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

Citation: Goranov, A. I., Breier, A. M., Merrikh, H. and Grossman, A. D. (2009), YabA of Bacillus subtilis controls DnaA-mediated replication initiation but not the transcriptional response to replication stress. Molecular Microbiology, 74: 454–466. doi: 10.1111/j.1365-2958.2009.06876.x

As Published: http://dx.doi.org/10.1111/j.1365-2958.2009.06876.x

Publisher: Blackwell Publishing

Persistent URL: http://hdl.handle.net/1721.1/59329

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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

Alexi I. Goranov, Adam M. Breier, and Alan D. Grossman*

Department of Biology

Building 68-530

Massachusetts Institute of Technology

Cambridge, MA 02139

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

running title: YabA and DnaA of *B. subtilis*

*Corresponding author:

phone: 617-253-1515

fax: 617-253-2643

E-mail: adg@mit.edu

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* {e.g., (Zakrzewska-Czerwinska et al., 2007)}.

Replication initiation in *B. subtilis*, and presumably other Gram-positive bacteria, is regulated, in part, by YabA (Noirot-Gros *et al.*, 2002, Noirot-Gros *et al.*, 2006, Hayashi et al., 2005, Cho *et al.*, 2008, Soufo *et al.*, 2008). *B. subtilis* YabA is a negative regulator of replication initiation. *yabA* null mutations cause increased and asynchronous replication (Noirot-Gros et al., 2002, Hayashi et al., 2005), and overexpression of *yabA* causes decreased replication (Hayashi et al., 2005). GFP-YabA forms foci within the cell and the positions of these foci correspond with those of the replication machinery (Noirot-Gros et al., 2002, Hayashi et al., 2005, Cho et al., 2008). YabA interacts with DnaA and the β-clamp (DnaN) of DNA polymerase and these interactions are thought to be important for YabA function and localization (Noirot-Gros et al., 2002, Noirot-Gros et al., 2006, Cho et al., 2008). In addition, YabA seems to be required for association of DnaA with the replication fork (Soufo et al., 2008). Because of these interactions and its subcellular location, it is thought that YabA regulates the activity of DnaA in response to replication status (Noirot-Gros et al., 2002, Noirot-Gros et al., 2006, Hayashi et al., 2005, Cho et al., 2008, Soufo et al., 2008).

In addition to its essential role in replication initiation, DnaA is also a transcription factor. In *B. subtilis*, replication stress causes multiple changes in transcription, and DnaA mediates many of these changes independently of the *recA*-dependent SOS response (Goranov *et al.*, 2005, Burkholder *et al.*, 2001). Genes controlled by DnaA are involved in many processes, including: replication, development, metabolism, and cell division (Messer & Weigel, 1997, Goranov et al., 2005, Collier *et al.*, 2006, Gon *et al.*, 2006, Burkholder et al., 2001, Wang & Kaguni, 1987, Atlung *et al.*, 1985, Braun *et al.*, 1985, Kucherer *et al.*, 1986, Ogura et al., 2001, Breier & Grossman, 2009). As is the case for replication initiation, it is thought that DnaA-ATP is the

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 ß-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 ß-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 (ß-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

133 also found to prevent formation of foci of GFP-YabA (Cho et al., 2008), consistent with the prior
134 findings. However, loss of interaction between YabA and DnaA is not the only effect of these
135 mutations. The mutant cells also over-initiate replication (Noirot-Gros et al., 2006, Cho et al.,
136 2008) and there could be other effects on YabA and DnaA as well. Therefore, it is possible that
137 the YabA:DnaA interaction may not actually be required for focus formation by GFP-YabA and
138 that the effects of the mutations on foci of GFP-YabA are indirect.

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

145 Maintenance of GFP-YabA foci was dependent on ongoing replication. We monitored GFP-
146 YabA foci in cells treated with HPUra, an inhibitor of DNA Polymerase III that blocks
147 replication elongation (Neville & Brown, 1972, Brown, 1970). Within 15 min of addition of
148 HPUra to cells, foci of GFP-YabA disappeared from >99% of cells in both the *dnaA* null mutant
149 initiating from *oriN* (Fig. 2B) and *dnaA*$^{+}$ cells initiating from *oriC* (compare Figs. 2C and 2D).

150 Loss of GFP-YabA foci correlated with loss of DnaN-GFP foci. We monitored the effects of
151 replication arrest on foci of the ß-clamp (DnaN-GFP), the catalytic subunit of DNA polymerase
152 (PolC-GFP), the HolB subunit of the clamp loader (HolB-GFP), and the clamp loader/τ-subunit
153 (DnaX-GFP). Foci of PolC-GFP (Fig. 2E, F), DnaX-GFP (Fig. 2G, H), and HolB-GFP (data not
154 shown) persisted for greater than 60 min after replication arrest. In contrast, foci of the β–clamp
155 (DnaN-GFP) disappeared from >98% of cells within 15 min after addition of HPUra (Fig. 2I, J).
156 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 ß-clamp, we tested whether alterations in expression of *dnaN* (ß-clamp) might also modulate replication initiation. We found that the ß-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 ß-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 ß-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 {(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 ß-clamp stimulates DnaA-dependent replication initiation from *oriC*

Since *dnaN* encodes the ß-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 ß-clamp on replication elongation, or due to an ability of ß-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 (Khodursky *et al.*, 2000, Simmons *et al.*, 2004, Wang *et al.*, 2007b, 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 {e.g., (Simmons et al., 2004)}.

The increase in replication initiation caused by overproduction of the ß-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* (ß-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 ß-clamp appear to the affect 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 ß-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

229 inhibited (Breier & Grossman, 2009). YabA could be required to couple the activity of DnaA to
230 replication elongation, perhaps by sequestering DnaA at active replication forks as previously
231 proposed (Noirot-Gros et al., 2006, Soufo et al., 2008). If this is the case, then the DnaA-
232 mediated transcriptional response to replication arrest should be severely compromised in a *yabA*
233 null mutant. Furthermore, expression of DnaA-regulated genes might be different in the absence
234 of *yabA*, even during ongoing replication.

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

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

251 Using the data from Figures 4A and 4B, we compared the fold change in mRNA levels
252 caused by replication arrest (treatment with HPUra) in *yabA*$^+$ cells to that in the *yabA* null mutant

253 (Fig. 4C). Expression of many genes increases in response to HPUra treatment and many of
254 these are part of the SOS regulon and depend on *recA* (Goranov et al., 2005, Goranov et al.,
255 2006). The changes in expression of these genes were similar in the *yabA*$^+$ and the *yabA* null
256 mutant cells (Fig 4C). In addition, most of the genes known or thought to be directly regulated
257 by DnaA (+'s and -'s in Fig. 4) also changed expression in response to HPUra, and those
258 changes were similar in the *yabA*$^+$ and *yabA* null mutant cells (Fig. 4C).
259 The magnitudes of the changes in gene expression in response to replication arrest in both
260 *yabA*+ and *yabA* null mutant cells initiating replication from *oriN* were somewhat less than those
261 previously reported for cells initiating replication from *oriC* (Goranov et al., 2005, Goranov et
262 al., 2006). In fact, there were many differences in gene expression simply comparing cells
263 initiating replication from *oriC* to those initiating from *oriN* in the absence of added replication
264 stress (unpublished results). These differences indicate that the apparently constitutive and
265 asynchronous replication initiation from *oriN* (Noirot-Gros et al., 2002, Hayashi et al., 2005)
266 might cause a small amount of replication stress. Since the basal expression of some of the
267 genes is already changed in the *oriN* cells, the magnitude of the effect of replication arrest is
268 muted and less dramatic in these cells compared to cells initiating from *oriC*. Nevertheless, for
269 many DnaA-regulated genes, there was a significant response to replication stress and there was
270 little or no effect of *yabA* on this response.
271
272 **Discussion**
273 Most of the studies on control of bacterial replication initiation have focused on *E. coli* and
274 its close relatives. However, many of the non-essential regulators characterized in *E. coli* are not
275 found in other organisms. YabA is one of the best characterized non-essential regulators of
276 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 (ß-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

301 required to modulate the activity of DnaA in response to replication stress and indicate that
302 YabA does not affect DnaA in a distributive manner. These findings are not consistent with
303 some of the previously proposed models for YabA function.

304 **YabA does not affect replication initiation from the DnaA-independent origin of**
305 **replication *oriN***

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

315 We found that neither a null mutation in nor overexpression of *yabA* affected the activity of
316 *oriN*. These findings indicate that the function of YabA in the negative control of replication
317 initiation is specific to the DnaA-dependent *oriC*. Like the effects of a *yabA* null mutation, we
318 found that overexpression of *dnaN* stimulates replication initiation from *oriC*, but not from *oriN*.
319 We suspect that this stimulation by increased production of DnaN is likely due to titration of
320 YabA away from *oriC*. Since the essential chromosomal replication initiation proteins DnaB
321 DnaD and the replicative helicase DnaC and the clamp DnaN are required for replication from
322 *oriN* (Hassan et al., 1997), these results indicate that YabA is not likely to affect the activity of
323 these other proteins, at least in the absence of DnaA. These findings also made it feasible to
324 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 (ß-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

397 the replicative helicase) (Ishigo-Oka *et al.*, 2001, Cho et al., 2008), formation of DnaA multimers
398 (Mott *et al.*, 2008), or another aspect of DnaA function that has not been yet appreciated. Both
399 of these models predict that the effects of YabA would be *oriC* specific, but differ in what aspect
400 of initiation is affected by YabA. It is not yet known how YabA inhibits replication initiation,
401 but it seems to be by a mechanism different from those described for the various factors that
402 regulate replication initiation in *E. coli* and its relatives {e.g., (Kaguni, 2006)}.
403
404 **Experimental procedures**
405 **Growth media and culture conditions**
406 For all experiments, cells were grown with vigorous shaking at 37°C in S7 defined minimal
407 medium with MOPS (morpholinepropanesulfonic acid) buffer at a concentration of 50 mM rather
408 than 100 mM (Jaacks *et al.*, 1989). The medium was supplemented with 1% glucose, 0.1%
409 glutamate, and required amino acids. In experiments utilizing expression from the xylose-
410 inducible promoter PxylA, (PxylA-*dnaN* and PxylA-*gfp-yabA*), glucose was replaced with 1%
411 arabinose and expression from PxylA was induced with 0.5% xylose. In experiments utilizing
412 expression from the IPTG-inducible promoters Pspac(hy) or Pspank(hy), expression was induced
413 with 1mM IPTG. Strains containing single crossover constructs were routinely grown in the
414 appropriate antibiotic to maintain selection for the integrated plasmid. Standard concentrations
415 of antibiotics were used (Harwood & Cutting, 1990). Where indicated, replication elongation
416 was blocked by addition of HPUra (stock in 50mM KOH) to a final concentration of 38 μg/ml.
417 Control cultures were mock treated with KOH. Samples were typically harvested 60 min after
418 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 spc*} 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*I/*Hin*dIII 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

514 was not flagged automatically as Not Found or manually as "bad" during gridding. Data were
515 normalized to set the global median to unity after removal of excluded spots and intergenic
516 regions. Analyses of mRNA levels were done with at least three independent biological
517 replicate.

518 **Microscopy**

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

523

524 **Acknowledgements**

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

530

531 **References**

532 Atlung, T., E. S. Clausen & F. G. Hansen, (1985) Autoregulation of the dnaA gene of
533 Escherichia coli K12. *Mol Gen Genet* **200**: 442-450.
534 Au, N., E. Kuester-Schoeck, V. Mandava, L. E. Bothwell, S. P. Canny, K. Chachu, S. A.
535 Colavito, S. N. Fuller, E. S. Groban, L. A. Hensley, T. C. O'Brien, A. Shah, J. T. Tierney, L. L.
536 Tomm, T. M. O'Gara, A. I. Goranov, A. D. Grossman & C. M. Lovett, (2005) Genetic
537 composition of the Bacillus subtilis SOS system. *J Bacteriol* **187**: 7655-7666.
538 Auchtung, J. M., C. A. Lee, R. E. Monson, A. P. Lehman & A. D. Grossman, (2005) Regulation
539 of a *Bacillus subtilis* mobile genetic element by intercellular signaling and the global DNA
540 damage response. *Proc Natl Acad Sci U S A* **102**: 12554-12559.
541 Berkmen, M. B. & A. D. Grossman, (2007) Subcellular positioning of the origin region of the
542 Bacillus subtilis chromosome is independent of sequences within oriC, the site of replication
543 initiation, and the replication initiator DnaA. *Mol Microbiol* **63**: 150-165.

544 Braun, R. E., K. O'Day & A. Wright, (1985) Autoregulation of the DNA replication gene dnaA
545 in E. coli K-12. *Cell* **40**: 159-169.
546 Breier, A. M. & A. D. Grossman, (2009) Dynamic association of the replication initiator and
547 transcription factor DnaA with the Bacillus subtilis chromosome during replication stress. *J*
548 *Bacteriol* **191**: 486-493.
549 Britton, R. A., P. Eichenberger, J. E. Gonzalez-Pastor, P. Fawcett, R. Monson, R. Losick & A. D.
550 Grossman, (2002) Genome-wide analysis of the stationary-phase sigma factor (sigma-H)
551 regulon of *Bacillus subtilis*. *J Bacteriol* **184**: 4881-4890.
552 Brown, N. C., (1970) 6-(p-hydroxyphenylazo)-uracil: a selective inhibitor of host DNA
553 replication in phage-infected Bacillus subtilis. *Proc Natl Acad Sci U S A* **67**: 1454-1461.
554 Burkholder, W. F., I. Kurtser & A. D. Grossman, (2001) Replication initiation proteins regulate a
555 developmental checkpoint in Bacillus subtilis. *Cell* **104**: 269-279.
556 Cho, E., N. Ogasawara & S. Ishikawa, (2008) The functional analysis of YabA, which interacts
557 with DnaA and regulates initiation of chromosome replication in Bacillus subtils. *Genes Genet*
558 *Syst* **83**: 111-125.
559 Collier, J., S. R. Murray & L. Shapiro, (2006) DnaA couples DNA replication and the expression
560 of two cell cycle master regulators. *EMBO J* **25**: 346-356.
561 Gon, S., J. E. Camara, H. K. Klungsoyr, E. Crooke, K. Skarstad & J. Beckwith, (2006) A novel
562 regulatory mechanism couples deoxyribonucleotide synthesis and DNA replication in
563 Escherichia coli. *Embo J* **25**: 1137-1147.
564 Goranov, A. I., L. Katz, A. M. Breier, C. B. Burge & A. D. Grossman, (2005) A transcriptional
565 response to replication status mediated by the conserved bacterial replication protein DnaA.
566 *Proc Natl Acad Sci U S A* **102**: 12932-12937.
567 Goranov, A. I., E. Kuester-Schoeck, J. D. Wang & A. D. Grossman, (2006) Characterization of
568 the global transcriptional responses to different types of DNA damage and disruption of
569 replication in Bacillus subtilis. *J Bacteriol* **188**: 5595-5605.
570 Gueiros-Filho, F. J. & R. Losick, (2002) A widely conserved bacterial cell division protein that
571 promotes assembly of the tubulin-like protein FtsZ. *Genes Dev* **16**: 2544-2556.
572 Harwood, C. R. & S. M. Cutting, (1990) *Molecular biological methods for Bacillus*. John Wiley
573 & Sons, Chichester, England.
574 Hassan, A. K., S. Moriya, M. Ogura, T. Tanaka, F. Kawamura & N. Ogasawara, (1997)
575 Suppression of initiation defects of chromosome replication in Bacillus subtilis dnaA and oriC-
576 deleted mutants by integration of a plasmid replicon into the chromosomes. *J Bacteriol* **179**:
577 2494-2502.
578 Hayashi, M., Y. Ogura, E. J. Harry, N. Ogasawara & S. Moriya, (2005) Bacillus subtilis YabA is
579 involved in determining the timing and synchrony of replication initiation. *FEMS Microbiol*
580 *Lett* **247**: 73-79.
581 Ishida, T., N. Akimitsu, T. Kashioka, M. Hatano, T. Kubota, Y. Ogata, K. Sekimizu & T.
582 Katayama, (2004) DiaA, a novel DnaA-binding protein, ensures the timely initiation of
583 Escherichia coli chromosome replication. *J Biol Chem* **279**: 45546-45555.
584 Ishigo-Oka, D., N. Ogasawara & S. Moriya, (2001) DnaD protein of Bacillus subtilis interacts
585 with DnaA, the initiator protein of replication. *J Bacteriol* **183**: 2148-2150.
586 Ishikawa, S., Y. Ogura, M. Yoshimura, H. Okumura, E. Cho, Y. Kawai, K. Kurokawa, T.
587 Oshima & N. Ogasawara, (2007) Distribution of Stable DnaA-Binding Sites on the Bacillus
588 Subtilis Genome Detected using a Modified ChIP-chip Method. *DNA Res* **14**: 155-168.
589 Jaacks, K. J., J. Healy, R. Losick & A. D. Grossman, (1989) Identification and characterization
590 of genes controlled by the sporulation-regulatory gene spo0H in Bacillus subtilis. *J Bacteriol*
591 **171**: 4121-4129.

592 Kadoya, R., A. K. Hassan, Y. Kasahara, N. Ogasawara & S. Moriya, (2002) Two separate DNA
593 sequences within oriC participate in accurate chromosome segregation in Bacillus subtilis. *Mol*
594 *Microbiol* **45**: 73-87.
595 Kaguni, J. M., (2006) DnaA: controlling the initiation of bacterial DNA replication and more.
596 *Annu Rev Microbiol* **60**: 351-375.
597 Kato, J. & T. Katayama, (2001) Hda, a novel DnaA-related protein, regulates the replication
598 cycle in Escherichia coli. *Embo J* **20**: 4253-4262.
599 Khodursky, A. B., B. J. Peter, M. B. Schmid, J. DeRisi, D. Botstein, P. O. Brown & N. R.
600 Cozzarelli, (2000) Analysis of topoisomerase function in bacterial replication fork movement:
601 use of DNA microarrays. *Proc Natl Acad Sci U S A* **97**: 9419-9424.
602 Kucherer, C., H. Lother, R. Kolling, M. A. Schauzu & W. Messer, (1986) Regulation of
603 transcription of the chromosomal dnaA gene of Escherichia coli. *Mol Gen Genet* **205**: 115-121.
604 Kurokawa, K., S. Nishida, A. Emoto, K. Sekimizu & T. Katayama, (1999) Replication cycle-
605 coordinated change of the adenine nucleotide-bound forms of DnaA protein in Escherichia
606 coli. *Embo J* **18**: 6642-6652.
607 Lee, P. S. & A. D. Grossman, (2006) The chromosome partitioning proteins Soj (ParA) and
608 Spo0J (ParB) contribute to accurate chromosome partitioning, separation of replicated sister
609 origins, and regulation of replication initiation in Bacillus subtilis. *Mol Microbiol* **60**: 853-869.
610 Lee, P. S., D. C. Lin, S. Moriya & A. D. Grossman, (2003) Effects of the chromosome
611 partitioning protein Spo0J (ParB) on oriC positioning and replication initiation in Bacillus
612 subtilis. *J Bacteriol* **185**: 1326-1337.
613 Messer, W., (2002) The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode
614 to initiate DNA replication. *FEMS Microbiol Rev* **26**: 355-374.
615 Messer, W., F. Blaesing, D. Jakimowicz, M. Krause, J. Majka, J. Nardmann, S. Schaper, H.
616 Seitz, C. Speck, C. Weigel, G. Wegrzyn, M. Welzeck & J. Zakrzewska-Czerwinska, (2001)
617 Bacterial replication initiator DnaA. Rules for DnaA binding and roles of DnaA in origin
618 unwinding and helicase loading. *Biochimie* **83**: 5-12.
619 Messer, W. & C. Weigel, (1997) DnaA initiator--also a transcription factor. *Mol Microbiol* **24**: 1-
620 6.
621 Moriya, S., A. K. Hassan, R. Kadoya & N. Ogasawara, (1997) Mechanism of anucleate cell
622 production in the oriC-deleted mutants of Bacillus subtilis. *DNA Res* **4**: 115-126.
623 Mott, M. L. & J. M. Berger, (2007) DNA replication initiation: mechanisms and regulation in
624 bacteria. *Nat Rev Microbiol* **5**: 343-354.
625 Mott, M. L., J. P. Erzberger, M. M. Coons & J. M. Berger, (2008) Structural synergy and
626 molecular crosstalk between bacterial helicase loaders and replication initiators. *Cell* **135**: 623-
627 634.
628 Neville, M. M. & N. C. Brown, (1972) Inhibition of a discrete bacterial DNA polymerase by 6-
629 (p-hydroxyphenylazo)-uracil and 6-(p-hydroxyphenylazo-)-isocytosine. *Nat New Biol* **240**: 80-
630 82.
631 Nievera, C., J. J. Torgue, J. E. Grimwade & A. C. Leonard, (2006) SeqA blocking of DnaA-oriC
632 interactions ensures staged assembly of the E. coli pre-RC. *Mol Cell* **24**: 581-592.
633 Nishida, S., K. Fujimitsu, K. Sekimizu, T. Ohmura, T. Ueda & T. Katayama, (2002) A
634 nucleotide switch in the Escherichia coli DnaA protein initiates chromosomal replication:
635 evidnece from a mutant DnaA protein defective in regulatory ATP hydrolysis in vitro and in
636 vivo. *J Biol Chem* **277**: 14986-14995.
637 Noirot-Gros, M. F., E. Dervyn, L. J. Wu, P. Mervelet, J. Errington, S. D. Ehrlich & P. Noirot,
638 (2002) An expanded view of bacterial DNA replication. *Proc Natl Acad Sci U S A* **99**: 8342-
639 8347.

Noirot-Gros, M. F., M. Velten, M. Yoshimura, S. McGovern, T. Morimoto, S. D. Ehrlich, N. Ogasawara, P. Polard & P. Noirot, (2006) Functional dissection of YabA, a negative regulator of DNA replication initiation in Bacillus subtilis. *Proc Natl Acad Sci U S A* **103**: 2368-2373.

Ogura, Y., Y. Imai, N. Ogasawara & S. Moriya, (2001) Autoregulation of the dnaA-dnaN operon and effects of DnaA protein levels on replication initiation in Bacillus subtilis. *J Bacteriol* **183**: 3833-3841.

Perego, M., G. B. Spiegelman & J. A. Hoch, (1988) Structure of the gene for the transition state regulator, abrB: regulator synthesis is controlled by the spo0A sporulation gene in Bacillus subtilis. *Mol Microbiol* **2**: 689-699.

Quisel, J. D., W. F. Burkholder & A. D. Grossman, (2001) In vivo effects of sporulation kinases on mutant Spo0A proteins in Bacillus subtilis. *J Bacteriol* **183**: 6573-6578.

Sekimizu, K., D. Bramhill & A. Kornberg, (1987) ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the E. coli chromosome. *Cell* **50**: 259-265.

Simmons, L. A., A. M. Breier, N. R. Cozzarelli & J. M. Kaguni, (2004) Hyperinitiation of DNA replication in Escherichia coli leads to replication fork collapse and inviability. *Mol Microbiol* **51**: 349-358.

Soufo, C. D., H. J. Soufo, M. F. Noirot-Gros, A. Steindorf, P. Noirot & P. L. Graumann, (2008) Cell-cycle-dependent spatial sequestration of the DnaA replication initiator protein in Bacillus subtilis. *Dev Cell* **15**: 935-941.

Wach, A., (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in S. cerevisiae. *Yeast* **12**: 259-265.

Wang, J. D., M. B. Berkmen & A. D. Grossman, (2007a) Genome-wide coorientation of replication and transcription reduces adverse effects on replication in Bacillus subtilis. *Proc Natl Acad Sci U S A* **104**: 5608-5613.

Wang, J. D., G. M. Sanders & A. D. Grossman, (2007b) Nutritional Control of Elongation of DNA Replication by (p)ppGpp. *Cell* **128**: 865-875.

Wang, Q. P. & J. M. Kaguni, (1987) Transcriptional repression of the dnaA gene of Escherichia coli by dnaA protein. *Mol Gen Genet* **209**: 518-525.

Yoshikawa, H. & N. Ogasawara, (1991) Structure and function of DnaA and the DnaA-box in eubacteria: evolutionary relationships of bacterial replication origins. *Mol Microbiol* **5**: 2589-2597.

Zakrzewska-Czerwinska, J., D. Jakimowicz, A. Zawilak-Pawlik & W. Messer, (2007) Regulation of the initiation of chromosomal replication in bacteria. *FEMS Microbiol Rev* **31**: 378-387.

676 **Table 1. *B. subtilis* strains used.**

Strains	Relevant Genotype
JH642	*trpC2 pheA1* (Perego *et al.*, 1988)
BB987	*trpC2 pheA1 amyE::{Pspac-() cat}* (empty vector)
MMB26	*trpC2 pheA1 amyE::{PxylA-dnaN cat}*
MMB170	*pheA1 (ypjG-hepT)122 spoIIIJ::{oriN repN kan} oriC-S*
AIG80	*trpC2 pheA1 amyE::{Pspank(hy)-yabA spc}*
AIG109	*trpC2 pheA1 ΔyabA::cat*
AIG185	*pheA1 (ypjG-hepT)122 spoIIIJ::{oriN repN kan} oriC-S ΔyabA::cat*
AIG208	*pheA1 (ypjG-hepT)122 spoIIIJ::{oriN repN kan} oriC-S amyE::{Pspank(hy)-yabA spc}*
AIG245	*trpC2 pheA1 ΔyabA::cat amyE::{PxylA-dnaN cat::tet}*
AIG261	*trpC2 pheA1 dnaN::{PxylA-dnaN cat}* (pAIG28)
AIG278	*trpC2 pheA1 spoIIIJ::{oriN repN kan} oriC-S amyE::{PxylA-dnaN cat}*
AIG371	*trpC2 pheA1 dnaN::{dnaN-gfp spc}*
AIG483	*trpC2 pheA1 amyE::{PxylA-gfp-yabA cat::mls}* (pAIG58)
AIG505	*pheA1 (ypjG-hepT)122 spoIIIJ::{oriN repN kan} oriC-S amyE::{PxylA-gfp-yabA cat}*
AIG573	*trpC1 pheA1 dnaX::{*Pspank(hy)*-dnaX spc}* (pAIG66)
AIG593	*pheA1 (ypjG-hepT)122 spoIIIJ::{oriN repN kan} oriC-S amyE::{PxylA-gfp-yabA cat::mls} dnaA::{Pspac(hy)-dnaN cat}* (*dnaA* null)
AIG595	*pheA1 (ypjG-hepT)122 spoIIIJ::{oriN repN kan} oriC-S amyE::{PxylA-gfp-yabA cat::mls} dnaA::{Pspac(hy)-dnaA-dnaN cat}*
KPL374	*trpC2 pheA1 polC::{polC-gfp spc}*
KPL382	*trpC2 pheA1 dnaX::{dnaX-gfp spc}*

677
678
679

679 **Figure legends**

680 **Figure 1. Effects of YabA on DNA replication are *oriC*-specific.** Strains were grown in
681 minimal medium, and exponentially growing cells were collected for analysis of total DNA and
682 protein. DNA to protein ratios are normalized to wild type (wt = 1). *yabA* overexpression, from
683 Pspank(hy)-*yabA*, was induced by growing cells for 4 generations in the presence of 1mM IPTG.
684 A) *oriC*$^{+}$ cells: *yabA*$^{+}$ wild type (BB987); *yabA* null mutant (AIG109); overexpression of
685 *yabA* (AIG80).
686 B) *oriC* mutant cells replicating from *oriN*: *yabA*$^{+}$ (MMB170); *yabA* null mutant (AIG185);
687 overexpression of *yabA* (AIG208).
688
689 **Figure 2. GFP-YabA focus formation does not depend on DnaA and correlates with**
690 **association of DnaN at replication foci.**
691 Cells containing the indicated GFP fusions were grown in defined minimal medium with
692 glucose, or with arabinose and xylose in the case of strains expressing GFP-YabA. Cultures were
693 either untreated (A, C, E, G, I) or treated with HPUra to block replication elongation (B, D, F, H,
694 J) and prepared for microscopy at indicated times after treatment.
695 A-B) GFP-YabA in a *dnaA oriC* null mutant (AIG593) 15 min after treatment with HPUra
696 C-D) GFP-YabA in *dnaA*$^{+}$ *oriC*$^{+}$ cells (AIG483) 15 min after treatment with HPUra
697 E-F) PolC-GFP (KPL374) 60 min after treatment with HPUra
698 G-H) DnaX-GFP (KPL382) 60 min after treatment with HPUra
699 I-J) DnaN(ß-clamp)-GFP (AIG371) 15 min after treatment with HPUra
700

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 PxylA-*dnaN* (MMB26). ↑*dnaX*: *dnaX* was overexpressed from Pspank(hy)-*dnaX* (**AIG573**). ↓*dnaN*: the endogenous copy of *dnaN* was placed under control of PxylA and cells were grown in arabinose without xylose to give only basal expression from PxylA-*dnaN* (AIG261). *oriN*$^{+}$*C*$^{-}$: (MMB170). *oriN*$^{+}$*C*$^{-}$ ↑*dnaN*: same as MMB170, but with *dnaN* overexpressed from PxylA-*dnaN* (AIG278). *yabA*-: *yabA* null mutant (AIG109). *yabA*- ↑*dnaN*: *yabA* null mutant with PxylA-*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.

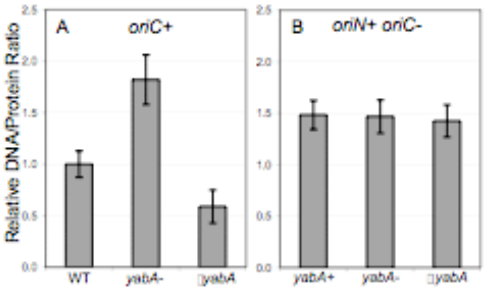
Cells replicating from the DnaA-independent *oriN* in the absence of a functional *oriC* (and *dnaA*$^{+}$), with and without *yabA* (MMB170 and AIG185, respectively) were grown to mid-exponential phase at 32°C, split, and treated with 38 μg/ml HPUra to block replication elongation, or mock-treated, 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 $\log_2$ 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.

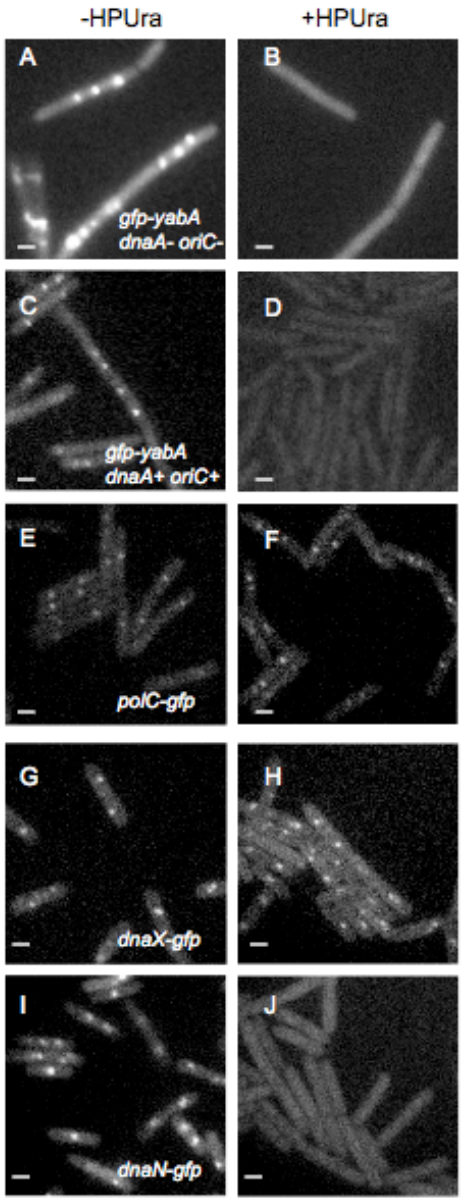
C) Change in gene expression between HPUra-treated and mock-treated cells. Changes in expression (+HPUra / -HPUra) are plotted as $\log_2$ values for yabA+ and yabA- cells. A position of (0,0) indicates no change in either strain; genes that were induced in both strains appear in the upper right quadrant, 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

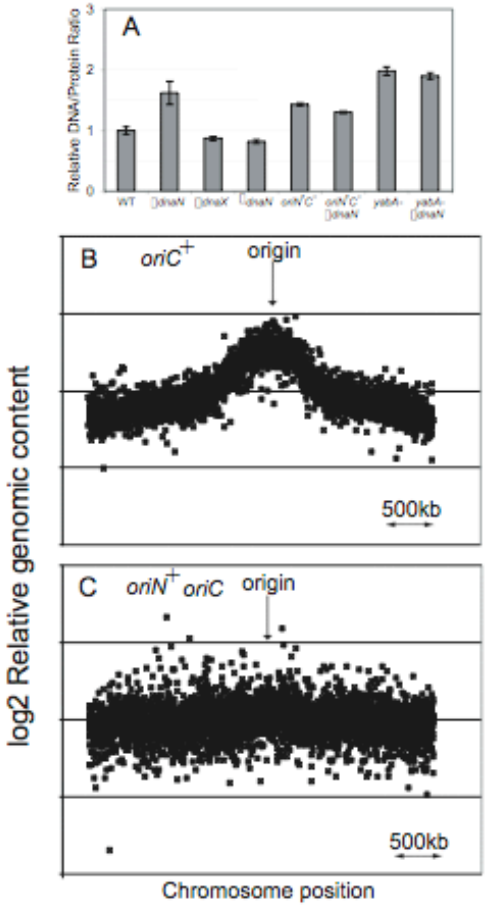

A
oriC+
B
oriN+ oriC-
Relative DNA/Protein Ratio
2.5
2.0
1.5
1.0
0.5
0.0
WT
yabA-
□yabA
yabA+
yabA-
□yabA

749

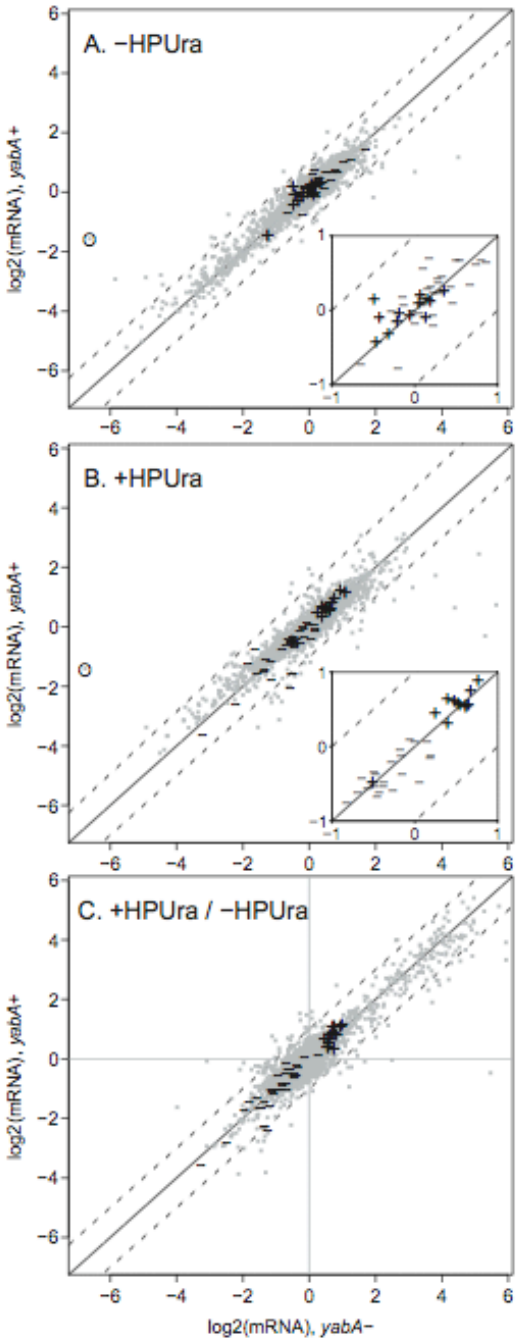
Figure 2


-HPUra
+HPUra
A
gfp-yabA
dnaA- oriC-
B
C
gfp-yabA
dnaA+ oriC+
D
E
polC-gfp
F
G
dnaX-gfp
H
I
dnaN-gfp
J

750


A
Relative DNA/Protein Ratio
0
1
2
3
WT
ΔdnaN
ΔdnaX
ΔdnaN
oriN'C'
oriN'C' ΔdnaN
yabA-
yabA- ΔdnaN
B
oriC+
origin
500kb
C
oriN+ oriC
origin
500kb
log2 Relative genomic content
Chromosome position

751



752