms such as the fission yeast *Schizosaccharomyces pombe*[52,53], underscoring the importance of understanding growth modes for homeostasis and cell cycle regulation. Determining how cells grow is crucial as the growth mode constrains which molecular mechanisms may be involved in cell growth[52,54,55]. Despite the critical role of the growth for tuberculosis pathogenesis and drug response, we have lacked an understanding of growth mode in Mtb.

In this study, we characterize the fundamental single-cell growth characteristics and growth mode of Mtb. Using time-lapse imaging, we measured single-cell growth and cell cycle parameters to describe Mtb growth mode and detailed growth behaviors, including cell size parameters and the origins of growth heterogeneity. We show that Mtb grows and divides asymmetrically and exhibits high levels of heterogeneity in cell size, interdivision time, and elongation speed. However, unlike *M. smegmatis*, accelerator and alternator subpopulations do not exhibit different growth behaviors in Mtb, suggesting that Mtb creates heterogeneity via novel mechanisms. We also observed that Mtb breaks the previously held polar growth model that elongation occurs first from the old pole. Instead, we find that Mtb cells can grow in three distinct patterns: from the old pole first (consistent with the prior models), from the new pole first, or from both poles together (Supplementary Figure 1). Furthermore, we found that Mtb's dominant growth mode is linear at the single-cell level throughout the cell cycle (Supplementary Figure 1). Together, our study establishes that Mtb growth behaviors cannot be learned from model organisms alone or by fixed-cell imaging and provides a quantitative framework to study growth behaviors and variation in Mtb.

Results

Single-cell analysis of Mtb growth and cell cycle timing

We conducted live-cell imaging experiments to generate growth and cell cycle timing data for Mtb at the single-cell level (movie set A, Supplementary Video 1). Annotation of these images allowed us to calculate many growth features at the single-cell level in 363 cells that were fully annotatable (from ~2700 cells imaged in biological triplicate movies), including cell length at birth (Lb) and division (Ld), growth rate, and elongation speed (Fig. 1a). We observed that cell size (medians of 2.3 μm at birth and 4.5 μm at division) and growth rates (median interdivision time of 16h) measured by live-cell imaging were consistent with those reported using bulk measurement (see Supplementary Notes Section 1). By tracking cell lineage, we identified sister cells (starting from the 2nd generation) and annotated which sister inherited the old and new poles (accelerator and alternator cells, respectively, starting from the 3rd generation) (Fig. 1a). To determine cell cycle timing, this base movie set was made with the Mtb strain CDC1551 carrying a fluorescent reporter of active DNA replication via a GFP-tag on an episomal copy of single-stranded binding protein (SSB) (Fig. 1b-1e and Extended Data Fig. 1a-1f)[56]. By annotating when SSB-GFP foci appear and disappear, we calculated the length of the B, C, and D periods for single cells, the average duration of each period in the total population (Fig. 1f,g), and SSB-foci location (Extended Data Fig. 1g). In Mtb, the B-C-D periods comprise 21%, 58%, and 20% of total cell cycle duration, respectively (Fig. 1f) compared to 4%, 69%, and 20%, respectively, in *M. smegmatis*, suggesting that the cell cycle periods were not proportionally similar in Mtb and *M. smegmatis* (Fig. 1f,g)[25,56,57].

Heterogeneity in Mtb growth characteristics

Studies of mycobacterial growth characteristics have been performed primarily in *M. smegmatis*[25,26,34,58,59]. In *M. smegmatis*, growth and division are asymmetric, giving rise to more heterogeneity in growth characteristics than in other bacterial species[4,25–28,60]. To describe Mtb growth characteristics and compare levels of heterogeneity to *M. smegmatis*, we compared distributions of lengths at birth and division, elongation speed, and interdivision time in Mtb to *M. smegmatis* (Fig. 2a,b). We observed that Mtb growth is heterogeneous to the same degree as in *M. smegmatis* for birth length (Mtb 19 % and *M. smegmatis* 19 % in CV, p-value = 1) and elongation speed (Mtb 22 % and *M. smegmatis* 23 % in CV, p-value = 0.41). Heterogeneity is increased in Mtb compared to *M. smegmatis* for division length (Mtb 17 % and *M. smegmatis* 15 % in CV, p-value = 0.018) and interdivision time (Mtb 28 % and *M. smegmatis* 21 % in CV, p-value = 1.42×10^{-7}). We also observed notable differences in growth and cell cycle behaviors of

Mtb compared to BCG, with significantly higher variation in interdivision time observed in Mtb compared to BCG (Extended Data Fig. 2 and Supplementary Table 1).

Growth properties are independent of pole age

Growth and division asymmetry in *M. smegmatis* leads to a deterministic heterogeneity in growth properties. For example, the accelerator cells are longer, elongate more, and elongate faster than the alternator cells (Fig. 2d)[25–28]. Because Mtb exhibits slightly more heterogeneous growth behaviors in cell sizes and interdivision time than *M. smegmatis*, we speculated that differences in growth characteristics between accelerator and alternator cells would be amplified in Mtb. Instead, we found no significant difference in their behaviors except for a slight difference in cell size where accelerator cells are born longer than alternator cells that did not remain different when we considered the batch effects of experimental replicates (Fig. 2c and Extended Data Fig. 3)[61]. Therefore, we conclude that accelerator and alternator cells do not exhibit different growth behaviors in Mtb as they do in *M. smegmatis*.

Mtb division asymmetry is variable

Because accelerator and alternator cells exhibit similar growth behaviors in Mtb, including cell size, we speculated that division might be relatively symmetric compared to *M. smegmatis*. We quantified the division asymmetry level by calculating the ratio of the length of the alternator cell at birth to the sum of the length of both sisters at birth. Accordingly, the distribution of division asymmetry would center around 0.5 for symmetric division and below 0.5 when the alternator cells are smaller than accelerator cells (Fig. 2e,f). Reflecting the relatively minor difference in birth sizes between accelerator and alternator cells in Mtb compared to *M. smegmatis*, we observed that the Mtb distribution is centered near 0.5 (0.49), whereas the *M. smegmatis* distribution is centered around 0.45 (Fig. 2e,f). We noted that the distribution of division asymmetry for Mtb, though narrower than in *M. smegmatis* (CV of 12% vs. 22%, p-value = 1×10^{-17}, Fig. 2f), is significantly more variable than in *E. coli* (CV 6%, p-value = 5×10^{-29}) (Fig. 2e). Together, these data suggest that growth heterogeneity is less systematically asymmetric in Mtb than it is in *M. smegmatis*, but the variation in the level of division asymmetry is still greater than other model organisms like *E. coli*.

Mtb growth is mildly asymmetric

Mycobacterial elongation is polar, and growth is faster (and earlier) from the old pole compared to the new pole in *M. smegmatis*[26,27,30,62–64]. In Mtb, fixed-cell imaging of growth patterns has suggested that Mtb growth is also asymmetric but less asymmetric than in *M. smegmatis*[27,28,30,64,65]. However, it has been challenging to quantify the asymmetric growth pattern and determine whether there is more growth from the old pole than the new pole without time-lapse imaging. By tracking cell surface features, Hannebelle and colleagues observed NETO (new end take-off), that is the old pole elongates before the new pole elongates, in both Mtb and *M. smegmatis*[64]. To measure growth asymmetry throughout entire division cycles in Mtb, we followed Mtb growth by time-lapse imaging for ~6 days (140 hours) after pulse labeling Mtb with a blue fluorescent D-alanine (HADA) for 24 hours (movie set B) (Fig. 3a,b and Supplementary Video 2). We used mathematical models to quantify growth by analyzing growth annotations over time. Based on previous results of NETO[64], we selected between linear and bilinear models, using Akaike information criterion (AIC) and the Bayesian information criterion (BIC) metrics to characterize the length growth at each pole as a function of time from birth (see Methods and Extended Data Fig. 4)[66]. A linear model implies constant elongation speed throughout the cell cycle, whereas a bilinear model implies a lag period followed by constant-speed growth.

For each cell, we calculated a growth asymmetry metric, which is the proportion of total growth over one cell cycle that is contributed by the old pole i.e., growth asymmetry = $\frac{\text{growth from old pole}}{\text{total growth from both poles}}$. A growth asymmetry score of 0.5 indicates symmetric growth (equal growth from the old and the new old), and scores <0.5 and >0.5 indicate more growth contribution by the new and the old poles, respectively. Using the polar growth data from linear and bilinear fits, we found the distribution of growth asymmetry to be centered around 0.54 (Fig. 3c), indicating that the old pole elongates more than the new pole in a larger subpopulation of Mtb.

Mtb growth is linear at the single-cell level

The similarity in the heterogeneity of growth characteristics, such as cell size at birth and division between Mtb and *M. smegmatis,* made us question whether they grow in a similar manner, qualitatively, at the single-cell level. In previous studies, *M. smegmatis* has been observed to grow exponentially[21,25,34]. Another study reported bilinear growth of *M. smegmatis* cells[64] where cells grow linearly for some time after which they change to a different elongation speed. To study the

mode of growth in Mtb, we use a differential method for the analysis of growth during the cell cycle, such as plots of changes in cell length dL/dt vs. time since birth (t), rather than cell length (L) vs. t (see Supplementary Notes Section 2)[67]. Recent works support the effectiveness of methods such as elongation speed ($\frac{dL}{dt}$) vs. age ($\frac{t}{Td}$) and growth rate ($\frac{1}{L}\frac{dL}{dt}$) vs. age plots for understanding the mode of growth[47,49,50]. Findings include *E. coli*'s super-exponential growth[47], biphasic growth in *B. subtilis*, and asymptotic linear growth in *C. glutamicum*[50,51]. In this study, we apply similar approaches to elucidate the mode of Mtb growth (see Supplementary Notes Section 3).

For exponentially growing cells, the growth rate ($\frac{1}{L}\frac{dL}{dt}$) is constant throughout the cell cycle while the elongation speed ($\frac{dL}{dt}$) increases with age (Supplementary Figure 1). The ideal linear fit of the growth rate vs. age plot would be a horizontal line with the y-intercept equal to the average growth rate. In contrast, for linear growth, the binned data trend for elongation speed will be constant throughout the cell cycle, and hence, the growth rate decreases with age (Supplementary Figure 1)[47].

On plotting the growth rate vs. age plot using data of Mtb grown in unbuffered medium (movie set A, as described in Fig. 1), the growth rate decreases with age (Fig. 4a) while the elongation speed vs. age plot stays constant (Fig. 4d). We found that the binned data trend (blue) from the experiments were largely consistent (barring small fluctuations) with the trend obtained using simulations of linear growth with measurement noise taken into account (Fig. 4a,d and see Methods). The mode of growth was also found to be linear when the experimental replicates were analyzed separately (Extended Data Fig. 5).

To investigate if Mtb's linear growth behavior extends beyond standard growth conditions (unbuffered), we examined Mtb growth in acidic (pH 5.9, resembling pH during macrophage infection) and neutral media[68,69]. We observed consistent linear growth in both acidic (movie set C, Supplementary Video 3) and neutral (movie set B, Supplementary Video 2) growth conditions, as evidenced by the trends of growth rate and elongation speed (Fig. 4).

Polar growth can start from either or both poles

A previous study on polar growth in mycobacteria indicated that Mtb elongates via NETO dynamics where the old pole starts growing linearly from birth while the new pole undergoes linear

growth after a certain lag time[64]. This NETO dynamics predicts a growth change when the new pole starts growing. In this section, we investigate how to reconcile polar growth with the roughly linear growth we observed in Fig. 4.

As introduced previously, pulse label experiments were used to observe growth at the old and new poles (Fig. 3a,b). Based on NETO, the old pole is expected to grow from birth and the new pole to be delayed before growing (Fig. 5a and Extended Data Fig. 4a and 4d)[64]. However, as shown in Fig. 5b and Extended Data Fig. 4b and 4e, approximately half of the cells started growing from both poles within 1 h of cell birth – 45.6% in neutral pH, 49.5% in acidic pH, from n = 147 and 101 cells in movie sets B and C, respectively (Fig. 5d and Supplementary Figure 1). Following the "NETO" nomenclature, we call these dynamics "BEITO" (both ends immediately take-off). The joint distribution for the time (relative to birth) at which the old pole and the new pole start growing peaks at short times on both axes in both neutral and acidic pH (Fig. 5d). Unlike NETO dynamics[64], we also find cases where the new pole starts growing before the old pole as shown in Fig. 5c and Extended Data Fig. 4c and 4f ("old end take-off", OETO). In neutral pH, we found that NETO was observed in 33.3% of the total cells, while OETO was observed in 21.1% of the total cells. In acidic pH, the proportions for NETO and OETO were 37.6% and 12.9%, respectively (Fig. 5d). The elongation speeds are greater for the old pole growth as compared to the new pole growth in both neutral and acidic pH conditions (Fig. 5e). The overall greater elongation from the old pole relative to the new pole may be due to a combined effect of slightly more cases of NETO than OETO and a faster elongation speed from the old pole.

Using simulations (Fig. 5f; Supplementary Notes Section 4), we reconcile the population-averaged linear growth across the cell cycle (Fig. 4) with the pattern of polar growth with different ends growing at different times (Fig. 5). This comes about primarily due to the early take-off times in NETO and OETO populations.

To conclude, the cells undergo polar growth, with both poles starting to grow from the beginning of the cell cycle in half of the cells (BEITO), and in the other half, either pole can start growing first. Using simulations based of our experimental data and distribution of cells that are BEITO, NETO, and OETO, we could reproduce the largely constant elongation speed vs. age curve observed in the experiments.

Discussion

A key feature of Mtb's pathogenesis is slow growth and phenotypic heterogeneity, which is thought to enable Mtb to reside, survive, and hide in multiple sites of infection in humans[4,36,70]. Therefore, we have an urgent need to understand how Mtb grows and regulates growth heterogeneity to understand their bet-hedging strategies that enable population-level drug and immune evasion. Using time-lapse imaging, we describe the growth behaviors and heterogeneity for tubercle bacilli in detail. Prior studies hinted at less asymmetric polar growth in Mtb compared to *M. smegmatis*, which would attenuate differences in growth behaviors between sister cells and overall variation in growth behaviors[30,64]. However, we show that cell-to-cell variation in cell size, interdivision time, and growth speed was similar and high compared to model rod-shaped bacteria. Because *M. smegmatis* generates two types of sister cells (accelerator and alternator) via their growth pattern that gives rise to subpopulations with different growth characteristics and drug susceptibility patterns[27,28], the tuberculosis field has sought to understand how accelerator and alternator cells contribute to drug tolerance in Mtb. However, our results here suggest that they do not exhibit deterministically different growth behaviors in Mtb, suggesting the need to explore alternative sources of growth heterogeneity in Mtb, potentially polar growth patterns, gene or protein distributions, or metabolic state, to better understand virulence and drug responses. Whereas numerous studies have established that *M. smegmatis* initiates growth from the old pole[25,27,28,64], we found that there are diverse polar growth types in Mtb: some cells grow from the new pole or old pole first and others elongate from both poles at once soon after birth. The three polar growth patterns observed in this study - NETO/OETO/BEITO - may play a role in driving phenotypic heterogeneity, including differences in drug and immune tolerance.

Combining single-cell growth data with mathematical modeling, we show that Mtb growth mode at the single-cell is linear throughout the cell cycle. Most bacterial species are thought to grow exponentially in biomass and volume[41,42,44,46,47]. In this study, we showed that Mtb growth was nearly linear not only in an unbuffered rich growth condition but also in slower, more stringent growth conditions in an acidic medium, mimicking a stressor the bacteria face during infection. The persistence of linear growth behavior in both rich and acidic conditions suggests that linear growth is the dominant growth mode of Mtb and not the exception.

Understanding bacterial growth at the single-cell level is crucial in bacterial physiology. While a prevalent framework implicates ribosome-centric growth models (see Supplementary Discussion), our discovery of linear growth in Mtb challenges this paradigm, suggesting the need for novel regulatory mechanisms of growth mode. Various mechanisms, including gene dosage and cell

wall growth limitations, may underlie this linear growth, highlighting the complexity of bacterial growth dynamics and the necessity for further investigation into Mtb's growth mechanisms.

In addition to exploring the question of how linear growth is observed in Mtb, one may also inquire about the underlying reasons for this phenomenon. In bacteria such as *E. coli*, the existing paradigm is that the selection pressure faced by cells during evolution has led them to grow as fast as possible (barring the presence of non-growing subpopulations such as persisters)[17,71]. Indeed, in long-term-evolution-experiments growth rate has significantly increased over the generations[72], and extensions of the ribosome-limited model for growth showed that by making ribosomes consist of numerous, smaller proteins leads to a significant growth-rate increase[73], thus interpreting the ribosome structure itself in terms of growth rate optimization. This picture appears invalid in Mtb, suggesting it faces different constraints and selection pressure. This could arise due to its constant battle with the immune system, making its main challenge evading it rather than optimizing growth rate.

Our work provides a baseline understanding of Mtb growth properties and their variations in a population of cells. These data include cell sizes, cell cycle timing, and elongation speeds. We find that the growth mode and level of heterogeneity are together unique and cannot be explained using models developed in model bacteria or other closely related species such as *M. smegmatis*. Given the critical role of growth, metabolic state, and adaptation to fluctuating environments that Mtb faces during infection to Mtb virulence and drug response, further studies on the growth behaviors of Mtb and other pathogens will enable us to develop improved therapeutic interventions.

Methods

Overview of the movie sets

For the baseline movie set (movie set A), we used Mtb CDC1551 strain harboring the SSB-GFP reporter, which was grown in unbuffered (pH ~6.8) standard (supplemented Middlebrook 7H9) growth medium at 37°C. Time-lapse imaging was conducted for 96 hours, with images taken every hour.

Two additional movie sets were made using the FDAA pulse-labeled CDC1551 wild-type strain. Movies were made in neutral (pH 7.0, movie set B) or acidic (pH 5.9, movie set C) standard (supplemented 7H9) growth medium at 37°C. Time-lapse imaging was conducted for 140 hours, with images captured every hour. All the movies are made inside a biosafety level-3 facility.

Bacterial strain

Mtb CDC1551 strain (movie sets B and C) and its transformant with a hygromycin-resistant replicating plasmid expressing single-stranded binding protein fused to a green fluorescent protein (SSB-GFP) and *smyc'*::mCherry (movie set A) are used in this study[56]. All live-cell work with these strains was performed inside a biosafety level-3 facility.

Bacterial culture

Mtb was grown in a standard medium consisting of 7H9 broth (ThermoFisher; DF0713-17-9) with 0.05 % Tween 80 (ThermoFisher; BP338-500), 0.2 % glycerol (ThermoFisher; G33-1), 10% Middlebrook OADC (ThermoFisher; B12351). For the SSB-GFP reporter strain (movie set A), 50 µg/ml of hygromycin (ThermoFisher; 10687010) was added to maintain SSB-GFP, *smyc'*::mCherry. Mtb strain was grown to OD_{600} of 0.5 - 1.0 from frozen aliquots at 37°C with mild agitation. Cultures were subcultured via back dilution to OD_{600} 0.05 and grown to the mid-log phase (OD_{600} 0.5-0.7) before experimental use. For movies where polar growth is assessed (movie sets B and C), Mtb cells were backdiluted to OD_{600} of 0.2 in 10 ml 7H9 media (unbuffered) supplemented with 100 µM HADA (see below) for 24 h. Labelled cells were washed twice with PBS (ThermoFisher; 20012-027) + 0.2 % Tween-80 (PBST) and resuspended in pH 5.9 or 7.0-adjusted fresh 7H9 media that was supplemented with sterile spent medium (50:50) for loading into the microfluidic devices for time-lapse imaging.

FDAA labeling (movie sets B and C)

The blue fluorescent D-amino acid (FDAA), HADA (Tocris; 6647), was used for movie set B and C. HADA powder was dissolved in DMSO to a stock concentration of 100 mM and stored short-term at -80ºC. Cells were incubated in 100 µM HADA for 24 h prior to the start of the imaging.

Microfluidic device

The highest throughput microfluidics for studying cell growth in rod-shaped bacteria require that the bacteria are loaded into thin channels[40,74,75]. Mycobacteria cannot be loaded into these channels because they are coated in a thick mycolic acid layer in their cell wall, making them too sticky for these devices (Fig. S1a).

We overcome this challenge by optimizing protocols and using a custom microfluidic device to achieve long-term time-lapse imaging of Mtb in a biosafety-level 3 laboratory. We used devices previously designed to study *M. smegmatis* to ensure freedom of movement in polar growth and v-snapping[23,25]. We observed that, whereas *M. smegmatis* grows with a new medium constantly flowing in the microfluidic devices, Mtb enters growth arrest. Culture filtrate is required to enable Mtb single cells to grow under constant flow; therefore, we supplement the new medium flowing into the device with culture filtrate at a ratio of 1:1 to avoid growth arrest. With these protocols, we were able to achieve a consistent growth pattern in Mtb over four days of imaging with a doubling time (~17h) that is consistent with the doubling times of Mtb in bulk culture[76–78].

Live-cell microscopy

Before loading Mtb cells into a custom polydimethylsiloxane (PDMS) microfluidic device, cells were filtered through a 10 µm membrane filter to remove clumps. Mtb cells were loaded into a microfluidic device through the outlet port using a syringe that contains filtered Mtb cell culture[23]. The devices contain a main microfluidic feeding channel with a height of 10-17 µm and viewing chambers with a diameter of 60 µm and a height of 0.8-0.9 µm. Fresh medium was delivered to cells at 5 µl/min flow using a microfluidic syringe pump. The device was placed on an automated microscope stage within an environmental chamber maintained at 37ºC. Mtb cells were imaged at 60x magnification using a widefield Deltavision PersonalDV (Applied Precision, Inc.) which is located inside a biosafety level-3 facility. For movie set A, cells were illuminated with an InsightSSI

Solid State Illumination system every hour for 96 hours. Cells were imaged using transmitted light brightfield microscopy, GFP (475 nm/525 nm), and mCherry (575 nm/625 nm). mCherry was imaged to ensure the presence of the plasmid and was not used for analysis. The movies were performed in biological triplicate, with each replicate performed separately in different microfluidic devices on different days. For FDAA pulse-labeled movies (movie sets B and C), cells were imaged every hour for 140 hours using transmitted light brightfield microscopy, and HADA was visualized with CFP filter (433 nm/475 nm).

Live-cell image segmentation

For movie set A, ImageJ plugin ObjectJ (version 1.03x) was used to hand-annotate cell length, growth, and cell cycle progression throughout the image stack (time-lapse). Single cells were annotated[23,25] for cell length by marking two points at each pole. Additional points (up to four points total within a cell) are annotated when foci were present. The SSB-GFP reporter forms a green fluorescent focus during DNA replication. Newly divided cells generally did not have SSB-GFP focus, labeled as the pre-replication period (B period). The period where one or two SSB-GFP foci were detected was defined as the replication period (C period), and the subsequent period where foci were absent was labeled as the post-replication period (D period). Some cells had one or two foci after the division period but before septation; in these cases, we labeled those occurrences as the pre-division period (E period)[25,56,57]. Cell poles and visible foci were annotated in each frame - two points at each pole of a single cell were annotated if no foci were detected, while three (one focus) or four (two foci) points were annotated when foci appeared. The localizations of foci were manually analyzed (custom code) and used to determine cell cycle timing. The annotation in each frame was extracted, containing information on cell length and cell cycle progression over time (1-hour time scale). The ObjectJ data was exported to an XML file. Information on mother-daughter and accelerator-alternator cell relationships was also collected from cell pedigree trees using custom scripts in MATLAB for analysis; specifically, these scripts calculate and collate single cell data - length at birth and division, duration of each cell cycle period, SSB foci presence and localization, accelerator/alternator status, and pedigree relationships between cells (ex. mother/daughter/sister cell relations). During time-lapse imaging, we observed a rare subpopulation in which the cells express a high intensity of GFP. We observed that these cells did not divide and entered growth arrest. This may be due to phototoxicity caused by high SSB-GFP abundance. These cells were excluded from annotation.

For FDAA pulse-labeled movies (movie sets B and C), using the HADA label as a marker of old cell wall material, we annotated growth from the new and the old poles in cells born during the movie (so that we could establish pole age) from birth to division (n = 248) (Fig. 3a,b). The cell poles and HADA labeling were hand-annotated in each frame using ImageJ (version 1.53f) with an ObjectJ plug-in. Whole-cell labeling was annotated at each pole in the first time point. When cells elongated, a non-labeled area appeared, representing the new growth site. In cases where cells elongated from only one pole, three points were annotated. When cells elongated from both poles, four points were annotated, starting from one pole (single point), then the HADA-labeled cell body (two points), and then the other pole (single point). After annotation, the x and y coordinates of each annotation point were extracted, and the distance was calculated using the Euclidean distance formula.

Simulations

In Fig. 4, we showed Mtb growth was largely consistent with linear growth simulations. Here, we describe the model used for the linear growth simulations and the simulation methodology. Simulations of linear growth were carried out for 500 cells. Elongation speeds were assumed to have a Gaussian distribution with mean $\langle\lambda_{lin}\rangle$ and standard deviation $CV_{\lambda,lin}\langle\lambda_{lin}\rangle$ determined using the experiments. They were calculated as mean and standard deviation of $\frac{Ld-Lb}{Td}$ where Lb, Ld and Td are lengths at birth, division and the generation time, respectively. Values for ($\langle\lambda_{lin}\rangle$ in μm/h, $CV_{\lambda,lin}$) are as follows – unbuffered medium (0.1442, 0.226), acidic medium (0.0741, 0.286), neutral medium (0.069, 0.240), replicate 1 (0.1663, 0.181), replicate 2 (0.134, 0.193), replicate 3 (0.1309, 0.220). The elongation speed was determined at the start of the cell cycle. The length at birth for each cell was sampled from a normal distribution with mean $\langle L_b\rangle$ and CV (CV_b) fixed using the experimental data. The values ($\langle L_b\rangle$ in μm, CV_b) are as follows- unbuffered medium (2.37, 0.19), acidic medium (2.34, 0.24), neutral medium (2.25, 0.18), replicate 1 (2.60, 0.19), replicate 2 (2.22, 0.18), replicate 3 (2.27, 0.17). The cells divided upon reaching size $Ld = 2(1-\alpha)Lb + 2\alpha\Delta + \eta$. Here, Δ is a constant and α is the size regulation strategy which can take any value from 0 to 1. When α is 1, cells divide on reaching a particular size 2Δ (sizer) and for $\alpha = \frac{1}{2}$, cells divide on adding a constant size Δ from birth. α and Δ are determined using the slope and intercept of the experimental Ld vs. Lb plot which are $2(1-\alpha)$ and $2\alpha\Delta$, respectively[79]. η is the size additive division noise with mean zero and standard deviation (σ_{bd}). Our results are independent of the nature of division noise. The values for (α, Δ in μm, σ_{bd} in μm) are as follows - unbuffered medium

(0.60, 2.28, 0.57), acidic medium (0.89, 2.77, 0.85), neutral medium (0.71, 2.26, 0.41), replicate 1 (0.61, 2.40, 0.66), replicate 2 (0.72, 2.26, 0.42), replicate 3 (0.65, 2.18, 0.37). The length of each cell is measured at 1-h intervals. The measured length is the sum of the actual length of the growing cell and a measurement error normally distributed with mean zero and standard deviation determined from experiments (see Supplementary Notes Section 5). The standard deviation values in μm are as follows - unbuffered medium (0.13), acidic medium (0.12), neutral medium (0.11), replicate 1 (0.14), replicate 2 (0.15), replicate 3 (0.12).

We also investigate the effects of NETO, OETO, and BEITO dynamics on the binned data trends of growth rate vs. age and elongation speed vs. age plots in Fig. 5f. We conducted simulations using the information of polar growth from the fluorescent d-alanine (HADA) labelled experiments in both neutral and acidic pH which we elaborate next. The simulations have the same number of cells as the experiments, i.e., n= 101 for acidic and n = 147 for neutral pH conditions. Each cell is initialized to be born at a length sampled from a normal distribution with mean (acidic – 2.30 μm, neutral – 2.29 μm) and CV (acidic – 0.24, neutral – 0.16) determined from the experiments. Each cell starts growing as BEITO with a probability equal to the fraction of BEITO cells in the experiments (~50% for acidic and ~46% in neutral pH). The elongation speed of old and new pole growth is drawn from independent normal distributions with mean (acidic- old: 0.044μm/h, new: 0.036μm/h; neutral- old: 0.045 μm/h, new: 0.034 μm/h) and CV (acidic- old: 0.38, new: 0.42; neutral- old: 0.35, new: 0.40) determined from the experiments. For the remaining non-BEITO cells, growth does not occur from the poles until a certain take off time. To accurately simulate the take-off time distributions, we sample the timings with equal probability and with replacement from the non-BEITO cells obtained in the experiments. It ensures that the distribution of take-off times for the old and new poles and the proportion of NETO and OETO cells are similar in simulations and experiments. The cell cycle ends once the cell reaches a particular division size (Ld) determined solely by the birth size (Lb) i.e., mathematically, $Ld = 2(1-\alpha)Lb + 2\alpha\Delta_{bd} + \zeta_{bd}$. The values of α and Δ_{bd} are obtained from the experiments (α = 0.83, Δ_{bd} = 2.89 μm for acidic and α = 0.8, Δ_{bd} = 2.34 μm for neutral pH) and ζ_{bd} is the normally distributed noise in division size with mean 0 and standard deviation determined such that the standard deviation of division size is same in simulations and experiments (acidic – 0.85 μm, neutral – 0.40 μm). The length of the cell is measured from cell birth until cell division at 1 h time intervals. A measurement noise term is added to each measurement with mean 0 and standard deviation determined using HADA labelled cells: in these experiments, the length of the HADA labelled part of the cell is assumed to be constant (non-growing) throughout the cell cycle and the deviation from the constancy

provides an estimate of the magnitude of measurement noise (acidic – 0.099 μm, neutral – 0.078 μm). In some of the cells, it is hard to visualize the growth of the new pole because of the finite resolution limit of imaging. In these cases, the new pole appears to be non-growing till some point in the cell cycle after which it experiences a sudden change in growth. We include these non-growth biases by sampling the times from experiments for which the new pole has no measured growth. We include this bias for completeness; however, we note that its exclusion does not change the results qualitatively.

Characterizing single trajectories using AIC and BIC values

We characterize the growth at the old and new poles using FDAA (HADA) labeling (Fig. 3). Fig. 3a (middle panel) shows that the cells have an unlabeled part at the old pole after the first generation. The old pole growth is measured by adding unlabeled parts to the existing unlabeled region. The growth at the new pole is marked by the appearance of an unlabeled region at the new pole and, in a few cases, the growth of unlabeled parts to the existing unlabeled region. The aim is to identify the amount of growth at each pole and the time at which the pole growth starts. However, precise measurement of single-cell polar growth is difficult because the clumping of Mtb cells (Fig. S1a) obscures the position of the poles and, thus, complicates the determination of the HADA unlabeled part. This section explains the statistical models used to determine the polar growth at both ends at a single-cell level.

Previous studies have determined growth at each individual pole to be linear[64]. Linear growth is also supported by our results in the main text (Fig. 4). Thus, to determine the growth at each pole, we fit two different models to the length vs. time trajectories – 1. Linear growth 2. Bilinear growth is where the length stays constant for a certain time and then increases linearly. The bilinear growth was used to characterize the NETO[64], where it was proposed that the new pole starts growing after a time delay from cell birth. We assume that some old poles may also grow bilinearly (Fig. 5c and Extended Data Fig. 4c and 4f).

In cells that already have an unlabeled part at the old pole, we calculate the amount of old pole growth at time t from birth as the difference between the length of the unlabeled HADA region at the old pole at time t from birth and the initial unlabeled part at birth. The measured length grown can be negative for the old pole as the unlabeled HADA region at birth can be inaccurate due to cell clumping or cell tilting along the z-plane in the microfluidic chamber. We show examples of length grown vs. time for the old pole (blue/green) and new pole (red) in Fig. 5 and Extended Data

Fig. 4. In most of the cells of the pulse label experiment, we do not observe a HADA unlabeled region at the new pole at the time of birth. In these cells, the length grown at the new pole is the length of the unlabeled HADA region at the new pole. The length grown is marked as zero when we do not observe the unlabeled HADA region at a pole.

Next, we fit the two models discussed above for each single cell to the length grown at each pole vs. time curves. The linear model is characterized by two parameters $y = ax + b$ where a is the elongation speed of the pole, and b is a measure of the unlabeled HADA region at cell birth. For the bilinear model, the underlying equation is,

$$y = \begin{cases} b, & x \le c \\ a(x - c) + b, & x > c \end{cases}, \qquad A1$$

where a, b, and c are the elongation speed of the pole, bias in determining the initial HADA unlabeled region and time when the pole starts growing (relative to birth), respectively. For fitting, we ignore those data points where the length grown is zero (the y-axis is zero). We minimize the squared sum of residuals (RSS); $RSS = \sum_{i=1}^{N}(y_i - \hat{y}_i)^2$, where y_i is the true value of the i^{th} data point of the dependent variable, and $\hat{y}_i$ is the predicted value from the model. To compare which of the two models better fits the single-cell trajectories, we use the Akaike information criterion (AIC) and the Bayesian information criterion (BIC). They are defined as,

$$AIC = 2k - 2\ln\left(\hat{L}\right), \qquad A2$$

$$BIC = \ln(N)\,k - 2\ln\ln\left(\hat{L}\right), \qquad A3$$

where k is the number of parameters in the model (k = 2 for linear, k = 3 for bilinear), N is the number of data points fitted and $\hat{L}$ is the maximum value of the model's likelihood function. In both AIC and BIC methods, a model is favored if it has a greater maximum likelihood value, and it is penalized for having a greater number of parameters, the penalty being different for AIC and BIC as shown in Equations *A2* and *A3*. Assuming that the errors $(y_i - \hat{y}_i)$ are drawn from an independent and identical normal distribution, the AIC and BIC values can be simplified to[66],

$$AIC = 2k + N\ln\left(\frac{RSS}{N}\right) + \frac{2k(k+1)}{N-k-1}, \qquad A4$$

$$BIC = k\ln(N) + N\ln\left(\frac{RSS}{N}\right). \qquad A5$$

The AIC and BIC values themselves have little significance. The relevant metric is the difference between AIC and BIC values of the two models being compared, in our case, they are $\Delta AIC =$

$AIC(linear) - AIC(bilinear)$, $\Delta BIC = BIC(linear) - BIC(bilinear)$. A model with a lower AIC and BIC value is preferred. In our case, the bilinear model is preferred if both ΔAIC and ΔBIC values are greater than zero, while linear is preferred if the ΔAIC and ΔBIC values are less than zero. In a few cases where ΔAIC and ΔBIC values have opposite signs, the model with fewer parameters, i.e., the linear model is preferred.

Having chosen the appropriate model using the ΔAIC and ΔBIC values obtained upon fitting both models to the length vs. time trajectories of each cell, we aim to calculate the time at which the growth at a particular pole starts, the elongation speed at that pole, and the amount of growth at that pole. We discuss the calculation of these quantities for different cases of fit results obtained- first for old pole growth and subsequently for new pole growth.

A bilinear curve best fits the trajectory for old pole growth shown in Extended Data Fig. 4c. In these cases, where the old pole growth is bilinear, the value of parameter c (Equation *A1*) of the best bilinear fit denotes the time at which the pole starts elongating. The slope a is the elongation speed of the pole and the constant b is the bias in determining the initial unlabeled HADA region. Thus, the amount of polar growth during the cell cycle is the length increase within time c and Td (the doubling time). Mathematically, the amount of polar growth = $a(Td - c)$. For the old pole growth trajectories in Extended Data Fig. 4a and 4b, where the best fits are linear, we assume that the growth starts from time 0. The y-intercept obtained (positive in (b) and negative in (c)) can be interpreted as the error in determining the initial unlabeled HADA region. The elongation speed is the slope of the best linear fit and the amount of growth is equal to $Slope \times Td$.

Next, we discuss the calculations of growth parameters for the new pole growth. We fit the non-zero length grown vs. time data to the two models- linear and bilinear- and choose the appropriate model based on ΔAIC and ΔBIC values. In the trajectory shown in Extended Data Fig. 4c, the best fit is linear with a negative y-intercept. On extrapolating the best fit to y = 0, we obtain a positive time T_{trans}. Since we do not observe the unlabeled HADA region before this time point, we interpret the time T_{trans} as the time when the pole starts growing. The raw data shows the new pole to have HADA label for some time after T_{trans} because the unlabeled HADA region is small and might be undetectable in the movies until it reaches a particular length. Examining the movies shows that this length is around 0.2-0.4 μm. This can also be seen in Extended Data Fig. 4a-4c trajectories, where there is a sudden jump in the length of the new pole. The elongation speed is the slope of the best linear fit and the amount of growth is $Slope \times (Td - T_{trans})$. In Extended Data Fig. 4b, the best linear fit has a positive y-intercept. In this case, we interpret the y-intercept as an initial undetectable HADA unlabeled region at the new pole. The new pole starts

growing from the beginning of the cell cycle with an elongation speed equaling the slope of the best linear fit. The amount of growth at the new pole during a cell cycle is $Slope \times Td$. In Extended Data Fig. 4a where a bilinear fit better explains the length grown as a function of time, the interpretation of the fit is similar to that of the old pole. The constant parameter b in Equation *A1* denotes the HADA unlabeled region at cell birth, c denotes the time when the new pole starts growing and the slope a denotes the elongation speed. The amount of growth is given by the same equation as the old pole, $a(Td - c)$.

In a few cases, we observed that the new pole had an unlabeled HADA region at cell birth. In such cases, we analyzed the new pole using the same method as the old pole, as discussed previously in this section.

We use the definition of BEITO as cells starting to grow from both poles within 1 h of birth. This is related to the error in estimating the value of c, the time when the pole starts growing. To calculate the accuracy in the estimation of the parameter c, we generated 100 trajectories using the model (either linear or bilinear) determined for each pole and each cell. To the deterministic growth component- elongation speed and growing time- we added a noise which is normally distributed with mean 0 and standard deviation determined by the residuals. For each of the 100 trajectories, we again undertook the fitting and model selection procedure described above and obtained estimates of elongation speed and time delay before growth at that pole starts. The standard deviation of the parameter c is obtained for the old pole and the new pole growth. The standard deviation for both poles in both acidic and neutral conditions is between 0.8-1.1 h. Note that this is close to the time between successive measurements (Δt = 1h) but much smaller than the mean interdivision time (35-45 h).

Statistics and Reproducibility

The scatter plots are presented with median values. The two sided, two-tailed Mann-Whitney test was performed throughout the features compared between accelerator and alternator cells within Mtb or *M. smegmatis* strains.

Significance between CV values was tested using an asymptotic test for the equality of coefficients of variation from k populations[80]. The test is commonly used to compare variation between k different groups with unequal sample sizes. In this work, we consider k=2 because we conduct pairwise CV comparisons. *P* values < 0.05 were considered statistically significant. The

test does quite well irrespective of the underlying distribution of the data (normal, log-normal, gamma) provided that the sample size is large (n = 20).

We used the package "cvequality" (Version 0.2.0) in R version 4.1.2 (202 1-11-01) to compare the coefficient of variations. Simulations and data analyses were performed using MATLAB version R202lb. 3D histograms were made using Python version 3.9.

Images from Fig. 1b,d and Fig. S1a,b are representative of three biological replicates. Images from Fig. 3a and Fig. S1c are representative of two biological replicates. Single cells loaded into 40-60 separate microfluidic chambers were used for imaging, generating 40-60 movies per biological replicate. The movies from two biological replicates, part of which were used for Fig. 3a, include a total of 1,089 annotated single cells.

Data Availability

The primary datasets and the referenced datasets processed from time-lapse imaging annotations are available in Figshare with the identifier https://doi.org/10.6084/m9.figshare.22930310.

Code Availability

Custom MATLAB code used in this study are available at https://github.com/pkar96/Mtb-growth

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Author Contributions

E.S.C, P.K., B.B.A., and A.A. conceptualized the project, E.S.C. and M.K. conducted the experiments and time-lapse imaging annotation, and P.K. ran mathematical analysis. E.S.C., P.K., B.B.A., and A.A. wrote the draft, and all authors reviewed and edited the manuscript. B.B.A. and A.A. acquired funding.

Competing Interests

The authors declare no competing interests.

Fig. 1. Measurement of growth characteristics in Mtb (movie set A). a, Schematic of growth parameters derived from Mtb across generations by time-lapse imaging. Cells loaded into the device (1st generation) establish the pole age for the next generations but are excluded from analysis due to incomplete cell cycle observation. In the 2nd and subsequent generations, all growth features are determined, including length at birth, DNA replication initiation, and division (Lb, Li, and Ld), interdivision time (Td), growth rate (λ), elongation speed ($\frac{dL}{dt}$), and identification of old and new poles. In the 3rd (and subsequent) generations, we identify accelerators (acc) (n = 173) and alternators (alt) (n = 130). In baseline experiments (movie set A), cell cycle timing at the single-cell level is determined using an SSB-GFP reporter strain of Mtb (total cell n = 363). **b**, Determination of cell cycle state using SSB-GFP. SSB binds to replication forks[81,82], with green foci indicating ongoing chromosome replication (C period); no visible foci before and after replication (B and D periods, respectively). Yellow arrows highlight foci. Scale bar = 2 μm. **c**, Single-cell traces of SSB-GFP localization with hourly time points corresponding to (**b**). The distance from the x-axis to the black circle represents cell length. Green circles indicate SSB-GFP foci localization along the cell body. Asterisks (*) correspond to the images shown in (**b**). **d**, Annotation of cell cycle state in a cell with an E period, where a small population (11 %) initiates replication before division (foci-positive)[25]. Daughter cells inheriting the foci enter directly into the C period. Yellow arrows highlight foci. Scale bar = 2 μm. **e**, Single-cell SSB-GFP traces with hourly time points corresponding to (**d**). Green circles indicate SSB-GFP foci. Asterisks (*) correspond to the images shown in (**d**). **f**,**g**, Cell cycle timing in Mtb (**f**) and *M. smegmatis* (**g**). The average time and proportion of each cell cycle period (B: pre-replication, C: DNA replication, D: post-replication, E: new DNA replication after a D period but before division). The *M. smegmatis* data are from a previous study[25].

Fig. 2. Growth properties in Mtb and *M. smegmatis*. a,**b**, Distributions of Lb and Ld, Td, and elongation speed ($\frac{dL}{dt}$) are shown in both Mtb (n = 363; movie set A) (green) (**a**) and *M. smegmatis* (n = 391) (orange) (**b**), respectively. The coefficient of variation is shown in the upper right corner of each plot. The center line of each box-and-whisker plot indicates median. The *M. smegmatis*

data are from a previous study[25]. **c**,**d**, Growth property distributions are compared between acc and alt cell in Mtb (n of acc = 173, n of alt = 130) (green) (**c**) and *M. smegmatis* (n of acc = 193, n of alt = 188) (orange) (**d**). Lb and Ld, elongation speed, growth amount, and Td are compared between acc and alt, respectively. Horizontal lines mark the median value for each sample. The *M. smegmatis* data are from a previous study[25]. P-values were calculated using the two-sided, two-tailed Mann-Whitney test. In Mtb, the p-values for each comparison are 0.024, 0.66, 0.39, 0.27, and 0.80, respectively (**c**). The p-values for *M. smegmatis* are $8.29*10^{-23}$, $6.66*10^{-20}$, $2.20*10^{-9}$, 0.0004, and 0.070, respectively (**d**). Mann-Whitney *U* = 9542, 10908, 10592, 10419, 11050 in Mtb and 7576, 8326, 11712, 14335, and 16207 in *M. smegmatis*, respectively. **e**,**f**, Distributions of division asymmetry in Mtb, *M. smegmatis* and *E. coli*. The distributions of division asymmetry in Mtb (green) (**e**) and *M. smegmatis* (orange) (**f**) are calculated as (Lb of alt cell (daughter cell) / Ld of mother cell). The division symmetry of *E. coli* (red) is calculated as (Lb of daughter cell / Ld of mother cell) (**e**). The *M. smegmatis* and *E. coli* data are from previous studies[25,83]. The dashed lines at 0.5 indicate perfect symmetry. The CVs of Mtb, *E. coli*, and *M. smegmatis* are 12 %, 6 %, and 22 %, respectively.

Fig. 3. Polar growth and growth symmetry in Mtb (measured in movie set B)**. a**, 5-hour-interval image sequence of FDAA-labeled Mtb cells. Individual cells were fully labeled with HADA at the beginning of the time-lapse imaging. The unlabeled area at the poles indicates a newly grown cell wall since the start of the imaging. Scale bar, 2 μm. **b**, Schematic diagram of polar growth quantification using FDAA-labeling Mtb. The blue area within the cell indicates FDAA (HADA) labeling. The white (unlabeled) area at the poles indicates the new cell wall. The white dashed line indicates the septum formation. **c**, Distribution of growth asymmetry in Mtb. The histogram is fit with a Gaussian. The gray dashed line (score of 0.5) represents symmetric growth. (mean value = 0.58, n = 147).

Fig. 4. Linear growth modes. a-c, The binned data trend (blue, orange, and purple dots for unbuffered, acidic, and neutral condition, respectively) of growth rate ($\frac{1}{L}\frac{dL}{dt}$) vs. age plot is shown for Mtb data (movie set A, n = 363 for unbuffered, movie set C, n = 135 for acidic, movie set B, n = 248 for neutral condition, using the cells that are born and divided during the movie only). The binned data trends decrease with age and are largely consistent with the predicted trends obtained using simulations of linear growth (red lines). **d-f**, The binned data trend (blue, orange,

and purple dots for unbuffered, acidic, and neutral condition, respectively) of elongation speed vs. age plot is shown for Mtb data. The binned data trends are nearly constant, consistent with linear growth simulations (red lines). The error bars are presented as mean ± SEM..

Fig. 5. Growth characteristics at old and new poles. a-c, Representative examples of different polar growth dynamics (movie set B, other examples are shown in extended data fig. 4). The elongation length at each pole is shown as a function of time. The lines represent the best fit to the data and can be either linear or bilinear. **a**, The new pole starts elongating later than the old pole (new end take off, NETO). **b**, Both poles elongate from the beginning of the cell cycle (both ends immediately take off, BEITO). **c**, The new pole starts elongating before the old pole (old end take off, OETO). **d**, Joint distribution of the timings when the old and new poles start growing in neutral (n = 147) and acidic pH (n = 101) (movie set B and C, respectively). Blue, red, and purple bars indicate NETO, OETO, and BEITO cells, respectively. **e**, Distribution of the elongation speed at each pole in neutral and acidic pH (movie set B and C, respectively). **f**, Growth rate and elongation speed vs. age in neutral (n = 147) and acidic (n = 101) growth conditions. Simulations of the proposed model are carried out using parameters derived from experimental data. Growth rate trends as a function of age and elongation speed vs. age are compared between simulations (black) and experiments (red) for neutral pH and acidic pH conditions. The error bars are presented as mean ± SEM.. Note that here we are showing numerical simulations with a comparable number of cells to that used in the experiment, leading to significant fluctuations. These fluctuations can obscure the deviations from linearity predicted by the full model, which accounts for different subpopulations. The model predictions, averaged over a larger population of cells, are shown in Fig. S4.

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