Amounts of proteins altered by mutations in the *dnaA* gene of *Escherichia coli*

Akiko Ohba, Tohru Mizushima, Tsutomu Katayama, Kazuhisa Sekimizu*

Faculty of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka 812-82, Japan

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Abstract We identified proteins whose amounts were altered in a temperature-sensitive *dnaA46* mutant of *Escherichia coli*. Proteins whose amounts were increased in the mutant were serine hydroxymethyltransferase, β-ketoacyl [acyl carrier protein] synthase II, long-chain fatty acid transport protein, and UDP-glucose 4-epimerase, while the decreased ones were flagellin and D-ribose-binding protein. Transformation of the mutant with a plasmid containing the wild type *dnaA* gene complemented the phenotype. As pulse-labeling experiments revealed that the rates of synthesis of the proteins were altered in the mutant, DnaA protein may be involved in expression of these proteins.

Key words: DnaA protein; Two-dimensional polyacrylamide gel electrophoresis; Gene expression; *Escherichia coli*

1. Introduction

DnaA protein is the initiation factor of chromosomal DNA replication in *Escherichia coli* [1,2]. The initiation of DNA replication is assumed to be regulated by alteration in the activity of DnaA protein, a protein with a high affinity for ATP and ADP [3]. The ATP-binding form is active in *oriC* DNA replication reconstituted from purified proteins, whereas the ADP-binding form is inert [3]. Recently, we reported that an organic compound designed to inhibit the ATP-binding capacity of DnaA protein inhibited in vitro *oriC* DNA replication [4]. These results suggest that the activity of DnaA protein is regulated by its adenine-nucleotide binding. Therefore, factors which affect inter-conversion between the ATP-binding form and the ADP-binding form of DnaA protein may regulate DNA replication. Acidic phospholipids which facilitate the exchange of ADP bound to DnaA protein with ATP may function as regulators [5–8].

In addition to initiation of DNA replication, DnaA protein negatively regulates expression of various genes, such as the *dnaA, mioC, rpoH, guaBA*, and *uvrB* genes, through specific binding to DnaA boxes located at the 5′-upstream regulatory region or coding region of these genes [9]. DnaA protein may also be involved in termination of transcription of some genes [9]. About 1600 DnaA boxes are estimated to be located on *Escherichia coli* chromosome DNA [10], suggesting that many other genes are regulated by DnaA protein through its specific binding to DNA. On the other hand, we reported that purified DnaA protein has activity which alters DNA topology through its non-specific binding to DNA [11]. We also found an increase in the DNA supercoiling of plasmid DNA in *dnaA* mutants [12]. These results suggest that DnaA protein affects DNA topology in cells. We also reported that expression of the *fliC* gene, encoding flagellin, is suppressed in various *dnaA* mutants [13]. As expression of the *fliC* gene is inhibited by stress which alters DNA supercoiling in cells [14,15], we proposed that the decrease in expression of the *fliC* gene in the mutants is caused by alteration in DNA supercoiling [12]. DNA supercoiling profoundly affects the transcription of various genes other than the *fliC* gene [16–18]. Therefore, expression of a number of genes may be regulated by DnaA protein via alteration in DNA supercoiling.

We assumed that proteins the expression of which is regulated by DnaA protein would be involved in regulation of the activity of DnaA protein. Therefore, screening of proteins whose expression is regulated by DnaA protein will yield pertinent information on regulatory proteins of DNA replication in *Escherichia coli*. We searched for proteins whose expression increases or decreases in *dnaA* mutants, using two-dimensional polyacrylamide gel electrophoresis, and six proteins with altered expression in *dnaA* mutants were identified.

2. Materials and methods

2.1. Bacterial strains

Temperature-sensitive mutants of the *dnaA* gene and the wild type strain were from our laboratory stock [19]. pHB10S plasmid, which carries only the *dnaA* gene as a insert [19], was used for plasmid complementation analysis.

2.2. Growth conditions

Escherichia coli cells were grown in LB medium containing 50 μg/ml thymine until optical density at 660 nm reached 0.5, then were harvested by centrifugation.

2.3. Two-dimensional gel analysis of proteins

Crude extracts of bacteria cells were prepared as described [20], but with some modifications. Cells suspended in sonication buffer [20] were disrupted by sonication and centrifuged. The supernatant was diluted in lysis buffer [20] to 2 mg/ml protein, following incubation with 50 μg/ml DNase I at 0°C for 10 min. Two-dimensional gel electrophoresis was performed as described elsewhere [21]: first dimension, non-equilibrium pH gradient electrophoresis (1000 V, 4 h), 2% pH 3.5–10 ampholine mixture; second dimension, SDS-polyacrylamide gel (10%) electrophoresis. Proteins were stained with Coomassie brilliant blue R-250.

2.4. Purification and identification of proteins

Proteins separated by two-dimensional gel electrophoresis were eluted from gel in SDS sample buffer (2% SDS, 4% 2-mercaptoethanol, 20% (v/v) glycerol, 20 mM Tris-HCl (pH 7.9), 0.16 mg/ml bromphenol blue). Samples were applied to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Millipore). Membranes were applied to an auto amino acid sequencer (473A protein sequencer, Applied Biosystems) and the obtained N-terminal sequences were analyzed using the Swiss-Prot data base.

*Corresponding author. Fax: (81) (92) 632-6648.
E-mail: sekimizu@bisei.phar.kyushu-u.ac.jp

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2.5. Protein pulse-labeling analysis

Cells were incubated with 5 mCi/ml ^{35}S-labeling mixture (70% methionine+30% cysteine) for 5 min. Samples were prepared as described in Section 2.4. The radioactivity of each sample was counted in a liquid scintillator (Beckman), and samples with the same amount of radioactivity were analyzed by two-dimensional gel electrophoresis. Gels were soaked in an enhancer solution (Beckman) for 30 min followed by fluorography.

3. Results

3.1. Two-dimensional gel analysis of proteins in dnaA mutants

A number of temperature-sensitive *dnaA* mutants have been isolated [22–24]. In addition to the growth-deficient phenotype at 42°C, these mutants show a decreased expression of the *fliC* gene [13] at 37°C, a permissive temperature for growth. The result suggested that mutant DnaA protein maintains the activity for DNA replication but its transcriptional activity is altered at 37°C. As we assumed that proteins whose expression is regulated by DnaA protein would be likely to increase or decrease in the temperature-sensitive *dnaA* mutants growing at 37°C, we compared the expression pattern of proteins in KS1003 (*dnaA46*) to that in KS1001 (wild type) using two-dimensional polyacrylamide gel electrophoresis. We identified four protein spots (spots #2, #3, #4, #6), the amounts of which were markedly higher in the *dnaA46* mutant (Fig. 1A). We also identified two protein spots (spots #1, #5) with amounts much lower in the mutant (Fig. 1A). The apparent molecular masses of these proteins were determined to be 52 kDa (#1), 45 kDa (#2), 44 kDa (#3), 43 kDa (#4), 30 kDa (#5), and 37 kDa (#6), based on their migration on gel electrophoresis. Densitometric scanning revealed that the amounts of these proteins were 4–7 times higher (#2, #3, #4, #6) or lower (#1, #5) in the mutant than in the wild type cells (Fig. 1B). At 28°C, the expression pattern of proteins was indistinguishable between the *dnaA* mutant and wild type cells (data not shown). Similar results were obtained for other temperature-sensitive *dnaA* mutants (*dnaA5, dnaA167, dnaA203, dnaA508, dnaA601, dnaA602,* and *dnaA604* mutants) (data not shown). To confirm that alteration in the expression of these proteins in the *dnaA* mutants was caused by *dnaA* mutation, plasmid complementation analysis was performed. When the pHB10S plasmid which contains the wild type *dnaA* gene [19] was introduced into the *dnaA46* mutant, the expression pattern of proteins became much the same as that of wild type cells (Fig. 1). The vector, pBR322, did not complement the phenotype of the *dnaA46* mutant (data not shown). Alteration of the protein pattern in other temperature-sensitive *dnaA* mutants was also complemented by pHB10S plasmid (data not shown). These results are taken to mean that alteration in the expression pattern of proteins in the *dnaA* mutants is due to the mutations in the *dnaA* gene.

3.2. Determination of the N-terminal amino acid sequence of the proteins

To identify the proteins with expression altered by the *dnaA* mutations, we recovered the proteins from the gel and determined their N-terminal amino acid sequences. More than 11 amino acid residues were determined for each protein and a homology search was done using the Swiss-Prot data base. The determined amino acid sequences of each protein were

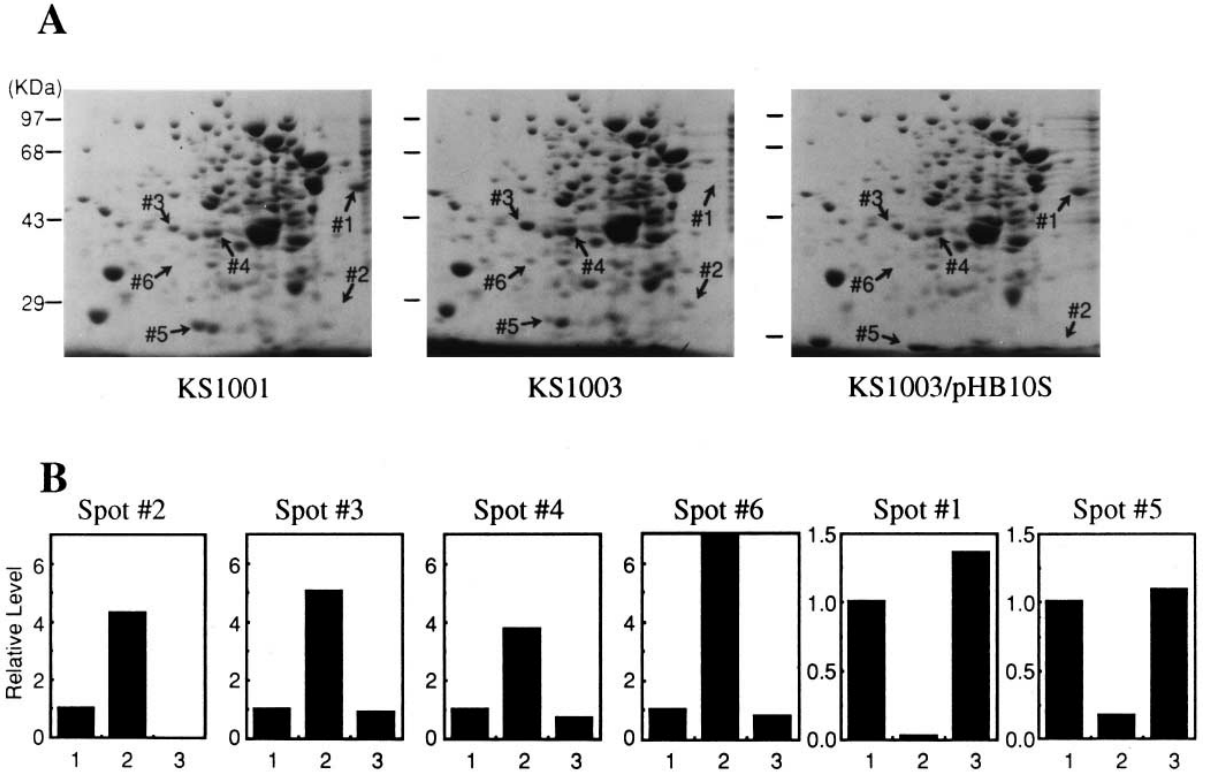

Fig. 1. Two-dimensional gel analysis of proteins in the *dnaA46* mutant. A: The crude extracts with the same amount of protein (120 μg) prepared from bacteria cells grown at 37°C were applied to two-dimensional gel electrophoresis. The arrows indicate the position of proteins, the amounts of which were altered in the mutant. B: The amount of each protein was determined by densitometric scanning of gel and relative values to KS1001 are shown. 1, KS1001 (wild type); 2, KS1003 (*dnaA46* mutant); 3, KS1003 harboring the pHB10S plasmid.

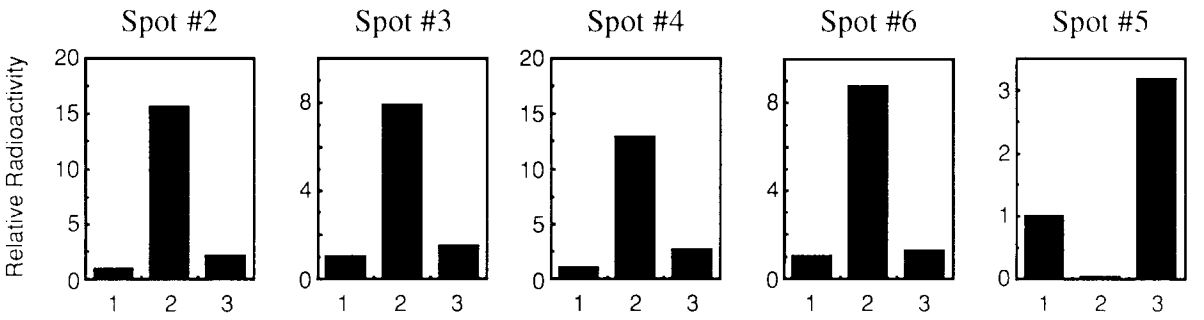

Fig. 2. Pulse-labeling analysis of proteins. Cells were incubated with 5 mCi/ml ^{35}S-labeling mixture (70% methionine+30% cysteine) for 5 min. Crude extracts with the same amount of radioactivity (240 000 cpm) were analyzed by two-dimensional gel electrophoresis. The radioactivity of each spot was determined and relative synthesis rate of each protein to KS1001 is shown. 1, KS1001 (wild type); 2, KS1003 (*dnaA46* mutant); 3, KS1003 harboring the pHB10S plasmid.

identical to those of proteins which were reported on earlier (Table 1). The molecular masses of these proteins were much the same as those we determined by gel electrophoresis (Table 1). From these results, we identified these proteins: flagellin (#1) [25], long-chain fatty acid transport protein (#2) [26], serine hydroxymethyltransferase (#3) [27], β-ketoacyl [acyl carrier protein] synthase II (#4) [28,29], D-ribose binding protein (#5) [30], and UDP-glucose 4-epimerase (#6) [31] (Table 1). We have earlier reported a decrease in the amount of flagellin in the *dnaA* mutants [13]. By examination of the DNA sequence of the 5′-upstream region and coding region of genes which encode these proteins, using the GenBank data base, one DnaA box (TTATACAAA) was present in the 5′-upstream region of the *fadL* gene which encodes a long-chain fatty acid transport protein. A DnaA box in 5′-upstream regions and coding regions of other genes was never evident.

3.3. *Pulse-labeling of proteins in dnaA mutants*

We reported a decrease in the synthesis of flagellin in *dnaA* mutants [13]. In the present study, we compared rates of synthesis of other proteins (#2, #3, #4, #5, #6) in the *dnaA46* mutant and in wild type cells by a pulse-labeling experiment. The amounts of radioactivity incorporated into the proteins #2, #3, #4, and #6 were 8–15 times higher and that into protein #5 was one-fiftieth in the *dnaA46* mutant compared with those in the wild type cells (Fig. 2). The result suggests that alteration in the amounts of these proteins in the *dnaA46* mutant (Fig. 1) is due to changes in rates of synthesis of these proteins.

4. Discussion

Two-dimensional gel analysis of proteins in *dnaA* mutants identified six proteins, the amounts of which were altered in the mutant. As alteration in expression pattern of proteins in *dnaA* mutants was complemented by a plasmid containing the wild type *dnaA* gene, the *dnaA* mutations are responsible for the events.

The DnaA box exists in the 5′-upstream region of the *fadL* gene which encodes a long-chain fatty acid transport protein. As the specific binding of DnaA protein to DnaA boxes which are located in the promoter region of various genes inhibits the initiation of transcription of the genes [9], the increase in the content of long-chain fatty acid transport protein in the *dnaA* mutations may be due to inhibition of the negative regulation of the *fadL* gene by DnaA protein. Examination of the effect of disruption of the DnaA box in the 5′-upstream region of the *fadL* gene on the expression of the *fadL* gene seems to be important to support this notion. On the other hand, a DnaA box was not evident in other genes. Therefore, a mechanism other than inhibitory action of DnaA protein on the transcription via DnaA box-dependent specific binding to

Table 1
Identification of proteins

No.[a]	Protein	Gene	Molecular mass[b]		Sequence[c]
			A	B	
1	Flagellin	*fliC*	52 000	51 163	AQVINTNSLSLITQ **********
2	Long-chain fatty acid transport protein	*fadL*	44 500	45 969	AGXQLNEFSXXGLGR **F******SS****
3	Serine hydroxymethyl transferase	*glyA*	44 000	45 625	MLKREMNLIADY **********
4	β-Ketoacyl [acyl carrier protein] synthase II	*fabF*	43 000	42 914	SSRRVVVTGLGMLSP **********
5	D-Ribose binding periplasmic protein	*rbsB*	29 500	30 950	KDTIALVVSTLNXPF **********N**
6	UDP-glucose 4-epimerase	*galE*	37 000	37 265	MRVLVTGGSGYIGSH **********

[a]Proteins are numbered as described in Fig. 1.
[b]A, the molecular mass of the proteins determined by their migration on gel electrophoresis. B, the molecular mass of the proteins previously reported.
[c]Upper row, amino acid sequence determined by direct protein sequence; lower row, amino acid sequence from Swiss-Prot data base. *, identical amino acid; X, amino acid not determined.

DNA is involved in alteration of the contents of these proteins in the mutants. An increase in supercoiling of DNA in *dnaA* mutants was apparent when cells were grown at 37°C but not at 28°C [12], thus corresponding to the effect of mutations on the expression pattern of proteins. DNA supercoiling greatly affects the expression of various genes [16–18]. Thus, we consider that perhaps the alteration of contents of these proteins other than long-chain fatty acid transport protein is caused by an increase in the supercoiling of DNA by the *dnaA* mutations. With regard to the *fliC* gene encoding flagellin, the expression is thought to be greatly affected by a change in DNA supercoiling [14,15]. There is a possibility that the *dnaA* mutations give adverse effects on DNA synthesis, even though the period of generation of cell growth is not prolonged [13]. Therefore, a notion that the alteration of content of these proteins is caused by adverse effects should be considered.

We proposed that the activity of DnaA protein is regulated by membrane phospholipids in cells [5,8], since acidic phospholipids, such as cardiolipin and phosphatidylglycerol, activate the ADP-binding form of DnaA protein in vitro [5–8]. This notion is supported by recent genetic studies [19,32,33]. From this point of view, the increase in the amounts of long-chain fatty acid transport protein and β-ketoacyl [acyl carrier protein] synthase II, both of which are involved in phospholipid metabolism, is important. We consider that alteration of expression of these two proteins is involved in the adaptation of the mutant to grow at 37°C through modulation of the activity of the mutant DnaA protein. Increase in the proteins may alter the fatty acid composition of the cell membrane which in turn affects interactions between DnaA protein and the membrane. Actually, we have data that the composition of fatty acids of phospholipids on membranes is drastically altered by *dnaA* mutations when cells were grown at 37°C but not at 28°C (Suzuki et al., manuscript in preparation).

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References

[1] Hirota, Y., Mordoh, J. and Jacob, F. (1970) J. Mol. Biol. 53, 369–387.
[2] Fuller, S.R. and Kornberg, A. (1983) Proc. Natl. Acad. Sci. USA 80, 5817–5821.
[3] Sekimizu, K., Bramhill, D. and Kornberg, A. (1987) Cell 50, 259–265.
[4] Mizushima, T., Sasaki, S., Ohishi, M., Kobayashi, M., Katayama, T., Miki, T., Maeda, M. and Sekimizu, K.J. (1996) Biol. Chem. 271, 25178–25183.
[5] Sekimizu, K. and Kornberg, A. (1988) J. Biol. Chem. 263, 7131–7135.
[6] Yung, B.Y. and Kornberg, A. (1988) Proc. Natl. Acad. Sci. USA 85, 7202–7205.
[7] Castuma, C.E., Crooke, E.C. and Kornberg, A. (1993) J. Biol. Chem. 268, 24665–24668.
[8] Mizushima, T., Ishikawa, Y., Obana, E., Hase, M., Kubota, T., Katayama, T., Kunitake, T., Watanabe, E. and Sekimizu, K. (1996) J. Biol. Chem., 271, 3633–3638.
[9] Skarstad, K. and Boye, E. (1994) Biochim. Biophys. Acta 1217, 111–130.
[10] Schaefer, C. and Messer, W. (1991) Mol. Gen. Genet. 266, 34–40.
[11] Mizushima, T., Katayama, T. and Sekimizu, K. (1996) Biochemistry 35, 11512–11516.
[12] Mizushima, T., Tanino, J., Miki, T. and Sekimizu, K. (1996) Biochem. Biophys. Res. Commun. 218, 137–141.
[13] Mizushima, T., Tomura, A., Shinpuku, T., Miki, T. and Sekimizu, K. (1994) J. Bacteriol. 176, 5544–5546.
[14] Shi, W., Li, C., Louise, C.J. and Adler, J. (1993) J. Bacteriol. 175, 2236–2240.
[15] Mizushima, T., Natori, S. and Sekimizu, K. (1993) Mol. Gen. Genet. 238, 1–5.
[16] Higgins, C.F., Dorman, C.J., Stirling, D.A., Waddell, L., Booth, I.R., May, G. and Bremer, E. (1988) Cell 52, 569–584.
[17] Dorman, C.J., Barr, G.C., NiBhrian, N. and Higgins, C.F. (1988) J. Bacteriol. 170, 2816–2826.
[18] Dorman, C.J., NiBhrian, N. and Higgins, C.F. (1990) Nature 344, 789–792.
[19] Shinpuku, T., Mizushima, T., Guo, L., Miki, T. and Sekimizu, K. (1995) Biochem. Biophys. Res. Commun. 212, 84–89.
[20] O'Farrell, P., H. (1975) J. Biol. Chem. 250, 4007–4021.
[21] O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) Cell 12, 1133–1142.
[22] Hansen, E.B., Atlung, T., Hansen, F.G., Skovgaard, O. and Meyenburg, K. (1984) Mol. Gen. Genet. 196, 387–396.
[23] Hansen, F.G., Koefoed, S. and Atlung, T. (1992) Mol. Gen. Genet. 234, 14–21.
[24] Hansen, F.G. and Meyenburg, K. (1979) Mol. Gen. Genet. 175, 135–144.
[25] Kuwajima, G., Asaka, J., Fujiwara, T., Fujiwara, T., Node, K. and Kondo, E. (1986) J. Bacteriol. 168, 1479–1483.
[26] Black, P.N. (1991) J. Bacteriol. 173, 435–442.
[27] Plamann, M.D., Stauffer, L.T., Urbanowski, M.L. and Stauffer, G.V. (1983) Nucleic Acids Res. 11, 2065–2075.
[28] Siggaard-Andersen, M., Wissenbach, M., Chuck, J.A., Svendsen, I., Olsen, J.G. and Wettstein-Knowles, P. (1994) Proc. Natl. Acad. Sci. USA 91, 11027–11031.
[29] Magnuson, K., Carey, M.R. and Cronan, J.E. (1995) J. Bacteriol. 177, 3593–3595.
[30] Groarke, J.M., Mahoney, W.C., Hope, J.N., Furlong, C.E., Robb, F.T., Zalkin, H. and Hermodson, M.A. (1983) J. Biol. Chem. 258, 12952–12956.
[31] Lemaire, H.G. and Muller-Hill, B. (1986) Nucleic Acids Res. 14, 7705–7711.
[32] Xia, W. and Dowhan, W. (1995) Proc. Natl. Acad. Sci. USA, 783–787.
[33] Mizushima, T., Shinpuku, T., Katayama, H., Kataoka, K., Guo, L., Miki, T. and Sekimizu, K. (1996) Mol. Gen. Genet. 252, 212–215.