Determination of the minimum domain II size of *Escherichia coli* DnaA protein essential for cell viability

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The DnaA protein is the bacterial initiator of replication at a unique chromosomal site, *oriC*. It is present in all bacterial species and has a conserved structure with four domains. The structures of domains I and III–IV have been solved recently for some bacterial species, and the molecular process leading to the initiation event has been investigated in detail. On the other hand, domain II appears to have no rigid structure and is assumed to be a flexible linker connecting the N-terminal domain I and the C-terminal domains III–IV. It differs significantly in length and amino acid sequence among bacterial species. Whether or not domain II has any function(s) to initiate replication is unknown. The precise borders at both of its ends as well as its essential portions for cell viability are also unknown. In this study, we introduced systematic deletions into the domain II region on the chromosomal *dnaA* gene of *Escherichia coli* and examined their effect on cell physiology. Stretches of 30–36 consecutive amino acid residues could be deleted from various portions between the 78th and the 136th residues without affecting cell viability. We propose that domain II of *E. coli* DnaA is from the 79th to the 135th residues and at least 21–27 residues are required as a spacer to keep domains I and III–IV in the correct positions.

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INTRODUCTION

The DnaA protein plays a key role in the initiation of DNA replication in prokaryotes (Kaguni, 2006; Leonard & Grimwade, 2005; Messer, 2002; Mott & Berger, 2007). In *Escherichia coli*, the association of 10–30 molecules of ATP-bound DnaA with the replication origin, *oriC*, is required in order to form an open complex, in which duplex DNA is unwound at the AT-rich 13-mer region within *oriC*. DNA helicase (DnaB protein) is loaded to the unwound region with the aid of DnaA, followed by the formation of a protein complex involved in the elongation step of replication. Duplex unwinding at the specific region is assisted by DNA architectural proteins such as HU and IHF (Dixon & Kornberg, 1984; Bramhill & Kornberg, 1988). After replication is initiated, the ATP-DnaA molecules are converted to inactive ADP-bound forms by a process termed RIDA (regulatory inactivation of DnaA) (Katayama *et al.*, 1998). At least a part of the inactive ADP-DnaA appears to be regenerated to the active ATP-bound form later in the cell cycle (Kurokawa *et al.*, 1999). In addition to the five originally identified 9 bp DnaA-binding sequences (DnaA boxes R1–R4 and M), five ATP-DnaA boxes (I1–I3, τ 1, τ 2) that specifically bind ATP-bound DnaA molecules have been found in *oriC* recently (Kawakami *et al.*, 2005; McGarry *et al.*, 2004). Furthermore, some other mechanisms have been revealed that control the timing of the initiation reaction (Kitagawa *et al.*, 1998; Lu *et al.*, 1994).

Despite extensive studies, it is still not clear how the association of DnaA protein takes place and what structural change is induced in *oriC* to trigger the initiation event at a specific time in the cell cycle.

DnaA is composed of four regions, called domains I–IV (Messer *et al.*, 1999). Domain I is involved in DnaA–DnaA interaction as well as in association with other proteins, including DnaB (Seitz *et al.*, 2000; Sutton *et al.*, 1998) and DiaA (Ishida *et al.*, 2004). The structure of domain I of *E. coli* (Abe *et al.*, 2007) and that of *Mycoplasma genitalium* (Lowery *et al.*, 2007) have been resolved by NMR analyses. In the former, a weak interaction has been suggested between domain I and *oriC* single-stranded DNA, and a model has been presented for the entry of the DnaB helicase to *oriC* (Abe *et al.*, 2007). Domain III is the conserved AAA + nucleotide-binding region and also contains sites for DnaB-binding and DnaA–DnaA interaction. Domain IV is the DNA-binding region, which is also conserved among bacterial species. It also contains residues suggested to interact with the membrane. Structural details of domains III and IV of the *Aquifex aeolicus* DnaA (Erzberger *et al.*, 2002, 2006) and the DNA-bound domain IV of *E. coli* (Fujikawa *et al.*, 2003) have been published, and a model has been presented in which the wrapping of origin DNA around a helical filament of DnaA oligomer promotes DNA unwinding for subsequent replication fork establishment (Erzberger *et al.*, 2006; Mott & Berger, 2007).

In contrast to domains I and III–IV, information about domain II is still ambiguous. It is generally assumed to be a flexible linker connecting domains I and III–IV, inasmuch as it appears to have no rigid structure and differs significantly in length and amino acid sequence among bacterial species (Messer *et al.*, 1999). Several insertion and deletion mutations in domain II have been reported that can complement *dnaA46* mutation at a non-permissive temperature (Schaper & Messer, 1997). It is stated in a review that a portion of domain II (amino acid residues 87–104 and 87–135) of *E. coli* DnaA can be deleted without loss of function (Messer *et al.*, 1999). Some reports have assigned sequences necessary for self-oligomerization (Simmons *et al.*, 2003; Weigel *et al.*, 1999) and DnaB interaction (Seitz *et al.*, 2000; Sutton *et al.*, 1998) to regions that might be contained in domain II. Precise borders at both of its ends, and of portions essential for cell viability, have not been determined.

To better understand its role in DnaA activity, we introduced systematic deletions into the domain II region on the chromosomal *dnaA* gene of *E. coli* and investigated the minimal essential region of the domain II of *E. coli* DnaA protein sufficient for cell viability. We were able to delete 30–36 consecutive amino acid residues at various positions between the 78th and the 136th residues without losing cell viability.

METHODS

Bacterial strains and media. The *E. coli* K-12 strains used were MC1061 (F[−] *araD139* Δ (*araA-leu*)7697 Δ (*codB-lacI*)3 *galK16 galE15* LAM[−] e14[−] *mcrA0 relA1 rpsL150 spoT1 mcrB1 hsdR2*) (Casadaban & Cohen, 1980), ON303 (MC1061 *zid::Tn10*) and GC2597 (*sfiA::Tn5 pyrD thr leu his lac gal malB srl::Tn10 sfiC str*) (Elledge & Walker, 1983). The media used were M9 and L broth (Kitagawa *et al.*, 1998).

Construction of deletion mutants. A deletion was introduced *in vitro* by overlap extension PCR (Sambrook & Russell, 2001) into the 0.85 kb *Bgl*III–*Pme*I fragment that contains the domain II-encoding region of the *dnaA* gene using the high-fidelity enzyme KOD-Plus DNA polymerase (TOYOBO). The fragment with a deletion was exchanged with the corresponding wild-type fragment of pKH5002SB*dnaA*, a plasmid that carries the 5.8 kb *Bam*HI–*Sma*I fragment encompassing the *dnaA* gene on vector pKH5002SB (Kitagawa *et al.*, 1998); this vector, which can be replicated in *rnhA* mutants but not in the wild-type strain, carries the *sacB* gene of *Bacillus subtilis*, which is lethal to *E. coli* cells in the presence of sucrose. The plasmid with a deletion in the *dnaA* domain II was introduced into *E. coli* ON303. Ampicillin-resistant transformants, confirmed by colony PCR and restriction enzyme analyses to be merodiploids – in which the plasmid is integrated into the chromosome and the deleted *dnaA* gene is separated from the wild-type gene by the vector sequence – were spread on sucrose-containing plates to select colonies that had lost a copy of *dnaA* in addition to the vector sequence via a second homologous recombination. The DNA sequence encoding the N-terminal region of the *dnaA* gene encompassing the deletion was confirmed with all viable mutants isolated after PCR amplification of the chromosomal sequence.

Microscopy. Cells were grown to exponential phase in M9 medium at 37 °C. Hoechst 33342, a DNA-binding fluorescent dye, was added to the culture at a final concentration of 5 μ g ml^{−1} 1 h before sampling for observation. Microscopic observations were performed

using an Axiovert 200M inverted microscope (Carl Zeiss) equipped with filter set 02 and MetaMorph ver. 6.1 software (MDS).

Western blotting. Exponentially growing cells in L broth were harvested, washed once with 0.85 % (w/v) NaCl, lysed in 1 × loading buffer and subjected to SDS-polyacrylamide (10 %) gel electrophoresis (Sambrook & Russell, 2001). Blotting of the proteins to a PVDF membrane (Millipore) and detection with DnaA antibody was carried out as described by Kitagawa *et al.* (1996). The polyclonal antibody used gave indistinguishable band intensities when DnaA with a 30-residue deletion in domain II was compared with full-length DnaA. The membrane was then washed with a solution containing 2 % SDS, 100 mM 2-mercaptoethanol and 50 mM Tris/HCl (pH 6.7) for 30 min at 50 °C to remove bound antibodies, followed by two successive washes with 20 mM Tris/HCl (pH 7.5), 137 mM NaCl, 0.05 % Tween 20 at room temperature. The washed membrane was reprobed using a rabbit antiserum raised against *E. coli* RNA polymerase. Intensity of the bands in the film was quantified using Intelligent Quantifier ver. 2.1.1 (Bio Image).

Flow cytometry. For the experiments in Fig. 4, cells were grown exponentially for about 10 generation times in M9 medium to an OD₆₅₀ of about 0.1, and then were treated with rifampicin (250 μ g ml^{−1}) and cephalexin (12 μ g ml^{−1}) for 4–5 generation times. Cells were harvested, washed with 10 mM Tris/HCl and 1 mM EDTA (pH 7.5) and fixed with 70 % ethanol. Flow cytometry was performed with a FACSCalibur (BD Biosciences) using the PicoGreen DNA-staining dye (Molecular Probes) at a 20 000-fold dilution. For the experiments in Table 1, treatments with rifampicin and cephalexin were not carried out. For estimating relative cell mass values, cellular protein content was determined by flow cytometry after staining with FITC as described by Wold *et al.* (1994).

RESULTS AND DISCUSSION

Isolation of viable deletion mutants in domain II

Our criterion to determine whether a region in domain II of DnaA is essential or not was based on the viability of cells in which the chromosomal *dnaA* is replaced by the mutant allele. In the experiment shown in Fig. 1, a plasmid carrying the *dnaA* gene with a deletion was introduced into the chromosomal *dnaA* region by homologous recombination. The merodiploid strain obtained was confirmed to have the wild-type and mutant alleles separated by a plasmid vector sequence. Subsequently a copy of either the wild-type or the mutant *dnaA* gene was deleted, together with the vector sequence, by homologous recombination. All deletion constructs that we made could be integrated into the chromosome to form a merodiploid, suggesting that deletion alleles have no dominant negative effect on the wild-type allele. We concluded that a deletion is lethal if all tested colonies isolated after final selection for plasmid-less cells have the wild-type *dnaA* gene only, as examined by colony PCR and agarose gel electrophoresis. At least 96 colonies were examined to draw this conclusion.

First, we confirmed a previous report that amino acid residues 87–104 are dispensable (Messer *et al.*, 1999). Indeed, we were able to obtain a strain in which DNA segments encoding these residues were deleted from the chromosomal *dnaA* gene. Furthermore, the mutant cells were indistin-

Fig. 1. Deletions in domain II of *E. coli* DnaA protein. Deletions of 30 amino acid residues or more in domain II are shown under the amino acid sequence. The range of deleted residues is shown in square brackets, and the number of deleted amino acids is shown in parentheses. The number of colonies that had deletions and the total number of colonies examined that were sucrose-resistant and ampicillin-sensitive are shown on the right. Viable and nonviable deletions are indicated by the lines between unfilled and filled rectangles, respectively.

guishable from the wild-type cells in growth rate and cell morphology as well as in replication initiation frequency and synchrony (data not shown). We then increased the size of the deletion in both directions and found that deletions up to 30 amino acid residues could be introduced without loss of viability (Δ[81–110], Fig. 1). Deletion of 31 residues, from either the 80th or 81st residue, did not support any colony recovery (Δ[80–110] and Δ[81–111], Fig. 1).

Deletion near the N-terminal region of domain II

To determine the N-terminal limit that can tolerate a deletion of 30 residues, we moved the position of deletion toward domain I. Cells carrying Δ[79–108] were viable. However, neither Δ[78–107] nor Δ[77–106] yielded viable cells (Fig. 1).

We assume that a deletion shorter than 30 residues would also not be allowed much beyond the 79th residue, since we have isolated a temperature-sensitive mutant caused by the F76S mutation (unpublished results). It should be pointed out that a recent NMR study identified the 78th residue as the C-terminal residue of the β3 sheet (residues 73–78) and residues 79–108 as a flexible and disordered chain (Abe *et al.*, 2007). Taking these results together, we propose to assign residues 1–78 of DnaA to domain I, instead of the previous proposal of residues 1–86 (Messer *et al.*, 1999).

Deletion near the C-terminal region of domain II

On the other hand, shifting the 30-residue deletion toward the C terminus revealed that sequences up to the 135th

Table 1. Cell growth and DNA replication characteristics

Cells were grown in M9 medium at 37 °C. Cell mass and cellular DNA content were measured by flow cytometry using exponentially growing cells.

Strain	Doubling time (min)	Cell mass	DNA per cell	DNA per mass
Wild-type	54 ± 1	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.03
Δ[79–108]	78 ± 7	1.83 ± 0.36	1.31 ± 0.25	0.73 ± 0.07
Δ[81–110]	76 ± 7	1.80 ± 0.30	1.33 ± 0.19	0.75 ± 0.12
Δ[91–120]	72 ± 4	1.47 ± 0.42	1.07 ± 0.09	0.75 ± 0.11
Δ[94–123]	62 ± 1	1.68 ± 0.22	1.23 ± 0.21	0.80 ± 0.22
Δ[99–134]	59 ± 2	1.30 ± 0.17	1.16 ± 0.19	0.90 ± 0.13
Δ[100–135]	63 ± 6	1.52 ± 0.25	1.24 ± 0.11	0.83 ± 0.13

residue are nonessential for cell viability (Fig. 1). Furthermore, to our surprise, deletions of up to 36 residues were tolerated in the C-terminal region of domain II ($\Delta[99-134]$ and $\Delta[100-135]$, Fig. 1). The C terminus of domain II of *E. coli* DnaA has been proposed to be the 129th or 134th residue (Messer *et al.*, 1999). If we define domain II as a spacer that connects domains I and III and has no specific residue for DnaA activity, domain II extends to at least the 135th residue. Since we focused on determining the minimum essential length of domain II, we did not examine whether or not a shorter deletion could be introduced beyond this residue.

Phenotypic characterization of the deletion mutants

Examination of the phenotypes of the deletion mutants revealed some remarkable features. Table 1 shows some parameters representing cell growth and DNA replication properties of mutants carrying deletions of various portions of domain II. Compared to the wild-type, all mutants with a deletion of 30 residues or more had longer doubling times. The doubling time was longest in the two mutants carrying a deletion in the N-terminal region of domain II ($\Delta[79-108]$ and $\Delta[81-110]$, Table 1). The slow growth was accompanied by formation of elongated cells (Fig. 2). In the elongated cells, the nucleoids appeared to be located at approximately regular intervals. The average cell mass of the mutant strains relative to that of the wild-type strain ranged from 1.3 to 1.8 (Table 1). The DNA content

per cell mass was lower in these mutants compared to the wild-type, as estimated by flow cytometry (Table 1) as well as by the assay using the diphenylamine reagent (Burton, 1956) (data not shown). Therefore, it is suggested that the initiation of DNA replication is inefficient in these mutant cells. Western blotting revealed that the content of DnaA protein in mutant cells was 63–79 % that in the wild-type cells (Fig. 3). The reduced DnaA level could have caused inefficient replication, slow growth, and formation of elongated cells as reported by other groups (Løbner-Olesen *et al.*, 1989; Zahn & Messer, 1979). The reduced DnaA levels in mutant cells may suggest that the mutant DnaA proteins are unstable compared to the wild-type protein. It appears that SOS induction is not involved in the formation of elongated cells, since the *sfiA*::Tn5 mutation had no effect on morphology (Fig. 2).

Fig. 4 shows DNA histograms obtained by flow cytometry of cells that were run out of replication by cultivation under conditions in which rifampicin inhibited new initiation events and cephalixin inhibited cell division (Kitagawa *et al.*, 1998; Løbner-Olesen *et al.*, 1989). The number of chromosomes represents the number of origins in a cell that were present at the time antibiotics were added. Compared to the synchronous initiation in wild-type cells, the occurrence of some asynchronous initiations was suggested in mutant cells, since a small amount of three-chromosome cells were present. In accordance with other altered phenotypes among the mutants with 30-residue deletions, asynchronous initiations were more conspicuous in the $\Delta[79-108]$ mutant carrying the deletion close to the N terminus of domain II. The histograms of the mutants with shorter deletions of domain II ($\Delta[87-104]$, $\Delta[85-104]$, $\Delta[87-106]$) were indistinguishable from that of the wild-type (data not shown). Also, these mutants displayed neither delayed growth nor abnormal cell morphology.

Concluding remarks

Thirty consecutive residues could be deleted from all tested regions in the 57 residues between V78 and H136 without affecting cell viability. Thirty-six consecutive residues near the C-terminal part of this region were not required for cell viability. The minimum length of domain II of *E. coli* DnaA essential for cell viability was 21–27 residues and depended on the region in the domain. The present results strongly suggest that the role of domain II is as a spacer connecting domains I and III. Both the N-terminal 21 residues and the C-terminal 27 residues in domain II appear to bring domains I and III–IV into the correct conformation for DnaA function. The N-terminal residues could take an expanded conformation compared to the C-terminal residues to accommodate longer deletions. The observed phenotypes of the deletion mutants could be due to the reduced cellular DnaA level. Alternatively, they may suggest that at least one of domains I, III, and IV is not fully active due to either constrained conformation or loss of specific

Fig. 2. Shapes of cells and nucleoids of deletion mutants in domain II. Merged images of phase-contrast and fluorescence (nucleoids) are shown. The wild-type strain is ON303, and all other strains are its derivatives.

Fig. 3. Estimation of cellular DnaA contents by Western blotting. Two different amounts of cells of each strain (0.008 OD₆₀₀ units in the left lanes and 0.016 OD₆₀₀ units in the right lanes) were applied to the gel. After detection of the DnaA protein, the bound antibodies were stripped from the membrane and used again to detect the α -subunit of *E. coli* RNA polymerase. The relative amounts of cellular DnaA, indicated at the bottom, were estimated assuming that the RNA polymerase level is the same in all strains.

residues that are required, although not essential, for full activity.

It is reported that there is a site involved in a transient interaction with DnaB helicase in residues 111–148 (Sutton *et al.*, 1998) or 130–148 (Seitz *et al.*, 2000). Residues 1–62 (Sutton *et al.*, 1998) or 24–86 (Seitz *et al.*, 2000) are also suggested to contain sequences for interaction with DnaB. Domain I is reported to be involved in self-oligomerization on *oriC* (Simmons *et al.*, 2003; Weigel *et al.*, 1999) as well as interaction with DiaA (Ishida *et al.*, 2004). The regions involved in these functions may be narrowed down from the present results, which indicate that no specific residue between the 78th and the 136th residues is required for cell viability.

It is suggested that the length of domain II of DnaA has a relationship with the spacing of DnaA boxes in *oriC*

(Zawilak-Pawlik *et al.*, 2005). For example, the *Helicobacter pylori* DnaA has a short domain II (66 residues) and exhibits the highest affinity towards closely spaced DnaA boxes in *H. pylori oriC*. In contrast, the presence of a long domain II (253 residues) allows the *Streptomyces coelicolor* DnaA to associate with the widely separated DnaA boxes in *S. coelicolor oriC*. Therefore, the length of domain II of *E. coli* DnaA may have evolved to optimally fit to the *E. coli oriC*.

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Fig. 4. Replication runaway histograms of DNA. Cells were grown exponentially in M9 minimal medium, treated with rifampicin and cephalixin for 4–5 generation times and analysed by flow cytometry. Strains are as described in Fig. 2.

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