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The primosomal protein DnaD inhibits cooperative DNA binding by the
replication initiator DnaA in *Bacillus subtilis*

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Running title: Primosomal protein DnaD affects DNA binding by DnaA

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27 **Abstract**

28 DnaA is a AAA+ ATPase and the conserved replication initiator in bacteria. Bacteria control
29 the timing of replication initiation by regulating the activity of DnaA. DnaA binds to multiple
30 sites in the origin of replication (*oriC*) and is required for recruitment of proteins needed to load
31 the replicative helicase. DnaA also binds to other chromosomal regions and functions as a
32 transcription factor at some of these sites. *Bacillus subtilis* DnaD is needed during replication
33 initiation for assembly of the replicative helicase at *oriC* and during replication restart at stalled
34 replication forks. DnaD associates with DnaA at *oriC* and at other chromosomal regions bound
35 by DnaA. Using purified proteins, we found that DnaD inhibited the ability of DnaA to bind
36 cooperatively to DNA and caused a decrease in the apparent dissociation constant. These effects
37 of DnaD were independent of the ability of DnaA to bind or hydrolyze ATP. Other proteins
38 known to regulate *B. subtilis* DnaA also affect DNA binding, whereas much of the regulation of
39 *E. coli* DnaA affects nucleotide hydrolysis or exchange. We found that the rate of nucleotide
40 exchange for *B. subtilis* DnaA was rapid and not affected by DnaD. The rapid exchange is
41 similar to that of *Staphylococcus aureus* DnaA and in contrast to the slow exchange rate of
42 *Escherichia coli* DnaA. We suggest that organisms in which DnaA has a rapid rate of nucleotide
43 exchange predominantly regulate the DNA binding activity of DnaA and those with slow rates of
44 exchange regulate hydrolysis and exchange.

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48 **Introduction**

49 Accurate and complete replication of DNA is essential for the propagation of genomic
50 information. DNA replication in bacteria initiates from a single origin of replication (*oriC*) and
51 depends on the conserved AAA+ ATPase DnaA (reviewed in 13, 25, 27, 29, 35, 45). DnaA
52 binds both ATP and ADP, and DnaA-ATP is required for replication initiation (2, 17, 33, 47,
53 57). The ATP bound form of DnaA forms oligomers that are important for promoting replication
54 initiation (14, 18, 35, 41, 58). DnaA-ATP binds to sites in *oriC* and promotes the unwinding of
55 the DNA Unwinding Element (DUE), which serves as a platform for assembly of the replicative
56 helicase and the rest of the replication machinery. Wherever examined, the nucleotide bound
57 state of DnaA controls its activity (e.g., 39, 42, 57, 63), and this can be affected by factors that
58 alter nucleotide hydrolysis and exchange (16, 26, 28, 64). The rate of nucleotide exchange for
59 purified *E. coli* DnaA is relatively slow, with a half-life of 45 min (57). This slow inherent rate
60 of exchange enables the modulation of DnaA activity by factors that stimulate the rate of
61 nucleotide hydrolysis and/or exchange (11, 16, 26, 64).

62 In contrast to the widespread conservation of DnaA, other proteins required for replication
63 initiation are less conserved. For example, steps involved in loading the replicative helicase at
64 *oriC* are different between *E. coli* and *Bacillus subtilis*. In *B. subtilis* and other low G+C Gram
65 positive bacteria, helicase loading requires the essential primosomal proteins DnaD, DnaB, and
66 DnaI, in addition to DnaA (5-8, 12, 36, 37, 54, 59, 62, 65). DnaD is associated with *oriC* and this
67 association depends on DnaA (54, 59). Association of DnaB with *oriC* depends on DnaD, and
68 finally, DnaI-mediated assembly of the helicase at *oriC* depends on DnaB. DnaD and DnaB bind
69 both double and single stranded DNA, which may help stabilize opening up of the origin of
70 replication (7, 38, 68).

71 In addition to binding to sites in *oriC*, *B. subtilis* DnaA binds to sites in chromosomal regions
72 outside of *oriC* (4, 20, 24). DnaA functions as a transcription factor at some of these secondary
73 binding regions (4, 9, 20, 24). Where tested, DnaD and DnaB are also found associated with
74 these secondary DnaA binding regions, and this association depends on DnaA (60). However, in
75 contrast to *oriC*, these secondary DnaA-binding regions do not function as origins of replication
76 and there is no indication that they are capable of loading the replicative helicase (60).

77 Because of the association of DnaD with DnaA at multiple regions throughout the
78 chromosome, we hypothesized that DnaD modulates the activity of DnaA. Some factors that
79 affect the activity of DnaA, predominantly in *E. coli*, are known to alter its nucleotide-bound
80 state (16, 26, 64). In contrast, regulators of *B. subtilis* DnaA, (e.g., YabA, Soj, and SirA) are
81 known to alter its DNA binding properties (40, 53, 55, 66). Using purified proteins, we tested for
82 effects of DnaD on both the ability of DnaA to bind DNA and on nucleotide exchange.

83 We found that the rate of nucleotide exchange for *B. subtilis* DnaA was relatively rapid,
84 similar to that of DnaA from *Staphylococcus aureus* (32), and in contrast to the slow rate of
85 exchange for DnaA from *E. coli* (57). DnaD had no effect on the rate of exchange of *B. subtilis*
86 DnaA. In contrast, DnaD had a marked effect on the ability of DnaA to bind DNA. Binding of
87 DnaA-ATP to DNA fragments that contain multiple binding sites is normally highly cooperative
88 (40, 41). We found that in the presence of DnaD, binding of DnaA to DNA was no longer
89 cooperative and the apparent dissociation constant for DnaA and DNA was reduced. We found
90 that the ATPase activity of DnaA was not needed for these effects by DnaD, indicating that
91 DnaD is not regulating the ATPase activity of DnaA. These effects of DnaD on the ability of
92 DnaA to bind DNA are similar to the effects of YabA (40) and Soj (55), two other regulators of
93 *B. subtilis* DnaA and replication initiation and further substantiate the notion that modulation of

94 cooperative binding and oligomerization of DnaA to DNA might be a common mechanism of
95 regulation (40, 55).

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98 **Materials and Methods**

99 **Purification of DnaA and DnaD**

100 *B. subtilis* DnaA (no tag) was produced in and purified from an *E. coli dnaA* null mutant,
101 using a clone and strain provided by A. Albuzzu and W. F. Burkholder, essentially as described
102 (17, 60). Protein was stored frozen (-80 °C) in buffer containing 45 mM Hepes pH 7.6, 0.5 mM
103 EDTA, 10 mM magnesium acetate, 1 mM DTT, 700 mM potassium glutamate, and 20%
104 sucrose. DnaD-his6 was produced in and purified from *E. coli*, essentially as described (60).
105 Protein was stored at -80°C in buffer containing 50 mM Tris pH 8, 0.1 mM EDTA, 1 mM DTT,
106 500 mM NaCl and 10% Glycerol. Proteins were quantified using absorbance at 280 nm.

107 **Nucleotide exchange reactions**

108 Nucleotide exchange was measured using alpha-³²P-ATP or ¹⁴C-ADP. Exchange reactions
109 contained 40 mM Hepes pH 7.5, 10 mM magnesium acetate, 0.5 mM EDTA, 1 mM DTT, 150
110 mM potassium glutamate, 100 µg/ml BSA, 10% glycerol and 1 µM alpha-³²P-ATP or 1 µM ¹⁴C-
111 ADP in the absence or presence of DnaD (600 nM). DnaA (300 nM) was incubated with either
112 nucleotide for 2 hours on ice in exchange buffer. Fifty microliters were removed at time zero to
113 measure binding and unlabeled ATP was added in excess (2 mM) and incubated at 37°C. Filter
114 binding was used to measure the amount of radio-labeled nucleotide still bound to DnaA at each
115 time point. Fifty microliter aliquots were removed and placed on equilibrated nitrocellulose
116 membranes (Millipore), washed with buffer (40 mM Hepes pH 7.5, 150 mM KCl, 10 mM

117 magnesium acetate, 0.5 EDTA, 10ug/ml BSA) and the amount of ^{32}P -ATP or ^{14}C -ADP
118 remaining on the filter was measured in triplicate and averaged. The half-life was calculated
119 using an exponential decay formula and plotted using GraphPad Prism 5 software.

120 **Gel Shift Assays**

121 The DNA template from the *oriC* region (*dnaA* promoter region) used for the gel shift assays
122 was an end-labeled 400 bp fragment, 382 bp of which correspond to chromosomal DNA from
123 the part of the *oriC* region that is upstream from *dnaA*. The fragment was generated by PCR
124 using primers OCB23 (5'-CCGGAATTCTTTTTTTAGTATCCACAGAGG-3') and OCB24 (5'-
125 CGCGGATCCCTTTTCTTAGAAAATGGC-3') and *B. subtilis* chromosomal DNA as template.
126 Allowing for one mismatch from the DnaA binding site consensus sequence (5'-TTATNCACA-
127 3'), this fragment contains eight consensus DnaA binding sites. The DNA template from
128 upstream of *yydA* used for gel shift assays was an end-labeled 228 bp fragment generated by
129 PCR using primers WKS167 (5'-CCCACAGCCTGTGAATTATG-3') and WKS168 (5'-
130 CGTAGGCCGAAAGTCGTTTG--3'). Allowing for 1 mismatch, this fragment contains four
131 consensus DnaA binding sites. It is important to note that the sequence requirements for binding
132 DnaA are not well defined and this estimate of the number of potential binding sites is likely an
133 underestimate as DnaA is also likely to bind sequences with more than one mismatch from
134 consensus (17).

135 The PCR products were purified on columns (Qiagen) and end-labeled with gamma- ^{32}P -ATP
136 using T4 polynucleotide kinase. The labeled DNA fragment was separated from free ATP with a
137 G50 Column (GE). DnaA was incubated with 2.5 mM ATP for two hours on ice before being
138 used in gel shift reactions containing 40 mM Hepes pH 7.6, 10 mM KCl, 140 mM potassium
139 glutamate, 10 mM magnesium acetate, 2.5 mM ATP, 0.5 mM EDTA, 1 mM DTT, 50 $\mu\text{g/ml}$

140 BSA, 20% glycerol, and 50 pM DNA probe in the presence or absence of DnaD-his6 (300 nM)
141 for 20 minutes at room temperature.

142 To determine an appropriate concentration of DnaD to use, we measured the effects of
143 different concentrations of DnaD-his6 on the electrophoretic mobility of the DNA fragment from
144 the *oriC* region in the presence of 10 nM DnaA-ATP, or with no DnaA (Fig. 1). DnaD-his6 was
145 used at 25, 50, 100, 200, 300 nM. At these concentrations of DnaD-his6, there was little or no
146 change in electrophoretic mobility of the DNA fragment in the absence of DnaA. However, in
147 the presence of DnaA, there was a change in the gel shift beginning at 50 nM DnaD-his6. We
148 chose to use 300 nM DnaD because there was a large change in the gel shift in the presence of
149 DnaA, but little or no change in its absence.

150 The binding reactions were run on a 5% polyacrylamide gel with 2.5% glycerol run in 0.5X
151 TBE at approximately 12 volts/cm for 3 hours. Gels were imaged on a Typhoon scanner (GE
152 Healthcare) and GraphPad Prism 5 software used to plot binding curves. Data were plotted and
153 fitted to the Hill equation $\{y=(m1*x^n)/(Kd^n+x^n)\}$ where y is the % DNA bound at any given
154 DnaA concentration, x is the DnaA concentration, m1 is maximal binding (100%), Kd is the
155 apparent dissociation constant (concentration at which 50% of DNA is bound determined from
156 data) and n is the Hill coefficient. All experiments were done in triplicate. Data presented are
157 averages of triplicates \pm standard error.

158 **ATPase Assays**

159 ATPase assays were carried out using gamma-³²P-ATP as substrate and products were
160 separated by thin layer chromatography (TLC). Reactions (50 μ l) contained 100 nM DnaA, 50
161 mM Tris pH 7, 5 mM magnesium acetate, 1 mM DTT, 100 ng/ml BSA, 10% glycerol, and 1 μ M
162 ATP (1/1000 gamma-³²P-ATP) and, where added, 1 μ M PCR product from *oriC* fragment

163 containing eight DnaA binding sites. Time points were stopped with 2 volumes of stop buffer
164 (0.5% SDS, 250 mM NaCl, 25 mM EDTA) and spotted on cellulose TLC plates. Products were
165 separated with 0.5 M LiCl, 1M formic acid. Plates were dried and exposed to a phosphostorage
166 screen. Free radiolabeled orthophosphate and ATP were measured and percent hydrolysis
167 calculated. All experiments were done in triplicate and data are presented as the averages \pm
168 standard error.

169 **Nucleotide Binding Assays**

170 Nucleotide binding was measured using alpha-³²P-ATP. DnaA (100 nM) and ATP (1 μ M)
171 were incubated for 30 min at room temperature in 50 μ l reactions containing 40 mM Hepes pH
172 7.5, 10 mM Mg Acetate, 0.5 mM EDTA, 1 mM DTT, 150 mM potassium glutamate, 100 μ g/ml
173 BSA, and 10% glycerol. The reactions were placed on equilibrated nitrocellulose membranes
174 (Millipore), washed with buffer (40 mM Hepes pH 7.5, 150 mM KCl, 10 mM magnesium
175 acetate, 0.5 mM EDTA, 10 μ g/ml BSA) and radioactivity was measured by filter binding as
176 described for the nucleotide exchange assay. All experiments were done in triplicate and data are
177 presented as the averages \pm standard error. The apparent Kd for ATP binding to DnaA was 29
178 nM (data not shown) similar to previous reports (17).

179

180

181

182 **Results**

183 **DnaD does not affect nucleotide exchange for DnaA**

184 We found that the rate of nucleotide exchange for DnaA was relatively rapid and that DnaD
185 had no detectable effect on this rate. To measure nucleotide exchange, we incubated purified
186 DnaA with radio-labeled ADP or ATP. The amount of radioactive nucleotide that remained
187 associated with DnaA was measured at various times after addition of excess unlabeled ATP at
188 37°C, and the amount of nucleotide that was released was calculated. For both DnaA-ADP (Fig.
189 2A) and DnaA-ATP (Fig. 2B), the radioactive nucleotide was released with a half-life of 5
190 minutes. The addition of DnaD-his6 (Materials and methods) had no detectable effect on this
191 half-life (Fig. 2). Based on these results, we conclude that the rate of nucleotide exchange for *B.*
192 *subtilis* DnaA is relatively rapid compared to the 45 min half-life of exchange for *E. coli* DnaA
193 (57) and that DnaA-ATP is regenerated from DnaA-ADP in the absence of any other cellular
194 factors. The relatively rapid rate of nucleotide exchange is similar to that of DnaA from *S. aureus*
195 (32).

196 **DnaD increases affinity and reduces cooperativity of DnaA-ATP binding to DNA**

197 Given the in vivo association of DnaD to DnaA binding sites around the chromosome (60),
198 we tested for effects of DnaD on the ability of DnaA to bind DNA using gel electrophoretic
199 mobility shift assays. DnaA-ATP bound to a DNA fragment from the *oriC* region with an
200 apparent dissociation constant (K_d) of 27 nM (Fig. 3A, C). Binding was highly cooperative and
201 had a Hill coefficient of 8. These results are consistent with previous findings (40).

202 We measured the effects of DnaD-his6 on the ability of DnaA to bind to DNA. DnaD-his6
203 alone (300 nM) did not have detectable binding activity under these assay conditions (Fig. 1,
204 3B), as previously reported (60). However, addition of DnaD-his6 to DNA and DnaA-ATP

205 substantially altered the binding properties of DnaA-ATP to DNA. The apparent dissociation
206 constant in the presence of DnaD was approximately 7 nM, compared to 27 nM in the absence of
207 DnaD. There was a concomitant loss of cooperative binding as the Hill coefficient decreased
208 from 8 in the absence of DnaD to 1 in its presence (Fig. 3, Table 1). This decrease in the Hill
209 coefficient and decrease in the apparent binding constant is consistent with DnaA-ATP binding
210 independently to multiple sites in the DNA fragment.

211 The DNA fragment used in these experiments was derived from sequences upstream of *dnaA*
212 in the *oriC* region. We found that DnaD had a similar effect on the ability of DnaA-ATP to bind
213 to a DNA fragment from a different chromosomal region. In addition to the *oriC* region, DnaA is
214 found associated with several chromosomal regions in vivo (20, 24), including the region
215 between *yydA* and *yydS* (4, 20, 24), two genes of unknown function. This region has also been
216 implicated in regulating DNA replication by recruiting DnaA away from *oriC* (51). Therefore,
217 we isolated a DNA fragment from the region upstream of *yydA* that contains four DnaA binding
218 sites with ≤ 1 mismatch to the consensus and tested the ability of DnaD to affect DnaA binding to
219 this region. DnaA-ATP bound to this fragment with an apparent dissociation constant of 25 (Fig.
220 3D). Binding was cooperative with a Hill coefficient of 6, indicative of binding to 6 possible
221 DnaA sites. Addition of DnaD-his6 (300 nM) to these reactions decreased the apparent
222 dissociation constant to 5 nM and reduced the Hill coefficient to 1, indicating that there was
223 essentially no cooperative binding in the presence of DnaD. These effects are similar to those on
224 the binding of DnaA-ATP to DNA fragment from the *oriC* region upstream from *dnaA* (Fig. 3).
225 Together, these results indicate that DnaD affects binding of DnaA to DNA fragments from the
226 *oriC* region and at least one origin-distal region. Since DnaD is found at multiple chromosomal

227 regions bound by DnaA in vivo (60), we suspect that DnaD similarly affects DnaA binding at
228 these regions.

229 We have not been able to detect any changes in the footprint of DnaA on DNA in the
230 presence compared to the absence of DnaD (unpublished results). This is likely because at the
231 high concentrations of DnaA needed to observe a footprint (17), the addition of DnaD has no
232 detectable effect on binding (Fig. 3). At lower concentrations of DnaA, where DnaD does
233 influence binding, we suspect that there is a population of DNA molecules with different sites
234 occupied by DnaA, thereby not producing any obvious protection in a footprint experiment, but
235 still capable of generating a change in electrophoretic mobility.

236 **Characterization of DnaA mutants defective in ATPase activity**

237 Since the rate of ADP exchange for ATP of *B. subtilis* DnaA is relatively rapid and
238 unaffected by DnaD, and DnaD affects the ability of DnaA-ATP to bind DNA, we hypothesized
239 that DnaD would affect DnaA mutants that are defective in nucleotide hydrolysis and/or binding.
240 To test this, we made two different mutations in *dnaA*. One mutation is in the conserved Walker
241 A motif and changes the lysine at amino acid 157 to alanine, DnaA(K157A), and is predicted to
242 reduce nucleotide binding (21). We also made a mutation in the conserved Walker B motif that
243 changes the glutamate at amino acid 215 to alanine, DnaA(D215A), and is predicted to alter
244 nucleotide hydrolysis (21). We purified the mutant proteins and tested them in vitro.

245 Both DnaA(K157A) and DnaA(D215A) were defective in ATP hydrolysis. We measured the
246 rate of ATP hydrolysis using gamma-³²P-ATP and measuring the release of ortho-phosphate
247 (Materials and Methods). The rate of ATP hydrolysis by wild type DnaA was 1.8 moles of ATP
248 hydrolyzed per mole of DnaA per hour. Upon addition of DNA, the rate of hydrolysis increased
249 approximately 6-fold to 12 moles of ATP hydrolyzed per mole of DnaA per hour. These rates of

250 ATP hydrolysis and the effects of DNA are consistent with previously published data for DnaA
251 from *E. coli* and *S. aureus* (32, 57). In contrast to the wild type protein, DnaA(K157A) and
252 DnaA(D215A) had rates of ATP hydrolysis of approximately 0.07 and 0.1 moles of ATP per
253 mole of DnaA per hour, respectively (Table 2).

254 As expected, the DnaA(K157A) mutant was defective and the DnaA(D215A) mutant had
255 normal ATP binding. At saturating ATP concentrations, we found that wild type protein bound
256 0.4 molecules of ATP per molecule of DnaA (Table 2), consistent with previous reports for
257 DnaA from *E. coli* (0.48 and 0.55) (10, 57) but greater than a previous report for *B. subtilis*
258 DnaA (0.17) (17). The DnaA(D215A) mutant had ATP binding (Table 2) that was
259 indistinguishable from that of the wild type protein. In contrast, the DnaA(K157A) mutant
260 appeared to bind 0.02 molecules of ATP per molecule of DnaA (Table 2), consistent with little or
261 no ATP binding.

262 **DnaD affects DnaA mutants defective in ATPase activity and nucleotide binding**

263 We determined the effects of DnaD on the ability of the mutant DnaA proteins to bind DNA
264 and compared the binding properties to those of wild type DnaA. The mutant DnaA that binds
265 ATP but is defective in hydrolysis {DnaA(D215A)} bound DNA with an apparent K_d of 12 nM,
266 compared to 27 nM for the wild type protein (Fig. 4A). Binding to DNA was cooperative and
267 had a Hill coefficient of approximately 5 (Fig. 4A). Addition of DnaD reduced the apparent K_d
268 to approximately 6 nM and the Hill coefficient to approximately 2 (Fig. 4A, Table 1). The
269 mutant DnaA that is defective in binding ATP {DnaA(K157A)} has an apparent K_d of 36 nM
270 and a Hill coefficient of approximately 5 (Fig. 4B). Addition of DnaD reduced the apparent K_d
271 to 17 nM and the Hill coefficient to approximately 3 (Fig. 4B, Table 1). Together, these results

272 indicate that the effects of DnaD on the ability of DnaA to bind DNA do not require ATP
273 binding or hydrolysis.

274 Previously, we found that YabA, a negative regulator of replication initiation, inhibited
275 cooperativity of DnaA while reducing the apparent K_d (40). We tested the effects of YabA on
276 the DnaA(K157A) mutant and found that the addition of YabA (700 nM) reduced the apparent
277 K_d from 36 nM to 14.7 nM and the Hill coefficient from 5.6 to 2 (Fig. 4C). These results are
278 consistent with the previous reports of the effects of YabA on the ability of DnaA to bind DNA
279 (40) and are comparable to the effects of DnaD, and suggest that they may regulate DnaA by
280 similar mechanisms.

281

282

283 **Discussion**

284 In many bacteria, the conserved replication initiator DnaA is a target for the control of
285 replication initiation. DnaA is also a transcription factor, and many of the factors that modulate
286 its activity in replication initiation are likely to affect its activity as a transcription factor.
287 Mechanisms regulating DnaA have been most studied with *E. coli* and its close relatives.
288 However, the proteins and mechanisms used by *E. coli* are largely limited to the proteobacteria
289 and are not found in Gram positive organisms like *B. subtilis*. Likewise, some of the proteins and
290 mechanisms used by *B. subtilis* are not found in *E. coli* and other proteobacteria. Results
291 presented here indicate that *B. subtilis* DnaD is a regulator of DnaA. Below, we discuss the
292 possible role of DnaD in regulation of DnaA and the different properties of DnaA that might lead
293 to differences in its regulation in different organisms.

294 **Role of DnaD in replication initiation and regulation of DnaA**

295 DnaD is found in *B. subtilis* and other low G+C-content Gram positive bacteria, but not in *E.*
296 *coli* and other Gram negative bacteria. DnaD is required for replication initiation (5, 6), interacts
297 with DnaA (23, 60), and is needed to recruit the helicase loading protein DnaB to *oriC* (7, 59).
298 DnaD is found associated with the *oriC* region of the chromosome (54, 59) and many other
299 chromosomal regions that also bind DnaA (60). The association of DnaD with these regions is
300 dependent on DnaA (59, 60).

301 We found that DnaD decreases the apparent K_d of DnaA-ATP for DNA, and also decreases
302 the cooperativity of DnaA binding to DNA. These effects could indicate that DnaD functions
303 either as an activator or repressor of DnaA, or both. For many regulators, the phenotype caused
304 by a null mutation typically indicates regulation is positive or negative. Unfortunately, *dnaD* is
305 essential and null mutations are not viable. Temperature sensitive and other conditional loss of
306 function *dnaD* mutants result in increased association of DnaA with chromosomal regions (4,
307 20), consistent with DnaD normally functioning to reduce binding of DnaA to DNA. However,
308 these mutations also lead to a decrease in replication initiation. Analyzing effects of
309 overexpressing DnaD is also problematic because overexpression of DnaD causes a severe
310 growth defect that is independent of replication initiation from *oriC* (38). Mutations in other
311 genes that cause a decrease in replication initiation also cause an increase in DnaA activity (4,
312 20) making it difficult to discern if the effects of DnaD are direct, due to changes in replication
313 initiation, or both.

314 It is well established that DnaD is essential and has a positive role in replication initiation.
315 During replication initiation, DnaA-ATP binds cooperatively to many sites in *oriC* (e.g., 18, 39,
316 41). By analogy to *E. coli*, it is likely that the temporal order of binding of DnaA-ATP to sites in

317 *oriC* is important for open complex formation (39, 41). In *B. subtilis*, association of DnaD with
318 the *oriC* region requires DnaA. DnaD is then required for association of DnaB and subsequent
319 loading of the replicative helicase (6, 7, 54, 59). It is possible that the effects of DnaD on the
320 binding of DnaA to *oriC* (increase in apparent affinity) could also stimulate replication initiation
321 by maintaining DnaA bound to *oriC*. However, we think that this is unlikely if ordered and
322 cooperative binding of DnaA is important for replication initiation.

323 In addition to its known positive role in replication initiation, we postulate that DnaD also
324 serves to negatively regulate replication initiation through its effects on the ability of DnaA to
325 bind DNA. The ability of DnaD to inhibit cooperative binding of DnaA-ATP to DNA is similar
326 to the effect of two other negative regulators of replication initiation, YabA (40) and Soj (55).

327 We postulate that immediately before or after replication initiation, DnaD helps keep DnaA
328 inactive at *oriC* by inhibiting cooperative binding. This activity of DnaD as a negative regulator
329 of DnaA could be modulated by changes in the amount of available DnaD during the replication
330 cycle. For example, the amount of DnaD available to interact with DnaA at *oriC* could change
331 during a replication cycle, perhaps due to association of DnaD with other proteins or
332 chromosomal regions (60), or possible changes in its synthesis or stability. We have not yet
333 tested these possibilities.

334 It is also possible that inhibition of DnaA by DnaD is relieved by the replication initiation
335 protein DnaB. That is, DnaD might be keeping DnaA inactive at *oriC* until proper assembly of
336 additional parts of the replication initiation complex. For example, DnaD is needed to recruit
337 DnaB (part of the helicase loader) to *oriC* (59) and other chromosomal regions bound by DnaA
338 (60). Association of DnaB might alter interactions between DnaA and DnaD, relieving the
339 putative inhibitory effect mediated by DnaD, enabling replication initiation. Preliminary

340 attempts to test this in vitro have not been successful, perhaps because of a possible role of the
341 membrane in interactions between DnaB and DnaD (54).

342

343 **Emerging theme in the regulation of DnaA**

344 One of the emerging themes of regulation of *B. subtilis* DnaA and replication initiation is the
345 role of regulators that directly alter the ability of DnaA to bind DNA. Including DnaD, there are
346 now at least four regulators of this type. The production and activities of the regulators are
347 differentially controlled and each regulator is likely to be important at different times during the
348 growth and replication cycles. The regulators are also likely to be partly redundant.

349 SirA. SirA is a negative regulator of DnaA that is produced during entry into stationary phase
350 and the initiation of sporulation (52, 66). SirA likely interacts with domain I of DnaA, and it
351 inhibits the ability of DnaA to bind sequences in *oriC* in vivo (53).

352 YabA. YabA was identified in a yeast two-hybrid screen for interactors with replication
353 proteins (48). YabA is produced during growth and interacts with both DnaA and DnaN, the
354 processivity clamp of DNA polymerase (48, 49). Like DnaD, YabA reduces the apparent K_d and
355 cooperativity of DnaA binding to DNA in vitro, and these effects are independent of the ATPase
356 activity of DnaA (40). Also like DnaD, YabA is found associated with chromosomal regions that
357 are bound by DnaA in vivo and this association is DnaA-dependent (40). In addition, YabA is
358 found associated with replication forks during ongoing replication (19, 49, 61), and this
359 association is likely due to interaction between YabA and DnaN. The interaction between DnaN
360 and YabA likely functions to reduce the ability of YabA to negatively regulate DnaA and
361 replication initiation (40, 49, 61) and could couple relief of YabA-mediated inhibition to the
362 release of DnaN from the replisome during replication termination (40).

363 Soj. *B. subtilis* Soj is expressed during growth and is a member of the ParA family of
364 chromosome partitioning proteins involved in chromosome and plasmid partitioning. Soj is a
365 negative regulator of replication initiation (34) and DnaA (46, 55, 56). Soj inhibits the ability of
366 DnaA to form a helix on DNA, independently of the ATPase activity of DnaA (55). The
367 inhibitory effects of Soj on replication initiation appear to be relieved by Spo0J (56), perhaps
368 coupling an aspect of replication control to chromosome organization or partitioning (46, 56).

369 **Diverse mechanisms controlling DnaA in different organisms**

370 The mechanisms used to control DnaA are diverse. Using *E. coli* and *B. subtilis* as examples,
371 there are some common mechanisms and some striking differences. In both of these organisms,
372 and many others, DnaA represses its own transcription (e.g., 1, 3, 20, 50, 67), establishing a
373 homeostatic regulatory loop. In addition, there are DnaA binding sites outside of *oriC* that
374 function to titrate DnaA away from *oriC* (51, 60). In *E. coli*, the *datA* locus binds DnaA and
375 appears to help limit the amount of DnaA available for replication initiation (31, 43, 44).
376 Similarly, in *B. subtilis*, there are six chromosomal regions outside of *oriC* that have clusters of
377 DnaA binding sites (20, 24, 51). At least one of these clusters seems to function to help limit the
378 amount of DnaA available for replication initiation (51).

379 One of the most notable differences between regulation of *E. coli* and *B. subtilis* DnaA is the
380 stimulation of nucleotide binding and hydrolysis in *E. coli*. One of the primary mechanisms used
381 by *E. coli* to inhibit the activity of DnaA is called RIDA (Regulatory Inactivation of DnaA) and
382 uses a protein called Hda (26, 28). Hda interacts with *E. coli* DnaN (β -clamp) and stimulates
383 nucleotide hydrolysis by DnaA, thereby stimulating conversion of the replication-competent
384 DnaA-ATP to the inactive DnaA-ADP (64). *E. coli* also has specific DnaA-reactivating
385 sequences that directly promote nucleotide exchange to generate DnaA-ATP from DnaA-ADP

386 (16). The stimulated rate of nucleotide exchange for *E. coli* DnaA (15) is about the same as the
387 basal rate for *B. subtilis* DnaA. *E. coli* also has a protein called DiaA that stimulates replication
388 initiation by stimulating binding by DnaA-ATP (22, 30).

389 In contrast to the mechanisms used by *E. coli* to regulate DnaA and replication initiation, *B.*
390 *subtilis* is not known to regulate nucleotide hydrolysis or exchange. Rather, the primary
391 mechanisms for controlling *B. subtilis* DnaA affect its binding to DNA (40, 53, 55), probably by
392 inhibiting formation of multimeric DnaA structures (helix formation) on the DNA and
393 preventing cooperative binding to sites in *oriC* (40, 55). No known regulator of DnaA in *B.*
394 *subtilis* affects nucleotide hydrolysis or exchange.

395 Clearly, different organisms use different mechanisms to control the activity of DnaA and
396 replication initiation. We suggest that the multiple mechanisms may have evolved in different
397 organisms, in part, due to the different rates of nucleotide exchange. For organisms like *E. coli*
398 where DnaA has a relatively slow rate of nucleotide exchange, stimulation of nucleotide
399 hydrolysis and exchange is likely to be a predominant mode of regulation. In contrast, for
400 organisms like *B. subtilis* and *S. aureus* where DnaA has a relatively rapid rate of nucleotide
401 exchange, the predominant modes of regulation of DnaA affect DNA binding and cooperativity.

402

403

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408

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602 **Table 1. Summary of DNA binding by wild type and mutant DnaA¹.**

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DnaD ²	DnaA		DnaA(K157A)		DnaA(D215A)	
	-	+	-	+	-	+
apparent Kd	27 ± 0.4	6.6 ± 1	36 ± 0.8	17 ± 1	12 ± 0.3	5.7 ± 0.3
Hill coefficient	8.6 ± 0.9	1.0 ± 0.1	5.3 ± 0.5	2.7 ± 0.4	5.2 ± 0.4	2.2 ± 0.2

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606 ¹ Results presented in Fig. 3C, 4A, and 4B are summarized. The DNA template was a fragment607 containing sequences upstream from *oriC* with eight DnaA binding sites (≤ 1 mismatch from

608 consensus).

609 ² DnaD-his6 was either added at 300 nM (+) or absent (-).

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Table 2. Summary of ATP hydrolysis and binding by wild type and mutant DnaA¹.

protein	ATPase	ATPase + DNA	ATP binding
DnaA	1.8 ± 0.25	11.9 ± 2.2	0.4 ± 0.05
DnaA(K157A)	0.07 ± 0.002	0.3 ± 0.04	0.02 ± 0.01
DnaA(D215A)	0.1 ± 0.2	0.3 ± 0.01	0.4 ± 0.11

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¹ The rate of ATP hydrolysis (ATPase) is presented as the number of moles of ATP hydrolyzed per mole of DnaA per hour. Where indicated, the 400 bp DNA fragment from the *oriC* region that was used for the gel shift assays was added (1 μ M). The amount of ATP bound (ATP binding) is presented as moles of ATP per mole of DnaA.

623 **Figure legends**

624

625 **Figure 1. Effect of different concentrations of DnaD on DnaA binding to *oriC*.**

626 Representative gel of the radiolabeled DNA probe from the *oriC* region with different amount of
627 DnaD-his, in the absence of DnaA (six lanes on the left) or presence of 10 nM DnaA-ATP (six
628 lanes on the right). Concentrations of DnaD-his are 0 (-), 25, 50, 100, 200, or 300 nM and are
629 indicated below each lane.

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632 **Figure 2. DnaD does not affect nucleotide exchange by DnaA.** The amount of ^{14}C -ADP
633 (A) or ^{32}P -ATP (B) bound to DnaA (300 nM) at various times after addition of unlabeled ATP (2
634 mM) at 37°C was measured by filter binding in the absence (open circles) and presence (filled
635 squares) of DnaD-his6 (600 nM). Data are averages of triplicates \pm standard error and are
636 normalized to the starting amount of radioactivity in the absence of unlabeled ATP.

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639 **Figure 3. DnaD inhibits cooperative binding of DnaA to DNA.** Representative gels and
640 binding curves measuring binding of DnaA-ATP to DNA (50 pM) with and without purified
641 DnaD-his6 (300 nM) are shown. DnaA concentrations used were: 0, 1, 2, 5, 10, 20, 30, 40, 50,
642 60, 80, 100, and 200 nM.

643 **A, B.** Representative gels with increasing concentrations of DnaA-ATP incubated with
644 template DNA from the *oriC* region in the absence (A) or presence (B) of DnaD-his6. Probe with
645 no added protein is shown in the first lane (A) or first lane (B). Probe with DnaD-his6 and no
646 DnaA is shown in the second lane of panel B.

647 **C, D.** Data from three independent gel shift assays using template DNA from the *oriC* region
648 (C) or the *yydA* region (D) are plotted as percent DNA bound vs. the concentration of DnaA-
649 ATP, in the absence (open circles) and presence (filled squares) of DnaD-his6.

650 In experiments with the DNA fragment from the *oriC* region (C), the calculated Hill
651 coefficient for DnaA-ATP was 8.6 in the absence of DnaD-his6 and 1 in the presence of DnaD-
652 his6. The apparent K_d for DnaA-ATP was 27 nM in the absence and 6.6 nM in the presence of
653 DnaD-his6. In experiments with the DNA fragment from the *yydA* region (D), the calculated Hill
654 coefficient for DnaA-ATP was 6 in the absence of DnaD-his6 and 1 in the presence of DnaD-
655 his6. The apparent K_d for DnaA-ATP was 25 nM in the absence and 5 nM in the presence of
656 DnaD-his6.

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660 **Figure 4. Effects of DnaD and YabA on DnaA binding to DNA are independent of**
661 **ATPase activity.** Binding curves of DnaA mutants defective in ATP hydrolysis, DnaA(D215A)-
662 ATP (A) and ATP binding, DnaA(K157A) (B, C) within the DNA fragment from the *oriC*
663 region. DnaA concentrations tested were: 0, 1, 2, 5, 10, 20, 30, 40, 50, 60, 80, 100, and 200 nM.
664 **A, B.** Binding in the absence (open circles) and presence (filled squares) of DnaD-his6 (300
665 nM).
666 **C.** Binding in the absence (open circles) and presence (filled diamonds) of his6-YabA (700
667 nM). For the DnaA mutant defective in ATP binding, DnaA(K157A), the presence of YabA
668 reduced the Hill coefficient from 5.6 to 2 and the apparent Kd from 36 nM to 14.7 nM.
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