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Stability and Replication Control of *Escherichia coli* Minichromosomes

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A stabilized minichromosome—a plasmid replicating from the chromosomal origin *oriC*—was constructed by cloning the *sopA,B,C* genes from plasmid F. This minichromosome had a loss frequency of less than 10^{-3}, while that of the nonstabilized parental plasmid was 2×10^{-2} to 4×10^{-2}. Both minichromosomes had the same average copy number per chromosomal origin, and the copy numbers were constant over an eightfold range of growth rates. Different mutations in the *mioC* gene and promoter, from which transcription enters *oriC*, were constructed, and their effects on minichromosome copy number and stability were tested. The results indicated that normal replication control at *oriC* was independent of the MioC protein and most of the sequences between the promoter and *oriC*, but required both transcription from the *mioC* promoter and probably also the presence of the DnaA box (DnaA protein-binding site) just upstream of the *mioC* promoter. Transcription from the *mioC* promoter was shown to be efficiently repressed in vivo after overproduction of DnaA protein and to be derepressed at the nonpermissive temperature in six different *dnaA*(Ts) mutants.

Replication of the *Escherichia coli* chromosome initiates at a fixed site, *oriC*, and proceeds bidirectionally (7, 37). Plasmids that replicate autonomously from *oriC*, minichromosomes, have been isolated (23, 49, 52). Minichromosomes are dependent on functional *dnaA* and *dnaC* gene products, de novo protein synthesis, and RNA polymerase-mediated transcription for their initiation of replication and thus resemble the *E. coli* chromosome (49). The DnaA protein concentration in the cell has been shown to be a limiting factor for initiation of both chromosome and minichromosome replication (2a). Recently, evidence has been presented that minichromosome replication occurs in the same discrete interval in the cell division cycle as initiation of chromosome replication (31). Despite their high copy number, the minichromosomes show segregational instability (41, 44, 49), suggesting that they lack a partition system.

The minimal sequence necessary for autonomous replication is 245 base pairs (bp) (42), but there are indications that DNA sequences to the right of this minimal origin sequence are needed for proper origin function in vivo and probably for bidirectional replication as well (38). This region encodes the 16-kilodalton (kDa) MioC protein of yet unknown function (21) and the 17-kDa AsnC protein, which has been identified as an activator of the *asnA* gene (29). In addition, the region contains one of the binding sites for a membrane protein, B′, the other one being in the minimal origin (25), and the incompatibility regions *incB* and *incC*, covering the *mioC* promoter and most of the *asnC* gene, respectively (44, 51). The weak incompatibility exerted by *incB* may be caused by binding of the DnaA initiator protein to a DnaA box situated in the *mioC* promoter (18, 19). Deletion of the nucleotide sequence covering the *mioC* promoter leads to decreased minichromosome copy number and increased segregational instability (44–46).

Transcription from the *mioC* promoter is negatively regulated by the DnaA protein in vitro (34), indicating that the DnaA protein acts as a repressor by binding to the DnaA box—in analogy with the autoregulation of the *dnaA* gene (2, 10).

In this work we show that under certain growth conditions minichromosomes can be partially stabilized by the *sop* genes from plasmid F and that the ratio of minichromosome copy number to chromosomal origins is the same at a wide range of growth rates. The implications of these results for minichromosomal stability are discussed.

A stabilized minichromosome proved useful in the study of replication control by the *mioC* promoter, which we found to be negatively regulated by the DnaA protein in vivo and to be essential for optimal minichromosome replication. The role of this transcription in the initiation event is also discussed.

MATERIALS AND METHODS

Bacterial strains. Strain CM987 (*asnA31 asnB32 recA1 relA1 spoT1 thi-1* [48]) was used for determination of copy number and stability of minichromosomes. Strains MC1000 (11) and NF1815 (= MC1000 *recA1*) were used as recipients in plasmid constructions. β-Galactosidase from plasmid-borne transcriptional fusions was assayed in NF1815, CM732 (*thi his metE trp fhu* Str^r T6^r *sup-38 galK ara lacY mtl* [22]), and CM734 (= CM732 *dnaA46* [22]). Cellular DNA and RNA contents at different growth rates were determined in strain L1 (*asnB32 lysA1 ilv pyrB* F′ *ilv*$^+$), a derivative of CM987. To test for origin activity of the cloned *sop* fragment, the isogenic strain pair W3110 *thyA* and W3110 *thyA polA1* (6) was used.

Growth media. Bacteria were grown in NY medium (50) or in AB minimal medium (13) supplemented with thiamine (1 μg/ml), and 0.2% glucose, 0.2% glycerol, or 0.4% acetate was used as the carbon source. Required amino acids were added to 50 μg/ml or, when indicated, Casamino Acids to 1%. Thymine was added at 50 μg/ml when required. MacConkey plates were prepared as described by the manufacturer (Difco Laboratories). X-Gal plates were prepared by supplementing the medium with 40 μg of 5-bromo-4-chloro-indolyl-β-D-galactoside (X-Gal) per ml. Concentrations of

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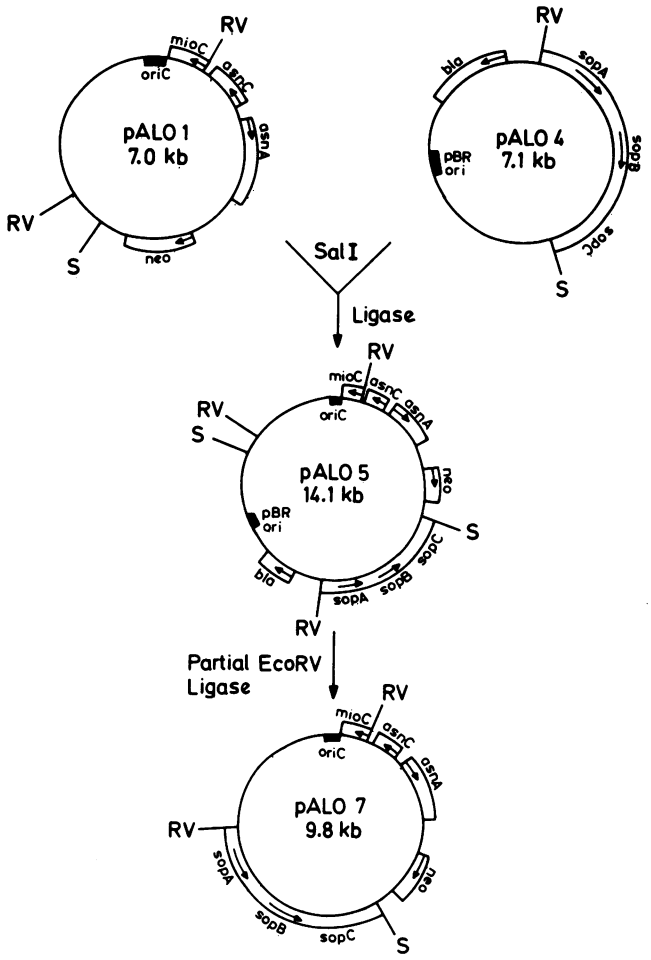

FIG. 1. Construction of the minichromosome pALO7. The composite *oriC*-pBR322 plasmid pALO5 was constructed by digesting pALO1 and pALO4 with *Sal*I, treatment with DNA ligase, and transformation of strain MC1000 to Amp^r Kan^r. The minichromosome pALO7 was constructed by digesting pALO5 partially with *Eco*RV, treatment with DNA ligase, and transformation of MC1000 to Kan^r. Colonies that were Kan^r Amp^s contained the desired minichromosome. Restriction enzyme abbreviations: S, *Sal*I; RV, *Eco*RV.

antibiotics used were: ampicillin, 200 μg/ml; tetracycline, 10 μg/ml; kanamycin and chloramphenicol, 25 μg/ml.

DNA technology. Plasmid DNA was prepared by the alkaline sodium dodecyl sulfate method described by Birnboim and Doly (8). Restriction enzymes, T4 DNA ligase, alkaline phosphatase, and DNA polymerase I (Klenow fragment) were used as recommended by the suppliers (New England BioLabs, Boehringer Mannheim, and Amersham International). For agarose gel electrophoresis agarose (Sigma Chemical Co. type II) was used, and gels were run in TBE buffer (36).

Construction of minichromosomes. The minichromosome pALO1 (Fig. 1) containing *oriC* as the only origin of replication was assembled from the two *Hin*dIII fragments spanning *oriC* from pFH271 (21), the *Hin*dIII-*Sal*I fragment of transposon Tn*5* carrying the kanamycin resistance gene (26), and the small *Hin*dIII-*Sal*I fragment from pBR322 (9). Plasmid pALO4 (Fig. 1) is a pBR322 derivative carrying the *sop* (stability of plasmid) genes from plasmid F (41) as an *Eco*RV-*Sal*I insert corresponding to positions 46.20 to 49.40 on the native F molecule (40). The minichromosome pALO7 was constructed from pALO1 and pALO4 via the composite *oriC*-pBR322 plasmid pALO5 as outlined in Fig. 1. pALO7 contains *oriC* as the only origin of replication, the kanamycin resistance gene from Tn*5*, and the *sop* genes from plasmid F.

To generate deletions and insertions in the minichromosomal *mioC* region, the *Hin*dIII-*Bam*HI fragment from position 244R to 2191R was cloned from plasmid pFH271 (21) into the *Hin*dIII-*Bam*HI site of pACYC184 (12) or into the *Hin*dIII-*Bam*HI site of pUC8 (47), yielding pALO29 and pALO30, respectively. The deletions and insertions are shown in Fig. 3. Deletions 1, 4, 5, 6, and 7 and insertion 3 were constructed in pALO29, giving plasmids pALO71, pALO46, pALO47, pALO48, pALO49, and pALO45, respectively, and deletions 2, 8, 9, and 10 in pALO30 yielded plasmids pALO33, pALO50, pALO51, and pALO52, respectively. The deletions were made as cutbacks with different enzymes, and the ends were treated with DNA polymerase I Klenow fragment and ligated. The correct 4-bp insertion in the *Mlu*I site at position 777R (see Fig. 3) was verified by the appearance of a new *Bss*HII site.

Subsequently, all modifications were transferred to both pALO1 and pALO7 by *Xho*I-*Mlu*I clonings, a method ensuring that exactly identical modifications exist in the derivatives of the minichromosomes pALO1 and pALO7.

Construction of *mioC-lacZ* transcriptional fusion plasmids. Plasmid pJL207 (32) is a *lacZ* transcriptional fusion vector containing the cloning sites for *Pst*I, *Bgl*II, and *Hin*dIII. The *mioC*-carrying *Hin*dIII-*Bam*HI fragment from pALO29 was inserted into the *Hin*dIII-*Bgl*II sites of pJL207, yielding pALO75, in which transcription from the *mioC* promoter proceeds across the *Hin*dIII site into *lacZ*.

The *Hin*dIII-*Bam*HI fragments from plasmids pALO33, pALO45, pALO46, pALO47, pALO48, pALO49, pALO50, pALO51, pALO52, and pALO71, carrying the described deletions or insertions in the *mioC* region, were also inserted into pJL207 to measure transcription from these across the *Hin*dIII (244R) site.

Additional plasmids used. Plasmid pTAC1534 is a pSC101 derivative carrying the temperature-sensitive lambda *c*I857 repressor gene (2). Plasmid pTAC1430 is a pBR322 derivative carrying the *dnaA* gene under control of the λ p_L promoter, and pTAC1431 is pTAC1430 with a 121-bp deletion internal in the *dnaA* gene (2a).

Determination of β-galactosidase activity. The levels of β-galactosidase activity in the *mioC-lacZ* transcriptional fusions, representing transcription from the right across the *Hin*dIII site at position 244R, were determined in strains NF1815, CM732, and CM734. Enzyme activity was determined by the method of Miller (39) in cells permeabilized with toluene. The differential rate of synthesis (units per cell mass) was determined from plots of enzyme activity against optical density of the culture (at 450 nm [OD_{450}], measured on a PMQ2 Zeiss spectrophotometer) at the time of sampling.

Copy number determination. Copy number was determined essentially by the method of Stüber and Bujard (43) and Atlung et al. (2). Exponentially growing cells (25 ml) were harvested at an OD_{450} of 0.5, and plasmid DNA was isolated by the alkaline sodium dodecyl sulfate method and digested with *Hin*dIII before agarose gel electrophoresis. A negative picture was taken (Polaroid type 665 film) and scanned on a Zeiss M4QIII scanner. Every gel contained lanes with plasmid DNA of known concentration to allow conversion of scanning units to micrograms of DNA. RNA concentration in the final plasmid preparation was deter-

mined by measuring the OD_{260} and used to correct for loss during the plasmid preparation. The RNA content was 40 μg/ml at an OD_{260} of 1 (36).

Determination of cellular RNA and DNA contents at different growth rates was done by uracil incorporation (L. Boe, Ph.D. thesis, University of Copenhagen, 1984). The DNA contents per ml at an OD_{450} of 1, corresponding to μ values of 2.10, 1.90, 1.80, 1.00, 0.90, 0.60, and 0.25, were 3.20, 3.30, 3.35, 3.75, 3.80, 4.00, and 4.20 μg, respectively. The RNA contents per ml at an OD_{450} of 1 for the same growth rates were 40.0, 37.0, 36.0, 25.0, 23.5, 19.5, and 17 μg, respectively. Knowing the amount of cells harvested, percent recovery, amount of DNA in the scanned fragment, fragment size, and the RNA and DNA amount per volume at an OD_{450} of 1 at each particular growth rate allowed the copy number/genome equivalent ratio to be calculated, assuming that the size of the chromosome is 4,500 kilobases (kb).

At the time of sampling, a portion of each culture was plated on nonselective plates after appropriate dilution and incubated at 37°C overnight. Replica-plating to selective plates and incubation of these overnight allowed the fraction of plasmid-containing cells to be determined. All calculations of copy number are based on this fraction only.

The copy number per origin and copy number per cell were calculated from the formulas

$$\text{origins per genome equivalent} = 2^{(C/t)} \ln \{[2^{(C/t)}]\}/[2^{(C/t)} - 1]$$

and

$$\text{origins per cell} = 2^{(C/t)}2^{(D/t)}$$

where C is the replication time, D is the interval from termination of replication to cell division, and t is the doubling time.

At generation times of less than 60 min the C period is set at 40 min and the D period at 20 min (14), and at generation times of more than 60 min the C period is set at the values determined by Kubitschek and Newman (30), while the D period is still 20 min.

Test for plasmid stability. Strain CM987 containing the plasmids to be tested for stability was inoculated into selective medium containing kanamycin from single colonies on selective agar plates and incubated at 37°C overnight. The culture was diluted with fresh nonselective medium (NY) and incubated at 37°C. To obtain exponentially growing cells for at least 20 generations, the culture was diluted at suitable intervals with fresh prewarmed nonselective medium. At intervals samples of the culture were plated on nonselective agar plates and incubated at 37°C overnight. The fraction of plasmid-containing clones was determined by replicating to selective plates. A loss curve was drawn for each plasmid tested, and the loss frequency (LF) can be calculated from $LF = -\Delta(\ln V_t)/\Delta G_t$, where V_t is the frequency of plasmid-carrying cells and G_t is the number of generations. Corresponding values of V and G can be read off the loss curve (see Fig. 2).

RESULTS

Stabilization of a minichromosome by *sop* genes. Despite their relatively high copy number, minichromosomes are rapidly lost from a cell population growing in the absence of selective pressure (49, 52), and construction of a stable minichromosome would facilitate the study of minichromosome replication. The *sop* genes from plasmid F, which are known to stabilize minichromosomes (41) and have been proposed to have a true partitioning function (4, 5), were chosen for minichromosome stabilization. First, the *sop* genes from plasmid F were cloned as a 3.2-kb DNA fragment into pBR322 (pALO4, Fig. 1) and shown not to carry any origin activity, since the resulting plasmid was unable to replicate in a *polA1* host. Subsequently, the *sop* genes were inserted into the kanamycin-resistant minichromosome pALO1, resulting in pALO7 (Fig. 1). The presence of the *sop* genes in pALO7 was found to increase the stability of the minichromosome more than 25-fold (Fig. 2; also see Table 2). It should be noted that the stabilizing effect observed was not due to an increase in minichromosome copy number (Table 1).

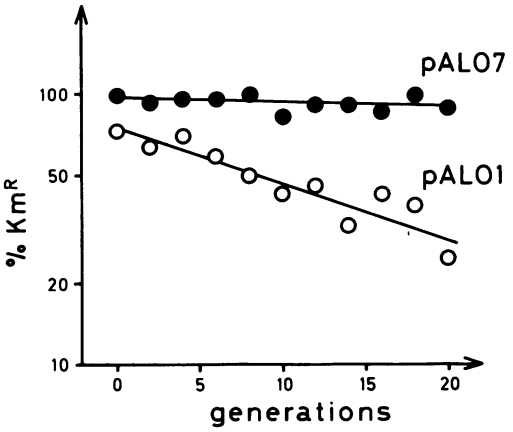

FIG. 2. Stability of plasmids pALO1 and pALO7. Exponentially growing cells of *E. coli* CM987 carrying pALO1 or pALO7 were examined for segregational stability during growth in nonselective NY medium as described in Materials and Methods. The marker used to determine the presence of the plasmid was kanamycin resistance (*neo*). Cells with and without plasmids had a doubling time of 29 min at 37°C.

Minichromosome copy number as a function of growth rate. The copy number per cell mass of both the unstable pALO1 and the stabilized pALO7 minichromosomes was virtually the same over an eightfold range of growth rates (Table 1). In this respect minichromosomes behaved like the chromosome, initiating on the average at the same cell mass per origin (15). The fraction of plasmid-free cells in a selectively grown culture reflected the instability of the plasmid, which again is related to the plasmid copy number per cell. Since the minichromosome copy number per cell decreases with decreasing growth rate, like that of the chromosomal origin, one would expect minichromosomes to show reduced stability at low growth rates. In accordance with this we found that the percentage of plasmid-containing cells was lowest in the slowly growing cultures (Table 1). A similar result was observed for CM987 containing the minichromosome pCM959 (49; T. Atlung, unpublished). A stability experiment confirmed that the minichromosomes showed increased loss frequencies at low growth rates (data not shown).

Even in the presence of kanamycin at 25 μg/ml, slowly growing cultures contained a large fraction of plasmid-free cells, which must eventually stop growing. But we have found that the plasmid-carrying cells are resistant to at least 1,000 μg of kanamycin per ml. Cells that fail to inherit the plasmid must therefore be expected to grow at the normal rate for several generations before becoming phenotypically sensitive to the antibiotic. A low percentage of nongrowing cells will have little influence on the measured growth rates and even less influence on the values of DNA per mass and origins per mass. When cultures were grown with selection

TABLE 1. Copy number of plasmids pALO1 and pALO7 as a function of growth rate

Plasmid[a]	Growth medium[b]	Growth rate[c] (μ)	Plasmid-bearing cells (%)[d]	Copy no./ml × OD_{450}	Copy no./genome equivalent	Copy no./origin	Copy no./cell
pALO1	NY*	2.10	62	10.0×10^9	13	9	38
	Glucose + CA	1.80	80	10.0×10^9	12	8	30
	Glucose	0.90	80	11.0×10^9	12	10	20
	Glycerol	0.60	36	13.0×10^9	13	11	18
	Acetate	0.25	55	9.7×10^9	10	8	12
pALO7	NY*	2.10	94	8.4×10^9	11	7	29
	Glucose + CA	1.80	98	9.8×10^9	12	8	30
	Glucose	1.00	97	9.7×10^9	11	9	18
	Glycerol	0.60	51	9.9×10^9	11	9	15
	Acetate	0.25	58	9.7×10^9	10	8	12

[a] The host strain was CM987.
[b] All media were supplemented with kanamycin (25 μg/ml) except where indicated with an asterisk (*). Growth temperature was 37°C. CA, Casamino Acids.
[c] Doublings per hour, determined by turbidity.
[d] Determined by replica-plating.

for Asn^+, similar values were obtained for growth rate and fraction of plasmid-carrying cells, again in accordance with the assumption that plasmid-free (Asn^-) cells grow for several generations. Furthermore, when the *asnA* gene was carried on a derivative of pBR322, the growth rate in glycerol minimal medium was 0.67 doublings per h with 100% plasmid-carrying cells, compared with 0.68 doublings per h with pALO1 and only 45% plasmid-carrying cells.

Surprisingly, the *sop* gene-carrying minichromosome pALO7 seemed to be stabilized only at high growth rates. In the slowly growing glycerol and acetate cultures, the plasmid was unstable despite an average copy number of 15 and 12 per cell, respectively. Since the *sop* genes from plasmid F are normally able to ensure stable inheritance of a plasmid at 2 copies per cell at division (5, 17), the reduced stability observed for pALO7 most probably reflects a mode of segregation very different from that of plasmid F.

Effects of transcription from the *mioC* promoter on the replication of minichromosomes. Several lines of evidence indicate that the *mioC* promoter has a regulatory role in initiation of minichromosome replication. However, it has never been clearly established whether the effects observed by removing or replacing the *mioC* promoter were caused by change in promoter activity or in some other feature of the region, like a specific DNA sequence.

To study the effect of different deletions and insertions in the *mioC* promoter region, we took advantage of the fact that any modification of a minichromosome that leads to a lower initiation frequency will also be expressed as a decrease in copy number and an increase in loss frequency. As anticipated, the stability differences were found to be amplified and therefore easier to demonstrate with derivatives of the stabilized minichromosome pALO7.

Figure 3 shows the deletions and insertion made in the

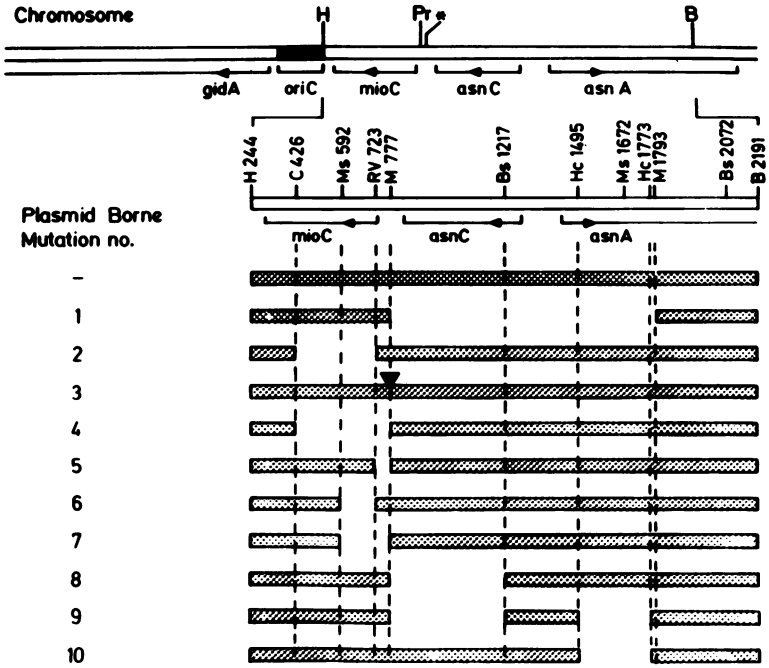

FIG. 3. Structure of the deletions and insertion generated in the *mioC* region of minichromosomes. Symbols: Pr, *mioC* promoter; *, *mioC*-DnaA box; ▼, 4-bp insertion in the *Mlu*I site at position 777R. Restriction enzyme abbreviations: H, *Hin*dIII; B, *Bam*HI; C, *Cla*I; Ms, *Mst*II; RV, *Eco*RV; M, *Mlu*I; Bs, *Bss*HII; Hc, *Hin*cII.

TABLE 2. Effect of deletions and insertion in the *mioC* region on copy number, stability of minichromosomes, and transcription into *oriC*

Plasmid	Mutation no.[a]	Relative copy no./ genome equivalent	LF (%/ generation)[b]	Relative transcription into *oriC* (%)[c]
pALO1 and derivatives				
pALO1		1.0	2.4	100
pALO25	1	0.5	4.6	8
pALO57	2	1.0	2.8	97
pALO59	3	0.7	3.5	6
pALO61	4	0.6	14.7	2
pALO63	5	0.6	4.7	1
pALO66	7	0.8	4.3	1
pALO68	8	0.5	4.6	12
pALO7 and derivatives				
pALO7		1.0	<0.1	100
pALO16	1	0.5	3.7	8
pALO56	2	1.1	<0.1	97
pALO58	3	0.8	0.7	6
pALO60	4	0.5	8.0	2
pALO62	5	0.3	3.0	1
pALO64	6	1.0	<0.1	36
pALO65	7	0.3	4.0	1
pALO67	8	0.4	2.8	12
pALO69	9	0.6	3.0	ND[d]
pALO70	10	1.0	<0.1	99

[a] See Fig. 3.
[b] Determined in strain CM987 as described in Materials and Methods. Cells were grown at 37°C in NY medium.
[c] Determined by transcriptional fusions to *lacZ*, the point of fusion being the *Hin*dIII site at position 244R. A value of 100% transcriptional activity corresponds to 118.9 U of β-galactosidase.
[d] ND, Not determined.

mioC promoter region. With the exception of mutations 6, 9, and 10, the modifications were made in both pALO1 (*sop*) and pALO7 (*sop*$^+$). To measure the degree of transcription entering *oriC* from the right in each derivative, the *Hin*dIII-*Bam*HI fragment extending from position 244R to 2191R, containing the deletion or insertion, was cloned into the *lacZ* transcriptional fusion vector pJL207 (32). Transcription across the *Hin*dIII site at position 244R could thus be measured as β-galactosidase activity (Fig. 3).

In Table 2 the effects of the deletions and insertion on copy number, stability (LF value), and relative transcription into *oriC* are summarized. Deletions within the *mioC* coding region had no effect on minichromosome copy number and stability (deletions 2 and 6), whereas all manipulations which destroyed the *mioC* promoter led to decreased copy number and decreased stability (deletions 1, 4, 5, 7, 8, and 9). The amount of transcription entering *oriC* could be reduced to 1/3 without effect (deletion 6). Specific disruption of the *mioC* promoter by a 4-bp insertion in the *Mlu*I site positioned at 777R between the −10 and −35 sequences of the promoter reduced transcription to 6%, but this was sufficient to sustain intermediate copy number and stability (insertion 3). Among the deletions destroying the *mioC* promoter, no difference was observed between deletions which destroyed only the promoter (4, 5, and 7) and deletions 1, 2, 8, and 9, which destroyed the promoter and removed the *mioC*-DnaA box.

When the transcriptional activity of deletion 8 was compared with those of deletions 4, 5, and 7, it was seen that the *asnC* transcript was normally efficiently terminated before reaching the *mioC* promoter. It was also clear from deletions 8 and 9 that *asnC* transcription cannot substitute for *mioC* transcription in sustaining the normal copy number and stability of the minichromosomes.

Deletion 1 generated a promoter containing the −10 sequence of the *mioC* promoter and a −35 sequence which is normally a coding region in the *asnA* gene. Transcription from this promoter was reduced to 8% and was probably not DnaA regulated since the *mioC*-DnaA box was deleted. This promoter was unable to sustain normal minichromosome copy number and stability.

Finally, there was not always a strict correlation between copy number and stability of the deletion derivatives of pALO1 and pALO7. For unknown reasons, the plasmid containing deletion 4 was lost at significantly higher rates than other deletion derivatives having the same copy number (5 and 7).

In vivo regulation of *mioC* promoter. Since the *mioC* promoter is involved in control of minichromosome replication and neither the *asnC* promoter (see above), which is autoregulated (29), nor the induced *lac* promoter (46) can replace it, regulation of this promoter seems to be a key feature in initiation of minichromosome replication. The occurrence of a DnaA box immediately upstream of the −35 sequence (18, 19) makes it a potential DnaA-regulated promoter. Previous experiments by Lother et al. (34) showed a derepression of the *mioC* promoter in a *dnaA46* strain at the nonpermissive temperature. A 6-bp deletion removing most of the *mioC*-DnaA box also led to a slight increase in transcription from this promoter (45). This suggests that the DnaA protein acts as a repressor of the *mioC* promoter. To study the effect of increased DnaA protein concentration on transcription from the *mioC* promoter in vivo, we used a transcriptional fusion to *lacZ* (pALO75), the point of fusion being the *Hin*dIII site at position 244R (Fig. 3).

The DnaA-overproducing plasmid was pTAC1430 (2a), carrying the *dnaA* gene under control of the thermoinducible λ p_L promoter. The control plasmid pTAC1431 carries a 121-bp internal deletion in the *dnaA* gene. In a strain carrying the temperature-sensitive λ repressor *c*I857, supplied by plasmid pTAC1534, only pTAC1430 directed the synthesis of functional DnaA protein (3).

Since pTAC1430 and pTAC1431 are pBR322 derivatives, pTAC1534 is a pSC101 derivative, and pALO75 is a p15A derivative, the three plasmids are compatible and able to coexist stably in the same cell.

Transcription from the *mioC* promoter after thermoinduction of the λ p_L promoter in front of the *dnaA* gene is shown in Fig. 4. The effect of DnaA overproduction was a total shutdown of the *mioC* promoter. The kinetics show that this happened within a few minutes after the temperature shift, suggesting that the DnaA protein is extremely efficient in repressing transcription from the *mioC* promoter (strain TC2185). No drastic effect was seen in the control strain TC2186.

Derepression of the *mioC* promoter in a *dnaA46* strain at the nonpermissive temperature observed previously (34) was verified by using plasmid pALO75 (Fig. 5). Derepression of the *mioC* promoter was also observed in host strains carrying the mutations *dnaA5*, *dnaA167*, *dnaA204*, *dnaA205*, and *dnaA602* when shifted to the nonpermissive temperature (A. Løbner-Olesen and K. Skarstad, unpublished).

DISCUSSION

Minichromosomes are unusual plasmids. Although they are replicons derived from the bacterial chromosome and are dependent on bacterial replication factors, their presence in

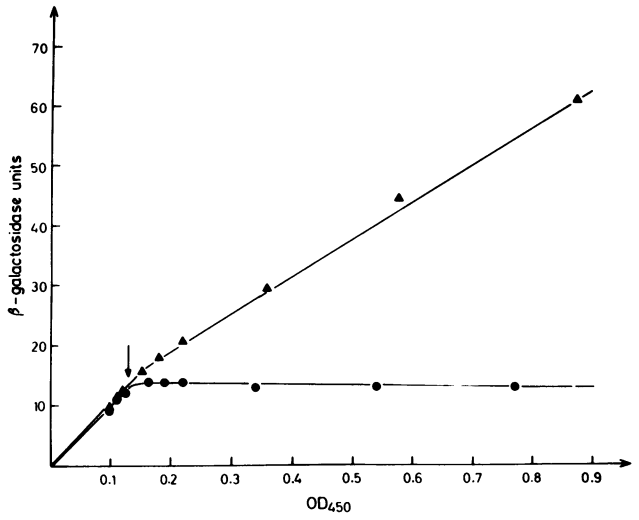

FIG. 4. Repression of *mioC* transcription after induction of DnaA protein overproduction. Strains TC2185 [NF1815 (pTAC1534, pTAC1430, pALO75)] (●) and TC2186 [NF1815 (pTAC1534, pTAC1431, pALO75)] (▲) were grown in AB medium supplemented with thiamine (1 μg/ml), 0.2% glucose, 1% Casamino Acids, ampicillin (100 μg/ml), kanamycin (25 μg/ml), and chloramphenicol (25 μg/ml). At the OD_{450} indicated by an arrow, cultures were shifted from 30 to 42°C to induce DnaA protein synthesis in TC2185 but not in TC2186. Samples were taken as indicated, and the level of β-galactosidase activity was determined. Since NF1815 is *lacZ*, the enzyme activity measured was synthesized from the *mioC-lacZ* transcriptional fusion carried on pALO75.

high copy numbers has little effect on the host cell. They initiate their replication in synchrony with the host chromosome. Their high loss frequencies might be taken as a sign of slight incompatibility, but the fact that they can be stabilized considerably by the *sop* genes from plasmid F, whose only function is proposed to be partitioning of plasmids at cell division, is a strong indication that the instability characteristic of minichromosomes is largely due to occasional segregation failure rather than to competition with the chromosome for replication factors.

At an average copy number of 38 plasmids per cell, corresponding to more than 50 copies at division, the nonstabilized minichromosome is lost at a frequency corresponding to random distribution of four to five plasmids to two sister cells. This indicates that the segregational unit may be an aggregate of as many as 12 plasmids, but knowledge of the single-cell copy number distribution is required to be more specific on this point. In slowly growing cells the average copy number might be so low that not all cells have two segregational units at division. This could explain why the stabilizing effect of the *sop* genes appears to break down at low growth rates. Also, fast-growing cells containing derivatives of pALO7 with deleted *mioC* promoters have an average copy number which is lower than the size of two segregational units as estimated above, and this may explain their instability.

In contrast to other plasmids, such as members of the IncFII and ColE1 families whose concentration in the cells increases with decreasing growth rate (16, 33), the copy number of minichromosomes was found to be constant per unit cell mass. This observation has its exact counterpart in the well-known constancy of the chromosomal "initiation mass" (the average mass per origin at initiation) and suggests that a common mechanism controls initiation at all *oriC* copies, chromosomal or minichromosomal. It should be noted, however, that for unknown reasons the same initiation mass permits initiation at one chromosomal origin and several minichromosomes.

The *mioC* promoter, which gives rise to transcription into the minimal origin (21), has a positive influence on minichromosome copy number. We have shown that the promoter itself, and neither a nearby site nor the MioC protein, is responsible for this effect.

The total amount of transcription entering *oriC* from the *mioC* promoter does not seem to be important for initiation frequency, since this can be reduced to one-third without effect and to 6% with only a slight reduction in copy number and stability. The latter effects are due to a 4-bp insertion between the −10 and −35 sequences of the *mioC* promoter; the small amount of transcription is probably still DnaA regulated since the DnaA box is intact and correctly positioned with respect to the −35 sequence. These observations, together with the fact that minichromosomes whose *mioC* promoters are substituted with promoters that are not DnaA regulated (*asnCp* [this work], *lacp* [46]) cannot maintain the high copy number, make us believe that it is the regulation by the DnaA protein of transcription from the *mioC* promoter that is important for initiation at *oriC*.

We have shown here that transcription from the *mioC* promoter is negatively regulated by the DnaA protein. Transcription was derepressed in six different *dnaA*(Ts) mutants after a shift to the nonpermissive temperature and was repressed shortly after induction of DnaA protein overproduction. Similar effects have been observed on transcription from the *dnaA* promoter (2), but the *mioC* promoter responded more quickly and the effects were stronger. This result is in accordance with the recent demonstration, by in vivo titration experiments, that the *mioC*-DnaA box is more efficient than the box in the *dnaA* promoter in binding the DnaA protein (20), and it suggests that regulation of the *mioC* promoter may be finely tuned to regulate initiation of replication.

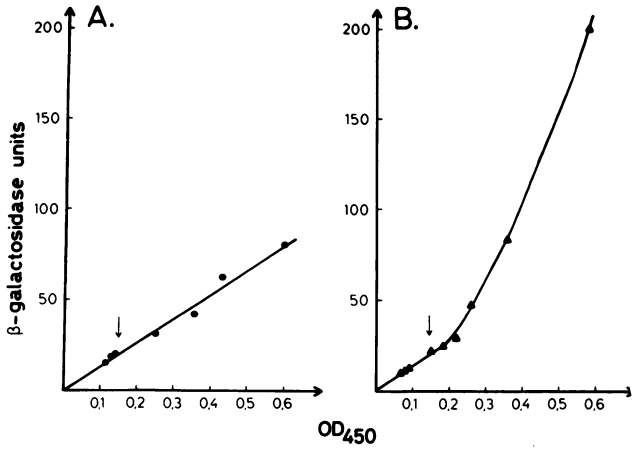

FIG. 5. Stimulation of *mioC* transcription in a *dnaA46* mutant. Strains CM732 (wild type) and CM734 (*dnaA46*) containing plasmid pALO75 were grown exponentially in AB medium supplemented with thiamine (1 μg/ml), 0.2% glucose, 1% Casamino Acids, tryptophan (50 μg/ml), and chloramphenicol (25 μg/ml). At the time indicated by an arrow, cultures were shifted from 30 to 39°C. Samples were taken as shown, and the level of β-galactosidase activity was determined. (A) CM732(pALO75); (B) CM734 (pALO75).

Overproduction of DnaA protein, leading to repression of the *mioC* promoter, was shown to increase minichromosome copy number 2- to 2.5-fold (2a). The effect of derepression of the *mioC* promoter on minichromosome copy number is less clear. However, site-directed mutagenesis of the *mioC*-DnaA box leading to a 25% increase in transcriptional activity reduced the copy number of the minichromosome to one-third (45).

Two models are proposed for the function of the *mioC* promoter and transcript in the initiation of minichromosome replication. First, *mioC* transcription into *oriC* could have an inhibitory effect on replication (46), presumably by a mechanism of promoter occlusion (1) on the origin promoters pORI-R and pORI-L, which have been proposed as the ones transcribing the RNA primers for initiation (35). In such a model, initiation will only occur when enough DnaA protein has accumulated to reduce the *mioC* promoter activity sufficiently to allow activation of the origin promoters. However, this model does not explain why deletion of the *mioC* promoter causes a reduction in minichromosome copy number.

A much more tempting model is that the *mioC* transcript itself, at a certain, strictly regulated transcription rate determined by the DnaA protein concentration in the cell, acts as the initial primer in the initiation of DNA replication. This notion is supported by the fact that RNA-DNA transition sites identified within *oriC* (24, 28) correspond to termination sites for the *mioC* transcript (27, 42a). The origin promoters pORI-R and pORI-L would thus be a backup system which has a lower capacity for initiation than the *mioC*-dependent pathway. This would explain the lower copy number of minichromosomes whose *mioC* promoter has been deleted.

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