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MINIREVIEW

Use of Thymine Limitation and Thymine Starvation To Study Bacterial Physiology and Cytology†

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A variety of very basic questions asked 30 to 40 years ago remain unanswered today. How do bacteria select and maintain a defined size, length, and width? How do they control the timing of chromosome replication? What is the mechanism by which they segregate their replicated (or replicating) chromosomes? How do they know where and when to divide? From the literature it is apparent that there is resurgence in studies aimed at answering these important questions. While much progress has been made, we still have a ways to go. This minireview revisits an old technique (thymine limitation) in a new light and illustrates how one can use this technique to manipulate some of the parameters of the cell cycle under balanced growth conditions, which should be helpful in addressing some of the above questions. Hopefully this review will spawn new interest and ideas about the old questions that can be tested by this technique.

Ever since the first thymine-requiring (so-called thymineless) strain of *Escherichia coli* was isolated in 1947 (described in reference 94), *thyA* mutants have been employed to follow DNA synthesis in vivo (60). Since thymine is a precursor of DNA only, radioactive isotope-labeled thymine and scintillation counters are used to track synthesis in bacteria and their viruses. Before the semiconservative nature of replication predicted by the double-helix model was demonstrated, running density gradients of DNA labeled with the heavy thymine analogue 5′-bromodeoxyuridine had been considered (43). However, saving on radiolabeled thymine isotope by using exceedingly low concentrations during the labeling period (for example, see reference 61) led to some flawed conclusions and discrepancies (for example, see reference 55). These were resolved by systematic investigations of pool sizes of thymine metabolites and of the rate of chromosome replication in relation to the external concentrations of thymine (for example, see reference 85). Those studies and their usefulness in getting to understand the composition, structure, and function of the bacterial cell are summarized here. This issue is of particular current importance, because the distinction between the two completely different physiological states of "thymine starvation" and "thymine limitation" has become somewhat vague (for example, see references 24 and 134). It is crucial to realize that the former is a pathological state of the cell, whereas the latter is not (Table 1). Thymine limitation is used as a means to dissociate the rate of DNA replication from the culture growth rate, i.e., to change the relative schedule of the replication and division cycles. This technique to dissociate the two rates differs from the classical method of nutritional shifts, because it does not affect the major metabolic pathways prevailing in the cell. To understand this method, one must know the unique modes by which thymine is metabolized. The earlier review by Ahmad et al. (2) presents an excellent account of the metabolic pathways involving thymine for both prokaryotes and eukaryotes (see the summary in Fig. 1).

Bacillus subtilis has not evolved an active transport mechanism to pump thymine from the environment (92), and indirect results demonstrate that this is the case for *Escherichia coli* as well (88). The passive, sheer diffusion-driven uptake of thymine causes its pool size, as well as the levels of its metabolites, to depend on the extracellular concentration (6). Changing the thymine concentration supplied in the growth medium thus changes the step-time in DNA synthesis (63) and the corresponding rate of chromosome replication (88), $1/C$ (44). This minireview describes (i) why the so-called "thymine limitation" state is totally different physiologically from that of "thymine starvation," the latter being caused by the complete removal of thymine from the medium, which blocks DNA synthesis entirely in *thyA* mutants, and (ii) how both methods can be exploited as powerful tools to study bacterial DNA and its interactions with other essential metabolic and structural aspects of cell physiology. These points are described below in the context of unanswered questions that relate DNA metabolism to the bacterial cell cycle, physiology, and cytology.

THYMINE STARVATION, THYMINE-LESS DEATH (TLD), AND THEIR USE IN BACTERIOLOGY

When a typical thymine auxotroph is deprived of thymine in an otherwise complete medium, its mass continues to increase in an "unbalanced" fashion (see the definition in reference 36) by synthesizing RNA and protein but not DNA for awhile (the duration of this period is strain dependent [23]). The cells stop dividing, thus forming prominent filaments of various lengths (5) that exponentially lose colony-forming ability on agar plates (19) but recover if thymine is added back before plating (known as the "liquid holding recovery" phenomenon [32]). The extent of kill-

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† Dedicated to Bob Pritchard on his 76th birthday.

TABLE 1. Major differences in cell physiological parameters reacting to thymine starvation and to thymine limitation

Phenomenon or parameter	Thymine limitation	Thymine starvation
Chromosome replication	*C* time varies between 40 and 120 min	Stops completely and indefinitely
Rate of culture mass growth	Identical indefinitely	Identical for ~2τ, then drops gradually
Rate of cell division	Identical after "rate maintenance" delay	Stops completely after a delay of *D* minutes
Colony-forming ability	Identical to the number of visible cells	Drops exponentially after a delay
Cell size, mass, volume, protein, RNA content	Larger; a function of $2^{(C+D)/\tau}$	Larger; a function of starvation period and growth rate
Cell dimentions (length and diameter)	Both increase, with significant branching	Filementation without diameter increase
Nucleoid position	Distributed around all cell surfaces	Extended along mid-cell
Nucleoid structure and complexity	Multiforked; function of $n = C/\tau$	Extended with lobules
Amount of DNA per cell	Increases as $(\ln 2/n)[2^{(C+D)/\tau} - 2^{D/\tau}]$	Remains constant after division inhibition
DNA concentration (DNA/mass)	Decreases as $(\ln 2/n)(1 - 2^{-n})$	Decreases with time (as inverse of cell size)
Amt of DNA per nucleoid	Increases as $[2^{(n-1)}](\ln 2/n)$	Remains constant
Time *D* from termination to division	Rises in proportion to *n* and cell diameter	Not applicable (no divisions)

ing is retarded by concomitant inhibition of protein synthesis (60), supporting the idea that plating efficiency is low because the cells are filamentous (see below). This so-called thymineless death (TLD) phenomenon seems to be widespread in the bacterial world beyond *E. coli*: by 1998, it had been observed in at least 8 gram-negative and 10 gram-positive species in addition to *Bacillus subtilis*, as well as in *Saccharomyces cerevisiae*, mammalian cells, and even in *thyA E. coli* infected with phage T4 (which encodes its own thymidylate synthetase) (Table 1 in reference 2).

A multitude of reactions to thymine starvation associated with TLD can be classified as molecular or cellular (see reference 2). Among the molecular reactions are mutagenesis, induction of harbored prophages, and curing of plasmids, as well as breakdown, structural changes, and undermethylation of DNA. Genetic recombination is enhanced, and nonconservative, repair replication takes place. On the cellular level, the SOS response is triggered (119), and SOS-independent cell division genes are repressed (58). TLD is energy dependent and was attributed to irreversible lesions induced under "unbalanced growth" (19). It has recently been reported (103) that a temperature sensitive mutant affected in the *cydA* gene, controlling cytochrome *bd* oxidase, protects a Δ*thy* strain from TLD under restrictive conditions without suppressing filamentation, but it does so only on rich Luria-Bertani (LB) medium. This observation links the respiratory pathways with cell survival and cytology, an association that should be further investigated and clarified. The apparent dissociations between TLD and UV sensitivity (22) or filamentation (38) do not ease the task of finding a common mechanism presumed for all of the phenomena associated with TLD. However, this is not the aim of this minireview.

In genetic studies, TLD has been exploited to enrich for double mutants (117), to increase the frequency of plasmid curing (111), and to study the physiological consequences of its relationships with *polA* (absence of lag period [7]) and temperature sensitive *dna* mutants (higher survival of initiation but not elongation mutants at the restrictive temperatures [12]). In a search for *E. coli* mutants resistant to TLD, *recQ* was discovered and characterized as more sensitive to UV and deficient in recombination (72). Helicases homologous to RecQ were later identified in eukaryotes and implicated in human hereditary diseases (54). The method devised to synchronize cell

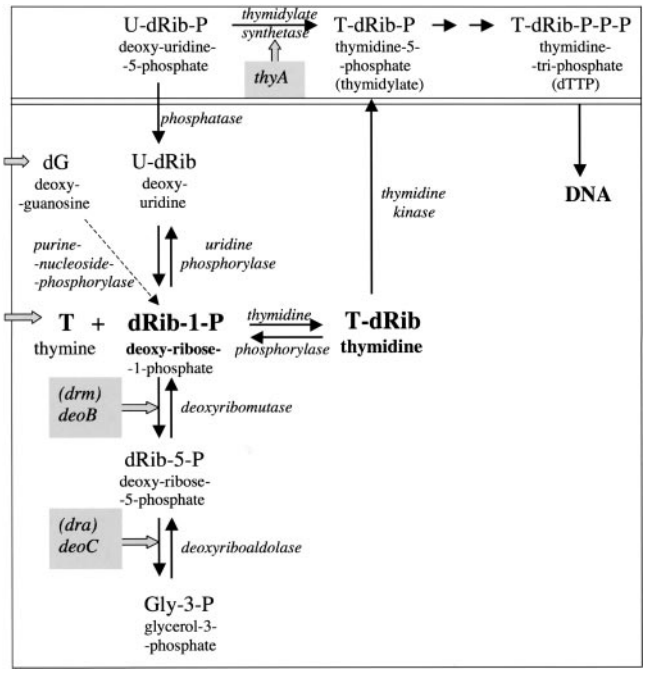

FIG. 1. The wild-type (top) and salvage (bottom) pathways for thymidylate synthesis. (Adapted from references 2, 75, and 85.) Wild-type species of bacteria are unable to pump thymine into the cells (92) and to incorporate it into DNA. Thy$^-$ auxotrophs lack thymidylate synthetase activity and, hence, cannot produce thymidine nucleotides by the natural route (top panel), while they gained the ability to incorporate thymine into DNA by condensing it with deoxyribose-1-phosphate (dRib-1-P) to thymidine, a reaction catalyzed by thymidine phosphorylase (bottom). The negligible pool of dRib-1-P in *thy*$^+$ cells that limits this condensation increases substantially in size in *thyA* mutants, since they accumulate U-dRib-P, which is degraded into dRib-1-P through U-dRib. Addition of a nucleoside such as 2′-deoxyguanosine (dG) similarly results in a wild-type pool size of dRib-1-P (6). Blocking further catabolism of the latter by *deoB* or *deoC* mutation results in a still higher pool size and, hence, lower thymine concentrations (2 rather than 20 μg ml^{-1}) are sufficient to support growth of *thyA* strains carrying at least one of these mutations; such double or triple mutants are "thymine-low-requirers." At least two additional mutations affect the regulation of thymidine phosphorylase to convert the cell to a "super-low requirer" (can grow on concentrations as low as 0.2 μg ml^{-1}). Mutants lacking thymidine phosphorylase cannot utilize thymine, and those lacking thymidine kinase fail to incorporate thymidine as well.

divisions by thymine starvation (4), on the other hand, was later shown to be a false synchrony (20), because cells continue to grow and hence to accumulate capacity for replication initiation during starvation (86, 120).

More recently, it was claimed that the regulatable chromosomal suicide module of *E. coli*, *mazEF*, which is triggered by various stress conditions, is also activated by thymine starvation and mediates TLD as well (99). Whether bacterial TLD indeed "has implications for both mammalian TLD and cancer research," as these authors propose, remains a moot question and is still under investigation (39). Thymine starvation has even been exploited to study experimental evolution during long-term adaptation to metabolic constraints (24). Dissociation between plasmid clustering and DNA synthesis or superhelicity has recently been achieved by disruption of localization but not clustering of plasmid RK2 in thymine-starved or gyrase-inhibited cells (46). All the above show that studies with thymine starvation and TLD are still not exhausted and how they can be exploited to better understand various aspects of bacterial cell physiology.

The complex and varied phenomena that the cell displays under complete removal of thymine from the medium, culminating in loss of colony-forming ability, are avoided by supplying it with limiting concentrations, as described below.

THYMINE LIMITATION AND ASSOCIATED PHYSIOLOGICAL CHANGES

In contrast to filamentation and loss of colony-forming ability when starved of thymine, it is crucial to realize that *thy* strains continue to grow indefinitely in a balanced mode, provided the external thymine concentration is above the minimal required according to its genetic background (Fig. 1) and environmental conditions. The rate at which a replication fork traverses the chromosome to complete a round from initiation to termination is slower at lower concentrations due to lower pool sizes of thymine metabolites that are DNA precursors. External concentrations of thymine that enters the cell by diffusion alone thus result in modified levels of nucleotides and rates of chromosome replication, with implications for the physiology of the bacterium (Table 1). Cellular changes occurring under thymine limitation resemble those taking place when growth rate is modified by the medium richness but avoid the marked difference in metabolic pathways in the latter case (see below).

Historical perspective. The extensive series of experiments of the so-called Copenhagen school that opened up the bacterial physiology field as a separate discipline (47, 100) showed how cell size and macromolecular composition of *Salmonella enterica* serovar Typhimurium (closely related to *E. coli*) vary systematically with the doubling time τ, manipulated by modifying the medium composition. Soon after the *E. coli* chromosome was demonstrated to be a closed circle (15, 130), "dichotomous replication" of the *B. subtilis* chromosome (i.e., containing multiple replication forks) was shown by measuring the frequencies of genetic markers in germinating spores (79) and in fast-growing cells in rich media (133). Initiation of replication before termination of the preceding cycle (unjustifiably named "pre-mature") was simultaneously exhibited in thymine-starved *E. coli* cells (86). This apparent peculiarity was later also demonstrated in fast-growing *E. coli* (44) (Fig. 2 and 3). It clearly distinguishes the replication pattern of fast-growing prokaryotes from that in eukaryotes.

Some peculiarities of DNA synthesis in cells grown on very low thymine concentrations, dissimilar to those observed when cells were completely deprived of thymine, were recorded in the 1960s (61, 62) but could not be understood until the Cooper-Helmstetter model for the cell cycle was conceived (44). The essence of the model (Fig. 2 and 3) is dissociation between the replication rate ($1/C$) and cell growth rate μ ($1/\tau$): the chromosome replication time C was found to be invariant ($\sim$40 min at 37°C) with growth rates μ greater than 0.85 h^{-1} ($\tau < 70$ min). Thymine limitation was designed, investigated, and implemented soon afterwards (85, 88, 139) to resolve inconsistencies in previous studies. The dissociation between C and τ enabled one further step, envisaging that these discrepancies could be settled by assuming that C can also vary according to the concentration of the external thymine supplied in the medium of *thyA* mutants. This hypothesis was corroborated in the following five independent ways, each based on a different set of assumptions: (i) percent residual DNA synthesized (ΔG) during inhibition of protein synthesis (so-called "run out," originally quantified in reference 104); (ii) average cell DNA concentrations (G/M; i.e., DNA/mass ratio) prevailing in the cultures growing in balanced states (both in reference 88, 139); (iii) degree of stimulation in the rate of DNA synthesis following inhibition lasting one τ (136); (iv) values of the cell cycle parameters measured directly in synchronous thymine-limited cultures (66); and (v) flow cytometry analyses (69).

The systematic changes in cell size and macromolecular composition with τ, manipulated by modifying the medium composition (62), is associated with substantial differences in the metabolic pattern between the various steady states of exponentially growing cultures (106). The changes occurring under thymine limitation, on the other hand, are independent of major metabolic variations, because the medium remains identical, except for the concentration of supplemented thymine. The changes in cell features are therefore a consequence of varied replication rate (88) and nucleotide pool sizes (6, 75, 78) and cannot be attributed to another primary cause. The only thymine metabolites other than those leading to DNA are dTDP sugars involved with synthesis of cell envelope antigens (77, 78); these may link cell shape to DNA replication (discussed in reference 138), but further studies are needed to clarify the possible connection (see below).

One crucial importance of the Cooper-Helmstetter model of the cell cycle (44) is that it can explain the size and DNA content of cells growing at different rates, as observed a decade earlier (100) (Fig. 3), with the additional corollary that cell mass (or volume) at the time of replication initiation is practically constant per chromosome origin (31, 87; but also see references 10, 120, and 148). Both modes of dissociation between rates of growth (μ) and of chromosome replication ($1/C$), by modifying one and not the other (reference 44 for the former; reference 88 for the latter), are complementary and reach the same conclusions. They form a sound quantitative framework for predicting cell sizes in steady states of exponential growth with various cycle parameters (τ, C, time between

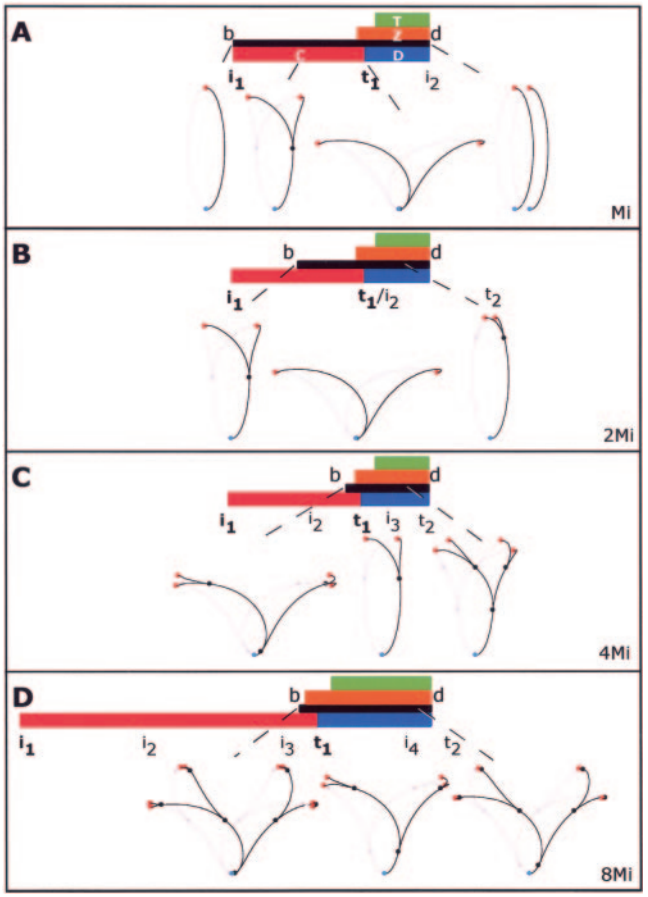

FIG. 2. Bacterial cell cycle, based on the Cooper-Helmstetter model (44), with additional data obtained in various laboratories, as follows. A cell initiates chromosome replication at *oriC* when it reaches a constant mass/*oriC* (M_i), terminates replication at *terC* after *C* min (red bar), and divides *D* min later (blue bar). The time spans to division from birth (τ) and from appearance of constriction (*T* [124]) and FtsZ ring (*Z* [26]) are depicted as black, green, and orange bars, respectively. The times of cell birth and division, and of initiation and termination of its chromosome replication in the current cycle, are labeled b, d, i_1, and t_1, respectively. The examples displayed are each of a newborn cell growing steadily with the following specific series of cell cycle parameters (τ, *C*, *D*, *T*, and *Z*, respectively): 60, 40, 20, 16, and 22 in A; 40, 40, 20, 16, and 22 in B; 25, 40, 20, 16, and 22 in C; and 40, 90, 35, 30, and 38 in D. In practice, A to C are obtained by nutritional changes, and D is obtained by thymine limitation. In conditions when cell cycles overlap, that is $\tau < N(C + D)$, and *N* is a positive, non-zero integer, initiation or termination occurs at the -*N*th cycle and the following events are labeled successively i_n and t_n. Under such circumstances, cell mass at initiation is a $2^{(N-1)}$ multiple of M_i (shown at the bottom right of each panel). Schematic chromosome structures at different cell cycle stages are shown, with relative positions of *oriC* (red dot), *terC* (blue dot), and replicating forks (black or gray dot, depending on its 3-dimensional position on the circle, viewed at an angle of 72.5° [perspective of 0.3]). Given are the structures at cell birth b (equivalent to one of two at division d [shown in panel A only]), during replication or at termination (in panels A and B), as well as between t_1 and i_3 or i_4 and between i_3/i_4 and d (in panels C and D, respectively).

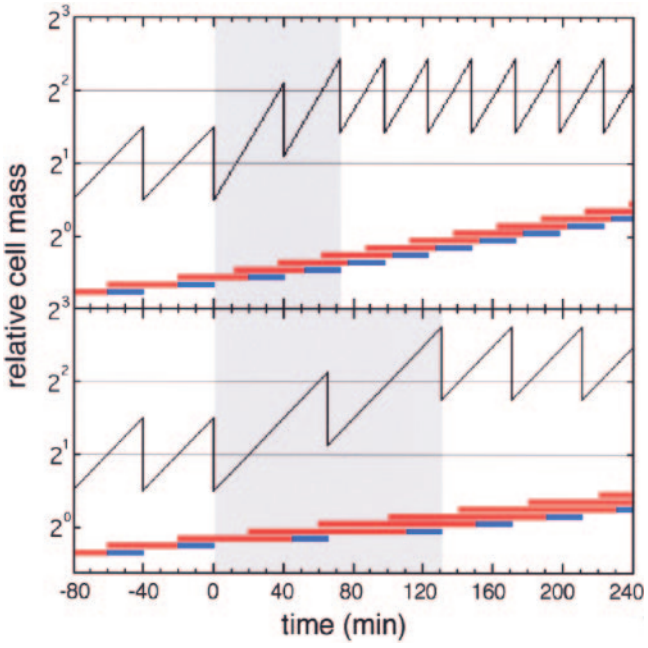

FIG. 3. Relative mass of a newborn cell during its cycle and transitions (at time zero; all in minutes) from growth conditions of $\tau = 40$, $C = 40$, and $D = 20$ to growth with $\tau = 25$, $C = 40$, and $D = 20$ (shift-up; top panel) and to growth with the same rate ($\tau = 40$) and $D = 20$ but $C = 90$ (step-down; bottom panel). Colors of bars conform to those in Fig. 2. Gray areas indicate the transition periods. Horizontal lines indicate $2^{(N-1)}$ multiples of initiation mass M_i (see the legend to Fig. 2). (Adapted from the schematic online simulation of Norbert Vischer, which is freely available at http://simon.bio.uva.nl.)

termination of a round of replication and cell division [*D*], and mass per number of *oriC* copies at the time of initiation M_i]) and during transitions between them (Fig. 2 and 3).

Thymine limitation was later extended both theoretically (13) and experimentally (17, 68, 89) and exploited to study the following aspects of cell physiology (in rough chronological order): (i) bidirectionality of chromosome replication (11); (ii) dependence of *D* (Fig. 2) on *C* (66, 140, 146); (iii) changes in cell shape with rate of chromosome replication (67, 137, 140, 141); (iv) kinetics of mutagenicity (29); (v) dependence of constitutive gene output at different DNA concentrations and relative gene dosages (16, 18); (vi) control of plasmid replication (81, 131); (vii) the minimum delay time (so-called "eclipse" period) before a round of replication can start following the start of the previous round (69, 136); (viii) changes in cell mass at initiation of replication (14); (ix) nucleoid segregation and occlusion in localization of the division plane (129, 143, 145); (x) changes in multiplication parameters of the virulent phage T4 (42); (xi) localization of replication forks by SeqA foci distribution (69). Our current investigation of how thymine limitation affects the kinetics of TLD may shed additional light on the difference between the two states and, furthermore, on the elusive mechanism of TLD (to be published elsewhere).

Thymine limitation, which subtly manipulates cell changes by varying the concentration of a small molecule, can thus be used as a tool to study many aspects of cell physiology and cytology. Relationships between the replication state of the chromosome and nucleoid position on the one hand and the plane of cell division as well as cell dimensions on the other hand are given particular attention below.

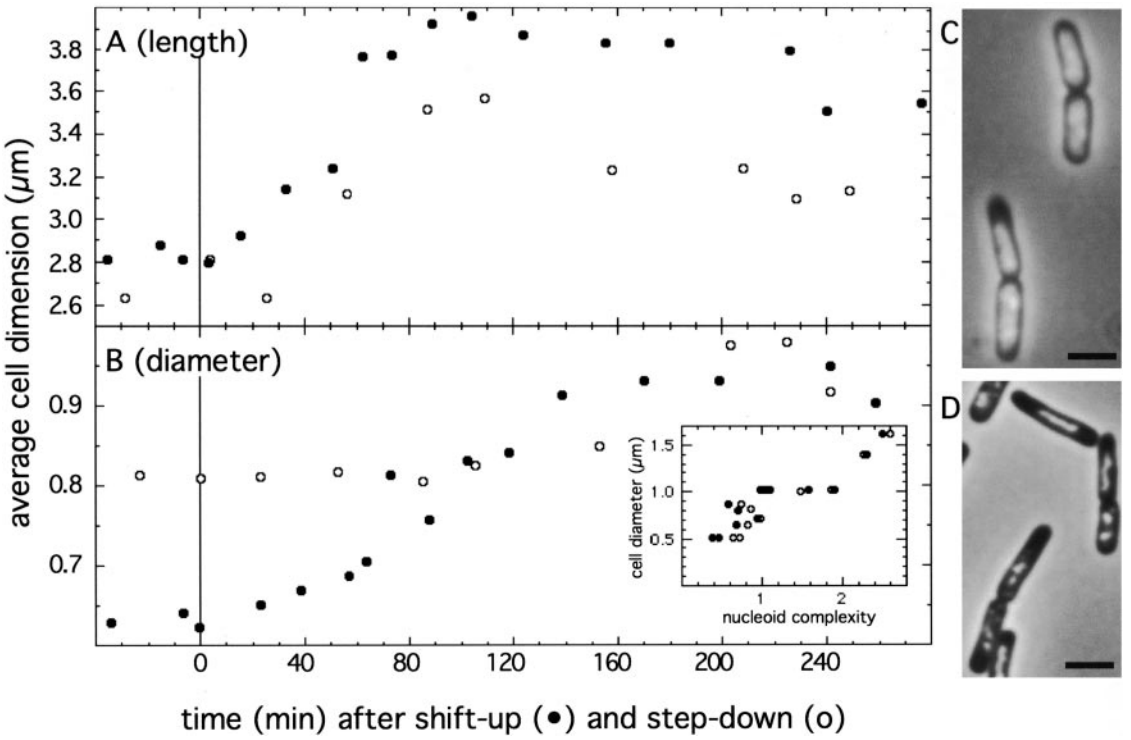

FIG. 4. Changes of relative average cell length (A) and diameter (B) during nutritional shift-up (filled circles) and thymine step-down (open circles) of cultures of *E. coli* B/r strain H266 (125) and K-12 strain CR34 (*thr-1 leuB6 thi thyA drm* [141–143]), respectively. Shift-up was performed from alanine-proline to glucose-Casa minimal medium (τ_1 = 72 min; τ_2 = 24 min). Step-down in glucose minimal medium (τ of ca. 53 min) from 10 μg thymine ml^{-1} and 100 μg ml^{-1} dG (yielding a C_1 of ~50 min) to 2.5 μg ml^{-1} thymine and no dG (by washing off thymine and dG using filtration and resuspending the cells in the same medium but a low [thymine] yielding C_2 of about 90 min). Culture densities were kept below A_{450} of 0.5 by successive four- to sixfold dilutions with prewarmed medium. (C and D) Cells were photographed at 180 min of the shift-up and step-down transitions of *E. coli* CR34, respectively. Bars, 2 μm. (Inset) Average cell diameter in various *E. coli* strains as a function of the nucleoid complexity (C/τ; filled circles) or size (DNA in genome equivalent units per *terC*; empty circles).

CELL DIMENSIONS AND NUCLEOID COMPLEXITY

The first clue that bacterial cell width changes with growth rate, observed in 1958 (100), was substantiated in a systematic way using appropriate equipment some 20 years later (121, 124). During the cell cycle, however, diameter changes very slightly (113). Differences in growth rates between mass and length (40, 85) or surface (95, 96) have been used to explain the large changes in cell diameter during transitions between growth rates (nutritional shifts [62]). The ratio of surface to mass (or surface to volume, because cell density remains constant [3]) is smaller when cells are wider, as during growth in richer media. The so-called linear-log series of models (for example, see references 85 and 144) are based on the fact that surface extension is linear and mass is synthesized exponentially. In these models, which have since been modified considerably (see below), emphasis was devoted to discrete events that happen during the cell cycle. Thus, peptidoglycan synthesis was assumed to proceed at a constant rate and to double once per cycle. The time of this doubling was linked to a discrete event or singularity in the cell: initiation of chromosome replication (33, 34) termination (141), or the replication fork (83). The differences between the presumed event or structure depends also on whether the rate of peptidoglycan synthesis per site varies according to the bacterial growth rate and whether the presumed growth zone is finite in existence (as is the replication fork) or goes on indefinitely (as with *oriC* or *terC*).

During thymine starvation, rod-shaped Thy$^-$ strains filament (5), largely maintaining cell diameter. It was therefore instructive to discover that at lower thymine concentrations, their increased volume is accommodated by increased width as well as length (140), as observed for faster growing cells. This discovery reinforced the ideas that couple cell dimensions to the chromosome replication cycle. Another encouraging observation was that cell length overshoots during the transition between slow-to-fast growth rates ("nutritional shift-up" [47]) and slowly approaches the new steady-state value at the same time as does cell diameter (125, 144) (Fig. 4, filled circles). A similar approach to the new steady-state cell dimensions was observed when thymine-limited cells are transferred from high to low thymine concentration ("stepped-down" [88]), both concentrations above the minimum required for normal growth (142) (Fig. 4, empty circles). The slower approach during step down than during shift up is due to the longer $C + D$ value under this transition and conforms to the Cooper-Helmstetter model (compare the top and bottom portions of Fig. 3). All these observations together led to a common explanation (127, 137) that couples cell diameter to the chromosome "complexity" (Fig. 4, inset): the common denominators for both cases are larger numbers of replication "positions" (originally de-

fined in reference 101), $n = C/\tau$ (filled circles), and larger amounts of the related DNA per nucleoid (127); G/terminus = $(\tau/C \cdot \ln 2) \cdot \{2^{[(C/\tau) - 1]}\} = 2^{(n-1)} \cdot (\ln 2/n)$ (empty circles). Both cell types, fast growing (with short τ and identical C [44]) and slow replicating (with long C and identical τ [88]), contain multiple-forked chromosomes. Under these circumstances, the number of simultaneously segregating origins in each chromosome (2^n) cannot exceed 4 in fastest growing Thy$^+$ cells (C = 40 min; τ = 20 min) but can exceed 8 in thymine-limited *thy* mutants (e.g., C = 90 min, $\tau < 30$ min). The real difference is even larger in practice, because in Thy$^+$ cells n does not exceed 3.14 ($2^{1.65}$ [10]). A simple, dynamic demonstration of the different situations in steady state of exponential growth and during transitions between such states can be seen and explicitly performed online at the interactive website of Norbert Vischer (http://simon.bio.uva.nl) (Fig. 2 and 3).

The "rate maintenance" phenomenon (where the rate of cell division does not change for a period of $C + D$ [44]), which had been disclosed by the "nutritional shift-up" regimen (47), was confirmed by the "thymine step-down" regimen (140). Similarly, the overshoot in cell length and slow approach to new steady-state dimensions (124) displayed during the former transition was confirmed during the latter transition as well (142) (Fig. 4A). The increase in cell diameter during both of these transitions (Fig. 4B) is not homogeneous along the cylinder: it starts at the site of constriction, where no nucleoid material is present, thus inducing tapered cells (125 and Fig. 4C and D, respectively). This seems to couple peptidoglycan synthesis and cell dimensions to nucleoid position and shapes.

The idea that cell diameter is correlated with nucleoid complexity, whereas length is passively determined by it and by the rate of volume growth, results in the interesting corollary that the aspect ratio (length/diameter) observed when wild-type cells are grown on different media is almost constant (124, 137).

A mechanism to account for the influence of nucleoid complexity on cell diameter was envisaged by the "nucleoid occlusion model" (123, 127, 128). It presumes that intense cytoplasmic activities (transcription and translation) around the nucleoid result in slower local rates of peptidoglycan synthesis (70, 71), thereby inhibiting constriction and hence influencing cell dimensions. Changes in cell shape associated with changes in appearance of the nucleoid that occur during thymine limitation are considered below and may be helpful in deciphering this phenomenon.

PLACEMENT OF DIVISION ARC/RING/PLANE BY TRANSERTION

Structure and segregation of the nucleoid has attracted the interest of bacteriologists ever since it was given serious attention as an "organelle" (reviewed in reference 93). The nucleoid can be visualized by light (56, 82) or electron microscopy (98) in fixed cells and by phase-contrast (65), confocal (114), or fluorescence microscopy (108) in vivo. Nucleoid structure and interactions with surrounding molecules and organelles (ribosomes, membrane, etc.) have recently been reviewed (123). The implications for placing the constriction (in gram-negative species) or septum (in gram positives) between newly segregated nucleoids are addressed below with the aid of the thymine-limitation method (for example, see reference 145).

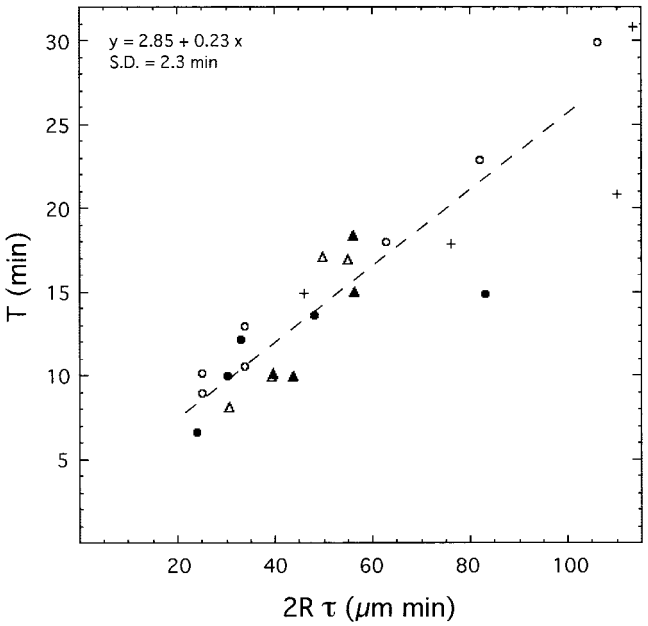

FIG. 5. Time before cell separation when constriction is visible (T min) as a function of cell diameter ($2R$) and doubling time (τ) in *E. coli* B/r strains A (filled circles) and K (empty circles) (124), in *E. coli* K12 strain CR34 (filled triangles) and its *repA* derivative (empty triangles) (141), and in *E. coli* K-12 strain LMC 500 (crosses) (1). Values of T were calculated from the percent of constricted cells visible in either a transmission electron microscope or a light microscope (as in reference 124 or 1, respectively) of steady-state growing cultures. Regression line and function was calculated with the lower 20 data points. S.D., standard deviation.

The first sign of the division process observed by phase contrast microscopy is a constriction or septum (in gram-negative or -positive species, respectively). The time T between this operational event and separation of the daughters is constant, ~12 min (in fast-growing *E. coli* cells at 37°C [124]). T seems to be determined by the cell circumference ($2\pi R$, where R is cell radius) and growth rate μ; the longer time it would take to synthesize a division ring of a wider cell is compensated by the faster rate at which the ring is synthesized. Since R is proportional to μ (40, 137), T turns out to be proportional to $2\pi R/\mu$ (or $2\pi R\tau$; Fig. 5). This correlation is consistent with the finding that, in fast-growing thymine-limited *thyA* mutants that are even wider (Fig. 6A and C), the D and T periods are also longer (146; see also Fig. 2 to 5). At a certain point, when cell diameter becomes almost as great as cell length under normal conditions, this balance seems to break down, and the cell branches by splitting the pole (138, 141) (Fig. 6B and F). Moreover, if both poles split simultaneously, the two branches seem to be tilted (see also Fig. 4 in reference 145, Fig. 3 in reference 80, and Fig. 2 in reference 143), as anticipated from the arrangement of the four nucleoids in the left-hand cell in Fig. 6C, predicted by the nucleoid occlusion model and discussed in reference 145). It is also consistent with lack of overlap between FtsZ rings or arcs and segregating nucleoids in spheroidal cells (Fig. 6D) using simultaneously the fluorochromatic molecules FtsZ-GFP and 4′,6′-diamidino-2-phenylindole (DAPI) (Fig. 1 in reference 143).

Genetic fusion of fluorescent proteins to FtsZ (59) and other

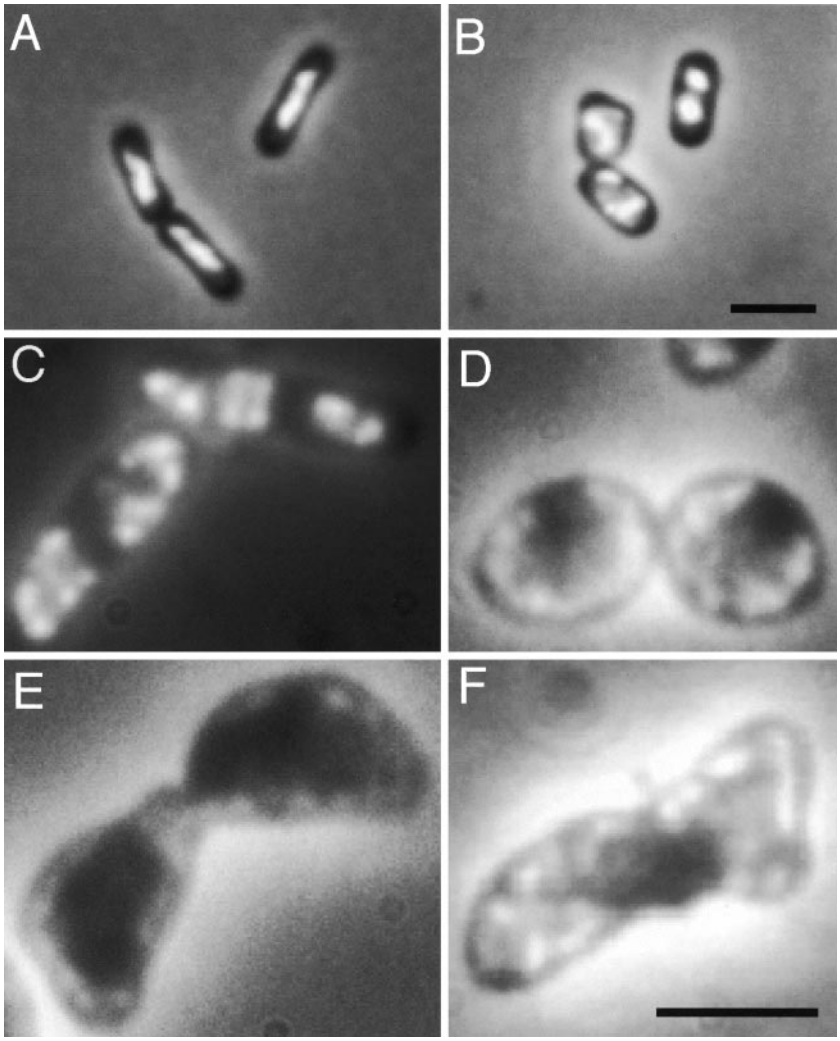

FIG. 6. Cell shapes and nucleoid arrangements under thymine limitation. Steady-state, exponentially growing *E. coli* K-12 cultures of strain CR34 (Fig. 4 and 5) in glucose minimal medium ($\tau = 60$ min) supplemented with (A) 10 μg ml^{-1} thymine and 20 μg ml^{-1} dG or (B) 0.4 μg ml^{-1} thymine without dG. (C to F) The same cells (selected from Fig. 3 in reference 145) growing exponentially in the same minimal medium, but supplemented with 1% Casamino Acids and 5 μg thymine ml^{-1} without dG (τ of ~30 min) (C), after 60 min of amdinocillin (1 μg ml^{-1}) treatment (D), and 60 min after washing off the drug and adding 15 μg ml^{-1} thymine and 100 μg ml^{-1} dG (E and F). Nucleoids labeled with 10 μM DAPI (4′,6′-diamidino-2-phenylindone dihydrochloridehydrate) are distributed along cell surface in "parachute"-like structures. Splitting tips by secondary constrictions are seen in panels B and F. Bars, 2 μm (A and B) and 5 μm (C to F).

proteins used to visualize the division ring in situ has been combined with the classical methods to investigate the timing of FtsZ ring assembly, thus tracing the division process back to its inception. This process starts shortly before termination of chromosome replication (26). It may be exploited together with thymine limitation to dissect more accurately the assembly of the "divisome" proteins (73). Manipulating the rate of replication rather than of growth rate is more informative because it changes *C* only without changing the general metabolism (as media do). The two-step model (1), in which mid-cell recruitment of proteins downstream of FtsK is substantially delayed over FtsZ ring formation, might well be better resolved by a suitable thymine limitation schedule to test the hypothesis that the delay in assembly depends on the length of the *C* period (or the ratio C/τ) or *D* (or D/τ), which are related (146). Such experiments are currently being performed (T. den Blaauwen, unpublished data).

The high accuracy of constriction/septum placement in the mid-cell of rod-shaped bacteria is intriguing (101, 112). The observed precision, with coefficients of variation as low as 4 to 10%, is surprising, because those of other cell cycle parameters that lead to the eventual division are larger, while such a sequence predicts the last event to be the least precise. Moreover, it is striking that positional information is more accurately fixed than the temporal information: cell sizes at initiation of DNA replication and at cell division, for example, vary less than cell ages at these events (48, 51). The variations in the timing and mid-cell localization of FtsZ are very small as well, in the same range as that of the constriction itself (T. den Blaauwen, personal communication).

The so-called "Tug of War" mechanism, which has been proposed for a cell to find its middle (50), relies on a difference between growth rates of the peptidoglycan and the cytoplasmic membrane. This difference is presumed to stress the membrane mainly at mid-cell, to be sensed by mechano-receptors and translated into a biochemical signal that activates FtsZ ring formation. Stress evolution has recently been related to interactions between the nucleoid and the membrane (90). The connection is attributed to the coupling between transcription and translation in bacteria, together with insertion (or translocation) of membrane proteins into (or through) the membrane, as described in the "transertion" model (123). This would exert a force on the membrane. Integration over the cell surface of the longitudinal component of this force produced by the transertion "strings" (composed of membrane protein genes, their mRNAs, the translating polyribosomes, and respective signal recognition particles [25]) yields a minimum stress at the mid-cell (90). As soon as the nucleoid separates, this minimum stress rises quickly to zero, and mechano-receptors are proposed to sense the rate of stress change, as described in reference 50.

Validity of this "Transertion-Dependent model for Division Plane Positioning" (TDP) does not exclude additional, superimposed mechanisms for placing the division ring/plane; the MinCDE system (e.g., see reference 91) is such a complementary mechanism (74, 135). Lack of oscillating MinCD in *B. subtilis* (64) may indicate the different balance between the two systems in positioning the division site in different bacterial species. The observed paths of MinD oscillations in spherical *rodA* cells (21) should be related to surface localizations of their existing nucleoids or of those in thymine-limited cells (143, 145). The advantage of the latter experimental condition is that it does not require mutants or long-term treatments with drugs that directly affect division or peptidoglycan synthesis. A brief pulse of amdinocillin (mecillinam) would convert the already-fat thymine-limited cells with multiple nucleoids (Fig. 6C) into spheroids (Fig. 6D) (see below).

The TDP model provides a means by which the nucleoid is stabilized symmetrically around the cell center by the force that the transertion strings exert on it, thus explaining the accuracy in placing the division plane right in the middle of the cell length (90). However, in wide cells that grow quickly and replicate their chromosome slowly (using thymine-limited cells in rich media), this symmetry breaks down so that each of the existing nucleoids is detached from one side of cell periphery and attaches to the other in a "parachute"-like form (143, 145) (Fig. 6B and C). The FtsZ ring position and division planes in such cells are consequently tilted as well, in agreement with the nucleoid occlusion model. The off-center position of a replicating and segregating nucleoid due to this detachment would alleviate the stress at the opposite side of the membrane to allow a constriction ring to initiate there as an arc. However, completion of this ring would be occluded by the nucleoid bound through the transertion strings at the other side. Consequently, an arc is formed leading to a branch (129, 138, 141). Primary constrictions are indeed clearly seen in such opposite-nucleoid positions (144), and secondary FtsZ arcs perpendicularly cross the primary in spheroidal cells (Fig. 2 in reference 80 and Fig. 1 in reference 143). The perpendicular crossing arcs in spherical cells induced by brief (60-min) amdinocillin treatment are likely to reflect the (tetrahedron-like) symmetrically positioned nucleoids. When the spheroids are allowed to recover and elongate, "butterfy-like" cells are observed that evolve into branched cells (Fig. 6E and F) (discussed in reference 145; see also Fig. 3 in reference 80). These observations are thus consistent with the idea that placement of the constriction plane for division is determined by the nucleoid spatial position in the cell.

IS BRANCHING RELATED TO NUCLEOID POSITION?

Cell branching has been reported in *E. coli* (105, 108) and *Agrobacterium tumefaciens* (37) and more recently in *Mycoplasma capricolum* (102) and *Rhizobium meliloti* (57) when DNA replication was inhibited, but the nucleoid positions were either not recorded or were unclear. The explanation for this phenomenon given here does not exclude other causes for branching, such as multiple sites of inert peptidoglycan (27) and abnormalities in cell wall elongation (41). The complex pathway of peptidoglycan synthesis (116 and references therein) must obviously be involved in any change in cell shape such as branching. However, branching observed during thymine limitation seems to originate at a higher level, either the nucleoid complexity and position as proposed above or a coupling between metabolism of DNA and of envelope components, which is still poorly understood. The observation should be taken into consideration that branching obtained by a slowed replication rate (during thymine limitation; e.g., Fig. 6B, E, and F) is much more frequent in the population than the occasional branches observed upon inhibition of DNA synthesis (during, e.g., thymine starvation; Fig. 7 and data not shown). Rhamnose-thymine metabolites (77, 78, 107) may be one such link, and the cyclic AMP connection (53) seems to be another. It is obvious, however, that existing cell poles are not as inert as they are thought to be (28, 29, 49) under certain circumstances: a polar cap can split when cell diameter approaches normal cell length (for example, see Fig. 6B, E, and F).

The recent observation of branched swarmer cells and their stalks in *rodA* and *mreB* mutants of *Caulobacter crescentus* (118) may resolve the branching mechanism of the peptidoglycan sacculus. The stalk cylinder is fivefold thinner than the cell body and does not contain DNA (84). It is thus conceivable that its diameter (of ~0.25 μm) is the default structure of MreB in vivo in cylinders devoid of the nucleoid (anticipated to be ~0.35 μm; extrapolation to zero complexity is shown in the inset of Fig. 4; see also Fig. 2A in reference 124). It would be useful to find conditions under which MreB is self assembled in vitro to measure its dimensions and to manipulate cells to force a nucleoid into the stalk and observe its morphogenesis. A natural tube resembling the stalk of *Caulobacter* swarmer cells, through which replicating nucleoids move to the daughter cell, is the prosthecum of *Hyphomonas* spp. Its diameter was estimated at 200 nm (149; more like 400 nm in hydrated material). Does the elongating prosthecum "pull" the DNA because its surface/volume ratio is larger and thus connects more transertion strings? Relating the biochemical and cytological observations to the complexity and position of the nucleoid may help to uncover the link between the metabolic pathways of DNA and peptidoglycan. The thymine limitation regimen can be used to achieve this aim.

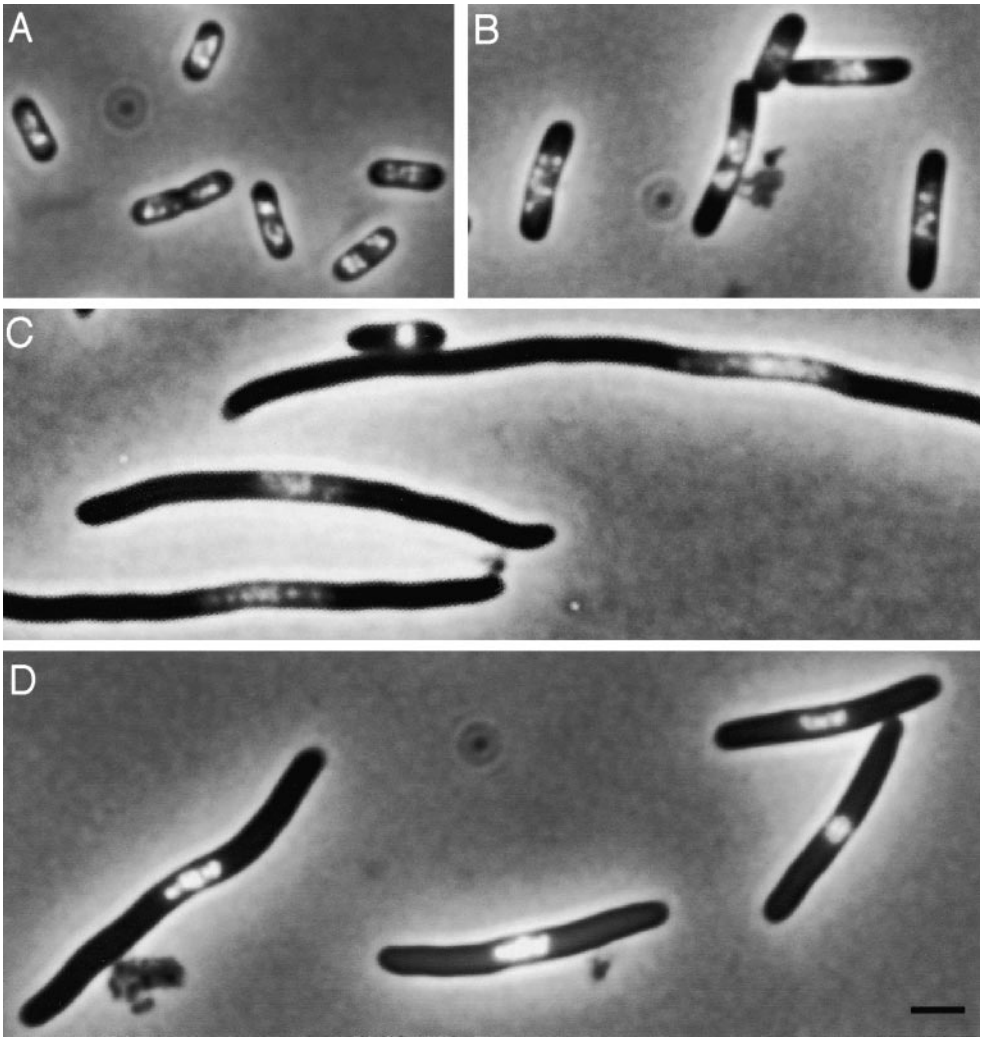

FIG. 7. Changes in nucleoid structure during thymine starvation. *E. coli* K-12 strain CR34 *thyA* was grown in glucose minimal medium supplemented with Casein hydrolysate (1%), thymine, and deoxyguanosine (dG) (20 μg ml^{-1} each) for more than 10 generations to achieve a steady state of growth. At A_{450} = 0.4, the culture was filtered, washed, and resuspended in fresh, prewarmed medium without thymine and dG. Cells were stained with DAPI (10 μM), fixed with 0.1% OsO_4, and concentrated by centrifugation. Cells were immobilized on a thin agar layer and observed under an Olympus BX60 microscope as described previously (115). Samples are shown from control, before starvation (A), 60 and 180 min afterwards (B and C, respectively), and 120 min after chloramphenicol treatment following 120 min of starvation (D). Bar, 2 μm.

Thymine limitation was instrumental in developing the TDP model, because it enabled manipulating cells growing under normal conditions to replicate their chromosomes with higher complexity (more multiple forks) than can be achieved by raising the growth rate alone, resulting in a more complex positioning of the FtsZ ring and division plane without mortal (or even observed deleterious) effect on cell multiplication and, hence, survival. This method is thus advantageous over the use of shape mutants (which are usually pleiotropic) or drugs (which are not specific) to obtain cells with more complicated shapes.

EVOLVING MODELS: "TRANSERTION" AND "NUCLEOID OCCLUSION"

Proposed mechanisms to regulate the necessary link between chromosome replication, nucleoid segregation, and cell division in bacteria have been debated for 40 years now. Linking structures like "Mesosomes" (membrane invaginations) and "periseptal annuli" (preformed mid-cell division sites) as well as "nucleoid jumps" turned out to be artifacts (35, 97, 115, 122). Existence of contractile proteins that pull the completed daughter nucleoids apart (e.g., see reference 110) is attractive but requires many assumptions to explain all of the observations, particularly under conditions of multifork replication and segregation (123). Even if the proposed temporary membrane connection of hemimethylated, just-initiated *oriC* (45, 76) can explain the presumed initial "pushing apart" of the daughter *oriC* (such as "Ribosomal Assembly Centers" [126, 147]), it certainly cannot explain the continued partition during the rest of the cell cycle.

The distinction between the transertion idea for nucleoid segregation and all models that involve motor proteins depends on the dissociation of DNA replication and cell growth

(in mass and surface). Differential inhibition of each separately would tell which is crucial, growth or replication: the former model predicts that the nucleoid will continue to expand during growth without replication and contract during replication without growth, while all other models predict the opposite. The fundamental assumption of the transertion model, that nucleoid segregates during replication by its link to cell membrane through genes coding for membrane-associated (or excreted) proteins (123), is supported by the observation that a partially replicated nucleoid is contracted to mid-cell when protein synthesis is specifically inhibited (by, e.g., adding chloramphenicol or by amino acid deprivation [9, 93, 129]). The only cells that complete the division process are those that had terminated replication before the block was imposed. Indeed, the proportion of cells that perform this so-called residual division has been used to determine the *D* period (30, 52, 109).

To complement this established observation, DNA synthesis was specifically inhibited by thymine starvation (Fig. 7). The cells grew in an unbalanced but exponential mode for about three mass doublings before slowing down (data not shown), and the nucleoid(s) that remained after residual divisions continued to extend over the cell length with no net replication (Fig. 7B and C), as predicted by the transertion model. While stretching, the original nucleoids, consisting of over one chromosome equivalent (replicating upon start of starvation), were pulled apart into small lobules (Fig. 7B and C). Such pulling apart of nucleoids upon inhibition of DNA synthesis has previously been described for a temperature sensitive *dnaX* mutant of *E. coli* (129). This behavior of nucleoid stretching may be connected to the biochemical and biophysical properties of DNA and its membrane interactions and should be investigated further. It is intriguing that the pulling apart of DNA stops around the time of slowed mass and length growth rate and is reversed when growth is blocked completely with chloramphenicol (Fig. 7D), concordant with the transertion model for segregation. Clusters of plasmid RK2 were also confined to a single or two nucleoid bodies in thymine-starved filaments (46). There, too, the nucleoid(s) was (were) not evenly distributed along the filament but remained near its center.

Inhibition of cell division as proposed by the nucleoid occlusion model has recently been supported by the discovery of nonspecific DNA binding proteins Noc (for "nucleoid occlusion") and Slm (for "synthetically lethal with a defective Min system") that prevent the division machinery of *B. subtilis* and *E. coli* (8, 132), respectively, from assembling in the vicinity of the nucleoid. Cell division was blocked in the absence of both the Min system and the Noc/Slm protein, presumably due to the accumulation of nonproductive division protein complexes. The puzzling mechanism of action of these accessory proteins, which seem to exert the nucleoid occlusion effect, is still not clear. In particular, the fact that Slm is not required for "growth on M9 minimal medium" even in the complete absence of the Min system but is necessary in LB (8) may reflect the difference in nucleoid complexity of cells between the two media (Fig. 2 to 5). This possibility can be tested by thymine limitation that simulates nucleoid complexity in fast growing cells without modifying the cell's metabolic situation (see above). If the TDP model is involved, the *slm127* (or *slm267*) Δ*minCDE* double mutant will only grow in minimal medium when supplied with high thymine concentrations (and 2′-deoxyguanosine) but not with low, limiting concentrations when nucleoid is more complex.

CONCLUDING REMARKS

Bacterial thymine-less mutants lose colony-forming ability when deprived of the required building block. A multitude of molecular and cellular processes are involved, but the major cause for "death" is still unknown. The possibility that it is a matter of plating efficiency of filaments with long, DNA-less ends (Fig. 7C) cannot be ruled out by existing data and should be tested.

Entering the cell by passive diffusion only, thymine concentrations supplementing *thyA* cultures determine intracellular levels of its metabolites and hence chromosome replication rate. Manipulating the concentration is a complementary means to dissociate between rates of cell growth μ and of chromosome replication $1/C$ and has been exploited to correlate various major cellular events and structures to DNA replication, nucleoid segregation, and chromosome complexity. The two physiological states, thymine limitation and thymine starvation, differ markedly, as discussed here and demonstrated morphologically in Fig. 6 and 7, respectively, and summarized in Table 1. The unfortunate claim that "thymine starvation of a *thy*$^-$ strain produce cellular monsters" (134) may mislead scientists to ignore this tool. The potential of both states to understand the bacterium is far from being exhausted.

Nucleoid complexity is defined as the number of replication positions per chromosome ($n = C/\tau$; the ratio between chromosome replication and cell doubling times) and manifested in multiforked replicating chromosomes. Thymine limitation was used to raise complexity beyond the limit possible in fast-growing Thy$^+$ cells. Cell diameter could thus be increased to a stage where the cell starts to split its pole. In the first instance, the segregating, multiforked nucleoids are displaced, hence the spatial position of the FtsZ ring assembling between them is tilted accordingly, resulting in a division plane that is not perpendicular to the cell axis. When complexity increases further and cell diameter becomes almost as large as its length, the pole splits to breed a branched cell. The results conform to the nucleoid occlusion model for division placement by the transertion mechanism. The model was tested, and substantiated, using thymine starvation, during which nucleoids extended, but only to a certain limit. This stretchability limit may provide clues to the delicate nucleoid structure.

The signals transduced between nucleoid structure and position and cell envelope, culminating in the phenomena described here in the framework of so-called nucleoid occlusion, might have evolved through this small molecule, thymine, and its metabolic intermediates that are only used in the processes of DNA and envelope syntheses. However, while these molecules are consumed during DNA synthesis, the sugar derivatives are used as mediators in envelope synthesis. Could it lead to filamentation and TLD? Deciphering this connection in biochemical and biophysical terms is a challenge for future studies in bacteriology.

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