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Distribution of minichromosomes in individual *Escherichia coli* cells: implications for replication control

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A novel method was devised to measure the number of plasmids in individual *Escherichia coli* cells. With this method, involving measurement of plasmid-driven expression of the green fluorescent protein gene by flow cytometry, the copy number distribution of a number of different plasmids was measured. Whereas natural plasmids had fairly narrow distributions, minichromosomes, which are plasmids replicating only from a cloned *oriC* copy, have a wide distribution, suggesting that there is no copy number control for minichromosomes. When the selection pressure (kanamycin concentration) for minichromosomes was increased, the copy number of minichromosomes was also increased. At up to 30 minichromosomes per host chromosome, replication and growth of the host cell was unaffected. This is evidence that there is no negative element for initiation control in *oriC* and that there is no incompatibility between *oriC* located on the chromosome and minichromosome. However, higher copy numbers led to integration of the minichromosomes at the chromosomal *oriC* and to initiation asynchrony of the host chromosome. At a minichromosome copy number of ~30, the cell's capacity for synchronous initiation is exceeded and free minichromosomes will compete out the chromosome to yield inviable cells, unless the minichromosomes are incorporated into the chromosome.

Keywords: chromosome replication/*Escherichia coli*/incompatibility/initiation capacity/minichromosomes

Introduction

Initiation of chromosome replication in *Escherichia coli* occurs at a fixed point, *oriC* (Marsh and Worcel, 1977). Minichromosomes are plasmids that replicate autonomously from *oriC* (Hiraga, 1976; Messer *et al.*, 1978; von Meyenburg *et al.*, 1979). They depend on functional DnaA and DnaC products, *de novo* protein synthesis and RNA polymerase mediated transcription for initiation of bi-directional replication, thereby resembling their chromosomal counterparts (for review see Messer and Weigel, 1996).

The cloned origin of replication has been used for detailed mechanistic studies of the different steps in the *oriC*-specific initiation and replication process *in vitro* (Kornberg and Baker, 1992). The first step in the initiation process is strand separation facilitated by the DnaA initiator protein binding to its recognition sequences (DnaA boxes) in *oriC* forming the 'initial complex' (Fuller *et al.*, 1984). Subsequently, duplex opening occurs at three adjacent AT-rich 13mers, allowing for entry of the DnaB and C proteins to form the 'pre-priming complex' that facilitates further strand separation (Bramhill and Kornberg, 1988) and allows for entry of the replication machinery.

A rate-limiting step for initiating DNA replication *in vivo* is governed by the amount of DnaA protein (Løbner-Olesen *et al.*, 1989), probably through formation of the initial complex. Initiations take place at a constant amount of DnaA protein per *oriC* (Hansen *et al.*, 1991b). The cell mass per *oriC* at initiation (the initiation mass; Donachie, 1968) was also shown in a number of studies to be constant (Hansen *et al.*, 1991b; Cooper, 1997; Bispatnath *et al.*, 1998) or to vary slightly (Wold *et al.*, 1994) over a range of growth rates where cell size varies >5-fold (Bremer and Dennis, 1996), indicating that the DnaA protein accumulates in proportion with cell growth. Several models have been proposed to account for the fairly constant initiation mass. These include the 'inhibitor dilution model' (Pritchard *et al.*, 1969) the 'autorepressor model' (Sompayrac and Maaløe, 1973) and more recently the 'initiator titration model' (Hansen *et al.*, 1991a; see below), which has received a great deal of attention.

In the initiator titration model, accumulation of DnaA protein to a critical level triggers initiation on one of the origins within the cell. The formation of a proposed low-affinity DnaA protein complex on *oriC* will only take place when all high-affinity DnaA boxes on the chromosome are bound. There are 308 consensus DnaA binding sites [TT(A/T)TNCACA; Schaper and Messer, 1995] on the chromosome (Blattner *et al.*, 1997), some of which have high affinity for the DnaA protein (Roth and Messer, 1998). The most prominent of these are the DnaA binding sites of the *datA* locus (Kitagawa *et al.*, 1996, 1998). As the origin is initiated the DnaA protein is released. Because the two newly formed origins are hemimethylated and membrane bound (Ogden *et al.*, 1988) and therefore inaccessible to the released DnaA protein (Landoulsi *et al.*, 1990), the ratio of free DnaA protein to the accessible origins will be momentarily increased. Consequently, initiations on 'old' origins will follow in a cascade-like manner (the initiation cascade; Løbner-Olesen *et al.*, 1994). This explains the almost simultaneous initiation on multiple origins contained within fast growing cells (Skarstad *et al.*, 1986). Following initiation, the hemimethylated origins are sequestered for approximately one-third of the doubling time (Campbell and Kleckner, 1990), a period when they presumably are inert for further initiations. Once *oriC* is liberated from sequestration, the nascent replication forks have proceeded far enough to replicate a number of high-affinity DnaA binding sites

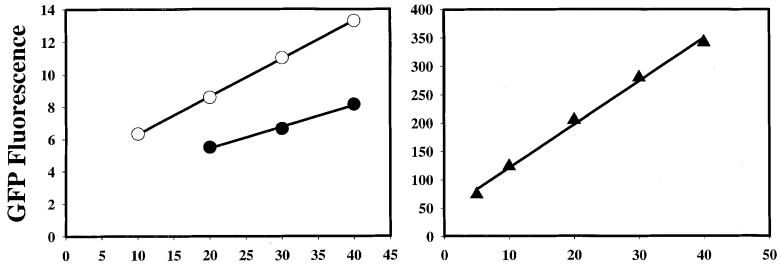

Fig. 1. Induction of GFP synthesis. Strain MC1000 containing plasmids: the F-based plasmid pALO280 (●), the R1-based plasmid pALO261 (○) or the pBR322-based plasmid pALO269 (▲) was grown at 37°C in AB minimal medium supplemented with 0.2% glycerol, 0.5 μg/ml thiamin and 50 μg/ml leucine. At t_0, arabinose was added to a final concentration of 0.2% to induce the pBAD promoter. Cell samples were take at the times indicated and rifampicin added to a final concentration of 300 μg/ml to stop protein synthesis and cephalexin to a final concentration of 10 μg/ml to stop cell division. Following 1 h incubation at 37°C cells were diluted in 0.9% NaCl and analysed for cellular GFP fluorescence (arbitrary units) as described in Materials and methods.

(Blattner *et al.*, 1997). These will titrate (bind) DnaA protein, thereby lowering the DnaA protein to *oriC* ratio to a level below the initiation threshold.

The ability of cells to maintain minichromosomes can be accounted for by the initiation cascade since in principle the number of origins that can be initiated is indefinite. In support of this it was found that minichromosomes replicate in synchrony with the chromosome (Leonard and Helmstetter, 1986; Koppes and von Meyenburg, 1987). Minichromosomes also display cell-cycle-specific replication in a strain where the chromosome is replicated from an integrated R1 plasmid by a mechanism not linked to the normal cell cycle (Eliasson and Nordstrøm, 1997). This demonstrates that the first initiation in an initiation cascade does not need to originate at the chromosomal origin. The accumulation of DnaA protein on minichromosomes and virtual simultaneous release by the initiation cascade is sufficient to create a cell-cycle-specific replication pattern. A precise timing of replication of chromosomal high-affinity DnaA binding sites, relative to initiation, is therefore not important for maintaining initiation synchrony (Eliasson and Nordstrøm, 1997; Kitagawa *et al.*, 1998).

If the initiation cascade is disrupted, for example by a *dam* mutation that no longer allows for sequestration of newly replicated origins, initiations become asynchronous (Boye *et al.*, 1988; Boye and Løbner-Olesen, 1990; Løbner-Olesen *et al.*, 1994). The asynchrony phenotype is accompanied by loss of the ability to sustain minichromosome replication (Løbner-Olesen and von Freiesleben, 1996).

In this work I have used a novel method to study the regulation of minichromosome replication and segregation, and the interrelationship between chromosome and minichromosome replication.

Results

GFP as a reporter for plasmid copy number

The green fluorescent protein (GFP) expression cassette (see Materials and methods) contains the arabinose inducible pBAD promoter (Guzman *et al.*, 1995) followed by the *GFP* gene and the *rrnB* T_1 and T_2 transcription terminators, and, in addition, the *araC* gene encoding the repressor of the pBAD promoter. Two versions of this cassette were constructed carrying either *GFPmut2* or the *GFPmut1* derivative *GFP-BioST* (Cormack *et al.*, 1996; Novo Nordisk A/S; for details see Materials and methods). The GFP cassette was introduced into derivatives of plasmids F, R1 and pBR322 (a member of the ColE1 family). Addition of arabinose resulted in a linear increase in GFP fluorescence with time for strain MC1000 containing any of the three plasmids, albeit at three different expression levels (Figure 1). The slopes of these curves can be taken as measures of plasmid content (see below) and indicate cellular copy numbers of plasmids R1 and pBR322 of 1.7 and 40–50, respectively, relative to plasmid F.

Minichromosome copy number distributions

In the individual cell, expression of GFP was expected to result in levels of fluorescence representing the number of plasmid copies carried by that particular cell, provided that the induction period was substantially shorter than the doubling time of the culture. An induction period of 30 min (culture doubling times of 110 min) was sufficient for detection of GFP produced from low copy number plasmids such as F and R1 derivatives, and was still well within the linear time range for GFP expression from high copy number plasmids such as the pBR322 derivative (Figure 1). During the 30 min induction period, cell growth as well as plasmid replication continued, and some cells divided. The GFP activity measured therefore represents an integration of plasmid copy number during this period, which ideally should be as short as possible. Induction of GFP from the pBR322-derived plasmid for 5 min resulted in a copy number distribution similar to but slightly wider than that obtained for a 30 min induction period (Figure 2A). The consequence of using a 30 min induction period may therefore be that the actual variation in cellular copy number for a given plasmid is slightly underestimated.

The GFP cassette was introduced into plasmids carrying the replicons listed in Table I. In strain MC1000, plasmids carrying the F, R1, P1Δ*incA*, pSC101, p15A or pBR322 replicons each had copy numbers (i.e. fluorescence) spanning approximately one logarithmic unit (Figure 2B). Differential induction of the pBAD promoter in individual cells was not likely to contribute to the variation in single cell fluorescence, since this is only

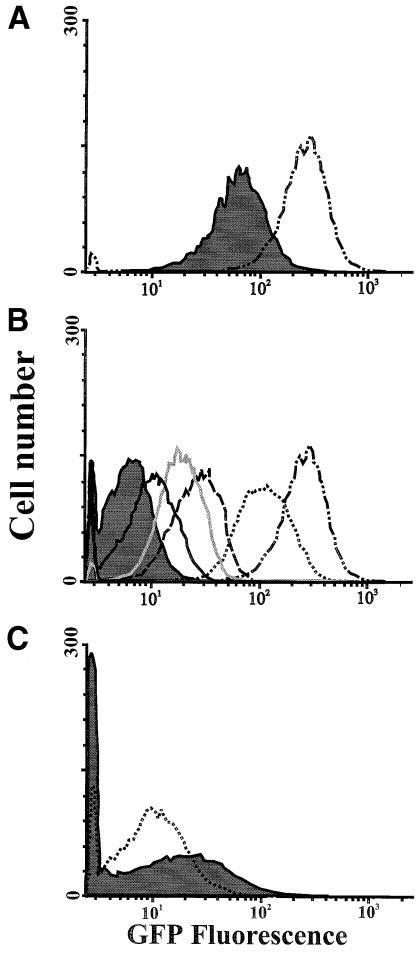

Fig. 2. Plasmid copy number distributions. Strain MC1000 containing the indicated plasmids was grown at 37°C in AB minimal medium supplemented with 0.2% glycerol, 0.5 μg/ml of thiamin and 50 μg/ml of leucine. GFP synthesis from the individual plasmids was measured as described (Materials and methods). Unless otherwise indicated the induction period was 30 min. (**A**) GFP synthesis was induced from the pBR322-derived plasmid pALO269 for 5 min (filled distribution) or for 30 min (dashed and double dotted line). (**B**) Natural *E.coli* replicons. The F-derived plasmid pALO280 (filled distribution); the R1-derived plasmid pALO261 (solid line); the pSC101-derived plasmid pALO284 (grey line); the P1ΔincA-derived plasmid pALO270 (dashed and dotted line); the p15A-derived plasmid pALO275 (dotted line); and the pBR322-derived plasmid pALO269. (**C**) *Esherichia coli* minichromosomes. The Sop^- minichromosome pALO264 (filled distribution) and the Sop^+ minichromosome pALO267 (dotted line).

observed at subsaturating inducer concentrations (Siegele and Hu, 1997). Cells carrying the plasmids listed above also appeared uniformly fluorescent when inspected by fluorescence microscopy (not shown). The coefficient of variation (CV) can describe the width of the distributions. All of the plasmids listed above had distributions with CV values ~40–50% (Table I). The distributions were wider than the cell size distributions for the same cells, which all had CV values ~21–24% (Table I).

The copy number distribution obtained from an *E.coli* minichromosome is different from those of the other plasmids, in that it was flat and it spanned more than two logarithmic units (Figure 2C), suggesting a large heterogeneity in single cell copy number. The distribution shows that there were cells with one plasmid copy or even zero copies, as well as cells with a copy number similar to that found for the pBR322-derived plasmid (compare Figure 2B and C). The wide copy number distribution of the minichromosome was not a result of heterogeneity in cell size, since all the cultures had similar cell size distributions (Table I).

A minichromosome derivative that had been stabilized by the *sopABC* partitioning function of plasmid F had a narrower copy number distribution than that of the parental minichromosome, but still wider than that of naturally occurring *E.coli* plasmids (Figure 2C; Table I).

The average copy number of plasmids determined by GFP fluorescence (Table I) was in good agreement with an alternative copy number determination based on DNA–DNA hybridization. For the pBR322-derived plasmid, the copy number determined by fluorescence was approximately twice as high as that determined by hybridization. However, the cells containing this plasmid were also 30% larger than cells containing the other plasmids, explaining some of the difference.

I conclude that the fluorescence-based assay gives a reasonably accurate estimate of the cellular copy numbers of individual plasmids. The copy number distribution of minichromosomes is much wider than that of natural *E.coli* plasmids, but can be narrowed by including a partitioning function on the plasmid. The wide copy number distribution of minichromosomes suggests that they have no means of controlling their own replication, so that they cannot ensure a well-defined copy number.

The copy number of minichromosomes can be altered

Minichromosome pALO2116 contains the *neo* gene as selective marker that confers gene dosage dependent kanamycin resistance on the host cells (Berg *et al.*, 1975). It also contains the GFP expression cassette containing *GFP-BioST* for low level GFP expression.

Cells containing minichromosome pALO2116 and grown in rich medium containing arabinose (for continuous induction of the *GFP* gene) at increasing kanamycin concentrations displayed a gradual reduction in growth rate (Table II). The copy number distributions of the minichromosome, visualized as GFP fluorescence, were moved towards higher copy numbers as the selective pressure increased (Figure 3A). There was also a tendency for distributions to become narrower at higher kanamycin concentrations. The higher fluorescence per cell at high kanamycin concentrations was not the result of increasing cell size (Table II).

When the kanamycin concentration was increased for cells carrying the Neo^R F-derived plasmid pALO280 the growth rate decreased (Table II). The growth inhibition at a given kanamycin concentration was more severe than that observed for minichromosome-containing cells, and suggests a lower resistance level conferred by the F plasmid. The copy number distributions were found to vary little with the kanamycin concentration (Figure 3B). In conclusion, minichromosome copy number increased as a function of the selective pressure, suggesting that they contain no mechanism to regulate their copy number. Plasmid F, on the other hand, had a very tight copy number control mechanism, and it was not possible to alter this copy number by increasing the selective pressure.

Table I. Plasmid copy numbers

Plasmid	Replicon	Relevant genotype	Copy No. (GFP)[a]	Copy No. (Southern)[b]	CV (%)[c] GFP	Cell size	CV (%)[c] cell size
pALO280	F	*oriS, oriV, sopABC, neo, araC,* pBAD-*GFPmut2*	1.0	1.0	42.9	1.0	21.5
PALO261	R1	*ori, bla, araC,* pBAD-*GFPmut2*	1.7	1.8	49.9	1.0	22.5
PALO270	P1 Δ*incA*	*ori,* Δ*incA, cat, araC,* pBAD-*GFPmut2*	4.4	5.9	47.8	1.0	21.8
PALO284	PSC101	*ori,* Km^R*, araC,* pBAD-*GFPmut2*	3.1	4.6	44.0	1.0	21.8
PALO275	P15A	*ori, cat, araC,* pBAD-*GFPmut2*	19.0	15.5	55.2	1.0	21.8
PALO269	PBR322	*ori, bla, neo, araC,* pBAD-*GFPmut2*	42.8	23.6	46.5	1.3	24.0
PALO264	*oriC*	*oriC, neo, araC,* pBAD-*GFPmut2*	3.8	4.1	>100	1.0	22.3
PALO267	*oriC sopABC*	*oriC, sopABC, neo, araC,* pBAD-*GFPmut2*	2.1	ND	70.8	1.0	22.1
PALO2116	oriC	*oriC, neo, araC,* pBAD-*GFP-BioST*	ND	50	ND	ND	ND

[a]Determined as the average fluorescence of cells (Figure 2). The average fluorescence of plasmid pALO280 was set at 1.0.
[b]Copy numbers were determined as described in Materials and methods. The copy number of plasmid pALO280 set to 1.0. This copy number corresponds to two copies per *oriC* or 3.6 copies/cell (not shown).
[c]The CV is a measure of peak width. It is defined as the standard deviation divided by the mean×100. The CV was found to give a reliable measure of peak width despite the fact that the distribution of the cellular fluorescence was not normal.
ND, not determined.

Table II. Size and DNA content of cells containing minichromosomes or F plasmids

Plasmid	Selection (μg/ml Km)	Doubling time (min)	Plasmid copies/oriC[a]	Cell size	DNA content	DNA/mass	Origins/cell
None	–	21	–	1.00	1.00	1.00	7.9
Minichromosome	50	22	10	1.02	0.97	0.95	8.1
(pALO2116)	250	25	32	1.08	1.03	0.95	7.7
	500	27	37	1.09	1.11	1.02	7.6
	1000	37	38	0.85	0.84	0.99	6.4
	1500	46	66	0.73	0.72	0.98	7.0
F plasmid	50	25	1.1	0.75	0.71	0.95	4.8
(pALO280)	500	33	0.9	0.57	0.53	0.96	3.5
	750	66	1.1	0.51	0.47	0.92	2.5

[a]Determined from data in Figure 4.

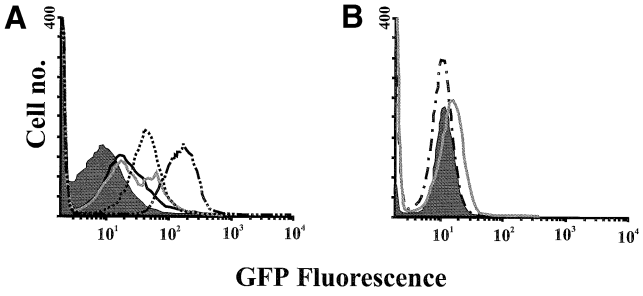

Fig. 3. Changing the minichromosome copy number distribution. Strain MC1000 containing either minichromosome pALO2116 (**A**) or the F-derived plasmid pALO280 (**B**) was grown exponentially in low salt LB medium containing varying amounts of kanamycin at 37°C. The single cell GFP distributions were determined as described (Materials and methods). The distributions are 50 μg/ml kanamycin (filled distribution), 250 μg/ml (solid line), 500 μg/ml (grey line), 750 μg/ml (dashed and dotted line), 1000 μg/ml (dotted line) and 1500 μg/ml (dashed and double dotted line).

High minichromosome copy number leads to incompatibility towards the chromosomal origin

The structure of the minichromosomes and the F plasmids contained within cells grown at increasing amounts of selective pressure was determined; cultures of cells were grown with different kanamycin concentrations and total DNA was isolated. Southern blot hybridization was done with a probe containing sequences homologous to *oriC* and the GFP expression cassette (see Materials and methods). For minichromosome-containing cells I observed the following: at the lowest kanamycin concentration used (50 μg/ml) fragments of ~9 kb, 7 kb and 2.1 kb in the *Eco*RI + *Sal*I digests were detected by the probe, representing the chromosomal *oriC*, the minichromosomal *oriC* and the minichromosomal GFP cassette, respectively (Figure 4). Quantitation of the bands indicated a minichromosome copy number of ~10 per chromosomal *oriC* (Table II), in agreement with an earlier report (Løbner-Olesen *et al.*, 1987). As kanamycin concentration was increased to 250 μg/ml, minichromosome copy number increased >3-fold, to 32 per chromosomal origin (Table II). A further increase in kanamycin concentration to 500 μg/ml resulted in the disappearance of some of the 9 kb chromosomal *oriC* fragment. It was replaced by two new fragments of ~6 and 10 kb, indicative of integration of one or more copies of the minichromosome into the chromosomal *oriC* region (for details on the integration see Løbner-Olesen and von Freiesleben, 1996). The population of cells was heterogeneous with approximately two-thirds of the cells containing one or more copies of the minichromosome integrated into the origin region of the chromosome. The total copy number had increased to 37 (Table II). At kanamycin concentrations above 500 μg/ml the original *oriC* fragment of 9 kb disappeared, indicating that all cells in the culture contained integrated copies of the minichromosome. Note that each of the new fragments of ~6 and 10 kb contain one copy of *oriC*, which allowed me to determine the total increase in the number of minichromosomes (Table II). From the

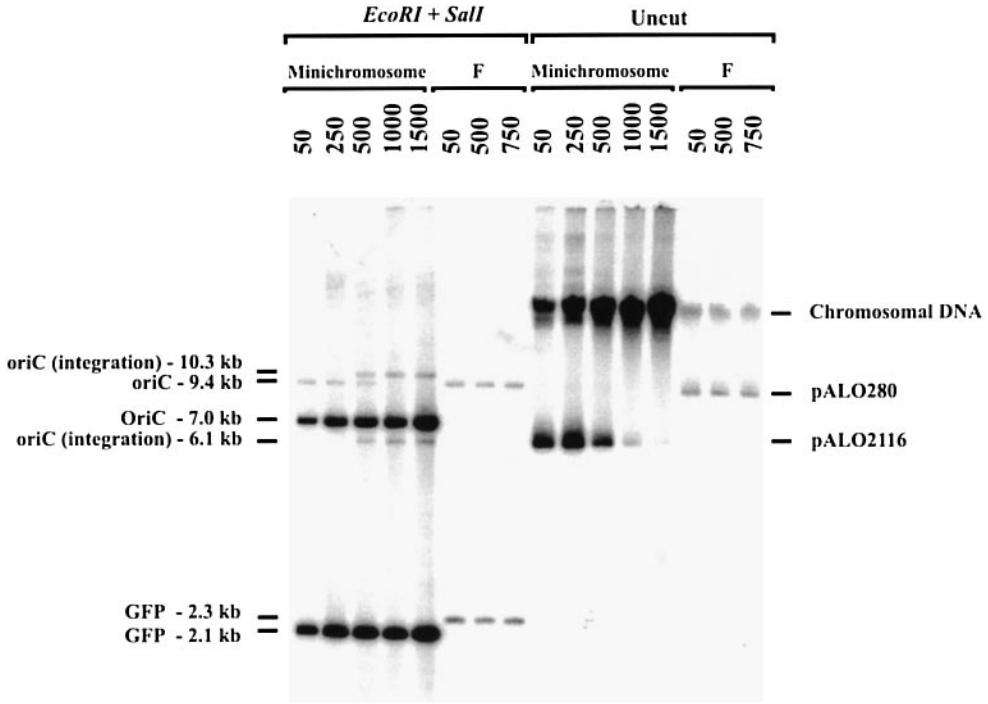

Fig. 4. Integration of minichromosomes into the chromosomal *oriC*. Strain MC1000 containing either minichromosome pALO2116 or the F-derived plasmid pALO280 was grown exponentially in low salt LB medium containing varying amounts of kanamycin at 37°C. Total DNA was isolated and a Southern blot hybridization performed as described in Materials and methods using a mixed *oriC/GFP* probe. The individual DNA samples were treated as indicated on the figure. Numbers above lanes refer to kanamycin concentrations in the growth medium. Sizes of fragments are also indicated on the figure.

undigested samples it was clear that the increase in total number of minichromosomes was accompanied by a reduction in the amount of free plasmid (Figure 4). At 1500 μg/l of kanamycin no free plasmid could be detected, and on average 66 minichromosome copies were integrated in the *oriC* region of each host chromosome (Table II).

For cells containing the F-derived plasmid the situation was different (Figure 4). In the digested samples, hybridization was always observed to two fragments of ~9 and 2.3 kb, representing the chromosomal origin and the plasmid GFP cassette, respectively. The proportion between them did not change with increasing kanamycin concentration (Figure 4; Table II), and the plasmid was always extrachromosomal.

It can be concluded that high minichromosome copy number caused interference with chromosome replication and minichromosomes were integrated into the chromosome. Integration of minichromosomes was not observed until the average copy number had increased from ~10 to more than 30 copies per chromosomal *oriC* (Figure 4; Table II). This level therefore represented the maximal number of free *oriC* copies that the cell could contain and still ensure replication of each chromosomal origin in each cell cycle.

The presence of minichromosomes does not affect the average initiation frequency

When cells containing minichromosomes were grown with increasing selection pressure and analysed for cell size and DNA content the following observations were made (Table II): cell size and DNA content increased ~10% when the kanamycin concentration was increased from 0 to 500 μg/ml. Higher kanamycin concentrations resulted in growth rate reduction and both cell size and DNA content decreased. Consequently, the DNA/mass ratio was the same at all kanamycin concentrations, i.e. irrespective of the minichromosome copy number. For the control cells containing the F-derived plasmid the average cell size and DNA content decreased by almost 50% as kanamycin concentration was increased. The DNA concentration was the same at all kanamycin concentrations (Table II).

I conclude that the presence of neither minichromosomes nor F-derived plasmids leads to an altered DNA concentration, suggesting that their presence in the cell does not affect the average initiation frequency of the chromosome. The changes in cell size and DNA content that were observed for cells containing either plasmid were similar to those observed when reducing growth rates by altering medium composition (Bremer and Dennis, 1996). It is therefore conceivable that the addition of sublethal concentrations of kanamycin that inhibit translation (Davies and Smith, 1978) mimics growth in a poorer medium.

High minichromosome copy number leads to asynchrony of initiation

When strain MC1000 was grown with a doubling time of 21 min without plasmid, or when containing minichromosomes at low kanamycin concentration (Figure 5A and B), the cells contained mainly four or eight fully replicated chromosomes after treatment with rifampicin and cephalexin (see Materials and methods), characteristic of synchronous initiations (Skarstad *et al.*, 1986). As the kanamycin concentration was increased, individual cells contained chromosome numbers different from 2^n at

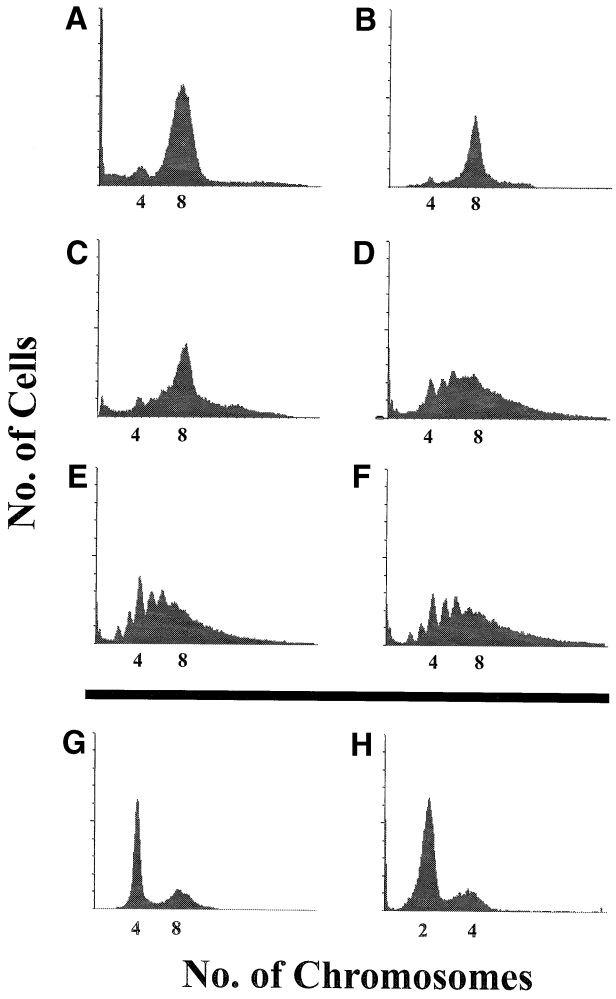

Fig. 5. Initiation synchrony of minichromosome-containing cells. Strain MC1000 (**A**), strain MC1000 containing either minichromosome pALO2116 (**B**–**F**) or the F-derived plasmid pALO280 (**G** and **H**) was grown exponentially in low salt LB medium containing varying amounts of kanamycin at 37°C. For determination of the cellular number of origins cells were treated with rifampicin and cephalexin as described in Materials and methods prior to FACS analysis. The amount of kanamycin in the individual cultures were as follows: (A), 0 μg/ml; (B), 50 μg/ml; (C), 250 μg/ml; (D), 500 μg/ml; (E), 1000 μg/ml; (F), 1500 μg/ml; (G), 50 μg/ml; (H), 750 μg/ml.

increasing frequency (Figure 5C–F). At 250 μg/ml of kanamycin, most cells contained eight chromosomes, but cells containing irregular numbers of chromosomes (different from 2^n) were present in significant amounts (Figure 5C), and at 500 μg/ml many cells contained five, six and seven chromosomes (Figure 5D). Kanamycin concentrations >500 μg/ml resulted in cells that contained all integral numbers of chromosomes ranging from two to more than 10 (Figure 5E–F).

Cells of strain MC1000 containing the F-derived plasmid contained four and eight chromosomes at 50 μg/ml of kanamycin (Figure 5G). As kanamycin concentration increased, so did the frequency of cells containing two chromosomes. At 750 μg/ml, 70% of the cells contained two chromosomes (Figure 5H; note the different scale of the abscissa in this panel). This histogram (Figure 5H) was similar to that expected from an unperturbed culture growing with a doubling time of 60–70 min.

I can thus conclude that the presence of minichromosomes leads to asynchrony of initiation when the copy number is high. Initiation synchrony is unaffected by the presence of plasmid F.

Discussion

Minichromosome and F plasmid replication

In spite of an average copy number of ~10 per chromosomal origin, minichromosomes are segregationally unstable. The copy number distribution suggests that random segregation gives rise to cells with few minichromosomes, from which plasmid-free cells might arise. In agreement with this, the *sopABC* genes of plasmid F can stabilize minichromosomes (Ogura and Hiraga, 1983; Løbner-Olesen *et al.*, 1987). Because minichromosomes are replicated once each cell cycle (Koppes and von Meyenburg, 1987), their high copy number arises from unequal segregation at cell division, provided that selection kills off plasmid-free segregants (Jensen *et al.*, 1990). Therefore, addition of the *sop* genes to minichromosomes decreases average copy number and narrows the copy number distribution. This indicates that the *sop* genes act by distributing equal numbers of plasmid molecules to daughter cells (equipartitioning), rather than distributing one pair of plasmids only; the latter is not expected to alter minichromosome copy number distribution significantly. The fact that simply increasing the selective pressure could increase the minichromosome copy number implies that no active copy number control mechanism is carried by these plasmids. However, interference with replication of the host chromosome is observed at very high copy numbers (see below).

In contrast, the copy number of an F plasmid could not be increased by increasing the selection pressure, indicating that it contained the necessary information for efficient copy number control. I failed to detect synchronous initiation of the F plasmids contained within each cell (on average 3.6 copies; Table I). Induction of GFP synthesis for one-quarter of a doubling time did not give rise to a distribution containing two peaks corresponding to non-initiated and initiated cells (2-fold difference in fluorescence), nor was the copy number distribution wider than for other plasmids. Detection of a 2-fold difference in fluorescence however, was near the detection limit of the method; compare distributions for F and an R1-derived plasmids, the latter having a copy number of 1.7 times that of F (Figure 2B). Previously, both cell-cycle-specific (Keasling *et al.*, 1992; Koppes, 1992; Cooper and Keasling, 1998) and random replication (Leonard and Helmstetter, 1988; Helmstetter *et al.*, 1997) has been reported. The data presented here lend support to the latter observations.

Minichromosomes interfere with initiation of replication of the host chromosome

The presence of minichromosomes did not lead to any significant increase in cell size, indicating that derepression of the autoregulated *dnaA* gene (Braun *et al.*, 1985) can compensate for the DnaA protein titration by the minichromosomal DnaA boxes. Minichromosomes had little effect on DNA concentration and initiation synchrony, as long as the copy number did not exceed ~30 per *oriC*. This demonstrates that the initiation cascade can

initiate a number of minichromosomal origins that are in more than 30-fold excess over chromosomal origins, in synchrony with the chromosome. As minichromosome copy numbers increased further, so did the number of copies of minichromosomes integrated into the chromosome. At the same time, initiations became asynchronous. The integrated state of minichromosomes *per se* is not sufficient to result in asynchrony since a moderate number (8–10) of integrated copies had little effect on initiation synchrony (Løbner-Olesen and von Freiesleben, 1996). It is therefore likely that it is the asynchrony of initiation that causes integration of minichromosomes.

Two explanations may be given for the initiation asynchrony of cells containing high numbers of minichromosomes. First, an important factor for initiation might become limiting and result in the occasional skipping of a chromosomal origin, such as proposed for certain *dnaATs* mutants where the availability of active DnaA protein is limiting (Skarstad *et al.*, 1988). However, cell size did not increase with increasing minichromosome content, which is observed when availability of DnaA protein is limiting (Løbner-Olesen *et al.*, 1989). The fact that initiations continued to be asynchronous in cells that containing >60 minichromosomes integrated head to tail in the origin region of the chromosome also argues against origin skipping as the cause of asynchrony. These cells contained the same average number of chromosomes as wild-type cells (Figure 5), and the initiation cascade has the capacity to initiate >30-fold this number of origins in synchrony. Because a single initiation in a multi-*oriC* region is sufficient to ensure replication of the entire chromosome, skipping replication of a chromosome becomes unlikely for statistical reasons.

Second, the initiation cascade may not be operating in cells containing high numbers of minichromosomes because of sequestration failure; initiation of replication in cells containing high numbers of minichromosomes results in high numbers of hemimethylated origins (chromosomal and extrachromosomal). If these hemimethylated *oriC*s cannot be sequestered efficiently, the newly replicated origins are capable of rebinding the DnaA protein released by initiation. Consequently, there will be no cascade, and a new period of accumulation is necessary before the next initiation can occur on a randomly picked origin. This would result in initiation asynchrony by a mechanism similar to that proposed for Dam$^-$ cells (Løbner-Olesen *et al.*, 1994). The asynchrony of Dam$^-$ cells also leads to the inability to host free minichromosomes (Løbner-Olesen and von Freiesleben, 1996).

Minichromosome and chromosome replication

It has been suggested that the DnaA boxes around the chromosome serve as negative elements for initiation of chromosome replication by lowering the availability of the DnaA initiator protein (Hansen *et al.*, 1991a), a function similar to that of *inc* regions (iteron repeats) of low copy number plasmids such as F and P1 (for review see Del Solar *et al.*, 1998). The DnaA protein also limits minichromosome replication (Atlung *et al.*, 1987). The chromosomal DnaA boxes therefore limit the availability of DnaA protein for initiation of both chromosome and minichromosome replication. Consequently, minichromosomes follow the replication control of the chromosome. This explains why minichromosome copy number per chromosomal origin is the same at different growth conditions (Løbner-Olesen *et al.*, 1987) and that they replicate in synchrony with the chromosome (Leonard and Helmstetter, 1986). Because minichromosomes do not contain DnaA boxes outside of *oriC*, an increase in copy number is not accompanied by any substantial increase in total DnaA boxes (only three of 308 consensus DnaA boxes are found in *oriC*), and concomitant turn-off of chromosome and minichromosome replication. Only when copy number is such that the cells' capacity for synchronous initiations is exceeded, is interference with the chromosomal origin (incompatibility) observed.

The similarities between minichromosomes and the chromosome, demonstrated by incompatibility when minichromosomes are present in numbers, suggest that *oriC* on minichromosomes behaves exactly like its chromosomal counterpart. Recently it was concluded that the requirements for initiation of minichromosome replication differed from those for the chromosome (Asai *et al.*, 1998). This conclusion was based on two lines of evidence. First, mutations in host genes such as those encoding proteins HU, IHF and Fis render cells viable but the ability to host minichromosomes is lost. Second, certain mutations in *oriC* that limit binding of DnaA protein can sustain perturbed chromosome replication, but minichromosomes containing the same mutations cannot replicate in wild-type cells. However, all of these observations may equally well result from competition for an essential factor for initiation, such as DnaA, between copies of *oriC* located on the chromosome and on minichromosomes. In HU, IHF and Fis deficient strains the initiation process is inefficient as demonstrated by asynchronous initiations. As minichromosomes are dispensable, they are lost from the population of such cells. Similarly, minichromosomes truncated in their DnaA binding ability, may require a level of DnaA protein that is never reached in wild-type cells, and consequently do not replicate.

Minichromosomes as model systems for chromosomal replication, are therefore well suited for regulatory studies of the initiation process as well as for mechanistic studies.

Materials and methods

Bacterial strains

Escherichia coli strain MC1000 [*thi*, *Δ*(*ara-leu*)7679, *araD*139, *lacΔX*74, *galU*, *galK*, *rpsL*; Casadaban and Cohen, 1980] was used as host cell for all plasmids.

Media

Cells were grown at 37°C in either low salt LB medium (containing 5g/l of NaCl; Bertani, 1951) or AB minimal medium (Clark and Maaløe, 1967) supplemented with 0.2% glycerol, 0.5 μg/ml of thiamin and 50 μg/ml of leucine. Unless otherwise indicated antibiotics were used at the following concentrations: ampicillin 50 μg/ml, kanamycin 50 μg/ml and cloramphenicol 10 μg/ml. For induction of the pBAD promoter, arabinose was added to a final concentration of 0.2%.

In order to grow cultures at fairly constant kanamycin concentrations, frequent dilutions of cells into fresh kanamycin-containing medium was necessary; the *neo* gene used encodes a 3′-phosphotransferase II (Berg *et al.*, 1975), which inactivates kanamycin.

Plasmids

The plasmids used are listed in Table I. Plasmid pALO240 was constructed by inserting the *Sal*I–*Xho*I fragment of plasmid pALO1

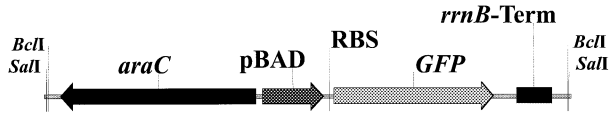

Fig. 6. Structure of the GFP expression cassette. The GFP expression cassette contains the *GFP* gene exclusively under control of the pBAD promoter, and followed by the *rrnB* T_1 and T_2 transcription terminators to prevent excessive transcription into other plasmid regions. The *araC* gene encoding the repressor of pBAD is under control of its own promoter. The *GFPmut2* allele of the GFP gene was used for all constructs except pALO2116 where the *GFP-BioST* allele was used. The ribosome-binding site preceding *GFP* is indicated (RBS).

containing the Tn*5* *neo* gene (Løbner-Olesen *et al.*, 1987) into the *Sal*I site of pBR322 (Bolivar *et al.*, 1977). Plasmid pALO277 was constructed by ligating the *Hin*dIII to *Sal*I fragment of pML31 (Lovett and Helinski, 1976) containing the *oriV, oriS* and *sopABC* region (plasmid F coordinates 41147–49729) to the *Hin*dIII–*Sal*I *neo* gene of Tn*5*. The latter was taken from plasmid pALO1.

The *araC*, pBAD-*GFPmut2* cassette (Figure 6) was constructed as follows: the *GFPmut2* (Cormack *et al.*, 1996) preceded by a T7 gene 10 ribosome binding site, nucleotide sequence TCTAGA*TTTAAGAAGGAG-ATATACAT***ATG**, was obtained from B.Cormack via P.De Boer as an *Xba*I–*Pst*I insert in pKEN (MacFerrin *et al.*, 1993). The *Xba*I site is underlined, the ribosome binding site in italics and the ATG codon of *GFPmut2* in bold. Plasmid pALO247 was constructed by inserting the *Xba*I–*Hin*dIII *GFPmut2* fragment into the *Nhe*I and *Hin*dIII digested plasmid pBAD18 (Guzman *et al.*, 1995). The region of plasmid pALO247 containing *araC*, pBAD-*GFPmut2* was PCR amplified using the primers 5′-GGGTACG-TCGACTGATCACCTATGCTACTCCGTCAAGCCG-3′ and 5′-GCG-CTCGTCGACTGATCAGTTCAAATCCGCTCCCGGCGG-3′, the PCR product was digested with *Sal*I and inserted into the *Sal*I site of plasmid pNEB193 (New England Biolabs, Inc.), resulting in plasmid pALO249. The structure of the *araC*, pBAD-*GFPmut2* cassette is shown in Figure 7.

Plasmids pALO264, pALO267, pALO269 and pALO280 were constructed by inserting the *Sal*I fragment containing *araC*, pBAD-*GFPmut2* of plasmid pALO249 into *Sal*I digested plasmids pALO1 (Løbner-Olesen *et al.*, 1987), pALO7 (Løbner-Olesen *et al.*, 1987), pALO240 (see above), pALO277 (see above), respectively.

Plasmids pALO261, pALO270, pALO275 and pALO284 were constructed by inserting the *Bcl*I fragment containing *araC*, pBAD-*GFPmut2* of plasmid pALO249 into *Bam*HI digested plasmids pJEL109 (Løbner-Olesen *et al.*, 1992), pSP102 (Pal *et al.*, 1986), pACYC184 (Chang and Cohen, 1978) and pLG339 (Stoker *et al.*, 1982), respectively.

The *GFP-BioST* gene preceded by a synthetic ribosome binding site was obtained from Sara Petersen Bjørn (Novo Nordisk A/S) as a *Kpn*I–*Eco*RI fragment. The nucleotide sequence preceding the *GFP-BioST* gene is as follows: GGTACC*AAGGAGGTAAGCTTT***ATG**. The *Kpn*I site is underlined, the ribosome binding site in italics and the ATG codon of *GFP-BioST* in bold. This fragment was inserted into the *Kpn*I–*Eco*RI digested plasmid pBADHisA (Invitrogen, Inc.), to yield pALO2062. The region of plasmid pALO2062 containing *araC*, pBAD-*GFP-BioST* was PCR amplified using the primers listed above, the PCR product was digested with *Sal*I and inserted into the *Sal*I site of plasmid pALO1 (Løbner-Olesen *et al.*, 1987), resulting in plasmid pALO2116. The orientation of the *neo* gene was the same as *GFP-BioST*. Eventual unterminated transcription from the *neo* promoter will therefore be terminated by the *rrnB* T_1 and T_2 transcription terminators before entering *oriC*.

Cell cycle analysis

Cells were fixed in 70% ethanol and proteins stained overnight at 4°C in a 0.1 M KH_2PO_4/K_2HPO_4 buffer pH 9.0 containing 1.5 μg/ml of fluoresceinisothiocyanate (FITC). Cells were washed twice with 0.02 M KH_2PO_4/K_2HPO_4 buffer pH 7.5 containing 130 mM NaCl, and DNA was stained for 1 h in the same solution containing 1.5 μg/ml of Hoechst 33258.

Flow cytometry was performed using a FACStar$^+$ instrument equipped with an argon laser and a UV laser (Becton Dickinson, Inc.). Excitation for FITC staining was at 488 nm and fluorescence was collected between 510 and 540 nm. For Hoechst 33258 staining excitation was at 351/356 nm and fluorescence collected between 410 and 470 nm.

For determination of numbers of origins per cell, samples were treated with 300 μg/ml of rifampicin to stop further initiations and 10 μg/ml of cephalexin to stop further cell division prior to flow cytometry. Cell size, cellular DNA content and DNA concentration was determined as described previously (Løbner-Olesen *et al.*, 1989), with the exception that protein content (FITC fluorescence) replaced light scatter as a measure of cell size.

Plasmid copy number distributions

Cells were grown exponentially in AB minimal medium using glycerol as carbon source as described above. At OD_{450} = 0.2 cells, arabinose was added to a final concentration of 0.2% to induce GFP synthesis from the pBAD promoter. After 30 min of induction rifampicin and cephalexin was added to final concentrations of 300 μg/ml and 10 μg/ml, respectively. Cells were incubated for an additional 60 min to chase immature GFP precursors into the mature form, before FACS analysis. Prior to FACS analysis cells were diluted 25-fold in ice-cold 0.9% NaCl.

Analysis for cellular GFP fluorescence

This was done using a FACStar$^+$ or a FacsCalibur instrument equipped with an argon laser (Becton Dickinson, Inc.). Excitation was at 488 nm and fluorescence was collected between 510 and 540 nm. Average cellular GFP fluorescence was determined using the Lysys$^{™}$II software (Becton Dickinson, Inc.).

Southern blot hybridization

This was done as described previously (Løbner-Olesen and von Freiesleben, 1996), except that cellular DNA was digested with *Eco*RI and *Sal*I prior to gel electrophoresis. For making the probe, plasmid pALO264 was digested with *Hin*dIII and *Sal*I. The *GFP* gene is located on a 2015 bp *Sal*I fragment and the majority of the *oriC* region on a 2135 bp *Hin*dIII fragment. These two fragments were copurified using the QiaEXII kit (Qiagen, Inc.) and labelled by the random primer method (Prime-A-Gene; Promega, Inc.) using [^{32}P]dTTP (NEN). Radioactive bands were visualized using a STORM 840 PhosphorImager (Molecular Dynamics, Inc.).

Copy number determination

Cellular copy numbers relative to that of plasmid F were determined by the average cellular GFP fluorescence following a 30 min pulse induction. Copy numbers of minichromosomes relative to *oriC* were determined as the amount of label in the minichromosome-specific *oriC* fragment relative to the chromosomal *oriC* fragment as described previously (Løbner-Olesen and von Freiesleben, 1996). For determination of copy numbers of other plasmids the method was modified slightly to determine the amount of label in the GFP-specific fragment relative to the *oriC* fragment; total DNA was digested with *Eco*RI and *Sal*I, and a mixed *oriC*/*GFP* probe used as described above. In such a digest, the chromosomal *oriC* region is located on a 9.4 kb fragment, whereas the *GFP* region for plasmids pALO280, pALO269, pALO264 and pALO267 is on 2.3 kb fragments. For plasmids pALO261, pALO270, pALO275, pALO284 and pALO2116 the *GFP* region is on fragments of 7.3 kb, 4.3 kb, 4.5 kb, 3.1 kb and 2.1 kb respectively. A sample of a strain MC1000 containing plasmid pALO264 from which the mixed probe was derived was included on all blots. This allowed for determination of the relative labelling/transfer efficiencies of the *oriC* fragment relative to the GFP fragment and all copy numbers were corrected for this. Copy numbers were also corrected for plasmid-free cells as described previously (Løbner-Olesen *et al.*, 1987).

For quantifying individual bands a STORM 840 PhosporImager was used along with the MD ImageQuant version 3.3 software (Molecular Dynamics, Inc.).

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