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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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Abstract

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

Introduction

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

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

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

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

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

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

Materials and Methods

Purification of DnaA and DnaD

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

Nucleotide exchange reactions

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

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

Gel Shift Assays

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

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

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

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

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

ATPase Assays

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

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

Nucleotide Binding Assays

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

Results

DnaD does not affect nucleotide exchange for DnaA

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

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

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

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

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

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

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

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

Characterization of DnaA mutants defective in ATPase activity

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

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

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

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

DnaD affects DnaA mutants defective in ATPase activity and nucleotide binding

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

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

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

Discussion

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

Role of DnaD in replication initiation and regulation of DnaA

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

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

It is well established that DnaD is essential and has a positive role in replication initiation. During replication initiation, DnaA-ATP binds cooperatively to many sites in *oriC* (e.g.,18, 39, 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

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

Emerging theme in the regulation of DnaA

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

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

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

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

Diverse mechanisms controlling DnaA in different organisms

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

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

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

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

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

Acknowledgments

We thank T. Baker, A. Olivares, and members of the Grossman lab for useful discussions, and C. Lee, J.L. Smith, C. Seid, M. Laub, and L. Simmons for comments on the manuscript.

This work was supported, in part, by Public Health Service grant GM41934 to A.D.G.

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Table 1. Summary of DNA binding by wild type and mutant DnaA[1].

	DnaA		DnaA(K157A)		DnaA(D215A)	
DnaD[2]	-	+	-	+	-	+
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

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

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

612
613 **Table 2. Summary of ATP hydrolysis and binding by wild type and mutant DnaA[1].**
614

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

615
616
617 [1] The rate of ATP hydrolysis (ATPase) is presented as the number of moles of ATP hydrolyzed
618 per mole of DnaA per hour. Where indicated, the 400 bp DNA fragment from the *oriC* region
619 that was used for the gel shift assays was added (1 μM). The amount of ATP bound (ATP
620 binding) is presented as moles of ATP per mole of DnaA.

621

622

Figure legends

Figure 1. Effect of different concentrations of DnaD on DnaA binding to *oriC*. Representative gel of the radiolabeled DNA probe from the *oriC* region with different amount of DnaD-his, in the absence of DnaA (six lanes on the left) or presence of 10 nM DnaA-ATP (six lanes on the right). Concentrations of DnaD-his are 0 (-), 25, 50, 100, 200, or 300 nM and are indicated below each lane.

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

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

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

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

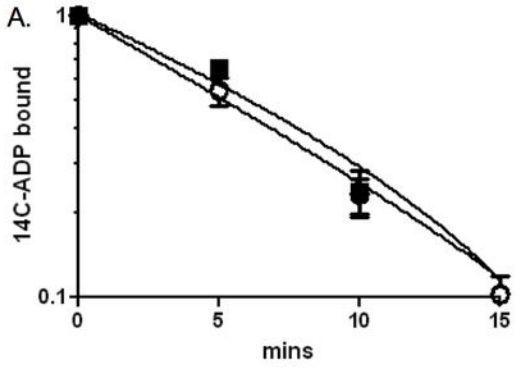
In experiments with the DNA fragment from the *oriC* region (**C**), the calculated Hill coefficient for DnaA-ATP was 8.6 in the absence of DnaD-his6 and 1 in the presence of DnaD-his6. The apparent Kd for DnaA-ATP was 27 nM in the absence and 6.6 nM in the presence of DnaD-his6. In experiments with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

Figure 4. Effects of DnaD and YabA on DnaA binding to DNA are independent of ATPase activity. Binding curves of DnaA mutants defective in ATP hydrolysis, DnaA(D215A)-ATP (**A**) and ATP binding, DnaA(K157A) (**B, C**) within the DNA fragment from the *oriC* region. DnaA concentrations tested were**:** 0, 1, 2, 5, 10, 20, 30, 40, 50, 60, 80, 100, and 200 nM.

A, B. Binding in the absence (open circles) and presence (filled squares) of DnaD-his6 (300 nM).

C. Binding in the absence (open circles) and presence (filled diamonds) of his6-YabA (700 nM). For the DnaA mutant defective in ATP binding, DnaA(K157A), the presence of YabA reduced the Hill coefficient from 5.6 to 2 and the apparent Kd from 36 nM to 14.7 nM.


- 25 50 100 200 300 - 25 50 100 200 300 nM DnaD
-DnaA
+DnaA 10nM


A.
1
14C-ADP bound
0.1
0
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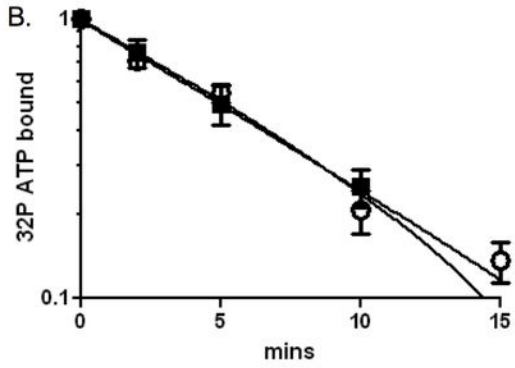
B.
1
32P ATP bound
0.1
0
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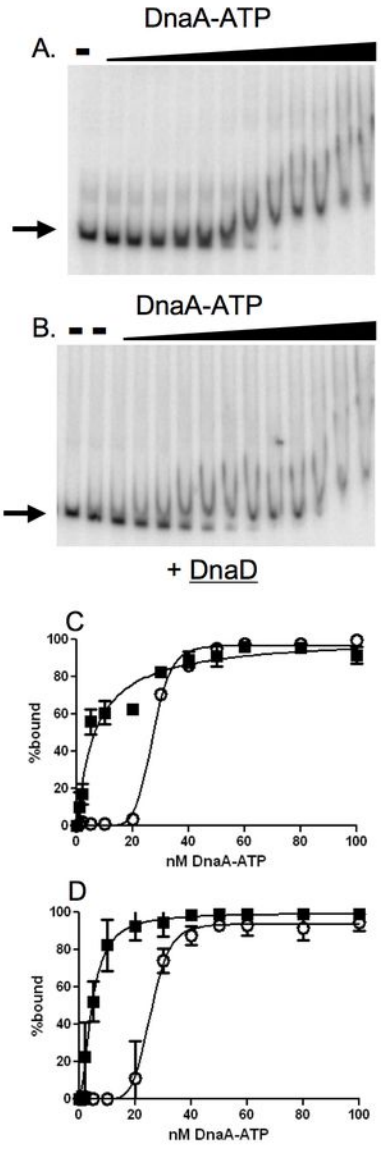
15
mins


DnaA-ATP
A.
DnaA-ATP
B.
+ DnaD
C
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D
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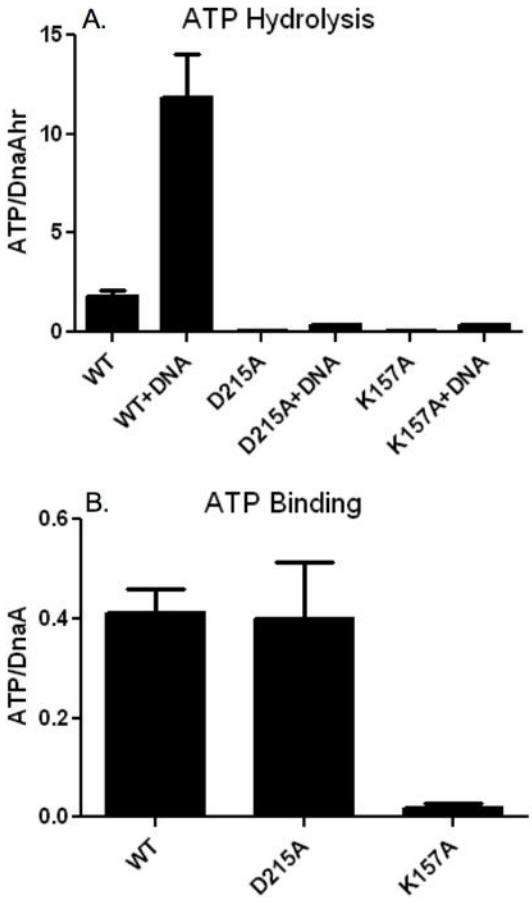
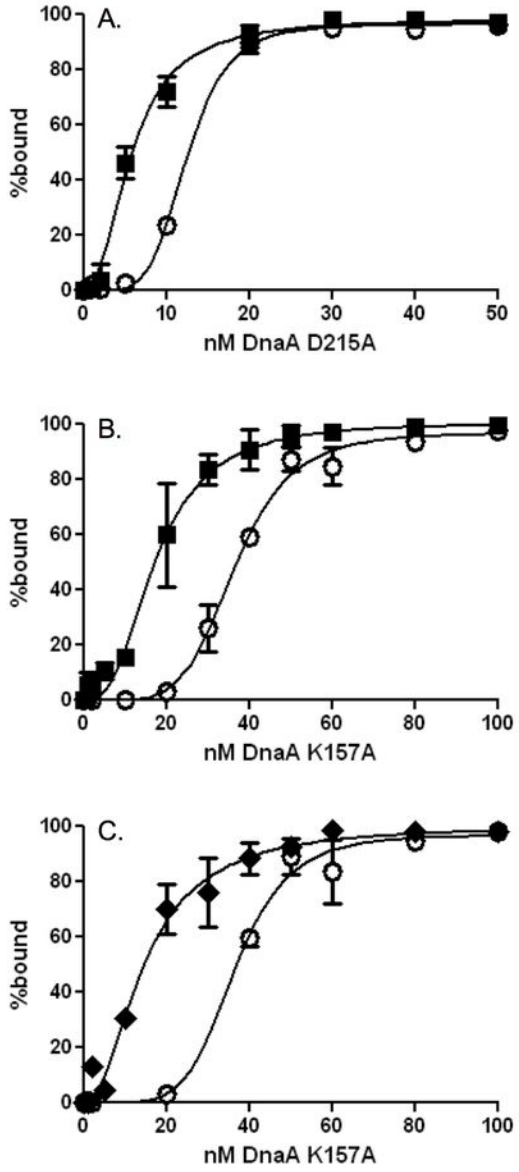
nM DnaA-ATP


A.
ATP Hydrolysis
ATP/DnaAhr
15
10
5
0
WT
WT+DNA
D215A
D215A+DNA
K157A
K157A+DNA
B.
ATP Binding
ATP/DnaA
0.6
0.4
0.2
0.0
WT
D215A
K157A


A.
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