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Changes of initiation mass and cell dimensions by the 'eclipse'

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Summary

The minimum time (*E*) required for a new pair of replication origins (*oriCs*) produced upon initiating a round of replication to be ready to initiate the next round after one cell mass doubling, the 'eclipse', is explained in terms of a minimal distance (I_{min}) that the replication forks must move away from *oriC* before *oriCs* can 'fire' again. In conditions demanding a scheduled initiation event before the relative distance $I_{min}/L_{0.5}$ (*L* being the total chromosome length) is reached, initiation is presumably delayed. Under such circumstances, cell mass at the next initiation would be greater than the usual, constant *Mi* (cell mass per copy number of *oriC*) prevailing in steady state of exponential growth. This model can be tested experimentally by extending the replication time *C* using thymine limitation at short doubling times τ in rich media to reach a relative eclipse $E/C < I_{min}/L_{0.5}$. It is consistent with results obtained in experiments in which the number of replication 'positions' n (= C/τ) is increased beyond the natural maximum, causing the mean cell size to rise continuously, first by widening, then by lengthening, and finally by splitting its poles. The consequent branching is associated with casting off a small proportion of normal-sized cells and lysing DNA-less cells. Whether or how these phenomena are related to peptidoglycan composition and synthesis are moot questions.

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Introduction

The bacterial cell manages to multiply faster than it duplicates its chromosome by initiating a successive replication round before the previous one has terminated (Sueoka and Yoshikawa, 1965; Helmstetter *et al*., 1968). To retain a steady state in a growing culture (Fishov *et al*., 1995), synchronous initiations from all existing *oriC*s occur once each cell cycle at a constant value of mass (or volume) per *oriC*, *Mi* (Donachie, 1968; Pritchard *et al*., 1969; Helmstetter, 1996; Cooper, 2006). Under optimal conditions (37°C in rich medium), the doubling time (τ) of *Escherichia coli* is about half the replication time (*C*), i.e. 20 and 40 min respectively (Helmstetter, 1996). Hence, initiation of a new round occurs when the bifurcating replication assemblies are midway between *oriC* and *terC* ($L_{0.5}$, half chromosome) (see also in http://simon.bio.uva.nl/cellcycle/index.html).

Control of replication and the eclipse

A delay between two successive initiation events was predicted (Helmstetter, 1971) and then validated (Zaritsky, 1971) following inhibition of replication for two mass doublings, during which the cells accumulated enough potential to initiate twice (i.e. they had reached mass of at least 2*Mi*). This so-called 'stacking' phenomenon (Zaritsky, 1971; 1975a), later termed 'eclipse' (Nordström, 1983), ensures that further initiations do not occur for a substantial fraction of the cell cycle, regardless of the degree of synchrony in 'firing' by all existing *oriC*s. This time delay (designated *E*) was originally visualized/considered to result from 'steric hindrance' by a bulky replisomal hyperstructure (see Guzmán *et al*., 2002; Molina and Skarstad, 2004) that prevented another one from forming in its vicinity. Studies during the 1970s with the physiological tools then available (reviewed in Helmstetter *et al*., 1979) estimated the eclipse time period *E* to be of the order of 12–20 min at 37°C (see also Eberle *et al*., 1982; Helmstetter and Krajewski, 1982; Helmstetter, 1996).

The R1 plasmid model system

Since the 1980s, the eclipse has been studied in plasmids, most extensively and rewardingly with R1 (e.g.

Nordström, 1983; reviewed in Nordström, 2006), and has been related to the mechanism regulating plasmid replication (e.g. Olsson *et al*., 2003). The Scandinavian school has suggested that the eclipse, at least in the R1 system, has two different causes, structural and copy-number control, and that the former depends on the degree of supercoiling in the daughter plasmid molecules (Gustafsson *et al*., 1978). At 42°C, these two causes contributed 3 and 10 min, respectively, to the eclipse period (Olsson *et al*., 2003), while the *E* period for the chromosome was estimated (Olsson *et al*., 2002) to span 0.6 of the culture generation time τ at different temperatures. Plasmid eclipse might thus not (fully or even partially) reflect that of the bacterial chromosome due to its structure and brevity of replication time. Even minichromosome replication was found not to be directly coupled to replication of the chromosome itself (Eliasson and Nordström, 1997). The dependence of division and, hence, cell size on the chromosome replication cycle, including the eclipse, is indeed considered as an important factor in survival (Boye *et al*., 2000; Grigorian *et al*., 2003; Zaritsky *et al*., 2006). Can *E* be eliminated (von Freiesleben *et al*., 2000) without affecting cell viability, for example?

The bacterial chromosome

By the end of the 1980s, a plausible mechanism to explain the chromosomal eclipse was based on the discovery that hemi-methylated DNA produced upon replication is associated with the cytoplasmic membrane (Ogden *et al*., 1988). Thus, newly replicated hemi-methylated *oriC* is assumed to be sequestered by binding to SeqA and, hence, unavailable for re-initiation during a substantial fraction of the cell cycle because it cannot return to the fully methylated state. The duration of this fraction is affected by the concentration of Dam methyl-transferase (reviewed in Boye *et al*., 2000). Titration of the replication initiator DnaA by *datA* (Kitagawa *et al*., 1998) and reduced DnaA activity by ATP/ADP exchange (Katayama *et al*., 1998) operate during the sequestration period to delay initiation further, but excess DnaA does not allow an eclipse to occur in the absence of sequestration and the eclipse can be longer than the sequestration interval (Bogan and Helmstetter, 1997). The *E* period without sequestration was indeed found to be significantly shorter in *seqA*, *dam* and *seqA dam* mutants, 0.16–0.4τ rather than 0.6τ in the isogenic wild-type *E. coli* (Olsson *et al*., 2002), but viability was not determined for these circumstances. The value of 0.6τ might be an over-estimate because wild-type cells in cultures growing at the maximum rate (with τ of 20 min at 37°C) and minimum *C* (of about 40 min) do attain steady-state mass. The observation that the actual maximal value of C/τ found is smaller than 2 (1.65; Bipatnath *et al*., 1989) is consistent with an *E* of about 0.6τ (= 1/1.65), however.

The reduced cell viability (operationally defined as 'plating efficiency') under 'premature' re-initiations observed in the cold-sensitive DnaA mutant *dnaAcos* was indeed suppressed by multiple *oriC*s on a multicopy plasmid (Katayama and Kornberg, 1994). These results indicate that over-initiation *per se* is specifically responsible for growth inhibition by DnaAcos. More recently, Simmons *et al*. (2004) concluded that increased initiations induce replication fork collapse, and that growth interference in *recB* (but not *recA*) mutants is due to failure to repair DNA double-strand breaks (DSB) caused by elevated *dnaA* or *dnaAcos* expression. These authors propose that the period of *oriC* sequestration after initiation allows collapsed replisomes to resume replication so that a fork formed by a new round of initiation is unlikely to run into the one ahead. Somewhat earlier, Grigorian *et al*. (2003) demonstrated that increased frequency of initiations effected by overexpression of *dnaA* resulted in stalled replication forks, filamentation and decreased viability, the latter being suppressed by simultaneous overexpression of *recF*. The observed reduction in the rate of replisome progression (longer *C* period) may reflect fork collapse and DSB recombinational repair that is required for survival of such cells. Under these circumstances, many chromosomes did not complete replication in run-out experiments. Similarly, fast outgrowing cultures of germinating Thy$^-$*Bacillus subtilis* in rich media release more DNA fragments nearer to *oriC*, a release that is closely correlated with 'proneness of the cell to lysis' (Borenstein and Ephrati-Elizur, 1969).

The minimum distance (l_{min}) hypothesis

It is well established that inactivated replication forks generated by various causes can be reversed or directly restarted by various recombination proteins (Table 1 in Michel *et al*., 2004), and that secondarily initiated forks collapse and form DSB when they run into the preceding forks. It is proposed here that this collapse is caused by 'premature' re-initiations that are scheduled (by the cells reaching *Mi* or producing excess DnaA) before the previous replication fork has moved a minimal physical distance ($l_{\min}$) away from *oriC*, and that the processes of repairing the ensuing DSB to restore viability delay the real time of this initiation event, hence modifying various cell biology parameters. This mechanism (being itself biochemical in nature) is interpreted here in terms of a minimal distance possible between two successive forks $l_{\min}$. As this distance is traversed by the kinetic reaction of replication, it can be expressed in time units *E*. The precise mechanism(s) governing the delayed initiation is (are) not crucial for the arguments presented here.

Changed superhelicity in the vicinity of *oriC* after initiation, for example, might have a long-range effect on local DNA structure and, hence, on re-initiation ability or replisome collapse. The recent observation that helicases modify stability of stalled replication fork (Tanaka and Masai, 2006) might also be relevant in this connection as well.

It can be concluded therefore that multiple components are involved in the eclipse, and that two major processes, sequestration and minimum distance, operate together. If the first becomes shorter than the time to reach I_{min}, and if initiation occurs, then the cells face a problem that must be corrected before a balanced growth status can be re-established. The bottom line is that *E* can never be shorter than the time to reach I_{min} without causing a problem such as fork collapse. Existing data support this concept, and new rigorous tests are proposed here.

The eclipse – structure model, implications and distinction

As discussed above, it seems likely that the bacterial eclipse time (*E*) results from a combination between time-consuming process(es) and a structure that limits formation of another replisome before the previous is I_{min} away from *oriC*. Assuming that I_{min} exists, specific questions arise such as 'What is its value?', 'Does it change with cell physiology?', 'What happens when it is artificially shortened?' and 'How would it affect cell physiology?' Can the I_{min} hypothesis be tested and distinguished from existing models?

To test the I_{min} hypothesis and to see whether *E* elongates when this limit is reached too early to fire initiation, growth conditions must be created in which *Mi* is attained before I_{min}. If I_{min} is below half the distance *oriC* – *terC* (i.e. $L_{0.25}$), this condition cannot be achieved with wild-type, thymine-independent (Thy^+) cells, in which the number of replication fork 'positions' ($n = C/\tau$; Sueoka and Yoshikawa, 1965) never exceeds 2. To reach $n > 2$, *C* must extend beyond 2τ, a condition that is achievable by the so-called 'thymine limitation' method (Pritchard and Zaritsky, 1970; Zaritsky *et al*., 2006): cultivating $ThyA^-$ mutants at low thymine concentrations without affecting the culture growth rate ($\mu = 1/\tau$).

Cell mass and DNA content and concentration (*Fig. 1 and http://simon.bio.uva.nl/cellcycle/index.html)*

If the forks do not reach I_{min} at the time when a new cycle should start (i.e. when cell mass surpasses *Mi*) to preserve steady state (Fishov *et al*., 1995), the scheduled initiation event would be delayed by a period Δt that is necessary for the previous set of forks to arrive at I_{min}. Thus, when $C/\tau > L_{0.5}/I_{min}$ (= *v*, defined as the maximum number of fork positions that the chromosome can contain), the next initiation event would occur Δt later, during which time cell mass would accumulate at the same rate to reach $Mi_1 = Mi \cdot 2^{\Delta t/\tau}$. This would result in a larger cell mass at division [$Md_1 = Mi_1 \cdot 2^{(C+D)/\tau}$, *D* being the time between termination and the subsequent cell division (Helmstetter *et al*., 1968)] and further increase in *Mi* at the next initiation event (to $Mi_2 = Mi \cdot 2^{2\Delta t/\tau}$), and so forth in the *g*th generation [$Mi_g = Mi \cdot 2^{g\Delta t/\tau}$ and $Md_g = Mi_g \cdot 2^{(C+D)/\tau}$ respectively]. Thus, when $C > \tau \cdot v$, a new steady state *cannot* be reached: mean cell mass *M* rises, while cell DNA content *G* remains constant; hence DNA concentration *G*/*M* drops with time *t* at a rate that depends on $I_{min}/L_{0.5}$ (see below).

The simulation program at http://simon.bio.uva.nl/cellcycle/index.html now includes the option to change $I_{min}/L_{0.5}$ (in terms of *E*/*C*), demonstrating conditions at which steady state of exponential growth (Fishov *et al*., 1995) cannot be achieved due to a structural eclipse, as described here. An example is shown in Fig. 1: a single *thyA* cell from a steady-state growing culture with $\tau = C = 40$ min is followed before and after transfer (at $t = 0$ min) to the same medium but with $C_2 = 90$ min, $D_1 = D_2 = 20$ min, and with relative eclipse E/C (= $I_{min}/L_{0.5}$) = 0.45. Although mean cell size continues to increase indefinitely under such circumstances, steady-state values of nucleoid complexity (*NC*; see below) and DNA content are finally attained (after periods of $C_2 + \tau$ and of $C_2 + D + \tau$ respectively) because (respectively) the distance between two successive sets of replication forks remains constant at its shortest possible I_{min} value, and the time span of the division processes *D* after termination is presumed not to change (but see in Meacock and Pritchard, 1975). (Longer *D* would only raise *M* and *G*.) The linear processes (*C*, *D*), together with exponential mass growth and the continuous rise in *Mi* due to maximal value of *E*/*C*, result in a constant *G* but monotonously reducing *G*/*M* at the same rate as *M* rises. The analytical expressions for the changes with time of the mean values [i.e. *M*(*t*), *G*(*t*) and *G*/*M*(*t*)] will be derived separately.

Estimating E/C *and* I_{min}/L

Continuous increases of *M* at rates that depend on the thymine concentration supplied have indeed been observed in fast-growing *thyA E. coli* cultures (Fig. 7 in Zaritsky and Pritchard, 1973), but *G*/*M* seemed to remain unchanged after a transition period that depends on C_2 (Fig. 8 in Zaritsky and Pritchard, 1973; but see also Meacock and Pritchard, 1975). However, *G*/*M* was measured for a time shorter than necessary to detect a slow change (Fig. 1A). The parameters in Fig. 1A were picked so as to emphasize this fact, albeit in a single cell: the rate of change of *G*/*M* is fast upon stepping the thymine concentration down due to the immediate slowing of the rate of replication at all forks. After a period of C_2 min, when the last (youngest) pre-step existing fork terminates, the

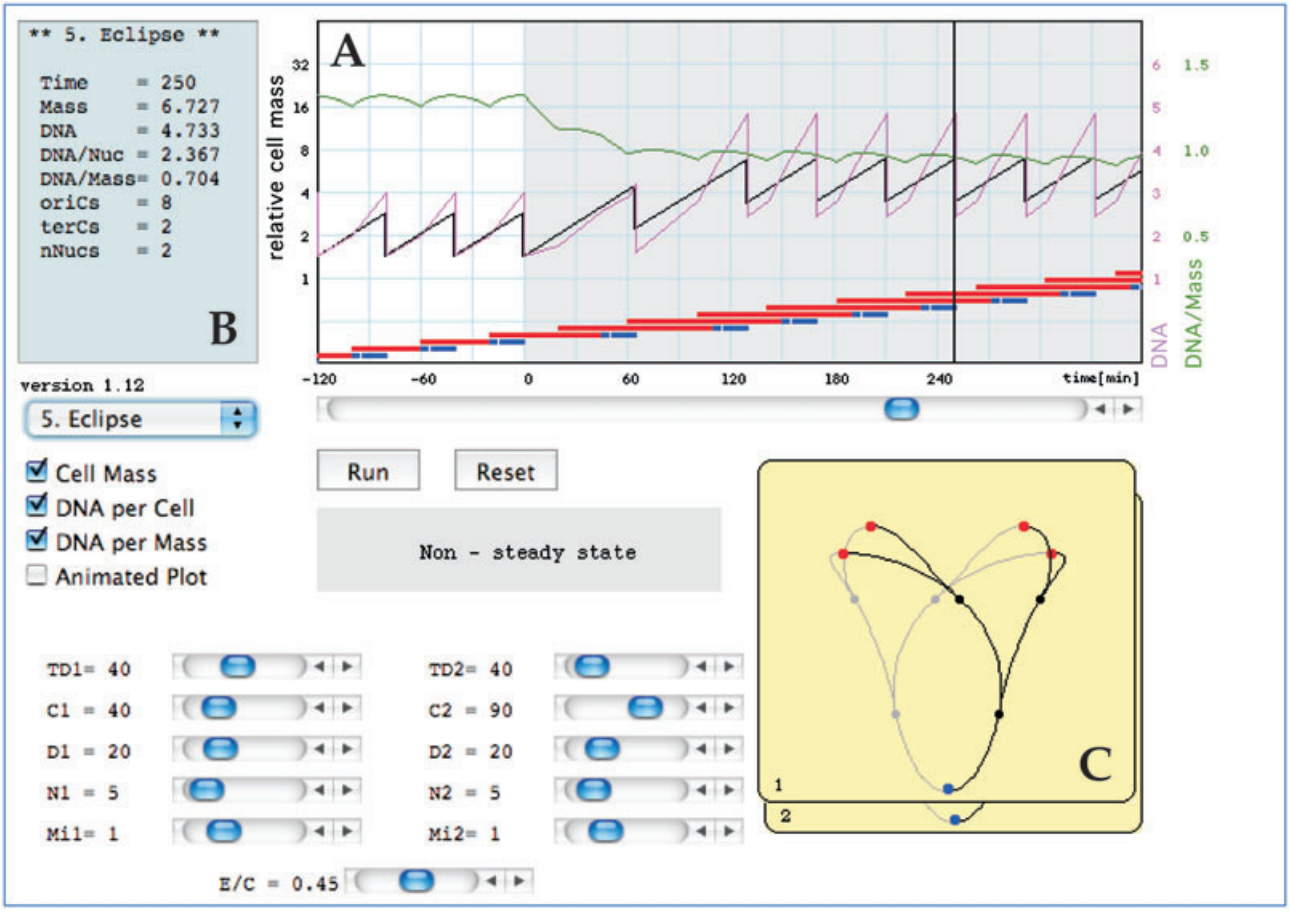

Fig. 1. Cell cycle simulation of the modified/refined Cooper–Helmstetter model (taken from the interactive program at http://simon.bio.uva.nl/cellcycle/index.html).
A. Relative mass in *Mi* units (black line), DNA content in genome equivalents (red) and DNA concentration (*G*/*M*; green) of a single cell (newborn at $t = 0$) during a step-down transition with the following parameters (shown in the scrollbars below): $\tau_1 = \tau_2 = 40$ min; $D_1 = D_2 = 20$ min; $C_1 = 40$ min; $C_2 = 90$ min; $E/C = 0.45$. (For other options, see in http://simon.bio.uva.nl/cellcycle/index.html) Horizontal red and blue bars indicate *C* and *D* periods respectively. *N* (white dots in the blue bars) is the time between termination of chromosome replication and nucleoid segregation.
B and C. Predicted parameters (B) and chromosome configuration (C) at $t = 250$ min after the step-down are displayed. (C) The yellow panels ('1' and '2') represent individual nucleoids; the red, blue and black dots represent, respectively, *oriC*s, *terC*s and replisomes, in perspective (as in Fig. 2 in Zaritsky *et al*., 2006).

rate of further reduction in *G*/*M* depends on the delay in initiation frequency Δt. At longer Δt values, i.e. in either longer *C*, shorter τ or longer I_{min} (larger *E*/*C*) (not shown but can be manipulated at http://simon.bio.uva.nl/cellcycle/index.html), the rate of continued change in *G*/*M* will be faster and in a predictable mode. It is noteworthy that this prediction is testable quantitatively.

The existing (observed) rates of increase in *M* that depend on the concentration of thymine in the medium (slopes of the lines in Fig. 7 in Zaritsky and Pritchard, 1973) can also be used to obtain first-approximation estimates of *E*-values (hence of I_{min}), because reasonable estimates of all other parameters (τ, *C*, *D*) are available (Pritchard and Zaritsky, 1970; Zaritsky, 1971; Meacock and Pritchard, 1975; Zaritsky *et al*., 1999). To this end, however, further analytical expressions of $M(t)$ will be necessary.

Nucleoid complexity and cell dimensions

The similar increase in diameter (2*R*) of rod-shaped cells such as *E. coli* and *Salmonella enterica* growing at fast growth rates μ (shorter τ) in rich media (Schaechter *et al*., 1958) or at slow replication rates (long *C*) in low thymine concentrations (Zaritsky, 1975b) is explained by a common denominator of the two different states: increased 'nucleoid complexity' (*NC*). This term was recently defined as the amount of DNA per nucleoid:

$$NC = G/terC = 2^{n-1} \cdot \ln 2/n \quad (1)$$

where $n = C/\tau$ (and see inset in Fig. 4 in Zaritsky *et al*., 2006). The biochemical or biophysical mechanism(s) that might govern the correlation between 2*R* and *NC* is (are) not known yet, but the apparently longer *D* that is corre-

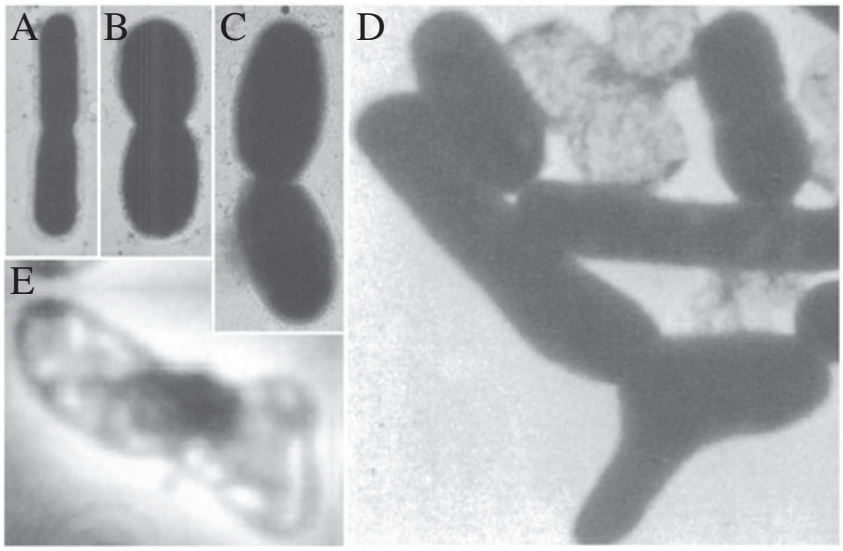

Fig. 2. Examples of cells in evolving cultures.
A–C. From Fig. 11a, d and j of Zaritsky and Pritchard (1973).
D. From Fig. 6 of Zaritsky and Woldringh (1978).
E. DAPI-stained; from Fig. 6F of Zaritsky *et al.* (2006).
Scales of (D) and (E) differ from that of (A)–(C) and between themselves.

lated with *C* (Zaritsky, 1971; Zaritsky *et al.*, 1999) might result from the larger surface area that a wider cell must synthesize at the same growth rate to complete the division septum (see Fig. 5 in Zaritsky *et al.*, 2006).

One consequence of such longer *D* periods at lower thymine concentrations is still larger *M* (and *G*), but no apparent positive feedback on 2*R* is predicted, as was previously thought (Zaritsky *et al.*, 2006), as new steady-state values of M ($= Mi \cdot 2^{n+D/\tau}$) and G [$= (2^{n+D/\tau} - 2^{D/\tau}) \cdot (n \cdot \ln 2)^{-1}$] are reached as long as *C* does not exceed $\tau \cdot v$. This is not the case under conditions (fast growth rate and slow replication rate) in which $C/\tau > v$; here, cell mass continues to rise due to an apparent positive feedback loop, as demonstrated in Fig. 1 and in the website at http://simon.bio.uva.nl/cellcycle/index.html. Thus, if 2*R* is indeed proportional to *NC* (Eqn. 1; and inset in Fig. 4 in Zaritsky *et al.*, 2006), it will stop increasing when the replication time *C* becomes longer than $\tau \cdot v$, because the limit of *NC*, $NC_{max} = \ln 2 \cdot v \cdot (2^v - 1)$, is reached, and more frequent acts of initiation than every $(\tau + \Delta t)$ are no longer possible.

Evolution of cell size and shape in thymine-limited cultures (Fig. 2)

Under the circumstances described above, the distance I_{min} between successive replication forks cannot shrink, extending *C* would extend *E* without affecting cell diameter, and the additional mass accumulated would then be accommodated by increased cell length. This prediction has also been observed, at least qualitatively in the evolution of a 'stepped-down' culture of thymine-less mutant (transferred to a lower thymine concentration) to grow with apparent $n > v$. This evolution occurs in several steps:

(a) An overshoot in cell length (Fig. 4 in Zaritsky *et al.*, 2006).
(b) A slow increase in cell diameter and little or no increase in cell length during the first stages of progressive increase in cell mass (Fig. 2A and B; taken from Fig. 11 in Zaritsky and Pritchard, 1973).
(c) When maximal nucleoid complexity is reached $C_2 + \tau$ min after the step, the cells stop widening and continue to elongate (Fig. 2C; and see Zaritsky, 1977).
(d) At this stage, some cell poles start to split by forming FtsZ arcs between replicating/segregating nucleoids (Fig. 2E; and see, for example, Fig. 6 in Zaritsky *et al.*, 2006) and these so-called 'split tips' develop branches (Fig. 2D; taken from Fig. 4 in Zaritsky and Woldringh, 1978).

In addition, some of the divisions produce normal-sized (large) cells that reiterate stages (c)–(d) and some seem to cast off DNA-less, non-multiplying cells. The latter observation (short cells and ghosts, respectively, in Fig. 2D) has yet to be rigorously quantified. A thorough analysis of cell dimensions during such a step-down experiment, qualitatively consistent with this description, is underway.

Branching, nucleoid complexity, and peptidoglycan synthesis and composition

Such 'split tip' formation can be enhanced in a culture at stage (c) after a pulse treatment with mecillinam (Fig. 2E; taken from Fig. 6 in Zaritsky *et al.*, 2006). Two different explanations for cell branching were recently proposed: one is based on asymmetrical FtsZ-ring formation in cells with large diameters resulting from the presence of complex nucleoids (Zaritsky *et al.*, 2006), the other

couples it to inert peptidoglycan (De Pedro *et al*., 2003). Both explanations are further complicated as follows. (i) When *C* durations are long (at low thymine concentrations), a set of replication forks arriving at *terC* is likely to lose whatever synchrony it had upon initiation, and this asynchrony may be reflected in asymmetrical deposition of FtsZ arcs, culminating in branch formation. (ii) The physiological response to changes in C/τ of different *E. coli* strains varies widely (Begg and Donachie, 1978). For example, a *thy* mutant of B/r seems to be so-called 'straight-jacketed' (data not shown): at limiting thymine concentrations it only grows to a certain size and then slows the total mass growth rate. These observations might reflect delicate differences in peptidoglycan composition/structure between these strains, differences that should be detectable (W. Vollmer, pers. comm.).

Concluding remarks

A generation-old series of puzzling results (Zaritsky and Pritchard, 1973; Zaritsky *et al*., 2006) seems to be resolved, at least partially, by the influence of structural eclipse on the timing of initiation of chromosome replication, and hence on cell size, shape and macromolecular composition. The model involves a minimal distance I_{min} between two successive replication forks, irrespective of the actual value, and can be tested under conditions that require a smaller distance for steady-state growth such as under thymine limitation. Careful quantitative testing of the predictions of this model might distinguish between such spatial/structural control of the eclipse and a temporal/chemical model. It is most likely that both mechanisms are related, operate simultaneously and assist/complement each other, in which case such tests/analyses would dissect them in time and space.

In parallel to the evolution of the bidirectional multi-forked replication mode in species such as *E. coli*, the natural value of I_{min} did not have to be much smaller than $0.25L$ because the number of replication positions never exceeds 2 ($= \nu = C/\tau_{min} = 40$ min/20 min). The un-natural situation of thymine limitation in fast-growing *thyA* strains (Zaritsky *et al*., 2006) elicits a similar response to that of overexpressing *dnaA* strains (an initiation event that occurs before *Mi* is reached): fork-collapse and DSB formation and repair that delay the actual initiation.

The description in different terms (kinetic and structural) of two models to explain the initiation eclipse reflects two different views, one based on biochemistry and the other based on biophysics/physiology. This debate is reminiscent of the one prevailing in mid-20th century about DNA replication (Cairns *et al*., 1966; Kornberg, 1974), a debate that culminated in a better understanding of the process under discussion. It is hoped that the current discussion will be equally fruitful.

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