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S. Marsin, S. Mcgovern, S.D. Ehrlich, Claude Bruand, Patrice Polard

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Early Steps of *Bacillus subtilis* Primosome Assembly*

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Stéphanie Marsin, Stephen McGovern, S. Dusko Ehrlich, Claude Bruand, and Patrice Polard‡

From the Laboratoire de Génétique Microbienne, INRA, Domaine de Vilvert, 78352 Jouy en Josas Cedex, France

Primosomes are nucleoprotein assemblies designed for the activation of DNA replication forks. Their primary role is to recruit the replicative helicase onto single-stranded DNA. The "replication restart" primosome, defined in *Escherichia coli*, is involved in the reactivation of arrested replication forks. Binding of the PriA protein to forked DNA triggers its assembly. PriA is conserved in bacteria, but its primosomal partners are not. In *Bacillus subtilis*, genetic analysis has revealed three primosomal proteins, DnaB, DnaD, and DnaI, that have no obvious homologues in *E. coli*. Interestingly, they are involved in primosome function both at arrested replication forks and at the chromosomal origin. Our biochemical analysis of the DnaB and DnaD proteins unravels their role in primosome assembly. They are both multimeric and bind individually to DNA. Furthermore, DnaD stimulates DnaB binding activities. DnaD alone and the DnaD/DnaB pair interact specifically with PriA of *B. subtilis* on several DNA substrates. This suggests that the nucleoprotein assembly is sequential in the PriA, DnaD, DnaB order. The preferred DNA substrate mimics an arrested DNA replication fork with unreplicated lagging strand, structurally identical to a product of recombinational repair of a stalled replication fork.

Chromosomal replication depends on the initial assembly of replication forks at defined origins and on the re-assembly of the ongoing replication forks in case of their arrest. Extensive genetic and biochemical studies in the Gram-negative bacterium *Escherichia coli* have unraveled two mechanisms for activating and reactivating DNA replication. The first occurs at the unique origin (*oriC*) of the circular chromosome and ensures the accurate timing of replication within the cell cycle (for reviews see Refs. 1 and 2). The triggering factor of this highly regulated initiation process is the DnaA protein, which specifically recognizes *oriC*. The second reactivation process has been described more recently (3–7). Its initiator is the PriA protein, which promotes replication restart by binding to particular DNA structures (8).

The replication routes opened by DnaA and PriA in *E. coli* have been reproduced *in vitro* with model DNA substrates and purified protein components (for review see Ref. 9). In both cases, these specialized proteins promote the recruitment of the replicative DnaB helicase on ssDNA.[1] This enzyme can be viewed as the keystone of the replication machinery, because it melts the DNA double helix, is tightly associated with the τ subunit of the DNA polymerase III holoenzyme, and interacts distributively with the DnaG primase that primes DNA synthesis (1). The proteins required for the recruitment of the DnaB-DnaG pair are known as primosomal proteins, and the nucleoprotein complex resulting from their assembly is designated the primosome. The DnaA-dependent primosome forms at *oriC* and includes DnaA, DnaB, and DnaG. DnaB loading onto ssDNA is assisted by the DnaC primosomal protein (10). The PriA-dependent primosome assembles on two distinct DNA substrates recognized by PriA. The first is a sequence designated *pas* (for primosome assembly site), discovered in the genome of the bacteriophage ϕX174 and required for the conversion of circular ssDNA to dsDNA (8). The second is a "D-loop" structure, which mimics the proposed product of recombinational repair of a stalled replication fork (4, 5, 11–13). The PriA-dependent primosome, also designated the "replication restart primosome" (7), contains the proteins PriA, PriB, PriC, and DnaT, which assemble sequentially in the order given to recruit the helicase DnaB, also with the help of DnaC, and the primase DnaG. PriA is a helicase moving on ssDNA with the 3′ → 5′ direction (8). Its DNA melting activity is dispensable for *in vitro* primosome assembly on the *pas* sequence and on an artificial D-loop structure (13, 14) but is essential on a nearly fully double-stranded forked DNA (15). In this particular case, it has been proposed that the PriA helicase activity generates the ssDNA substrate required for the installation of the DnaB helicase. Nevertheless, the helicase activity of PriA appears dispensable for its pivotal role in the "coordinated processing of damaged replication forks" (5, 14, 16).

DnaA, PriA, the replicative helicase, and the primase are conserved in bacteria, arguing for generalization of the *E. coli* DNA replication initiation schemes to these microorganisms. Genetic and biochemical analyses conducted on DnaA and PriA of the Gram-positive bacterium *Bacillus subtilis* have confirmed their primosomal function (17).[2] However, there is ample evidence for a striking difference in the composition of *B. subtilis* primosomes relative to their *E. coli* counterparts. First, no obvious homologues of PriB, PriC, and DnaT are encoded by the *B. subtilis* genome (19). Second, three *B. subtilis* essential genes, *dnaB*,[3] *dnaD*, and *dnaI*, are required for the PriA-dependent primosome activity (20). Two of these, *dnaB* and *dnaD*, encode proteins that have no homologues in *E. coli*. The product of the third, DnaI, has a marginal sequence similarity with the *E. coli* DnaC (21, 22), interacts with the *B. subtilis* DnaC[3] helicase and could be the

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‡ Member of the CNRS staff. To whom correspondence should be addressed. Tel.: 33 1 34 65 25 08; Fax: 33 1 34 65 25 21; E-mail: polard@biotec.jouy.inra.fr.

[1] The abbreviations used are: ssDNA, single-stranded DNA; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; dsDNA, double-stranded DNA.

[2] P. Polard, S. Marsin, S. McGovern, M. Velten, D. Wigley, S. D. Ehrlich, and C. Bruand, submitted for publication.

[3] Note that the *B. subtilis* DnaB protein is distinct from the *E. coli* DnaB helicase. The *B. subtilis* helicase is named DnaC.

counterpart of the *E. coli* helicase loader (23). However, suppressors of PriA deficiency in *E. coli* map in the C-terminal moiety of DnaC (24), whereas in *B. subtilis* they map in the C-terminal part of DnaB and not in DnaI (25). These observations point to the intriguing differences between the *E. coli* and *B. subtilis* systems, further underlined by the fact that none of the PriA-dependent primosome components is required for initiation of DNA replication in *E. coli*, whereas all of the components but PriA are essential for this process in *B. subtilis* (25).[2]

We report an *in vitro* study of the assembly of the *B. subtilis* replication restart primosome. We have purified the DnaB and DnaD proteins and studied their individual and concerted activities in the presence of various DNA substrates related to arrested DNA forks. We present evidence that DnaB and DnaD are multimeric and display affinity for DNA. Furthermore, DnaD stimulates the binding of DnaB to ssDNA and to DNA molecules carrying a 5′ ssDNA tail. In the presence of PriA, which is a better ssDNA and forked DNA-binding protein, DnaD alone and the DnaD/DnaB pair assemble specifically and preferentially on the fork structures with 5′ ssDNA tails. These molecules include the correct ssDNA strand for the proper loading of the replicative helicase. Therefore, we suggest that the assembly of PriA, DnaD, and DnaB promote loading of the replicative helicase DnaC, possibly assisted by DnaI, at the arrested replication forks. Finally, we speculate that DnaD and DnaB play similar roles in the DnaA-dependent primosome that promotes initiation of chromosomal replication.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media—*E. coli* strain MiT898 (26) was used for all plasmid constructions except that of the $PriA_{Ec}$ expressing plasmid, for which the *E. coli* strain JC19008 *priA2*::*kan* harboring the suppressive mutation *dnaC809* (24) was used. Strains were grown in Luria broth supplemented with 25 $mg \cdot ml^{-1}$ of thymine (27) and ampicillin (100 $\mu g \cdot ml^{-1}$). $PriA_{BS}$ and DnaB overexpression was carried out in MiT898, $PriA_{Ec}$ in JC19006 (24), and DnaD in *E. coli* strain B834(DE3) (28).

DNA Manipulations, Enzymes, Nucleotides, Oligonucleotides, and Compounds—All standard DNA manipulations were carried out as described in Sambrook *et al.* (29). Radiolabeled nucleotide [γ-^{32}P]ATP was from ICN (specific activity of 4500 Ci $mmol^{-1}$). Oligonucleotides were from Genset (France). All enzymes used for DNA cloning and modification were purchased from Roche Molecular Biochemicals or New England Biolabs and used as recommended. Chitin resin was from New England Biolabs.

Plasmid Constructions—For constructing pSMG24, $priA_{Ec}$ was PCR-amplified from plasmid pAPJ42[2] with Osmg1-Osmg49 as primers, cleaved by *Nde*I, and inserted between the *Nde*I and *Sap*I sites of pCYB1 (New England Biolabs). The *SapI* site was filled in with Klenow polymerase prior to ligation. Most of the cloned $priA_{Ec}$ gene was exchanged by that of plasmid pAPJ42[2] by *Nde*I/RsrII restriction. The remaining sequence of $priA_{Ec}$ carried by pSMG24 originating from the PCR fragment has been verified as follows: Osmg1, 5′ GAGCGGATAACAATTTCACACAGG 3′; Osmg49, 5′ ACCCTCAATCGGATCAACATCCA3 ′.

For constructing pSMG6, *dnaB* was PCR-amplified from chromosomal DNA of *B. subtilis* strain 168 with Osmg9-Osmg10 as primers, cleaved by the *Nde*I and *Sap*I, and inserted in pCYB1 cleaved by the two enzymes. *dnaB* sequence cloned in pSMG6 has been verified as follows: Osmg9, 5′ GAATTCCATATGGCTGACTATTGGAAAGAT 3′; Osmg10, 5′ GAATTCGCTCTTCCGCAATAGGCAGAGTATTTTTTCAGTT 3′.

Construction of pSMG22 was in two steps. First, *dnaD* was PCR-amplified from chromosomal DNA of *B. subtilis* strain 168 with Osmg13 and Osmg14 primers, digested with *Nde*I and *Sap*I, and inserted in pCYB1 cleaved by the two enzymes. This generated plasmid pSMG8. The sequence of *dnaD* cloned in pSMG8 has been verified. DnaD expression from this plasmid in MiT898 strain gave mainly the expected protein accompanied, however, by two proteins originating from internal initiation of translation within *dnaD* (not shown). To reduce synthesis of these two products, DnaD translation was placed under the control of the stronger translational signals of the ϕ10 gene carried by the pTYB1 vector (New England Biolabs). The exchange (using *Nde*I-*Sap*I restriction), which gave pSMG22, placed the expression of DnaD under the transcriptional control of the T7 RNA polymerase. As expected, the synthesis of the shorter DnaD derivatives was highly diminished. Osmg13, 5′ GAATTCCATATGAAAAAACAGCAATTTATTG 3′; Osmg14, 5′ GAATTCGCTCTTCCGCATTGTTCAAGCCAATTGTAAAAAG 3′.

$PriA_{Bs}$, $PriA_{Ec}$, DnaB, and DnaD Purification—All protein manipulations were at 4 °C. The purification columns used were all Hitrap from Amersham Pharmacia Biotech, as was the desalting PD10 column.

The four proteins were purified from fusion proteins with the *in vitro* excisable Intein-Chitin-Binding-Domain Tag (New England Biolabs). $PriA_{Bs}$ purification will be described elsewhere.[2] The same procedure was used for $PriA_{Ec}$, except that expression was in strain JC19008 carrying plasmid pSMG24.

DnaD overexpression was in *E. coli* strain B834 (DE3), cultivated at 30 °C in 1 liter of LB until A_{600} reached ~1, and then transferred to 24 °C for 4 h in the presence of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside to induce protein expression. Cells were harvested, resuspended in 12 ml of ice-cold HEN_{500}-T buffer (20 mM Hepes, 0.1 mM EDTA, 500 mM NaCl, 0.1% Triton X-100, pH 7.6), and broken by sonication (Vibracell 72408 sonicator from Bioblock was used). The lysate was centrifuged at 20,000 × *g* for 1 h, and supernatant was loaded onto 2 ml of chitin beads and washed with HEN_{500}; the protein was separated from intein by addition of 30 mM dithiothreitol. After overnight incubation, the eluted proteins were further purified by conventional chromatography. DnaD was diluted three times with buffer Q50 (buffer Q: 50 mM Tris, 0.1 mM EDTA, 1 mM dithiothreitol, pH 8, supplemented with 50 mM NaCl) and applied to a Q column equilibrated with the same buffer. The flow-through containing DnaD was bound to a heparin column equilibrated with buffer Q50. DnaD was eluted with a linear NaCl gradient in buffer Q and collected at 250 mM NaCl.

To produce DnaB, *E. coli* strain MiT898 harboring pSMG6 was grown at 30 °C for 15 h without isopropyl-1-thio-β-D-galactopyranoside induction. The first steps of DnaB purification were as for DnaD, except that the sonication was carried out in L buffer (20 mM Tris, 500 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, pH 8). DnaB was eluted from chitin beads, precipitated with 45% saturation ammonium sulfate, resuspended in buffer Q50, and desalted on PD10 column equilibrated in the same buffer. DnaB was bound to a Q column and washed with Q buffer supplemented with 120 mM NaCl, and DnaB was eluted in Q buffer with 250 mM NaCl. DnaB-containing fractions were diluted twice with Q50 and loaded on a heparin column. DnaB was eluted in Q buffer supplemented with 400 mM NaCl, desalted on PD10 in Q50, and finally bound on a SP column. DnaB was eluted with a linear NaCl gradient in Q buffer and collected at 400 mM NaCl. A polypeptide of about 110 kDa, co-purified with DnaB, represented less than 2% of the total protein preparation. This unknown protein could not be separated from DnaB either by gel filtration or by sucrose gradient centrifugation at high ionic strength (data not shown), suggesting a stable interaction of DnaB with an unknown *E. coli* protein.

Purified $PriA_{Bs}$, $PriA_{Ec}$, DnaB, and DnaD proteins were stored at −20 °C in the presence of 50% glycerol. They kept their characteristic "protein-DNA" and "protein-protein" interacting activities for at least 6 months.

Standard Protein Manipulations—Protein concentrations were estimated by Bradford analysis using the Bio-Rad Protein Assay. Electrophoretic analysis of protein samples was carried out by the SDS-PAGE method of Laemmli (30). Coomassie staining of the gels was performed as described before (29).

Molecular Weight Estimation—Experiments to determine Stokes radius (Å) and the sedimentation coefficient (*s*) were carried out in TM buffer (20 mM Tris, 0.1 mM EDTA, 50 mM NaCl, 0.01% Triton X-100, pH 8). The Stokes radius of the different proteins was determined using a Superose 12 HR 10/30 gel filtration column (Amersham Pharmacia Biotech). The column was standardized with proteins of known Stokes radius: thyroglobulin (669 kDa, 85 Å), ferritin (444 kDa, 61 Å), catalase (232 kDa, 52 Å), bovine serum albumin (67 kDa, 35 Å), and ovalbumin (43 kDa, 30 Å). Sedimentation coefficients were estimated using a 12-ml linear 5–24% sucrose gradient centrifuged for 16 h at 38,000 rpm in a Beckman SW40 rotor. Internal standards included for calculating sedimentation coefficients were catalase (11.3 *s*), aldolase (7.4 *s*), bovine serum albumin (4.2 *s*), and ovalbumin (3.5 *s*). Sedimentation coefficients, Stokes radius, and molecular weights were estimated as previously described (31), using the equation from Siegel and Monty (32).

DNA Substrates—Nine DNA substrates were used for the gel shift assays as follows: a ssDNA fragment of 90 nucleotides designated Ost4; five forked DNA molecules FI, FII, FIII, FIV, and FV; three dsDNA

substrates harboring a ssDNA tail O-5′, O-3′, and O-3′40; a dsDNA of 98-base ds98.

The forked and tailed molecules were prepared by annealing of the following purified oligonucleotides: Ost4, 5′ GCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTG 3′; Ost6, 5′ CAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGCCAGCCACAGTCGTGGCCATTGCCATATGGCCCG 3′; Ost7, 5′ GGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGC 3′; Ost9, 5′ CAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGCCAGCCACAGTCGTGGCCATTGCCATATGGCCCGGTCTAC 3′; Ost24, 5′ CGGGCCATATGGCAATGGCCACGACTGTGGCTGGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGC 3′; Ost25, 5′ CGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTG 3′; Ost26, 5′ CAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCG 3′; and Oflo7, 5′ CGGGCCATATGGCAATGGCCACGACTGTGGCTGG 3′. FI results from the annealing of Ost4 and Ost6; FII from Ost4, Ost6, and Oflo7; FIII from Ost4, Ost6, and Ost7; FIV from Ost4, Ost6, Ost7, and Oflo7; and FV from Ost4, Ost6, and Ost24. O-5′ results from the annealing of Ost4 and Ost26; O-3′ from that of Ost6 and Ost25; and O-3′40 from that of Ost9 and Ost25.

Prior to annealing, one oligonucleotide was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs as recommended by the supplier). Ost4 was labeled prior to construction of FI, FII, FIII, FIV, and O-5′; Ost24 prior to construction of FV; Ost6 prior to construction of O-3′; and Ost9 prior to construction of O-3′40. Unlabeled oligonucleotides were added in a 5-fold excess in the annealing buffer A (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8). The mixture was heated at 95 °C for 5 min and cooled slowly (~3 h) to room temperature. The annealed products were loaded on an 8% PAGE (30:1) and electrophoresed in 1× TBE buffer. The properly assembled products were eluted in buffer E (10 mM Tris, 1 mM EDTA, 0.2% SDS, 300 mM NaCl, pH 8) overnight at 37 °C and recovered by ethanol precipitation. They were resuspended and stored in buffer A. The correct oligonucleotide assembly was ascertained by radiolabeling of the final substrates and separation on a sequencing gel in denaturing conditions.

ds98 was made by PCR amplification of pUC19 plasmid with 1201 and 1211 primers (New England Biolabs) using *Taq* polymerase (Promega), and the obtained fragment was blunted with Klenow fragment and T4DNAPol (Promega). DNA was loaded on an 8% polyacrylamide (30:1) gel, migrated in 1× TBE buffer, and purified by passive elution (33). ds98 was then labeled as Ost4.

The concentrations of DNA substrates were estimated by monitoring the specific activity of radiolabeled Ost4 and the final ratio activity of purified substrates.

Gel Mobility Shift Assay—Different proteins were incubated at concentration indicated in the figures with labeled DNA substrates (0.1 nM) in 20 μl of R buffer (50 mM Hepes, 1 mM dithiothreitol, 1 mM EDTA, 0.1 mg/ml bovine serum albumin, 50 mM NaCl, 12.5% glycerol, pH 7.4) at 30 °C for 10 min. At the end of incubation, 5 μl of loading buffer (50% glycerol, 0.4% cyanol, 0.1 mg/ml bovine serum albumin) were added, and the samples were loaded on a 5% polyacrylamide (30:1) gel containing 5% glycerol and 0.25× TBE and migrated in 0.25× TBE. After electrophoresis, gels were dried under vacuum, revealed with a Storm Apparatus (Molecular Dynamics), and the results were quantified with the ImageQuant Software and the apparent K_D value was determined according to Riggs *et al*. (34).

RESULTS

B. subtilis DnaD and DnaB Proteins Are Multimeric—PriA$_{Bs}$ (92 kDa) has been previously overproduced in *E. coli* and purified.[2] By using a similar procedure, DnaB (55 kDa), DnaD (28 kDa), and PriA$_{Ec}$ (82 kDa) were purified to a level exceeding 95% (Fig. 1; see "Experimental Procedures").

The native molecular weights of PriA$_{Ec}$, PriA$_{Bs}$, DnaB, and DnaD have been estimated by the combination of the Stokes radius and sedimentation coefficient values, determined by gel filtration chromatography and sucrose gradient centrifugation, respectively. These experiments have shown that the four purified polypeptide preparations were composed of a single homogeneous protein form. PriA$_{Bs}$ and PriA$_{Ec}$ were monomeric, as it was already known for PriA$_{Ec}$ (Table I; see Ref. 35). In contrast, DnaB and DnaD were multimeric, apparently self-associating as a tetramer and between a dimer and a trimer,

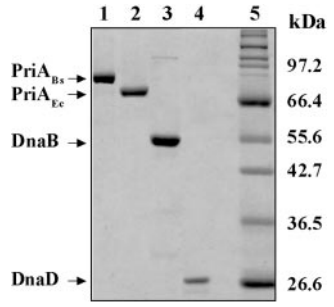

FIG. 1. **Primosomal proteins PriA$_{Ec}$, PriA$_{Bs}$, DnaD, and DnaB.** Coomassie Blue-stained 10% SDS-polyacrylamide gel of the four primosomal proteins used in this study, PriA$_{Bs}$ (*lane 1*), PriA$_{Ec}$ (*lane 2*), DnaB (*lane 3*), and DnaD (*lane 4*). Standards are shown in *lane 5*, and their molecular masses are indicated on the *right* of the gel.

respectively (Table I). These results are consistent with the recent report about DnaD multimerization state as a dimer (36), and the multimerization of DnaB was predictable from reported double-hybrid interaction (23). However, it should be noted that seemingly incoherent results were obtained for DnaB, which appeared to be trimer by sedimentation coefficient and close to a heptamer by the Stokes radius. The Siegel and Monty equation (32), which includes both the Stokes radius value and the sedimentation coefficient values, indicates that DnaB is a tetramer. Glutaraldehyde cross-linking of DnaB generated a homogeneous form composed of at least four monomers, which had a sedimentation coefficient identical to that of untreated DnaB (data not shown). We conclude that DnaB is a tetramer, of the shape greatly different from globular.

Gel Shift Analysis of DNA Binding Activities of PriA$_{Bs}$, DnaB, and DnaD Proteins—To determine whether the three *B. subtilis* proteins interact with each other, the appropriate mixtures were made with pairs of proteins and analyzed by gel filtration, sucrose gradient sedimentation, and cross-link experiments. No protein-protein interaction was detected, even at low ionic strength (data not shown). We then tested whether the three proteins interact in the presence of different synthetic forked DNA. These substrates supposedly mimic the chromosomal sites targeted by PriA to promote DNA replication restart. Before carrying out the mixing experiments, proteins were individually assayed with various DNA substrates.

The substrates used were the 90-nucleotide single-stranded oligonucleotide Ost4 and the five Y-shaped DNA structures presented in Fig. 2. FI, FII, FIII, and FIV mimic particular forks that may be encountered during chromosomal DNA replication. FI represents a half of an open duplex, which could form at *oriC* by the action of DnaA. FII, FIII, and FIV have a sequence identical to FI but with the leading, the lagging, or both strands replicated, respectively. FII mimics the forked structure on which PriA$_{Ec}$ promotes replication *in vitro* (13). A double-stranded DNA of 98 base pairs (ds98) and the FV fork, which is identical to FIV but with no strand interruption, were also used as controls and known to interact poorly with PriA$_{Ec}$ (12). We have observed previously a stable and specific binding of PriA$_{Bs}$ to an artificial D-loop structure.[2] Here we observed PriA$_{Bs}$ binding to all substrates with almost the same apparent K_D, except to ds98 and to FV (Table II and Fig. 2*C*). Two nucleoprotein complexes were detected, apparently formed successively with increasing amounts of PriA$_{Bs}$ (Fig. 2*A*). Low amounts of a third complex were detected with FI, FIII, and FIV at the highest protein concentration. The lack of substrate preference of PriA$_{Bs}$ could be due to the high affinity of the protein for ssDNA and to the low ionic strength used in our experiments. However, the efficient binding to FIV, which is fully double-stranded, demonstrates recognition of nicked

TABLE I
Physical characteristics of PriA$_{Bs}$, PriA$_{Ec}$, DnaB, and DnaD

The Stokes radius and sedimentation coefficient values of each protein were determined from gel filtration and sucrose gradient analyses. Native molecular masses were calculated from the estimated values (see "Experimental Procedures").

Protein	Stokes radius	Sedimentation coefficient	Estimated native mass	No. of subunits	Calculated mass of a monomer	Calculated mass
	Å	*S 10^{-13} s*	*kDa*		*kDa*	
PriA$_{Bs}$	35	4.6	71	1	91.3	91.3
PriA$_{Ec}$	32	5.8	81	1	81.8	81.8
DnaB	65	8.0	227	4	54.9	220
DnaD	39	4.3	73	2–3	27.6	55.2–82.8

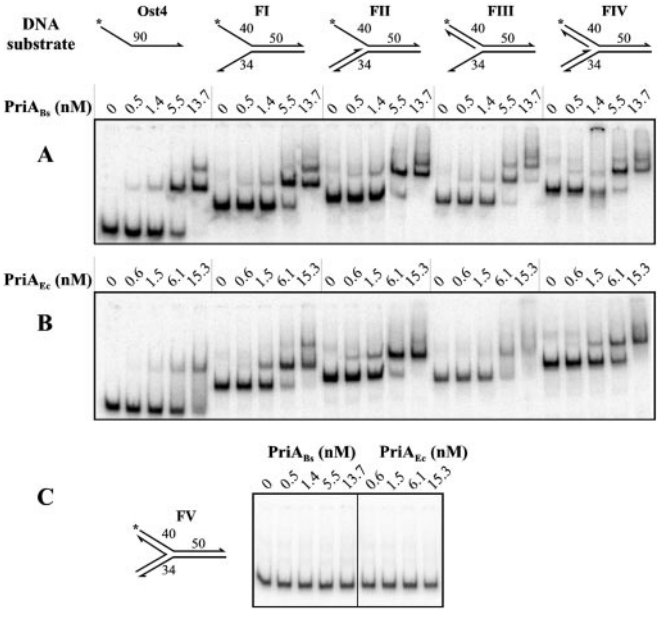

FIG. 2. **Binding of the PriA$_{Bs}$ and PriA$_{Ec}$ proteins to ssDNA and different forked DNA substrates.** The different DNA substrates are schematically represented at the *top* of the figure. *Arrows* indicate the DNA 3′ ends, and *asterisks* indicate the radiolabeled 5′ end of the Ost4 oligonucleotide common to all molecules. *Numbers* indicate the size (in nucleotides) of the ss- and dsDNA parts of the molecules. Radiolabeled DNA was incubated with the indicated concentrations of each protein. Nucleoprotein complexes were separated by native PAGE, as described under "Experimental Procedures." *A* and *B,* PriA$_{Bs}$ and PriA$_{Ec}$ binding to Ost4, FI, FII, FIII, and FIV, respectively. *C,* PriA$_{Bs}$ and PriA$_{Ec}$ binding to FV.

TABLE II
Apparent K_D values of PriA$_{Bs}$, PriA$_{Ec}$, DnaB, and DnaD

The apparent K_D values were determined (34) for each protein and each substrate. The last column corresponds to the apparent K_D of DnaB in the presence of 91 nM of DnaD. In that case, DnaD alone partially binds to the DNA. Therefore, K_D values were estimated by measuring the decrease of the sum of free DNA and DnaD bound DNA. ND, not determined.

	Apparent K_D (nM)				
	PriA$_{Bs}$	PriA$_{Ec}$	DnaD	DnaB	DnaB/D
Ost4	4	20	140	>4000	140
FI	4	3	165	750	160
FII	3	3	180	540	180
FIII	3	4	285	605	>1500
FIV	2	6	445	540	>1500
ds98	18	20	500	370	480
FV	27	30	ND	ND	ND

Y-shaped molecules by PriA$_{Bs}$, because this protein binds 4–5-fold less efficiently to ds98 and to FV in these conditions (Table II and Fig. 2*C*).

For comparison, we also examined binding of PriA$_{Ec}$. The protein interacted with the forked structures and dsDNA as efficiently as PriA$_{Bs}$, whereas its affinity for Ost4 (ssDNA) was 5-fold lower (Fig. 2*B* and Table II). PriA$_{Ec}$ binding to ssDNA appeared unstable, as indicated by the presence of the smear below the shifted substrate. Similarly, PriA$_{Ec}$ interaction with FIII was less stable than with the three other forked substrates. This could be due to a different binding mode of PriA$_{Ec}$ to these DNA substrates (37). Supporting this hypothesis, a 4-fold increase of the ionic strength abolished PriA$_{Ec}$ binding to ssDNA, strongly diminished that observed to the FIII and FIV forks, but did not affect the binding observed with FI and FII (data not shown). In contrast, PriA$_{Bs}$ binding was not affected by similar changes of the ionic strength (data not shown). As reported previously (12), PriA$_{Ec}$ proved to bind less efficiently to dsDNA and to the FV fork, with an efficiency similar to that of PriA$_{Bs}$ in those experimental conditions (Table II and Fig. 1*C*).

Next, we observed that DnaD exhibited binding activities similar to PriA$_{Bs}$ but displayed a 35–200-fold lower affinity for the different substrates (Fig. 3*A* and Table II).The DnaD binding to the ssDNA substrate resulted in a particular gel shift pattern (Fig. 3*A*). At low DnaD concentrations a single discrete band was observed, whereas at high protein concentrations all the material remained in the well. This indicates formation of large aggregates unable to enter the gel, which could be due to a simultaneous binding of ssDNA molecules to several DnaD oligomers. Moreover, DnaD did bind to the four forked DNA substrates but with a lower affinity than to the ssDNA substrate and decreasing from FI to FIV (Table II). Two types of gel shift patterns were observed (Fig. 3*A*), suggesting different modes of interaction with the different substrates. A fast migrating complex, predominant at low DnaD concentration, was detected with all substrates. Several complexes of lower mobility and aggregates were observed at higher DnaD concentrations with Ost4, FI, and FII, whereas only one additional complex was detected at high concentrations with FIII and FIV; the formation of aggregates was very limited (Fig. 3*A*). Finally, DnaD also bound to dsDNA but not stably (data not shown) and with a lower affinity than for the other substrates, except FIV (Table II).

DnaB was also found to bind to the five DNA substrates (Fig. 3*B*) and to dsDNA (data not shown). Its affinity for all forked DNA and for dsDNA was weak but was higher than for ssDNA (Table II). Several discrete complexes of very low and similar mobility were detected in all cases (Fig. 3*B*). A faint and fast-migrating complex, observed with ssDNA, was due to a contaminating ssDNA-binding protein (data not shown). No substrate aggregation was noticed with DnaB, even at the very high protein concentrations (3-fold higher than with DnaD).

In conclusion, PriA$_{Bs}$, DnaD, and DnaB are three DNA-binding proteins, but the affinity of the last two for the DNA was much weaker than that for PriA$_{Bs}$.

DnaD Binds Specifically to the "PriA$_{Bs}$-FII Forked DNA" Complex—To test the hypothesis that PriA$_{Bs}$ triggers a protein-protein interaction cascade upon binding to the DNA, we first analyzed the gel shift patterns of PriA$_{Bs}$ in the presence of DnaD. At the concentrations used, PriA$_{Bs}$ generated one main complex (I) and one or two minor complexes (I′ and I″), depend-

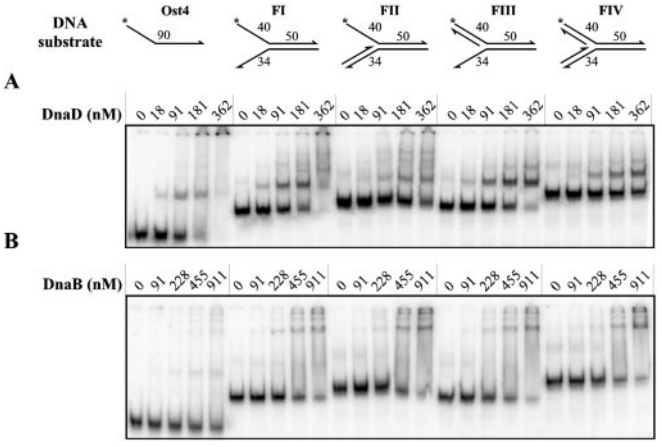

FIG. 3. **Binding of the DnaD and DnaB proteins to ssDNA and different forked DNA substrates.** Experiments were conducted as indicated in Fig. 2. *A* and *B,* DnaD and DnaB binding to Ost4, FI, FII, FIII, and FIV, respectively.

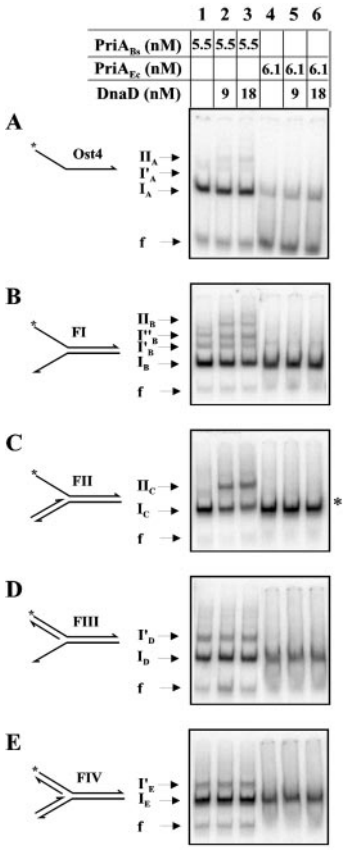

FIG. 4. **Interaction between PriA$_{Bs}$ and DnaD in the presence of DNA.** The different DNA substrates, drawn on the *left* of the figure, were incubated with the proteins at the indicated concentrations, as described under "Experimental Procedures." *f,* free substrate; *I*, *I′*, and *I″*, complexes generated with PriA$_{Bs}$ or PriA$_{Ec}$; *II*, complexes observed in the presence of both PriA$_{Bs}$ and DnaD. The *asterisk* indicates the expected position of a FII-DnaD complex.

ing on the substrate (Fig. 4, *lane 1*). DnaD concentrations used gave rise to barely detectable binding when assayed alone (Fig. 3). When the two proteins were mixed, interaction took place on the substrate FII, as deduced by the appearance of a new retarded band (Fig. 4*C*). Interaction probably also occurred on substrates Ost4 and FI (Fig. 4, *A* and *B*), although to a lower extent. The complex formed with FII might contain both proteins, because its mobility was different from that of the complexes formed with either protein alone. Separate incubation of either protein with the five DNA substrates prior to the addition of the second protein resulted in an identical gel shift pattern (data not shown). Interestingly, incubation of DnaD with PriA$_{Ec}$, which bound to the DNA substrates similarly to PriA$_{Bs}$ (Figs. 3 and 4), did not lead to the formation of an additional nucleoprotein complex (Fig. 4, *lanes 5* and *6*). This strongly indicates that PriA$_{Bs}$ and DnaD interact specifically. The apparent lack of interaction between DnaD and PriA$_{Bs}$ bound to FIII and FIV indicates that the structure of the DNA substrate is determinant for the specific DNA-dependent association of the two proteins.

No interaction between PriA$_{Bs}$ and DnaB in the presence of the five DNA substrates was detected, as judged by the lack of modification of the gel shift patterns obtained following mixing of the two proteins (data not shown and Fig. 6). This result further supports the conclusion of a DNA-mediated protein-protein interaction between DnaD and PriA$_{Bs}$, because DnaB which has weak DNA binding activities, like DnaD (Fig. 3), did not interact with PriA$_{Bs}$.

DnaD Selectively Stimulates Binding of DnaB to Substrates Harboring a 5′ ssDNA Tail—As DnaD and DnaB exhibited DNA binding activities (Fig. 3), we carried out the mobility shift assays with the DNA substrates in the presence of the two proteins. DnaD strongly stimulated DnaB binding to ssDNA, FI, and FII but not to FIII, FIV, and ds98 (Fig. 5 and Table II), as judged by the appearance of several low mobility bands resembling those obtained with DnaB alone but at higher protein concentrations (see Fig. 3). The stimulatory effect required a minimal concentration of DnaD, where at least a part of DNA substrates was bound (91 nM, see Fig. 3 and Fig. 5). Under these conditions, DnaB bound to the substrates at a much lower concentration than when assayed alone (Fig. 5, *A–C*, compare *lanes 3* and *4* with *5* and *6*). Quantification of the binding indicated that DnaD lowered substantially the apparent K_D of DnaB for Ost4, FI, and FII (Table II). In contrast, the K_D increased with FIII and FIV (Table II), indicating that DnaB cannot bind to these substrates in the presence of DnaD. On the ds98, DnaD did not modify DnaB binding. These results suggest that DnaD assists DnaB binding to substrates that include an ssDNA 5′ tail.

Sequential Binding of PriA$_{Bs}$, DnaD, and DnaB to DNA Substrates—Experiments described above revealed specific DNA-dependent "PriA$_{Bs}$-DnaD" and "DnaD-DnaB" interactions. Consequently, we analyzed interactions between the three proteins in the presence of five DNA substrates (Fig. 6). At the concentrations used, PriA$_{Bs}$ generated one main (I) and one or two minor complexes (I′ and I″), depending on the substrate (Fig. 6, *lane 2*). DnaB and DnaD did not bind to any of the substrates, either alone (*lanes 3* and *4*) or together (*lane 6*); the concentration of DnaD was too low for its DNA-dependent interaction with DnaB. Addition of DnaB to PriA$_{Bs}$ did not change significantly the binding pattern (compare *lanes 2* and *5*).

PriA$_{Bs}$ and DnaD interacted on substrates FI and FII, as deduced from the appearance of an additional complex of unique mobility (II, *lane 7*). An interaction involving the three proteins, PriA$_{Bs}$, DnaD, and DnaB, took place on Ost4, FI, and FII, as judged by the appearance of a unique complex of very low mobility (III; Fig. 6, *A–C*, *lanes 8* and *9*), which was absent with FIII and FIV (Fig. 6, *D* and *E*). FII was the best substrate for complex III formation. Interestingly, in the case of FI and FII, complex III arose from complex II, as indicated by the decrease of the amount of complex II upon DnaB addition. This indicates that the three proteins assemble sequentially on the two substrates. In contrast, complex II was not observed with Ost4, whereas the complex III was. It is possible that the protein assembly on this substrate was also sequential but that

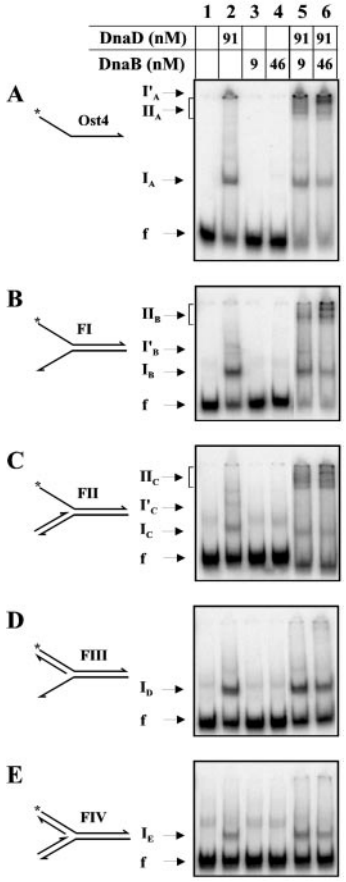

FIG. 5. **Binding of DnaB is stimulated by DnaD.** The different DNA substrates, drawn to the *left* of the figure, were incubated with the proteins at the indicated concentrations, as described under "Experimental Procedures." *f,* free substrate; *I,* complexes generated with DnaD; *II,* complexes observed in the presence of DnaD and DnaB.

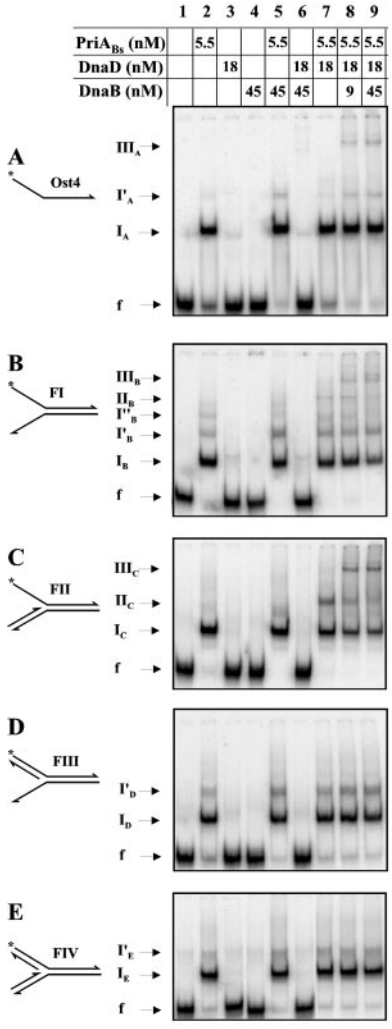

FIG. 6. **Simultaneous binding of PriA$_{Bs}$, DnaB, and DnaD on DNA.** The different DNA substrates, drawn on the *left* of the figure, were incubated or not with PriA$_{Bs}$ and/or DnaB and/or DnaD proteins. Protein concentrations are indicated on the *top* of the figure. *f*, free substrate; *I, I′*, and *I″*, complexes generated with PriA$_{Bs}$; *II,* complexes observed in the presence of PriABs and DnaD; *III,* complexes observed in the presence of PriA$_{Bs}$, DnaD, and DnaB.

the stability of complex II was too low to be detected by the gel shift assay used. As expected, PriA$_{Ec}$ protein did not substitute for PriA$_{Bs}$ in the formation of complex III (data not shown).

Importance of the 5′ ssDNA Tail for the DNA-dependent DnaD-DnaB and PriA$_{Bs}$-DnaD-DnaB Interactions—Previous experiments indicated that preferred substrates carry a 5′ ssDNA tail for the DNA-dependent DnaD-DnaB and PriA$_{Bs}$-DnaD-DnaB interactions. To probe this conclusion further, the O-5′, O-3′, and O-3′40 substrates have been assayed (Fig. 7). O-5′ and O-3′ are FII and FIII derivatives, respectively, without one dsDNA tail. O-3′40 carries a ssDNA tail of identical size to that of O-5′ (40 nucleotides long). As shown in Fig. 7*A*, the stimulation of DnaB binding activity by DnaD occurred on O-5′, as judged by the formation of the nucleoprotein complexes II of low mobility. The efficiency of this stimulation was identical to that measured with Ost4, FI, and FII (see Fig. 5). In contrast, no stimulation was observed with O-3′ and O-3′40 (Fig. 7*A*) and proved to be equivalent to FIII. Therefore, a 5′ ssDNA tail is required for the stimulation of DnaB binding to DNA by DnaD. As expected, the affinity of PriA$_{Bs}$ for O-5′, O-3′, and O-3′40 substrates was equivalent to ssDNA and to the forked DNA substrates FI to FIV (compare Fig. 7*B* with Fig. 2). Mixing PriA$_{Bs}$ with DnaD and DnaB generated new nucleoprotein complexes of low mobility only with O-5′ (complexes III, Fig. 7*B*). The efficiency was as low as that observed with FI and Ost4 (see Fig. 6). Therefore, the specific interaction revealed on DNA between PriA$_{Bs}$ and DnaD required a 5′ssDNA tail and took place preferentially on a forked molecule of the FII shape.

DISCUSSION

The *B. subtilis* essential proteins DnaB and DnaD have been genetically characterized as being involved in initiation of chromosomal DNA replication at *oriC* (for reviews see Refs. 17, 38, and 39). More recently, we have shown that DnaB and DnaD are also involved in a distinct initiation pathway of chromosomal DNA replication, required for the restart of arrested forks (20, 25). However, no homologues of DnaB and DnaD are encoded by the *E. coli* genome, and their primosomal function can therefore not be deduced from previous studies with *E. coli* proteins. This disparity between *E. coli* and *B. subtilis* is particularly intriguing, because the proteins beginning (DnaA and PriA) and ending (the helicase and primase) the initiation processes are conserved in these two and, to the best of our knowledge, all other bacteria.

In this report, we present biochemical characterization of the DnaB and DnaD proteins, which confirms their primosomal identity and reveals their function in the early stages of replication fork activation. Both are weak DNA-binding proteins, which interact in the presence of DNA. Furthermore, they appear to assemble sequentially and specifically with PriA$_{Bs}$ on a particular three-stranded DNA molecule, mimicking a fork that can be generated by the recombinational repair of arrested

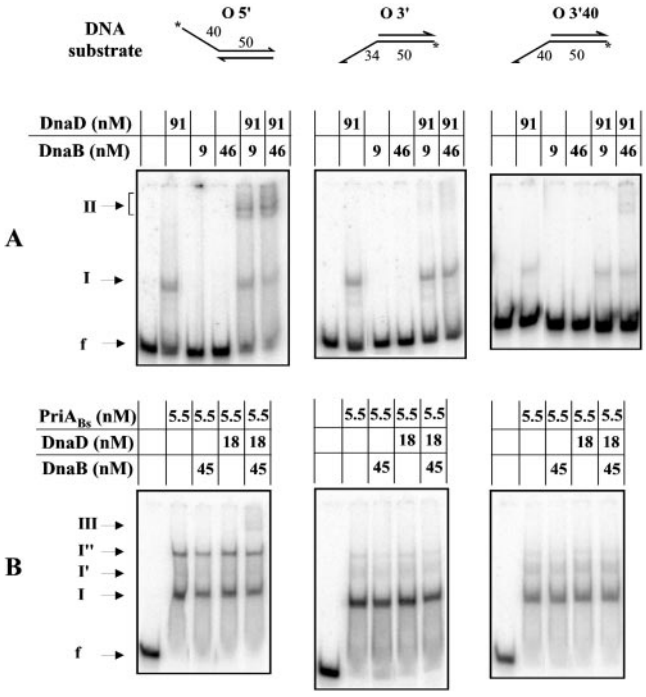

FIG. 7. **Simultaneous binding of PriA$_{Bs}$, DnaB, and DnaD to dsDNA substrates harboring a 5′ ssDNA tail.** The different DNA substrates, drawn on the *top* of the figure, were incubated or not with PriA$_{Bs}$ and/or DnaB and/or DnaD proteins. Protein concentrations are indicated on the *top* of the figure. *A,* stimulation of DNA binding activity of DnaB by DnaD. *f,* free substrate; *I,* complex generated with DnaD; *II,* complexes observed in the presence of DnaB and DnaD (experiments were conducted as described in Fig. 5). *B,* simultaneous binding of PriA$_{Bs}$, DnaB, and DnaD; *f,* free substrate; *I*, *I′*, and *I″*, complexes generated with PriA$_{Bs}$; *II,* complexes observed in the presence of PriA$_{Bs}$, DnaD, and DnaB (experiments were conducted as described in Fig. 6).

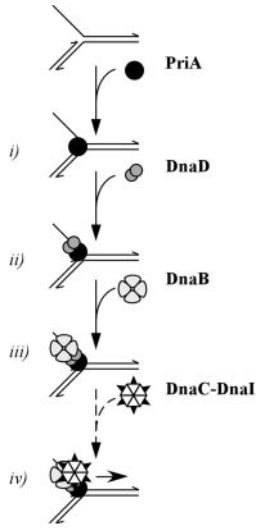

FIG. 8. **Model for assembly of the *B. subtilis* replication restart primosome.** (*i*) The arrested DNA fork is first recognized and bound by PriA$_{Bs}$. (*ii*) PriA$_{Bs}$ assists DnaD fixation. (*iii*) DnaD activates DnaB fixation on the neighboring ssDNA. (*iv*) The putative DnaC-DnaI complex is recruited onto the DNA leading to replication re-start. PriA is represented as a monomer, DnaD as a dimer, and DnaB as a tetramer, as deduced from their native molecular weight. Six monomers of DnaI (*black triangle*) are represented as interacting with one hexamer of the helicase DnaC (*white triangle*), by analogy with the stoichiometry of the *E. coli* DnaC-DnaB complex.

replication (5). Consequently, we propose that these interactions represent the initial steps of the assembly of the *B. subtilis* replication-restart primosome.

The model of the PriA-primosome assembly, based on the present study, is represented in Fig. 8. PriA$_{Bs}$, which has higher affinity than DnaD and DnaB for all substrates tested, triggers the process by stable binding onto an arrested replication fork. This nucleoprotein complex attracts DnaD, by lowering the amount of DnaD required for binding. PriA$_{Ec}$ cannot replace PriA$_{Bs}$ in this process, although the two proteins have similar binding properties on various substrates, which shows the specificity of the assembly of the *B. subtilis* primosome. The PriA/DnaD pair attracts in turn the DnaB, most probably via a contact with DnaD. Indeed, we have shown that DnaD and DnaB interact on several substrates, whereas PriA$_{Bs}$ and DnaB do not. We have also shown that the ordered assembly of PriA$_{Bs}$, DnaD, and DnaB takes place most efficiently on the FII substrate. The selectivity for this substrate is highly interesting in regard to the function of these proteins in the replication fork activation process. This Y-shaped molecule with a 5′ ssDNA tail is the expected product of recombinational repair of a broken replication fork, as well as a stalled replication fork with the lagging strand unreplicated. We suggest that complex promotes the loading of the DnaC helicase with the help of another *B. subtilis* primosomal protein, DnaI, which interacts with DnaC and should be involved in its loading (17, 20, 23, 40). DnaI has a weak sequence similarity with the *E. coli* DnaC helicase loader, overlapping the nucleotide-binding motif (21). Consequently, DnaI could deliver the helicase onto the proper ssDNA in an ATP-dependent manner, as does *E. coli* DnaC. However, all mutations that suppress lack of PriA in *B. subtilis* map in *dnaB* (25), whereas in *E. coli* they map in *dnaC* (24), which suggests that DnaB might be the helicase loader in *B. subtilis*. Alternatively, DnaB and DnaI might act together to bring the helicase onto the DNA. Indirect support for this hypothesis stems from the co-localization of the two proteins in the cell (23), the tandem organization of the *dnaB* and *dnaI* genes (22, 41), and the requirement for DnaI in the DnaB suppressor mutants lacking PriA (25). We propose that a putative DnaI-DnaC complex contacts the complex formed by PriA$_{Bs}$, DnaD, and DnaB on a forked DNA molecule that carries a 5′ ssDNA tail (Fig. 8). Following this encounter, DnaC is delivered onto the proper ssDNA strand and triggers replication restart.

This study underlines the similarities and the differences with the assembly of the *E. coli* replication restart primosome. In both cases, each protein partner can be added individually to build sequentially the primosome onto an appropriate DNA substrate. As quoted above, there is, however, a clear difference, due to the sequence disparity of the proteins acting immediately after PriA. *B. subtilis* DnaB and DnaD proteins appear to fulfill the same linkage function as the *E. coli* PriB, PriC, and DnaT proteins between PriA and the replicative helicase. It is not known, however, if PriB and/or PriC and DnaT have the same activities as DnaB and DnaD. In the case of the *E. coli* replication restart primosome, it is not known if its assembly is arrested immediately after PriA$_{Ec}$ binding onto DNA substrates that are not productive for the loading of the replicative helicase. Nevertheless, the participation of PriA$_{Ec}$ in the remodeling of the DNA substrate for mediating primosome assembly has recently been reported for the *E. coli* replication restart primosome, through the study of the replicative transposition of the bacteriophage Mu (11, 15). In this system, the DNA forks recognized by PriA$_{Ec}$ are constructed at the Mu ends by the Mu-encoded transposase. They are structurally close to the FIV fork used in this study. It has been proposed that the Mu forks are reshaped by the 3′ → 5′-helicase activity of PriA$_{Ec}$ to unmask the lagging strand template for the loading of the replicative helicase DnaB (15). We have shown that PriA$_{Bs}$ is also a helicase.[2] Therefore, by analogy with the action of PriA$_{Ec}$ on the Mu forks, the helicase activity of PriA$_{Bs}$ may

convert FIII and FIV, unproductive for the concerted binding of $PriA_{Bs}$, DnaD, and DnaB, into FI and FII, active for this assembly (Fig. 6). Such a remodeling of the DNA substrate by $PriA_{Bs}$ would be dedicated to the recruitment of the DnaD/DnaB pair and, in turn, the *B. subtilis* replicative helicase DnaC on the liberated lagging strand template.

Biochemical properties of DnaD and DnaB might explain the observation that $PriA_{Bs}$ is not essential in slow growing cells,[2] where the number of the replication forks, and presumably of their arrest, is lower than in the fast growing cells. The two proteins bind individually to ssDNA and forked DNA molecules, albeit with a low affinity, and the binding of DnaB to substrates with a 5′ ssDNA tail is stimulated by DnaD. We suggest that the DnaD-DnaB complex is able to attract the putative DnaI-DnaC complex, delivering the helicase on the appropriate strand and thus promoting the replication restart in the absence of PriA. This alternative restart pathway would target the same DNA sites as $PriA_{Bs}$, but less efficiently, which could be sufficient in slow but not in fast growing cells. The existence of such $PriA_{Bs}$-independent pathway in *B. subtilis* was recently suggested by our genetic analyses. Indeed a mutation that reduces the amount of DnaD cannot be combined with *priA* mutations (25). Mutations in *dnaB* that suppress the absence of $PriA_{Bs}$ but require DnaD and DnaI might improve the efficiency of this salvage pathway and make it efficient enough even in fast growing cells (25).

Interestingly, it has been reported that DnaA interacts with DnaD (17, 36). It is possible that the recruitment of the replicative helicase at *oriC* and at arrested forks proceeds through similar series of interactions but with DnaA and $PriA_{Bs}$ as initiators, respectively. Indeed, we have observed that FI substrate, which is structurally similar to the product expected from the action of DnaA at *oriC*, allows formation of the DnaD-DnaB complex. Taken together, these results lead us to propose that the recruitment of the replicative helicase in *B. subtilis* is under the control of a common protein core, made of DnaD and DnaB. The characterized initiators, DnaA and PriA, would attract this loading machinery at specific DNA sites by contacting DnaD in both cases.

As its *E. coli* counterpart, the *B. subtilis* replication restart primosome is a multiprotein complex that assembles on a particular forked DNA substrate. In both cases, each protein partner can be added individually to sequentially build the primosome. However, the proteins acting immediately after PriA have no sequence homology. Because DnaD and DnaB from *B. subtilis* appear to fulfill the same linkage function between PriA and the replicative helicase as the DnaT, PriB, and/or PriC from *E. coli*, we conclude that the two systems have evolved differently to serve a universal cellular function and that DnaB and DnaD are two proteins enlarging the repertoire of primosomal proteins involved in chromosomal DNA replication. Interestingly, bacteriophage T4 uses a very different primosomal system to initiate replication at forked DNA, where a single protein, gp59, loads the phage helicase onto the DNA (for a review see Ref. 18). This contrasts with the complexity of the PriA-dependent primosomes in the two distant bacteria where they were characterized and raises questions about the reasons for this complexity. One possibility is that the bacterial primosomal proteins have functions other than promoting initiation of DNA replication, such as providing links to other cellular events. In this respect, it is worth mentioning the fixed subcellular localization of DnaB (23), a protein proposed to be associated to the membrane (39). Characterization of the *B. subtilis* primosomal proteins opens future research avenues, aiming to unravel not only their involvement in replication via recruitment of the replicative helicase but also of their other possible and specialized cellular functions.

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Early Steps of *Bacillus subtilis* Primosome Assembly

Stéphanie Marsin, Stephen McGovern, S. Dusko Ehrlich, Claude Bruand and Patrice Polard

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