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Decrease in the Linking Number of Plasmid DNA in *dnaA* Mutants of *Escherichia coli*

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We made use of agarose gel electrophoresis in the presence of chloroquine to examine the linking number of plasmids in temperature-sensitive *dnaA* mutants, including *dnaA5* and *dnaA46* mutants. The linking number of DNA prepared from *dnaA* mutants growing at 37°C was lower than that from wild type cells yet there was no significant difference when cells were grown at 28°C. Complementation analysis with a plasmid containing the wild type *dnaA* gene and phage P1-mediated transduction confirmed that mutations in the *dnaA* gene were responsible for the decrease in the linking number of DNA.

DnaA protein is the initiation factor of chromosomal DNA replication in *Escherichia coli* (1,2). About 20 molecules of DnaA protein are required for the formation of the initial complex on the *oriC* sequence, the initiation site of chromosomal DNA replication (3,4). DnaA protein opens-up the *oriC* sequence and guides the entrance of DnaB helicase to form the prepriming complex (5). The high abundance of DnaA protein (more than 1000 molecules per one cell) (6) suggests that DnaA protein has functions other than that at the *oriC* region. DnaA protein negatively controls transcription of various genes, including the *dnaA* gene, through specific binding to DnaA boxes located at the 5′-upstream regulatory region of the genes (7–10).

We reported that expression of the *fliC* gene which encodes flagellin, a subunit of flagella, decreased in *dnaA* mutants growing at 37°C, a permissive temperature for cell growth (11). As there is no DnaA box in the 5′-upstream region of the *fliC* gene or of the genes regulating expression of the *fliC* gene (12), the involvement of DnaA protein in the regulation of *fliC* gene expression cannot be explained by its specific binding to the DnaA boxes. DNA supercoiling profoundly affects the transcription of various genes (13–15). Expression of the *fliC* gene is inhibited by environmental stress which alters the extent of DNA supercoiling in cells (16,17). One explanation is that the level of DNA supercoiling in the *dnaA* mutants may differ from that in wild type cells and that topological change of DNA represses transcription from the promoter of the *fliC* gene.

Recently, we found that purified DnaA protein has *oriC*- and DnaA box-independent DNA unwinding activity (T. Mizushima *et al.*, unpublished results). DnaA protein has the capacity to bind DNA in a sequence non-specific manner (18). As non-specific DNA binding proteins, such as HU protein, were seen to be involved in regulation and maintenance of negative supercoiled structure of DNA *in vivo* (19–21), DnaA protein may possibly participate in DNA supercoiling events. This notion was given evidence by findings of Schaper and Messer who described the DNA bending activity of DnaA protein (22).

When we compared the extent of negative supercoiling of plasmid DNA in temperature-sensitive *dnaA* mutants to that in wild type cells, we found that the linking number of plasmids had decreased in *dnaA* mutants.

MATERIALS AND METHODS

Analysis of the linking number of plasmid DNA in cells. The linking numbers of pUC118 plasmids were examined, as described previously (23,24). Exponentially growing *Escherichia coli* cells harboring plasmid were harvested by centrifu-

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gation and washed with 0.9% NaCl. Plasmid DNA was extracted and purified by the alkaline method (25). The samples were analyzed by agarose gel electrophoresis in the presence of chloroquine. Electrophoresis was carried out in a TBE buffer at 40V for 16 hrs. Gels were rinsed in deionized water for 12 hrs to remove chloroquine and DNA was stained with ethidium bromide. DNA bands were visualized on a UV illuminator and photographs were taken with a Polaroid apparatus.

Bacterial strains. Following temperature-sensitive mutants of the *dnaA* gene and their parent strain (26–28) were kindly provided by Dr. T. Kogoma (University of New Mexico) through Dr. T. Katayama (Kyushu University). AQ5425 [*metE46 trp-3 his-4 thi-1 galK2 lacY1 or lacZ4 mtl-1 ara-9 tsx-3 ton-1 rpsL8 or rpsL9 supE44*] *l-dnaA*$^+$ AQ4370, AQ5425 *dnaA5;* AQ5448, AQ5425 *dnaA602;* AQ5449, AQ5425 *dnaA601;* AQ5450, AQ5425 *dnaA604;* AQ5480, AQ5425 *dnaA46;* AQ5481, AQ5425 *dnaA205;* AQ5482, AQ5425 *dnaA204;* AQ5483, AQ5425 *dnaA203;* AQ5484, AQ5425 *dnaA508.* P1 transduction of these AQ strains to W3110 cells was done as described elsewhere (15,29). P1 phages were grown in the *dnaA* mutants, transduced into W3110 cells and the transductants were selected on LB agar plates containing 10μg/ml tetracycline. Sensitivity to temperature (42°C) was examined to select *dnaA* mutants with the genetic background of W3110.

Plasmid. pHB10S plasmid which carries only the *dnaA* gene as a insert (29) was used for plasmid complementation analysis.

RESULTS

DNA Supercoiling in Various dnaA Mutants

Temperature-sensitive *dnaA* mutants have been isolated and areas of mutation were identified (26–28). In addition to growth phenotype at 42°C, these mutants carry the immotile phenotype at 37°C, a permissive temperature for growth (11). We examined the extent of DNA supercoiling in mutants growing at 37°C. We transformed the mutants and the wild type strain with pUC118, a plasmid which was used as a reporter for analysis of the DNA supercoiling (21,22). The plasmid was extracted using alkaline preparations (25) and the extent of supercoiling was analyzed by agarose gel electrophoresis in the presence of chloroquine. pUC118 DNA from all *dnaA* mutants tested migrated more slowly than that from the wild type cells in the presence of 15 μg/ml chloroquine (Figure 1(a)), thereby indicating that the linking number of plasmids in the *dnaA* mutants differed from that in the wild type cells. Furthermore, plasmids from the *dnaA* mutants migrated more rapidly than did those from the wild type cells, as seen using agarose gel electrophoresis in the presence of 1.5 μg/ml chloroquine (data not shown). DNA is negatively supercoiled in the gel containing 1.5 μ g/ml chloroquine and positively supercoiled in the gel containing 15 μg/ml chloroquine (22). Therefore, DNA from the *dnaA* mutants has a lower linking number than does DNA from the wild type cells. In other words, DNA is more negatively supercoiled in the *dnaA* mutants than in the wild type cells. Figure 1(a) also shows that the extent of DNA supercoiling differed among the *dnaA* mutants; AQ5481 harboring *dnaA205* mutation showed a less

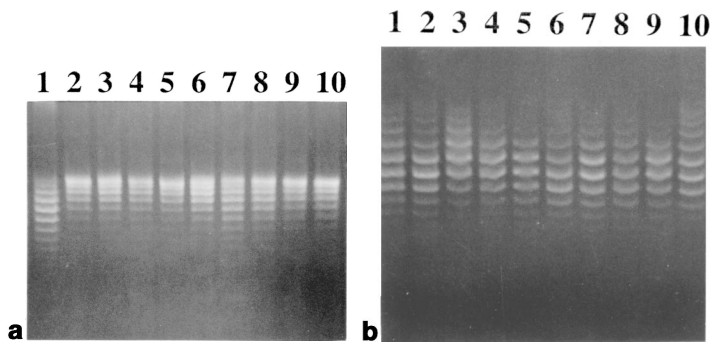

FIG. 1. DNA supercoiling in *dnaA* mutants and its isogenic parent. Exponentially growing cells harboring pUC118 plasmid at 37°C (a) or 28°C (b) were harvested by centrifugation when the optical density value of culture reached 1.5. DNA was extracted and analyzed by agarose gel electrophoresis in the presence of 15 μg/ml chloroquine. Lane 1, AQ5425 (*dnaA*$^+$); lane 2, AQ4370 (*dnaA5*); lane 3, AQ5448 (*dnaA602*); lane 4, AQ5449 (*dnaA601*); lane 5, AQ5450 (*dnaA604*); lane 6, AQ5480 (*dnaA46*); lane 7, AQ5481 (*dnaA205*); lane 8, AQ5482 (*dnaA204*); lane 9, AQ5483 (*dnaA203*); lane 10, AQ5484 (*dnaA508*).

negative supercoiling than seen in other mutants. Growth temperature greatly affected the linking number of DNA in the *dnaA* mutants. There was no significant difference in the linking number of plasmid DNA between *dnaA* mutants and wild type cells growing at 28°C (Figure 1(b)).

We next examined the influence of growth phases on DNA supercoiling in the *dnaA* mutants. The difference in the linking number of DNA between AQ4370, harboring the *dnaA5* mutation and wild type cells was apparent when cells were in a late log stage (Figure 2). The linking number in the *dnaA* mutant in an early log phase was much the same as that in the wild type cells. As growth proceeded, the linking number of DNA in the wild cells gradually increased, as noted by other workers (30, 31). Similar results were obtained for AQ5480, harboring the *dnaA46* mutation (data not shown).

Plasmid Complementation and P1-Mediated Transduction for the Phenotype of Highly Negative Supercoiling of DNA in the dnaA Mutants

To confirm that *dnaA* mutations are responsible for the highly negative supercoiling of DNA, we carried out P1-mediated transduction experiments for AQ4370 (*dnaA5*) and AQ5480 (*dnaA46*). P1 phages were grown in both *dnaA* mutants in which Tn10 was inserted close to the *dnaA* gene, and were used to transduce the mutant *dnaA* genes to W3110, carrying the wild type *dnaA* gene. Tetracycline-resistant transductants were selected and analyzed for temperature-sensitivity. Temperature-sensitive and temperature-resistant transductants were obtained, with the predicted ratio concerning the distance from the insertion position of Tn10 to the *dnaA* gene (26–28). We then examined the extent of DNA supercoiling in the transductants, and found that the linking number of plasmid DNA in temperature-sensitive transductants decreased compared to that in temperature-resistant transductants (data not shown). These observations suggest that mutations in the *dnaA5* and the *dnaA46* can explain decrease in the linking number of plasmid DNA with the genetic background of both AQ5425 and W3110.

To gain support for the notion that phenotype of the *dnaA* mutants for extensively negative DNA supercoiling was caused by *dnaA* mutations, we carried out complementation studies using the pHB10S plasmid carrying the wild type *dnaA* gene (29). When introduced into AQ4370 (*dnaA5*) and AQ5480 (*dnaA46*), pHB10S but not the vector pBR322 complemented the temperature-sensitive phenotype (data not shown). We also examined the linking number of plasmids in the transformants on agarose gel electrophoresis in the presence of 15 μg/ml chloroquine. The pHB10S plasmids in AQ4370 showed much the same linking number as that from AQ5425 (*dnaA*$^+$) (Figure 3) and similar results were observed with AQ5480 (data not shown). On the other hand, the linking

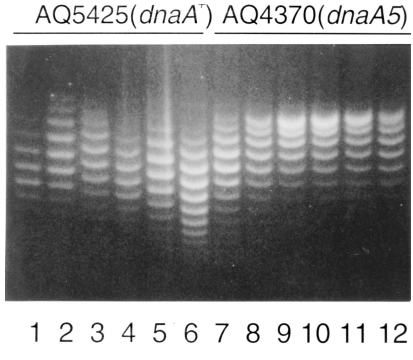

FIG. 2. DNA supercoiling in *dnaA5* mutants and the wild type cells at various stages of growth. Exponentially growing AQ5425 (*dnaA*$^+$) (lanes 1–6) and AQ4370 (*dnaA5*) (7–12) harboring pUC118 plasmid at 37°C were harvested by centrifugation when optical density of the culture reached 0.5 (lanes 1, 7), 1.2 (lanes 2, 8), 2.4 (lanes 3, 9), 3.1 (lanes 4, 10), 3.7 (lanes 5, 11), or 3.9 (lanes 6, 12). DNA was extracted and analyzed by agarose gel electrophoresis in the presence of 15 μg/ml chloroquine.

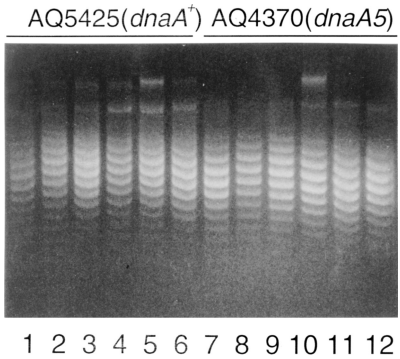

FIG. 3. Complementation analysis with a plasmid, pHB10S, carrying the wild type *dnaA* gene, for the phenotype of highly negative supercoiling of DNA in the *dnaA* mutant. Exponentially growing AQ5425 (*dnaA*$^+$) (lanes 1–6) and AQ4370 (*dnaA5*) (lanes 7–12) cells harboring pHB10S at 37°C were harvested by centrifugation when optical density of the culture reached 0.5 (lanes 1, 7), 1.0 (lanes 2, 8), 1.6 (lanes 3, 9), 2.2 (lanes 4, 10), 2.8 (lanes 5, 11), or 3.4 (lanes 6, 12). DNA was extracted and analyzed by agarose gel electrophoresis in the presence of 15 μg/ml chloroquine.

number of pBR322 in the *dnaA* mutants was lower than that in the wild type cells (data not shown). Thus, pHB10S but not pBR322 complemented the phenotype of the *dnaA* mutants for extensively negative DNA supercoiling. Based on the results of P1-mediated transduction and plasmid complementation, it seems apparent that highly negative supercoiling of DNA in the *dnaA* mutants is caused by mutations in the *dnaA* gene.

DISCUSSION

We obtained evidence that the linking number of plasmid DNA was lower in temperature-sensitive *dnaA* mutants than that in wild type cells. Complementation analysis with the plasmid which contains the wild type *dnaA* gene, and P1-mediated transduction analysis, revealed that the phenotype is caused by mutations in the *dnaA* gene.

The phenotype of the *dnaA* mutants for DNA supercoiling was apparent when cells were grown at 37°C. The linking number of DNA in the *dnaA* mutants was much the same as that in the wild type strain when cells were grown at 28°C. Expressions of some genes are greatly influenced by *dnaA* mutations when cells are grown at 37°C but not at 28°C (A. Ohba *et al.*, unpublished results). For example, we reported earlier that expression of the *fliC* gene is repressed in various *dnaA* mutants growing at 37°C (11). As expression of the *fliC* gene is inhibited by stress which alters the linking number of DNA in cells (16, 17, 23), the extensively negative supercoiling of DNA in the *dnaA* mutants may lead to repression of transcription of the *fliC* gene.

DnaA protein binds to DnaA boxes in the *oriC* region and opens-up the duplex DNA to lead the entrance of DnaB and DnaC proteins (3–5). The torsion stress driven be negative supercoiling of DNA may stimulate this opening-up reaction by DnaA protein. In other words, the highly supercoiled structure of DNA in the *dnaA* mutants might complement the weak activity of mutant DnaA protein to open-up the *oriC* region. This notion is supported by the finding that temperature-sensitive phenotype of *dnaA46* mutant is suppressed by the *topA* deletion mutation which decreases the linking number of DNA in cells (32). The phenotype of the *dnaA* mutants sensitive to nalidixic acid (33), an inhibitor of DNA gyrase, also can be explained by this notion. The possibility that the highly negative supercoiling of DNA in *dnaA* mutants is required for the initiation of chromosomal DNA replication that depends on function of DnaA protein deserves serious consideration.

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