YabA of Bacillus subtilis controls DnaA-mediated replication initiation but not the transcriptional response to replication stress

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key words: DNA replication; transcription; DnaA; YabA; Bacillus subtilis

running title: YabA and DnaA of B. subtilis

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Summary

*yabA* encodes a negative regulator of replication initiation in *Bacillus subtilis* and homologues are found in many other Gram-positive species. YabA interacts with the β-processivity clamp (DnaN) of DNA polymerase and with the replication initiator and transcription factor DnaA. Because of these interactions, YabA has been proposed to modulate the activity of DnaA. We investigated the role of YabA in regulating replication initiation and the activity of DnaA as a transcription factor. We found that YabA function is mainly limited to replication initiation at *oriC*. Loss of YabA did not significantly alter expression of genes controlled by DnaA during exponential growth or after replication stress, indicating that YabA is not required for modulating DnaA transcriptional activity. We also found that DnaN activates replication initiation apparently through effects on YabA. Furthermore, association of GFP-YabA with the replisome correlated with the presence of DnaN at replication forks, but was independent of DnaA. Our results are consistent with models in which YabA inhibits replication initiation at *oriC*, and perhaps DnaA function at *oriC*, but not with models in which YabA generally modulates the activity of DnaA in response to replication stress.
**Introduction**

Bacteria use multiple mechanisms to regulate the initiation of replication and to alter gene expression in response to changes in replication status. The chromosomal origin of replication, oriC, and the replication initiation protein DnaA are key targets for controlling replication initiation in bacteria {Kaguni, 2006, Zakrzewska-Czerwinska *et al.*, 2007, Mott & Berger, 2007}, and references therein. DnaA is highly conserved (Messer, 2002). It is a member of the AAA+ family of proteins, binds ATP or ADP, and has a weak ATPase activity. In *E. coli*, and presumably other bacteria, DnaA is active for replication initiation only when in the ATP-bound form (Sekimizu *et al.*, 1987). During replication initiation, DnaA binds to sequences in oriC and can cause melting of a portion of oriC to generate ssDNA. The ssDNA serves as an assembly region for the replication machinery {reviewed in (Kaguni, 2006, Mott & Berger, 2007, Zakrzewska-Czerwinska *et al.*, 2007, Messer, 2002, Messer *et al.*, 2001)}.

Much of what we know about the control of replication initiation by DnaA comes from work with *E. coli*. There are multiple mechanisms for controlling the activity of *E. coli* DnaA and its ability to bind to its sites in oriC {e.g., (Kato & Katayama, 2001, Ishida *et al.*, 2004, Nievera *et al.*, 2006, Kaguni, 2006, Zakrzewska-Czerwinska *et al.*, 2007)}. However, despite the conservation of DnaA and its binding sites and the similar overall regulation of replication initiation in many organisms, the proteins used to regulate replication initiation in *E. coli* are not widely conserved.

Replication initiation in *Bacillus subtilis* is also highly regulated and DnaA is part of this regulation (Yoshikawa & Ogasawara, 1991, Ogura *et al.*, 2001, Hayashi *et al.*, 2005). As in *E. coli*, transcription of dnaA is auto-repressed and overexpression of dnaA causes excessive replication initiation and reduces the size of cells at the time of initiation (Ogura *et al.*, 2001). However, *B. subtilis* does not contain homologs of any of the well-characterized proteins known
to modulate replication initiation and DnaA activity in *E. coli* \(\text{e.g., (Zakrzewska-Czerwinska et al., 2007)}\).


active form for transcriptional regulation (Kurokawa et al., 1999, Kaguni, 2006, Zakrzewska-Czerwinska et al., 2007, Gon et al., 2006).

Because of its ability to interact with both DnaA and the β-clamp of DNA polymerase, YabA has been proposed to modulate the activity of DnaA, both as a replication initiator and transcription factor, in response to alterations in replication status (Noirot-Gros et al., 2006, Soufo et al., 2008). We investigated the role of YabA in regulating replication initiation and in regulating the activity of DnaA as a transcription factor. We found that YabA function is mainly limited to replication initiation at oriC. Loss of YabA did not significantly alter expression of genes controlled by DnaA nor the broader class of genes whose expression is affected in response to inhibition of replication elongation. Our results indicate that YabA is not required for modulating the activity of DnaA as a transcription factor in response to replication stress. We also found that the β-clamp of DNA polymerase regulates replication initiation and that this regulation appears to be mediated by YabA.

Results

Effects of YabA on DNA replication require the DnaA-dependent oriC

Null mutations in yabA cause an increase in replication initiation (Noirot-Gros et al., 2002, Noirot-Gros et al., 2006, Hayashi et al., 2005, Cho et al., 2008). We found that this increase was not observed in cells initiating replication from a heterologous origin inserted into the chromosome. We compared the effects of deletion and overexpression of yabA on replication in cells initiating from either oriC or the heterologous origin oriN. Initiation from oriN requires its cognate initiator protein RepN and is independent of DnaA (Hassan et al., 1997). We monitored replication relative to cell growth by measuring the DNA to protein ratio (DNA/protein) of cells in culture (Kadoya et al., 2002).
In cells growing in minimal medium and initiating replication from oriC, a yabA null mutation caused an increase in the DNA to protein ratio of nearly 2-fold relative to that of yabA⁺ cells (Fig. 1A). Conversely, overexpression of yabA from a heterologous promoter caused a decrease in the DNA to protein ratio (Fig. 1A). The effect in the yabA deletion mutant was more severe in minimal medium than in rich medium (data not shown). These results are consistent with previous findings that YabA is a negative regulator of replication (Noirot-Gros et al., 2002, Noirot-Gros et al., 2006, Hayashi et al., 2005, Cho et al., 2008).

In contrast to the effects of yabA on replication from oriC, there was little or no effect when replication initiated from the DnaA-independent oriN. We constructed oriC-mutant strains by integrating the heterologous origin of replication, oriN, and its specific replication initiator, repN (Hassan et al., 1997, Kadoya et al., 2002, Berkmen & Grossman, 2007, Goranov et al., 2005), close to the location of the endogenous origin, and deleting part of oriC (oriC⁻). Replication from oriN does not require DnaA, but appears to require all other known replication initiation factors that are needed at oriC (Hassan et al., 1997). Neither deletion nor overexpression of yabA had any detectable effect on the DNA to protein ratios in oriN⁺ oriC⁻ strains (Fig.1B).

**Subcellular localization of YabA**

The use of strains capable of initiating replication from oriN makes dnaA dispensable for replication and viability (Hassan et al., 1997, Kadoya et al., 2002, Berkmen & Grossman, 2007, Goranov et al., 2005) and allowed us to determine if dnaA is required for formation of foci of YabA. Previous cell biological analyses indicate that GFP-YabA forms foci that correspond to the subcellular positions of the replisome (Noirot-Gros et al., 2006, Hayashi et al., 2005).

Analyses of YabA mutants defective in interacting with either DnaA or DnaN (β-clamp) indicate that YabA needs to interact with both DnaA and the β-clamp in order to form foci (Noirot-Gros et al., 2006). Mutations in dnaA that cause altered interactions between DnaA and YabA were
also found to prevent formation of foci of GFP-YabA (Cho et al., 2008), consistent with the prior findings. However, loss of interaction between YabA and DnaA is not the only effect of these mutations. The mutant cells also over-initiate replication (Noirot-Gros et al., 2006, Cho et al., 2008) and there could be other effects on YabA and DnaA as well. Therefore, it is possible that the YabA:DnaA interaction may not actually be required for focus formation by GFP-YabA and that the effects of the mutations on foci of GFP-YabA are indirect.

To test directly if DnaA and oriC are required for formation of GFP-YabA foci, we monitored the formation of GFP-YabA foci in oriC mutant cells initiating replication from oriN. We disrupted dnaA in the oriN+ oriC− cells by integrating a plasmid into dnaA and placing dnaN, the gene downstream, under control of the IPTG inducible promoter Pspac(hy). GFP-YabA still formed foci in the dnaA null mutant strain (Fig. 2A). Thus, DnaA is not required for GFP-YabA focus formation.

Maintenance of GFP-YabA foci was dependent on ongoing replication. We monitored GFP-YabA foci in cells treated with HPUra, an inhibitor of DNA Polymerase III that blocks replication elongation (Neville & Brown, 1972, Brown, 1970). Within 15 min of addition of HPUra to cells, foci of GFP-YabA disappeared from >99% of cells in both the dnaA null mutant initiating from oriN (Fig. 2B) and dnaA+ cells initiating from oriC (compare Figs. 2C and 2D). Loss of GFP-YabA foci correlated with loss of DnaN-GFP foci. We monitored the effects of replication arrest on foci of the β-clamp (DnaN-GFP), the catalytic subunit of DNA polymerase (PolC-GFP), the HolB subunit of the clamp loader (HolB-GFP), and the clamp loader/τ-subunit (DnaX-GFP). Foci of PolC-GFP (Fig. 2E, F), DnaX-GFP (Fig. 2G, H), and HolB-GFP (data not shown) persisted for greater than 60 min after replication arrest. In contrast, foci of the β–clamp (DnaN-GFP) disappeared from >98% of cells within 15 min after addition of HPUra (Fig. 2I, J). These results indicate that after replication arrest with HPUra, several of the replisome
components remain assembled, but that the β-clamp is largely dissociated. Taken together, our results indicate that formation of foci of GFP-YabA is independent of dnaA and oriC, but correlates with foci of DnaN-GFP, and that YabA and DnaN are released from the replication complex following replication arrest.

**Overproduction of β-clamp (DnaN) stimulates DNA replication**

Since both DnaA and YabA regulate replication initiation and YabA interacts with both DnaA and the β-clamp, we tested whether alterations in expression of dnaN (β-clamp) might also modulate replication initiation. We found that the β-clamp stimulates replication initiation, likely by inhibiting the activity of YabA. In these experiments, we manipulated transcription of dnaN using a xylose-inducible PxylA-dnaN fusion. Induction of PxylA-dnaN, in the presence of a wild type copy of dnaN, caused an approximately 8-fold increase in the amount of dnaN mRNA as determined using DNA microarrays (data not shown). We were unable to measure differences in levels of β-clamp protein since antibodies were not available. Nonetheless, alterations in expression of dnaN caused changes in replication.

Increased transcription of dnaN caused an increase of approximately 60% in the DNA to protein ratio relative to that of wild type (Fig. 3A), indicating that an increase in β-clamp stimulated DNA synthesis. This effect was not general for overexpression of any replisome component as overexpression of dnaX (the clamp loader/tau–subunit of DNA polymerase holoenzyme) for 3–4 generations caused a decrease in the DNA to protein ratio (Fig. 3A).

We also found that decreased transcription of dnaN caused a decrease in replication initiation. By placing the only copy of dnaN under the regulation of PxylA and growing without inducer (xylose) for 3 generations, the level of dnaN mRNA was reduced to ~60% of normal. The reduced transcription of dnaN caused a decrease of 18 ± 3% in the DNA to protein ratio (Fig. 3A). Severe decreases in expression of *B. subtilis* dnaN can cause replication fork stalling
and induction of the SOS response \(\text{(Ogura et al., 2001) and data not shown}}\). Under the
conditions in which mRNA levels of \(dnaN\) were reduced to \(~60\%\) of normal and replication was
decreased, there was no detectable increase in mRNA levels of SOS genes (data not shown),
indicating that the decrease in replication is either due to a very mild decrease in replication
elongation or a decrease in replication initiation.

**Overproduction of β-clamp stimulates DnaA-dependent replication initiation from oriC**

Since \(dnaN\) encodes the β-clamp of DNA polymerase holoenzyme and is necessary for
replication elongation, it is possible that the increase in DNA synthesis from overexpression of
\(dnaN\) is due to either an effect of β-clamp on replication elongation, or due to an ability of β-
clamp to modulate replication initiation. We found that the increase in DNA synthesis due to
overexpression of \(dnaN\) was due to an increase in replication initiation. We monitored DNA
replication in asynchronous populations of exponentially growing cells using DNA microarrays,
essentially as described \(\text{(Khodursky et al., 2000, Simmons et al., 2004, Wang et al., 2007b,}\)
\(\text{Wang et al., 2007a, Goranov et al., 2006}}\). Overexpression of \(dnaN\) for 25 (data not shown) or 50
min (Fig. 3B) in an \(oriC^+\) strain caused an increase in the amount of origin region DNA
compared to other chromosomal regions. This type of increase is typically seen in cells
overinitiating DNA replication \(\text{\{e.g., (Simmons et al., 2004}}\)).

The increase in replication initiation caused by overproduction of the β-clamp was dependent
on replication initiation from \(oriC\). We tested whether overexpression of \(dnaN\) affects
replication initiation in a strain initiating solely from the heterologous origin \(oriN\). We found
that overexpression of \(dnaN\) (β-clamp) in \(oriN^+ oriC^+\) mutant strains had no significant effect on
the DNA to protein ratio (Fig. 3A). In addition, overexpression of \(dnaN\) for 25 (data not shown)
or 50 min (Fig. 3C) in the \(oriN^+ oriC^-\) mutant strains had no detectable effect on replication
initiation as monitored using DNA microarrays to profile DNA content. These results indicate
that the regulatory effects of β-clamp on replication depend on initiation from oriC, and that
overexpression of β-clamp, to the levels tested here, stimulates replication initiation. There did
not appear to be any significant effects on replication elongation as those are expected to be
independent of the origin of replication.

YabA and β-clamp appear to affect the same rate-limiting step in replication initiation
Since YabA and β-clamp directly interact (Noirot-Gros et al., 2002, Noirot-Gros et al.,
2006), affect replication initiation { (Noirot-Gros et al., 2002) and above}, and both require
DnaA-dependent initiation of replication from oriC to manifest their effects (see above), it is
plausible that β-clamp and YabA affect the same rate-limiting step in replication initiation. If this
is true, then overexpression of dnaN in a yabA null mutant should not cause an increase in
replication above that of the yabA mutant alone. We constructed a strain that contains a deletion
of yabA and overexpresses β-clamp (PxyLA-dnaN). Each single allele causes overreplication as
determined by DNA to protein ratios (Fig. 3A). Overexpression of dnaN in the yabA null mutant
did not increase the DNA to protein ratio above that in the yabA null mutant alone (Fig. 3A),
indicating that these effects were not additive and that YabA and the β-clamp are likely affecting
the same rate-limiting step in replication initiation.

Gene expression in a yabA null mutant
YabA is an attractive candidate for regulating the activity of DnaA in response to replication
status. YabA affects replication initiation from the DnaA-dependent oriC, it interacts with both
DnaA and DnaN, and its association with the replisome correlates with that of DnaN.
Expression of genes known or thought to be controlled by DnaA changes in response to
replication stress (Goranov et al., 2005, Breier & Grossman, 2009). DnaA binds to the promoter
regions of many of the proposed target genes in vivo (Goranov et al., 2005, Ishikawa et al., 2007,
Breier & Grossman, 2009) and binding to some of these regions increases when replication is
inhibited (Breier & Grossman, 2009). YabA could be required to couple the activity of DnaA to replication elongation, perhaps by sequestering DnaA at active replication forks as previously proposed (Noirot-Gros et al., 2006, Soufo et al., 2008). If this is the case, then the DnaA-mediated transcriptional response to replication arrest should be severely compromised in a yabA null mutant. Furthermore, expression of DnaA-regulated genes might be different in the absence of yabA, even during ongoing replication.

To determine if YabA is required for the regulation of DnaA in response to replication stress, we analyzed global gene expression in a yabA null mutant, both during growth and after replication arrest with HPUra. We compared the results to those in isogenic yabA+ cells. To eliminate potential pleiotropic effects due to overreplication in yabA null mutants, we did these experiments in strains initiating replication from oriN and containing an inactive oriC. yabA has no effect on replication in these conditions (Fig. 1B).

We found that the yabA null mutation had little or no effect on gene expression during exponential growth (Fig. 4A) nor in response to replication arrest (Fig. 4B). We plotted the relative mRNA levels for each gene in yabA+ cells versus those in the yabA null mutant (Fig. 4A, B). Genes known or thought to be regulated directly by DnaA are indicated with black + or - symbols, depending on whether expression increases (+) or decreases (-) in response to replication arrest. All other genes are indicated with gray dots. Most of the values from yabA+ cells are the same as or similar to those from the yabA null mutant, both during ongoing replication in exponential growth (Fig. 4A) and after replication arrest in cells treated with HPUra (Fig. 4B). These results indicate that during exponential growth and after replication arrest, there is little or no effect of loss of yabA on gene expression.

Using the data from Figures 4A and 4B, we compared the fold change in mRNA levels caused by replication arrest (treatment with HPUra) in yabA+ cells to that in the yabA null mutant
Expression of many genes increases in response to HPUra treatment and many of these are part of the SOS regulon and depend on *recA* (Goranov et al., 2005, Goranov et al., 2006). The changes in expression of these genes were similar in the *yabA*⁺ and the *yabA* null mutant cells (Fig 4C). In addition, most of the genes known or thought to be directly regulated by DnaA (+’s and -’s in Fig. 4) also changed expression in response to HPUra, and those changes were similar in the *yabA*⁺ and *yabA* null mutant cells (Fig. 4C).

The magnitudes of the changes in gene expression in response to replication arrest in both *yabA*⁺ and *yabA* null mutant cells initiating replication from ori*N* were somewhat less than those previously reported for cells initiating replication from ori*C* (Goranov et al., 2005, Goranov et al., 2006). In fact, there were many differences in gene expression simply comparing cells initiating replication from ori*C* to those initiating from ori*N* in the absence of added replication stress (unpublished results). These differences indicate that the apparently constitutive and asynchronous replication initiation from ori*N* (Noirot-Gros et al., 2002, Hayashi et al., 2005) might cause a small amount of replication stress. Since the basal expression of some of the genes is already changed in the ori*N* cells, the magnitude of the effect of replication arrest is muted and less dramatic in these cells compared to cells initiating from ori*C*. Nevertheless, for many DnaA-regulated genes, there was a significant response to replication stress and there was little or no effect of *yabA* on this response.

**Discussion**

Most of the studies on control of bacterial replication initiation have focused on *E. coli* and its close relatives. However, many of the non-essential regulators characterized in *E. coli* are not found in other organisms. YabA is one of the best characterized non-essential regulators of replication initiation in a Gram-positive organism. YabA is a negative regulator of replication
initiation in *B. subtilis* (Noirot-Gros et al., 2002, Noirot-Gros et al., 2006, Hayashi et al., 2005, Cho et al., 2008, Soufo et al., 2008). Null mutations in *yabA* cause increased replication initiation and overexpression of *yabA* causes decreased replication initiation (Hayashi et al., 2005). YabA was identified because of its ability to interact with DnaA and DnaN (β-clamp) in a yeast two-hybrid assay (Noirot-Gros et al., 2002).

Several different models have been proposed to explain how YabA controls replication initiation. One enticing model (Noirot-Gros et al., 2002) was based on a comparison of YabA to the function of Hda in *E. coli*. Even though YabA and Hda are not homologous, they have several properties in common. Like YabA, Hda is a negative regulator of replication initiation that interacts with both DnaA and DnaN (Kato & Katayama, 2001). When associated with an active replication fork via DnaN, Hda stimulates the intrinsic ATPase activity of DnaA to generate DnaA-ADP, a form of DnaA that is not active for replication initiation (Kato & Katayama, 2001, Nishida et al., 2002). In this way, Hda couples inactivation of DnaA to active replication forks, and YabA might do so too, although there are recent data indicating that YabA functions differently than Hda (Cho et al., 2008). An alternative model proposed that YabA functions to tether DnaA to active replication forks and release it during replication stress (Soufo et al., 2008). Both of these models for YabA function strongly predict that YabA should affect DnaA in a distributive manner and that in the absence of YabA, DnaA should be more active throughout the cell. These models also predict that *yabA* is required for the DnaA-mediated part of the cellular response to replication stress (Soufo et al., 2008).

Our results demonstrate that, under conditions in which *yabA* had no effect on DNA replication, it had no significant effect on expression of known and putative transcriptional targets of DnaA. There were no significant effects on expression of these genes either during exponential growth or during replication stress. These results demonstrate that YabA is not
required to modulate the activity of DnaA in response to replication stress and indicate that
YabA does not affect DnaA in a distributive manner. These findings are not consistent with
some of the previously proposed models for YabA function.

YabA does not affect replication initiation from the DnaA-independent origin of

Models proposing that YabA modulates the activity of DnaA in response to replications
stress {e.g., (Soufo et al., 2008)} are quite reasonable. Unfortunately, analyses of the effects of
yabA on the activity of DnaA are complicated by the fact that yabA affects replication initiation
and alterations in replication initiation or elongation alter the activity of DnaA (Noirot-Gros et
al., 2002, Goranov et al., 2005). Therefore, to test the effects of YabA on the activity of DnaA, it
was helpful to eliminate the effects of YabA on replication while maintaining the ability to
monitor the activity of DnaA as a transcription factor. This was accomplished by integrating the
heterologous DnaA-independent origin of replication oriN into the chromosome and inactivating
oriC.

We found that neither a null mutation in nor overexpression of yabA affected the activity of
oriN. These findings indicate that the function of YabA in the negative control of replication
initiation is specific to the DnaA-dependent oriC. Like the effects of a yabA null mutation, we
found that overexpression of dnaN stimulates replication initiation from oriC, but not from oriN.
We suspect that this stimulation by increased production of DnaN is likely due to titration of
YabA away from oriC. Since the essential chromosomal replication initiation proteins DnaB
DnaD and the replicative helicase DnaC and the clamp DnaN are required for replication from
oriN (Hassan et al., 1997), these results indicate that YabA is not likely to affect the activity of
these other proteins, at least in the absence of DnaA. These findings also made it feasible to
determine the effects of yabA on the activity of DnaA under conditions in which yabA had no
detectable effect on replication and to directly determine the effects of dnaA on the subcellular
positioning of YabA.

Subcellular location of YabA
Analyses of GFP-YabA fusions indicated that YabA is found associated with the replisome
during ongoing replication (Noirot-Gros et al., 2006, Hayashi et al., 2005, Cho et al., 2008).
This association appeared to depend on DnaA based on analysis of YabA mutants unable to
interact with DnaA (Noirot-Gros et al., 2006) and DnaA mutants unable to interact with YabA
(Cho et al., 2008). YabA was also found be required to “tether” DnaA to the replisome (Soufo et
al., 2008).
We also found that GFP-YabA appeared to be associated with the replisome. However, this
association was not dependent on DnaA, nor was it dependent on oriC. These findings are not
consistent with the interpretation that DnaA is required for the association of YabA with the
replisome (Noirot-Gros et al., 2006, Soufo et al., 2008, Cho et al., 2008). Previous analyses used
point mutations in yabA or dnaA that alter interactions between the two gene products (Noirot-
Gros et al., 2006, Soufo et al., 2008, Cho et al., 2008). These point mutations are known to
affect replication initiation (Noirot-Gros et al., 2006, Cho et al., 2008), which likely causes small
amounts of replication stress. The point mutations in yabA and dnaA could also have other
effects on those gene products.

The use of the heterologous origin of replication, oriN, allowed us to compare directly the
ability of GFP-YabA to form foci in cells with and without dnaA. We observed no difference,
indicating that neither oriC nor dnaA is required for association of YabA with the replisome. We
also found that after inhibition of replication elongation (replication fork arrest), several
replisome subunits were still present in foci, but that DnaN (β-clamp) and YabA were no longer
associated with these foci. These findings are consistent with previous results indicating that
association of YabA with the replisome requires interaction with DnaN (Noirot-Gros et al., 2006).

**YabA does not affect the activity of DnaA as a transcription factor**

Using strains in which *yabA* does not affect replication, we found no evidence that it affects the activity of DnaA or the ability of cells to respond to replication stress. In cells deleted for *oriC* and initiating replication from the DnaA-independent origin *oriN*, there was no significant effect of *yabA* on expression of genes known or thought to be regulated by DnaA during exponential growth. These findings are consistent with conclusions in a previous report that transcription of *dnaA* and association of DnaA with several chromosomal targets did not change significantly in a *yabA* null mutant (Cho et al., 2008). In addition, we found that genes known or thought to be regulated by DnaA still respond to replication stress in the absence of *yabA*. These results indicate that *yabA* is not required for modulating the activity of DnaA in response to disruptions in replication and are inconsistent with the model that YabA functions to tether DnaA at the replication fork and release it during replication stress (Soufo et al., 2008). Taken together, the simplest model for YabA function is that YabA acts locally at *oriC* to inhibit replication initiation. It is also formally possible that YabA affects DnaA globally, and that this effect is only manifest at *oriC* and does not alter the ability of DnaA to act as a transcription factor. Although we can not rule it out, we think this possibility is unlikely.

**Models for the function of YabA and its interactions with DnaA and DnaN**

YabA does not affect replication initiation from the DnaA-independent *oriN*, indicating that its function is specific to some aspect of *oriC* and/or DnaA. Since YabA does not affect DnaA in a distributive manner, that is, it does not appear to affect the ability of DnaA to function as a transcription factor, we favor models in which YabA functions at *oriC* to inhibit replication initiation.
It seems likely that there are at least three aspects to the ability of YabA to inhibit replication initiation from oriC without affecting the global activity of DnaA. First, YabA, although in bulk appears to be with the replication elongation machinery, must get to oriC. Second, it somehow inhibits replication initiation from oriC. Third, that inhibition is somehow relieved, or not complete, so that replication can initiate at the appropriate time in the cell cycle. Assuming that the interactions between YabA and both DnaA and DnaN are important for YabA function, then these interactions could be related to any of the three aspects of the ability of YabA to inhibit replication initiation.

There are many possible models that accommodate these three aspects of YabA function. For example, YabA could get to the origin via its interaction with DnaN, and then function to locally inhibit the activity of DnaA or some aspect of DnaA function at oriC. Versions of these types of models have been proposed (Cho et al., 2008, Hayashi et al., 2005). Missense mutations in yabA that cause altered interaction with either DnaA or DnaN cause phenotypes similar to those of a yabA null mutation (Noirot-Gros et al., 2006). If these mutant phenotypes are not due to general defects in YabA and are indicative of loss of YabA function, and are not due to secondary consequences of altered replication initiation, then the phenotypes appear to be most consistent with a model in which DnaN brings YabA to the oriC region, and then YabA inhibits a function of DnaA at oriC. For example, YabA might prevent the proper oligomerization or assembly of DnaA on oriC or prevent DnaA-mediated melting of oriC.

Alternatively, the interaction between YabA and DnaN could function to move YabA away from the oriC region and YabA could get to oriC through its interaction with DnaA. Once at oriC, YabA could regulate a step in replication initiation that is downstream of but not directly involving DnaA. YabA could also regulate some aspect of DnaA function that is required for the initiation of DNA replication, for example interaction between DnaA and DnaD (required to load
the replicative helicase) (Ishigo-Oka et al., 2001, Cho et al., 2008), formation of DnaA multimers (Mott et al., 2008), or another aspect of DnaA function that has not been yet appreciated. Both of these models predict that the effects of YabA would be oriC specific, but differ in what aspect of initiation is affected by YabA. It is not yet known how YabA inhibits replication initiation, but it seems to be by a mechanism different from those described for the various factors that regulate replication initiation in E. coli and its relatives {e.g., (Kaguni, 2006)}.

Experimental procedures

Growth media and culture conditions

For all experiments, cells were grown with vigorous shaking at 37°C in S7 defined minimal medium with MOPS (morpholinepropanesulfonic acid) buffer at a concentration of 50 mM rather than 100 mM (Jaacks et al., 1989). The medium was supplemented with 1% glucose, 0.1% glutamate, and required amino acids. In experiments utilizing expression from the xylose-inducible promoter PxylA, (PxylA-dnaN and PxylA-gfp-yabA), glucose was replaced with 1% arabinose and expression from PxylA was induced with 0.5% xylose. In experiments utilizing expression from the IPTG-inducible promoters Pspac(hy) or Pspank(hy), expression was induced with 1mM IPTG. Strains containing single crossover constructs were routinely grown in the appropriate antibiotic to maintain selection for the integrated plasmid. Standard concentrations of antibiotics were used (Harwood & Cutting, 1990). Where indicated, replication elongation was blocked by addition of HPUra (stock in 50mM KOH) to a final concentration of 38 µg/ml. Control cultures were mock treated with KOH. Samples were typically harvested 60 min after treatment with HPUra.
Strains and alleles

*B. subtilis* strains are listed in Table 1 and specific alleles are described below. Genetic manipulations were performed using standard protocols (Harwood & Cutting, 1990).

(ypjG-hepT)122 is a deviation in sequences of the ~24 kb chromosomal region from ~ypjG (201.4°) to ~hepT (203.5°) and was described previously (Berkmen & Grossman, 2007). Briefly, the *ypjG-hepT* region contains the tryptophan biosynthesis genes (*trpABFCDE*), and the (ypjG-hepT)122 variant likely encodes a heterologous tryptophan operon as strains containing it were transformed to tryptophan-prototropy.

Δ*yabA::cat* is a deletion-insertion that inactivates *yabA* by replacing it with *cat*. The allele was generated by the long-flanking homology PCR method (Wach, 1996). The deletion starts at the 1st codon (TTG) and ends 50bp downstream of the translational stop, removing a total of 407bp. The deletion stops 13bp upstream of the next gene, *yabB*. The *yabA* ORF is substituted with the 994bp chloramphenicol resistance cassette from pGEMcat. The cassette contains the *cat* ORF, 322bp upstream to include the promoter, and 20bp downstream of the stop codon, and does not include the transcriptional terminators. mRNA levels of *yabB* and other downstream genes in the *yabA::cat* mutant AIG109 were indistinguishable from those in wild type (*yabA*) cells as assessed by microarray analysis (data not shown).

*amyE::*{Pspank(hy)-yabA spe} is a fusion of *yabA* to the IPTG-inducible promoter Pspank(hy) at *amyE* and was used to overexpress *yabA*. AIG80 was constructed by cloning the entire ORF of *yabA* with its endogenous ribosome binding site into a plasmid containing the Pspank(hy) promoter (pDR66, a gift from David Rudner) thus generating plasmid pAIG10. The Pspank(hy)-*yabA* construct was integrated into the genome of JH642 through a double crossover at the *amyE* locus to generate strain AIG80.
spoIIIJ::\{oriN repN kan\} is an insertion of the plasmid origin of replication, oriN, along with the plasmid initiator gene repN, into the chromosome at spoIIIJ, near oriC (Goranov et al., 2005, Berkmen & Grossman, 2007). The presence of this replication origin allowed the inactivation of oriC.

oriC-S inactivates oriC replication functions by deleting ~150 bp of the sequence downstream of dnaA, including many essential DnaA binding sites and most of the AT-rich region that is normally unwound during replication initiation (Hassan et al., 1997, Kadoya et al., 2002, Berkmen & Grossman, 2007).

dnaN::\{PxylA-dnaN cat\} is a fusion of the only full copy of dnaN to the xylose-inducible promoter PxylA. This was constructed by amplifying a region of the genome of MMB26 that included PxylA and the 5’ end of dnaN. The amplified fragment was cloned into plasmid pGEMcat resulting in plasmid pAIG28. pAIG28 was integrated in the genome of JH642 by a single crossover to generate strain AIG260.

amyE::\{PxylA-dnaN cat\} is a fusion of PxylA to dnaN (encoding ß-clamp) at amyE (Goranov et al., 2005, Berkmen & Grossman, 2007) and was used to ectopically express dnaN.

amyE::\{PxylA-gfp-yabA cat\} expresses a fusion of GFP to the N-terminus of YabA under control of PxylA and integrated at amyE in the chromosome. The gfp-yabA construct was obtained by cloning the entire yabA ORF in frame with gfp in the pEA18 plasmid (Gueiros-Filho & Losick, 2002). The resulting plasmid (pAIG58) was integrated into the chromosome through double cross over at the amyE locus. {The GFP-YabA fusion protein was at least partly functional as it complemented phenotypic characteristics of a yabA null mutant (data not shown) (Noirot-Gros et al., 2006)}.

dnaA::\{Pspac(hy)-dnaN cat\} disrupts dnaA while inserting Pspac(hy) to drive expression of dnaN. A 450bp DNA fragment internal to dnaA was PCR amplified and cloned into the
Sph/HindIII cloning site of pJQ43 (Quisel et al., 2001) downstream of the Pspac-hy promoter to yield plasmid pAIG37. Integration of pAIG37 into the chromosome by a single crossover disrupts dnaA and places dnaN under the regulation of Pspac-hy. dnaA is normally essential, but it can be deleted in strains capable of initiating chromosomal replication from a heterologous origin such as oriN (Hassan et al., 1997, Berkmen & Grossman, 2007, Moriya et al., 1997, Kadoya et al., 2002). dnaA was in fact disrupted as evidenced by loss of detectable protein by Western blots and alterations in gene expression consistent with loss of DnaA (data not shown).

**DNA/protein ratio determination**

The ratio of DNA to protein was determined as previously described (Lee & Grossman, 2006, Kadoya et al., 2002). Briefly, 25ml of exponentially growing cells were collected at an OD600 ≤ 0.6. DNA and protein were extracted and the concentrations were determined using the diphenylamine reaction (DNA) and the Lowry BioRad DC Protein Assay Kit, with appropriate standards. The ratios for all strains were normalized to wild type (wt = 1.0) grown on the same day and under the same conditions. The average of three biological replicates is presented with error bars representing standard deviation.

**DNA microarrays**

DNA microarrays were prepared either using PCR products from >99% of the annotated B. subtilis open reading frames, or 65-mer oligonucleotide library representing all of the annotated ORFs in the B. subtilis genome (Sigma-Genosys) spotted onto Corning GAPS slides essentially as described previously (Goranov et al., 2005, Au et al., 2005, Britton et al., 2002, Auchtung et al., 2005). Culture samples were added to an equal volume of ice cold methanol and processed as described previously (Goranov et al., 2006).

**Use of microarrays to analyze DNA replication**. Chromosomal DNA was prepared essentially as described previously (Goranov et al., 2006, Wang et al., 2007b). Briefly, DNA
was extracted and purified by using G-100 QIAGEN genomic DNA purification columns, fragmented by digestion with HaeIII, and purified again with QIAGEN QiaQuick PCR purification columns. DNA was mixed with random hexamers and aminoallyl-dUTP was incorporated during primer extension reactions. DNA was then labeled with Cy3 or Cy5 fluorescent dyes. The amount of DNA from each open reading frame (spot on the microarray) for experimental samples was determined relative to that from a sample of reference DNA taken from cells in stationary phase (non-replicating). Experimental and reference DNA samples were coupled to Cy5 and Cy3 dyes respectively, mixed, and hybridized to a microarray as previously described. The ratios of experimental to reference samples for each chromosomal locus were then determined. Different experimental samples were then compared to each other using these normalized ratios. Microarray scanning, analysis, and normalization was preformed as previously described (Goranov et al., 2006, Wang et al., 2007b). The results presented are from a single representative experiment.

Use of microarrays for analysis of mRNA levels. Experimental samples of RNA were purified using RNEasy kits (Qiagen). A reference sample was made by pooling total RNA from cultures grown in defined minimal medium and cultures treated with DNA damaging agents, thus ensuring that all genes expressed under those conditions are represented in the reference sample. Experimental and reference RNA samples were mixed with Superscript II reverse transcriptase (Invitrogen), random hexamers, and aminoallyl-dUTP (Sigma) to make cDNA. The samples were then labeled by conjugation to monofunctional Cy3 or Cy5. The experimental and reference samples were mixed and hybridized to a DNA microarray. GenePix 3.0 (Axon Instruments) was used to analyze microarray images. We included every spot that has ≥70% of the pixels at least one standard deviation over background and has an overall median intensity at least threefold higher than the global background level in one or both Cy3 or Cy5 channels, and
was not flagged automatically as Not Found or manually as “bad” during gridding. Data were normalized to set the global median to unity after removal of excluded spots and intergenic regions. Analyses of mRNA levels were done with at least three independent biological replicate.

**Microscopy**

Microscopy was performed essentially as described (Lee *et al.*, 2003). Briefly, cells were placed on 1% agarose pads, and images were captured with a Nikon E800 microscope equipped with a Hamamatsu digital camera. Improvision OpenLabs 2.0 software was used to process images.

**Acknowledgements**

We thank C. Lee for measuring the effects of *yabA* on mRNA levels; T. Baker, S. P. Bell, F. Solomon, A. Wright, C. Lee, J. D. Wang, and J. Auchtung for useful suggestions and discussions, and C. Lee, H. Merrikh and M. Berkmen for comments on the manuscript. This work was supported, in part, by Public Health Service grant GM41934 to A.D.G. and NIH Kirschstein NRSA postdoctoral fellowship 5 F32 G-076950 to A.M.B.

**References**


Table 1. *B. subtilis* strains used.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Genotype</th>
</tr>
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<tbody>
<tr>
<td>BB987</td>
<td><em>trpC2 pheA1 amyE:</em>{Pspac-} cat (empty vector)</td>
</tr>
<tr>
<td>MMB26</td>
<td><em>trpC2 pheA1 amyE:</em>{PxylA-dnaN cat}</td>
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<td>MMB170</td>
<td><em>pheA1</em> (ypjG-heap)I22 spoIII::oriN repN kan oriC-S</td>
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<td>AIG80</td>
<td><em>trpC2 pheA1 amyE:</em>{Pspank(hy)-yabA spc}</td>
</tr>
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<td>AIG109</td>
<td><em>trpC2 pheA1 ΔyabA::cat</em></td>
</tr>
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<td>AIG185</td>
<td><em>pheA1</em> (ypjG-heap)I22 spoIII::oriN repN kan oriC-S ΔyabA::cat</td>
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<td>AIG208</td>
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<td>AIG245</td>
<td><em>trpC2 pheA1 ΔyabA::cat amyE:</em>{PxylA-dnaN cat::tet}</td>
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<td>AIG261</td>
<td><em>trpC2 pheA1 dnaN:</em>{PxylA-dnaN cat} (PAIG28)</td>
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<td>AIG278</td>
<td><em>trpC2 pheA1 spoIII::oriN repN kan</em> oriC-S amyE:*{PxylA-dnaN cat}</td>
</tr>
<tr>
<td>AIG371</td>
<td><em>trpC2 pheA1 dnaN:</em>{dnaN-gfp spc}</td>
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<td>AIG483</td>
<td><em>trpC2 pheA1 amyE:</em>{PxylA-gfp-yabA cat::mls} (PAIG58)</td>
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<td>AIG595</td>
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<td>KPL374</td>
<td><em>trpC2 pheA1 polC:</em>{polC-gfp spc}</td>
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<tr>
<td>KPL382</td>
<td><em>trpC2 pheA1 dnaX:</em>{dnaX-gfp spc}</td>
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Figure legends

Figure 1. Effects of YabA on DNA replication are oriC-specific. Strains were grown in minimal medium, and exponentially growing cells were collected for analysis of total DNA and protein. DNA to protein ratios are normalized to wild type (wt = 1). yabA overexpression, from Pspank(hy)-yabA, was induced by growing cells for 4 generations in the presence of 1mM IPTG.

A) oriC\(^+\) cells: yabA\(^+\) wild type (BB987); yabA null mutant (AIG109); overexpression of yabA (AIG80).

B) oriC mutant cells replicating from oriN: yabA\(^+\) (MMB170); yabA null mutant (AIG185); overexpression of yabA (AIG208).

Figure 2. GFP-YabA focus formation does not depend on DnaA and correlates with association of DnaN at replication foci.

Cells containing the indicated GFP fusions were grown in defined minimal medium with glucose, or with arabinose and xylose in the case of strains expressing GFP-YabA. Cultures were either untreated (A, C, E, G, I) or treated with HPUra to block replication elongation (B, D, F, H, J) and prepared for microscopy at indicated times after treatment.

A-B) GFP-YabA in a dnaA oriC null mutant (AIG593) 15 min after treatment with HPUra

C-D) GFP-YabA in dnaA\(^+\) oriC\(^+\) cells (AIG483) 15 min after treatment with HPUra

E-F) PolC-GFP (KPL374) 60 min after treatment with HPUra

G-H) DnaX-GFP (KPL382) 60 min after treatment with HPUra

I-J) DnaN(\(\beta\)-clamp)-GFP (AIG371) 15 min after treatment with HPUra
Figure 3. DnaN positively regulates DNA replication in an oriC-dependent manner.

A) DNA to protein ratios were measured as in Fig. 1. Where used, inducers were present for at least 4 generations before samples were collected. ↑dnaN: dnaN was overexpressed from PxyIA-dnaN (MMB26). ↑dnaX: dnaX was overexpressed from Pspank(hy)-dnaX (AIG573).

↓dnaN: the endogenous copy of dnaN was placed under control of PxyIA and cells were grown in arabinose without xylose to give only basal expression from PxyIA-dnaN (AIG261). oriN+C: (MMB170). oriN+C ↑dnaN: same as MMB170, but with dnaN overexpressed from PxyIA-dnaN (AIG278). yabA-: yabA null mutant (AIG109). yabA- ↑dnaN: yabA null mutant with PxyIA-dnaN (AIG245).

B-C) The effect of overproduction of β-clamp (DnaN) on initiation of replication was assessed with DNA microarrays. The log_2 of the relative abundance of chromosomal DNA 50 min (~1 generation) after addition of inducer is compared to uninduced samples and plotted as a function of the position on the chromosome. The position of the functional origin of replication is indicated by an arrow. dnaN mRNA levels (as determined by DNA microarrays) were similar in each of the strains used.

B) Cells replicating from the endogenous DnaA-dependent oriC (MMB26)

C) Cells replicating from oriN in the absence of a functional oriC (AIG278) (dnaA+)

Figure 4. Effects of yabA on gene expression in the absence and presence of HPUra.

Cells replicating from the DnaA-independent oriN in the absence of a functional oriC (and dnaA+), with and without yabA (MMB170 and AIG185, respectively) were grown to mid-exponential phase at 32°C, split, and treated with 38 µg/ml HPUra to block replication elongation, or mock-treated, for 60 min. Cells were harvested and RNA was purified, labeled, and mixed with a differently labeled reference RNA for normalization. RNAs from cells grown...
under several different conditions were pooled to make the reference (Goranov et al., 2005). The mixture of experimental and reference RNA was hybridized to whole genome DNA microarrays and fluorescence signals for each gene were determined. Data are presented as log₂ values on scatter plots of mRNA from yabA+ cells (vertical axis) versus mRNA from yabA null mutant (yabA-) cells (horizontal axis). Solid diagonal lines indicate the main y=x diagonal, and the dashed lines represent two-fold deviations. Points appearing near the main diagonal had very similar expression in the yabA+ and yabA- strains. Genes previously found to be regulated independently of recA and known or postulated to be directly controlled by DnaA are plotted as + and - symbols, with + indicating those whose expression increases and - indicating those whose expression decreases in response to HPURa and replication arrest (Goranov et al., 2005). All other genes are indicated as gray dots.

A, B) Gene expression in mock-treated exponentially growing cells (A) and in cells treated with HPURa for 60 min to arrest replication elongation (B). Values are relative to the pooled reference and are considered arbitrary, although very high or very low values indicate that mRNA from that gene is significantly different from the level in the pooled reference. The expression level of yabA, which was essentially undetected with a value below -6 in the yabA- strain, is circled. The inset includes the area from -1 to +1 on each axis with only the known and putative DnaA-regulated genes shown for clarity.

C) Change in gene expression between HPURa-treated and mock-treated cells. Changes in expression (+HPURa / -HPURa) are plotted as log₂ values for yabA+ and yabA- cells. A position of (0,0) indicates no change in either strain; genes that were induced in both strains appear in the upper right quadrant, and genes that were repressed in both strains appear in the lower left quadrant. That most genes fall on or near the line y=x indicates that there is little or no effect of yabA on the response to HPURa.
Figure 1

(A) onC+  
(B) onN+ onC-  

Relative DNA:Protein Ratio
Figure 2

-HPUra          +HPUra

A               B

\text{gfp-yabA}
\text{dnaA- oriC-}

C               D

\text{gfp-yabA}
\text{dnaA+ oriC+}

E               F

\text{polC-gfp}

G               H

\text{dnaX-gfp}

I               J

\text{dnaN-gfp}