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Graphical Abstract



Authors

Mats Wallden, David Fange, Ebba Gregorsson Lundius, Özden Baltekin, Johan Elf

Correspondence

johan.elf@icm.uu.se

In Brief

Cell-to-cell variation in division timing and cell size in E. coli is due to differences in growth rate, whereas the timing of replication is triggered at an invariant fixed volume per chromosome.

Highlights

- Replication initiates at a nearly fixed volume per chromosome for all growth rates
- The time from initiation to division depends on the individual cell's growth rate
- · Variation in growth rate sets the variation in generation time and division size
- E. coli appears as a "sizer" at slow growth and an "adder" at fast growth





The Synchronization of Replication and Division Cycles in Individual *E. coli* Cells

Mats Wallden,^{1,2} David Fange,^{1,2} Ebba Gregorsson Lundius,¹ Özden Baltekin,¹ and Johan Elf^{1,*}

¹Department of Cell and Molecular Biology, Science for Life Laboratory, Uppsala University, Husargatan 3, 75124 Uppsala, Sweden ²Co-first author

*Correspondence: johan.elf@icm.uu.se

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SUMMARY

Isogenic E. coli cells growing in a constant environment display significant variability in growth rates, division sizes, and generation times. The guiding principle appears to be that each cell, during one generation, adds a size increment that is uncorrelated to its birth size. Here, we investigate the mechanisms underlying this "adder" behavior by mapping the chromosome replication cycle to the division cycle of individual cells using fluorescence microscopy. We have found that initiation of chromosome replication is triggered at a fixed volume per chromosome independent of a cell's birth volume and growth rate. Each initiation event is coupled to a division event after a growth-rate-dependent time. We formalize our findings in a model showing that cellto-cell variation in division timing and cell size is mainly driven by variations in growth rate. The model also explains why fast-growing cells display adder behavior and correctly predict deviations from the adder behavior at slow growth.

INTRODUCTION

Balanced growth requires that DNA replication keeps up with cell-division events (Schaechter et al., 1958). This may, at first, seem hard to achieve for rapid-growth *Escherichia coli*, where it takes more time to replicate a chromosome than to double the biomass. The solution to the apparent paradox was first described by Cooper and Helmstetter (Cooper and Helmstetter, 1968). In their model, new rounds of DNA replication are started before the previous round has finished (Figure 1A). As long as cells, on average, initiate one round of replication per chromosomal origin per generation and divide only once following each replication termination, the model produces stable cell cycles at all growth rates. An example of a deterministic, i.e., noise free, simulation of replication and division cycles including up and downshifts in growth rates is given in Figure 1B.

A missing component of Cooper's and Helmstetter's model is how the cell manages to trigger replication initiation once per generation. An answer proposed by Donachie (1968) is that replication initiates at a critical volume per origin. This guarantees that, on average, the concentration of origins is constantly maintained as the cells' birth and division volumes exponentially change in response to the growth rate (Schaechter et al., 1958) (Figure 1B). However, Donachie's (1968) proposal was later refuted on the basis that the introduction of extra copies of the origin of replication region does not disrupt the cell cycle (Helmstetter and Leonard, 1987) and evidence gathered from cells synchronized at the time of division using the baby-column technique (Bates and Kleckner, 2005). It was instead proposed that division issues a license for an initiation event to occur at a well-defined time later. Recently, Hill et al. (2012) used the rifampicin (rif) run-out technique (Skarstad et al., 1986) in a number of size mutants to re-establish the constant volume model. Unfortunately, it is not possible in these experiments to correlate the replication initiation volume in one cell to its growth rate or division size. Without these correlated measurements, it is not possible to determine what drives the variability and accuracy of the division and replication cycles.

Recent developments in microscopy, microfluidics, and image analysis techniques help us answer this question by circumventing the need to experimentally synchronize cell cycles; synchronization can be achieved in post-processing of the images (Sliusarenko et al., 2011; Ullman et al., 2012; Wang et al., 2010). These techniques provide direct observations of sizes and lifespans of individual bacteria growing exponentially under well-controlled conditions. For example, Wang et al. (2010) demonstrate that no clear dependence can be inferred between the age of a cell, as defined by the number of divisions since the establishment of the oldest pole and the growth rate.

The data from Wang et al. (2010) was later used by Osella et al. (2014) to investigate the mechanisms governing cell division. The authors conclude that the observed correlations are inconsistent with either a purely time-dependent or purely size-dependent control mechanism of division. Instead, they defined a phenomenological description that included both time and size dependence. The composite control scenario for division has been recently explained by an "adder" model in which the added volume between one replication initiation event and the next is independent of cell size (Amir, 2014). Soon after, an alternative version of the "adder" model was presented by Campos et al. (2014) and (Taheri-Araghi et al. (2015). Here, the volume added between birth and division is independent of the individual cells birth size; this model has shown to be consistent with an overwhelming body of additional experimental data. Despite that the "adder" model displays a striking predictability for cell-cycle-related distributions over a large range of growth conditions (Taheri-Araghi et al., 2015), it is not known what gives rise to the adding.



Here, elucidation of the molecular mechanism underlying the "adder" model is the main focus. Such a mechanism should explain why cells behave as "adders", what gives rise to the observed correlations in division sizes and generation times, and when deviations from the "adder" model should be expected.

Our approach is to make a single-cell version of the classical Cooper-Helmstetter (CH) model for the coupling of replication and division cycles in *E. coli* and test if the model accounts for the observed cell-to-cell variation. To do this, we revisit the assumptions of the CH model with the following questions: what determines the point in the cell cycle at which replication starts? How long does it take to replicate the genome and divide? How are these two stochastic parameters correlated and dependent on the growth rate of an individual cell?

In order to acquire the data we need to answer these questions, we combine tracking of growing cells throughout their division cycles (Ullman et al., 2012) with fluorescence microscopy of labeled DNA replication components (Adiciptaningrum et al., 2015; Reyes-Lamothe et al., 2010). Following parameterization, the model was validated based on its ability to predict the variation in division timing and cell size. Finally, we have used the model to explain the "adder" behavior previously observed in fast growing cells, and we also accurately predict a deviation from the "adder" model at slow growth.

RESULTS

Characterizing Replication and Division in Individual Cells

To directly study the coordination of the replication and division cycles, we imaged a fluorescently labeled epsilon subunit of DNA polymerase (Pol) III, named DnaQ (Reyes-Lamothe et al., 2010). Because of the low diffusivity of the Pol IIIs engaged in

Figure 1. Coupling of Replication and Division Cycles in *E. coli*

(A) An illustration of the Cooper-Helmstetter model, where division events are scheduled a fixed time after replication initiates, is shown for slow (top) and intermediate growth (bottom). Initiation once per generation results in overlapping replication cycles at fast growth.

(B) Simulated volume expansion and division for an idealized cell lineage going through an upshift and a downshift in growth rate. Replication is initiated at a fixed volume per chromosome (red circle), and the cells divide a fixed period of time later, including the required time for replication (C-period) and chromosome segregation and septum formation (D-period). A dashed green arrow indicates the relation between initiation of replication and its corresponding division.

replication, individual replisomes can be localized by using single-molecule fluorescence imaging (Figure 2A). We ensure that labeling does not influence growth (Figures S1A and S1B) or replication initiation (Figures S1C–S1E).

Cell size and division events were determined by time-lapse phase-contrast microscopy of cells grown in a microfluidic device

(Ullman et al., 2012) (Figure 2D). This device keeps the E. coli cells in a state of exponential growth for the days during which they are simultaneously imaged in the microscope. The individual cell's growth rate, generation time, lineage, and size were obtained from phase-contrast images by using customized image analysis and tracking tools (Magnusson et al., 2015; Sadanandan et al., 2014). The precision in estimating the division time using phase contrast was found to be 2 min based on a comparison to the division time established using a fluorescent segmentation marker (Figure S2). Individual cell's growth rates determined by phase-contrast microscopy were very similar to those determined by fluorescence microscopy (Figure S3D). Individual cells grew exponentially, independent of the position within the device and unperturbed by imaging laser exposure (Figures S3A-S3C). The individual cell's growth rate is defined by a fit of an exponential function $V_B e^{\mu t}$ to the cell's volume expansion, where V_B is the birth volume and µ is the growth rate. We observed significant cell-to-cell variation in division size, generation time, and growth rate (Figures 2E-2G). A model of the cell cycle should explain how these distributions are related and what drives the variation. Based on the assumption that the cells do not limit their growth rate to reach a particular size or division time, we hypothesized that the division time and size depend on the growth rate. We therefore used growth rate as the basis for the single-cell version of the CH model and tested if it could predict the variation in division time and cell size when the variation in growth rate was used as an input.

Initiation of Replication Occurs at a Constant Volume

The single-cell CH model requires that we determine when replication is initiated. Figures 3A–3C shows the localization of the replication complexes, the replisomes, along the long axis of the cells as a function of the cell volume for three different growth



Figure 2. Characterizing Growth and Chromosome Replication at the Single-Cell Level (A) A fluorescence image with identified DnaQ-Ypet. (B and C) Fluorescence and phase-contrast time lapse of an individual cell with fluorescently labeled replisomes.

(D) Automatically segmented phase-contrast image of *E. coli* growing in a microfluidic device.

(E) Distributions of single-cell growth rates for fast (*black*), intermediate (*blue*), and slow (*red*) conditions (see the Experimental Procedures for definitions) fitted to normal distributions (corresponding solid lines). Estimated averages for the growth rate distributions are $< \mu > = 0.0241 \text{ min}^{-1}$, $< \mu > = 0.0111 \text{ min}^{-1}$, and $< \mu > = 0.0043 \text{ min}^{-1}$ and their corresponding SDs $\sigma_{\mu} = 0.0039$, $\sigma_{\mu} = 0.0026$, and $\sigma_{\mu} = 0.00098$ for the fast, intermediate, and slow growth conditions, respectively. The number of cells included in the distributions is 5,385, 1,217, and 1,683 for fast-, intermediate, and slow-growth

conditions, respectively. The data were collected from six microfluidics traps in one microfluidic chip for each condition. Filtering criteria are described in the Experimental Procedures.

(F) Distributions of generation times corresponding to (E).

(G) Distributions of birth volumes corresponding to (E).

See also Figures S1, S2, S3, and S5.

conditions. New rounds of replication were observed to start at defined cell sizes (Figures 3A-3C, red dashed line). If the cells, instead, were aligned by the time from division, the distribution of replisomes was less coherent (Figures S4A-S4C). This suggests that control of the replication cycle is related to size rather than to the time from division (Donachie, 1968; Hill et al., 2012). The origin of replication locus, oriC, co-localized with the replisomes at replication initiation, as shown by a fluorescently labeled Mall transcription factor bound at the oriC-proximal bgLG locus (Figures 3D-3F). Furthermore, the number of ongoing replication cycles was validated by replication runout experiments (Skarstad et al., 1986) adapted to the microfluidic environment. For slow, intermediate, and fast growth, the number of origins was typically 1 or 2, 2 or 4, and 4 or 8, respectively (Figure S4D). The observed initiation volumes (Figures 3A-3C, red dashed lines), divided by the corresponding number of origins, are relatively invariant for the different growth conditions $(0.9-1.0 \ \mu m^3)$. This is particularly clear for slow growth, where a significant fraction of the cells initiated one round of replication at 0.9 µm³ and another at 1.8 µm³ (Figure 3A). Replication initiations at integer multiples of a fixed volume, i.e., just after division and just before division as seen in the slow growth case, invalidates any model in which replication is initiated at a certain time or added volume following division.

Initiation Volume Is Uncorrelated with Volume at Birth and Growth Rate

Next, we asked if the small cell-to-cell variation in initiation volume per chromosome depends on the individual cell's birth volume or growth rate. The detection of DnaQ does not allow us to reliably monitor the individual replication initiation events in individual cells due to fluorophore maturation, blinking and/or bleaching. For this reason, we also studied replication using a strain with a chromosomal *seqA-ypet* fusion (Babic et al., 2008) (courtesy of the Waldminghaus lab). A large number of SeqA molecules bind the hemimethylated DNA, which trails the replication forks (Waldminghaus et al., 2012), resulting in highly fluorescent dots at the sites of replication that can be detected throughout the cell cycle of individual cells (Figure 4A) (Adiciptaningrum et al., 2015). The SeqA fusion strain did not display any significant alteration in the location of replication events over the cell cycle (Figure S5). However, different regions of the chromosome are hemimethylated for different periods of time (Campbell and Kleckner, 1990), and the SeqA-Ypet signal will therefore not be directly proportional to the number of replisomes. Given this limitation, we only used this strain to determine the timing of replication initiation in relation to the division events.

By studying the SeqA strain, we could determine that the cells initiate replication at 0.92 μ m³ in the slow-growth condition with one chromosomal origin, *oriC*, and at 1.73 μ m³ in the intermediate growth condition with two *oriCs*. The SD for the cell-to-cell variation in initiation volume is 0.07 μ m³ for slow growth and 0.17 μ m³ for intermediate growth. At fast growth, there are too many ongoing rounds of replications to unambiguously identify the initiation events. Although the variation in initiation volume per *oriC* is only 10%, it should be seen as an upper limit since any error in estimating the cell volume will contribute to this number.

The small variation in initiation volume between cells is not correlated with the individual cell's growth rate or volume at birth (Figures 4B and 4C). However, lack of correlation can be due to many factors, including measurement errors in the birth volume, V_B. For this reason, we test the more specific prediction that cells born small spend more time, τ_B , between birth and initiation. In fact, if the initiation volume V_I is constant, we would expect $\tau_B \approx (ln(V_I) - ln(V_B))/\mu$. Using our measured average growth rates, we have found that the birth volumes and initiation times are related as expected (Figure 4D). This confirms that the lack of correlation between V_B and V_I is not a consequence of inaccurate measurements. The fixed initiation volume per chromosome



Figure 3. Distributions of Replication Timing (A–C) The distributions of localized DnaQ along the long axis of the cell (x axis) for cells of different volumes (y axis) are shown. Panels correspond to slow- (A), intermediate (B), and fast-growth (C) conditions (see the Experimental Procedures for definitions). Replication initiation is indicated by red dashed lines. White dashed lines indicate average volumes at birth and division. Note that the two replication forks initiated from the same origin of replication are spatially too close to be visualized as separate distributions. The number of cells included in the distributions is 10,774, 5,946, and 4,257, for fast-, intermediate, and slow-growth conditions, respectively.

(D–F) Same as (A)–(C), but with localization of Mallvenus bound to *malO* sites proximal to *oriC*. See also Figures S1, S4, and S5.

can therefore be seen as a reset point for the otherwise correlated variations in the cell cycle.

Time from Replication Initiation to Division Is Growth-Rate Dependent

The next step in making a single-cell version of the CH model was to determine how much time cells spend in replication, segregating their chromosomes, and dividing (the C and the D periods). Cooper and Helmstetter have assumed this time to be constant for generation times faster than 60 min, and the main question is thus if this holds at all growth conditions. By mapping replication to the division cycle using the DnaQ data, we determined the C and D periods for the different growth conditions (Figures 5A–5C, "Determining the C+D periods in DnaQ-Ypet strain" in the Supplemental Experimental Procedures). We have found that the average C+D-period, τ , is relatively constant for the fast and intermediate growth conditions, but is much longer at slow growth (Figure 5C).

Previous replication run-out experiments in bulk (Michelsen et al., 2003) suggest a functional form for the growth-rate dependence as $\tau = \alpha \mu^{-\beta} + \gamma$. In this equation, γ can be thought of as the minimal time for chromosome replication, chromosome segregation, and septum formation at fast-growth rates. Conversely, at slow growth, these processes are limited by factors related to growth, where a 1% decrease in growth rate results in approximately a β % increase in τ . Fitting the data from the DnaQ strain, which has been stratified based on growth rates in the different media, gives $\alpha = 1.3$, $\beta = 0.84$, and $\gamma = 42$ (min). This implies that τ is strongly dependent on growth rate at slow growth.

To test if the τ dependence estimated from the population averages applies also for individual cells, we used the data from the SeqA-YFP strain, where initiation of replication could be accurately determined in individual cells (Figure 5D). Our result shows two things. First, cells with the same growth rate in different media have different τ and therefore τ does not only depend on growth rate. The difference in τ for cells growing at the same rate in different media in combination with a fixed initiation volume will result in difference cell sizes, which is in agreement

with the observations by (Taheri-Araghi et al., 2015). Second, there is also a strong growth-rate dependence on τ at the single-cell level; in the same media individual cells growing slower display a longer τ than individuals growing faster. For cells growing under the slow-growth condition, the cell-to-cell variation is $\tau \sim \alpha/\mu$, i.e., an increase in generation time by 1% results in an increase of τ by 1%. This strong response to the individual cell's grow before they divide than more rapid-growth cells in the same media.

Single-Cell Cooper-Helmstetter Model

On the basis of our data we can recast the Cooper and Helmstetter model (Figure 1A) in a stochastic single cell setting. The two basic assumptions of the model are that replication is initiated when the cell has grown to a fixed volume per origin (Figures 3 and 4) and that the cell divides at a growth rate dependent time after initiation (Figure 5). The initiation volume, V_I, and the time between initiation and division, τ , are parameterized according to our experimental measurements (Figures 3, 4, and 5). To account for the experimentally observed cell-to-cell variation we can introduce variability in the growth rate, μ , the initiation volume, V_{I} , the C+D period, τ , or in combinations of the three. In Figure 6A we illustrate a few simulated cell cycles where the growth rates of new-born cells are sampled from the experimental distribution. When a cell grows past the replication initiation size, which is independently sampled from the distribution of initiation sizes, a division event is scheduled at a time τ later. (See "Modeling" in the Supplemental Experimental Procedures for a more detailed description of the simulation.) When a large number of cell cycles have been simulated, the statistical properties of these can be compared to the experimental distributions.

In Figure 6B we show the experimentally measured distributions of cell sizes at birth and division as a function of generation times and growth rates respectively. The same distributions were also calculated from the model where the cell-to-cell variability is introduced in the growth rate only (Figure 6C). There is an approximate agreement with respect to the experimental



Figure 4. Initiation of Replication in Individual Cells

(A) Fluorescence image time-lapse series of the SeqA-Ypet strain. Top: the division cycle of one cell at slow growth. The frames are separated by 18 min. Bottom: one-and-a-half division cycles for a cell at intermediate growth. The frames are separated by 14 min. See also Movies S1 and S2 for additional examples of fluorescence image time-lapse series.

(B) Scatter plot of birth volumes and initiation volumes for individual cells at the slow-growth rate (red) and intermediate growth rate (blue). No initiations can be observed below the dashed line since this would correspond to initiation in the mother cell. The inset shows the fraction of cells that are born with ongoing replication for different birth volumes.

(C) The correlation of the individual cells' growth rate and initiation volume visualized in a scatter plot.

(D) The individual cells' birth volume plotted against the time to initiation. The dashed lines correspond to the theoretical predictions $\tau = (ln(V_I) - ln(V_B))/ < \mu >$, assuming a constant initiation volume. The number of cells included is 605 for intermediate and 401 for slow-growth conditions. See also Figure S5 and Movies S1 and S2.

distributions, especially in the marginal distribution (Figure 6E), but the cell size distributions are too narrow, especially for slow growth (Figure 6C, red curves). This is because of the fixed initiation volume and a perfect compensation in τ to variations in growth rate. In Figures 6D and 6F, the variation in replication initiation volume and the C + D period was also included. With these additional sources of variation, there is an overall excellent agreement with the experimental data, suggesting that the model captures the most important aspects of cell cycles variations.

Basis for the "Adder" Mechanism and When It Breaks Down

Given the accuracy of the model in describing the division timing and cell sizes, we could use it to understand the mechanisms underlying the "adder" model (Amir, 2014; Campos et al., 2014; Taheri-Araghi et al., 2015; Voorn and Koppes, 1998). We started by looking at what the single-cell CH model predicts under the growth conditions for which the "adder" has been described. Here, the growth is relatively fast; replication is initiated in the mother or grandmother generation and τ is, as a first approximation, constant. This means that, regardless of its birth size, a cell will divide a fixed time after a replication initiation event that occurred in a previous generation (Figure 7A). This results in a volume expansion per generation that is uncorrelated to the birth volume (Figure 7B) and instead depends on the growth rate (Figure 6B), i.e., it is an "adder".

The situation is different in slowly growing cells where replication initiation occurs in the same generation as the corresponding division event and τ is strongly dependent on growth rate. This implies that slowly growing cells have more time to grow from the initiation volume to cell division than more rapid-growth cells (Figure 7C). Since $\tau \approx \alpha/\mu$, the division volume will be proportional to the initiation volume, i.e., $V_D = V_I e^{\mu \tau} = V_I e^{\alpha}$. This striking relation is seen for the slow-growing cells (Figure 7D). (The dashed line in 7D is not a fit, but a theoretical prediction with slope e^{α} , where α estimated by a regression of the C+D periods for slow growth in Figure 5D to $\tau = \alpha/\mu$.) Furthermore, since the initiation volume does not depend on the growth rate or the birth volume (Figure 4), the division volume will be independent of growth rate and the birth volume. This implies that the slowly growing cells behave more like "sizers", although the main reason is not an explicit size sensor at division but rather at initiation of replication. Furthermore, cells born small are predicted to add a relatively greater volume compared to cells born large resulting in negative correlations between birth volume and added volume. This is also observed in the experimental data (Figure 7B, red curves).

The negative correlation of the added volume to the birth volume in slow-growing cells (Figure 7B) is a clear deviation from



Figure 5. The C+D Period for Different Growth Conditions

(A and B) Illustrations of the procedure used to compute the C+D periods for slow (A) and intermediate (B) growth. The distributions of DnaQ as functions of volume from Figures 3A and 3B are concatenated based on division time to allow for C+D period determination. The inferred C and D periods are shown as gray bars. As in Figure 3, the red dashed line indicates initiation volumes, and the white dashed lines indicate division volumes.

(C) The C+D periods determined in the DnaQ-Ypet strain plotted against the growth rate. The data are fitted with a power-law curve, $1.3\mu^{-0.84}$ +42 min (dashed line). The data for slow (red circles) and intermediate growth (blue circles) are stratified based on growth rate (see "Determining the C+D period for DnaQ-Ypet strain" in the Supplemental Experimental Procedures). The number of cell cycles used in each point is, in order of increasing growth rate: 593, 1.427, 1.464, 595, 814, 2.023, 2.020, 844, 3,758, and 10,774.

(D) C+D periods measured for individual cells in the SeqA-YFP strain. The fitted curves are given in the inset.

the "adder" model. This negative correlation would however also be observed if the measured birth and division volumes were uncorrelated due to measurement errors. To reduce such errors as much as possible, we repeated the slow-growth experiment with cells expressing high concentrations of a fluorescent segmentation marker. By using a sensitive, high-pixel density sCMOS camera, we could image magnified cells at low-laser power, which allowed for high-resolution segmentation without using active contour modeling. In Figure 7E we show that the slowgrowing cells still display deviation from what would be expected in the "adder" model.

DISCUSSION

The single cell version of the Cooper and Helmstetter model has two basic components: an initiation volume per chromosome that does not depend on the birth size of the cell or its growth rate, and a growth rate dependent time for replication and division. The model explains the "adder" behavior at fast growth and sizer behavior at slow growth. Here we discuss what these observations say about the underlying mechanisms.

Replication Initiation Size

We find that the cell-to-cell variation in volume at which DNA replication initiates is approximately 10% (SD/mean). The obser-

vation that replication initiates at a relatively constant volume per *oriC* is not novel in itself (Donachie, 1968; Hill et al., 2012; Wold et al., 1994), but the single cell time-lapse measurements also allow us to determine that the initiation volume is not correlated to the birth size of the cell or its growth rate. Our findings therefore imply that it is meaningful to think of replication initiation as the starting point of the cell cycle.

Although we find that coordination between replication and division is assured by initiating DNA replication at a constant size per chromosome origin, it is not likely that it is the *oriC* locus itself that is regulated (Helmstetter and Leonard, 1987) but rather some closely linked locus. A likely candidate is *datA* that binds the active form of the replication initiation protein DnaA-ATP and promotes its hydrolysis to its inactive form DnaA-ADP (Donachie and Blakely, 2003; Kasho and Katayama, 2013). Therefore, when *datA* is replicated, the initiation potential drops, which makes *datA* a good candidate for how the control system for initiation senses chromosome copy number. For a review on DnaA mediated replication initiation please see (Skarstad and Katayama, 2013).

At fast growth, i.e., when the cell should initiate replication at multiple origins, it is also important that this occurs synchronously (Skarstad et al., 1986). All origins have to fire within the period when newly replicated origins are hemimethylated and protected for re-initiation by sequestration for approximately 13 min (Lu et al., 1994). In Figure S5L, we show that at least 90% of the origins fire within this time at the intermediate growth rate where it is possible to identify the appearance of replication forks at quarter position in the same cell.

What does the accuracy in initiating replication at a fixed volume say about the underlying mechanism? The data show that in 95% of the cells, initiation is triggered within in a range corresponding to a 50% change in volume. This implies that the initiation rate, whatever determines it, has to increase sharply in a narrow volume range. For example, if the instantaneous initiation rate, r, responds to the volume, V, as a Hill function $r = a(V/V_I)^n / (1 + (V/V_I)^n)$, the exponent, *n*, needs to exceed 20, in order to initiate in the observed volume range (see "Initiation Sensitivity Analysis" in the Supplemental Experimental Procedures). To achieve this, the cell needs a very sensitive control system (Paulsson and Ehrenberg, 2001; Savageau, 1976). In a simple control system, in which a repressor of replication would dissociate from oriC due to dilution by volume growth, the repression would at the most decrease by 1% for a 1% volume growth. The experimental data instead requires a mechanism that can respond by 20% to a 1% change in volume. This cannot be achieved by models that rely on simple titration (Hansen et al., 1991; Pritchard, 1968; Sompayrac and Maaloe, 1973). Plausible mechanisms are instead based on energy dependent cycling of for example DnaA between its ATP and ADP forms (Kurokawa et al., 1999; Sekimizu et al., 1987). This could for example be a zero-order modification-demodification scheme (Koshland et al., 1982) sensitive to the chromosome to volume ratio, or an irreversible multi-step processes (Paulsson and Ehrenberg, 2000) where DnaA-ATP builds up an initiation complex that is interrupted by the incorporation of DnaA-ADP. The requirement to reach the experimentally observed sensitivity should guide further thinking about possible mechanisms.



Figure 6. The Growth Rate Variation Propagated to Other Variables and Compared to Experimental Observations

(A) The cell volume (black solid line) of a single cell lineage simulated according to the single-cell CH model throughout an upshift in growth conditions. The growth rates are sampled from the observed distributions. When the cell passes the initiation volume(s) (red lines), a division event is triggered (green arrow) after a time period corresponding to the C and D periods (gray bars). Variation can be introduced in growth rates, initiation volume, or C+D period.

(B) Top: The joint distribution of generation time and cell size at birth and division as observed in the DnaQ-YPet experiments. Bottom: The same data plotted as a function of growth rate.

(C) Model predictions of the distribution in B when cell-to-cell variation is introduced in growth rate only. SD and means are as estimated from experiments (Figure 2F). Here, SD/mean = 0.16, 0.23, and 0.23 were used for fast-, intermediate, and slow-growth rate, respectively.

(D) Same as (C), but cell-to-cell variation is also introduced into the initiation volume (SD/mean = 0.095) and C+D period (SD/mean = 0.05 for fast and intermediate and SD/mean = 0.1 for slow growth).

(E) Marginal distributions for generation time and volume at birth from (C) shown as solid lines. Corresponding experimental distributions from (B) added for comparison (dashed lines).

(F) Marginal distributions for generation time and volume at birth from (D) shown as solid lines. Corresponding experimental distributions from (B) added for comparison (dashed lines). The cells included in experimental data are the same as in Figure 2. See also Figures S5, S6, and S7.

Cell-to-Cell Variation in Growth Rate

We have found that the cell-to-cell variation in generation time and division size is mainly caused by variation in growth rate. A remaining question is what determines the cell-to-cell variation in growth rate? Why do not all cells grow as fast as the fastest cell for a specific growth medium, when cells with this composition



Figure 7. The Added Volume over One Division Cycle

(A) An example of how division control according to the model at intermediate growth appears as an "adder". The division volumes for rapid- (gray solid line) and slow-growth (black solid line) siblings will be different since the initiation occurred in the mother generation and the C+D period is insensitive to growth rate.

(B) The joint distribution of volume added during one generation and the birth volume are shown for slow, intermediate, and fast growth as red, blue, and black solid lines. The cells included are the same as in Figure 2.

(C) An example of how division control according to the model at slow growth with non-overlapping replication cycles appears as a "sizer." This effect is illustrated with two siblings following division. In the rapid growth case (gray solid line) the C+D period, following initiation, is shorter than in the slow-growth case (black solid line), and thus they will divide at the same size.

(D) The division volumes and initiation volumes of the SeqA-Ypet strain are plotted for slow (red) and intermediate growth (blue). The black line represent the prediction from V_D = V_I e^{α}, where α is based on fitting the C+D period in Figure 5B to $\tau = \alpha/\mu$ ($\alpha = 0.518$).

(E) Same as the experimental data for slow growth in (B), but here size (in area) is determined by using a strain expressing a fluorescent segmentation marker (inset). The number of cells included in the distribution is 849. See also Figure S5.

would presumably outcompete their siblings? The answer is likely related to a high fitness cost of the hypothetical control systems that would be required to tune the cell composition to the maximal growth in each specific condition. Instead, it appears that the cell uses a slightly sloppy control system that results in important components getting into suboptimal balance. Since the growth-rate correlation is rapidly lost over generations (Figure S6), it appears as if cell division itself causes the suboptimal composition (Huh and Paulsson, 2011), but we do not know which components are out of balance. Recently, it has been reported that ribosomes are more unevenly inherited between sisters than expected by randomly putting each ribosome in one of the daughter cells (Chai et al., 2014). Thus, we tested if the difference in ribosome inheritance impacts the growth rate of the sisters, but found only limited correlation (Figures S7A-S7D). It appears the situation is more complicated.

A possible key is that different experimental conditions display different ranges of cell-to-cell variation in growth rate. Our setup typically gives similar cell-to-cell variation in the growth rate as those observed in Campos et al. (2014), but larger than those observed by Taheri-Araghi et al. (2015). Although occasional experiments also in our setup, for example, the ribosome-labeled strain (Figures S7E–S7J), displayed smaller variation in growth rates, we have not been able to determine a common denominator that explains the differences.

Growth-Rate-Dependent C+D Period

Our version of the Cooper-Helmstetter model explains the observation that cells add a growth-condition-dependent volume each generation independently of birth size at fast and intermediate growth (the "adder" mechanism) (Campos et al., 2014; Taheri-Araghi et al., 2015). In essence, cells with overlapping replication cycles will complete the replication-division program that was started in a previous generation at a time that is independent of their birth sizes. Hence, the division volume will not depend on the birth volume in these cases. The model also predicts a deviation from the "adder" model under conditions, which prior to our work, had not been previously experimentally studied. At slow growth, where the replication-division program is started and completed in the same generation, the program takes a much longer for cells growing slowly than for those growing fast. It appears as if cell division is limited by making a certain number of specific components in order to divide and that this number is only reached at a specific size.

Perspective

The model we present structures our understanding of the *E. coli* cell cycle and clarifies the mechanisms underlying the cell-to-cell variation with respect to cell division. It also suggests three directions in which further studies are needed: (1) what causes the sensitivity to be high enough to trigger replication at the

experimentally observed volume; (2) what causes the cell-to-cell variation in growth rate; and (3) what are the factors limiting replication and division at the slow-growth rate?

Answering these questions will guide us toward a molecular understanding of the *E. coli* cell-cycle control system.

EXPERIMENTAL PROCEDURES

Strains

DnaQ localization was investigated using the MG1655 strain JJC5350 (a gift from the lab of Benedict Michelle; Reyes-Lamothe et al., 2010), carrying a genetic fusion of the replication factor DnaQ and the yellow fluorescent protein YPet, encoded in the native *dnaQ* locus. The construct was also transferred to the MG1655 strain BW25993 (Datsenko and Wanner, 2000) using a P1 phage. We found the average growth rate in bulk experiments (Figures S1A and S1B) and the growth-rate distribution and DnaQ localization (Figures S1I and S1J) in microfluidics experiments to be very similar. Based on these similarities, we used the longest high-quality imaging data series regardless of strain origin (JJC5350 in fast- and intermediate growth conditions and BW25993 in slow-growth conditions) when comparing cell physiology to simulations (Figures 2, 6, and 7B). To increase the number of imaged cell cycles in the fast- and intermediate growth conditions, we used both strains for DnaQ localizations in Figures 3A–3B.

SeqA localization (Figures 4, 5D, and 7D) was investigated using an MG1655 strain DS116 carrying a SeqA-YPet fusion. The strains were a kind gift from the Waldminghaus lab, who replaced the native *seqA* gene with the *seqA-ypet* fusion, constructed in the Radman lab (Babic et al., 2008).

The location of origins was investigated using strain JE200 (Figures 3D–3F), in which a genetic fusion of the transcription factor *mall* and the gene encoding the yellow fluorescent protein variant *Venus* was introduced in the origin-proximal *bgLG* locus in strain BW25993 using the lambda-red protocol (Datsenko and Wanner, 2000). The construct contains two tandem operator sites, *malO*, to which Mall-Venus binds tightly. Further, the native *mall* gene, as well as the native *malO* sites, was deleted using the lambda-red method. This minimal construct was selected to avoid the risks associated with having a large number of operator-transcription factor complexes present in the cell (Lau et al., 2003).

Precision in the determination of division timing (Figure S2; see "Validation of the phase contrast division classifier" the Supplemental Experimental Procedures) was investigated in strain EC442 (Söderström et al., 2014), containing a genetic fusion of the division factor, *ftsQ*, and a green fluorescent protein, *gfp*, introduced in MG1655.

Accuracy in determining individual growth rates (Figure S3D; see "Comparing growth rates in phase contrast and fluorescence" in the Supplemental Experimental Procedures) and the control experiment for deviation from "adder" behavior (Figure 7E) was investigated in strain JE201, in which a gene encoding a red fluorescence protein, *tRFP*, regulated by the constitutive ribosomal RNA promoter *P2rmB* was introduced at the *intC* locus using the lambda-red method in BW25993.

The dependence of the growth rate on ribosome content (Figure S7; see "Dependence of growth rate and ribosome content and partitioning at birth" in the Supplemental Experimental Procedures) was studied in strain JE202, in which gene *rpsB* was genetically fused to yellow fluorescence protein, *Venus*, and gene *rpll* to a red fluorescent protein, *mCherry*, by using the lamda-red protocol. In both cases the constructs replaced the native genes. The *rpsB* and *rpll* genes express the proteins S2 and L9 that associate to the small and large subunit of the ribosome, respectively.

Growth Conditions

Growth conditions are as follows: fast: M9 minimal medium and 0.4% glucose supplemented with RPMI 1640 amino acids (R7131, Sigma-Aldrich) at 37° C; intermediate: M9 minimal medium and 0.4% succinate supplemented with RPMI 1640 amino acids (R7131, Sigma-Aldrich) at 30° C; and slow: M9 minimal medium and 0.4% acetate at 37° C.

All strains were grown in M9 minimal media, except for the experiment that determines the accuracy in individual growth rates (Figure S3D), in which the strain JE201 was grown in Luria-Bertani liquid medium (LB) at 37°C. Strain EC442 was grown under fast conditions with 5 μ M IPTG present in the medium to induce the expression of FtsQ-GFP molecules. For the control experiment showing deviations from "adder" behavior (Figure 7E), strain JE201 was grown in slow-growth conditions. Strain JE202 was studied under fast-growth conditions. All media used in the microfluidic experiments contained a surfactant, Pluronic F108 (CAS 9003-11-6, Sigma-Aldrich), at a final concentration of 0.85 gLl⁻¹.

Microfluidic Sample Management

The preparation and operation of the microfluidic devices used in experiments with strains JJC5350, JE200, DS116, EC442, JE201, and JE202 were performed as described in Ullman et al. (2012). For experiments under slow-and intermediate growth conditions, the trap depth used was 800 nm. For all other microfluidic experiments, 900 nm was used.

Microscopy and Imaging Conditions

All microscopy experiments were performed using an inverted microscope (Nikon Ti-E) with 100× oil-immersion objectives (either an Apo TIRF 1.49 na or a 100× Plan Apo λ 1.45 na). For phase-contrast imaging, a CFW-1312M (Scion), a DMK 23U274 (the Imaging Source) or an Infinity 2-5M (Lumenera) camera was used. Fluorescence and bright-field images were recorded on Andor Ixon EMCCD cameras, unless otherwise stated. The Andor cameras were equipped with an additional 2× (Diagnostic instruments DD20NLT) or 2.5× lens (Nikon Instruments).

JJC5350, DS116, and JE200 Imaging: phase-contrast images were acquired with a 125-ms exposure at a rate of 2 min^{-1} . For fluorescence imaging, a 514-nm laser (Coherent Genesis CX STM) was used. Laser exposure time and power were 1 s and 3.0 Wcm⁻² per frame for experiments using JJC5350, 4 s and 0.75 Wcm⁻² for JE200, and 1 s and 0.3 Wcm⁻² for DS116. Fluorescence and bright-field imaging frequency were 1/3 min⁻¹.

EC442 Imaging: phase-contrast images were acquired with a 125-ms exposure and at a rate of 2 min⁻¹. For fluorescence imaging, a 488-nm laser (Cobolt MLD) was used. Laser exposure time was 100 ms. Fluorescence and bright-field imaging frequency were 1 min⁻¹.

JE201 Imaging: for Figure S3D, phase-contrast images were acquired with a 125-ms exposure at a rate of 1 min⁻¹. For fluorescence imaging, a 561-nm laser (Genesis MX, Coherent) was used. Laser exposure time was 300 ms. Fluorescence and bright-field imaging frequency were 1 min⁻¹. For Figure 7E, phase-contrast images were acquired with a 500-ms exposure at a rate of 1/3 min⁻¹. For fluorescence imaging, a 561-nm laser (Genesis MX, Coherent) was used. Laser exposure time and power were 200 ms and 10 Wcm⁻². Fluorescence and bright-field imaging frequency were 1/3 min⁻¹. Here, a Zyla 4.2 PLUS sCMOS (Andor) was used.

JE202 Imaging: phase-contrast images were acquired with 125-ms exposure at a rate of 6 min⁻¹. For fluorescence imaging, a 514-nm laser (Fandango 150, Cobolt) and 580-nm laser (F-04306-03, MBP Com.) were used. Laser exposure time and power were 30 ms and 18.5 Wcm⁻² for 514 nm and 200 ms at 61.5 Wcm⁻². Fluorescence and bright-field imaging frequency were 1/3 min⁻¹.

Rif Run-out in Microfluidics: a Spectra light engine (Lumencor) with a bandpass filter transmitting light between 352 and 402 nm was used for fluorescence imaging (see "Microfluidic Replication Run-Out Experiments" in the Supplemental Experimental Procedures for details).

The microscope was controlled using μ -Manager (Edelstein et al., 2014), and automated acquisitions were performed using in-house micro-manager plugin. Unless otherwise stated time-lapsed acquisitions were performed in parallel at multiple microfluidic trap regions, one of which was not exposed to laser. Exceptions are JE201 and JE202 imaging, in which all traps were laser exposed, and only one trap was imaged in the JE201 case. The duration of the acquisition varied from 2–24 hr, depending on the growth conditions. In all cases, cells were grown in the microfluidic devices for at least 24 hr prior to imaging to ensure steady-state exponential growth before the start of image acquisition. The temperature of the microfluidic device was maintained using a cage incubator (either OKO lab or Haison) encapsulating the microscope stage.

Cell Segmentation, Tracking, and Detection of Single Molecules

A custom-written, fully automated analysis pipeline written in MATLAB was used to analyze the time-lapsed microscopy data. Cells in each

phase-contrast image were segmented using the method described in Sadanandan et al. (2014). An active contour model based on Sliusarenko et al. (2011) was developed, and a contour was computed for each segmented object. Cells were tracked between frames using the method described in Magnusson et al. (2015). The output was filtered so that each cell cycle must have a parent and two children and that the maximum displacement of that cell between any consecutive frames was less than the cell width. Growth-condition-dependent filter criteria were added so that only cells with a cycle time of 50, 30, and 15 min were retained for slow, intermediate, and fast growth, respectively. To attain high-precision volume estimates, the estimated volumes from the active contours were fitted to $V(t) = V_B \exp(\mu t)$, where t is the time from cell birth, V_B is the birth volume, and μ is the growth rate. Cell generations were filtered based on root-mean-squared errors of the fit being smaller than 5% of the average cell volume. The fitted volumes at times of division and initiation were used in all figures, except for Figure 3, where a large number of cell generations were required and it is sufficient to know the size of the cell regardless of division parameters. The analysis of the precision in division timing is described in the Supplemental Experimental Procedures (see "Validation of the phase contrast division classifier") (Figure S2). The determination of length, areas, volumes, and widths was based on the contour model as in Sliusarenko et al. (2011).

Phase-contrast and fluorescent images of strain JE202 and the fluorescent images of JE201 were segmented and tracked as described above, but not fitted with active contours.

Replisomes and origins were detected in the raw fluorescence images using the method described in Hammar et al. (2014). The coordinates were set in a cellular reference frame using the contour model derived from the phasecontrast image taken in conjunction with the fluorescence image.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.06.052.

AUTHOR CONTRIBUTIONS

M.W., J.E., and D.F. designed the experiments. D.F., J.E., and M.W. developed the model. J.E., D.F., and M.W. wrote the paper; M.W., D.F., E.G.L., and O.B. made the experiments; M.W. and D.F. made the analysis codes and performed analysis; and D.F. built the setup and made the simulation code and the simulations.

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