Analyses for mechanisms in formation of the replication initiation complex and for functional characteristics of the DNA unwinding region at the replication origin of hyperthermophilic bacterium Thermotoga maritima

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for functional characteristics of the DNA unwinding region at the replication origin
of hyperthermophilic bacterium *Thermotoga maritima*
高度好熱性真正細菌 *Thermotoga maritima* の複製起点における
開始複合体の形成メカニズムと開裂部位の特性の解析

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【Introduction】

Chromosomal DNA replication is a central process in all cellular organisms. To initiate DNA replication, a specific nucleoprotein complex called the initiation complex is constructed at the origin of replication, promoting recruitment of the replisome components including replicative DNA helicases and DNA polymerases. If the regulation of replication initiation is disturbed, it can inhibit normal cell proliferation and differentiation resulting from abnormal chromosome content. To avoid these problems, the processes of the initiation complex formation are highly organized in order to ensure the replication of chromosomal DNA to occur only once at a specific time during the cell cycle progression. Thus, the study addressing the common principles underlying initiation complexes plays a fundamental role in the fields of pharmaceutical biochemistry and molecular biology.

In most eubacteria, the origin *oriC* contains DnaA oligomerization region (DOR) bearing multiple DnaA box sequences to which the ubiquitous DnaA initiators bind (Fig. 1A). For unwinding mechanism of DNA unwinding element (DUE) of *oriC* in well-characterized *Escherichia coli*, the single-stranded (ss)DUE-recruitment mechanism has been proposed (1-4). In this mechanism, DnaA boxes sustain construction of higher-order complexes via DnaA-DnaA interactions, promoting the unwinding of DUE. The left-part of DOR also contains a specific binding site for the nucleoid-associated protein IHF and the IHF-dependent DOR bending facilitates formation of the DnaA complexes on the left-DOR, promoting DUE unwinding activity (Fig. 1B and C). Alternatively, IHF-structural homolog HU, a ubiquitous nucleoid-associated protein, binds to the similar site instead of IHF when the DOR-DnaA complex is formed, promoting DOR bending and DUE unwinding activity (5). The upper strand of the resulting ssDUE is then recruited to the DUE-proximal DnaA subcomplex via the DOR bending and directly binds to specific DnaA molecules in the subcomplex (Fig. 1B and C).

E. coli DnaA consists of four domains and the central domain III containing AAA+ (ATPases associated with various cellular activities) motifs (Fig. 1B) plays essential roles in ssDUE recruitment mechanisms. The AAA+ arginine-finger motif Arg285 predominantly recognizes ATP bound to the adjacent DnaA protomer, promoting co-operative ATP-DnaA binding onto the DOR in a head-to-tail orientation (Fig. 1C). Moreover, H/B-motifs (Val211 and Arg245) in this domain bind ssDUE in a sequence-specific manner (Fig. 1C). Also, the AAA+ domain III of DnaA is linked via a flexible hinge to its C-terminal domain IV, which is responsible for DnaA box-specific DNA binding (Fig. 1B and C) (3). These functional structures are proposed to be conserved among various DnaA orthologs. By contrast, the *oriC* sequences are highly diverse in terms of the number and orientation of DnaA boxes and general features of DUE also still remain to be elucidated. (Fig. 1A).

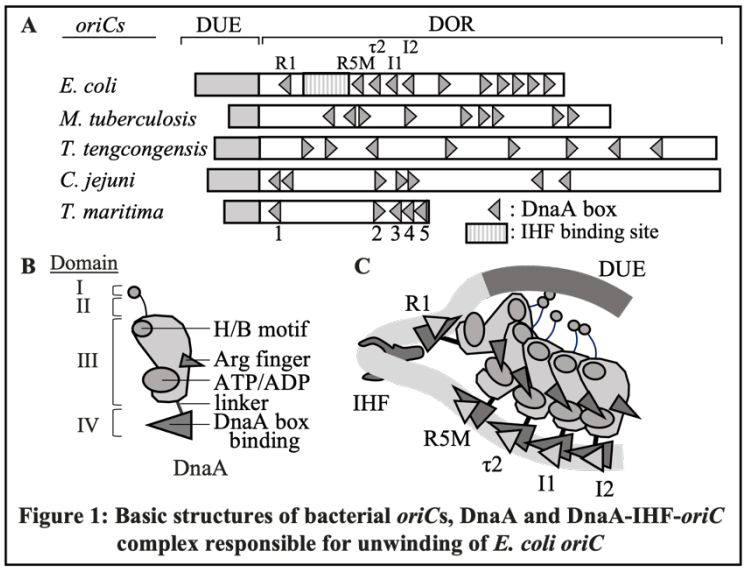

Figure 1: Basic structures of bacterial *oriC*s, DnaA and DnaA-IHF-*oriC* complex responsible for unwinding of *E. coli oriC*

In this study, to elucidate the basic mechanisms underlying DNA unwinding at DUE and DnaA oligomerization in DOR, I

focused on the hyperthermophilic eubacterium *Thermotoga maritima* as a model organism. *T. maritima* is placed at a deep branch in the evolutional tree of life (6). The 149-bp minimal *oriC* (*tma-oriC*) region of this bacterium contains a 24-bp AT-rich DUE (*tma*DUE) and a flanking DOR (*tma*DOR) with five *tma*DnaA boxes (Fig. 1A) and supports open complex formation by the cognate DnaA initiator (*tma*DnaA) and HU (7). In this study, I analyzed using purified proteins and various *in vitro* reconstituted systems the unwinding motifs within *tma*DUE and the mode of *tma*DnaA oligomerization.

【Methods】

Open complex formation assay using P1 nuclease

A wild-type *tma-oriC* bearing plasmid (pOZ14) and its mutant derivatives were individually incubated with ATP-*tma*DnaA and *E. coli* HU for 10 min at 48°C as described (7). The unwound DUE regions were digested using the single-strand-specific endonuclease P1 and the resulting samples were subjected to 1% agarose gel electrophoresis. DNA bands were visualized by ethidium bromide staining and the band intensities were quantified using the FIJI software.

Assays for *tma*DnaA-*tma*DOR complex formation and ssDUE binding

A 230 bp DNA fragment containing *tma*DOR and its mutant derivatives were individually incubated with ATP-*tma*DnaA for 5 min at 48°C, followed by electrophoretic mobility shift assay (EMSA) as described (7). When ssDUE binding activities were assessed, *tma*DOR and *tma*DnaA were incubated in the presence of ^{32}P-labeled ss-*tma*DUE, followed by EMSA. *tma*DOR and ^{32}P-labeled ss-*tma*DUE were visualized using GelStar DNA dye and phosphorimaging, respectively.

Head-to-tail dimerization assay

A set of 30 bp DNA fragments containing direct or inverted repeats of *tma*DnaA boxes with different interspacing distance was incubated with ATP-*tma*DnaA for 5 min at 48°C, followed by EMSA as described (7). DNA was visualized using GelStar DNA dye and the band intensities were quantified using the FIJI software.

【Results】

Identification of a novel motif within *tma-oriC* required for DUE unwinding

*tma*DUE contains two potential ssDUE-binding motifs: TTATT motif from *E. coli* DUE (1) and the DnaA-trio motif (AAATAATA) from *B. subtilis* DUE (8). Besides, the three tandem repeats of TAG (hereafter referred to as eTrio) reside between the two motifs (Fig. 2B). To examine if these sequence motifs are important for DUE unwinding, I constructed a set of mutant *tma-oriC* plasmids bearing base-substitutions within *tma*DUE and assessed their activities in the open complex formation assay. Consistent with the previous reports (7), wild-type *tma-oriC* plasmid pOZ14 formed open complex in the presence of ATP-*tma*DnaA and HU, exhibiting the *tma*DUE unwinding activity. By contrast, mutations in eTrio completely abolished the *tma*DUE unwinding activity. Scrambling of the TTATT sequence or DnaA-trio moderately inhibited *tma*DUE unwinding. In addition, when the TTATT and DnaA-trio sequences were simultaneously scrambled, the inhibition levels were additive, but slight unwinding activity remained. These observations suggested that both the TTATT motif and the DnaA-trio play a supportive, but not essential, role in open complex formation while eTrio was essential.

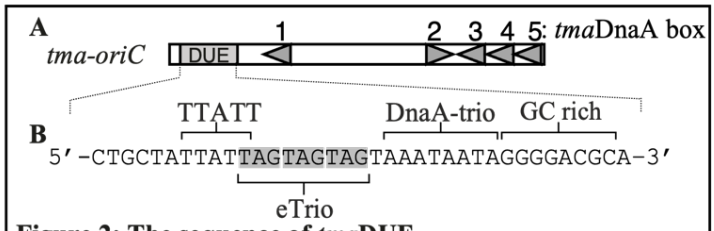

Figure 2: The sequence of *tma*DUE

ATP-*tma*DnaA oligomers on DOR bind ssDUE through eTrio

To further dissect the roles of the sequence motifs within DUE, interactions between ss-*tma*DUE and ATP-*tma*DnaA oligomers constructed on *tma*DOR were analyzed by ssDUE binding assay. As reported previously (7), ATP-*tma*DnaA oligomers constructed on *tma*DOR (ATP-*tma*DnaA-*tma*DOR complexes) specifically interacted with the ligand TMA28, an upper strand fragment of ss-*tma*DUE in a manner depending on *tma*DOR and ATP-bound, but not ADP-bound, *tma*DnaA. Mutant analysis demonstrated that dC-substitutions in TTATT and DnaA-trio fully sustained the binding activity. By contrast, mutations in eTrio completely abolished the binding activity. These findings indicated that eTrio is strictly required for binding, and the DnaA-trio within *tma*DUE is dispensable for binding to ATP-*tma*DnaA-*tma*DOR

complexes but plays only a supportive role in open complex formation.

Individual *tma*DnaA boxes within minimal *tma*DOR are crucial for unwinding

*tma*DOR contains *tma*DnaA boxes 1 to 5 (Figure 2A). Although *tma*DnaA box 2 is oriented in the opposite direction, the *tma*DOR is associated with the formation of ATP-*tma*DnaA oligomers responsible for DUE unwinding. To address the requirements for individual *tma*DnaA boxes 1 to 5, I conducted open complex formation assays using a set of minimal *tma-oriC* plasmid pOZ14 derivatives in which each of *tma*DnaA box sequence was randomized. As a result, randomization of one of *tma*DnaA box 2-5 moderately inhibited unwinding activity, and randomization of *tma*DnaA box 1 almost completely abolished the unwinding activity. These results suggested that each of the five *tma*DnaA boxes plays a crucial role for full DUE unwinding activity.

Intact DNA helical turn between DUE and DOR is crucial for unwinding

To investigate the mechanisms required for open complex formation, importance of DNA helical turn differences in the *tma*DnaA box 1-box 2 intervening region for *tma*DUE unwinding was analyzed. This analysis is based on a hypothesis that if the ssDUE recruitment mechanism was responsible for open complex formation, then inserting a full (10 bp) turn of a DNA helix would allow this region to retain its phasing, resulting in sustained unwinding activity. Conversely, inserting a half (5 bp) turn of a DNA helix would alter the phasing and then altering the interaction modes among the ATP-*tma*DnaA complexes and impairing interactions between the *tma*DUE and DnaA complexes. Supporting this hypothesis, DUE unwinding activity was fully preserved by insertion of a 10 bp fragment but was reduced by insertion of a 5 bp fragment. These observations are in good agreement with the ssDUE recruitment mechanism, in that appropriate phasing between the DnaA complexes promotes open complex formation.

Involvement of the Arg-finger in open complex formation

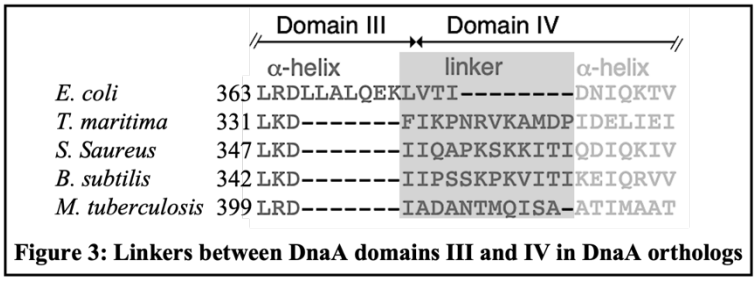

Figure 3: Linkers between DnaA domains III and IV in DnaA orthologs

I next examined if a conventional mechanism of head-to-tail oligomerization underlies formation of the open complex in *tma-oriC*. In *E. coli*, DOR contains direct repeats of the DnaA boxes that facilitate head-to-tail oligomerization of ATP-DnaA proteins depending on the interaction between the DnaA Arg285 Arg-finger and ATP bound to the adjacent protomer (4) (Fig. 1B and C). This head-to-tail interaction is essential for open complex formation. However, given *tma*DnaA box 2 is oriented in the opposite direction, it remains unclear whether the similar mechanisms underly formation of the open complex in *tma-oriC*. At first, I noted that unlike *E. coli* DnaA where DnaA domains III and IV are connected by a presumable short linker of four amino acid residues (Fig. 1B), *tma*DnaA, as well as other representative DnaA orthologs, is predicted to have a corresponding linker with a longer length consisting of 11-12 amino acid residues (Fig. 3). I then reasoned that the flexibility of the longer linker might allow for greater swiveling of the domains, bringing the Arg-finger close to the ATP at the adjacent protomer bound to *tma*DnaA box 3. To test this idea, I examined the requirement of the *tma*DnaA Arg251 residue that corresponds to *E. coli* DnaA Arg285 for open complex formation. The assay demonstrated that ATP-*tma*DnaA R251A is virtually inactive for unwinding DUE. By contrast, a filter retention assay and a head-to-tail dimerization assay demonstrated that *tma*DnaA R251A retained the intact affinities for ATP and for a DNA carrying a single consensus *tma*DnaA box sequence. These observations indicated that the Arg251 Arg-finger is essential for *tma-oriC* open complex formation, which is consistent with the idea that head-to-tail oligomerization of ATP-*tma*DnaA proteins underlies open complex formation in *tma-oriC*.

Inverted *tma*DnaA boxes permit ATP-*tma*DnaA interactions in a head-to-tail manner

To obtain the mechanistic insight into formation of head-to-tail oligomerization of ATP-*tma*DnaA proteins in *tma-oriC*, we performed EMSA using a DNA fragment with oppositely oriented *tma*DnaA boxes 2 and 3. When wild-type ATP-*tma*DnaA was used, the predominant DNA complex contained two *tma*DnaA molecules (C2 complex), whereas the complex containing one *tma*DnaA molecule was barely detected. By contrast, the complex containing one *tma*DnaA molecule was the major product when wild-type ADP-*tma*DnaA or ATP-*tma*DnaA R251A was used. Thus, both ATP and the Arg-finger are involved in the efficient formation of C2 complex. Further analyses using a set of direct

or inverted repeats of *tma*DnaA boxes with different interspacing distance showed that formation of C2 complex was most efficient when two identically oriented *tma*DnaA boxes were separated by 2 bp, as shown in the arrangements of *tma*DnaA boxes 3-4 and boxes 4-5. By contrast, maximum C2 complex formation by two inverted *tma*DnaA boxes was observed when they were separated by 4 bp interspace, as observed for *tma*DnaA boxes 2-3. These results suggested that formation of C2 complex depends on appropriate spacing between two *tma*DnaA boxes and their orientation.

Reversed *tma*DnaA box 2 permits open complex formation

To confirm the concept that the inverted repeats of *tma*DnaA boxes allow for head-to-tail ATP-*tma*DnaA interactions to form an open complex in *tma-oriC*, an open complex formation assay was performed using a *tma-oriC* mutant derivative bearing the reversed *tma*DnaA box 2, aligning *tma*DnaA boxes 2-4 in the same direction with a 2-bp interspace. As a result, the unwinding activity of this mutant plasmid was comparable to that of the wild-type *tma-oriC* plasmid. These observations suggest a tunable nature of DnaA box orientation in the formation of bacterial initiation complexes at the origin.

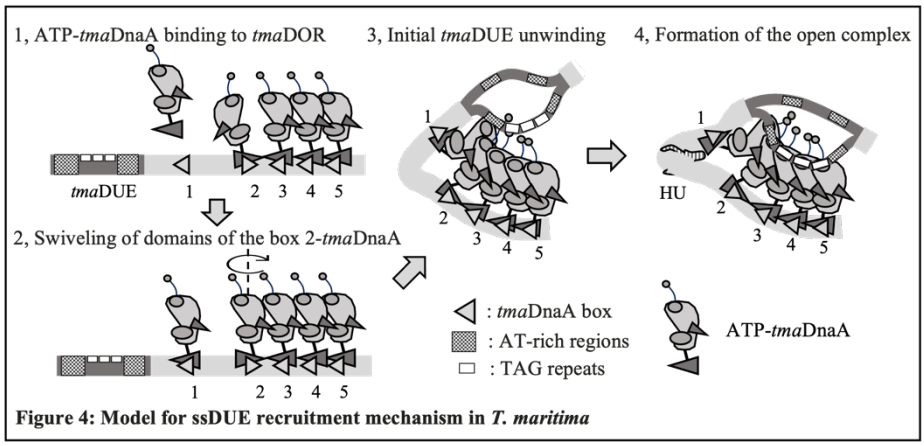

Figure 4: Model for ssDUE recruitment mechanism in *T. maritima*

【Discussion】

This study provides concrete biochemical evidence that the ssDUE recruitment mechanism operates in open complex formation of the ancient bacterium *T. maritima*. Based on the current results we propose the mechanistic model as follows: 1, ATP-*tma*DnaA binds co-operatively to the *tma*DOR; 2, *tma*DnaA protomer bound to the inverted *tma*DnaA box 2 swivels its AAA+ domain around to bring the Arg-finger close to the ATP on the adjacent protomer bound to *tma*DnaA box 3; 3, When *tma*DnaA boxes 1 to 5 are all occupied by ATP-*tma*DnaA, the ATP-*tma*DnaA protomers bound to *tma*DnaA boxes 1 and 2 would interact transiently as a result of Brownian motion, inducing *tma*DUE unwinding; 4, The unwound ssDUE would be stabilized though direct interaction of eTrio with the ATP-*tma*DnaA trimer bound to *tma*DnaA boxes 3 to 5 (Fig. 4). This model is also supported by latest report showing that HU binds to *tma*DOR in an ATP-*tma*DnaA-dependent manner and that *E. coli* can utilize HU-promoted ssDUE recruitment mechanism (5). Given the facts that two evolutionally-distal *E. coli* and *T. maritima* share the similar mechanisms and that DnaA residues responsible for ssDUE binding and HU are highly conserved among bacterial species (5), the proposed model is likely applicable to diverse bacterial organisms.

The current study also opens up the opportunity for developing novel antibiotics targeting replication initiation. In bacteria, the origin sequences differ substantially in the number of DnaA boxes and their spatial arrangements, which has hindered understanding of a general mechanism for open complex formation in various bacteria including pathogens. Here, I determined the optimal arrangement of the *tma*DnaA boxes, including their orientation and spacing, which argues that a tunable nature of DnaA box orientation is a general principle in the formation of bacterial initiation complexes. Given *T. maritima* and many pathogenic bacteria share the similar principles of replication initiation, I propose that the model of *T. maritima* open complex can be useful to design a novel compound that inhibits duplex unwinding of the pathogenic bacteria.

【References】

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【Publication】

Lu *et al.* (2023) *J. Biol. Chem.* 299: 104888.