Biodata of **Arieh Zaritsky** and his team contributors of ***Surviving Escherichia coli in Good Shape: The many Faces of Bacillary Bacteria.***

Dining in Amsterdam, August 1997. From left to right: Conrad L. Woldringh (b. 1940), Robert H. Pritchard (1930), Arieh Zaritsky (1942), Itzhak Fishov (1949)

The team consists of three generations of scientists who have spent thirty years exploring what the bacterial cell is trying to tell us about itself. Arieh Zaritsky came to the lab of his mentor Robert Pritchard at Leicester University soon after the cell-cycle model was announced by colleagues and friends Charles Helmstetter and Steve Cooper late in 1968. Immersion in the Leicester cell-cycle tumult resulted in thymine-limitation and step methodology dissociating rates of replication and growth under constant nutritional conditions. They were not aware of Conrad Woldringh's simultaneous struggle with *E. coli* cell and nucleoid shapes in Amsterdam. A fortuitous encounter in Lunteren (1974), just when our respective research lines crossed yielded a long lasting friendship associated with productive collaboration. Among other joint ventures, Conrad and Arieh organized the first two EMBO Workshops on The Bacterial Cell Cycle, in 1980 (Noordwijkerhout, with Nanne Nanninga) and 1984 (Sede-Boker, with Eliora Ron), following the 1978 example of Bob (Leicester, with Kurt Nordström, on plasmid replication). Itzhak Fishov complemented the team upon emigrating to Be'er-Sheva from Moscow in 1991. The ring was closed when Itzhak visited Conrad and met Bob at the Chorin workshop (1997) and at Leicester (visiting Vic Norris).

Many of the raw ideas stemming from the so-called "Copenhagen school" (led by Ole Maaløe) shaped our own. Bob visited Copenhagen as a plant geneticist in 1953 and Arieh (1972), as a bacterial physiologist. Together with the multi-faceted artistic talents and microscopic approach of Conrad and the biophysical skills of Itzhak, we clarified and crystallized ideas about the bacterial cell, some of which are summarized here.

For more about three of us, see our respective Web Sites:

http://www.bgu.ac.il/life/zaritsky.html (Arieh Zaritsky)
http://wwwmc.bio.uva.nl/~conrad/ (Conrad L. Woldringh)
http://www.bgu.ac.il/life/fishov.html (Itzhak Fishov)



*J. Seckbach (ed.), Journey to Diverse Microbial Worlds,* 347-364.

# SURVIVING *ESCHERICHIA COLI* IN GOOD SHAPE

*The Many Faces of Bacillary Bacteria*

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## 1. Introduction

One characteristic defining a bacterial species is its cell shape (van Leeuwenhoek, 1684). Our work deals with simple symmetrical shapes that can be defined by the ratio between two dimensions, length $L$ and diameter $2R$. There are two such possible forms, an approximation to a prolate (including a sphere, where $L/2R = 1$) and a cylinder (the length of which equals $L\text{-}2R > 0$) with hemispherical polar caps. Cells of the latter (bacilli) are often transformed into spheroids (cocci), such as upon entrance to the stationary phase (in gram negative species) or sporulation (in gram positives). The most familiar rod-shaped bacterium, which can easily become sphere-like, is *Escherichia coli*. Being the species of preference in the investigations conducted during the last 3 decades in our respective laboratories, it is the focus of this Chapter. The general laws derived are valid for other bacterial species and seem universal, albeit with slight modifications. Related subjects that are not thoroughly discussed here have frequently been reviewed in the literature (e.g., Kornberg and Baker, 1992; Koch, 1995; Messer and Weigel, 1996; Höltje, 1998; Nanninga, 1998).

We start by defining states of bacterial culture growth and describe the dependence of cell growth (mass and surface) and division and of nucleoid duplication and segregation on nutritional conditions. We specify the peculiar properties of thymine (a unique DNA precursor) and how they can be exploited to study chromosome replication and cell duplication, viability and shape. The possible induction of membrane heterogeneity by the bacterial nucleoid and the role of membrane domains in regulating cell shape and cycle events are discussed. Morphological modifications obtained by various means are compared, and a "unifying model" that attempts to couple these and other physiological parameters is advanced.

## 2. The Bacterial Cell Cycle

### 2.1. STATES OF GROWTH AND TRANSITIONS

"Steady-state growth" was defined as a situation in which "the distribution of each intensive random variable" (any attribute of individual cells) is time-invariant (Painter and Marr, 1968). The more limited term "balanced growth" is a condition in which "every extensive property of the system" (attributes of the whole population) "increases by the same factor over a time interval" (Campbell, 1957). A balanced culture is also in "exponential growth" if the factor is constant over time, and it is in "steady-state growth" if that same factor applies to cell number as well (Fishov *et al.*, 1995). The confusion over these terms in the realm of microbiology was a major reason for the poor understanding of the mechanisms governing bacterial adaptability and shape determination (Henrici, 1928; Pritchard, 1974).

The first systematic study relating cell size and macromolecular composition to the nutritional conditions (Schaechter *et al.*, 1958) opened up the field of bacterial physiology but yielded a limited amount of information. It was followed by studies of transitions between growth rates (Kjeldgaard *et al.*, 1958), which led to the discovery of the "rate maintenance" phenomenon. Delay in adjustment of the rate of protein synthesis during nutritional up-shifts was later explained by the need to raise ribosomal concentrations (Maalφe and Kjeldgaard, 1966). The longer and constant-time delay in the adjustment of cell division rate was understood ten years later, when Helmstetter *et al.* (1968) came up with their cell cycle control (C-H) model. This classical model stemmed from an extensive series of experiments with age-selected synchronous (not synchronized) cultures in a "quasi steady state" (Campbell, 1957; Maalφe, 1963; Cooper, 1991; Fishov *et al.*, 1995).

### 2.2. GROWTH, CHROMOSOME REPLICATION AND CELL SHAPE

#### 2.2.1. *Dissociation between Rates of Growth and of Chromosome Replication*

The C-H model (Helmstetter *et al.*, 1968; Helmstetter, 1996) relates chromosome replication to cell growth and division. It readily explains the exponential dependence of average cell size ($M_{avg}$) and macromolecular composition on the culture doubling time $\tau$ as follows. Chromosome replication initiates when cell mass reaches a threshold, constant value per *oriC* ($M_i$), and proceeds at a constant rate to terminate $C$ min later, irrespective of $\tau$ over a wide range of growth rates $\mu$ (inversely related to $\tau$). The time $D$ between replication-termination and the subsequent cell division is also relatively constant. Thus, cells growing faster (with shorter $\tau$) are larger ($M_{avg} = M_i \ln 2\ 2^{(C+D)/\tau}$) because more mass accumulates before they divide during the constant time ($C+D$ min) following initiation at a constant $M_i$ (Helmstetter *et al.*, 1968; Donachie, 1968; Pritchard *et al.*, 1969). During growth in rich media supporting $\tau < C$, the cell compensates by a strategy not used in eukaryotes: it initiates a new round of replication before the proceeding round has terminated. The idea that the rate of DNA synthesis is not tightly coupled to growth rate nor to rate of chain elongation (Helmstetter *et al.*, 1968) had existed earlier (Maalφe, 1961; Pritchard, 1965), and gained support from autoradiography (Cairns, 1963), genetics (Oishi *et al.*, 1964), and physiological (Pritchard and Lark, 1964) techniques. The amount (in genome equivalents) of DNA per cell, $G_{avg} = (\tau/C \ln 2)\ [2^{(C+D)/\tau} - 2^{D/\tau}]$, thus increases with $\mu$. On the other hand, DNA

concentration (Zaritsky and Pritchard, 1973), $G_{avg}/M_{avg} = [\tau/M_i \ C \ (\ln 2)^2] \ (1-2^{-C/\tau})$, decreases with $\mu$ because $G_{avg}$ changes slower than $M_{avg}$. A dynamic, heuristic version of the model with some of its consequences and implications, including specific mutants with modified values of $M_i$, $C$ and $D$, was developed by Norbert Vischer in Amsterdam and can be downloaded from ftp://simon.bio.uva.nl/pub2/

2.2.2. *Cell Dimensions*

Under steady-state of exponential growth, a cylindrical bacterium extends during the cell cycle by elongation only (Marr *et al.*, 1966; Trueba and Woldringh, 1980). A larger cell obtained in richer medium supporting faster growth rate, might be expected to accommodate its excess mass (or volume, because cellular density is also essentially constant (Rosenberger *et al.*, 1978a)) in the length dimension. Unpredictably, cell diameter also changes with $\mu$ (Schaechter *et al.*, 1958) such that its axial ratio remains relatively constant (Zaritsky, 1975a). The molecular mechanism responsible for the systematic change of cell diameter is still unknown (Cooper, 1991), but slow 'adaptation' to new dimensions following nutritional shifts (Woldringh *et al.*, 1980; Zaritsky *et al.*, 1993; Woldringh *et al.*, 1995a) suggest that remodeling of the peptidoglycan sacculus is involved. It could be argued that the increase of cell diameter with growth rate is caused by drastic alterations in de-repression pattern during growth at different media (Kumar, 1976). An experimental leverage was given by the discovery of a simple means to manipulate the number of replication "positions" without changing the growth rate (Pritchard and Zaritsky, 1970).

A replication "position" $n$, defined (Sueoka and Yoshikawa, 1965) as a set of forks moving on a chromosome following a simultaneous initiation event at *oriC*, is equal to the ratio $C/\tau$ (= $C\mu$) (Zaritsky, 1975b). Values larger than 1 are allowed by the multi-forked replication strategy (Helmstetter *et al.*, 1968). It was useful to find conditions in which $n$ can be modified at will over a wide range without affecting cellular growth rate, by manipulating the concentration of thymine in thymineless mutants (Zaritsky and Pritchard, 1971; Ephrati-Elizur and Borenstein, 1971). Thus, instead of the 'natural' but complex change in one component of $n$ ($\tau$), it became possible to vary the other component ($C$) using exceedingly simple procedure (Pritchard and Zaritsky, 1970).

2.2.3. *Models for Cell Shape Determination*

Cell length has been considered for a long time to be used as a 'ruler' that can somehow trigger division when reaching two "unit cell lengths" (Donachie and Begg, 1989; Koch and Höltje, 1995). Other models have been proposed that predict the observed changes in cell shape (relative dimensions), based on the relationships referred to above. They assume that cell surface area (and also length under steady-state conditions) increases linearly (Previc, 1970) with a discrete doubling in rate at a particular point in the cell cycle (Zaritsky and Pritchard, 1973; Pritchard, 1974). Two possible explanations for a linear increase in surface area were proposed. New envelope material could be laid down at one or more discrete annular sites (like building a chimney) which double in number at a particular point in the cell cycle. Alternatively, a rate-limiting envelope component could be produced from a constitutive gene, output of it doubling when the gene replicated. If the site (or gene copies) doubled late in the cell cycle (triggered by termination, or because the presumed gene was located near the chromosome terminus), an increase in mass accumulation could only be accommodated without a change in density by an increase in cell diameter during the transition. It was postulated that the

change in diameter was a physical response to the increase in turgor that would occur during a transition to a higher growth rate, implying that diameter is passively derived (reviewed in Zaritsky *et al.*, 1982). Other models dealt with cell elongation only, neglecting the second (width) dimension. They tied elongation rate to the number per cell of *oriC*s, replication forks or *terC*s (Donachie *et al.*, 1973; Sargent, 1975).

Extensive statistical analysis performed could not distinguish between the various models due to similarity of their predictions for cell dimensions under steady-state of exponential growth (Grover *et al.*, 1977; 1980; Rosenberger *et al.*, 1978b). They do predict substantial differences for changes in dimensions during transitions between steady-states (Grover *et al.*, 1980). The data are best fit to the model that presupposes a zonal surface synthesis at a rate proportional to the instantaneous $\mu$ which doubles when the hypothetical controlling gene is replicating $d$ min before cell division (Pritchard, 1974; Woldringh *et al.*, 1980; Zaritsky *et al.*, 1982). A serious reservation stems from the unreasonably long estimated $d$ (ca. 40 min); assembly of FtsZ ring has never been observed that early (Den Blaauwen *et al.*, 1999).

Based on extensive observations of nucleoids under various growth conditions (Woldringh, 1976; Woldringh *et al.*, 1977), it occurred to us that replicating chromosomes with $n > 1$ (i.e., multi-forked) need more space in the width dimension to segregate properly before the cell can divide (Zaritsky and Woldringh, 1987). We have thus proposed that the nucleoid complexity (i.e., its physical size and shape) actively determined cell diameter, predicting a relationship between cell diameter and number of genome equivalents per terminus (DNA content/nucleoid) (Woldringh *et al.*, 1990), $G/T = G_{ter}/T_{ter} = (\tau/C \ \ln 2) \ (2^{C/\tau}-1)$. (The average number of termini (completed chromosomes) per cell is $T_{ter} = 2^{D/\tau}$ (Zaritsky and Pritchard, 1973).) The available data on average diameter as a function of either $\tau$ or $C$ are not sufficiently precise to distinguish between the different models. Nevertheless, we shall discuss below the possibility that the self-organizing properties of bacterial DNA and its direct relationship to the inner membrane may influence cell shape and division.

## 3. Thymine Metabolism—Cell Division, Size and Composition, Viability and Shape

### 3.1. THYMINE METABOLISM

The unique role of thymine in cell metabolism derives from the fact that it is a building block of one macromolecule only, DNA, and is not included as a constituent in any other cellular component, though its derivatives (e.g., thymidine-rhamnose) are used in lipopolysaccharide biosynthesis (Ohkawa, 1976). However, elucidation of thymine metabolism and estimation of pool sizes did not throw any light on the regulation of chromosome replication. On the contrary, it seems possible that it is the regime of replication that influences nucleotide concentration. Thus initiation of replication would draw on the pool reducing its size and termination might result in a temporary rise.

Since it is not a normal metabolite in *E. coli* and *Bacillus subtilis*, these species have not evolved an active system to take thymine up (Rinehart and Copeland, 1973). It is a breakdown product and can only be incorporated into DNA through salvage pathway in the presence of deoxyribonucleotides or in *thyA* mutants. Thymine auxotrophs lack thymidylate synthetase thus cannot produce any thymidine nucleotide endogenously; they incorporate thymine by converting it to thymidine and subsequently to dTMP using

deoxyribose-1-phosphate (Pritchard, 1974; Ahmad *et al.*, 1998). A pool of the latter is generated from dUMP, which is prevented from conversion to dTMP by the mutation.

Manipulating the concentration of thymine and its derivatives used as DNA precursors in *thyA* mutants is a powerful tool to study nucleoid replication and partition, as well as the presumed coupling between these processes with cell division and shape.

## 3.2. THYMINELESS DEATH AND RELATED PHENOMENA

### 3.2.1. *Thymine Starvation and Thymineless Death*

Thymineless death (TLD) was discovered during studies of T-even phages (Watt and Cohen, 1953). Under thymine deficiency in an otherwise complete medium, cells lose the ability to form colonies on agar plates exponentially (following a brief delay) (Barner and Cohen, 1954; Cohen and Barner, 1955). This energy-dependent killing phenomenon (Freifelder and Maaløe, 1964) was ascribed to irreversible lesions induced under unbalanced growth (Cohen and Barner, 1954): inhibition of DNA synthesis while other processes continue normally. The rate of killing is retarded by concomitant inhibition of RNA or protein synthesis (Barner and Cohen, 1957, 1958). Nalidixic acid (Deitz *et al.*, 1966), methionine starvation (Breitman *et al.*, 1971), cytosine arabinoside (Atkinson and Stacey, 1968), and 5-fluorouracil (5-FU) (Cohen *et al.*, 1958) are examples of other means reducing colony-forming ability by inhibiting DNA synthesis. Low levels of cytosine arabinoside not causing TLD shorten the lag before onset of death upon thymine removal. Thymidine analogs cannot be assumed to affect DNA metabolism exclusively: osmotic damage and lysis provoked by 5-FU (Tomasz and Borek, 1962) may be related to conversion of glucose to the cell wall constituent rhamnose, via thymidine diphospho derivatives.

Ahmad *et al.* (1998) has recently reviewed the multitude of molecular and cellular reactions to thymine starvation (leading to TLD). Major examples are cell filamentation (Bazill, 1964; Donachie, 1969), mutagenesis (Smith *et al.*, 1973), DNA breakdown (Freifelder, 1969), structural changes (Nakayama *et al.*, 1994) and lack of methylation (Freifelder, 1967), induction of plasmids and prophages (Korn and Weissbach, 1962; Melechen and Skaar, 1962; Mennigmann, 1964). Integration of the information about these effects may eventually explain the immediate reasons for TLD.

### 3.2.2. *"Liquid Holding Recovery" and "Resurrection" of Viability*

Whatever the primary target is, TLD depends on the definition of bacterial "death". Operationally, it is loss of colony-forming ability on an agar plate. However, the definition of colony forming units is ambiguous: a bacterial culture can lose a substantial fraction of its viable cells without reduced metabolism under certain conditions, such as upon spreading *lon* mutants from a minimal growth medium on rich agar plates (e.g., Walker and Pardee, 1967; Berg *et al.*, 1976). Failure to generate a colony under one set of conditions is thus not sufficient to define a cell as dead. Furthermore, the number of particles (determined by an electronic counter) during TLD remains constant. It would seem that among the necessary conditions for a cell to be defined as "dead" is lysis or loss of its single DNA complement.

The most striking discrepancy is found when thymine is restored to a thymine-starved culture (Barner and Cohen 1956; Donachie and Hobbs, 1967). The number of colonies increases much quicker than by continued division rate of surviving bacteria. The kinetics of divisions demonstrates that a fraction of cells that lost their colony-

forming ability during the starvation period regains this ability while held in liquid medium (thus defined as 'sensitive to plating'). A possible explanation for this plating sensitivity may be found in our previous proposal (2.2.3.) that during transitions between growth rates there is an imbalance between the rate of mass increase and the increase in surface area (and volume). This imbalance could become extreme in the case of thymine starvation because the rate of cell envelope synthesis would be frozen but mass continues to increase quasi-exponentially for some time. This could generate sufficient turgor stress to lead to cell fragility.

3.2.3. *Does Cyt1Aa Exert TLD on E. coli Cells?*
The TLD-like phenomenon (Douek *et al.*, 1992) caused by expressing in *E. coli* of Cyt1Aa (a component of the mosquito larvicidal activity from the entomopathogenic bacterium *B. thuringiensis* subsp. *israelensis*) has recently been associated with compaction of the nucleoid (Manasherob *et al.*, in preparation). Thymine starvation leading to TLD, on the other hand, does not result in compact nucleoids (Woldringh *et al.*, 1994, and see section 6.1.). The lethal effect of Cyt1Aa is associated with growth inhibition, apparently due to perforation of the cytoplasmic membrane, does not imitate TLD, and can thus not be used as a lever to understand the killing mechanism of thymine starvation. Taken together, both observations support the transertion model (Norris, 1995; Woldringh *et al.*, 1995b; Binenbaum *et al.*, 1999), to be dealt with below.

## 3.3. THYMINE LIMITATION VS. THYMINE STARVATION

As with precursors of other macromolecules, cultures of *thy* mutants continue to grow and multiply indefinitely at the same rate when the medium is supplemented with a wide range of thymine concentrations. However, manipulating the intracellular levels of thymine and its metabolites by varying the external concentration (Pritchard, 1974) is possible due to lack of active transport for this DNA precursor (Rinehart and Copeland, 1973). The resultant physiological state, defined as "thymine limitation", is completely different than that reached by thymine starvation. Thymine limitation reduces the rate of DNA chain elongation without altering the overall rate of DNA synthesis under steady state conditions since this is determined by the frequency of initiation of rounds of replication. The assumption that thymine concentrations could be changed with impunity provided there was no change in growth rate led many scientists to misinterpret their data. For example, to isolate or follow high specific radioactive DNA and save on the radioisotope, exceedingly low concentrations have been used during the labeling period (e.g., Maalφe and Rasmussen, 1963), thus leading to flawed conclusions (e.g., Lark and Lark, 1965). The discrepancies were resolved by systematic investigations relating the length of $C$ to the external concentration supplied (Pritchard and Zaritsky, 1970; Beacham *et al.*, 1971; Zaritsky and Pritchard, 1971; 1973; Zaritsky, 1971; 1975b; Pritchard, 1974; Bremer *et al*, 1977; Molina *et al.*, 1998). This series of studies confirmed the concept, that the rate of DNA chain elongation is not coupled to growth rate, and that prokaryotes regulate DNA synthesis by the initiation rather than elongation process (Maalφe and Kjeldgaard, 1966). Helmstetter *et al* (1968) first demonstrated a constant $C$ over a wide range of $\mu$'s in wild-type strains, while our studies manipulated $C$ without changing $\mu$ in *thy* mutants. This technique has since been proved useful for several purposes (e.g., Bird *et al.*, 1972; Zaritsky and Pritchard, 1973;

Pritchard *et al.*, 1975; Chandler and Pritchard, 1975; Meacock *et al.*, 1977; Woldringh *et al.*, 1994; Hadas *et al.*, 1997; Zaritsky *et al.*, 1999a, b).

Slowing the rate at which replication fork traverses the chromosome to terminate by short supply of thymine metabolites (Beacham *et al.*, 1971) delays subsequent cell division. As happens at fast growth rates, cells are consequently larger with higher DNA content (though lower DNA concentration), and the nucleoid is more complex, containing a larger number of *oriC* and forks thus more DNA (Zaritsky and Pritchard, 1973; Pritchard, 1974). Filaments were anticipated as found under thymine starvation leading to TLD (Bazill, 1964; Donachie, 1969). But surprisingly, as with faster growth rates, the increase in cell size was accommodated by an increase in cell diameter (Zaritsky and Pritchard, 1973; Zaritsky and Woldringh, 1978). The connection between cell diameter and the nucleoid complexity, membrane heterogeneity and peptidoglycan synthesis will be discussed below.

## 4. The Transertion Model, Membrane Domains, and Cell Cycle Events

The cytoplasmic membrane has been implicated in crucial cell cycle events (Marvin, 1968; Funnell, 1993; Nanninga, 1998). For instance, rejuvenation of DnaA following replication initiation (Crooke *et al.*, 1991) requires acidic phospholipids (Castuma *et al.*, 1993), and assembly of FtsZ in a "cytokinetic ring" between daughter chromosomes anchored to the membrane preceding division (Lutkenhaus, 1993) needs phosphatidylethanolamine (Mileykovskaya *et al.*, 1998).

On a more general level, the highest activities of transcription, translation and insertion or transport ("transertion") of inner and outer membrane proteins expressed from many different genes scattered on the chromosome could be envisaged to occur around the nucleoid (Norris, 1995; Woldringh *et al.*, 1995b; Binenbaum *et al.*, 1999). This view is depicted schematically in Figure 1B. The "transertion" activity could affect enzymes involved in peptidoglycan synthesis, resulting in decreased rate at the surface surrounding the nucleoid, as has been observed by autoradiography (Mulder and Woldringh, 1991). Such a crowding effect can also result in membrane domains determined by the position(s) of nucleoid(s) and its (their) interactions with the plasma membrane, as previously suggested by Norris (1992). Sequestration of newly-replicated, hemi-methylated *oriC* is one such possibility that is widely entertained in current literature (Ogden *et al.*, 1988; Campbell and Kleckner, 1990; Bogan and Helmstetter, 1996). It may thus be the mechanism exploited to block a second round of replication-initiation (so-called "stacking") during ca. 10 min following an initiation event ("eclipse period" or inter-initiations "dead time") despite accumulated capacity for initiation (Zaritsky, 1975b).

The crowding effect may be the mechanism used to prevent premature cell division, as suggested in the nucleoid occlusion model (Mulder and Woldringh, 1989). When sister nucleoids segregate, a new less crowded membrane domain with a different phospholipid and protein composition seems to be created between them, which can signal the assembly of the FtsZ ring and the subsequent recruitment of cytoplasmic (FtsA) and membrane-bound proteins (ZipA, FtsQ, FtsL and PbpB) (Fig. 1D). These proteins have been shown by immuno-fluorescence studies (Ma *et al.*, 1996; Yu *et al.*, 1998) to be sequentially recruited to the divisome (Nanninga, 1998). An indicator for the appearance of such membrane domains at the site of separating nucleoids has recently been obtained with the fluorochrome FM4-64 (Fishov and Woldringh, 1999).

## 5. Peptidoglycan Synthesis, Assembly of the Division Ring and Cell Shape

### 5.1. DETERMINATION OR MAINTENANCE OF CELL SHAPE

Bacterial cell shape must have genetic determinants because it is characteristic for any strain and perpetuates between generations. The mechanism(s) to accomplish this is (are) completely unknown. The rigid peptidoglycan sacculus maintains cell shape, but is it the shape-determining molecule as well? One view is that the sacculus plays the role of a simple template that is duplicated (Goodell and Schwarz, 1975). It has been schematized in Höeltje's (1998) "three-for-one" growth model, proposing that the holoenzyme murein-replicase duplicates the sacculus according to a "make-before-break strategy" (Koch, 1982). The complex synthesizes three new murein strands and attaches them on both sides of a docking strand via the peptide cross-bridges. The docking strand is simultaneously degraded by transglycosylases and endopeptidases incorporated in the back of the sliding complex. This mode of shape determination is only possible, however, if the glycan strands lie in an ordered fashion and their lengths can be precisely copied by the murein synthesizing machinery (as in the Hoop theory of Cooper, 1989). This model for bacterial morphogenesis does not solve the problem of division mechanism nor of changes observed in cell diameter. A murein-based model becomes very difficult to imagine if the glycan strands are perpendicularly oriented, as recently suggested by Dmitriev *et al.* (1999). According to such a model, the necessity for order of glycan strands functioning as a template has vanished. Our preferred, alternative view, that cell shape is determined by physico-chemical interactions between cytoplasm and the nucleoid, will be described below. Obviously, the genetic background constrains these interactions through variations in composition of cellular cytoplasm and structures.

### 5.2. PENICILLIN-BINDING PROTEINS

The final steps in peptidoglycan synthesis and maturation are catalyzed by 12 penicillin-binding proteins (PBPs) (Spratt, 1978; 1983; Henderson *et al.*, 1997). Inactivating them either by specific $\beta$-lactam antibiotics or growth at the restrictive temperature of *ts* mutants is instructive for understanding their respective roles in bacterial physiology. For example, inactivating PBP2 yields spheroidal cells, while PBP3 is obligatory for division. However, among the series of 192 mutants with all possible combinations of deletions of eight PBP genes that has recently been constructed (Denome *et al.*, 1999), several mutants continued to grow as enlarged spheres when both genes for PBPs 2 and 3 were specifically inactivated. The only lethal combinations were those lacking PBPs 1a and 1b.

Cell division is inhibited by inactivating PBP3, by growing mutants at restrictive conditions or in the presence of specific antibiotics (e.g., furazlocillin or cephalexin). Existing constrictions (formed at the cell center) in the growing filaments are deformed into so-called blunt constrictions. The extra peptidoglycan synthesis needed for the constriction indeed takes place at these positions (Wientjes and Nanninga, 1989). In addition, FtsZ rings can form in the absence of PBP3 activity (Pogliano *et al.*, 1997). It thus seems that the enzymes responsible for the initial peptidoglycan synthesizing activity are penicillin insensitive (Nanninga, 1991).

Pritchard *et al.*, 1975; Chandler and Pritchard, 1975; Meacock *et al.*, 1977; Woldringh *et al.*, 1994; Hadas *et al.*, 1997; Zaritsky *et al.*, 1999a, b).

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The crowding effect may be the mechanism used to prevent premature cell division, as suggested in the nucleoid occlusion model (Mulder and Woldringh, 1989). When sister nucleoids segregate, a new less crowded membrane domain with a different phospholipid and protein composition seems to be created between them, which can signal the assembly of the FtsZ ring and the subsequent recruitment of cytoplasmic (FtsA) and membrane-bound proteins (ZipA, FtsQ, FtsL and PbpB) (Fig. 1D). These proteins have been shown by immuno-fluorescence studies (Ma *et al.*, 1996; Yu *et al.*, 1998) to be sequentially recruited to the divisome (Nanninga, 1998). An indicator for the appearance of such membrane domains at the site of separating nucleoids has recently been obtained with the fluorochrome FM4-64 (Fishov and Woldringh, 1999).

### 5.3. ASSEMBLY OF THE DIVISION RING

The abundant cytoplasmic protein FtsZ has been shown to polymerize as a ring surrounding the cell center to direct the division process (Bi and Lutkenhaus, 1991). Many other Fts proteins involved in the so-called "divisome" (Nanninga, 1998) lie in the periplasm anchored to the membrane and some are cytoplasmic. The interactions between them (spatial and temporal) are currently being intensively studied (e.g., Ghigo *et al.*, 1999; and see Fig. 1D), but the consensus is that FtsZ is the first to be active and recruits the others to the division site (e.g., Taschner *et al.*, 1988; Addinall and Lutkenhaus, 1996; Liu *et al.*, 1999).

## 6. Nucleoid Replication and Segregation—Cell Division and Shape

### 6.1. COUPLING BETWEEN REPLICATION AND DIVISION

Coupling between cell division and replication of the genetic-information complement (in this case, the bacterial chromosome) is a necessary condition for survival of a species. The mechanism involved has still not been deciphered. The C-H model (Helmstetter *et al.*, 1968) raises an attractive idea, that termination of a round of chromosome replication triggers cell division by an abrupt rise of the local concentrations of dNTPs upon instantaneous cessation of their use in replication. This coupling is envisaged to be a consequence of the inter-relationships and connections between metabolism of DNA and cell wall precursors (Woldringh *et al.*, 1991).

Our approach couples the timing of cell division and location of the division ring (septum in gram positive species, constriction in gram negatives) to certain physical parameters of the cell and the nucleoid by influencing local cellular biochemistry. This view is difficult to envisage but represents a flexible and dynamic mechanism for morphogenesis. The model assumes that the self-organizing properties of DNA generate a compact, spherical nucleoid through the interactions of DNA supercoils with themselves and with the cytoplasmic proteins exerting molecular crowding forces (Woldringh and Odijk, 1999). Depending on its biosynthetic activity (concomitant transcription, translation and protein transport) the nucleoid assumes a prolate shape, which during multifork replication can change into dumbbell and multi-lobular shapes (Woldringh *et al.*, 1995b). The "transertion" activity (Norris, 1995; Woldringh *et al.*, 1995b; Binenbaum *et al.*, 1999) could crowd out or push aside the PBPs involved in peptidoglycan synthesis. This would result in the decreased rate of murein synthesis at the surface surrounding the nucleoid, as has been observed by autoradiography (Mulder and Woldringh, 1991). The above ideas have led to the model described below and in Figure 1.

### 6.2. THE MODEL (Figure 1, reproduced from http://wwwmc.bio.uva.nl/~conrad/)

Our model is based on the observations that nucleoid segregation in *E. coli* (Woldringh, 1976; Van Helvoort *et al.*, 1996) as well as in *B. subtilis* (Nanninga, unpublished) takes place gradually during replication. The idea that *oriC* functions as a centromer-analog was originally proposed by Ogden *et al.* (1988) and is implicit in the theoretical considerations of Dingman (1974) and in the nucleoid-formation model of Lϕbner-

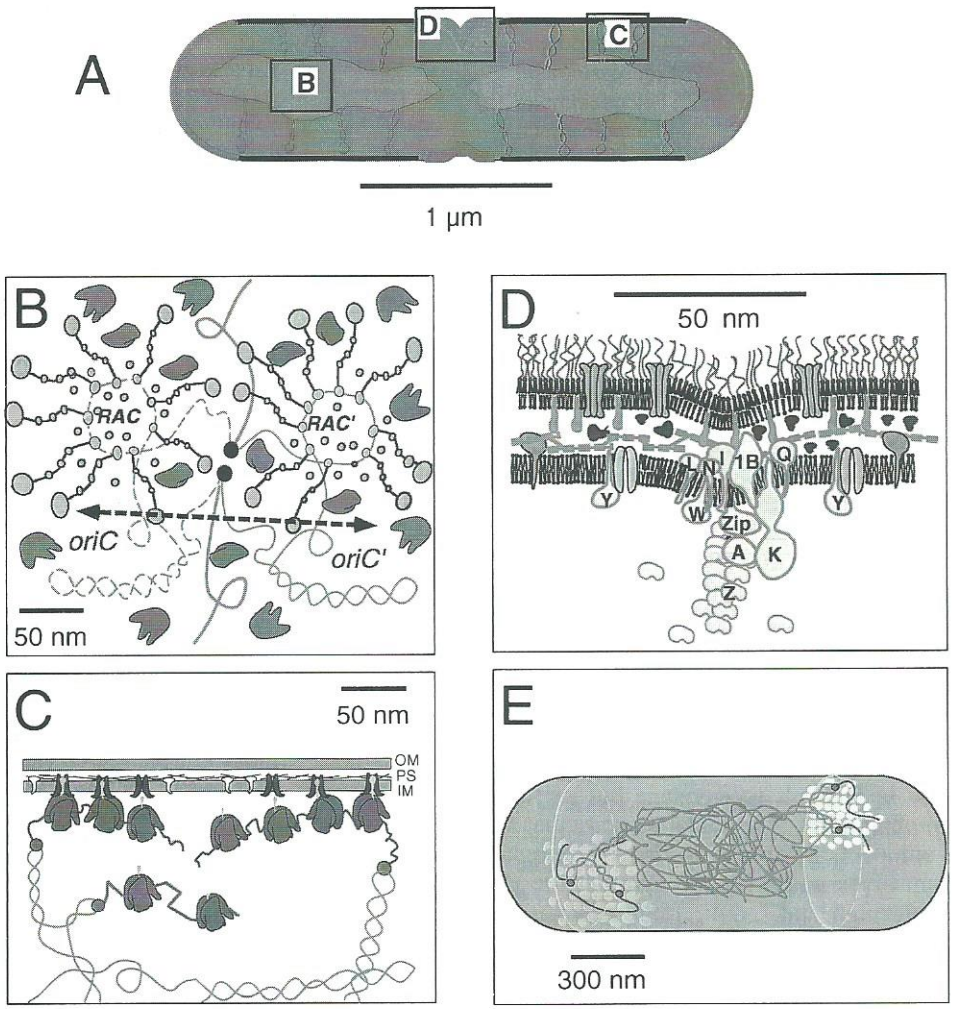


**Figure 1** A schematic representation of the model relating nucleoid segregation to cell division.

A. Overview of an *E. coli* cell grown in glucose minimal medium (length 3 µm, diameter 0.7 µm). Three membrane domains generated by the transertion activity of the nucleoid have been distinguished: polar, lateral and central. Boxes indicate three processes that have been depicted in more detail in B, C and D.

B. Initial displacement of the newly replicated sister origins (*oriC* and *oriC'*) within the nucleoid. The replicated daughter strands are indicated by thin, dashed and full lines. The origins (represented as loops) are assumed to be pushed apart by formation of two ribosome assembly compartments (RAC and RAC') around the *rrnC* operon. An imaginary nucleoid segregation axis (NSA, indicated by the dashed line) is assumed to develop between the RACs. The filledblack circles represent the two replication forks.

C. Formation of a proteolipid domain by transertion. Co-transcriptional and co-translational insertion of membrane proteins (transertion) indirectly links DNA to the inner membrane (IM) via RNA polymerase, mRNA, ribosomes, nascent polypeptides and (here) the Sec translocase. RNA polymerase drags the DNA at the apical loop of its supercoils, thereby forcing it to rotate (ten Heggeler-Bordier *et al.*, 1992). PS, periplasmic space; OM, outer membrane.

D. The divisome. After FtsZ ring formation between the segregating nucleoids, the following membrane proteins have been recruited: FtsA, ZipA, FtsI (PBP3), FtsQ, FtsN, FtsW, FtsL, FtsK and PBP1B. FtsY forms part of the translocase complex.

E. Formation of two proteo-lipid membrane domains (300 x 300 nm) by the transertion mechanism. The two domains, located at the front and back of the cell, consist of 50 inserted proteins (center indicated by the circles, proteins not to scale) transcribed from two different genes. Such transertion structures exist temporarily for many different genes coding for membrane proteins.

-Olesen and Kuempel (1992). The analogy with a centromer is only applicable if the replicated *oriC* is the first region to be separated. Since there is no structural analog for the eukaryotic spindle to give directionality to this initial outward movement, we have postulated that an imaginary Nucleoid Segregation Axis (NSA) is formed between the two sister *oriC*s (Woldringh and Nanninga, 1985; Woldringh *et al.*, 1994; 1995a). One possibility for such a segregation axis is formation of two Ribosomal Assembly Compartments (RACs) that push away the newly-replicated *oriC*s and may thus be regarded as a nucleolar-like structure.

6.2.1. *Ribosomal Assembly Compartments and Nucleoid Segregation Axis (Fig. 1B, C)*
According to this concept, diffusion of new ribosomes is limited by their functioning on nearby mRNAs. The centers of the two new RACs are considered as the ends of an imaginary line, the NSA, which extends with mass growth and pushes the nascent DNA outward. The lifetime of a RAC is equal to inter-initiation time ($\tau$), but an old one is not just "dissolved" and replaced by new ones starting from size zero; it persists, but with a duplicated "active center". During this "reorganization", the replicated *oriC*s are pushed apart, thus used as the initial force in their partitioning (Fig. 1B). Following this initial separation, newly replicated DNA-loops are sequentially pulled into the different cell halves by the transertion activity (Fig. 1C). Instantaneous replacement of an old RAC by two new ones is necessary because a RAC is the motor that drives cell growth continuously. If duplication is inhibited (e.g., by inhibiting DNA synthesis), the RACs continue to enlarge "indefinitely" pulling the nucleoid apart into small lobules (Woldringh *et al.*, 1994).

The model further assumes that secondary axes develop during multifork replication under conditions of rapid growth. Initially these lie perpendicular to the previous axis (which is the long cell axis when $C < \tau$). In a cylindrical cell, the NSA is forced to rotate over 90° during its lifetime to parallel the cell's long axis. It then induces a division plane perpendicular to the longest axis (Woldringh et al., 1995a). Thus, even after reinitiation, the old axis continues to rotate as if initiation had not occurred.

The initial strand separation by formation of the nucleolar-like RACs requires the presence of rRNA genes (*rrn*) near *oriC*. This requirement is fulfilled in many genomes (Cole and Saint Girons, 1994). The RAC idea has recently gained support from a completely new experimental angle: in a systematic study inactivating sequentially from one to all seven existing rRNA (*rrn*) operons, Asai *et al.* (1999) observed "a pronounced morphological change during exponential growth; the cells became more and more elongated....not completely reversed in a Δ7 strain..., suggesting that cellular processes other than *rrn* gene dosage are still perturbed". One of those presumed "other processes" may be the initial separation of *oriC*s.

6.2.2. *The Partitioning System*
Another mechanism to partition sister replicons, which is found on both chromosomes and plasmids, consists of a cis-acting DNA-site and two trans-acting proteins (Par). In *B. subtilis*, several DNA sites (*parS*) have been found near *oriC*, with high affinity for SpoOJ (Lin and Grossman, 1998). It has been suggested (Glaser *et al.*, 1997; Wu and Errington, 1997) that SpoOJ forms in *B. subtilis* a nucleoprotein complex displacing the segregating origins. The above two mechanisms, i.e., formation of RACs and the Par system, may function together and complement each other. It should be noted, however, that Par is not found on the *E. coli* chromosome (although it exists on its P and F

plasmids), whereas *Caulobacter crescentus* lacks an rRNA gene close to its origin. The *par* system in the latter appears to be essential (Marczynski *et al.*, 1990; Mohl and Gober, 1997), whereas mutations in the *par*-like genes of *B. subtilis*, in which an *rrn* occurs close to *oriC*, only lead to mild effects (Webb *et al.*, 1997).

6.2.3. *Summary and corollaries*

Soon after initiation of replication, an initial displacement of the newly replicated daughter strands occurs by either one or a combination of the two mechanisms described above, the RACs (Fig. 1B) or the *par*-system. This displacement creates a direction of movement through an imaginary axis (NSA), which helps to distinguish replicated DNA regions of the two daughter strands. Subsequently, these regions are pulled to either cell half by the transertion mechanism (Fig. 1C): a promoter-containing DNA loop is translated co-transcriptionally. If it encodes plasma membrane or excreted protein, the nascent peptide is targeted by one of the known insertion or translocation mechanisms. Co-translational insertion anchors the polysome to the membrane, together with the RNA polymerase, through which the DNA tracks. The membrane-pulled DNA-loop increases the likelihood that other RNA polymerase-mRNA-ribosome complexes find an available anchoring site there. Numerous locally inserting (integral) membrane proteins with high affinity to specific phospholipids may form a distinct proteo-lipid domain (Fig. 1E).

In rapidly growing cells, the transertion-mediated pulling force acting on the replicating DNA causes its separation into lobules (as happens during replication inhibition). The perpendicular and tilted planes in which the lobules often lie confirm the existence of axes along which DNA moves. Although these structures represent characteristic shapes, recognizable in most cells of a rapidly growing population, they do not necessarily reflect the position and rotation of the hypothetical segregation axes between the RACs.

By minimal energetic considerations, the NSA initially lies perpendicular to the plane of the previous one (Zaritsky *et al.*, 1999a, b). During extension, it is forced by the rigid cell envelope to rotate until it parallels cell length. The division plane is eventually positioned perpendicularly to that of the NSA when the signal for constriction is activated, presumably around the time of replication-termination at *terC*.

## 6.3. TESTING THE MODEL BY MULTIPLE CONSTRICTIONS

Successive divisions are usually parallel in bacillary bacteria, whether growing under steady-state conditions or following filamentation (brought about by various means; Taschner *et al.*, 1988). In spheroids containing multi-forked replicating nucleoids, where the spatial constraint (the cylindrical rigid wall) forcing them to rotate is relieved, NSA would continue to extend in the same plane in which the duplicated *oriC*s were pushed away by the RACs following initiation. The segregating *oriC*s would be located under such conditions in the apexes of a regular tetrahedron, implying a perpendicular axis to the previous axis and to each other (as in Fig. 6c of Zaritsky *et al.*, 1999a). Our model predicts that resultant secondary constrictions are laid down in planes perpendicular to the previous and to their sister division planes, in 3 dimensions. This prediction can be confirmed by obtaining successive constrictions simultaneously on the same cell before separation to two daughters, a condition requiring enhancement (at least temporarily) of the division signals. We have recently developed a method to meet

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both requirements, to relieve the constraint and to enhance division signals (Zaritsky *et al.*, 1999a, b).

A brief inhibition of PBP2 activity, transforming the bacillary cell into a coccus, can be obtained either by a *pbpA ts* mutant in the restrictive conditions or by mecillinam, the latter avoids complications incurred by the temperature shift. Enhanced frequency of division signals was achieved by manipulating the nucleoid replication rate using the defined transitions ("steps") between various concentrations of thymine. Secondary constrictions in such cells were visualized by scanning electron microscopy and by confocal scanning laser microscopy of cells stained with FM 4-64 (Zaritsky *et al.*, 1999a). More recently, division rings were probed by specific anti-FtsZ monoclonal antibodies. Only partial rings (arcs) were observed, probably due to shortage of FtsZ to complete them over the wide cells, but their planes were tilted (Zaritsky *et al.*, 1999b), supporting our model.

The ultimate solution will follow FtsZ ring assembly in such multi-constricted cells by visualizing the rings with FtsZ-GFP fusion proteins in vivo. To this end, the chimeric construct *ftsZ-gfp* is cloned on the multi-copy pRRE7 for high expression (Einav *et al.*, in preparation).

## 7. Conclusions

The self-organizing properties of bacterial DNA and its indirect relationship to the inner membrane may determine cell shape and division.

In this view, cell diameter, and thus cell shape, may be related to the nucleoid complexity, membrane heterogeneity and peptidoglycan synthesis, determined by the vertical displacement of the segregation axes from the longitudinal axis of the cell.

There seem to be reciprocal inter-relationships between two forces as follows:
(a) the cylindrical cell wall forcing the NSA to rotate, and (b) the increased amount of DNA per nucleoid, which leads to increased membrane crowding (more genes coding for envelope proteins expressed in the vicinity of the nucleoid).

The two forces probably slow down peptidoglycan synthesis, causing decreased surface/volume ratio and thus, increased diameter.

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