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Loss of Flagellation in *dnaA* Mutants of *Escherichia coli*

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A *dnaA46* mutant of *Escherichia coli* showed loss of motility at 37°C, a permissive temperature for cell growth of this mutant. Other *dnaA* mutations near the middle of the gene also caused an immotile phenotype. The amount of flagellin was much less in the *dnaA46* mutant than in the wild-type control, as was the promoter activity. DnaA protein may play an important role in expression of the *fliC* gene.

Bacteria swim by the propeller function of flagella, and this motility is important for the cell to acquire nutrients (1, 2, 8). The formation of flagella is strictly regulated and is coupled with stages of cell proliferation. For example, flagellum formation initiates at a late period of exponential growth (17) and ceases when cells are exposed to various stresses which inhibit growth (11). Flagellin, a subunit of flagella, suppresses its own synthesis under conditions which are adverse. Therefore, cell proliferation and flagellum formation may be coregulated.

Nishimura and Hirota reported that some temperature-sensitive mutants of cell growth and cell division showed a defective phenotype of motility (13). Repression of flagellin synthesis as a result of mutations in heat shock protein genes, the *dnaK* and *dnaJ* genes, which are essential for chromosomal DNA replication, has also been reported (16). Therefore, mechanisms which regulate chromosomal DNA synthesis may affect the synthesis of flagellin. DnaA protein is the initiator of chromosomal DNA synthesis in *Escherichia coli* (5, 7). The abundance of this protein in cells (800 to 1,200 molecules per cell) (15) and the presence of DnaA boxes to which DnaA protein specifically binds in the upstream regions of various genes, including the *dnaA* gene itself (3, 4, 10, 19), suggest that DnaA protein acts as a transcription factor of various genes. On the basis of the thesis that DnaA protein may be involved in the cooperative regulation of cell growth and flagellin synthesis, we did a series of experiments, the descriptions of which are given herein.

dnaA mutants and their isogenic parent, AQ5425 (*metE46 trp-3 his-4 thi-1 galK2 lacY1* or *lacZ4 mtl-1 ara-9 tsx-3 ton-1 rpsL8* or *rpsL9 supE44 tnaA*::Tn*10* λ^-) (6), were kindly provided by T. Kogoma (University of New Mexico) via T. Katayama. pHB10 DNA with the wild-type *dnaA* gene (14) was kindly provided by Y. Sakakibara. *pfliC-lacZ* DNA and its vector were kindly provided by K. Kutsukake. *pfliC-lacZ* DNA was constructed by fusion of pFZY1 (9) and the promoter region of the *fliC* gene of *Salmonella typhimurium* (10a). Formation of swarm rings was observed in semisolid agar containing 1% tryptone, 0.5% NaCl, 50 μg of thymine per ml, and 0.3% agar (2). Each strain was cultured to the stationary phase in Luria-Bertani (LB) medium supplemented with 50 μg of thymine per ml. At the time of full growth, the suspension (2 μl) was placed on the swarm plate, and the preparation was incubated at 37°C for 6 h.

We compared the motility phenotype of the AQ5480 strain, with the *dnaA46* mutation, with that of AQ5425, the parent strain with the wild-type *dnaA* gene, by measuring diameters of swarm rings in soft agar plates. As shown in Fig. 1, the swarm ring formed with the *dnaA46* mutant was much smaller than that formed with the wild-type strain.

To confirm that the loss of motility in the AQ5480 strain was caused by the *dnaA* mutation, we transformed the strain with plasmid pHB10 DNA having the wild-type *dnaA* gene and examined the motility of the transformants. The transformants were highly motile in the swarm assay (Fig. 1), and recovery of motility was nil when the cells were transformed with the vector, pBR322 (data not shown). Transformation of the wild-type strain with pHB10 did not affect motility (data not shown).

We then examined whether the motility phenotype of the *dnaA46* mutation would be cotransducible with the temperature-sensitive phenotype in P1 transduction experiments. In the AQ5480 cells, Tn*10* with the tetracyline resistance marker is located at a position close to the *dnaA* gene. P1 phages were grown in AQ5480 cells, whose growth was restricted at 42°C, and transduced into W3110 cells, and the transductants were selected on tetracycline plates; the temperature sensitivity and motility phenotypes were then examined. Of 100 independent transductants, 21 were high temperature resistant and motile, and all of the remaining 79 were temperature sensitive and immotile. There was no transductant with either high-temperature-resistant and immotile or temperature-sensitive and motile phenotypes. Thus, the temperature-sensitive and immotile phenotypes of AQ5480 cells were cotransducible.

At 28°C, strain AQ5480 had much the same motility as wild-type cells in the swarm assay (data not shown). Since growth of the AQ5480 strain is temperature sensitive, there remained the possibility that the loss of motility was the result of inhibition of cell proliferation at 37°C. We then compared the growth of AQ5480 at 37°C with that of AQ5425. The time required for generation of these strains in liquid medium, determined either by measuring the number of viable cells which formed colonies on agar plates (Fig. 2) or by measuring culture absorbance, was approximately 60 min, and the size of colonies on agar plates of these strains grown at 37°C was indistinguishable. We isolated more than 10 independent suppressor mutants from the AQ5480 strain, with high-temperature-resistant (42°C) and immotile phenotypes. We carried out a transductional backcross of the *dnaA* region in the four independent revertants to W3110 cells having the wild-type *dnaA* gene. P1 phage was proliferated in the revertants and transduced into W3110 cells. Fifty independent transductants were isolated on tetracycline plates, and their temperature sensitivity and motility were examined. In all cases, 12 to

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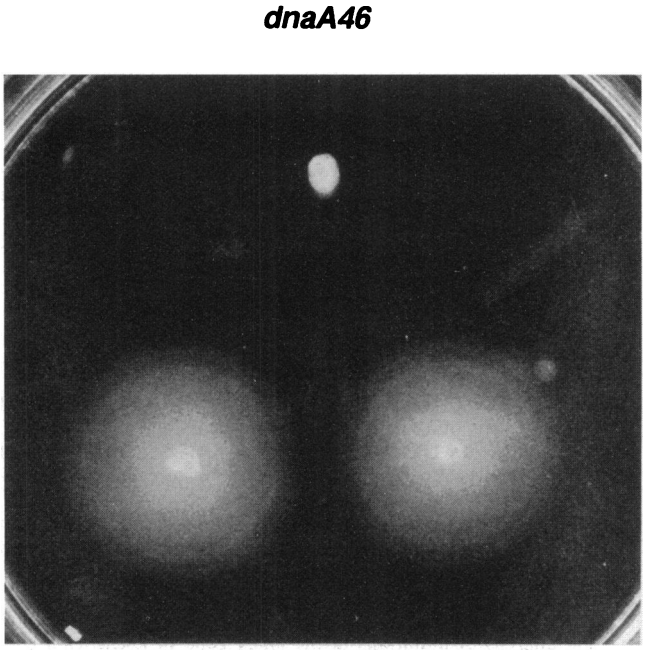

FIG. 1. Swarm plate assay. Full-growth suspensions of AQ5480 (*dnaA46*), AQ5425 (*dnaA*$^+$), and AQ5480 transformed with pHB10 (*dnaA46/dnaA*$^+$) were placed on a soft agar plate, incubated at 37°C, and then photographed.

15 transductants showed temperature-resistant and motile phenotypes, and other transductants showed temperature-sensitive and immotile phenotypes. These results suggest that all of these four revertants having high-temperature-resistant and immotile phenotypes carry an intergenic suppressor mutation of the *dnaA* gene. We also isolated motile revertants from the AQ5480 strains and examined their temperature-sensitive growth phenotype. Of 33 independent revertants, 8

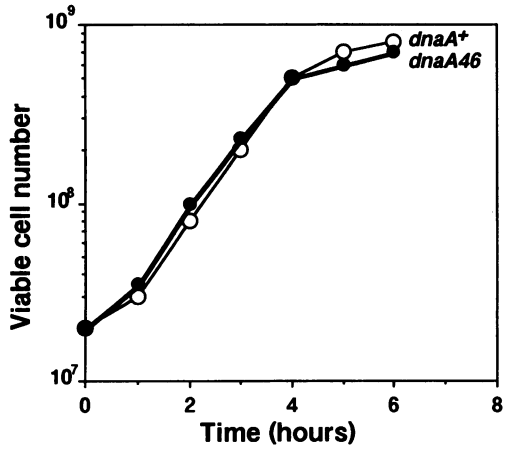

FIG. 2. Growth of AQ5480 (*dnaA46*) and AQ5425 (*dnaA*$^+$) cells at 37°C. Cells were grown in LB medium supplemented with 50 μg of thymine per ml at 37°C for the indicated time period. Appropriate dilutions of the culture were plated to monitor growth.

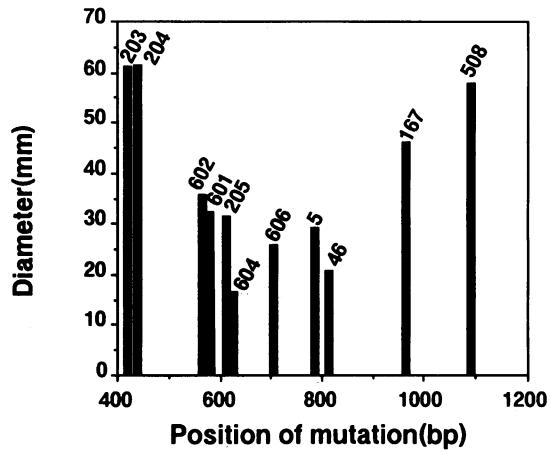

FIG. 3. Swarming abilities of various *dnaA* mutants. Diameters of swarm rings on the soft agar plates at 37°C after 6 h were measured. The diameter of the motility ring of the wild-type cells was 60 mm. The mutation positions of each *dnaA* mutant are from reference 6.

were temperature sensitive and 25 were temperature resistant. From these results, we concluded that the immotile phenotype had no accompanying temperature-sensitive phenotype.

dnaA mutants have been isolated, and their mutated sequence positions were determined. To observe the relationship between the location of mutations in the *dnaA* gene and the extent of the loss of motility phenotype, we examined the motility phenotypes of various *dnaA* mutants at 37°C. All of these mutants grew normally at 37°C but not at 42°C. As shown in Fig. 3, some *dnaA* mutations, including *dnaA46* and *dnaA604*, showed a clear phenotype of the loss of motility, whereas other *dnaA* mutations, including *dnaA203*, *dnaA204*, and *dnaA508* were motile. This means that the N- and C-terminal regions of DnaA protein are not likely to be required for the motile phenotype.

We then asked whether the immotility phenotype of AQ5480 is due to the lack of formation of flagella. Flagella can be released from cells by vigorous vortexing of the bacterial cell suspension (18). We analyzed flagellum fractions of the AQ5480 (*dnaA46*) and the AQ5425 (wild-type) cells grown at various temperatures. As shown in Fig. 4, the amount of flagellin, a subunit of flagella, in the AQ5480 cells growing at 37°C was much less than that in the AQ5425 cells. In the AQ5425 cells, the amount of flagellin was constant at temperatures between 28 and 37°C, and at temperatures below 31°C, a significant amount of flagellin was observed in the AQ5480 cells.

The synthesis of flagellin is regulated at the level of transcription (8). We examined the promoter activity of the *fliC* gene in AQ5480 cells by using the transcriptional fusion plasmid *pfliC-lacZ* containing the promoter of the *fliC* gene fused with the *lacZ* gene. Cells transformed with the fusion plasmid were grown at various temperatures, and β-galactosidase activities in the cell extracts were determined. As shown in Fig. 5, the promoter activity of the *fliC* gene in the AQ5480 cells decreased concomitant with temperature shift-up and yet was constant in the AQ5425 cells, despite a change in temperature. Extracts prepared from cells transformed with the vector showed a low level of enzyme activity, thereby indicating that β-galactosidase was synthesized, dependent on transcription from the *fliC* promoter of the fusion plasmid. These results suggest that the inhibition of flagellum synthesis in the *dnaA46*

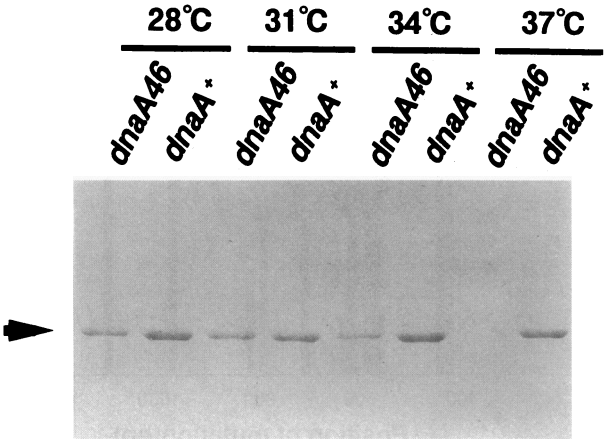

FIG. 4. Analysis of flagellum fractions by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. AQ5480 (*dnaA46*) and AQ5425 (*dnaA*$^+$) cells were grown in LB medium supplemented with 50 μg of thymine per ml at the various temperatures indicated until the optical density at 600 nm reached 0.5. The flagellum fraction was prepared by vigorous vortexing of cell suspensions and analyzed by SDS-polyacrylamide gel electrophoresis (18). The arrow indicates the migration position of flagellin.

mutant is due to the inhibition of transcription of the *fliC* gene. The possibility that the *lacZ* expression is regulated by some sort of posttranscriptional control would need to be ruled out.

In this article, we suggest that DnaA protein plays an important role in expression of the *fliC* gene. With regard to the manner in which DnaA protein participates in transcription of the *fliC* gene, two possibilities are being entertained:

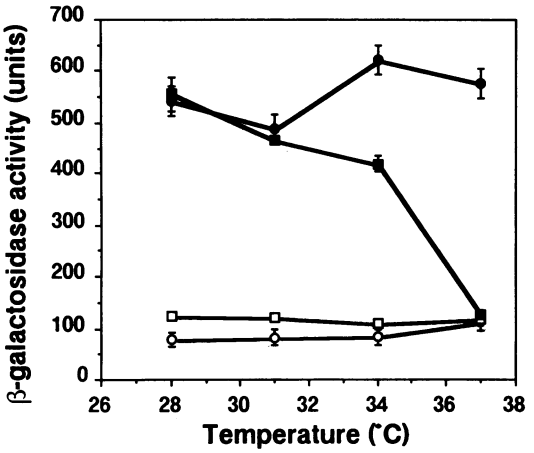

FIG. 5. Promoter activity of the *fliC* gene in a *dnaA46* mutant. AQ5480 (*dnaA46*) and AQ5425 (*dnaA*$^+$) cells were transformed with plasmid *pfliC-lacZ* with the promoter of the *fliC* gene fused to the structure gene of the *lacZ* gene. Cells were grown at various temperatures in LB medium and treated with toluene to extract β-galactosidase. β-Galactosidase activity in the extracts were determined by the method described by Miller (12). Average values of triplicates with standard deviations are presented. Symbols: ●, AQ5425 with *pfliC-lacZ*; ■, AQ5480 with *pfliC-lacZ*; ○, AQ5425 with the vector; □, AQ5480 with the vector.

one is that DnaA protein acts as a transcription factor of the *fliC* gene or other regulatory genes of flagellation, and the other is that adverse effects on DNA synthesis caused by *dnaA* mutations reduce expression of the *fliC* gene, even though the period of generation of cell growth is not prolonged.

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