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Initiator (DnaA) Protein Concentration as a Function of Growth Rate in *Escherichia coli* and *Salmonella typhimurium*

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The DnaA protein concentration was determined in five different *Escherichia coli* strains and in *Salmonella typhimurium* LT2 growing at different growth rates. The DnaA protein concentration was found to be invariant over a wide range of growth rates in the four *E. coli* K-12 strains and in *S. typhimurium*. In *E. coli* B/r the DnaA protein concentration was generally higher than in the K-12 strains, and it increased with decreasing growth rates. For all the strains, there appears to be a correlation between the DnaA protein concentration and the initiation mass. This supports the concept of the conentration of DnaA protein setting the initiation mass and, thus, that the DnaA protein is a key molecule in the regulation of initiation of chromosome replication in members of the family *Enterobacteriaceae*.

The DnaA protein is an essential factor for initiation of chromosome replication in *Escherichia coli*, and possibly also in other bacteria, since the DnaA protein shows a very high degree of homology among different bacteria (12, 28, 35, 36).

The DnaA protein has been shown to interact in vitro with the origin of replication, *oriC*, by binding to the four DnaA boxes (13, 26). Dimethyl sulfate treatment of intact *E. coli* cells demonstrated that three of the four DnaA boxes show alterations in their methylation patterns, indicating that DnaA proteins are bound to and are protecting these boxes during most of the cell cycle (32). The binding of DnaA protein to *oriC* is one of the first events leading to formation of the replication fork. The binding of DnaA protein facilitates the opening of the double-stranded *oriC* structure in the region containing three 13-meric AT-rich sequences, preceding the formation of the primosome (4). The DnaA protein contains nucleotide-binding sites and relatively stable ATP and ADP forms of the protein have been described (33). The ATP form of DnaA is essential for initiation at *oriC* in vitro (4). The DnaA protein also binds cyclic AMP (16).

The *dnaA* gene is transcribed from two promoters, $dnaAp_1$ and $dnaAp_2$. The $dnaAp_2$ promoter is the stronger and yields 70 to 80% of the total amount of *dnaA* transcript and thus the total DnaA protein; both promoters seem to be repressed by high DnaA protein levels in the cell and derepressed in *dnaA*(Ts) mutants at nonpermissive temperatures (1, 5, 20). The *dnaA* gene expression is also derepressed when extra DnaA boxes carried on pBR322-derived plasmids are introduced into *E. coli* cells (14).

Initiation of chromosome replication is a molecular process which takes place only once per *oriC* per cell cycle. Donachie (11) pointed out that at the time of initiation there is a constant origin-to-cell mass ratio, the initiation mass, or critical volume (31). Experimentally it has been found that deliberate changes in DnaA protein concentration change the initiation mass (2, 24). Intuitively, we have for a long time thought that when the DnaA protein approached a favorable threshold concentration in the cell cycle, it would result in the final modulation of *oriC* before initiation. We took this to indicate that the DnaA protein concentration would be constant in cells at the time of initiation and independent of the growth rate of the cells, in correspondence with the constant initiation mass. Surprisingly, Chiaramello and Zyskind (7) reported that the DnaA protein concentration was proportional to the growth rate. Their results imply that the total number of DnaA proteins per *oriC* varies widely with growth rate. To accommodate the constancy of initiation mass, it was proposed that the active ATP form of the DnaA protein has a constant concentration per *oriC* at the time of initiation (25).

In preliminary experiments using antibody precipitation of cytoplasmic DnaA protein, we obtained conflicting results, indicating the DnaA protein concentration to be constant at different growth rates (13a). These studies have now been extended by using immunoblotting to include different *E. coli* strain backgrounds, namely four K-12 strains with widely different histories and one B/r strain; *Salmonella typhimurium*, the DnaA protein of which is also recognized by our antibodies, was included as well. In contradiction of the results of Chiaramello and Zyskind (7), constancy of DnaA protein concentration, i.e., independence from growth rate, could unequivocally be established.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. Bacteria were grown exponentially in the following media, which yield different growth rates: Luria-Bertani medium supplemented with 0.2% glucose; A+B medium (9) supplemented with 0.5% Casamino Acids and either 0.2% glucose or 0.2% glycerol; or A+B medium with any one of the following carbon sources: 0.2% glucose, 0.2% glycerol, 0.4% succinate, or 0.4% acetate. Auxotrophic requirements were met by adding amino acids in concentrations of 10 to 50 μg/ml and uracil to 20 μg/ml. Thiamine was always present at 2 μg/ml.

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TABLE 1. Strains

Bacterium	Strain	Genotype	Source or reference
E. coli K-12	FH1218	*trp-3 his-4 pyrB*::Tn*5 thi-1 galK2 lacY1* or *lacZ4 mtl-1 ara-9 tsx-3 ton-1 rpsL8* or *9 supE44* λ(c^+)	24
E. coli K-12	RB210	*galU galK rpsL* Δ*lac174* Δ(*leu-ara*)*7697* λRB1	MC1000 λRB1; 5, 6
E. coli K-12	LJ24	*thi-1 leu-6 lacY1 lacI-ZΔ*(*Mlu*) *supE44 tonA21 rpsL rfb*Δ*1*	C600 derivative; obtained from Lene Juel Rasmussen
E. coli K-12	EMG2	λ(c^+) F^+	
E. coli K-12	TC3480[a]	RB210; *rnh-373 dnaA*::*cat*	This study
E. coli B/r	Cp14	Prototroph	38
S. typhimurium	LT2	Prototroph	Laboratory strain collection

[a] The *dnaA*^{0} strain TC3480 was constructed as follows. The *cat* gene was isolated on a *Taq*I fragment from plasmid pACYC184 and inserted into the *Pvu*II-*Sph*I sites of the *dnaA* gene on plasmid pFHC539 (36). The resulting *dnaA*::*cat* mutation was recombined in vivo onto λ*tna*330 (15) and then crossed into the chromosome of an *rnh-373* (37) derivative of strain RB210 in order to create a *dnaA*^{0} strain in analogy with Kogoma and von Meyenburg (18). The construction was verified by Southern blot analysis with a *dnaA* gene fragment as probe.

Determination of origin concentration by flow cytometry. Samples were prepared, and flow cytometry was performed as described by Løbner-Olesen et al. (24). The average cell mass was determined from samples taken directly from exponentially growing cultures. The average number of origins per cell was determined from parallel samples incubated for 4 h with rifampin (300 μg/ml) to block initiation of replication and cephalexin (12 μg/ml) to block cell division. This treatment results in fully replicated chromosomes, the number of which is equivalent to the number of origins per cell, and which can be visualized directly by flow cytometry.

Enzyme measurements. Cell extracts prepared by treatment with toluene were used to determine the β-galactosidase activity as described by Miller (27).

Immunological procedures. Samples were prepared for immunoblot analysis as follows. Steady-state exponentially growing cells (grown for more than 10 mass doublings) were harvested by centrifugation (15,000 × *g* for 10 min). Extreme care was taken not to lose cells when the supernatant was removed. The pellet was dissolved in standard loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and boiled for 5 min. Alternatively, cells were processed exactly as described by Chiaramello and Zyskind (7). Approximately 30 μg of total protein was loaded per lane for SDS-PAGE. Our standard gels contained 15% acrylamide and a low bisacrylamide concentration (0.075%) (15), but in special cases, the gels contained 10 and 15% acrylamide, with bisacrylamide in concentrations of 0.27 and 0.4%, respectively. After electrophoresis (20 cm of migration), proteins were transferred to Immobilon-P membranes (Millipore) by using a semi-dry blotting procedure (21). Protein transfer was for 2 h, and quantitative transfer was verified by Coomassie Brilliant Blue G staining of the gel. The Immobilon-P membrane was blocked in a TBS buffer (0.05 M Tris, 0.15 M NaCl [pH 10.3]) containing 2% Tween 20, washed for 1 min in TBS, and incubated with primary DnaA antibody (22) in TBS plus 0.05% Tween 20 for 1 or 2 h. After three 5-min washes in TBS, the filters were incubated with alkaline phosphatase-conjugated swine immunoglobulin to rabbit immunoglobulins (Dakopats, Copenhagen, Denmark) for 2 h. After three additional washes, the filter was stained with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium essentially as described by Blake et al. (3). For all immunoblots, a DnaA protein standard curve was produced by running lanes with different amounts (from 0.5 to 62.5 ng) of purified DnaA protein (obtained from Satoko Maki in Arthur Kornberg's laboratory). The Western immunoblots were quantified by using an LKB ultrascan laser scanner.

RESULTS

***dnaA* gene expression at different growth rates studied with a *dnaA'-'lacZ* fusion.** Strain RB210, an MC1000 derivative which carries λRB1 (5) integrated at *att*λ, was grown in a variety of different media to vary the growth rate. λRB1 carries a *dnaA'-'lacZ* fusion gene; therefore, *dnaA* gene expression can be monitored by measuring DnaA–β-galactosidase activity. Figure 1 (open symbols) shows that the specific β-galactosidase activity increases slightly with decreasing growth rate. Since the cultures were grown at

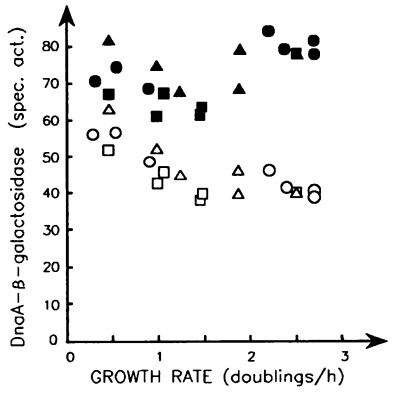

FIG. 1. *dnaA* gene expression as a function of growth rate. Strain RB210 was grown in different media to provide different growth rates. Different symbols represent independent sets of experiments. Open symbols: specific β-galactosidase activity (Miller units) (27) of DnaA–β-galactosidase. Closed symbols: as above but corrected for the chromosomal gene position, i.e., representing the activity, which would be expected, had the *dnaA'-'lacZ* fusion gene been located at the normal *dnaA* gene position on the chromosome. This correction was based on (i) Kubitschek and Newman's (19) data on the chromosome replication time (C) at different doubling times (t_D) using their formula: $C = 0.357t_D + 27$ (min), and (ii) Collins and Pritchard's formula (10) for the dependence of the relative gene dosage on C and t_D. The specific β-galactosidase units were therefore multiplied by $2^{0.67C}/t_D$ to correct for the gene position.

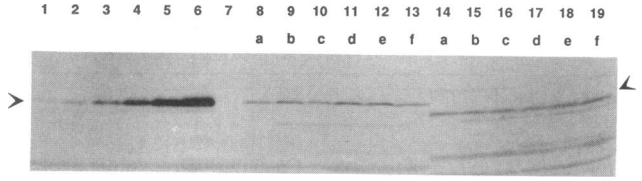

FIG. 2. Immunoblot analysis of Dna protein in *E. coli* K-12 (strain EMG2) and *S. typhimurium* in different growth media (a, Luria-Bertani medium + 0.2% glucose; b, A+B + 0.2% glycerol + 0.5% Casamino Acids; c, A+B + 0.2% glucose + 0.5% Casamino Acids; d, A+B + 0.2% glycerol; e, A+B + 0.2% glucose; f, A+B + 0.4% succinate). Lanes 1 through 6: strain TC3480 (*E. coli*, DnaA^0) with addition of twofold increasing amounts of purified DnaA protein (from 2 to 64 ng). Lane 7, strain TC3480 with no addition. Lanes 8 through 13: *E. coli* K-12 (strain EMG2). Lanes 14 through 19: *S. typhimurium* LT2. DnaA protein position indicated by arrowheads.

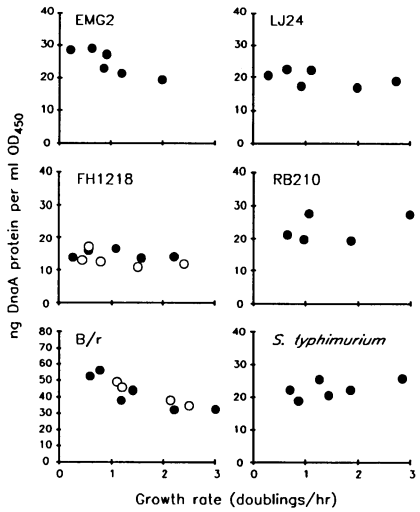

FIG. 3. DnaA protein concentration as a function of growth rate. Western blots were quantified by laser scanning, and the amount of DnaA protein in a given sample was found by using the DnaA protein standards (see Fig. 2, lane 1 to 6) which were always included in the analyses. DnaA protein concentration is expressed as nanograms per milliliter of culture at an optical density at 450 nm of 1.0. Every symbol represents at least two and most often three independently processed samples from each growth rate. The different symbols for FH1218 and B/r represent independent sets of experiments to demonstrate the day-to-day reproducibility.

widely different growth rates, these primary measurements were corrected for the gene position effect, as gene dosage of the normal chromosomal *dnaA* gene relative to the *dnaA'-'lacZ* gene increases with increasing growth rate. In other words, we had to calculate what the expression of the *dnaA'-'lacZ* gene would be if it were at the normal *dnaA* gene position. The formula derived by Collins and Pritchard (10) allows such a correction. The values thus corrected for gene position and replication time variation (19) clearly suggest that the DnaA–β-galactosidase fusion protein is present in a practically constant concentration at different growth rates (Fig. 1).

Direct measurements of DnaA protein concentration at different growth rates. DnaA protein was quantified in *E. coli* strains growing exponentially in different growth media as described in Materials and Methods. Figure 2 shows a typical Western blot. The polyclonal DnaA antibody preparation we used also recognizes a number of other proteins on the filter. However, lane 7, which represents a cell extract from a *dnaA*^{0} strain of *E. coli* (TC3480) (Table 1), shows that no other proteins migrating at the position of the DnaA protein are recognized by the antibody. Lanes 1 through 6 represent a dilution series of purified DnaA protein mixed with a constant amount of *dnaA*^{0} cell extract. These lanes demonstrate the ease with which differences that are less than twofold can be distinguished. A quantitation of the DnaA protein by scanning the Western blot on a laser scanner shows a linear relationship between the input DnaA protein and the scanning signal over a 30- to 40-fold difference in DnaA protein concentration (data not shown). Lanes 8 to 13 (Fig. 2) indicate that the DnaA protein content of strain EMG2 grown at six different growth rates is fairly constant. The amount of DnaA protein in these cells is within the linear range of the DnaA protein standard curve.

There might exist proteins recognized by our polyclonal antibody which comigrate with the DnaA protein. To exclude this possibility, we ran the samples on SDS-polyacrylamide gels with different compositions, since the relative migration rate of DnaA protein varies considerably with the acrylamide and bisacrylamide concentrations (15). However, we obtained a constant DnaA protein concentration at the different growth rates, irrespective of the composition of the gel (data not shown).

A quantitation of the data from Fig. 2, representing the *E. coli* K-12 strain EMG2, and similar data from four other *E. coli* strains are shown in Fig. 3, which demonstrates that DnaA protein concentration appears to be fairly constant and independent of the growth rate of the cells for all the K-12 strains. We also grew the *dnaA*^{0} strain in the growth media we used for the other strains. Immunoblot analysis of cell extracts from these cultures showed no cross-reacting material at the DnaA protein position regardless of the growth medium (data not shown). This substantiates the notion that there are no other proteins which contribute to the band that we see and quantify in our immunoblots.

A significant difference is found in *E. coli* B/r, which at all growth rates has a higher DnaA concentration than the four K-12 strains, and in which the DnaA protein concentration increases with decreasing growth rate (Fig. 3).

The DnaA antibody used also recognizes the DnaA proteins from other enteric bacteria. From experiments with *E. coli* carrying a plasmid with the *S. typhimurium dnaA* gene under *lacp* control, we know that this DnaA protein is recognized by our antibody as efficiently as the *E. coli* DnaA protein (Fig. 4). Full induction with isopropyl-β-D-thiogalactopyranoside (IPTG) gives the same amount of DnaA protein in the two strains. Figure 4 also demonstrates that we can easily record changes in *E. coli* DnaA concentrations by the immunoblot procedure. Overproduction of the *S. typhimurium* DnaA protein results in a threefold repression of the *E. coli dnaA* gene.

The data in Fig. 2, lanes 14 to 19, which are summarized in Fig. 3, show that DnaA protein concentration is also constant in *S. typhimurium*, with the caveat that we have no *dnaA*^{0} derivative of this bacterium.

Our results concerning the DnaA protein concentration as a function of the growth rate are different from those obtained by Chiaramello and Zyskind (7), who used strain EMG2. Therefore, we have repeated the experiment with their protocol for harvesting and processing the samples for gel electrophoresis, but we got results identical to those

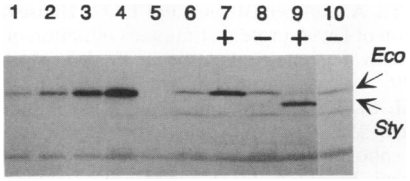

FIG. 4. Immunoblot analysis of *E. coli* and *S. typhimurium* DnaA protein in *E. coli* K-12 (strain FH1218) grown in A+B + 0.2% glycerol + 0.5% Casamino Acids. Lanes 1 through 4: strain TC3480 with twofold increasing amounts of purified DnaA protein; lane 5, strain TC3480 with no addition; lane 6, strain FH1218 with no plasmid; lanes 7 and 8, strain FH1218 carrying pALO15 (*lacI*) and pALO12 (*lacp* regulated *E. coli dnaA*) (=CM3873; 24); lanes 9 and 10, strain FH1218 carrying pALO15 and pFHC1492 (*lacp* regulated *S. typhimurium dnaA*). Plasmid pFHC1492 is identical to pALO12 except that the codons 23 to 455 of the *E. coli dnaA* gene have been replaced with the corresponding region from *S. typhimurium*. The positions of the DnaA proteins are indicated with arrows marked *Sty* and *Eco*. + and − indicate IPTG induction of DnaA protein synthesis and no induction, respectively.

obtained by our standard procedure (used for the immunoblot shown in Fig. 2).

In contrast to the experiments of Chiaramello and Zyskind (7), our DnaA protein determinations have been made with cultures which have been in steady-state exponential growth for more than 10 generations (balanced growth) before the samples were taken. In addition, we have used the proper controls to be sure that we measured only the DnaA protein and that we measured it quantitatively.

Our experience with the immunoblot procedure we are using is very positive. Parallel samples from the same culture always give similar DnaA protein concentrations, and the reproducibility is convincing from one experiment to another (see strains FH1218 and B/r in Fig. 3). We also find proportionality between loadings of different volumes of the sample onto the gel and the corresponding scanning signals (data not shown). Although the reproducibility is high, we occasionally observe up to 30% variation in the measures of DnaA protein from the same sample run on different gels.

Correlation between DnaA protein and origin concentration. We have determined the origin concentration at four different growth rates for two of the *E. coli* strains we have used in our analyses. Table 2 shows that the origin concentration is fairly constant in the K-12 strain FH1218, whereas in the B/r strain Cp14, it increases somewhat with decreasing growth rate. The result for the K-12 strain fits well with the proposal by Donachie (11) that the origin-to-mass ratio is independent of growth rate. The result for our B/r strain, on the other hand, is in accordance with data for a B/r strain presented by Lin-Chao and Bremer (23). By using the data for DnaA protein concentration shown in Fig. 3, we calculated the number of DnaA protein molecules per origin (Table 2). For both *E. coli* strains, we find that the number of DnaA molecules per origin is constant over the range of growth rates examined.

TABLE 2. Number of DnaA protein molecules per *oriC* in *E. coli*

E. coli strain	μ (no. of doublings/h)	No. of: Origins (10^8)/ml at OD_{450} 1.0[a]	Cells (10^8)/ml at OD_{450} 1.0[b]	Origins/cell[c]	DnaA molecules/cell[d]	DnaA molecules/origin[e]
K-12	0.58	7.8	4.9	1.6	330	210
FH1218	0.90	7.0	3.7	1.9	430	230
	1.67	9.4	2.3	4.1	700	170
	2.31	8.3	1.6	5.2	1,020	200
B/r	0.77	18.0	4.4	4.1	1,390	340
	1.00	20.8	5.2	4.0	1,060	270
	2.00	12.2	3.3	3.7	1,290	350
	3.16	9.8	1.6	6.1	2,200	360

[a] Values were obtained by multiplying the values in the fourth and fifth columns. OD_{450}, optical density at 450 nm.

[b] The number of cells per ml was determined as viable cell counts.

[c] The number of origins per cell was determined directly by flow cytometry.

[d] DnaA protein concentration expressed as the number of molecules per ml of culture at an optical density at 450 nm of 1.0 was calculated from the immunoblot analysis and divided by the cell concentration in the fourth column. For strain FH1218, the average concentration of 14 ng/ml of DnaA protein was used; for the B/r strain, we read and calculated the actual concentrations from the data in Fig. 3.

[e] The number of DnaA molecules per origin was calculated by dividing the sixth column by the fifth column.

DISCUSSION

We have used five different *E. coli* strains—four K-12 derivatives and one B/r strain—to analyze the DnaA protein concentration at different growth rates. Recently, Chiaramello and Zyskind (7) have reported that DnaA protein concentration increases proportionally with increasing growth rate. They used the *E. coli* K-12 strain EMG2, which we have included in our analyses as well. We found in contrast to Chiaramello and Zyskind (7) that the DnaA protein concentration in all K-12 strains is constant at different growth rates. Polaczek and Wright (30) have reported intermediate results, although they did not quantify their immunoblots. Interestingly, the *E. coli* B/r strain behaves differently; the DnaA protein concentration increases up to twofold with decreasing growth rate over the range measured, i.e., showing a pattern even more in contrast to the results of Chiaramello and Zyskind (7).

We do not understand and cannot explain the difference between our results and the results of Chiaramello and Zyskind (7). The methods are seemingly identical. It is, however, important to note that the DnaA protein concentrations we find for the slower growth rates are significantly higher than those found by Chiaramello and Zyskind (7). The DnaA protein concentration we find at all growth rates is identical to that found by Chiaramello and Zyskind at their fastest growth rate, i.e., the growth rate at which they find the highest DnaA concentration. The numbers of DnaA molecules per cell for fast-growing cells (Table 2) are similar to those reported for fast-growing (as well as stationary phase) cells by Sekimizu et al. (34). We also found that the direct measurement of the concentration of DnaA protein yields results identical to those obtained with a *dnaA'-'lacZ* fusion. This indicates that *dnaA* gene expression is regulated independently of the position on the chromosome. In accordance with this finding, Petersen and Hansen (29) present data indicating that an RNA polymerase mutant (*rpoC*) increases its DnaA protein as well as its DnaA–β-galactosidase synthesis fivefold at a semipermissive temperature.

Chiaramello and Zyskind (8) and Polaczek and Wright (30) have found that the *dnaA* gene transcripts, and especially those coming from the $dnaAp_2$ promoter, vary with growth rate. These data are in full agreement with our finding of a constant DnaA protein concentration at all growth rates. Ingraham et al. (17) state that "to double a given quantity of protein, a fixed number of polysomes must therefore be

initiated and express themselves per doubling time." This statement is based on the assumption that both the mRNA half-life and the translation initiation frequency are constant at different growth rates. If we assume that the *dnaA* mRNA obeys these rules, our finding of invariant DnaA protein concentrations would indicate that the concentration of *dnaA* mRNA, and therefore the *dnaA* transcription initiation frequency, should be proportional to the growth rate. This means that with the sixfold difference in growth rates between the fastest- and the slowest-growing cultures in the experiments of Polaczek and Wright (30), one should expect a sixfold difference in *dnaA* mRNA concentration. The *dnaA* mRNA data presented in references 8 and 30 are in both cases given as the amount of *dnaA* mRNA per total RNA and thus do not allow a direct comparison with the theoretical expectations presented above.

Chiaramello and Zyskind (8) have also shown by using a K-12 strain that *dnaA* gene transcription, and especially that from the $dnaAp_2$ promoter, responds to the ppGpp concentration in the cell. We do not think that there is any discrepancy between these data and the present finding of a constant DnaA protein concentration at different growth rates. The stringent control signal molecule, ppGpp, might have a rather important role, together with the DnaA protein itself through autoregulation (1, 5, 20), in keeping DnaA protein concentration constant at different growth rates.

The finding that the DnaA protein concentration increased with decreasing growth rate in B/r prompted us to investigate the concentration of replication origins in the B/r strain and in one of the K-12 strains (FH1218) at different growth rates (Table 2). In both strains, we found proportionality between the DnaA concentration and the origin/mass ratio. The higher average number of DnaA proteins per origin found in the B/r strain might reflect a different DnaA protein distribution between various compartments of the cell.

We feel confident that the five strains we have chosen for our experiments adequately represent commonly used *E. coli* strains. Also, the methodology used seems to yield reproducible results. We conclude that the DnaA protein concentration is constant as a function of growth rate in *E. coli* K-12. We also included *S. typhimurium* in our analyses and found a DnaA concentration independence from the growth rate. Also, the actual DnaA protein concentration in *S. typhimurium* is similar to that of most of the *E. coli* K-12 strains.

Our results are not in conflict with the concept that it might be the total concentration of DnaA protein (in its different forms) which determines the initiation mass in *E. coli* and *S. typhimurium*.

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