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The initiation mass for DNA replication in *Escherichia coli* K-12 is dependent on growth rate

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It is widely accepted that the initiation mass of *Escherichia coli* is constant and independent of growth rate, and therefore is an important parameter in the regulation of initiation of DNA replication. We have used flow cytometry to measure the initiation mass of *E.coli* K-12 cells as a function of growth rate. The average initiation mass was determined by two methods: (i) from a mathematical relationship between average cell mass, cell age at initiation and number of origins present in the cells, and (ii) directly from the cell mass distribution. The light scattering signal from individual cells and the protein content per cell were employed as measures of cell mass. The initiation mass was found to increase monotonically with decreasing growth rate, being 1.6 times higher (light scattering) or 2.1 times higher (protein content) at 0.3 than at 2.5 doublings per hour. We conclude that the initiation mass is dependent on growth rate. This finding indicates that the control for timing of initiation is not governed by a direct connection between mass accumulation and the molecule(s) determining initiation of replication.

Key words: Escherichia coli/flow cytometry/initiation of DNA replication/initiation mass

Introduction

All cells, both prokaryotic and eukaryotic, grow and divide at a rate determined by the constituents of the growth medium. Cells divide when they attain a certain size (Fantes *et al.*, 1975; Koppes *et al.*, 1978; Nurse, 1990). Cells that are larger than average at birth have a shorter subsequent cycle, while those smaller than average at birth have a longer subsequent cycle. Thus, cell sizes deviating from the average at division are compensated for in the following cell cycle to avoid a divergence in cell size. This illustrates that the control of the cell cycle and cell division is coupled to the rate at which the cells accumulate their mass.

A prerequisite for a cell to divide is that its DNA is replicated, which makes the initiation of replication a key event in the cell cycle. In *Escherichia coli*, the biochemistry of initiation of replication from the chromosomal origin *oriC* has been worked out in detail (reviewed in Kornberg and Baker, 1992). Initially, the initiator protein DnaA binds to its four recognition sites in *oriC* (Fuller and Kornberg, 1983; Funnel *et al.*, 1987). This leads to the formation of an open complex where the DNA is unwound in a certain AT-rich region (Bramhill and Kornberg, 1988; Sekimizu *et al.*, 1988). With the help of DnaC, DnaA recruits the DnaB protein to the unwound region, which is then enlarged by the helicase activity of DnaB (Baker *et al.*, 1986; Lebowitz and McMacken, 1986). Subsequently, the primers are synthesized and replication forks in both directions are established. Under suboptimal conditions, initiation may be activated by nearby transcriptional activity (Baker and Kornberg, 1988; Skarstad *et al.*, 1990). However, although the proteins and cofactors necessary for initiation to occur *in vitro* are known, the rate limiting steps or possible regulatory functions have not been identified.

In bacteria, the time required for replication of the chromosome may exceed the doubling time. Rapidly growing cells get around this problem by initiating replication in the preceding generation at two origins instead of one, or even two generations before at four origins (Cooper and Helmstetter, 1968). Thus, fast-growing cells have overlapping replication cycles and contain more replication origins than slow-growing cells. Fast-growing cells are also larger than slow-growing cells. By combining data for the size of *Salmonella typhimurium* (Schaechter *et al.*, 1958) with the model for DNA replication in *E.coli* B/r (Cooper and Helmstetter, 1968), the cell mass per origin at the time of initiation, the initiation mass, was found to be constant (Donachie, 1968). The concept that the initiation mass is the same at different growth rates was attractive and brought novel and interesting aspects to our thinking about how initiation of replication is regulated. For 25 years, the initiation mass has been a central concept for cell cycle regulation in *E.coli* [see, for example, Helmstetter *et al.* (1968), Pritchard *et al.* (1969), von Meyenburg and Hansen (1987) and Donachie (1993)].

Insight into the mechanism of coupling between bacterial cell growth and the cellular 'clock' that regulates initiation of chromosome replication is important for our understanding of the cell cycle control. A constant initiation mass would indicate a direct connection between mass accumulation and the molecule(s) controlling initiation of replication. In contrast, a variation of the initiation mass with growth rate would imply no such direct coupling, meaning that the controlling molecule(s) is not produced, or converted into an active form, in direct proportion to the cell mass.

There are few reports where the initiation mass has actually been measured over a range of growth rates. In spite of a lack of experimental support for a constant initiation mass, it is commonly stated in the literature that initiation takes place at a fixed cell mass. We therefore considered it important to establish methods by which initiation mass can be accurately measured, and to perform a systematic examination of the theoretically deduced growth rate invariance. In this work, the average initiation mass of *E.coli* K-12 was measured over a wide range of growth rates by two sensitive methods based on flow cytometry using: (i)

a mathematical expression involving the average cell mass during steady-state growth, the cell age at initiation and the number of origins in the cells; and (ii) the initiation age and the cell mass distribution. The results showed that the initiation mass was not constant, but decreased monotonically with increasing growth rate.

Results

Cells of strain AB1157 were grown in media containing different carbon sources and amino acids, yielding growth rates ranging from 0.3 to 2.5 doublings per hour. The average initiation mass in each culture was determined by two different methods, both based on flow cytometry of individual cells. Two alternative measures of cell mass were employed: the flow cytometric light scattering signal and the cellular protein content.

Initiation mass calculated from average cell mass and initiation age

In an exponentially growing culture, the initiation mass (M_i) can be calculated from the expression (derived in Appendix):

$$M_i = \frac{M_{av} \cdot 2^{a_i}}{2ln \cdot O_i} \qquad (1)$$

where M_{av} is the average cell mass in the population, a_i is the cell age at initiation of a new round of replication and O_i is the number of origins per cell at initiation. All three parameters were obtained by flow cytometry. The number of origins was determined from samples treated with rifampicin and cephalexin (Figure 1A). Rifampicin stops initiation of DNA replication, but allows already started replication forks to continue to the termini. Cephalexin rapidly stops cell division (Boye and Løbner-Olesen, 1991). Hence, a combined addition of these drugs followed by further incubation results in cells containing an integral number of fully replicated chromosomes, the number of which is equal to the number of origins per cell at the time of drug addition (Skarstad *et al.*, 1986; Boye and Løbner-Olesen, 1991). The initiation age was calculated from the age distribution function as described in Appendix. Briefly, the fraction of cells that had not initiated replication was obtained from the DNA histograms of the samples treated with rifampicin and cephalexin (shaded area in Figure 1A). This fraction equals the fraction of cells having an age between 0 and a_i. Thus, integrating the age distribution function from 0 to a_i and solving for a_i yielded the initiation age explicitly. M_{av} was measured as the average light scattering per bacterial cell.

Employing Equation 1, the initiation mass was calculated for five parallel and independent series of experiments. The results taken together (Figure 2A) showed that the initiation mass was dependent on the growth rate, decreasing from ~1.3 to 0.8 relative units when the growth rate increased from 0.3 to 2.5 doublings per hour. For comparison, average cell mass (scattered light) increased ~4-fold within the same range of growth rates (data not shown).

Linear regression analysis of the results of each individual series of experiments revealed that the slope of the regression line (regression coefficient) varied somewhat between the series, but was significantly <0 ($P < 0.05$) in four out of five experiments. Data from the fifth series, represented by

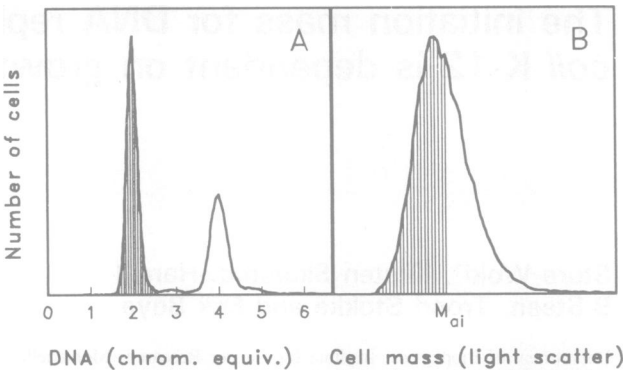

Fig. 1. Measurement of initiation mass from cell mass distribution. (**A**) DNA histogram of cells treated with rifampicin and cephalexin. The shaded area represents the cells that have not yet initiated replication at the time of drug addition. The positions of the peaks reflect the content of fully replicated chromosomes, i.e. the number of origins present in the cells. (**B**) Light scattering histogram of cells from the same culture as in panel A before treatment with rifampicin and cephalexin. The shaded area is equal to the shaded fraction in panel A and therefore corresponds to the fraction of cells that have not yet initiated a new round of replication. M_{a_i} is the average cell mass at the time of initiation.

open circles in Figure 2A, did not give a regression coefficient significantly different from 0 solely due to the left-most point. Linear regression analysis was also carried out for all the experimental points taken together, revealing a highly significant ($P < 0.001$) negative regression coefficient. Analyses of the residuals (see Materials and methods) showed that the differences in regression coefficient between the series can be ascribed to random errors (data not shown).

An alternative way of determining the initiation mass is to use the same equation as above (Equation 1), but to use average cellular protein content instead of average light scattering for M_{av}, i.e. to calculate the protein content per origin at initiation. The protein content was measured as fluorescence intensity of individual cells stained with the protein-specific dye fluorescein isothiocyanate (FITC). Two independent series of experiments were performed with cell samples from two of the previous experiments. The dependence on growth rate was confirmed; the initiation mass decreased from 1.6 to 0.75 relative units when the growth rate increased from 0.3 to 2.5 doublings per hour (Figure 2B). The larger variation in initiation mass compared with that in Figure 2A is due mainly to higher initiation mass values at the lowest growth rates in Figure 2B.

Initiation mass determined directly from the cell mass distribution

The initiation mass can also be estimated without using Equation 1. The DNA histogram of cells treated with rifampicin and cephalexin yields the fraction (F) of cells that have not yet initiated replication at the time of drug addition (Figure 1A). Assuming that cell size increases monotonically with cell age, cells that have not yet initiated replication are smaller than those that have. Thus, knowing fraction F, the cell mass at initiation can be determined directly from the mass distribution (light scattering histogram) of untreated cells (drugs not added) from the same culture. In other words, if the left-most fraction of the light scattering histogram is set equal to fraction F, i.e. the shaded areas

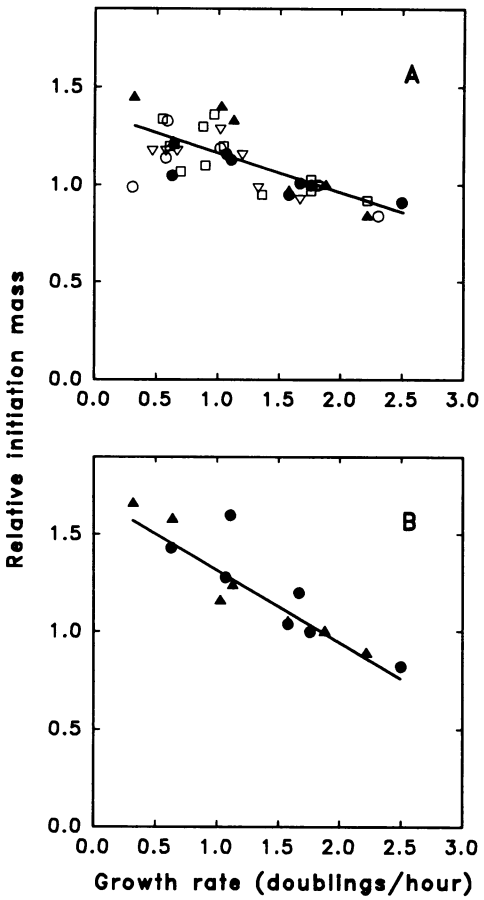

Fig. 2. Initiation mass calculated by Equation 1 using light scattering (**A**) or FITC fluorescence (**B**) as measures of cell mass. Different symbols represent separate series of experiments. The samples used in panel B were the same as in two of the (A) series (corresponding symbols used). The regression lines for all experimental points taken together are shown. The initiation mass is measured in relative units and has been normalized to 1.0 at a growth rate of 1.75 doublings per hour.

in the two histograms (Figure 1A and B) are equal, the cell mass at initiation can be read directly from the light scattering histogram (Figure 1B). The initiation mass is obtained when dividing by the number of origins to be initiated. The use of this alternative method revealed a variation in the initiation mass (Figure 3) similar to that in Figure 2A, further confirming that the initiation mass is dependent on growth rate.

Measurement of the delay in rifampicin action

In the above methods it is assumed that cell division and initiation of DNA replication are stopped simultaneously. Cephalexin inhibits cell division in <1 min (Boye and Løbner-Olesen, 1991). If rifampicin needs a longer time to inhibit initiation, simultaneous addition of the two drugs would yield an underestimation of the initiation mass. Therefore the delay in the effect of rifampicin inhibition of initiation was measured. In the method used (Bremer and Churchward, 1977), the difference between the total amount of DNA accumulated in the presence and absence of rifampicin (γ) is measured; $\sqrt{\gamma}$ is plotted against time, and the intersection between the regression line and the abscissa is determined by extrapolation (see legend to Figure 4 and

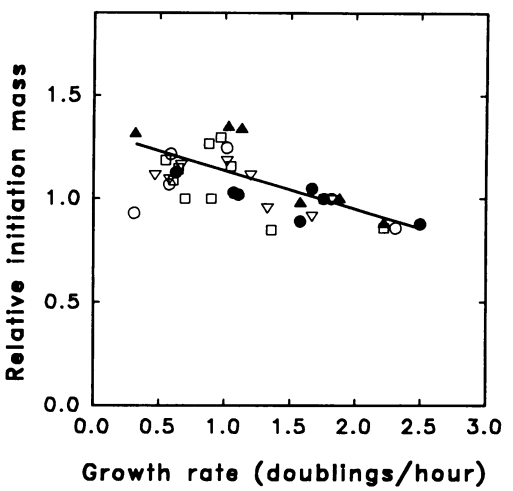

Fig. 3. Relative initiation mass determined directly from the light scattering histograms, as illustrated in Figure 1. Data are from the same experiments as in Figure 2A and are indicated by corresponding symbols. The common regression line is shown. The initiation mass has been normalized as in Figure 2.

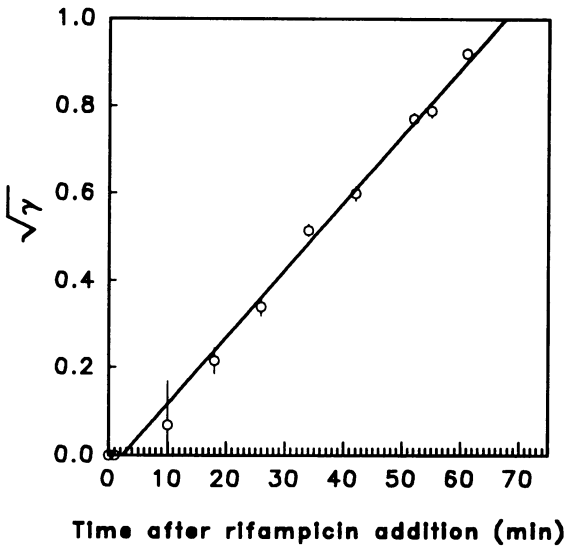

Fig. 4. Inhibition of DNA replication by rifampicin. Strain EBO81 was grown in AB + glucose supplemented with [^{14}C]uracil, and rifampicin was added at $t = 0$. The intersection value for the $\sqrt{\gamma}$ regression line with the time axis shows the delay in inhibition of initiation (Bremer and Churchward, 1977). The vertical bars through the symbols indicate the standard deviation (two measurements).

Materials and methods). Three different media yielding different growth rates were used: LB + glucose, 2.5 doublings per hour; AB + glucose, 1.1 doublings per hour; AB + glycerol, 0.7 doublings per hour. The estimated delay in rifampicin action was 2 min or less in all three media (Figure 4; data shown for AB + glucose only). Hence, the combined addition of cephalexin and rifampicin stops cell division and initiation of DNA replication almost immediately.

Measurements of initiation age

The most critical parameter when calculating the initiation mass by Equation 1 is the age at initiation, a_i. The data in

Figure 2 are based on a_i values obtained by flow cytometry of cultures treated with rifampicin and cephalexin. We wanted to measure the initiation age by another independent method. The C period is the time from initiation to termination of DNA replication, whereas the D period is the time from termination to cell division. If the duration of C and D is known, a_i can be derived from the expression:

$$a_i = 1 + n - (C + D)/\tau \quad (2)$$

where n is the next lower integer value of $[(C + D)/\tau]$, and τ is the doubling time. This formula simply expresses that initiation occurs $(C + D)$ min before cell division. The C and D periods in some of the cultures from the above experiments were determined by computer simulation of the DNA distributions obtained from flow cytometry, as described previously (Skarstad *et al.*, 1985; Allman *et al.*, 1991). This method employs the Cooper and Helmstetter (1968) model to predict the DNA content of individual cells in an exponentially growing population. The parameters C and D are adjusted to make the predicted DNA distribution fit the experimentally obtained distribution.

The simulations showed that for the DNA histograms in which a good fit was obtained, i.e. with no major deviations between the simulated and the experimental DNA distributions, the initiation ages calculated from Equation 2 were similar to those obtained by analysis of cells treated with rifampicin and cephalexin (data not shown). However, the experimental uncertainty in the C and D values obtained by the simulation routine gave variations in a_i that were larger than those obtained when measuring a_i after treatment with rifampicin and cephalexin. Therefore, we favour the latter method.

Discussion

We have shown that the initiation mass of the *E.coli* K-12 strain AB1157, as measured by two different flow cytometric methods, was not constant, but increased monotonically with decreasing growth rate. Within the range of growth rates investigated (0.3 to 2.5 doublings per hour), the initiation mass varied by a factor of 1.6 (when measuring light scattering) or 2.1 (when measuring protein contents).

The age at initiation was derived from the number of fully replicated chromosomes present after run-out of DNA replication in the presence of rifampicin. Initiation of DNA replication is therefore defined as attainment of the rifampicin-resistant stage, and the initiation mass is derived from the cell mass at entrance to this stage. Our other method of determining initiation age, by analysing DNA histograms from exponentially growing cultures, gave essentially the same results as those obtained with rifampicin treatment, indicating that the replication forks actually leave *oriC* shortly after rifampicin resistance is achieved.

The light scattering signal from individual cells was employed as one estimate of cell mass. The amount of scattered light from each cell passing the excitation focus in the flow cytometer has been shown to reflect cell size (Boye *et al.*, 1983). However, the light scattering signal is a complex function of the size, shape and structure of the cell. Scattered light is therefore not a strictly linear function of cell mass, even though it increases monotonically with cell mass. A convenient and widely used measure of the biological mass of a bacterial culture is its optical density (OD). The culture OD, as measured in a spectrophotometer, is mainly a measure of the amount of light being scattered out of the primary beam by individual cells in the light path [see, for example, Koch (1981)]. Thus, the average flow cytometric light scattering signal from individual bacterial cells is strongly related to the OD of a population of cells.

The total cellular protein, measured as FITC fluorescence intensity, was used as another estimate of cell mass. The initiation mass based on FITC fluorescence intensity showed a larger variation with growth rate than the initiation mass based on light scattering as cell mass. This may be due to a decrease in the protein:mass ratio with increasing growth rate. In the B/r strain the amount of protein constitutes ~70% of the total cell mass at a growth rate of 0.6 doublings per hour compared with only ~50% at 2.5 doublings per hour (Bremer and Dennis, 1987). Assuming equivalent conditions in strain AB1157, an adjustment of the initiation mass in Figure 2B according to this variation in relative protein content yields a similar variation in the initiation mass as shown in Figure 2A.

It might be argued that the average initiation mass would be expected to vary somewhat with growth rate, but to be essentially constant compared with the change in absolute cell mass. However, the variation in initiation mass found here is almost half as large as the variation in average cell mass measured within the same range of growth rates. Thus, the variation observed is large compared with what could be expected from random fluctuations.

An increase in initiation mass with decreasing growth rate is opposite to an earlier finding by Churchward *et al.* (1981), who reported that B/r cells decrease their initiation mass ~2-fold when the growth rate is decreased from 1.6 to 0.6 doublings per hour, whereas for higher growth rates it remains constant. In their work, OD_{460} was used as a measure of cell mass, whereas the number of origins was estimated from the increase in DNA contents after rifampicin addition. We do not know whether this difference reflects strain-specific properties or is due to different measuring methods.

A criterion that must be met by a mechanism regulating initiation of replication is that the initiation event must be triggered at the correct time, corresponding to the rest of the cell cycle and to the growth rate. It is difficult to envisage how cell mass *per se*, as the total sum of DNA, RNA, proteins, lipids, carbohydrates and other components, could determine when to initiate replication. A more likely candidate is some mass-related parameter, e.g. a protein synthesized or regulated in a growth-dependent manner. Our data indicate that the total amount of protein in the cell is not the determinant of initiation. This fact does not, however, exclude the possibility of a specific protein playing this crucial role in the control of initiation. Two different models have been proposed for how accumulation of DnaA protein might trigger initiation of replication (Mahaffy and Zyskind, 1989; Hansen *et al.*, 1991). These models are flexible and can account for the experimental observations on the control and regulation of DNA replication. However, few critical experiments designed to prove or disprove the models have been published.

The signal nucleotide guanosine tetraphosphate (ppGpp) may be involved in determining the time of initiation. ppGpp

modulates the ratio of stable to total RNA, directing transcription such that cells in rich media have a higher relative amount of ribosomes than cells in poor media (Ryals *et al.*, 1982). Furthermore, ppGpp affects the expression of several promoters, including the main promoter of the *dnaA* operon (Chiaramello and Zyskind, 1990). Transcription from the *gid* and *mioC* promoters, which flank *oriC*, can activate replication of *oriC* plasmids, and the activity of these promoters is inhibited *in vitro* by ppGpp (Ogawa and Okazaki, 1991). The initiation mass of ppGpp-less bacteria is, compared with the wild-type, larger at low growth rates and smaller at high growth rates (Hernandez and Bremer, 1993), suggesting that ppGpp affects the control of DNA replication. At a certain intermediate growth rate the initiation mass of the two strains is the same; at this growth rate the ratio of stable to total RNA is also the same in the wild-type and the ppGpp-less cells. Thus, the main effect of ppGpp on initiation of replication seems to be indirect, through changes affecting the protein synthesis machinery (Hernandez and Bremer, 1993).

Note that the initiation mass as defined here refers to the average initiation mass of the cells in a culture. Donachie (1968) speculated that, in addition to being growth rate-invariant, the initiation mass is constant from cell to cell within a population of exponentially growing bacteria. It is important to distinguish between these two concepts. An invariability from cell to cell within a culture relates to the initiation control itself, whereas the variation in the average initiation mass with growth rate relates to the regulation of synthesis of the factor(s) controlling the initiation. Although individual cells were measured in our study, the calculated initiation masses represent the average initiation mass in each culture. Our experiments do not, therefore, address the question of a constancy of the individual initiation mass within a cell population.

Materials and methods

Bacterial strain and growth conditions

The *E.coli* K-12 strain AB1157 [F$^-$, *thr-1*, *ara-14*, *leuB6*, *Δ(gpt-proA)62*, *lacY1*, *tsx-33*, *supE44*, *galK2*, λ$^-$, rac$^-$, *hisG4* (Oc), *rfbD1*, *mgl-51*, *rpsL31*, *kdgK51*, *xyl-5*, *mtl-1*, *argE3*, *thi-1*; Bachmann, 1987] was exponentially grown in shaker flasks at 37°C in one of the following media: (i) Luria Broth (LB) supplemented with 0.2% glucose; (ii) AB minimal medium (Clark and Maaløe, 1967) supplemented with 0.5% casamino acids and either 0.2% glucose or 0.2% glycerol; (iii) AB with one of the following carbon sources: 0.2% glucose, 0.2% glycerol, 0.4% succinate, 0.4% acetate or 0.6% proline; or (iv) AB with 0.2% glucose and either four or nine non-essential amino acids (100 μg/ml asparagine, 100 μg/ml glutamine, 100 μg/ml glycine and 20 μg/ml isoleucine; or the previous four plus 100 μg/ml alanine, 20 μg/ml methionine, 100 μg/ml serine, 20 μg/ml tryptophan and 40 μg/ml valine). Essential amino acids (22 μg/ml arginine, 22 μg/ml histidine, 30 μg/ml proline, 20 μg/ml leucine and 80 μg/ml threonine) were always present in AB without casamino acids. Thiamine was always present at 1 μg/ml. Exponentially growing cultures were routinely obtained by a 2000-fold dilution of overnight cultures and incubation under constant conditions for at least seven generations before samples were taken. Bacterial growth was followed by measuring OD at 450 nm for minimal medium and at 600 nm for LB. Strain EBO81 (AB1157 *pyrB*::Tn*5*) was constructed by P1 transduction from strain FH1218 *pyrB*::Tn*5* (F.G.Hansen, unpublished data), and was used to measure the kinetics of rifampicin action in slow-growing cells.

Rifampicin and cephalexin treatment

The fraction of cells that had not initiated DNA replication at a given time and the corresponding number of origins per cell were measured by flow cytometry of cells incubated with rifampicin and cephalexin before fixation and staining. At an OD of ~0.15, rifampicin was added to 150 μg/ml and cephalexin to 10 μg/ml, whereafter incubation was continued for three to four doubling times to complete ongoing rounds of replication. This treatment yields cells containing an integral number of fully replicated chromosomes, the number of which is equal to the number of origins present at drug addition (Boye and Løbner-Olesen, 1991).

Fixation and staining of cells

Fixation and DNA staining of cells for flow cytometry was performed as described (Skarstad *et al.*, 1985, 1986). For protein staining, fixed cells were washed and resuspended in 0.1 M phosphate buffer, pH 9.0, and the samples diluted to equal protein concentration (5 μg/ml), as measured chemically (Bradford, 1976). A fresh solution of FITC (3 μg/ml) dissolved in the same buffer, was added dropwise to a final concentration of 1.5 μg/ml. FITC was allowed to bind covalently to protein during overnight storage at 4°C before unbound dye was washed off by centrifugation. The cells were resuspended in cold 0.02 M phosphate buffer, pH 9.0, containing 0.13 M NaCl, and kept in the dark at 0°C for at least 3 h before flow cytometry.

Flow cytometry

Flow cytometry was performed with an Argus flow cytometer (Skatron AS, Lier, Norway) as described previously (Skarstad *et al.*, 1983). To monitor the stability of the instrument, fluorescent latex beads (1.5 μm diameter; Polysciences Inc.) were run between each sample. All flow cytometric parameters used were corrected for variation in instrument performance. The protein content of cells stained with FITC was measured in a FACScan flow cytometer (Becton Dickinson).

Statistical analyses of the data in Figure 2A

Linear regression analyses were performed for each series of experiments and for all experimental points taken together. To justify the regression procedure we examined the distribution of the residuals (the distances from the experimental points to the regression line) of the initiation masses. The residuals were also subjected to a one-way analysis of variance with respect to the different series of experiments.

Residual DNA synthesis after addition of rifampicin

LB medium containing glucose and 2 μCi/ml methyl-[^{3}H]thymidine, or AB containing 50 nCi/ml 2-[^{14}C]uracil (both obtained from Amersham, UK), essential amino acids and either glucose or glycerol, were inoculated with overnight cultures of AB1157 or its uracil-requiring derivative EBO81, respectively. Rifampicin (150 μg/ml) was added at an OD of 0.15 and 1 ml samples were withdrawn at intervals. The [^{14}C]uracil-labelled samples were diluted into equal volumes of 1 N NaOH, incubated for 90 min at 45°C to degrade the RNA, whereafter the DNA was precipitated in 10% trichloroacetic acid (TCA). The [^{3}H]thymidine-labelled samples were directly precipitated in 10% TCA. The samples were stored at 4°C overnight. Unlabelled uracil or thymidine (2 mg/ml) was added before the precipitates were collected on Whatman GF/C filters. The filters were washed with cold 0.15% TCA, dried and the radioactivity was measured by liquid scintillation counting.

The delay in inhibition of initiation by rifampicin was found by determining the intersection of the regression line for $\sqrt{\gamma_t}$ with the time axis, as described by Bremer and Churchward (1977). $\gamma_t = 2^{(t/\tau)} - \Delta G_t$, where t is time after addition of rifampicin, τ is the doubling time and ΔG_t is the DNA content per unit volume of culture at time t divided by the DNA content at the time of drug addition.

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Appendix

Calculation of time of initiation and initiation mass

The age distribution of an exponentially growing culture is given by the function (Figure 5):

$$n(a) = 2 \cdot \ln 2 e^{-a\ln 2}, \quad 0 \le a \le 1 \tag{A1}$$

where a is the age and $n(a)$ is the probability density for a cell to be of age a. Newly divided cells have age $a = 0$ and dividing cells have age $a = 1$. The function can be normalized as follows:

$$\int_0^1 n(a)da = 1, \quad 0 \le a \le 1 \tag{A2}$$

and $n(0) = 2 \cdot n(1)$.

The fraction (F) of cells that have not initiated replication corresponds to the integral of $n(a)$ from 0 to the age of initiation, a_i (Figure 5):

$$F = \int_0^{a_i} n(a)da = \int_0^{a_i} 2\ln 2 e^{-a\ln 2} da \tag{A3}$$

$$\Rightarrow \quad F = -2(e^{-a_i \ln 2} - 1) \tag{A4}$$

$$\Rightarrow \quad a_i = -\frac{\ln(1-\frac{F}{2})}{\ln 2}, \quad 0 \le a_i \le 1 \tag{A5}$$

Hence, by substituting F by the fraction of cells that have not initiated replication (obtained from the DNA histogram of cells treated with rifampicin and cephalexin), Equation A5 gives the age at initiation explicitly. Assuming that individual cells grow exponentially from cell mass M_0 at birth to $2M_0$ at division (Cooper, 1988), cell mass at initiation (M_{a_i}) is given by:

$$M_{a_i} = M_0 2^{a_i} \tag{A6}$$

Since $M_0 = M_{av}/(2\ln 2)$, where M_{av} is the average cell mass, it follows that:

$$M_{a_i} = \frac{M_{av}}{2\ln 2} 2^{a_i} \tag{A7}$$

The initiation mass, M_i, is by definition M_{a_i} divided by the number of origins present at initiation, O_i:

$$M_i = \frac{M_{av} \cdot 2^{a_i}}{2\ln 2 \cdot O_i} \tag{A8}$$

Thus, the initiation mass can be derived from the time of initiation, the average cell mass and the number of origins present.

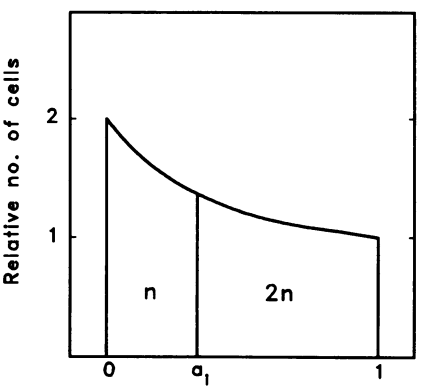

Fig. 5. The age distribution of an exponentially growing culture. A hypothetical age of initiation, a_i, is indicated, dividing the population into cells containing n and $2n$ origins (n = 1, 2, 4 or 8 depending on the growth rate).