Plasmid DNA Supercoiling and Gyrase Activity in *Escherichia coli* Wild-Type and *rpoS* Stationary-Phase Cells

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Stationary-phase cells displayed a distribution of relaxed plasmids and had the ability to recover plasmid supercoiling as soon as nutrients became available. Preexisting gyrase molecules in these cells were responsible for this recovery. Stationary-phase *rpoS* cells showed a bimodal distribution of plasmids and failed to supercoil plasmids after the addition of nutrients, suggesting that *rpoS* plays a role in the regulation of plasmid topology during the stationary phase.

DNA supercoiling is essential for DNA metabolism. Supercoiling is introduced into DNA molecules by enzymes called DNA topoisomerases, which break, pass DNA strands through the break, and rejoin DNA (6, 24, 30). In *Escherichia coli* the level of supercoiling depends mainly on the activities of DNA topoisomerase I (TopI) and TopII (gyrase) and, to a lesser extent, on TopIV (6, 30, 32). TopI introduces DNA single-strand breaks and relaxes DNA molecules. Gyrase, an ATP-dependent enzyme composed of two GyrA and two GyrB subunits, makes double-strand breaks and introduces supercoils into DNA. TopIV is an ATP-dependent enzyme that makes double-strand breaks and contributes to DNA relaxation (24, 30, 32). The level of DNA supercoiling is regulated by a complex homeostatic control (19, 28, 32). Transcription of *topA*, which encodes TopI, increases when the level is high, whereas transcription of the *gyr* genes increases when the level is low (19, 28). It is well established that changes in the level of DNA supercoiling influence the activity of many promoters (9, 30) and that environmental variations alter this level (2, 4, 8, 22, 23). The role of DNA topoisomerases during the cellular response to these variations is not completely understood.

E. coli stationary-phase cells in minimal or rich media, or after extreme nutrient downshifts, display a relaxation of DNA (2, 8, 26, 28). In general, stationary-phase cells exhibit a number of morphological and physiological changes in order to survive starvation. These changes include resistance to several harmful conditions, condensation of the nucleoid, increased protein degradation, and a general decrease in transcription and translation (13). In *E. coli* many of these characteristics depend on the product of *rpoS*, σ^S , a global regulator responsible for the induction of more than 50 genes (11, 12). It has been shown that σ^S -dependent promoter recognition is more efficient on relaxed DNA templates, suggesting that the DNA

relaxation that occurs in stationary-phase cells plays a role in the transcription of σ^S -dependent genes (14).

As a first step to a better understanding of the regulation of DNA supercoiling during nutritional stress, the level of supercoiling and the expression of the *gyr* genes were determined along the growth curve and in stationary-phase cells after dilution into fresh medium. The role of σ^S on the level of DNA supercoiling was also explored.

Variations in the level of DNA supercoiling in exponentially growing cells, cells in stationary phase, and cells starved for several hours were determined by using plasmid pMS01, a tetracycline-sensitive derivative of pBR322 (17), as a reporter. This plasmid was used to avoid interference of the expression of the membrane protein TetA (18) and the topological effects generated by transcription of divergent promoters on the level of plasmid supercoiling.

Rich Luria-Bertani (LB) medium (20) containing 40 mM morpholinepropanesulfonic acid (LB-MOPS; pH 7.4) was used throughout the present study. LB-MOPS medium was used to avoid exposure of cells to alkaline stress due to the high pH reached by stationary-phase cultures in LB medium (29). The pH of LB-MOPS cultures remained neutral along the stationary phase studied. Cultures (10 ml) in 125-ml Erlenmeyer flasks were grown at 37°C with shaking (180 rpm). The optical density at 550 nm (OD_{550}) was used to monitor cell growth and identify the onset of stationary phase. Stationary phase was defined as the point where the OD_{550} levels off, 6 h after the culture was started under the experimental conditions used. The bacterial strain MC4100 $\Delta(argF-lac)205 araD139 rpsL150 thiA1 relA1 flb5301 deoC1 ptsF25 rbsR$ (5) was used in most experiments. Plasmid DNA was isolated by the clear alkaline lysate method (25), and topoisomers were separated by electrophoresis through 1% agarose gels containing chloroquine at 12 μ g/ml. At this concentration of chloroquine, the topoisomers that were more supercoiled prior to electrophoresis migrated more rapidly through the gel. Electrophoresis was run in Tris-borate-EDTA buffer (25) at 1.7 V/cm for 22 h.

The results show that the level of plasmid supercoiling decreased as cells entered into stationary phase (Fig. 1A), a finding in agreement with previous reports from other groups

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FIG. 1. Agarose gel electrophoresis separation of reporter plasmid pMS01 isolated from MC4110 stationary-phase cells. Plasmid DNA topoisomers were isolated and separated through 1% agarose gels containing chloroquine at 12 $\mu\text{g}/\text{ml}$ as described in the text. Migration was from top to bottom. (A) Distribution of plasmid topoisomers as a function of growth phase. (B) Plasmid supercoiling of 18-h stationary-phase cells after dilution into fresh medium. SC, supercoiled plasmids; EP, exponential phase; SP, stationary phase; R, time (in minutes) after the addition of fresh medium to 18-h stationary-phase cultures.

(8, 26, 28). Plasmids became more relaxed after 72 h than after 18 h of stationary phase, as shown in Fig. 4. To study the capacity of stationary-phase bacteria to recover the high level of plasmid supercoiling typical of sustained growth conditions, cultures were diluted 1:10 into fresh medium after 18 h in stationary phase. Plasmids from these cells reached a level of supercoiling similar to that observed in exponentially growing cells after 0.5 to 1 min (Fig. 1B). Similar results were obtained with cells after 72 h in stationary phase (data not shown).

The fast recovery of supercoiling in cells after dilution into fresh medium was not prevented by the addition of transcriptional (rifampin, 300 $\mu\text{g}/\text{ml}$) or translational (chloramphenicol, 200 $\mu\text{g}/\text{ml}$) inhibitors. However, this recovery was prevented by the gyrase inhibitor novobiocin (200 $\mu\text{g}/\text{ml}$) (data not shown). These results suggest that gyrase recovers its activity as soon as new nutrients are available to cells.

To verify that gyrase molecules already present in stationary-phase cells were involved in the recovery of plasmid supercoiling after addition of fresh medium, the level of transcription of the *gyr* genes and the amount of the Gyr proteins were determined in stationary-phase cells and in cells during recovery of growth.

The level of transcription was studied by using MC4100 merodiploid strains bearing the chromosomal transcriptional fusion $\lambda\Phi(\text{gyrA-lacZ})$ or $\lambda\Phi(\text{gyrB-lacZ})$. The transcriptional fusions were constructed as described elsewhere (27). The regulatory regions of genes *gyrA* and *gyrB* were PCR amplified and cloned in the multicopy operon fusion vectors pRS415 and pRS528, respectively (27). The amplified regulatory region of *gyrA* included 1,190 bp upstream of the *gyrA* initiation codon, whereas that of *gyrB* included 902 bp upstream of the *gyrB* initiation codon. The transcriptional fusions $\Phi(\text{gyrA-lacZ})$ and $\Phi(\text{gyrB-lacZ})$ were then transferred from the plasmid to the phage vector λRS45 by in vivo recombination (27). Recombinant phages were used to infect strain MC4100, and single lysogens carrying the gene fusions were isolated. The activity of β -galactosidase was assayed in sodium dodecyl sulfate (SDS)-permeabilized cells by using ONPG (*o*-nitrophenyl- β -D-galac-

FIG. 2. Expression of transcriptional $\lambda\Phi(\text{gyrA-lacZ})$ or $\lambda\Phi(\text{gyrB-lacZ})$ fusions along the growth curve and in 18-h stationary-phase cells after transfer to fresh medium. MC4100 derivatives bearing the transcriptional fusions were grown in LB-MOPS medium. Similar results were obtained in three independent experiments. Solid symbols, cell growth (at OD_{550}); open symbols, β -galactosidase levels expressed in Miller units as described in the text. (A and B) Expression of the transcriptional *gyrA-lacZ* (A) and *gyrB-lacZ* (B) fusions along the growth curve. (C and D) Expression of the transcriptional *gyrA-lacZ* (C) and *gyrB-lacZ* (D) fusions during the recovery of growth induced in stationary-phase cells by the addition of fresh medium.

topyranoside) as a substrate. Enzyme units were calculated with data from readings at OD_{420} , OD_{550} , and OD_{600} and are expressed as Miller units as described elsewhere (20).

The *gyr-lacZ* fusions showed that the levels of transcription of *gyrA* and *gyrB* increased when growing cells began to enter into stationary phase, and then the level of β -galactosidase activity remained the same or decreased slightly (Fig. 2A and B). After the dilution of 18-h stationary-phase cells into prewarmed medium, the level of transcription of the *gyr* genes dropped and increased only after ca. 60 min (Fig. 2C and D). These results showed an increase in the transcription of the *gyr* genes before cells enter into stationary phase and that these genes continued to be transcribed during this phase. The delay in the increase of transcription of the *gyr* genes during growth recovery is probably due to the fast and high increase in the cellular concentration of Fis observed after the addition of fresh nutrients (26). This protein is a negative regulator of the transcription of the *gyr* genes (26).

To determine the amount of the Gyr proteins present in stationary-phase cells, a Western blot analysis was performed with monoclonal antibodies against the Gyr proteins (John Innes Enterprises, Ltd.). Proteins were separated by electrophoresis through 7.5% SDS-polyacrylamide gels (15) and electrophoretically transferred to a nitrocellulose filter (0.45- μm

FIG. 3. Immunoblot analyses of extracts from exponential and stationary-phase MC4100 cells with anti-*gyrA* and anti-*gyrB* monoclonal antibodies. Total protein samples were separated by electrophoresis through 7.5% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes as described in the text. Similar results were obtained in three independent experiments. EP, exponential phase; SP, stationary phase.

pore size; Schleicher & Schuell). Similar concentrations of protein from growing and stationary-phase cells and from cells during recovery of growth were loaded onto the gels. Horseradish peroxidase-conjugated anti-mouse IgGAM was used as secondary antibody (Zymed Laboratories, Inc.) for detection of the Gyr proteins with the ECL Western blotting detection system (Amersham Life Sciences). As shown in Fig. 3, the levels of the Gyr proteins were similar in cells in the exponential and stationary phases. A similar result was obtained with cells after 72 h of starvation (data not shown). Although stationary-phase cells increase protein degradation (13), Gyr proteins are not degraded even after a long stationary phase (72 h). This is relevant, since transcription of the *gyr* genes did not increase until ca. 60 min after the dilution of stationary-phase cells into fresh medium (Fig. 2C and D).

In an attempt to further explore the regulation of DNA supercoiling during starvation, the role of the stationary-phase σ factor, σ^S , was evaluated. The global regulator σ^S is involved in the general cellular stress response, which allows cells to adapt to changing conditions, such as nutrient deprivation or hyperosmolarity (11, 13). Among the genes regulated by σ^S are genes whose products could be involved in DNA topology, e.g., *topA*, *dps*, and *gyrI* (*sbmC*) (1, 3, 7, 12, 21). The *dps* gene encodes a protein that binds with no specificity to DNA (1, 13), and *gyrI* encodes an inhibitor of gyrase (21). To this end, the level of plasmid supercoiling in stationary-phase wild-type and *rpoS*::Tn10 cells was determined. The capacity of these cells to recover plasmid supercoiling after dilution of stationary-phase cultures into prewarmed growth medium was also studied.

As described above, plasmids from wild-type cells became more relaxed as the stationary phase advanced, and the recovery of a level of supercoiling similar to that of plasmids from exponentially growing cells takes place in ca. 1 min (Fig. 1A and B). However, under the same experimental conditions, the topology of plasmids from the *rpoS* strain showed a different behavior. Plasmids isolated from mutant cells after 18 h of stationary phase showed a bimodal distribution of topoisomers that became more clearly established after 72 h in stationary phase (Fig. 4). The recovery of plasmid supercoiling of wild-type cells after 18 and 72 h of stationary phase was complete 10 min after the transfer to fresh medium (Fig. 4). On the other hand, the recovery of plasmid supercoiling in *rpoS* cells starved for 18 h was partial after 30 min, and in mutant cells starved for 72 h no recovery of supercoiling was observed (Fig. 4). There

FIG. 4. RpoS influences the level of relaxation of plasmids isolated from stationary-phase cells and the capacity of these cells to supercoil plasmids after dilution into fresh medium. Plasmids were isolated, and topoisomers were separated as described for Fig. 1. Abbreviations are as defined for Fig. 1. wt, wild type.

were no changes in the number of CFU per milliliter in wild-type stationary-phase cultures. On the other hand, the number of CFU of *rpoS* stationary-phase cultures per milliliter decreased from ca. 7.0×10^9 at the onset of stationary phase to 1.0×10^9 after 72 h in stationary phase. A possible role of cell death in the generation of the bimodal distribution of plasmid topoisomers displayed by *rpoS* stationary-phase cells is currently being studied.

To rule out any effect of the *relA1* mutation present in strain MC4100 on the results described above, similar experiments were performed with an *rpoS*::Tn10 mutant constructed by P1vir transduction (20) into strain CF1648 (MG1655), which is *relA*⁺ (31). The *relA*-null mutation *relA251::kan* present in strain CF1652 (31) was also studied. The RelA protein participates in the synthesis of ppGpp, a molecule that positively modulates the expression of *rpoS* (16). The results obtained with these strains were similar to those observed for the *relA1* strain MC4100 (data not shown). The level of transcription of the *gyr* genes and the amount of the Gyr proteins in *rpoS*::Tn10 strains along the growth curve and during the recovery of growth of stationary-phase cells were similar to those observed in wild-type strains (data not shown).

Although care must be taken in interpreting results obtained with *rpoS* mutants because of their pleiotrophic phenotype, the results obtained with the *rpoS*::Tn10 strains suggest that genes regulated by σ^S could be implicated in the regulation of plasmid topology during the stationary phase. This σ factor seems not to be important in the regulation of transcription of the *gyr* genes or in the stability of Gyr proteins. These results also show that *relA* does not play an important role in the regulation of plasmid topology under the experimental conditions used. Finally, at least two of the genes regulated by *rpoS*, *dps*, and *gyrI* (*sbmC*) are not involved in the regulation of the plasmid topology under the conditions studied here (data not shown).

Stationary-phase cells display different strategies to ensure survival under conditions of nutritional stress and to regain growth quickly once nutrients become available. In this context, the recovery of DNA supercoiling and thus of gyrase activity is essential to reinitiate growth after starvation. Gyrase activity is essential for the transcription and replication needed to reinitiate growth and reestablish the cell cycle (6, 24, 30). The results presented here show that the regulatory mechanisms underlying this recovery appear to include an increased

transcription of the *gyr* genes before cells enter into stationary phase and conditions to protect the integrity of the Gyr proteins throughout this phase. The extent of contribution of other topoisomerases, mainly TopI, of the ATP/ADP ratio, and of proteins CRP and Fis, two regulators of the transcription of the *gyr* genes (10, 26), to the regulation of DNA supercoiling during stationary phase and growth recovery needs to be evaluated.

Finally, it could be important to elucidate if stationary-phase cells, independently of the nutrient composition of the medium and growth conditions, regulate DNA topology during stationary phase and after transfer to fresh medium in a manner similar to that described here.

Y.R.-D. and G.C.-F. contributed equally to this study.

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