Suppression of temperature-sensitivity of a *dnaA46* mutant by excessive DNA supercoiling

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We report here that the high-temperature sensitivity of a *dnaA46* mutant was suppressed by addition of high concentrations of NaCl into the culture medium. This suppression was also observed with other high-temperature-sensitive *dnaA* mutants, except *dnaA167* and *dnaA508* mutants, which have mutations in the N-terminal region of DnaA protein. Since high concentrations of NaCl in the medium increased negative DNA supercoiling in a *dnaA46* mutant, we hypothesized that the increase in DNA supercoiling is involved in the suppression of the temperature-sensitivity of the *dnaA46* mutant by high concentrations of NaCl.

This hypothesis was supported by *in vitro* and *in vivo* results as follows. A low DNA replication activity of purified DnaA46 protein at high temperatures was increased in line with an increase in DNA supercoiling of template DNA. The *dnaA46* mutant showed higher sensitivity to nalidixic acid, a DNA-relaxing drug, than did the wild-type cells under the conditions of high temperatures and high concentrations of NaCl.

Key words: DNA replication, DnaA protein, *Escherichia coli*, linking number, nalidixic acid.

INTRODUCTION

Initiation of chromosomal DNA replication is regulated by various mechanisms, including the control of the activity of initiator proteins. In *Escherichia coli*, control of the activity of DnaA protein (the initiator of chromosomal DNA replication) by its adenine-nucleotide binding seems to play an important role in the regulation of the initiation of DNA replication [1]. DnaA protein has high affinities for ATP and ADP, and the ATP-binding form is the active form for DNA replication [2]. Recent genetic and biochemical studies strongly suggest that adenine-nucleotide binding to DnaA protein regulates the initiation of chromosomal DNA replication in *E. coli* cells [3–10]. However, some other mechanisms should be investigated in order to understand completely the regulation of DNA replication. To identify such a novel mechanism, one approach would be to identify conditions under which temperature-sensitivity of a *dnaA* mutant is suppressed.

In cells, chromosomal DNA is negatively supercoiled. The extent of DNA supercoiling is altered according to changes in intracellular or extracellular conditions, resulting in changes in various DNA transactions, such as transcription and recombination [11–13]. Recent genetic and biochemical studies strongly suggest that changes in DNA supercoiling affect the initiation of DNA replication in cells, as follows. *In vitro*, the DNA replication reaction requires the negative supercoiling of template DNA [14]. *In vivo*, various environmental stresses, which decreased DNA supercoiling, inhibited DNA replication [15]. We suggested a close relationship between DnaA protein and DNA supercoiling, as follows. Some *dnaA* mutants show much higher DNA supercoiling than do the wild-type cells [16]. DnaA protein itself has an ability to change DNA supercoiling *in vitro* [17].

DnaA protein specifically binds to the origin of DNA replication and forms oligomers [1]. ATP binding to DnaA protein is required for the oligomerization [2,6]. The torsional stress on DNA caused by this oligomerization is thought to open up the duplex DNA at *oriC* (the origin of chromosomal DNA replication)

to initiate DNA replication. An increase in negative DNA supercoiling also put a torsional stress on DNA, which may open up duplex DNA. Thus we considered that excessive DNA supercoiling assists DnaA protein to open up the duplex DNA at the origin. In other words, inhibition of initiation of DNA replication caused by a low duplex-opening activity of DnaA proteins (such as in some high-temperature-sensitive *dnaA* mutants at high temperatures) may be suppressed by an increase in DNA supercoiling of chromosomal DNA. In the present study we showed that addition of high concentrations of NaCl in the medium caused suppression of the high-temperature-sensitivity of a *dnaA46* mutant and excessive DNA supercoiling in cells. Our genetic and biochemical results suggest that excessive DNA supercoiling by high concentrations of NaCl causes this suppression in cells.

MATERIALS AND METHODS

Materials

A crude extract for an *oriC* complementation assay was prepared from the WM433 strain of *E. coli* as previously described [18]. The wild-type DnaA and DnaA46 proteins were purified as described [19]. KS1001 (W3110 *dnaA*⁺ *tnaA*::Tn10 *lac*⁺), KS1002 (W3110 *dnaA5*), KS1003 (W3110 *dnaA46*), KS1004 (W3110 *dnaA167*), KS1006 (W3110 *dnaA205*), KS1007 (W3110 *dnaA508*), KS1008 (W3110 *dnaA601*), and KS1009 (W3110 *dnaA604*) were from our laboratory stocks [20–22].

Luria-broth (LB) medium [1% tryptone and 0.5% yeast extract (both from Difco)] was used for all experiments. In the present paper, the concentration of salt stated is the total concentration, not that added.

oriC DNA replication in a crude extract

Replication of minichromosomes in a crude extract (Fraction II) was assayed as described in [18] with some modifications.

Abbreviation used: LB, Luria broth.

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Template DNA (pTB101) [23] (600 pmol as nucleotides), 240 μ g of Fraction II from WM433 (*dnaA204*) and DnaA protein were mixed with reaction cocktails [18] and incubated for replication at 40 °C for 10 min. The reaction was terminated by chilling on ice and adding 10% trichloroacetic acid. Samples were passed through Whatman GF/C glass-fibre filters. The amount of radioactivity on the filters was measured in a liquid-scintillation counter, and the amount of DNA synthesized (pmol of nucleotides) was calculated [18].

Analysis of supercoiling of plasmid DNA in cells

Supercoiling of plasmid DNA in cells was measured by an agarose-gel-electrophoresis method [24,25]. Briefly, *E. coli* cells harbouring the plasmid pBR322 were harvested by centrifugation at 4 °C for 5 min, followed by washing with 0.9% NaCl. Plasmid DNA was extracted and purified by the alkaline method. The samples were analysed by 1%-agarose-gel electrophoresis in the presence of chloroquine. Electrophoresis was carried out in TBE buffer [0.089 M Tris/borate (pH8.0)/2 mM EDTA] at 60 V for 16 h. Gels were rinsed in deionized water for 5 h to remove chloroquine, and DNA was stained with 1 μ g/ml ethidium bromide. DNA bands were revealed under a UV illuminator, and photographs were taken with a Polaroid apparatus.

RESULTS AND DISCUSSION

Suppression of a high-temperature-sensitivity of a *dnaA46* mutant

In order to test the hypothesis that excessive DNA supercoiling suppresses a low activity of DnaA protein for the duplex DNA opening, we used a temperature-sensitive *dnaA46* mutant and a culture medium containing high concentrations of NaCl. DnaA46 protein has mutations in the ATP-binding site, and ATP binding to DnaA protein is essential for the duplex opening at the origin [1,2,6]. High concentrations of NaCl in the medium were shown to induce excessive DNA supercoiling in wild-type cells [26]. The colony-formation abilities of the wild-type (KS1001) and the *dnaA46* (KS1003) strains were examined at 30 or 40 °C in the presence of various concentrations of NaCl. As

Figure 1 Suppression of the temperature-sensitivity of the *dnaA46* mutant by high concentrations of NaCl

KS1001 (*dnaA*⁺), KS1003 (*dnaA46*), and KS1008 (*dnaA508*) strains were grown to the stationary phase at 30 °C in LB medium without NaCl. The suspension was diluted with the same medium and spread on LB agar plates containing the indicated concentrations of NaCl and incubated at 30 or 40 °C for 24 h. The number of viable cells per ml of original culture suspension was calculated and is shown as the colony-formation ability (10¹ = 10¹ etc.).

Figure 2 Effect of high concentrations of NaCl on the temperature-sensitivities of various *dnaA* mutants

The colony-formation ability of each *dnaA* mutant was examined on LB agar plates with or without 0.51 M NaCl at 40 °C as described in the legend to Figure 1. The ratio of the colony-formation ability (with 0.51–0 M NaCl) is shown as the relative colony-formation ability (10⁻⁴ = 10⁻⁴ etc.). Mutants were arranged in order of their mutation positions (left, N-terminal; right, C-terminal).

shown in Figure 1, the colony-formation ability of the *dnaA46* mutant at 40 °C was increased depending on the concentration of NaCl in the medium. In the presence of 0.68 M NaCl, the colony-formation ability was almost indistinguishable between the *dnaA46* and the wild-type strains. The colony-formation abilities of the *dnaA46* mutant at 30 °C and of the wild-type cells both at 30 °C and 40 °C were not affected by the presence of NaCl in the medium (Figure 1). Suppression was also observed at 39 °C and 41 °C in the *dnaA46* mutant (results not shown). These results indicate that the temperature-sensitivity of the *dnaA46* mutant is suppressed by high concentrations of NaCl.

We next asked whether the suppression of the temperature-sensitivity of the *dnaA46* mutant is specific for NaCl. The change in DNA supercoiling by high concentrations of NaCl is due to the alteration in osmotic pressure in the medium, because the alteration in osmotic pressure by other reagents (such as glucose) also caused a change in DNA supercoiling in cells [26]. We examined the effect of KCl, ammonium sulphate and glucose on the colony-formation ability of the *dnaA46* mutant at 40 °C and found that they all could suppress the temperature-sensitivity of the *dnaA46* mutant to much the same extent as NaCl (results not shown). These results suggest that a change in osmotic pressure in the medium suppresses the temperature-sensitivity of the *dnaA46* mutant by these chemicals and thus that the suppression is due to the increase in DNA supercoiling in cells.

Effect of high concentrations of NaCl on the temperature-sensitivity of other *dnaA* mutants

A number of temperature-sensitive *dnaA* mutants have been isolated and the mutations have been identified [1]. In order to examine the allele specificity of the suppression of temperature-sensitivity of *dnaA* mutants by high concentrations of NaCl, we investigated the effect of high concentrations of NaCl on the temperature-sensitivities of various *dnaA* mutants (Figure 2). Temperature-sensitivities of mutants with mutations in the ATP-binding site (*dnaA5*, *dnaA601* and *dnaA604*) were suppressed by

Figure 3 DNA supercoiling by high concentrations of NaCl in cells

KS1001 (*dnaA*⁺), KS1003 (*dnaA46*), and KS1008 (*dnaA508*) strains harbouring the pBR322 plasmid were grown at 40 °C in LB medium containing the indicated concentrations of NaCl until the A_{600} reached 1.0. Cells were collected by centrifugation and plasmid DNA was extracted and analysed by agarose-gel electrophoresis in the presence of 3 μ g/ml chloroquine as described in the Materials and methods section.

0.51 M NaCl as much as was the *dnaA46* mutant, which also has mutations in this region. The temperature-sensitivity of the *dnaA205* mutant, which has a mutation in the DNA-binding domain (C-terminal region), was partly suppressed. In contrast, the *dnaA508* and *dnaA167* mutants, which have mutations in the N-terminal region of DnaA protein, showed the opposite results. The colony-formation abilities of these mutants were decreased in the presence 0.51 M NaCl (Figure 2). The decrease in the colony-formation ability of the *dnaA508* mutant is dependent on the concentration of NaCl and was not observed at 30 °C (Figure 1). These results clearly show the allele specificity of *dnaA* mutants for the suppression by high concentrations of NaCl. High concentrations of NaCl may affect the function of DnaA protein, which is related to the ATP-binding activity of this protein (such as the duplex DNA opening).

Effect of high concentrations of NaCl on DNA supercoiling in the *dnaA46* and *dnaA508* mutants

As mentioned above, we consider that excessive DNA supercoiling suppresses the temperature-sensitivities of *dnaA* mutants by high concentrations of NaCl. Thus we tried to confirm that the extent of DNA supercoiling is increased by high concentrations of NaCl in the *dnaA46* mutant at high temperatures. To determine why NaCl did not suppress the temperature-sensitivity of the *dnaA508* mutant, we also examined DNA supercoiling in this mutant. The wild-type, *dnaA46* and *dnaA508* cells harbouring pBR322 (a reporter plasmid for DNA supercoiling in cells) were incubated with various concentrations of NaCl at 40 °C for 4 h. The extent of DNA supercoiling of plasmids prepared from these cells was analysed by agarose-gel electrophoresis in the presence of 3 μ g/ml chloroquine. Under these conditions, more negatively supercoiled DNA migrates more rapidly [27]. To an extent depending on the concentration of NaCl, DNA in the *dnaA46* mutant became more negatively supercoiled, as is the case in the wild-type cells (Figure 3). The extent of change in DNA supercoiling in the wild-type cells was consistent with a previous paper [26]. Even in the *dnaA508* mutant, NaCl increased DNA supercoiling in a dose-dependent manner (Figure 3), suggesting that the inability of NaCl to suppress the temperature-sensitivity

B

Figure 4 Effect of excessive DNA supercoiling of template DNA on the replication activity of DnaA46 protein *in vitro*

pTB101, a plasmid with *oriC*, was constructed as described in [23]. Plasmids with various extents of DNA supercoiling were prepared as described in [33]. The linking number of each plasmid was determined on the basis of the results of agarose-gel electrophoresis with various concentrations of chloroquine (one of them is shown as Figure 4A, 1.5 μ g/ml). Δ Linking number shows the difference in the linking number of each plasmid in comparison with that of the normal plasmid (plasmid extracted from cells, without topoisomerase I treatment). The plasmids of + values of Δ linking number are more relaxed and those of - values are more negatively supercoiled compared with the normal plasmid. All plasmids are negatively supercoiled in the absence of chloroquine. DNA replication with each plasmid as a template was done in the presence of 100 ng of DnaA46 as described in the Materials and methods section.

of *dnaA508* is not due to its inability to increase DNA supercoiling. DNA in *dnaA46* and *dnaA508* mutants in the absence of NaCl was more negatively supercoiled than in the wild-type cells, which is consistent with our previous results [16]. The results in Figures 1 and 2 were reproduced with *dnaA* mutants harbouring pBR322 (results not shown).

Effect of excessive DNA supercoiling of template DNA on the replication activity of DnaA46 *in vitro*

We next tested our hypothesis using an *in vitro* DNA replication system. Since high-temperature-dependent inhibition of DNA replication with DnaA46 was reproduced in an *in vitro oriC* complementation assay using a crude extract, but not in a system reconstituted from purified proteins [3], we used a system consisting of a crude extract. A problem with this system is that the extent of DNA supercoiling changed upon incubation at high temperatures even without dNTP (without DNA replication reaction), which is due to the presence of DNA topoisomerases (results not shown). We solved this problem by shortening the

Figure 5 A phenotype of the *dnaA46* mutant sensitive to nalidixic acid.

The colony-formation abilities at 40 °C of the *dnaA46* and the wild-type strains on LB agar plates with 0.51 M NaCl and the indicated concentrations of nalidixic acid were examined as described in the legend to Figure 1.

reaction period of DNA replication. Under the conditions of an *in vitro* *oriC* DNA replication system in a crude extract (but without dNTP), we found the extent of DNA supercoiling of plasmid DNA was maintained up to 10 min at 40 °C (results not shown). Thus we examined the effect of the extent of DNA supercoiling of template DNA on DNA replication with purified DnaA46 protein by incubating at 40 °C for 10 min. As shown in Figure 4(A), we prepared *oriC* plasmids with various extents of DNA supercoiling. The DNA-replication reaction rate with DnaA46 protein at 40 °C was higher when the more negatively supercoiled DNA was used as a template (Figure 4B). The DNA-replication activities of DnaA46 at 30 °C or of the wild-type DnaA protein both at 30 °C and 40 °C were not affected by an increase in DNA supercoiling above the normal level of DNA supercoiling (DNA extracted from cells), whereas a decrease in DNA supercoiling inhibited the DNA replication reaction (results not shown), which is consistent with previous results [14]. Since the extent of change in DNA supercoiling by addition of 0.51 M NaCl in the *dnaA46* mutant cells was about –6 of the Δ linking number (see the legend to Figure 4), the results shown in Figure 4 suggest that the increase in DNA supercoiling by high concentrations of NaCl can increase the activity of Dna46 for DNA replication in cells.

A phenotype of the *dnaA46* mutant sensitive to nalidixic acid

On the basis of the notion that DNA replication in the *dnaA46* mutant, but not in the wild-type cells at 40 °C in the presence of high concentrations of NaCl, is maintained by excessive DNA supercoiling, we predicted that, under these conditions, the *dnaA46* mutant would show a higher sensitivity to DNA relaxation. We used nalidixic acid, which is known to relax DNA in cells [15]. Full-growth suspensions of the wild-type and the *dnaA46* mutant were spread on LB agar plates containing 0.51 M NaCl and various concentrations of nalidixic acid and incubated at 40 °C. As shown in Figure 5, the *dnaA46* mutant showed a phenotype that was more sensitive to nalidixic acid than the wild-type cells at all the concentrations tested. The *dnaA46* mutant showed a phenotype that was more sensitive to nalidixic acid,

even at 30 °C, although the difference in the sensitivity between the wild-type and the mutant was not as apparent as it was at 40 °C (results not shown). These data suggest that excessive DNA supercoiling is required to maintain DNA replication in the *dnaA46* mutant.

In the present study we tested the hypothesis that excessive DNA supercoiling suppresses a low activity of DnaA protein and obtained supporting evidence as follows. High concentrations of NaCl suppressed the temperature-sensitivity of the *dnaA46* mutant (Figure 1), in which DNA supercoiling in cells increased (Figure 3). The low DNA-replication activity of DnaA46 protein was stimulated by increasing the DNA supercoiling of template DNA *in vitro* (Figure 4). The *dnaA46* mutant showed a greater sensitivity to nalidixic acid, a DNA-relaxing drug, than did the wild-type cells under the conditions of high temperature and high concentrations of NaCl (Figure 5). It would be very noteworthy if *E. coli* cells were to change the extent of DNA supercoiling to maintain the proper frequency of DNA replication. Our previous finding, that DNA in temperature-sensitive *dnaA* mutants was more negatively supercoiled at semi-permissive temperature than was the DNA in the wild-type cells [16], suggest such a mechanism.

It was reported that genetic inactivation of topoisomerase I suppresses the temperature-sensitivity of the *dnaA46* mutant [28]. Since topoisomerase I relaxes DNA, mutations in the *topA* gene cause an increase in DNA supercoiling in cells [27,29]. Thus this previous observation supports the notion that the increase in DNA supercoiling by high concentrations of salts is involved in the suppression of the temperature-sensitivity of the *dnaA46* mutant.

The suppression of temperature-sensitivity by high concentrations of NaCl showed an allele specificity of *dnaA* mutants (Figure 2). The temperature-sensitivities of *dnaA* mutants with mutations in the ATP-binding site or DNA-binding sites, but not in the N-terminal region, were suppressed by high concentrations of NaCl. The function of DnaA protein can be divided into two categories. One is duplex opening at *oriC* and the other is loading DnaB protein into the *oriC* region. Excessive DNA supercoiling may assist the former function, but not the latter one. ATP binding and DNA binding are involved in the former function. As for the N-terminal region of DnaA protein, several functions (oligomerization, loading of DnaB, and retention of DnaB) have been suggested [30–32]. DnaA508 and DnaA167 proteins may have a defect in DnaB loading and retention, which may cause the inability of excessive DNA supercoiling to suppress the temperature-sensitivity of these mutants.

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