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Copy-number control of the *Escherichia coli* chromosome: a plasmidologist's view

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The homeostatic system that sets the copy number, and corrects over-replication and under-replication, seems to be different for chromosomes and plasmids in bacteria. Whereas plasmid replication is random in time, chromosome replication is tightly coordinated with the cell cycle such that all origins are initiated synchronously at the same cell mass per origin once per cell cycle. In this review, we propose that despite their apparent differences, the copy-number control of the *Escherichia coli* chromosome is similar to that of plasmids. The basic mechanism that is shared by both systems is negative-feedback control of the availability of a protein or RNA positive initiator. Superimposed on this basic mechanism are at least three systems that secure the synchronous initiation of multiple origins; however, these mechanisms are not essential for maintaining the copy number.

Keywords: chromosome replication; copy-number control; DnaA; *Escherichia coli*; synchronous replication

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Introduction

During the exponential growth of bacteria, such as *Escherichia coli*, the single chromosome is replicated from a unique origin of replication (*oriC*) with the same generation time as the cell mass, which gives rise to a true steady state. Replication is initiated at a defined stage (that varies with the generation time) during the cell cycle. Replication control is exerted at the initiation of replication.

In addition to the chromosome, bacteria often contain other DNA molecules, called plasmids, which replicate in harmony with their hosts. During exponential growth, plasmids are present in defined copy numbers (the number of copies per cell or mass, or chromosome equivalent). They are independent replicons and control their own replication. Replication control is also, in this case, exerted at the level of initiation of replication from an origin of replication. Hence, plasmids are able to both measure their concentration (copy number) and adjust the replication frequency accordingly to maintain a controlled copy number. The systems that control the copy number are well known for some plasmids.

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Studies of chromosome and plasmid replication have given the impression that their respective control systems are different. Therefore, there has been little communication between the two research fields. In this review, we compare the replication control of well-known plasmids and the chromosome, and propose that the two systems are basically similar.

The cell cycle and chromosome replication

The cell cycle is defined by events that occur only once per generation: chromosome duplication and segregation, and cell division. Analogous to that of eukaryotes, the *E. coli* cell cycle is divided into three phases: before chromosome replication (B), the duration of chromosome replication (C) and the time from the completion of chromosome duplication to complete cell division (D). For the *E. coli* B/r strain, the C and D periods last for about 40 and 20 min, respectively, at 37 °C and remain constant over a wide range of generation times from 25 to more than 180 min (Cooper & Helmstetter, 1968; Helmstetter, 1996). Generation times that are shorter than the C+D periods are accommodated by overlapping replication cycles such that each cell receives 2, 4 or 8 origins at birth (Fig 1). All origins in a cell fire synchronously (Skarstad *et al*, 1986) at a fixed cell size per origin (initiation mass) that is independent of the growth rate (Bipatnath *et al*, 1998; Donachie, 1968).

The first recognized event during the initiation of replication is the binding to *oriC* of about 20 molecules of the rate-limiting initiator protein DnaA, the active form of which is DnaA–ATP (Sekimizu *et al*, 1988). The expression of DnaA is autoregulated, with the protein functioning as its own repressor (Messer, 2002; Sekimizu *et al*, 1988; Skarstad & Boye, 1994). In our view, this is the most important step in the copy-number control of the *E. coli* chromosome. At least three systems are responsible for the coordinated initiation.

The first system is sequestration. The hemimethylated GATC sites in the origin, *oriC*, and the *dnaA* promoter are sequestered by the SeqA protein and are not methylated until about one-third of the generation time has passed after initiation (Campbell & Kleckner, 1990). Hence, new initiations cannot occur and new DnaA is not synthesized during this period.

The second system is the titration of DnaA. At least 300 DnaA-binding sites (DnaA boxes) are spread over the entire chromosome (Messer, 2002) with a special high-affinity cluster of DnaA boxes

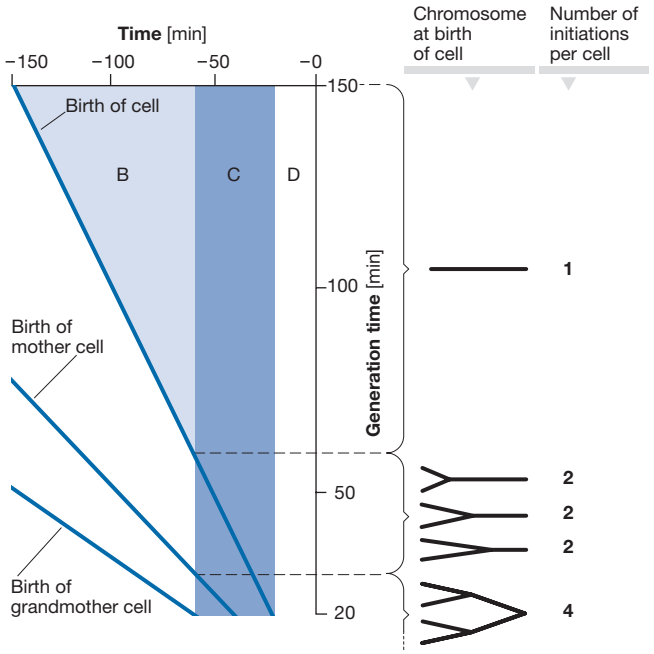

Fig 1 | Overlapping replication cycles in *Escherichia coli* during rapid growth. The time of initiation of the cell cycle at different generation times, in which cell division takes place at time zero, is shown. At generation times greater than 60 min (C, D), there is a period (B) without replication in the young cells; the cells are born with a non-replicating chromosome (shown as a horizontal line). At generation times between 60 and 30 min, DNA replication starts in the mother cell and the cells are born with a partly replicated chromosome containing two origins of replication (*oriC*s; shown as Y-shaped structures). Finally, at generation times less than 30 min, DNA replication starts in the grandmother cell and the cells are born with chromosomes in which there are two consecutive replication cycles (shown as a structure with three branch points); the initiation of replication takes place at four *oriC*s during each cell cycle.

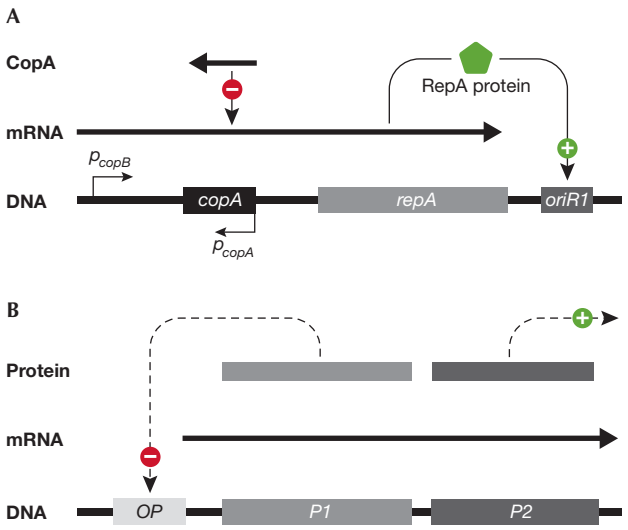

Fig 2 | Examples of replication-contrrol systems. (**A**) Simplified version of the basic replicon of plasmid R1. The replicon contains the following elements: an origin of replication, *oriR1*; the structural gene, *repA*, which encodes the initiator protein RepA that binds to *oriR1*; CopA, which negatively regulates the translation of the *repA* mRNA; and the constitutive P_{copA} and P_{copB} promoters. (**B**) The autorepressor model proposed by Sompayrac & Maaløe (1973). The two genes (*P1* and *P2*) in the regulatory circuit form an operon (*OP*, operator and promoter). The *P1* product is an autorepressor of the operon and the product of *P2* is the initiator protein of replication.

(DnaA titration or *datA* site) located about 470 kb clockwise of *oriC* (Kitagawa *et al*, 1996). This makes the whole chromosome into a titration sink for free DnaA protein.

The third system is the regulatory inactivation of DnaA (RIDA). The homologous to DnaA (Hda) protein catalyses the hydrolysis of the active DnaA–ATP to the inactive DnaA–ADP through a replication-dependent reaction (Camara *et al*, 2003; Kato & Katayama, 2001).

Together, these systems limit the amount of time when DnaA–ATP is available for initiating replication from *oriC* to once per generation, resulting in an eclipse of about two-thirds of a generation time during which *oriC* cannot reinitiate (Olsson *et al*, 2002). This defines the minimum inter-replication time, whereas the average is, of course, exactly one generation. Inactivation of any one of the controls leads to the asynchronous initiation of replication and reduced eclipse.

In conclusion, the control of initiation of chromosome replication in *E. coli* is characterized by two properties: first, it occurs at a defined initiation mass (that is, it couples replication to growth of the population); and second, multiple initiations are synchronized. The thesis of this review is that the key parameter is the initiation mass, and that the synchrony is an addition that contributes little to the copy-number control—it just upholds the 'once and only once' (Boye *et al*, 2000) initiation from each *oriC*. Below, we compare the replication control of the chromosome with that of plasmids.

Plasmid replication

Plasmids are extrachromosomal replicons that live in harmony with their host bacteria. During steady-state growth, they are present in defined copy numbers, which vary from a few to hundreds of copies per cell. The copy-number control of plasmids operates by either limiting the supply of initiation factors (RNA or protein) or inactivating the initiator through dimerization and iteron binding (Das *et al*, 2005; Nordström, 1990). For most plasmids, these negative-control systems set the level of synthesis of a rate-limiting initiator protein (Rep). Fig 2A shows the basic replicon of plasmid R1 in which the copy number is determined by an antisense RNA. In a bimolecular reaction with the upstream region of the mRNA of the *repA* gene, this antisense RNA regulates the rate of synthesis of the RepA protein (Nordström, 2005; Nordström & Wagner, 1994). The copy number is essentially determined by the ratio between the rate constants for the synthesis of the *repA* mRNA and the antisense RNA (Nordström & Wagner, 1994). This control system corrects deviations from the controlled copy number (Fig 3). For plasmid R1, the rate of replication per plasmid copy is inversely proportional to the copy number (Nordstöm *et al*, 1984).

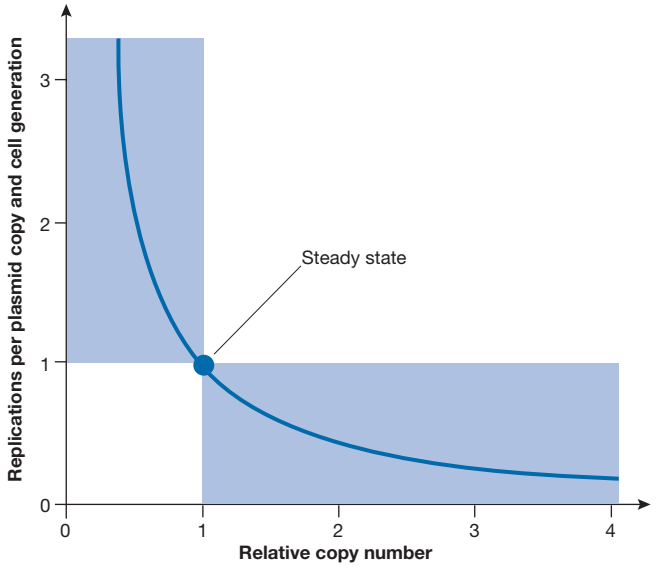

Fig 3 | Kinetics of control of plasmid replication (Nordström *et al*, 1984). Control curves lie within the shaded areas. The control curve of plasmid R1 (blue line) follows an inverse proportionality between the frequency of replication (replications per plasmid copy and cell generation) and the relative copy number (Nordström *et al*, 1984; Nordström & Wagner, 1994).

Plasmid replication is random both in time—spread over the entire cell cycle (Bogan *et al*, 2001; Gustafsson *et al*, 1978)—and with respect to which copy is replicated at each instance (Table 1; Gustafsson *et al*, 1978). Hence, during one cell cycle, some plasmid copies do not replicate at all, whereas others replicate two or more times; however, the average for the population is one replication per plasmid copy and cell generation.

In conclusion, control of plasmid replication is characterized by the control of the average copy number, and the correction of deviations from this controlled copy number.

Comparison of plasmid and chromosome replication

It is clear that there are distinct differences between plasmids and chromosomes (Table 1). As mentioned above, initiation of replication of the chromosome takes place at a cell size that is defined by the initiation mass (cell mass at initiation per *oriC*); this might imply that the chromosome also measures its copy number. However, the introduction of minichromosomes (plasmids that replicate from *oriC*) affects neither the timing of initiation nor the initiation mass (Helmstetter & Leonard, 1987), which might cause a problem with control models as discussed by Donachie & Blakely (2003).

Furthermore, the copy number of minichromosomes varies markedly in the population, and the variation increases during growth of the population due to the uneven distribution of the minichromosomes in the daughter cells (Løbner-Olesen, 1999). This study showed that the content of *oriC* is not measured, but did not exclude the possibility that the copy number of the chromosome is measured indirectly. Below we re-address this question, and propose that the *E. coli* chromosome measures as well as corrects deviations in its copy number.

Table 1 | Comparison of key characteristics of replication of the *Escherichia coli* chromosome and plasmids

Characteristics	Synchronous chromosome	Plasmids	Asynchronous chromosome
Replication during the cell cycle	Fixed time	Random	Random
Replication of an origin during one cell generation	Only once	Random	Random
Fixed copy numbers	Yes (see text)	Yes	Yes (see text)
Corrections of copy-number deviations	Probably yes (see text)	Yes	Probably yes (see text)

Models for control of replication

Several models have been proposed to explain the control of replication. The classical replicon theory (Jacob *et al*, 1963) suggested positive control. The Pritchard group proposed the extremely influential inhibitor-dilution model (Pritchard *et al*, 1969), which introduced negative-feedback control of replication. In the autorepressor model there is an operon for the synthesis of a rate-limiting initiator protein (Fig 2B; Sompayrac & Maaløe, 1973). The operon contains a gene that autoregulates transcription of the operon. This ensures that the correct amount of the initiator protein is formed during each cell cycle, and that deviations in the concentration of the operon/replicon are corrected. Elaborate mathematical models have been proposed for the control of replication of the *E. coli* chromosome (Browning *et al*, 2004; Hansen *et al*, 1991a; Mahaffy & Zyskind, 1989). Donachie & Blakely (2003) suggested that the ratio between DnaA–ATP and DnaA–ADP, and not their amounts and/or concentrations, determines the timing of initiation.

During steady-state growth, all components of a population are present in constant proportions. However, the concentrations of origins are never measured in the plasmid systems. The key component of the plasmid R1 system is the antisense RNA, *copA*. As CopA is formed constitutively and is unstable (with a half-life of about 90 s), its concentration is always proportional to the plasmid concentration. The control system keeps the concentration of CopA constant and corrects fluctuations. Hence, the control of replication is indirect. Similarly, the other models and real systems use indirect measures of the concentration of the replicons as shown in Table 1, and act by regulating the availability of a rate-limiting initiator protein. This is analogous to the initiation of replication of the chromosome controlled by the DnaA protein, as is implicit in the models of Hansen *et al* (1991a) and Browning *et al* (2004), and was specifically stressed by Herrick *et al* (1996). The synthesis of DnaA is autoregulated (Messer & Weigel, 1997), which makes the system similar to the Sompayrac–Maaløe model with DnaA combining autorepressor and initiator functions.

Minichromosomes have posed a problem for the construction of copy-number-control models (see, for example, Donachie & Blakely, 2003). However, as stated above (and in Table 2), the concentration of origins has never been measured in any system. *E. coli* minichromosomes do not carry the *dnaA* gene and would not be able to replicate in the absence of a DnaA donor. The control of replication of minichromosomes was recently reviewed by Dasgupta & Løbner-Olesen (2004).

Asynchronous replication and over-replication

As the *E. coli* chromosome has an elaborate system for maintaining the synchronous initiation of replication, here we compare chromosomes that have asynchronous initiation of replication with plasmids that replicate randomly.

Loss of synchrony by inactivation of, for example, sequestration gives a shortened eclipse and random replication with respect to both timing and selection (Table 1; Olsson *et al*, 2002). Hence, some *oriC*s initiate two or more times per cell generation. Although this has been described as over-initiation because some origins fire more than once per cell generation, it is merely a logical consequence of random initiation of replication and is not necessarily indicative of a higher copy number—the copy number is still maintained by the autoregulation of the *dnaA* gene. To a plasmidologist, over-initiation is an increase in the copy number. For plasmids, random replication leads to variations in the copy number in individual cells (Løbner-Olesen, 1999). Hence, asynchronous chromosomes replicate in the same manner as plasmids. It should therefore be stressed that the terms 'over-initiation' and 'asynchrony' are often used synonymously in the literature, which is unfortunate and misleading.

Minichromosomes can become established in wild-type bacteria, whereas they are severely incompatible with deoxyadenosine methyltransferase (*dam*) mutants; this is another plasmid-like feature (Løbner-Olesen & von Freiesleben, 1996).

DnaA sets the chromosome copy number

The DnaA protein is not only the key element in the initiation of replication of the *E. coli* chromosome, but is also responsible for linking this initiation of replication to cell growth. Hansen *et al* (1991b) reported that the amount of DnaA per *oriC* is about 200 molecules at growth rates ranging from 0.6 to 2.3 generations per hour. As the initiation mass is constant over this range of growth rates, they concluded that DnaA determines the initiation mass.

However, in contrast to plasmids, for which increased production of the initiation protein can lead to runaway replication (Nordström & Uhlin, 1992), large increases in the DnaA concentration lead to only marginal changes in the DNA concentration (Atlung *et al*, 1987; Churchward *et al*, 1983). Atlung & Hansen (1993) extended these studies by measuring the concentration of *oriC* in addition to the total amount of chromosomal DNA. At DnaA concentrations above the normal level, there was a proportional increase in *oriC*/mass up to at least 50%, whereas the DNA/mass increased only slightly and then levelled off. This was because many initiations were abortive and replication never reached the terminus, or because the rate of fork movement was reduced. Atlung & Hansen (1993) concluded that the initiation mass was determined by the availability of DnaA (see also Herrick *et al*, 1996).

Negative control of chromosome replication

The copy number of most plasmids is determined by the rate of synthesis of a rate-limiting initiator protein (Rep). Deviations from the copy number are corrected by the presence of a negative loop.

The *dnaA* promoter is autoregulated (Messer & Weigel, 1997), which allows deviations in copy number of the chromosome to be corrected. Hansen *et al* (1987) inserted a lambda phage carrying a *dnaA–lacZ* fusion into *λatt* and introduced plasmids carrying different numbers of *dnaA* boxes into the strain; this caused a marked increase in *dnaA* expression. Hence, there is a strong potential for de-repression of the *dnaA* promoter, and the concentration of DnaA–ATP is within the range that gives effective control of expression of the *dnaA* gene.

Table 2 | Substances that are measured by different replication-control systems

Model or system	Measured control substance
Pritchard inhibitor-dilution model	Putative inhibitor
Sompayrac–Maaløe autorepressor model	Autorepressor
Plasmid λdv	Cro/Tof protein
Plasmids with antisense-RNA control	Antisense RNA
Atlung–Hansen inhibitor-titration model	Initiator protein DnaA
Escherichia coli chromosome	Initiator protein and autorepressor DnaA–ATP

There do not seem to be any direct experiments showing that copy numbers are corrected. However, the autoregulation of the *dnaA* gene strongly suggests that copy-number deviations are corrected. Such experiments have been performed with plasmids in shifts between different copy-number levels (Gustafsson & Nordström, 1980).

Effect of the synchrony systems on initiation mass

Strains with asynchronous initiation of replication (for example *dam* mutants) grow at steady states; hence, the three synchrony systems are not vital for the functioning of the cells (Boye & Løbner-Olesen, 1990; Kato & Katayama, 2001; Kitagawa *et al*, 1998; Olsson *et al*, 2003; von Freiesleben *et al*, 1994); the main effect seems to be that cell division is disturbed, which results in broader cell-size distributions. Hence, the systems that maintain synchrony are not important for the maintenance of copy number, although the copy number of the chromosome might be different in some of the asynchronous mutants compared with the wild type. The systems might function to secure better co-ordination between replication and cell division.

A newly replicated *oriC* cannot take part in a new initiation during an eclipse period of about two-thirds of a generation time (Olsson *et al*, 2002). The eclipse is caused by sequestration (which makes a newly replicated *oriC* unavailable for initiation and, presumably, DnaA binding for one-third of a generation time), titration of DnaA by the *datA* locus, and the RIDA system. Sequestration prohibits the synthesis of DnaA, and DnaA titration and RIDA prolong the period of relatively low concentrations of free DnaA–ATP. Inactivation of any of these functions therefore leads to asynchronous initiation.

Inactivation of the *dam* gene reduces the length of the eclipse to about one-third of a generation time (Olsson *et al*, 2002); therefore, the period during which the concentration of DnaA–ATP is extremely low is shortened compared with the wild type, because the *dnaA* promoter is not sequestered and is active throughout. This causes asynchronous replication.

Loss of sequestration results in the production of DnaA during the entire cell cycle, whereas DnaA is not produced during the first one-third of the wild-type cell cycle. However, this might be compensated by de-repression of the *dnaA* promoter in the wild type, due to the extremely low concentration of DnaA at the end of the sequestration period. Therefore, a similar amount of DnaA might be formed per cell cycle in a *dam* mutant and in the wild type. The situation is different for *seqA* mutants because of the change in superhelicity of the chromosome (Olsson *et al*, 2002).

The *datA* locus titrates DnaA, and its deletion increases the origin copy number by about 30% (Kitagawa *et al*, 1998). Hence, the rest of the chromosome binds about 70% of the DnaA. This might fit with the doubling of the initiation mass caused when a threefold increase in *datA* is caused by an introduced plasmid (Morigen *et al*, 2003).

Loss of RIDA (inactivation of the *hda* gene) also causes some degree of asynchrony (Camara *et al*, 2003). This might be due to the release of DnaA–ATP from the DnaA boxes, and its re-use in initiation of replication. Consequently, there is an increased *oriC* concentration in *hda* strains (Camara *et al*, 2003).

Conclusions

This review has compared the control of replication of the *E. coli* chromosome with that of some plasmids. The main conclusion is that the two control systems are similar.

First, the copy number of the replicons is measured indirectly by the availability of a rate-limiting initiator protein. The synthesis of this protein is controlled negatively, which enables a given copy number to be set and variations to be adjusted. For the chromosome, negative control is exerted by autoregulation of the *dnaA* gene by the DnaA protein. We claim that this is the main system that controls copy number. Hence, the control system is analogous to the Sompayrac–Maaløe model.

Second, plasmids replicate randomly during the entire cell cycle, whereas chromosome replication occurs at a fixed time; during rapid growth, multiple initiations occur and are highly synchronized. However, this synchrony is ensured by several systems: sequestration of *oriC* and the *dnaA* promoter, titration of DnaA, and RIDA. Loss of synchrony by mutation is not lethal to the bacteria, which means that these systems are not crucial for maintaining controlled chromosome replication. Chromosome replication in strains with asynchronous replication is similar to plasmid replication. It should also be stressed that the synchrony systems all operate by affecting either the synthesis or the fate of the DnaA protein.

Third, the copy number of some mutants with asynchronous chromosome replication is different from that of the wild type. This is due to effects on the synthesis and fate of the DnaA protein. However, the new copy number is maintained by autoregulation of the *dnaA* gene.

Fourth, during asynchronous—that is, random—replication, some origins fire more than once per cell cycle. This is a logical consequence of randomness, but has often been called over-initiation in the literature. For plasmidologists this is not an appropriate term, as it implies an increased copy number. This is, for example, evident from the behaviour of the *dnaA46* mutant, which, at permissive temperatures, has a reduced copy number compared with the wild type but replicates its chromosome asynchronously.

The emerging picture of the homeostatic control of chromosome copy number is that the DnaA protein is the key regulator of both timing and frequency of the initiation of chromosome replication, and their coordination with cell growth. Several additional elements of control (SeqA, Dam, *datA*, Hda and so on) are used for fine-tuning, but are neither necessary nor sufficient for initiation. By contrast, loss or excess of the initiator activity of DnaA causes cell death. From this perspective, the copy-number control of the chromosome seems similar to that of plasmids, with superimposed mechanisms that coordinate replication with the cell cycle.

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