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The *Bacillus subtilis* DnaD protein: a putative link between DNA remodeling and initiation of DNA replication

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Abstract The *Bacillus subtilis* DnaD protein is an essential protein and a component of the *oriC* and PriA primosomal cascades, which are responsible for loading the main replicative ring helicase DnaC onto DNA. We present evidence that DnaD also has a global DNA architectural activity, assembling into large nucleoprotein complexes on a plasmid and counteracting plasmid compaction in a manner analogous to that recently seen for the histone-like *Escherichia coli* HU proteins. This DNA-remodeling role may be an essential function for initiation of DNA replication in the Gram +ve *B. subtilis*, thus highlighting DnaD as the link between bacterial nucleoid reorganization and initiation of DNA replication.
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Keywords: DNA replication; Atomic force microscopy; DnaD; Nucleoid

1. Introduction

Bacterial chromosomal DNA is organized into a high order condensed structure called the nucleoid [1]. Histone-like DNA remodeling proteins form the structural scaffolds of the nucleoid, which is a dynamic nucleoprotein structure that changes during cell growth [2–4]. Such architectural transitions are promoted by growth-phase dependent variations in the histone-like protein (and other DNA-binding proteins) composition of the bacterial proteome [5,6]. Global nucleoid remodeling is associated with crucial biological functions such as regulation of gene expression [7], chromosome segregation [8] and initiation of DNA replication [9,10].

The *Bacillus subtilis* DnaD protein has been implicated in a primosomal DNA initiation cascade that involves DnaB and DnaI and loads the replicative ring helicase, DnaC, onto the *oriC* or non-specific DNA as part of the PriA-mediated restart primosome [11–13]. The essential role of DnaD has also been confirmed genetically in *Staphylococcus aureus* [14]. It interacts with both DnaA [15] and PriA [16] in the early steps of the primosomal cascade to set the stage for helicase loading. The primosomal cascade is sequential in the DnaA (or PriA)–DnaD–DnaB order to set up an initial complex that then recruits DnaC [16]. DnaC exists in a complex with DnaI [17,18], whilst the role of DnaB is still unclear. It may act together with DnaI forming a pair of helicase loaders [18] or alternatively as a membrane attachment protein to regulate initiation of DNA replication by regulating the recruitment of DnaD to the membrane [19,20]. The role of the cell membrane in the initiation of DNA replication in *B. subtilis* is well documented [21], but the precise molecular mechanisms that underpin this function have just started to be unraveled [22].

We provide evidence that in addition to its role in the initiation of DNA replication, DnaD also functions as a global regulator of DNA architecture in a manner similar to that seen for the HU and H-NS proteins of *Escherichia coli*. It forms a variety of higher order nucleoprotein structures that can remodel a supercoiled plasmid into an open circular form. The DNA binding activity of DnaD is resistant to high NaCl concentration and appears to induce the assembly of higher order DnaD oligomers. Finally, DnaD disrupts the stable interaction between the helicase and DnaI in vitro, a property that may be functionally relevant during helicase loading. The role of DnaD in the initiation of DNA replication and its ability to alter the global architecture of DNA make it an obvious candidate for a link between nucleoid reorganization and DNA replication in *B. subtilis*. A speculative model for combining this dual role is presented.

2. Materials and methods

2.1. Protein purifications

The *dnaD* gene was cloned by PCR from *B. subtilis* (strain 168 EMG50) genomic DNA into the *Nde*I-*BamH*I sites of pET22b (Novagen). Overexpression of DnaD was carried out in B834 (DE3) *E. coli* with 1 mM IPTG for 7–8 h from exponentially growing cultures (OD_{600} = 0.6). DnaD overexpressing *E. coli* grew noticeably slower than un-induced cells. Cells were harvested in 20 mM Tris, pH 7.5, 2 mM EDTA, 1 mM DTT, 500 mM NaCl, and 20 w/v sucrose, sonicated incubated for 30 min at 37°C with benzonase (Novagen) and the cell extract was clarified by centrifugation at 17 500 rpm for 30 min. Total protein in the clarified supernatant was precipitated with ammonium sulfate (2.9 mg per 10 ml) and centrifuged at 17 500 rpm, 30 min. The protein pellet was re-suspended in GE (20 mM glycine, pH 9.8, 2 mM EDTA, and 1 mM DTT) buffer until the conductivity reached 40 mS and loaded onto a sourceQ column, pre-equilibrated in GE, at 10–12 mS. The column was washed with GE/100 mM NaCl and DnaD was eluted with a 100–400 mM NaCl gradient in GE. Relevant fractions were pooled and DnaD was precipitated with ammonium sulfate, as described before. The protein pellet was re-suspended in BisTE (Bis-Tris, pH 6.7, 2 mM EDTA, and 1 mM DTT) buffer maintaining the conductivity at 40 mS and loaded onto a HiTrap 5 ml Heparin, pre-

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equilibrated in BisTE, at 15–20 mS. The column was washed with BisTE/100 mM NaCl and DnaD was eluted with 100–400 and stepping up to 700 mM NaCl. The relevant fractions were pooled and loaded onto a superdex S75 gel filtration column pre-equilibrated in TE/300 (20 mM Tris, pH 7.5, 2 mM EDTA, 1 mM DTT, and 300 mM NaCl) buffer. The molecular weight of DnaD is 27.5 kDa and its elution profile indicated an oligomer. Typically, 8–9 mg of DnaD per liter of bacterial culture was obtained. Purity was assessed by SDS–PAGE and concentration was determined spectrophotometrically.

2.2. Sedimentation velocity ultracentrifugation experiments

Analytical ultracentrifugation sedimentation velocity experiments were carried out in a Beckman XL-I analytical ultracentrifuge using cells with two channel centerpieces, loaded into a four-hole AnTi 60 rotor. All experiments were carried out at 40 000 rpm. Cells were scanned every 5 min at 280 nm and the temperature was set at 20 °C. DnaD concentrations were 4.9, 2.4 and 1.2 μM.

Sedimentation coefficient distributions were generated using the program SEDFIT [23] using the c(s) method of analysis. Errors in the distribution were estimated at the 67% and 95% confidence levels using 1000 runs of a Monte Carlo simulation. Frictional coefficients for theoretical distributions of oligomers were estimated using the program SEDNTERP [24].

Experiments designed to observe the DNA dependent behavior of aggregation were carried out with a 5′ HEX labeled 33mer oligonucleotide (5′-HEX-TGCATGCCTGCAGGTCGACTCTAGAGGATCCCC-3′) and data were obtained at a wavelength of 536 nm.

2.3. Gel shift assays

DNA binding reactions were carried out using a radioactively labeled 33mer oligonucleotide (5′-TGCATGCCTGCAGGTCGACTCTAGAGGATCCCC-3′) at 2.5 nM in 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and increasing concentrations of DnaD (123–738 nM). Mixtures were incubated for 10 min at room temperature and complexes were resolved by non-denaturing PAGE. Gels were dried and radioactive bands were visualized using a phosphorimager.

2.4. Analytical gel filtration

Gel filtration experiments were carried out as described before [17] using mixtures of *B. stearothermophilus* DnaB (4.52 μM), DnaD (1.96 M) and DnaI (11.22 μM), as appropriate. Proteins were incubated in 50 mM Tris, pH 7.5, 250 mM NaCl, 1 mM EDTA, and 1 mM DTT for 10 min on ice before fractionated through a superdex S200 10/30 column (Amersham Pharmacia Biotech). Fractions from the peaks were analyzed by SDS–PAGE. Mixtures of DnaB/DnaD/DnaI, DnaB/DnaI, DnaB/DnaD, DnaI/DnaD as well as the individual proteins alone were analyzed in a similar manner.

2.5. Atomic force microscopy imaging

DnaD samples were diluted to between 0.5 and 0.005 μg/ml in 20 mM Tris, pH 7.5, 2 mM EDTA, and 300 mM NaCl. Plasmid pBR322 was purchased from Sigma-Aldrich. Plasmid samples were made up to a stock solution of 10 μg/ml in dH_2O before being diluted further to a desired concentration in 10% v/v PBS containing 0.5mM $MgCl_2$. All solutions were filtered through 0.2 μM pore size filters (Sartorius) prior to use. Protein DNA complexes were prepared by mixing protein samples diluted to 0.25–0.0025 μg/ml in 20 mM Tris, pH 7.5, 2 mM EDTA, 300 mM NaCl, with between 0.5 and 0.15 μg/ml of pBR322 diluted in 10% v/v PBS containing 0.5 mM $MgCl_2$ to generate varying molar ratios of protein to DNA.

For imaging in air, samples of 5–10 μl were incubated with freshly cleaved mica (Agar scientific) for 5–10 s and rinsed with 5 ml of dH_2O and allowed to dry under a gentle flow of nitrogen gas. For imaging in liquid, samples of 30 μl were spotted as a single aliquot onto the underside of a cantilever, placed inside a liquid cell and placed directly onto freshly cleaved mica.

All atomic force microscopy (AFM) imaging was carried out with a Digital Instruments Nanoscope III Mutlimode AFM with a type E scanner (Veeco) in tapping mode. The cantilevers used were silicon tapping probes with a spring constant of 34.4–74.2 N/m (Olympus, OMCL-AC160TS). The tapping set point was adjusted to minimize probe–sample interactions. Images were recorded in both topography and phase modes with a pixel size of 512 × 512, flattened and analyzed with WSxM software 3.0 Beta 2.3 (Nanotec Electronic S.L, Spain).

3. Results and discussion

3.1. DnaD forms higher order oligomers in a concentration dependent manner

DnaD forms mainly dimers or trimers and also a smaller proportion of higher order oligomers [15,16]. A variety of techniques ranging from yeast two hybrid technology, glycerol density and sucrose density centrifugation, to gel filtration and glutaraldehyde fixing were used to establish the DnaD oligomeric state, but in all cases purified DnaD was tagged with either a His-tag or an intein tag to facilitate purification. We purified untagged DnaD and subjected it to analytical gel filtration through a calibrated superdex S-75 gel filtration column. DnaD eluted as an apparent tetramer (data not shown). However, oligomerization estimations by gel filtration may be inaccurate because they depend on a combination of shape and size [25].

Sedimentation velocity experiments over three concentrations revealed sedimentation coefficient distributions consistent with three species in solution that interact in a concentration dependent manner (Fig. 1A). At the lowest concentration (1.2 μM), frictional coefficients expected for each oligomeric species (Table 1) reveal that only a monomer (sedimenting at around 2.0 *s*) has a physically reasonable value ($f/f_0 = 1.488$). Increasing the concentration leads to a second peak at around 4.5 *s* (corresponding to a compact dimer or an

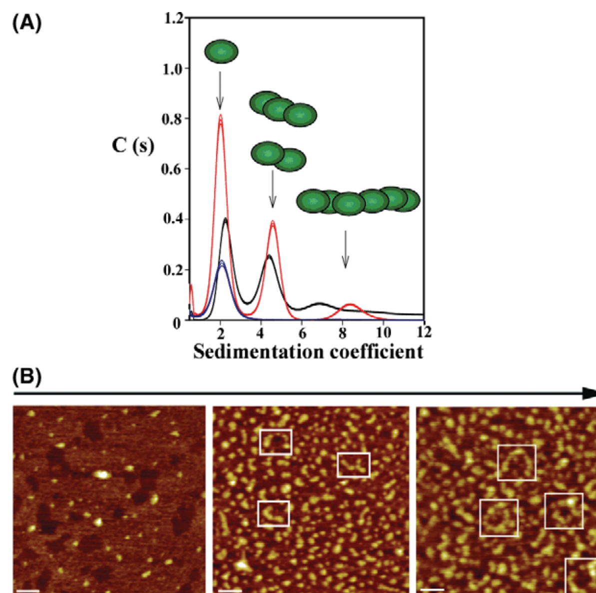

Fig. 1. (A) Analysis of DnaD oligomers by sedimentation velocity ultracentrifugation. Data were analyzed by the c(s) method of Schuck [23] and errors were plotted at the 95% confidence limit. At low 1.2 μM (blue lines), there is a species corresponding to a monomer of DnaD. At 2.4 μM (black) and 4.9 μM (red), species that correspond to dimers or trimers and to higher order aggregates of at least pentamers and hexamers are apparent. (B) AFM images in air of DnaD at increasing concentrations. The arrow indicates increasing concentrations (0.49, 4.9 and 49 nM). The formation of 'chains' of individual DnaD molecules can be seen clearly with higher oligomers evident as concentration increases. Scale bars represent 100 nm.

Table 1
Table of possible frictional coefficients versus species type for DnaD

Species	2*s*	4*s*	6*s*	9*s*
Monomer	1.488			
Dimer	2.363	1.182		
Trimer	3.059	1.549	1.032	
Tetramer	3.750	1.875	1.250	
Pentamer	4.352	2.176	1.451	
Hexamer	4.914	2.457	1.638	1.092
Heptamer	5.446	2.723	1.815	1.210
Octamer	5.953	2.977	1.984	1.323
Nonamer	6.440	3.220	2.147	1.431
Decamer	6.908	3.454	2.3027	1.535

Physically unreasonable values are in grey. Frictional coefficients are calculated using the program SEDNTERP [24]. Higher values indicate a more elongated molecule.

extended trimer; see Table 1), with higher order species in excess of 6 *s* (pentamer and hexamer, as judged by reasonable frictional coefficients) also present. Separation of the species into distinct sedimentation coefficient distributions also reveals a specific type of behavior. It is known both theoretically and experimentally that it is impossible to separate out a rapidly reversible monomer/dimer equilibrium using sedimentation analysis; a weighted average distribution is produced [26]. If the second species is a dimer in the presented studies, this would mean that the rate of dimerization is slow on the timescale of sedimentation. A second possibility is that the samples undergo irreversible aggregation, and hence separate into distinct species as there is no appreciable off rate of aggregation. This, however, is unlikely as dilution of the sample for sedimentation yields only one small species: irreversible aggregation would yield the same proportion of species, irrespective of concentration, upon dilution. A third scenario is that the species are in a monomer/*n*-mer rapidly reversible equilibrium, where $n > 2$. Theory and experiment show that separation of species is physically possible if one accepts that there is a cooperative transition from monomer to trimer or tetramer. These would be present as highly extended conformations (as judged by physically reasonable frictional coefficients). AFM images of DnaD at various concentrations verified the formation of higher extended oligomeric species at high concentrations, giving weight to this third hypothesis. Elongated strand-like and also circular and semi-circular structures were visible at high concentrations (Fig. 1B). The smallest features representing DnaD monomers were approximately 25 nm in their longest dimension and larger features of 50 nm (dimers), 79 nm (trimers) and also longer oligomers were also apparent.

3.2. DnaD-mediated nucleoprotein complexes

We proceeded to investigate the binding of DnaD to DNA with a HEX-tagged synthetic oligonucleotide so as to follow independently the sedimentation of the protein (280 nm) or the Hex-oligonucleotide (536 nm). We observed that upon addition of the oligonucleotide to DnaD, an extremely large nucleoprotein complex was formed (1–2 million Daltons) that was impossible to investigate with analytical ultracentrifugation. Instead, the DNA binding activity was investigated by gel shifts using the same untagged but radioactively labeled oligonucleotide (Fig. 2A). DnaD bound to the oligonucleotide producing a clear shifted band and as the DnaD concentration increased a streak started to appear higher up the gel until the shifted band ended up in the well at the highest DnaD concentration. The remarkable observation was that additional DnaD molecules were directed exclusively to the initial nucleoprotein complex rather than to the free oligonucleotide, although at very high DnaD concentrations all of the substrate could be shifted into the well (data not shown). The stoichiometry of DnaD versus oligonucleotide in the initial complex is not certain, but comparisons with the shifted bands from other proteins implied that it may be either two or three DnaD molecules bound to one oligonucleotide (data not shown). The simplest interpretation of these data is that once DnaD forms an initial nucleoprotein complex, it acts as the nucleus to recruit additional DnaD molecules eventually forming a very large complex. Furthermore, the interaction of DnaD with DNA is salt resistant, with stable DnaD–DNA complexes observed at NaCl concentrations as high as 800 mM (data not shown). This is a feature that has been observed in other nucleoid associated proteins such as the H-NS protein in *E. coli* [27].

3.3. AFM imaging of DnaD-mediated nucleoprotein complexes

We investigated the nature of the nucleoprotein complexes by AFM in liquid and air using pBR322 plasmid. We analyzed 149 images of DnaD–pBR322 complexes and observed that when DnaD binds to pBR322 it opens up the supercoiled plasmid into an open circular shape (Fig. 2B and C). This remarkable global structural effect on pBR322 resembles a similar effect observed for the HU binding to supercoiled pUC19 that reflects its ability to constrain DNA supercoils

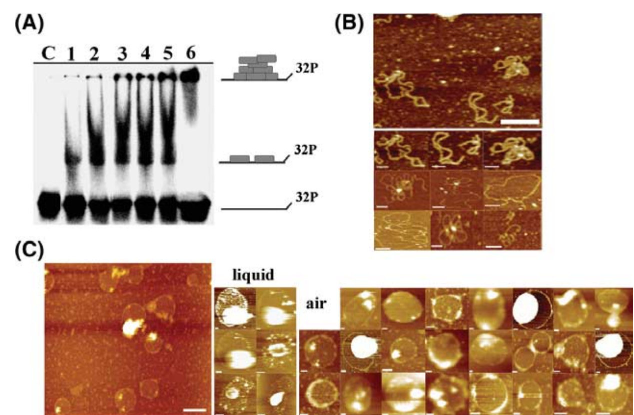

Fig. 2. (A) Gel shift analysis of DnaD binding to an oligonucleotide at increasing concentrations. Binding reactions were carried out at 2.5 nM oligonucleotide substrate and 123, 246, 369, 492, 615 and 738 nM DnaD, lanes 1–6, respectively. Lane C shows the control radioactively labeled oligonucleotide. (B) Control AFM images of naked pBR322 plasmid in air. A typical field view and a few representative individual pBR322 molecules adopting a variety of compact supercoiled structures are shown. The scale bars in the field view and individual images represent 250 and 50 nm, respectively. (C) AFM topography images of DnaD–pBR322 complexes. A typical field view in air and a few representative individual complexes in air and liquid are shown. All complexes show the DNA in an open circular conformation around the periphery of the circular structures with DnaD molecules forming large scaffolds inside the circles. In several images, DnaD also appears to form large foci on the pBR322 plasmid around the periphery of the circles. The scale bars in the field views and individual images represent 1 μm and 100 nm, respectively.

[28]. HU counteracts the H-NS mediated global DNA compaction effect in *E. coli*. A similar structural transition from a supercoiled to a relaxed state has also been observed in the case of the replication initiator RepE54 protein encoded by the mini-F plasmid and in this case this relaxation is not the result of DNA strand break or local melting of the double strand [29]. Whether the DnaD-induced relaxation is the result of strand break or not is not known and is the subject of current investigations in our laboratory. Two remarkable features were prominent in the DnaD–pBR322 structures. In many images, recorded in both air and liquid, there was a build up of DnaD inside the circular plasmid and also at foci on the pBR322 plasmid (Fig. 2C). Similar features have been observed for the HU protein [28]. Our data thus suggest that DnaD can impose global DNA architectural transitions, a property that may also be biologically important during primosomal assembly. DnaD may be forming large scaffolds for global (or localized in the vicinity of *oriC*) remodeling of the bacterial nucleoid rendering it replication proficient, while at the same time attaching the bacterial chromosome to the membrane via its interaction with the membrane associated DnaB primosomal protein (see below). In this respect, the DnaD–DnaB interaction may be functionally analogous to the RacA–DivIVA interaction near the *oriC* that attaches the chromosome to the membrane during *B. subtilis* sporulation [30].

3.4. DnaD disrupts the helicase–DnaI complex in vitro

The precise role of DnaD in primosomal assembly is still unknown. It appears to act early on in primosomal assembly [15,16,31], while its interaction with the membrane associated DnaB protein may provide a mechanism for regulating the initiation of DNA replication in *B. subtilis* [20]. We have previously reported a stable interaction in vitro between the putative *B. subtilis* helicase loader DnaI and the main replicative ring helicase DnaB from *B. stearothermophilus* [17]. In order to avoid confusion with the nomenclature, we should make it clear that the main replicative helicase in *B. subtilis* is known as DnaC and is homologous to *B. stearothermophilus* DnaB (82% identity, 92% similarity). We investigated the effect of DnaD on the DnaB–DnaI complex and found that in vitro DnaD disrupts the complex (Fig. 3A). Incubating all three DnaD, DnaB, and DnaI proteins together disrupted the DnaB–DnaI interaction whilst, no complex could be detected between either DnaB–DnaD (Fig. 3B) or DnaI–DnaD (data not shown). The biological significance of this observation may be relevant to the process of helicase loading. The interaction of the DnaI–DnaC complex with DnaD may facilitate DnaC release and loading onto the DNA.

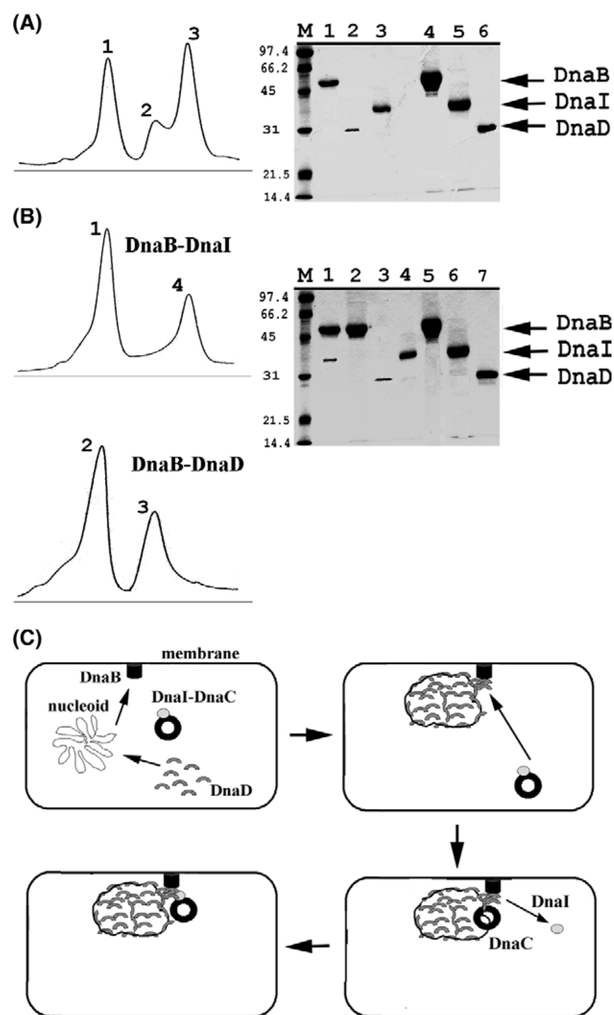

Fig. 3. (A) Gel filtration analysis of a DnaB/DnaI/DnaD mixture showing that no complexes can be detected when all three proteins are incubated together. (B) Gel filtration analysis of DnaB/DnaI and DnaB/DnaD mixtures showing the formation of a DnaB–DnaI complex but no stable complex between DnaB and DnaD. In a similar manner DnaI did not form a stable complex with DnaD (data not shown). In all cases, samples from the peaks were analyzed by SDS–PAGE and the numbers on the peaks correspond to the numbers of the lanes. Lanes M, 4, 5, 6 indicate molecular weight markers and controls of DnaB, DnaI and DnaD proteins, respectively. In both panels A and B, DnaB refers to the replicative helicase from *B. stearothermophilus*. (C) Speculative model for the coordination of nucleoid remodeling and initiation of DNA replication in *B. subtilis*. Indicated stoichiometries of complexes and the membrane attachment site are not precise and have not been established. The sizes of various components depicted are not drawn to size. Although DnaD is depicted to remodel the whole of the nucleoid, also localized remodeling in the vicinity of the *oriC* may be the case. DnaB refers to the primosomal protein from *B. subtilis*.

4. Model

We have provided evidence that DnaD forms large nucleoprotein assemblies affecting global DNA architecture and that it disrupts a complex between a ring helicase and a helicase loader. We propose that DnaD is the link between nucleoid reorganization and initiation of DNA replication in *B. subtilis* (Fig. 3C). It has been suggested previously that initiation of DNA replication is regulated by recruitment of DnaD to the membrane [20]. Maintaining DnaD in the cytoplasm prevents excessive over-initiation and its regulated recruitment to the membrane by DnaB (referring to the primosomal protein from *B. subtilis* and not the replicative helicase from *B. stearothermophilus*) activates initiation of DNA replication [20]. DnaD may bind to chromosomal DNA forming a scaffold to open up the nucleoid (either locally in the vicinity of *oriC* or globally) and render it replication proficient. DnaC is recruited from the DnaI–DnaC complex in the cytoplasm to the membrane attachment site by a direct interaction with DnaB [16–18]. DnaD

will then disrupt the DnaI–DnaC complex thus facilitating its loading onto the *oriC*. DnaD provides a possible link between nucleoid reorganization and initiation of DNA replication. Its interaction with the membrane associated DnaB may be the prime regulatory event that couples chromosomal remodeling to DNA replication.

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