Mechanism for the TtDnaA–Tt-oriC cooperative interaction at high temperature and duplex opening at an unusual AT-rich region in *Thermoanaerobacter tengcongensis*

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**ABSTRACT**

*Thermoanaerobacter tengcongensis* is an anaerobic low-GC thermophilic bacterium. To further elucidate the replication initiation of chromosomal DNA at high temperature, the interaction between the replication initiator (TtDnaA) and the putative origin (Tt-oriC) in this thermophile was investigated. We found that efficient binding of TtDnaA to Tt-oriC at high temperature requires (i) at least two neighboring DnaA boxes, (ii) the specific feature of the TtDnaA Domain IV and (iii) the self-oligomerization of TtDnaA. Replacement of the TtDnaA Domain IV by the counterpart of *Escherichia coli* DnaA or disruption of its oligomerization by amino acid mutations (W9A/L20S) abolished the oriC-binding activity of TtDnaA at 60 °C, but not at 37 °C. Moreover, ATP-TtDnaA, but not ADP-TtDnaA or the oligomerization-deficient mutants was able to unwind the Tt-oriC duplex. The minimal oriC required for this duplex opening *in vitro* was demonstrated to consist of DnaA boxes 1–8 and an unusual AT-rich region. Interestingly, although no typical ATP-DnaA box was found in this AT-rich region, it was exclusively bound by ATP-TtDnaA and acted as the duplex-opening and replication-initiation site. Taken together, we propose that oligomerization of ATP-DnaA and simultaneously binding of several DnaA boxes and/or AT-rich region may be generally required in replication initiation at high temperature.

**INTRODUCTION**

The replication initiation of bacterial chromosomes requires two basic elements: the initiator protein DnaA and the replication origin oriC (1,2). In *Escherichia coli*, the DnaA sequentially binds to the 9-mer DnaA boxes (1,3), including the R-boxes that can be bound by both ATP-DnaA and ADP-DnaA (4), and the more recently identified I-sites that show strong preference for ATP-DnaA (5). Using the adjacent DnaA boxes as an anchor, the ATP-DnaA then cooperatively binds to the AT-rich region at the special sites named ATP-DnaA boxes (6,7), which overlap the Dam-methyltransferase-recognizing sites that are involved in the regulation of replication initiation by SeqA (8). In the presence of DNA-structuring proteins Fis/IHF and HU, ATP-DnaA then unwind the AT-rich region, leading to the DnaC-dependent loading of the DnaB helicase and the successive priming and chain elongation (2,5,9). Recently, the structure of AMP-PCP-bound *E. coli* DnaA and the conserved core of *Aquifex aeolicus* DnaA have been resolved, indicating that the mechanistic elements of origin processing may be conserved across the bacterial, archaeal and eukaryotic domains (10,11).

While research on replication initiation of bacterial chromosome has been well documented in several mesophilic model bacteria, such as *E. coli* or *Bacillus subtilis* (2,12), many murky aspects exist in the corresponding process in thermophilic bacteria. To adapt to the high-temperature condition, the replication-related proteins should be thermostable, and the interactions between these proteins and the oriC should also have higher affinity to prevent the quick dissociation at high-temperature. Thus, in thermophilic bacteria, the new
mechanism for assembly of the replication initiation complex at high temperature awaits to be discovered.

*Thermoanaerobacter tengcongensis* is an anaerobic, rod-shaped and low-GC (33%) thermophilic eubacterium, which is isolated from a freshwater hot spring in China and grows well at ~75°C (13). Although empirical definitions (such as staining) suggest that *T. tengcongensis* is Gram-negative, genome analysis reveals that it shares many genes that are characteristic of Gram-positive bacteria but lacks molecular components unique to Gram-negative bacteria (14). More interestingly, 86.7% of the chromosomal genes are found to be located on the leading strand of DNA replication (14). Recently, comparative genomics has revealed that the oriC of *T. tengcongensis* (Ti-oriC) is located between the dnaA and rrmH genes, and 12 DnaA boxes have been predicted within this Ti-oriC region. This is a little different from the oriC of the high-GC (64–65%) thermophilic bacterium *Thermus thermophilus* where the oriC is located between the dnaA and dnaN genes, and contains 13 DnaA boxes and an apparent AT-rich region located downstream of the DnaA boxes (16). Compared to *T. thermophilus*, the T. *tengcongensis* is a low-GC thermophile and the AT content in Ti-oriC is >65%, and neither Dam-methyltransferase-recognizing site nor typical ATP-DnaA box is found therein. These features make it difficult to predict the functional AT-rich region where the duplex begins to open during replication initiation. Therefore, it remains to be explored how the Ti-oriC duplex opens to initiate chromosome replication and how this process becomes adapted to the high temperature in this thermophilic bacterium.

In the present study, we have identified the precise replication initiation point (RIP) of the *T. tengcongensis* chromosome at a unique AT-rich region by RIP mapping and duplex opening assay, and also defined the minimal oriC required for open complex formation *in vitro*. Moreover, we have investigated the cooperative interactions of the TtDnaA with Ti-oriC, and revealed for the first time that interplay of the DnaA boxes and the oligomerization of the ATP-TtDnaA were absolutely required for the initial step of replication in *T. tengcongensis*.

**MATERIALS AND METHODS**

**Bacteria, plasmids and oligonucleotides**

*E. coli* DH5α was used as a host for the cloning experiments, and *E. coli* BL21(DE3) for overproduction of the recombinant proteins. *T. tengcongensis* was grown in the modified MB medium at 70°C without shaking (13,14). The plasmids and oligonucleotides used in the present study were described in Tables 1 and 2, respectively.

**DNA manipulations**

For overexpression of TtDnaA, the dnaA gene of *T. tengcongensis* was amplified by PCR using the genomic DNA as the template and P1/P2 as the primers (Table 2). The PCR fragment was then cloned into the expression vector pET28a (Novagen) to generate the expression plasmid p28TtDnaA. In a similar way, the p28TtDnaA-DM and p23TtDnaA-IV were also constructed to produce the double-site mutant of TtDnaA (W9A/L20S, TtDnaA-DM) and the Domain-IV (TtDnaA-IV), respectively, with the corresponding primers (Table 2). To construct the plasmid p28MuDnaA for expression of the TtEcDnaA, the domain IV of TtDnaA was replaced with the counterpart of the *E. coli* DnaA. To do so, one PCR with primers P7/P8 was used to amplify the sequence encoding the domain IV of *E. coli* DnaA and another with primers P1/P6 to obtain the sequence coding for the TtDnaA-DM and p23TtDnaA-IV, respectively.

**Table 1. Plasmids used in this study**

| Plasmids | Description | Sources |
|----------|-------------|---------|
| pUCm-T   | Amp¹, T-vector for cloning PCR-amplified fragments | Sangon, China |
| pET-28a  | Kan¹, expression vector, His-tag coding sequence | Novagen |
| pET-23b  | Amp¹, expression vector, His-tag coding sequence | Novagen |
| pOC-T    | pUCm-T derivative plasmid containing the 653-bp oriC region | This work |
| p28TtDnaA| Expression plasmid derived from pET-28a for *T. tengcongensis* TtDnaA | This work |
| p28MuDnaA| Expression plasmid derived from pET-28a for the hybrid Tt/EcDnaA protein | This work |
| p28TtDnaA-DM| Expression plasmid derived from pET-28a for the double-site mutant (W9A/L20S, DM) of TtDnaA | This work |
| p23TtDnaA-IV| Expression plasmid derived from pET-23b for the Domain IV of TtDnaA | This work |
| pOC-1    | pUCm-T derivative containing the 472-bp oriC region | This work |
| pOC-2    | The subclone of pOC-1 without DnaA boxes 9–12 | This work |
| pOC-3    | The subclone of pOC-1 without DnaA boxes 9–12 and AT-rich region | This work |
| pOC-4    | The subclone of pOC-1 with the DnaA boxes 1–8 and AT-rich region | This work |
| pOC-5    | The subclone of pOC-4 without DnaA box 1 | This work |
| pGBK7T7  | Yeast two-hybrid DNA-binding domain vector | Clontech |
| pGAD7T7  | Yeast two-hybrid activation domain vector | Clontech |
| pGBK7T7-TtDnaA| pGBK7T7 derivative for expression of the TtDnaA of *T. tengcongensis* | This work |
| pGAD7T7-DM| pGBK7T7 derivative for expression of the double-site mutant (W9A/L20S, DM) of TtDnaA | This work |
| pGAD7T7-DM| pGAD7T7 derivative for expression of the double-site mutant (W9A/L20S, DM) of TtDnaA | This work |
| pGAD7T7-IV| pGBK7T7 derivative for expression of the Domain IV of TtDnaA | This work |
| pGAD7T7-IV| pGAD7T7 derivative for expression of the Domain IV of TtDnaA | This work |
DnaA was cloned into the pET28a to generate p28MuDnaA.

To clone the chromosomal replication origin region of *T. tengcongensis*, the putative Tt-oriC region located between the rpmH (TTE2802) and dnaA (TTE0001) genes was amplified by PCR with primer T1 and T2 (Table 2). This Tt-oriC fragment was inserted into the pUCm-T (Sangon, Shanghai, China; Table 1), resulting in the plasmid pOC-T, which was used for subcloning or as the templates for Tt-oriC sequencing. Similarly, the subsequent pOC-1, pOC-2, pOC-3, pOC-4 and pOC-5 were constructed by cloning the corresponding sequence of Tt-oriC fragments into the pUCm-T with the indicated primers (Table 2).

For all of these constructs, the PCR-amplified sequences were verified by DNA sequencing.

**TtDnaA expression and purification**

_E. coli_ BL21(DE3) harboring the p28TtDnaA was used to overproduce the His-tagged TtDnaA protein by induction with 0.5 mM IPTG for 3 h. TtDnaA purification was performed as previously described, with minor modifications (17–19). Briefly, the cells were suspended in ice-cold buffer A [0.5 M NaCl, 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, 200 mM potassium glutamate] and treated with lysozyme (1 mg/ml) for 30 min on ice, and then further lysed by sonication. The cell lysates were centrifuged at 12 000 g for 20 min and the cleared supernatants were incubated at 60°C for 30 min, followed by centrifugation at 12 000g for 25 min. The soluble protein extracts were loaded onto the Ni-NTA (Ni²⁺-nitritriacetae)-agarose column (Novagen) according to the manufacturer’s instruction. The elution solution was precipitated with 0.34 g/ml ammonium sulfate. The precipitated proteins were centrifuged at 27 000g for 30 min, the pellets were suspended in 2.5 ml of LG buffer [45 mM HEPES/KOH (pH 7.6), 200 mM potassium glutamate, 10 mM magnesium acetate, 0.5 mM EDTA and 5 mM 2-mercaptoethanol]. The purified TtDnaA proteins were desalted by Amicon desalt column using buffer B [25 mM HEPES (pH 7.6), 200 mM potassium glutamate, 1 mM DTT]. Samples (1 ml) of TtDnaA protein (1.5 mg/ml) were incubated with 3 mM ATP or ADP for 24 h at 4°C. The solution was then concentrated to 200 μl using Centricon 30 tubes (Amicon). Dialysis was performed for 3 h at 4°C against 20 ml of LSE buffer [40 mM HEPES (pH 7.6), 10 mM MgSO₄, 1 mM EDTA, 1 mM Na-glutamate, 200 mM potassium glutamate, 1 mM DTT] containing 3 mM ATP (or ADP) with a 0.25-μm MF-Millipore membrane. TtDnaA aggregates, if any, are always removed by a high-speed centrifugation step. Other proteins were prepared with the similar method. Protein concentrations were determined by using the BCA™ protein assay kit (PIERCE).

**Yeast two-hybrid assay**

The GAL4-based yeast two-hybrid system (Clontech) was employed. The *dnaA* gene and its mutants were

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### Table 2. Partial oligonucleotides used in this study

| Names | Sequences (5' to 3') | Purposes |
|-------|---------------------|----------|
| P1    | TACATATGATTACCGTCAAATTTGGG | P1/P2- p28TtDnaA |
| P2    | TACTTCATACCTTCTCCTCCT | P2/P3- p28TtDnaA-DM |
| P3    | TAATGACCTCTTTTATCCTTTTTTAAG | P4/P5- p23TtDnaA-IV |
| P4    | TACTTCATACCTTCTCCTCCT | P6/P7 plus P8 -p28MuDnaA |
| P5    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | H1/H2-pGBKT7-TtDnaA, pGADT7-TtDnaA |
| P6    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | H3/H2- pGBKT7-DM, pGADT7-DM |
| P7    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | H1/H2- pGBKT7-TtDnaA, pGADT7-TtDnaA |
| P8    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | H3/H2- pGBKT7-DM, pGADT7-DM |
| T1    | AGTCTGTCTCCTCCCTTTAATC | T1/T2- pOC-T |
| T2    | AACTTCTACTATCTCCTCCTCCT | Primer extension |
| T3    | TCTCAGAACCCTAAACCAAGGGTTATAAGG | T3/T4- pOC-1 |
| T4    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | T3/T5- pOC-2 |
| T5    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | T3/T6- pOC-3 |
| T6    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | T5/T7- pOC-4 |
| T7    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | T7/T8- pOC-5 |
| T8    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | H1/H2- pGBKT7-TtDnaA, pGADT7-TtDnaA |
| H1    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | H3/H2- pGBKT7-DM, pGADT7-DM |
| H2    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | H1/H2- pGBKT7-TtDnaA, pGADT7-TtDnaA |
| H3    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | H3/H2- pGBKT7-DM, pGADT7-DM |
| H4    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | H4/H2- pGBKT7-IV, pGADT7-IV |
| NP    | AACCTCTACTATCTCCTCCTCCT | Primer extension |
| S1    | GAGGTCAGAAATTGAAATGTAACCACAACTCCTCGTAAAAGGAGGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | RIP mapping |
| S2    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | RIP mapping |
| S3    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | EMSA³ |
| S4    | TACATATGATTACCGTCAAATTGCGGAGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | EMSA³ |
| 1Box  | GAGGTCAGAAATTGAAATGTAACCACAACTCCTCGTAAAAGGAGGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | EMSA³ |
| 2Box3D | GAGGTCAGAAATTGAAATGTAACCACAACTCCTCGTAAAAGGAGGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | EMSA³ |
| 2Box2SD | GAGGTCAGAAATTGAAATGTAACCACAACTCCTCGTAAAAGGAGGAGGATAGTAGAAGTTATAAAAAGTGAGAGTACCC | EMSA³ |

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*Restriction sites are underlined; DnaA boxes are boxed.*

*Prior to using in EMSA assay, the oligonucleotides were annealed with their complement oligonucleotides.*
amplified by corresponding primers (Table 2) and then cloned into the pGADT7 and pGBK7T vectors, resulting in the plasmids pGBK7-TtDnaA/pGADT7-TtDnaA, pGBK7T7-DM/pGADT7-DM, and the pGBK7T7-IV/ pGADT7-IV (Table 1), respectively. The AH109 yeast strain was transformed with the appropriate plasmids using the lithium acetate procedure, and grown on SD plates in the absence of Leu and Trp (SD−Leu/−Trp). Protein interactions were tested on SD plates without Leu, Trp and His (SD−Leu−Trp−His).

Electrophoretic mobility shift assay (EMSA)

Unless otherwise noted, 32P-labeled DNA oligonucleotides (1Box; 2Box3D and 2Box25D, Table 2) and full-length Tt-oriC fragment (purified 472-bp PCR product amplified with primers T3 and T4, Table 2) were incubated with TtDnaA protein in the presence of non-specific competitor [0.05 mg/ml poly(dI-dC), Sigma] at 37 or 60°C for 30 min, in the binding buffer [20 mM HEPES/KOH (pH 7.6), 5 mM magnesium acetate, 1 mM EDTA, 4 mM dithiothreitol, 0.2% Triton X-100, 3 mM ATP, 25 μg/ml bovine serum albumin and 5% glycerol]. The samples were run on a 6% polyacrylamide gel in 0.5×TBE (preheated to 37 or 60°C) at 140 V for 2–3 h. The gels were analyzed by autoradiography.

RIP mapping

RIP mapping was performed as previously described (20,21) with minor modifications. Briefly, total genomic DNA was denatured for 4 min at 100°C in 10 mM Tris-HCl (pH 8.3) and 0.1 mM EDTA, followed by rapidly chilled at 0°C. All 5'-OH termini existing in denatured DNA were first phosphorylated by treatment with T4 polynucleotide kinase and ATP at 37°C. Afterwards, the replication intermediates were enriched by a benzooylated naphtholyated DEAE (BND)-cellulose column and then treated with λ-exonuclease (New England Biolabs) to digest the nicked DNA at 37°C for 24 h, whereas the nascent DNA was protected by its RNA primer and not digested. For primer extension, ~500 ng of template DNA, 25 ng of the radio-labeled primer and 2 U of Vent (exo-) DNA polymerase were incubated in 25-μl buffer provided by the manufacturer (New England Biolabs). After 30 cycles of primer extension reaction (1 min at 94°C, 1 min at 70°C and 1.5 min at 72°C) with the primers S1 and S2 (Table 2), samples were analyzed on an 8% denaturing polyacrylamide gel. The corresponding sequencing reactions with the same primers using pOC-T as the template were performed and analyzed side by side on the same gel.

Surface plasmon resonance (SPR) assay

SPR assay was performed on the BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden) as described by Speck and Messer (7) with minor modifications. The streptavidin-coated sensor chip SA was conditioned with 3–5 1-min injections of 1 M NaCl in 50 mM NaOH at 10 μl/min (until a stable baseline was observed) and then washed twice with 0.05% SDS in TES (10 mM Tris pH 8.0, 300 mM NaCl, 1 mM EDTA) for 3 min. Biotinylated double-stranded oligonucleotides (Box-AT, Boxm-AT and Box-ATm in Figure 6A) were diluted in TES and immobilized to 100 RU respectively using TES as the flow buffer. The TtDnaA protein was diluted in HKM buffer (25 mM HEPES pH 7.6, 100 mM potassium acetate, 1 mM magnesium acetate, 0.005% BIAcore surfactant P20) containing 33 ng/μl of poly(dI-dC). Samples in a concentration range of 1–200 nM of TtDnaA were injected with the K-inject command. At the end of each cycle, bound TtDnaA was removed with a 5-μl pulse of 0.005% SDS in TES. For data analysis, the BIAevaluation 3.0 program (BIAcore AB, Sweden, Uppsala) was used.

Open complex formation assay

The open complex formation assay was performed by nuclease P1 digestion and primer extension analysis as previously described with minor modifications (18,19). Briefly, the indicated amounts of TtDnaA samples were incubated for 3 min at 48°C in pre-warmed buffer (50 μl) containing 60 mM HEPES-KOH (pH 7.6), 0.1 mM zinc acetate, 8 mM magnesium acetate, 30% (v/v) glycerol, 0.32 mg/ml BSA, 16 ng E. coli HU protein, 5 mM ATP and supercoiled pOC-1 or other plasmids (400 ng), followed by incubation for 100 s at the same temperature in the presence of nuclease P1 (10 U, Yamasa). DNA was then digested with AlwNI, followed by 1% agarose gel electrophoresis and ethidium bromide staining. Primer extension analysis was performed by using the DNA polymerase I Large Fragment with 32P-end labeled primers, T3 and NP (Table 2), respectively.

RESULTS

Characterization of the replication origin and the initiator protein in T. tengcongensis

The deduced replication origin (Tt-oriC) region of T. tengcongensis is located immediately upstream of the replication initiator gene (dnaA) (14,15). It is composed of 12 unusually arranged DnaA boxes and 7 AT clusters (Figure 1A). The consensus sequence for these T. tengcongensis DnaA boxes is 5'-TTATCNACA-3', resembles those of E. coli (5'-TTATNCACA-3') and T. thermophilus (5'-TTATCCACA-3'). The T. tengcongensis DnaA boxes 1–8 and 9–12 are clustered in two groups, with an 89-bp spacing containing the longest AT cluster IV (18 mer). The DnaA boxes 9–12 overlapped the promoter region of the dnaA gene and thus might be involved in regulation of gene expression, while the boxes 1–8 may contribute to duplex opening. These predictions need to be experimentally elucidated.

Besides the DnaA boxes, another important element in oriC is the AT-rich region where the oriC duplex is unwound. It is well established that such an AT-rich region contains an AT cluster plus three 13-mer repeats in E. coli (4). However, due to the high AT content
of the Tt-oriC, and the presence of many AT clusters but absence of any characteristic repeats (Figure 1A), it is hard to predict the functional AT-rich region. Thus, it is necessary to design functional assays to localize it in T. tengcongensis.

The dnaA gene is located downstream of the Tt-oriC region, and expresses a DnaA protein (TtDnaA) of 433 amino acid residues (\(48.4\) kDa). The TtDnaA is most similar to the DnaA from Clostridium acetobutylicum (61% identity) and distantly related with the counterpart of E. coli (32.9% identity). Based on structural and functional analysis of the DnaA homologs (11,22), four domains of TtDnaA were deduced (Figure 1B). The key elements of the DNA-binding Domain IV including the DnaA basic loop (B-loop) and DnaA signature sequence (A-sig) are conserved among T. tengcongensis, E. coli and another thermophilic bacterium A. aeolicus. Two conserved amino acids, W9 and L20 (corresponding to the W6 and L17 in E. coli DnaA), important for DnaA oligomerization (23,24) were also found in the N-terminus of the TtDnaA domain I.

Efficient binding of TtDnaA at high temperature requires at least two DnaA boxes

To reveal the mechanism of replication initiation at high temperature in T. tengcongensis, EMSA was used to study the complex formation between TtDnaA and various DnaA boxes of the Tt-oriC at 60°C. The following DNA fragments were used (Table 2): (i) oligonucleotides containing one typical T. tengcongensis DnaA box (TTATCCACA in 1Box); (ii) oligonucleotides containing two T. tengcongensis DnaA boxes with 3-bp distance (2Box3D); (iii) oligonucleotides containing T. tengcongensis two DnaA boxes with 25-bp distance (2Box25D). Interestingly, although the DNA fragment containing single DnaA box could be efficiently bound by TtDnaA at 37°C (data not shown), it was not bound at 60°C even with a high concentration of TtDnaA (1800 nM) (Figure 2A). However, when the DNA fragment containing two DnaA boxes with 3-bp spacing was used, TtDnaA could achieve the efficient binding at a much lower protein concentration (8 nM) (Figure 2B). It is noteworthy that the binding of the TtDnaA to the 31-bp oligonucleotides containing two T. tengcongensis
DnaA boxes resulted in multiple complex bands (Figure 2B), implying that TtDnaA may be oligomerized. When the distance between the two DnaA boxes was increased to 25 bp, the DNA fragment was still bound by TtDnaA, although the binding affinity decreased a little (Figure 2C). These results suggest that specific binding of the TtDnaA protein at high temperature requires at least two neighboring repeated DnaA boxes. Moreover, our results also explain why TtDnaA can efficiently bind onto the whole Tt-oriC at high temperature since the distance between two neighboring DnaA boxes is usually <25 bp in T. tengcongensis.

The TtDnaA domain IV is important for efficient binding of Tt-oriC at high temperature

When the full-length Tt-oriC fragment was used in the binding assay, TtDnaA displayed a similar binding affinity to Tt-oriC at both low (37°C) and high (60°C) temperatures (Figure 3A). To understand such adaptive ability of TtDnaA in oriC binding at high temperature, the domain IV of TtDnaA (the predicted DNA-binding domain, Figure 1B) was analyzed. When this domain in TtDnaA was replaced with the counterpart of the E. coli DnaA, EMSA revealed that the hybrid protein (Tt/EcDnaA) could bind to the Tt-oriC at 37°C, but not at 60°C (Figure 3B). This result suggests that the TtDnaA Domain IV is thermo-adaptive, and is important for efficient binding of Tt-oriC at high temperature.

Another important feature observed during complex formation between the TtDnaA protein and the entire Tt-oriC region was that such interaction gave rise to only 1–2 main high-molecular-mass bands (Figure 3A), which is different from the similar interaction in E. coli where the interaction leads to the formation of multiple discrete nucleoprotein complexes appeared as a ladder of retarded bands (25,26). The formation of discrete nucleoprotein complexes can be explained by monomeric binding of the E. coli DnaA to multiple DnaA boxes at lower concentration of DnaA (25). Unlike that in E. coli but similar to Mycobacterium tuberculosis (17), incubation of the T. tengcongensis DnaA protein with the Tt-oriC led to the formation of only 1–2 high-molecular-weight complexes from low TtDnaA concentration, indicating that the complex might be assembled by cooperative binding of the TtDnaA protein to the DnaA boxes and the TtDnaA might also be highly oligomerized.

The TtDnaA self-oligomerization is essential for efficient binding to Tt-oriC at high temperature

To examine whether the TtDnaA self-oligomerization is essential for specific binding at high temperature, the oligomerization-deficient mutants of TtDnaA were constructed and subjected for the binding affinity assay. Yeast two-hybrid assay showed that the wild-type TtDnaA could self-interact, but the TtDnaA-DM mutant (W9A/L20S, Figure 1B) and TtDnaA-IV (the TtDnaA-binding domain only) lost the capability of self-interaction and hence should be oligomerization-deficient (Figure 4A). When the purified TtDnaA and mutant proteins were used to bind the entire Tt-oriC fragment, we found that unlike the wild-type TtDnaA that can bind to the Tt-oriC efficiently at both 37 and 60°C (Figure 3A), the oligomerization-deficient DnaA mutant proteins, TtDnaA-DM and TtDnaA-IV, could only bind to the Tt-oriC at 37°C, but not at 60°C even at their high concentrations (Figure 4B and C). These results clearly
indicated that the TtDnaA self-oligomerization is absolutely required for TtDnaA binding to Tt-oriC at high temperature.

Taking into account the above results of TtDnaA/Tt-oriC interaction, it could be concluded that the TtDnaA protein requires at least two DnaA boxes for efficient binding at high temperature, and the specific Domain IV as well as the self-oligomerization of TtDnaA are both essential for this binding, thus should also be required for replication initiation in this thermophile.

**The replication initiation point (RIP) is located at an unusual AT-rich region in Tt-oriC**

To precisely localize the RIP in the chromosome of *T. tengcongensis*, the technique of RIP mapping (20) was employed. This technique has been successfully used to detect the start sites of DNA replication at the nucleotide level in chromosomes (21,27) or plasmid (28) in many organisms. After isolation of the DNA samples from the rapidly dividing cells of *T. tengcongensis*, the replication intermediates were enriched and subjected to the λ-exonuclease treatment to remove the nicked DNA. The RNA–DNA junction in the RNA-primed nascent DNA was then detected by primer extension, and the transition point (TP, which represents the RIP) of the
leading and lagging strands was finally determined. It was revealed that the replication was initiated in the longest AT cluster IV located 25-bp downstream of the neighboring DnaA box 8. The TPs at the top and bottom strands spanned only \( /C24 \) bp (Figure 5A and B), thus represented the RIP of *T. tengcongensis*. Although the AT cluster is located in an AT-rich region (Figure 6A), this functional AT-rich region is distinct from that of *E. coli*, as neither typical repeat nor ATP-DnaA box was found therein.

**ATP-TtDnaA specifically binds the AT-rich region**

Since no typical ATP-DnaA box was found in the AT-rich region where replication normally initiated, it would be interesting to investigate whether the TtDnaA protein interacts with this region to open the *Tt-oriC*. For this purpose, a more sensitive method named SPR technique was used to measure the binding activities of ATP-TtDnaA or ADP-TtDnaA to the following DNA fragments, which contain the AT-rich region and its adjacent DnaA boxes 6–8 or respective mutants (Figure 6A): (i) DnaA boxes 6–8 and AT-rich region (Box-AT); (ii) DnaA boxes 6–8 and mutated AT-rich region (Box-ATm); (iii) Mutated DnaA boxes 6–8 and AT-rich region (Boxm-AT).

Representative binding results were shown in Figure 6B–G, which indicated that ATP-TtDnaA (Figure 6B), but not ADP-TtDnaA (Figure 6C), could weakly but specifically bind to the AT-rich region (Ka 250 nM). Significantly, although the ATP-TtDnaA and ADP-TtDnaA could almost equally bind to the DnaA boxes 6–8 (Figure 6D and E), ATP-TtDnaA also has a higher affinity with the BOX-AT fragment than ADP-TtDnaA, with the corresponding Ka of 12 and 15 nM, respectively (Figure 6F and G), confirming that the ATP-DnaA bound to the AT-rich region. Interestingly, when the neighboring DnaA boxes 6–8 existed, this interaction was enhanced, as the maximum binding response (3250 RU, Figure 6F) of BOX-AT bound by ATP-TtDnaA is obviously higher than the sum of that of BOXm-AT (300 RU, Figure 6B) and BOX-ATm (1800 RU, Figure 6D).

**ATP-TtDnaA but not ADP-TtDnaA nor oligomerization-deficient mutants unwinds the *Tt-oriC* duplex in vitro**

With the purified DnaA and oriC, the nucleoprotein initiation complexes that could undergo site-specific DNA unwinding in vitro have been reconstituted in *E. coli* (4) and *Thermotoga maritima* (19). Since ATP-TtDnaA could bind both *Tt-oriC* DnaA boxes and the AT-rich region, we then used the nuclease P1 assay to determine whether the initial opening of the DNA duplex could occur at this AT-rich region. The plasmid pOC-1 (3245 bp) containing the *Tt-oriC* region (Figure 7A) was incubated with ATP-TtDnaA in the presence of *E. coli* HU protein (29), and then treated with nuclease P1 and AlwNI. As nuclease P1 only breaks DNA at single-stranded site, so if the ATP-TtDnaA unwinds the DNA duplex at the *Tt-oriC* AT-rich region, digestion with nuclease P1 and AlwNI will produce two fragments of \( /C24 \) 1.0 and 2.2 kb (Figure 7A). We found that when TtDnaA and the supercoiled pOC-1...
plasmid were incubated at 48°C in the presence of *E. coli* HU protein and 6mM ATP, the *Tt-oriC* region was unwound specifically. The unwinding site is very likely located at the AT-rich region, as the characteristic fragments (~1.0 and 2.2 kb) were generated after P1/AlwNI digestion (Figure 7B, ATP form).

In contrast, the ADP-TtDnaA is deficient to unwind the *Tt-oriC*, as no P1-digested products were generated in this assay (Figure 7B, ADP form). The requirement of ATP form of TtDnaA for site-specific unwinding at the *Tt-oriC* region is similar to those in *E. coli* and *T. maritima* (4,19), further supporting that the ATP-dependent activation of DnaA in replication initiation is highly conserved in eubacteria. Significantly, when the oligomerization-deficient mutants were subjected to this site-specific duplex unwinding assay, it was revealed that both the TtDnaA-DM and TtDnaA-IV completely lost the capability for open complex formation (Figure 7C), indicating that oligomerization of TtDnaA, as revealed in *E. coli* (24), is also strictly required for *Tt-oriC* unwinding.
The DNA duplex opens at the RIP-located AT-rich region

To further confirm and precisely determine that the unwinding site of Tt-oriC is within the AT-rich region, the primer extension experiments were performed with the unwound pOC-1 as the template after nuclease P1 digestion (see above). The primer extension products from both the top and bottom strands pointed the same AT-rich region between the DnaA boxes 8 and 9 (Figure 8A and B). The dominant nuclease P1-cut borders of the unwinding region are very close to the AT cluster where the RIP is located (Figure 8B), implying that the ATP-TtDnaA might unwind the Tt-oriC at the AT-rich cluster, and then spread up to ~40 bp, thus providing enough space for the subsequent entry of the DnaB–DnaC complex (4) for replication initiation.

The minimal Tt-oriC region for open complex formation in vitro contains DnaA boxes 1–8 and an AT-rich region

As the functional AT-rich region for duplex opening is located in the middle of the 12 DnaA boxes, it should be interesting to investigate the function of the DnaA boxes located up- or downstream of the RIP region in replication initiation. For this purpose, the minimal Tt-oriC sequence required for open complex formation was determined by deletion analysis. The capability of each deleted fragment of Tt-oriC in ATP-TtDnaA-induced duplex opening was examined by the nuclease P1 assay. Our results revealed that the deletion of the DnaA boxes 9–12 did not affect the open complex formation (Figure 9, pOC-2), but deletions of the AT-rich region, DnaA box 1 or a short sequence upstream of the DnaA box 1 resulted in loss of the capacity for duplex opening (Figure 9, pOC-3 to pOC-5). Thus the minimal Tt-oriC for open complex formation in vitro would include the AT-rich region and its upstream sequence containing the DnaA boxes 1–8. It is noteworthy that the short sequence between the rpmH and the DnaA box 1, with an AT-cluster but without DnaA boxes or repeats, is also essential (Figure 9, pOC-2). In contrast, the DnaA boxes

Figure 7. Activities of the TtDnaA and its mutants in open complex formation at Tt-oriC. (A) Structure of the Tt-oriC-bearing plasmid pOC-1. The AT cluster in Tt-oriC corresponding the RIP (see Figure 5) is indicated. The supercoiled form of this plasmid will be used in the following reactions. (B) Nuclease P1 assay of the DNA-duplex-opening activities of the ATP- or ADP-form of TtDnaA. The indicated amounts (3, 5 or 8 pmol) of TtDnaA were incubated for 10 min at 48°C in buffer (50 μl) containing pOC-1 and E. coli HU protein (16 ng), followed by digestion with nuclease P1 and AlwNI. ck, the plasmid pOC-1 without TtDnaA incubation was directly digested by nuclease P1 but not AlwNI. (C) Nuclease P1 assay of the DNA-duplex-opening activities of wild-type TtDnaA (WT), oligomerization-deficient double-site-mutated (W9A/L20S) TtDnaA (DM) and Domain IV only of TtDnaA (IV), respectively. ck, the plasmid pOC-1 without TtDnaA incubation was directly digested by nuclease P1 and AlwNI. The samples (both in B and C) were analyzed by 1% agarose gel electrophoresis.

Figure 8. Determination of the precise unwinding sites within the Tt-oriC after open complex formation. (A) The unwinding reaction and nuclease P1 digestion were performed as described in Figure 7. The DNA was then isolated and subjected to primer extension analysis with the 32P-labeled T3 and NP primer, respectively. The products were analyzed with 8% denaturing polyacrylamide gel. Sequencing reactions (AGTC) with the same primers were run side by side on the same gel. The arrowheads indicate the nuclease P1-digested sites. The AT-cluster and the DnaA boxes 6–9 are also indicated. (B) The sequence of the Tt-oriC region unwound by ATP-TtDnaA is shown. The RIP and the AT cluster are indicated, the big arrowheads indicate the major borders of unwinding region and the small arrowheads indicate the minor ones.
arranged DnaA boxes, located upstream of the dnaA gene, and the consensus sequence (5'-TTATCNACA-3') indicated as shown in Figure 8B, while the minimal sequence needed for open complex formation in this in vitro system, although its function for replication initiation in vivo remains to be explored.

**DISCUSSION**

Much of our knowledge on replication initiation of bacterial chromosome is based on model organisms such as E. coli and B. subtilis, and the corresponding process at high temperature remains to be further investigated. T. tengcongensis would be an interesting thermophilic bacterium for this investigation. It is a low-GC Dam-negative thermophile, and the predicted replication origin (Tt-oriC) is different from that of E. coli or B. subtilis in both the size and structure. It has been suggested that the quantity, localization, orientation and distance of the DnaA boxes and other elements are important for efficient replication initiation in a given bacteria (1,30-32). Therefore, establishment of the replication initiation model in T. tengcongensis would not only provide novel insights about the replication initiation in a specific Dam-negative bacterium, but also shed light on how replication initiates at high temperature.

The Tt-oriC region is composed of 12 unusually arranged DnaA boxes, located upstream of the dnaA gene, and the consensus sequence (5'-TTATCNACA-3') for TtDnaA boxes is similar to those of T. thermophilus and E. coli. However, the high-AT feature of T. tengcongensis makes it very difficult to predict the functional AT-rich region where replication may start. Using RIP mapping and open-complex formation assay, in the present study, we have demonstrated the exact position of replication initiation in T. tengcongensis (Figures 5 and 8). It was precisely located in the middle of the Tt-oriC region, with the DnaA boxes 1–8 at the upstream and the boxes 9–12 at the downstream. The replication of T. tengcongensis was shown to initiate from an AT cluster in this AT-rich region. To our knowledge, this is the first experimental demonstration of the chromosomal RIP in thermophilic bacteria at nucleotide level.

This AT-rich region is distinct from the counterpart of E. coli as no typical ATP-DnaA boxes or repeats were found there. However, our results indicated that ATP-TtDnaA, but not ADP-TtDnaA, indeed bound the Tt-oriC AT-rich region. Interestingly, although the AT-rich region of T. tengcongensis is located in the middle of the DnaA boxes between the DnaA boxes 8 and 9 while that of T. thermophilus is located downstream of all the DnaA boxes, open complex assay indicated that the downstream DnaA boxes 9–12 in T. tengcongensis may not be necessary for duplex opening. Moreover, the distance between the AT cluster and the neighboring DnaA box are both 25 bp in these two thermophiles (16), indicating that the oriC features of T. tengcongensis and T. thermophilus are more similar and the location of the AT-rich region might also be important for replication initiation in thermophile bacteria.

It is noteworthy that the function of DnaA boxes 9–12 in DNA replication should not be underestimated. As these four DnaA boxes are located in or immediately upstream of the dnaA promoter region, DnaA binding of these boxes might regulate the gene expression of dnaA, and thus control the T. tengcongensis replication initiation. In addition, there are three other DnaA boxes located downstream of the dnaA gene (15). These boxes may also be involved in the dnaA gene expression regulation. As the T. tengcongensis is a Dam-negative bacterium, and the mechanisms for replication regulation that are established in E. coli, including the SeqA pathway (8), RIDA pathway (33-37) or the datA-mediated DnaA titration (38,39), seem not to be applied to this bacterium (corresponding gene homologs were not found in T. tengcongensis genome), so these DnaA boxes may play an important role in control of the replication by regulating the adjacent dnaA gene.

For T. tengcongensis, the binding requirements of TtDnaA to Tt-oriC at high temperature are another important question that should be addressed. Using EMSA, we revealed that it requires the thermo-adaptive feature of TtDnaA Domain IV and at least two DnaA boxes. Although the DnaA-binding Domain IV is usually well conserved in bacteria, there still exist many amino acids that are conserved only in the two thermophiles (T. tengcongensis and A. aeolicus) but different from those of E. coli (Figure 1B), thus it is understandable why TtDnaA lost the binding activity at high temperature when its binding domain was replaced by the counterpart of E. coli, as those different amino acids might result in different binding behavior especially at high temperature (Figure 3B). It is also possible that the structure of TtDnaA Domain IV is more stable and functional at high temperature than that of E. coli.

Likewise, the efficient binding of TtDnaA to Tt-oriC at high temperature requiring at least two neighboring DnaA boxes is very interesting. We found that even the TtDnaA box is very typical (TTATCCACA) in T. tengcongensis, single box cannot be bound by TtDnaA at high temperature (Figure 2A). Perhaps, cooperative interaction of DnaA with more DnaA boxes could
enhance the binding intensity especially at high temperature. It has been reported that EMSA will not detect binding to a single lower affinity box in *E. coli*, unless cooperation occurs with a DnaA that is bound to a perfect DnaA box close by (25). Similarly, if the DnaA box is not typical, the DnaA will require two DnaA boxes for efficient binding in *M. tuberculosis* (17), *Helicobacter pylori* (40) and *Streptomyces lividans* (41,42). But significantly, even the DnaA boxes are very typical, the DnaA will require two DnaA boxes for efficient binding (16). These results suggested that cooperative interaction of DnaA with more DnaA boxes may be a conserved mechanism in thermophiles.

Significantly, we also found that the DnaA self-oligomerization is absolutely required for Ti-DnaA binding to *Tt*-oriC at high temperature. Although the oligomerization-deficient DnaA mutants could still bind to *Tt*-oriC at 37°C, it lost the oriC-binding activity at a high temperature (60°C) (Figure 4), suggesting that DnaA self-oligomerization can enhance the binding affinity at the *Tt*-oriC. This is the first observation that the DnaA oligomerization affects its binding affinity at different temperature. Moreover, we have also revealed that oligomerization of ATP-Ti-DnaA is required for open complex formation at high temperature (Figure 7C), which indicates that oligomerization may also assist the efficient assembly of ATP-Ti-DnaA on *Tt*-oriC at high temperature to unwind the DNA duplex.

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