The functional analysis of YabA, which interacts with DnaA and regulates initiation of chromosome replication in *Bacillus subtilis*

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The initiation of bacterial chromosome DNA replication and its regulation are critical events. DnaA is essential for initiation of DNA replication and is conserved throughout bacteria. In *Escherichia coli*, hydrolysis of ATP-DnaA is promoted by Hda through formation of a ternary complex with DnaA and DnaN, ensuring the timely inactivation of DnaA during the replication cycle. In *Bacillus subtilis*, YabA also forms a ternary complex with DnaA and DnaN, and negatively regulates the initiation step of DNA replication. However, YabA shares no structural homology with Hda and the regulatory mechanism itself has not been clarified. Here, in contrast to Hda, we observed that *dnaA* transcription was stable during under- and overexpression of YabA. ChAP-chip assays showed that the depletion of YabA did not affect DNA binding by DnaA. On the other hand, yeast two-hybrid analysis indicated that the DnaA ATP-binding domain interacts with YabA. Moreover, mutations in YabA interaction-deficient mutants, isolated by yeast two-hybrid analysis, are located at the back of the ATP-binding domain, whereas Hda is thought to interact with the ATP-binding pocket itself. The introduction into *B. subtilis* of a *dnaA*Y144C mutation, which disabled the interaction with YabA but did not affect interactions either with DnaA itself or with DnaD, resulted in over-initiation and asynchronous initiation of replication and disabled the formation of YabA foci, further demonstrating that the amino acid on the opposite side to the ATP-binding pocket is important for YabA binding. These results indicate that YabA indeed regulates the initiation of DNA replication by a different mechanism from that used by Hda in the *E. coli* RIDA system. Interestingly, all DnaA mutants deficient in YabA binding also displayed reduced DnaD binding in yeast two-hybrid assays, suggesting that YabA can inhibit replication initiation through competitive inhibition of DnaD binding to DnaA.

**Key words:** DnaA protein, YabA protein, *Bacillus subtilis*, replication initiation

**INTRODUCTION**

Bacterial chromosomal replication is initiated at a site called oriC (origin of chromosome) by binding of the highly conserved origin recognition protein DnaA (Messer, 2002). Biochemical and structural studies have shown that DnaA consists of four domains: domain I, which binds the replicative helicase DnaB (DnaC in *Bacillus subtilis*); a linker domain II, which is not essential for cell viability and is highly variable in length; domain III, a highly conserved ATP-binding domain, homologous to AAA+ superfamily proteins, that comprises a large and small subdomain (IIIA and IIIB, respectively); and domain IV, another highly conserved DNA-binding domain that specifically recognizes DnaA-binding sequences (Erzberger et al., 2002). In *Escherichia coli*, DnaA binds via domain IV to multiple 9-base-pair sequences (TTa/tTNCACA) within oriC, termed the DnaA box (Messer et al., 2001), to form a nucleoprotein complex spanning approximately 250 base pairs with 20–30 DnaA monomers (Fuller et al., 1984; Matsui et al., 1985). The complex then opens an adjacent (A+T)-rich region referred to as a DNA-unwinding element (DUE), generating the single DNA strands necessary for sequential loading of DnaB helicase and DnaN, the β clamp subunit of DNA polymerase III holoenzyme. Although both ATP-
and ADP-bound DnaA (ATP-DnaA and ADP-DnaA) bind to DnaA boxes with a similar affinity, only ATP-DnaA induces melting of the DUE, by interacting with additional weaker-affinity DnaA boxes in oriC, which are only recognized by ATP-DnaA, and homo-oligomerization of DnaA proteins (Messer et al., 2001). Recently, a unique higher-order structure of DNA bound by ATP-DnaA, a right-handed DNA wrap around a large oligomeric DnaA complex, has been proposed on the basis of the crystal structure of DnaA bound with AMP-PCP (the non-hydrolyzable ATP analog adenosine 5’-(β, γ-methylene)-triphosphate) (Erzberger et al., 2006).

In *B. subtilis*, the basic system of replication initiation is the same as in *E. coli*: *B. subtilis* oriC also has multiple DnaA boxes and (A+T)-rich stretches, and DnaA binding induces melting at one of the (A+T)-rich stretches between dnaA and dnaN, leading to DNA replication in vitro (Fukuoka et al., 1990; Moriya et al., 1994). In addition, the in vitro replication of plasmids containing *B. subtilis* oriC has been shown to be strictly dependent on the presence of ATP (Moriya et al., 1994). However, the system by which DnaC helicase (the counterpart of *E. coli* DnaB) is loaded onto the single DNA strands is different. In *E. coli*, this is accomplished solely by DnaC (a homolog not of *B. subtilis* DnaC but of Dna1), but in *B. subtilis*, as well as Dna1, it has been suggested that DnaD and DnaB, essential proteins for replication initiation, work co-operatively in the helicase-loading reaction (Braund et al., 2005). In addition, yeast two-hybrid analysis revealed an interaction between DnaA and DnaD, and genetic studies suggested the importance of this interaction for replication initiation (Ishigo-Oka et al., 2001).

To ensure that the chromosome is replicated only once per cell cycle, two systems for regulating replication initiation operate in *E. coli*. One sequesters oriC to the cell membrane while reducing levels of active DnaA, which is accomplished by SeqA binding to hemimethylated oriC after initiation (Lu et al., 1994; von Freiesleben et al., 1994; Brendler et al., 1995; Slater et al., 1995; Brendler and Austin, 1999; Skarstad et al., 2000). The other is RIDA (regulatory inactivation of DnaA), which is accomplished by Hda (homologous to DnaA) (Katayama, 2001; Camara et al., 2003) and has been shown as the major mechanism for preventing over-initiation of DNA replication in *E. coli* (Camara et al., 2005). In this latter system, hydrolysis of ATP-DnaA is promoted when ATP-DnaA interacts in the presence of DNA-loaded DnaN with Hda, which can form a stable complex in vitro, ensuring the timely inactivation of DnaA during the replication cycle (Katayama et al., 1998; Kurokawa et al., 1999; Katayama, 2001; Kato and Katayama, 2001; Su‘etsugu et al., 2004, 2005; Gon et al., 2006; Kawakami et al., 2006). It has also been suggested recently that RIDA and dnaA gene autoregulation act in concert to secure once-per-cell-cycle initiation, based on the observations that Hda deficiency leads to reduced dnaA gene expression and that overproduction of Hda leads to DnaA overproduction (Riber et al., 2006).

In Gram-positive bacteria, including *B. subtilis*, however, homologs of the Hda and SeqA proteins have not been identified. On the other hand, in *B. subtilis*, YabA, which is conserved in low-(G+C) Gram-positive bacteria, was identified by yeast two-hybrid screening as a protein that interacts with DnaA and DnaN, as does Hda (Noirot-Gros et al., 2002), and a yabA deletion mutant exhibits overinitiation and replication asynchrony (Noirot-Gros et al., 2002; Hayashi et al., 2005). Recent functional analysis of two YabA mutants, in which the interaction with DnaA or DnaN was disrupted independently, showed that initiation control and the formation of YabA foci were abolished in both mutants, indicating that interactions with both DnaA and DnaN are essential for YabA function (Noirot-Gros et al., 2006). In addition, purified YabA oligomerized and formed a ternary complex with DnaA and DnaN, indicating that YabA negatively regulates initiation through formation of this complex and that it enables the functional association of the complex with the cell cycle via replication, as is the case for Hda (Noirot-Gros et al., 2006). In contrast to Hda, however, YabA shares no structural homology with the AAA+ family of ATPases, including DnaA, and lacks a DnaN-binding consensus motif (Noirot-Gros et al., 2002). These observations suggest that the regulatory mechanisms of YabA are different from those of Hda, and it is therefore not yet well understood how YabA regulates replication initiation via direct interactions with DnaA and DnaN in *B. subtilis*.

In this study, we demonstrate that the regulatory mechanisms of YabA in *B. subtilis* and Hda in *E. coli* are indeed different. Western blotting analysis showed that, in contrast to Hda in *E. coli*, the amount of YabA in *B. subtilis* cells does not affect the expression level of DnaA. Moreover, ChAP-chip (Chromatin Affinity Precipitation coupled with tiling chip) assays (Ishikawa et al., 2007) of DnaA indicated that depletion of YabA does not affect the binding of DnaA to eight DnaA-binding regions on the *B. subtilis* genome. Supporting these observations, yeast two-hybrid analysis showed that YabA interacts with DnaA on the opposite side of the ATP-binding pocket in domain III, in contrast to Hda, which has been suggested to interact with the ATP-binding pocket itself. The introduction into *B. subtilis* of a dnaA*Y144C* mutation, which disabled the interaction with YabA but did not affect interactions either with DnaA itself or with DnaD, resulted in over-initiation and asynchronous initiation of replication, as observed in YabA-depleted cells, and disabled the formation of YabA foci. In addition, in vitro binding assays between DnaA and YabA by zinc precipitation also revealed that the DnaA*Y144C* mutant protein showed reduced binding activ-
ity to YabA, demonstrating that the amino acid on the opposite side to the ATP-binding pocket is important for YabA binding. Interestingly, all DnaA mutants deficient in YabA binding also displayed reduced DnaD binding in yeast two-hybrid assays, suggesting that YabA can inhibit replication initiation through competitive inhibition of DnaD binding to DnaA.

MATERIALS AND METHODS

**Bacterial strains and primers** *B. subtilis* CRK6000 (Moriya et al., 1990) and its derivatives used in this study are listed in Table 1. *E. coli* strains DH5α and C600 were used throughout as cloning hosts. All primers used for PCR are listed in Table 2. Structures of plasmids and strains constructed in this study were confirmed by DNA sequencing to avoid PCR mutations.

**Construction of strains expressing C-terminally histidine-tagged DnaA** Strains expressing C-terminally histidine-tagged DnaA from its original position without plasmid integration between dnaA and dnaN were constructed as previously described (Ishikawa et al., 2006). Briefly, CRK6000 and NIS6051 were transformed with the integration plasmid pMUTinAhisN (Ishikawa et al., 2007), and transformants were selected on PAB medium supplemented with 0.5 μg/ml erythromycin and 1 mM IPTG to induce essential dnaN expression from the spac promoter of the integration plasmid downstream of the integration position. The resultant transformants were then cultured in competent medium containing 1 mM IPTG, to induce a second recombination event for removal of the integrated plasmid, and selected on PAB medium without erythromycin and IPTG. Finally, insertion of the 12 x His coding sequence at the 3′ end of dnaA and removal of the integrated plasmid were confirmed by sequencing, and the transformants were designated as UC6000AH and UC6051AH, respectively.

**ChAP-chip analysis of DnaA** ChAP-chip analysis was performed as previously described (Ishikawa et al., 2007) with the following modifications. UC6000AH and UC6051AH cells were cultured in PAB at 30°C and DnaA complexes were purified in UT buffer without DTT (100 mM HEPES, 50 mM imidazole, 8 M urea, 0.5 M NaCl, 1% Triton X-100, 1 mM PMSF, pH 7.4) using TALON Magnetic Beads (Clontech). In addition, a newly designed Genechip with probes at 9-base intervals on both strands was used. The signal intensities of mismatched probes were subtracted from those of perfectly matched probes. If the resultant values were negative, the probes were omitted from further analysis. The signal intensities of DNA in the affinity-purified fraction and those of DNA isolated from the whole cell extract fraction before purification (control DNA) were adjusted to confer a signal average of 500. Signal intensities of DNA in the affinity-purified fraction were then divided by those of control DNA to obtain DnaA-binding signals, and plotted along the genome coordinates.

**Quantitative PCR** DNA fragments co-purified with DnaA were extracted as for ChAP-chip analysis. The eluate was diluted 32-fold, and used as templates for quantitative PCR after further 4-fold serial dilutions. The oriC sequence between rpmH and dnaA was amplified by KOD-plus- DNA polymerase (Toyobo) in the presence of 1.5 mM MgSO4 using rpmH-dnaA.f and rpmH-dnaA.r primers. PCR was performed for 30 cycles using 1 μl of the diluted templates in 10 μl of reaction mixture, and 6 μl of the products were separated in a 2% agarose gel, followed by ethidium bromide staining.

**Yeast two-hybrid assay** All procedures essentially followed the Yeast Protocols handbook (Clontech). For library screening, *Saccharomyces cerevisiae* JH109 was used as a host, and *S. cerevisiae* PJ69-4A and PJ69-4c were used for mating experiments. YPDA medium was used for wild-type strains and SD medium for selecting

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### Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRK6000</td>
<td>purA16 metB5 hisA3 guaB 33</td>
<td>Moriya et al., 1990</td>
</tr>
<tr>
<td>NIS6051</td>
<td>CRK6000 yabaA::spec amyE::cat-Pspac-yabA</td>
<td>Hayashi et al., 2005</td>
</tr>
<tr>
<td>NIS6052</td>
<td>CRK6000 yfp-yabaA-cat yabaA::spec</td>
<td>Hayashi et al., 2005</td>
</tr>
<tr>
<td>UC6000AH</td>
<td>CRK6000 dnaA-12his</td>
<td>This study</td>
</tr>
<tr>
<td>UC6051AH</td>
<td>CRK6000 dnaA-12his yabaA::spec amyE::cat-Pspac-yabA</td>
<td>This study</td>
</tr>
<tr>
<td>UC6002A</td>
<td>CRK6000 amyE::Pxytl-dnaA-cat</td>
<td>This study</td>
</tr>
<tr>
<td>UC6001</td>
<td>CRK6000 amyE::Pxytl-tet</td>
<td>This study</td>
</tr>
<tr>
<td>UC6013</td>
<td>CRK6000 amyE::Pxytl-tet dnaA_{Y144C}</td>
<td>This study</td>
</tr>
<tr>
<td>UC6001Y</td>
<td>CRK6000 yfp-yabaA-cat yabaA::spec amyE::tet</td>
<td>This study</td>
</tr>
<tr>
<td>UC6013Y</td>
<td>CRK6000 yfp-yabaA-cat yabaA::spec amyE::tet dnaA_{Y144C}</td>
<td>This study</td>
</tr>
</tbody>
</table>

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*CRK6000, NIS6051, and NIS6052 were used throughout as cloning hosts. All primers used for PCR are listed in Table 2. Structures of plasmids and strains constructed in this study were confirmed by DNA sequencing to avoid PCR mutations.*
and maintaining plasmids in the hosts. When necessary, 3-amino-1, 2, 4-triazole (3-AT) was added to suppress *HIS3* expression and obtain an accurate His<sup>–</sup> phenotype, and 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal) was added as a substrate for *MEL1* (secretable α-galactosidase, a reporter for the yeast two-hybrid system) to evaluate the strength of interactions in SD medium.

**Construction of dnaA domain library for yeast two-hybrid assay**

To construct the plasmids for this purpose, the Gateway system was used as previously described (Ishikawa et al., 2006). The ten fragments shown in Fig. 3 were amplified using primers listed in Table 2 and recombined with pDONR201 (Invitrogen) by the BP reaction, generating Entry clones (pENTR dnaA<sub>1</sub> to dnaA<sub>10</sub>). The resultant plasmids were then recombined with pDBGW to transfer the integrated sequences to pGBT9 (Clontech) by the LR reaction, generating pGBT-dnaA<sub>1</sub> to -dnaA<sub>10</sub>. The method used to detect the interaction between partial DnaAs and YabA is described in the legend of Fig. 3.

**Construction of dnaA mutation library**

A mutation library of *dnaA* was constructed in pGBT9 as follows. In the first step, mutations were introduced into *dnaA* by PCR mutagenesis. The PCR reaction was carried out by ExTaq DNA polymerase (Takara Bio) in the presence of 0.15 mM MnCl<sub>2</sub>, using pGBT-dnaA<sub>1</sub> as a template and pGBT9-F2 and pGBT9-R2 primers (Table 2), which anneal on the outside of essential sequences for the recombination reaction, attL<sub>1</sub> and attL<sub>2</sub>, respectively. The resultant PCR product was used in the LR reaction with pBDGW (Ishikawa et al., 2006). *E. coli* DH5α cells were transformed with the reaction solution and transformants were selected on LB medium containing 100 μg/ml of ampicillin in the presence of 0.5 mM IPTG and 50 μg/ml of X-α-gal.

**Table 2. Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location on <em>B. subtilis</em> genome&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
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<td>agaactgtagctgtagc</td>
<td>Outside multi cloning site in pGBT9</td>
</tr>
<tr>
<td>pGBT-R</td>
<td>tgcgggaattgctg</td>
<td>Outside multi cloning site in pGBT9</td>
</tr>
<tr>
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<td>735 ... 713</td>
</tr>
<tr>
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<td>734 ... 756</td>
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<td>1234 ... 1214</td>
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</table>

<sup>a</sup> Capital and lowercase letters indicate sequences derived from the *B. subtilis* genome and others, respectively.

<sup>b</sup> Numbers refer to the SubtiList database at the Pasteur Institute (http://genolist.pasteur.fr/SubtiList/index.html).

<sup>c</sup> attB1 and attB2 sequences are indicated in bold and italic letters, respectively.

<sup>d</sup> Underlined sequences indicate recognition sites of restriction enzymes.
ampicillin. Plasmids were extracted directly from the transformants on the plate. JH109 harboring pGAD424-yabA (Ogura, 2002) was transformed with the plasmid library, and transformants were selected on SD medium without tryptophane and leucine in the presence of X-α-gal to monitor interactions between DnaA and YabA. Colonies without blue haloes, indicating a reduction of the DnaA-YabA interaction, were used for the next assay.

**Purification of DnaA and YabA proteins** The IMPACT system (New England Biolabs (NEB)) was used to purify DnaA and YabA. The dnaA and yabA genes were amplified from genomic DNA of *B. subtilis* CRK6000 with CBdnaA5′–CBdnaA3′ and IMPACTyabA5′–IMPACTyabA3′ primer sets, respectively, and cloned into *NdeI* and *SacI* sites of pTYB1 (NEB), which enables fusion at the C terminus to a self-cleavable intein-CBD affinity tag. To purify the proteins, *E. coli* strain BL21 (DE3 pLysS) was transformed with the resultant plasmids (pTYB1-dnaA and pTYB1-yabA).

To purify DnaA, the cells were cultured in LB medium supplemented with 100 μg/ml ampicillin and 10 μg/ml chloramphenicol at 37°C. When the OD600 reached 0.6, IPTG was added to 0.5 mM to induce expression of the fusion protein, and the culture was incubated for a further 2 h at 30°C. All subsequent steps were carried out at 4°C. The cells were pelleted and resuspended in buffer A (45 mM HEPES-KOH, pH7.6, 0.5 mM EDTA, 10 mM magnesium acetate, 100 mM potassium glutamate, 1 M NaCl, 0.1% Triton X-100, 20% sucrose), which was used throughout the purification process, and were disrupted by sonication and centrifuged at 40,000 × g for 30 min. The supernatant was loaded onto a chitin column (NEB) and washed with buffer A. After cleavage of the affinity tag in the presence of 50 mM DTT, the untagged DnaA was eluted, and the purified DnaA was stored in aliquots at −80°C.

Purification of YabA protein was carried out essentially as for DnaA, with the following modifications. The cells were cultured at 30°C in LB supplemented with 25 μg/ml thymine, 100 μg/ml ampicillin and 10 μg/ml chloramphenicol, and the subsequent culture after addition of IPTG was performed at 20°C overnight. The harvested cells were then suspended in buffer B (50 mM Tris-HCl, pH8.0, 0.5 M NaCl) and cleaved YabA was eluted with buffer C (50 mM Tris-HCl, pH8.0, 50 mM NaCl). The purified YabA aliquots were stored at −80°C in the presence of 20% glycerol.

**Construction of dnaA<sub>Y144C</sub> strain** Construction of dnaA<sub>Y144C</sub> strain is schematically shown in Supplementary Fig. S1. Since dnaA is essential for cell growth, a dnaA-inducible strain with another copy of dnaA under a xylose-dependent promoter (Pxyl) at amyE locus was first constructed. The dnaA gene with the SD sequence was amplified by the adaptor PCR method using the dnaAS-DgwF–dnaAgwR primer set (Ishikawa et al., 2006) and recombinated with pXGW (S. Ishikawa and N. Ogasawara, unpublished), a derivative of pX, which is a vector for integration at the amyE locus for xylose-inducible gene expression (Kim et al., 1996). The resultant plasmid (pX-dnaA) was then used for transformation of CRK6000, and the resultant strain was designated as UC6002A (amyE::Pxyl-dnaA-cat).

To introduce the dnaA<sub>Y144C</sub> mutation into the original dnaA gene, the internal region of dnaA in pGBT9-dnaA1 (for wild-type control) and pGBT9-dnaA<sub>Y144C</sub> (selected as described above) were amplified with HindIII-dnaA7/13 and BamHI-dnaA7/13 primers and cloned into the HindIII and BamHI sites of pMUTinNC (Morimoto et al., 2002). The resultant plasmids (pMUTinNC-dnaA and pMUTinNC-dnaA13) were then introduced into strain UC6002A and transformants were selected on PAB medium supplemented with 0.5 μg/ml erythromycin, 1% xylose and 1 mM IPTG to induce the downstream dnaN from the *spac* promoter on pMUTinNC. Transformants having an integration at the original dnaA position were selected by IPTG dependency. The strains were then cultured in competent medium containing 1 mM IPTG to induce a second recombination event for removal of the integrated plasmid, and selected on PAB medium without erythromycin and IPTG. After checking sensitivity to erythromycin, the mutation in dnaA was confirmed by sequencing. To remove the dnaA gene from the amyE locus, the strains were transformed with pXtt, a pX derivative having the *tet* gene instead of the *cat* gene (S. Ishikawa and N. Ogasawara, unpublished), and transformants, confirmed as lacking the dnaA gene at the amyE locus by PCR, were designated as UC6001 and UC6013 for the wild-type control strain and the dnaA<sub>Y144C</sub> mutant, respectively.

**Immunoblotting** Cell lysates were prepared as described (Ishikawa et al., 2006). Proteins in the lysates were separated by SDS–polyacrylamide gradient gel electrophoresis and were blotted onto a Hybond-P polyvinylidene difluoride membrane. Rabbit anti-DnaA and anti-YabA antibodies and a goat anti-rabbit IgG horse-radish peroxidase conjugate (Bio-Rad) were used as first and second antibodies, respectively. Chemiluminescent signals were detected with ECL Plus western blotting detection reagents (Amersham).

**Fluorescence microscopy** Live cells were examined on agarose slides (Price and Losick, 1999). Cells were grown in ST<sub>50</sub> minimal medium supplemented with 0.5% sodium fumarate, 0.1% potassium glutamate and auxotrophic requirements (adenine and guanosine at 20 μg/ml and histidine and methionine at 50 μg/ml) at 30°C as previously described (Hayashi et al., 2005). Cell morphology and nucleoid distribution were examined by
fluorescence microscopy after DAPI (4',6-diamidino-2-phenylindole) staining, as described previously (Ishikawa et al., 2006), using a DMRE-HC microscope (Leica) with a cooled digital CCD camera (1300Y, Roper Scientific) and a YFP filter set (Omega). Images were analyzed to determine the intracellular locations of the signals using MetaMorph software (Universal Imaging).

Flow cytometry Cells from the CRK6000 background were grown exponentially in PAB medium and treated with chloramphenicol (200 μg/ml) to inhibit new rounds of initiation (Ogura et al., 2001). Incubation was continued for 5 h to complete ongoing chromosome replication. The cells were then fixed with ethanol, treated with 0.25 mg/ml RNase A at 37°C for 1 h in TE buffer, stained with Syto16 (Molecular Probes) and analyzed using a FACScan (BD Biosciences).

RESULTS

YabA level does not affect expression level of DnaA

Despite their lack of sequence similarity, B. subtilis YabA and E. coli Hda are functionally analogous: both proteins bind DnaA and the sliding clamp (DnaN), negatively regulating the initiation of DNA replication by forming a ternary complex with DnaA and DnaN (Kato and Katayama, 2001; Noirot-Gros et al., 2006). Additionally, overexpression of YabA and Hda causes a delay in replication initiation (Hayashi et al., 2005; Riber et al., 2006). In the case of Hda, its absence and overexpression decrease and increase, respectively, the expression level of DnaA, probably as a result of Hda-dependent RIDA changing the amount of ATP-DnaA and thus its binding affinity to the promoter region of dnaA (Riber et al., 2006). Since expression of the dnaA gene is autoregulated in both E. coli and B. subtilis (Ogura et al., 2001; Messer, 2002), we speculated that depletion and overproduction of YabA might likewise affect the expression level of the dnaA gene, if YabA also stimulates the hydrolysis of DnaA-bound ATP.

To examine the effect of YabA levels on dnaA expression, and the interaction between DnaA and YabA, we introduced a 12 x His tag at the 3’ end of the dnaA gene, which has no significant effect on replication initiation in vivo (Ishikawa et al., 2007), into a yabA-inducible mutant (NIS6051; yabA::spec amyE::cat-Pspac-yabA) as previously described (Ogura et al., 2001; Hayashi et al., 2005; Ishikawa et al., 2007). The resultanf mutant, designated as UC6051AH (dnaA-12his yabA::spec amyE::cat-Pspac-yabA), was cultured at various concentrations of IPTG. The expression level of YabA in UC6051AH cells was lower than that in wild-type cells at 30 mM IPTG, but increased significantly at higher concentrations of IPTG, as previously reported for the parental strain NIS6051 (Hayashi et al., 2005). Western blotting demonstrated that the amount of DnaA was not affected by overexpression of YabA (Fig. 1), while a pull-down assay indicated that increasing amounts of YabA copurified with DnaA as the expression level increased (Fig. 1). The level of dnaA transcription in wild-type (CRK6000) and YabA-depleted (NIS6051) cells was also analyzed by quantitative PCR, and the results from two independent experiments showed that, in contrast to about 2-fold induction by HdaA overexpression in E. coli (Riber et al., 2006), the difference was less than 1.4-fold, indicating that dnaA transcription was comparable in both strains, in agreement with protein levels (data not shown).

Absence of YabA does not affect the DnaA-binding profile on the B. subtilis genome Eight intergenic regions on the B. subtilis genome containing closely clustered DnaA boxes have been identified as stable DnaA-binding regions by ChAP-chip analysis (Ishikawa et al., 2007). To determine the effect of the absence of YabA on DnaA binding to these regions, we performed ChAP-chip analysis using DNA fragments copurified with DnaA from UC6000AH (CRK6000; dnaA-12his) and UC6051AH (dnaA-12his yabA::spec amyE::cat-Pspac-yabA) cells cultured without IPTG (Fig. 2A). The DnaA-binding patterns in both cells were essentially identical, and the minor differences observed were within the range of experimental error. Although we normalized hybridization intensities to Genechip probes to a signal average of 500, to compare DnaA-binding signals in UC6000AH and UC6051AH cells, it is difficult to strictly compare the signal intensities in differently prepared samples. Therefore, the amounts of DNA bound by DnaA at the oriC region were compared by quantitative PCR analysis. For this purpose, DNA fragments were extracted from DnaA complexes containing the same amounts of DnaA (Fig. 2B), and used as templates for quantitative PCR. No significant differences in binding of DnaA to the oriC region could be detected between wild-type and YabA-depleted cells (Fig. 2C). Thus, we conclude that the absence of YabA does not significantly affect the profile of DnaA binding to the genome.

YabA binds to DnaA domain IIIa The results described above suggested that the molecular mechanism by which YabA prevents re-initiation in B. subtilis is distinct from that of Hda in E. coli. Hda, which contains a region homologous to the ATP-binding domain (domain III) of DnaA, is thought to hydrolyze ATP bound to DnaA through direct interaction between the ATP binding pocket of DnaA and the conserved Arg168 of Hda, forming the ATPase catalytic center (Su’etsugu et al., 2005). Next, we examined the YabA-interacting sites in B. subtilis DnaA by yeast two-hybrid assay. The domain structure of B. subtilis DnaA was inferred based on
Functional analysis of *B. subtilis* YabA

sequence similarity with that of *E. coli* DnaA, as shown in Fig. 3. Ten fragments containing various DnaA domains were fused with the DNA-binding domain of Gal4 (DB) and YabA was fused with the activation domain of Gal4 (AD). The two-hybrid assay results showed that fragments 1, 3, 6, 8, 9 and 10 could interact with YabA; all six of these fragments contain domain IIIa, strongly suggesting that YabA, like Hda, interacts with the DnaA domain IIIa (Fig. 3).

**DnaA<sub>F120S</sub> and DnaA<sub>Y144C</sub> fail to interact with YabA** To determine the amino acid residues in DnaA responsible for YabA binding, we randomly mutagenized the dnaA sequence by PCR, and screened for mutants defective in interaction with YabA by yeast two-hybrid assay, as described in Materials and Methods. The dnaA sequences in selected clones were determined, and mutants having a single point mutation were identified (Table 3). Since loss of correct ternary structure was expected in some of the mutants defective in YabA interaction, we also examined their interaction with DnaA itself and DnaD, thereby obtaining four YabA interaction-defective mutants that retained DnaA or DnaA/DnaD interaction(s) (Table 3). In good agreement with yeast two-hybrid screening of the domain library, all four mutations having a single base substitution and retaining self-interaction are located within domain IIIa (Table 3). In addition, mapping of the mutations on the 3-D structure of *Aquifex aeolicus* DnaA (Erzberger et al., 2002) revealed that they are located on the opposite surface of the ATP-binding pocket of domain III (Fig. 4), suggesting that YabA interacts with a different region of domain IIIa from that contacted by Hda. Interestingly, mutants that lost their interaction with YabA also showed a reduced or disabled interaction with DnaD (Table 3).

**Interaction of YabA with DnaA<sub>F120S</sub> and DnaA<sub>Y144C</sub> in vitro** To confirm the results of yeast two-hybrid analysis, the interaction between mutant DnaA and YabA was also analyzed by zinc precipitation, as described previously (Noirot-Gros et al., 2006), for DnaA<sub>F120S</sub> and DnaA<sub>Y144C</sub>, which retained some degree of interaction with DnaD albeit reduced. Purified YabA, DnaA, DnaA<sub>F120S</sub> and DnaA<sub>Y144C</sub> remained soluble in the buffer we used (Fig. 5a). Only YabA was precipitated when incubated with zinc (Fig. 5b) and most of the wild-type DnaA was co-precipitated with YabA in the presence of zinc (Fig. 5c). In contrast, half of the DnaA<sub>Y144C</sub> incubated with

<table>
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<th>UC6051AH (dnaA-12his yabA::spec amyE::Pspac-yabA)</th>
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![Fig. 1. Expression levels of DnaA in YabA-depleted and -overproducing cells. DnaA and YabA levels in CRK6000 (wild-type) and UC6051AH (dnaA-12his yabA::spec amyE::Pspac-yabA) cells were analyzed by Western blotting using anti-DnaA and anti-YabA sera. The strains were pre-cultured in PAB medium in the absence of IPTG overnight at 30°C and inoculated into 400 ml PAB with various concentrations of IPTG, as indicated, to give an initial OD<sub>600</sub> of 0.005. When the OD<sub>600</sub> reached 0.3, 10 ml was used for extraction of crude cell lysate. The remainder was treated with 1% formaldehyde for purification of DnaA complex, as described previously (Ishikawa et al., 2006). DnaA complexes were finally eluted with 50 µl elution buffer. To detect YabA in the DnaA complexes, 10 µl was used after heating to reverse the cross-linking; to detect DnaA, the eluate was diluted 100-fold and 10 µl was used.](image)
YabA and zinc remained soluble, indicating a reduced interaction with YabA (Fig. 5c). Interestingly, when DnaA_F120S was mixed with YabA in the presence of zinc, it remained mostly soluble and also prevented the precipitation of YabA (Fig. 5c), although the reason for this is not clear (Fig. 5c). Thus, DnaA mutants screened by...
Asynchronous replication in dnaA<sup>Y144C</sup> cells

The yabA-inducible mutant NIS6051 (yabA:spec amyE:cat-Pspac-yabA) showed over-initiation and asynchronous replication in the absence of IPTG (Hayashi et al., 2005). Since DnaA<sub>F120S</sub> and DnaA<sub>Y144C</sub> proteins possessed impaired YabA interaction activity, it was expected that cells expressing mutant DnaA as the sole DnaA source would show a similar phenotype to that of the YabA-depleted cells. To assess the effect of our dnaA mutations on replication initiation, we introduced the dnaA<sub>F120S</sub> or dnaA<sub>Y144C</sub> mutations into the dnaA gene on the genome, as described in Materials and Methods (see Supplementary Fig. S1). We first placed the wild-type dnaA gene under the control of a xylose-inducible promoter at the genomic amyE locus, and sequences containing mutations cloned in an integrative plasmid were then integrated into the original dnaA gene by a single crossing over. Next, the integrated plasmid sequence was removed by a second recombination to create the mutated dnaA gene at the original position, and finally the wild-type dnaA gene at the amyE locus was removed. Attempts to construct a dnaA<sub>F120S</sub> mutant failed at the step to remove the integrated plasmid: all sequences in the dnaA gene at the original position after the second recombination were wild-type, even in the presence of xylose to express wild-type DnaA from the amyE locus. Thus, dnaA<sub>F120S</sub> seems to have a dominant negative effect on wild-type DnaA. We did succeed, however, in constructing a dnaA<sub>Y144C</sub> mutant (UC6013; dnaA<sub>Y144C</sub> amyE::tet) and a wild-type control strain (UC6001; amyE::tet). The dnaA<sub>Y144C</sub> mutant showed no apparent

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**Fig. 3.** Interaction between YabA and various domains of DnaA. (A) Similarities between DnaA domains I to IV in E. coli and B. subtilis are shown. The amino acid identity (%) of each domain is also indicated. The fragments cloned into pGBT9 are numbered 1 to 10, corresponding to pGBT-dnaA1 to -dnaA10. (B) Diploid strains were obtained by mating PJ69-4A-containing pGBT9 derivatives with PJ69-4α-containing pGAD424 derivatives. pGBT9-dnaA (Ishigo-Oka et al., 2001) and pGBT9-yabA (Ogura, 2002) were used as positive controls. Interactions between proteins were detected on selection medium (SD-LWH) supplemented with 1 mM 3-AT after a 3-day incubation.
growth defect in rich medium (PAB) at 30, 37 and 48°C (data not shown). Using UC6013 (dnaA_{Y144C} amyE::tet), UC6001 (amyE::tet), and NIS6051 (yabA::spec amyE::cat-Pspac-yabA) strains, cultured at 30°C in PAB medium in the absence of IPTG, and after a 5-h incubation with chloramphenicol to inhibit new rounds of initiation and complete ongoing replication, we compared their DNA histograms to that of the wild-type strain (CRK6000) by flow cytometry. Under the conditions used, 2, 4 and 8 origins per cell were observed in wild-type cells (Fig. 6A, B), and over-initiation and asynchronous replication were observed in YabA-depleted cells (Fig. 6C) as previously reported (Hayashi et al., 2005). In dnaA_{Y144C} mutant cells (CRK6013), synchrony of initiation was severely impaired and over-initiation (more than 8 origins per cell) was also detected, as in YabA-depleted cells (Fig. 6D). However, there is one notable difference: the highest peak position in the DNA histogram of the dnaA_{Y144C} mutant was 4 origins per cell while it was 8 in YabA-depleted cells, and additional peaks representing 1 to 3 origins per cell, which were not detected in YabA-depleted cells, were observed for the dnaA_{Y144C} mutant (Fig. 6C, D). These results indicate that the dnaA_{Y144C} mutation indeed

| Protein fused to Gal4 AD | domain | Protein fused to Gal4 BD | wild-type DnaA | YabA | DnaD | Self-interaction$^b$
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$^a$Interactions between proteins were detected on selection medium (SC-LWH) supplemented with 5 mM 3-AT after a 3-day incubation. ++, yeast grew to the level of wild-type DnaA; +, yeast formed smaller colonies than wild-type DnaA; –, no colony formation was detected.

$^b$The dnaA genes with the indicated mutations were cloned into the Gal4 BD expression vector pGBT9, and the interactions between the mutant proteins fused to Gal4 AD and BD were examined.

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**Table 3. Interaction of DnaA mutants, selected by first screening, with DnaA, YabA and DnaD**

**Fig. 4.** Mapping of YabA interaction-deficient mutations on the 3-D structure of DnaA. The 3-D crystallograph was created by Cn3D (NCBI), using data for *A. aeolicus* DnaA (Erzberger et al., 2002), and modified by Adobe Photoshop. Mutations buried in DnaA are depicted (green), as well as ADP (dark brown), domain IIIa (red), domain IIIb (yellow), domain IV (cyan) and box VII (blue). The box VII motif, which includes a conserved arginine finger and is required for interaction with ATP-binding pocket of neighboring DnaA, and a magnesium ion in the ATP-binding pocket are shown as a blue line and a blue ball, respectively. Front (A) and back (B) views of the ATP-binding pocket are shown.
Functional analysis of *B. subtilis* YabA

impairs the DnaA interaction with YabA *in vivo*, and that
this impairment induces over- and asynchronous initia-
tion of genome replication, although the extent of over-
initiation seemed to be reduced in *dnaA*Y144C mutant cells
compared to that in YabA-depleted cells.

YabA does not form foci in *dnaA*Y144C mutant cells
YabA localizes as foci at cell-center and -quater positions
in *B. subtilis* cells (Hayashi et al., 2005). Additionally,
cells expressing a YabA mutant deficient in DnaA or
DnaN interaction fail to form YabA foci, suggesting that
focus formation by YabA is dependent on its interactions
with DnaA and DnaN (Noirot-Gros et al., 2006). We con-

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<tr>
<td>WT</td>
<td>F120S</td>
<td>Y144C</td>
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<tr>
<td>YabA</td>
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<td>-</td>
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<tr>
<td>DnaA</td>
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<td>+</td>
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<tr>
<td>Zn2+</td>
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| s | p | s | p | s | p | s | p |

Fig. 5. Interactions among YabA, wild-type DnaA, and DnaA mutants (DnaA_F120S and DnaA_Y144C) *in vitro* using a zinc precipitation assay. Purified YabA (14 kDa) and DnaA (51 kDa) proteins (a). YabA precipitation upon addition of Zn$^{2+}$ (b) was assayed in the presence of wild-type or mutant DnaA proteins (c). Soluble fractions (s) and precipitates (p) were analyzed by Coomassie-stained 5–20% SDS/PAGE.

Fig. 6. Flow cytometry analysis of DNA content in *yabA* mutant cells. Histograms of DNA content in CRK6000 (A), UC6001 (amyE::tet) (B), NIS6051 (*yabA::spec Papac-yabA*) (C) and mutant UC6013 (*dnaA*Y144C amyE::tet) (D) cells are shown.

Origin/cell

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firmed the direct involvement of DnaA in YabA focus formation by examining YabA localization in dnaA<sub>Y144C</sub> mutant cells. To this end, yfp-yabA of NIS6052 (yfp-yabA-cat amyE::P<sub>xyl-tet</sub>) was introduced into wild-type control (UC6001) and dnaA<sub>Y144C</sub> mutant (UC6013) strains, to create strains UC6001Y (yfp-yabA-cat amyE::P<sub>xyl-tet</sub>) and UC6013Y (dnaA<sub>Y144C</sub> yfp-yabA-cat amyE::P<sub>xyl-tet</sub>). In wild-type strains (NIS6052 and UC6001Y), YabA foci were clearly observed at cell-center and -quater positions, as previously reported (Fig. 7A, B). In contrast, YabA did not form such foci and the fluorescence was dispersed over the cell in dnaA<sub>Y144C</sub> mutant cells (UC6013Y), as shown in Fig. 7C.

**DISCUSSION**

Although *B. subtilis* YabA has no sequence similarity with *E. coli* Hda, it seemed possible that YabA might be a functional homolog of Hda, since the two proteins have several features in common: they interact with DnaA and DnaN (the β clamp of DNA polymerase III) (Noirot-Gros et al., 2002), and their absence and overproduction result
in over-initiation of replication and delayed replication initiation, respectively (Hayashi et al., 2005; Noirot-Gros et al., 2006; Riber et al., 2006). Since *B. subtilis dnaA* transcription is also autoregulated, we speculated that the concerted effects of YabA and *dnaA* autoregulation seen in *E. coli* might also occur in *B. subtilis*. In contrast to Hda, however, we found that depletion and overproduction of YabA have no effect on transcription of *dnaA* or expression of DnaA protein (Fig. 1). In addition, DnaA-binding profiles at eight DnaA-binding regions on the genome were not affected significantly when YabA protein was depleted (Fig. 2). These results clearly showed that YabA has no inhibitory activity toward DNA binding of DnaA* in vivo*, indicating that YabA regulates replication initiation by a different mode from that of Hda.

Yeast two-hybrid analysis using a DnaA domain library revealed that domain IIIa is the YabA-interacting domain of *B. subtilis* DnaA (Fig. 3). This was also supported by the results of a yeast two-hybrid screen using a random mutation library, which showed that all the positions of DnaA mutants, selected by their deficiency in YabA binding and retention of an interaction with DnaA, were within the domain IIIa region (Table 3). Mapping of these mutations on the 3-D structure of *A. aeolicus* DnaA (Erzberger et al., 2002) placed them on the opposite surface of the ATP-binding pocket of domain IIIa (Fig. 4), further supporting the idea of different modes of interaction with DnaA for YabA and Hda, as discussed below. Impairment of the interaction with YabA was confirmed *in vitro* by the zinc precipitation method for DnaA*1428* and DnaA*144C* mutant proteins (Fig. 5).

The *dnaA*<sub>144C</sub> mutation was successfully introduced into the *B. subtilis* genome, and the mutant strain showed an over-initiation and asynchronous replication phenotype as observed in YabA-depleted cells (Fig. 6). In *dnaA*<sub>144C</sub> mutant cells, YabA foci, which have been suggested to form as a result of interactions with both DnaA and DnaN, were dispersed over the cell (Fig. 7), strongly suggesting that the effects of the *dnaA*<sub>144C</sub> mutation on replication initiation resulted directly from the loss of interaction between DnaA and YabA *in vivo*. Thus, our results further demonstrated that the DnaA-YabA interaction is involved in negative regulation of initiation of replication and YabA focus formation. Moreover, we have clearly identified tyrosine 144 of DnaA as being critically important for the interaction with YabA, providing a further indication that domain IIIa of DnaA is the interacting interface for YabA binding.

In contrast to *dnaA*<sub>144C</sub>, attempts to construct a *dnaA*<sub>1208</sub> mutant failed at the stage of removal of the inserted plasmid region. This indicates that the mutation would exert a dominant negative effect on wild-type DnaA protein. Interestingly but unexpectedly, the purified DnaA<sub>1208</sub> mutant protein inhibited even YabA precipitation (Fig. 5). YabA comprises two distinct domains, a DnaA-binding leucine zipper domain at the N terminus and a zinc-binding domain at the C terminus, that enable the co-precipitation of DnaA by YabA induced by zinc binding (Noirot-Gros et al., 2006). Thus, the result of the *in vitro* experiment suggests that DnaA<sub>1208</sub> binds to the C-terminal zinc-binding domain of YabA rather than to the N-terminal DnaA-binding domain because of an unexpected conformational change that could disturb an essential interaction *in vivo*, for example self-interaction of wild-type DnaA and/or the interaction between wild-type DnaA and DnaD. Further analysis of the dominant negative effect of the DnaA<sub>144C</sub> mutant protein should clarify this question.

It has been suggested that *E. coli* Hda interacts directly with the ATP-binding pocket on DnaA, based on the observation that the highly conserved Arg168 is essential for ATP-DnaA hydrolysis. Upon formation of multimers of AAA+ proteins, to which both DnaA and Hda belong, the arginine finger motif plays an important role in ATP hydrolysis at the interface between the ATP-binding pocket and the arginine residue of two neighboring subunits (Su‘etsugu et al., 2004). In contrast to Hda, the positions of the mutations that we isolated in YabA interaction-deficient mutants, including the *dnaA*<sub>144C</sub> mutation, were all on the opposite surface to the ATP-binding pocket (Fig. 4). This difference again suggests that the inhibitory mechanisms in YabA and Hda are distinct. Interestingly, all four YabA interaction-deficient mutants that we obtained here were also impaired in their ability to interact with DnaD, an event thought to be essential to the progression from initiation of replication to elongation (Ishigo-Oka et al., 2001). This observation suggests that YabA and DnaD recognize overlapping regions on the same surface on DnaA, and that YabA competitively inhibits the DnaA-DnaD interaction to suppress overinitiation. In *dnaA*<sub>144C</sub> mutant cells, over-initiation and asynchronous replication occurred in the same way as they did in YabA-depleted cells. However, the number of genomes in *dnaA*<sub>144C</sub> cells was lower than that in YabA-depleted cells (Fig. 6). Considering that this mutation did not affect the self-interaction of DnaA, this phenotype seems to reflect a diminished interaction between DnaA and DnaD.

The intrinsic ATPase activity of *B. subtilis* DnaA is very low *in vitro*, and we have not yet been able to find a condition to activate it. Therefore, we have no data on the effect of YabA on DnaA ATPase activity. However, the location of the YabA interaction surface on DnaA and the lack of an effect on *dnaA* autoregulation of YabA depletion strongly suggest that YabA would not directly affect DnaA ATPase activity. Based on new observations reported here and the fact that, in addition to the interaction with DnaA, the interaction with DnaN is essential for YabA function (Noirot-Gros et al., 2006), we propose the following model for the role of YabA in regulation of
initiation of replication. YabA is introduced into the ini-
tiation complex through an interaction with DnaN, where
it forms a stable complex with DnaA. This mechanism
ensures that the interaction of YabA with DnaA only
occurs after assembly of the elongation complex at oriC.
Next, YabA removes DnaD from the initiation complex,
and inhibits further association between the two to pre-
vent premature initiation. In support of this idea, the
location of YabA foci appears to overlap with that of the
replication machinery for only a limited period during the
cell cycle (Hayashi et al., 2005). The DnaA-YabA com-
plex would dissociate from the replication machinery
after initiation. According to this hypothesis, DnaA
should form foci in association with YabA. However, we
could not detect DnaA foci, possibly because of a high
background of DnaA protein, both associated with regions
other than oriC and also dispersed in its free form
throughout the cell. We also anticipated that the amount of DnaD complexed with DnaA would be
repressed when YabA protein was overexpressed. How-
ever, since the amount of DnaD detected by the pull-down
assay shown in Fig. 1 was barely distinguishable from
background levels, we were unable to detect any dif-
fERENCE that correlated with the expression level of YabA
protein (data not shown). This may indicate that the
DnaA-DnaD interaction is transient compared to the
YabA-DnaA interaction, consistent with our model. Fur-
ther studies are necessary to provide direct evidence in
support of our model for competitive inhibition of the
DnaA-DnaD interaction by YabA in *B. subtilis*.

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script.

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