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Different effects of *mioC* transcription on initiation of chromosomal and minichromosomal replication in *Escherichia coli*

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ABSTRACT

The *mioC* gene, which neighbors the chromosomal origin of replication (*oriC*) in *Escherichia coli*, has in a number of studies been implicated in the control of *oriC* initiation on minichromosomes. The present work reports on the construction of cells carrying different *mioC* mutations on the chromosome itself. Flow cytometry was employed to study the DNA replication control and growth pattern of the resulting *mioC* mutants. All parameters measured (growth rate, cell size, DNA/cell, number of origins per cell, timing of initiation) were the same for the wild type and all the *mioC* mutant cells under steady state growth and after different shifts in growth medium and after induction of the stringent response. It may be concluded that the dramatic effects of *mioC* mutations reported for minichromosomes are not observed for chromosomal replication and that the *mioC* gene and gene product is of little importance for the control of initiation. The data demonstrate that a minichromosome is not necessarily a valid model for chromosomal replication.

INTRODUCTION

Replication of the *Escherichia coli* chromosome is initiated at a unique locus called *oriC* and proceeds bidirectionally around the circular chromosome (1,2). The rate of DNA replication is largely controlled at the level of initiation from *oriC* (for reviews, see refs. 3,4).

Plasmids replicating autonomously from *oriC* have been isolated (5–7) and such minichromosomes were shown to have the same requirements for initiation as the chromosome itself (8).

When several chromosomal origins are present in a cell all origins are initiated simultaneously (9). Minichromosomes are initiated in synchrony with the chromosome (10,11) and each *oriC* copy is initiated only once per cell cycle (12,13). Minichromosomes have therefore been extensively used as model systems for chromosome replication.

The gene located clockwise of *oriC* is transcribed towards *oriC* (Fig. 1) and encodes a 16kD protein of unknown function. There is general consensus in the literature that presence of the 16kD gene promoter on a minichromosome stimulates initiation of replication. For this reason the gene has been given the name *mioC* (*m*odulation of *i*nitiation from *oriC*). Deletion of the *mioC* promoter or replacement with other promoters lead to a decrease in minichromosome copy number and segregational instability (14–17), to lack of replication in the absence of Integration Host Factor (18), and to feeble replication activity in vitro (19). Also, the *mioC* promoter strongly stimulates initiation of minichromosomes under suboptimal conditions, i.e. in gyrase mutants (20), in mutants lacking the histone-like protein HU (21) and in *dnaA*(Ts) mutants (22). On the other hand, a lack of effect of transcription from p*mioC* has also been reported (23).

The *mioC* promoter region contains a consensus box for binding of the initiation specific protein DnaA and transcription from p*mioC* is negatively regulated by the DnaA protein both *in vivo* (17, 24, 25) and *in vitro* (15, 26). Absence of this DnaA box on a minichromosome results in a five-fold decrease in copy number (24).

A transcriptional event is essential (27, 28) and rate limiting for *oriC* initiation. There are at least two possible ways in which transcripts from p*mioC* could influence initiation. First, the transition points between RNA and DNA synthesis, which represent the start sites for DNA replication within *oriC*, and the termination sites for *mioC* transcripts have been reported to coincide (29, 30); this has been taken to suggest that the transcripts supply the primer for leading strand synthesis in *oriC*. Second, initiation of DNA replication is strongly stimulated in vitro by transcription close to *oriC* (31, 32). Transcription from the p*gid* promoter located immediately to the left of *oriC*, has also been reported to stimulate *oriC* initiation (19, 23, 33).

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An important regulator of protein synthesis and of transcription in *E. coli* is the nucleotide guanosine 3',5'-bispyrophosphate (ppGpp, for a review see ref. 34). The intracellular ppGpp concentration is inversely correlated to growth rate (35–37), and ppGpp is the mediator of the stringent response after shifts in growth rate or amino acid starvation (34). Stringent control of the *mioC* promoter (30) and of initiation of minichromosome (38, 39) and chromosome (40) replication in *E. coli* suggest the possibility that *mioC* transcription may provide a necessary but still unidentified coupling mechanism between general cell growth and the rate of initiation.

The evidence for a regulatory connection between *mioC* and initiation of DNA replication stems from work with small minichromosomes. In this work we describe for the first time detailed studies of the DNA replication kinetics in cells carrying *mioC* mutations on the chromosome itself. We have constructed mutants with different deletions in the *mioC* promoter region or with a deletion within the *mioC* coding region. A very sensitive method (flow cytometry) was employed to monitor growth and DNA replication kinetics of the mutant cells and their wild type parent under steady state conditions as well as after shifts in the growth conditions, which elicit changes in the intracellular ppGpp concentration. Under all conditions tested the mutants behaved exactly like their wild type parent, and we conclude that the *mioC* gene is of little, if any, importance for the control of DNA replication in *E. coli*.

MATERIALS AND METHODS

Media

For steady state growth experiments, cells were grown at 37°C in AB minimal medium (41) containing 0.0001% thiamine and either 0.5% succinate, for slow growth, or 0.2% glucose and 0.5% casamino acids (CAA), for fast growth. When required, 20μg/ml leucine was added for growth of the leucine auxotroph strain LJ24 and derivatives. Growth of the cultures was monitored by measuring optical density at 450nm (OD_{450}).

A shift up in growth rate was obtained by growing cells in steady state in the slow-growth medium above, with glycerol substituted for succinate. At an OD_{450} = 0.1, glucose and C-AA were added to final concentrations of 0.2% and 0.5%, respectively.

A shift-down in growth rate was obtained by growing cells in steady state in the fast-growth medium above, with only 0.1% glucose and 0.5% casamino acids. At an OD_{450} = 0.15, α-methylglucoside was added to a final concentration of 1% to compete with glucose for uptake into the cell (42, 43).

Starvation for the amino acid isoleucine was achieved by growing cells in steady state in the fast-growth medium above without CAA, and at an OD_{450} = 0.15, valine was added to a final concentration of 20μg/ml (44).

Bacterial strains

The bacterial strains were *Escherichia coli* K-12 and are listed in Table 1.

Strain constructions

The source of *mioC* mutant genes were the pALO7 minichromosome derivatives containing the *oriC-mioC* region with different *mioC*$^-$ mutations (Fig. 1) as described earlier (17). Strain CM1559 harboring plasmid pALO58 *mioC4*, pALO60 *mioC351*, pALO62 *mioC54*, pALO67 *mioC440*, or pALO64 *mioC131* were infected with a mixed lambda lysate of λ*asn20*::Tn*10-590*/λh derived from strain CM1290. Phage λ*asn20*::Tn*10-590* is a λ*asn20* containing a Tn10 inserted in the proximal part of the *asnA* gene (45; Fig. 2). Transductants yielding high frequency of transduction lysates for Tet^R when using strain CM987 as recipient were isolated at 30°C and named ALO56, ALO58, ALO48, ALO51 and ALO609, respectively.

After heat induction of the λ phages contained in strains ALO56, ALO58, ALO48, ALO51 and ALO609, some of the resultant phage progeny recombined with the minichromosome coresiding in the strain, giving rise to λ*asn*$^+$ transducing phages (Fig.2, step A). Some of these transducing phages will also have acquired the *mioC* deletion from the minichromosome.

Phage lysates of strains ALO56, ALO58, ALO48, ALO51 and ALO609 were tested using CM987 as a recipient strain, selecting Asn$^+$ colonies at 30°C. About 99% of the λ*asn*$^+$ transducing phages obtained were also carrying a kanamycin resistance determinant as would be the expected result of a single crossover event between the phage and the plasmid. Approximately 1% of the λ*asn*$^+$ transducing phages transduced neither Tet^R nor Kan^R. These were presumably the result of a double crossover event. Transductants with this latter phenotype, and which yielded high-frequency-of-transformation Asn$^+$ lysates using CM987 as recipient at 30°C, were chosen for further analysis (Fig. 2, step B).

Isolation of the specialized transducing phage DNA from a number of these strains and subsequent restriction enzyme

Table 1. Bacterial Strains.

Name	Relevant Genotypes	Reference
CM1559	*asnA, asnB, relA1, spoT1*	46
LJ24	*leu*	56
CM789	λasn20/λh	57
CM987	*asnA, asnB, recA1, relA1, spoT1*	57
CM1290	pλ*asn20*::Tn*10-590*/λh	45
CM1XXXX	λ*asn105Δ1221*/λh	46
ALO18[ab]	pALO60 *mioC351*	17
ALO19[ab]	pALO62 *mioC54*	17
ALO21[ab]	pALO67 *mioC440*	17
ALO240[ab]	pALO64 *mioC131*	17
ALO282[ab]	pALO58 *mioC4*	17
ALO48[ab]	pALO62, λ*asn20*::Tn*10-590*/λh	This work
ALO51[ab]	pALO67, λ*asn20*::Tn*10-590*/λh	"
ALO56[ab]	pALO58, λ*asn20*::Tn*10-590*/λh	"
ALO58[ab]	pALO60, λ*asn20*::Tn*10-590*/λh	"
ALO609[ab]	pALO64, λ*asn20*::Tn*10-590*/λh	"
ALO60[c]	λ*asn20 mioC54*/λh	"
ALO61[c]	λ*asn20 mioC440*/λh	"
ALO650[c]	λ*asn20 mioC131*/λh	"
ALO86[c]	λ*asn20 mioC351*/λh	"
ALO238[c]	λ*asn20 mioC4*/λh	"
ALO252[a]	*asnA*$^+$, *mioC54*	"
ALO258[a]	*asnA*$^+$, *mioC440*	"
ALO264[a]	*asnA*$^+$, *mioC351*	"
ALO270[a]	*asnA*$^+$	"
EBO21[d]	*mioC4*	"
EBO24[d]	*mioC131*	"
ALO659[d]	*mioC54*	"
ALO663[d]	*mioC440*	"
ALO667[d]	*mioC351*	"

[a] Bacterial genotype of CM1559
[b] Plasmid derived from the minichromosome pALO7 (ref. 17)
[c] Bacterial genotype of CM987
[d] Bacterial genotype of LJ24

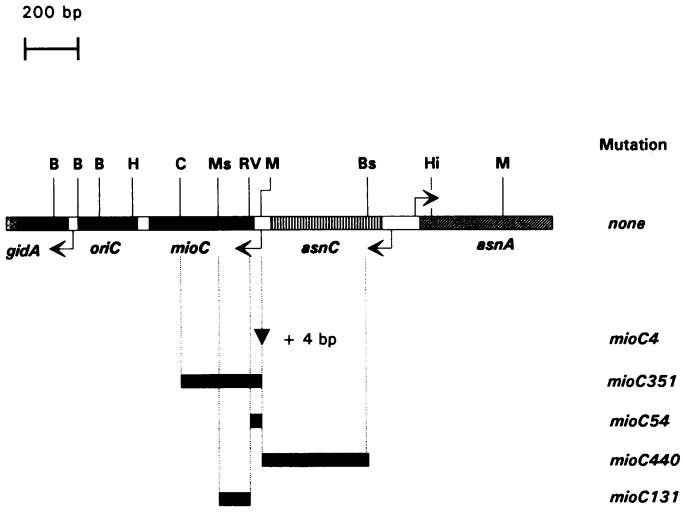

Figure 1. Map of the *oriC−mioC* region of the *E. coli* chromosome. Coding sequences are marked by shaded boxes, intergenic regions are open boxes, and the start sites and direction of transcripts are denoted by arrows. The *mioC* deletions are marked by black rectangles, the 4bp insertion by an arrow and +4bp. Relevant restriction sites are: B, *Bam*HI; Bs, *Bss*HII; C, *Cla*I; RV, *Eco*RV; H, *Hind*III; Hi, *Hind*II; M, *Mlu*I; Ms, *Mst*II.

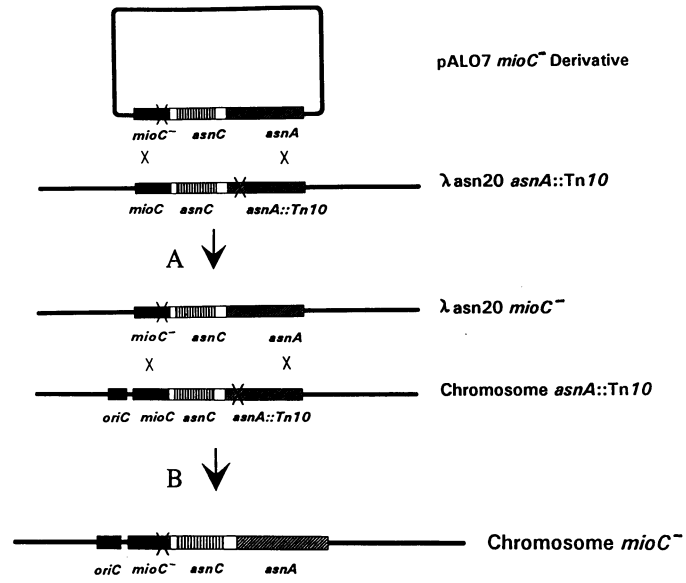

Figure 2. Schematic representation of the method for transferring the *mioC* mutations from plasmid to λ phage (step A) and from the phage onto the chromosome (step B). See text for details.

analyses, identified the strains harboring phages which contained the desired *mioC* deletions. Strains ALO238, ALO86, ALO60, ALO61 and ALO650 contain λ*asn20* derivatives carrying insertion *mioC4*, and deletions *mioC351*, *mioC54*, *mioC440*, *mioC131* respectively.

Mixed lambda lysates of strains ALO238, ALO86, ALO60, ALO61, ALO650 and CM789 were used to infect strain CM1559. Asn$^+$ colonies were selected at 42°C; at this temperature selection against maintenance of the λ DNA is imposed due to the temperature sensitivity of the *cI*857 allele. The resulting Asn$^+$ colonies should thus be the result of double crossover events between the lambda transducing phage and the bacterial chromosome. Due to the size of the chromosomal DNA carried on λ*asn20* and available for homologous recombination, about 75% of the Asn$^+$ colonies selected were expected to have acquired the *mioC* gene from the phage as well (Fig. 2).

Two different approaches were used to verify that the *mioC* deletions had in fact been transferred to the chromosome. First, *oriC* and *mioC* from the putative *mioC*-defective strains were transferred to the specialized transducing phage λ*asn105Δ1221* (46) after infecting the strains with the defective phage and a λ helper. The *oriC* deletion of the phage extends into *asnA*, which makes the phage unable to transduce strains to Asn$^+$. Phages transducing Asn$^+$ that were furthermore capable of autonomous replication had acquired the entire *oriC−mioC−asnA* region from the chromosome of the host strain. Restriction enzyme analyses of such phages revealed that strains ALO282, ALO264, ALO252, ALO258 and ALO652 carry mutations *mioC4*, *mioC351*, *mioC54*, *mioC440* and *mioC131*. respectively.

Second, P1 transduction of the *mioC* mutations into strain LJ24 resulted in strains EBO21, ALO667, ALO659, ALO663, and EBO24, carrying mutations *mioC4*, *mioC351*, *mioC54*, *mioC440* and *mioC131* respectively. Appropriate restriction enzyme digestion of the chromosomal DNA, electrophoresis, and genomic blotting confirmed the presence of the mutations.

Genomic blotting and hybridization

Isolation of chromosomal DNA, capillary transfer to Genescreen membranes, hybridization with ^{32}P-labelled probe and autoradiography were performed as described (47).

Treatment with rifampicin and cephalexin

At an OD_{450} of 0.1 rifampicin (RIF) was added to the culture at a final concentration of 300μg/ml to inhibit initiation of replication, and 10μg/ml cephalexin (Eli Lilly, Basingstoke, England) was added to inhibit cell division (48). After drug addition incubation was continued for 3−4 doubling times to allow completion of ongoing rounds of replication.

Flow cytometry

Cell preparation and flow cytometry with an Argus flow cytometer (Skatron, Tranby, Norway) was performed as described previously (49). Average cell mass and cellular contents of DNA and origins were determined from dual parameter fluorescence (DNA) versus scattered light (cell mass) histograms as described previously (50). The synchrony of initiation of DNA replication was determined from the fraction of cells containing 2^n fully replicated chromosomes after RIF and cephalexin treatment (9, 48).

RESULTS

Construction of chromosomal *mioC* mutations

In a previous study of minichromosome replication, the construction of a number of plasmid borne *mioC* mutations was described (17). Five of these were chosen for transfer to the bacterial chromosome (Fig. 1). Below is a brief description of the mutations and of the level of transcription into *oriC* and the minichromosome copy number characteristic of each, as measured when the mutations are located on a minichromosome

Figure 3. Genomic blotting and hybridization of the different *mioC* mutants and the wild type. Cellular DNA was purified and cut with *MluI* (A to E) or with *Bam*HI and *Eco*RV (F, G) before gel electrophoresis, blotting, and hybridization to a radioactively labeled *Hind*III-*Hind*II fragment from the *mioC* region (Fig. 1). A=ALO659, B=ALO663, C=ALO667, D=EBO21, E=LJ24, F=EBO24, G=LJ24. The numbers to the right mark the length and position of molecular weight marker DNA molecules.

Table 2.

Strain	Growth nutrient	Doubling time (min)	DNA[1]	LS[1]	DNA/LS
LJ24 (wt)	Succinate	110	100	100	1.00
EBO21 (*mioC4*)	Succinate	110	104	98	1.06
EBO24 (*mioC131*)	Succinate	110	107	97	1.10
ALO659 (*mioC54*)	Succinate	108	98	89	1.10
ALO663 (*mioC440*)	Succinate	110	99	105	0.94
ALO667 (*mioC351*)	Succinate	108	98	95	1.03
LJ24 (wt)	Glucose/CAA	38	100	100	1.00
EBO21 (*mioC4*)	Glucose/CAA	38	107	97	1.10
EBO24 (*mioC131*)	Glucose/CAA	38	101	97	1.04
ALO65 (*mioC54*)	Glucose/CAA	38	101	99	1.02

[1] Average DNA or light scatter (LS) per cell, as measured by flow cytometry and normalized to 100 for strain LJ24.

and compared to transcription and copy number of *mioC*$^+$ minichromosomes (17).

The *mioC4* mutation is a 4 bp insertion between the −10 and −35 sequences of the promoter, which reduces the amount of transcription entering *oriC* from the right to 6% and copy number by 20% (17). Mutations *mioC351* and *mioC54* remove the −10 sequence of the promoter as well as part of the *mioC* coding region. Residual transcription into *oriC* in plasmids with these mutations are 2% and 1% of the wild type level, respectively, while the copy numbers are reduced by 50% and 30%, respectively. Mutation *mioC440* removes the DnaA box, the −35 sequence of the *mioC* promoter, the transcription terminator immediately downstream of *asnC*, and part of the *asnC* coding region. In this construct the *mioC* gene is transcribed from the autoregulated *asnC* promoter (51) at a level that is 12% of that of the *mioC* promoter, with an accompanying reduction in copy number of 60%. Mutation *mioC131* is an internal deletion in the *mioC* structural gene leaving the promoter intact. Transcription into *oriC* on plasmids with this mutation is reduced to 36%, while the copy number is unchanged.

Transfer of the mutations from the different minichromosomes to the chromosome was performed with two steps of homologous recombination: In the first step (Fig. 2, step A), the *mioC* mutations were recombined onto a specialized transducing phage, λ*asn20 asnA*::Tn*10* by selection for Asn$^+$ transducing phages and subsequent screening for the *mioC* mutation by restriction enzyme analysis of DNA from the resultant phages.

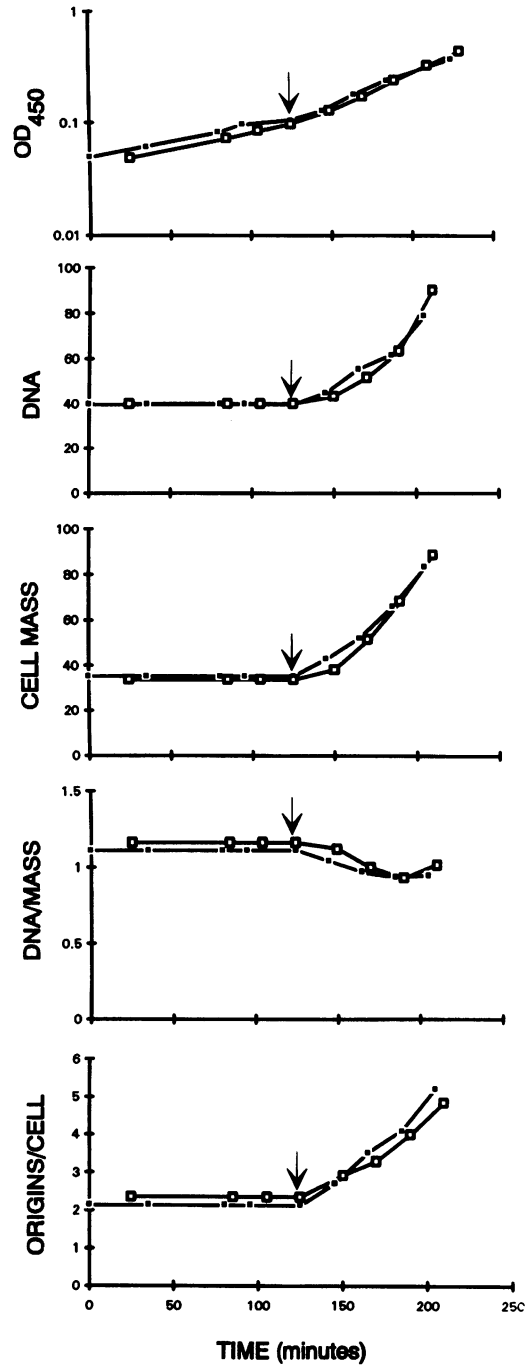

Figure 4. Results of flow cytometric analyses of a shift up in growth rate. Strains LJ24 (open squares) and ALO659 (closed squares) were grown in glycerol minimal medium before addition of glucose (0.2%) and CAA (0.5%) were added at 125 min (arrow). The figure shows OD of the culture (upper panel) and average single-cell parameters.

In the second step (Fig. 2, step B), the *mioC* mutations were recombined onto the chromosome of an *asnB*, *asnA*::Tn*10* host strain by infecting with the different *asn20 mioC*$^-$ λ phages and selecting for asparagine prototrophy under conditions that do not allow propagation of the phage (for details see Materials and Methods).

The presence of the different *mioC* mutations on the bacterial chromosome was verified by genomic blotting and hybridization

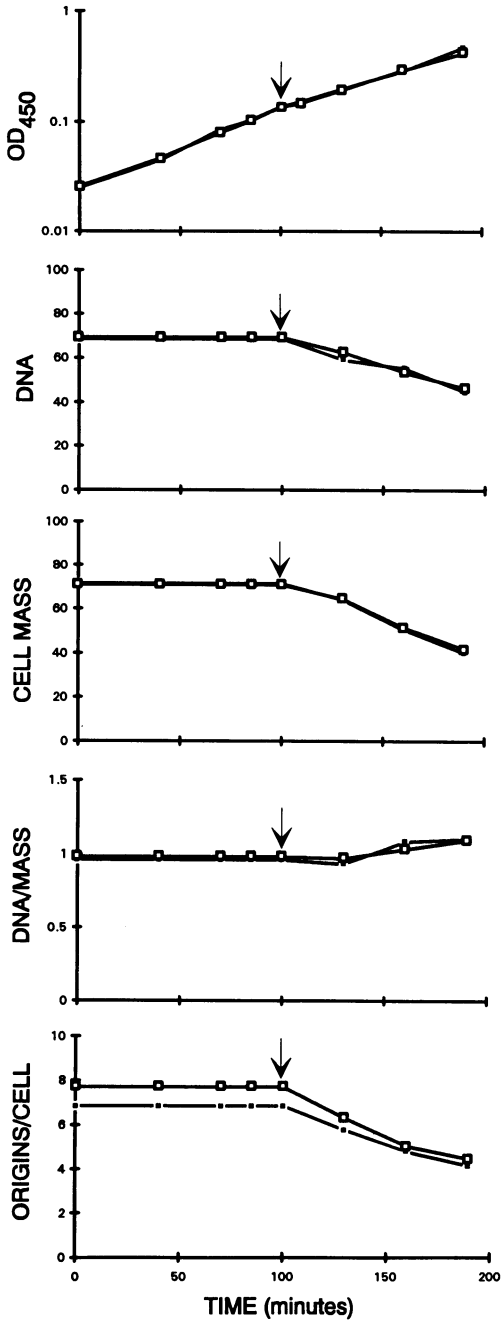

Figure 5. Results of flow cytometric analyses of a shift down in growth rate. Strains LJ24 (open square) and ALO659 (closed square) were grown in minimal medium supplemented with 0.1% glucose and 0.5% CAA before addition of 1% α-methylglucoside at 100 min (arrow). The figure shows OD of the culture (upper panel) and average single-cell parameters.

(Fig. 3). Chromosomal DNA from strains ALO659 *mioC54*, ALO663 *mioC440*, ALO667 *mioC351*, EBO21 *mioC4*, and LJ24 (wild type) was digested with *Mlu*I and the fragments were separated by gel electrophoresis, blotted and probed with a radioactively labelled 1251 bp *Hind*III−*Hind*II fragment from the *mioC* region (Fig. 1). In the wild type, two fragments of 3117 and 1016 bp were detected, whereas the mutants had lost the *Mlu*I site in the *mioC* promoter and consequently gave rise to only one fragment each, with sizes 4079, 3693, 3782, and 4137 bp for mutations *mioC54*, *mioC440*, *mioC351* and *mioC4*, respectively (Fig. 3, lanes A to E).

Similarly, the presence of mutation *mioC131* in strain EBO24 was verified by genomic blotting, except that chromosomal DNA from strains EBO24 and LJ24 was digested with *Eco*RV and *Bam*HI. Two major fragments of 631 and 1468 bp were recognized in the wild type DNA (Fig. 3G). The *mioC131* mutation resulted in the loss of the *Eco*RV site within *mioC*, and consequently only one fragment of 1968 bp was detected in DNA from strain EBO24 (Fig. 3F).

We conclude that strains EBO21, ALO667, ALO659, ALO663 and EBO24 carry, respectively, mutations *mioC4*, *mioC351*, *mioC54*, *mioC440* and *mioC131* on their chromosomes. Since the mutants are viable it may also be concluded that the *mioC* gene or the *mioC* gene promoter do not serve vital functions in the cell.

Steady state DNA replication pattern

To study the effect of different *mioC* mutations on the control of DNA replication the mutant and wild type strains were grown in different media under steady state conditions. Strains carrying *mioC* mutations grew with doubling times similar to those of the wild type, both at high and low growth rate (Table 2).

Cellular DNA contents and cell sizes of the mutants were indistinguishable from those of the wildtype (Table 2). Also, the number of origins per cell and the synchrony of initiations were the same in all strains (data not shown). Since cells of wild type and *mioC* mutant strains have the same size, the same DNA content per cell, the same average number of origins per cell, and the same high synchrony of initiations, it follows that they also have the same initiation mass and the same duration of the DNA replication period.

Shifts in growth rate

The *mioC* mutant strains were investigated for possible defects in DNA replication control after a shift in growth conditions. Shifts in growth rate elicit dramatic effects on the intracellular ppGpp concentration (34, 35), which can be expected to strongly influence *mioC* transcription.

A nutritional shift up was accomplished by adding glucose and casamino acids to cultures pregrown on a minimal glycerol medium. The shift was rapidly followed by an increase in the rate of DNA and protein synthesis. The rounds of DNA replication induced by the shift were synchronous within each cell (data not shown). The changes in growth rate, DNA content, cell mass, DNA/mass and number of origins per cell caused by the shift were similar for the wild type strain LJ24 as well as for all the *mioC* mutant strains. The deviations between the mutants and wild type were small and not reproducible. As an example, the data for the wild type and one mutant strain are shown in Fig. 4. The shift in growth rate was almost instantaneous, while DNA content, cell mass, and origins per cell took more than 90 minutes to reach their new values. DNA/mass decreased after the shift, but only by 15−20%.

A nutritional shift down was obtained by growing the cells with glucose as carbon source before adding α-methylglucoside to inhibit glucose uptake (42, 43). As for the shift up experiment, the new growth rate was established rapidly, while the values for DNA, mass, and number of origins per cell were adjusted to their new values over a longer period of time (Fig. 5). Again there was no difference between the wild type and the different *mioC* mutant strains.

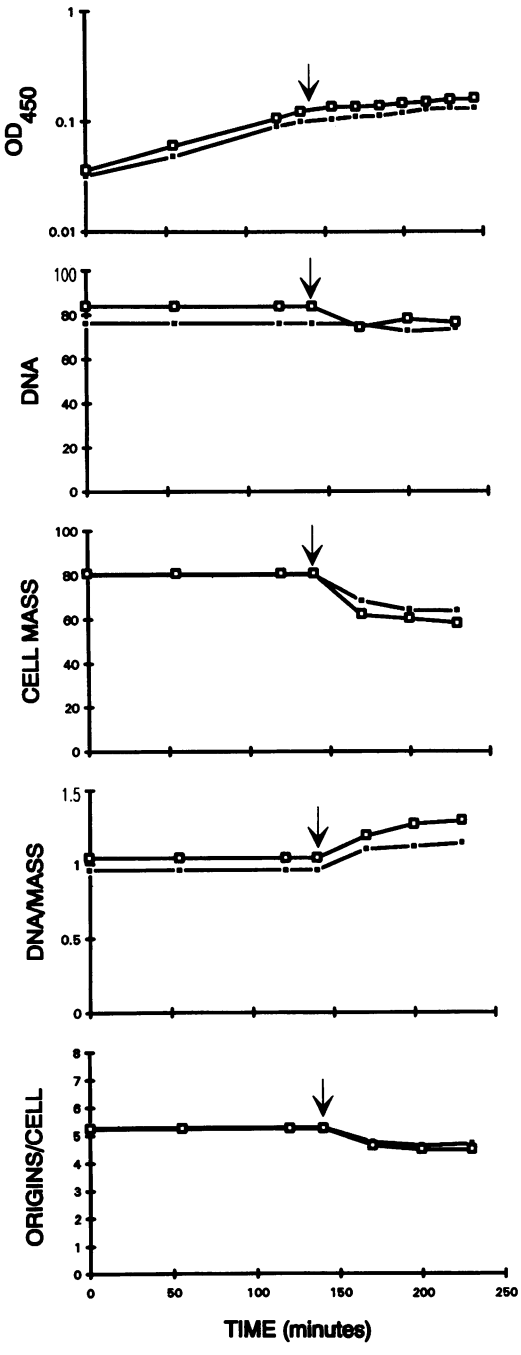

Figure 6. Results of flow cytometric analyses of valine addition to exponentially growing cells. Strains LJ24 (open squares) and ALO659 (closed squares) were grown in glycerol minimal medium before the addition of valine (20μg/ml) at 140 min (arrow). The figure shows OD of the culture (upper panel) and average single-cell parameters.

In the third shift experiment the cells were subjected to isoleucine starvation, which can be induced by addition of valine (44). The cells were pregrown in minimal medium with glucose and addition of valine rapidly stopped cell growth (Fig. 6), probably due to induction of the stringent response. The contents of DNA per cell did not change much after the shift. Cell mass and the number of origins per cell decreased significantly, while DNA/mass increased. This can be attributed to cell division in the absence of initiation of new rounds of DNA replication. For none of the parameters measured was there a difference between the wild type cell and the *mioC* mutant cells. The isoleucine starvation was reversed by adding back isoleucine, which stimulated a rapid resumption of growth. All the parameters measured behaved similarly for all strains also under this shift up situation (data not shown).

Shifts in *relA mioC* strains

Strains deficient in the RelA gene product can not elicit the stringent response, and with a *spoT* mutation in addition the cells are devoid of ppGpp (52, 53). Experiments involving shifts in growth rate was performed with the *relA spoT mioC* derivative strains ALO252, ALO258, and ALO264 together with their parent ALO270 *mioC*$^+$. Detailed flow cytometric measurements in search of a possible phenotype revealed no evidence that the *relA spoT mioC*$^-$ cells behaved differently from their parent *mioC*$^+$ strain (data not shown).

In all the experiments involving shifts in nutrient availability we have been unable to demonstrate any deficiency in the *mioC* strains in regulation of DNA replication or cell growth.

DISCUSSION

The data presented strongly suggest that the *mioC* gene has no necessary or stimulatory function in initiation of chromosomal DNA replication. Mutations affecting transcription from the *mioC* promoter as well as those altering the coding sequence of the *mioC* gene were shown to exert no influence on DNA replication or cell division under many different growth conditions, including shifts in growth rate and induction of the stringent response. This demonstrates that regulation of *mioC* transcription does not provide a link between initiation of DNA replication and general cell growth. In contrast to previous studies linking *mioC* transcription to *oriC* initiation, the present work has been performed with the *mioC* mutations located on the chromosome. Exactly the same *mioC* mutations as used here have been shown to significantly reduce the copy number of minichromosomes (17). It is not known whether the reduction in transcriptional activity caused by the different *mioC* mutations on a minichromosome (see Results) is exactly the same when the mutations are present on the chromosome. However, it can be assumed that p*mioC* activity is severely reduced and in some cases abolished, and a dramatic effect should be expected if transcription from p*mioC* were important for *oriC* initiation.

Most of what we know about the biochemicals details of *oriC* initiation has been derived from experiments with minichromosomes. Nonetheless, the present data show that there are differences between initiation of a chromosome and a minichromosome, maybe primarily at the level of regulation. Therefore, the validity of using minichromosomes as models may be questioned, at least for certain aspects of initiation.

The possibility must be considered that *mioC* deletions confer a significant growth disadvantage which may be suppressed by secondary mutations in our constructs. An argument against the existence of such secondary mutations is that the *mioC* mutations were easily obtained and were easily transferred to other strains by P1-mediated transduction. If *mioC* transcription were important for activation of *oriC*, it is difficult to imagine how distant suppressor mutations in our *mioC* mutants could substitute for this transcription. Suppressor mutations within the *mioC* gene

itself are highly unlikely, since we have investigated the effects of several different, and some rather extensive, *mioC* deletions.

There is one report in the literature showing an effect of a chromosomal *mioC* mutation (10). The mutant strain contained a transposon in the *mioC* structural gene and was shown to have a higher DNA/mass ratio than wild type cells, particularly at low growth rates. The *mioC* mutant strain used, as opposed to the wild type strain, contained an integrated F factor, which carries its own origin of replication. The F origin is initiated once per cell cycle and is sufficient to keep the bacterium alive even in the absence of *oriC* (integrative suppression). We suggest that the increased DNA/mass ratio observed may be caused by an increase in the average initiation frequency at the F origin.

If *mioC* has no effect on initiation from a chromosomal *oriC*, why is there an effect on minichromosome replication? It is known that transcription changes the local supercoiling of DNA (54, 55). Removing one promoter on a small minichromosome may therefore alter the local superhelical density, which exerts a strong influence on *oriC* initiation in vitro (31). However, on larger minichromosomes the effect on supercoiling is lower, and the effect of *mioC* on the copy number should be comparably reduced. To investigate this possibility we have measured the copy number of small minichromosomes (pALO1 derivatives (17), about 7kb) and of large minichromosomes (λ*asn20* derivatives, about 40 kb), containing the different *mioC* alleles listed in Table 1. For each individual *mioC* allele the reduction in copy number was about the same, independent of the minichromosome size. Thus, the earlier minichromosome results can probably not be explained by an effect of *mioC* transcription on supercoiling.

Recently, the *gid* promoter has been shown to exert a stimulatory effect on *oriC* initiation on minichromosomes (23, 33). In addition, a promoter inside *oriC* positively affects minichromosome replication (33). These promoters are more likely candidates than p*mioC* for providing transcriptional activation of *oriC*, and their transcripts may represent the RIF sensitive step in *oriC* initiation.

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