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Control of the replication initiator DnaA by an anti-cooperativity factor

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Summary

Proper coordination of DNA replication with cell growth and division is critical for production of viable progeny. In bacteria, coordination of DNA replication with cell growth is generally achieved by controlling activity of the replication initiator DnaA and its access to the chromosomal origin of replication, *oriC*. Here we describe a previously unknown mechanism for regulation of DnaA. YabA, a negative regulator of replication initiation in *Bacillus subtilis*, interacts with DnaA and DnaN, the sliding (processivity) clamp of DNA polymerase. We found that in vivo, YabA associated with the *oriC* region in a DnaA-dependent manner and limited the amount of DnaA at *oriC*. In vitro, purified YabA altered binding of DnaA to DNA by inhibiting cooperativity. Though previously undescribed, proteins that directly inhibit cooperativity may be a common mechanism for regulating replication initiation. Conditions that cause release of DnaN from the replisome, or overproduction of DnaN, caused decreased association of YabA and increased association of DnaA with *oriC*. This effect of DnaN, either directly or indirectly, is likely responsible, in part, for enabling initiation of a new round of replication following completion of a previous round.

Keywords

DNA replication; *Bacillus subtilis*; YabA; DnaN; processivity clamp

Introduction

DNA replication is highly regulated and essential for normal growth and production of viable progeny. Bacteria like *Escherichia coli* and *Bacillus subtilis* regulate the frequency of replication initiation in response to growth rate to ensure segregation of at least one complete intact genome to dividing cells. This is particularly important under conditions of fast growth when cells can double in less time than it takes to duplicate the genome. To successfully accomplish this, a cell initiates multiple rounds of replication sequentially such that each sister cell receives a complete genome that is also partly duplicated. During slow growth, when the doubling time is greater than the time for genome duplication, replication initiation is inhibited to prevent over-replication and consequent problems with cell division.

In bacteria, the frequency of replication initiation is typically regulated by controlling the activity of the conserved initiator protein DnaA and its access to the origin of replication *oriC* (reviewed in: Kaguni, 2006; Mott & Berger, 2007; Zakrzewska-Czerwinska *et al.*, 2007; Katayama *et al.*, 2010; Leonard & Grimwade, 2010). DnaA is a AAA+ ATPase that binds both ATP and ADP. DnaA bound to ATP (DnaA-ATP) is active for replication

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initiation (Sekimizu *et al.*, 1987), binds cooperatively to multiple binding sites in *oriC*, and causes local melting (unwinding) of the DNA unwinding element (DUE) in *oriC* (Fig. 1). The single stranded DNA generated by the melting of *oriC* serves as an assembly platform for the replication machinery (the replisome). In addition to binding *oriC*, DnaA also binds many sites around the chromosome and functions as a transcription factor for several genes, including its own (Atlung *et al.*, 1985; Braun *et al.*, 1985; Kucherer *et al.*, 1986; Wang & Kaguni, 1987; Messer & Weigel, 1997; Ogura *et al.*, 2001; Goranov *et al.*, 2005; Hottes *et al.*, 2005; Collier *et al.*, 2006; Gon *et al.*, 2006; Ishikawa *et al.*, 2007; Breier & Grossman, 2009; Scholefield *et al.*, 2011a).

Several mechanisms that control the activity of DnaA and replication initiation from *oriC* are well characterized in *E. coli* (reviewed in: Kaguni, 2006; Mott & Berger, 2007; Katayama *et al.*, 2010; Leonard & Grimwade, 2010). These include sequestration of the origin by SeqA, titration of DnaA by the *datA* locus, and regulated inactivation of DnaA (RIDA) mediated by Hda. However, the regulatory factors in *E. coli* are largely limited to proteobacteria and do not have homologues in Gram-positive bacteria.

Replication initiation in *Bacillus subtilis*, representative of many Gram-positive bacteria, is also highly regulated (Moriya *et al.*, 1999; Katayama *et al.*, 2010), although the mechanisms of regulation are not known. One of the regulatory factors, YabA, inhibits replication initiation in *B. subtilis* and likely other Gram-positive bacteria. It was identified based on its ability to interact with DnaA and DnaN, the sliding (processivity or beta-) clamp of DNA polymerase (Noirot-Gros *et al.*, 2002). *yabA* null mutations cause increased replication initiation (Noirot-Gros *et al.*, 2002; Hayashi *et al.*, 2005) and overproduction of YabA causes decreased replication initiation (Hayashi *et al.*, 2005; Goranov *et al.*, 2009). YabA affects DnaA-dependent replication from *oriC*, but not DnaA-independent replication from a heterologous origin introduced into the chromosome (Goranov *et al.*, 2009). Visualization of fusions of YabA to fluorescent proteins indicate that some (but not necessarily all or even most) YabA is associated with the replisome during active replication, most likely through its interaction with the sliding clamp (Hayashi *et al.*, 2005; Noirot-Gros *et al.*, 2006; Cho *et al.*, 2008; Goranov *et al.*, 2009). The interaction of YabA with DnaA and DnaN and its inhibition of replication initiation make it somewhat analogous to Hda from *E. coli* (Kato & Katayama, 2001; Katayama *et al.*, 2010). Although it is not known how YabA inhibits replication initiation, previous studies indicate that YabA and Hda function by different mechanisms (Cho *et al.*, 2008).

Two different models for YabA function have been proposed, each predicting different mechanisms. One model proposes that a YabA-DnaN complex associated with the replisome functions to recruit DnaA to the replisome, thereby titrating it away from *oriC* and inhibiting replication initiation (Noirot-Gros *et al.*, 2006; Soufo *et al.*, 2008; Katayama *et al.*, 2010). Support for this model comes from the finding that some DnaA is located with the replisome, although this finding is somewhat controversial (Imai *et al.*, 2000; Soufo *et al.*, 2008; Wagner *et al.*, 2009). In this model and others (Hayashi *et al.*, 2005; Cho *et al.*, 2008; Goranov *et al.*, 2009), DnaN facilitates YabA-mediated inhibition of replication initiation. In contrast, other work indicated that DnaN antagonizes (rather than stimulates) the ability of YabA to inhibit replication initiation (Goranov *et al.*, 2009).

We investigated the mechanism by which replication initiation is regulated by YabA. We found that in vitro, purified YabA alters binding of DnaA to DNA by inhibiting cooperativity. The presence of YabA made DNA binding by DnaA-ATP similar to that of DnaA-ADP. In vivo, YabA was associated with the *oriC* region and other chromosomal regions bound by DnaA, and DnaA was required for this association. The presence of YabA in vivo inhibited association of DnaA with *oriC* and other chromosomal regions, consistent

with inhibition of cooperativity observed *in vitro*. Inhibition of replication elongation or initiation causes release of the sliding clamp (DnaN) from the replisome (Goranov et al., 2009; Su'etsugu & Errington, 2011) and caused decreased association of YabA and increased association of DnaA with *oriC* and other DnaA targets *in vivo*. Similarly, overproduction of DnaN also caused decreased association of YabA and increased association of DnaA with *oriC* and other DnaA targets *in vivo*, indicating that DnaN antagonizes YabA function. Our results indicate that *B. subtilis*, and likely other Gram-positive bacteria, regulate replication initiation, in part, by using YabA as an anti-cooperativity factor to inhibit binding of DnaA to *oriC*, thereby preventing over-replication. Our findings also indicate that the interaction between the sliding clamp and YabA might be one of the mechanisms that enables replication initiation following termination of a previous round. We postulate that protein-mediated direct inhibition of cooperativity of DnaA is a common, though previously undescribed, mechanism to control replication initiation in *B. subtilis* and other organisms.

Results

YabA inhibits DnaA binding to *oriC* and other chromosomal regions *in vivo*

Some of the models for YabA function predict that it should cause a reduction in the amount of DnaA bound to the *oriC* region, and perhaps other chromosomal regions. To test this prediction, we measured association of DnaA with the *oriC* region and three other chromosomal regions (Fig. 1E–H) *in vivo* in *yabA*⁺ and *yabA* mutant cells. Because replication initiation from *oriC* is more frequent and mis-regulated in *yabA* mutants, the replication status of the two comparison strains (*yabA*⁺ vs. *yabA* mutant) would be quite different. Since alterations in replication can affect the activity of DnaA (Goranov et al., 2005; Breier & Grossman, 2009), and DnaA also affects replication, possible effects of YabA on DnaA might be masked in cells replicating from *oriC*. To eliminate this complication, we analyzed the effects of a *yabA* null mutation in strains that had the bidirectional DnaA-independent origin of replication *oriN* (Hassan *et al.*, 1997; Moriya *et al.*, 1997), and its cognate initiator *repN*, inserted into the chromosome near *oriC* (Berkmen & Grossman, 2007). These strains also contained a small deletion in one part of *oriC* (Fig. 1B) that abolishes *oriC* function. This mutation leaves intact the DnaA-box region that is upstream from *dnaA* and is part of *oriC* (Moriya *et al.*, 1992). We refer to this region as "PdnaA" since it is in the *dnaA* promoter region as well as being part of *oriC*. In all experiments, cells were grown in defined minimal medium where the strains replicating from *oriN* have apparently normal chromosome partitioning and DNA to protein ratios (Moriya *et al.*, 1997), and have growth rates indistinguishable from those of cells replicating from *oriC* (data not shown). Since *yabA* has no effect on replication from *oriN* (Goranov *et al.*, 2009), we could analyze the effects of *yabA* on DnaA binding independently of effects on replication. Any small changes in cell physiology due to replication from *oriN* rather than *oriC* would be the same in cells with or without *yabA*.

Loss of *yabA* caused an increase in the amount of DnaA associated with the *oriC* region (indicated as PdnaA) and the three other chromosomal regions tested (*yydA*, *ylwC* and *ywcI*) that have multiple DnaA binding sites (Fig. 1) and are known to associate with DnaA *in vivo* (Goranov *et al.*, 2005; Ishikawa *et al.*, 2007; Cho *et al.*, 2008; Breier & Grossman, 2009; Smits *et al.*, 2011). In these experiments, cells were grown to exponential phase in defined minimal medium at 30°C, formaldehyde was added to crosslink protein and DNA, DnaA was immunoprecipitated with anti-DnaA antibodies (ChIP), and the amounts of specific chromosomal regions in the immunoprecipitates were determined by quantitative real time PCR (qPCR). DnaA binding to the *oriC* region and the other secondary DnaA-binding regions increased 6- to 10-fold in the absence of YabA (Fig. 2). YabA could limit the amount of DnaA on DNA either by titrating DnaA away from the binding regions as was

proposed previously (Noirot-Gros et al., 2006; Soufo et al., 2008), or it could modulate DnaA binding directly at the binding sites. If YabA alters DnaA binding directly on DNA, then YabA might be associated with DNA, perhaps through its interaction with DnaA.

YabA associates with *oriC* and other DnaA targets in a DnaA-dependent manner

We found that YabA associated preferentially with the *oriC* region and the three other regions tested (*yydA*, *ywlC* and *ywcI*) that bind DnaA (Fig. 3A). Cells were grown at 30°C (Fig. 3A, black bars) in defined minimal medium and samples were prepared as described above. YabA was immunoprecipitated with anti-YabA antibodies and the amounts of specific DNA regions in the immunoprecipitates were determined by qPCR. The immunoprecipitation was specific to YabA as there was little or no enrichment of these chromosomal regions in analogous ChIP-qPCR experiments from a *yabA* null mutant (Fig. 3A, gray bars). For all regions tested, there was also association of YabA in cells grown at 48°C (Fig. 3A white bars; see below). We do not know why there is increased association, especially with the *oriC* region (*PdnaA*), at high temperature.

Association of YabA with these four regions was dependent on DnaA. In order to delete *dnaA*, we used a strain containing the DnaA-independent *oriN*, and a deletion of the *oriC* region that removes *dnaA*, *oriC*, and *dnaN*, but leaves the *dnaA* promoter region with multiple DnaA binding sites (Fig. 1C). *dnaN* was expressed from a xylose-inducible promoter (*Pxyl-dnaN*). For comparison, we used the same strain but expressed *dnaA* from an IPTG-inducible promoter (*Pspank-dnaA*). YabA was associated with the DnaA targets in the strain expressing DnaA (Fig. 3B, black bars). In contrast, association of YabA with these regions was reduced to background levels in the absence of DnaA (Fig. 3B, gray bars). YabA is likely associated with these regions through its interaction with DnaA.

There was more YabA associated with DnaA targets in cells replicating from *oriC* (Fig. 3A, black bars) than in cells replicating from *oriN* and expressing *dnaN* from *Pxyl-dnaN* (Fig. 3B, black bars). This could be due, in part, to the differences in replication from the highly regulated *oriC* and the apparently un-regulated *oriN*. In addition, there is ~3.5-fold more DnaN in cells with *Pxyl-dnaN* compared to that in wild type cells, and overproduction of DnaN affects YabA (see below).

Together, our results indicate that some of the cellular YabA is associated with chromosomal regions bound by DnaA, the presence of YabA at these regions depends on DnaA, and YabA causes a decrease in the amount of DnaA detectably associated with these regions. Since YabA and DnaA interact directly (Noirot-Gros et al., 2002; Noirot-Gros et al., 2006), these results are consistent with a model in which YabA directly modulates DnaA binding to DNA.

YabA inhibits cooperative binding of DnaA to DNA in vitro

To directly test for effects of YabA on the ability of DnaA to bind DNA, we purified both DnaA and YabA (Experimental procedures) and tested for effects on mobility during gel electrophoresis (Fig. 4). We prepared DnaA in the ATP-bound form and used a DNA fragment from the *oriC* region (Fig. 1D) that contains multiple DnaA binding sites. Incubation of DnaA-ATP with the ³²P-labelled DNA fragment led to a change in mobility of the DNA (Fig. 4A). A plot of the fraction of the DNA probe bound versus the concentration of DnaA indicated cooperative binding (affinity of DnaA for DNA increases when some DnaA is already bound) with a Hill coefficient between 6 – 7 (Fig. 4C), and an apparent binding constant of ~30 nM. The DNA fragment has 8 putative DnaA binding sites with ≤1 mismatch from the consensus binding site (5'-TTATNCACA-3'). Perfectly cooperative binding to all these sites is expected to give a Hill coefficient of 8. The measured Hill

coefficient of approximately 7 for DnaA binding is indicative of a highly cooperative system where at least 7 sites are occupied interdependently. This cooperative binding is consistent with that exhibited by DnaA from other organisms (Fuller *et al.*, 1984; Majka *et al.*, 2001; Ozaki *et al.*, 2006).

We then measured the effects of YabA on DNA binding. YabA alone was unable to bind DNA as judged by lack of a shift in mobility of the DNA fragment (Fig. 4B, first lane). However, the presence of YabA had a dramatic effect on the binding of DnaA to DNA. There was still substantial binding by DnaA, as judged by the change in mobility of the DNA fragment (Fig. 4B). However, this binding was no longer cooperative (Fig. 4C). The Hill coefficient in the presence of YabA was 1.4, indicative of a strong inhibition of cooperativity, and the apparent dissociation constant in the presence of YabA was ~5 nM. Thus, YabA dramatically alters how DnaA binds to DNA, both increasing its affinity for DNA and nearly abolishing cooperativity. The mobility of bound DNA fragments in the presence of DnaA alone (Fig. 4A) was different from that in the presence of both DnaA and YabA (Fig. 4B). This difference is likely due to the presence of YabA in the complex, consistent with the *in vivo* findings that YabA is associated with chromosomal regions bound by DnaA. The decrease in the apparent dissociation constant is consistent with DnaA binding independently to multiple sites in the DNA fragment.

Effects of YabA were also evident when DnaA was bound to the non-hydrolyzable ATP analogue AMP-PNP (Fig. 4D). Binding of DnaA-AMP-PNP to DNA (Fig. 4D) was cooperative (Hill coefficient of 3.4), but less so than that of DnaA-ATP (Fig. 4C). Similar to the results with DnaA-ATP, binding of DnaA-AMP-PNP was no longer cooperative in the presence of YabA (Hill coefficient of 1.4) (Fig. 4D). Since AMP-PNP is non-hydrolyzable, these results indicate that the effects of YabA are independent of DnaA-mediated ATP hydrolysis, consistent with previous findings that indicate YabA is not likely to affect the ATPase activity of DnaA (Cho *et al.*, 2008).

We also tested for effects of YabA on binding of DnaA-ADP to DNA. Binding of DnaA-ADP to DNA (Fig. 4E) was much less cooperative than that of DnaA-ATP (Fig. 4C) or DnaA-AMP-PNP (Fig. 4D). In the presence of YabA, binding of DnaA-ADP to DNA was no longer cooperative (Fig. 4E). Our results indicate that YabA causes loss of cooperative binding by DnaA to DNA independent of nucleotide hydrolysis and irrespective of the nucleotide bound by DnaA.

Together, our results from *in vitro* and *in vivo* analyses indicate that the interactions between YabA and DnaA enable YabA to associate with DnaA at chromosomal regions normally bound by DnaA and that YabA directly alters the binding of DnaA to DNA by interfering with the cooperativity of DnaA. *In vivo*, the effect of YabA is to reduce the amount of DnaA that is associated with the *oriC* region, consistent with the genetically defined function of YabA as a negative regulator of replication initiation.

Inhibition of replication causes decreased association of YabA with the *oriC* region and other DnaA targets

Inhibition of replication elongation or initiation causes increased binding of DnaA to various chromosomal regions (Goranov *et al.*, 2005; Breier & Grossman, 2009; Smits *et al.*, 2011). This increase in DnaA binding could recruit more YabA. Alternatively, it could be correlated with decreased association of YabA. To distinguish between these possibilities, we measured association of YabA with several chromosomal regions following inhibition of replication elongation or initiation.

Association of YabA with the *oriC* region (*PdnaA*) and at least some of the other chromosomal regions bound by DnaA was reduced by 50 min. after inhibition of either replication elongation (Fig. 5A) or initiation (Fig. 5B). We used 6-(*p*-hydroxyphenylazo)-uracil (HPUra) to block replication elongation (Fig. 5A). HPUra binds to PolC, the catalytic subunit of DNA polymerase, and blocks replication elongation (Brown, 1970). Replication initiation was blocked by shifting a *dnaBts* mutant (Fig. 5B) to non-permissive temperature (inactivating DnaB, a component of the helicase loading machinery), preventing initiation yet allowing ongoing replication to finish. Similar results were obtained with other temperature sensitive mutants (*dnaDts* and *dnaItts*) blocked in replication initiation (data not shown). As a control, we verified that YabA did not dissociate from these sites at high temperature (48°C) in wild type cells (Fig. 3A, white bars). Together, these results indicate that inhibition of replication elongation or initiation causes decreased association of YabA with chromosomal regions bound by DnaA. There is an increase in association of DnaA under similar conditions (Goranov et al., 2005; Breier & Grossman, 2009; Smits et al., 2011).

Overproduction of DnaN, the sliding clamp, causes removal of YabA from *oriC* and other DnaA targets and stimulates DnaA binding to DNA

The replisome complex, including DnaN, disassociates following completion of a round of replication, both during normal growth and after inhibition of replication initiation. Inhibition of replication elongation also causes release of DnaN from the replisome while at least some other replisome subunits remain associated (Goranov et al., 2009; Su'etsugu & Errington, 2011). In addition, changes in levels of DnaN affect replication initiation from *oriC*, and these changes are epistatic to *yabA* (Goranov et al., 2009). Based on this information and the known interaction between YabA and DnaN (Noirot-Gros et al., 2002; Noirot-Gros et al., 2006), it seemed that effects on YabA and DnaA following completion or interruption of replication (Fig. 5) (Goranov et al., 2005; Breier & Grossman, 2009; Smits et al., 2011) were likely mediated by DnaN.

We found that increasing the amount of DnaN caused a decrease in the association of YabA and increase in the association of DnaA with chromosomal regions. These experiments were done in a strain initiating replication from *oriN*, where over-production of DnaN does not have any detectable effect on replication (Goranov et al., 2009). We found that over-production of DnaN (~3.5-fold) caused decreased association of YabA with the *oriC* region (*PdnaA*) and other DnaA targets (Fig. 6A). In addition, although levels of DnaA were unaffected, DnaA binding to *oriC* and some of the other regions increased when DnaN was over-expressed (Fig. 6B). These results support the model that DnaN, likely separate from the replisome, removes YabA from association with DnaA on the chromosome, enabling increased association of DnaA with its targets. Attempts to determine the effects of DnaN on the binding of DnaA and YabA to DNA in vitro were unsuccessful due to binding of DnaN to DNA, complicating any interpretation of possible effects on DnaA and YabA. The effects of DnaN in vivo are most likely due to the known direct interaction between DnaN and YabA (Noirot-Gros et al., 2002; Noirot-Gros et al., 2006). At *oriC*, the relief of YabA-mediated inhibition of DnaA likely stimulates replication initiation when a round of replication is completed and the replisome is disassembled. These results are also consistent with the increase in DnaA binding to *oriC* and other chromosomal regions after arrest of replication elongation or initiation (Breier & Grossman, 2009; Smits et al., 2010; Smits et al., 2011).

Discussion

YabA, a negative regulator of replication initiation, interacts directly with DnaA and the sliding clamp, DnaN (Noirot-Gros et al., 2002; Noirot-Gros et al., 2006). Two types of

models have been proposed for the function of the YabA-DnaA interaction: 1) YabA functions to titrate DnaA away from *oriC*, thereby limiting replication initiation (Noirot-Gros et al., 2006; Soufo et al., 2008; Katayama et al., 2010), and 2) YabA works directly on DnaA to affect its activity at *oriC* (Hayashi et al., 2005; Cho et al., 2008; Goranov et al., 2009). These possibilities are not mutually exclusive.

YabA and DnaA

Our results indicate that YabA works directly on DnaA at *oriC* and other chromosomal regions. We found that, in vivo, YabA associates with *oriC* and other chromosomal regions bound by DnaA, and that this association depends on DnaA. Also, the presence of YabA causes a reduction in binding of DnaA to these regions (Fig. 7). We found that in vitro, YabA directly alters cooperative binding of DnaA to DNA, independently of nucleotide hydrolysis and irrespective of the nucleotide bound to DnaA. Non-cooperative binding by DnaA-ATP to DNA in the presence of YabA could limit formation of DnaA-ATP oligomers needed for replication initiation. Together, our results from in vivo and in vitro experiments indicate that YabA is recruited to chromosomal regions bound by DnaA and limits the amount of DnaA associated with these regions by inhibiting cooperative binding of DnaA. Our results also indicate that DnaN relieves YabA-mediated inhibition of replication initiation (Fig. 7).

Previous experiments detected little or no effect of YabA on DnaA binding in vivo (Cho et al., 2008). These experiments were done in cells replicating from *oriC*. We also found modest effects of YabA on DnaA binding under these conditions. However, interpretations of these results are confounded by the effects of YabA on replication initiation and possible effects of replication on the activity of DnaA. Here, we monitored the effects of YabA on DnaA under conditions in which there are no detectable effects on replication initiation by using strains that replicate from the heterologous origin *oriN*. Replication from *oriN* is unaffected by YabA (Goranov et al., 2009) and in our experiments, the replication status of the strains with and without *yabA* is unchanged. Use of these strains revealed a significant effect of YabA on DnaA binding to various chromosomal regions.

Some of the chromosomal regions bound by DnaA are in promoters that affect expression of downstream genes (Burkholder *et al.*, 2001; Goranov et al., 2005; Ishikawa et al., 2007; Breier & Grossman, 2009). YabA is also associated with these regions, at least the ones analyzed. Expression of these genes changes in response to inhibition of replication elongation or initiation (Burkholder et al., 2001; Goranov et al., 2005; Breier & Grossman, 2009). Although YabA is associated with these regions, it is not required for gene expression to change in response to replication stresses (Goranov et al., 2009). Also, the absence of YabA does not appear to substantially effect expression of these genes under the conditions tested (Goranov et al., 2009). DnaA activity at these regions is probably affected by other factors, including the need for new synthesis to generate DnaA-ATP (Goranov et al., 2005; Breier & Grossman, 2009) and perhaps the presence of DnaD and DnaB (Smits et al., 2011). Effects of these other factors might be partially redundant with YabA, helping to mask effects of loss of *yabA*.

In addition to its association with chromosomal regions bound to DnaA, YabA is also associated with the replisome and appears as foci visualized using fusions to GFP (or its derivatives) (Hayashi et al., 2005; Noirot-Gros et al., 2006; Cho et al., 2008; Goranov et al., 2009). In general, these types of cell biological findings should never be interpreted as indicating the location of all of a given protein in the cell, and sometimes do not even indicate the location of biologically active protein. Together, the previously published cell biological analyses of YabA and the results presented here indicate that some YabA is associated with the replisome (Hayashi et al., 2005; Noirot-Gros et al., 2006; Cho et al.,

2008; Goranov et al., 2009) independently of DnaA (Goranov et al., 2009), and some YabA is associated with chromosomal regions through interaction with DnaA. It is also possible that YabA is present elsewhere in the cell. Using Western blots with anti-YabA antibodies and comparing amounts of YabA in cell lysates with known amounts of purified protein, we found that there are >20,000 molecules of YabA per colony forming unit, at least 5–10-fold greater than the amount of DnaA (HM, C. Lee, ADG, unpublished results), consistent with the notion that YabA is found at several subcellular locations.

YabA limits the amount of DnaA bound to DnaA boxes, including those in *oriC*. Much of this inhibition is likely direct since YabA alters cooperative binding of DnaA to DNA in vitro. This loss of cooperativity results in a decrease in the apparent dissociation constant, consistent with relatively independent binding of DnaA to different sites in the DNA fragment. If there is any importance to the order and geometry of DnaA binding to sites in *oriC* in *B. subtilis*, as there is for *E. coli* (Messer *et al.*, 2001; Speck & Messer, 2001; Leonard & Grimwade, 2010), then the decreased cooperativity in the presence of YabA is very likely what enables YabA to inhibit replication initiation. The absence of YabA likely enables increased cooperative binding and causes over-replication in *yabA* null mutants.

YabA and DnaN

In addition, two types of models have been proposed for the function of the YabA-DnaN interaction. This interaction could: 1) relieve, or 2) enable or stimulate YabA-mediated inhibition of DnaA. DnaN could stimulate YabA-mediated inhibition of DnaA either by recruiting YabA to the replisome and enabling titration of DnaA away from the origin, or by delivering YabA to the origin where it can directly inhibit DnaA function.

Our results indicate that treatments that cause release of DnaN from the replisome cause increased association of DnaA and decreased association of YabA with *oriC* and other chromosomal regions (Fig. 7). Increased production of DnaN also has the same effects. Previous work showed that an increase in DnaN causes an increase in replication initiation that depends on the presence of *yabA*, and conversely, that a decrease in DnaN causes a decrease in replication initiation (Goranov et al., 2009). These results are most consistent with DnaN functioning as an activator of replication initiation. Since YabA and DnaN are known to interact directly, the simplest interpretation is that DnaN likely functions directly as an antagonist of YabA-mediated inhibition.

Data presented here are most consistent with a role for DnaN in antagonizing (rather than enabling) YabA-mediated inhibition of DnaA. Previous models predicted that DnaN enables (rather than antagonizes) YabA-mediated inhibition of DnaA, either by helping YabA titrate DnaA away from *oriC* (Noirot-Gros et al., 2006; Soufo et al., 2008; Katayama et al., 2010) or by delivering YabA to *oriC* (Hayashi et al., 2005; Cho et al., 2008; Goranov et al., 2009). In these cases, DnaN is an inhibitor of replication initiation by virtue of being an activator of YabA. The most compelling data in support of a positive role for DnaN in YabA function are YabA mutants that reduce its interaction with DnaN. These YabA-nim mutants have a loss-of-function phenotype. That is, they are similar to a YabA null mutant. The rationale is that if the loss of interaction with DnaN is the only defect of these mutants, then YabA-DnaN interaction is required in some way for YabA to inhibit replication initiation. However, the amino acid changes in the YabA-nim mutants are close to amino acids implicated in YabA interaction with DnaA (Noirot-Gros et al., 2006). Preliminary experiments indicate that the YabA-nim mutant is partly defective in association with DnaA at chromosomal regions in vivo (data not shown), consistent with the notion that the null phenotype of this mutant could be due to decreased interaction with DnaA and not decreased interaction with DnaN.

Comparisons of YabA to replication regulators of *E. coli*

The cell biology of YabA is somewhat similar to that of SeqA in *E. coli*. Like YabA, SeqA is a negative regulator of replication initiation. Visualization of SeqA with a fluorescent reporter or immunofluorescence microscopy (Hiraga *et al.*, 1998; Onogi *et al.*, 1999) indicates that it is associated with replication forks. However, like YabA, SeqA clearly associates with *oriC* to affect replication initiation (e.g., Nievera *et al.*, 2006) and also associates with other chromosomal regions. Unlike YabA, SeqA binds directly to hemimethylated DNA. This occurs at methylation sites in newly replicated chromosomal regions that become hemimethylated. The highest concentration of newly replicated and thus hemimethylated DNA in the cell is near the active replication fork. At *oriC*, binding by SeqA sequesters *oriC* (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994; Waldminghaus & Skarstad, 2009), making it inaccessible for replication initiation while the newly replicated origin regions remains hemimethylated. Methylation sites become fully methylated by the activity of the DNA adenine methylase (Dam). Thus, the cell biological visualization of both YabA and SeqA do not provide the proper view of where they act to control replication initiation.

YabA also shares some properties with Hda, a well-studied replication regulator in *E. coli*. Like YabA, Hda interacts with DnaA and DnaN and is a negative regulator of replication initiation (Kato & Katayama, 2001; Su'etsugu *et al.*, 2004). Unlike YabA, Hda is homologous to DnaA and stimulates the ATPase activity of DnaA, thereby inhibiting DnaA activity by stimulating conversion to the inactive (or less active) DnaA-ADP. The role of *E. coli* DnaN is to stimulate the activity of Hda when DnaN is part of the active replisome, thereby enabling the inhibitory function of Hda and coupling it to ongoing replication (Katayama *et al.*, 1998; Kato & Katayama, 2001; Su'etsugu *et al.*, 2004; Katayama *et al.*, 2010). In contrast, *B. subtilis* DnaN inhibits YabA function and YabA is not known to affect the ATPase activity of DnaA (Cho *et al.*, 2008). Our results indicate that YabA directly affects the ability of DnaA to bind DNA, and this effect is seen with DnaA-ATP, DnaA-AMP-PNP, and DnaA-ADP.

In addition to Hda, there are several other mechanisms that regulate the activity of DnaA and replication initiation in *E. coli*. Even though DnaA is found in virtually all bacteria, and its binding site is largely conserved, the proteins known to regulate DnaA and replication initiation in *E. coli* are not widely conserved. Nonetheless, Gram-positive bacteria also have multiple mechanisms for regulating DnaA and replication initiation.

Other mechanisms regulating DnaA and replication initiation in *B. subtilis*

YabA is not the only regulator of replication initiation in *B. subtilis*. Several other proteins have been identified that alter replication initiation. As cells enter stationary phase and begin to sporulate, a negative regulator of replication initiation is induced (Rahn-Lee *et al.*, 2009; Wagner *et al.*, 2009). This protein, SirA, interacts directly with DnaA, apparently inhibiting its ability to bind DNA (Wagner *et al.*, 2009; Rahn-Lee *et al.*, 2011). In addition, the chromosome partitioning proteins Soj (ParA) and Spo0J (ParB) somehow modulate replication initiation (Lee *et al.*, 2003; Ogura *et al.*, 2003; Lee & Grossman, 2006; Murray & Errington, 2008; Scholefield *et al.*, 2011b), and Soj interacts directly with DnaA, although the mechanism by which replication is inhibited is not yet known.

In addition to these and other proteins that affect replication initiation, the levels of DnaA and its rate of synthesis are important. DnaA is autoregulated and its overproduction leads to overreplication in *B. subtilis* (Ogura *et al.*, 2001) and other organisms (Atlung *et al.*, 1985; Braun *et al.*, 1985; Kucherer *et al.*, 1986; Berenstein *et al.*, 2002; Salazar *et al.*, 2003). The combination of DnaA autoregulation and the activities of several regulatory proteins

contributes to the complex control of replication initiation (e.g., Riber *et al.*, 2006). Cells have adopted multiple ways of controlling the activity of DnaA with various organisms using different mechanisms to achieve similar types of regulation. We predict that other proteins in *B. subtilis* might also inhibit the cooperativity of DnaA, limiting the amount of DnaA that is bound to *oriC*. Furthermore, we expect that other organisms also use this mechanism to control replication initiation.

Experimental procedures

B. subtilis strains and alleles

B. subtilis strains (Table 1) are isogenic with the laboratory strain JH642, and contain the *trpC2* and *pheA1* alleles, unless indicated otherwise. Strains that are *trp*⁺ contain a deletion linked to the *trp* region, $\Delta(\text{ypjG-hepT})122$, described previously (Berkmen & Grossman, 2007). *dnaB134* confers a temperature-sensitive phenotype, preventing initiation but not elongation of replication at the non-permissive temperature (48°C). The transposon insertion *zhh83::Tn917* is linked to the *dnaB* operon. The heterologous origin, *oriN*, and its initiator, *repN*, were inserted near *oriC* in *spoIIIJ* (Moriya *et al.*, 1997; Berkmen & Grossman, 2007) and support replication in the absence of *oriC* and *dnaA* (Hassan *et al.*, 1997). The DnaA binding sites and part of the DUE between *dnaA* and *dnaN* are deleted in the *oriC-S* allele (Fig. 1) (Kadoya *et al.*, 2002; Berkmen & Grossman, 2007). $\Delta(\text{dnaA-oriC-dnaN})::\text{spc}$, also called $\Delta(\text{oriC-L})::\text{spc}$, is a deletion-insertion, with *dnaA* and most of *dnaN* missing with a *spc* cassette inserted. It still contains the *dnaA* promoter region with the DnaA binding sites upstream of *dnaA* (Goranov *et al.*, 2005; Berkmen & Grossman, 2007).

Media and growth conditions

Cells were grown at 30°C, in defined minimal medium with 1% glucose, 0.1% glutamate and required amino acids. Arabinose (1%) and xylose (1%) were substituted for glucose when inducing expression from xylose-inducible promoter Pxyl, in strains TAW5 and AIG278. TAW5 was grown in the presence of 0.1 mM IPTG to allow for production of DnaA from the LacI-repressible-IPTG-inducible promoter Pspank. Replication elongation was arrested by treating cells with 38 µg/ml of 6-(*p*-hydroxyphenylazo)-uracil (HPUra) for 50 minutes. HPUra binds to the catalytic subunit of DNA polymerase, PolC, and blocks replication (Brown, 1970). For inactivation of initiation, the *dnaB134* was shifted to the non-permissive temperature (48°C) for 50 min. The temperature shift prevents initiation of new rounds of replication from *oriC* while allowing ongoing rounds of replication to finish.

Purification of DnaA and YabA

B. subtilis DnaA (no tag) was produced in and purified from an *E. coli dnaA* null mutant, essentially as described (Smits *et al.*, 2011). *yabA* was cloned into pET14b (generating pWKS666) to encode a recombinant YabA that has a hexa-histidine tag at its N-terminus (his-YabA). The construct was transformed into *E. coli* BL21-AI and expression was induced for 2 to 3 hours using a final concentration of 0.2% arabinose. *E. coli* cells were washed with cold wash buffer (50 mM Tris-HCl pH 8 and 300 mM NaCl), pelleted, and lysed in lysis buffer (300 mM NaCl, 20 mM Tris-HCl pH 8 and 10 mM MgCl₂) containing 1 mg/ml lysozyme and CellLytic™ lysis reagent (SIGMA) by incubation at 23°C for 30 minutes and 3×10 sec pulses with a sonicator. The lysates were cleared by centrifugation, and his-YabA was bound to Ni-NTA beads (QIAGEN) by rotation in a conical tube at 4°C for 1 hour. The protein-bound beads were packed into a column and washed 5 times with 20 column volumes of lysis buffer containing increasing amounts of imidazole (from 10 mM to 50 mM in 10 mM increments). His-YabA was then eluted in six 1 ml fractions with lysis buffer containing 250 mM imidazole. The fractions containing the protein with 95% or more purity as determined by SDS-PAGE were combined and dialyzed at 4°C overnight against

storage buffer (45 mM HEPES, pH 7.6, 0.5 mM EDTA, 10 mM Mg acetate, 1 mM DTT, 20% sucrose, 700 mM potassium glutamate). Aliquots were frozen at -80°C .

Gel shift experiments

A 381 bp region upstream of *oriC*, containing the *dnaA* promoter region and 8 putative DnaA binding sites, was generated using primers CB23 and CB24 (sequences available upon request) and *B. subtilis* genomic DNA. The PCR product was purified using a PCR purification column (QIAGEN) and end labeled with radioactive gamma- ^{32}P -ATP using T4 Polynucleotide Kinase (New England Biolabs). The end-labeled fragment was purified using a PCR purification column. This DNA (1 nM) was used in each gel shift reaction.

To bind the desired nucleotide, purified DnaA was incubated with 5 mM ATP, AMP-PNP, or ADP on ice for 2 hours. DNA binding assays were done with DnaA-ATP, DnaA-AMP-PNP, and DnaA-ADP at the concentrations indicated in the figure legends, with and without 700 nM his-YabA in gel shift buffer (45 mM HEPES, pH 7.6, 10 mM KCl, 140 mM KGLu, 10 mM Mg acetate, 2.5 mM ATP or AMP-PNP or ADP, 0.5 mM EDTA, 1 mM DTT, 50 $\mu\text{g}/\text{ml}$ bovine serum albumin (BSA), and 20% glycerol). The DNA binding reactions were run on a 5% polyacrylamide gel (37:1 acrylamide/bisacrylamide) in 0.5x Tris-borate-EDTA (TBE) and 2.5% glycerol and run in 0.5x TBE at approximately 12 V/cm for 3 h. Gels were imaged on a Typhoon scanner (GE Healthcare) and signals were quantified using ImageQuant software. The fraction of DNA bound was determined by subtracting the signal for “free” unbound DNA in each lane from the total signal in that lane.

ChIP-qPCR

Chromatin immunoprecipitations (ChIPs) of DnaA, followed by quantitative real-time PCR (qPCR) were performed essentially as described (Smits et al., 2010). The anti-DnaA antibodies are specific for DnaA (Goranov et al., 2005; Smits et al., 2010). YabA ChIPs were performed essentially as described for DnaA, except that immunoprecipitations from cross-linked lysates were done for 2 h at 23°C with rabbit anti-YabA antibodies. DNA in the ChIPs was quantified using qPCRs essentially as described, using *yhaX* (a region that does not bind DnaA) for normalization (Merrikh et al., 2011). Similar results were obtained with other control regions that do not bind DnaA (unpublished results). ChIP-chip and ChAP-chip experiments with DnaA (Ishikawa et al., 2007; Cho et al., 2008; Breier & Grossman, 2009; Smits et al., 2011) indicate that there are many chromosomal regions that do not bind DnaA and validate the use of specific regions as controls in ChIP-qPCR. Primers for the promoter region of *dnaA* (part of *oriC*) amplified a fragment depicted in Fig. 1E.

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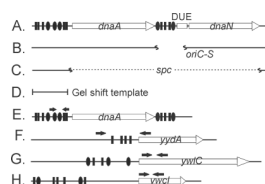


Figure 1. Map of *oriC* and other regions tested that are bound by DnaA

A. The *oriC* region includes *dnaA* and *dnaN*, the DnaA binding sites upstream and downstream of *dnaA* and the DNA unwinding element (DUE). Filled ovals represent sequences that match the consensus DnaA binding site (5'-TTATNCACA-3') and filled boxes represent sequences with one mismatch. The DUE is indicated by an open rectangle. The region upstream of *dnaA* and the region between *dnaA* and *dnaN* are required for *oriC* function (Moriya et al., 1992).

B. The region deleted in the *oriC-S* (a.k.a., *oriC-6*) allele is indicated. This mutation leaves intact the DnaA binding sites upstream of DnaA that are part of *oriC*, as well as *dnaA* and *dnaN* (Kadoya et al., 2002; Berkmen & Grossman, 2007).

C. The region deleted in the $\Delta(dnaA-oriC-dnaN)::spc$ {a.k.a., $\Delta(oriC-L)::spc$ } is indicated. This mutation leaves the *dnaA* promoter region with the DnaA binding sites intact (Goranov et al., 2005; Berkmen & Grossman, 2007).

D. The 381 bp fragment that was used as template in the in vitro gel shift assays (Fig. 4). This region contains the indicated binding sites upstream of *dnaA* and was amplified by PCR with appropriate primers.

E–H. Cartoons of the chromosomal regions, *dnaA* (**E**), *ydaA* (**F**), *ywIC* (**G**), *ywIC* (**H**) that were tested for interaction with DnaA and YabA by ChIP-qPCR are indicated. Sequences that match the consensus DnaA binding site are indicated as in panel A. The lines with arrows above the gene regions represent the approximate locations of the primers used for qPCR. Drawings are not to scale.

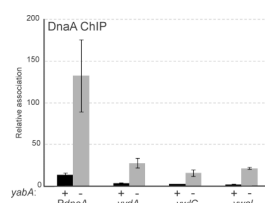


Figure 2. YabA inhibits association of DnaA with chromosomal regions in vivo

The relative amounts of the indicated chromosomal regions that were associated with DnaA were determined in ChIP-qPCR experiments from cells with and without *yabA*. PCR primers were for chromosomal regions in *oriC* (indicated as "PdnaA" since this part of *oriC* is in the promoter region for *dnaA*) and three other chromosomal regions known to bind DnaA (*yydA*, *ywlC* and *ywcI*) and were normalized to a control region (*yhaX*) to which DnaA does not bind (Experimental procedures). A value of 1 indicates no enrichment relative to the control region. Strains were replicating from the heterologous origin *oriN*, contained a small deletion in *oriC* (*oriC-S*) and either contained wild type *yabA* (black bars; strain MMB170) or a *yabA* null mutation (gray bars; strain AIG185). Data are averages from of at least 3 independent experiments and error bars represent the standard error. Enrichment values (\pm standard errors) in the *yabA*⁺ strain (black bars) for the PdnaA, *yydA*, *ywlC* and *ywcI* regions were 13.7 ± 2.3 , 3.8 ± 0.5 , 2.4 ± 0.3 , and 2.3 ± 0.4 , respectively.

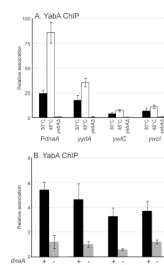


Figure 3. YabA is associated with chromosomal regions bound by DnaA and association depends on DnaA

The relative amounts of the indicated chromosomal regions that were associated with YabA were determined in ChIP-qPCR experiments. A value of 1 indicates no enrichment relative to the control region (Fig. 2). PCR primers used to analyze chromosomal regions were as in Fig. 2. Data are averages of at least 3 independent experiments and error bars represent the standard error. **A.** YabA is associated with chromosomal regions bound by DnaA. Relative association of YabA with the indicated chromosomal regions in wild type cells (AG174) at 30°C (black bars) and 48°C (white bars) and a *yabA* null mutant at 30°C (AIG109) (gray bars). The lack of a signal in the *yabA* null mutant indicates specificity of the immunoprecipitation. **B.** Association of YabA with the *oriC* region and other DnaA targets depends on DnaA. Strains were replicating from the heterologous origin *oriN* and contained a deletion extending from *dnaA* through *dnaN*, removing both genes. *dnaN* was expressed from P_{xyl}-*dnaN*. Strain with no *dnaA* (gray bars; strain AIG200). Strain with *dnaA* expressed from P_{spank}-*dnaA* (black bars; strain TAW5; 0.1 mM IPTG).

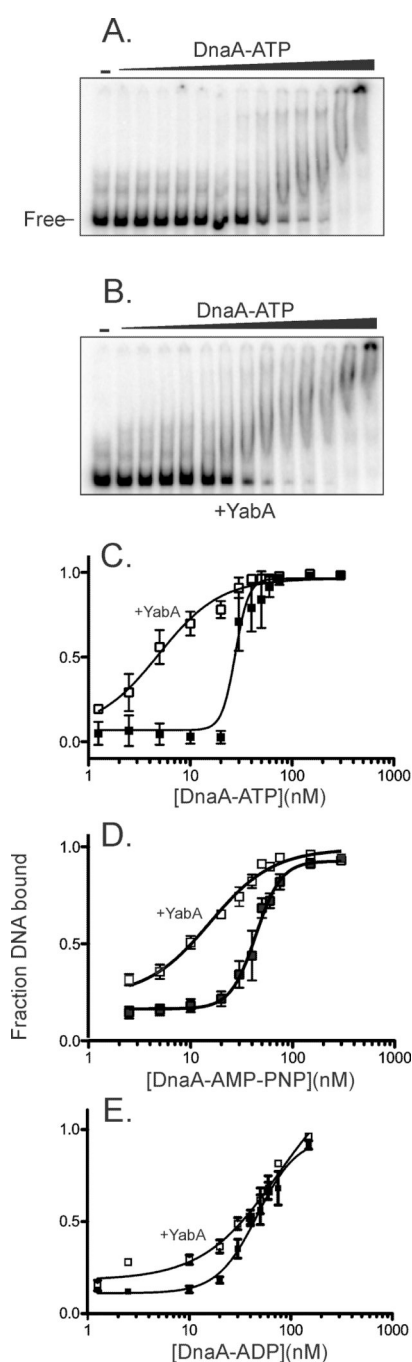


Figure 4. YabA makes DnaA binding to DNA non-cooperative

Representative gels and binding curves measuring binding of DnaA to DNA with and without purified YabA are shown. Purified DnaA was pre-bound with ATP (A, B, C), AMP-PNP (D), and ADP (E) as indicated.

A, B. A representative gel with increasing concentrations of DnaA-ATP incubated with 1 nM of template DNA in the absence (A) or presence (B) of YabA (700 nM) is shown. ^{32}P -labeled DNA without added protein (0) or with His-YabA (700 nM) is shown in the first lanes of panels A and B respectively. The other lanes contain DnaA-ATP concentrations: 0.25, 0.5, 1, 2, 4, 8, 16, 30, 40, 50, 60, 120, and 240 nM, in that order.

C, D, E. Data from at least three independent gel shift experiments with each nucleotide (including the ones shown above) are shown for **(C)** DnaA-ATP, **(D)** DnaA-AMP-PNP, and **(E)** DnaA-ADP either without (filled squares) or with (open squares) 700 nM YabA in the reactions. Data were plotted and fitted to the Hill equation $\{\theta = (L)^n / K_d^n + (L)^n\}$, where θ is the fraction of DNA bound, (L) is the DnaA concentration for a given θ , K_d is the apparent dissociation constant (concentration at which 50% of DNA is shifted, determined from the data), and n is the Hill coefficient. The calculated Hill coefficient for DnaA-ATP was ~6.6 in the absence of YabA and ~1.4 in the presence of YabA. The apparent dissociation constant for DnaA-ATP was ~28 nM in the absence and ~5 nM in the presence of YabA. The calculated Hill coefficient for DnaA-AMP-PNP was ~3.4 in the absence and ~1.4 in the presence of YabA. The apparent dissociation constant for DnaA-AMP-PNP was ~46 nM in the absence and ~13 nM in the presence of YabA. DNA binding by DnaA-AMP-PNP was somewhat less cooperative than that of DnaA-ATP, indicating that AMP-PNP was not a true mimic of ATP. The calculated Hill coefficient for DnaA-ADP was ~2.2 in the absence and ~1.1 in the presence of YabA. The apparent dissociation constant for DnaA-ADP was ~81 nM in the absence and ~47 nM in the presence of YabA.

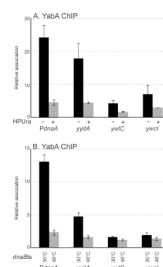


Figure 5. Replication status influences association of YabA with chromosomal regions

The relative amounts of the indicated chromosomal regions that were associated with YabA were determined in ChIP-qPCR experiments. PCR primers used to analyze chromosomal regions were as in Fig. 2. Data are averages of at least 3 independent experiments and error bars represent the standard error.

A. Blocking replication elongation with HPURA causes dissociation of YabA from the chromosomal regions tested. Wild type cells (AG174) without (black bars) and 50 min after addition of HPURA to block replication elongation (gray bars).

B. Blocking replication initiation in a *dnaB134ts* mutant (KPL69) causes dissociation of YabA from the chromosomal loci tested. growth at permissive temperature, 30°C, (black bars) and after 50 minutes of incubation at non-permissive temperature, 48°C, (gray bars). There is some reduction in association of YabA even at the permissive temperature in this strain background compared to wild-type cells most likely due to the partial defect observed in this mutant at the permissive temperature. Increasing the temperature to 48°C in wild-type cells did not lead to dissociation of YabA (see Fig. 3A). Similar results were obtained (YabA dissociated after blocking initiation) in two other mutant backgrounds tested (*dnaD23* and *dnaI2*).

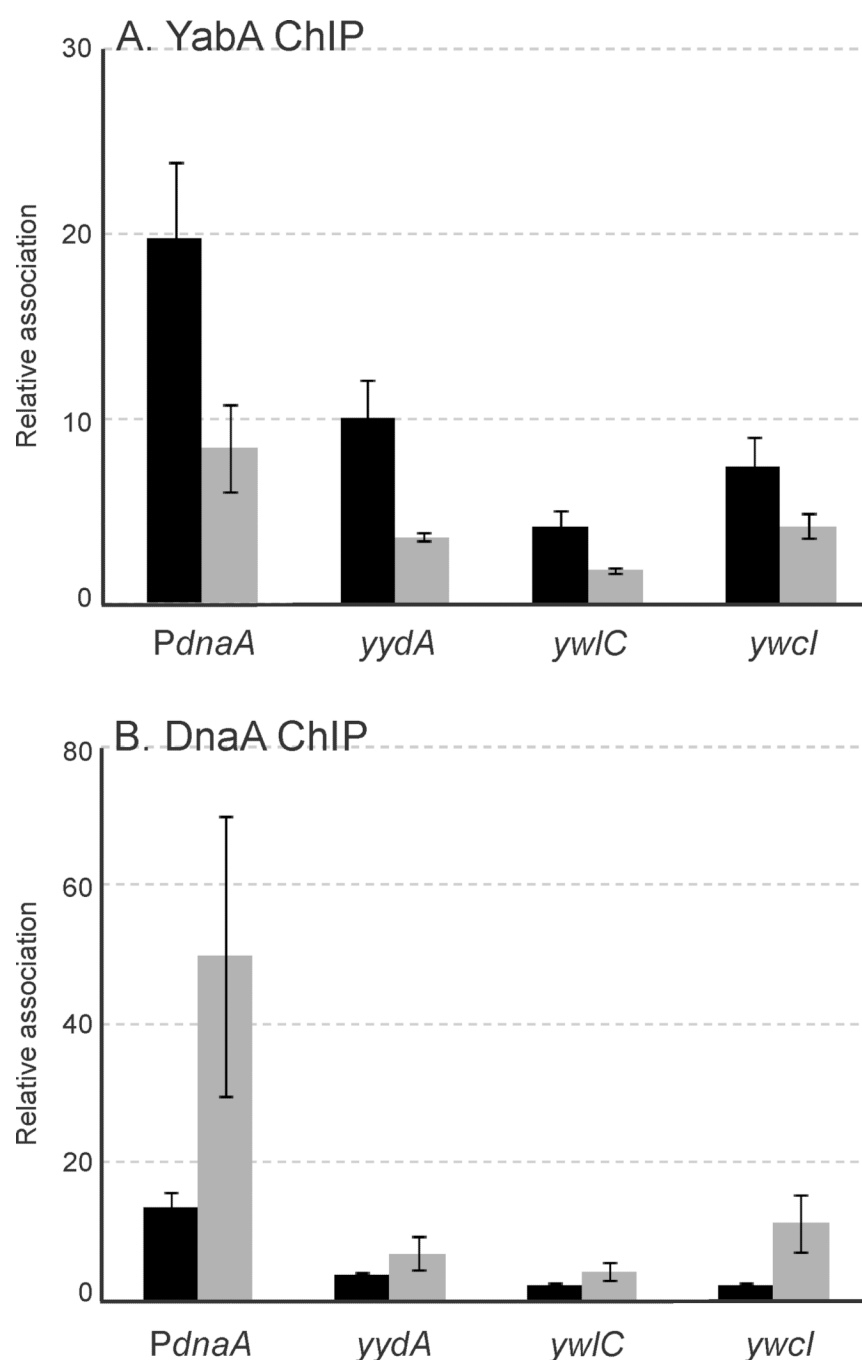
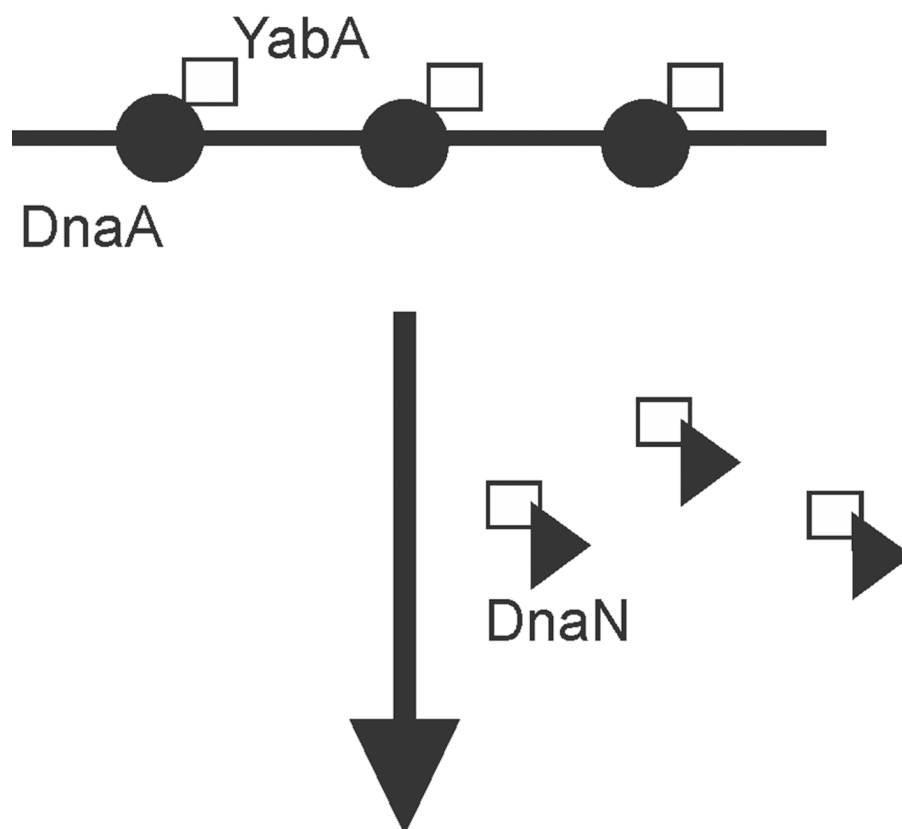


Figure 6. Increased production of DnaN causes decreased association of YabA and increased association of DnaA

The relative amounts of the indicated chromosomal regions that were associated with YabA (A) and DnaA (B) were determined in ChIP-qPCR experiments. Samples were taken from cells initiating replication from *oriN* and deleted for *oriC* and either producing normal levels of DnaN (black bars, strain MMB170) or ~3.5-fold more DnaN, from *Pxyl-dnaN*, (gray bars, strain AIG278). The amount of DnaA was not detectably different between these two strains. PCR primers used to analyze chromosomal regions were as in Fig. 2. Data are averages of at least 3 independent experiments and error bars represent the standard error.

DnaA binding non-cooperative



DnaA binding cooperative



Figure 7. Model for effects of YabA and DnaN on cooperative binding by DnaA

Binding of DnaA (filled circles) to DNA (black line) is non-cooperative in the presence of YabA (open squares), thereby limiting the amount of DnaA associated with *oriC* and at least several other chromosomal regions. The presence of DnaN (filled triangles), separate from the replisome, removes YabA from DnaA, enabling cooperative binding and oligomerization of DnaA to *oriC* and other chromosomal regions. The stoichiometry of these interactions is not known.

Table 1*B. subtilis* strains.

Strain	Relevant Genotype ^a (reference)
AG174	<i>trpC2 pheA1</i> (wild-type, JH642)
AIG109	$\Delta yabA::cat$ (Goranov et al., 2009)
KPL69	<i>dnaB134</i> (Ts)- <i>zhh83:Tn917 (mls)</i> (Rokop et al., 2004)
MMB170	<i>trp</i> ⁺ <i>spoIIIJ::oriN repN kan</i> $\Delta oriC-S$ (Berkmen & Grossman, 2007)
AIG278	<i>trp</i> ⁺ <i>spoIIIJ::oriN repN kan</i> $\Delta oriC-S amyE::PxylA-dnaN cat$ (Goranov et al., 2009)
AIG185	<i>trp</i> ⁺ <i>spoIIIJ::oriN repN kan</i> $\Delta oriC-S \Delta yabA::cat$ (Goranov et al., 2009)
TAW5	<i>trp</i> ⁺ <i>spoIIIJ::oriN repN kan</i> $\Delta(dnaA-oriC-dnaN)::spc amyE::PxylA-dnaN cat lacA::Pspank-dnaA tet$
AIG200	<i>trp</i> ⁺ <i>spoIIIJ::oriN repN kan</i> $\Delta(dnaA-oriC-dnaN)::spc amyE::PxylA-dnaN cat$ (Goranov et al., 2005)

^a All strains are derived from AG174 and contain the *trp phe* alleles unless otherwise indicated.