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

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

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21

21        **Summary**

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

36

37

## 37 Introduction

38 Bacteria use multiple mechanisms to regulate the initiation of replication and to alter gene  
39 expression in response to changes in replication status. The chromosomal origin of replication,  
40 *oriC*, and the replication initiation protein DnaA are key targets for controlling replication  
41 initiation in bacteria {(Kaguni, 2006, Zakrzewska-Czerwinska *et al.*, 2007, Mott & Berger,  
42 2007), and references therein}. DnaA is highly conserved (Messer, 2002). It is a member of the  
43 AAA+ family of proteins, binds ATP or ADP, and has a weak ATPase activity. In *E. coli*, and  
44 presumably other bacteria, DnaA is active for replication initiation only when in the ATP-bound  
45 form (Sekimizu *et al.*, 1987). During replication initiation, DnaA binds to sequences in *oriC* and  
46 can cause melting of a portion of *oriC* to generate ssDNA. The ssDNA serves as an assembly  
47 region for the replication machinery {reviewed in (Kaguni, 2006, Mott & Berger, 2007,  
48 Zakrzewska-Czerwinska *et al.*, 2007, Messer, 2002, Messer *et al.*, 2001)}.

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

56 Replication initiation in *Bacillus subtilis* is also highly regulated and DnaA is part of this  
57 regulation (Yoshikawa & Ogasawara, 1991, Ogura *et al.*, 2001, Hayashi *et al.*, 2005). As in *E.*  
58 *coli*, transcription of *dnaA* is auto-repressed and overexpression of *dnaA* causes excessive  
59 replication initiation and reduces the size of cells at the time of initiation (Ogura *et al.*, 2001).  
60 However, *B. subtilis* does not contain homologs of any of the well-characterized proteins known

61 to modulate replication initiation and DnaA activity in *E. coli* {e.g., (Zakrzewska-Czerwinska et  
62 al., 2007)}.

63 Replication initiation in *B. subtilis*, and presumably other Gram-positive bacteria, is  
64 regulated, in part, by YabA (Noirot-Gros *et al.*, 2002, Noirot-Gros *et al.*, 2006, Hayashi *et al.*,  
65 2005, Cho *et al.*, 2008, Soufo *et al.*, 2008). *B. subtilis* YabA is a negative regulator of replication  
66 initiation. *yabA* null mutations cause increased and asynchronous replication (Noirot-Gros *et al.*,  
67 2002, Hayashi *et al.*, 2005), and overexpression of *yabA* causes decreased replication (Hayashi *et al.*  
68 *et al.*, 2005). GFP-YabA forms foci within the cell and the positions of these foci correspond with  
69 those of the replication machinery (Noirot-Gros *et al.*, 2002, Hayashi *et al.*, 2005, Cho *et al.*,  
70 2008). YabA interacts with DnaA and the  $\beta$ -clamp (DnaN) of DNA polymerase and these  
71 interactions are thought to be important for YabA function and localization (Noirot-Gros *et al.*,  
72 2002, Noirot-Gros *et al.*, 2006, Cho *et al.*, 2008). In addition, YabA seems to be required for  
73 association of DnaA with the replication fork (Soufo *et al.*, 2008). Because of these interactions  
74 and its subcellular location, it is thought that YabA regulates the activity of DnaA in response to  
75 replication status (Noirot-Gros *et al.*, 2002, Noirot-Gros *et al.*, 2006, Hayashi *et al.*, 2005, Cho *et al.*  
76 *et al.*, 2008, Soufo *et al.*, 2008).

77 In addition to its essential role in replication initiation, DnaA is also a transcription factor. In  
78 *B. subtilis*, replication stress causes multiple changes in transcription, and DnaA mediates many  
79 of these changes independently of the *recA*-dependent SOS response (Goranov *et al.*, 2005,  
80 Burkholder *et al.*, 2001). Genes controlled by DnaA are involved in many processes, including:  
81 replication, development, metabolism, and cell division (Messer & Weigel, 1997, Goranov *et al.*,  
82 2005, Collier *et al.*, 2006, Gon *et al.*, 2006, Burkholder *et al.*, 2001, Wang & Kaguni, 1987,  
83 Atlung *et al.*, 1985, Braun *et al.*, 1985, Kucherer *et al.*, 1986, Ogura *et al.*, 2001, Breier &  
84 Grossman, 2009). As is the case for replication initiation, it is thought that DnaA-ATP is the

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

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

98

## 99 **Results**

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

101 Null mutations in *yabA* cause an increase in replication initiation (Noirot-Gros *et al.*, 2002,  
102 Noirot-Gros *et al.*, 2006, Hayashi *et al.*, 2005, Cho *et al.*, 2008). We found that this increase was  
103 not observed in cells initiating replication from a heterologous origin inserted into the  
104 chromosome. We compared the effects of deletion and overexpression of *yabA* on replication in  
105 cells initiating from either *oriC* or the heterologous origin *oriN*. Initiation from *oriN* requires its  
106 cognate initiator protein RepN and is independent of DnaA (Hassan *et al.*, 1997). We monitored  
107 replication relative to cell growth by measuring the DNA to protein ratio (DNA/protein) of cells  
108 in culture (Kadoya *et al.*, 2002).

109 In cells growing in minimal medium and initiating replication from *oriC*, a *yabA* null  
110 mutation caused an increase in the DNA to protein ratio of nearly 2-fold relative to that of *yabA*<sup>+</sup>  
111 cells (Fig. 1A). Conversely, overexpression of *yabA* from a heterologous promoter caused a  
112 decrease in the DNA to protein ratio (Fig. 1A). The effect in the *yabA* deletion mutant was more  
113 severe in minimal medium than in rich medium (data not shown). These results are consistent  
114 with previous findings that YabA is a negative regulator of replication (Noirot-Gros et al., 2002,  
115 Noirot-Gros et al., 2006, Hayashi et al., 2005, Cho et al., 2008).

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

#### 124 **Subcellular localization of YabA**

125 The use of strains capable of initiating replication from *oriN* makes *dnaA* dispensable for  
126 replication and viability (Hassan et al., 1997, Kadoya et al., 2002, Berkmen & Grossman, 2007,  
127 Goranov et al., 2005) and allowed us to determine if *dnaA* is required for formation of foci of  
128 YabA. Previous cell biological analyses indicate that GFP-YabA forms foci that correspond to  
129 the subcellular positions of the replisome (Noirot-Gros et al., 2006, Hayashi et al., 2005).  
130 Analyses of YabA mutants defective in interacting with either DnaA or DnaN ( $\beta$ -clamp) indicate  
131 that YabA needs to interact with both DnaA and the  $\beta$ -clamp in order to form foci (Noirot-Gros  
132 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*<sup>+</sup> *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*<sup>+</sup> 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  $\beta$ -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/ $\tau$ -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  $\beta$ -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



157 components remain assembled, but that the  $\beta$ -clamp is largely dissociated. Taken together, our  
158 results indicate that formation of foci of GFP-YabA is independent of *dnaA* and *oriC*, but  
159 correlates with foci of DnaN-GFP, and that YabA and DnaN are released from the replication  
160 complex following replication arrest.

### 161 **Overproduction of $\beta$ -clamp (DnaN) stimulates DNA replication**

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

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

176 We also found that decreased transcription of *dnaN* caused a decrease in replication  
177 initiation. By placing the only copy of *dnaN* under the regulation of PxylA and growing without  
178 inducer (xylose) for 3 generations, the level of *dnaN* mRNA was reduced to ~60% of normal.  
179 The reduced transcription of *dnaN* caused a decrease of  $18 \pm 3\%$  in the DNA to protein ratio  
180 (Fig. 3A). Severe decreases in expression of *B. subtilis dnaN* can cause replication fork stalling

181 and induction of the SOS response {(Ogura et al., 2001) and data not shown}. Under the  
182 conditions in which mRNA levels of *dnaN* were reduced to ~60% of normal and replication was  
183 decreased, there was no detectable increase in mRNA levels of SOS genes (data not shown),  
184 indicating that the decrease in replication is either due to a very mild decrease in replication  
185 elongation or a decrease in replication initiation.

### 186 **Overproduction of $\beta$ -clamp stimulates DnaA-dependent replication initiation from *oriC***

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

198 The increase in replication initiation caused by overproduction of the  $\beta$ -clamp was dependent  
199 on replication initiation from *oriC*. We tested whether overexpression of *dnaN* affects  
200 replication initiation in a strain initiating solely from the heterologous origin *oriN*. We found  
201 that overexpression of *dnaN* ( $\beta$ -clamp) in *oriN*<sup>+</sup> *oriC*<sup>-</sup> mutant strains had no significant effect on  
202 the DNA to protein ratio (Fig. 3A). In addition, overexpression of *dnaN* for 25 (data not shown)  
203 or 50 min (Fig. 3C) in the *oriN*<sup>+</sup> *oriC*<sup>-</sup> mutant strains had no detectable effect on replication  
204 initiation as monitored using DNA microarrays to profile DNA content. These results indicate

205 that the regulatory effects of  $\beta$ -clamp on replication depend on initiation from *oriC*, and that  
206 overexpression of  $\beta$ -clamp, to the levels tested here, stimulates replication initiation. There did  
207 not appear to be any significant effects on replication elongation as those are expected to be  
208 independent of the origin of replication.

### 209 **YabA and $\beta$ -clamp appear to affect same rate-limiting step in replication initiation**

210 Since YabA and  $\beta$ -clamp directly interact (Noirot-Gros et al., 2002, Noirot-Gros et al.,  
211 2006), affect replication initiation {(Noirot-Gros et al., 2002) and above}, and both require  
212 DnaA-dependent initiation of replication from *oriC* to manifest their effects (see above), it is  
213 plausible that  $\beta$ -clamp and YabA affect the same rate-limiting step in replication initiation. If this  
214 is true, then overexpression of *dnaN* in a *yabA* null mutant should not cause an increase in  
215 replication above that of the *yabA* mutant alone. We constructed a strain that contains a deletion  
216 of *yabA* and overexpresses  $\beta$ -clamp (PxylA-*dnaN*). Each single allele causes overreplication as  
217 determined by DNA to protein ratios (Fig. 3A). Overexpression of *dnaN* in the *yabA* null mutant  
218 did not increase the DNA to protein ratio above that in the *yabA* null mutant alone (Fig. 3A),  
219 indicating that these effects were not additive and that YabA and the  $\beta$ -clamp are likely affecting  
220 the same rate-limiting step in replication initiation.

### 221 **Gene expression in a *yabA* null mutant**

222 YabA is an attractive candidate for regulating the activity of DnaA in response to replication  
223 status. YabA affects replication initiation from the DnaA-dependent *oriC*, it interacts with both  
224 DnaA and DnaN, and its association with the replisome correlates with that of DnaN.  
225 Expression of genes known or thought to be controlled by DnaA changes in response to  
226 replication stress (Goranov et al., 2005, Breier & Grossman, 2009). DnaA binds to the promoter  
227 regions of many of the proposed target genes in vivo (Goranov et al., 2005, Ishikawa *et al.*, 2007,  
228 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*<sup>+</sup> 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*<sup>+</sup> 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*<sup>+</sup>  
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*<sup>+</sup> 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*<sup>+</sup> 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*<sup>+</sup> 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*<sup>+</sup> 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

277 initiation in *B. subtilis* (Noirot-Gros et al., 2002, Noirot-Gros et al., 2006, Hayashi et al., 2005,  
278 Cho et al., 2008, Soufo et al., 2008). Null mutations in *yabA* cause increased replication  
279 initiation and overexpression of *yabA* causes decreased replication initiation (Hayashi et al.,  
280 2005). YabA was identified because of its ability to interact with DnaA and DnaN ( $\beta$ -clamp) in  
281 a yeast two-hybrid assay (Noirot-Gros et al., 2002).

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

297 Our results demonstrate that, under conditions in which *yabA* had no effect on DNA  
298 replication, it had no significant effect on expression of known and putative transcriptional  
299 targets of DnaA. There were no significant effects on expression of these genes either during  
300 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

325 detectable effect on replication and to directly determine the effects of *dnaA* on the subcellular  
326 positioning of YabA.

### 327 **Subcellular location of YabA**

328 Analyses of GFP-YabA fusions indicated that YabA is found associated with the replisome  
329 during ongoing replication (Noirot-Gros *et al.*, 2006, Hayashi *et al.*, 2005, Cho *et al.*, 2008).

330 This association appeared to depend on DnaA based on analysis of YabA mutants unable to  
331 interact with DnaA (Noirot-Gros *et al.*, 2006) and DnaA mutants unable to interact with YabA  
332 (Cho *et al.*, 2008). YabA was also found be required to “tether” DnaA to the replisome (Soufo *et*  
333 *al.*, 2008).

334 We also found that GFP-YabA appeared to be associated with the replisome. However, this  
335 association was not dependent on DnaA, nor was it dependent on *oriC*. These findings are not  
336 consistent with the interpretation that DnaA is required for the association of YabA with the  
337 replisome (Noirot-Gros *et al.*, 2006, Soufo *et al.*, 2008, Cho *et al.*, 2008). Previous analyses used  
338 point mutations in *yabA* or *dnaA* that alter interactions between the two gene products (Noirot-  
339 Gros *et al.*, 2006, Soufo *et al.*, 2008, Cho *et al.*, 2008). These point mutations are known to  
340 affect replication initiation (Noirot-Gros *et al.*, 2006, Cho *et al.*, 2008), which likely causes small  
341 amounts of replication stress. The point mutations in *yabA* and *dnaA* could also have other  
342 effects on those gene products.

343 The use of the heterologous origin of replication, *oriN*, allowed us to compare directly the  
344 ability of GFP-YabA to form foci in cells with and without *dnaA*. We observed no difference,  
345 indicating that neither *oriC* nor *dnaA* is required for association of YabA with the replisome. We  
346 also found that after inhibition of replication elongation (replication fork arrest), several  
347 replisome subunits were still present in foci, but that DnaN ( $\beta$ -clamp) and YabA were no longer  
348 associated with these foci. These findings are consistent with previous results indicating that



349 association of YabA with the replisome requires interaction with DnaN (Noirot-Gros et al.,  
350 2006).

### 351 ***yabA* does not affect the activity of DnaA as a transcription factor**

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

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

368 YabA does not affect replication initiation from the DnaA-independent *oriN*, indicating that  
369 its function is specific to some aspect of *oriC* and/or DnaA. Since YabA does not affect DnaA in  
370 a distributive manner, that is, it does not appear to affect the ability of DnaA to function as a  
371 transcription factor, we favor models in which YabA functions at *oriC* to inhibit replication  
372 initiation.

373 It seems likely that there are at least three aspects to the ability of YabA to inhibit replication  
374 initiation from *oriC* without affecting the global activity of DnaA. First, YabA, although in bulk  
375 appears to be with the replication elongation machinery, must get to *oriC*. Second, it somehow  
376 inhibits replication initiation from *oriC*. Third, that inhibition is somehow relieved, or not  
377 complete, so that replication can initiate at the appropriate time in the cell cycle. Assuming that  
378 the interactions between YabA and both DnaA and DnaN are important for YabA function, then  
379 these interactions could be related to any of the three aspects of the ability of YabA to inhibit  
380 replication initiation.

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

392 Alternatively, the interaction between YabA and DnaN could function to move YabA away  
393 from the *oriC* region and YabA could get to *oriC* through its interaction with DnaA. Once at  
394 *oriC*, YabA could regulate a step in replication initiation that is downstream of but not directly  
395 involving DnaA. YabA could also regulate some aspect of DnaA function that is required for the  
396 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.

419       **Strains and alleles**

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

422       (*ypjG-hepT*)122 is a deviation in sequences of the ~24 kb chromosomal region from ~*ypjG*  
423 (201.4°) to ~*hepT* (203.5°) and was described previously (Berkmen & Grossman, 2007). Briefly,  
424 the *ypjG-hepT* region contains the tryptophan biosynthesis genes (*trpABFCDE*), and the (*ypjG-*  
425 *hepT*)122 variant likely encodes a heterologous tryptophan operon as strains containing it were  
426 transformed to tryptophan-prototropy.

427        $\Delta yabA::cat$  is a deletion-insertion that inactivates *yabA* by replacing it with *cat*. The allele  
428 was generated by the long-flanking homology PCR method (Wach, 1996). The deletion starts at  
429 the 1<sup>st</sup> codon (TTG) and ends 50bp downstream of the translational stop, removing a total of  
430 407bp. The deletion stops 13bp upstream of the next gene, *yabB*. The *yabA* ORF is substituted  
431 with the 994bp chloramphenicol resistance cassette from pGEMcat. The cassette contains the *cat*  
432 ORF, 322bp upstream to include the promoter, and 20bp downstream of the stop codon, and does  
433 not include the transcriptional terminators. mRNA levels of *yabB* and other downstream genes in  
434 the *yabA::cat* mutant AIG109 were indistinguishable from those in wild type (*yabA*<sup>+</sup>) cells as  
435 assessed by microarray analysis (data not shown).

436       *amyE::{Pspank(hy)-yabA spc}* is a fusion of *yabA* to the IPTG-inducible promoter  
437 Pspank(hy) at *amyE* and was used to overexpress *yabA*. AIG80 was constructed by cloning the  
438 entire ORF of *yabA* with its endogenous ribosome binding site into a plasmid containing the  
439 Pspank(hy) promoter (pDR66, a gift from David Rudner) thus generating plasmid pAIG10. The  
440 Pspank(hy)-*yabA* construct was integrated into the genome of JH642 through a double crossover  
441 at the *amyE* locus to generate strain AIG80.

442 *spoIIIJ::{oriN repN kan}* is an insertion of the plasmid origin of replication, *oriN*, along with  
 443 the plasmid initiator gene *repN*, into the chromosome at *spoIIIJ*, near *oriC* (Goranov et al., 2005,  
 444 Berkmen & Grossman, 2007). The presence of this replication origin allowed the inactivation of  
 445 *oriC*.

446 *oriC-S* inactivates *oriC* replication functions by deleting ~150 bp of the sequence  
 447 downstream of *dnaA*, including many essential DnaA binding sites and most of the AT-rich  
 448 region that is normally unwound during replication initiation (Hassan et al., 1997, Kadoya et al.,  
 449 2002, Berkmen & Grossman, 2007).

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

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

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

464 *dnaA::{Pspac(hy)-dnaN cat}* disrupts *dnaA* while inserting Pspac(hy) to drive expression of  
 465 *dnaN*. A 450bp DNA fragment internal to *dnaA* was PCR amplified and cloned into the

466 *SphI/HindIII* cloning site of pJQ43 (Quisel *et al.*, 2001) downstream of the Pspac-hy promoter to  
467 yield plasmid pAIG37. Integration of pAIG37 into the chromosome by a single crossover  
468 disrupts *dnaA* and places *dnaN* under the regulation of Pspac-hy. *dnaA* is normally essential, but  
469 it can be deleted in strains capable of initiating chromosomal replication from a heterologous  
470 origin such as *oriN* (Hassan *et al.*, 1997, Berkmen & Grossman, 2007, Moriya *et al.*, 1997,  
471 Kadoya *et al.*, 2002). *dnaA* was in fact disrupted as evidenced by loss of detectable protein by  
472 Western blots and alterations in gene expression consistent with loss of DnaA (data not shown).

#### 473 **DNA/protein ratio determination**

474 The ratio of DNA to protein was determined as previously described (Lee & Grossman,  
475 2006, Kadoya *et al.*, 2002). Briefly, 25ml of exponentially growing cells were collected at an  
476 OD600  $\leq$  0.6. DNA and protein were extracted and the concentrations were determined using the  
477 diphenylamine reaction (DNA) and the Lowry BioRad DC Protein Assay Kit, with appropriate  
478 standards. The ratios for all strains were normalized to wild type (wt = 1.0) grown on the same  
479 day and under the same conditions. The average of three biological replicates is presented with  
480 error bars representing standard deviation.

#### 481 **DNA microarrays**

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

488 Use of microarrays to analyze DNA replication. Chromosomal DNA was prepared  
489 essentially as described previously (Goranov *et al.*, 2006, Wang *et al.*, 2007b). Briefly, DNA

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

503 Use of microarrays for analysis of mRNA levels. Experimental samples of RNA were  
504 purified using RNEasy kits (Qiagen). A reference sample was made by pooling total RNA from  
505 cultures grown in defined minimal medium and cultures treated with DNA damaging agents,  
506 thus ensuring that all genes expressed under those conditions are represented in the reference  
507 sample. Experimental and reference RNA samples were mixed with Superscript II reverse  
508 transcriptase (Invitrogen), random hexamers, and aminoallyl-dUTP (Sigma) to make cDNA.  
509 The samples were then labeled by conjugation to monofunctional Cy3 or Cy5. The experimental  
510 and reference samples were mixed and hybridized to a DNA microarray. GenePix 3.0 (Axon  
511 Instruments) was used to analyze microarray images. We included every spot that has  $\geq 70\%$  of  
512 the pixels at least one standard deviation over background and has an overall median intensity at  
513 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. Improvise OpenLabs 2.0 software was used to process  
522 images.

523

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530

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676

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

<u>Strains</u>	<u>Relevant Genotype</u>
JH642	<i>trpC2 pheA1</i> (Perego <i>et al.</i> , 1988)
BB987	<i>trpC2 pheA1 amyE::</i> { <i>Pspac</i> (-) <i> cat</i> } (empty vector)
MMB26	<i>trpC2 pheA1 amyE::</i> { <i>PxylA-dnaN cat</i> }
MMB170	<i>pheA1 (ypjG-hepT)I22 spoIIIJ::</i> { <i>oriN repN kan</i> } <i>oriC-S</i>
AIG80	<i>trpC2 pheA1 amyE::</i> { <i>Pspank(hy)-yabA spc</i> }
AIG109	<i>trpC2 pheA1 ΔyabA::cat</i>
AIG185	<i>pheA1 (ypjG-hepT)I22 spoIIIJ::</i> { <i>oriN repN kan</i> } <i>oriC-S ΔyabA::cat</i>
AIG208	<i>pheA1 (ypjG-hepT)I22 spoIIIJ::</i> { <i>oriN repN kan</i> } <i>oriC-S amyE::</i> { <i>Pspank(hy)-yabA spc</i> }
AIG245	<i>trpC2 pheA1 ΔyabA::cat amyE::</i> { <i>PxylA-dnaN cat::tet</i> }
AIG261	<i>trpC2 pheA1 dnaN::</i> { <i>PxylA-dnaN cat</i> } (pAIG28)
AIG278	<i>trpC2 pheA1 spoIIIJ::</i> { <i>oriN repN kan</i> } <i>oriC-S amyE::</i> { <i>PxylA-dnaN cat</i> }
AIG371	<i>trpC2 pheA1 dnaN::</i> { <i>dnaN-gfp spc</i> }
AIG483	<i>trpC2 pheA1 amyE::</i> { <i>PxylA-gfp-yabA cat::mls</i> } (pAIG58)
AIG505	<i>pheA1 (ypjG-hepT)I22 spoIIIJ::</i> { <i>oriN repN kan</i> } <i>oriC-S amyE::</i> { <i>PxylA-gfp-yabA cat</i> }
AIG573	<i>trpC1 pheA1 dnaX::</i> { <i>Pspank(hy)-dnaX spc</i> } (pAIG66)
AIG593	<i>pheA1 (ypjG-hepT)I22 spoIIIJ::</i> { <i>oriN repN kan</i> } <i>oriC-S amyE::</i> { <i>PxylA-gfp-yabA cat::mls</i> } <i>dnaA::</i> { <i>Pspac(hy)-dnaN cat</i> } ( <i>dnaA</i> null)
AIG595	<i>pheA1 (ypjG-hepT)I22 spoIIIJ::</i> { <i>oriN repN kan</i> } <i>oriC-S amyE::</i> { <i>PxylA-gfp-yabA cat::mls</i> } <i>dnaA::</i> { <i>Pspac(hy)-dnaA-dnaN cat</i> }
KPL374	<i>trpC2 pheA1 polC::</i> { <i>polC-gfp spc</i> }
KPL382	<i>trpC2 pheA1 dnaX::</i> { <i>dnaX-gfp spc</i> }

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*<sup>+</sup> cells: *yabA*<sup>+</sup> wild type (BB987); *yabA* null mutant (AIG109); overexpression of  
685 *yabA* (AIG80).

686 B) *oriC* mutant cells replicating from *oriN*: *yabA*<sup>+</sup> (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*<sup>+</sup> *oriC*<sup>+</sup> 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( $\beta$ -clamp)-GFP (AIG371) 15 min after treatment with HPUra

700

701 **Figure 3. DnaN positively regulates DNA replication in an *oriC*-dependent manner.**

702 A) DNA to protein ratios were measured as in Fig. 1. Where used, inducers were present for  
 703 at least 4 generations before samples were collected.  $\uparrow dnaN$ : *dnaN* was overexpressed from  
 704 P<sub>xylA</sub>-*dnaN* (MMB26).  $\uparrow dnaX$ : *dnaX* was overexpressed from P<sub>spank(hy)</sub>-*dnaX* (AIG573).  
 705  $\downarrow dnaN$ : the endogenous copy of *dnaN* was placed under control of P<sub>xylA</sub> and cells were grown  
 706 in arabinose without xylose to give only basal expression from P<sub>xylA</sub>-*dnaN* (AIG261). *oriN*<sup>+</sup>*C*:  
 707 (MMB170). *oriN*<sup>+</sup>*C*  $\uparrow dnaN$ : same as MMB170, but with *dnaN* overexpressed from P<sub>xylA</sub>-  
 708 *dnaN* (AIG278). *yabA*<sup>-</sup>: *yabA* null mutant (AIG109). *yabA*<sup>-</sup>  $\uparrow dnaN$ : *yabA* null mutant with  
 709 P<sub>xylA</sub>-*dnaN* (AIG245).

710 B-C) The effect of overproduction of  $\beta$ -clamp (DnaN) on initiation of replication was  
 711 assessed with DNA microarrays. The log<sub>2</sub> of the relative abundance of chromosomal DNA 50  
 712 min (~1 generation) after addition of inducer is compared to uninduced samples and plotted as a  
 713 function of the position on the chromosome. The position of the functional origin of replication is  
 714 indicated by an arrow. *dnaN* mRNA levels (as determined by DNA microarrays) were similar in  
 715 each of the strains used.

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

717 C) Cells replicating from *oriN* in the absence of a functional *oriC* (AIG278) (*dnaA*<sup>+</sup>)

718

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

720 Cells replicating from the DnaA-independent *oriN* in the absence of a functional *oriC* (and  
 721 *dnaA*<sup>+</sup>), with and without *yabA* (MMB170 and AIG185, respectively) were grown to mid-  
 722 exponential phase at 32°C, split, and treated with 38  $\mu$ g/ml HPUra to block replication  
 723 elongation, or mock-treated, for 60 min. Cells were harvested and RNA was purified, labeled,  
 724 and mixed with a differently labeled reference RNA for normalization. RNAs from cells grown

725 under several different conditions were pooled to make the reference (Goranov et al., 2005). The  
726 mixture of experimental and reference RNA was hybridized to whole genome DNA microarrays  
727 and fluorescence signals for each gene were determined. Data are presented as  $\log_2$  values on  
728 scatter plots of mRNA from *yabA*<sup>+</sup> cells (vertical axis) versus mRNA from *yabA* null mutant  
729 (*yabA*<sup>-</sup>) cells (horizontal axis). Solid diagonal lines indicate the main  $y=x$  diagonal, and the  
730 dashed lines represent two-fold deviations. Points appearing near the main diagonal had very  
731 similar expression in the *yabA*<sup>+</sup> and *yabA*<sup>-</sup> strains. Genes previously found to be regulated  
732 independently of *recA* and known or postulated to be directly controlled by DnaA are plotted as  
733 + and - symbols, with + indicating those whose expression increases and - indicating those  
734 whose expression decreases in response to HPUra and replication arrest (Goranov et al., 2005).  
735 All other genes are indicated as gray dots.

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

743 C) Change in gene expression between HPUra-treated and mock-treated cells. Changes in  
744 expression (+HPUra / -HPUra) are plotted as  $\log_2$  values for *yabA*<sup>+</sup> and *yabA*<sup>-</sup> cells. A position  
745 of (0,0) indicates no change in either strain; genes that were induced in both strains appear in the  
746 upper right quadrant, and genes that were repressed in both strains appear in the lower left  
747 quadrant. That most genes fall on or near the line  $y=x$  indicates that there is little or no effect of  
748 *yabA* on the response to HPUra.

Figure 1

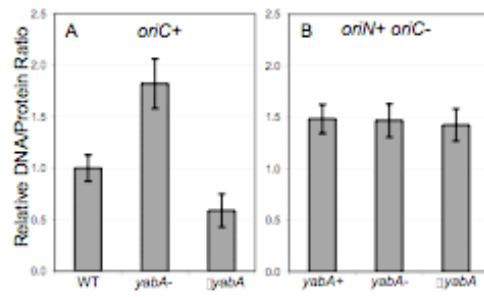




Figure 2

