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The *Escherichia coli minB* mutation resembles *gyrB* in defective nucleoid segregation and decreased negative supercoiling of plasmids

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Summary. Nucleoid segregation in the *Escherichia coli minB* mutant and in cells that over-produce *minB* gene products appeared defective as measured from fluorescence micrographs. Electrophoretic resolution of topoisomers of plasmid isolates from the *minB* strain revealed a decreased level of negative supercoiling; in addition, multimerization was observed. Over-production of the *minB* gene product also resulted in a decreased level of negative supercoiling. This phenotype is typical of the *gyrB*(ts) mutant, which is known to be affected in chromosome decatenation and supercoiling. We propose that the *minB* mutation and over-production of the *minB* gene products cause a defect in nucleoid segregation, which may be related to the decrease in negative supercoiling. As in the *gyrB*(ts) mutant, retardation of nucleoid segregation is proposed to inhibit constriction initiation in the cell centre and to give rise to nucleoid-free cell poles. As a consequence, these cells divide between nucleoid and cell pole, resulting in minicell and (sometimes) in anucleate cell formation.

Key words: *Escherichia coli* – *minB* – Minicells – Segregation – Supercoiling

Introduction

Minicells result from aberrant positioning of constrictions near one of the cell poles. The division process itself appears normal with respect to morphology (Adler et al. 1967), the dependence on Fts gene products (Khachatourians et al. 1973) and the locally increased rate of peptidoglycan synthesis as the constriction site (Mulder et al., in preparation). The minicell producing strain P678-54 was originally derived from P678 by triethylenemelamine treatment and selection for resistance to ionizing irradiation (Adler et al. 1967). The *minB* mutation responsible for the minicell phenotype (Davie et al. 1984) has been mapped in the 25.6 min region of the *Escherichia coli* chromosome (Schaumberg and Kuempel 1983).

A model to explain minicell formation was devised by Teather et al. (1974). These authors suggested that the *minB* protein is involved in the cell division process by inactivating division sites at newly formed cell poles. In the *minB* mutant, the cell poles of the newborn cell remain active as potential division sites. Divisions may thus occur either at the constriction site in the cell centre or at the cell poles. Further assumptions are that the probability of division occurring at the cell pole or centre is the same and that every mass-doubling of a unit cell generates a certain quantum of division potential that suffices for one division.

The *minB* locus contains three reading frames *minC*, *D* and *E* (de Boer et al. 1989). Deletion of the *minB* locus and over-production of the *minE* gene product from a plasmid result in the minicell forming phenotype. From their analysis of the *minB* locus, De Boer et al. extended the above model, stating that proper placement of constrictions requires the coordinate regulation of the *minC*, *D* and *E* genes: the *minC* and *D* gene products act in concert to form a nonspecific inhibitor capable of blocking cell division at all potential division sites and *minE* as a topologically specific factor that, in wild-type cells, prevents the cell division inhibitor from acting at internal division sites while permitting it to block septation at polar sites.

Jaffé et al. (1988) describe *min* mutants that, apart from minicells, produce anucleate rods. The *min* populations contained filaments with an abnormal distribution of nucleoids. The *minB* strain PB104 (de Boer et al. 1988) also occasionally pinches off short DNA-less rods (this paper). This phenotype is reminiscent of gyrase mutants [*gyrA*(am), Hussain et al. 1987; *gyrB*(ts), Orr et al. 1979], which probably through a defect in nucleoid segregation divide between nucleoid(s) and cell pole and, apart from DNA-less rods, also produce minicells. Could this resemblance indicate a functional similarity of the two gene products?

Gyrase is involved in the decatenation of daughter chromosomes after termination of replication (Steck and Drlica 1984). Another activity of this enzyme complex is the introduction of negative supertwists into the DNA double helix (Gellert et al. 1976; Drlica et al. 1986). Together with topoisomerase I, an enzyme that relaxes negative supertwists (Gellert et al. 1983), gyrase is involved in regulation of the level of DNA superhelical tension. The level of superhelical tension has been shown to be of vital importance for chromosome replication, recombination, transcription and gene expression (Drlica 1984, 1987; Wang 1985; Higgins et al. 1988).

The phenotypical similarities between *gyr* and *minB* mutants with respect to DNA-less cell formation, led us to compare nucleoid structure and supercoiling in both mutants. As in the *gyrB*(ts) mutant, nucleoid segregation in the *minB* cells appeared retarded, suggesting that minicell formation and DNA-less cell formation of the *gyr* mutants may have a similar origin. Also negative supercoiling of reporter plasmids (Higgins et al. 1988) was affected in both

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the *minB* and *gyrB*(ts) mutants. Cells that over-produce the *minB* gene products (de Boer et al. 1988) showed similarly affected nucleoid segregation and negative supercoiling.

Materials and methods

Bacterial strains and growth conditions. E. coli strains used in this study are listed in Table 1. The cells were cultured under steady-state conditions at 30° or 37° C in minimal medium of 300 mOsm (Taschner et al. 1988), supplemented with glucose (0.4%) or glycerol (0.2%) as sole carbon source, thiamine (4 μg/ml) and the required amino acids (50 μg/ml). When appropriate, the osmolality was adjusted to 100 mOsm by omission of NaCl. For induction of the *lac* promoter on pDB103, isopropyl-β-D-thio-galactopyranoside (IPTG) at a final concentration of 0.5 mM was added to the medium. Antibiotics used were: ampicillin (100 μg/ml), tetracycline (20 μg/ml), kanamycin (40 μg/ml) and chloramphenicol (30 μg/ml). Temperature shifts to 42° C were carried out by diluting the steady-state culture ($OD_{450\ nm}$ = 0.2–0.3) four times into prewarmed medium. The $OD_{450\ nm}$ was never allowed to increase above 0.3. For the isolation of plasmids, cells were grown in Luria broth (1% tryptone, 0.5% yeast extract and 0.5% salt).

Microscopic techniques. For the study of nucleoid morphology by fluorescence microscopy, exponentially growing cells ($OD_{450\ nm}$ = 0.1–0.2) were harvested and fixed in 0.1% OsO_4. The cells were concentrated × 20–40 by centrifugation and resuspension in veronal-acetate buffer (pH 6.0) containing 0.12 M NaCl and 0.01 M $MgCl_2$. The nucleoids were specifically stained with fluorochrome 33342 (Hoechst-Roussel Pharmaceuticals) at a final concentration of 16 μg/ml; 5 μl of the cell suspension was left to dry on a polylysine coated coverslip (50 μl, 0.01%) and covered with 5 μl 3% Methocel (methylcellulose, Sigma). The fluorochrome was irradiated at 365 nm in a Zeiss fluorescence microscope. The cells with the fluorescent nucleoids were photographed with Tmax films (400 ASA, Kodak) which were developed with acu-1 developer (Acufine). Nucleoids were measured from fluorescence micrographs, projected on a digitizing screen (Summagraphics) at a final magnification of × 3200. Dimensions were measured by tipping the respective extremities with an electronic pen with a measurement error of about 5% (Trueba and Woldringh 1980).

Table 1. Bacterial strains, phage and plasmids

Strain	Genotype	Source or reference
Escherichia coli K-12		
MC4100	F⁻ *araD139* Δ(*argF-lac*)U169 *rpsL150 flbB5301 ptsF25 deoC1 rbsR relA1*	I.B. Holland via D. Sinden
LMC500	MC4100 *lysA*	P1(JE5510) × MC4100
LMC1011	LMC500 *minB*	P1(GC7115) × LMC500
GC7106	F⁻ *thy deo sfiA85*	Jaffé et al. (1988)
GC7115	GC7106 *minB zcf*::Tn*5*	Jaffé et al. (1988)
PB103	*dadR trpE61 trpA62 tna5*	de Boer et al. (1988)
PB104	PB103 *minB*	de Boer et al. (1988)
LE316	F⁻ *argE gyrB*(ts)*ilv leu metB rpsL supE thi tna xyl*	Orr et al. (1979)
Plasmids		
pGG26	Amp^r *pbpB*$^+$, derived from λCharon-Eco*sep*$^+$-1	Irwin et al. (1979)
pBR322	Amp^r Tet^r	laboratory stock
pACYC184	Cam^r Tet^r	Chang and Cohen (1978)
pDB103	P_{lac}::*minDE* Amp^r	de Boer et al. (1988)
Phage		
P1-*vir*		laboratory stock

Genetic techniques. P1 *vir*-mediated transduction of *E. coli* was carried out as described by Miller (1972). The DMSO method described by Kushner (1978) was used for transformations. To induce the expression of antibiotic resistance, transformed cells were incubated in Luria broth for 1 h before plating on selective media.

Plasmid isolation and analysis. Plasmid DNA was isolated using the cleared lysate method described by Maniatis et al. (1982). To limit re-coiling of DNA in the 42° C culture of the *gyrB*(ts) mutant during cooling (Steck and Drlica 1984), the cells were cooled rapidly by mixing with an equal volume of frozen growth medium. Plasmids and restriction fragments were analysed on 0.8% agarose gels with ethidium bromide, using a TRIS-borate buffer (Maniatis et al. 1982). The superhelical tension of plasmids was analysed on 0.9% agarose gels in TRIS-phosphate buffer, pH 7.2, with 13 μg/ml chloroquine (Sigma) as intercalating ligand (Pruss 1985). In this way supercoiled topoisomeric plasmids that vary by one linking number are separated (Pruss et al. 1982). Proteinase K treatment of plasmid isolates was performed as described by Maniatis et al. (1982). Plasmid structure was analysed by electron microscopy of Kleinschmidt preparations (Kleinschmidt 1968).

Results

Comparison of nucleoid structure in minB *and* gyrB *(ts) mutant cells*

Nucleoid structure in the *minB* mutant strain, LMC1011 (Fig. 1a), differed markedly from that of the wild-type parent strain, LMC500 (Fig. 1c). Figure 1a shows that especially the longer filaments contain chains of unseparated nucleoids. Similar nucleoids were observed in the *gyrB*(ts) mutant (Fig. 1b), which is known to be defective in nucleoid segregation (Steck and Drlica 1984). A clear effect of the temperature shift on nucleoid segregation was obtained after incubation for 2 h at 42° C. This incubation time was applied in all subsequent experiments. The similarity in nucleoid structure between the *minB* and *gyrB*(ts) mutants suggests retardation of segregation in the *minB* mutant.

Comparison of plasmid topology and superhelical tension in the minB *and* gyrB *(ts) mutant cells and in cells that over-produce the* minB *gene products*

The phenotypic similarities between the *minB* and *gyrB*(ts) mutants led us to investigate whether plasmids isolated

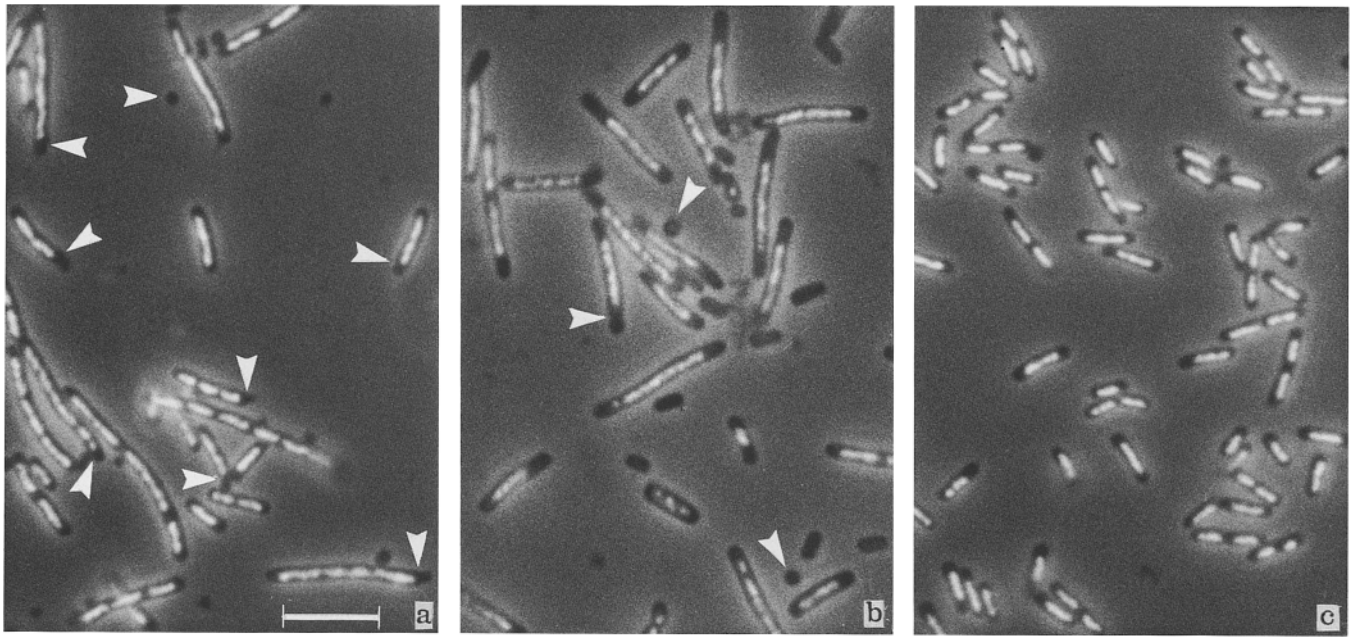


Fig. 1a–c. Nucleoid morphology and segregation of **a** strain LMC500 *minB*, grown in minimal medium with 0.2% glycerol, in steady state at 37° C; **b** *gyrB*(ts) strain in glucose medium, after 2 h at 42°; and **c** strain LMC500 *minB*$^+$ in glycerol medium, in steady state at 37°, visualized by fluorescence microscopy. The *arrows* indicate free minicells, and those being formed; the *bar* represents 5 μm

from the *minB* strain show a decrease in negative supercoiling, similar to the *gyrB*(ts) mutant at restrictive temperature (Steck and Drlica 1984). Also the topology of plasmids in *minB* over-producing cells was investigated (*minB* over-production means over-production of *minDE* proteins; de Boer et al. 1988).

Figure 2 illustrates the effect on plasmid superhelical density of *gyrB*(ts) and *minB* mutations, and *minB* over-production by chloroquine-agarose gel electrophoresis. Below the bright '4.8 kb band', presumably containing relaxed closed circular DNA, the supercoiled topoisomers and linear plasmid DNA (4.0 kb) are separated. In Fig. 2, lanes a and b show the decrease in negative supercoiling of pACYC184 plasmid DNA in the *gyrB*(ts) mutant, when these cells were shifted to restrictive temperature. Plasmid pACYC184 DNA isolated from the *minB* mutant (LMC1011) also showed a decreased level of negative supercoiling (lane d) compared to that isolated from the parent strain (lane c, LMC500). The negative supercoiling of pACYC184, transformed into cells containing the pDB103 plasmid with inducible *minB* genes, also decreased upon induction with 0.5 mM IPTG (Fig. 2, lanes e and f). DNA at the top of lanes e and f is pDB103, which multimerized (see below). The decreased level of negative supercoiling of pACYC in LMC500/pDB103 in the absence of IPTG (Fig. 2, lane e) compared to that in the parent strain (lane c) may be due to a low level of *minB* over-production in these cells.

The decrease in negative supercoiling in the *minB* mutant was also observed with pBR322 (not shown). However, a pBR322-related plasmid, pGG26 (Table 1) in which the *tet* gene is replaced, showed a dramatically different plasmid topology on agarose gel electrophoresis. In pGG26 isolates from the *minB* mutant no supercoiled monomers could be detected (Fig. 3, lane d), whereas pGG26 from the *gyrB*(ts) mutant showed a decrease in negative supercoiling (Fig. 3, lanes a and b) which was similar to that of pACYC184 (Fig. 2, lanes a and b). The large aggregates in pGG26 isolates from *minB* cells did not disintegrate upon treatment with 50 μg/ml proteinase K for 1 h at 37° C (result not shown). Kleinschmidt preparations of pGG26 isolated from LMC500 (wild type) and LMC1011 (*minB*) show that the isolates from the wild-type strain primarily contained small plasmids (Fig. 4a). By contrast, the *minB* isolates mainly consisted of large DNA molecules, which often appeared as large circles (Fig. 4b). Length measurements of these DNA molecules showed that small DNA molecules in the isolates from the parent were monomers and dimers, whereas the multimeric plasmids in the *minB* isolates consisted of up to 16 plasmid molecules (result not shown). Restriction analysis of wild-type and *minB* pGG26 isolates resulted in similar fragments, indicating head-to-tail arrangement in the multimers. Plasmid pDB103 which, like pGG26, is pBR322 related and lacks the *tet* gene, showed a pGG26-like phenotype on gel electrophoresis (Fig. 2, lanes e and f); this indicates that apart from the *minB* mutation, low levels of *minB* over-production may induce the plasmid phenotype. In conclusion plasmid topology suggests that, like the *gyrB*(ts) mutation, the *minB* mutation and *minB* over-production cause a decrease in plasmid superhelical tension and may cause plasmid multimerization.

Retardation of segregation in minB *and* minB *over-producing cells*

The extent of retardation of segregation in different *minB* populations appeared to vary. Nucleoid segregation capacity was quantified as the average nucleoid length in all cells of the population. As segregation of nucleoids occurs along the length axis of the cell, retardation of segregation would cause the average nucleoid length to increase. We may compare nucleoid segregation in *minB* and *minB*$^+$ populations

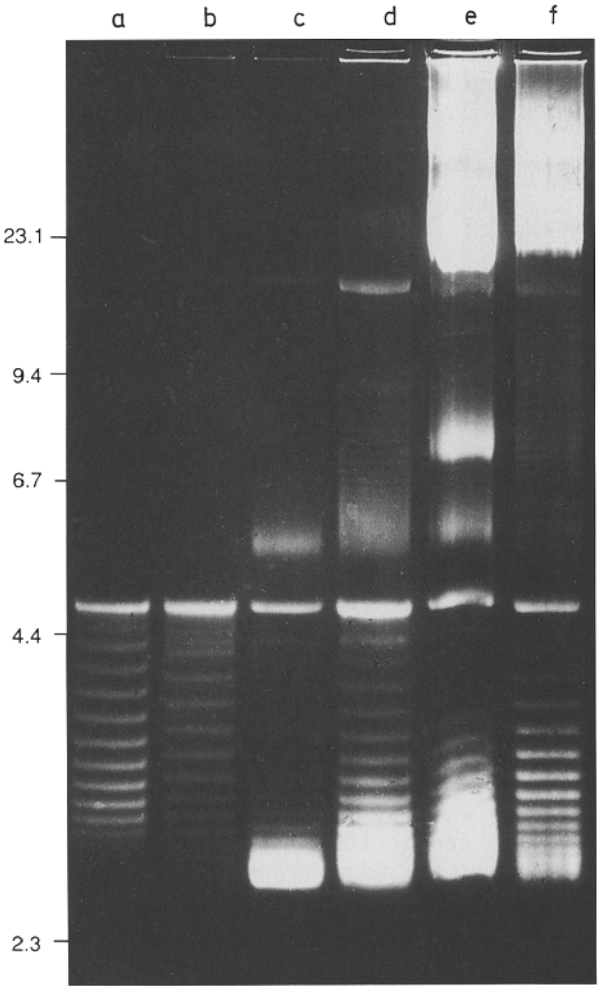


Fig. 2. The effect of *gyrB*(ts) and *minB* mutations and of overproduction of the *minB* gene products on superhelical density of pACYC184, demonstrated by chloroquine-agarose gel electrophoresis. Lane a, *gyrB*(ts) 30° C; lane b, *gyrB*(ts) 2 h at 42° C; lane c, LMC500(WT); lane d, LMC1011 *minB*; lane e, LMC500/pDB103; lane f, LMC500/pDB103 grown in the presence of 0.5 mM IPTG. More highly supercoiled topoisomers migrate more rapidly. The size markers indicate linearDNA lengths in kb

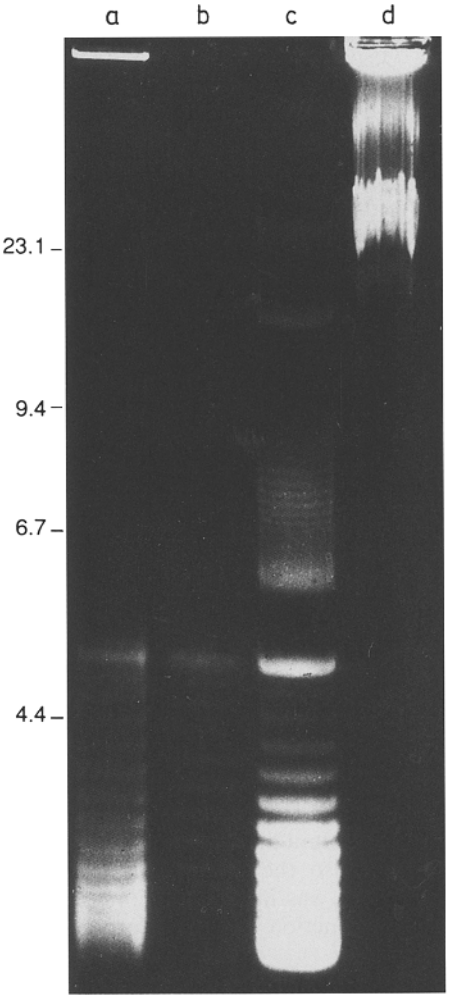


Fig. 3. The effect of *gyrB*(ts) and *minB* mutations on superhelical density of pGG26. Lane a, *gyrB*(ts) 30° C; lane b, *gyrB*(ts) 42°; lane c, LMC500 (wild type); lane d, LMC1011 (*minB*). The size markers indicate linear DNA lengths in kb

by their respective average nucleoid lengths, because nucleoids in both strains appear to be of similar shape (see Fig. 1). Also the *minB* and parent populations cultured under similar conditions grew at similar rates (Table 2) and, as DNA replication is not affected by the *minB* mutation (Frazer and Curtiss 1975), nucleoids of parent and *minB* cells are expected to be of similar size. Table 2 shows that the average nucleoid length of the *minB* strains is larger than that of the respective parent strains, suggesting retardation of nucleoid segregation. The increase in average nucleoid length due to the *minB* mutation varied from 30% in the LMC1011 strain, to 75% in the GC7115 strain. Also *minB* over-producing cells form minicells (de Boer et al. 1988) and, like the *minB* mutant, showed retarded nucleoid segregation: the average nucleoid length increased 25% after induction with 0.5 mM IPTG (Table 2). The larger average nucleoid length in *minB* and *minB* over-producing populations was not due to a few long nucleoids in the longer filaments, but occurred over the whole range of cell lengths. The coefficient of variation and range of the nucleoid length distributions are presented in Table 2.

No steady-state value for the average nucleoid length of the *gyrB*(ts) mutant could be obtained, because, unlike the *minB* mutant, the *gyrB*(ts) mutant could not be cultured in steady state at restrictive temperature (Fig. 5). However, qualitatively the pictures of Fig. 1 indicate that nucleoid segregation in the *gyrB*(ts) mutant is more hampered than in the *minB* mutant.

DNA-less cell formation in the minB *mutant*

From fluorescence micrographs we found that *gyrB*(ts) cells, probably as a result of the segregation defect, pinched off both anucleate cells and minicells, whereas the LMC500 *minB* cells only produced minicells. Most of the *minB* populations that were studied exclusively formed minicells; only PB104 (*minB*) occasionally pinched off short DNA-less rods. This observation deviates from that of Jaffé et al. (1988), who report the occurrence of 10% anucleate rods in strain GC7115. The length distributions of the DNA-less cells produced by the *gyrB*(ts) and *minB* (PB104) mutants are depicted in Fig. 6a and b, respectively. The length distri-

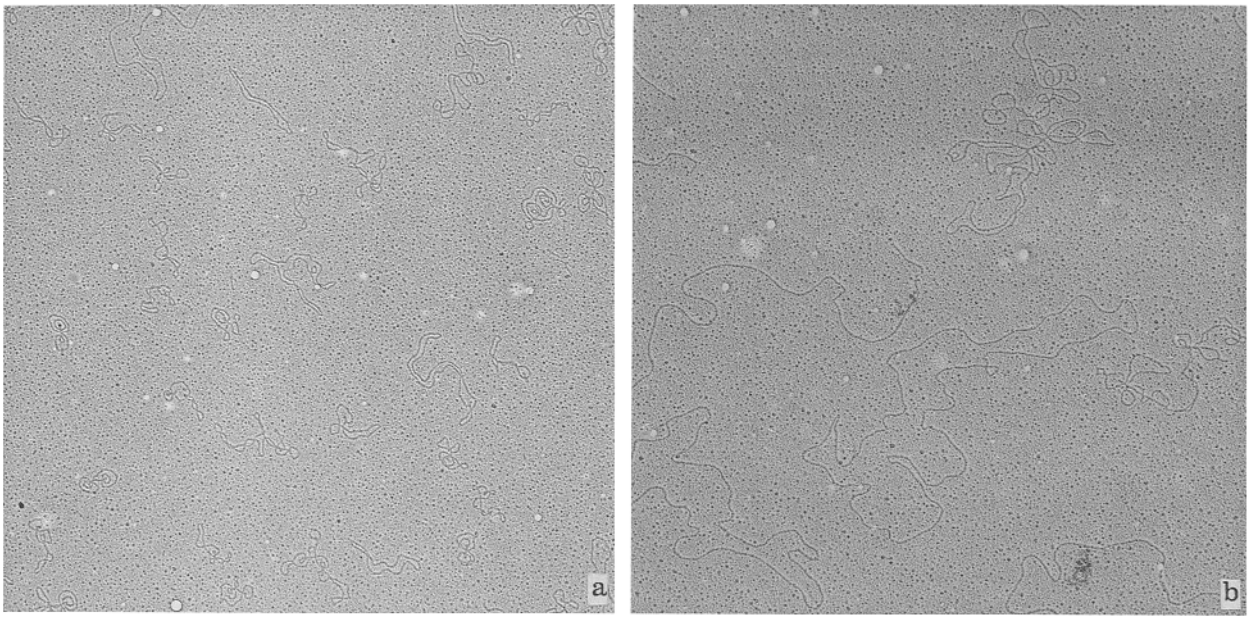


Fig. 4a and b. Plasmid morphology of pGG26 isolated from **a** the parental strain LMC500 (wild type) and **b** LMC1011 (*minB*) visualized by electron microscopy of Kleinschmidt preparations

Table 2. Average nucleoid length ($n=100$) in *minB*$^+$ and *minB* cells and in cells that over-produce *minB* gene products, measured from fluorescence micrographs at a final magnification of $\times 3200$

Strain/growth conditions	Carbon source	Culture doubling time (min)	Nucleoid length[a] (μm)
LMC500 *minB*$^+$	glucose	56	1.2 (30, 0.5–2.6)
LMC1011 *minB*		54	1.6 (34, 0.6–4.7)
LMC500/pDB103 + 0.5 mM IPTG[b]		55	1.5 (41, 0.6–4.2)
LMC500 *minB*$^+$	glycerol[c]	76	1.6 (29, 0.6–2.8)
LMC1011 *minB*		84	2.4 (60, 0.7–9.5)
PB103 *minB*$^+$	glucose	105	1.6 (31, 0.7–2.9)
PB104 *minB*		108	2.1 (32, 0.6–3.7)
GC7106 *minB*$^+$	glucose	50	1.2 (25, 0.5–2.4)
GC7115 *minB*		52	2.1 (58, 0.8–9.2)

[a] Average nucleoid length and, between brackets, the coefficient of variation and the range of the length distribution
[b] Isopropyl-β-D-thio-galactopyranoside added to induce over-production of *minB* gene products from pDB103
[c] In contrast to the osmolality of the glucose medium (300 mOsm), the glycerol medium was 100 mOsm

butions show some overlap, and confirm that the *gyrB*(ts) mutant produced DNA-less rods and some minicells, the *minB* mutant minicells and a few short rods.

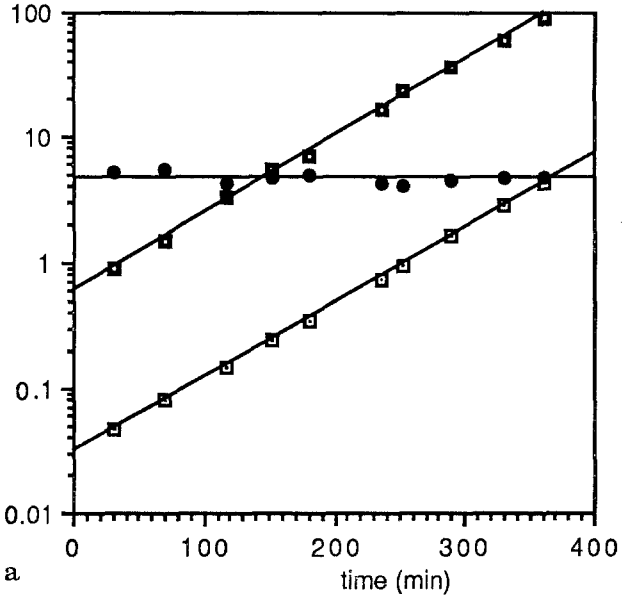
Discussion

Nucleoid segregation and supercoiling are similarly affected in the minB *and* gyrB *(ts) mutants and in cells that over-produce the* minB *gene products*

The chains of unseparated nucleoids in *minB* filaments (Fig. 1a) suggest retardation of nucleoid segregation and probably relate to the abnormal DNA distributions in *min* mutants observed by Jaffé et al. (1988) Quantification of nucleoid segregation capacity from fluorescence micrographs indicated large differences between different *minB* populations (Table 2). Retardation of the segregation of chromosomes is a typical phenotype of *gyr* mutants. The average nucleoid length in the *gyrB*(ts) mutant could not be measured at 42° C, because these cells could not be cultured in steady state (Fig. 5). However, from the micrographs of Fig. 1, nucleoid segregation appeared more retarded in *gyrB*(ts) than in *minB* cells.

Gyrase mutations affect both chromosome and plasmid pBR322 superhelical tension (Pruss et al. 1982): they become less negatively supercoiled. Superhelical tension of plasmids isolated from gyrase mutants and analysed on chloroquine-agarose gels thus reflects chromosomal superhelical tension within the cell (Pruss et al. 1982; Higgins et al. 1988). Plasmids isolated from the *minB* mutant also showed a decreased level of negative supercoiling upon chloroquine agarose gel electrophoresis, which therefore may reflect an effect of the *minB* mutation on chromosomal supercoiling.

The phenotypic similarities between the *gyrB*(ts) and *minB* mutants with respect to nucleoid segregation and DNA supercoiling suggest involvement of the *minB* gene products with gyrase activity. Apart from the possibility that the *minB* products themselves may be responsible for negative supercoiling and decatenation, the proteins could either interfere with the gyrase enzyme or with its substrate. The observations of cells that over-produce the *minB* gene products, indicating retarded nucleoid segregation and a decrease in plasmid superhelical tension, suggest that balanced expression of *minB* proteins in the cell is essential for the proper segregation of nucleoids. In this way minicell formation is a secondary effect of the retardation of segregation (see below). Another indication that *minB* affects DNA directly is the multimerization of pBR-related plasmids in *minB* and *minB* over-producing strains.



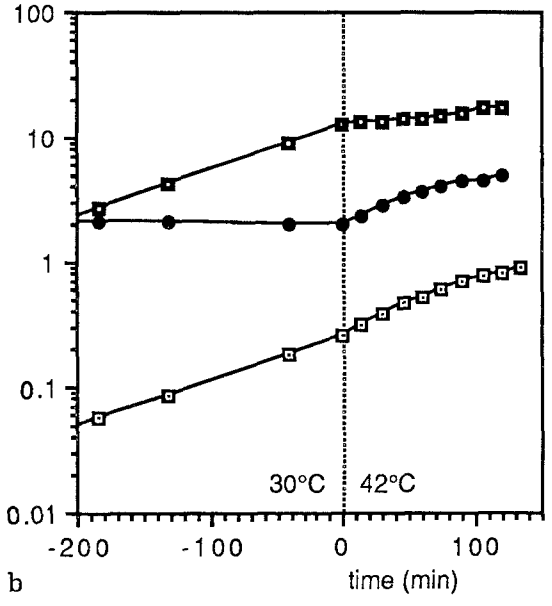


Fig. 5a and b. Growth and cell division of **a** the LMC500 *minB* mutant growing in steady state at 37° C and **b** the *gyrB*(ts) mutant during a temperature shift experiment (30°–42° C), illustrated by changes in $OD_{450\ nm}$ (□), cell number (■) and average cell mass (●, $OD_{450\ nm}$/cell number)

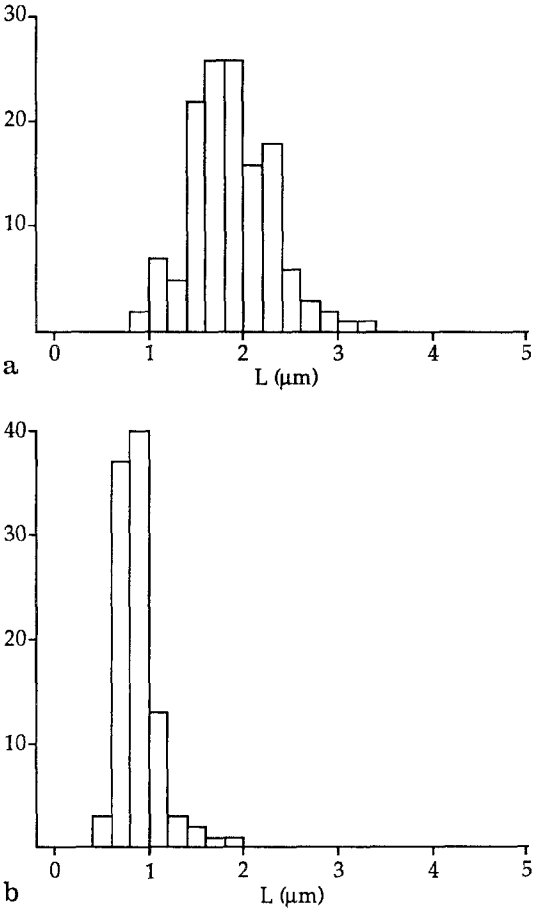


Fig. 6a and b. Frequency distributions of the length of DNA-less cells formed by **a** *gyrB*(ts) (LE316) after 2 h at 42° C, and **b** *minB* (PB104) in steady state at 37°, measured from fluorescence micrographs (final magnification ×3200)

The above observations suggest another function for a balanced expression of *minB* gene products in the cell division cycle of *E. coli* other than that given by de Boer et al. (1989). They proposed a direct interaction of *minB* products with cell division sites in the cell membrane. If, indeed, *minB* proteins are directly involved in membrane processes, the *minB* mutation might affect membrane integrity. As Dorman et al. (1989) suggested, a change in membrane structure is likely to affect the intra-cellular osmolality and indirectly DNA supercoiling (Higgins et al. 1988).

Minicell and DNA-less cell formation

The defect at the level of segregation of nucleoids observed in the *minB* mutant suggests that the production of minicells resembles DNA-less cell formation in other segregation mutants, e.g. *gyrB*(ts). Why does the *minB* mutant mainly produce minicells whereas the *gyrB*(ts) mutant mainly produces rods? In our concept of the regulation of cell division (Mulder and Woldringh 1989), the timing and positioning of constrictions is dependent on the balance between a short-range negative effector, originating from the nucleoids, and a long-range positive effector, a cell division signal (e.g. a 'termination protein'; Jones and Donachie 1973), generated upon termination of replication. This termination signal is only able to induce initiation of constriction outside the inhibitory area of the nucleoids. Retardation of segregation in the *minB* mutant would result in inhibition of constriction in the cell centre. As DNA replication, and thus termination, is not affected in the *minB* mutant (Frazer and Curtiss 1975), a constriction can be initiated as soon as enough room is available at one of the cell poles, resulting in minicell formation. In the *gyrB*(ts) mutant, nucleoid segregation appears more retarded than that of the *minB* mutant (Fig.1). Moreover, the *gyrB*(ts) mutant is defective in DNA replication (Cozzarelli 1980; Gellert 1981). Retardation of replication would result in postponement of the termination signal, which together with the defect in nucleoid segregation would result in the creation of longer nucleoid-free cell poles. As the constrictions are preferentially initiated close to the nucleoids (Mulder and Woldringh 1989), on average longer anucleate cells are

formed. In conclusion, our observations indicate that nucleoid segregation is defective in the *minB* mutant. The segregation defect, which may be related to the observed decrease in negative supercoiling of reporter plasmids, may indirectly cause this mutant to form minicells.

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