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Plasmid DNA Supercoiling and Survival in Long-Term Cultures of *Escherichia coli*: Role of NaCl

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The relationship between the survival of *Escherichia coli* during long-term starvation in rich medium and the supercoiling of a reporter plasmid (pBR322) has been studied. In aerated continuously shaken cultures, *E. coli* lost the ability to form colonies earlier in rich NaCl-free Luria-Bertani medium than in NaCl-containing medium, and the negative supercoiling of plasmid pBR322 declined more rapidly in the absence of NaCl. Addition of NaCl at the 24th hour restored both viability and negative supercoiling in proportion to the concentration of added NaCl. Addition of ofloxacin, a quinolone inhibitor of gyrase, abolished rescue by added NaCl in proportion to the ofloxacin added. This observation raises the possibility that cells had the ability to recover plasmid supercoiling even if nutrients were not available and could survive during long-term starvation in a manner linked, at least in part, to the topological state of DNA and gyrase activity.

The ability to maintain cellular integrity during long-term starvation is essential for the survival of bacteria. Such starvation occurs upon entry into stationary phase. Once thought to be characterized by metabolic inactivity, stationary phase is now recognized as a period during which a series of metabolic changes take place over time (17, 19, 24).

The enteric bacterium *Escherichia coli* has to cope with continuous change in its environment. One of the variable parameters is the osmolarity of the surrounding medium. In *E. coli*, the level of supercoiling is maintained by the opposing actions of DNA gyrase and topoisomerase I, and it is increased when cells are cultured in medium of high salt concentration (16, 22) or in anaerobiosis (15). This laboratory previously reported that the level of negative supercoiling of pBR322 changed during growth according to the absence or presence of NaCl in the medium. Relaxation of pBR322 was observed at entry into stationary phase in the presence or absence of NaCl, but this relaxation was followed by an increase of supercoiling in NaCl-free Luria-Bertani (LB) medium (8). Many authors have described a DNA plasmid relaxation as cells enter into stationary phase (1, 9, 26). Recently, stationary-phase cells were shown to display different strategies to ensure survival under conditions of nutritional stress and to regain growth quickly once nutrients become available (26); the recovery of DNA supercoiling and gyrase activity are essential to reinitiate growth. Gauthier et al. (13) studied the cultivability of *E. coli* in seawater and found that long-term protection was afforded to cells by growth in medium whose osmotic pressure was increased by NaCl. Survival in seawater was correlated to the topological state of the DNA. Apart from a study of cultivability of *E. coli* in river water (12), little attention has been paid to long-term survival in the absence of NaCl. We previously reported poor long-term survival of *E. coli* grown in NaCl-free LB medium compared to the good viability of cells grown in rich media supplemented with either 170 or 400 mM NaCl (7). We report here a correlation between long-term survival and the level of supercoiling of a reporter plasmid, pBR322, in cells grown with or without NaCl and the effect of ofloxacin, a DNA gyrase inhibitor (10), on the ability to survive during starvation.

The bacterial strain MC4100 F$^-$ *araD* Δ(*argF-lac*)*205 araD139 deoC1 rpsL150 relA flbB5301 ptsF25 rbsR* (5) was used in this study. When suitable, plasmid pBR322 was introduced by transformation. Bacteria were grown in rich LB medium (10 g of tryptone/liter plus 5 g of yeast extract/liter; Difco Laboratories) (20) with no NaCl (LB0) or supplemented with 400 mM NaCl (LB400). We avoided use of buffered medium in order to test only the presence or absence of NaCl and because external pH phase was not significantly different after growth under identical conditions in NaCl-containing or NaCl-free medium (7).

For each experiment, an overnight culture (25 ml of medium in a 125-ml Erlenmeyer flask) was diluted 1,000-fold into Erlenmeyer flasks containing 200 ml of the same preheated medium. Cultures were grown at 37°C in a water bath, with shaking (200 rpm). Viability was determined by plating 100 μl or by spotting 5 μl of serial dilutions of starved cultures on three LB agar plates (with and without ampicillin at 100 μg/ml) and counting the colonies after 24 h of incubation at 37°C. No significant difference was found when cultures were plated on LB0 or LB400 agar, with or without antibiotic.

We measured the variations of DNA supercoiling of reporter plasmid pBR322 during starvation in parallel with the ability to form colonies in the same culture. Plasmid DNA was isolated, and topoisomers were separated by electrophoresis through 1% agarose gels containing chloroquine at a suitable concentration. The gels were run for 16 h at 4°C, washed for 3 h, and stained in ethidium bromide (1 μg/ml) for 2 h. After being rinsed twice with 1 mM $MgSO_4$, the gels were photographed under UV illumination. The degree of negative or positive supercoiling of pBR322 molecules was estimated by comparing the migration in gels containing two different con-

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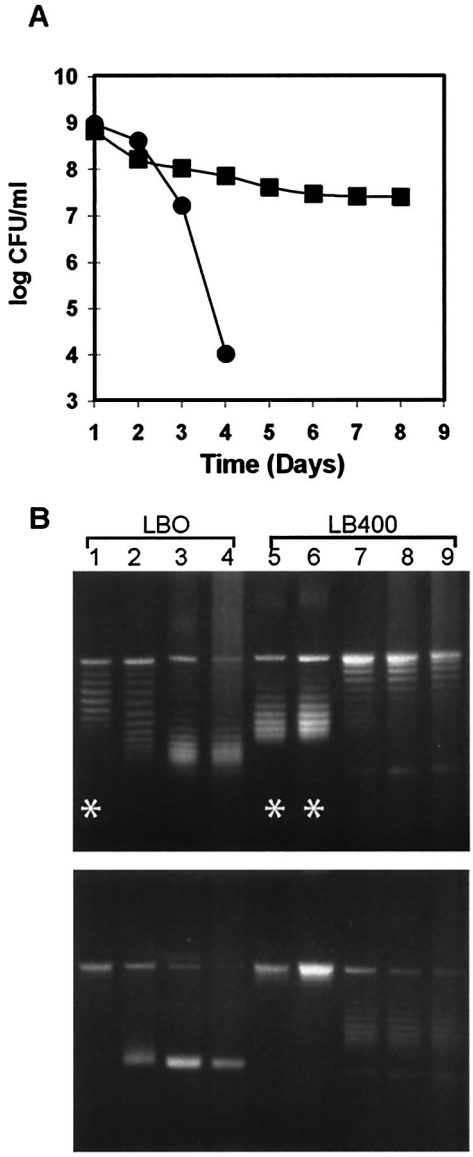

FIG. 1. Long-term survival of MC4100 and plasmid supercoiling in rich media. At time zero, an overnight culture was diluted 1,000-fold in 200 ml of preheated medium and incubated at 37°C with continuous shaking in LB0 and LB400. Cultures reached stationary phase after 6 h. Similar results were obtained in three independent experiments, and the results of one experiment are shown. (A) Survival. Aliquots removed every day from day 1 (24 h after inoculation, 18 h in stationary phase) were diluted, and CFU were counted after plating in triplicate on LB agar dishes. Circles, LB0; squares, LB400. (B) Plasmid supercoiling. Results shown are from chloroquine-agarose gel electrophoresis of pBR322 extracted from the same continuously shaken cells grown in LB0 (from day 1 to 4; lanes 1 to 4) or in LB400 (from days 1, 2, 3, 4, and 8; lanes 5 to 9). Plasmid supercoiling was compared by electrophoresis in the presence of chloroquine at 5 μg/ml (upper gel) and 15 μg/ml (lower gel). Negatively supercoiled pBR322 molecules (in lanes indicated by a star) were identified by a decrease in gel mobility after raising the chloroquine concentration. All other DNA species were relaxed or positively supercoiled.

centrations of chloroquine. In the presence of increasing chloroquine concentrations, negative supercoiled DNA species shift up while positive supercoiled species increase in mobility.

The viability of *E. coli* strain MC4100 was monitored each day during stationary phase in rich medium without NaCl

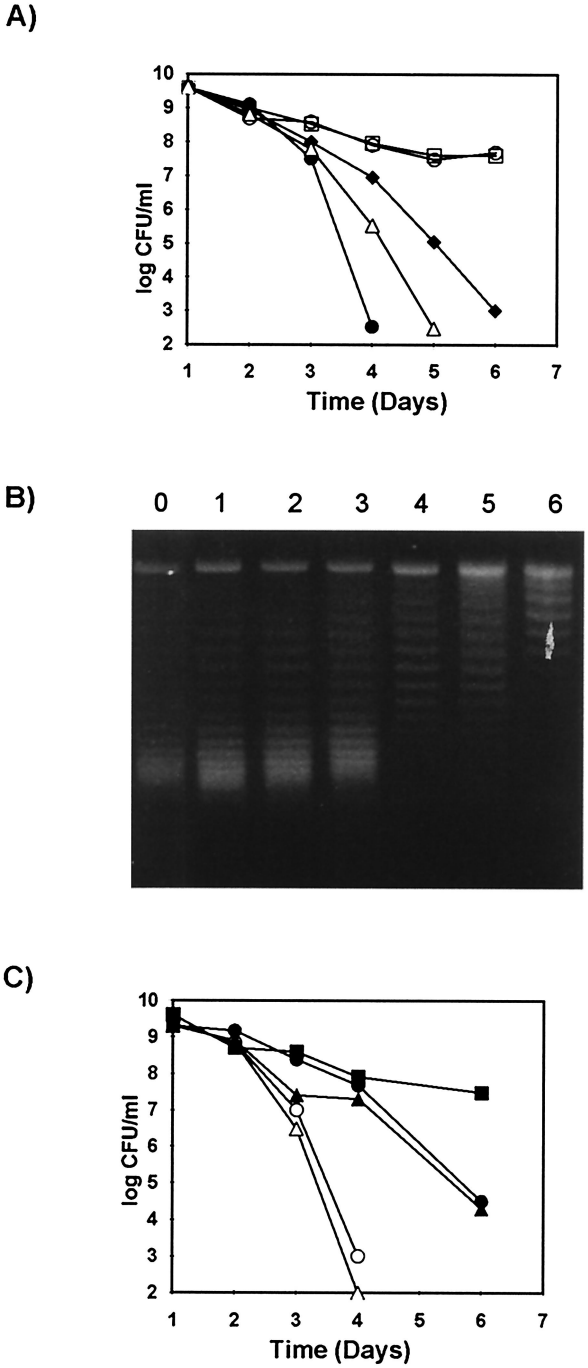

FIG. 2. Effect of NaCl addition on survival and supercoiling. (A) Survival following NaCl addition at 24 h after inoculation in LB0 with shaking. NaCl was added to final concentrations of 0, 12.5 mM (closed circles), 25 mM (triangles), 50 mM (diamonds), 100 mM (open squares), and 200 or 400 mM (open circles; only one curve is shown, since results were identical). Two separate experiments were performed. (B) Plasmid supercoiling after NaCl addition, with chloroquine (7 μg/ml)-agarose gel electrophoresis of pBR322 extracted 2 h after addition of NaCl at the 24th hour to the LB0-grown cultures shown in panel A. Under these conditions, pBR332 molecules were positively supercoiled (the higher chloroquine concentration increased mobility for all lanes [data not shown]). NaCl was absent (lane 0) or was added at final concentrations of 12.5 mM (lane 1), 25 mM (lane 2), 50 mM (lane 3), 100 mM (lane 4), 200 mM (lane 5), or 400 mM (lane 6). (C) Survival following addition of 200 mM NaCl at different times after inoculation. Results shown are for no NaCl (open triangles) or NaCl added at 24 h (closed squares), 32 h (closed circles), 48 h (closed triangles), or 60 h (open circles).

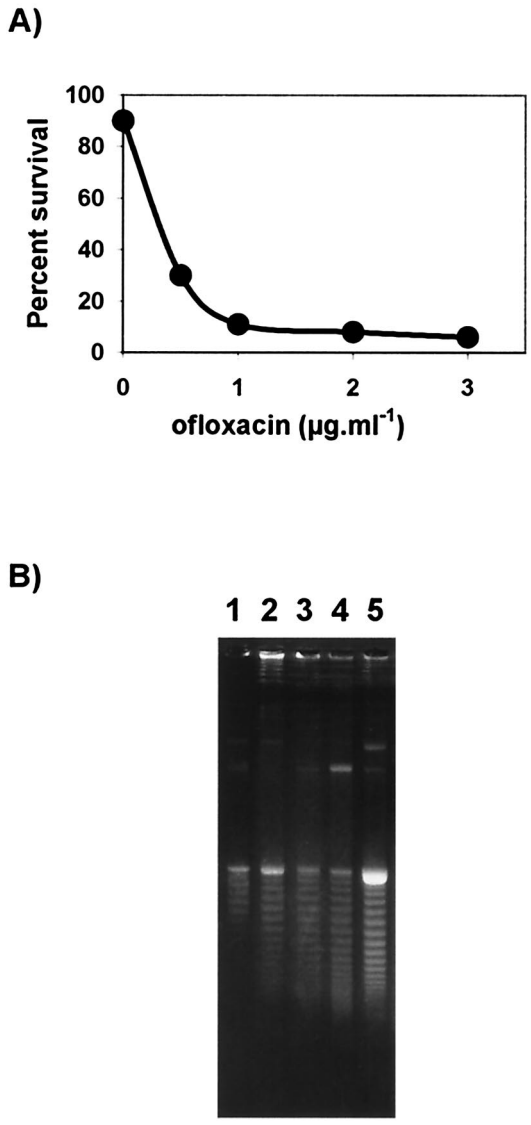

FIG. 3. The effect of ofloxacin on NaCl rescue of a 24-h MC4100 culture. (A) Percent survival of MC4100 2 days after addition of 200 mM NaCl and ofloxacin to cells cultured in LB0. The experiment was done as described in the legend for Fig. 1. The 100% survival level corresponds to the viable cell count at 24 h. (B) Effect of ofloxacin on the supercoiling of pBR322 extracted from cultures treated for 2 h. Plasmid supercoiling was determined by gel electrophoresis in the presence of 7 μg of chloroquine/ml. At this concentration, all pBR322 molecules were positively supercoiled. In lanes 1 to 4, cultures were treated with 200 mM NaCl and either no ofloxacin (lane 1) or 0.5, 1, or 3 μg of ofloxacin/ml (lanes 2 to 4). In lane 5, the culture received neither NaCl nor ofloxacin.

(LB0) or supplemented with 400 mM NaCl (LB400) (Fig. 1). In LB400, viability dropped about 1 log over the first 2 days and then remained fairly constant for at least 8 days. In contrast, viability in rich medium without NaCl remained constant throughout the first 48 h in stationary phase and then abruptly fell to less than 10^3 CFU/ml at day 5. Plasmids extracted from LB0-grown cells exhibited lower negative supercoiling (about four turns) than those from LB400-grown cells at 24 h (Fig. 1B, lanes 1 and 5). A sharp decrease in supercoiling of the plasmid (about 12 turns) occurred between 24 and 48 h in NaCl-free LB cultures, leading to molecules which were positively supercoiled in 5 μg of chloroquine/ml (lanes 2 to 4). A decrease in negative supercoiling also occurred in LB400-grown cultures. This decrease, which occurred to a lesser extent (eight turns), was observed later (between days 2 and 3), and the level of supercoiling then remained constant up to day 8 (lanes 7 to 9).

Our results indicate that the loss of viability of LB0-grown cells in stationary phase was preceded by an abrupt relaxation of plasmid DNA during the second day. Cells grown in the presence of NaCl exhibited a relatively mild relaxation of pBR322 DNA on the third day, which was then maintained together with viability. Addition of NaCl at various concentrations in cultures grown for 1 day in LB0 could restore viability identical to that of cultures continuously grown in NaCl-containing medium (7). To investigate the relationship between viability and DNA supercoiling, we added different concentrations of NaCl to continuously shaken LB0 cultures at the 24th hour (Fig. 2A), and the level of supercoiling of pBR322 was measured (Fig. 2B). Rescue by NaCl was a function of the NaCl concentration and was maximal above 100 mM (Fig. 2A). The level of supercoiling of pBR322 at 2 h after NaCl addition also increased in proportion to the NaCl added (Fig. 2B). Note that all the DNA samples appeared positively supercoiled at this chloroquine concentration (7 μg/ml), which is higher than that used in Fig. 1. Therefore, as negative supercoils are added, the DNA mobility decreases. A transition in colony-forming rates and in negative supercoiling occurred between 50 and 100 mM NaCl (lanes 3 and 4). NaCl (200 mM final concentration) was also added to cultures at different times of starvation (Fig. 2C). Viability was completely rescued when NaCl was added at 24 h (Fig. 2C) and partially rescued when NaCl was added between 24 and 48 h (Fig. 2C). At later times of addition, no rescue was observed.

The ability to form colonies on LB plates depended on NaCl being present by the second day and was correlated with the increased level of negative supercoiling of plasmid during this time, independently of its initial level.

If the rescue of NaCl-free LB cultures by NaCl addition were correlated to an increase in negative supercoiling, DNA gyrase activity would be necessary. This was investigated by using ofloxacin, a quinolone inhibitor of gyrase (10). The addition of ofloxacin alone had no effect in the absence of NaCl (data not shown). The addition of ofloxacin at 0.5 to 3 μg/ml together with 200 mM NaCl to LB0-grown cells suppressed the rescue (Fig. 3A). The increase in negative supercoiling of pBR322 after NaCl (200 mM) addition (Fig. 3B, lanes 5 and 1) was eliminated by addition of ofloxacin at concentrations greater than 1 μg/ml (Fig. 3B, lanes 2 to 4). The same results were obtained with ciprofloxacin, another quinolone (data not shown) (3). Thus, gyrase activity was required for the NaCl rescue. The advantage of NaCl for survival in stationary-phase cultures could be correlated to the maintenance of a suitable level of superhelicity by DNA gyrase.

In an *rpoS* mutant strain, no rescue was seen with NaCl, but the plasmid topology was difficult to measure in *rpoS* mutant cultures due to poor survival rates (1% of cells survive in LB without NaCl [data not shown]). RpoS is thus required for NaCl-mediated rescue. The reinitiation of culture growth after

starvation was also shown to require RpoS (26). NaCl addition was not specifically required for long-term survival; exposure to short-chain *n*-alcohols also prevents viability loss in an *rpoS*-dependent fashion (27). Cells grown in minimal medium A (20) exhibited both 100% survival and a constant level of negative supercoiling during at least 8 days of starvation (data not shown). Anaerobiosis or arrest of shaking was another growth condition that ensured good survival (7). The difference in negative supercoiling between unshaken and shaken cultures was about eight turns at 24 h (data not shown), and this result was in good agreement with the increase in supercoiling during a shift to low oxygen or to anaerobiosis (15, 21).

It is possible that any factor contributing to a suitable level of negative supercoiling would favor long-term survival. The DNA binding HU proteins are one example of factors that contribute both to survival and supercoiling (6).

When cells were cultured under conditions leading to plasmid DNA relaxation (NaCl deprivation, aeration, or gyrase inhibitor), they could not maintain a suitable level of supercoiling over the long term and died (references 8 and 18 and this work). In a recent publication, Reyes-Dominguez et al. showed that Gyr proteins are not degraded even after a long stationary phase (26) and that the preexisting gyrase molecules in cells were responsible for the recovery of supercoiling as soon as nutrients became available. The present results elucidate the ability of cells to regulate topology through gyrase activity even late after entry into stationary phase and independently of fresh nutrient addition.

This confirms that there is a relationship between the topological state of the plasmid DNA in *E. coli* and the ability of cells to survive and maintain their cultivability in late stationary phase.

The regulation of DNA topology that permits proper gene expression (2, 7, 14, 23), as well as protein synthesis in the early hours of stationary phase, is required for long-term survival (4, 25). It has also been shown that the life of growth-arrested wild-type *E. coli* cells could be significantly extended by omitting oxygen and that a significant number of genes induced by stasis are associated with protection against endogenously generated oxidative damage (11). These observations are compatible with the requirement for suitable DNA supercoiling levels that permit the transcription of stress response genes induced when cells stop growing in stationary phase.

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