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Does the eclipse limit bacterial nucleoid complexity and cell width?

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ABSTRACT

Cell size of bacteria *M* is related to 3 temporal parameters: chromosome replication time *C*, period from replication-termination to subsequent division *D*, and doubling time τ. Steady-state, bacillary cells grow exponentially by extending length *L* only, but their constant width *W* is larger at shorter τ's or longer *C*'s, in proportion to the number of chromosome replication positions n ($= C/\tau$), at least in *Escherichia coli* and *Salmonella typhimurium*. Extending *C* by **thymine limitation** of **fast-growing *thyA* mutants** result in continuous increase of *M*, associated with rising *W*, up to a limit before branching. A set of such puzzling observations is qualitatively consistent with the view that the actual cell mass (or volume) at the time of replication-initiation *Mi* (or *Vi*), usually relatively constant in growth at varying τ's, rises with time under thymine limitation of fast-growing, thymine-requiring *E. coli* strains. The hypothesis will be tested that presumes existence of a minimal distance l_{min} between successive moving replisomes, translated into the time needed for a replisome to reach l_{min} before a new replication-initiation at *oriC* is allowed, termed Eclipse *E*. Preliminary analysis of currently available data is inconsistent with a constant *E* under all conditions, hence other explanations and ways to test them are proposed in an attempt to elucidate these and other results. The complex hypothesis takes into account much of what is currently known about Bacterial Physiology: the relationships between cell dimensions, growth and cycle parameters, particularly nucleoid structure, replication and position, and the mode of peptidoglycan biosynthesis. Further experiments are mentioned that are necessary to test the discussed ideas and hypotheses.
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1. Introduction

1.1. The bacterial cell cycle: temporal and spatial aspects

The conventional Bacterial Cell Division Cycle (BCD) is defined by four parameters, 3 of which are temporal: mass doubling time τ, chromosome replication time C and the time D between replication-termination and cell division, the latter two are relatively constant (about 40 and 20 min, respectively) under steady-state exponential growth at fast rates (τ < 70 min) in 37 °C, modulated by nutritional conditions [20]. The 4th parameter, also relatively constant, is the strain-dependent cell mass *Mi* (or volume *Vi*, since density does not change with τ_m [27] per *oriC* at which chromosome replication is initiated [2,13,39,47], synchronously at all existing *oriC*s [7]. Together, these 4 parameters couple cell size *M* to the linear processes of the BCD: cells are larger at shorter τ's [46,57] because they grow (exponentially so) more during the fixed time ($C + D$) between replication-initiation (at *Mi*) and the consequent division (reviewed in Ref. [72]). Put in an equation, average cell size $<M> = Mi \times (\ln 2) \times 2^{(C+D)/\tau}$. Size-control is thus coupled to temporal aspects (rates) of mass growth and nucleoid replication leading to division [67]. Regulation of replication-initiation has largely been resolved [29]; the molecular meaning of the constant *D* period, on the other hand, is still enigmatic (and see below). Under faster growth rates μ ($= \tau^{-1}$), initiation occurs in the mother or grandmother cell when $\tau < (C + D)$. Furthermore, when $\tau < C$, a replication cycle starts before the previous one has terminated [20,22], to form a multi-forked replicating nucleoid with a higher complexity *NC* [23]. *NC* is defined as the culture-average amount of DNA in genome equivalents associated with a single *terC* [$NC = (2^n - 1)/n\ln 2$] [60,67,76] where $n = C/\tau$ (the number of replication positions; [49], irrespective of the value of *D*.

During steady-state of exponential growth conditions [16], cells enlarge by elongation and divide in a perpendicular plane; cell width *W* is strikingly constant, in the culture and during individual cycle [51]. The simple prediction that the larger, faster-growing cells in richer media are proportionately longer is not fulfilled: they are wider as well (Fig. 1)! A fundamental question thus arises: **how does cell width change during transfer to a richer medium**, so-called nutritional shift-up [24]; Fig. 2)? This question interfaces the major spatial aspects of the cell (placing the FtsZ-ring exactly in mid-cell, fixing and changing cell dimensions under different growth conditions) with the temporal aspects (rates of growth, DNA replication and division processes). The long-standing puzzle of the crucial coordination between nucleoid structure and FtsZ-ring assembly to form the divisome is elusive because "several partially redundant mechanisms to achieve this task" seem to be involved [31] as safeguards for species survival. The *primary signal* delivered from the nucleoid to assemble a divisome for cell division

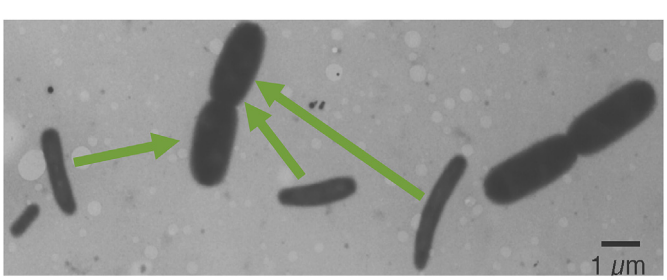

Fig. 1. Electron micrograph of a mixture of two *E. coli* B/r cultures on agar filters. The big cells were grown with a doubling time τ = 22 min; the small cells, with τ = 150 min. Adapted from Zaritsky & Woldringh [72]. Arrows indicate the transition enigma.

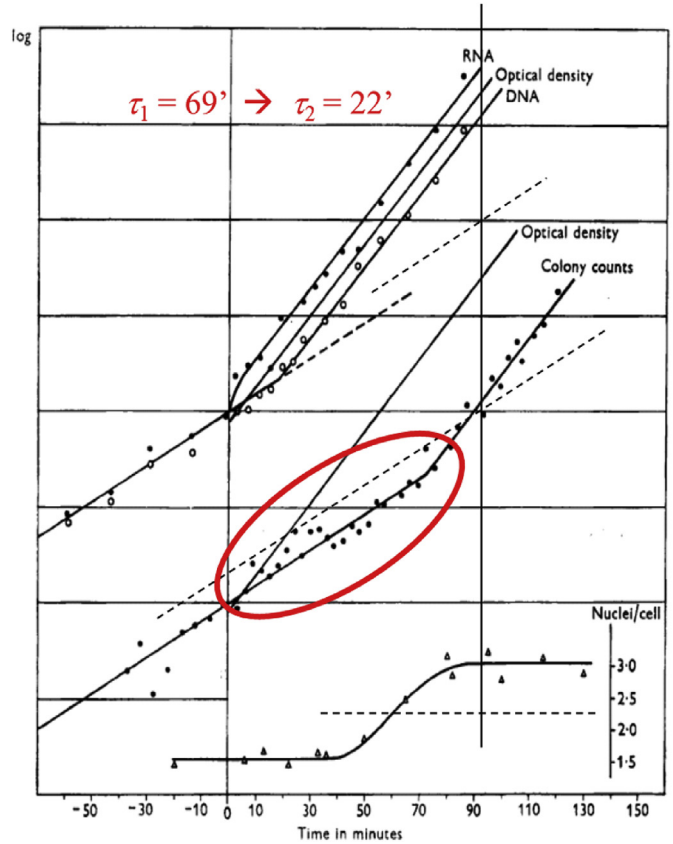

Fig. 2. The classic nutritional shift-up experiment (Adapted from Ref. [24].). The red oval depicts the maintenance period (~65 min) of cell division rate.

in the right place and time cannot be simply a protein-set because the question of their expression is analogous to the "enzyme-cannot-make-enzyme paradox" [48]. As discussed by Kirschner et al. [25]: "This picture of self-organization to a thermodynamic minimum at steady state is likely applicable to many, perhaps all, cellular assemblies". - Isn't the divisome one? A physics-based mechanism for division site-selection was therefore proposed [41]; and see below). Repeating waveform pattern of cell surface undulations along the long axis was just observed in mycobacteria that lack both Min and NO systems [14], but a mechanism for coordinating the FtsZ-ring assembly with the nucleoid is missing. Here, we succinctly summarize the current knowledge about this sought for signal.

The classical upshift experiment (Fig. 2) discovered the then-enigmatic "rate maintenance" phenomenon: cell divisions remain at pre-shift rate for ~65 min before abruptly soaring to the post-shift rate. This exciting observation was resolved by a series of experiments with the so-called "baby machine" [19] to yield the BCD Dogma (reviewed in Ref. [72]). This rate maintenance time roughly equals the period ($C + D$) thus resulting in a corresponding change of average cell size $<M>$ (= total mass/cell number in a withdrawn sample). This understanding however does not answer the main question posed here about the *primary* signal(s) for cell division and width determination.

1.2. Cell dimensions under steady-state growth and during nutritional shift-up

The BCD Dogma, which explained the rate maintenance phenomenon (Fig. 2) and resolved the temporal aspects of the cell

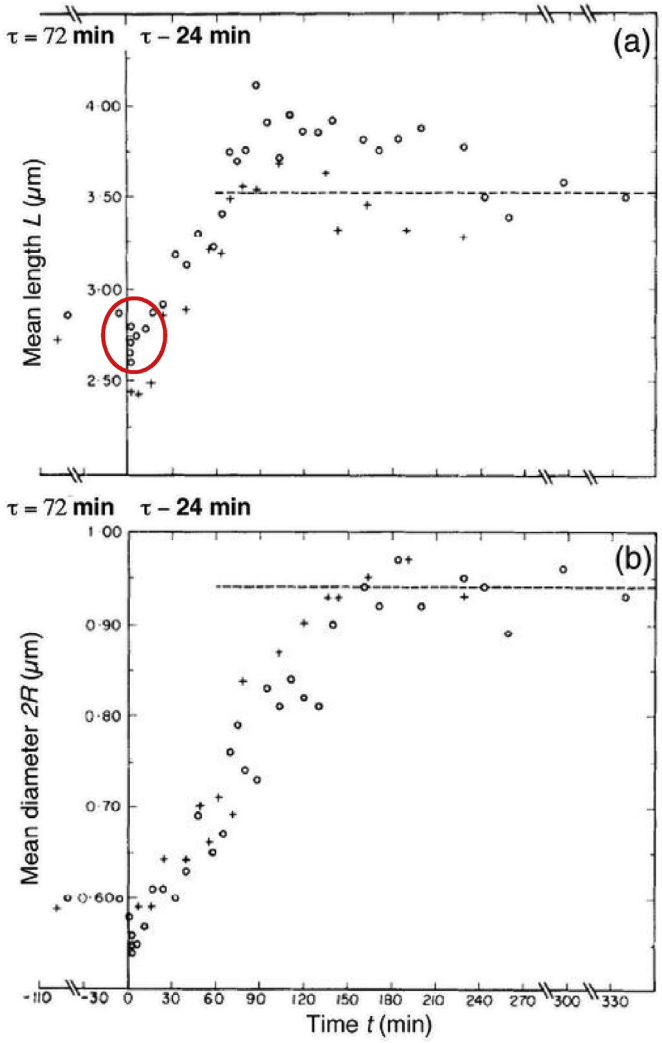

Fig. 3. Dimensional Rearrangement during nutritional shift-up (Adapted from Ref. [59].). The red oval depicts a temporary enhanced rate of cell division upon the upshift.

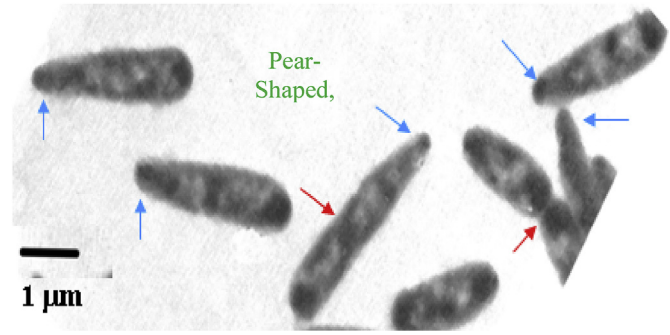

Fig. 4. Electron micrographs of cells 60 min after a nutritional shift-up from $\tau_1 = 72' \rightarrow \tau_2 = 24'$. The nucleoids appear as electron-transparent regions. Red arrows indicate constriction sites, blue arrows—tapered tips. From Ref. [72].

cycle, did not elucidate the mechanism governing the apparent relationship between cell dimensions and the nucleoid's structure and replication state [67], which is a major aim of this analysis. To achieve this goal, the scarce number of articles describing the upshift perturbation will now be scrutinized further.

The long division-rate maintenance (65 min ≈ $(C + D)$) distracted attention from several other phenomena observed during the upshift transition that should carefully be re-examined, *e.g.* strange fluctuations in division rate during the first 20 min (Figs. 2 and 3). More striking and important for the present analysis are the long times needed to (a) reach the new mean value of the calculated cell volume (**about 2 h**; [73]), and (b) attain steady-state cell length L and width W (**over 3 h!**; [59]), during which L overshoots its final new value (Fig. 3, top panel). The latter observation was accompanied by cell images during the transition: these clearly show that W changes exclusively **during** cell division and **at** its constricting ring thus creating temporarily pear-shaped, tapered cells (Fig. 4). A new set of shift-up data that includes nucleoids, under the mother machine that enables following-up single cells is sorely lacking.

The first account relating steady-state cell dimensions L and W to the 4 parameters (τ_m, Mi, C, D) of the BCD [65] considered them within the then-current concept: W was thought to be passively regulated by active control over cell volume V (exponential mass synthesis) and L (linear zonal growth of the envelope (e.g. Refs. [69,73]). This view was abandoned when the shape-determining peptidoglycan PG was proven to be synthesized diffusely along cell length during elongation [11]. Many studies since then concluded that a separate mechanism exists to constrict/septate in a perpendicular plane, organized in the hyper-structure [34] named divisome [43], a process that is initiated nearly simultaneously with the start of the D-period [1,12]. Thus, two systems exist for PG synthesis [10,38]: one (termed elongasome), during growth, that keeps W constant, and the divisome, allowing change in W as well. PG's elasticity equalizes W forming temporarily tapered, pear-shaped cells during the transition (Fig. 4). A fundamental question in bacterial cell biology is the mechanism governing the determination of W.

1.3. Are cell dimensions related to nucleoid structure and replication?

The satisfactory correlation observed [60,67,76] between W and NC for two species of *Enterobacteriaceae* (Table 1; column 5) led to the alternative idea, that ***W*** (rather than L) is **actively** determined and that an elusive signal is transmitted by the nucleoid structure to the divisome to widen cell diameter during nutritional upshift and keep it constant under steady-state growth conditions.

This presumed new task for DNA through nucleoid structure and replication should be further explored using physiological manipulations. The difference (74%) between the two NC/W averages (1.36 for *Salmonella typhimurium*, top, Table 1; 2.37 for *E. coli*, bottom) may be either real or due to the different methods of measurements or of growth conditions. We believe that it is a real difference between strains/species (and see Refs. [5,45], depending on the PG biosynthesis in/by the divisome. The question whether cell diameter is a spandrel such as cell size is [3] remains moot, but if the $NC \rightarrow W$ idea is confirmed, another puzzle arises as follows. The structure of the nucleoid, explicitly expressed in terms of NC, changes continuously during the cell cycle as a function of the relationships between τ, C and D [22]; lack of similar fluctuations in W during the cycle means that it is fixed either at the time of division-determination or continuously by the weighted average, in the population or during the cell cycle.

Another quantitative value that describes nucleoid structure in terms of the temporal parameters of BCD (τ, C, D) is *oriC*/*terC* ratio. Its relevance to the determination of W was tested by computing the relationships between these two (columns 4 and 5 in Table 1), and found not suitable; the differences in the fit are more than 50%

Table 1
Cell Width *W*, Length *L*, *oriC*/*terC* ratio o/t and Nucleoid Complexity *NC*.

μ (hr^{-1})[a]	τ (min)[a]	*W* (μm)[a]	*oriC*/*terC*[c]	(*o*/*t*)[c]/*W*	*NC*[b]	*NC*/*W*	*L*	L_{meas}	L_{meas}/L
2.73	22	1.43	3.53	2.47	2.01	1.41	3.42	600[d]	175
1.85	32	1.22	2.38	1.95	1.60	1.31	4.40	430[d]	98
1.00	60	0.93	1.59	1.71	1.28	1.38	3.61	400[d]	111
0.61	98	0.87	1.33	1.53	1.18	1.36	2.36	359[d]	152
1.17	51.35	0.55	1.72	3.13	1.33	2.42	2.69	2.055	0.76
1.18	50.85	0.56	1.73	3.09	1.33	2.37	2.66	2.200	0.83
1.60	37.70	0.64	2.08	2.08	1.48	2.31	2.90	2.140	0.74
2.00	30.15	0.71	2.51	3.54	1.64	2.31	3.07	2.380	0.78
2.25	26.65	0.72	2.83	3.93	1.76	2.44	3.21	2.890	0.90
2.67	22.50	0.85	3.43	4.04	1.98	2.32	3.39	3.305	0.97
3.51	17.10	1.04	5.06	4.87	2.51	2.41	3.77	3.945	1/05

[a], data from Schaechter et al ([46]; top 4) and Taheri-Araghi et al ([50]; bottom 7);
[b], calculated $NC = (2^n—1)/n\ln 2$, assuming $C = 40$ min [67];
[c], calculated ratio $oriC/terC = 2^{C/\tau}$ (e.g., [23,22]);
[d], calculated in Zaritsky [65].

over the ranges studied so far, precluding the possibility that *W* is related to the *oriC*/*terC* ratio.

Assuming that *W* is indeed determined by *NC* through a still-unknown mechanism and is proportional to its weighted average, $NC = (2^n—1)/n\ln 2$, approximating cell shape to a perfect cylinder with volume $V = \pi W^2 L/4$, and accepting exponential growth $V = kM_i 2^{(n+m)}$ (where $n = C/\tau$, $m = D/\tau$, and $k = \rho^{-1}$, *ρ being the density*), the cylinder's length *L* would be proportional to $V/W^2 = [n^2 2^{(n+m)}/(2^n—1)^2]$, thus resolving the inconsistency detected earlier [67]. This contradiction resulted of the obviously wrong assumption that *L* is also actively determined by some aspect of the nucleoid; "wrong" because the simple geometrical relation between *V*, *W* and *L* has no more than two degrees of freedom. Table 1 (column 6–8) summarizes also the currently available data for wild-type cells growing at 37 °C, where $C = 40'$ and $D = 20'$. [Under such circumstances, $(C + D) = 60'$ and hence $(n + m)/\tau = \mu$ thus $2^{(n+m)/\tau} = 2^{\mu}$.] The large variation in the ratio between measured/calculated *L* of *S. typhimurium* (top 4) may result of the fact that L_{meas} was also calculated (as in Ref. [65]), but the systematic, substantial increase in faster growing *E. coli* cells (bottom 3) may have been caused by the mode of measurement [50]: cells were grown and measured inside micro-channels and therefore have distorted dimensions due to elasticity of PG [55], that may vary with growth rate. This possibility should be tested in a series of parallel experiments – in batch and in micro-channels. In addition, a second approximation of cell shape (cylinder with two polar caps, hemispherical or ellipsoidal) will be calculated. Alternatively, if *W* is determined for a whole cell division cycle at the time of constriction-initiation, when the assembled divisome starts the division process, a different computation is necessary. The predictions of these possibilities should be examined against experimental results, existing (Table 1) and (sorely missing) to be newly obtained. We encourage bacterial physiologists to perform experiments to this effect and analyze their results accordingly thus testing the hypothesis presented here. Meanwhile, however, a crucial lesson must be learned from this sequence of scientific events: observed correlations between parameters, which frequently expose interesting phenomena (e.g. [13]), may be fortuitous [3] and lead to wrong conclusions.

A resting, non-growing bacterium (at the so-called stationary phase) is smallest, containing a single, non-replicating nucleoid, the complexity of which [$NC (= (2^n—1)/n\ln 2)$] is not trivial to calculate because both rates C^{-1} and τ^{-1} (components of *n*) are equal to zero (times $C = \tau = \infty$). In order to provide a meaning to *n* under these conditions one must consider the limit in the approach to the stationary phase. Since inhibiting mass growth blocks further replication -initiations but ongoing -elongation continue to 'run-out' [28], *n* approaches zero and hence, $NC = \lim_{n\to 0}[\frac{(2^n-1)}{(n \ln 2)}] = \lim_{n\to 0} \frac{2^n \ln 2}{\ln 2} = 1$. This result is consistent with the presence of a single chromosome complement in stationary phase cells. Another open question here concerns the mode of *W*-change during such nutritional shift-down perturbations; would the growth 'history' (τ before the inhibition) affect the final stationary value of *W*? Upon transfer to fresh medium, the cell starts to grow in mass at a rate that corresponds to the medium-quality, and until *Mi* is reached without chromosome replication, during which time *NC* cannot be appropriately calculated because $C = 0$. Such cells are anticipated to overshoot *L* and reach the new steady-state dimensions after 3–4 h, as they do during a classical upshift conditions. We shall not be surprised if *B. subtilis* cells are also wider at short τ or long *C* provided they are kept growing exponentially for much longer because their PG is much thicker [55].

On the other hand, when DNA replication is blocked (by e.g., thymine starvation of *thyA* mutants; [9]), mass continues to grow but nucleoid structure is 'frozen', at least for a while (until DNA breakdown commence) hence its complexity remains identical to the *NC* value at the instant of treatment. This latter situation results, indeed, in filamentation [76], i.e., growth by elongation without change in *W*, as is also predicted by the $NC \rightarrow W$ hypothesis.

The classic nutritional shift-up paradigmatic perturbation has two snags: it (i) causes a large change in cellular gene expression profile [26] thus hampers comparisons between growth states, and (ii) is limited to a maximum number (~2) of replication positions *n* ($= C/\tau$) since *C* (~40 min) = ~ $2\tau_{\min}$ (minimum doubling time of ~20 min), and hence the presumed $l_{\min}$ (minimal distance necessary for an ongoing replisome-set to pass before the next set can actually be initiated) is not reached (and see the "natural" eclipse of [23]). The idea of such a limit was proposed by Zaritsky [66] consequent to results obtained by 'primitive' methods in *thyA* strains of *E. coli*, and substantiated a decade ago by considering various investigations with *dnaA* and other mutants (summarized in Ref. [77]). The ability to modulate *C* with no change in τ by changing the external concentration of thymine supplied [T] to *thyA* mutants [40] overcomes both snags: (i) the medium composition remains identical except for [T], which is anticipated to retain

gene expression profile [26], and (ii) it extends C to levels that bring n to values > 3, thus enabling to test existence of eclipse shorter than ~ 45% chromosome-length Λ and validity of the $NC \rightarrow W$ signal hypothesis. The time-consuming processes required to repair DNA double-strand breaks due to arrest of fork progression and hence restart replication [32] can readily explain the apparent initiation delay caused by "bumping" too-close successive forks under over-expressed *dnaA* (enhancing initiation frequency) or extended C (slowing ongoing replisomes), both shortening the distance between successive replisomes.

1.4. Lack of steady-state under thymine limitation - preliminary analyses

Cultures of *E. coli* strains requiring thymine, which behave according to the BCD model when growing relatively slow (τ_m > ~60 min [69]; or when C is short (with e.g., deoxyguanosime; [4]), do not reach steady-state when growing fast (τ < ~50 min) in media supplemented with low [T] (Fig. 5); the mean inter-division time τ_d is longer than mass doubling time τ_m and hence average cell mass increases with time. In this series of studies, τ_m = ~40 min regardless of [T] and C depends on [T] [68], whereas D is unknown. Preliminary analysis assumes that D does not depend on [T], but higher-order approximations will deal with the way it may change with W and hence, with time (see below).

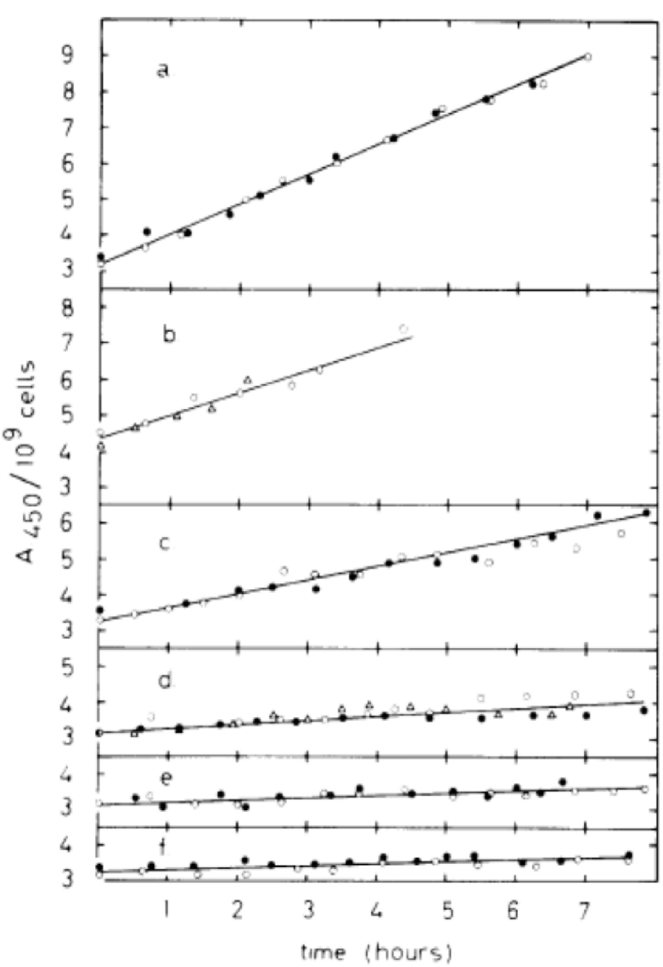

Fig. 5. Rate of increase in average cell mass with time (Adapted from Ref. [69].). *E. coli* strain P178 was grown in glucose M9 containing the following [T] (in μg ml^{-1}): (a) 0.4, (b) 0.5, (c) 1, (d) 2, (e) 5, (f) 30.

The existing data (Fig. 5) is used to test the eclipse hypothesis that a minimum fraction of chromosomal length $l_{\min}/\Lambda$ must be passed by a replisome-set before replication of the next set can actually be initiated [77], irrespective of the mechanism involved. This distance was translated to time units that depend on the rate of replication C^{-1} and on the inter-initiation time τ_m. When $\tau_m/C < l_{\min}/\Lambda$, initiation of each replication cycle, which usually occurs at a constant cell mass per existing *oriC*, *Mi* (or *Vi*) is delayed by (E—τ_m), where E is the time needed for a replisome to reach $l_{\min}$, referred to as the *E*clipse. Under such circumstances, E is equal to the time between two successive initiation events, usually (in wild-type strains) shorter than τ_m. If $E > \tau_m$, the number of cells in the population increase at a slower rate than total mass, so that the average cell mass (<M>) rises with time, exponentially so because $\langle M\rangle(t) = M^{population}(t)/N(t) = M_0 2^{t/\tau m}/2^{t/E} = M_0 2^{(1-\tau m/E)\ (t/\tau m)}$, where t is the time after a rate maintenance period ($C + D$) following transfer to a medium with a lower [T] (resulting in a longer C), M_0 is the average cell mass at $t = 0$, $M^{population}$ is the total mass of the population, and N is the number of cells in the population. Note that for $l_{\min}/\Lambda < \tau_m/C$, the slope is zero. A value for E was calculated for each experiment. The set of simulations of this equation, performed by Po-Yi Ho (pers. commun.), results in a slight decrease of E with increasing [T] for the reported values of C [40], inconsistent with this simplistic model that predicts a constant $l_{\min}/\Lambda$. The calculated E values change less than C whereas the model presumes a linear relationship between the two periods [$E = (l_{\min}/\Lambda)C$]. The significant, systematic change of $l_{\min}/\Lambda$ with higher [T] (shorter C) precludes the validity of this simple model as such; other ideas are proposed and discussed, the validity of which should be examined against existing data and designed experiments.

2. Proposed explanations and future analyses

2.1. Sequestration of Hemi-Methylated DNA

The term Eclipse in this context, of the time needed for the replisome to transit some minimal distance $l_{\min}$ along the chromosome length Λ before re-initiation is allowed at *oriC*, was coined by Kurt Nordstrom [33]; and qualitatively explained by the discoveries that hemi-methylated DNA (i) does not replicate [44] and (b) as soon as it is produced upon replication, it is bound to SeqA for a substantial fraction of the cell cycle, during which *oriC* is not available for re-initiation [35]. Quantitative aspects of DNA sequestration has never been considered as a factor in dimensional determination of the cell except briefly in the framework of thymine limitation, qualitatively so [77]. The sequestration mechanism involved has further been resolved (recently in Ref. [37]), but the unpredictable variability in the value of E in cells growing under different τ's precludes a rigorous, analytic expression of this phenomenon, at least now. The mechanism will anyway have to take into account the longer sequestration times at slower replication rates (at lower [T]'s), and moreover, in a way consistent with all the available data gathered during decades about these physiological conditions.

2.2. Is thymine metabolite involved?

DNA is the only macromolecule to which thymine is incorporated, but thymidine di-phosphate rhamnose is a precursor of rhamnose moiety in the O antigen of lipopolysaccharide LPS in many gram-negative bacteria [52]. It is unlikely that LPS metabolism can affect the rate of dTTP incorporation into DNA thus changing C, but other derivatives of DNA and RNA precursors are

involved in PG biosynthesis; various interactions between the pathways leading to 4 macromolecules (DNA, RNA, PG, LPS) may therefore not be excluded [36]. The current knowledge in this arena is also not sufficient to warrant analytic expressions for these relationships.

2.3. Can cell width be related to the eclipse?

Lacking another mechanism that can predict quantitative aspects of the bizarre phenomenon—continuous rise of cell size (Fig. 5) and diameter [69] under thymine limitation at fast growth rates [78], and to at least partially reconcile this discrepancy (Section 1.4 above), we envision two physiological-plausible possibilities:

(1) Partial loss of viability due to formation of DNA-less cells (*minor contribution*), and
(2) Continuous change in the value of *D* due to slow rise in cell width *W* (*major cause*).

These may not cover all possibilities and do not preclude other causes that may be involved and arise later. [An example for another explanation, ignored here, is connected to the relatively high concentration of *rrn* genes (transcribed to rRNA to compose the backbone of ribosomes), several of them are located near *oriC*, as explained in http://ariehz.weebly.com/uploads/2/9/6/1/29618953/proposel.pdf.]

Each of these two will be dealt with separately and their relative contributions evaluated. An attempt should be made to merge the results to a single solution as best possible with the meagre existing data-set, and further experiments needed to finalize the analysis will be suggested.

(1) *Loss of Viability of DNA-Less Cells*

Under the *E*-existing conditions analyzed here, mean DNA concentration [*G*] (= <G>/<V>), in genome equivalents per unit volume or mass) drops with time at slow DNA replication rate due to cumulative initiation-delays by the Eclipse with no change in mass growth rate. (See *e.g.*, the *E-pre-set* option in the program CCSim http://sils.fnwi.uva.nl/bcb/cellcycle/, explained in Refs. [67,78]). This causes occasional casting off DNA-less cells (can be seen) due to asymmetrical divisions in the monstrous cells developing during a long time under such thymine limitation conditions [70,74], which would decelerate their individual growth rate down to zero (i.e., 'dead'); they do however contribute to the measured mass because they usually do not lyse, hence the real mass growth rate of live cells in the culture is somewhat faster than the measured rate. This may have been undetected in practice likely due to the then-low resolutions of mass and DNA determinations. Percent dead cells can however easily be experimentally determined. When a new set of similar experiments is performed to test the model's predictions, more reliable *C* values will be determined by modern methods (e.g., ratios of *oriC*/*terC* using PCR; [21].

A word of caution must explicitly be expressed here: if even a small proportion of dead cells do lyse, they release their contents to the surrounding medium thus enriching it and hence enhance growth rate of the remaining majority of living cells, at least for a while, thus perturbing the steady-state of exponential growth. This possibility is not simple to even evaluate and would therefore be ignored, at least for the time being.

(2) *NC-Correlation with W, and hence with T and D*

The apparent relationship between cell width *W* and nucleoid complexity *NC* (related to $n = C/\tau_m$) in several unrelated strains (Table 1; [76]) is reflected by increased *W* at either fast growth rate (short τ in rich media) or slow replication rate (long *C* at low [T] in *thyA* mutants). In both cases, increased *NC* is presumed to influence peptidoglycan synthesis through the so-called nucleoid occlusion phenomenon [61,62]: metabolic activities around the nucleoid disturb the polymerization of FtsZ and MreB in the plasma membrane. During steady-state growth, FtsZ-ring assembly in cell center is postponed until the daughter nucleoids have sufficiently separated thus affecting the transertion forces [41]. The FtsZ protofilaments are then stabilized through interactions with ZipA and FtsA (Fig. 7 in Ref. [54]); only after ring-stabilization, the divisome matures to synthesize cell pole PG leading to visible constriction during the so-called *T*-period [6,38,42,58].

Operating mainly in the elongasome [15], MreB forms at the membrane small patches of filaments that surround the cell circumference, probably driven by PG synthesis (Fig. 2 in Ref. [53]). Z-ring stabilization is likely more sensitive to a disturbance by transertion-activities, but MreB complexes may also be hampered by a presumed sudden rise, soon after replication-initiation, of expressing secreted proteins-encoded genes that are located near *oriC* (to be analyzed *ala* [56], causing elongation to slow down and therefore *W* increases accordingly. Similarly, when MreB is titrated away, cells slow elongation without changing mass growth rate hence increase *W*, and the *D* period increases in parallel [79].

It is noteworthy here that *D* was originally defined as the difference [$(C + D)$—C] without suggesting a mechanism [20]. It remains constant at different growth rates μ, but is proportional to extended *C* at slow growth [18]. The longer *D* values found when *W* increases by titrating *mreB* expression at identical growth rates μ [79] assigns a molecular explanation to at least part of *D*. The question whether the sequence of processes leading to division that seems to inaugurate upon termination of DNA replication (Z-ring assembly, divisome maturation, polar cap formation) is μ-dependent or not has yet to be determined. The *T*-period of many *E. coli* strains growing at different rates is linearly proportional to cell circumference and μ (cf. Fig. 5 in Ref. [76]): "the longer time it would take to synthesize a division ring of a wider cell [growing faster] is compensated by the faster rate at which the ring is synthesized… This correlation is consistent with the finding that, in fast-growing thymine-limited *thyA* mutants that are even wider, [both] the *D* and *T* periods are also longer". Thus, in wider cells at increased growth rates (after nutritional shift-up), the *D*-period remains constant likely because the rates of the processes leading to divisome maturation and division are also increased. Under thymine-limitation at a constant τ_m however, as is studied here, the time to complete the division processes *D* is linearly proportional to septum's circumference [76] hence *D* (and *T*) may similarly depend on *NC* as well. The increased *W* without a change in μ [69] seems to cause relative shortage of FtsZ, which may be the reason for delayed divisions [79]. In a series of experiments [64], rates of cell division were followed in *E. coli* 15T− during growth transitions from low concentrations of thymine to higher concentrations (nicknamed '[T] step-up'). The pre-step rate was maintained for a period, defined as "apparent *D*" that depended on the post-step concentration. The relationship between this apparent *D* to the post-step *C* (Fig. 2 in Ref. [75]) may be related to enhancement of expression from *ftsZ* (or another gene the product of which precedes FtsZ to trigger the division process) following onset of the transition, as other genes do [8].

Fig. 6 summarizes the four currently-studied physiological situations at which cell dimensions (*L* and *W*) change: nutritional shift-up (a), and titrating down MreB (b), FtsZ (c) and [T] (d).

The values of *W* and hence *D* however, do not change abruptly upon stepping-down [T] because the cell modulates its width

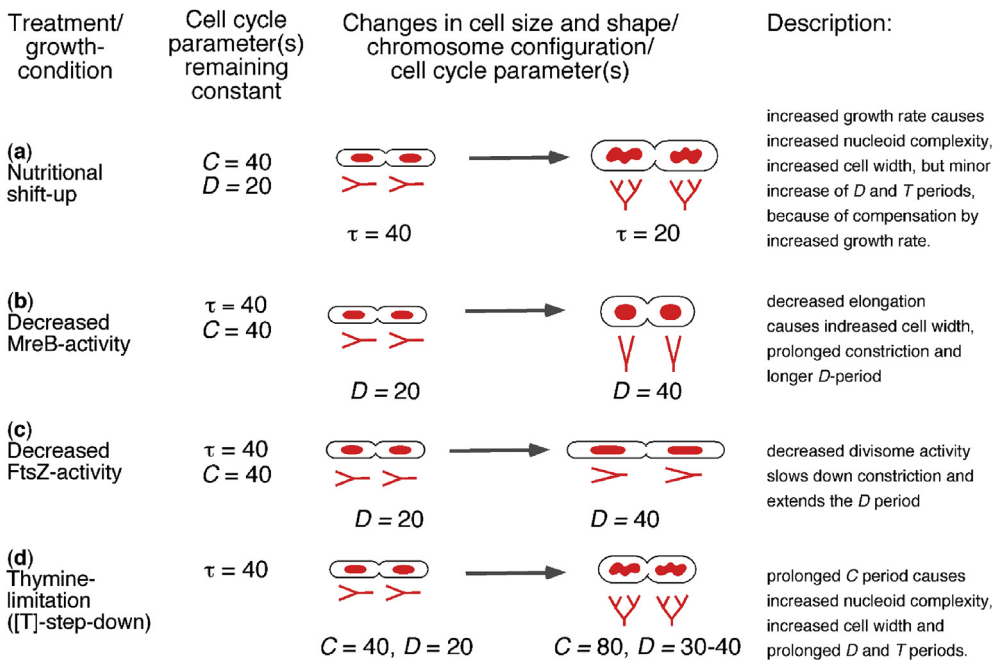

Fig. 6. Schematic overview of 4 physiological states of *E. coli* cells with different sizes, shapes and chromosome configurations. The initial state represents a cell with (τ, C, D) = (40, 40, 20) min. The drawings are only roughly to scale. Note: nucleoid complexity (*NC*) only changes in states (a) and (d), from a chromosome with 4 to one with 8 origins at the end of the cell cycle. For exact chromosome configurations, see Norbert Vischer's CCSim program [67,78]; http://sils.fnwi.uva.nl/bcb/cellcycle/).

during the division process only (Figs. 3 and 4; [59]). The immediate response to slower replication rate (extending C) by reduced [T] is delayed terminations of the ongoing replication cycles and hence the forthcoming divisions. Just as after a nutritional shift-up, when cell mass grows faster without an immediate rise in the divisions frequency ("rate maintenance"), the first reaction to [T]-step-down is increased length, overshooting the final value. Inversely here, the frequency of divisions is lower with no change in growth rate, resulting in delaying the processes at which W expands. The rise in W, and hence of D [58,79], therefore develops during a very long time; the final new steady-state dimensions following shift-up of wild-type *E. coli* cells, from $\tau = 72$ to 24 min for example, takes at least 3 h (Fig. 3), whereas the steady-state for mass is reached within about 1 h [59]. The apparent straight lines in Fig. 5 were obtained after a relatively long cultivation time, but obviously not long enough to reach a steady-state hence we can approximate it to a so-called "quasi-steady-state" with a longer D period.

To complicate matters further, cell branching occurs at later stages under such circumstances, seemingly due to asymmetrical assembly of FtsZ in arcs rather than rings (e.g. [74]), consequent to asymmetrical arrangement of the segregating nucleoid [71]. Our $NC \rightarrow W$ hypothesis, together with existence of $E > \tau_m$, is consistent with branching because the model predicts a maximum value of W (related to the maximum value of NC) when n_{max} is breached; intuitively, it looks as though appearance of branched cells is coincidental with reaching a maximum W—a qualitative observation that should also be tested quantitatively.

3. Evolution of cell dimensions under eclipse-breached conditions

The temporal order of the complex, spatial cell dimensions-modifications reacting to a [T]-step-down at relatively fast growth rate can be summarized as follows:

1. Immediate slowing replication rate (extending C), that
2. Delays both, subsequent (i) divisions due to postponed terminations of ongoing replication cycles and (ii) actual initiations by the existent eclipse that breaches the minimal distance between two successive replisomes, which further delays later divisions (i), and moreover, cumulatively so (item # 7 below);
3. Cell length overshoots because volume (or mass) continues to grow exponentially with delayed change in W until the forthcoming divisomes assemble and ensue the division processes;
4. Cell width rises in the divisome during the divisions only, according to its relationship to the NC;
5. Values of D rise due to wider cells growing at the same rate, thus
6. Delaying further divisions;
7. The sequence of events (items # 2–6) repeats, but with wider cells hence longer D.
   At a certain level after several such iterations,
8. Cell diameter reaches a maximum W_{max} due to the limited NC under conditions of $E > \tau_m$, and
9. Constrictions symmetry is broken resulting in occasional branched and DNA-less cells ((1) above).

During this series of modifications, the cells lose their cylindrical symmetry, bulge in all directions, and later branch (see e.g., Fig. 4 in Ref. [70]). The segregating nucleoids in such wide, almost-spherical cells are arranged as tetrahedron-heads to maximize the distances between themselves (Fig. 6 in Ref. [74]).

Reverting the long thymine-limitation by restoring high [T] dramatically enhances division frequency and restores normal-sized cells [78], strongly supporting a prediction of this model, that the 'deficit' in actual initiations caused by breaching the maximum possible n, is 'paid back' due to enhanced rate of DNA replication (short C). At least 5 simultaneous divisions during 100 min ($2.5 \times \tau_m$) seem to reflect the minimal distance possible between successive replisomes, about half chromosome-length Λ (translated to 20 min under C of 40 min). More direct means to confirm our complicated model are needed, of course.

4. Concluding remarks

The idea that a *primary signal* for the PG-synthesizing systems to switch from elongasome to divisome, necessarily emitted from the nucleoid to couple its replication to cell division while ensuring equal distribution to daughter cells upon division is physical rather than a protein, is further developed and explored here. Slowing DNA replication by thymine-limiting *thyA* mutants without a change in cell mass growth rate serves as a powerful tool to manipulate, in a reversible manner, the presumed signal involved, breaching physical limits that have evolved during millennia.

The intricate model detailed here to explain a set of strange, seemingly not related, existing observations is highly speculative at the moment. The intertwined series of inherent assumptions that lead to likely conclusions, requires many tests of the predictions by independent experimental regimes, some of which are mentioned. Profiles of genetic marker frequency gradients, transcriptomic and proteomic of cells growing fast during long periods under thymine-limitation (titrating the *C* period), as well as values of *T*, *D*, DNA/mass ratios and % dead cells, are just a few examples mentioned above of necessary (though not sufficient) parameters to be obtained.

A word of caution is appropriate here: One lesson from this study is that any perturbation to a steady-state exponentially growing culture, by a drug or well-defined mutation, opens up a whole series of physiological changes (a Pandora box?) that reflect modifications of biochemical and genetic circuits and interactions between them such as described here, with merely a simple mutation apparently affecting a single reaction: producing thymine nucleotide and channeling it to DNA replication.

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