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Visualizing multiple constrictions in spheroidal *Escherichia coli* cells

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Abstract — An *Escherichia coli* cell grows by elongation and divides in a perpendicular plane. Alternating planes of successive divisions in three dimensions can only be ascertained when multiple constrictions exist simultaneously in large, spheroidal cells (with extended constriction process), if the division signals are enhanced. Large, spheroidal cells are obtained by a brief mecillinam treatment, and more frequent divisions are achieved by manipulating the rate of chromosome replication without affecting cell mass growth rate. Such a procedure has recently been performed by thymine-limitation of *E. coli* K12 strain CR34 (Zaritsky et al., Microbiology 145 (1999), 1052–1022). Enhancing the replication rate in cells with multi-forked replicating chromosomes (by addition of deoxyguanosine) shortens the intervals between successive terminations and thus triggers divisions more frequently. Monoclonal antibodies against FtsZ were used to visualize the rings of secondary constrictions, but apparent shortage of FtsZ to complete rings over wide cells allowed assembly of arcs only. The arcs observed were not parallel nor perpendicular; the tilted constriction planes are consistent with our 3-D 'nucleoid segregation'model for division under conditions which relieve the cylindrical constraint for nucleoid segregation by the bacillari peptidoglycan sacculus (Woldringh et al., J. Bacteriol. 176 (1994) 6030–6038). The shortage in FtsZ may explain the longer time required to complete the division process in wide cells with long circumferences, observed during thymine step-up. Overexpression of fusion protein FtsZ-GFP on a multi-copy plasmid should circumvent the shortage. © 1999 Société française de biochimie et biologie moléculaire/Éditions scientifiques et médicales Elsevier SAS

FtsZ rings and arcs / division planes / fluorescent microscopy / thymine limitation / mecillinam / rate of cell division

1. Introduction

Manipulating the macromolecular composition, size and shape of bacterial cells can be achieved by mutations, by drugs, and by physiological means. Numerous mutants with modified morphologies or sizes have been described in bacillary species such as *Escherichia coli* over the years. Cells harboring mutated penicillin binding proteins (PBPs) either filament or turn into spheroids at the restrictive temperature (e.g., for *pbpB* or *pbpA*, respectively [1, 2], and *rodA* [3]), mutants in any of the *fts* loci filament [4, 5], and other mutations cause distorted divisions and strange morphologies [6-9]. Similar results are obtained by applying penicillin derivatives that specifically interact with PBPs [1]. In all these cases, however, the cultures cannot be maintained in a steady-state of exponential growth [10] and the cells eventually stop dividing and sometimes even lyse at the restrictive conditions.

Changes in cell composition, size and shape, without interfering with unrestricted growth, can be achieved only by manipulating the composition of the medium. The first systematic investigation was performed 40 years ago by scientists who established the so-called 'Copenhagen school' and opened up a new field, namely bacterial physiology [11]. Moreover, these scientists realized that the amount of information gained from steady-state of exponential growth is limited. Their classical nutritional upshift experiment [12] disclosed the so-called 'rate maintenance' in cell division. This phenomenon could only be understood a decade later, when Helmstetter et al. [13] came up with their cell cycle control (C-H) model. Thus, the quantitative dependence of cell size and macromolecular composition on the culture growth rate μ [11] was explained by the constant rate of chromosome replication starting at a constant cell mass, while cell mass itself grows exponentially [13, 14].

It has naively been predicted that the larger size of faster-growing cells is accommodated in the length dimension because a growing cell extends primarily by elongation [15, 16]. The puzzling observation that cell girth changes in parallel to cell length [11, 17] has been ignored for a long time [18]. A hint to the mechanism was given

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Abbreviations: C, the time it takes to complete a round of chromosome replication; D, the time between termination of a replication cycle and subsequent cell division; dG, 2'-deoxyguanosine; μ, culture growth rate (h^{-1}); PBP, penicillin binding protein.

when the number of replication 'positions' [19], defined as the ratio between chromosome replication time (*C*) and culture doubling time ($C \times \mu$, where growth rate μ is inversely proportional to the doubling time), could be modified and over a wider range, without affecting the latter [20]. The trick used, changing the concentration of external thymine supplied to *thyA* mutants, is based on understanding thymine metabolism in *E. coli* [21] and has been exploited since for various purposes [22-25]. It has recently been used to obtain cells with secondary constrictions [26].

When temporary inhibition of division in *E. coli*, such as at low penicillin concentration [27], is relieved, the enhanced divisions of the filaments [28] occur in parallel. The dispute about the relative planes of subsequent divisions in spheroidal *E. coli* [26, 29, 30] can be resolved by visualizing the nascent FtsZ rings [31] in such cells. This is the aim of the investigation described here.

2. Materials and methods

2.1. Bacterial strains and growth

E. coli K12 (CR34; *thr-1 leuB6 thyA* [26, 32]) was the major strain used in this study. One series of experiments was performed with *E. coli* 15T$^-$ (555-7*; trp met arg thy drm* [20, 33]). Cultures were grown at 37 °C in glucose minimal medium [32], with or without casamino acids (1% w/v; Difco), and thymine as indicated. For fast replication, 2'-deoxyguanosine (dG) (100 μg mL^{-1}; Sigma) was added [34]. The OD_{450} was measured with a spectrophotometer (LKB Ultraspec II), and cell numbers with an electronic particle counter (30 μm orifice). Balanced cultures were grown 'normally' [10] for at least 10 doublings by successive dilutions ($OD_{450} < 0.4$).

2.2. Multi-constricted cells

Large CR34 cells with multi-forked chromosomes were obtained [26] with 5 μg thymine mL^{-1} ($C \approx 80$ min [33, 34]). Treatment with mecillinam (1 μg mL^{-1}) resulted in spheroids. To obtain secondary-constricted cells, dG (100 μg mL^{-1}) and thymine (to 10 μg mL^{-1}) were added at 50–60 min, to enhance replication rate [34] and to let the ongoing forks terminate, thus triggering successive divisions more frequently [26]. Samples were fixed (below) at various times; best results were found between 20–35 min.

2.3. In situ immunofluoresence labeling of FtsZ rings

Cells were fixed for 15 min in 2.8% formaldehyde and 0.04% glutaraldehyde, centrifuged (7000 rpm for 5 min) and permeabilized by washing twice in PBS, pH 7.2, and incubation in 0.1% Triton X-100/PBS for 45 min, all at room temperature. The cells were washed thrice in PBS and incubated in PBS containing 100 mg mL^{-1} lysozyme and 5 mM EDTA for 45 min, then washed thrice again in PBS. Non-specific binding sites were blocked by incubation in 0.5% blocking reagents (Boehringer) in PBS for 30 min at 37 °C. Reaction with the primary antibody (a monoclonal antibody against FtsZ diluted in blocking buffer) was performed during 60 min at 37 °C [35]. The cells were washed thrice (PBS containing 0.05% Tween-20), incubated with secondary antibody (Goat-anti-mouse conjugated with AlexaTM 546 (Molecular Probes, Eugene, USA), diluted in blocking buffer) for 30 min at 37 °C and washed again (three times in PBS/Tween-20). The nucleoids were stained with DAPI (4,6-diamino-2-phenylindole) at a final concentration of 0.5 μg mL^{-1} in H_2O. The cells were washed in H_2O and resuspended in PBS.

3. Results and discussion

It has been proposed that planes of successive divisions in spheroidal *E. coli* cells lie either parallel [29] or perpendicular [30] to each other, restricted to one or two dimensions, respectively. To test the hypothesis that divisions can occur in planes alternating in three dimensions [32], we have recently developed a method to generate spheriodal cells with secondary constrictions during growth in suspension [26]. The method involves a combination of mecillinam treatment (to inhibit PBP2) and thymine limitation (to manipulate rate of chromosome replication). The spheroidal cells thus obtained display secondary constrictions after adding dG (accelerating replication rate), that temporarily enhances division signals (by increased frequency of terminations). The successive constrictions develop in planes that are tilted relative to each other, but are seen only superficially (i.e., on cell surface). We have decided to demonstrate these secondary constrictions by visualizing their intracellular FtsZ rings at earliest appearance [31].

3.1. Immunolabeling of FtsZ

Cells of *E. coli* K12 (strain CR34) were manipulated physiologically to enhance division signals in spheroids [26], and immunolabeled for FtsZ visualization. Rings corresponding to deep primary constrictions did not show up; they had probably disassembled by the time secondary constrictions appeared as FtsZ arcs [36], sometimes in tilted planes *(figure 1)*. Such arc-shaped FtsZ incomplete rings have recently been observed in different strains under other conditions [36, 37], and can be explained by shortage of FtsZ to complete a ring in thick cells.

3.2. Longer constriction in wider cells

Bacterial cell division occurs a constant time (*D*) after termination of DNA replication over a wide range of

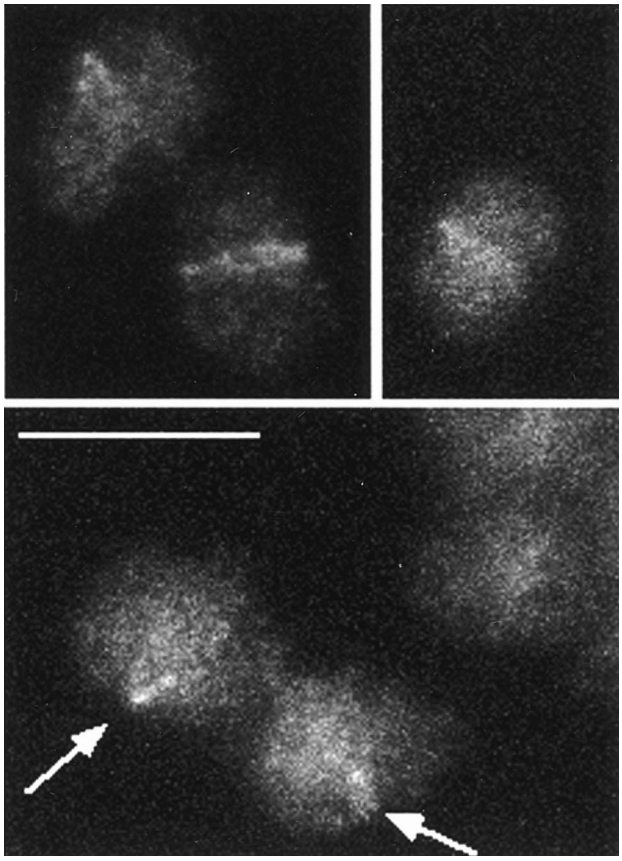

Figure 1. Immunofluorescent-labeled FtsZ on fixed *E. coli* K12 CR34 cells, following mecillinam treatment and thymine step-up ([26], and *Materials and methods*). Magnification bar, 2 μm.

μs [13]. Constriction is apparently initiated by self-assembly of the FtsZ ring [31], the length of which (cell circumference) is μ-dependent [27]. A constant *D* period is thus expected if the ring is produced at a rate proportional to μ [27, 38]. Under thymine-limitation, however, the increased cell diameter without a change in μ [39] seems to cause relative shortage of FtsZ, which may be the reason for delayed divisions. In a series of experiments [33], rates of cell division were followed in *E. coli* 15T⁻ during growth transitions from various concentrations of thymine to higher concentrations (nicknamed '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* period (*figure 2*; reproduced from [33]) may be related to enhancement of expression from *ftsZ* (or another gene the product of which precedes FtsZ in triggering the division process) following onset of the transition, as other genes do [24].

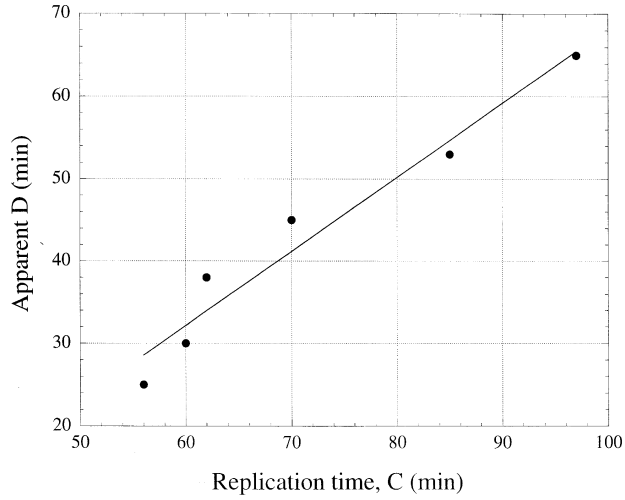

Figure 2. Apparent coupling between the time necessary for division *D* and chromosome replication time *C*. Values of *C* and apparent *D* have previously been derived for *E. coli* 15T⁻ (in [20] and [33], respectively).

The interpretation that FtsZ arcs *(figure 1)* [36, 37]) are completed to rings slower because FtsZ is limiting can be tested by over-producing it, such as over-expressing *ftsZ* cloned on a multi-copy expression vector [40].

4. Conclusion

Successive constrictions do not overlap in *E. coli* as do successive replication cycles due to the brevity of the time *D* needed to separate in two daughters under usual conditions. To circumvent this difficulty, *D* was extended and division signals enhanced in the present work. Under these circumstances, the division plane in spheroidal cells is not limited to a single or to two dimensions.

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